Activities on Osteoblastic Cells and Anti-allergy of Thai Herbs in Longevity Herbal Preparation

Sawanee Kraithep

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

Master of Pharmacy in Pharmaceutical Science

Prince of Songkla University

2008

ACKNOWLEDGEMENT

This thesis could not have been successfully completed without the help of many individuals. First of all, I would like to express my most sincere gratitude and deep appreciation to my major advisor, Assist. Prof. Dr. Kwunchit Oughbho, and my co-advisor, Assoc. Prof. Dr. Supinya Tewtrakul for all of the valuable advice, guidance, suggestion, constructive criticism, comments and help throughout this study.

I would like to thank Dr. Premjit Arpornmaeklong, Faculty of Dentistry, Prince of Songkla University, for her kind provision of MC3T3-E1 pre-osteoblastic cells. I offer special thanks to all staffs of Department of Pharmaceutical Technology and Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University for cell culture laboratory and scientific equipments facilities throughout this study.

I would like to express my thanks to the staffs in the Scientific Equipment Center, Prince of Songkla University for their suggest in microplate reader of my work. Additionally, special thanks to Graduate School, Prince of Songkla University for the grant support during my study.

Finally, great respect to my parents for their support and encouragement throughout my study and I would like to give my special thanks to my friends for their help and take care during my study.

Sawanee Kraithep

Thesis Title	Activities on Osteoblastic Cells and Anti-allergy of Thai Herbs in Longevity Herbal Preparation
Author	Miss Sawanee Kraithep
Major Program	Pharmaceutical Sciences

Major Advisor

.....
(Asst. Prof. Dr. Kwunchit Oungbho)

Co-advisor

.....
(Assoc. Prof. Dr. Supinya Tewtrakul)

Examining Committee :

.....Chairperson
(Assoc. Prof. Dr. Pharkphoom Panichayupakaranant)

.....Committee
(Asst. Prof. Dr. Kwunchit Oungbho)

.....Committee
(Assoc. Prof. Dr. Supinya Tewtrakul)

.....Committee
(Assoc. Prof. Dr. Arunporn Itharat)

The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirements for the Master of Pharmacy Degree in Pharmaceutical Sciences

….....
(Assoc. Prof. Dr. Krerkchai Thongnoo)
Dean of Graduate School

ชื่อวิทยานิพนธ์ ฤทธิ์ต่อเซลล์สร้างกระดูกและแก้แพ้ของพืชสมุนไพรไทยในตำรับยาอายุวัฒนะ

ผู้เขียน นางสาว สวนีย์ ไกรเทพ

สาขาวิชา เภสัชศาสตร์

ปีการศึกษา 2550

บทคัดย่อ

วัตถุประสงค์ในการศึกษานี้เพื่อจำแนกฤทธิ์สร้างกระดูกและฤทธิ์แก้แพ้ของสมุนไพรไทยในตำรับยาอายุวัฒนะ ตำรับยาอายุวัฒนะ(Longevity formulation: LF) ที่คัดเลือกมาศึกษาประกอบด้วยสมุนไพรแห้ง 6 ชนิดในสัดส่วนโดยน้ำหนักที่เท่ากัน ได้แก่ ทิ้งถ่อน (*Albizia procera* (Roxb.) Benth.) แห้วหมู (*Cyperus rotundus* Linn.) ตะโกนา (*Diospyros rhodocalyx* Kurz.) พริกไทย (*Piper nigrum* Linn.) ข่อย (*Streblus asper* Lour.) และบอระเพ็ด (*Tinospora crispa* Linn.) โดยใช้คำย่อคือ AP, CR, DR, PN, SA, and TC ตามลำดับ สารสกัดหยาบชั้นน้ำและชั้นเอทานอลของสมุนไพรทั้ง 6 ชนิดได้ถูกเตรียมขึ้นและศึกษาฤทธิ์สร้างกระดูกและฤทธิ์แก้แพ้ที่ความเข้มข้น 3-100 มคก./มล. สารสกัดหยาบไม่เป็นพิษต่อเซลล์ MC3T3-E1 (pre-osteoblastic cells) ยกเว้น AP-W ที่ความเข้มข้น 30-100 มคก./มล. และ AP-E ที่ความเข้มข้น 10-100 มคก./มล. แล สารสกัดหยาบชั้นเอทานอลอื่นๆ ที่ความเข้มข้น 100 มคก./มล. การวิเคราะห์ปริมาณโปรตีนทั้งหมด (total protein content) และ alkaline phosphatase (ALP) activity เมื่อเพาะเลี้ยงเซลล์ MC3T3-E1 และเหนี่ยวนำด้วยสารสกัดหยาบ พบว่าผลต่อเซลล์ของสารสกัดบางชนิด อาทิ DR-W, TC-W, LF-W, SA-E และ TC-E ที่ความเข้มข้น10 มคก./มล. และ PN-W ที่ความเข้มข้น 100 มคก./มล. มีนัยสำคัญสูงกว่าผลของ 10^{-6}M dexamethasone ที่เป็นตัวควบคุมผลบวก ทำการศึกษาฤทธิ์แก้แพ้ของสารสกัดหยาบโดยวิเคราะห์การปลดปล่อยเอนไซม์ β-hexosaminidase พบฤทธิ์แก้แพ้ของ PN-E โดยมี IC_{50} เป็น 14.0 มคก./มล. เทียบกับ 16.0 มคก./มล. ใน piperine และพบการเสริมฤทธิ์ของ PN-E ที่ 10 มคก./มล. ต่อฤทธิ์สร้างกระดูกของ DR-W ที่ 10 มคก./มล. และ PN-W ที่ 100 มคก./มล. โดย ALP activity ของสารผสม 1:1 PN-E/DR-W และ 1:10 PN-E/PN-W เป็น 1.61 และ 1.60 เท่าของ 10^{-6}M dexamethasone ตามลำดับ ฤทธิ์แก้แพ้ของสารผสม 1:1 PN-E/DR-W และ 1:10 PN-E/PN-W มี IC_{50} 28.7 และ23.8 มคก./มล. ตามลำดับ และสูงกว่า DR-W, PN-W และLF (IC_{50} เท่ากับ 40.8, 65.8 และ66.6 มคก./มล. ตามลำดับ) ผลการศึกษานี้อาจเป็นประโยชน์ต่อการใช้

ยาอายุวัฒนะตามแบบโบราณ และเป็นประโยชน์ในการศึกษาและการพัฒนายาแผนโบราณนี้ในรูปแบบสากลต่อไป

Thesis Title	Activities on Osteoblastic Cells and Anti-allergy of Thai Herbs in Longevity Herbal Preparation
Author	Miss Sawanee Kraithep
Major Program	Pharmaceutical Sciences
Academic Year	2007

ABSTRACT

The aim of this study was to identify osteogenic and anti-allergic activities of Thai herbs in longevity herbal preparation. Thai longevity formulation (LF) selected in this study was composed of equal ratio by weight of six dried herbs, i.e. *Albizia procera* (Roxb.) Benth., *Cyperus rotundus* Linn., *Diospyros rhodocalyx* Kurz., *Piper nigrum* Linn., *Streblus asper* Lour., and *Tinospora crispa* Linn., using AP, CR, DR, PN, SA, and TC, respectively as their abbreviations. Water (-W) and ethanolic (-E) extracts of these dried herbs and formulation were prepared and evaluated for their osteoblastic and anti-allergic activities. All crude water extracts at 3-100 μg/ml, except AP-W at 30-100 μg/ml, and all ethanolic extracts at 100 μg/ml, except AP-E at 10-100 μg/ml, were compatible to MC3T3-E1 cells. Determination of total protein content and alkaline phosphatase activity (ALP) in this preparation were performed. It was indicated that some extracts i.e. DR-W, TC-W, SA-E, TC-E, and LF-W at 10 μg/ml, and PN-W at 100 μg/ml, resulted in significantly higher ALP activity of MC3T3-E1 cells compared with 10^{-6} M dexamethasone as positive control. Evaluation of anti-allergic activity of crude extracts by determination of β-hexosaminidase released in rat basophilic leukemia cells (RBL-2H3) confirmed

potent anti-allergic activity of PN-E with IC_{50} of 14.0 μg/ml compared with 16.0 μg/ml of piperine. Synergistic effect of PN-E (10 μg/ml) on osteogenic activity of DR-W (10 μg/ml) and PN-W (100 μg/ml) was observed. Increased ALP activity was found as 1.61 and 1.60 folds of dexamethasone in 1:1 mixture of PN-E/DR-W and 1:10 mixture of PN-E/PN-W, respectively. The anti-allergic activity of these mixtures was found as IC_{50} values of 28.7 and 23.8 μg/ml, in 1:1 PN-E/DR-W and 1:10 PN-E/PN-W mixtures respectively, more potent than DR-W, and PN-W, and LF (IC_{50} = 40.8, 65.8, and 66.6 μg/ml, respectively). The results from this study may be useful for traditional use of longevity herbal preparations and further study and development of this traditional medicine in modern dosage forms.

CONTENTS

CONTENTS (Continued)

CONTENTS (Continued)

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ALP	Alkaline phosphatase
BMP	Bone morphogenetic protein
cont.	Continued
°C	Degree Celsius
CO_2	Carbondioxide
Dex	Dexamethasone
e.g.	Exempli gratia, for example
et al.	Et alii, and others
etc.	Et cetera, and other things
FBS	Fetal bovine serum
FCS	Fetal calf serum
IL	Interleukin
g	Gram (s)
h	Hour (s)
µg	Microgram (s)
µl	Microliter (s)
mg	Milligram (s)
MW	Molecular weight
nm	Nanometer (s)
No.	Number

%	Percent
pH	The negative logarithm of the hydrogen ion concentration
r^2	Coefficient of determination
rpm	Revolutions per minute
s	Second (s)
S.D.	Standard deviation
S.E.M.	Standard error of mean
w/w	Weight by weight

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

General introduction

Longevity herbal preparations display an important role of medical system in Thailand to cure illness and enhance wellness. They balance particular parts of the body and controll immune system (Shinwari and Khan, 2000; Kishikawa and Sakae, 1997). Immunodeficiency can cause various diseases such as common cold, asthma, food allergies, infections, rheumatoid arthritis, and others (Srivastava *et al*., 2005; Lam and Ng, 2002; Lam *et al*., 2000). Many disorders are associated with immune systems including bone disease and allergy. In this study, we are interested in the bioactivity of longevity herbal preparations related to osteogenic activity and anti-allergic activity which are connected with immune systems (Zhang *et al*., 2006; Huh *et al*., 2006; Mühlbauer *et al*., 2003; Srivastava *et al*., 2005; Novak *et al*., 2001; Blanc *et al*., 1997).

Cell culture models have provided information regarding the basic biological and physiological processes of organisms. Over the past several years, advances in modeling of tissues with defined cell systems, and advances in molecular biology have greatly enhanced our ability to resolve complex biological processes down to their cellular and molecular components (Quaroni and Hochman, 1996).

The immune balance controlled by T helper 1 (Th1) and T helper 2 (Th2) is crucial for immunoregulation and its imbalance causes various immune

diseases including allergic disorders and asthma. It has been recently proposed that allergic disorders may result from an imbalance between Th1 and Th2 cells. Allergic disorders are result from excess production of Th2 cytokines (IL-4, IL-5, IL-13) which are related to Th1 cytokine (IFN-γ) (Factor, 2003). Th1 cells are characterized by the prevalent production of IL-2, IFN-γ, and TNF-β, without IL-4, IL-5, IL-9, and IL-13 production. In contrast, Th2 cells are characterized by the prevalent production of IL-4, IL-5, IL-9, and IL-13 in the absence of IFN-γ (Lee and Kim, 2008).

The dominance of either one of the Th1 or Th2 responses is shown to correlate with severity of immune disease such as fungal infection, bacterial infection or viral. Cytokines are glycoproteins that are recognized as important mediators in allergic and inflammatory responses after antigen stimulation (Plaut *et al*., 1989). They are produced by various cells, such as fibroblasts, epithelium, endothelium and inflammatory cells (Plaut *et al*., 1989; Stevens and Austen, 1989). The roles of cytokine correlate with disease including allergic symptoms. Commonly, mast cells produce histamine, as well as proinflammatory cytokine, especially TNF-α, IL-4 and IL-6 (Zhao *et al*., 2005). These cytokines have a critical biological role in the allergic reaction (Lee *et al*., 2007). Degradation of mast cells in the endothelium is abrogated by the inhibition of the action of TNF-α (Kempuraj *et al*., 2003). The inhibition of proinflammatory cytokines expression from mast cells must be one of the key indicators of reduced allergic symptoms by inhibition of degranulation (Lee *et al*., 2007).

Futhermore, other cytokines are related to bone formation of a complex process involving proliferation, expansion, differentiation and mineralisation. This sequence of events requires the up-regulation and inhibition of several genes that encode phenotypic proteins, e.g. collagen type 1, alkaline phosphatase (ALP) and osteocalcin (Aubin and Lin, 1996). Therefore, traditional herbal medicines have been developed against bone diseases, and their effectiveness has received increasing bone formation. Several herbs have been reported to be effective in the prevention of postmenopausal osteoporosis (soybean (Choi *et al*., 2001), licorice root (Choi, 2005) and *Drynaria fortunei* (Sun *et al*., 2002; Jeong *et al*., 2005). Cytokines correlated with stage of bone formation during osteogenesis such as IL-11 and TGF-β1 which induce high level of alkaline phosphatase activity (ALP) (Clark-Greuel *et al*., 2007).

Folk medicines have been accepted as one of the main sources of drug and still widely used in lifestyle. In Thailand, several drugs isolated from plants and herbal medication have been developed in the ancient tradition such as curcumin, roselle, *Centella asiatica* (Varalakshmi *et al*., 2008; Choi *et al*., 2008; Amin and Hamza, 2005; Faraji *et al*., 1999; Cheng *et al*., 2004; Gupta *et al*., 2003). In this study, we examined bioactivities of Thai longevity herbal preparation and its composition. Osteogenic activity and anti-allergic activity were evaluated. The six components in this preparation were derived from *Albizia procera, Cyperus rotundus, Diospyros rhodocalyx*, *Piper nigrum*, *Streblus aspers and Tinospora crispa*. There has not been any report of these herbs on osteoblastic cells and anti-allergic activities. In our study,

osteogenic activity of water and ethanolic extracts of these herbs was determined for their cytotoxicity using the tetrazolium salt (MTT) assay, alkaline phosphatase activity (ALP) and total protein content on MC3T3-E1 pre-osteoblastic cells. Anti-allergic activity was evaluated on the enzyme activity of β-hexosaminidase from mast cell or basophil (RBL-2H3 cells). Scientific found in this study were to support several indications and applications of the herbal preparations and integrate knowledge of the traditional and modern medicine.

Objectives

The aim of the present study was to identify osteogenic and anti-allergic activities of longevity herbal preparation and its compositions on MC3T3-E1 pre-osteoblastic cells and RBL-2H3 cells by release of β-hexosaminidase enzyme.

Literature review

1. Herbs in Longevity herbal preparations

1.1 *Albizia procera* (Roxb.) Benth (Leguminosae)

A. procera is known as White Siris and Sit. It is native to tropical Asia and Australia, and widely cultivated in the tropics. Trees are often planted for shade or decoration along roads. Commonly *A. procera* has been used in traditional medicine. The bark contains tannins and a reddish gum. It has been given with salt as a medicine for water buffalo. The leaves are used to treat ulcers and have insecticidal properties. In India, leaves are poulticed onto ulcers. In the Philippines, the cooked leaves are eaten as a vegetable. Bark, leaf, and root of *A. procera* contain saponin. Leaf and fruit have been used for haemolysis (List & Horhammer, 1969–1979). Pentacyclic triterpenic acid, procera acid, from the seed has been report (List & Horhammer, 1969–1979). The gum contains aldobiuronic acid and the disaccharide 3-0-D-galactopyranosyl-L-arabinose. Degraded gum from *A. procera* contains D-galactose, D-mannose, D-glucuronic acid and 4-0-methyl-D-glucuronic acid. Complete methylation and subsequent hydrolysis of the product give 2,4-di-0-methyl-D-galactose (3 moles), 3,4,6-tri-0-methyl-L-arabinose. Perceragenin, $C_{30}H_{46}O_4$, was reported from the seed (List & Horhammer, 1969–1979).

1.2 *Cyperus rotundus* Linn. (Cyperaceae)

C. rotundus (coco-grass, nut sedge, nut-grass, purple nut sedge, red nut sedge) is a species of sedge (Cyperaceae) native to africa, southern and central europe (north to France and Austria), and southern asia. Arabs of the land of Israel

traditionally use roasted tubers, while they are still hot, or hot ashes from burned tubers, to treat wounds, bruises, carbuncles, etc (http://en.wikipedia.org). In India, rhizomes of *C. rotundus* have been used in ancient medicine for fever, dysentery, pruritis, pain, vomiting and various blood disorders (Yusuf *et al*., 1994; Uddin *et al*., 2006). Modern alternative medicine recommends this plant for treatment of nausea, fever and inflammation (Gupta *et al*., 1971; Uddin *et al*., 2006), pain reduction, muscle relaxation and many other disorders. Modern ayurvedic medicine uses this plant for treatment of fevers, digestive system disorders, dysmenorrhea. It has been known as an important ingredient of anti-aging Ayurvedic nutraceutical Chyavanprash and other maladies (Sharma and Gupta, 2007).

C. rotundus has been used as a anti-diarrhoeal, anti-pyretic, anti-inflammatory, and antimalaria and also for treatment of stomach and bowel disorders (Uddin *et al*., 2006; Gupta *et al*., 1971; Jagtap *et al*., 2004), diabetes, anti-oxidant (Raut and Gailwad, 2006), anti-inflammatory and antifungal (Tam *et al*., 2007). *C. rotundus* has been reported to contain oils, alkaloids, glycosides, saponins, flavonoids, tannins, starch and carbohydrates. Several pharmacologically active substances have been identified in *C. rotundus*: α-cyperone, β-selinene, cyperene, cyperotundone, patchoulenone, sugeonol, kobusone, and isokobusone.

1.3 *Diospyros rhodocalyx* Kurz (Ebenaceae)

Diospyros, the largest genus of the family Ebenaceae, comprises about 400 species widespread mainly in the tropics. Plants in this genus have long been

known for their medicinal uses. It is interesting that almost all parts of these plants were found to possess therapeutic properties (Mallavadhani *et al*., 1998). Phytochemical investigation of more than 130 *Diospyros* species led to the isolation of a variety of compounds, the majority of which is triterpenoids and naphthoquinones (Mallavadhani *et al*., 1998). Several compounds of these two groups have been found to exhibit interesting bioactivities (Mallavadhani *et al*., 1998). In Thailand, sixty species of *Diospyros* have been found, excluding two foreign species (Phengklai *et al*., 1981). About ten of them have been recorded of their uses in Thai traditional medicine. Twelve species of Thai *Diospyros* plants have been reported for their chemical constituents (Mallavadhani *et al*., 1998). Some of them have been biologically examined and several biological activities have been demonstrated (Mallavadhani *et al.*, 1998).

In Thai traditional medicine, the fruits are used for treatment of diarrhea, bleeding, abdominal discomfort, parasitic infection, abscess, and renal disease; the bark is used for symptomatic relief of leucorrhea, and as anti-diuretic. Lupeol and betulin have been reported to exhibit anti-inflammatory activity (Akihisa *et al*., 2002; Recio *et al*., 1995), antiviral (Pavlova *et al*., 2003). Other bioactivities of these compounds have also been documented e.g. antitumor activity of lupeol and betulin. However, some species of *Diospyros* (*D. glandulosa*) have been reported for bioactivities such as antihepatotoxic and antifertility activities of oleanolic acid, and antileukemic and hepatoprotective activities of ursolic acid (Duke, 1992). Although there have been several reports on triterpenes, naphthoquinones (Marston and

Hostettmann, 1984), and lignans among others in many *Diospyros* species, the chemical constituents of this plant have however not been investigated.

1.4 *Piper nigrum* Linn (Piperaceae)

P. nigrum is a flowering vine in the family Piperaceae, cultivated for its fruit, which is usually dried and used as a spice and seasoning. The same fruit is also used to produce white pepper, red or pink pepper, and green pepper. Black pepper is native to South India and elsewhere in tropical regions. The dried fruit is a small drupe five millimetres in diameter and dark red when fully mature.

Some uses of black pepper in folk medicine are supported by modern science consist of an antipyretic, anti-inflammatory (Szallasi, 2005), antioxidant activity (Mujumadar *et al*., 1990), antimutagenic (EI *et al*., 2003) and antibacterial activities (Dorman and Deans, 2000). Increasing popular modern-day uses of active compound of its compounds especially piperine that it has been used to stimulate metabolism, aid absorption of nutrients and determination the efficacy of drugs such as Viagra® (Szallasi, 2005). Whereas other uses are puzzling such as the treatment for epilepsy and snake venom poisoning (Szallasi, 2005).

The pepper, the fruits of *P. nigrum*, is an important as spice and flavoring agents, and has also been used in the treatment of cholera and dyspepsia, as well as a variety of gastric ailments and arthritic disorders (Jung and Shin, 1998). Terpenes, steroids, lignans, flavones, and alkaloids/alkamides have been identified as

the primary constituents of the peppers (Navickene *et al*., 2000; Parmar *et al*., 1997). Alkamides are particularly interesting, due to their various biological activities, including insecticidal (Kiuchi *et al*., 1988; Park *et al*., 2002), anti-bacterial (Reddy *et al*., 2004) and anti-inflammatory properties (Mujumdar *et al*., 1990).

Black pepper oil is formed by monoterpenes, sesquiterpenes, and oxygenated compounds (Ferreira, 1996). The most important constituents in the essential oil of *P. nigrum* are limonene, β-caryophyllene, sabinene and β-pinene (Martin *et al*., 1998). Piperine is an alkaloid that contributes towards the pungency of the pepper and varies from 2 to 7.4% among the cultivars with the volatile levels being within the range 0.4–7.0% (Ravindran, 2001).

1.5. *Streblus asper* Lour (Moraceae)

S. asper is a small tree which is indigenous to tropical countries such as India, Sri Lanka, Southern China, Malaysia, the Philippines and Thailand. It is known by various names, e.g. Bar-inka, Berrikka, Rudi, Sheora, Koi, Siamese rough bush and Tooth brush tree (Glasby, 1991). It has been used for several pharmaceutical purposes. For example its bark extract is used for relief of fever, dysentery, toothache and gingivitis (Gaitonde *et al*., 1964). The branch has been used as a toothbrush for strengthening teeth and gums (Lewis, 1980). The seeds are reported to be beneficial in epistaxis and diarrhea. The twigs have been used as a 'toothbrush' for strengthening teeth and gums (Lewis, 1980). The root has been applied to unhealthy ulcers, sinuses, epilepsy and inflammation and locally as an antidote to snake bite.

The milky juice has been used as antiseptic and astringent for chapped hands and cracked heels (Mukherjee and Roy, 1983). Stem bark has been reported to be effective against lymphoderma, chyluria and other manifestations of filariasis. Two glycosides, asperoside and strebloside were found to possess promising microfilaricidal activity (Chatterjee *et al*., 1992). The leaf extract has demonstrated insecticidal activity towards mosquito larvae (Kritsaneepaiboon, 1989), and antimicrobial activity (Hashim and Devi, 2003). Root has been found to be useful in epilepsy and inflammation (Nadkarni, 1976). Stem bark has been reported to be effective against lymphoderma, chyluria and other manifestations of filariasis (Hashim and Devi, 2003), and anti-malarial of an extract of the plant *S. asper* in murine malaria (Das and Beuria, 1991).

1.6 *Tinospora crispa* Linn (Menispermaceae)

T. crispa has been used in many Thai traditional medicines. It is one of ingredients in Thai folk medicine for health promotion. Stems of *T. crispa* are drunk as a vermifuge and a decoction of the whole plant is used to treat cholera, diabetes (Burkill, 1966; Perry, 1980; Umi Kalsom *et al*.,1999), fever, jaundice, cholera, malaria and against worms in children (Umi Kalsom *et al*., 1999). The roots have been used to treat rheumatism (Rahman *et al*., 1999). Leaves can be applied to wounds and also made into a poultice for treatment of itching (Burkill, 1966; Quisumbing, 1978). The stem has been registered in the Thai Pharmacopoeia, and commonly used in hospital to treat type II diabetes (Noor and Ashcroft, 1998). The chemical constituents in *T. crispa* have been reported such as borapetol A, borapetol B, borapetoside A, borapetoside B, tinocrisposide, N-formylanondine, N-

formylnornuciferine, N-acetyl nornuciferine, γ-sitosterol, picrotein and tinotubride (Pathak *et al.*, 1995).

Summary of chemical constituents of Thai herbs in the longevity herbal preparation used in this study is shown in Table 1.

Table 1 Chemical constituents of Thai herbs in the longevity herbal preparation

Plants	Part-used	Active compounds	Activities	References
A. procera	Stem bark	Triterpenoid saponins		
		3-*O*-[β-D-xylopyranosyl-(1 → 2)-α-L-arabinopyranosyl-(1 → 6)-2-acetamido-2-deoxy-β-D-glucopyranosyl] echinocystic acid	n/a	Melek *et al*., 2007
		3-*O*-[α-L-arabinopyranosyl-(1 → 2)-β-D-fucopyranosyl-(1 → 6)-2-acetamido-2-deoxy-β-D-glucopyranosyl] echinocystic acid	n/a	Melek *et al*., 2007
		3-*O*-[β-D-xylopyranosyl-(1 → 2)-α-L arabinopyranosyl-(1 → 6)-2-acetamido-2-deoxy-β-D-glucopyranosyl]acacic acid lactone	n/a	Melek *et al*., 2007
C. rotundus	Bulb	Essential oil		
		Cyperone	n/a	Tam *et al*., 2007
		Cyperene	n/a	Tam *et al*., 2007
		Patchoulenone	Antimalarial (against *Plasmodium falciparum*)	Thebtaranonth *et al*., 1995;
		β-selinene	Antimicrobial, antibacterial and weak antioxidant activity	Juteau *et al*., 2002;
		Volatile oil		
		α-copaene	n/a	Tam *et al.,* 2007
		β-cyperone	n/a	Tam *et al*., 2007
		Tannin alkaloids	n/a	Raut and Gaikwad, 2006
		Glycosides	n/a	Raut and Gaikwad, 2006
		Saponins	n/a	Raut and Gaikwad, 2006
		Flavonoids	n/a	Raut and Gaikwad, 2006
		Phenolic compound		
		Gallic acid	Antioxidant	Kim *et al*., 2007
		p-coumaric acid	Antioxidant	Zang et al., 2000; Kim *et al*., 2007;

n/a = not applicable

Plants	Part-used	Active compounds	Activities	References
C. rotundus	Bulb	Epicatechin	Neuroprotection	Ban *et al*., 2006
			Anti-cancer and anti-angiogenic effects	Matsubara *et al*., 2007
			Antimutagenic and antioxidant activities	Geetha *et al*., 2004
			Inhibit lipid peroxidation	Kondo *et al*., 1996
			Immunoregulatory effects	Sanbongi *et al*., 1997
			Suppress peroxynitrite induced nitration of tyrosine	Pannala *et al*., 1997
			Protect fibroblasts	Spencer *et al*., 2001;
			Striatal neurons against apoptosis induced by oxidative stress.	Schroeter *et al*., 2000
		Sesquiterpenes		
		α-cyperone	Antimalarial activity	Thebtaranonth *et al*., 1995
			Insecticidal activity	Morimoto *et al*., 1999
D. rhodocalyx	Stem bark	Betulin	Antilithic	Vidya and Varalakshmi, 2000
			Anti-tumor agents	Akihisa *et al*., 2002; Kvasnica *et al*., 2005
			Antiviral properties	Pavlova *et al*., 2003
			Antitumor activity against the Walker Carcinoma–256 tumor system	Recio *et al*., 1995
		Betulinic acid	Anti-inflammatory inhibitory effects on Epstein-Barr virus early antigen (EBV-EA) activation and Anti-tumor activity	Akihisa *et al*., 2002
			Anticancer	Kvasnica *et al*., 2005
			Anthelmintic activity	Enwerem *et al*., 2001

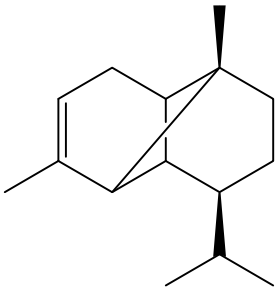
Plants	Part-used	Active compounds	Activities	References
D. rhodocalyx	Stem bark	Betulinic acid	Anti-HIV	Hashimoto *et al*., 1997; Fujioka *et al*., 1994; Li *et al*., 2003; Sun *et al*., 1998
			Antimalarial	Steele *et al*., 1999; Bringmann *et al*., 1997
			Antitumor activity against the Walker Carcinoma–256 tumor system	Recio *et al*., 1995
		Lupenone	n/a	Sutthivaiyakit *et al*., 1995
		Lupeol	Anti-urolithiatic	Vidya and Varalakshmi, 2000
			Anti-inflammatory	Geetha and Varalakshmi, 1998
			Antitumor activity	Recio *et al*., 1995 Saleem *et al*., 2001
			Antiarthritic	Kweifio-Okio *et al*., 1995; Geetha *et al*., 1998
			Antimutagenic	Guevara *et al*., 1996
			Antihepatotoxic, antioxidant and antitumor activities	Nagaraj *et al*., 2000, Saleem *et al*., 2001
			Hepatoprotective	Sunitha *et al*., 2001
		Stigmasterol	n/a	Sutthivaiyakit *et al*., 1995
		Taraxerol	Anti-inflammatory	Singh *et al*., 2002; Gupta *et al*., 1969
		Taraxerol acetate	n/a	Sutthivaiyakit *et al*., 1995
		Taraxerone	n/a	Sutthivaiyakit *et al*., 1995
		β-sitosterol	Antipyretic, anti-inflammatory, and analgesic activity	Erazo *et al*., 2006; Gupta *et al*., 1980
			Anti-edematogenic	Delaporte *et al*., 2004
			Myeloperoxidase activity	G´omez *et al*., 1999
P. nigrum	Fruit	Piperine	Antihyperlipidemia activity	Gereltu *et al*., 2004
			Gastric emptying inhibited activity	Bajad *et al*., 2001
			Anti-inflammatory	Mujumdar *et al*., 1990

n/a = not applicable

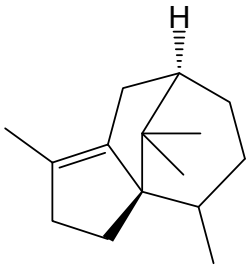
Plants	Part-used	Active compounds	Activities	References
P. nigrum	Fruit	Piperine	Analgesic, antipyretic and antifeedant activities	Miyakado *et al*., 1979
			Antiasthmatic property	
			Antifertility	
		Chavicine	n/a	Clause *et al*., 1973
		Piperidine	Insecticidal	Bandara *et al*., 2000
			Antidepressant activity	Ahmad *et al*., 1997
		Piperic acid	n/a	Clause *et al*., 1973
		Volatile oil		
		α-pinene	Antimicrobial activity	Dorman and Deans, 2000
			Anti-inflammatory and analgesic activity	Erazo *et al*., 2006
		β-pinene	Anti-inflammatory and analgesic activity	Erazo *et al*., 2006
		α-phellandrene	n/a	Youngken, 1950
T. crispa	Stem bark	N-trans-feruloyltyramine	Stimulatory effect on insulin secretion	Tomohiro *et al*., 2007
			Antioxidant, radical scavenging properties towards DPPH radical	Dweck and Cavin, 2004
		N-cis-feruloyltyramine	Antioxidant, radical scavenging properties towards DPPH radical	Dweck and Cavin, 2004
		Tinotuberide	n/a	Dweck and Cavin, 2004
		Phytosterol	Decrease LDL cholesterol	NCEP, 2001
			Antiatherogenic, reduction of proinflammatory cytokine production	Nashed *et al*., 2005
			Decreased prostaglandin release and modulated leukocyte function in murine models of inflammation	Navarro *et al*., 2001; Park *et al*., 2001
		Cycloeucalenol	n/a	Dweck and Cavin, 2004

