การทดสอบความเป็นพิษของสารสกัดจากกวาวเครือขาว *Pueraria mirifica* กวาวเครือแดง *Butea superba* และกวาวเครือดำ *Mucuna collettii* ที่มีต่อเซลล์ Hep-G2

นางสาววรรณรวี สังขพงษ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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CYTOTOXICITY TESTS OF EXTRACTS FROM WHITE KWAO KRUA *Pueraria mirifica,* RED KWAO KRUA *Butea superba* AND BLACK KWAO KRUA *Mucuna collettii* ON Hep-G2 CELLS

MissWanrawee Sangkapong

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By	Miss Wanrawee Sangkapong
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.....Dean of the Faculty of Science
(Professor Piamsak Menasveta, Ph.D.)

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.....Chairman
(Associate Professor Kingkaew Wattanasermkit, Ph.D.)

.....Thesis Advisor
(Associate Professor Wichai Cherdshewasart, D.Sc.)

..... Member
(Associate Professor Sirirat Rengpipat, Ph.D.)

.....Member
(Associate Professor Nattaya Ngamrojanavanich, Ph.D.)

วรรณรวี สังขพงษ์: การทดสอบความเป็นพิษของสารสกัดจากกวาวเครือขาว (*Pueraria mirifica*) กวาวเครือแดง (*Butea superba*) และกวาวเครือดำ (*Mucuna collettii*) ที่มีต่อเซลล์ Hep-G2 (CYTOTOXICITY TESTS OF EXTRACTS FROM WHITE KWAO KRUA *Pueraria mirifica*, RED KWAO KRUA *Butea superba* AND BLACK KWAO KRUA *Mucuna collettii* ON Hep-G2 CELLS) อ. ที่ปรึกษา: รศ.ดร.วิชัย เชิดชีวศาสตร์ จำนวน 77 หน้า ISBN 974-14-3246-1

ในงานวิจัยครั้งนี้ได้ศึกษาผลของสารสกัดจากกวาวเครือขาว กวาวเครือแดง และกวาวเครือดำ ต่อการกระตุ้นและยับยั้งการแบ่งตัวของเซลล์ตับมนุษย์ (Hep-G2) ในสภาวะนอกร่างกายสิ่งมีชีวิต หลังจากบ่มเซลล์ตับกับสารสกัดพืชทั้ง 4 ชนิดที่ความเข้มข้น 1, 10, 100 และ 1000 ไมโครกรัม/มิลลิลิตรเป็นเวลา 3 วัน นำมาวัดปริมาณเซลล์มีชีวิตด้วยสารเอ็มทีทีเปรียบเทียบเป็นเปอร์เซ็นต์กับกลุ่มควบคุม ($P < 0.05$) พบว่า ตัวอย่างกวาวเครือขาวจากจังหวัดน่านที่ความเข้มข้น 1000 ไมโครกรัม/มิลลิลิตรแสดงผลกระตุ้นการเจริญเติบโตของเซลล์มากที่สุด และตัวอย่างกวาวเครือแดงจากจังหวัดราชบุรีที่ความเข้มข้น 1 ไมโครกรัม/มิลลิลิตรแสดงผลยับยั้งการเจริญเติบโตของเซลล์มากที่สุด ตัวอย่างกวาวเครือแดงจากจังหวัดเชียงใหม่ที่ความเข้มข้น 1000 ไมโครกรัม/มิลลิลิตรแสดงผลกระตุ้นการเจริญเติบโตของเซลล์มากที่สุด และตัวอย่างกวาวเครือแดงจากจังหวัดขอนแก่นที่ความเข้มข้น 1000 ไมโครกรัม/มิลลิลิตรแสดงผลยับยั้งการเจริญเติบโตของเซลล์มากที่สุด และตัวอย่างกวาวเครือดำจากจังหวัดกาญจนบุรีที่ความเข้มข้น 1000 ไมโครกรัม/มิลลิลิตรแสดงผลกระตุ้นการเจริญเติบโตของเซลล์มากที่สุด และตัวอย่างกวาวเครือดำจากจังหวัดเชียงใหม่ที่ความเข้มข้น 1000 ไมโครกรัม/มิลลิลิตรแสดงผลกระตุ้นและยับยั้งการเจริญเติบโตของเซลล์มากที่สุด เป็นที่น่าสนใจว่าการกระตุ้นการเจริญเติบโตของเซลล์ตับนั้นอาจแสดงถึงฤทธิ์ในเชิงชะลอความแก่ของเซลล์ซึ่งสามารถช่วยยืดอายุการตายของเซลล์ลงได้ ส่วนผลการยับยั้งการเจริญเติบโตของเซลล์นั้นอาจนำไปศึกษาต่อเพื่อนำไปพัฒนาเป็นยาฆ่าเซลล์มะเร็ง

สาขาวิชา.....เทคโนโลยีชีวภาพ.....ลายมือชื่อนิสิต.....

ปีการศึกษา.....2548.....ลายมือชื่ออาจารย์ที่ปรึกษา.....

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KEYWORD: Cytotoxicity test / Proliferative / Anti-proliferative / *Pueraria mirifica* / *Butea superba* / *Mucuna collettii* / Hep-G2 cell / human liver

WANRAWEE SANGKAPONG: CYTOTOXICITY TESTS OF EXTRACTS FROM WHITE KWAO KRUA, *Pueraria mirifica*, RED KWAO KRUA, *Butea superba* AND BLACK KWAO KRUA, *Mucuna collettii* ON Hep-G2 CELLS.

