CHAPTER II
REVIEW OF THE LITERATURE

2.1 Phytoestrogens; chemical approach

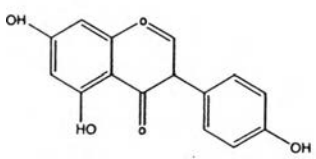
2.1.1 Origin and classification

A phytoestrogen is any plant derived compound that induces biological responses in vertebrates and can mimic or modulate the actions of endogenous estrogens usually by binding to estrogen receptors. It was firstly found in the outbreak of infertility in sheep grazing on pastures rich in subterranean clover (Bennets et al., 1946). Phytoestrogen is subdivision of flavonoids and classified as isoflavones, lignans and coumestans, which distributed in various plants (Table 2.1). Isoflavones are found predominately in soybeans (*Glycine max* L.), whereas coumestans have been preliminary identified by genus of *Trifolium* such as clovers (Franke et al., 1994) and species of *Pueraria* and Glycyrrhiza (Dewick, 1993). Genistein and daidzein is the major isoflavones found in soybeans and their products. The available data especially in epidemiological study are examined the relationship between the consumption of natural food containing phytoestrogens and the reduced risk of cardiovascular symptoms, cancer and osteoporosis.

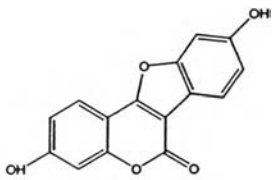
Chemical structure of phytoestrogen is diphenolic (better bisphenolic) compounds that comprised of 2 benzene rings (A and B) linked through a heterocyclic pyran or pyrone ring (C) in the middle (Figure 2.1). The basic structure of phytoestrogens is closely similar to natural and synthetic estrogens and antiestrogens such as resorcyclic acid lactones (e.g. zearalenone). When both structures of phytoestrogen and estradiol are superimposed, the distance between the hydroxyl groups is identical (Figure 2.1). Isoflavones are often present as glucoside conjugates (glycones) such as genistin, daidzin and glycitin. These glycosides can be further metabolized in gut to aglycone such as genistein, daidzein and glycitein. Based on the structural similarities, phytoestrogens can bind to estrogen receptors (ERs) (Setchell, 1998 and Hopert et al., 1998) and act as a weak estrogen (Setchell, 1998).

Table 2.1 Classification and sources of phytoestrogens (adapted from Krazeisen et al., 2001 and Cornwell et al., 2004)

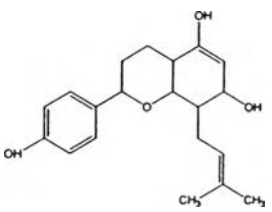
Class	Examples	Sources
Flavonoid		
Isoflavonoids	• Genistein	• Soybeans, beer
	• Daidzein, Equol	• Clover, beer, soybeans
	• Glycitein	• Soybeans
	• Biochanin A	• Red clover, beer, bourbon
	• Formonoetin	• Red clover
Coumestans	• Coumestrol	• Clover, alfalfa, beans, *Pueraria* sp.
Prenyl flavonoids	• 8-Prenylnarigenin	• Grapefruit
	• 6-Prenylnarigenin	• Grapefruit
Non-flavoniods		
Lignans	• Isolaricriresinol	
	• Matairesinol	• Flaxseed, black gram, tomato, strawberries
	• Secoisolariciresinol	
	- (Enterodiol)	• Oilseed, flaxseed, black gram, tomato, strawberries
	- (Enterolactone)	• Linseed, flaxseed, cereal bran, whole cereals, vegetable, fruits, legumes



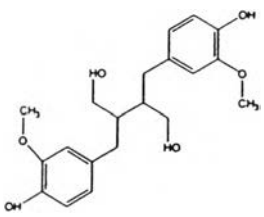
Genistein



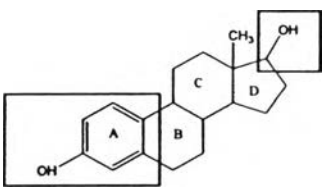
Coumestrol



8-Prenylnaringenin



Secoisolariciresinol



Estradiol

Figure 2.1 The similarity of the structure of the human hormone, estradiol and phytoestrogen examples. Outlined in boxed on estraidol structure of phenolic and hydroxyl moieties. The distance between the two groups in each compound is similar.

2.1.2 Analysis of phytoestrogens

Generally, phytoestrogens and their metabolites are present in part per billion to part per million in plants, foodstuff as well as in biological fluids such as urine, plasma and feces. Initially, phytoestrogens were analyzed using simply techniques such as thin-layer and paper chromatography. However, the development of increasingly sensitive technologies has advanced phytoestrogen analysis considerably. The most widely used methods for quantify of phytoestrogens are high performance liquid chromatography with ultraviolet detection (HPLC-UV) (Wang et al., 1990; Franke et al., 1994 and Thomas et al., 2001), gas chromatography with mass spectrometric detection (GC-MS) (Mazur et al, 1996; Tekel et al., 1999 and Nesbitt, Lam and Thompson, 1999) and liquid chromatography with mass spectrometric detection (LC-MS) (Coward et al., 1996; Cimino et al., 1999 and Doerge, Churchwell, and Delclos et al., 2000).

These developments have also been useful in pharmacological and toxicological studies. Prior to analysis and their metabolites, phytoestrogens must be isolated from matrices. The extraction of phytoestrogens is required. During extraction, phytoestrogen might be lost; an appropriate internal standard must be added prior to extraction. A general schematic of the steps involved in extraction and anyalyse of phytoestrogens is shown in Figure 2.2

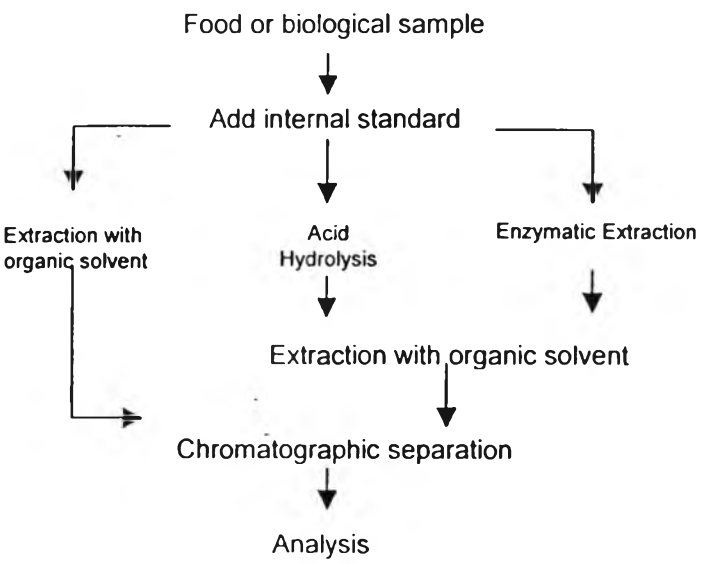


Figure 2.2 A general schematic of the steps involved in extraction and analysis of phytoestrogens (Adlercreutz et al., 1986 and Franke et al., 1994).

2.2 Phytoestrogens; physiological approach

2.2.1 Phytoestrogen action

Numerous studies have shown that phytoestrogens can exert multi-functions through genomic and non-genomic mechanisms of cellular regulation by competing to estrogen receptor binding or interfering to estrogen biosynthesis and metabolism.

2.2.1.1 Cellular and molecular mechanisms

According to the classic concept, estrogens are steroid hormones, which involved in the important functions for the sexual processes and act through protein, estrogen receptors which distributed in reproductive tissues such as ovary, mammary, uterus and vagina. Until recently, ER has been identified into two subtypes, ERβ and ERα. ERβ, discovered only a few years ago, is heterodimers in DNA binding domain (over 95% amino acid identity) and splicing variants of ERα (Kuiper et al., 1996 and Mosselman et al., 1996). ERs are predominately present in nucleus where is formed the complex with heat shock proteins when received the stimulating (Figure 2.3 and 2.4). Bound ERs are activated to the specific DNA-binding sites called estrogen receptor response elements (ERE) or AP-1 site. After binding, the target gene transcription in initiated or repressed which ultimately elicits biological responses as agonist or antagonist characters (Clark, et al., 1996; Fitzpatrick, 1999 and Diel, Smolnikar, and MIchna, 1999) depending on the phytoestrogens concentration and target organ (Setchell et al., 1998). If phytoestrogens induce biological effect as estradiol, they are considered as agonist, however, the potency is too weak that require much higher concentrations to boost the responses as well as estradiol. On the other hand, phytoestrogens can act as antagonists by block the binding of estrogen that caused an interrupted hormone response.

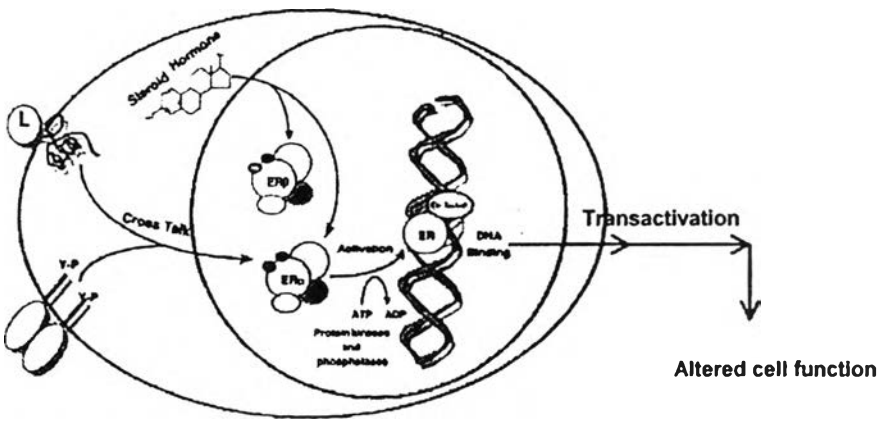


Figure 2.3 Mechanisms of estrogen receptor activation on target cells (Adapted from Diel, Schmidt, and Vollmer, 2002)

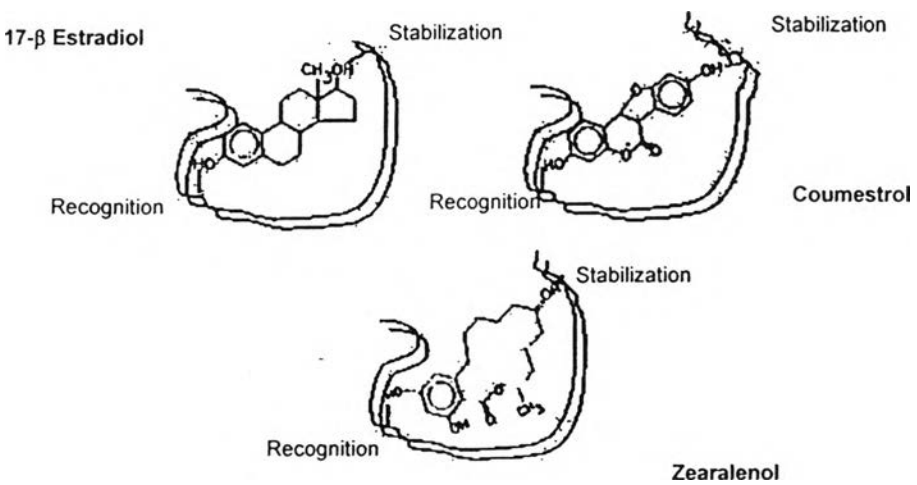


Figure 2.4 Structure-activity relationships of estrogen and some phytoestrogens (coumestrol and zearalenol) on estrogen receptor lignands (Clarke et al., 1996)

2.2.1.2 Biotransformation and metabolism

The metabolisms of isoflavonoids and lignans show similar patterns in animal (Price and Fenwick, 1985) and human (Adlercreutz et al., 1991) whereas coumestan have not been identities. After consumption, isoflavone and lignan glycosides are probably hydrolyzed within gastrointestinal tract by gastric acid (Xu et al., 1995) and intestinal microflora hydrolysis enzymes. The precursor of genistein and daidzein are biochanin A and formononetin, respectively (Figure 2.5). After absorption, isoflavonoids are transported to the liver, reconjugated and then excreted in urine and bile. The reconjugation of aglycone with glucuronic acid and sulfuric acid is function by hepatic phase II enzymes (Morton et al., 1994 and Adlercreutz et al., 1993). However, genistin was partly absorbed without previous cleavage (Andlauer, Kolb, and Fürst, 2000). In human, aglycones were absorbed faster and in greater amounts than their glycosides (Izumi et al., 2000). The maximum peak of isoflavonoids is range at 7- 8 hr after consuming a single soy meal (King and Bursill, 1998). Those isoflovones have been detected in biological fluid including plasma (Adlercreutz et al., 1994), amniotic fluid (Adlercreutz et al., 1999), urine (Adlercreutz et al., 1991), feces (Adlercreutz, et a., 1995), milk (Franke and Custer, 1996), saliva, breast aspirate (Hargreaves et al., 1999) and prostatic fluid (Finlay et al., 1991).