Plants	Part-used	Active compounds	Activities	References
T. crispa	Stem bark	Cycloeucalenone	n/a	Dweck and Cavin, 2004
		Picroretin	n/a	Dweck and Cavin, 2004
		Tinosporine	n/a	Dweck and Cavin, 2004
		Picroretin	n/a	Dweck and Cavin, 2004

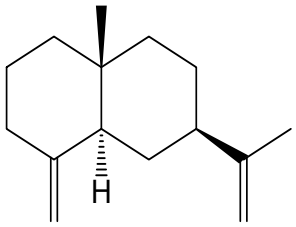
n/a = not applicable



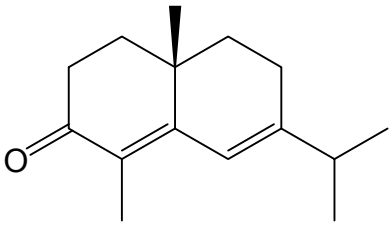
(a) α-copaene



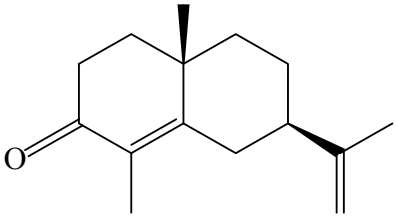
(b) cyperene



(c) β-selinene

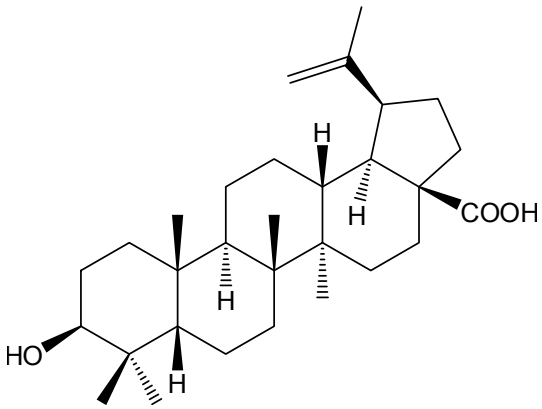


(d) β-cyperone

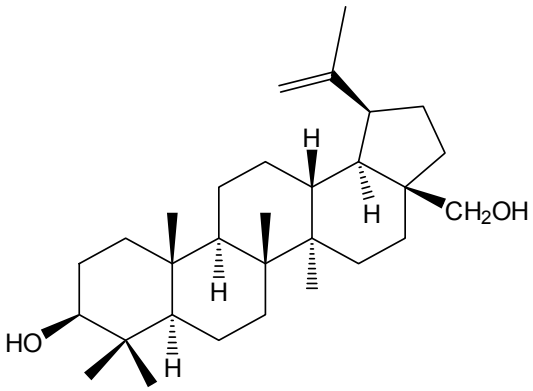


(e) α-cyperone

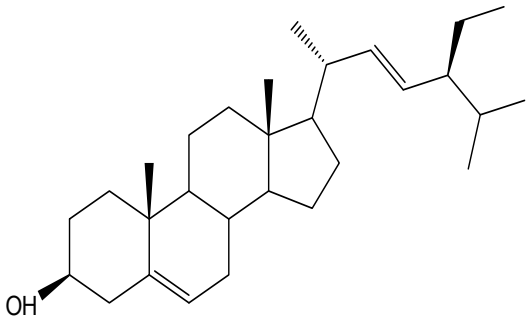
Figure 1 Structures of five identified compounds in *C. rotundus* (Tam *et al.*, 2007)



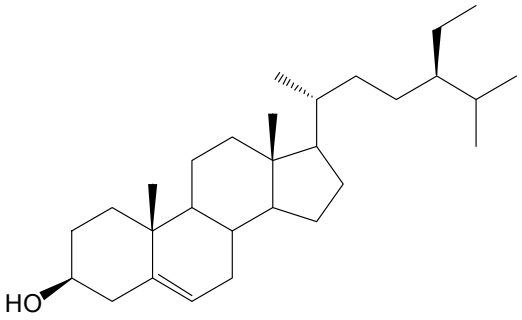
(a) Betulinic acid



(b) Betulin

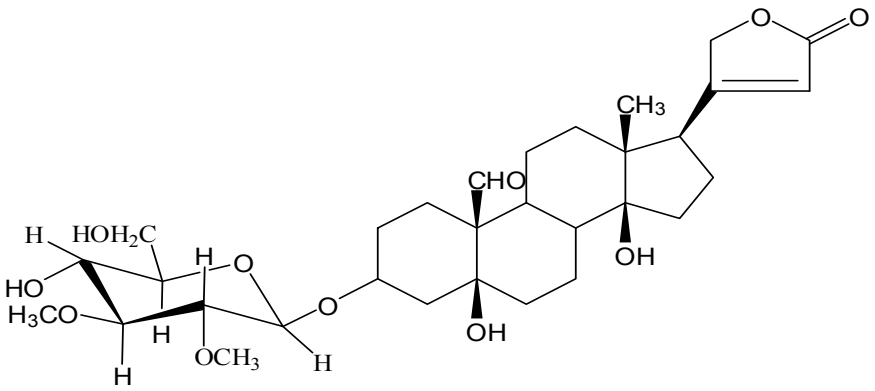


(c) Stigmasterol

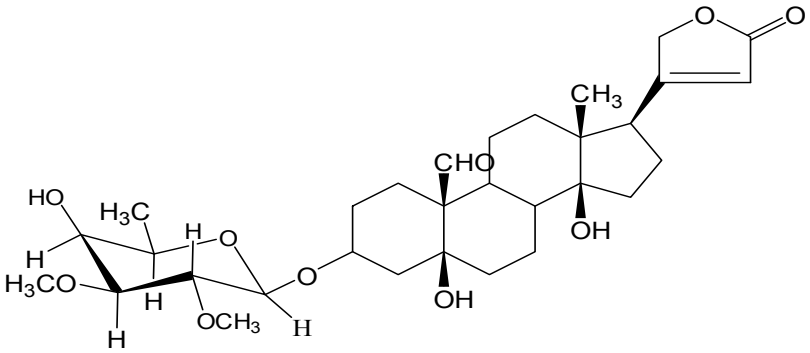


(d) β-Sitosterol

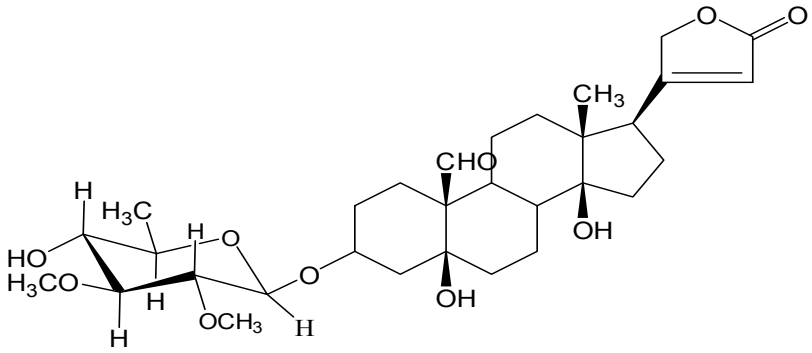
Figure 2 Structures of biologically active compounds in *D. rhodocalyx* (Kuo *et al*., 1997; Kvasnica *et al*., 2005)



(a) Asperoside

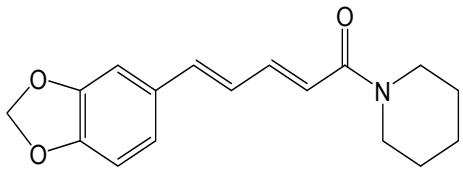


(b) Strebloside



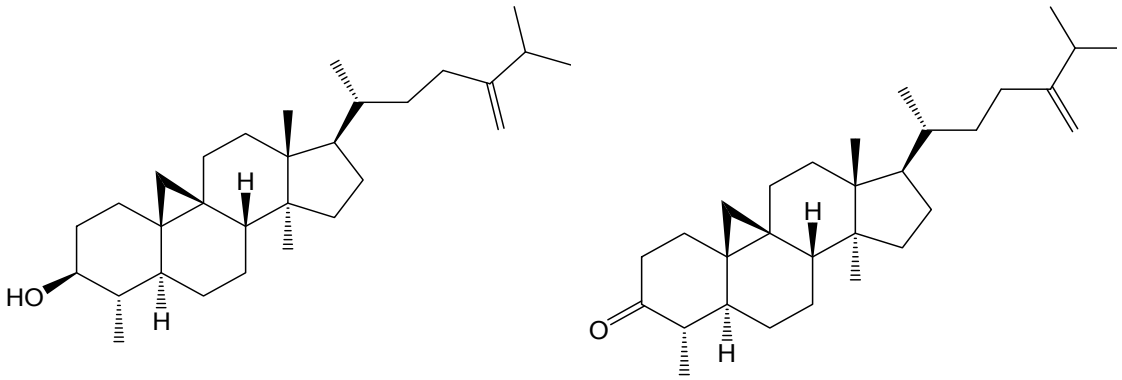
(c) Pansonin

Figure 3 Structures of biologically active compounds in *S. asper*. (Rastogi *et al*., 2006)



Piperine

Figure 4 Structure of piperine, major compound of *Piper* spp. (Siddiqui *et al*., 1997)



(a) Cyclocucalenol (1) (b) Cyclocucalenone (2)

Figure 5 Structures of cyclocucalenol and cyclocucalenone from dried stems of *T. crispa* (Kongkathip *et al*., 2002)

2. Osteogenic activity

2.1 Osteogenic cells

The matrix of bone is uniquely elaborated by populations of osteogenic cells derived from mesenchymal, or stromal, stem cells that differentiate into fully secretorily active mature osteoblasts. Like other matrix-product in connective tissue cells, bone cells are derived from so-called mesenchymal stem cells (MSC). The critical issue is that the proliferative capacity of these undifferentiated cells provides the opportunity to create large autologous cell populations by expansion, *in vitro*, which a number of cells harvested from the host. MSCs can differentiate into a number of connective tissue phenotypes include bone and muscle (da Silva Meirelles *et al*., 2006; Bochev *et al*., 2008).

Two strategies can be employed to win MSC-mediated tissue repair. First, MSC populations are expanded, in an assumed undifferentiated state and the presence of local environmental, *in vivo*. The MSCs will differentiate into osteogenic cells, which will be responsible for tissue regeneration process. Second, culture-expanded, MSCs are directed *ex vivo* into the osteogenic lineage prior to implantation, to accelerate the regeneration of bone. In fact, it has been shown that the cells responsible for bone nodule formation arise from single colonies (Atala and Lanza, 2002).

Thus, both the expansion phase of each strategy and the proliferative capacity of these cells are important for tissue engineering. It has been estimated that

less than 1% of the stromal layer of rat and mouse marrow cells is osteogenic (Atala and Lanza, 2002). Intracellular markers of osteogenic differentiation include alkaline phosphatase, osteocalcin and collagen.

2.2 Factors induced bone formation

Bone forming osteoblasts arise from mesenchymal stem cell precursors and undergo differentiation in response to a number of factors including the bone morphogenetic proteins (BMPs), transforming growth factor-β (TGF-β), and glucocorticoids (Spelsberg *et al*., 1999) or hormones; estrogen, parathyroid hormone (Yamaguchi, 2000). Among them, insulin-like growth factor (IGF) and transforming growth factor-β (TGF-β) are the important targets of glucocorticoid in bone formation (Iu *et al.*, 2005). TGF-β is produced by osteoblasts and appears to regulate bone metabolism in various ways, including skeletal development and bone remodeling (Sowa *et al*., 2002). It modulates the proliferation, differentiation and production of bone matrix proteins of osteoblasts (Centrella *et al.*, 1994). TGF-β also induces bone formation when locally administered into bone tissues in rats (Noda and Camilliere, 1989).

Bone morphogenetic proteins (BMPs) promote osteoinduction both *in vivo* and *ex vivo* (Biase and Capanna, 2005) and stimulate osteoblast functional markers, including ALP activity, osteocalcin (OC) secretion, and type I collagen synthesis *in vitro*. BMPs have been revealed as the most potent inducers and stimulators of osteoblast differentiation (Yamaguchi, 2000).

It was reported that rat bone marrow stromal cell supplemented with 10^{-5} M dexamethasone or BMP-2 produced a mixed population of cells with low ALP and high ALP levels (Rickard *et al*., 1994), while the combination of dexamethasone and BMP-2 yielded very few dim cells with higher ALP levels compared with either inducer alone or the interactions between dexamethasone and 1, 25-Dihydroxyvitamin D3 by treatment on normal human osteoblast like cells (Subramaniam *et al*., 1992). Other factors affected the cell differentiation are serial passage, passage number, source of serum, and plating density (Franceschi and Iyer, 1992)

2.3 Supplementation of osteogenic differentiation

Dexamethasone is essential for osteogenic differentiation of mesenchymal stem cells (Abe *et al*., 2000; Gundle *et* al., 1995; Maniatopoulos *et* al., 1988). It promotes osteoprogenitor cells in an early state of osteoblastic differentiation into osteoblast-like cells (Abe *et al*., 2000). Bone formation were stimulated by dexamethasone as concentration-dependent (range from 10^{-9} M to 10^{-7} M) of marrow stromal populations, *in vitro* (Atala and Lanza, 2002). The stimulation was maximum at 1 μM of dexamethasone in human bone marrow-derived mesenchymal cells (hBMMCs), while a concentration as low as 1 nM was effective (Cheng *et al*., 1994). In addition, ascorbic acid promotes osteogenic differentiation of undifferentiated cells (stromal cell line ST2) and osteoblast precursor cells (MC3T3 mouse calvaria-derived cell line) by inducing a formation of type I collagen matrix, which is essential for the induction of osteoblast differentiation (Otsuka *et al*., 1999). This study, supplementation of 10^{-6} M dexamethasone and ascorbic acid in culture medium of

MC3T3-E1 pre-osteoblastic cells measured ALP activity, to ensure the earliest and maximum osteogenic differentiation of bone cells.

2.4 Biocompatibility determination

Biocompatibility is determined by a number of cells or cell viability using MTT assay or SRB assay. Based on MTT reduction by mitochondrial dehydrogenases and to a lower extent by cytosolic enzymes, MTT assay is widely used to reliably gather quantitative data on cells surviving toxic insults (Monti *et al*., 2007).

2.5 Determination of ALP activity

Alkaline phosphatase (ALP) is a glycoprotein associated with the formation of calcified tissues. It catalyzes the hydrolysis of phosphate esters at an alkaline pH (Harris 1989). In humans, ALP occurs thoroughly and three isoenzymes, the tissue nonspecific, intestinal and placental have been identified (Sabokbar *et al*., 1994). The tissue non-specific isoenzyme occurs in three forms that they were derived from bone, kidney and liver. ALP is isoenzyme of bone and liver which can be useful in diagnosis of osteoblastic bone and cholestatic hepatic disorders, respectively (Epstein *et al*., 1985).

On a cellular level, ALP is the most widely recognized marker of osteoblast phenotypes (Rodan and Rodan, 1983; Sabokbar *et al*., 1994). ALP activity is strongly associated with the membrane and the matrix vesicles (Ecarot-Charrier *et*

al., 1988; Pizauro *et al*., 1992). Thus, also in cell cultures, ALP seems to be closely linked to the mineralization process (Ecarot-Charrier *et al*., 1988). Expression of ALP activity was measured by determination of osteoblastic markers during the proliferative state (Franceschi and Iyer, 1992; Lecoeur and Ouhayoun, 1997; Rickard *et al*., 1994). This enzyme begins immediately following the cessation of cell proliferation and reaches a maximum level during the phase of matrix maturation (Risteli and Risteli, 1993; Stein *et al*., 1990). ALP plays a major in the progression of mineralization (Woltgens *et al*., 1982).

2.6 Determination of total protein

The BCA protein assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. The BCA method is not a true end-point method, i.e. the final color continues to develop. However, following incubation, the rate of continued color development is sufficiently slow to allow large numbers of samples to be assayed together.

The macromolecular structure of protein, the number of peptide bonds and the presence of four particular amino acids (cysteine, cystine, tryptophan and tyrosine) are reported to be responsible for color formation with BCA (Wiechelman *et al*., 1988). The extent of color formation caused by more than the only sum of individual color producing functional groups of di-, tri-, and tetrapeptides (Wiechelman *et al*., 1988). Accordingly, protein concentrations are generally calculated and reported with reference to standards of a common protein such as

bovine serum albumin (BSA). Each value was normalized to the protein concentration.

3. Allergic activity

3.1 Allergy

Allergy is an immunological reaction to a foreign antigen (allergen) such as dust mites, pollen, cosmetics, food and mold spores that causes tissue inflammation and organ dysfunction (Nakatani *et al.*, 2002). Allergic reactions of types I, II and III are antibody mediated, and that of type IV is T-cell mediated. Type I allergy is induced antigens and the production of antigen-specific IgE antibodies that bind to receptors on mast cells or basophils (Matsuda *et al*., 2004b). The allergic mediators induce various physiological effects that cause allergic diseases (Lee *et al.*, 2004). The mediators are released from mast cells and basophils such as histamine and serotonin within minutes, the early phase reaction. These mediators induce vasodilation, mucous secretion and bronchoconstriction (Matsuda *et al*., 2004a,b)

Allergy diseases are characterized of T_H2-based immune responsed to allergens. Allergen-specific T_H2 cells are key players in allergic immune reactions, initiating and propagating inflammation through release of a number of T_H2 cytokines, such as IL-4 (Wijk and Knippels, 2007;Gutermuth *et al*., 2007). Some studies suggest allergen-dependent mechanisms evaluated at the dendritic cell (DC) level because of particular attributes of the specific protein. Other studies suggest T cell-dependent or individual factors leading to a predominance of the T_H2 response

(Gutermuth *et al.*, 2007). Protein fragments are processed by antigen-presenting cells (APCs) and displayed on their surface in association with major histocompatibility complex (MHC) class II molecules that can be recognized by a specific T cell receptor. In the presence of interleukin (IL-4), allergic sensitization is subsequently initiated by the differentiation of antigen-specific T helper (T_H) cells into effector T_H2 cells. T_H2 cells produce a cocktail of cytokines such as IL-4 encourage B cells to develop into IgE-producing plasma cells. Secreted antigen-specific IgE antibodies are distributed systemically and bind to the high-affinity receptor FcεRI on mast cells and basophils (Gutermuth *et al*., 2007). Mechanism of type 1 hypersensitivity reaction (Allergic reaction) as shown in Figure 6.

3.2 Mast cells

Rat basophilic leukemia (RBL-2H3) cells are mucosal-type mast cells. The 2H3 cells contain several hundred thousand IgE receptors on the membrane surface after that sensitization with monoclonal IgE. The cells respond to antigen and release histamine (Ikawati *et al*., 2001) that is major model for study of IgE-mediated degranulation (Yamashita *et al*., 2000).

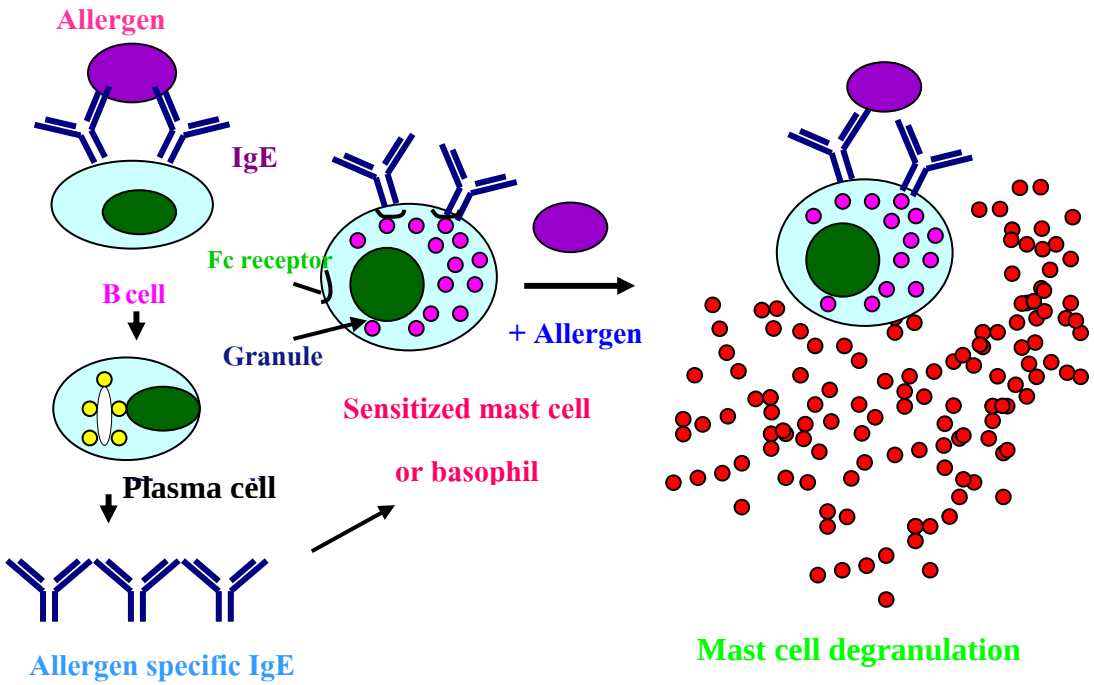


Figure 6 Mechanism of type 1 hypersensitivity reaction (Allergic reaction)

CHAPTER 2

MATERIALS AND METHODS

Plant materials

Six dried herbs commonly used in Thai longevity preparation were determined for osteogenic activity and anti-allergic effect. The selected herbs were *Albizia procera* (stem bark), *Cyperus rotundus* (bulb), *Diospyros rhodocalyx* (stem bark), *Piper nigrum* (fruit), *Streblus asper* (seed) and *Tinospora crispa* (stem bark). These plant materials were bought from Thai traditional drug store, Hat-Yai, Songkhla, Thailand in January 2006.

Chemicals and reagents

1. MTT assay

Thiazolyl blue tetrazolium bromide (Sigma-Aldrich, MO, USA)

Dimethyl sulfoxide (Sigma-Aldrich, MO, USA)

2. Alkaline phosphatase activity

Triton X-100; *t*-Octylphenoxypoly-ethoxyethanol

(Sigma-Aldrich, MO, USA)

Magnesium chloride hexahydrate (Ajax Finechem, Australia)

Glycine (Ajax Finechem, Australia)

p-Nitrophenyl phosphate disodium salt hexahydrate (Fluka, Austria)

p-Nitrophenol (Sigma-Aldrich, MO, USA)

Sodium hydroxide (Fluka, China)

Dexamethasone (Fluka, China)

3. BCA protein

Bicinchoninic acid, BCA (Sigma-Aldrich, MO, USA)

Copper (II) sulfate solution (Sigma-Aldrich, MO, USA)

Protein standards, Micro standard, Liquid (Sigma-Aldrich, MO, USA)

4. β-hexosaminidase release

Anti-DNP IgE, Monoclonal anti-DNP (Sigma-Aldrich, MO, USA)

Dinitrophenylated bovine albumin, DNP-BSA (Sigma-Aldrich, MO, USA)

Sodium chloride, Potassium chloride, Magnesium chloride, Sodium hydroxide, Calcium chloride, Bovine serum albumin (BSA) and piperazine-N,N′-bis (2-ethanesulfonic acid) (PIPES), (Wako)

5. MC3T3-E1 cell culture

α-Modified eagle's minimum essential medium; α-MEM

(Biochrom KG seromed®, Germany)

10% Bovine fetal serum; FBS (Gibco™, UK)

100 U/ml Penicillin (Gibco™, Germany)

6. RBL-2H3 cell culture

Minimum essential medium eagle; MEM

(Sigma-Aldrich, MO, USA)

Fetal calf serum, FCS (Sigma-Aldrich, MO, USA)

Penicillin-streptomycin (Gibco™, Germany)

Instrument

Instrument	Model	Company
Laminar flow	Faster Ultrasafe 48	44100 Ferrara, Italy
Microplate reader	Power Wave X	Bio-TEK Instruments Inc.
Freeze dryer	Eyela	Tokyo Rikakikai CO., LTD.
Vacuum rotary evaporator	Eyela	Tokyo Rikakikai CO., LTD.
Hot air oven	DIN 12880-KI	Memmert, Germany
pH meter	PHM 82	Radiometer, Denmark
Water bath	WB-14	Memmert, Germany
Centrifuge	Hermle z323k	Hermle Labortechnic
Refrigerator (-20^{o}C, -80^{o}C)		Germany

Methods

1. Preparation of crude extract

The dried materials (10 g) were refluxed with water or 95% ethanol for 3 h. The water extracts and ethanolic extracts were filtered through cotton and then dried using rotary evaporator. The yields of the crude extracts (calculated on the dried weight) were shown in Table 5. The extracts were kept in tight glass containers in a refrigerator at −20 °C for futher studies.

2. Cytotoxicity using MTT assay

Mitochondrial function was measured by the ability of viable cells to convert soluble 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into an insoluble dark blue formazan reaction product.

Crude extracts were dissolved in PBS to obtain a stock solution (1 mg/ml). The stock solutions were then diluted at four dilutions (3, 10, 30 and 100 μg/ml).

MC3T3-E1 cells (1×10^4 cells/well) were cultured in 96-well plates in a humidified atmosphere with 5%CO_2 at 37^{o}C. After 24 h the media was aspirated and replaced with 90 μl of fresh media and 10 μl of crude extract solutions. After incubation for 24 h, 10 μl of 5 mg/ml MTT solution (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenylterazolium bromide) were added. After 4 h, the MTT solution was replaced with 100 μl of dimethyl sulfoxide (DMSO) and then agitated to dissolve the blue formazan salts. The absorbance at 570 nm of the solutions in 96-well plate was determined using a BIO-TEK microplate reader. The relative activity of MC3T3-E1 cells for each sample solution was expressed as the average percentage cell viability (WU *et al*., 2003; Li *et al*., 2005; Trentz *et al*., 2003; Cerroni *et al*., 2002; Xu and Simon Jr, 2005). Cytotoxicity was assumed when cell viability was lower than 80% (Gomes *et al*., 2001; Melendez *et al*., 2004). Percentage of cell viability was calculated as the following equation:

$$\% \text{ Viability} = [A_{sample} / A_{control}] \times 100$$

A_{sample} = Absorbance of sample with crude extract solution

$A_{control}$ = Absorbance of sample without crude extract solution

3. Determination of total protein content

This method combines the well-known reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (the biuret reaction) with the highly sensitive and the selective colorimetric detection of the cuprous cation (Cu^{+1}) using a unique reagent containing bicinchoninic acid, BCA (Smith *et* al., 1985). The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion.

Table 2 Preparation of protein standard solutions.

Protein stock 1 mg/ml	Lysis buffer (µl)	Final concentration (mg/ml)
200	0	1.0
160	40	0.8
120	80	0.6
80	120	0.4
40	160	0.2
0	200	0.0

The cell lysates were stored at −20°C. Protein quantitation (25 µl) was performed with BCA protein assay reagent (200 µl) (Sigma-Aldrich, MO, USA). The samples were mixed with the working reagent at the ratio of 1:8 (v/v), and then incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air for 30 min to allow color formation. The absorbance of the samples at 562 nm was measured. The total protein was calculated based on the standard curve and expressed as mg/ml. These data were normalized by the number of cells counted at day 1, 3, 7 and 9. Then,

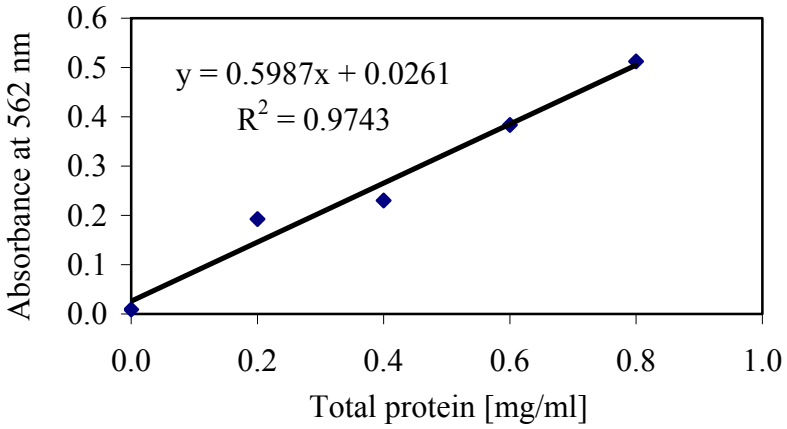
the absolute ALP (mU/ml) of crude extracts by the test samples was calculated by the following equation:

$$\text{Total Protein (X)} = [A_{Sample} - A_{Blank}] = Y_i \text{ (mg/ml)}$$

Where;

Yi = Y

X = Data was calculated from standard curve



ALP (mU/mg of protein) was calculated by the following equation:

$$A = [B / X] \times 1000$$

where;

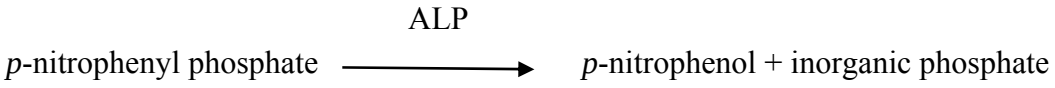
A = absolute alkaline phosphatase activity (mU/mg of protein)

B = alkaline phosphatase activity (U/ml)

X = total protein calculated from a standard curve (mg/ml)

4. Measurement of alkaline phosphatase (ALP) activity

This measurement is based on the basic that the alkaline phosphatase (ALP) enzyme catalyses the hydrolysis of monophosphates at an alkaline pH. ALP presenting in the sample catalyses the hydrolysis of *p*-nitrophenyl phosphate (substrate) and *p*-nitrophenol and phosphate are released. The increase in absorbance at 405 nm is correlated with the enzyme activity.