THESIS ADVISOR: ASSOC. PROF. WICHAI CHERDSHEWASART, D.Sc. 77 pp. ISBN 974-14-3246-1

This experiment is designed to evaluate the proliferative and antiproliferative effect in vitro of the plant crude extracts (white Kwao Krua, *Pueraria mirifica*, red Kwao Krua, *Butea superba* and black Kwao Krua, *Mucuna collettii*) to human hepatoblastoma, Hep-G2 cell line. The cells were incubated with the 4 plant extracts at the concentration of 1,10, 100 and 1000 μg/ml for 3 days and assay for the percent of cells viability by MTT compared with the control ($P < 0.05$). Samples of *P. mirifica* extracts collected from Nan (1000 μg/ml) and Ratchaburi provinces (1 μg/ml) exhibited most proliferative and anti-proliferative effect, respectively. Samples of *B. superba* extracts collected from Chaing Mai (1000 μg/ml) and Khon Kaen provinces (1000 μg/ml) exhibited most proliferative and anti-proliferative effect, respectively. Samples of *M. collettii* extracts collected from Kanchanaburi (1000 μg/ml) and Chaing Mai provinces (1000 μg/ml) exhibited most proliferative and anti-proliferative effect, respectively. Very interesting, proliferative effect to Hep-G2 itself may refer to rejuvenating effect of the plant extracts as normally the hepatocytes can do rapid cell division if the hepatic tissue is damaged. Besides, the antiproliferative effect to the cells may enable establishment of study to develop the plant product into a therapeutic medicine for cancer cells.

Field of study...Biotechnology .....Student's signature.....

Academic.....2005..... .Advisor's signature.....

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CHAPTER I

Introduction

Among the popular Thai herbal plants, Kwao Krua plants are the most interesting ones. This group of plants comprising of white Kwao Krua *(Pueraria mirifica* Airy Shaw & Suvatabandhu), red Kwao Krua (*Butea superba* Roxb.) and black Kwao Krua (*Mucuna collettii* Lace). The plants have long-time been used in traditional medicine and folklore. *Pueraria mirifica* has been used as a rejuvenating medicine suggested for adult. Several indications of this plant were suggested for a traditional purpose such as skin enrichment, thickening and blackening hair, a relief of weakness, an increase of an appetite, treatment of insomnia and breast enlargement in women (Charoenkul, 2001). *B. superba* and *M. collettii* have been consumed among Thai males for potency purposes (Suntara, 1931).

The use of cells models is an indispensable tool of current research into the biological activity of plant extracts. The liver is known to be the main site of xenobiotic biotransformation due to the ability of this organ to express a plethora of enzymes, both quantitatively and qualitatively. Therefore, cell lines of liver origin are widely used in biomedical research involving xenobiotic metabolism including genotoxicity studies. Of these cell lines, the Hep-G2 cell line is the most versatile one. This cell line retains many of the specialized functions normally lost by primary hepatocytes in culture (Knowles *et al.*, 1980; Aden *et al.* 1979) In the fields of experimental toxicology and pharmacology, isolated human hepatocytes have been extensively used for the development and testing of new drugs at both preclinical and clinical stage because of the special *in vivo* role of human hepatocytes in drug metabolism and detoxification (Dvorak *et al.*, 2003).

At presents study, plant-derived products are rapidly developed into dietary supplements and cosmetic products for both domestic consumption and exportation. The basal data should be rapidly and fully established to support the product development. Our study will focus on evaluation of proliferative and anti-proliferative of the 3 plant extracts to Hep-G2 cells. The data should establish quantity of active compound in plants that safety for consumers. The plants with high proliferaive might

increase the rejuvenate activity. The plants with anti-proliferative effects are also interesting as they might be good sources of materials that could be developed into anti-liver cancer drugs.

Purpose of the study is as followed:

To evaluate the cytotoxicity of collected white, *P. mirifica,* red, *B. superba* and black, *M. collettii* Kwao Krua extracts on the growth of human liver hepatoblastoma (Hep-G2) cells.

CHAPTER II

Literature Review

2.1 Botanical characteristics of Kwao Krua plants

Pueraria mirifica, *Butea superba* and *Mucuna collettii* were classified into the family of Leguminosae (Ridley, 1967; Pengklai, 1977; Suvatti, 1978). The name of Kwao Krua was commonly applied to plants in different genus. At least three kinds of Kwao Krua, the white, red and black Kwao Krua, were commonly found and long term consumed in Thailand (Suntara, 1931)

2.1.1 *Pueraria mirifica*

P. mirifica Airy Shaw & Suvatabandhu is an indigenous herb of Thailand, known as "Kwao Krua" or Kwao Krua Kao" (white Kwao Krua). The plant is a long-living twinning wood. Leaves are pinnately three foliate stipulate; terminal leaflet. Tuberous roots are varied in sizes and shapes. Flower are bluish-purple legume shaped, flowering occurred during late January to early April. The length of the inflorescence of certain flowers is approximately 15-40 cm. The flower contained five sepals and the petals are one standard with two keels. Pods are slender typically short or elongate, smooth or hairy, including 1-10 single seed when fully matured and dried which turned into various color (Smitasiri and Wungjai, 1986 ; Cherdshewasart unpublished data, Fig. 2.1).

Figure 2.1 (a) Leaves, (b) flowers, (c) tuberous roots and (d) pods of *P. mirifica* from Chiang Mai Province, photos courtesy by W. Cherdshewasart

P. mirifica extracts were characterized into classes of compounds (Table 2.1) with some defined biological function and the chemical structures of these compounds were shown in Fig 2.2.