Biochanin A and formononetin are metabolized by gut microflora to genistein and daidzein, respectively. Genistein can be further metabolized to 4-ethylphenol and daidzein to equol, dihydrodaidzein and *O*-desmethylangolensin (Anderson and Garner, 1997). The data suggest that equol has a greater antioxidant effect than other phytoestrogens, which often found in highest level in biological matrices and exert significant biological effects (Hodgson et al., 1996).

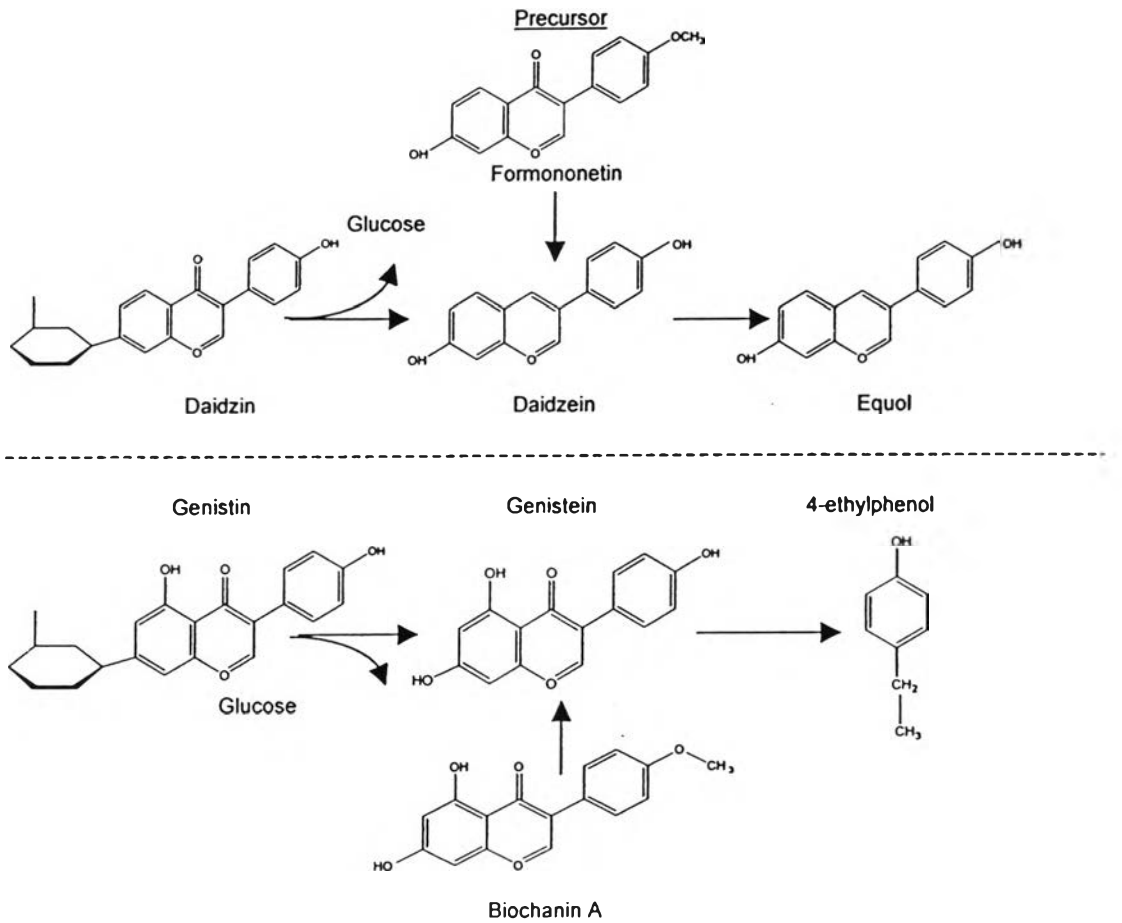


Figure 2.5 Schematic of some phytoestrogen metabolism in intestine (Anderson and Garner, 1997).

2.2.1.3 Phytoestrogens potency and active concentration

Bound compound to both type of ERs can stimulate transcription of ERE, with different tissue expression patterns. The splicing variants lead to different relative binding affinities in different compounds. Assessment of estrogenic potency varies across assays such as receptor binding affinity, transcriptional activation, cell proliferation and *in vivo* assays. It is derived from the difference in copies of EREs in used cells. Generally, a low concentration of phytoestrogen (1-100 nM) stimulates cell growth, whereas, a high concentration (5-100 μM) shows suppression. This is called a biphasic pattern.

Phytoestrogens can bind with higher affinity to ERβ on transcriptional level than to ERα (in which amount %, should mention) (Kuiper, et al., 1997 and 1998 and

Casanova et al., 1999). Phytoestrogens can affect endogenous hormonal production by inhibiting the key enzyme of hormonal biosynthesis (Evans, Griffiths and Morton, 1995; Kao et al, 1998 and Makela et al., 1998) or stimulating of sex hormone binding globulin (SHBG) (Adlercreutz et al., 1987), however, the estrogenic potency is less than estradiol. The proliferation action occurs at nanomolar concentration level. *In vitro* study, the concentration of phytoestrogens to elicit a response on ERα and ERβ site are 0.145 and 0.0084 μM, respectively (IC_{50} values for competition of estradiol-recepter binding) (Kuiper et al., 1998). Compared with all phytoestrogens, coumestrol has the highest estrogenic potency (Kuiper et al., 1997), however, an affinity to ERα is ten times less than estradiol. It is noticed that the methoxy derivative of genistein and biochanin A does not bind ER, but they can show estrogenic activity *in vivo*. This might be the role of hydroxyl substituents at 4' and 7' positions in phytoestrogen groups (Figure 2.4). The ranking of phytoestrogens potency as compared with estradiol is estradiol>coumestrol>8-prenylnaringenin>equol≥genistein> biochaninA> daidzein>genistin>glucoronide>daidzin>glucoronide>formononetin (Kuiper et al., 1997 and Milligan et al., 2000) (Table 2.2).

Table 2.2 Relative binding activity of estrogen and various phytoestrogens binding to ERα and ERβ

Compound	**Binding at ERα**	**Binding at ERβ**
17- β Estradiol[a]	100	100
Estrone[a]	60	37
Estriol[a]	14	21
Progesterone[a]	<0.001	<0.001
Testosterone[a]	<0.01	<0.01
Coumestrol[a]	94	185
Genistein[a]	5	36
Daidzein[a]	0.2	1
8-Prenylnaringenin[b]	10	10
β-Sitosterol[a]	<0.001	<0.001
Tamoxifen[a]	7	6

([a]Kuiper et al., 1997 and [b]Milligan et al., 2000)

2.2.2 Biological and pharmacological effects

2.2.2.1 Hormonal effects

The hormonal effects have been shown in both animal and human, however, the results have been inconsistency depending on the analytical methods. Phytoestrogens are quite weak according to both *in vitro* and *in vivo* assays, which possessing less than activity of estradiol between 1,000 and 10,000 (Folman and Pope, 1966 and Markiewicz et al., 1993). The phytoestrogen-rich plant, red clover demonstrated hyperestrogenize and infertility in grazing animals (Bennets, Underwood and Shier 1946 and Shutt and Braden, 1968). In uterine growth of female mice, subcutaneous injection of genistein could inhibit the stimulation of estrone (Folman and Pope, 1966). The length of the follicular phase in pre-menopausal women is increased when administered within isoflavone-rich diet (Cassidy, Bingham and Setchell, 1995 and Lu et al., 1996). On the other hand, progesterone (Lu et al., 1996 and Lu et al., 2000) and testosterone could be decreased (Strauss et al., 1998). Estradiol-17β in serum is also affected (Cassidy, Bingham, and Setchell, 1995). At high dosages of isoflavonoids, the feedback-regulating system of the hypothalamus-pituitary gland axis is interrupted and affected to hormonal status.

Genistein has been shown to exert both estrogenic and antiestrogenic activity in human cell lines (MCF-7) (Wang, Sathyamoorthy, and Phang, 1996 and Sathyamoorthy, Wang, and Phang, 1994). In cell culture, the low concentration of those compounds to stimulate estrogen-dependent receptor gene activity is 10-1000 nM (Kuiper et al., 1998). Moreover, some reports have shown that soy isoflavonoids could increase nerve growth factor mRNA and brain-derived neurotropic factor mRNA in rats (Pan, Anthony and Clarkson, 1999) and also affect the cell signaling conduction on receptor expression. At high concentration of genistein, EGF receptor expression is inhibited in rat uterus and vagina (Akiyama et al., 1987 and Brown and Lamartiniere, 2000). Phytoestrogens may involve in estrogen synthesis and metabolism through interfering and/or inhibiting estrogen related enzyme such as 17-β-hydroxysteroid oxidoreductase; the conversion catalyst of estrone to estradiol (Makela et al, 1995), aromatase (Kellis and Vickery, 1984 and Pelissero et al., 1996) and steroid sulphatase (Murkies, Wilcox, and Davis, 1998 and Krazeisen et al., 2001). Aromatase converts the androgens, dehydroepiandosterone and testrone to

estrogen and estradiol, repectively. Inhibition of these enzymes would alter the balance between estradiol and the less potent of estrone. Genistein and daidzein also suppressed glucocorticoid and stimuated androgen production in human adrenal cortical cells cultures (Mesiano et al., 1999).

In HepG2 liver cancer cells culture, isoflavonoids have been reported to stimulate the biosynthesis of SHBG. It is noticed that the change of SHBG concentration affect in relatively large changes in amount of free and bound hormones whereas the change of total hormone concentration affect in relatively small changes (Loukovaara et al., 1995 and Mousavi and Adlercreutz, 1993). To sum up, the action of phytoestrogen depends on the hormonal status of animal and human.

2.2.2.2 Anticarcinogenic effect

There are evidences in human studies, pointing to the potency of soy products or phytoestrogen such as genistein or daidzein to inhibit and prevent on various cancers such as endometrial (Goodman et al., 1997), prostate, (Jacobsen, Knutsen and Fraser, 1998; Herbert et al., 1998; Kolonel et al., 2000 and Strom et al., 1999), stomach (Nagata, 2000 and Nagata et al., 2002), colon (Nagata, 2000), thyroid (Horn-Ross, Hoggatt and Lee, 2002), lung (Seow et al., 2002) and mammary (Lee et al., 1991; Yuan et al., 1995; Hirose et al., 1995; Wu et al., 1996; Ingram et al., 1997; Witte et al., 1997; Key et al., 1999; Dai et al., 2001 and Shu et al., 2001). It is noticed that all these cancers are hormone-dependent.

Understandably, anticancer effects of soybean have primarily attracted attention on breast cancer. The low incidence of breast cancer in East Asia was found associated with consumption of typically Asian/oriental diet (Tham, Gardner and Haskell, 1998). Animal studies support the notion that phytoestrogen have also been found to exert the inhibition of chemically induced mammary cancer (Barnes et al., 1990; Lamartiniere et al., 1995a, Murrill et al., 1996 and Gotoh et al., 1998a and 1998b). It was confirmed *in vitro* studies, isoflavonoids inhibit cancer cell growth including prostate cancer cell line (Davis et al., 1998 and Hillman et al., 2001) and MCF-7 human breast cancer cell line (Hsu, Ying, and Chen, 2000).

There is a correlation of reduced risk of breast cancer with a high plasma level of phytoestrogens such as mammalian lignan, enterolactone (Pietinen et al., 2001). Several reports have shown that genistein can inhibit the growth of both hormone-dependent and hormone-independent cancers with IC_{50} at 5 - 100 μM/L (2-25 ug/mL) (reviewed in Messina, 1999). However, genistein can stimulate those cancers at physiologically concentrations (<5-6 μmol/L) (Zhang et al., 1999 and Ren et al., 2001).

The numerous mechanisms may be involved such as the inhibition of angiogenesis (Fotsis et al., 1993), protein tyrosine kinases (Akiyama et al., 1987) and related hormonal enzymes. The preventive effect of isoflavonoids may involve the decreasing synthesis and altering metabolism form of estrogen (Xu et al., 1998 and 2000). In addition, genistein has been shown to inhibit the metastatic activity of breast cancer (Scholar and Toews, 1994) and prostate cancer cells (Santibanez, Navarro and Martinez, 1997). Cell cycle progression at G_2-M is arrested by genistein that result to the differentiation and apoptosis of various cancer cell lines including human gastric cancer (Yanagihara et al., 1993), human breast carcinoma (Shao et al., 1998), leukemia (Spinozzi, et al., 1994), melanoma (Rauth, Kichina, and Green, 1997) and colon (Kuo, 1996).