ALP activity was calculated from a standard curve. Results were expressed as mU/ml *p*-nitrophenol normalized by total protein content. The number of cells excludes the effect of proliferation, so the observed difference could resulted from both a higher differentiated number of cells and a higher cellular activity (Rosa and Beloti, 2003)

Table 3 Standard curve of *p*-nitrophenol

1 mM *p*-nitrophenol (ml) in lysis buffer solution	Lysis buffer solution (ml)	Final *p*-nitrophenol concentration (μg/ml)
0.0	1.0	0.0
0.05	0.95	0.0167
0.2	0.8	0.0667
0.6	0.4	0.2
0.9	0.1	0.3

The crude extracts were dissolved in DMSO to obtain stock solutions at 10 mg/ml. The stock solutions were further diluted 3, 10, 30 and 100 μg/ml as the final concentrations.

MC3T3-E1 cells ($1x10^5$ cells/ml in α-MEM) were cultured in 24-well plate for 24 h. The cells were conditioned with 900 μl of fresh media (900 μl) and 100 μl of crude extract solutions. The final concentrations of the extracts in the condition media were 3, 10, 30 and 100 μg/ml, respectively. After 24 h, the cells were lysed with 200 μl of 0.1% Triton X-100 under freezing and thawing at −80 °C for 1.5 h. The cell lysates were then centrifuged at 25,000 rpm for 10 min at 4°C. ALP activity was determined using a colorimetric method. The supernatant (50 μl) was transferred into 96-well plate and incubated with 50 μl of substrate (*p*-nitrophenyl phosphate) in substrate buffer solution for 60 min at 37°C. The reaction was stopped with 50 μl of 0.2N NaOH. The absorbance was measured using microplate reader at 405 nm. ALP activity was calculated from standard curve of *p*-nitrophenol. Each value was normalized with the total protein content (Mouries *et al*., 2002). The ALP (U/ml) was calculated by the following equation:

$$\text{Alkaline phosphatase activity (U/ml)} = [(A/B)x\ (C/D]$$
$$= [(A/\ 60)\ x\ (200\ /\ 50)]$$
$$= [(A/15)]$$

Where; A = Data calculated from standard curve

B = Times (60 min)

C = Volume of lysis buffer (200 µl)

D = Volume of supernatant (50 µl)

5. Examination of β-hexosaminidase release from RBL-2H3 cells

This examination based on the colorimetric assay of the mediators released from tha degranulated mast cells and basophils after binding of antigen-specific IgE antibodies to receptors on mast cells or basophils. The colorimetric assay is to determine *p*-nitrophenol derived from *p*-nitrophenyl-N-acetyl- β-D-glucosaminide (Ozaka *et al*., 1993; Lee *et al*., 2004). The results are expressed as the percentage of intracellular β-hexosaminidase released into the medium.

Crude extracts were dissolved in DMSO to obtain a stock solution (10 mg/ml). The test samples were prepared by dilution of the stock solution in Siraganian buffer (buffer A) to 3, 10, 30, and 100 µg/ml, respectively (Matsuda *et al*., 2004 a,b). Briefly, RBL-2H3 cells ($5x10^5$ cells/ml) cultured in 24-well plates in culture media (MEM) were sensitized with anti-DNP IgE (0.45 µg/ml). The cells were washed with Siraganian buffer (buffer A) supplemented with glucose, 1 mM $CaCl_2$, and 0.1% bovine serum albumin (BSA) as incubation buffer, and then incubated in 160 µl of the incubation buffer at 37°C. After incubation for 10 min, 20 µl of the test sample solution was added to each well and incubated for 10 min, followed by an addition of 20 µl of antigen (DNP-BSA of which the final concentration was 10 µg/ml) for 20

min at 37°C to stimulate the cells to degranulate. The reaction was stopped by cooling the samples in an ice bath for 10 min. The supernatant (50 µl) was transferred into 96-well plate and incubated with 50 µl of substrate (1 mM *p*-nitrophenyl-N-acetyl-β-D-glucosaminide) in 0.1 M citrate buffer, pH 4.5 at 37 °C for 2.5 h. The reaction was stopped by adding 200 µl of stop solution (0.1 M Na_2CO_3/$NaHCO_3$, pH 10.0). The absorbance was measured using a microplate reader at 405 nm. The test sample was dissolved in dimethylsulfoxide (DMSO). The solution was added to incubation buffer (final DMSO conc. was 0.1%). The percent inhibition of the release of β-hexosaminidase by the test samples was calculated by the following equation, and IC_{50} values were determined graphically:

$$\text{Inhibition (\%)} = [1 - (T - B - N) / (C - N)] \times 100$$

where; Normal (N) = DNP-BSA (–), test sample(–)

Control (C) = DNP-BSA (+), test sample (–);

Test (T) = DNP-BSA (+), test sample (+);

Blank (B) = DNP-BSA (–), test sample (+)

6. Measurement of β-hexosaminidase inhibitory activity

This measurement is to clarify that the samples exhibite the potent anti-allergic activity by inhibiton of cell degranulation, but not a false positive result from the inhibition of β-hexosaminidase activity.

The cell suspension (5×10^6 cells) in 10 ml of PBS was sonicated. The suspension was then centrifuged and the supernatant was diluted with Siraganian buffer to adjuste the sample to equal the enzyme activity of the degranulation tested above. The enzyme solution (45 μl) and test sample solution (5 μl) were transferred into a 96-well microplate and incubated with 50 μl of the substrate solution at 37°C for 1 h. The reaction was stopped by adding 200 μl of the stop solution (0.1 M Na_2CO_3/$NaHCO_3$, pH 10.0). The absorbance was measured using a microplate reader at 405 nm.

Statistics

1. Evaluation of total protein and ALP activity

The results were shown as mean ± S.E.M. (n = 4). The difference between various test samples was evaluated by an analysis of variances statistic method. The post hoc test was performed using Scheffe test. The level of statisticance was defined as $*P < 0.05$ and $**P < 0.01$.

2. Statistical analysis of anti-allergic activity

The results were expressed as mean ± S.E.M. of four determinations at each concentration for each sample. The IC_{50} values were calculated using the Microsoft Excel program. Statistical significance was calculated by one-way analysis of variance (ANOVA), followed by Dunnett's test.

CHAPTER 3

RESULTS AND DISCUSSION

Part 1: Preparation of crude extracts

Dried materials were refluxed with water and 95% ethanol for 3 h. The yield of herbs in longevity herbal preparation was shown as percentage by weight in Table 4. The water and ethanol extracts were observed for bioactivities on osteogenic, anti-allergic and synergistic effects. Thai people traditional use folk medicine by boiling dried herbs in water or maceration in ethanol. The crude water and ethanolic extracts were obtained either in the forms of powder or sticky mass.

Table 4 Crude water and ethanolic extracts of six herbs in longevity herbal preparation

Botanical name	Water extract		Ethanolic extract	
	Code	[%] Yield (w/w)	Code	[%] Yield (w/w)
Albizia procera (Roxb.) Benth.	AP-W	18.2	AP-E	12.7
Cyperus rotundus Linn.	CR-W	11.3	CR-E	19.8
Diospyros rhodocalyx Kurz.	DR-W	11.7	DR-E	11.7
Piper nigrum Linn.	PN-W	8.80	PN-E	7.70
Streblus asper Lour.	SA-W	20.3	SA-E	4.80
Tinospora crispa Linn.	TC-W	12.9	TW-E	6.00
Longevity formulation	LF-W	18.7	LF-E	6.70

Part 2: Evaluation of osteogenic activity of crude extracts

2.1 Cytotoxicity of crude extracts

To investigate the cytotoxicity on MC3T3-E1 preosteoblastic cells of crude extracts in longevity herbal preparation at the concentrations of 3-100 μg/ml. Cell viability less than 80% and change in cell morphology are criteria of cytotoxicity (Gomes *et* al., 2001; Melendez *et* al., 2004). Effects of the crude extracts on the percentage of cell viability of MC3T3-E1 cells after 24 h are shown in Figures 7-8. The amount of formazan produced is proportional to the number of living cells present in cultures.

From Figure 7, the results indicated compatibility to the cells of all crude water extracts, except AP-W at 100 μg/ml (72.2% cell viability). From Figure 8, all crude ethanolic extracts were compatible to MC3T3-E1 cells at low concentrations (3-30 μg/ml). At high concentration (100 μg/ml), CR-E, DR-E, and SA-E were compatible to the cells, while cytotoxicity of the crude extracts was significant in AP-E (66.9% ± 10.2), TC-E (73.6% ± 5.3), and LF-E (76.0% ± 14.9), $p < 0.01$. Cytotoxicity of PN-E at 100 μg/ml was not significant, $p < 0.01$ (cell viability 79.3%± 7.6).

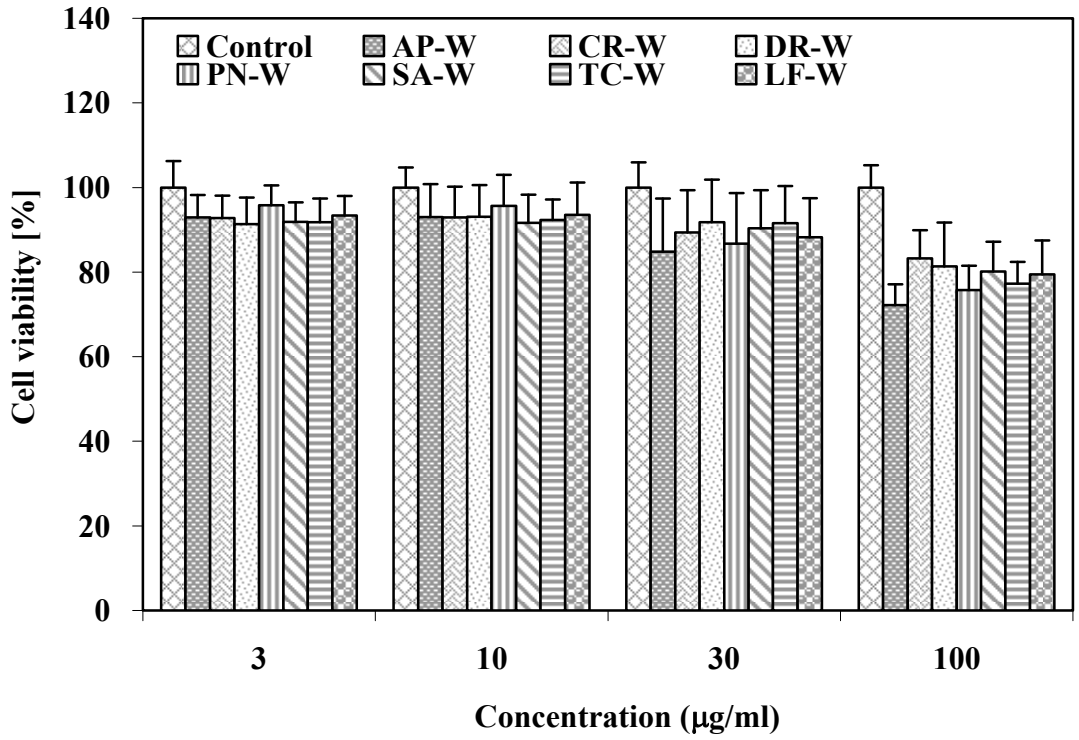


Figure 7 Effects of crude water extracts on cell viability. MC3T3-E1 cells were conditioned with the crude water extracts at 3, 10, 30 and 100 μg/ml for 24 h. Cell viability was determined by a colorimetric MTT assay. Data are expressed as percentage of control. Results are shown as the mean ± S.D. of duplicate (n = 8 in each replicate).

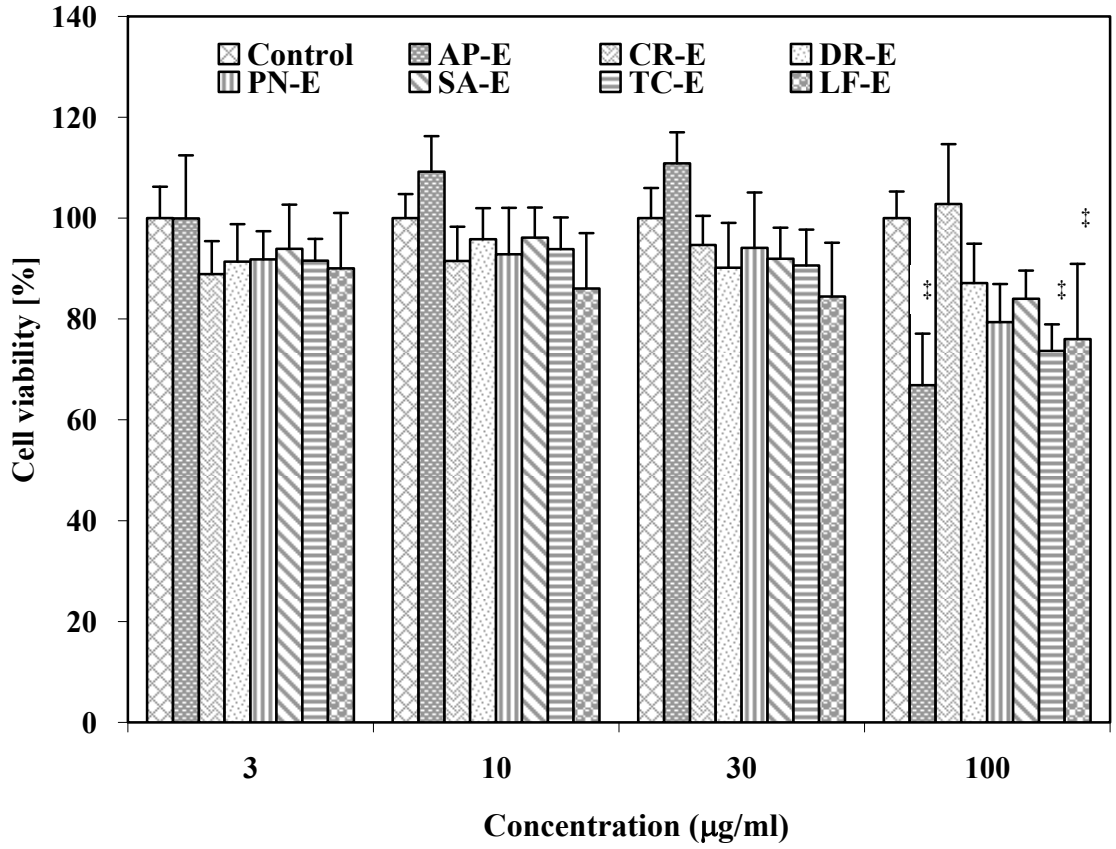


Figure 8 Effects of crude ethanolic extracts on cell viability. MC3T3-E1 cells were conditioned with the crude ethanolic extracts at 3, 10, 30 and 100 μg/ml for 24 h. Cell viability was determined by a colorimetric MTT assay. Data are expressed as percentage of control. Results are shown as the mean ± S.D. of duplicate (n = 8 in each replicate). $^{‡}p < 0.01$. Data are shown as the mean ± S.D. of duplicate (n = 8 in each replicate).

There have been a few reports on cytotoxicity of the herbs comprising in the longevity formulation. Piperine isolated from *P. nigrum* was reported as dose-dependent toxic to *Trypanosoma cruzi* on epimastigotes and amastigotes with IC_{50} of 7.36 and 4.91μM, respectively (Ribeiro *et* al., 2004). Cytotoxicity of AP-W and AP-E was observed in our study may be the results of saponins as reported by Melek and co-workers (Melek *et* al., 2007). Saponins derived from *Albizia procera***,** as echinocystic acid and triterpenoid saponins, were published for their cytotoxicity to HEPG2 cell line with IC_{50} 9.13 μg/ml and 10 μg/ml, respectively (Melek *et* al., 2007). Other compounds extracted from *Albizia procera* were found to exhibit cytotoxicity on cancer cells (Kaskiw *et al*., 2008). Diosgenyl saponins exerted antitumor effect by inducing apoptosis in human cancer cells. Terpenoids (lupeol and two diterpenes, diosphenol 2), isolated from *Spirostachys africana*, expressed IC_{50} value 300.9 and 308.9 μg/ml, respectively. Lupeol has been reported to have inhibitory activity on HUVEC tube formation while it did not affect the growth of tumor cell lines such as SK-MEL-2 and B16-F10 melanoma (You *et al*., 2003, Mathabe *et al*., 2008). Some authors (Chaturvedula *et al*., 2002; Lui *et al*., 2004; Mathabe *et al*., 2008) have reported weak cytotoxicity of lupeol against melano B16 cells ($IC_{50} \geq 100$ μg/ml) and human ovarian cancer cell lines.

2.2 Morphology of MC3T3-E1 cells

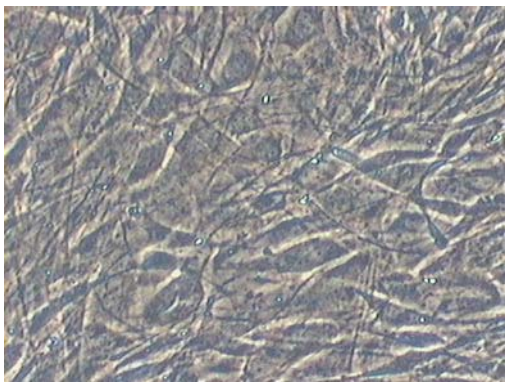
Figures 9-22 show morphology of MC3T3-E1 cells conditioned with crude extracts at 9 days. Determination of cell morphology was to ensure that the crude extracts did not affect cell characteristic (size and shape). Generally, ALP activity expressed after 2-3 days of treatment and reached a maximum at 7-14 days

(Cheng *et al*., 2007). Morphology of the cell monolayers was determined under optical microscope (magnification x100) at day 9 before determination of ALP activity.

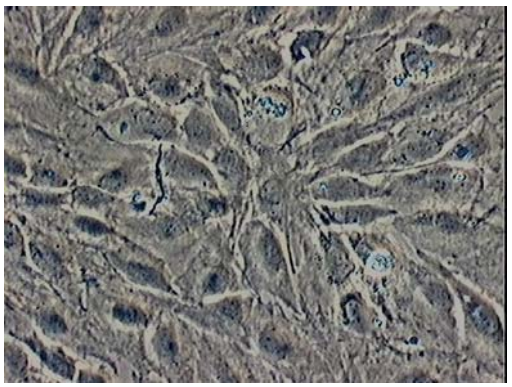
Figures 9-15 present effects of the crude water extracts on morphology at day 9 of MC3T3-E1 cells. Biocompatibility was observed in all crude water extracts, except AP-W at the concentration 3-100 μg/ml. Cytotoxicity was found in AP-W. From Figure 9, morphology change was observed in AP-W at the concentrations 30-100 μg/ml which related to the data from MTT assay of cell viability as discussed above.

Figure 16-22 show effects of the crude ethanolic extracts on morphology at day 9 of MC3T3-E1 cells. Biocompatibility was observed in CR-E, DR-E, and SA-E at the concentrations of 3-100 μg/ml. Other crude ethanolic extracts, i.e. PN-E, TC-E, and LF-E were compatible to the cells at low concentrations (3-30 μg/ml). AP-E was compatible at the concentration 3 μg/ml (Figure 16). Morphology change was observed in PN-E, TC-E, and LF-E at high concentrations (100 μg/ml) and AP-E at 10-100 μg/ml. The change in cell morphology confirmed the data in cell viability determined by MTT assay as described above.

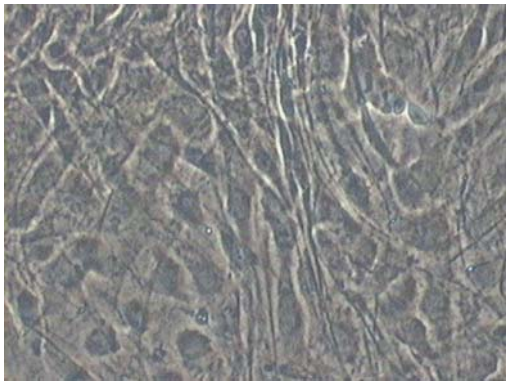
(a) Control



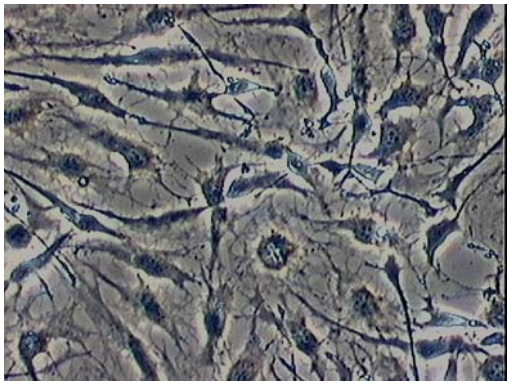
(b) 10^{-6} M dexamethasone



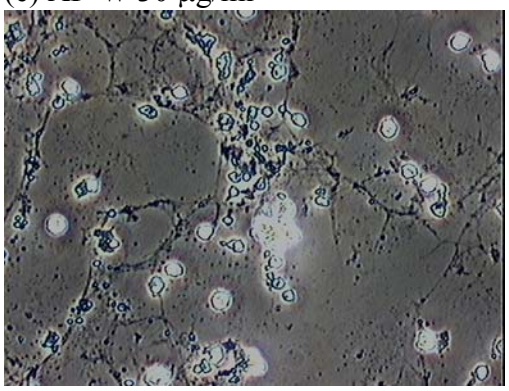
(c) AP-W 3 μg/ml



(d) AP-W 10 μg/ml



(e) AP-W 30 μg/ml



(f) AP-W 100 μg/ml

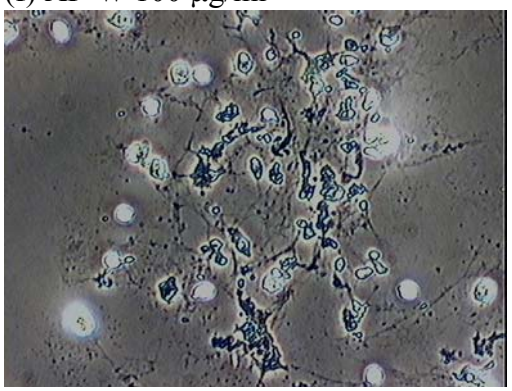
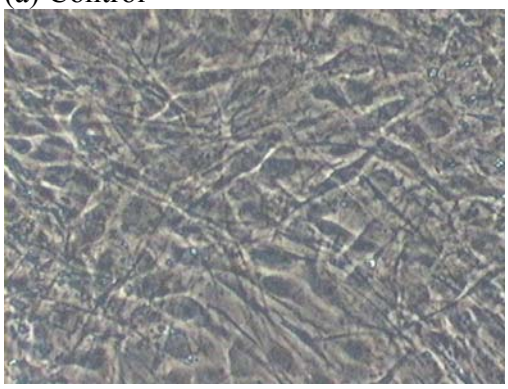
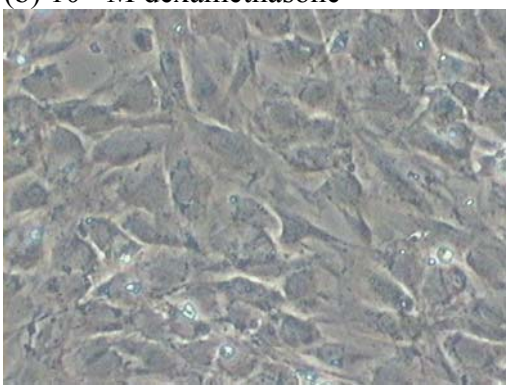


Figure 9 Morphology of MC3T3-E1 pre-osteoblastic cells conditioned with AP-W and dexamethasone at 10^{-6} M for 9 days but expressed cell morphology on day 9. Cell morphology indicated biocompatibility of AP-W correlated with ALP activity.

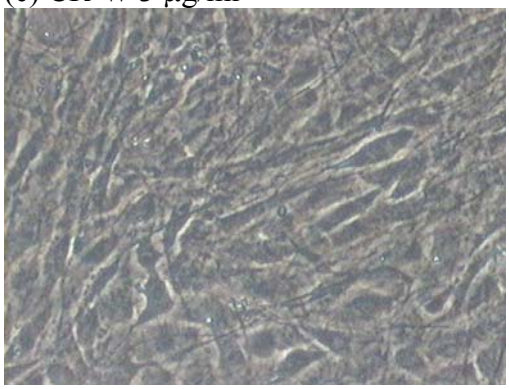
(a) Control



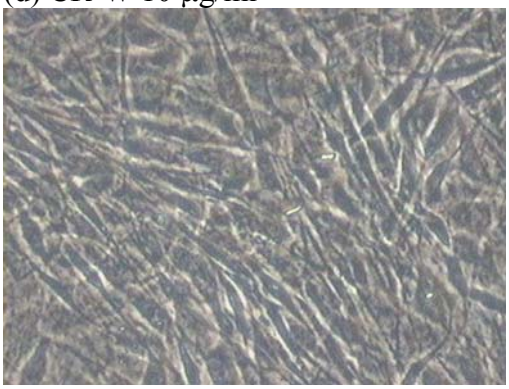
(b) 10^{-6} M dexamethasone



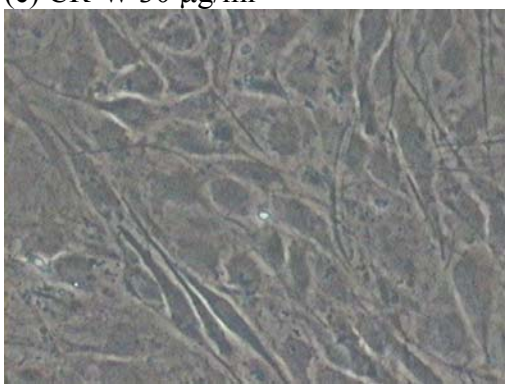
(c) CR-W 3 μg/ml



(d) CR-W 10 μg/ml



(e) CR-W 30 μg/ml



(f) CR-W 100 μg/ml

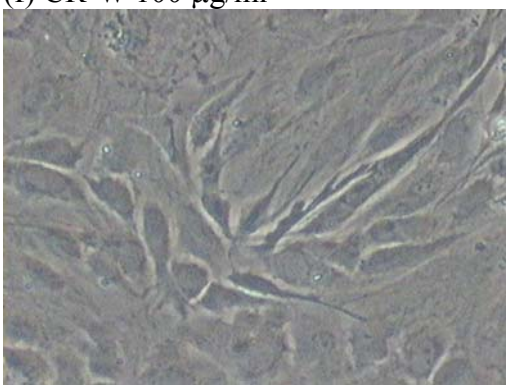
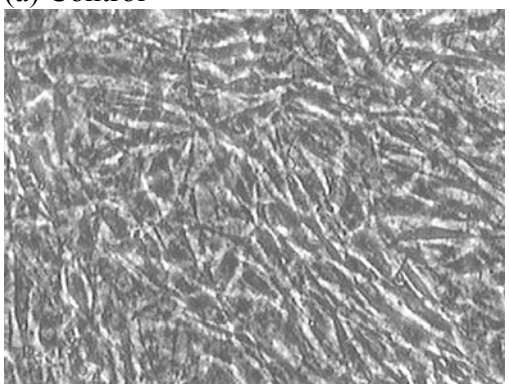
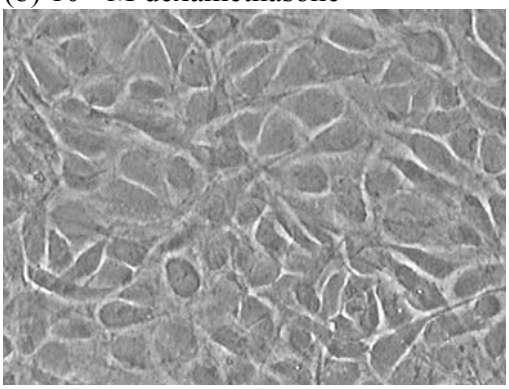


Figure 10 Morphology of MC3T3-E1 pre-osteoblastic cells conditioned with CR-W at day 9. Cell morphology indicated biocompatibility of crude extracts on MC3T3-E1 cells. This figure observed ALP activity were released from cells and % cell viability of CR-W were compatible with all concentrations.

