Chapter II

Literature review

P. mirifica, B. superba and M. collettii were classified into the family of Leguminosae. The common name for these plants was varied in various part of Thailand as shown in Table 1

Table 1. Common name of *P. mirifica, B. superba,* and *M collettii* in various part of Thailand (Panriansaen, 2000)

Species	Common name	Location
P. mirifica	- Tong Kwao, Thong Krua, Hua Kwao, Tan Krua, White Kwao Krua, Chan Krua	- Northeast Thailand
	- Potagu, Thao Thong	- Kanchanaburi
B. superba	- Thong Khrua,	- Central Thailand
M. collettii	- Tao Hom, Haen Hao Hon	- Loei
	- Beng-ke	- Kanchanaburi (Karen)
	- Maba Maeng	- Chiengmai
	- Yang Dam	- Nakhornratchasima
	- Saba Ling	- Kanchanaburi
	- Saba Ling Dam	- Central Thailand
	- Mak Ba Luem Dam	- Sukhothai

I. Botanical background and chemical constituents

1.1 P. mirifica Airy Shaw et Suvatabandhu

The climber tuberous plant was a long live twining wood. The leaves were pinnately 3 foliate; terminal leaflet. The tuberous roots were varied in sizes and shapes. The flower was bluish-purple typically legume shaped. Flowering occurred during late January to early April. The purple flower contained five petals and the petals were one stand with two keels. The pod was slender typically short or elongate, hairy or smooth, including 1-10 single seeds when fully matured and dried which turn into various color. (Cherdshewasart unpublished data)

There are at least 20 chemicals in *P. mirifica* that were classified into a group of phytoestrogens. The first isolated chemical was miroestrol (Bound and Pope, 1960), was believed to be the most active compound, found in approximately 15 mg/kg of the dried tuber. Miroestrol was found to act as estrogen evens the chemical structure was not steroid (Cain, 1960). Miroestrol was recently claimed to be an artifact of deoxymiroestrol, an unstable compound. (Chansakaow *et. al.*, 2001. The other compounds, mainly found in *P. mirifica* were coumarins, isoflavones, chromenes, sterols. The list of found chemicals in *P. mirifica* tuberous root was shown in **Table2**

P. mirifica was interesting not only in term of research but also in term of product development. The tubers were long-term consumed in Thailand as traditional remedies (Suntara, 1931). The clinical trail to evaluate the estrogenic effects of the crude drug derived from *P. mirifica* in five female volunteers showed the crude drug improved the signs and symptoms related to menopause such as hot flushes, frustration, sleep disorder, skin dryness, high blood cholesterol, oligomenorrhoea and amenorrhoea.(Muangman and Cherdshewasart, 2001) The acute toxicity in mice, a skin irritation test and applied Draize test revealed no meaningful allergic response.(Cherdshewasart, 2003)

Table 2. Summary of the chemical constituents of *P. mirifica*
(After Panriansaen, 2000)

Category	Chemical	Reference
Coumarin	Coumestrol	Ingham, Tahara and Dziedzic, 1986, 1988
	Mirificoumestan	Ingham, Tahara,and Dziedzic, 1988
	Mirificoumestan glycol	Ingham, Tahara,and Dziedzic, 1988
	Mirificoumestan hydrate	Ingham, Tahara,and Dziedzic, 1988
Isoflavone	Daidzein	Ingham *et. al.*, 1986
	Daidzin (daidzein-7-o-glucoside)	Ingham, Tahara,and Dziedzic, 1986
	Genistein	Ingham, Tahara,and Dziedzic, 1986
	Genistin (genistein-7-o-glucoside	Ingham, Tahara,and Dziedzic, 1986, 1989
	Kwakhurin	Ingham, Tahara,and Dziedzic, 1986
	Kwakhurin hydrate	Ingham, Tahara,and Dziedzic, 1989
	Mirificin (puerarin 6"-o-β-apiofuranoside)	Ingham, Tahara,and Dziedzic, 1986 Ingham *et. al.*, 1986
	Puerarin (daidzein-8-glucoside)	Nilandihi *et. al.*, 1957, Ingham, Tahara, and Dziedzic, 1986, 1989 Ingham *et. al.*, 1986
	Puerarin 6"-monoacetate	Ingham *et. al.*, 1989
Chromene	Miroestrol	Schoeller, Dohrn and Hohweg, 1904 Bound and Pope, 1960 Jones and Pope, 1960
	Deoxymiroestrol	Chansakaew *et. al.*, 2000
Sterol	β- sitosterol	Hoyodom, 1971
	Stigmasterol	Hoyadom, 1971