In vitro and *in vivo* studies, at micro molar concentration level of phytoestrogens exert various non-hormonal related effects. Genistein was shown to inhibit DNA topoisomerase I and II (Yamashita, Kawada, and Nakono, 1990; Ji et al., 1999; Martin et al., 2000 and Salti et al., 2000) that effected to DNA damage and epidermal growth factor-induced phosphatidylinositol turnover (Imoto et al., 1988). Biochanin A is linked to increase nitric oxide level that later induction of cell apoptosis (Hsu et al., 1999). In addition, phytoestrogens may exert their effects by decreasing the activity of enzymes that activate procarcinogens, such as cytochrome P450 (CYPs) (Roberts-Kirchhoff et al., 1999).

2.2.2.3 Other effects

Epidemiological observations and laboratory animals and *in vitro* investigations have revealed a number of biological properties suggesting a prevention of western diseases such as cardiovascular, atherosclerosis,

hypercholesterolemia, menopausal symptoms and osteoporosis (review in Kurzer and Hu 1997; Bingham et al., 1998 and Tham et al., 1998).

In vivo and epidemiologic studies have demonstrated that soy protein reduced risk of coronary heart disease and atherosclerosis (Anderson et al., 1999; Anthony, 2000, and van der Schouw et al., 2000). The effects result from a reduction of plasma low-density lipoprotein (LDL) (Tovar-Palacio et al., 1998; Crouse et al., 1999 and Ashton and Ball, 2000) cholesterol and triglycerides (Anderson, Johnstone, and Cook-Newell, 1995 and Ho et al., 2000). Soy could reduce absorption of dietary (Greaves et al., 2000), arterial permeability, concentration and delivery of LDL (Wagner et al., 2000) and increased LDL receptor quantity and activity (Baum et al., 1998).

Many studies suggest that phytoestrogens play role in maintaining bone density in postmenopausal women (Dalais et al., 2003; Alekel et al., 2000 and Kim et al., 2002). In animal studies, isoflavonoids could prevent bone loss that occurs as a result of estrogen deficiency in ovariectomized rats (Fanti et al., 1998; Vincent and Fitzpatrick, 2000; Picherit et al., 2000 and Uesugi et al., 2001). Postmenopausal woman seems to benefit the most from consumption of soy phytoestrogens. Bone mineral density (BMD) of the lumbar spine is increased with the treatment of 90 mg. isoflavonoids per day for 24 weeks (Potter et al., 1998). Osteoclastic bone resorption is inhibited by genistein and daidzein (Ono, Ma, and Yamaguchi, 2000) but stimuted osteoblastic bone formation (Yamaguchi, Gao, and Ma, 2000). However, data available in human about the effect of isoflavonoids on osteoporesis is limited.

Since estrogen has an important effect on the immune system. A changing of estrogen level result to autoimmune diseases that commonly occurs in women (Enmark and Gustafsson 1998 and 1999). Isoflavonoids have also exerted an anti-inflammatory potential in various animal models. High dosages of daidzein (20 and 40 mg/kg) can enhance several immonologic function (Zhang, Li, and Wang, 1997) and is proven to increase the activation of murine lymphocytes (Wang, Higuchi, and Zhang, 1997). Moreover, isoflavone glucuronides are able to activate natural killer cells to increase the immune defenses of the body against cancer (Zhang et al., 1999).

2.3 Phytoestrogens; breast cancer approach

2.3.1 An essential issue of breast cancer

Breast cancer is the second leading cause of cancer death. It is the most common cancer among Western females, especially U.S and Western Europe. The incidence rate of breast cancer seems to have been increasing gradually over the past 5-10 years (Murphy, 1998), which may be related to the change of lifestyle and diet. In Thai female cancer patients, it is found at a second rate following cervical cancer. Even though, the incidence of breast cancer in Thailand is low, with an age-specific rate similar to those in developing countries (see Appendix A) (Ferlay, Parkin, and Globocan, 1998).

Now it is accepted that breast cancer is a complex disease and the specific cause of breast cancer remains unknown. Various risk factors such as genetic, reproductive and life-style are recognized. Endogenous factor, genetic or family history, play role for 5% of all cases (Easton et al., 1993). The breast cancer gene BRCA1 confers a 59% risk of developing breast cancer by the age of 50 as compared to only 2% risk in the non-gene carrying population (Ford et al., 1994). While up to 75% of women with breast cancer has no significant family history of the disease. Epidemiologic studies have been shown that reproductive and exogenous factors as menarche (Ford et al., 1994 and Ritter and Richter, 1995), environmental exposures especially to xeno-estrogens (Higson and Muir, 1979 and Kuiper et al, 1998) and diet are also strongly related (Ishimoto, Nakamura, and Miyoshi, 1994 and Ferranoi et al., 1993).

2.3.1.1 Mammary gland biology

Regulation of mammary cell proliferation is complex and unique. Understanding the biology of mammary gland has to be useful in assessing the development and testing of the novel therapeutic approaches for mammary cancer. Rat mammary gland consists nipple and ducts which contains four cell types; myoepithelial, alveolar, basal lamina and ductal cell. In human, breast consists of 15-20 lobules of corpus mammae that connect to milk duct and embedded in adipose tissue (Figure 2.6). The functional myoepithelial cell involves in contractile element

of milk while alveolar involve in the synthesis of milk product and passage to ductal cells. Those cells are lined on basal lamina (reviewed in Young and Hallowes, 1973).

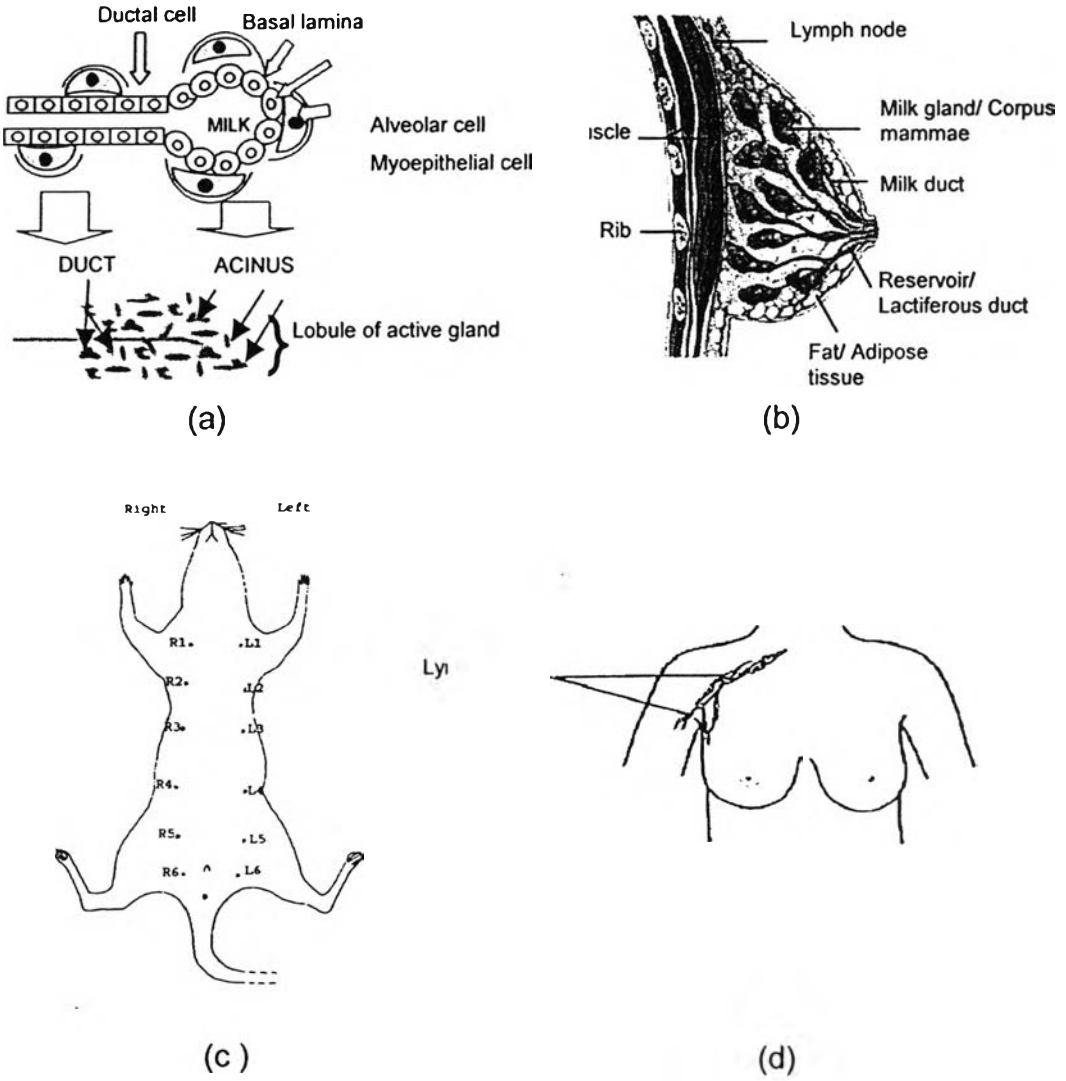


Figure 2.6 Rat mammary gland (a,c) and human breast anatomy (b,d)

In adult animals, exception of pregnant and lactating periods, the structure of the mammary glands is similar in both sexes. The development of male and female mammary glands is qualitatively similar which can be divided into five distinct stages, embryo, prepuberty, puberty, pregnancy, lactation, and involution. Most proliferation and differentiation occur after birth and are regulated by steroid and peptide hormones (reviewed in Hennighausen and Robinson, 2001). At the onset of puberty, mammary ducts elongate and branch. Terminal ends buds (TEBs) appear (Figure 2.7) (Clarke et al., 1996). There are two distinct cell types: body cells, which give rise

to mammary epithelial cells, and cap cells, which are precursors of myoepithelial cells (Humphreys et al., 1996) in the TEB. Functional differentiation of the secretary epithelial cell occurs at parturition and lactation. Ductal arborization is initiated at the highly proliferate TEBs (Daniel and Siberstein, 1987) by regulated cell proliferation and apoptosis. A single layer of luminal epithelial cells lines the mammary gland ducts and the myoepithelial cells form a collar around the primary ducts but are discontinuous around secondary and tertiary ducts and TEBs.

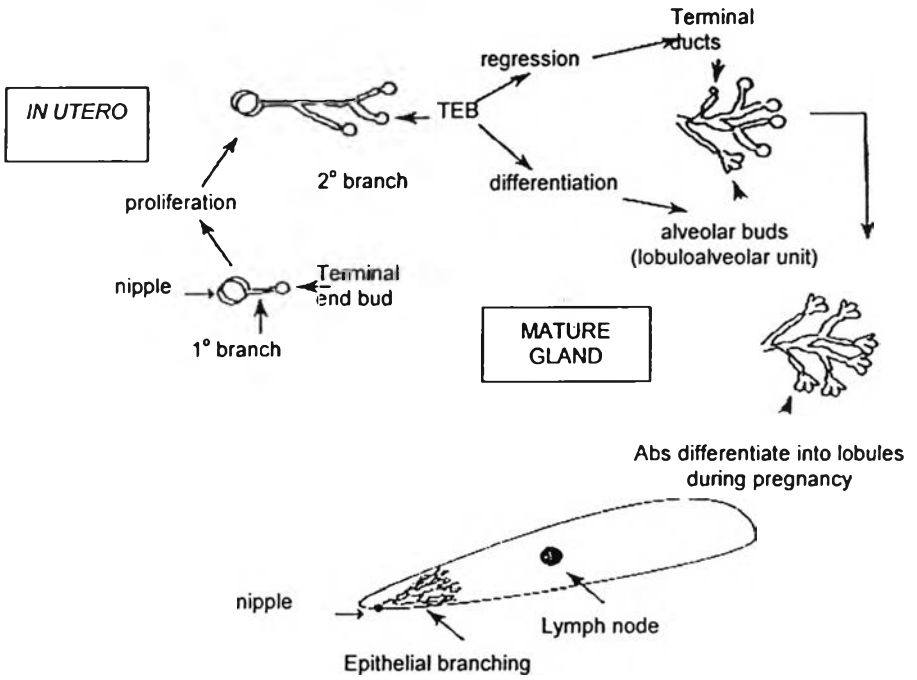


Figure 2.7 The development of the rodent mammary gland (Clarke et al., 1996)

2.3.1.2 Carcinogenesis of mammary gland

According to the carcinogenesis theory, breast cancer arises from normal cell through the accumulation of multiple mutation, lost function of tumor suppressor gene; *p53* and or activation of oncogenes; Ha-*ras*, BRCA1, BRCA2 (Sukumar et al., 1995 and Yokota, 2000). The normal cells are transformed to initiate cells that can be promoted the expression of preneoplastic phenotype by endogenous or exogenous promotional agents. In appropriate environment, those initiated cells can form mutant cells or neoplastic cells and then transform to malignant cells or tumors and progress more (Figure 2.8).