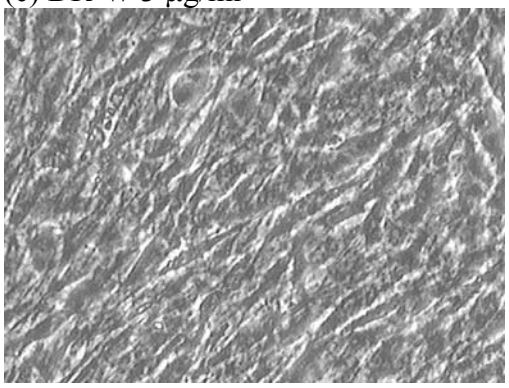
(a) Control



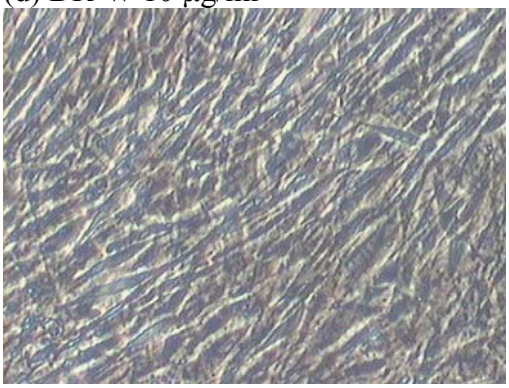
(b) 10^{-6} M dexamethasone



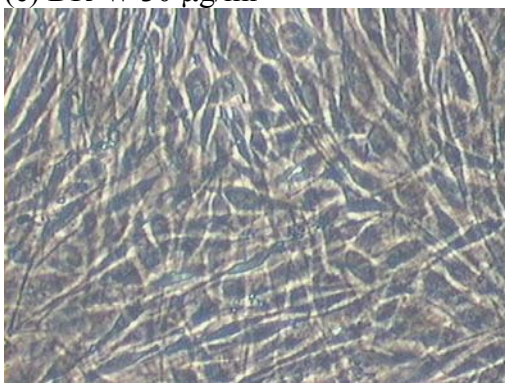
(c) DR-W 3 μg/ml



(d) DR-W 10 μg/ml



(e) DR-W 30 μg/ml



(f) DR-W 100 μg/ml

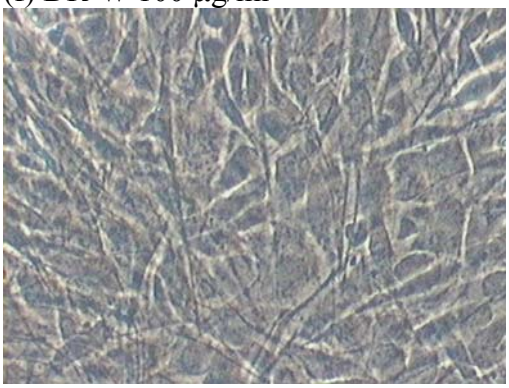
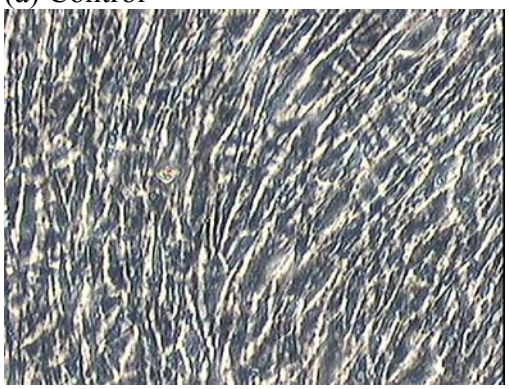
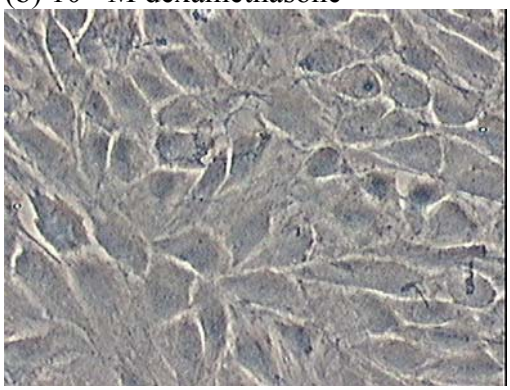


Figure 11 Morphology of MC3T3-E1 pre-osteoblastic cells conditioned with DR-W at day 9.

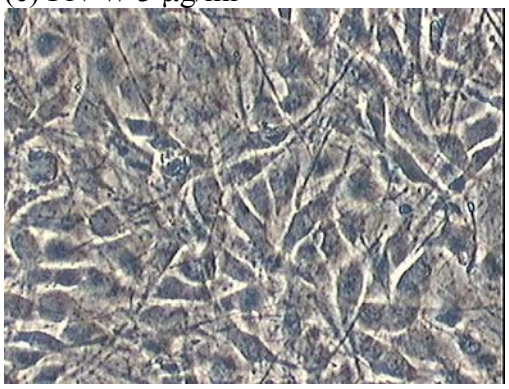
(a) Control



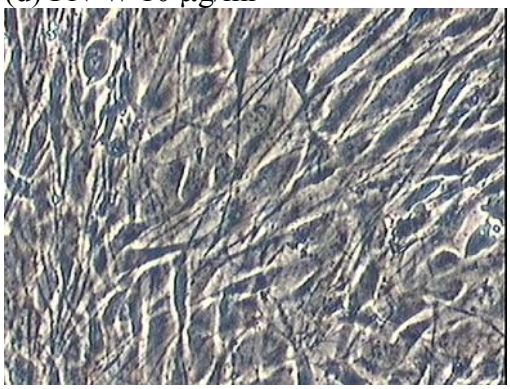
(b) 10^{-6} M dexamethasone



(c) PN-W 3 μg/ml



(d) PN-W 10 μg/ml



(e) PN-W 30 μg/ml

(f) PN-W 100 μg/ml

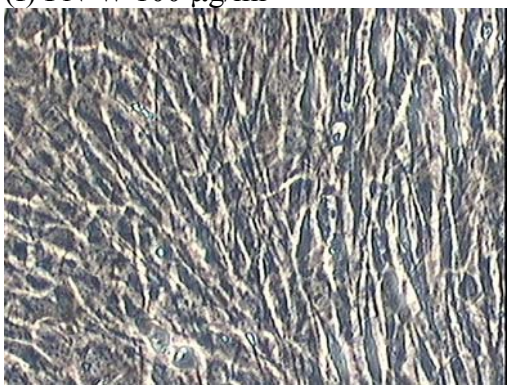
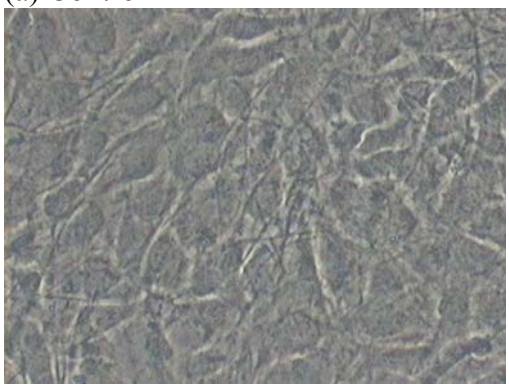
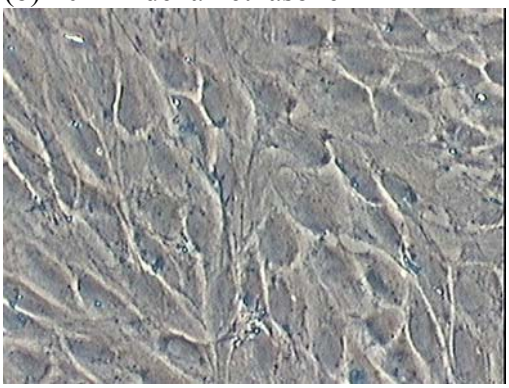


Figure 12 Morphology of MC3T3-E1 pre-osteoblastic cells conditioned with PN-W at day 9.

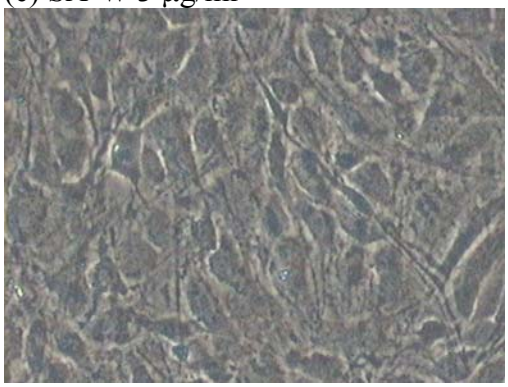
(a) Control



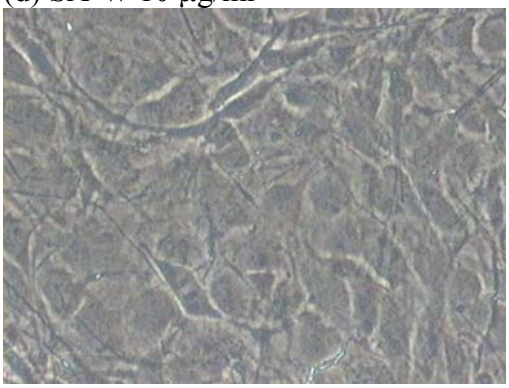
(b) 10^{-6} M dexamethasone



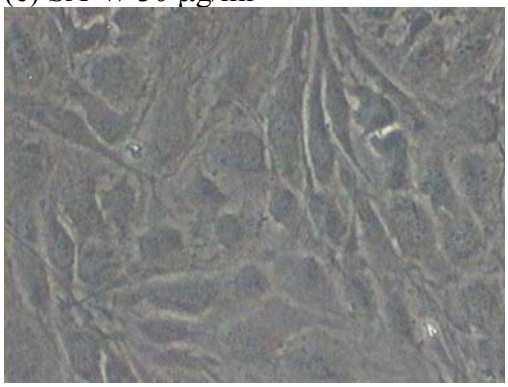
(c) SA-W 3 μg/ml



(d) SA-W 10 μg/ml



(e) SA-W 30 μg/ml



(f) SA-W 100 μg/ml

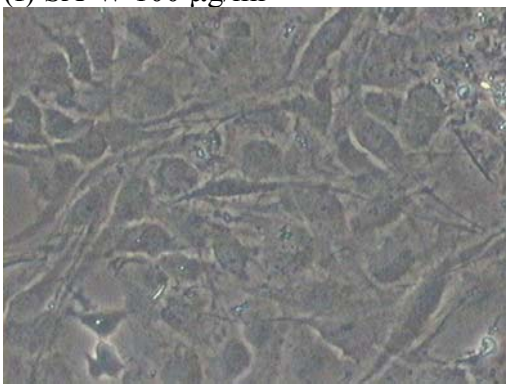
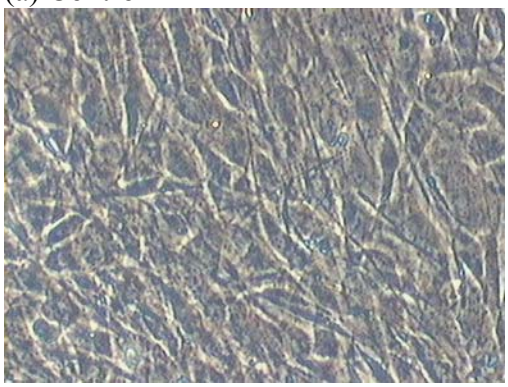
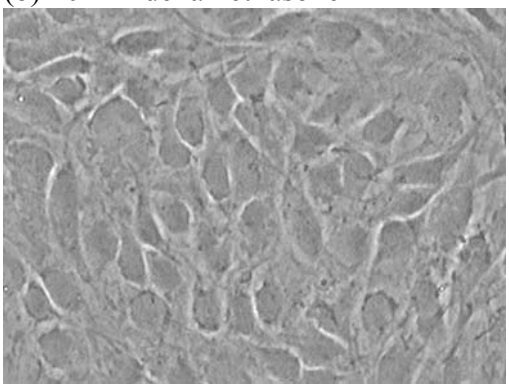


Figure 13 Morphology of MC3T3-E1 pre-osteoblastic cells conditioned with SA-W at day 9.

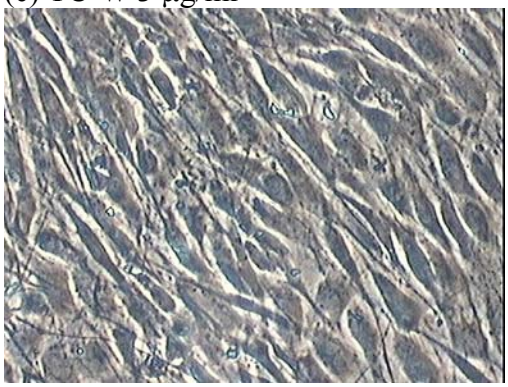
(a) Control



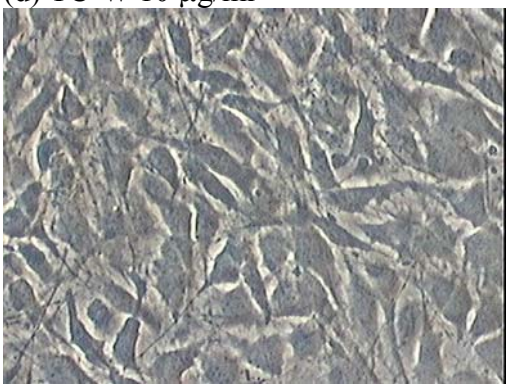
(b) 10^{-6} M dexamethasone



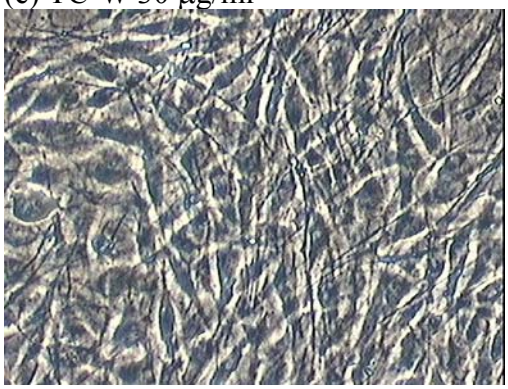
(c) TC-W 3 μg/ml



(d) TC-W 10 μg/ml



(e) TC-W 30 μg/ml



(f) TC-W 100 μg/ml

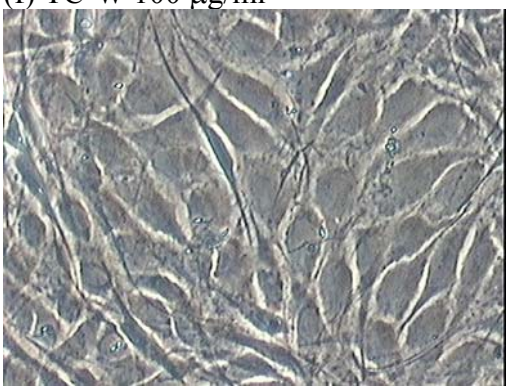
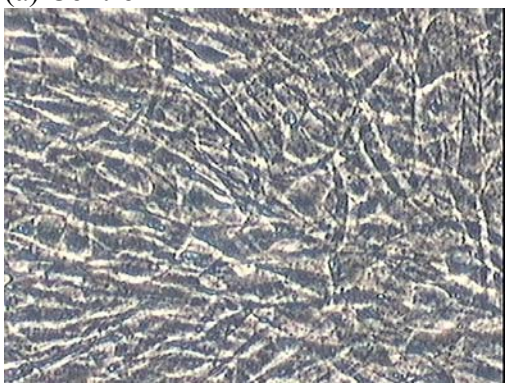
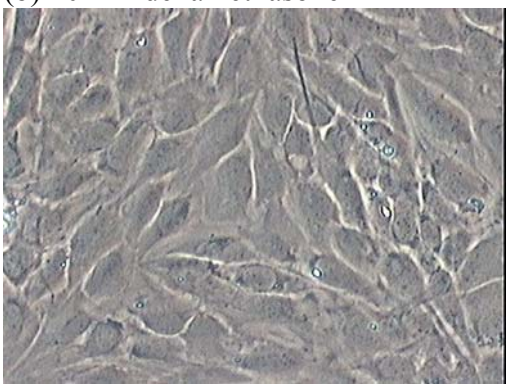


Figure 14 Morphology of MC3T3-E1 pre-osteoblastic cells conditioned with TC-W at day 9.

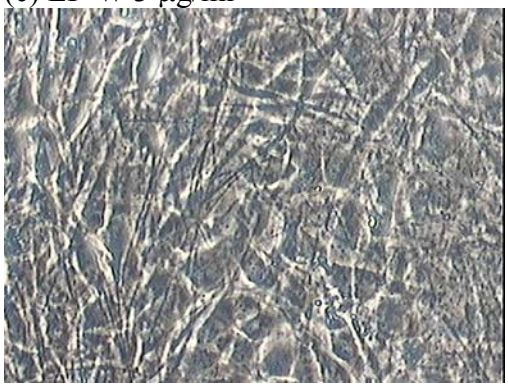
(a) Control



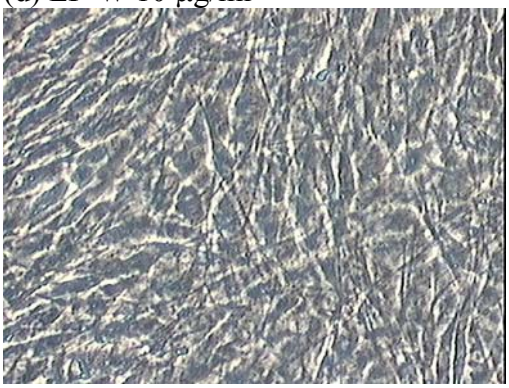
(b) 10^{-6} M dexamethasone



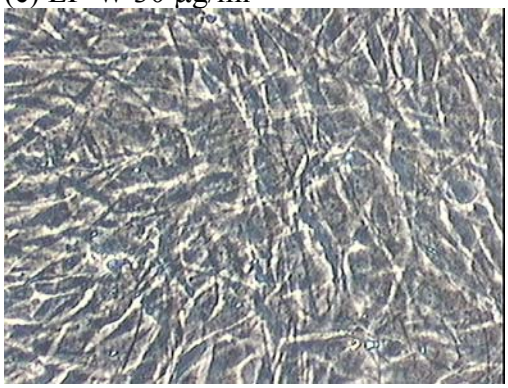
(c) LF-W 3 μg/ml



(d) LF-W 10 μg/ml



(e) LF-W 30 μg/ml



(f) LF-W 100 μg/ml

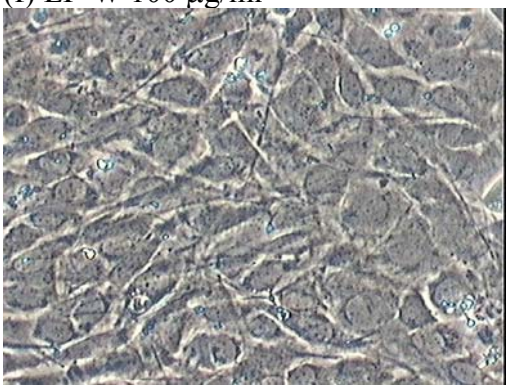
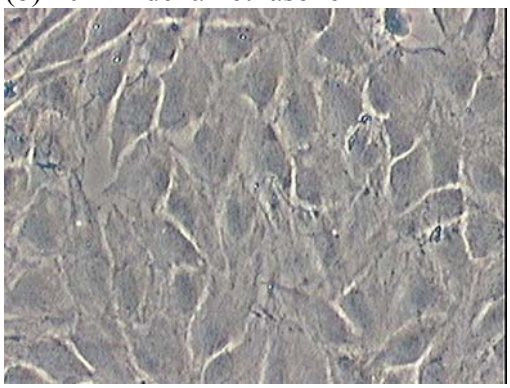


Figure 15 Morphology of MC3T3-E1 pre-osteoblastic cells conditioned with LF-W at day 9.

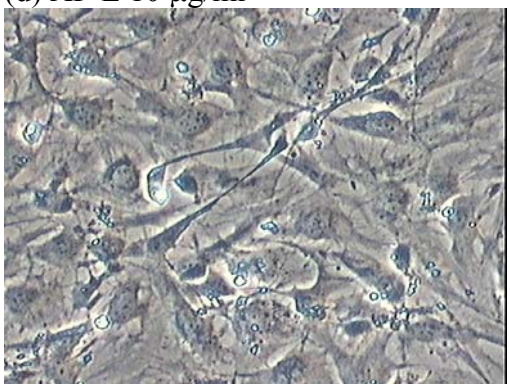
(a) Control

(b) 10^{-6} M dexamethasone

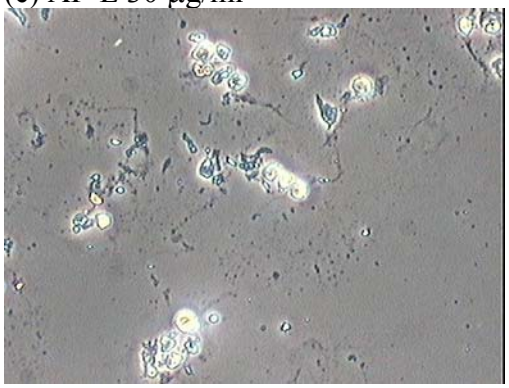


(c) AP-E 3 μg/ml

(d) AP-E 10 μg/ml



(e) AP-E 30 μg/ml



(f) AP-E 100 μg/ml

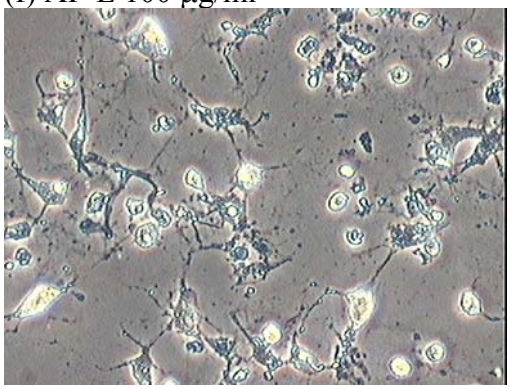
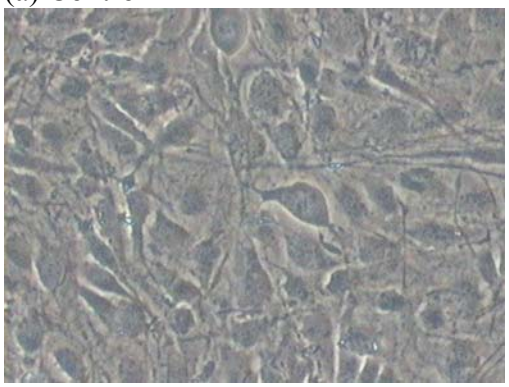
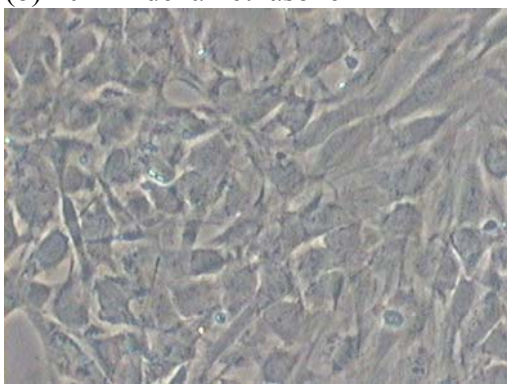


Figure 16 Morphology of MC3T3-E1 pre-osteoblastic cells conditioned with AP-E at day 9.

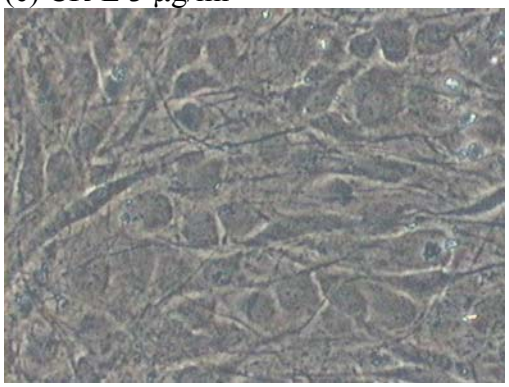
(a) Control



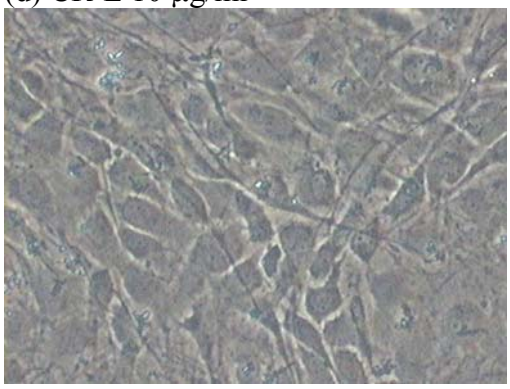
(b) 10^{-6} M dexamethasone



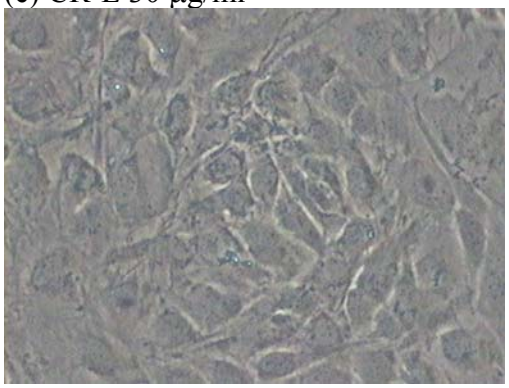
(c) CR-E 3 μg/ml



(d) CR-E 10 μg/ml



(e) CR-E 30 μg/ml



(f) CR-E 100 μg/ml

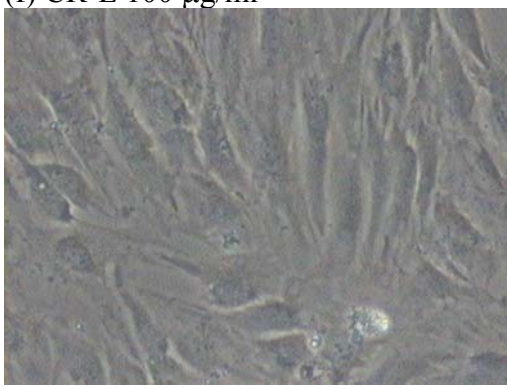
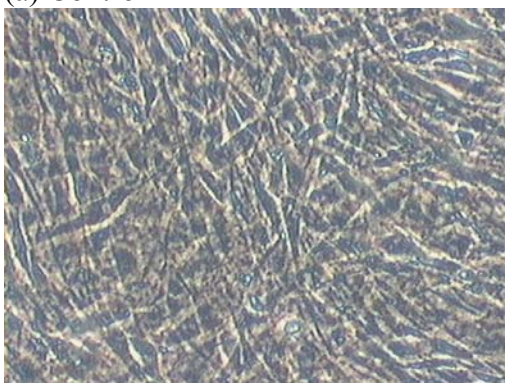
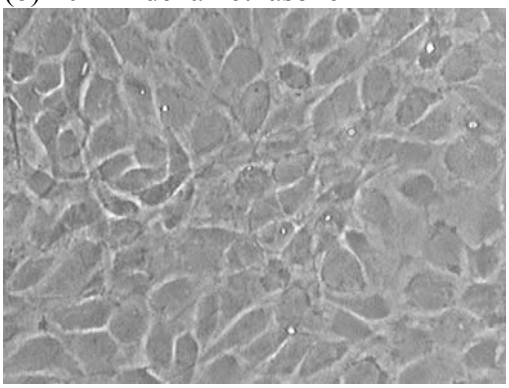


Figure 17 Morphology of MC3T3-E1 pre-osteoblastic cells conditioned with CR-E at day 9.

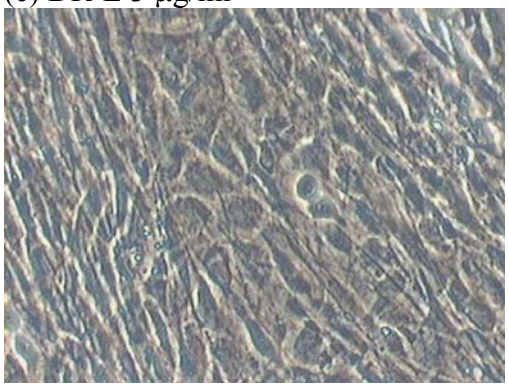
(a) Control



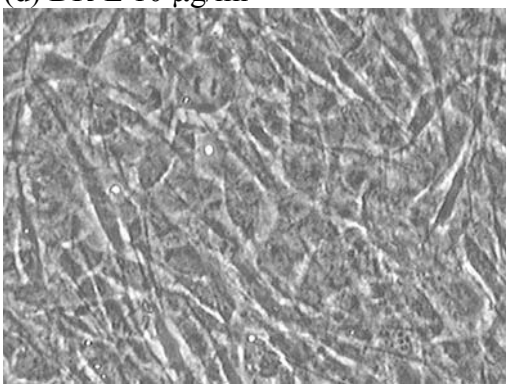
(b) 10^{-6} M dexamethasone



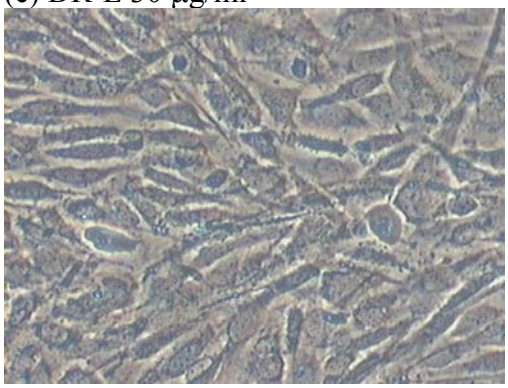
(c) DR-E 3 μg/ml



(d) DR-E 10 μg/ml



(e) DR-E 30 μg/ml



(f) DR-E 100 μg/ml

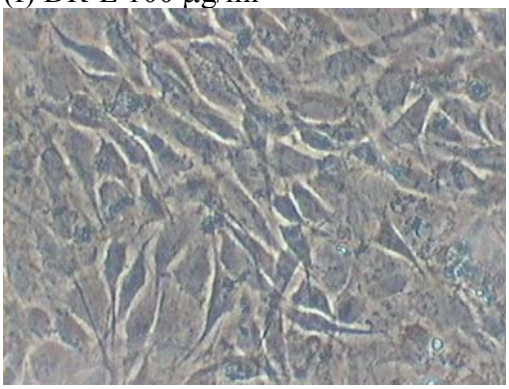
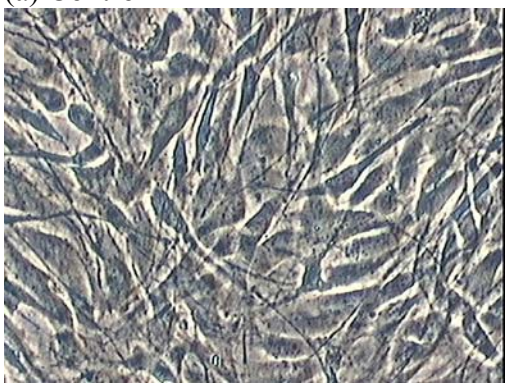
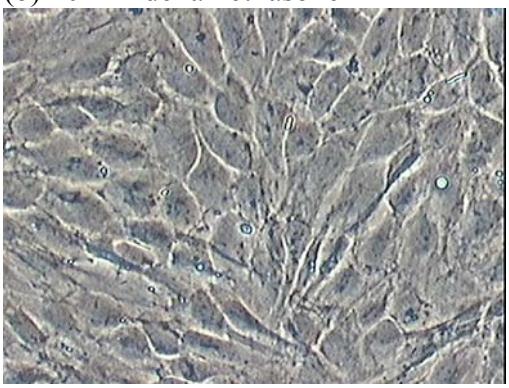


Figure 18 Morphology of MC3T3-E1 pre-osteoblastic cells conditioned with DR-E at day 9.

