CHAPTER 2

LITERATURE REVIEW

2.1 *Vanilla siamensis* Rolfe ex Downie

The genus Vanilla belongs to the family Orchidaceae when the pods mature aroma of vanillin. Vanilla is a perennial tropical orchid with succulent stem, sessile leaves and aerial adventitious roots at the nodes. This climbing orchid can be grown in humid tropics from sea level up to an altitude of 1500 m and optimum temperature range is 21 – 32 ^{0} C. Vanilla beans originated in Mexico, and in some Central American countries as Costa Rica and Honduras. Its flavor is well-known and appreciated in the world and is widely used in the food industry. Species with commercial value, there are only three species *Vanilla planifolia* Jacks ex Andrews, *Vanilla pompon,* and *Vanilla tahaitensis*. The most popular commercially cultivated *Vanilla planifolia* Jacks ex Andrews as well as commercial varieties of vanilla (Verma *et al*., 2009). Thai traditional Vanilla scattered four species is *Vanilla siamensis* Rolfe ex Downie, *Vanilla albida* Blume, *Vanilla pilifera* Holttum, and *Vanilla aphylla* Blume. Thailand was known as the vanilla trade to grow by more than 30 years and it has grown in the experimental Station of the Department of Agriculture, Ministry of Agriculture and Cooperatives. The Royal Project Foundation has studied the growing commercial varieties of vanilla since 2002 supported by research grants to scholars of the Institute of Scientific and Technological Research. Scientific studied at the Royal Project was the first time and came to the Royal Project Pa Miang, Doi Saket, Chiang Mai, which is found up in the forest and growing native

of vanilla (Pool Chang) *Vanilla simensis* Blumes. This has led to commercial varieties of vanilla (*Vanilla planifolia* Jacks ex Andrews) and used to study plant growth. Under the shade of forest trees and Saran shading from 50 to 70 percent, it was able to grow and flower. In the third year of planting the seedlings, cuttings can be obtained from the tree. It will bloom in late March and April which is faster than native species, about a month. The vanilla plant is able to glide over the last several years. The study of the materials used to hold the island. The key is to have a tree shading can be 50 to 70 percent, which is a legume tree, found that the use of cement to hold the best choice for the commercial cultivation of vanilla. Comparison of materials, mulching with chopped coconut, legumes, and grasses, there was no difference in the growth of vanilla. And it was now expanding to the promotion and development centers such as Khun Wang Royal Project, where was the largest producer in the country and a learning center for the production of vanilla, Pa Meang Royal Project, Teen Tak Royal Project, Monnger Royal Project, and expansion projects for the Royal Project Pongkum, Tue peace, etc. They were funded the research results to farmers from the National Innovation Agency.

2.1.1 Taxonomical classification

Kingdom	*Plantae*	**Phylum**	*Tracheophyta*
Class	*Magnoliopsida*	**Order**	*Asparagales*
Family	*Orchidaceae*	**Subtribe**	*Vanillinae*
Genus	*Vanilla*		
Specific epithet	*siamensis* - Rolfe ex Downie		
Botanical name	*Vanilla siamensis* Rolfe ex Downie		
Province	Chiang Mai	**District**	Mae Wang
Location	Royal Project, Doi Intanon		
Elevation	1,200 m	**Date**	7 March 2012
Habitat	cultivated in a shade house: native species from primary, evergreen, seasonal, hardwood forest		
Note	evergreen vine; stem, immature capsules dull dark green; pedicels bright dark green, base cream; tepals bright yellow-green outside, light yellow inside; bracts green; midlobe of lip with fine, red, parallel line inside; operculum & column pale light yellow; slightly fragrant; leaf blade dull dark green above, same underneath.		

Figure 1 *Vanilla siamensis* Rolfe ex Downie

Figure 2 Morphology of *Vanilla siamensis* Rolfe ex Downie

2.1.2 Green Vanilla

Given the ripening characteristics of the beans it has been considered that a minimum waiting period to start harvest could be nine months from pollination (Figure 3). Vanilla fruits are gathered when they are fully mature but before they are too ripe. When picked immaturely, the fruits do not develop the requisite full-bodied aroma and proper color during processing. When harvested at the over-ripe stage, the fruits tend to split and lose some of their aroma (Figure 4).

2.1.3 Vanilla composition

Natural vanilla is a complex mixture of flavor components extracted from the beans. The bean contain more than 250 compounds and over one hundred volatile compounds have been detected, including aromatic carbonyls, aromatic alcohols, aromatic esters, phenols and phenols ethers, aliphatic alcohol, carbonyls, acids, esters and lactones, of which the aldehyde vanillin is the most abundant. The level of aldehydes, 4-hydroxy-3-methoxybenzaldehyde (vanillin), *p*-hydroxybenzaldehyde and their respective acids (vanillic acid, 4-hydroxy-3-methoxybenzoic acid and *p*-hydroxybenzoic acid), in cured vanilla bean is used as an indicator of cured vanilla bean quality for commercial purpose (Adedeji, Hartman, & Ho, 1993; Riley & Kelyn, 1989). Although they are important, these compounds alone do not account for the flavor strength and extremely complex structure of this flavoring (Ranadive, 1994). The balance between nonaromatic compounds, such as lipids that contain steroid compound and the vanillin content should distinguish natural, adulterated vanilla products. Most of these components have been reported on Table 1 (Rao & Ravishankar, 2000).

Figure 3 The pollination of vanilla

Figure 4 Stages in vanilla maturation

Table 1 Chemical composition of processed vanilla beans

COMPONENT	**g/kg bean** (d.m.)
Vanillin	20
vanillic acid	1
p-hydroxybenzaldehyde	2
p-hydroxybenzoic acid	0.2
Sugars	250
Lipids	150
Cellulose	150 – 300
Minerals	60
Water	350

(Rao & Ravishankar, 2000)

2.1.3.1 Lipid composition

Lipids can be considered to be biological molecules which are soluble in organic solvents, such as chloroform/methanol. They are sparingly soluble in aqueous solutions. There are two major classes, saponifiable and nonsaponifiable. Saponification is the process that produces soaps from the reaction of lipids and a strong base. The saponifiable lipids contain long chain carboxylic acids, or fatty acids, esterified to a "backbone" molecule, which is either glycerol or sphingosine. The nonsaponifiable classes include the "fat-soluble" vitamins (A, E) and cholesterol.