1.2 B. superba, Roxb.

B. superba is a large size climber. The leaflet is acuminate chartaceous. The flowers are large with gorgeous orange color. The peticels are three times longer than the calyx. The pods are 3-4 inches long, oblong shaped with silvery silky short hair and only one seed is present (Cherdshewasart, unpublished data)

B. superba tuberous root was found to contain 5 groups of chemical constituents namely: carboxylic acid, steroid, steroid glycoside, flavonoid and flavonoid glycoside (Thanatip, 1995) (Table 3). The flavonoid glycoside could inhibit cAMP Phosphodiesterase activity (Roengsamran *et. al.*, 2000). β-sitosterol, campesterol and stigmasterol were reported to be the anti-inflamatory agent, enhancing activity of T-Helper-1 (TH-1) cell, reduce blood sugar by increase insulin secretion. β-sitosterol, campesterol and stigmasterol might offer protection and treatment for the common cancers such as colon, prostate and breast cancer (Awad and Fink, 2000 and Tapiero, Townsend and Tew, 2003). *Butea superba* crude appears to improve the erectille function in ED patients without apparent toxicity. Cherdshewasart and Nimsakul, 2003).

Table 3. Summary of the chemical constituents of *B. superba* (Yavada and Reddy, 1998; Roengsamran *et. al.*, 2000)

.Category	Chemical
Carboxylic acid	Straight chain carboxylic acid (C_{22}-C_{26})
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, 5'-trihydro-4'-methoxyflavone
Flavonoid glycoside	3-5'-dihydroxy-4'-methoxyflavone-7-o-β-D-glucopyranoside

1.3 M. collettii, Lace.

M. collettii is a large woody climber, 30-40 m height scattered by stems in evergreen forest. The leaves were trifoliate; leaflets 4-8 by 2-4 inches sparsely hairy, entire margin; petiole 5-10 cm long, base stout. The flowers were hanging on the stem up to 12 inches long with 5 sepals covered with brown rough hair and unite into a bell-shaped tube. The petals were blackish-purple pea-like shaped. The stamens were two bundles. The pods were linear-oblong shaped up to 16 inches long. The seeds were hard and flattened. The flowers were blooming during January to March (Cherdshewasart unpublished).

The whole stem of *M. collettii* was found to contain 3 interested chemical constituents namely: Kaempferol, Quecertin and Hopeaphenol. The median inhibitory concentration (IC_{50}) for cAMP Phosphodiesterase inhibiting effect was found to be 281.83, 80.91 and 22.75 µg/ml, respectively (Roengsamran *et. al.*, 2000). The *in vivo* study indicated that *M. collettii* affected the reproductive system by causing the abnormality of spermatozoa (Wutteeraphon *et. al.*, 2001)

II Phytoestrogens

Phytoestrogens are the group of non-steroidal plant chemicals naturally that structure similarity to natural animal estrogens, such as estradiol, and estrogen-like bioactivity. The compounds could regulate gene expression mediated by an estrogen responsive element (ERE), in a manner either agonistic or apparently antagonistic to 17 β-estradiol, as a result of binding to estrogen receptor (ER). (Murkies, Wilcox and Devis, 1998). The use of some plants in traditional medicine and remedy could show evidence on their estrogenic effect. The consumption of diets containing large amount of phytoestrogens such as soybean and unrefined gain products, may be associated with low risk of breast and prostate cancer, (Strauss *et. al.*, 1998). It could also prevent other estrogen-related condition; cardiovascular disease, menopausal symptoms and post-menopausal osteoporosis (Messina *et. al.*, 1994 and Strauss *et. al.*, 1998).

Base on their chemical structure, phytoestrogens are divided into 3 main classes; namely isoflavones (e,g. genistein, daidzein) coumestans (e.g. coumestrol) and lignans (e.g. enterolactone). A single plant often contains more than one class of phyrtoestrogens for example, soybean was found to be rich in isoflavone (genistein and daidzein) while its sprout was a potent source of coumestan (coumestrol) (Murkies, Wilcox and Devis, 1998 and USDA, 1999)

2.1 Sources of phytoestrogens

Phytoestrogens could be found in various plants including beans, peas, clover sprouts, alfalfa seeds, flaxseeds and tea (USDA, 1999) or even in cabbage. (Ju *et. al.*, 2000). The most famous source of phytoestrogens was soybean. *P. mirifica* was also reported to contain high amount of isoflavones. (Cherdshewasart, unpublished data).