The initiation stage is an irreversible step. The fixation of mutations in critical target genes in the originated cell of cancer (stem cells) is the ultimate end point. This genetic change apparently predisposes the cells to subsequent development of cancer. However, initiation alone does not lead to the development of cancer. The promotion stage involves the clonally expansion of initiated cells, such that the total number of cells processing the initiating lesion is greatly increased in number. The promotion stage is reversible, at least at the early stage. This increase in the number of cells, which possess the initiating lesion, increases the probability of an additional (complementing) genetic changes occurring in cell population. The acquisition of additional genetic changes is the basis for the concept of cancer progression, which also involves a loss of genotypic stability and the development of a heterogeneous cell population (review in Mehta, 2000).

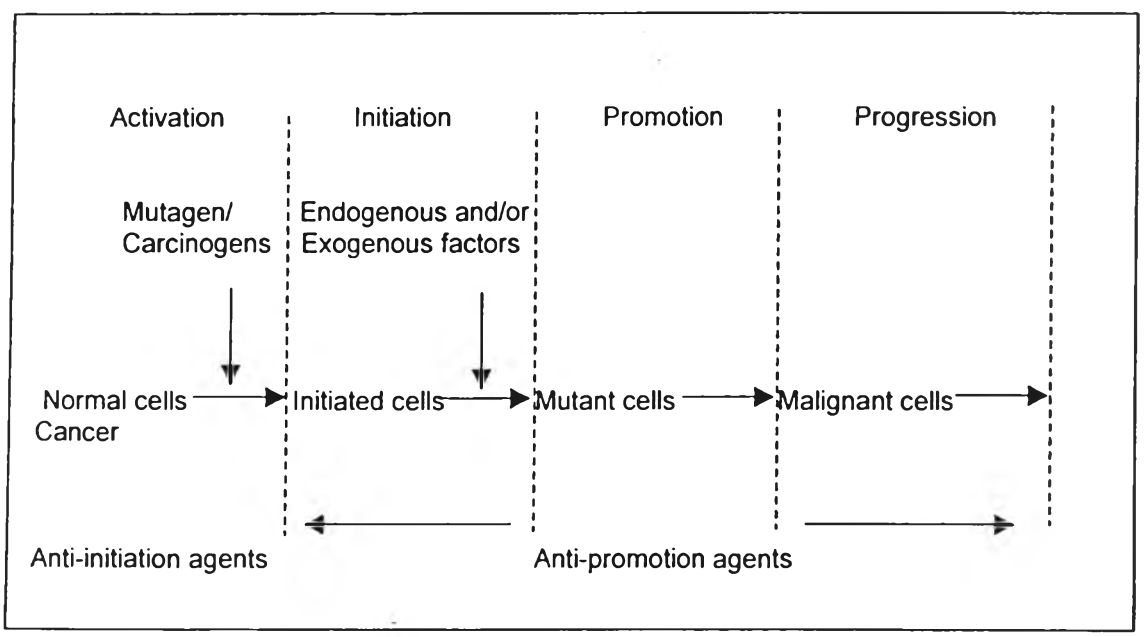


Figure 2.8 Schematic diagram of mammary carcinogenesis and potential sites of chemoprevention in each stepwise (adapted from Mehta, 2000).

2.3.1.3 Induction of mammary cancer

In rats, mammary tumors may occur spontaneously and can be induced in increased incidence by various means, including hormones, chemical carcinogens, transplantation and ionizing radiation. The critical factors are the nature and the dosage of carcinogen, species, strain, age and hormonal status of the recipient (Huggins, Ford, and Jensenm, 1965)

There are many groups of chemical compound that can induce mammary cancer such as aminofluorenes, polycyclic hydrocarbon and alkylating agents. 2-aminofluorene was found carcinogenic to various tissues, including mammary epithelium but susceptibility of occurrence was not equally in all strains (Symeonidis, 1954). Polycyclic hydrocarbon such as 3-methylcholanthrene (MC) and 7,12-dimethlybenz-a-anthracene (DMBA) and N-methyl-N-nitrosourea (MNU) can develop mammary carcinoma. For rat, 7, 12-DMBA has been proved to be more effective than all others (Robert et al., 1977).

DMBA-induced mammary carcinogenesis in rat: A remarkable experiment in mammary cancer research has been done in animal model especially rat and mouse. To set up an experimental model of mammary and other carcinogenesis, it has to concern the similarity of tumor on histological and biological character and induced tumor by a single dose or only few exposures of carcinogen and specific to target organ.

In rat mammary carcinogenesis, DMBA and MNU is mostly used. DMBA does as an indirect-acting while MNU does as a direct-acting to mammary cancer. DMBA might be called as a "proximate" carcinogen that must be metabolically activated to an "ultimate" carcinogen before it can adduct to DNA and other cellular macromolecules. DMBA adducts can be removed by a cellular DNA repair process, but if they are not, they may be exert as mutagen in period of DNA synthesis. It is believed that DMBA causes mutation in newly synthesized DNA strands, which are passed as heritable manner to daughter cell and all progeny. Thus, if the cell has a high reproductive capacity (a stem cell), it can potentially produce a large number of daughter cells, each one containing mutation originally imposed by its parent's encounter with the carcinogen (reviewed in Smart, 2004.).

Comprising DMBA and MNU-induced mammary carcinogenesis, they are similarity on tumor induction. Both are specific to mammary gland and developed tumor without any systematic toxicity. Mammary adenocarcinoma is induced by a single dose. Induction time and multiplicity of tumor are dose-dependence. On the other hand, mechanisms of tumor induction are differences. DMBA requires metabolic activation whereas MNU is not. It might be said that DMBA is suitable to study on initiation and promotion stage of chemopreventive agent. Largely

adenocarinomas are induced by MNU whereas DMBA-induced mammary cancer consists of 60% adenocarinomas and 40% benign fibroadenomas (McCormick et al., 1981 and Moon and Mehta, 1990). In addition, DMBA-induced tumor are neither locally invasive nor metastasis to remote organ.

Metabolism of 7,12-DMBA is occurred in liver by the mixed function of oxidases. Primarily, the metabolic pathway is oxygenation of methyl group and extension in the ring structure. Metabolic products are the isomeric monohydroxymethly derivatives (7-hydroxymethly-12-methly-BA and 7-methly-12-hydroxymethly-BA) and small amounts of phenol, diols, exopides and dihydrodihydroxy compounds (Huggins, 1979). At the age of 50 days, female Sprague-Dawley rat is used for DMBA-induced mammary tumors by gastric intubations with the dose ranges of 12-20 mg. After administration, mammary tumor occurred within 3-4 months with 100% tumor incidence (Mehta, 2000).

Hormone and mammary carcinogenesis: A numerous of endogenous hormones such as estrogen and pituitary hormones can act as tumor promoters *in vivo* if expressed inappropriately or at chronic high levels. Several groups of research administered natural estrogens to rats by implantation of pellets or subcutaneous injection. Mammary tumors can be developed in many animals in various characteristic depending on the dosage of estrogen and induction period (Geschinkter and Byrnes, 1942 and Noble and Collip, 1941).

Hormones play role in the genesis of mammary cancers, which mostly originate in lining epithelial of mammary duct and alveoli. According to their requirements of hormone for proliferation, these cancers are classified as hormone-dependent tumors (HDTs) and hormone-independent tumors (HITs). The detection of mammary cancers in rat is mostly of the HDTs type (Cutts, 1964 and Noble and Collips, 1941) whereas in human in both types were found (Harris et al., 1991 and Stoll, 1969).

Hormones act as a key regulator for mammary proliferation (Imagawa, Bandyopadhyyay, and Nandi, 1990 and Imagawa et al., 1994) by stimulating on stromal compartment cells (adipocyted, fibroblasts and vascular cells) and epithelial cells (including luminal epithelial cells and myoepithelial cells). These cells produced

and secreted mammogenic growth factors to response for the proliferation. Changes in ovarian hormones can result in increasing or decreasing of the tumor incidence. It is revealed that pituitary tumors induce mammary fibroadenoma whereas hypophysectomy in animal can against the induction of mammary tumor (Furth and Clifton, 1957). Tumor regression is induced by ovarectomy or combination of ovarectomy and adrealectomy. The regression time is varied upon type of mastectomy and individually of each tumor.

Over the last decades, breast cancer research has rapidly developed. Age, race, tumor size, histological tumor type, auxiliary nodal status, standardized pathological grade, and hormone-receptor status are accepted as established prognostic and/or predictive factors for selection of systemic adjuvant treatment of breast cancer. Research today is focusing more and more on natural substances and synthetic analogues with mechanisms for cell function alteration (Osborne, 1999).

2.3.1.4 Characteristics of rat mammary cancers

Mammary tumors are nodules that occurred in subcutaneous of the mammary gland areas that originated from the clotting of fat in luminal mammary epithelial cells lining duct and alveoli. The first detection of tumor can be palpable. Some tumors are softer and more rubbery than others. Tumor may be grown in irregular and lobular shape. Normally, tumor is adherent to skin than body wall (reviewed in Young and Hallowes, 1973).

Tumor or neoplasia literally means new growth and implies an abnormal proliferation of cells. If the proliferating cells do not invade surrounding tissues, the resultant tumor is benign; if they do, it is malignant. The term cancer usually implies a malignant tumor (malignancy). Morphologic classification of spontaneous and induced mammary tumors has been focused in three criteria; cellular origin or differentiation, grades of malignancy and subcategories. The type of classification depends on the goal to be achieved. Ideally, tumor classifications should be simple and easy to apply as benign fibroepithelial tumors (including adenoma and fibroadenoma), malignant epithelial tumors (classified upon the pattern of tumor growth; including adenocarcinoma, papillary carcinoma, cribriform carcinoma, comedocarcinoma, and squamous cell carcinoma) (Figure 2.9) (Rehm and Liebelt,

1996) and malignant mesenchymal tumors (including sarcoma) (Russo et al, 1990 and Russo and Russo, 1996)

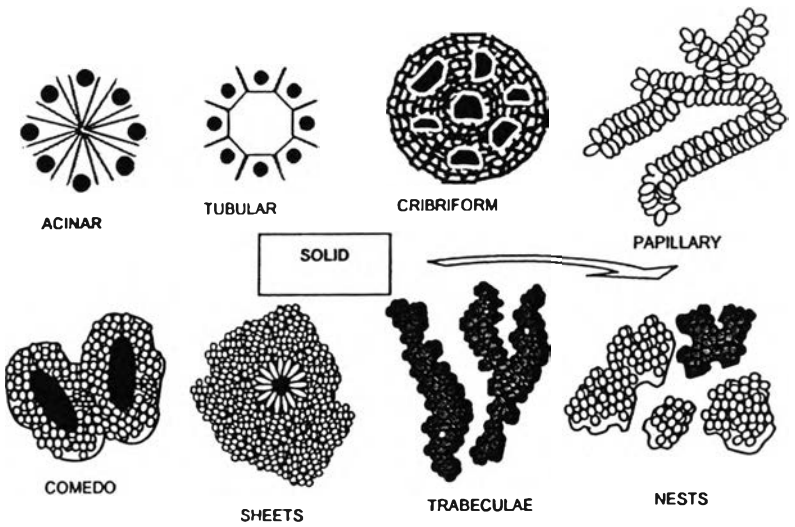


Figure 2.9 The patterns of tumor growth (Rehm, and Liebelt, 1996)

2.3.2 Phytoestrogen influences in breast cancer

2.3.2.1 Epidemiological studies

Soy or phytoestrogen product intake has been effected on breast cancer risk. In cohort study, the miso soup (soybean paste soup) consumption group of Japanese and Japanese-Hawaii were statistically significant decreased on the breast cancer risk (Nomura, Henderson, and Lee, 1978 and Key et al., 1999). In case-control study, urinary levels of lignans and isoflavonoids are assumed to be reflection of their intake and associated risk of developing breast cancer. Newly diagnosed breast cancer cases in Australian (Ingram et al., 1997), Finlandian (Aldercreutz et al., 1992) and English (Grace et al., 2004) women, the data showed substantially lower excretion of major as equol, enterolactone, genistein and daidzein in the breast cancer cases compared with their individually matched controls.