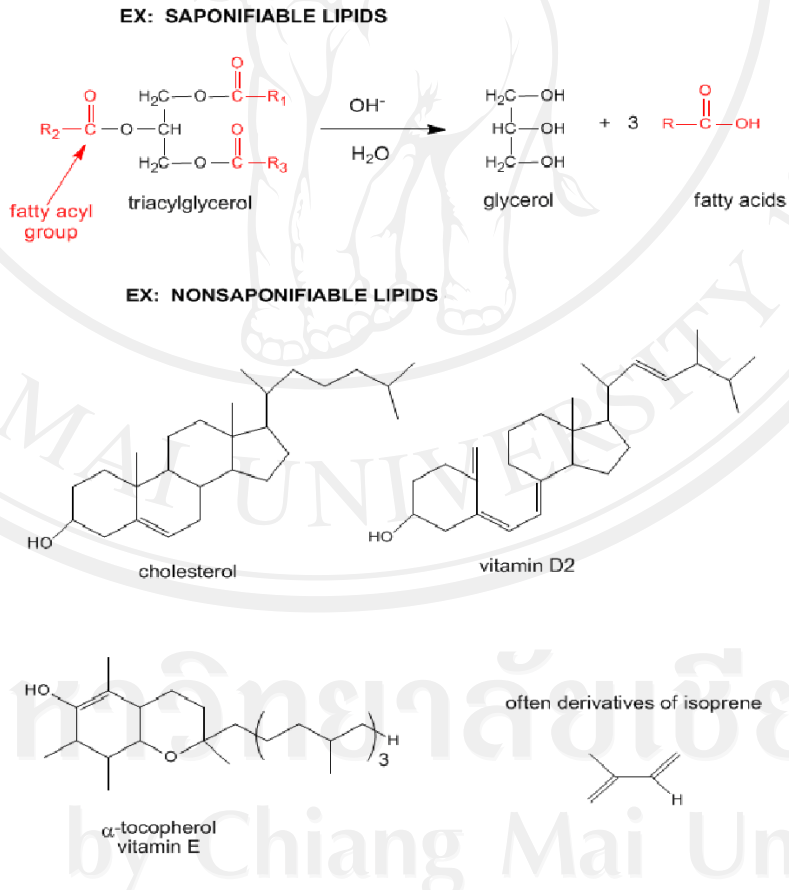

Figure 5 Sapo[illegible] (http://employees.csbsju.edu/hjakubowski/classes/ch331/lipidstruct/ollipidintro1.html access date, 15 April 2012).

2.1.3.2 Nonsaponifiable matters

The nonsaponifiable matters have important bioactive, nutritional, and characteristic compositional properties that affect the quality of individual oils and fats (Bailey, 1951). They present usually composed of sterols, fatty alcohols, tocopherols, triterpene alcohols, and hydrocarbon (squalene) which have individual biological importance. The most common plant sterols are the 4-desmethysterols, β-sitosterol, campesterol, and stigmasterol, which are the end products of plant sterol synthesis from squalene. β-sitosterol are structurally similar to cholesterol, all having a cyclopentenophenanthrene ring with 3-beta hydroxy substitution and a 5-6 double bond, and the vertebrate reproductive steroid such as 17β- estradiol (Sharpe, Drolet, & MacLatchy, 2006) (Figure 6). The phytosterol β-sitosterol (β-sit) very closely resembles cholesterol, differing only by an ethyl group on carbon 24 (Figure 6B).

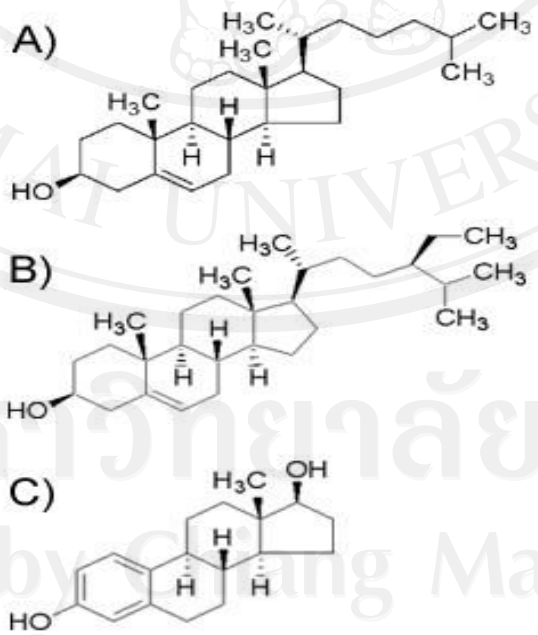

Figure 6 Chemical structures of (A) cholesterol (B) β-sitosterol (β-sit), and (C) 17β-estradiol (E_2) (Sharpe, Drolet, & MacLatchy, 2006).

2.1.4 Vanilla Analysis

Because of natural vanilla is so costly and has a limited supply. Vanillin synthetic is readily available which efforts are being made to both better synthetically match natural vanilla, and also to find ways of detecting adulteration. For both purposes it is vital that reliable and practical analytical techniques for determining chemical compounds in vanilla are found (Sinha, Sharma, & Sharma, 2008). Several chromatographic methods for the identification and quantification of chemical compounds have been used in vanilla research including: Thin layer chromatography (TLC) (Anwar, 1963), gas chromatography (GC), high performance liquid chromatography (HPLC), and capillary electrophoresis (CE) (Sinha, *et al*., 2008).

Vanillin and its derivatives was separated and identification using paper chromatography techniques (Anwar, 1963). Vanilla flavor metabolites were successfully identified using silica plates (Rao & Ravishankar, 2000). TLC was used to identify vanillin and ethyl vanillin in food flavorings (Gerasimov, Gornova, & Rudometova, 2003) because of TLC is rapid, easy to handle, and economical. High performance TLC (HPTLC), however, has largely replaced TLC as an analytical method (Sinha, *et al.*, 2008).

GC has advantages over other chromatographic techniques for separating volatile mixtures such as high efficiency and high resolution. It can also be used for quantitative analysis. GC complemented with mass spectrometry (MS) allows for better quantitation and analysis of vanilla volatiles, and the volatile compounds including odor active compounds using GC-MS and GC olfactometry (Perez-Silva *et al*., 2006). The volatile compounds have shown on Table 2.

HPLC is one of the most powerful and preferred techniques for quantifying organic molecules because of its simplicity, sensitivity, precision and selectivity. HPLC can be made more selective by the use of different stationary phases, mobile phases, and detectors (Sinha, *et al.*, 2008). The compounds in vanilla samples were used to HPLC (Dignum, *et al.*, 2004). Generally when working with vanilla compounds, a reverse phase column is used with a polar organic solvent as the mobile phase (Sinha *et al*., 2008). Elution times from literature vary between 7-36 min at detection wavelengths of 254-340. HPLC has been used successfully in both compositional analysis as well as adulteration detection (Ehlers, Pfister, & Bartholomae, 1994).

Vanillin, vanillic acid, *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid were the major compounds in the pentane/diethyl ether aroma extract from vanilla beans and are shown on Table 3.

Table2 Volatile compounds detected in aroma extracts from cured *Vanilla planifolia* beans obtained using various organic solvents

Compounds	ether	pentane/ether	pentane/dichloromethane
Phenol			
Guaiacol	*	*	*
4-Methylguaiacol		*	*
Phenol	*	*	
p-Cresol	*	*	*
4-Vinylguaiacol		*	
Vanillyl methyl ether		*	*
4-Vinylphenol	*	*	
Vanillin	*	*	*
Acetovanillone	*	*	*
Vanillyl alcohol	*	*	*
Vanilloylmethyl cetone	*	*	
p-Hydroxybenzaldehyde	*	*	*
p-Hydroxybenzyl alcohol	*	*	
Vanillic acid	*	*	*
p-Hydroxybenzyl alcohol	*	*	*
Aliphatic acids			
Acetic acid	*	*	*
Propanoic acid	*	*	*

Table 2 Continued

Compounds	ether	pentane/ ether	pentane/dichloromethane
Isobutyric acid	*	*	
Butyric acid	*	*	
Isovaleric acid	*	*	*
Valeric acid	*	*	*
Hexanoic acid	*	*	*
Heptanoic acid	*	*	*
Octanoic acid	*	*	*
2-Heptenoic acid	*	*	*
Nonanoic acid	*	*	*
Dodecanoic acid	*	*	
Myristic acid	*	*	
Pentadecanoic acid	*	*	
Hexadecanoic acid	*	*	*
9-Hexadecanoic acid		*	
Heptadecanoic acid	*	*	
Stearic acid	*	*	
Oleic acid	*	*	
Linoleic acid	*	*	