Table 2.1 Summary of the chemical constituents of *P. mirifica*

Categories	Chemical constituents	References
Isoflavonoids	Daidzein	Ingham *et al.*, 1986
	Genistein	Ingham *et al.*, 1986
	Kwakhurin	Ingham *et al.*, 1986
	Kwakhurin hydrate	Ingham *et al.*, 1989
Isoflavone glycosides	Daidzin (daidzein-7-o-glucoside)	Ingham *et al.*, 1986
	Genistin (genistein-7-o-glucoside)	Ingham *et al.*, 1986 ; Ingham *et* al., 1989
	Mirificin (puerarin6'-o-β-apiofuranoside)	Ingham *et al.*, 1986
	Puerarin (daidzein-8-glucoside)	Nilandihi *et al.*, 1957 Ingham *et al.*, 1986 ; Ingham *et* al., 1989
	Puerarin 6''- monoacetate	Ingham *et al.*, 1989
Chromenes	Miroestrol	Schoeller *et al.*, 1940 Bound and Pope, 1960 Jones and Pope, 1961
	Deoxymiroestrol	Chansakaew *et al.*, 2000[a]
	Isomiroestrol	Chansakaew *et al.*, 2000[a]
Coumestans	Coumestrol	Ingham *et al.*, 1986 ; Ingham *et* al., 1988
	Mirificoumestan	Ingham *et al.*, 1988
	Miricoumestan glycol	Ingham *et al.*, 1988
	Miricoumestan hydrate	Ingham *et al.*, 1988
Sterols	β-sitosterol	Hoyodom, 1971
	Stigmasterol	Hoyodom, 1971
Pterolcapans	Pueriicapene	Chansakaew *et al.*, 2000[b]
	Tuberosin	Chansakaew *et al.*, 2000[b]
Acid	Tetracosanoic acid	Chansakaew *et al.*, 2000[b]

Modified from Panriansaen, 2005; Subtang, 2002

Isoflavone and Isoflavone glycosides

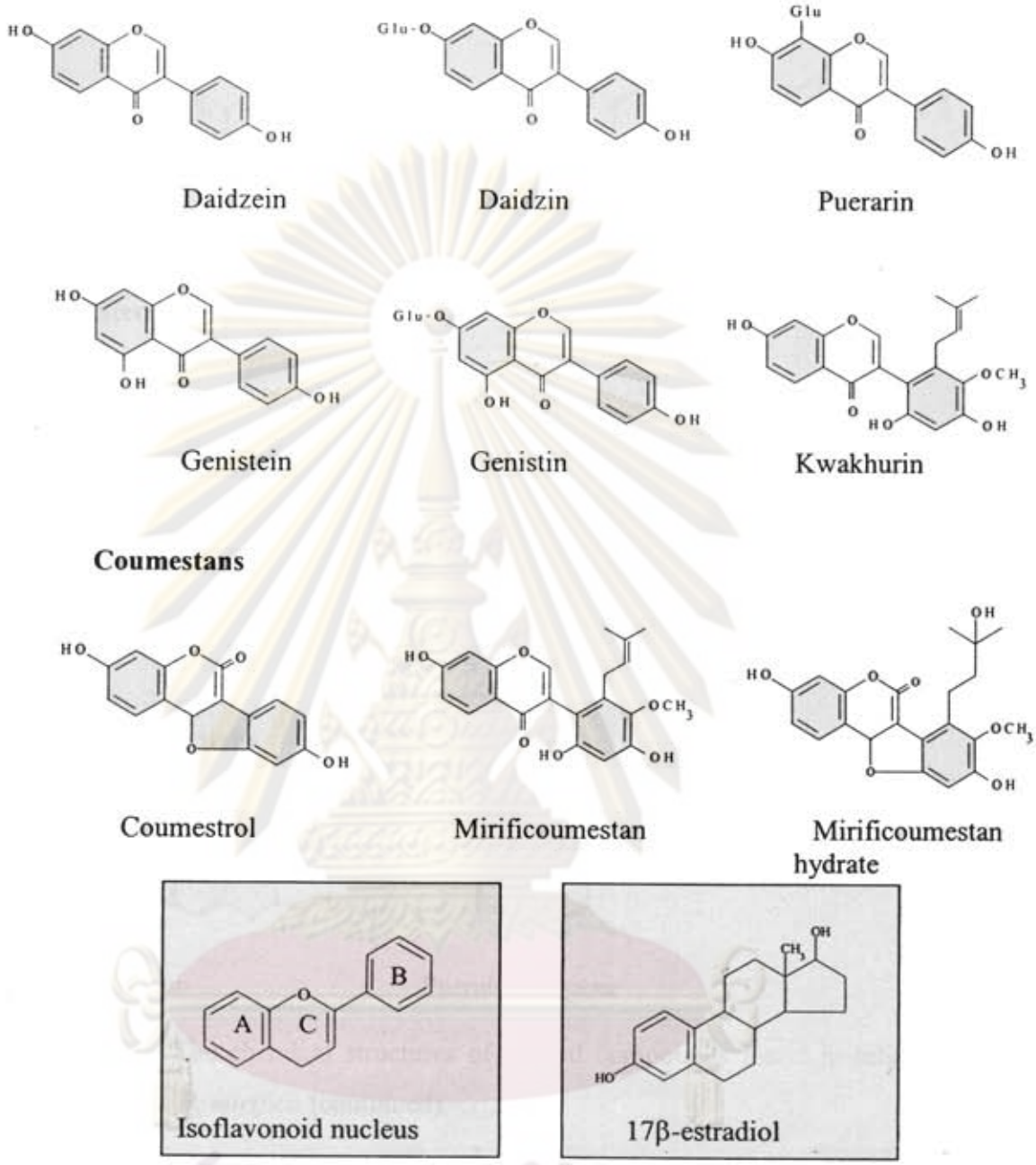
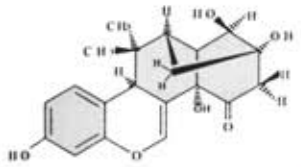
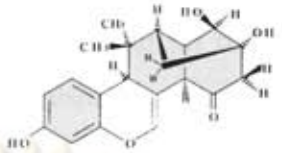