2.2 Effects of phytoestrogens on cancer

Phytoestrogens have been suggested to be a preventive chemical against various cancers. They might reduce the risk of development of many cancer types such as breast cancer. (Pagliacci *et. al.*, 1994: Barnes *et al*, 1996: Peterson *et. al.*, 1996: Zava and Duwe, 1997: Constantinou *et. al.*, 1998), colon cancer (Messina *et. al.*, 1994), prostate cancer (Steiner, Raghow and Neubauer, 2001). The case-control study in breast cancer patients showed that the increased excretion of some phytoestrogens was associated with a substantial reduction in breast cancer risk (Ingram *et. al.*, 1997).

The effects of phytoestrogens on breast cancer were mostly emphasized. Many phytoestrogens showed anti-proliferation of the estrogen receptor positive (ER^+) mammary cancer cell line, MCF-7 and T47D at high dose but also showed the proliferation effect at low dose (as summarized in Table 4).

Table 4 Summary of the study of the effects of phytoestrogens on breast cancer cell lines.

(*Estrogen receptor positive breast cancer cell line, **Estrogen receptor negative breast cancer cell line)

Chemical	Cell	Dosage	Results	Reference
Genistein	MCF-7*	not described	- Relative binding affinity to SBG = 27% compared with E_2 - Markedly enhance tumor cell proliferation. - Competed with E_2, resulted in rapid ER decrement.	Martin *et. al.*, 1978
	MCF-7*	0, 1, 5, 50, 500 µM/L	- Inhibited cell growth in dose dependent manner - ID_{50} = 4µM/L after 72 hr. of incubation. - The cell exhibited DNA content decrement and nuclear fragmentation characteristic of apoptosis.	Pagliacci *et. al.*, 1994
	MCF-7*	1, 10, 100 nM 1, 10, 100 µM	- Stimulated growth at 1 nM – 10 µM but inhibited growth at >10 µM - The maximal growth stimulation (0.1 –1 µM) was equal to that of estradiol at 1 nM. - *pS2* level in the growth medium was rose steadily (in dose dependent manner) and peaking at 20 µM	Zava and Duwe, 1997

Table 4 (Continued) Summary of the study of the effects of phytoestrogens on breast cancer cell lines.

(*Estrogen receptor positive breast cancer cell line, **Estrogen receptor negative breast cancer cell line)

Chemical	Cell	Dosage	Results	Reference
Genistein (continued)	MDA-MB 468** and HMEG**	1, 10, 100 nM 1, 10, 100 μM	- genistein had little effect or was slightly growth inhibition at10 nM – 1μM	Zava and Duwe, 1997
	T47D*	1, 10, 100 nM 1, 10, 100 μM	- Increased growth from 10nM to 10 μM but inhibited growth at >20 μM	
		100 nM-20μM	- Markedly inhibited cell growth at 20 μM	
		1, 10, 100 nM 1, 10, 100 μM + 0.3 nM E_2	- Genistein had little effect on the growth-promoting effects of 0.3 nM E_2 over the concentration range from 0.3-10 nM (but was slightly inhibit E_2 action from 80-300 nM	
		1, 10, 100 nM 1, 10, 100 μM + 1 mM TAM	- The dose-response curve was shift 1 log to the right (from genistein only curve)	

Table 4 (Continued) Summary of the study of the effects of phytoestrogens on breast cancer cell lines.

(* Estrogen receptor positive breast cancer cell line, ** Estrogen receptor negative breast cancer cell line)

Chemical	Cell	Dosage	Results	Reference
Genistein (continued)	T47D*	1, 10, 100 nM 1, 10, 100 μM + 100 nM HTAM	- The dose-response curve was shift 2 log to the right (from genistein only curve)	Zava and Duwe, 1997
	MCF-7*	0-100 μ mol/L	- Stimulated growth at low concentration (5 μmol/L) but inhibited growth at higher concentrations in dose dependent manner. - IC_{50} = 31 μ mole/ L - Caseine, lipid and the membrane protein ICAM1 were optimally expressed after the treatment. - The cells became differentiated in response to the treatment.	Constantinous *et. al.*, 1998
	MCF-7* nude mice xenograph	30 μ mol/L	- Diminished the cells tumorigenic potential.	