Table 2 Continued

Compounds	ether	pentane/ether	pentane/dichloromethane
Aromatic acids			
Benzoic acid	*	*	
Benzene propanoic acid	*	*	*
Cinnamic acid *(isomer 1)*	*	*	*
Cinnamic acid *(isomer 2)*	*	*	*
Anisic acid	*	*	
Alcohols			
1-Octen-3ol	*	*	
2,3-Butanediol *(isomer 1)*	*	*	*
1-Octanol	*	*	
2,3-Butanediol *(isomer 2)*	*	*	*
1,2-Propanediol	*	*	
Benzyl alcohol	*	*	*
2-Phenylethanol	*	*	*
Benzene propanol	*	*	
Anisyl alcohol	*	*	*
Cinnamyl alcohol	*	*	*
Aldehydes			
2-Heptenal	*	*	*
(*E*)-2-decenal		*	*

Table 2 Continued

Compounds	ether	pentane/ether	pentane/dichloromethane
(*E*,*Z*)-2,4-decadienal	*	*	*
(*E*,*E*)-2,4-decadienal	*	*	*
Esters			
Methyl salycilate		*	
Methyl cinnamate		*	
Anisyl formate		*	
Ethyl linolenate	*	*	*
Hydrocarbons			
Tricosane		*	*
Pentacosane		*	*
Heterocyclics			
Furfural	*	*	*
γ-Butyrolactone	*	*	*
Pantolactone		*	*
1H-pyrrole-2,5-dione, ethyl-4-methyl	*	*	*
Ketone			
3-Hydroxy-2-butanone	*	*	*

(Perez-Silva, *et al*., 2006)

Table 3 Concentrations of volatile compounds in pentane/ether extract from cured *Vanilla planifolia* beans

Compounds (quantified by HPLC)	**Concentration, ppm(mg/kg of cured vanilla)**	**Compounds (quantified by HPLC)**	**Concentration, ppm(mg/kg of cured vanilla)**
Phenol		Isobutyric acid	1.7
Guaiacol	9.3	Butyric acid	<1
4-Methylguaiacol	3.8	Isovaleric acid	3.8
Phenol	1.8	Valeric acid	1.5
p-Cresol	2.6	Hexanoic acid	<1
4-Vinylguaiacol	1.2	Heptanoic acid	1.9
Vanillyl methyl ether	<1	Octanoic acid	5.5
4-Vinyl phenol	1.8	2-Heptenoic acid	1.7
Vanillin	19,118	Nonanoic acid	15.7
Acetovanillone	13.7	Dodecanoic acid	2.2
Vanillyl alcohol	83.8	Myristic acid	12.4
Vanilloyl-methyl cetone	2.2	Pentadecanoic acid	13.4
p-Hydroxybenzaldehyde	873.3	Hexadecanoic acid	126.6
p-Hydroxybenzyl alcohol	65.1	9-Hexadecanoic acid	5.7
Vanillic acid	1,315	Heptadecanoic acid	5.7
p-Hydroxybenzoic acid	255	Stearic acid	13.9
Aliphatic acids		Oleic acid	16.3
Acetic acid	124.3	Linoleic acid	225.6
Propanoic acid	1.7		

Table 3 Continued

Compounds (quantified by HPLC)	**Concentration, ppm(mg/kg of cured vanilla)**	**Compounds (quantified by HPLC)**	**Concentration, ppm(mg/kg of cured vanilla)**
Aromatic acids		(*E*)-2-decenal	1.8
Benzoic acid	2.6	(*E,Z*)-2,4-decadienal	1.4
Benzene propanoic acid	3.9	(*E,E*)-2,4-decadienal	1.2
Cinnamic acid *(isomer 1)*	3.4	*Esters*	
Cinnamic acid *(isomer 2)*	9.5	Methyl salycilate	<1
Anisic acid		Methyl cinnamate	1.1
Alcohols		Anisyl formate	2.3
1-Octen-3ol	<1	Ethyl linolenate	13.5
2,3-Butanediol *(isomer 1)*	16.5	*Hydrocarbons*	
1-Octanol	1.1	Tricosane	15.9
2,3-Butanediol *(isomer 2)*	8.0	Tricosane	15.9
1,2-Propanediol	<1	Pentacosane	19.9
Benzyl alcohol	2.7	*Heterocyclics*	
2-Phenylethanol	1.0	Furfural	<1
Benzene propanol	<1	γ-butyrolactone	<1
Anisyl alcohol	2.4	Pantolactone	1.4
Cinnamyl alcohol	<1	1H-pyrrole-2,5-dione, ethyl-4-methyl	1.8
Aldehydes		*Ketone*	
2-Heptenal	2.1	3-Hydroxy-2-butanone	14.6

(Perez-Silva, *et al.*, 2006)

The best method of the determination for *Vanilla tahitensis* was study using GC–O (Gas Chromatography–Olfactometry) analysis between CHARM (Combined Hedonic of Aromatic Response Measurement) and OSME (Brunschwig *et al*., 2012). The analysis by CHARM showed that Tahitian vanilla extract was characterized by a predominance of two main odor classes: "spicy-anise" and "phenolic" odors. The other odor classes ("buttery", "sulfury", "earthy" and "floral") were of less importance with values (figure 7).

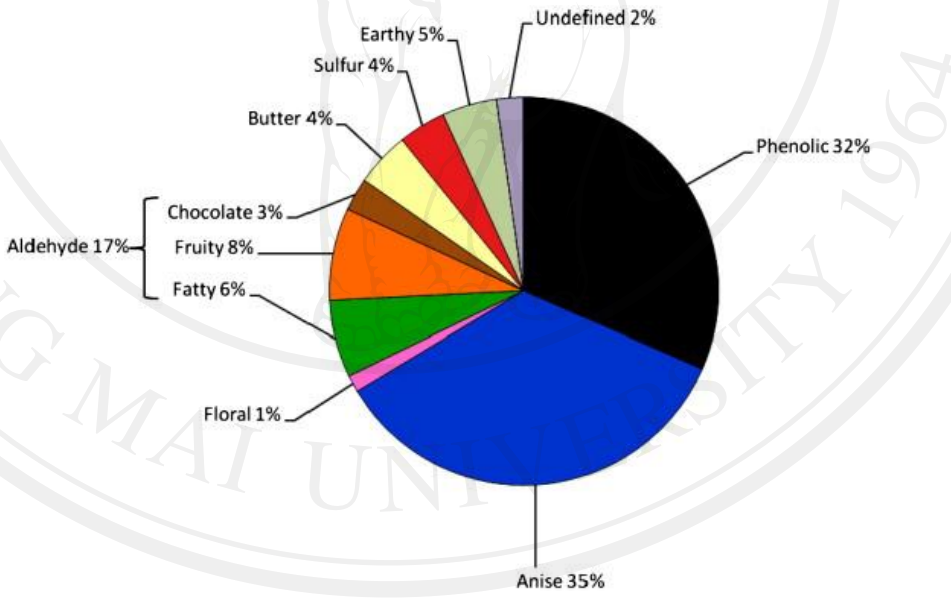

Figure 7 Distribution of the odor classes of Tahitian vanilla flavor by CHARM analysis (Brunschwig *et al*., 2012).

2.2 Phytoestrogen

Phytoestrogens are biologically active phenolic compounds of plant origin structurally mimic the principal mammalian estrogen 17 β-estradiol (Piironen *et al.*, 2000) . The term ‘phenolic’ or ‘polyphenol’ can be defied chemically as a substance, which this possesses an aromatic ring bearing one or more hydroxyl substituents, including functional derivatives (esters, methyl esters, glycosides etc.) (Harbome, 1989). All are polyphenols that have a structural similarity to estradiol and possess estrogenic activity due to having a similar ‘A’ ring to that of estradiol and possessing two hydroxyl groups at positions that afford the correct distance between them to facilitate binding to the estrogen receptor. Shared structures include a pair of hydroxyl groups and a phenolic ring, which is required for binding to ERα and/or ERβ, and the position of these hydroxyl groups appears to be an important factor in determining their abilities to bind the ERs and activate transcription (Bail *et al.*, 2000).