(a) Control

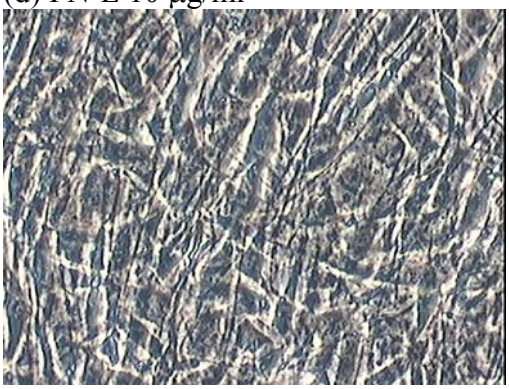


(b) 10^{-6} M dexamethasone

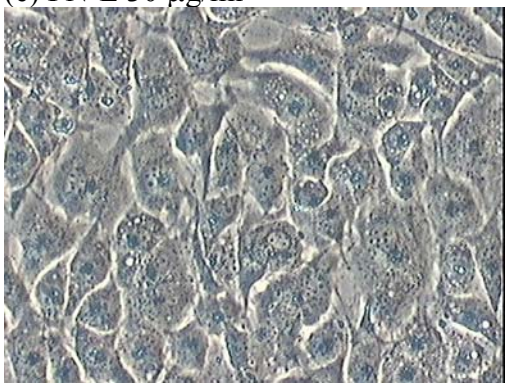


(c) PN-E 3 μg/ml

(d) PN-E 10 μg/ml



(e) PN-E 30 μg/ml



(f) PN-E 100 μg/ml

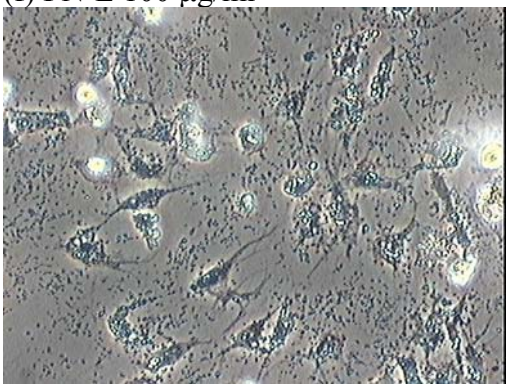
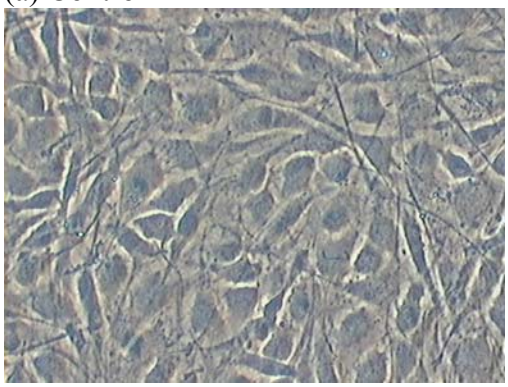
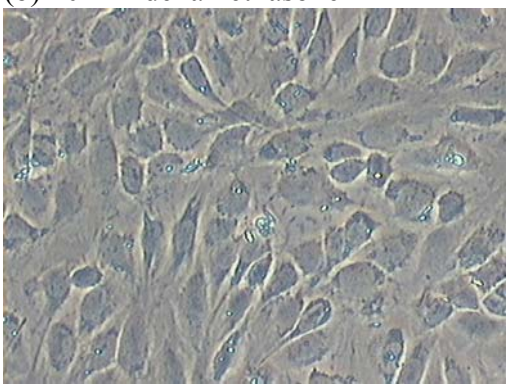


Figure 19 Morphology of MC3T3-E1 pre-osteoblastic cells conditioned with PN-E at day 9.

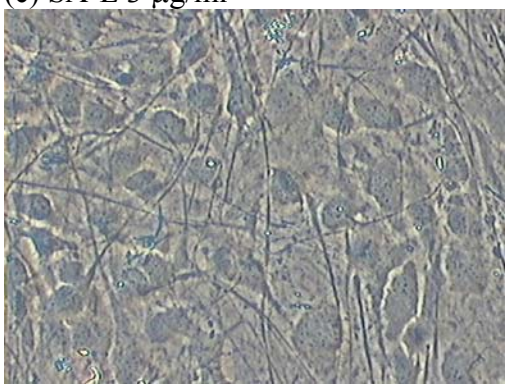
(a) Control



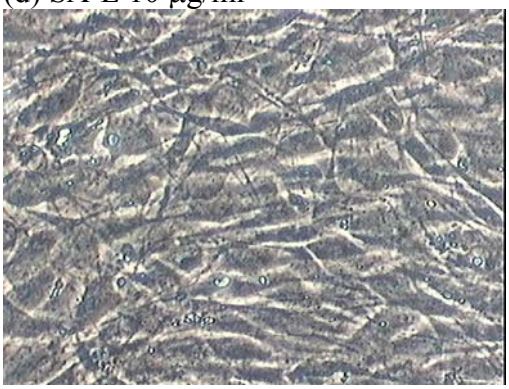
(b) 10^{-6} M dexamethasone



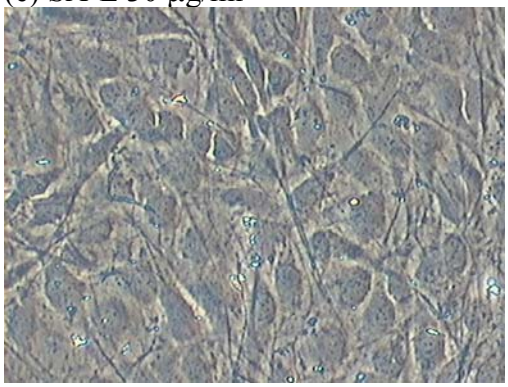
(c) SA-E 3 μg/ml



(d) SA-E 10 μg/ml



(e) SA-E 30 μg/ml



(f) SA-E 100 μg/ml

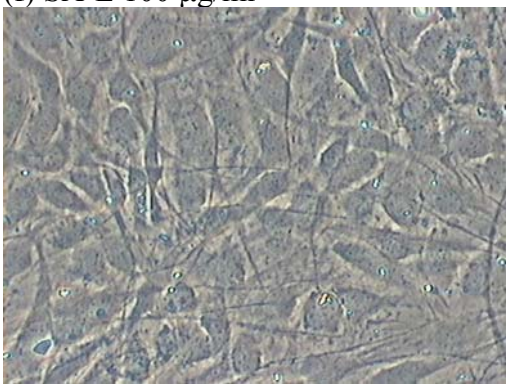
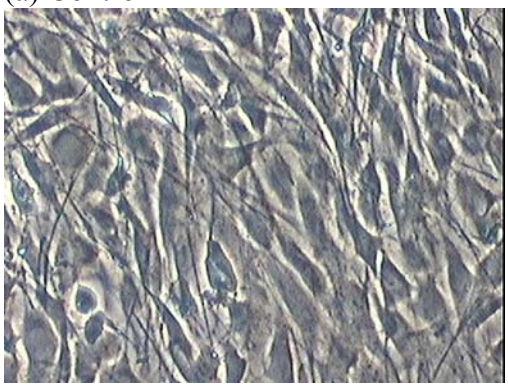


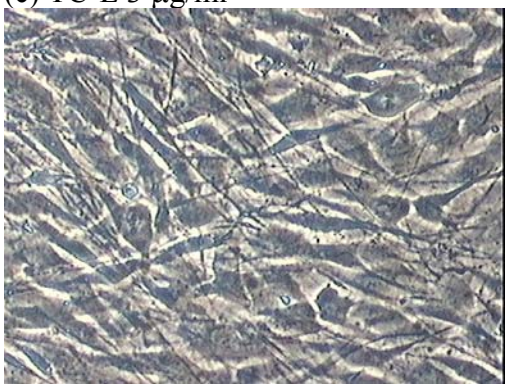
Figure 20 Morphology of MC3T3-E1 pre-osteoblastic cells conditioned with SA-E at day 9.

(a) Control

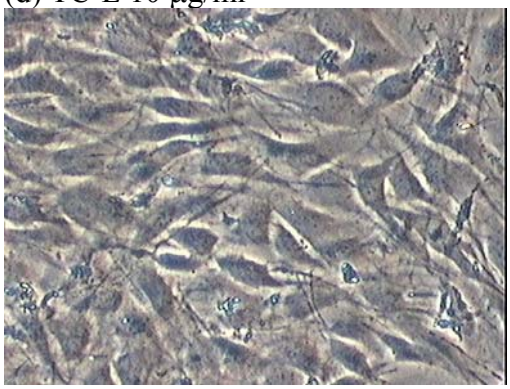


(b) 10^{-6} M dexamethasone

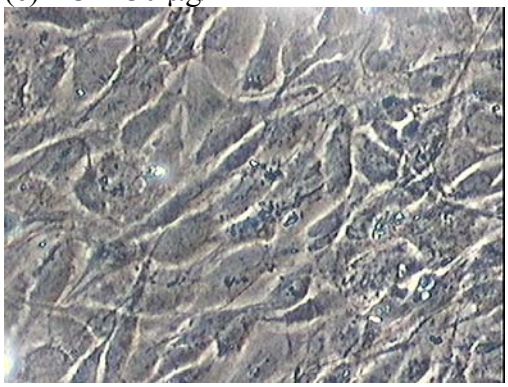
(c) TC-E 3 μg/ml



(d) TC-E 10 μg/ml



(e) TC-E 30 μg/ml



(f) TC-E 100 μg/ml

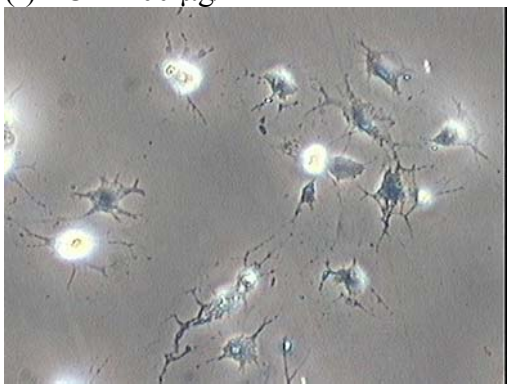
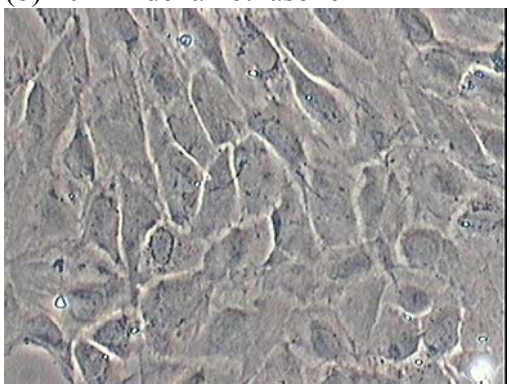


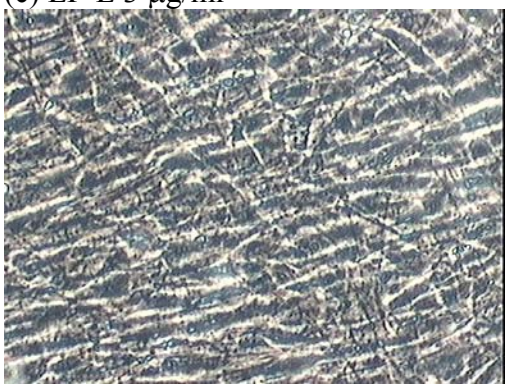
Figure 21 Morphology of MC3T3-E1 pre-osteoblastic cells conditioned with TC -E at day 9.

(a) Control

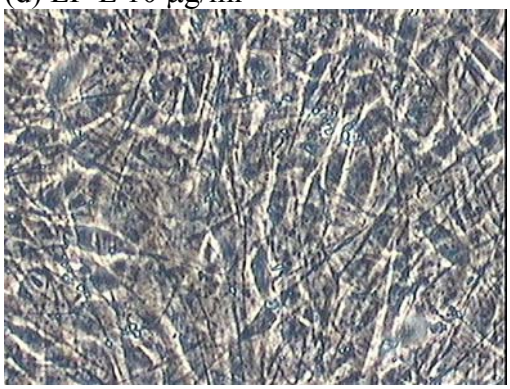
(b) 10^{-6} M dexamethasone



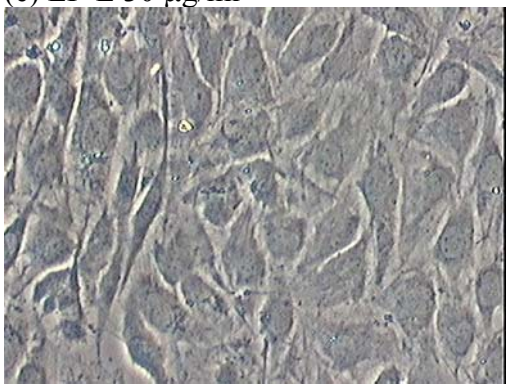
(c) LF-E 3 μg/ml



(d) LF-E 10 μg/ml



(e) LF-E 30 μg/ml



(f) LF-E 100 μg/ml

Figure 22 Morphology of MC3T3-E1 pre-osteoblastic cells conditioned with LF -E at day 9.

2.3 Determination of total protein

The total protein content was directly associated with the number of cells (Rosa and Beloti, 2003; Gugala and Gogolewski, 2004). Total protein was carried out and the ALP activity of the cells was normalized by total protein content (Mouries *et al*., 2002). Effects of crude water and ethanolic extracts on the expression of protein content during the maturation and differentiation of osteoblasts was studied (Figure 23-24). After MC3T3-E1 cells were conditioned with 3-100 μg/ml of the crude extracts, the total protein content was examined at day 9.

Effects of the crude water extracts on the expression of total protein content was found as dose-independent (Figure 23). The highest total protein content was found in DR-W and TC-W. Total protein contents were 0.55 mg/ml in DR-W at 3 μg/ml, and 0.56 and 0.59 mg/ml in TC-W at 3 and 10 μg/ml, respectively.

The crude ethanolic extracts affected dose-independently the expression of total protein content (Figure 24). The highest total protein content was found in SA-E, followed by DR-E. Total protein contents were 0.78 mg/ml in SA-E at 3 μg/ml, and 0.52 mg/ml in DR-W at 3 μg/ml.

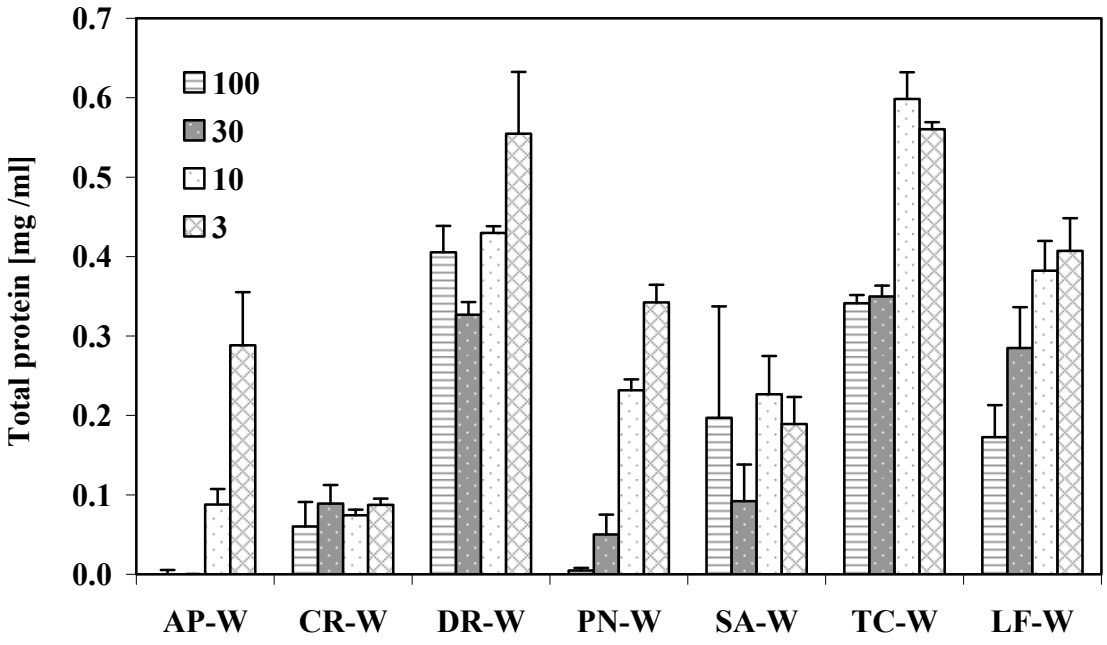


Figure 23 Effects of crude water extracts on the expression of total protein content in MC3T3-E1 cells. Crude water extracts at 100, 30, 10 and 3 μg/ml were added to MC3T3-E1 cells for 9 day. Total protein was measured using BCA kit from whole cell extracts residuals of ALP activity. Each samples are expressed as mean ± S.D. of four determinations at each concentration.

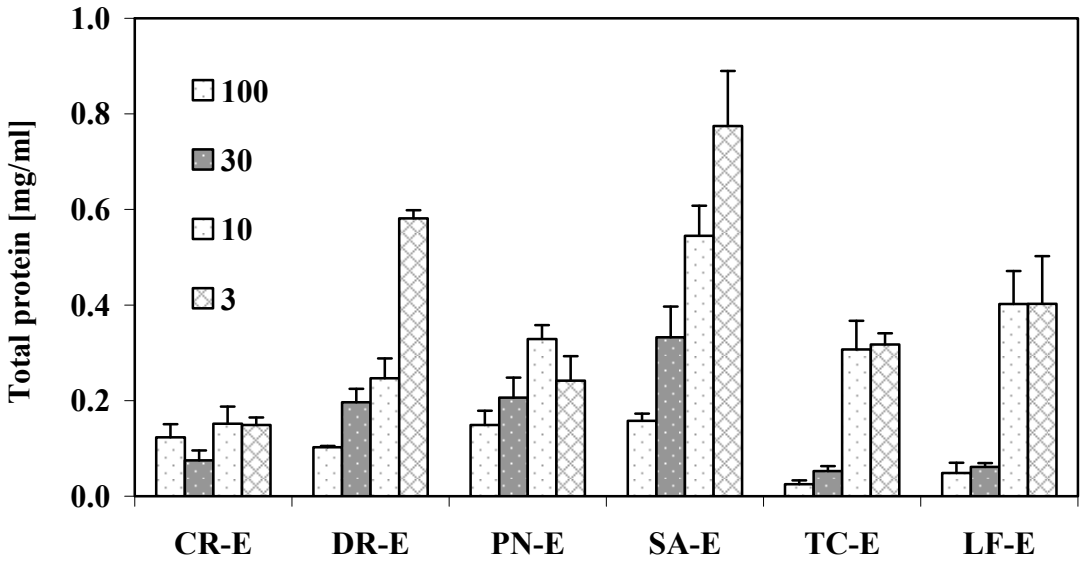


Figure 24 Effects of crude ethanolic extracts on the expression of total protein content in MC3T3-E1 cells. Crude ethanolic extracts at 100, 30, 10 and 3 μg/ml were added to MC3T3-E1 cells for 9 day. Total protein was measured using BCA kit from whole cell extracts residuals of ALP activity. Each samples are expressed as mean ± S.D. of four determinations at each concentration.

2.4 Determination of alkaline phosphatase activity

Alkaline phosphatase (ALP) activity has been reported as a parameter determining bone cell activity (Mouries *et al*., 2002). ALP level depends on several factors, such as state of differentiation of cells, the numbers of differentiated cells, growth hormone, cytokines, steroid drugs, and nutritions (Cheng *et al*., 1994).

The ALP activity and total protein amount are the parameters indicative of activity and amount of cells (http://courses.washington.edu). Thus, measurement of ALP activity not only indicates the cell proliferation but also the osteoblastic phenotype expression seem to be promoted by the presence size of cells and ALP level. With the action of crude extracts, the cells showed a relative higher activity of ALP and a higher proliferating activity ($p<0.01$) than treatment without crude extracts. These bone formation markers have been shown to correlate with bone formation measured by bone histomorphology in MC3T3-E1 cells (http://courses.washington.edu).

In our study, after MC3T3-E1 cells were conditioned with the crude water and ethanolic extracts at 3-100 μg/ml for 9 days, the ALP activity was determined and normalized with protein content using 10^{-6} M dexamethasone as a positive control. Figures 25-26 show the ALP activity of the cells normalized with total protein content and expressed as folds of the ALP activity of the dexamethasone treatment in Figures 27-28.

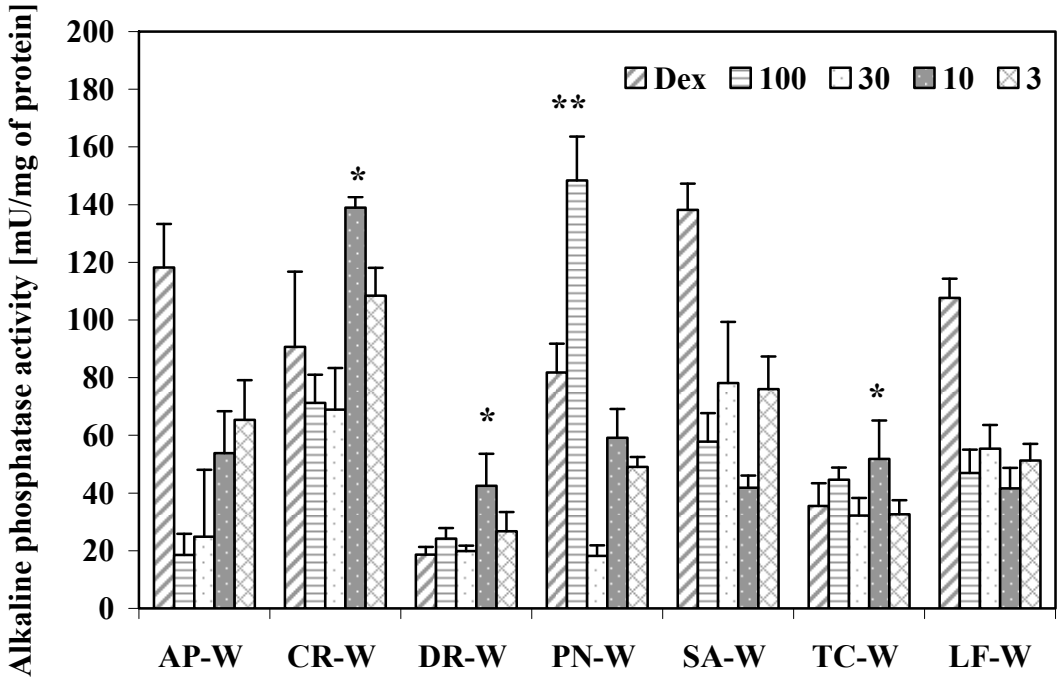


Figure 25 Alkaline phosphatase activity [mU/mg protein] was measured using colorimetric method from whole cell extracts at day 9. MC3T3-E1 cells conditioned with water extracts of various concentrations (100, 30, 10 and 3 μg/ml). Results are shown as the mean ± S.D. of four determinations at each concentration for each sample. *$p < 0.05$, **$p < 0.01$ compared with dexamethasone for each sample.

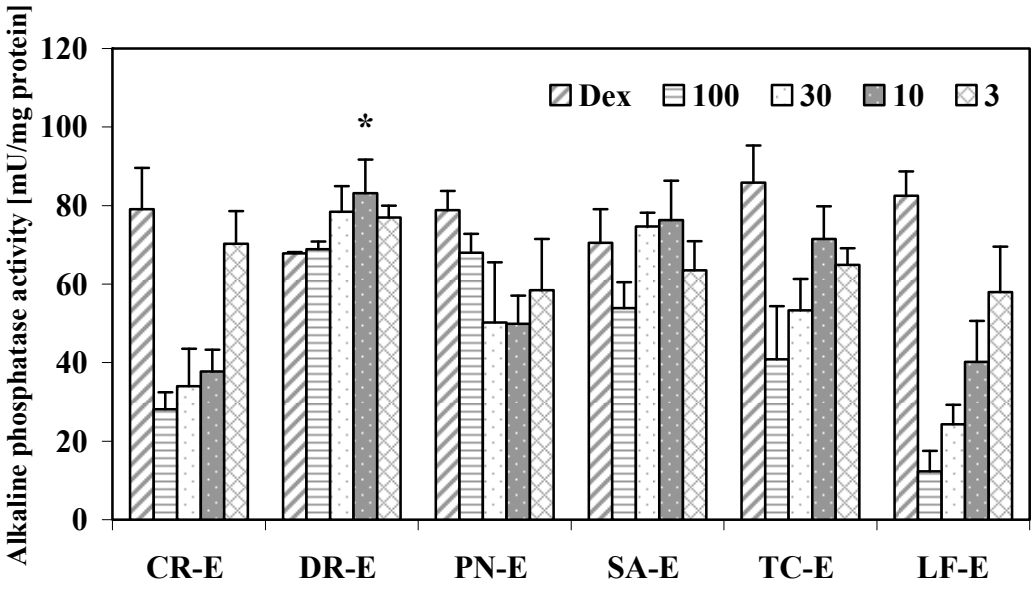


Figure 26 Alkaline phosphatase activity [mU/mg protein] was measured using colorimetric method from whole cell extracts at day 9. MC3T3-E1 cells conditioned with ethanolic extracts of various concentrations (100, 30, 10 and 3 μg/ml). Results are shown as the mean ± S.D. of four determinations at each concentration for each sample. *$p < 0.05$ compared with dexamethasone for each sample.

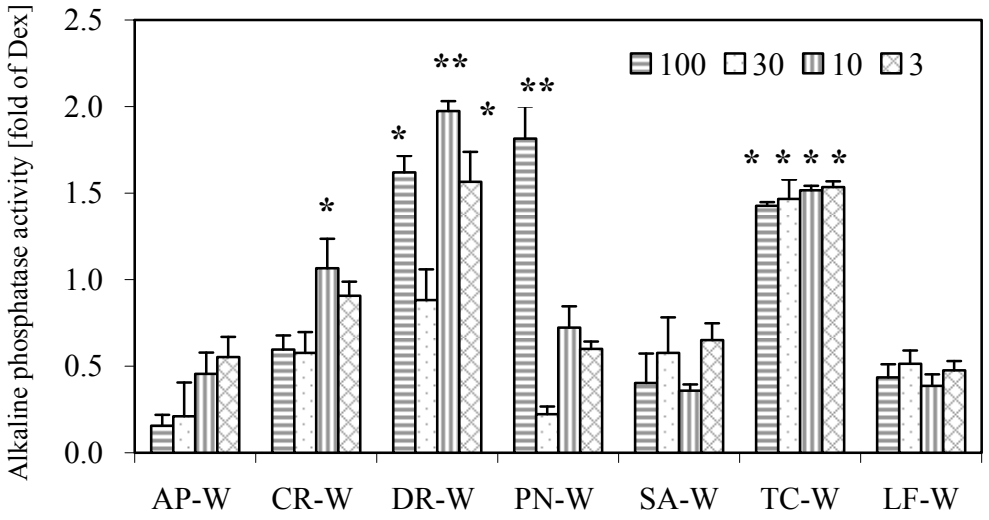


Figure 27 Effect of crude water extracts on alkaline phosphatase activity of MC3T3-E1 cells at day 9. MC3T3-E1 cells were cultured in combination with crude water extracts. Data are shown mean ± S.D. of four determinations at each concentration for each sample,*p< 0.05, **p<0.01 and expressed as fold of 10^{-6} M dexamethazone as positive control .

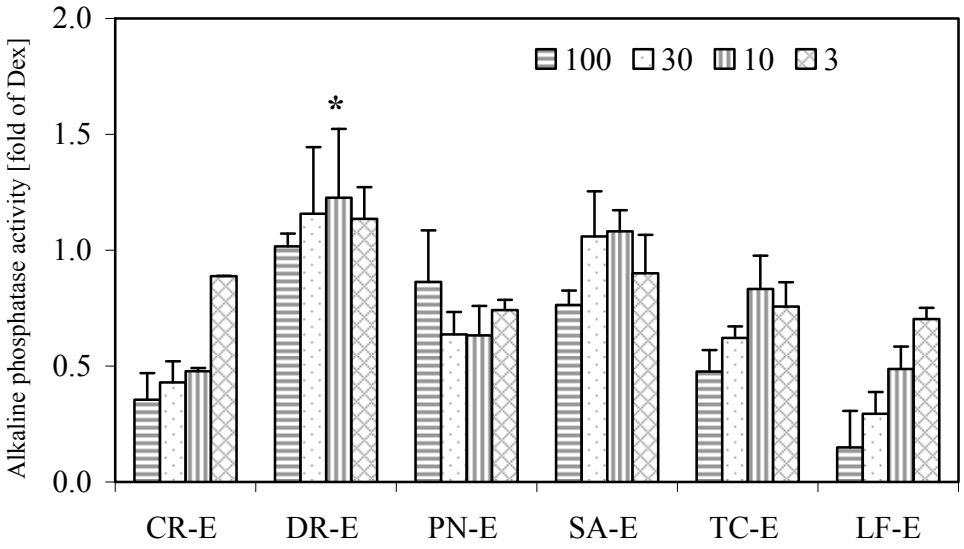


Figure 28 Effect of crude ethanolic extracts on alkaline phosphatase activity of MC3T3-E1 cells at day 9. MC3T3-E1 cells were cultured in combination with crude ethanolic extracts. Results shown are mean ± S.D. of four determinations at each concentration for each sample, *$p< 0.05$ and expressed as fold of 10^{-6} M dexamethazone as positive control .

ALP enzyme has been reported to bind with several substrates to induce the ALP activity (Doublet *et al*., 1975). Induction on stimulation of ALP activity has been known to play a critical role for the calcification of the bone matrix (Xujuan *et al*., 2005). In our study *D. rhodocalyx* was found as the most effective herb in the longevity formulation to induce the ALP activity of MC3T3-E1 cells. Both water and ethanolic extracts of *D. rhodocalyx* were active to MC3T3-E1 cells dose-independently. From Figures 27-28, the highest ALP activity was observed in DR-W at 10 μg/ml (1.97 folds), followed by TC-W at 10 μg/ml (1.52 folds), and DR-E at 10 μg/ml (1.23 folds), $p<0.05$. The high total protein content resulted from these extracts shown in Figure 23-24 confirmed the effects of these extracts on high ALP activity at high cell numbers. On the other hand, the ALP activity observed in PN-W at high concentration (100 μg/ml) was found as 1.81 folds of dexamethasone. This false high value was obtained after normalization of the ALP activity by the low total protein at the toxic concentration. Betulinic acid, betulin, and triterpenes found in *D. rhodocalyx* have been reported as active constituents to promote cell proliferation and secretion of intracellular of UMR 106 cells (Xujuan *et al*., 2005; Hata *et al*., 2002).