Table 4 (Continued) Summary of the study of the effects of phytoestrogens on breast cancer cell lines.

(* Estrogen receptor positive breast cancer cell line, **Estrogen receptor negative breast cancer cell line)

Chemical	Cell	Dosage	Results	Reference
Genistein (continued)	MDA-MB-468**	0-100 μ mol/L	- Genistein showed more efficient in inhibiting MDA-MB-468 cell growth than MCF-7 cell with no stimulatory effect. - IC_{50} = 21 m mol/L - The cells become differentiated in response of the treatment.	Constantinous *et. al.*, 1998
	MDA-MB-468** nude mice xenograph	30 μ mol/L	- Diminished the cells tumorigenic potential.	
	MCF-7*, T47D*, 549*	0–40 μg/ml+10^{-9}M E_2	- Stimulated growth at lower concentrations (1-5 μg/ml) but inhibited growth at higher concentrations (20-40 μg/ml). - Resulting in down regulation of ER mRNA level in dose dependent manner. - Anti-proliferative effects are estrogen dependent.	

Table 4 (Continued) Summary of the study of the effects of phytoestrogens on breast cancer cell lines.

(* Estrogen receptor positive breast cancer cell line, ** Estrogen receptor negative breast cancer cell line)

Chemical	Cell	Dosage	Results	Reference
Genistein (continued)	MCF-7*, T47D*, 549*	0–40 µg/ml+10^{-9}M E_2	- Inhibited PTK activity in Phenol Red media. - Inhibited E_2 up-regulation of *pS2* and TGF-α mRNA at high concentration - In the absence of E_2, increased ERE-CAT activity at lower concentration (<20µg/ml). - In the presence of E2, at both low and high concentrations shown decrement of ERE-CAT activity. - Inhibited growth at high concentration (>10 µg/ml) with no stimulatory effect at any concentration. - Anti-proliferative effects are estrogen independent. - Showed no effect on PTK activity - Showed no effect on ERE-CAT activity	Shao *et. al.*, 2000

Table 4 (Continued) Summary of the study of the effects of phytoestrogens on breast cancer cell lines.

(*Estrogen receptor positive breast cancer cell line, **Estrogen receptor negative breast cancer cell line)

Chemical	Cell	Dosage	Results	Reference
Genistein (continued)	MCF-7* nude mice xenograph	15, 150, 300 ppm supplemented in diet.	- Cell proliferation was greatest in tumors of animals given 150, 300 ppm. - The dosage 150, 300 ppm resulted in the increment of pS2 expression	Allred *et. al.*, 2001
Daidzein	MCF-7*and MDA-MB231**	0-100 μmol/L	- No growth stimulatory effect at low concentration but show growth inhibitory effect at higher concentration. - Did not induce cell differentiation.	Constantinou *et. al.*, 1998
Quercertin	MCF-7*	5.2 μg/ml (IC_{50}) + 100 nM E_2	- The addition of E_2 was unaffected to the cells treated with quercertin.	So *et. al.*, 1997
	T47D*	100 nM-20□M	- Markedly inhibited cell growth at 20 μM	Zava and Duwe, 1997
Kaempherol	T47D*	100 nM-20□M	- Markedly inhibited cell growth at 20 μM	Zava and Duwe, 1997

Table 4 (Continued) Summary of the study of the effects of phytoestrogens on breast cancer cell lines.

(* Estrogen receptor positive breast cancer cell line, ** Estrogen receptor negative breast cancer cell line)

Chemical	Cell	Dosage	Results	Reference
Others	MCF-7*	Daidzein and Equol 10^{-11},10^{-10},10^{-9},10^{-8}, 10^{-7},10^{-6},10^{-5} M	- Equol is a 100-fold more potent than Daidzein is stimulating an oestrogenic response. - Equol compound stimulated the growth of MCF-7 cells - Both compound stimulated the growth o f MCF-7 cells in a concentration-dependent manner	Sathyamoorthy, N. and Wang, T.T.Y., 1997
	MCF-7*	Galangin 4.2 µg/ml Baicalein 5.3 µg/ml Hesperetin 12.0 µg /ml Naringenin 18.0 µg /ml	- The addition of E_2 was unaffected to the cell treated with these flavonoids at their IC_{50} concentration.	So *et. al.*, 1997
	MCF-7* nude mice xenograph.	Genistin 750, 1200 ppm. Supplemented in diet.	- Increased tumor growth, *pS2* expression and cellular proliferation - Removal .of genistin from the diet caused tumor regression.	Allred *et. al.*, 2001

III The interaction of phytoestrogens with estrogen receptor

3.1 Estrogen receptor

Estrogen receptor (ER) was a family of hormone-activated transcription factors that can initiate or enhance the transcription of genes containing specific hormone response elements (Macgregor and Jordan, 1998). The Estrogen receptor exists as two subtypes, ERα and ERβ (Gustafsson, 1999).