Consequently, many plant-derived bioactive substances with considerable therapeutic benefits have attracted interest in the scientific community over the last two decades. Among these, phytochemical is the broad class of polyphenols including non-steroidal estrogen called phytoestrogens. The scientific interest lies in the potential of phytoestrogens for medical use like in hormone replacement therapy (HRT) either as registered drug or mostly as dietary supplement. The use of certain plant derivatives might be related to their ability to bind estrogen receptor and estrogenic effects. Phytoestrogens display estrogenic effects by binding to the estrogenic receptors. It might also provide selective action on reproductive and non-reproductive estrogen target tissues depending on the expression of estrogenic receptor (ER) α and/or ERβ (Morito *et al.*, 2002). They are non-steroidal naturally

occurring phenolic compounds that can be divided into two groups: firstly, the flavonoids that are further subdivided into isoflavones, coumestans and prenyl flavonoids (figure 8). The flavonoid has a common core, the flavane nucleus, consisting of two benzene rings linked by an oxygen-containing pyrane ring (Figure 9) and secondly the non-flavonoids, comprising the lignans. The non-flavonoids, phenols with only one aromatic ring, are derivatives of hydroxycinnamic acid and hydroxybenzoic acid (Figure 10).

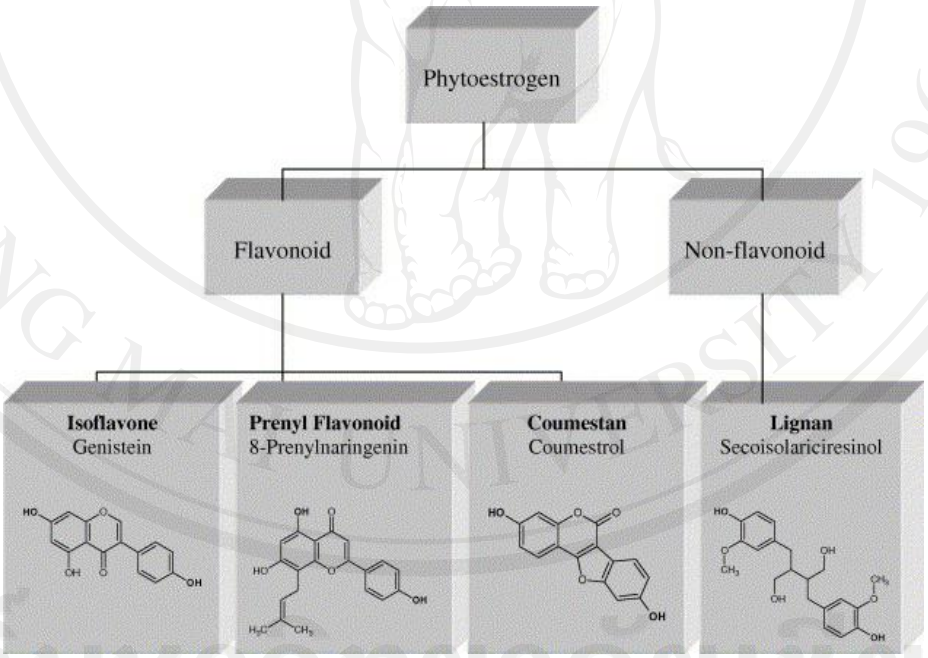

Figure 8 The different classes of phytoestrogens (Martin *et al.*, 2007).

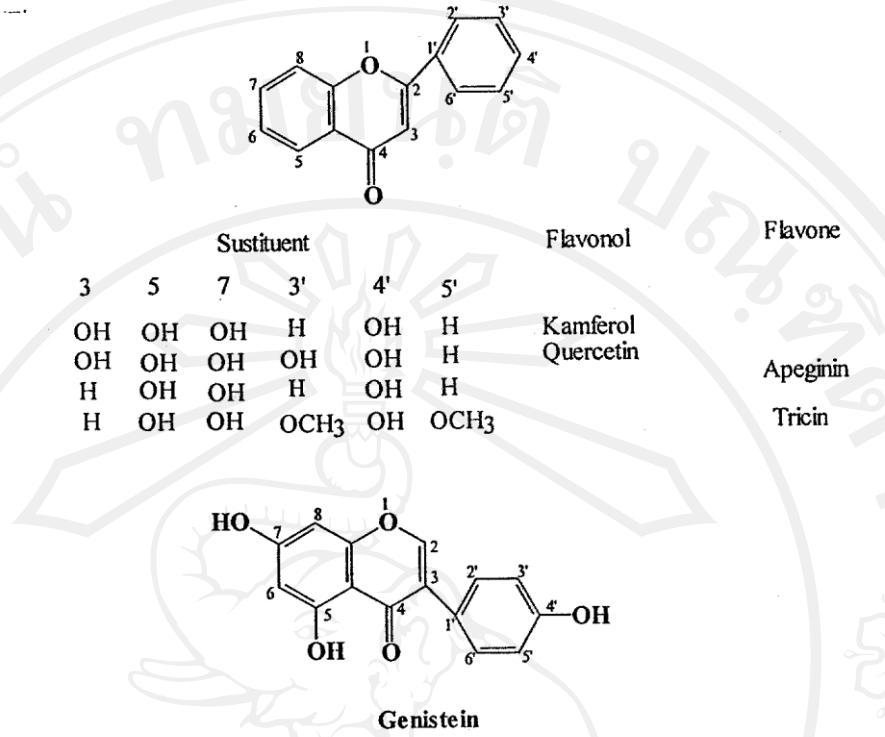

Figure 9 The core structure of flavonoids (Martin *et al.*, 2007).

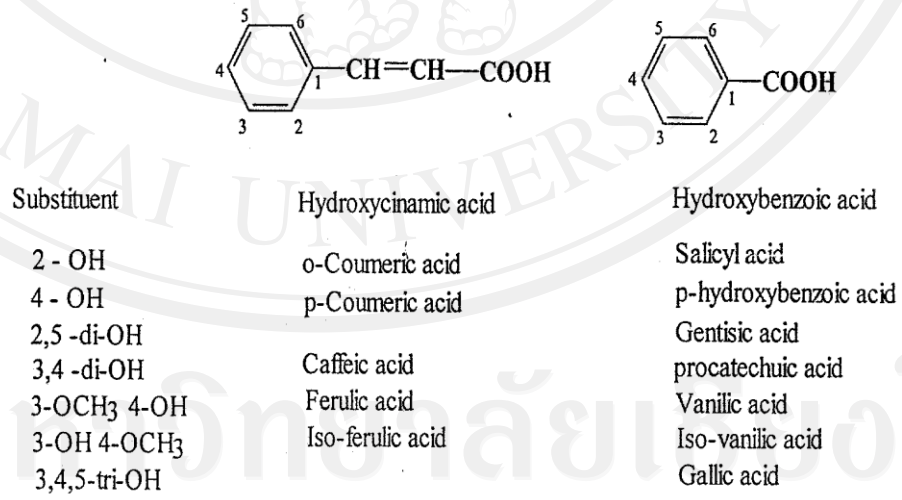

Figure 10 The phenolic compound structures of non-flavonoids (Martin *et al.*, 2007).