Effects of other natural products on ALP activity have been studied. Enhanced ALP activity by 3-hydroxybutyrate at lower than 0.01 g/L has been reported (Zhao *et al*., 2007). Stimulatory effect of *Drynariae Rhizoma*, traditional Chinese and Korean folk medicine, was observed at relatively low doses of 50–150 μg/ml (Jeong *et al*., 2004). The active components of *Drynariae Rhizoma* were reported as flavonoids, phenylpropanoids and triterpenes (Jeong *et al*., 2005 a,b).

Part 3: Evaluation of anti-allergic activities of crude extracts

3.1 Effects of crude extracts on the release of β-hexosaminidase from RBL-2H3 cells

Longevity herbal formulation, together with water and ethanolic extracts were determined for their anti-allergic effects. Inhibitory effects of the crude extracts on the release of β-hexosaminidase in RBL-2H3 cells and β-hexosaminidase activity and inhibition against the enzyme activity were determined (Tables 5-6).

As shown in Table 5, PN-E exhibited the most potent activity with an IC_{50} value of 14.0 μg/ml, followed by DR-W (40.8 μg/ml), TC-W (53.4 μg/ml), CR-W (61.0 μg/ml), PN-W (65.8 μg/ml), LF-W (66.6 μg/ml) and SA-E (82.2 μg/ml),whereas the other extracts were inactive (IC_{50} > 100 μg/ml). It was indicated that anti-allergic effect of PN-E (IC_{50} = 14.0 μg/ml) was higher than that of ketotifen fumarate, a positive control (IC_{50} = 20.2 μg/ml). The crude extracts were also examined on the enzyme activity of β-hexosaminidase. As a result, they showed weak inhibition against this enzyme activity at 100 μg/ml (Table 5). The result indicated that these extracts inhibited the antigen-induced degranulation but not substantially affected the activity of β-hexosaminidase.

Table 5. Inhibitory effects of Thai medicinal plants on the release of β-hexosaminidase in RBL-2H3 cells

Samples	Inhibition (%) at various concentration (μg/ml)					IC_{50} (μg/ml)	%Enzyme inhibition at 100 μg/ml
	0	3	10	30	100		
AP-E	0.0 ± 5.6	–	–	–	-95.8 ± 11.3**	>100	-
AP-W	0.0 ± 5.6	–	–	–	-25.2 ± 10.2	>100	-
CR-E	0.0 ± 2.5	-7.7 ± 2.1	2.2 ± 3.2	19.1 ± 1.7**	43.7 ± 2.9**	>100	-
CR-W	0.0 ± 5.1	–	-3.5 ± 8.3	31.1 ± 6.2	63.6 ± 4.3**	61.0	32.6
DR-E	0.0 ± 6.7	–	-3.7 ± 6.5	4.2 ± 7.4	38.4 ± 5.8*	>100	-
DR-W	0.0 ± 6.6	–	-2.3 ± 12.4	36.5 ± 9.5	84.9 ± 3.4**	40.8	28.6
PN-E	0.0 ± 6.1	-14.4 ± 8.6	62.6 ± 6.5**	81.3 ± 4.4**	95.8 ± 1.0**	14.0	16.3
PN-W	0.0 ± 4.4	–	–	22.4 ± 6.6	64.6 ± 0.2**	65.8	36.6
SA-E	0.0 ± 7.0	–	-40.7 ± 5.9	2.4 ± 8.9	60.2 ± 7.1**	82.2	40.4
SA-W	0.0 ± 7.0	–	–	–	38.2 ± 7.1*	>100	-
TC-E	0.0 ± 6.0	–	-11.9 ± 7.6	16.0 ± 2.1	44.2 ± 2.9**	>100	-
TC-W	0.0 ± 7.0	–	18.9 ± 5.4	33.0 ± 4.9*	65.2 ± 5.0**	53.4	37.7
LF-E	0.0 ± 3.3	-1.1 ± 2.3	10.1 ± 3.5	21.5 ± 5.7	43.5 ± 5.9*	>100	-
LF-W	0.0 ± 5.5	–	-5.0 ± 5.0	23.3 ± 5.4	63.5 ± 2.5**	66.6	38.2
Ketotifen fumarate	0.0 ± 6.9	–	12.8 ± 0.5	38.3 ± 3.2**	68.2 ± 1.5**	47.5 μM (20.2 μg/ml)	15.8

Each value represents the mean ± SEM. Significantly different from control, *p<0.05, **p<0.01.

Table 6. Inhibitory effects of ethanolic extract of *P. nigrum* and piperine on the release of β-hexosaminidase

Samples	Inhibition (%) at various concentrations (μg/ml)					IC_{50} (μg/ml)	%Enzyme inhibition at 100 μg/ml
	0	3	10	30	100		
PN-E	0.0 ± 6.1	-14.4 ± 8.6	62.6 ± 6.5**	81.3 ± 4.4**	95.8 ± 1.0**	14.0	16.3
Piperine	0.0 ± 4.9	-2.8 ± 13.6	34.1 ± 5.8*	90.1 ± 6.3**	88.1 ± 3.6**	16.0	36.6
Ketotifen fumarate	0.0 ± 6.9	–	12.8 ± 0.5	38.3 ± 3.2**	68.2 ± 1.5**	47.5 μM (20.2 μg/ml)	15.8

Each value represents the mean ± SEM. Significantly different from control, *$p < 0.05$, **$p < 0.01$

Piperine has been reported as a major compound of *P. nigrum*. It has been studied for the inhibition of β-hexosaminidase release. IC_{50} values of PN-E and piperine were 14.0 and 16.0 μg/ml, respectively (Table 6). The information from this study may be useful for using piperine as an anti-histamine drug.

Part 4: Effect of mixtures of selected crude extracts on ALP activity and anti-allergic activity

In our study, DR-W (10 μg/ml), and PN-W (100 μg/ml) were selected as the most effective crude extracts to promote of ALP activity, while PN-E (10 μg/ml) was selected as the most effective crude extract on anti-allergic activity. Effects of the 1:1 mixture of DR-W/ PN-E, and the 1:10 mixture of PN-E/ PN-W on the ALP activity and anti-allergic activity were observed.

4.1 Determination of cytocompatibility

The 1:1 and 1:10 mixtures of PN-E/DR-W and PN-E/PN-W were prepared and examined by MTT assay for the compatibility to MC3T3-E1 cells. Effects of the crude extract mixtures on cell viability are shown in Figures 29. All mixtures were compatible to MC3T3-E1 cells with cell viability over 80% (Gomes *et* al., 2001; Melendez *et* al., 2005).

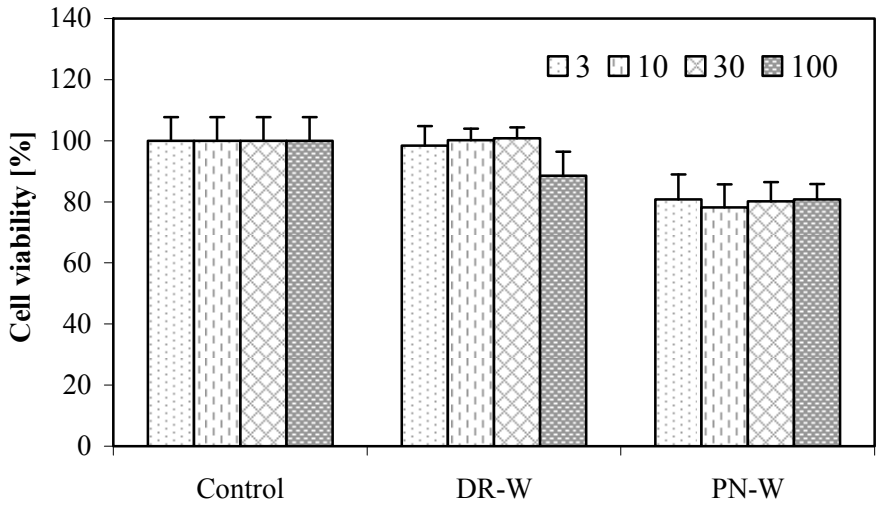


Figure 29 Effect of 1:1 and 1:10 mixtures of PN-E/DR-W and PN-E/PN-W (3-100 μg/ml) on the growth of MC3T3-E1 cells. Data expressed as a percentage of control. Results are shown as mean ± S.D.of duplicate (n = 8 in each replicate).

4.2 Morphology of MC3T3-E1 cells

Morphology of MC3T3-E1 cells conditioned with 1:1 and 1:10 mixtures of PN-E/DR-W and PN-E/PN-W at day 9 is shown in Fig. 30. No change in cell morphology confirmed cytocompatibility of these mixtures to the cells.

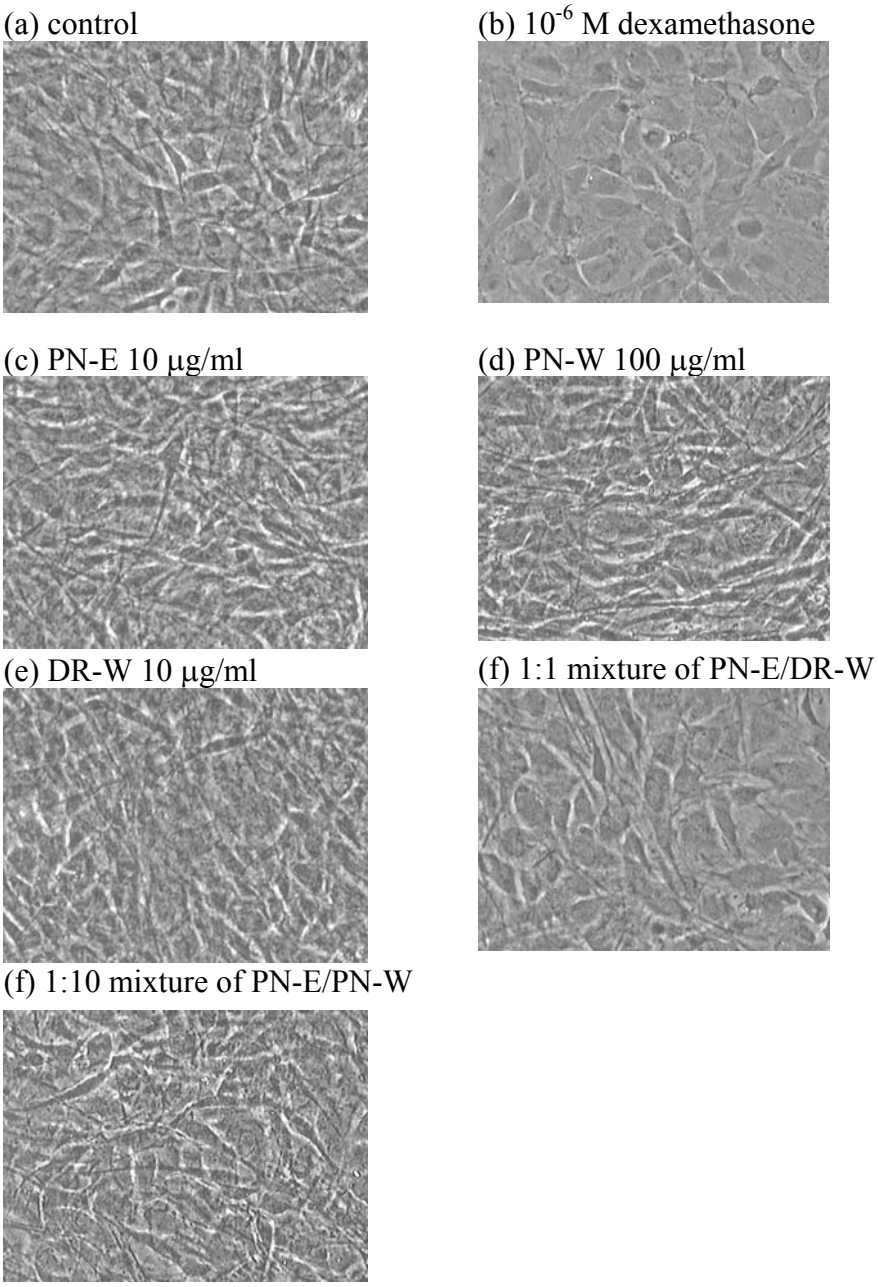


Figure 30 Morphology of MC3T3-E1 cells conditioned with 1:1 and 1:10 mixtures of PN-E/DR-W and PN-E/PN-W at day 9.

4.3 Determination total protein content

Total protein contents of MC3T3-E1 cells conditioned with mixtures of crude extracts were determined at day 9. As shown in Figure 31, total protein content observed in the 1:1 mixture of PN-E/DR-W was 0.52 ± 0.04 mg/ml, significantly higher than PN-E alone (0.47 ± 0.01 mg/ml), but not different from DR-W alone (0.56 ± 0.05 mg/ml), $p<0.05$. In addition, total protein content determined in 1:10 mixture of PN-E/PN-W was 0.42 ± 0.03 mg/ml, significantly higher than PN-W alone (0.35 ± 0.02 mg/ml), but not different from PN-E alone (0.52 ± 0.04 mg/ml), $p<0.05$.

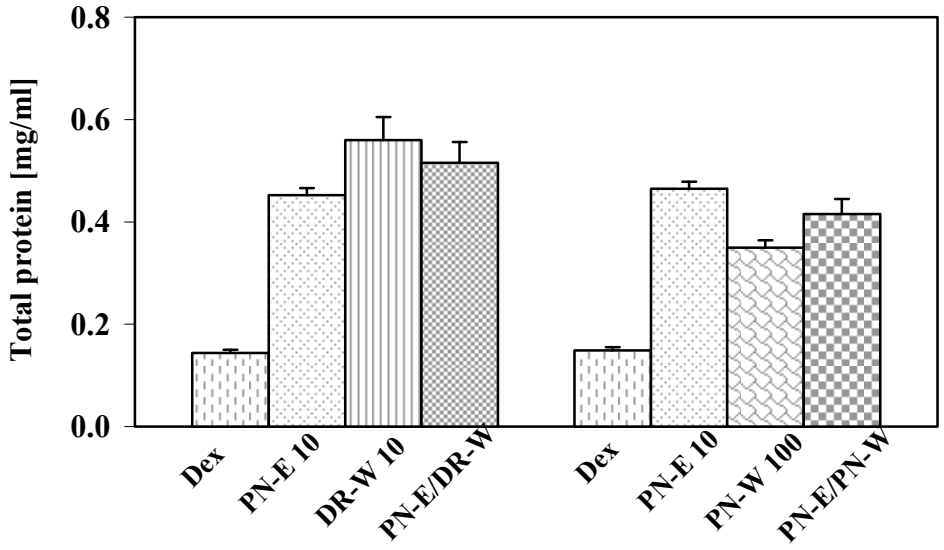


Figure 31 Effect of 1:1 mixture of PN-E/DR-W and 1:10 mixture of PN-E/PN-W on the total protein content of MC3T3-E1 cells at day 9. Total protein was measured using BCA kit. The results are expressed as mean ± S.D. of four determinations in each replication.

4.4 Determination of ALP activity

ALP activity of 1:1 mixture of PN-E/DR-W and 1:10 mixture of PN-E/PN-W were determined at day 9 (Figure 32). Effects of PN-E on enhanced ALP activity of DR-W and PN-W was observed in both crude extract mixtures. The ALP activity of 1:1 PN-E/DR-W mixture was 1.61 folds of dexamethasone, while that of 1:10 PN-E/PN-W mixture was 1.60 folds of dexamethasone (Figure 33). The two mixtures of crude extracts were more osteogenic active than crude extracts alone, and the longevity herbal preparation.

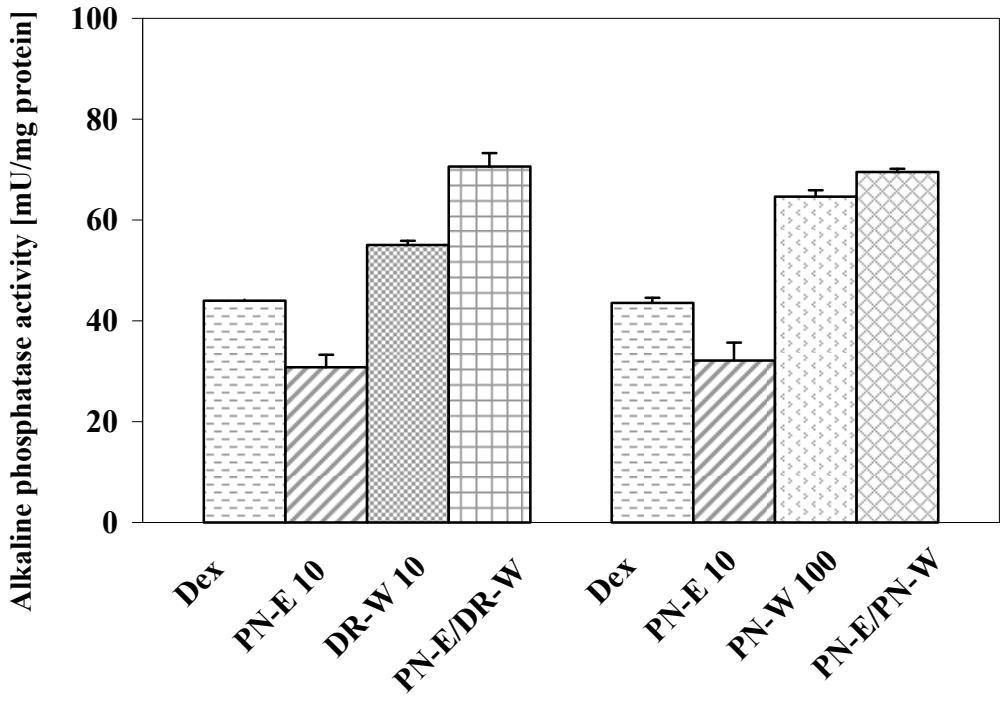


Figure 32 Alkaline phosphatase activity [mU/mg protein] was measured using colorimetric method from whole cell extracts at day 9 and normalized with total protein content. The results are shown as mean ± S.D. of four determinations in each replication.

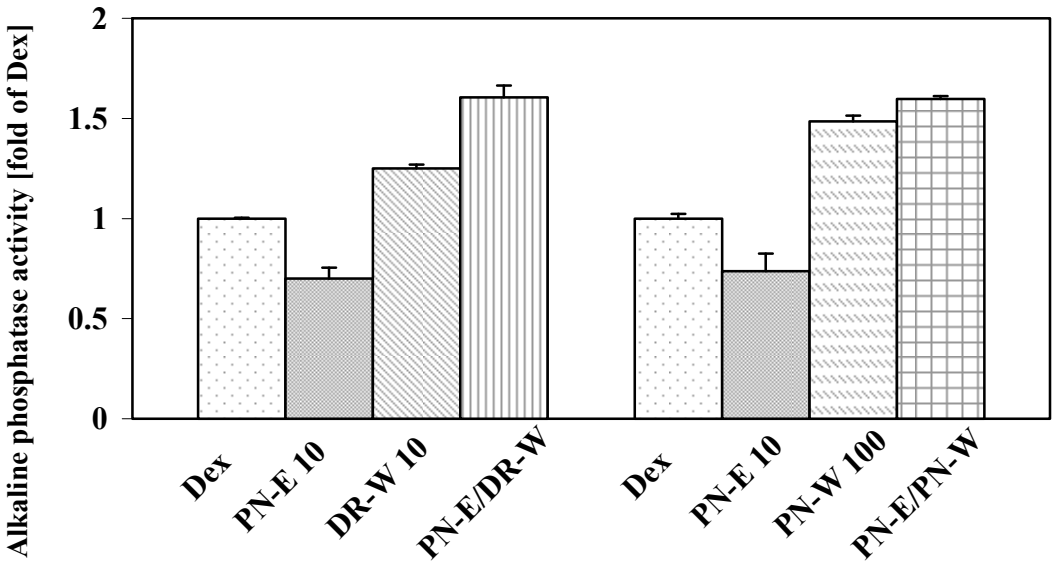


Figure 33 Alkaline phosphatase activity [mU/mg protein] was measured using colorimetric method from whole cell extracts at day 9 and normalized with total protein content. The ALP activity was expressed as folds of 10^{-6} M dexamethasone treatment. The results are shown as mean ± S.D. of four determinations in each replication.

4.5 Determination of β-hexosaminidase released from RBL-2H3 cells

Effect of PN-E on enhanced anti-allergic activity of DR-W and PN-W was observed in both crude extract mixtures. Inhibitory effects of 1:1 mixture of PN-E/DR-W and 1:10 mixture of PN-E/PN-W on the release of β-hexosaminidase in RBL-2H3 cells and β-hexosaminidase activity were determined. As shown in Table 7, inhibition of antigen-induced degranulation in RBL-2H3 cells was observed in the mixtures of PN-E/DR-W and PN-E/PN-W with IC_{50} values of 28.7 and 23.8 μg/ml, respectively. The anti-allergic effects of these two mixtures were higher than that of DR-W, and PN-W alone, and higher than that of the longevity formulation (IC_{50} = 66.6 μg/ml).

Table 7. Inhibitory effects of 1:1 mixtures of selected crude extracts on the release of β-hexosaminidase in RBL-2H3 cells

Mixture	Inhibition (%) at various concentrations (μg/ml)					IC_{50} (μg/ml)	% Enzyme inhibition at 100 μg/ml
	0	3	10	30	100		
PN-E	0.0 ± 6.1	-14.4 ± 8.6	62.6 ± 6.5**	81.3 ± 4.4**	95.8 ± 1.0**	14.0	16.3
DR-W	0.0 ± 6.6	–	-2.3 ± 12.4	36.5 ± 9.5	84.9 ± 3.4**	40.8	34.0
PN-W	0.0 ± 4.4	–	–	22.4 ± 6.6	64.6 ± 0.2**	65.8	36.6
PN-E/DR-W	0.0 ± 4.7	-4.9 ± 1.7	9.2 ± 3.3	40.1 ± 4.8**	97.1 ± 0.4**	28.7	28.8
PN-E/PN-W	0.0 ± 7.3	–	-16.2 ± 15.2	76.7 ± 4.2**	111.1 ± 1.8**	23.8	23.3
Ketotifen fumarate	0.0 ± 6.9	–	12.8 ± 0.5	38.3 ± 3.2**	68.2 ± 1.5**	20.2	15.8

Each value represents the mean ± SEM. Significantly different from control, *$p < 0.05$, **$p < 0.01$.

Concentration of ketotifen fumarate 20.2 μg/ml was equivalent to 47.5μM.

CHAPTER 4

CONCLUSIONS

This study was focused on osteogenic and anti-allergic activities of Thai herbs in longevity herbal preparations. The crude water extracts and the ethanolic extracts of six herbs, i.e. *Albizia procera* (Roxb.) Benth. (AP), *Cyperus rotundus Linn.*(CR), *Diospyros rhodocalyx* Kurz. (DR), *Piper nigrum* Linn. (PN), *Streblus asper* Lour. (SA), and *Tinospora crispa* Linn. (TC), and the longevity formulation (LF), were prepared. Effects of the crude water (-W) and ethanolic (-E) extracts of these extracts on the ALP activity in MC3T3-E1 pre-osteoblastic cells (MC3T3-E1 cells) and inhibitory effects on the release of β-hexosaminidase in rat basophilic leukemia (RBL-2H3 cells) were determined. Effects of the crude extracts on viability of bone cells were evaluated using MTT assay. All crude extracts, except AP-E, were compatible to MC3T3-E1 cells at 3-100 μg/ml. Total protein content was dose-independent at day 9 of culture. High total protein was found in TC-W at 3 and 10 μg/ml as 0.56 and 0.59 mg/ml, respectively, and in DR-W at 3 μg/ml as 0.55 mg/ml. Alkaline phosphatase (ALP) activity in MC3T3-E1 cells conditioned with crude extracts was studied. The ALP activity of MC3T3-E1 cells by the treatment of these extracts was dose-independent. The crude extracts DR-W, TC-W, LF-W, SA-E, and TC-E each at 10 μg/ml and PN-W at 100 μg/ml revealed the higher ALP activity compared with effect of 10^{-6} M dexamethasone. DR-W was the most effective in osteogenesis. The ALP activity induced by DR-W at 10 μg/ml was higher than 1.5 folds of dexamethasone. Evaluation of anti-allergic activity of crude extracts by

determination of β-hexosaminidase released in RBL-2H3 cells confirmed potent anti-allergic activity of *Piper nigrum* ethanolic extract (PN-E) with an IC_{50} value of 14.0 μg/ml compared with 16.0 μg/ml of piperine. The data implied the anti-allergic activity of piperine in PN-E as an active constituent in the longevity herbal preparation. The finding supports the traditional use of *Piper nigrum* fruits for treatment of allergy and allergy-related diseases. Synergistic effect of PN-E on enhanced ALP activity was observed in the combination extracts of PN-E and DR-W or PN-W. The ALP activity of the 1:1 PN-E/DR-W and 1:10 PN-E/PN-W mixtures were found as 1.61 and 1.60 folds of 10^{-6} M dexamethasone, respectively. The anti-allergic effects of these mixtures on the inhibition of antigen-induced degranulation in RBL-2H3 cells were observed. The IC_{50} values of the mixtures of 1:1 PN-E/DR-W and 1:10 PN-E/PN-W were 28.7 and 23.8 μg/ml, respectively. This result indicated that PN-E enhanced anti-allergic activities of DR-W, PN-W, and LF-W. The anti-allergic activity of these mixtures was more potent than that of DR-W, and PN-W alone, and higher than that of the longevity formulation (IC_{50} = 66.6 μg/ml). The information from this study may be useful for further studies and development of this traditional medicine as modern products for treatment of allergy and bone-related diseases.

BIBLIOGRAPHY

Abe, E., Yamamoto, M., Taguchi, Y., Lecka-Czernik, B., O'Brien, C.A., Economides, A.N., Stahl, N., Jilka, R.L., and Manolagas, S.C. 2000. Essential requirement of BMPs2/4 for both osteoblast and osteoclast formation in murine bone marrow cultures from adult mice: antagonism by noggin. *Journal of Bone and Mineral Reserarch*. 15: 663-673.

Ahmad, Y.E., Maillet, P., Laurent, E., Talab, A., Teste, J.F., C6dat, M.J., Fiez-VandaU, P.Y., Dokhan, R. and Ollivier, R. 1997. New N-(benzhydryloxyalkyl)-4-(carboxy/carbamoylmethyl) piperidine derivatives with antidepressant activity. *Eur*opean. *Journal of Medicinal Chemistry*. 32: 205-218.

Akihisa, T., Takamine, Y., Yoshizumi, K., Tokuda, H., Kimura, Y., Ukiya, M., Nakahara, T., Yokochi, T., Ichiishi, E.and Nishino, H. 2002. Microbial transformations of two lupane-type triterpenes and anti-tumor-promoting effects of the transformation products. *Journal of Natural Products*. 65: 278-282.

Ali, A.T., Penny, C.B., Paiker, J.E., Psaras, G., Ikram, F., Crowther, N.J. 2006. The relationship between alkaline phosphatase activity and intracellular lipid accumulation in murine 3T3-L1 cells and human preadipocytes. *Analytical Biochemistry*. 354: 247-254.

Amin, A. and Hamza, A.A. 2005. Hepatoprotective effects of *Hibiscu*, *Rosmarinus and Salvia* on azathioprine-induced toxicity in rats. *Life Sciences*. 77: 266-278.

Aubin, J.E. and Liu, F. 1996. The osteoblast lineage. In: Bilezikian J, Raisz L, Rodan G, editors. Principles of bone biology. San Diego (CA, USA): *Academic Press*. 51–68.

Bajad, S., Bedi, K.L., Singla, A.K. and Johri, R.K. 2001. Piperine Inhibits Gastric Emptying and Gastrointestinal Transit in Rats and Mice. *Planta Medica*. 67: 176-179.

Ban, J.Y., Jeon, S.Y., Bae, K.W., Song, K.S. and Seong, Y.H. 2006. Catechin and epicatechin from *Smilacis chinae* rhizome protect cultured rat cortical neurons against amyloid β protein (25–35)-induced neurotoxicity through inhibition of cytosolic calcium elevation. *Life Sciences*. 79: 2251-2259.

Bandara, K.A.N.P., Kumar, V., Jacobsson, U., Molleyres, L.P. 2000. Insecticidal piperidine alkaloid from Microcos paniculata stem bark. *Phytochemistry*. 54: 29-32.

Biase, P.D. and Capanna, R. 2005. Clinical application of BMPs. *Injury International Journal of the care of the injured*. 36: 43-46.

Blanc, P.D., Kuschner, W.G., Katz, P.P., Smith, S. and Yelin, E.H. 1997. Use of herbal products, coffee or black tea, and over-the-counter medications as self-treatments among adults with asthma. *Journal of Allergy and Clinical Immunology*. 100: 789-791.

Bretaudière, J.P., Vassault, A., Amsellem, L., Pourci, M.L., Thieu-Phung, H., Bailly, M. 1977. Criteria for establishing a standardized method for determining alkaline phosphatase activity in human serum. *Clinical Chemistry*. 23: 2263–2274.

Bringmann, G., Saeb, W., Assi, L.A., François, G. and Sankara, A.S. 1997. Betulinic Acid: Isolation from *Triphyophyllum peltatum* and *Ancistrocladus heyneanus*, Antimalarial Activity, and Crystal Structure of the Benzyl Ester. *Natural Product Chemistry*. 63: 255-257.

Burkill, I. H. 1966. A Dictionary of the Economic Products of the Malay Peninsula. *Ministry of Agriculture and Co-operatives*. Kuala Lumpur. 1-2: 2444.

Centrella, M., Horowitz, M.C., Wozney, J.M. and Mccarthy, T.L. 1994. Transforming growth factor β (TGF-β) family members and bone. *Endocrine Research*. 15: 27–39.

Cerroni, L., Filocamo, R., Fabbri, M., Piconi, C., Caropreso, S. and Condo, S.G. 2002. Growth of osteoblast-like cells on porous hydroxyapatite ceramics: an in vitro study. *Biomolecular Engineering*. 19: 119-124.