Figure 2.2 The chemical structures of natural compounds found in tuberous root *P. mirifica*.

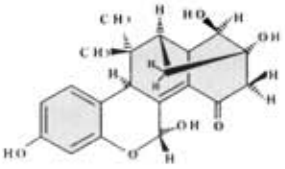
Chromenes



Miroestrol

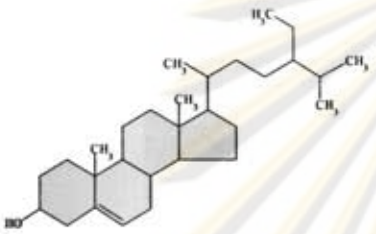


Deoxymiroestrol

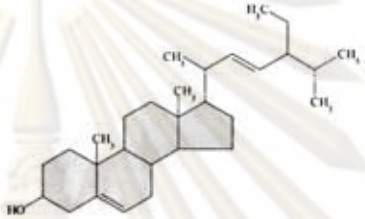


Isomiroestrol

Sterols

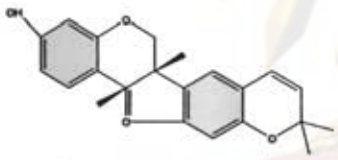


β-sitosterol

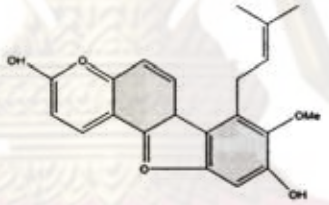


Stigmasterol

Pterocarpans



Tuberosin



Puemirificarpene

Figure 2.2 The chemical structures of natural compounds found in tuberous root *P. mirifica* (continued).

Genistein increases the sex hormone-binding globulin (SHBG) production by Hep-G2 cells, and also suppresses the proliferation of these cells already at a stage when SHBG production continues to be high (Mousavi and Adlercreutz, 1993)

2.1.2 *Butea superba*

B. superba or red Kwao Krua is a large size climber. Leaves are pinnately three foliate, acuminate leaflet and long leafstalk. Flowers are large with yellowish orange color. Petals are three times longer than the calyx. Pods are 3-4

inches long, oblong shaped with silvery silky short hair (Kruz, 1877 ; Brandis, 1990) but only one seed present (Cherdshewasart, unpublished data, Fig. 2.3).

The plants were found abundant in the forests of the north, middle, northeastern regions of Thailand (Cherdshewasart unpublished). The tuber and stem of plant has been used in medicines and believed to promote strength and male potency (Suntara, 1931).

Figure 2.3 (a) Leaves, (b) tuberous root, (c) tuberous cross section and (d) flowers of *B. superba* from Lumpang Province, photos courtesy by W. Cherdshewasart

B. superba tuberous root extracted contained five groups of chemical constituents namely; carboxylic acid, steroid, steroid glycoside, flavonoid and flavonoid glycoside (Roengsamran *et al.*, 2000; Ngamrojanavanich *et al.*, 2006 in preparation). The chemical constituents and their structural formula found in the tuberous root is summarized and shown in Table 2.2.

Table 2.2 Summary of the chemical constituents of *B. superba**

Categories	Chemical constituents	References
Carboxylic acid	Straight acid carboxylic acid (C_{22}-C_{26})	Rakslip, 1995
	3-hexacosanoloxy-propane-1,2- diol	Ngamrojanavanich *et al.*, 2006 in preparation
Steroid	Campesterol	Ngamrojanavanich *et al.*, 2006 in preparation
Steroid glycoside	β-sitosteryl 1-3-O-β-D-glucopyranside	Ngamrojanavanich *et al.*, 2006 in preparation
	Stigmasteryl 1-3-O-β-D-glucopyranside	Ngamrojanavanich *et al.*, 2006 in preparation
Flavonoid	3,7,3'-trihydro-4'-methoxyflavone	Rakslip, 1995
	Prunetin (5,4'-dihydroxy-7-methoxy-isoflavone)	Ngamrojanavanich *et al.*, 2006 in preparation
	Medicarpin (3-hydroxy-9-methoxypterocarpan)	Ngamrojanavanich *et al.*, 2006 in preparation
	Formononetin (7-hydroxy-4'-methoxy-isoflavone)	Ngamrojanavanich *et al.*, 2006 in preparation
Flavonoid glycoside	7-hydroxy-6-4'-dimethoxyisoflavone	Subba and Seshadri, 1949
	3,5,7,3',4'-pentahydroxy-8-methoxy-flavonol-3-O-β-D-xylopyranosyl-(1,2)- α-L- rhamnopyransoside	Yavada and Reddy, 1998[a]
	3,7-dihydroxy-8-methoxyflavone-7-O-α-L-rhamnopyransoside	Yavada and Reddy, 1998[b]

*Modified from Panriansaen, 2005; Subtang, 2002

B. superba is the Thai traditional remedy known as "rejuvenating" and "neurotonic" agent which have been popular elderly. It is believed that the remedy can prevent forgetfulness and improve memory. Acetylcholinesterase inhibitor is one of most promising approaches for treating Alzheimer's disease by enhance the acetylcholine level in the brain (Enz *et al.*, 1993) and methanolic extract of *B. superba*

at concentration 0.1 mg/ml also could inhibit activity of acetylcholinesterase 55.87% (Ingkaninan, 2003).