2.2.1 Chemical Structure

All are polyphenols that have a structural similarity to estradiol (Figure 11) and possess estrogenic activity due to having a similar ‘A’ ring to that of estradiol and possessing two hydroxyl groups at positions that afford the correct distance between them to facilitate binding to the estrogen receptor. The isoflavone phytoestrogens share a common structure (Figure 11), with genistein having the important –OH groups at positions 7, and 4′. Biochanin A has a methoxy group at position 4′ and Prunetin has a methoxy group at position 7 resulting in less estrogenic activity as the methoxy groups hinder binding to the estrogen receptor. In Quercetin the ‘B’ ring is attached to position 2 and there is an –OH group at position 3.

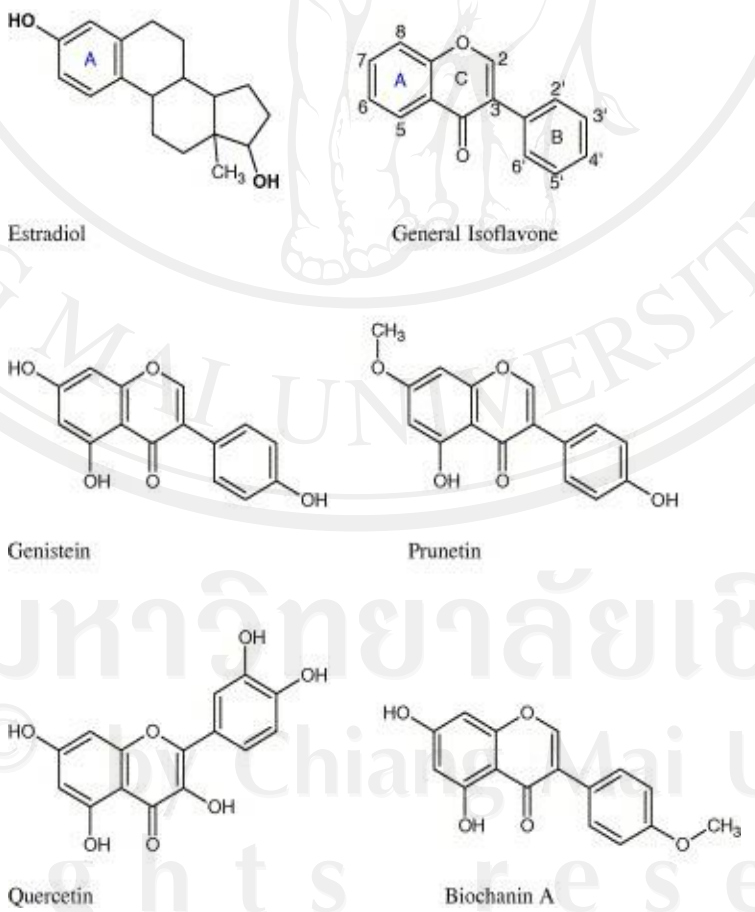

Figure 11 The structure of estradiol and selected phytoestrogens (Martin *et al.*, 2007).

2.2.2 Source of Phytoestrogen

Phytoestrogen are found in many food products as glycosidic conjugates. In fermented foods, they are deconjugated to their aglycones. Once consumed, phytoestrogens undergo several metabolic transformations. Intestinal hydrolases remove the glycosidic groups allowing for rapid uptake of the aglycones and distribution to peripheral sites. In the liver and certain other tissues, phytoestrogens are converted to β-glucuronides, sulfateesters, and methylated products. The most extensively studied phytoestrogens are isoflavones, which are found in high concentration in soybeans (0.2-1.6 mg /g dry weight). Depending on diet, human plasma concentrations of isoflavones can vary by more than 100-flods, and can reach 6 μM (Cassidy, 2003). The soy and soy products have been a tradition food for eastern Asian populations for thousands years. Daidzein, genistein and their glycoside conjugates represent the major isoflavones, along with small amounts of glycitein and its glycoside derivatives. These compounds are also present in significant concentrations in various beans and sprouts (Hendrich, 2002). However, dietary estrogen (phytoestrogen) can be also found in wide variety of food products (including herbs), even though the level varies depending on the source. Below is a short list of phytoestrogen food sources analyzed by researchers in Canada (Huang *et al*., 2012).

2.2.3 Effects of Phytoestrogen

In addition to being a source of compounds necessary for human nutrition, certain plant foods also contain compounds that may have long-term effects on human and animal health which among the most important are the phytoestrogen. Some plants contain steroidal estrogens, but as these are essentially based on the same structures that occur naturally in animals, they are not considered as phytoestrogen because of the confined definition. Estrogenic compounds appear in more than 300 plant species, but few of these are consumed by animals or humans. Almost of these compounds occur in the plant in glycosylated forms, and importantly, the bioavailability of the glycoconjugates might be different forms that of the unsubstituted aglycones, which are often, used in animal feeding and model cell culture studies. The effects of phytoestrogens are related not only to the interaction with estrogen receptors (ER), but also to other action which is not mediated by the ER in a similar fashion to estrogen. In addition to the classical ligand-dependent action (figure 12), there are three non-classical actions; ligand-independent; DNA binding-independent; cell-surface (non-genomic) signaling (Hall, Couse, & Korach, 2001). The non-classical actions seem particularly important in explaining the selective action of phytoestrogens on bone.

The biological activity of osteoblast and osteoclasts are regulated by many factors including growth factors, cytokines, and hormones and also the mechanical stress (Ducy, Schinke, & Karsenty, 2000). The model for estrogen regulation on bone cells have been shown both in osteoblasts and osteoclasts (Sundeep, Merry , & David, 2012) (figure 13).

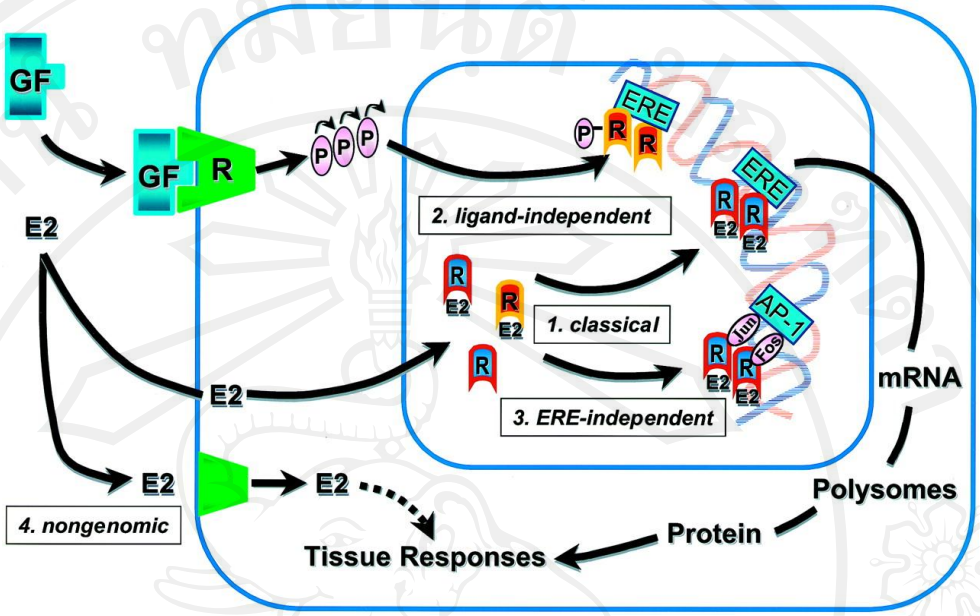

Figure 12 Estrogen signaling (Hall, Couse, & Korach, 2001).