Chatterjee R.K., Fatma, N., Murthy, P.K., Sinha, P., Kulshrestha, D.K., Dhawan, B.N. 1992. Macrofilaricidal activity of the stembark of *Streblus asper* and its major active constituents. *Drug Development Research*. 26: 67-78.

Chatterjee, S., Niaz, Z., Gautam, S., Adhikari, S., Variyar, P.S. and Sharma, A. 2007. Antioxidant activity of some phenolic constituents from green pepper (*Piper nigrum* L.) and fresh nutmeg mace (Myristica fragrans). *Food Chemistry*. 101: 515-523.

Chaturvedula, V.S., Schilling, J.K., Miller, J.S., Andriantsiferana, R., Rasamison, V.E. and Kingston, D.G. 2002. Two new triterpene esters from the twigs of *Brachylaena ramiflora* from the Madagascar rainforest. *Journal of Natural Products*. 65: 1222-1224.

Cheng, C.L., Guo, J.S., Luk, J. and Koo, M.W.L. 2004. The healing effects of Centella extract and asiaticoside on acetic acid induced gastric ulcers in rats. *Life Sciences*. 74: 2237-2249.

Cheng, S.L., Yang, J.W., Rifas, L., Zhang, S.F., and Avioli, L.V. 1994. Differentiation of human bone marrow osteogenic stromal cells *in vitro*: induction of the osteoblast phenotype by dexamethasone. *Endocrinology*. 134: 277-286.

Choi, B.H., Kim, C.G., Lim, Y., Shin, S.Y. and Lee, Y.H. 2008. Curcumin down-regulates the multidrug-resistance mdr1b gene by inhibiting the P13K/Akt/NFκB pathway. *Cancer Letters*. 259: 111-118.

Choi, E.M., Suh, K.S., Kim. Y.S., Choue, R.W. and Koo. S.J. 2001. Soybean ethanol extract increases the function of osteoblastic MC3T3-E1 cells. *Phytochemistry* 56: 733-739.

Choi, E.M. 2005. The licorice root derived isoflavan glabridin increase the function of osteoblastic MC3T3-E1 cells. *Biochemical Pharmacology*. 70: 363-368.

Chung, C.Y., Klein, A.I., Wyatt, L.E., Rudkin, G.H., Ishida, K., Yamaguchi, T. and Miller, T.A. 1999. Serial passage of MC3T3-E1 cells alters osteoblastic function and responsiveness to transforming growth factor-β1 and bone morphogenetic protein-2. *Biochemical and Biophysical Research Communications*. 265: 246-251.

Clark-Greuel, J.N., Connolly, J.M., Sorichillo, E., Narula, N.R., Rapoport, H.S., Mohler III, E.R., Gorman III, J.H., Gorman, R.C. and Levy, R.J. 2007. Transforming growth factor-β1 mechanisms in aortic valve calcification:

Increased alkaline phosphatase and related events. *The Annals of Thoracic Surgery*. 83: 946-953.

Clause, E.P., Tyler, V.E. and Brady, L.R. 1973. Textbook of Pharmacognosy, Lea and Febiger, Philadelphia.

Da Silva Meirelles, L., Chagastelles, P.C., Nardi, N.B. 2006. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *Journal of Cell Science*. 119: 2204-2213.

Das, M.K. and Beuria, M.K. 1991. Anti-malarial property of an extract of the plant *Streblus asper* in murine malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. 85: 40-41.

Delaporte, R.H., Sarragiotto, M.H., Takemura, O.S., Sanchez, G.M., Filho, B.P. and Nakamura, C.V. 2004. Evaluation of the anti-edematogenic, free radical scavenging and antimicrobial activities of aerial parts of *Tillandsia streptocarpa* Baker-Bromeliaceae. *Journal of Ethnopharmacology*. 95: 229–233.

Dorman, H.J. and Deans, S.G., 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *Journal of Applied Microbiology*. 88: 308-316.

Doublet, M.O., Olomucki, A., Baici, A. and Luisi, P.L. 1975. Investigations on the kinetic mechanismof octopine dehydrogenase. *European Journal of Biochemistry*. 59: 185-191.

Duke, J.A. 1992. A Handbook of biologically active phytochemicals and their activities, CRC Press, Ann Arbor. pp. 17, 65, 101, 122, 171.

Dweck, A.C. and Cavin, J.P. 2004. Andawali (*Tinospora crispa*)-a review. 1-7.

Ecarot-Charrier, B., Shepard, N., Charette, G., Grynpas, M. and Glorieux, F.H. 1988. Mineralization in osteoblast cultures: A light and electron microscopic study. *Bone*. 9:147-154.

El, H.R., Idaomar, M., Alonso, M.A. and Munoz, S.A., 2003. Antimutagenic properties of bell and black peppers. *Food and Chemical Toxicology*. 41: 41-47.

Enwerem, N.M., Okogun, J.I., Wambebe, C.O., Okorie, D.A. and Akah, P.A. 2001. Anthelmintic activity of the stem bark extracts of *Berlina grandiflora* and one of its active principles, Betulinic acid. *Phytomedicine*. 8: 112–114.

Epstein, E., Keichie, F.L., Zak, B. 1985. Alkaline phosphatase isoenzymes. *Clinical Chemistry News*. l1: 6-7.

Erazo, S., Delporte, C., Negrete, R., Garc, R., Zald´ıvar, M., Iturra, G., Caballero, E., L´opez, J.L. and Backhouse, N. 2006. Constituents and biological activities of *Schinus polygamus*. *Journal of Ethnopharmacology*. 107: 395–400.

Factor, P. 2003. Gene therapy for asthma. *Molecular Therapy*. 7: 148–152.

Faraji, M.H., Tarkhani, A.H.H. 1999. The effect of sour tea (*Hibiscus sabdariffa*) on essential hypertension. *Journal of Ethnopharmacology*. 65: 231-236.

Ferreira, S. R. S. 1996. Cinetica de Transfer^encia de Massa na Extrac~ao Supercritica deOOleo Essencial de Pimenta-do-reino. Ph.D. *Thesis, UNICAMP*, *Campinas*; 218

Fleischer, G.A., Eickelberg, E.J., Elveback, L.R. 1977. Alkaline phosphatase activity in the plasma of children and adolescents. *Clinical Chemistry*. 23: 469–472.

Francescchi, R.T., and Iyer, B.S. 1992. Relationship between collagen synthesis and expression of the osteoblast phenotype in MC3T3-E1 cells. *Journal of Bone and Mineral Research*. 7: 235-246.

Fujioka, T., Kashiwada, Y., Kilkuskie, R.E., Cosentino, L.M., Ballas, L.M., Jiang, J.B., Janzen, W.P., Chen, I.S. and Lee, K.H. 1994. Anti-AIDS agents. 11. Betulinic acid and platanic acid as anti-HIV principles from *Syzigium claviflorum* and the anti-HIV activity of structurally related triterpenoids. *Journal of Natural Product*s. 57: 243–247.

Gaitonde, B., Vaz, A., Patel, J., 1964. Chemical and pharmacological study of root bark of *Streblus asper*. *Indian Journal of Medical Sciences* 18, 191–199.

Geetha, T., Garg, A., Chopra, K. and Kaur, I.P. 2004. Delineation of antimutagenic activity of catechin, epicatechin and green tea extract. *Mutation Research/ Fundamental and Molecular Mechanisms of Mutagenesis*. 556: 65-74.

Geetha, T. and Varalakshmi, P. 1998. Anti-inflammatory activity of lupeol and lupeol linoleate in adjuvant-induced arthritis, *Fitoterapia*. 69: 13–19.

Geetha, T, Varalakshmi, P, Marylatha, R.1998. Effect of triterpene from *Crataeva nurvala* stem bark on lipid peroxidation in adjuvant induced arthritis in rats. *Pharmacology Research*. 37: 191–195.

Gereltu, B., Nashengsang, Jingzhuang, Zhao, R.G., Gao, J., Songlin. 2004. Applictions of piperamide compounds in preparation of anti-hyperlipidemic drug or health dietary. Patent NO, ZL.

Glasby, J.S., 1991. Dictionary of Plants containing secondary metabolites. London Taylor and Francis. pp. 307.

Gomes, W.A. and Kessler, J.A. 2001. Msx-2 and p21 mediate the pro-apoptotic but not the anti-proliferative effects of BMP4 on cultured sympathetic neuroblasts. Developmental Biology. 237: 212–221.

G´omez, M., Saenz, M., Garc´ıa, M., Fern´andez, M., 1999. Study of topical anti-inflammatory activity of *Achillea ageratum* on chronic and acute inflammation models. *Zeitschrift fur Naturforschung*. 54: 937–941.

Guerreiro, S., Monteiro, R., Martins, M.J., Calhau, C., Azevedo, I., Soares, R. 2007. Distinct modulation of alkaline phosphatase isoenzymes by 17b-estradiol and xanthohumol in breast cancer MCF-7 cells. *Clinical Biochemistry* 40:268-273.

Guevara, A.P., Amor. E. and Russel,G. 1996. Antimutagenic from *Plumeria acuminata*. *Mutation Research*. 361: 67–72.

Gugala, Z. and Gogolewski, S. 2004. Protein adsorption, attachment, growth and activity of primary rat osteoblasts on polylactide membranes with defined surface characteristics. *Biomaterials*. 25: 2341-2351.

Gundle, R., Joyner, C.L. and Triffitt, J.T.1995. Human bone tissue formation in diffusion chamber culture in vivo by bone-derived cells and marrow stromal fibroblastic cells. *Bone*. 16: 597-601.

Gupta, M. B., Bhalla, T. N., Gupta, G. P., Mitra, C. R. and Bhargava, K. P. 1969. Anti-inflammatory activity of natural products (I) triterpenoids *European Journal of Pharmacology*. 6: 67-70.

Gupta, M.B., Palit, T.K., Singh, N., Bargawa, K.P., 1971. *Indian Journal of Medicine Research*. 59:76-82.

Gupta, M.B., Nath, R. Srivastava, N. Shanker, K., Kishor, K. and Bhargava, K.P. 1980. Anti-inflammatory and antipyretic activities of beta-sitosterol, *Planta Medicine*. 39: 157–163.

Gupta, Y.K., Kumar, M.H.V. and Srivastava, A.K. 2003. Effect of Centella asiatica on pentylenetetrazole-induced kindling, cognition and oxidative stress in rats. *Pharmacology Biochemistry and Behavior*. 74: 579-585.

Gutermuth, J., Bewersdorff, M., Traidl-Hoffmann, C., Ring, J., Mueller, M.J., Behrendt, H. and Jakob, T. 2007.Immunomodulatory effects of aqueous birch pollen extracts and phytoprostanes on primary immune responses *in vivo*. *Journal of Allergy Clinic Immunology*. 120: 293-299.

Harrris, H. 1989. The human alkaline phosphatases: what we know and what we don't know. *Clinical Oral Implants Research*. 7:38-47.

Hartwell, S.K., Somprayoon, D., Kongtawelert, P., Ongchai, S., Arppornchayanon, O., Ganranoo, L., Lapanantnoppakhun, S. and Grudpan, K. 2007. Online assay of bone specific alkaline phosphatase with a flow injection-bead injection system. *Analytica Chimica Acta*. 600: I88-I93.

Hashim, M.S. and Devi, K.S. 2003. Insecticidal action of the polyphenolic rich fractions from the stem bark of *Streblus asper* on *Dysdercus cingulatus*. *Fitoterapia*. 74: 670-676.

Hashimoto, F., Kashiwada, Y., Cosentino, L.M., Chen, C.H., Garrett, P.E. and Lee, K.H. 1997. Anti-AIDS agents—XXVII. Synthesis and anti-HIV activity of betulinic acid and dihydrobetulinic acid derivatives. *Bioorganic & Medicinal Chemistry*. 5: 2133– 2143.

Hata, K., Hori, K. and Takahashi, S. 2002. Differentiation- and Apoptosis-Inducing Activities by Pentacyclic Triterpenes on a Mouse Melanoma Cell Line. *Journal of Natural Products*. 65: 645-648.

Huh, J.E., Yang, H.R., Park, D.S., Choi, D.Y., Baek, Y.H., Cho, E.M., Cho, Y.J., Kang, K., Kim, D.Y. and Lee, J.D. 2006. Puerariae radix promotes differentiation and mineralization in human osteoblast-like SaOS-2 cells. *Journal of Ethnopharmacology*. 104: 345-350.

Ikawati, Z., Wahyuono, S. and Maeyama, K. 2001. Screening of several Indonesian medicinal plants for their inhibitory effect on histamine release from RBL-2H3 cells. *Journal of Ethnopharmacology*. 75: 249-256.

Iu, M., Kaji, H., Sowa, H., Naito, J., Sugimoto, T. and Chihara, K. 2005. Dexamethasone suppresses Smad3 pathway in osteoblastic cells. *Endocrine*. 185: 131-138.

Jagtap, A.G., Shirke, S.S. and Phadke, A.S. 2004. Effect of polyherbal formulation on experimental models of inflammatory bowel diseases. *Journal of Ethnopharmacology*. 90: 195–204.

Jeong, J.C., Lee, J.W., Yoon, C.H., Lee, Y.C., Chung, K.H., Kim, M.G. and Kim, C.H. 2005a. Stimulative effects of *Drynariae Rhizoma* extracts on the proliferation and differentiation osteoblastic MC3T3-E1 cells. *Journal of Ethnopharmacology*. 96: 489-495.

Jeong, J.C., Lee, J.W., Yoon, C.H., Kim, H.M. and Kim, C.H. 2005b. Effect of *Drynariae Rhizoma* on the proliferation of human bone cells and the immunomodulatory activity. *Pharmaceutical research*. 51: 125-136.

Jeong, J.C., Lee, J.W., Yoon, C.H., Kim, H.M. and Kim, C.H. 2004. *Drynariae Rhizoma* promotes osteoblast differentiation and mineralization in MC3T3-E1 cells through regulation of bone morphogenetic protein-2, alkaline phosphatase, type I collagen and collagenase-1. *Toxicology in Vitro*. 18: 829-834.

Jung, B.S. and Shin, M.K. 1998. Encyclopedia of illustrated Korean natural drugs, Araliaceae. In: Kwon, S.B. (Ed.), Young Lim Sa, Seoul: 439–443.

Juteau, F., Masotti, V., Bessie`re, J.M. 2002. Antibacterial and antioxidant activities of *Artemisia annua* essential oil. *Fitoterapia*. 73: 532–535.

Kang, J. 2007. The strong correlation between alkaline phosphatase activity and cell viability. *Cell Biology International*. 1-2.

Kaskiw, M.J., Tassotto, M.L., Th'ng, J. and Jiang, Z.H. 2008. Synthesis and cytotoxic activity of diosgenyl saponin analogues. *Bioorganic & Medicine Chemistry*. 16: 3209-3217.

Kempuraj, D., Huang, M., Kandere-Grzybowska, K., Basu, S., Boucher, W., Letourneau, R. 2003. Azelastine inhibits secretion of IL-6, TNF-alpha and IL-8 as well as NF-kappa B activation and intracellular calcium ion levels in normal human mast cells. *International Archives of Allergy and Immunology*. 132: 231-239.

Kim, J.H., Kang, N.J., Lee, B.K., Lee, K.W. and Lee, J. 2008. Gallic acid, a metabolite of the antioxidant propyl gallate, inhibits gap junctional intercellular communication via phosphorylation of connexin 43 and

extracellular-signal-regulated kinase1/2 in rat liver epithelial cells. *Mutation Research*. 638: 175-183.

Kim, S.I., Jeong, Y.I., Jung, I.D., Lee, J.S., Lee, C.M., Yoon, M.S., Seong, E.Y., Kim, J.I., Lee, J.D. and Park, Y.M. 2007. *p*-coumaric acid inhibits indoleamine 2, 3-dioxygenase expression in murine dendritic cells. *International Immunopharm*aceutical. 7: 805–815.

Kishikawa, M. and Sakae, M. 1997. Herbal medicine and the study of aging in senescence-accelerated mice (SAMP1TA/Ngs). *Experimental Gerontology*. 32: 229-242.

Kiuchi, F., Nakamura, N., Tsuda, Y., Kondo, K., Yoshimura, H., 1988. Studies on crude drugs effective on visceral larvar migrans. IV. Isolation and identification larvicidal principles in pepper. *Chemistry Pharm*aceutical *Bull*. 36: 2452–2465.

Kondo, K., Hirano, R., Matsumoto, A., Igarashr, O. and Ltahura, H. 1996. Inhibition of LDL oxidation by cocoa. *The Lancet*. 348: 1514.

Kongkathip, N., Dhumma-upakorn, P., Kongkathip, B., Chawananoraset, K., Sangxhomkaeo, P. and Hatthakitpanichakul, S. 2002. Study on cardiac contractility of cycloeucalenol and cycloeucalenone isolated from *Tinospora crispa*. *Journal of Ethnopharmacology*. 83: 95-99.

Kritsaneepaiboon, S. 1989. Effect of plant extracts on insects. *Songklanagarin Journal of Science and Technology*. 11: 107–112.

Kuo, Y.H., Chang, C.I. and Kuo, Y.H. 1997. Triterpenes from *Diospyros maritime*. *Phytochemistry*. 46: 1135-1137.

Kvasnica, M., Sarek, J., Klinotova, E., Dzubak, P. and Hajduch, M. 2005. Synthesis of phthalates of betulinic acid and betulin with cytotoxic activity. *Bioorganic & Medicine Chemistry*. 13: 3447–3454.

Kweifio-Okio, G., Field, B., Ruble, B.A., Macrides, T.A., Demunk, F. 1995. Esterfication improves antiarthritic effectiveness of lupeol. *Drug Development Research*. 35: 137–141.

Lam, S.K. and Ng, T.B. 2002. A xylanase from roots of sanchi ginseng (*Panax notoginseng*) with inhibitory effects on human immunodeficiency virus-1 reverse transcriptase. *Life Sciences*. 70: 3049-3058.

Lam, T.L., Lam, M.L., Au, T.K., Ip, D.T.M., Ng, T.B., Fong, W.P. and Wan, D.C.C. 2000. A comparison of human immunodeficiency virus type-1 protease inhibition activities by the aqueous and methanol extracts of Chinese medicinal herbs. *Life Sciences*. 67: 2889-2896.

Lecoeur, L. and Ouhayoun, J.P. 1997. In vitro induction of osteogenid differentiation from non-osteogenic mesenchymal cells.*Biomaterials*. 18: 989-993.

Lee, C.Y. and Kim, S.H. 2008. Immunomodulatory effect of Juglans sinensis, Psoralea corylifolia, Cheong-a-hwan extract and cyclosporine A on Th1(IFN-g)/Th2(IL-4) cytokine balance, eosinophil accumulation in a murine model. *Phytochemistry Letter*. 1: 6-10.

Lee, J.H., Seo, J.Y., Ko, N.Y., Chang, S.H., Her, E., Park, T., Lee, H.Y., Han, J.W., Kim,Y.M. and Choi, W.S. 2004. Inhibitory activity of *Chrysanthemi sibirici* herbal extract on RBL-2H3 mast cells and compound 48/80 induced anaphylaxis. *Journal of Ethnopharmacology*. 95: 425-430.

Lee, S.H., Bae, E.A., Park, E.K., Shin, Y.W., Baek, N.I., Han, E.J., Chung, H.G. and Kim, D.H. 2007. Inhibitory effect of eupatilin and jaeosidin isolated from Artemisia princes in IgE-induced hypersensitivity. *International Immunopharm*. 7: 1678-1684.

Lewis, W. 1980. Plants used as chewing sticks. *Journal of Preventive Dentistry* 6, 71–73.

Li, F., Goila-Gaur, R., Salzwedel, K., Kilgore, N.R., Reddick, M., Matallana, C. Castillo, Zoumplis, A.D., Martin, D.E., Orenstein, J.M., Allaway, G.P., Freed, E.O. and Wild, C.T. 2003. PA-457: a potent HIV inhibitor that disrupts core condensation by targeting a late step in gag processing. *Product Natural Academic Science USA*. 100: 13555–13560.

Li, J., Liu, Y. an Ru, B. 2005. Effect of metallothionein on cell viability and its interactions with cadmium and zinc in HEK293 cells. *Cell Biology International*. 29: 843-848.

List, P.H. and Horhammer, L. 1969–1979. Hager's handbuch der pharmazeutischen praxis. vols 2–6. Springer-Verlag, Berlin.

Lui, W., Ho, J.C.K., Cheung, F.W.K., Liu, B.P.L., Ye, W. and Che, C. 2004. Apostotic activity of betulinic acid derivatives on murine melanoma B 16 cell lines. *Journal of Ethnopharmacology*. 498: 71-78.

Mallavadhani, U.V., Panda, A.K. and Rao, Y.R. 1998. Review article number 134 pharmacology and chemotaxonomy of diospyros. *Phytochemistry*. 49: 901-951

Maniatopoulos, C., Sodek, J., and Melcher, A.H. 1988. Bone formation *in vitro* by stromal cells obtained from bone marrow of young adult rats. *Cell Tissue Research*. 254: 317-330.

Marston, A. and Hostettmann, K. 1984. High-performance liquid chromatography of some naturally occurring naphthoquinones. *Journal of Chromatography A*. 295: 526-529.

Martins, A.P., Salgueiro, L., Vila, R., Tomi, F., Canigueral, S., Casanova, J., Cunha, A.P.D. and Adzet, T. 1998. Essential oils from four piper species. *Phytochemistry*. 49: 2019-2023.

Mathabe, M.C., Hussein, A.A., Nikolova, R.V., Basson, A.E., Meyer, J.J.M. and Lall, N. 2008. Antibacterial activities and cytotoxicity of terpenoids isolated from *Spirostachys africana*. *Journal of Ethnopharmacology*. 116: 194-197.

Matsubara, K., Saito, A., Tanaka, A., Nakajima, N., Akagi, R., Mori, M. and Mizushina, Y. 2007. Epicatechin conjugated with fatty acid is a potent inhibitor of DNA polymerase and angiogenesis. *Life Sciences*. 80: 1578-1585.

Matsuda, H., Tewtrakul, S., Morikawa, T. and Yoshikawa, M. 2004a. Anti-allergic activity of stilbenes from *Korean rhubarb* (*Rheum undulatum* L.): structure requirements for inhibition of antigen-induced degranulation and their effects on the release of TNF-α and IL-4 in RBL-2H3 cells. *Journal of Bioorganic Medicine Chemistry*. 12: 4871-4876.

Matsuda, H., Tewtrakul, S., Morikawa, T., Nakamura, A. and Yoshikawa, M. 2004b. Anti-allergic principles from Thai zedoary: structural requirements of curcuminoids for inhibition of degranulation and effect on the release of TNF-

α and IL-4 in RBL-2H3 cells. *Journal of Bioorganic Medicine Chemistry*. 12: 5891-5898.

Melek, F.R., Miyase, T., Ghaly, N.S. and Nabil, M. 2007. Triterpenoid saponins with *N*-acetyl sugar from the bark of *Albizia procera*. *Phytochemistry*. 68: 1261-1266.

Melendez, R.F., Kumar, N., Maswadi, S.M., Zaslow, K. and Glickmank, R.D. 2004. Photodynamic actions of indocyanine green and trypan blue on human lens epithelial cells in vitro. *American Journal of Ophthalmology*. 140: 132-134.

Miyakado, M., Nakayam, I., Yoshioka, H. and Nakatani, N. 1979. The piperaceae amides I: structure of pipercide, a new insecticidal amide from *Piper nigrum* L. *Agricology Biology Chemistry*. 43: 1609–1611.

Morimoto, M., Fujii, Y. and Komai, K. 1999. Antifeedants in Cyperaceae: coumaran and quinones from Cyperus spp. *Phytochemistry*. 51: 605-608.

Moss, D.W. 1982. Alkaline phosphatase isoenzymes [Review]. *Clinic Chem*istry. 28: 2007-2016.

Mouries, L.P., Almeida, M.J., Milet, C., Berland, S. and Lopez, E. 2002. Bioactivity of nacre water-soluble organic matrix from the bivalve mollusk *Pinctada maxima* in three mammalian cell types: fibroblasts, bone marrow stromal cells and osteoblasts. *Biochemistry and Molecular Biology*. 132: 217-229.

Mühlbauer, R.C., Lozano, A., Palacio, S., Reinli, A. and Felix, R. 2003. Common herbs, essential oils, and monoterpenes potently modulate bone metabolism. *Bone*. 32: 372-380.

Mujumdar, A.M., Dhuley, J.N., Deshmukh, V.K., Raman, P.H. and Naik, S.R. 1990. Anti-inflammatory activity of piperine. *Japan Journal of Medicine Sci*ence *Biology*. 43: 95–100.

Mukherjee, K. and Roy, L., 1983. Chemical examination of *Streblus asper* leaves. *International Journal of Crude Drug Research*. 21: 189–190.

Nadkarni, K.M. 1976. Indian materia medica. Bombay: The Karnataka Printing Press and The Popular Press Ltd. 1171.

Nagaraj, M., Sunitha, S. and Varalakshmi, P. 2000. Effect of lupeol, a pentacyclic triterpene, on the lipid peroxidation and antioxidant status in rat kidney after chronic cadmium exposure. *Journal of Apply Toxicology*. 20: 413–417.

Nakatani, K., Atsumi, M., Arakawa, T., Oosawa, K., Shimura, S., Nakahata, N. and Ohizumi, Y. 2002. Inhibitions of histamine release and prostaglandin E_2 synthesis by mangosteen, a thai medicinal plant. Journal of *Pharmacology Society of Japan*. 25: 1137-1140.

Nashed, B., Yeganeh, B., HayGlass, K.T., Moghadasian, M.H. 2005. Antiatherogenic effects of dietary plant sterols are associated with inhibition of proinflammatory cytokine production in Apo E-KO mice. *Journal of Nutrition*. 135: 2438–2444.

Navarro, A., De las Heras, B. and Villar, A. 2001. Anti-inflammatory and immunomodulating properties of a sterol fraction from Sideritis foetens Clem. *Biology and Pharmaceutical Bulletin*. 24: 470–473.

Navickene, H.M.D., Alecio, A.C., Kato, M.J., Bolzani, V.S., Young, M.C.M., Cavalheiro, A.J., Furlan, M., 2000. Antifungal amides from *Piper hispidum* and *Piper tuberculatum*. *Phytochemistry*. 55: 621–626.

NCEP, 2001. Executive summary of the third report of the national cholesterol education program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (adult treatment panel III). *JAMA*. 285: 2486–2497.

Noda, M. and Camilliere, J.J. 1989. In vivo stimulation of bone formation by transforming growth factor-beta. *Endocrine*. 124: 2991-2994.

Noor, H. and Ashcroft, S.J.H. 1998. Pharmacological characterisation of the antihyperglycaemic properties of *Tinospora crispa* extract. *Journal of Ethnopharmacology*. 62: 7-13.

Novak, N., Haberstok, J., Kraft, S., Siekmann, L., Allam, J.P. and Bieber, T. 2001. Standardized extracts from Chinese herbs induce IL-10 production in human monocyte-derived dendritic cells and alter their differentiation in vitro. *Journal of Allergy and Clinical Immunology*. 108: 588-593.

Okamoto, T., Seo, H., Mano, H., Furuhashi, M., Got, S., Tomoda, Y., 1990. Expression of human placental alkaline phosphatase in placenta during pregnancy. *Placenta*.11: 319–327.

Oshina, H., Sotome, S., Yoshii, t., Torigoe, I., Sugata, Y., Maehara, H., Marukawa, E., Omura, K., and Shinomiya, K. 2007. Effects of continuous dexamethasone treatment on differentiation capabilities of bone marrow-derived mesenchymal cells. *Bone*. 41: 575-583.

Otsuka, E., Yamaguchi, A., Hirose, S., and Hagiwara, H. 1999. Characterization of osteoblastic differentiation of stromal cell line ST2 that is induced by ascorbic acid. *Journal of Physiology*. 277: C132-C138.

Owen, C.E. 2007. Immunoglobulin E: role in asthma and allergic disease: lessons from the clinic. *Pharmacology Therapy*. 113: 121-133.

Ozaka, K., Szallasi, Z., Kazanietz, M.G., Blumberg, P.M., Mischak, H., Mushinski, J.F. and Beaven, M.A. 1993. Ca^{2+}-dependent and Ca^{2+}-independent isozymes of protein kinase C mediate exocytosis in antigen-stimulated rat basophilic RBL-2H3 cells: reconstitution of secretory responses with Ca^{2+} and purified isozymes in washed permeabilized cells. *Journal of Biology Chemistry*. 268: 1749-1756.

Park, E.H., Kahng, J.H., Lee, S.H., Shin, K.H., 2001. An anti-inflammatory principle from cactus. *Fitoterapia*. 72: 288–290.

Park, I.K., Lee, S.G., Shin, S.C., Park, J.D., Ahn, Y.J., 2002. Larvicidal activity of isobutylamides identified in Piper nigrum fruits against three mosquito species. *Journal Agricology of Food Chemistry*. 50: 1866–1870.

Parmar, V.S., Jain, S.C., Bisht, K.S., Jain, R., Taneja, P., Jha, A., Tyagi, O.D., Prasad, A.K., Wengel, J., Olsen, C.E., Boll, P.M., 1997. Phytochemistry of the genus Piper. *Phytochemistry*. 46: 597–673.

Pathak, S.K., Jain, D.C. and Sharma, R.P., 1995. Chemistry and biological activities of the Genera Tinospora, a review. *International Journal of Pharmacuetics*. 33: 277-287.

Pannala, A., Rice-Evans, C.A., Halliwell, B. and Singh, S.1997. Inhibition of peroxynitrite-mediated tyrosine nitration by catechin polyphenols. *Biochem and Biophysical Research Communications*. 232: 164-168.

Pavlova, N.I., Savinovaa, O.V., Nikolaevaa, S.N., Borekoa, E.I. and Flekhterb, O.B. 2003. Antiviral activity of betulin, betulinic and betulonic acids against some enveloped and non-enveloped viruses. *Fitoterapia*. 74: 489–492.

Perry, L.M. 1980. Medicinal Plants of East and Southeast Asia: Attributed Properties and Uses. *The MIT Press*. Cambridge, England. 620.

Phengklai, C. 1978. Ebenaceae of Thailand. Thai Forest Bulletin (Botany). Vol. 11. *Edited by* Forest Herbarium, Royal Forest Department, Bankok. Published by the Secretariat of the Cabinet Printing Office. pp. 1-103.