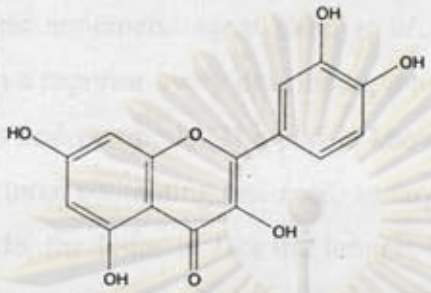
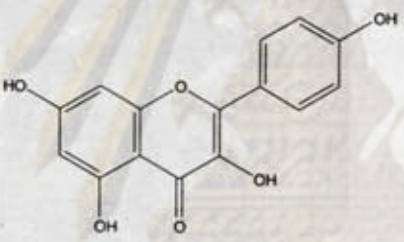
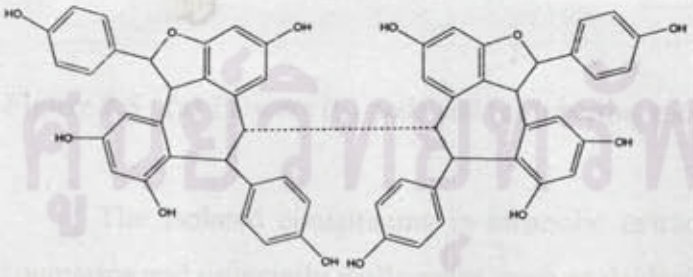
2.1.3 *Mucuna collettii*

M. colletti or black Kwao Krua is a large woody climber, 30-40 m. in height scattered by stems in evergreen forest. Leaves are trifoliate; leaflets 4-8 by 2-4 inches sparsely hairy, entire margin; petiole 5-10 cm. in long. Flowers are hanging on the stem up to 12 inches in long with 5 sepals covered with brown rough hair and unite into a bell-shaped tube. Petals are blackish-purple pea-like shaped. Stamens are two bundles. Pods are linear-oblong shaped up to 16 inches in long. Seeds are hard and flat. Flowers are blooming during January to March (Pengklai, 1997 ; Cherdshewasart unpublished, Fig. 2.4).

Figure 2.4 (a) Leaves and (b) stems of *M. collettii*, Photos of *M. collettii*

The whole stem of *M. collettii* contained three interested chemical constituents in ethyl acetate crude extracts namely; Kaempferol, Quercetin and Hopeaphenol (Sookkongwaree *et al.*, 2006, in preparation, Table 2.3).

Table 2.3 The structure and bioactivity effects of chemical constituents in *M. collettii*

Category	Chemical compound structures	Bioactivity
Flavonoid	 Quercetin M.W. 302.24 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one)	• Accelerated the tumor necrosis factor-alpha induced growth inhibition and apoptosis in MC3T3-E1 osteoblastic cells (Son *et al.*, 2006) • Up regulated expression of several tumor suppressor genes (Erk *et al.*, 2005) • Reduced superoxide dismutase activity and increase the malonaldehyde content in BNL SV A.8 cells (Son *et al.*, 2004)
	 Kaempferol M.W. 286.24 (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one)	• Inhibited apoptosis in vascular smooth muscle induced by a component of oxidized LDL (Ruiz *et al.*, 2006) • Exhibited the strong antioxidant activity (Yu *et al.*, 2005) • Exhibited the strong antibacterial and antiviral (HSV-1) activity (Ulanowska *et al.*, 2006; Lyu *et al.*, 2005) • Inhibited aflatoxin B(1) biosynthesis in *Aspergillus flavus* (Norton, 1999)
	 Hopeaphenol M.W. 906.94	• Exhibited murine leukemia P-388 cells with IC_{50} of 5.7 μM (Sahidin *et al.*, 2005) • Exhibited moderate activity against methicillin - resistant *Staphylococcus aureus* and *Mycobacterium smegmatis* (Pols *et al.*, 2002) • Showed potent inhibition on the biosynthesis of leukotriene B4 and strong antagonism of the histamine acceptor (Huang *et al.*, 2001)

2.2 *Pueraria montana* var. *lobata*

Pueraria montana var. *lobata* (Kudzu) is classified in the genus *Pueraria* , Family Leguminosae and subfamily Papilionoideae. It is one of the earliest medicinal plants to be used in China. Root of this plant was used as an antipyretic, antidiarrhetic, diaphoretic and antiemetic agent (Yan *et al.*, 2004). The fruit is a legume, a pod, resulting from a superior ovary developing into a bivalved usually dehiscent structure, such as beans and pea pods. The pod is known in a multitude of shapes, from tiny rounded structures containing one seed, to large woody legumes up to 2 m long and 10-20 cm wide, the latter in fact the longest fruit structures in the world. Kudzu is widely spread over China and Japan (Keung, 2002) (Fig. 2.5).