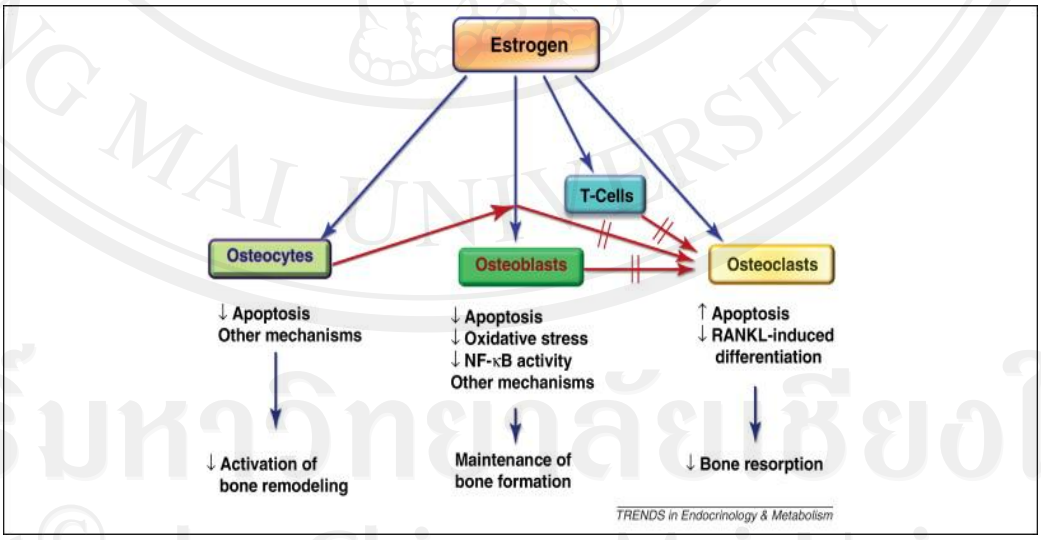

Figure 13 Model for estrogen regulation of bone cells

(http://dx.doi.org/10.1016/j.tem.2012.03.008 access date, 15 May 2012).

2.3 Bone cell

Bone is a complex tissue composed of cells, collagenous matrix, and inorganic elements. It provides many essential functions, including mechanical support, protection of vital organs, a microenvironment for hematopoiesis, and a depot for calcium and other minerals. The growth, development, and maintenance of bone is a highly regulated process (Nijweide, Burger, & Feyen, 1986). During development and growth, the skeleton is sculpted to archive its shape and size by the removal of bone from one site and deposition at a different one; this process is termed remodeling.

Bone remodeling is the process by which the catabolic effects (bone resorption) of one cell type of bone, osteoblasts, are balanced by the anabolic effects (bone formation) of a second cell type, osteoclasts. Normal bone remodeling had proceeded in a highly regulated cycle in which osteoclasts adhere to bone and subsequently remove it by acidification and proteolytic digestion. Once osteoclasts leave the removal site, osteoblasts enter and secrete osteoid, which is a matrix of collagen and other protein that is calcified into new bone

Both osteoclasts and osteoblasts are derived from precursor origination in the bone marrow. The precursors of osteoblasts are multipotent mesenchymal stem cells, which also give rise to bone marrow stromal cell, chondrocytes, muscle cell, and adipocytes. The precursors of the osteoclasts are hematopoietic cells of the monocyte /macrophage lineage. The genesis and differentiation of either cell type is regulated by growth factors and cytokines produced in the local microenvironment as well as by systemic hormones that control the production and/or action of growth factors,

inflammatory mediators and cytokines, and adhesion molecules (Manolagas & Jilka, 1995; Mundy, 1992, 1993). In spite of the fact that millions of small packets of bone are continually remodeled, bone mass is preserved in the healthy skeleton tanks to a remarkably tight balance between the amount of bone resorbed and formed during each cycle of remodeling (figure 14).

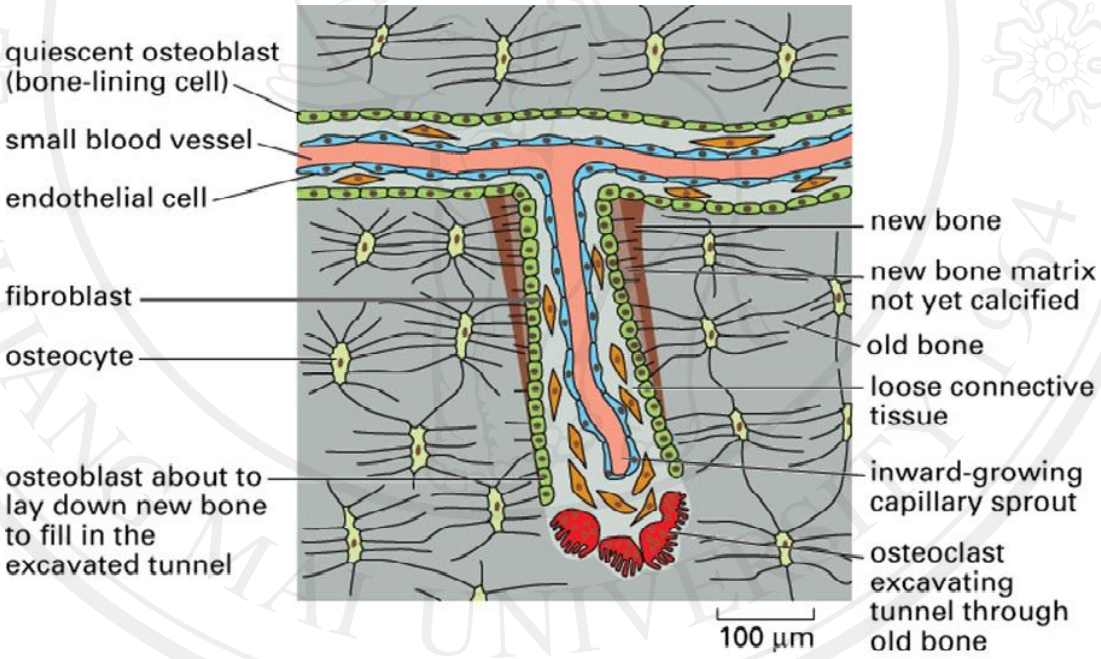

Figure 14 The remodeling of compact bone

(http://www.ncbi.nlm.nih.gov/books/NBK26889/ access date, 30 June 2012).

2.3.1 Osteoporosis

Osteoporosis is a condition, characterized by low bone mass and structural deterioration of bone tissue, leading to bone fragility and increased susceptibility to fractures of the hip, spine, and wrist. This condition is generally associated with old age and women with menopause (Bahram & Arjmandi, 2001) (figure 15). Erosion of the trabecular bone has resulted in enlargement of the spaces between trabecular. It is well known that estrogen deficiency in postmenopausal and ovaricetomy leads to acceleration of bone resorption and rapid bone loss, resulting in the development of osteoporosis. The pathologic bone loss from this condition can largely be prevented by early estrogen replacement therapy, but the mechanism by which estrogen exert their bone sparing effect is still unclear. Recent advances suggested that the female hormone 17 β-estradiol (E_2) regulates the circuitry of cytokine action which controlled bone remodeling, potentially providing a more precise understanding of how E_2 exerts its action in bone. Advanced bone tissue and cell culture techniques were lead to the identification of estrogen receptors in the bone forming (osteoblast) and bone resorbing (osteoclasts), which in part, explained the role of estrogen on bone as a target tissue (Kennedy *et al.*, 2005; Turner, Riggs, & Spelsberg, 1994). Although the mechanism of 17 β-estradiol action, it represented the major estrogen in human, which was documented at the molecular level. Like other steroid hormones, estrogens bind to specific intracellular receptors and regulate the transcription of defined sets of responsive genes. Thus, estrogen promoted the expression of traits associated with the formation of bone while reducing cellular responsiveness to hormones that may trigger the resorption of bone (Sundeep *et al.*, 2012). Currently available treatments

for post-menopausal osteoporosis include hormone replacement therapy, bisphosphonates, calcitonin, calcium products and selective estrogen receptor modulators (SERMs), such as raloxifene. Moreover, clinical practice has found that women undergoing treatment for osteoporosis require long-term dosing regimen that offer non symptomatic relief and may cause side effects. As with other chronic disease, continuance and compliance with therapy are poor (Radford *et al.*, 2002).