However, data from several observational studies have revealed non-consistent association between soy intake and breast cancer risk (see Appendix B). In combined subject (not separately premenopausal or postmenopause) in American (Horn-Ross et al., 2002), Dutch (Keinen-Booker et al., 2004), Japanese (Hirohata et al., 1985), Chinese (Yuan et al., 1995) exhibited no relationship between breast

cancer and intake of soy product and isoflavonoids. One additional study in premenopausal Dutch women were also reported as well (den Tonkelaar et al., 2001) while a further four studies in Chinese-Singapore (Lee et al., 1991 and 2003), Chinese-China (Hirose et al., 1995) and American women (McCann et al., 2002) suggested that there were low breast cancer risk in high intake of soy and isoflavonoids in premenopausal women. More recently, in both premenopausal and postmenopausal Asian American women were reported with 3-folds lowering of breast cancer risk associated with high tofu intake (Wu et al., 1996) and harmonized with an observation in the US and Canada (Witte et al., 1997).

Only two studies have directly examined isoflavone influences on the breast in women. In nipple aspirate fluid of Chinese and Japanese women who consequently soy isoflavone consumption had lower volume, lower mean levels of gross cystic disease fluid protein, fewer hyperplasic cells, and fewer atypical epithelial cells than American women (Petrakis et al., 1996). However, supplementation of the diets of premenopausal American women with soy protein, providing genistein and daidzein in the dosage of 0.6 and 0.4 mg/kg/day, respectively stimulated breast epithelial cell proliferation and 2 to 6 folds increased in nipple aspirate fluid volume approximately 30% (Petrakis et al., 1996). Monthly plasma collections suggested that E_2 was also erratically elevated. Other study examined the proliferation rate of breast epithelium in biopsy samples from women with benign or malignant breast disease treated with and without a soy supplement providing an isoflavone dose of 0.75 mg/kg/day (McMichael-Phillips, et al., 1998). Short-term dietary soy stimulates breast proliferation. After 14 days of treatment, the proliferation rates of the lobular epithelium and progesterone receptor expression were significantly increased when both the day of menstrual cycle and the age of patient were accounted for. Further studies are required to determine whether this is due to estrogen agonist activity and to examine the long-term effects of soy supplementation on the pituitary gland and breast.

2.3.2.2 *In vitro* studies

Phytoestrogens shows biphasic effect depending on both hormone-dependent; MCF-7 and hormone independent breast cancer cells; MDA-MB-231. IC_{50} of Genistein is range from ~10 to 50 μM/L (Le Bail et al., 2000, Pagliacci et al., 1994, Peterson and Barnes, 1996; Hsieh et al., 1998; Shao, et al., 2000; Twaddle et

al., 1999 and Li et al., 1999). At physiological doses (100 nM/L to 1 μM/L), genistein stimulates ER dependent cellular proliferation. At pharmacological doses (>10 μM/L), genistein exhibits cytotoxic effect *via* inhibition of growth factor receptor tyrosine kinase activity (Wang, Sathymoorthy, and Phang, 1996 and Wang and Kurzer, 1997) and DNA synthesis in MCF-7 cell culture (Wang and Kurzer 1997). In contrary, DNA synthesis was stimulated by the low concentration of genistein and coumestan.

Twenty μM/L of genistein inhibits MDA-MB-231 cell proliferation by as much as 50%. Nevertheless, no significant changes in tumor size or morphology were seen on the MDA-MB-231 inoculated mice that fed on diet containing genistein rich (750 μg/g) (Santell et al., 2000). Genistein may act as estrogen agonist and was able to enhance the growth of xenografted MCF-7 tumors, it was shown that genistein can affected both *in vitro* and *in vivo* (Hsieh et al., 1998)

2.3.2.3 *In vivo* studies

Phytoestrogen treatment showed protective effect on experimental mammary cancer (Table 2.3). The experiment was determined by various phytoestrogen sources such as whole soybean, soy isolated protein, soy hypocotyls, and soy products as miso, fermented soymilk and pure phytoestrogen compound such as genistein, daidzein, and zearelenone. However, most of the studies provided support for a protective effect of phytoestrogen on mammary cancer. The protective effect was significant promise in reducing the tumor development and progression, which is favorably monitored by tumor number, incidence, metastasis and latency.

Of the three studies using raw soybean, a protective effect was found by reducing tumor incidence and increasing tumor latency in rat (Troll et al., 1980; Barnes et al., 1990 and Gotoh et al., 1998b). Moreover, dietary soybeans and soybean hypocotyls were capable of suppressing tumor promotion. (Zaizen, et al., 2000). Each of the studies were determined by using soy protein. No effect on spontaneous tumor latency period was found in mice (Gridley et al., 1983). However, the protective effect was found in chemical-induced mammary tumor. Tumor incidence was 50% reduced in rat fed on diet containing soy protein (Hawrylezwicz, Huang, and Blair, 1991) while the latency period was increased (Hawrylezwicz, Huang, and Blair, 1991 and Barnes et al., 1990).

Four studies were incorporated with the soy products such as miso, fermented soymilk (FSM) and soy extract (Gallo et al., 2001). The protective effect was found in all experiments. Feeding of 10% FSM and 0.02 or 0.04% isoflavone mixture reduced the sizes of 2-amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine (PhIP)-induced rat mammary carcinogenesis (Ohta et al., 2000). In miso and fermented soy product treated rats, the number of mammary tumor was 20% decreased (Baggot et al., 1990 and Ito et al., 1996).

Many reports were focused on a single compound of phytoestrogen and isoflavone extraction on mammary tumor. Fifteen out of nineteen studies attributed the protective effect, three studies were found in adversely and the left one was not affected. In the 50 mg/kg of biochanin A-supplemented diet groups, the result of mammary cancer development was 32% decreased compared to the control (80%) ($P<0.01$). Moreover, the multiplicity and proliferative cell nuclear antigen-labeling index of mammary tumors were also significantly decreased in both 10 mg/kg and 50 mg/kg diet groups (Gotoh et al., 1998b). In addition, MMTV-neu transgenic mice AIN-93G fed diets containing an isoflavone mixture (NovaSoy, equivalent to 250 mg genistein/kg) from 7 wk of age. Mammary tumor latency was significantly delayed. Once tumors formed, however, isoflavones did not reduce the number or size of tumors. Hence, in the MMTV-neu transgenic mouse, soy isoflavones delayed mammary tumorigenesis (Jin and McDonald, 2002).

Phytoestrogen can exert the preventive effect in various periods as pre-initiation, initiation, post-initiation and concomitant. The protective effect was predominantly exhibited when animals were pretreated with genistein during neonatal or prepubertal period (Lamartiniere et al., 1995b; Murrill et al., 1996 and Hilakivi-Clarke et al., 1999a). Three-day treatments with a much higher genistein dose (500 mg/kg/day) during the neonatal (Lamartiniere et al., 1998) or prepubertal period resulted in more marked reductions (50%) in tumor number in DMBA-treated rats. Transitory increases in mammary gland weight were also produced with three subcutaneous doses of 500 mg/kg/day genistein in prepubertal and neonatal rats (Murrill et al., 1996).

Physiological levels of genistein in the diet enhance cell differentiation, resulting in programming of mammary gland cells for reduced susceptibility to

mammary cancer (Fritz et al. 1998). Similarly the study in cell proliferation revealed that 50 day old genistein-treated rats had lower percentages and total numbers of cells in the S-phase of the cell cycle in the terminal end buds, terminal ducts, lobules I and lobules (Lamartieniere et al., 1995 and Murrill et al., 1996)

A high estrogenic in estrogenic environment *in utero* may increase subsequent breast cancer risk. *In utero* exposure to genistein, dose-dependently increased the incidence of DMBA-induced mammary tumors. Tumor growth characteristics were not altered. The number of ER binding sites was significantly elevated in the mammary glands, but the mammary protein kinase C activity was significantly reduced in the genistein offspring. Maternal exposure to subcutaneous administration of genistein could increase mammary tumorigenesis in the offspring, mimicking the effects of *in utero* estrogenic exposures (Hilakivi-clarke et al., 1999b).

Conflicting data of protective effect were in three studies as shown in Table 2.3. Sixty female Lewis rats injected with mammary tumor (MAC-33) were randomized to receive injection of soy extract (18 mg), 5 times per week for 30 days. It was found significant increased in tumor weight, tumor volume (cm^3), and tumor: carcass ratio. Soy protease inhibitors are not responsible for the increase in tumor growth and number of lung metastases (Charland et al., 1998). Furthermore, rats were fed with 20% isoflavone-depleted soy protein diets by prior to initiate NMU administration (at 50 days of age) for one week and continued for another 18 weeks. No significant differences were found among the five groups when assessed in terms of tumor incidence, latency, multiplicity or volume (Cohen et al., 2000).

Table 2.3 Effects of some phytoestrogens on spontaneous, chemically and transplant of breast cancers in laboratory animal studies

Treatment	Dose	Animal/ Strain	Age	Carcinogen	Method	Result	References
Soybean	500 g/kg diet	SD rat	Adult	X-ray radiation	P	Reduced tumor incidence (60%)	Troll, et al., 1980
Soybean	50, 100 and 200 g/kg diet	SD rat	Adult	NMU	C	Increased tumor latency period	Barnes, et al., 1990
Soybean	10 g/kg diet	CD/Crj rat	Adult	MNU	P	Reduced tumor number (50%)	Gotoh, et al., 1998b
Soy hypocotyls	5 g/kg diet	F344 rat	Adult	NMU	C	Decreased tumor incidence	Zaizen, et al., 2000
Soy protein	-	C3H/HeJ mice	Adult	Spontaneous	B	Tumor latency period is not affected	Gridley, et al., 1983
Soy protein	190 g/kg diet	SD rat	Adult	NMU	P	Reduced tumor incidence and Increasing latency period (50%)	Hawrylewicz, Huan, and Blair, 1991
Soy protein	100 or 200 g/kg diet	SD rat	-	DMBA	C	Tumor incidence was not affected. Increasing tumor latency period	Barnes, et al., 1990
Miso	100 g/kg diet	SD rat	-	DMBA	B+I+P	Reduced tumor incidence and Increasing latency period (20%)	Baggot, et al., 1990
Miso	200 g/kg diet	CD/Crj rat	Adult	MNU	P	Reduced of tumor per rat (50%)	Gotoh, et al., 1998a and 1998b
Fermented soy milk	10 g/kg diet	SD rat	Adult	PhIP	I+P	Reduced tumor size	Ohta, et al., 2000
Soy extract	0.35 g/kg diet 0.7 g/kg diet	SD rat	-	DMBA	C	Increased latency period	Gallo et al., 2001
Genistein	5 mg	SD rat	PND 2,4,6	DMBA	B	Reduced tumor number (50%)	Lamartiniere et al., 1995b
Genistein	500 µg/g BW	SD rat	PND 16,18,20	DMBA	B	Reduced tumor number (50%)	Murrill el al., 1996
Genistein	25 and 250 mg/kg diet	SD rat	PND 21	DMBA	B+I+P	Decreased tumor per rat by dose dependent	Fritz et al., 1998

PhIP = 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine, B = Before initiation, I = At initiation, P = Post-initiation, C = Concomitant (whole period)

Table 2.3 Effects of some phytoestrogens on spontaneous, chemically and transplant of breast cancers in laboratory animal studies. (Continued)

Treatment	Dose	Animal/ Strain	Age	Carcinogen	Method	Result	References
Genistein	1 mg/kg BW	SD rat	PND 7, 20	DMBA	B	Reduced multiplicity	Hilakivi-Clarke et al., 1999a
Genistein	20,100,300 µg	SD rat	Adult	DMBA/ Transplacental	B	Promoted tumor incidence	Hilakivi-Clarke et al., 1999b
Genistein	2-8 mg/kg diet	SD Rat	PND 25-155	DMBA	B	Reduced tumor number (20-40 %)	Barnes et al., 1994
Genistein	3-8 mg/kg diet	SD rat	PND 35-215	MNU	B	Reduced tumor number (27%)	Constantinou, Mehta and Vaughan, 1996
Genistein	11 mg/kg diet	Rat	PND 28	Implanted tumor cell	B	Increased mammary and tumor size	Hsieh et al., 1998
Genistein	4 mg/ kg BW	Rat	Adult	MNU		Tumor incidence and tumor per rat were not affected	Constantinou, Mehta, and Vaughan, 1996
Daidzein	1-3 mg/kg diet	SD rat	PND 25-155	DMBA	B	Reduced tumor number (20-40 %)	Barnes et al., 1994
Daidzein	3-8 mg/kg diet	Rat	PND 25-215	MNU	B	Reduced tumor number (27%)	Constantinou, Mehta, and Vaughan, 1996
Daidzein	4 mg/kg BW	Rat		MNU		Tumor incidence and tumor per rat were not affected	Constantinou Mehta, and Vaughan, 1996
Biochanin A	10 and 50 mg/kg diet	CD/Crj rat	Adult	MNU	P	Reduced tumor incidence and muliplicity	Gotoh et al., 1998a
Zearalenone	10 mg/kg/day	Rat	PND 7,14	Spontaneous	-	Increased tumor incidence	Schoental, 1985
Zearalenone	1 mg/kg BW	SD rat	PND 7, 20	DMBA	B	Reduced tumor incidence and multiplicity	Hilakivi-Clarke et al., 1999a
Isoflavones mixture	250 mg /genistein/kg	MMTVneu mice	Adult	Spontanous	C	Increased tumor latency	Jin and McDonald, 2002
Soy isoflavone	200 g/kg diet	F344 rat	Adult	NMU	B+I+P	No effect	Cohen et al., 2000
Soy extract	18 mg	Lewis rat	Adult	Implanted tumor cell	B	Increased tumor growth	Charland, Hui, and Torosian, 1998

PhIP = 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine, B = Before initiation, I = At initiation, P = Post-initiation, C = Concomitant (whole period)

2.4 The evidence of the phytoestrogens rich plant; *Pueraria mirifica* and *Butea superba* candidates on breast cancer prevention.