Figure 2.5 (a) Flowers (b) pods and (c) tuberous roots of *P. lobata*.

The isolated constituents in ethanolic extracts of *P. lobata* are flavonoids, coumarins and especially isoflavones, such as daidzein, daidzin, puerarin and daidzin-4',7-diglucoside (Guerra *et al.*, 2000). But the crude aqueous extracts of *P. lobata* are daidzein, daidzin, puerarin, 5-hydroxypuerarin, 3'-hydroxypuerarin and 3'-methoxypuerarin (Jiang *et al.*, 2005).

2.3 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 identified. 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 2.6). 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 ; Adlercreutz *et al.*, 1993). However, genistin was partly absorbed without previous cleavage (Andlauer *et al.*, 2000). In human, aglycones were absorbed faster and in greater amounts than their glycosides (Izumi *et al.*, 2000). The maximum peak of isoflavonoids is found at the range of 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 al.*, 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 are often found in highest level in biological matrices and exert significant biological effects (Hodgson *et al.*, 1996).

Precursor

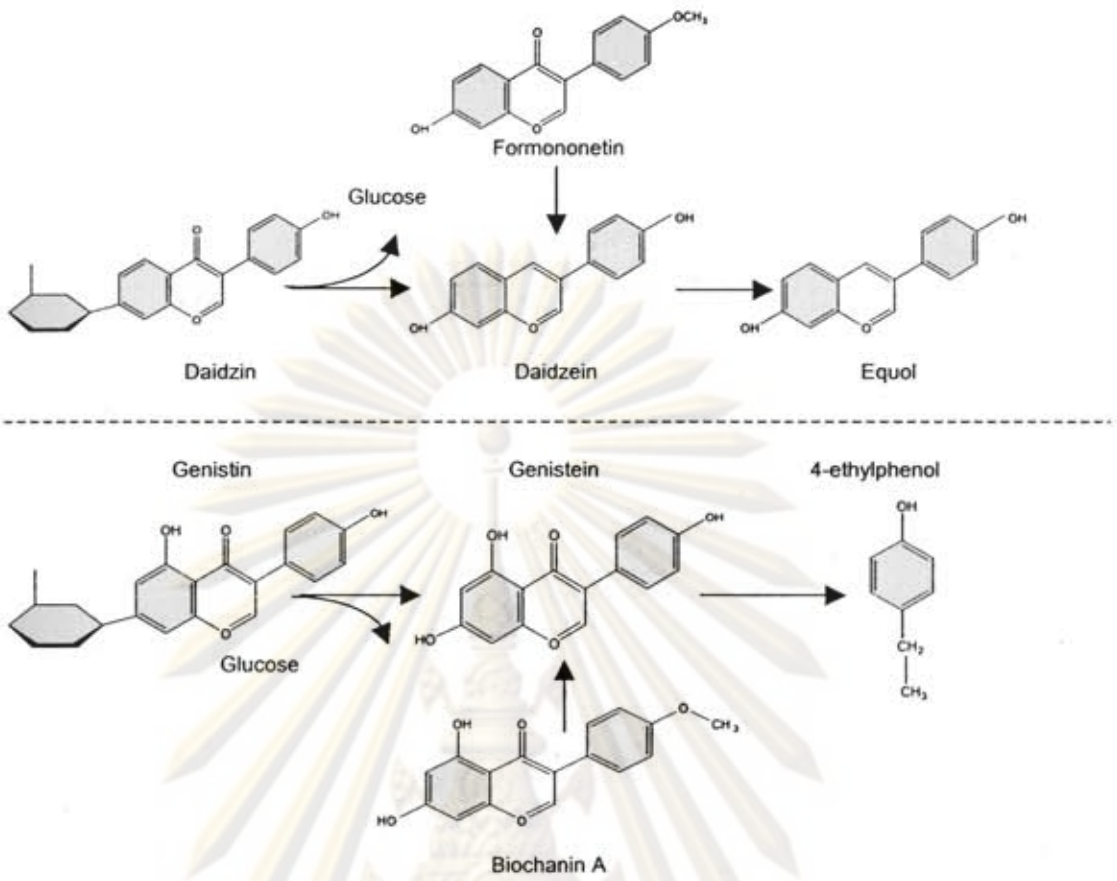


Figure 2.6 Schematic of some phytoestrogen metabolism in intestine (Anderson and Garner. 1997).

2.4 Cytotoxicity

Cell death can be defined as the irreversible loss of essential cellular functions and structures. It has long been used as an endpoint to assess both drug efficacy and drug toxicity *in vitro*. In terms of drug toxicity, cytotoxicity assays have been used to assess the formation of chemically reactive intermediates from drugs that have been implicated in idiosyncratic drug reactions (Park *et al.*, 1992, Fig. 2.7).