Pizauro, J.M., Ciancaglini, P.and Leone, F.A. 1992. Phosphotransferase activity associated with rat osseous plate alkaline phosphatase: a possible role in biomineralization. *Internation of Journal Biochemistry*. 24: 1391–1396.

Plaut, M., Pierce, J.H., Whatson, C., Hanley-Hyde, J., Nordan, R.P., Paul, W.E. 1989. Mast cell lines produce lymphokines in response to cross-linkage of Fc epsilon RI or to calcium ionophore. *Nature*. 339: 64-67.

Quaroni, A. and Hochman, J. 1996. Development of intestinal cell culture models for drug transport and metabolism studies. *Advanced Drug Delivery Reviews*. 22: 3-52.

Rahman, N.N.N.A., Furuta, T., Kojima, S., Takane, K. and Mohd, M.A. 1999. Antimalarial activity of extracts of Malaysian medicinal plants. *Journal of Ethnopharmacology*. 64: 249–254.

Ravindran, P.A. 2001. Black Pepper, *Piper nigrum*. Medicinal and Aromatic Plants-Industrial Profiles. Harwood Academic Publishers, The Netherlands, Book review. *Phytochemistry*. 58: 827-829.

Raut, N.A. and Gailwad, N.J. 2006. Antidiabetic activity of hydro-ethanolic extract of Cyperus rotundus in alloxan induced diabetes in rats. *Fitoterapia*. 77: 585-588.

Raut, N.A. and Gaikwad, N. J. 2006. Antidiabetic activity of hydro-ethanolic extract of *Cyperus rotundus* in alloxan induced diabetes in rats. *Fitoterapia*. 77: 585–588.

Recio, M., Giner Recio, R.M., Manez, S. and Rios, J.L. 1995. Structural requirements for the anti-inflammatory activity of natural triterpenoids. *Planta Medicine*. 6: 182–185.

Reddy, S.V., Srinivas, P.V., Praveen, B., Kishore, K.H., Raju, B.C., Murthy, U.S. and Rao, J.M. 2004. Antibacterial constituents from the berries of *Piper nigrum*. *Phytomedicine*. 11: 697–700.

Ribeiro, T.S., Freire-de-Lima, L., Previato, J.O., Mendonca-Previato, L., Heise, N. and Lima, M.E.F. 2004. Toxic effects of natural piperine and its derivatives on epimastigotes and amastigotes of *Trypanosoma cruzi*. *Bioorganic & Medicine Chemistry Letter*. 14: 3555-3558.

Rickard, D.J., Sullivan, T.A., Shenker, B.J., Leboy, P.S. and Kazhdan, I. 1994. Induction of rapid osteoblast differentiation in rat bone marrow stromal cell cultures by dexamethasone and BMP-2. *Development Biological*. 161: 218-228.

Risteli, L. and Risteli, J. 1993. Biochemical markers of bone metabolism. *Annals Medicine*. 25: 385-393.

Rodan, G.A. and Rodan, S.B. 1983. Expression of the osteoblastic phenotype. In: Peck, W.A., ed. *Bone and mineral research*. Annual. 2: 244-285.

Rosa, A.L. and Beloti, M.M. 2003. Effect of cp Ti surface roughness on human bone marrow cell atteachment, proliferation, and differentiation. *Journal of Brazil Dentis*. 14: 16-21.

Sabokbar, A., Millertt, P.J., Myer, B. and Rushton, N. 1994. A rapid, quantitative assay for measuring alkaline phosphatase activity in osteoblastic cells in vitro. *Bone and Mineral*. 27: 57-67.

Saleem, M., Alam, A., Arifin, S., Shah, M.S., Ahmed, B. and Sultana, S. 2001. Lupeol, a triterpene, inhibits early responses of tumor promotion induced by benzoyl peroxide in murine skin. *Pharmacology*. *Research*. 43: 127–134.

Sanbongi, C., Suzuki, N. and Sakane, T Sakane. 1997. Polyphenols in chocolate, which have antioxidant activity, modulate immune functions in humans *in vitro*. *Cellular Immunology*. 177: 129-136.

Schroeter, H., Williams, R.J., Matin, R., Iversen, L. and Rice-Evans, C.A. 2000. Phenolic antioxidants attenuate neuronal cell death following uptake of oxidized low-density lipoprotein. *Free Radical Biology and Medicine*. 29: 1222-1233.

Sharma, R. and Gupta, R. 2007. *Cyperus rotundus* extract inhibits acetylcholinesterase activity from animal and plants as well as inhibits

germination and seedling growth in wheat and tomato. *Life Sciences*. 80: 2389-2392.

Shi, Y.C., Worton, L., Esteban, L., Baldock, P., Fong, C., Eisman, J.A. and Gardiner, E.M. 2007. Effects of continuous activation of vitamin D and Wnt response pathways on osteoblastic proliferation and differentiation. *Bone*. 41: 87-96.

Shinwari, M.I. and Khan, M.A. 2000. Folk use of medicinal herbs of Margalla Hills National Park, Islamabad. *Journal of Ethnopharmacology*. 69: 45-56.

Siddiqui, B.S., Begum, S., Gulzar, T., Noor, F. 1997. An amide from fruits of *Piper nigrum*. *Phytochemistry*. 45: 1617-1619.

Singh, B., Sahu, P.M. and Sharma, M.K. 2002. Anti-inflammatory and antimicrobial activities of triterpenoids from *Strobilanthes callosus* Nees. *Phytomedicine*. 9: 355–359.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. 1985. Measurement of protein using bicinchoninic acid. *Analytical Biochemistry*. 150:76-85.

Sowa, H., Kaji, H, Yamaguchi, T., Sugimoto, T. and Chihara, K. 2002. Smad3 promotes alkaline phosphatase activity and mineralization of osteoblastic MC3T3-E1 cells. *Journal of Bone Mineral Research*. 17: 1190-1199.

Spelsberg, T.C., Subramaniam, M., Riggs, B.L., Khosla, S. 1999. The actions and interactions of sex steroids and growth factors/cytokines on the skeleton. *Molecular Endocrinology*. 13: 819–828.

Spencer, J.P.E., Schroeter, H., Kuhnle, G., Kaila, S., Srai, S., Tyrrell, R.M., Hahn, U. and Rice-Evans, C. 2001. Epicatechin and its in vivo metabolite, 3'-O-methyl epicatechin, protect human fibroblast from oxidative-stress-induced cell death involving caspase-3 activation. *Journal of Biochemistry*. 354: 493-500.

Srivastava, K.D., Kattan, J.D., Zou, Z.M., Li, J.H., Zhang, L., Wallenstein, S., Goldfarb, J., Sampson, H.A. and Li, X.M. 2005. The Chinese herbal medicine formula FAHF-2 completely blocks anaphylactic reactions in a murine model of peanut allergy. *Journal of Allergy and Clinical Immunology*. 115: 171-178.

Steele, J.C.P. Warhurst, D.C., Kirby, G.C. and Simmonds, M.S.J. 1999. In vitro and in vivo evaluation of betulinic acid as an antimalarial. *Phytotherapy Research*. 13: 115–119.

Stein, G.S., Lian, J.B. and Owen, T.A. 1990. Relationship of cell growth to the regulation of tissue-specific gene expression during osteoblas differentiation. *FASEB Journal*. 4: 3113-3123.

Stevens, R.L. and Austen, K.F. 1989. Recent advances in the cellular and molecular biology of mast cells. *Immunology Today*. 10: 381-386. Review.

Sun, I.C., Shen, J.K., Wang, H.K., Cosentino, L.M. and Lee, K.H. 1998. Anti-aids agents. 32.1 synthesis and anti-HIV activity of betulin derivatives. *Bioorganic & Medicine Chemistry Letters*. 8: 1267-1272.

Sun, J.S., Lin, C.Y., Dong, G.C., Sheu, S.Y., Lin, F.H., Chen, L.T. and Wang, Y.L. 2002. The effect of Gu-Sui-Bu (Drynaria fortunei J. Sm) on bone cell activities. *Biomaterial*. 23: 3377-3385.

Sunitha, S., Nagaraj, M. and Varalakshmi, P. 2001. Hepatoprotective effect of lupeol and lupeol linoleate on tissue antioxidant defence system in cadmium-induced hepatotoxicity in rats. *Fitoterapia*. 72: 516–523.

Sutthivaiyakit, S., Pakakatsama, P., Kraus, W. and Vogler, B. 1995. Constituents of *Diospyros rhodocalyx*. *Planta Medicine*. 61: 295.

Szallasi, A. 2005. Piperine: researchers discover new flavor in an ancient Spice. *TRENDS in Pharmacological Sciences*. 26: 437-439.

Tam, C.U., Yang, F.Q., Zhang, Q.W., Guan, J. and Li, S.P. 2007. Optimization and comparison of three methods for extraction of volatile compounds from *Cyperus rotundus* evaluated by gas chromatography–mass spectrometry. *Journal of Pharmacological Biomedical Analytical*. 44: 444–449.

Thebtaranonth, C., Thebtaranonth, Y., Wanauppathamkul, S. and Yuthavong, Y. 1995. Antimalarial sesquiterpenes from tubers of Cyperus rotundus: structure of 10, 12-peroxycalamenene, a sesquiterpene endoperoxide. *Phytochemistry*. 40: 125-128.

Tietz, N.W., Rinker, A.D., Shaw, L.M. 1983. IFCC methods for the measurement of catalytic concentration of enzymes. Part 5. IFCC method for alkaline phosphatase. *Journal Clinical Chemistry Clinic Biochemistry*. 21: 731–748.

Trentz, O.A., Hoerstrup, S.P., Sun, L.K., Bestmann, L., Platz, A. and Trentz, O.L. 2003. Osteoblasts response to allogenic and xenogenic solvent dehydrated cancellous bone in vitro. *Biomaterials*. 24: 3417-3426.

Uddin, S.J., Mondal, K., Shilpi, J.A. and Rahman, M.T. 2006. Antidiarrhoeal activity of *Cyperus rotundus*. *Fitoterapia*. 77: 134-136.

Umi Kalsom, Y., Horsten, S.F.A.J. and Lemmens, R.H.M.J. 1999. *Tinospora* Miers. In: dePadua, L.S., Bunyapraphatsara, N., Lemmens, R.H.M.J. (Eds.), PROSEA—Plant Resources of Southeast Asia No. 12(1). *Medicinal and Poisonous Plants 1*. *Backhuys Publishers*, Leiden, the Netherlands. 479–484.

Van Hoof, V.O., Van Campenhout, C.M., De Broe, M.E., Lepoutre, L.G. 1990. Variations in measured alkaline phosphatase activity: influence of isoenzymes and buffer systems. *Clinical Chemistry*. 36: 2012–2013.

Varalakshmi, C., Ali, A.M., Pardhasaradhi, B.V.V., Srivastava, R.M., Singh, S. and Khar, A. 2008. Immunomodulatory effects of curcumin: In-vivo. *International Immunopharmacology*. 8: 688-700.

Vidya, L. and Varalakshmiu, P. 2000. Control of urinary risk factors of stones by betulin and lupeol in experimental hyperoxaluria. *Fitotrapia*. 71: 535-543.

Wiess, M.J., Henthorn, P.S., Lafferty, M.A., Slaughter, C., Raducha, M.and Harris, H. 1986. Isolation and characterization of a cDNA encoding a human liver/ bone/ kidney-type alkaline phosphatase. *Product of Natural Academic Science USA*. 83: 7182-7186.

Wiechelman, K.J., Braun, R.D. and Fitzpatrick, J.D. 1988. Investigation of the bicinchoninic acid protein assay: Identification of the groups responsible for color formation. *Analytical Biochemistry*. 175: 231-237.

Wijk, F. and Knippels, L. 2007. Initiating mechanisms of food allergy: Oral tolerance versus allergic sensitization. *Biomedical & Pharmacotherapy*. 61: 8-20.

WU, W.B., Peng, H.C. and Huang, T. 2003. Disintegrin causes proteolysis of β-catenin and apoptosis of endothelial cells. Involvement of cell-cell and cell-

ECM interactions in regulating cell viability. *Experimental Cell Research*. 286: 115-127.

Xujuan, Y., Naili, W., Wenxiu, H., Xinzi, C. and Xinsheng, Y. 2005. Studies of triterpenoids isolated from Sambucus williamsii Hance and their effects on UMR106 cell proliferation and alkaline phosphatase activity. *Journal written in Chinese*. 22: 449-452, 457.

Yamaguchi, M. and Sugimoto, E. 2000. Stimulatory effect of genistein and daidzein on protein systhesis in osteoblastic MC3T3-E1 cells: activation of aminoacyl-tRNA synthetase". *Molegular Cell Biochem*. 214: 97-102.

Yamashita, K., Suzuki, Y., Matsui, T., Yoshimaru, T., Yamaki, M., Karasaki, M.S., Hayakawa, S. and Shimizu, K. 2000. *Epigallocatechin Gallate* inhibits histamine release from rat basophilic leukemia (RBL-2H3) cells: role of tyrosine phosphorylation pathway. *Biochemistry*. *Biophysic*. *Res*earch *Communucation*. 274: 603-608.

You, Y.J., Nan, H.H., Kim, Y., Bae, K.H. and Ahn, B.Z. 2003. Antiangiogenic activity of lupeol from Baombax ceiba. *Phytotheraphy Research*. 17: 341-344.

Youngken, H.W. 1950. Textbook of Pharmacognosy, 6th ed, The Blakiston Company, Philadelphia.

Yusuf, M., Chowdhury, J.U., Yahab, M.A. and Begum, J. 1994. *Medicinal plants of Bangladesh*, BCSIR Laboratories, Bangladesh. 87.

Zang, L.Y., Cosma, G., Gardner, H., Shi, X., Castranova, V., Vallyathan, V. 2000. Effect of antioxidant protection by p-coumaric acid on lowdensity lipoprotein

cholesterol oxidation. *Am Journal of Physiology Cell Physiology*. 279: C954–C960.

Zhang, G., Qin, L., Hung, W.Y., Shi, Y.Y., Leung, P.C., Yeung, H.Y. and Leung, K.S. 2006. Flavonoids derived from herbal *Epimedium Brevicornum Maxim* prevent OVX-induced osteoporosis in rats independent of its enhancement in intestinal calcium absorption. *Bone*. 38: 818-825.

Zhao, W., Oskerizian, C.A., Pozez, A.L., Schwartz, L.B. 2005. Cytokine production by skin-derived mast cells: endogenous proteases are responsible for degradation of cytokines. *Journal of Immunol*ogy. 175: 2635-2642.

Zhao, Y., Zou, B., Shi, Z., Wu, Q. and Chen, G.Q. 2007. The effect of 3-hydroxybutyrate on the *in vitro* differentiation of murine osteoblast MC3T3-E1 and in vivo bone formation in ovariectomized rats. *Biomaterials*. 28: 3063–3073.

http://courses.washington.edu./bonephys/opmark.html

APPENDIX-A

PREPARATION OF REAGENTS

1. Minimum Essential Medium Alpha Medium (α-MEM)

1.1 Incomplete media

Ingredients	**1 L**	**Storage**
Alpha-MEM	1 pack	2-8°C
Sodium bicarbonate	2.2 g	RT
Sterile water	1 L	RT
1 N NaOH or 1 N HCl	Adjusted to pH 7.1-7.2	RT
Filtered at a pore size of 0.22 μm and stored at 2-8°C		

1.2 Complete media

Ingredients	500 ml	Storage
Incomplete media	450 ml	2-8°C
10% Fetal bovine serum	50 ml	-20°C
2% Penicillin-Streptomycin	10 ml	Below 0°C (-20°C)
Stored the complete media at 2-8°C		

2. Ascorbic acid solution 5 mg/ml

Ingredients	10 ml	40 ml
Ascorbic acid	50 mg	200 mg
Sterile water q.s.	10 ml	40 ml
Steriled by filtration (0.22 μm) and stored at -20°C.		

3. Medium supplements with ascorbic acid solution* (Fresh medium)

Ingredients	10ml	20 ml
Complete media	10 ml	20 ml
Ascorbic acid solution	100 μl	200 μl

4. Reagent for determination ALP activity

4.1 Preparation of *p*-Nitrophenol standard solutions

1 mM *p*-nitrophenol (ml) in lysis buffer solution	Lysis buffer solution (ml)	Final *p*-nitrophenol concentration (μg/ml)
0.0	1.0	0.0
0.05	0.95	0.0167
0.2	0.8	0.0667
0.6	0.4	0.2
0.9	0.1	0.3

(*Standard solution was stored at 2-8°C)

4.2 Lysis buffer solution

Ingredients	
Triton X-100	0.2 ml
Magnesium chloride hexahydrate (MW= 203.3)	0.1017 g
Distilled water	100 ml

4.3 Substrate buffer solution

Ingredients	
Glycine	1.88 g
Magnesium chloride hexahydrate (MW=203.3)	0.1017 g
Distilled water	500 ml
Adjust pH 10.5 with 1 N NaOH	

4.4 *p*-nitrophenol (substrate) solution 1 mg/ml

Ingredients	
p-nitrophenyl phosphate (phosphatase substrate)*	5 mg
Substrate buffer solution	5 ml
Wrapped foil, stored at refrigerator	

4.5 Preparation of control and 10^{-6} M dexamethasone, as positive control

4.5.1 Control

Ingredients	
DMSO (µl)	10
PBS (µl)	90
Media (µl)	900

4.5.2 10^{-6} M dexamethasone

Ingredients	
Dexamethasone (10^{-5} M) (µl)	100
Media (µl)	900
Stored at 2-8°C	

4.5.3 10^{-5} M dexamethasone

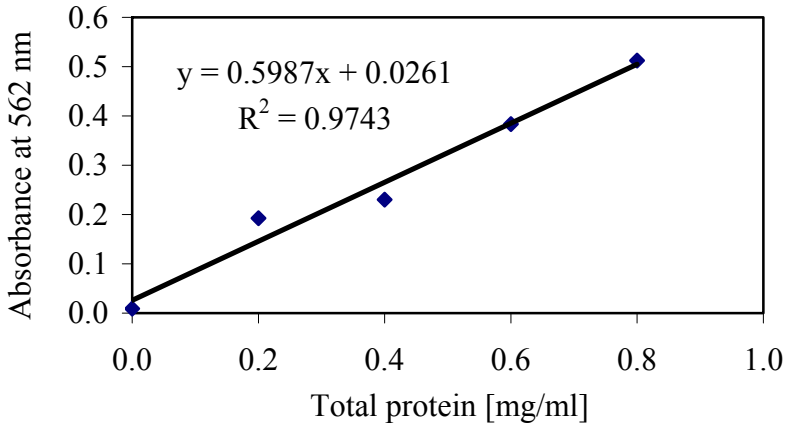
Ingredients	µl	Storage
Dexamethasone (10^{-3} M in absolute ethanol)	10	-20°C
DMSO	90	RT
PBS (µl)	900	RT

5. MTT solution 5 mg/ml

Ingredients	
Thiazolyl blue tetrazolium bromide	200 mg
PBS	40 ml

6. Total protein kit (BCA kit)

6.1 Protein standard solution, Micro standard, Liquid 1 ml/ampule, consists of 1 mg BSA/ml in 0.15 M NaCl, 0.05% NaN_3 and stored at 2-8°C



6.2 Working reagent solution

Ingredients	
Bicinchoninic acid solution (BCA)	20 ml
Copper (II) sulfate solution	0.4 ml
Wrapped foil, stored at refrigerator (limited 24 h)	

7. Minimum Essential Medium Eagle (MEM) of RBL-2H3 cells

7.1 Incomplete media

Ingredients	1 L	Storage
MEM	1 pack	2-8°C
Sodium bicarbonate	2.0 g	RT
1 N NaOH or 1 N HCl	Adjusted to pH 7.0	RT
Sterile water	Adjusted to1 L	

7.2 Complete media

Ingredients	
Incomplete media	900 ml
10% Fetal calf serum (FCS)	100 ml
1% Penicillin-streptomycin	10 ml
Steriled by filtration (0.22 μm) and stored at 2-8°C	

8. Trypsin-EDTA solution

0.25 trypsin	0.5 g
0.02% EDTA·2Na	0.04 g
PBS	200 ml

9. Phosphate buffer saline (PBS)

Ingredients	
NaCl	80 g
$Na_2HPO_4 \cdot 12H_2O$ (Na_2HPO_4 11.5 g , NaH_2PO_4 9.7 g, Add water 1,000 ml)	29 g
KCl	2 g
KH_2PO_4 (K_2HPO_4 2.55 g)	2 g
Ultra water pure	900 ml
Autoclave 121°C, 30 min	

10. Reagent for determination β-hexosaminidase

10.1 Siraganian buffer (Buffer A)

Ingredients		g/l
NaCl	119 mM	6.594 g
KCl	5 mM	0.373 g
Glucose	5.6 mM	1.00 g
$MgCl_2 \cdot 6H_20$	0.4 mM	0.081 g
$CaCl_2 \cdot 2H_2O$	1 mM	0.147 g
PIPES	25 mM	7.559 g
BSA	0.1%	1.00

10.2 0.1 M Citric buffer (pH 4.5)

Ingredients		g/500ml
Citric acid monohydrate	0.1 M	10.51
Trisodium citrate dehydrate	0.1 M	14.71

10.3 0.1 M Na_2CO_3 buffer solution (pH 10.0)

Ingredients		g/500 ml
Na_2CO_3	0.1 M	5.3 g
$NaHCO_3$	0.1 M	4.2 g

10.4 DNP-IgE solution 50 μg/ml

Ingredients	
DNP-IgE	0.5 ml
PBS	9.5 ml

10.5 DNP-BSA solution

Ingredients	
BSA fraction	1 g
0.9% NaCl solution	50 ml
2,4-DNBS	0.5 g
Adjusted pH to 10.5	

APPENDIX B

Data

1. Biocompatibility determination

	Cell viability (%)				Standard deviation			
Conc.(μg/ml)	3	10	30	100	3	10	30	100
Control	100.0	100.0	100.0	100.0	6.2	4.8	6.0	5.3
AP-W	92.9	93.0	84.9	72.2	5.3	7.8	12.5	5.0
CR-W	92.8	92.9	89.4	83.3	5.3	7.2	10.0	6.7
DR-W	91.4	93.0	91.8	81.4	6.2	7.5	10.0	10.3
PN-W	95.8	95.7	86.7	75.8	4.7	7.3	12.0	5.7
SA-W	91.9	91.6	90.4	80.1	4.6	6.7	9.0	7.0
TC-W	91.8	92.4	91.6	77.3	5.6	4.8	8.8	5.2
LF-W	93.4	93.5	88.2	79.4	4.6	7.6	9.3	8.0

	Cell viability (%)				Standard deviation			
Conc.(μg/ml)	3	10	30	100	3	10	30	100
Control	100	100	100	100	6.23	4.8	6.0	5.3
AP-E	99.9	109.2	110.8	66.9	12.5	7.0	6.2	10.2
CR-E	88.9	91.5	94.6	102.8	6.6	6.8	5.8	11.8
DR-E	91.4	95.8	90.1	87.1	7.4	6.1	8.9	7.8
PN-E	91.8	92.9	94.1	79.3	5.6	9.2	10.9	7.6
SA-E	93.9	96.1	91.9	84.0	8.8	6.0	6.1	5.6
TC-E	91.6	93.8	90.6	73.6	4.3	6.3	7.1	5.3
LF-E	90.0	86.0	84.4	76.0	11.0	11.0	10.7	14.9

2. Total protein of water extracts

Crude extracts		Total Protein [mg/ml]				
		Conc. (μg/ml)				
		Dex	100	30	10	3
AP-W	Mean	0.076	-0.006	-0.011	0.088	0.288
	SD	0.012	0.011	0.011	0.019	0.067
CR-W	Mean	0.041	0.060	0.089	0.074	0.087
	SD	0.018	0.031	0.023	0.007	0.008
DR-W	Mean	0.180	0.405	0.327	0.430	0.555
	SD	0.020	0.033	0.016	0.009	0.078
PN-W	Mean	0.104	0.005	0.050	0.232	0.342
	SD	0.011	0.003	0.025	0.014	0.022
SA-W	Mean	0.047	0.197	0.092	0.227	0.189
	SD	0.023	0.140	0.046	0.048	0.034
TC-W	Mean	0.165	0.341	0.350	0.598	0.560
	SD	0.007	0.010	0.014	0.034	0.009
LF-W	Mean	0.105	0.173	0.285	0.382	0.407
	SD	0.010	0.040	0.051	0.038	0.041

3. Total protein of ethanolic extracts

Crude extracts		Total Protein [mg/ml]				
		Conc. (μg/ml)				
		Dex	100	30	10	3
CR-E	Mean	0.030	0.123	0.075	0.152	0.149
	SD	0.010	0.027	0.021	0.036	0.016
DR-E	Mean	0.131	0.102	0.197	0.247	0.581
	SD	0.026	0.003	0.028	0.041	0.017
PN-E	Mean	0.090	0.149	0.206	0.329	0.242
	SD	0.013	0.030	0.042	0.029	0.051
SA-E	Mean	0.117	0.158	0.333	0.545	0.775
	SD	0.023	0.015	0.064	0.063	0.115
TC-E	Mean	0.088	0.025	0.053	0.307	0.317
	SD	0.023	0.008	0.010	0.060	0.024
LF-E	Mean	0.105	0.048	0.061	0.402	0.402
	SD	0.010	0.022	0.008	0.069	0.100

4. ALP activity of water extracts (fold of dexamethasone, 10^{-6} M)

Crude extracts		ALP activity (fold of dex)				
		Conc. (μg/ml)				
		Dex	100	30	10	3
AP-W	Mean	1	0.16	0.21	0.46	0.55
	SD	0.13	0.06	0.20	0.12	0.12
CR-W	Mean	1	0.60	0.58	1.07	0.91
	SD	0.08	0.08	0.12	0.17	0.08
DR-W	Mean	1	1.62	0.88	1.97	1.57
	SD	0.05	0.09	0.18	0.06	0.17
PN-W	Mean	1	1.81	0.22	0.72	0.60
	SD	0.12	0.19	0.04	0.12	0.04
SA-W	Mean	1	0.40	0.58	0.36	0.65
	SD	0.32	0.17	0.21	0.04	0.10
TC-W	Mean	1	1.43	1.47	1.52	1.54
	SD	0.02	0.02	0.11	0.03	0.03
LF-W	Mean	1	0.44	0.51	0.39	0.48
	SD	0.06	0.08	0.08	0.07	0.05

5. ALP activity of ethanolic extracts (fold of dexamethasone, 10^{-6} M)

Crude extracts		ALP activity (fold of dex)				
		Conc. (μg/ml)				
		Dex	100	30	10	3
AP-E	Mean	1	0.42	0.23	0.16	0.61
	SD	0.17	0.11	0.09	0.01	0.00
CR-E	Mean	1	0.36	0.43	0.48	0.89
	SD	0.48	0.06	0.29	0.30	0.14
DR-E	Mean	1	1.02	1.16	1.23	1.13
	SD	0.03	0.22	0.10	0.13	0.04
PN-E	Mean	1	0.86	0.64	0.63	0.74
	SD	0.06	0.06	0.20	0.09	0.17
SA-E	Mean	1	0.76	1.06	1.08	0.90
	SD	0.12	0.09	0.05	0.14	0.11
TC-E	Mean	1	0.48	0.62	0.83	0.76
	SD	0.11	0.16	0.09	0.10	0.05
LF-E	Mean	1	0.15	0.29	0.49	0.70
	SD	0.07	0.06	0.06	0.13	0.14

6. ALP activity of crude water extracts normalized with total protein content

Crude extracts		ALP activity [mU/mg of protein]				
		Conc. (µg/ml)				
		Dex	100	30	10	3
AP-W	Mean	118.26	18.50	24.90	53.85	65.43
	SD	15.05	7.39	23.13	14.56	13.72
CR-W	Mean	90.66	71.28	68.91	139.00	108.41
	SD	26.15	9.69	14.41	3.59	9.66
DR-W	Mean	38.32	62.08	33.79	75.63	59.99
	SD	1.99	3.63	6.81	2.20	6.62
PN-W	Mean	81.75	148.37	18.19	59.11	49.03
	SD	10.04	15.17	3.63	10.07	3.48
SA-W	Mean	138.15	57.81	78.18	41.86	76.01
	SD	9.08	9.89	21.15	4.22	11.35
TC-W	Mean	42.42	60.50	62.18	64.33	65.12
	SD	0.77	0.89	4.77	1.08	1.38
LF-W	Mean	107.64	46.92	55.38	41.59	51.29
	SD	6.70	8.08	8.24	7.13	5.73

7. ALP activity of crude ethanolic extracts normalized with total protein content

Crude extracts		ALP activity [mU/mg of protein]				
		Conc. (µg/ml)				
		Dex	100	30	10	3
CR-E	Mean	79.07	28.10	33.98	37.73	70.23
	SD	10.52	4.39	9.55	5.53	8.34
DR-E	Mean	67.81	68.90	78.45	83.17	76.96
	SD	0.36	1.91	6.51	8.55	3.00
PN-E	Mean	78.81	68.00	50.19	49.90	58.43
	SD	4.91	4.82	15.38	7.18	13.05
SA-E	Mean	70.51	53.90	74.65	76.27	63.52
	SD	8.58	6.57	3.52	10.05	7.41
TC-E	Mean	85.80	40.88	53.33	71.52	64.90
	SD	9.50	13.47	7.96	8.27	4.21
LF-E	Mean	82.52	12.31	24.33	40.21	58.00
	SD	6.14	5.25	4.97	10.44	11.53

VITAE

Name Miss Sawanee Kraithep

Student ID 4852027

Education Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Science (Medical Technology)	Walailak University	2005

List of Publication and Proceeding

Kraithep, S., Oungbho, K. and Tewtrakul, S. 2006. Anti-allergic activity of Thai medicinal plants on inhibition of β-hexosaminidase release from RBL-2H3 cells. The 6th National Symposium on Grad-Research, (Chulalongkorn University, Bangkok, Thailand, October 13-14, 2006) 380.

Kraithep, S., Tewtrakul, S. and Oungbho, K. 2007. Alkaline Phosphatase Activity of Thai Herbs in Longevity Herbal Medicine. The 1st International Bone and Dental Technology Symposium 2007, (Grand Mercure Fortune Hotel, Bangkok, Thailand, November 12-13, 2007) 135-136.