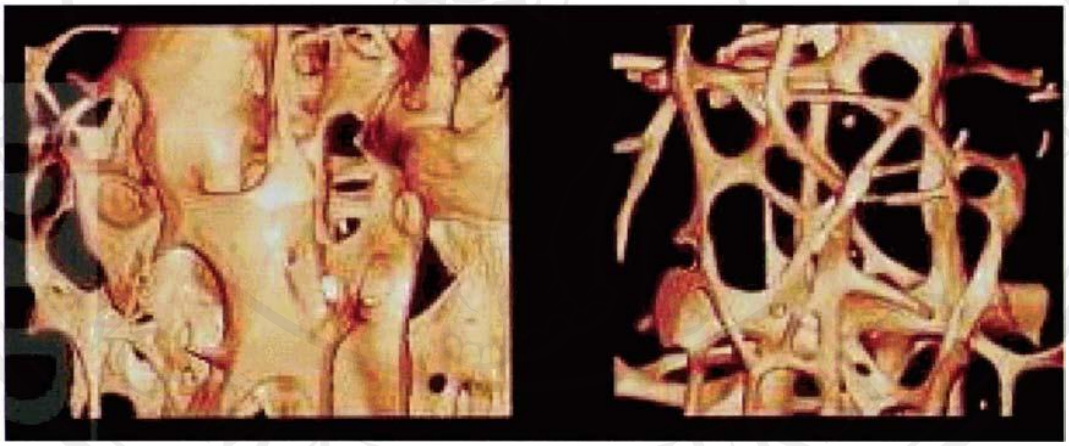

Figure 15 Structure of the vertebrae of a healthy 50 year old (left) and an osteoporotic 70 year old (right) as visualised by x-ray microtomography; Photo courtesy of ESRF -CREA TIS / Photo Researchers, Inc.

2.3.2 Effects of Phytoestrogen on Bone cell

In vitro studies indicate that phytoestrogens such as genistein are able to stimulate osteoblastic activity and inhibit osteoclast formation and action at range of concentration (10^{-5}-10^{-7}M) consistent with the levels observed in human subjects after ingestion of genistein. The effects of genistein significantly increased breaking strength, bone quality, serum bone-alkaline phosphatase, osteoprotegerin (OPG), reduced collagen C-telopeptide (CTX) and soluble receptor activator of nuclear factor-κB ligand (sRANKL) compared with alendronote, raloxifene and oestradiol in an established osteoporosis of ovariectomized rat model (Bitto *et al.*, 2008).The results strongly suggest that genistein aglycone might be a new potential therapy for the management of postmenopausal in humans combining a powerful bone-forming as well as an anti-resorptive activity.

Another reported effects of phytoestrogen in bone are ipriflavone (7-isopropoxyisoflavone; IP), which is derived from the soy and has important effects on bone metabolism (Albanese *et al.*, 1994). The effect of IP on bone formation *in vivo* was studied in rat peri-alveolar bone after surgically producing a hole in the mandible; the result was consistent with a role of IP in stimulating osteogenesis and suggested that IP is a potential therapeutic tool for promoting the repair of injured peri-alveolar bone (Martini *et al*., 1998). Furthermore, a study of effect of IP on augmented bone using guided bone regeneration (GBR), within the limitations of a rabbit experimental model, concluded that the daily intake of IP before or after GRB inhibited the resorption of augmented tissue and would be useful for improving the quality of newly generated bone beyond the skeletal develop (Ito *et al*., 2007). In some studies,

the tested phytoestrogen mostly displayed positive profiles in osteoblasts and osteoblast-like cell lines. Therefore a feasible potential of these compounds to effectively prevent osteoporosis in vivo might be concluded in some instances, these effects are even stronger than those of 17 β-estradiol. Importantly, they also influence markers of bone metabolism to a similar extent as17 β-estradiol. The tested compounds can therefore be declared phytoestrogens because they display estrogenic activity in several independent assays. Phytoestrogens such as genistein inhibited bone loss caused by estrogen deficiency in mice (Ishimi), and an analogue of coumestrol increased alkaline phosphatase (ALP) activities. This is one of the osteoblastic phenotype markers in osteoblast-like cell. Biochanin A, an isoflavone, which has significantly influenced the differentiation of MC3T3-E1 cell, an osteoblast-like cell line, as in terms of significant elevation of cell growth, alkaline phosphatase (ALP) activity, collagen content, and osteocalcin secretion (Tripathi *et al*., 2008). These results suggest that phytoestrogens can reproduce some of the developmental events of bone formation, mineralization, and organization of a bone-like extracellular in osteoblast culture models.

The hFOB 1.19 human fetal-osteoblast cell line displays osteoblast-specific phenotypic marker and mineralizes extracellular matrix (ECM). The gene coding for the human wild – type estrogen receptor (ER) was stably transfected into the human fetal-osteoblast cell line hFOB 1.19. The subclone with the highest level of functional (nuclear bound) ER, hFOB/ER9, was examined further for17 β-estradiol (E_2) responsiveness. The ER expressed in hFOB/ER9 cells was shown to be function using luciferase construct. These construct increased 25-fold in hFOB/ER9 cells

following 10^{-9} M E_2 treatment (Harris *et al*., 1995). *In vitro* study showed that the expression and production of dietary isoflavone-mediated osteoclastogenesis-regulatory cytokines, such as interleukin-6 (IL-6) and osteoprotegerin (OPG), are related to the different levels of estrogen receptors expressed in hFOB osteoblastic cell lines. The increased expression of OPG and decreased IL-6 production by isoflavones, such as genistein, or daidzein, were dose-dependent. Responses to isoflavones were much stronger in hFOB/ER9 cells, which express the estrogen receptor 20 times higher than those in hFOB1.19 cells (Chen, Garner, & Anderson, 2002). Both genistein and E_2 increased the endogenous gene expression of the progesterone receptor (PR), the proteoglycan versican, and alkaline phosphatase (AP), but inhibited osteopontin (OP) gene expression and interleukin-6 (IL-6) protein levels in hFOB/ERα9 cells. The establishment of the estrogen responsive human osteoblastic cell line should provide an excellent model system for the study of estrogen action on osteoblast function.

2.4 Biological Assay for phytoestrogenicity

In order to measure the estrogenicity of a plant-derived chemical compound, several factors must be taken into account: 1) affinity of the compound for the estrogen receptor, 2) accumulation of the compound in the environment and the body, 3) degradation or metabolism of the compound in the environment and body, and 4) the availability of the compound to the target cell. So far there are no publications about the experiments on the estrogenic activity towards the different glycosides naturally occurring in vanilla beans. *In vivo* and *in vitro* showed that had been

developed to test estrogenic substances, the binding of a ligand to estrogen receptor was the simplest and suitable for screening of estrogen and anti-estrogenic substance.