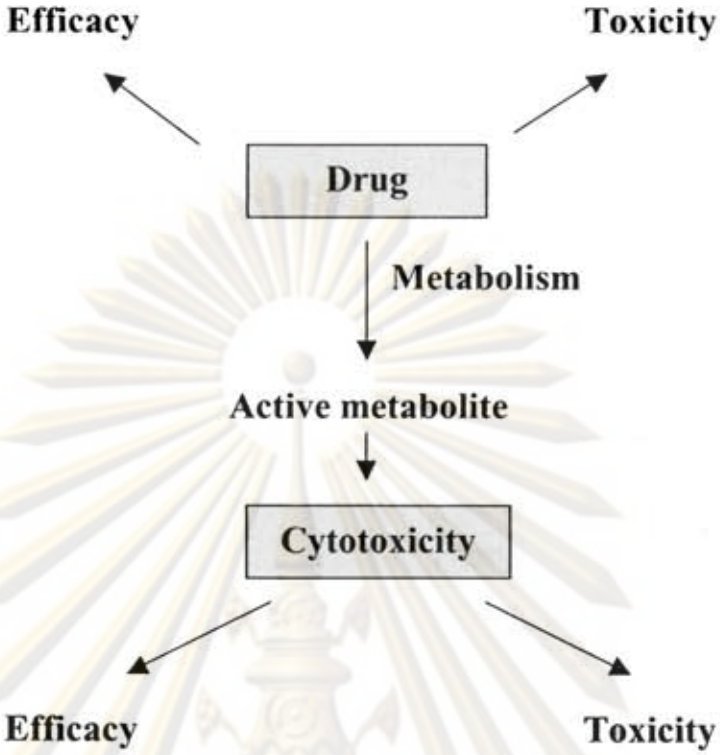


Figure 2.7 The role of metabolism in mediating drug (metabolite) cytotoxicity, which may, in turn, be important for efficacy or toxicity.

2.5 Methods for the assessment of cell viability

Many methods for the assessment of cell viability have been described (Table 2.4). These can be divided into direct and indirect methods (Sellers *et al.*, 1994). The direct assays include clonogenic assays and assays that back extrapolate cell densities in exponentially proliferating cultures; they are sensitive and directly measure surviving fractions of cells, but suffer from the major disadvantages of being labor-intensive, technically difficult, and require several weeks to obtain results. In view of these limitation, various indirect, more rapid assays have been developed. These include measurement of (Woolf, 1999)

- Inhibition of incorporation of labeled RNA or DNA precursors, amino acids, or other metabolites
- Exclusion of dyes or enzymes
- Decrease of intracellular ATP content
- Formation of a colored or fluorescent product from a precursor

- Release of a labeled compound or an enzyme from damaged cells
- Total cell biomass

Table 2.4 Some of the methods used to assess cell death (Woolf, 1999)

Assay	Type
Trypan blue dye exclusion	Exclusion of dye
Lactate dehydrogenase (LDH) release assay	Release of intracellular enzyme
MTT assay (tetrazolium salt reduction)	Formation of colored product
BCECF proliferative assay	Formation of fluorescent product from precursor
Tritiated thymidine (^{3}H-TdR) release assay	Release of labeled compound
^{51}Chromium (Cr) release assay	Release of radiolabeled compound
Europium (Eu^{3+}) release assay	Release of fluorescent compound
ATP bioluminescence	Release of ATP from damaged cell

Almost any cell can be used as a target in cytotoxicity assays. When toxicity of an agent to a particular organ is being assessed, ideally human cells derived from that organ should be used as targets because they will reflect the content of activating and detoxifying enzymes in that organ. However, this is often not possible to directly extrapolate the data obtained from the animal to humans. So an alternative is to use readily accessible human cells. (Spielberg, 1980)

2.6 Mode of cell death

There are two fundamental types of cell death : apoptosis and necrosis (Alison and Sarraf, 1995). They can be differentiated as indicated in Table 2.5.

Necrosis occurs when the cell is exposed to severe and sudden injury, such as physical and chemical trauma. Because cell injury is extensive, there is often an inflammatory response that is required to clear the debris.

Apoptosis is more subtle, and it is the most important form of programmed cell death. On exposure to the noxious substance, the cell undergoes a complex series of molecular changes (which are not fully understood) that ultimately result in its death in the absence of an inflammatory response. However, in the late stages of apoptosis, changes similar to necrosis may be seen within the cell; hence, simple dye exclusion assays cannot be used to differentiate the two forms of cell death (Majno and Joris, 1995)

Table 2.5 Morphological distinction between apoptosis and necrosis

Apoptosis	Necrosis
• Death of isolated cells	• Death of contiguous patches or areas of tissue
• Chromatin condensation, nuclear shrinkage and cell shrinkage	• Nuclear and organelle swelling and whole-cell swelling
• Budding of plasma membrane	• Blebbing of plasma membrane
• Late loss of membrane integrity	• Early loss of membrane integrity
• No inflammatory infiltrate	• Inflammatory infiltrate present
• Phagocytosis of dying cells by neighboring cells	• Phagocytosis of dying cells by professional phagocytes
• DNA laddering on gel electrophoresis	• Nonspecific DNA degradation as a late event

Apoptosis is an energy-dependent process that can run a very fast course (34 min) from the onset of budding to complete break-up has been reported (Majno and Joris, 1995). A biochemical feature of most forms of apoptosis is DNA fragmentation. Initially, DNA fragmentation occurs at 300 or 50 kilobase (Kb) pair intervals; this is followed by cleavage into 180 to 200 bp internucleosomal-sized fragments, which can be visualized on agarose gel as DNA laddering (Que and Gores, 1996). This is caused by activation of calcium-magnesium-sensitive nucleases, which have not yet been fully characterized. The complex series of molecular events underlying apoptosis are not fully understood; several genes appear to be involved (Table 2.6 ; White, 1996).