2.4.1 Plant backgrounds

2.4.1.1 *Pueraria mirifica*

Ethnobotany and application: *P. mirifica* have been long recorded as domestic consumption to promote youth in both male and female (Suntara, 1931). Traditionally, women in some area of Thailand consume a traditional Thai remedy that includes both tuberous powder of *P. mirifica* and Tripala; *Terminalia bellerica*, *Terminalis chebula* and *Phyllanthus emblica* to relieve vasomotor symptoms (hot flashes and night sweats) associated with menopause. Recently, *P. mirifica* powder is extracted and used in cosmetic industry such as breast cream, eye gel and skin moisturizer. The beneficial of skin application is used for breast firming and anti-wrinkle. Moreover, the plant powder packed in capsule was manufactured and distributed as food supplement for aging (Dweck, 2002).

Botanical characteristics and habitats: *Pueraria mirifica* Airy Shaw et. Suvatabandhu (synonym: *Pueraria candollei* Wall. ex Benth var. *mirifica* Airy Shaw et Suvatabandhu), is classified in the family Leguminosae, subfamily Papilinoideae, the same as soy, bean and pea (Ridley, 1967 and Suvatti, 1978). The plant is known as "Kwao Krua" or "Kwao Krua Kao (White Kwao Krua)". *P. mirifica* was previously referred as *B. superba* until February 1947, it was identified as a new species of *Pueraria* and was named *Pueraria mirifica* Airy Shaw et Suvatabandhu (Lakshnankara and Suvatabandhu, 1952).

The plant is a long-living twinning wood. The leaves were pinnately three foliate stipulate; terminal leaflet. The tuberous roots were varied in sizes and shapes. The flower was bluish-purple legume shaped, blooming during late January to early April. The length of the inflorescence of certain flowers was approximately 15-40 cm. The flower contained five sepals and the petals were one standard with two keels. The pod was slender typically short or elongate, smooth or hairy, including 1-10 single seeds when fully matured and dried which turned into various color. (Smitasiri and Wungjai, 1986 and Cherdshewasart unpublished) (Figure 2.10).

P. mirifica is found in the deciduous forest of the northern, western and northeastern parts of Thailand at the 80-800 meters level (Panriansaen, 2000 and Lakshnakara and Suvatabandhu,1952). Recent survey revealed that the plant is distributed in at least 28 provinces (Subtaeng and Cherdshewasart, 2003). Variation within province is also found. The co-habitated plant of *P. mirifica* was typically teak and bamboo. The plant was not found in the forest with high-density trees. The vine of *P. mirifica* elongated for climbing over the trees while spreads on the ground in an open area (Panriansaen, 2000 and Panriansaen and Cherdshewasart, 2003). Attempts had been made to establish *in vitro* multiplication and plantation of *P. mirifica*. It was found that the plant tissue was responsed to plant hormones and plantlets could initiated from *in vitro* (Cherdshewasart et al., 1996). The derived plants could initiate tubers (Sompornpailin et al., 2003).

Chemical constituents: *P. mirifica* had been found to contain at least 20 chemicals in the group of phytoestrogen with similar effects to estrogen. Miroestrol was the first isolated chemical and found in the amount of approximately 1.5 mg/ 100 g dry weight (Bound and Pope, 1960) and also shown estrogenic activity in rat vaginal cornification test (Jones and Pope, 1961). The chemical structure was not classified as steroid (Benson, Cowie and Howsking, 1961). The other compounds, mainly found in *P. mirifica* were isoflavonoids, chromenes, coumarins, sterols (Table 2.4 and figure 2.11) and macromolecule such as protein, lipid and starch (Appendix C). Deoxymiroestrol was isolated and found to be the compound with higher estrogenic potency than miroestrol, approximately 10-folds. However, it was easily oxidized by the air and converted to miroestrol and isomiroestrol (Chansakaow et al., 2000[a]).

The determination of isoflavone content, puerarin, daidzin, genistin, daidzein and genistein in the extracts of *P. mirifica* root from various location of Thailand by HPLC fingerprint assay revealed a great diversity of both total and individual assayed-isoflavone (Subtang, 2002 and Subtaeng and Cherdshewasart, 2003). The five isoflavonoids and isoflavone glycosides, daidzein, genistein puerarin, daidzin, and genistin had been used as markers. Whereas miroestrol cannot be used for quantitative standardization of *P.mirifica* root extract because no available commercialized standard.

Toxicity of *P. mirifica*: Several animal toxicology studies had been completed on *P. mirifica* using both crude powders and standardized extracts. *In vitro* study, *P. mirifica* root extract is not mutagenic by AMES test. (Julsiri and Cherdshewasart, 2003). LD_{50} of *P. mirifica* root extract, a single dose of 40 g/kg BW failed to induce signs of acute or subacute toxicity in mice (Chivapat et al., 2000). In long-term feeding experiments, a chronic toxicology study in rats treated orally with *P. mirifica* root extract at daily doses of 10, 100 and 1,000 mg/kg for 90 consecutive days revealed that at doses of 10 mg/kg BW or 100 mg/kg BW exhibited no toxic effects. However, in one study, a daily dose of 1000 mg/kg BW for 90 days induced reversible anemia and pathologic changes in the kidneys and testicles (Chivapat et al., 2000). The later study was found that plant powder and extract were evaluated and no toxicity was found (Cherdshewasart et al., 2000 and Cherdshewasart, 2003).

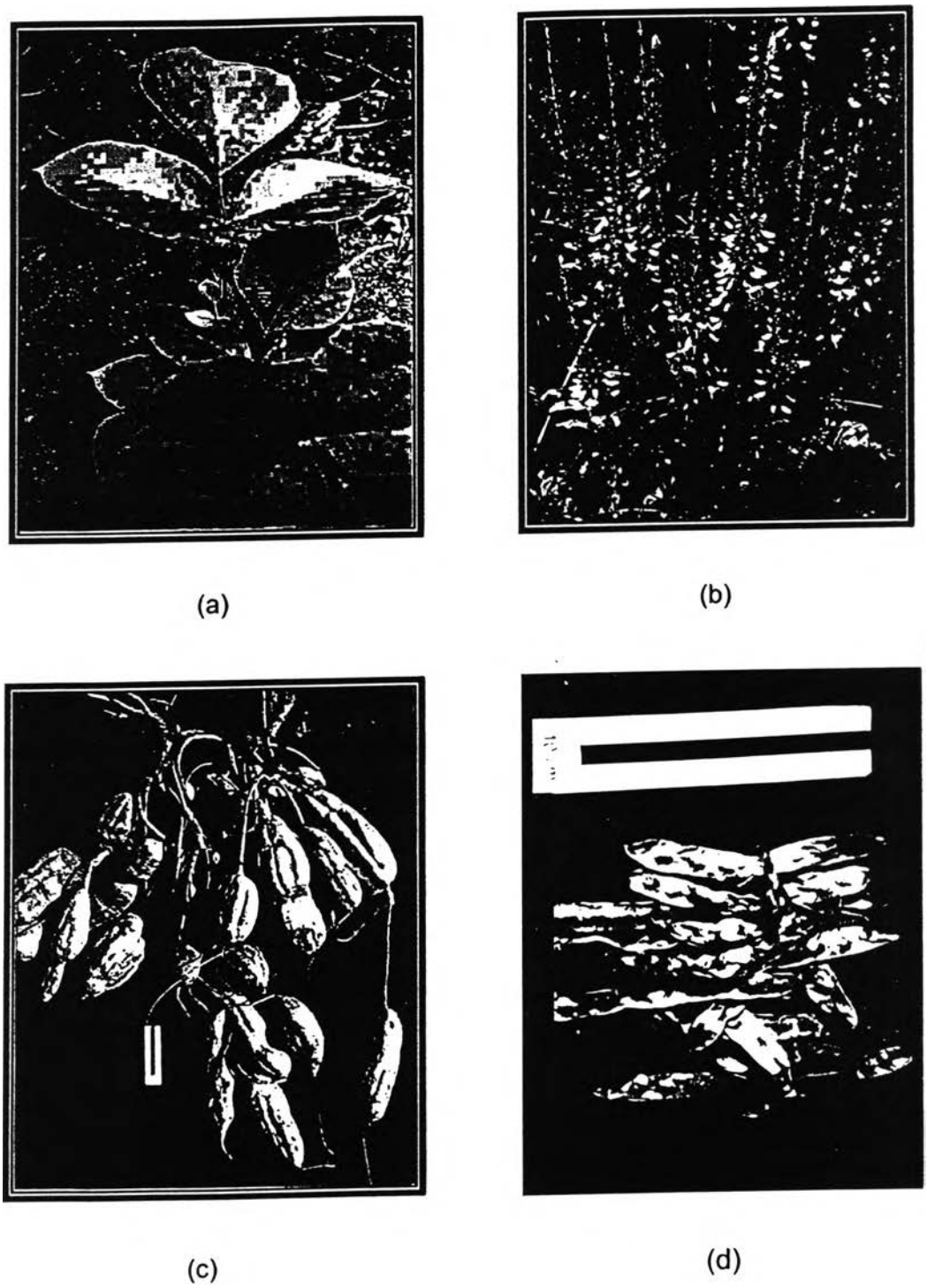
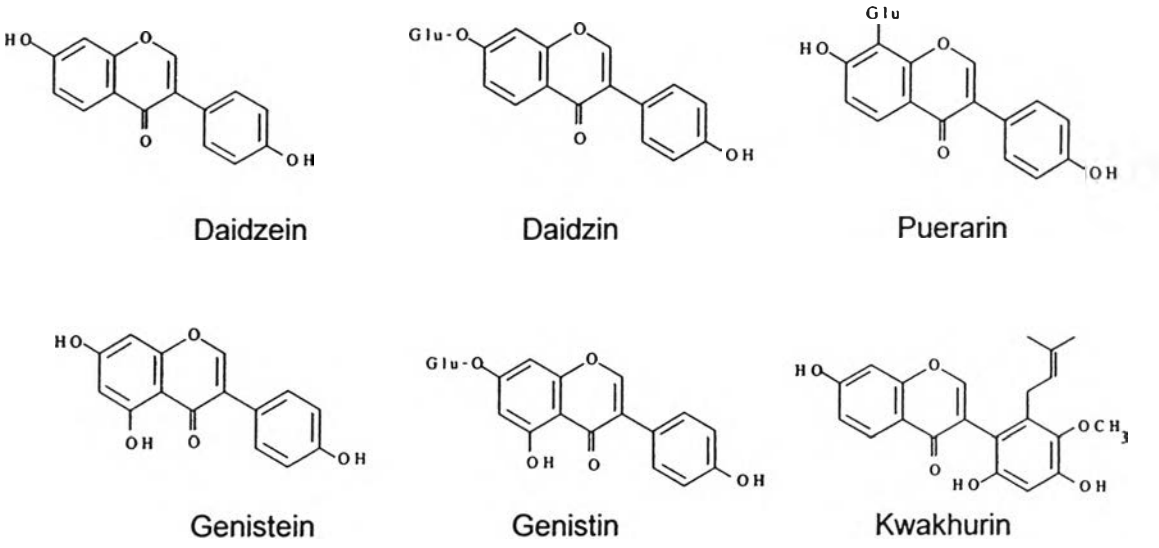


Figure 2.10 Leaves (a), flower (b), tuberous root (c) and pod (d) of *P. mirifica*

Table 2.4 Summary of the chemical constituents of *P. mirifica* (adapted from Panriansaen, 2000)