The first type of ER was named ERα, consisted of 595 amino acids that separated into six different functional regions. The second type of ER was cloned from a rat prostate complementary DNA (cDNA) library and was named ERβ. (Kuiper *et. al.*, 1996). The ERβ protein consisted of 485 amino acids and separated into six functional regions.

Both ER subtypes seem to be important roles in the reproductive system (Gorodeski and Pal, 2000), cardiovascular system (Makela *et. al.*, 1999); development (Cassanova *et. al.*, 1999), reproduction-related behaviors (Ogawa *et. al.*, 1998).

3.1.1 The distribution of ER

Both ER subtypes could be found through out the body including cardiovascular system, central nervous system, reproductive system, gastrointestinal system, breast and bone (Gustafsson, 1999 and Weihua *et. al.*, 2003). The amount of each ER subtype was depended upon the tissue type. For example, ERα was found predominately in the reproductive system (Gustafsson, 1999)

3.1.2 Functional region of ER

There are six functional regions of both type of ER had been defined based on the putative functions that were contained in each area (A-F region). (Macgregor and Jordan, 1998)

A/B	C	D	E	F
AF-1	DBD	Hinge	LBD/AF-2	C-terminal

Figure 1. Schematic structural of ER (A-F region)

1) A/B region

The A/B region contained one of the two transcriptional AFs presented in ER. Activation Function –1 (AF-1) activated transcription in a cell and motor context specific manner. (Weihua *et. al.*, 2003)

2) C region

The C region, the DNA binding domain (DBD), contains a two zinc finger structure, which plays an important role in receptor specific DNA binding and dimerization domain. Another basic requirement for DBD activity, the C region may bind the heat shock protein 90 and would be responsible for nuclear localization of the receptor. (Macgregor and Jordan, 1998)

3) D region

The D region was the hinge region. When the ER conformation change, this region would swing in and out to bear DBD to bind to DNA.

4) E region and F region

The E/F region or the ligand binding domain (LBD) were sites for cofactor binding, transactivation (AF-2), nuclear localization and interactions with heat shock proteins. Functionally, these receptor form dimers and transcription (Weihua *et. al.*, 2003). The F region was the C-terminus. (Macgregor and Jordan, 1998)

3.1.3 Estrogen action on ER

Estrogen diffused through the plasma membrane of cell and bound to ER (Headley, 1996), resulted in the dissociation of the heat shock protein. The ER conformation was then altered into active form by phosphorylation. The hormone-receptor dimerization was subsequently occurred. The dimer bound to EREs represented in the promoter region of the estrogen-activated genes and resulted in activation of the transcription (Macgregor and Jordan, 1998).

3.2 Phytoestrogens action on estrogen receptor

Due to the fact that ER was described as a nuclear transcription, this function was depend on the association with an appropriate ligand that bind to an ERE or AP-1. The ER was capable to bind several structurally diverse compounds with different affinities (Martin *et. al.*, 1978). Some phytoestrogens such as coumestrol, genistein and kaempferol could compete stronger with E_2 for binding to ERβ than to ERα (Kuiper *et. al.*, 1998: Speirs and Kerin, 2000).

3.3 Selective estrogen receptor modulators

Selective estrogen receptor modulators (SERMs) or estrogen analogs (previously called "anti-estrogen") were the agents that could maintain the benefits of estrogen but avoid the risk of estrogen. They were the additional alternative for prevent or treat disease caused by estrogen deficiency, such as osteoporosis. SERMs was interested in using medication for overall health maintenance after menopause. Some of these agents could act as antagonists in human reproductive tissues, with partial agonists on the skeletal system and on serum lipoproteins. Each agent had its own unique activities, with quantitative and qualitative variability in its agonists and antagonist properties at different target tissues. The mechanisms involved with differential binding to different estrogen receptor subtypes, different conformations produced with each agent when bound to the estrogen receptor (Cosman and Linsay, 1999). The SERMs are chemically diverse compounds that lack the steroid structure of estrogen but posses a structure that allowed them to bind to the Estrogen receptor (Wood, 2004). The examples for SERMs were Tamoxifen and Raloxifen (Diel *et. al.*, 2001) It was found that phytoestrogens showed some hints to act like SERMs.