2.4.1 Yeast Estrogen Screen (YES)

Yeast cells carrying the human estrogen receptor (hER) gene, estrogen response elements (ERE) and *Escherichia coli* β-galactosidase gene (lacZ) are very suitable for large-scale screening and sensitive analysis for estrogenic compound. This model system has been proven as useful an assay for discovering novel estrogenic substances in Oriental medicinal plants (Arnold *et al.*, 1996).

This system is on the ligand-dependent interaction of two proteins, a hormone receptor and a coactivator. The hormonal activity is detected by β-galactosidase activity (Figure 16).

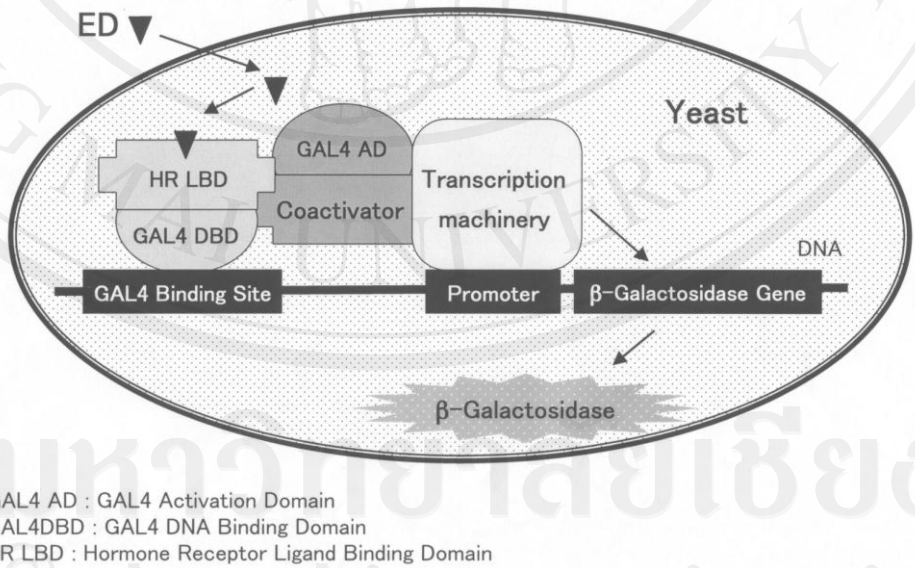

Figure 16 Outline and Principle of Yeast Two-Hybrid Assay for Estrogenic activity (Haeng *et al.* , 2002).

According to the scheme, the compound tested (ligand) penetrated the cells and bound at LBD which fused to GAL4-DBD to form the ligand – receptor complex. Since the yeast strain used for the creation of the test system contained GAL4-binding site at the promoter region of the lacZ reporter gene, if the ligand – receptor complex interacts with the coactivator fused to GAL4-AD, the transcription mechanism is triggered by activating the lacZ gene promoter, which eventually led to the expression of the enzyme β-galactosidase, the final product. For this reason, the activity of *de novo* enzyme synthesized β-galactosidase reflects the effectiveness of the interaction between estrogen, ER and the coactivator. The protein – protein interaction between the ER receptor complex and the coactivator directly depended on the coactivator nature (Haeng *et al.*, 2002).

This assay is a test method with high repeatability, and has tested the estrogenic activity of more than 500 chemicals including natural substances, medicines, pesticides, and industrial chemicals. Sixty- four compounds were evaluated as positive, and most of these demonstrated a common structure; phenol with a hydrophobic moiety at the para-position without bulky groups at the ortho-position (Nishihara *et al*., 2000) . On the basis of this knowledge, it has been screened a selection of commercially used phenolic additives. The activity of 73 phenolic additives that are used as sunscreens, preservatives, disinfectants, antioxidants, flavoring, or for perfumery was used a recombinant yeast estrogen assay. The major criteria for activity appear to be the presence of an unhindered phenolic OH group in *para* position and a molecular weight of 140-250 Da (Miller *et al.*, 2001). These results are expected to facilitate further risk assessment of chemicals.

2.4.2 β-galactosidase enzyme activity

β-Galactosidase is able to hydrolyze (cleave) β-D-galactosides. This enzyme facilitates growth on carbon sources like lactose by cleaving into a molecule of glucose and a molecule of galactose which the cells can catabolize and grow on. In the assay described above, the substrate o-nitrophenyl-β-D-galactopyraniside (ONPG) is used place of lactose. When β-galactosidase cleaves ONPG, o-nitrophenol is released. This compound has a yellow color, and absorbs 420 nm light.

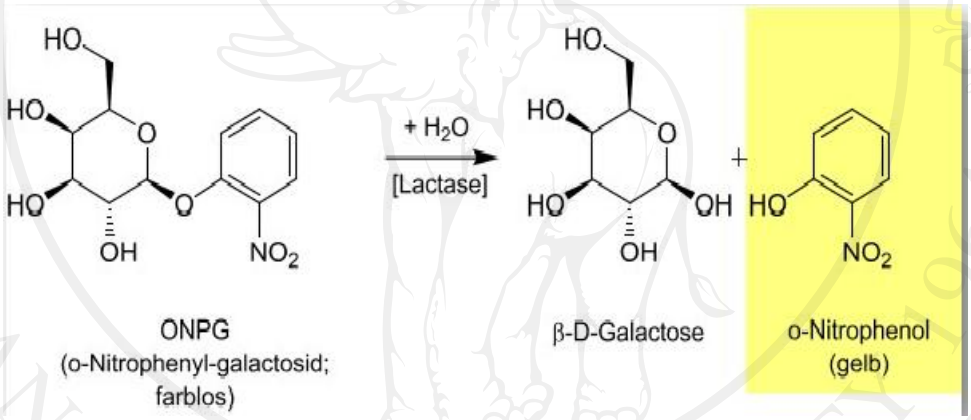

Figure 17 β-galactosidase enzyme activity

http://rothlab.ucdavis.edu/protocols/beta-galactosidase-3.html

access date, 7 July2012).

2.4.3 Osteoblast alkaline phosphatase Activity

Osteoblasts are protein-secreting cells. Their secreted proteins play an important role in bone formation. One of them is collagen protein, which is the major component in osteoid, another one is alkaline phosphatase (ALP), which is of great importance in bone mineralization. During the process of mineralization, ALP reacts with phospholipid and releases inorganic phosphoric acid locally. The inorganic

phosphoric acid then binds with calcium to form hydroxyapatite crystal and deposit between collagens, facilitating osteoid mineralization (Sugawara *et al*., 2002). The ALP expressed strongly in active osteoblasts. ALP activity was measured by p-nitro-phenyl phosphate (PNPP) method, and normalized to total protein. The ALP activity = (ALP activity in drug group minus ALP activity in control group)/ALP activity in control group x 100).

2.4.4 Osteoblast Mineralization

Osteoblasts form mineralized nodules *in vitro* in the presence of ascorbate and *β*- sodium glycerophosphate. Mineral nodules can be stained positively with alizarin red. Mineralized nodules are one of the main parameters indicating the bone formation function of osteoblasts. The mineralization nodules stained positively with alizarin red and they were quantified histomorphometric. Mineralization capacity = (Number or area of mineralization nodules in drug group - Number or area of mineralization nodules in control group)/ Number or area of mineralization nodules in control group x100).

2.4.5 Osteoblast Proliferation Rate

The MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) method can be used to show the proliferation rate of osteoblasts in vitro. Osteoblasts were cultured in 96-cell plates with drug for 1–10days. Absorbance value for the osteoblasts was measured dynamically using auto-microplate reader .The proliferation rate = (OD value in drug group minus OD value in control group)/OD value in control group x100).