Category	Chemical	Reference
Isoflavonoids	Daidzein	Ingham et al., 1986
	Genistein	Ingham, Tahara and Dziedzic, 1986
	Kwakhurin	Ingham, Tahara and Dziedzic, 1986
	Kwakhurin hydrate	Ingham, Tahara and Dziedzic, 1989
Isoflavone	Daidzin	Ingham, Tahara and Dziedzic, 1986
glycosides	(daidzein-7-o-glucoside)	
	Genistin (genistein-7-o-glucoside)	Ingham, Taharaand Dziedzic, 1986 and 1989
	Mirificin	Ingham, Tahara and Dziedzic, 1986 and
	(puerarin 6"-o-β-apiofuranoside)	Ingham et al., 1986
	Puerarin (daidzein-8-glucoside)	Nilandihi et al., 1957
		Ingham, Tahara and Dziedzic, 1986 and 1989, Ingham et al., 1986
	Puerarin 6"-monoacetate	Ingham et al., 1989
Chromenes	Miroestrol	Schoeller, Dohrn and Hohweg, 1940
		Bound and Pope, 1960
		Jones and Pope, 1960
	Deoxymiroestrol	Chansakaew et al., 2000[a]
	Isomiroestrol	Chansakaew et al., 2000[a]
Coumestans	Coumestrol	Ingham, Tahara and Dziedzic, 1986 and 1988
	Mirificoumestan	Ingham, Tahara, and Dziedzic, 1988
	Mirificoumestan glycol	Ingham, Tahara, and Dziedzic, 1988
	Mirificoumestan hydrate	Ingham, Tahara, and Dziedzic, 1988
Sterols	β- sitosterol	Hoyodom, 1971
	Stigmasterol	Hoyadom, 1971
Pterocapans	Pueriicapene	Chansakaew et al., 2000[b]
	Tuberosin	Chansakaew et al., 2000[b]
Acid	Tetracosanoic acid	Chansakaew et al., 2000[b]

Isoflavone and Isoflavone glycosides



Coumestans

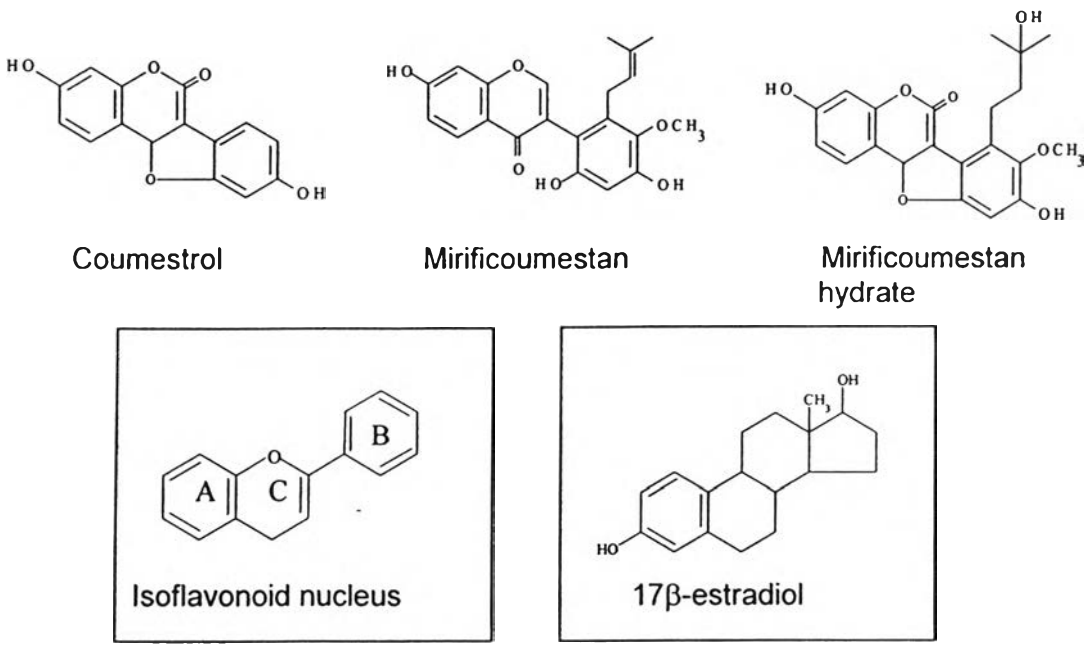
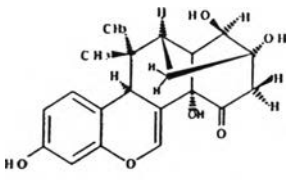
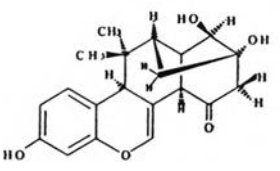


Figure 2.11 The structure of chemical compounds in *P. mirifica* and isoflavonoid nucleus compare with estrogen

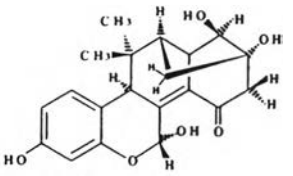
Chromenes



Miroestrol

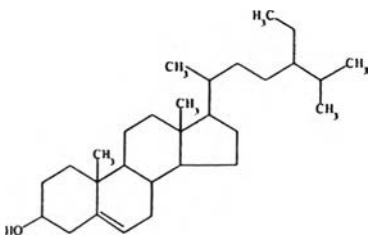


Deoxymiroestrol

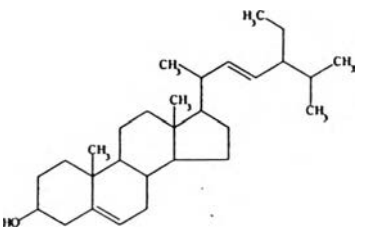


Isomiroestrol

Sterols

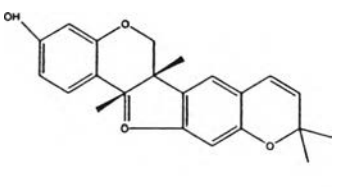


β-sitosterol

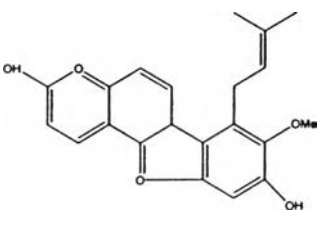


Stigmasterol

Pterocarpans



Tuberosin



Puemirificarpene

Figure 2.11 The structure of chemical compounds in *P. mirifica* and isoflavonoid nucleus compare with estrogen (continued)

Hormonal effect of *P. mirifica*: Many studies on *P. mirifica* were mostly evaluated in estrogenic activity on the reproductive system. In animal model, *P. mirifica* showed many effects depend on the method of bioassay such as dosage and period of exposure. The biological effects have been identified in Table 2.5. It was revealed that Miroestrol had 0.7 time of Estradiol on mammary gland proliferation activity (Pope et al., 1958). In ovariectomized rat, *P. mirifica* root extraction induced proliferation of the cornifined cell, increased uterus weight (Sukhavachana, 1941) and exhibited strong estrogenic activity in uterotrophic assay

(Kim et al., 2003). Uterus and vagina weight of *P. mirifica*-treated immature rat was significantly increased (Sawatdipong, 1981). *P. mirifica* could influence the reproductive functions in both sex of rats, but the response was greater in male than female (Malaivijitnond et al., 2004; 2003a; 2003b and Kaitthaipipat, 2001). In monkey, a single dose of 1,000 mg/kg BW of *P. mirifica* disrupted ovarian function, menstrual cycle and also decreased the Parathyroid hormone and serum calcium level (Trisomboon, et al. 2004). In clinical trial, miroestrol (Cain, 1940) and the crude drug showed the effectiveness in treatment of menopausal symptom (Muangman and Cherdshewasart, 2001; Lamlertkittikul and Chandeying, 2004)

Estrogenic activity of *P. mirifica* was responded in dose-dependent manner on MCF-7 cells and HepG2 cells. The mechanism of action of the plant extract was evaluated. It was found that the chemicals needed a metabolic activation to promote their actions within human cells. Recombinant yeast exhibited no estrogenic activity because it lacked metabolic enzyme (Lee et. al., 2002). Crude extract of *P. mirifica* showed biphasic effect on the growth of MCF-7 as well as 17β-estradiol with proliferative effect at low concentration and antiproliferative effect at high concentration with ED_{50} value of 642.83 μg/mL (Cheewasopit, 2001; Cheewasopit et al., 2003; Trisap et al., 2003 and Cherdshewasart, Cheewasopit, and Picha, 2004a). While the crude extract of *P. mirifica* indicated no proliferative and anti-proliferative effect in HeLa cells at 100 and 1,000 μg/mL (Cherdshewasart, Cheewasopit and Picha, 2004[b])

To compare the estrogenic activity in each compound on MCF-7 system, it was found that those compounds had different degree of estrogenic activity (17β-estradiol (lower 10^{-12} Check Unit) > deoxymiroestrol (10^{-10}-10^{-9})>miroestrol (10^{-8})>coumestrol (10^{-7}) ≈ genistein (10^{-7}) > daidzein (10^{-6}) ≈ kwakhurin (10^{-3})). Whereas daidzin, puerarin, puermicapene, tuberosin and isomiroestrol had no estrogenic activity (Chansakaow et al., 2000[a] and 2000[b]) as shown in Table 2.6.

Table 2.5 Summary of the recent reports of the biological effects of *P. mirifica* on animal model

Effects	References
1. Reproductive system	
1.1 Reproductive organ development	
• Promoted mammary duct and breast enlargement in mice, rat, pig	• Pope et al., 1960, Smitasiri, Pangjit and Anatalabhochai, 1986
• Proliferated the uterus and vagina	• Sukhavachana, 1941, Sawatdipong, 1981
1.2 Fertilization and birth control	
• Increased mating behavior	• Smitasiri, 1988
• Anti-fertilization	• Smitasiri and Pangjit, 1986, Smitasiri, 1988
• Induce abortion	• Sangkaew and Smitasiri, 1985, Smitasiri et al., 1986
• Reduction of sperm	• Langkalichan and Smitasiri, 1985
2. Others	
• Cholesterol level	• Thaiyanun et al., 1992b, Chivapat et al., 2000
• Calcium level	• Anuntalabhochai and Jersrichai, 1986, Bulintanthikul, 1978, Trisomboon, 2004

Table 2.6 The growth-promoting effects of chemical compound extracts of *P. mirifica* on MCF-7 human breast cancer cells. (Chansakaow et al., 2000[a] and 2000[b])

Compounds	Content (mg/100 g powder)	Growth-promoting effects on MCF-7 (Minimal concentrations)
Isoflavone and glycoside		
Genistein	0.6	10^{-7}
Genistin	data not shown	Data not shown
Daidzein	46.1	10^{-6}
Daidzin	8.5	No activity
Kwakhurin	0.6	$>10^{-6}$
Chromenes		
Miroestrol	3.0	10^{-8}
Deoxymiroestrol	2.0	10^{-10}-10^{-9}
Isomiroestrol	2.2	no activity
Coumestrol	0.07	10^{-7}
Pterocarpens		
Tuberosin	0.3	No activity
Puemiricarpene	1.8	No activity
Acid		
Tetracosanoic acid	15.3	-
17β-estradiol (control)	-	$<10^{-12}$

* Minimal concentration of compounds that caused 50% MCF-7 breast caner cells growth when compared to the control

2.4.1.2 *Butea superba*

Ethnobotany and application: *B. superba* has been popular among Thai males for the purpose of aphrodisiac and rejuvenating as well as maintaining sexual performance ability, sex enhancer or prevention of erectile dysfunction. Traditionally, men in some area of Thailand consume a traditional Thai formulation that includes both tuberous powder of *B. superba* and Tripala; *Terminalia bellerica*, T*erminalis chebula* and *Phyllanthus emblica*. All plant power was mixed with honey and done as pepper pill. Consumption was two third of pill size for tonic and rejuvenating (Suntara, 1931). While the commercial Erectile Dysfunction-treated drug is a great interested product despite its serious adverse effect which some are related to death,

a herbal product such as *B. superba* with soft but safe action should be an alternative (Cherdshewasart and Nimsakul, 2003).

Botanical Characteristics. *Butea superba* Roxb, is an indigenous herb of Thailand, known as "Kwao Krua Daeng" (Red Kwao Krua). The leaves are pinnately three foliate, acuminate leaflet and long leafstalk. The flowers are large size with yellowish orange color. The petals are three times longer than the calyx (Figure 2.12). The pods are 3-4 inches long, oblong shaped with silvery silky short hair (Kruz, 1877 and Brandis, 1990) but only one seed present. (Cherdshewasart, unpublished). *B. superba* is a large size twinning wood found in the deciduous forest of the north, west and northeast parts of Thailand. The plant was found in the same habitat with *P. mirifica* and also the mountainous area. Due to the large dispersal habitats of the plant, variation in leaf form and size as well as tuber shape was found. A cultivar had been studied and selected for commercialized plantation.