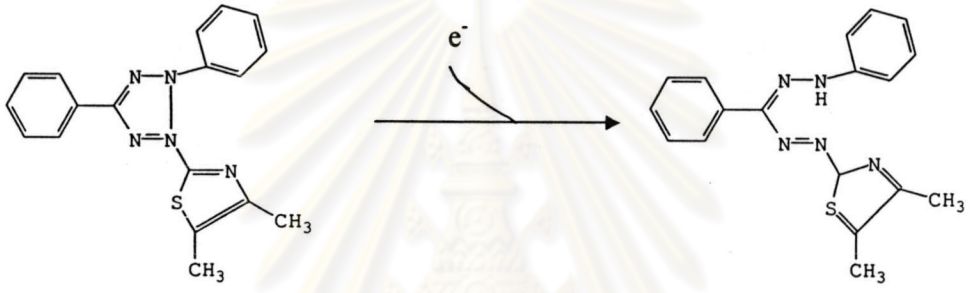
A SERMs could bind to either ERα and ERβ and the complexes could exhibit recruit co-activators or co-repressors (Paech *et. al.*, 1997 and Wood, 2004). The complex might be homo- or hetero-dimerized and modulated genes by either ERE and AP-1. The different in AF-1 altered the SERM-ER complex, resulted in increased or decreased estrogenictity. It was now cleared that the ligan program the shape of the ER complex. Then the co-activators or co-repressors could bind to a SERM-ER complex. When the transcriptional complex was formed, the SERM-ERα and SERM-ERβ complexes must dimerized to be homo- or heterodimer, and resulted in initiation of the gene transcription. Thus SERMs could modulate gene transcription via two pathways, like estrogen. (Paech *et. al.*, 1997),

The dietary phytoestrogens were claimed to be the alternative choice for hormonal replacement therapy (HRT), cancer prevention and treatment (An *et. al.*, 2001). The other phytoestrogen-rich, *P. mirifica* might exhibit the higher potential to use in the same purpose. In addition, *P. mirifica* and *B. superba* herbal products were available in the markets whereas their effects on cancer were still uncleared. The

study of the effects upon some human cancer cell line would provide the evidence *in vitro* and thus resulted in a confidence of the consumers to make a choice on these products.

IV MTT assay (Mosmann, 1983).

The MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay is a simple colorimetric method to measure proliferation, cell viability and cytotoxicity. MTT is a yellow, water-soluble, tetrazolium salt. Metabolically active cell are able to convert this dye into a water-insoluble dark blue formazan by reductive cleavage of the tetrazolium ring (Mosmann, 1983).



Tetrazolium bromide (MTT) Formazan crystals

Figure 2 Conversion of Tetrazolium bromide (MTT) to Formazan crystals (Mossman, 1983)

The assay detects living but not dead cells and the signal generated is dependent on the degree of activation of the cells. Cells uptake, converted to a colored water insoluble formazan crystals by mitochondrial dehydrogenase activity and subsequent quantitative extraction of a dye. The results can be read on a multiwells scanning spectrophotometer (ELISA reader) at 570 nm. and show a high degree of precision. The number of viable cells is then determined indirectly by MTT dye reduction. (Freshner, 2000)

The main advantages of the colorimetric assay are its simple, reproducibility, rapid, precision and can be semi-automated to process a large number of samples and provide a rapid measurement of cell number. Therefore this method has been used for adopt several organisms such as cell line (Oliver *et. al.*, 1989), fungal (Levitz and Diamond, 1985), human sperm (Nasr-Esfahani *et. al.*, 2002), yeast (Kjertstedt *et. al.*, 1998) and Parasites (Mukherjee, Misra and Chatterjee, 1997).

In this study, MTT assay was assessed to study proliferative and anti-proliferative effect of *P. mirifica*, *B. superba* and *M. collettii* extracts with MCF-7. The results will enable us to screen for plant with high proliferative/anti-proliferative effect or high estrogenic effect.