Chemical constituents: The plant tuber exhibited some chemicals closely related to that of *P. mirifica* but some chemicals are different. *B. superba* tuberous root was found to contain 5 groups of chemical constituents including, carboxylic acid, steroid, steroid glycoside, flavoncid and flavonoid glycoside (summarized in Table 2.7, Rugsilp, 1999). Macromolecule such as protein, lipid and starch (Appendix C) were also found. Flavonoid glycoside (3, 7-dihydroxy-8-methoxyflavone 7-O-α-L-rhamnopyranoside) was found in stem of Indian *B. superba* (Yadava and Reddy, 1998).

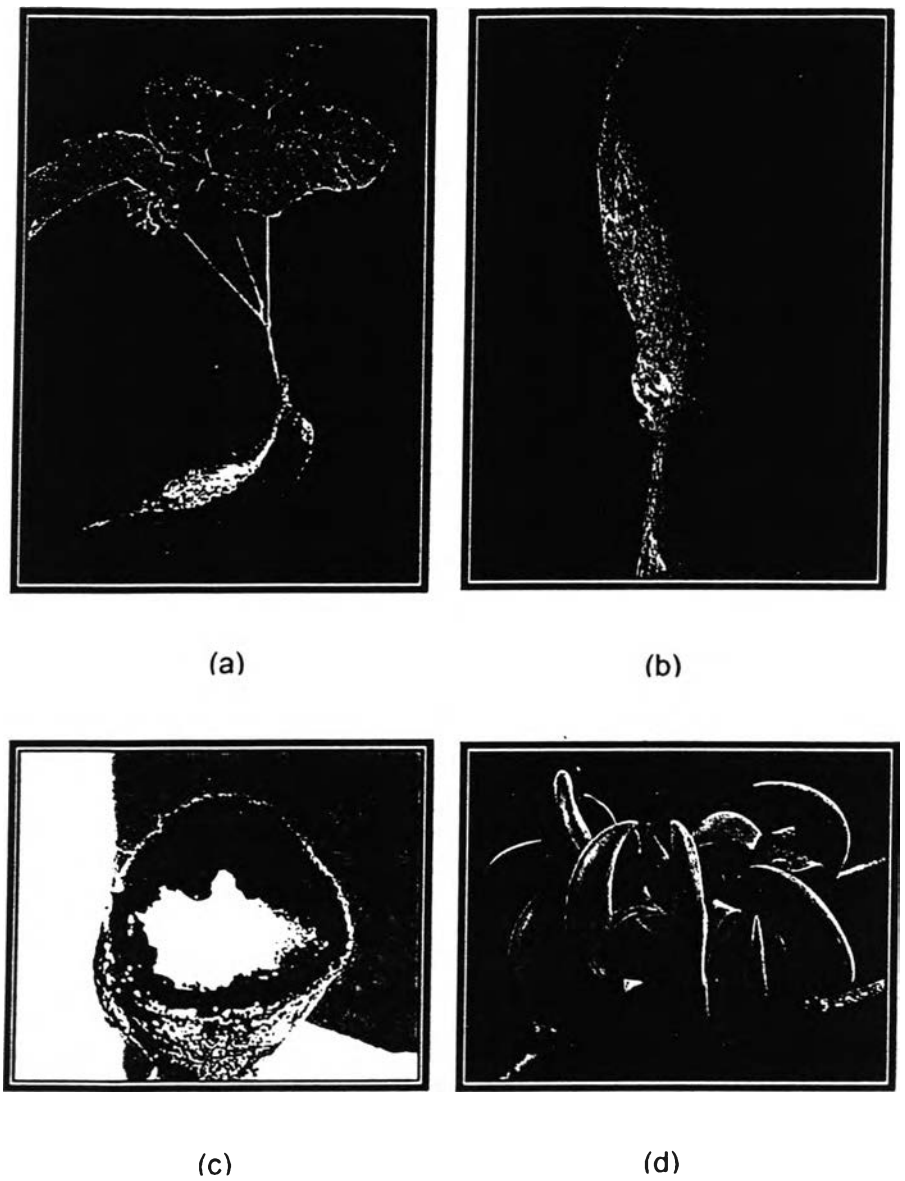
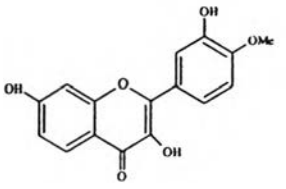


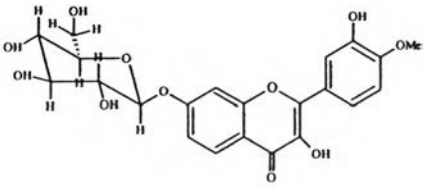
Figure 2.12 Plant (a), tuber (b), tuber cross section (c) and flower (d) of *B. superba*

Table 2.7 Chemical constituents of *B. superba*

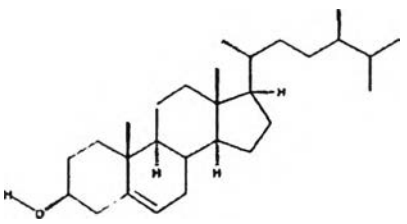
Category	Chemical	References
Carboxylic acid	Straight chain carboxylic acid (C_{22}-C_{26})	Raksilp, 1995
	3-hexacosanoyloxy-propane-1,2-diol	Am-orn unpublished data
Steroid	Campesterol, stigmasterol, β-sitosterol	
Steroid glycoside	β-sitosteryl 1-3-o-β-D-glucopyranoside, Stigmasteryl 1-3-o-β-D-glucopyranoside	
Flavonoid	3,7,3-trihydro-4'-methoxyflavone	Raksilp, 1995
Flavonoid glycoside	3-3'-dihydroxy-4'-methoxyflavone-7-o-β-D-glucopyranoside	Raksilp, 1995
	3,7-dihydroxy-8-methoxyflavone-7-O-α-L-rhamnopyransoside	Yavada and Reddy, 1998
	5,4'-dihydroxy-7-metoxy-isoflavone (Prunetin)	Aim-Orn, unpublished data
	3-Hydroxy-9-methoxypterocarpan (Medicarpin)	Aim-Orn, unpublished data
	7-Hydroxy-4'-methoxy-isoflavone (Pormononetin)	Aim-Orn, unpublished data
	7-Hydroxy-6-4'-dimethoxyisoflavone	Aim-Orn, unpublished data
	7, 4'-Dimethoxyisoflavone	
	Butein, Butin	Subba and Seshadri, 1949



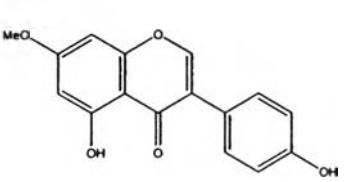
3,7,3'- trihydroxy-4'-methoxyflavone



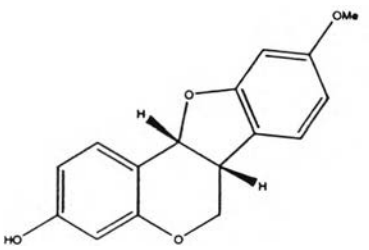
3,5'-dihydroxy-4'methoxyflavone-7-O-β-D-glucopyranoside



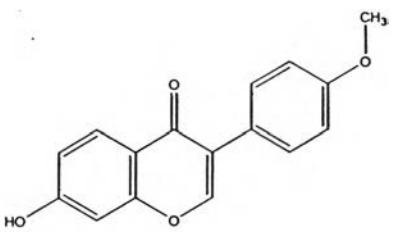
Campesterol



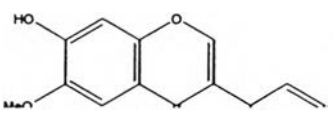
5,4'-dihydroxy-7-metoxy-isoflavone (Prunetin)



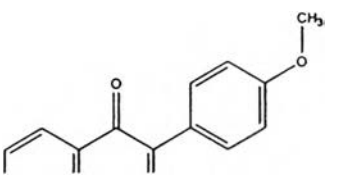
3-Hydroxy-9-methoxypterocarpan (Medicarpin)



7-Hydroxy-4'-methoxy-isoflavone (Pormononetin)



7-Hydroxy 6-4'-dimethoxyisoflavone



7, 4'-Dimethoxyisoflavone

Figure 2.13 Some structure of chemical compound in *B. superba*

Biological and pharmacological effects: The preliminary chronic toxicity study of *B. superba* in rat, at the doses of 5 and 100 mg/ kg BW showed no adverse effect on essential organs but the sperm count was increased. Abnormal sperms were found at the treatment of 5 mg/kg BW (Manosroi, Saowadon and Manosroi, 2001). The aqueous solution of *B.* superba dry powder at the doses of 2, 20, 200 and 1,000 mg/kg/day were undertaken on male rats by micronucleus test for the purposes of dominant lethal tests. The treatment of 1,000 mg/kg/day *B. superba* solution exhibited significantly more effective in inducing the formation of micronuclei in polychromatic erythrocytes than the control. Whereas, dominant lethal test indicated that none of the doses had a toxic effect on male reproduction. There were no abnormal changes in body weight of treated rats and the number of implantation sites and the number of dead fetuses produced by females that had mated with *B. superba* (Pongpanparadon, Artajat, and Saenphet, 2002).

In the rat uterotrophic assay, the treatment with 40 mg/kg BW of *B. superba* extract caused the significant increase of the vagina weight but no response for uterus weight (Kim et. al., 2003). Alcoholic extract of *B. superba* increased intracavernous pressure in male rat (Tochaus et al., 2003). The extract of *B. superba* root barks showed 50-60% inhibitory acitivity on Acetylcholinesterase, one investigating for the treatment of Alzheimer's disease (Ingkaninan et al., 2003).

Flavonoid (3,7,3'-trihydroxy-4'-methoxyflavone) and flavonoind glycoside (3,5'-dihydroxy-4'-methoxyflavone-7-β-D- glucopyranoside) in the tuber were found to be the inhibitor of cAMP phosphodiesterase at IC_{50} value of 190 and 58 μg/ml, respectively, which were capable of stimulating the function of the central nervous system, the cells and the aldosterone hormone which attributes in increasing the male sexual performance (Roengsamran et al., 2000 and 2001). The result was clearly shown in clinical trial that the crude drug showed over 75% effectiveness for erectile dysfunction treatment without apparent toxicity (Nimsakul and Cherdshewasart, 2001 and Cherdshewasart and Nimsakul, 2003). The crude extracts of the herb show vasodilatation effect. One flavonol glycoside (7-o-β-D-glucopyranoside3,7-dihydroxy-8-methoxyflavone-7-O-α-L-rhamnopyransoside) from the stem of the Indian *B. superba* has antimicrobial activity against several organisms including plant pathogenic fungi (Yavada and Reddy, 1998).

2.4.2 Strength of the evidence for breast cancer relation

There has been much optimism in the literature regarding the breast cancer preventing potential of phytoestrogens. Diets high in soy, which also contain high levels of phytoestrogens, have been associated with a decreased risk of breast cancer, particularly in pre-menopausal women. Animal studies support the notion that phytoestrogens *per se* may be active anti-cancer components. *In vitro* studies have identified a number of possible mechanisms, not all reliant on estrogenic/ antiestrogenic activity, by which these compounds may exert their anticancer effects. The most impressive evidence comes from the animal studies showing that exposure to genistein during the early stage of life reduces the later development of chemically induced mammary cancer. Genistein inhibits both estrogen-dependent and estrogen-independent breast cancer cells *in vitro*.

There were reports on the strong relation of soyabean consumption containing phytoestrogens with low risk of breast cancer. It might be possible that *P. mirifica* may benefit on breast cancer protection. *P. mirifica* had proved to be extremely rich in isoflavonoids, which was found over 50 mg/100 g dry weight. Besides, chromene, the most important active compound, was found at 7 mg/100 g dry weight (Chansakaew 2000b) and is unique to this species with strong estrogenic effect. Various phytoestrogens in the plant powder might play role as estrogenic antogonist and affect to alteration of breast cancer. In addtition, β-sitosterol, campesterol and stigmasterol were presented in the plants. The chemicals also show protection and effective treatment for the common cancers (Awad and Fink, 2000 and Awad et al., 2000) such as colon (Awad et al., 1996), prostate (Awad et al., 2001) and breast cancer (Awad, Downie and Fink, 2000; Awad et al., 2000[b] and Awad, Williams, and Fink, 2001). *B. superba* extract showed exhibited only anti-proliferation effects on the growth of MCF-7 cells in relation with a possible anti-estrogen mechanism at low concentration but a potent cytotoxic effect at high concentration. At high concentration (100 and 1,000 μg/ml), it showed markedly inhibition of MCF-7 and HeLa cell proliferation (Cherdshewasart, Cheewasopit, and Picha, 2004a and 2004b)