Table 2.6 Gene products influencing apoptosis

Enhancers of apoptosis	Inhibitors of apoptosis
Bcl-x_s	*Bcl*-2
Bax	*Bcl*-x_L
Bak	*Bcl*-w
Bad	*Mcl*-1
Nbk	p53
Bik 1	Colony-stimulating factors
TNF-α	
Fas/Apo1/CD95	
Interleukin-1β-converting enzyme (ICE)	
c-*myc*	

Necrosis in contrast to apoptosis is an energy-independent process, and is characterized by a progressive reduction in the cellular ATP content (Corcoran *et al.*, 1994). Membrane damage seems to be the key event in the pathogenesis of necrosis; as the injury becomes irreversible, there is a progression from subtle changes in the membrane ionic pumps to nonspecific increase in membrane permeability and, ultimately, to physical membrane disruption.

To date, most of the cytotoxicity assays used in pharmacology and toxicology have used cell death as the endpoint, with differentiating between apoptosis and necrosis.

2.7 The role of metabolism in drug cytotoxicity

In general, drug metabolism can be considered a detoxification process, in that it converts therapeutically active compounds to inactive metabolites that can then be excreted harmlessly from the body. This process may require one or more than one drug-metabolizing enzyme that may be a phase I and phase II enzyme (Fig. 2.8). Metabolism can lead to the formation of chemically reactive intermediates that, if not adequately detoxified, can bind to various essential cellular macromolecules *in vivo* and, thereby, result in different forms of toxicity. *In vitro* the formation of chemically reactive metabolites will often be manifested as cellular death, irrespective of the

nature of the macromolecule to which the toxic metabolite binds (Woolf and Jordan, 1987). A drug may undergo sequential phase I and phase II metabolism, or alternatively, it may undergo only phase I or phase II metabolism (Tephly and Burchell, 1990).

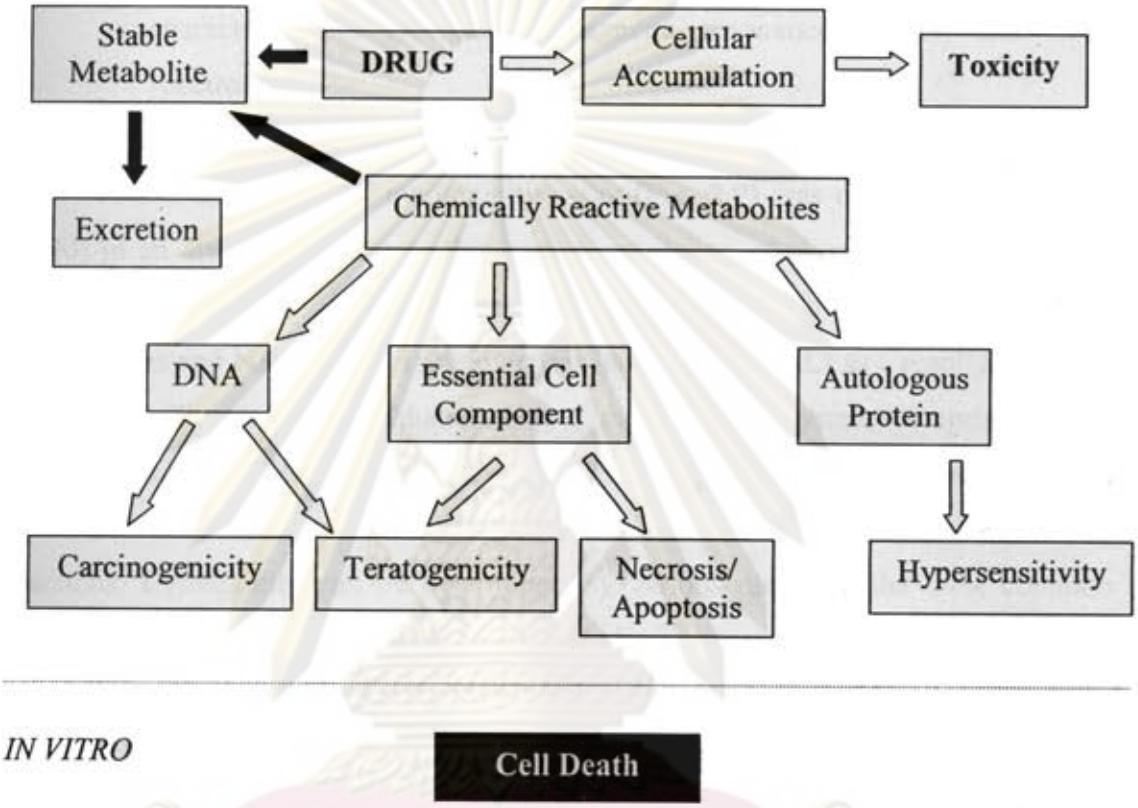


Figure 2.8 The role of metabolism in drug toxicity:

The pharmaceutical industry has also made use of the body's drug-metabolizing enzymes by developing products that are metabolized (by phase I, phase II, or both, enzymes), either within the liver or in the target tissue, to their active components that then mediate the drug's therapeutic action.

2.8 The use of cytotoxicity as an indicator of drug toxicity

By definition, a chemically reactive metabolite is unstable and it may not be possible to characterize it directly by routine analytical methods, such as high-performance liquid chromatography (HPLC) and mass spectrometry (MS). To assess

the formation of these metabolites and their possible role in idiosyncratic drug toxicity, various methods have been used as indirect markers for their formation (Pirmohamed and Park, 1996). These include the following:

- Covalent binding to proteins
- Immunological detection of drug protein conjugates
- Trapping of the reactive metabolites as thiol adducts and subsequent characterization by nuclear magnetic resonance (NMR) and mass spectroscopy
- Cytotoxicity assays

A two-stage *in vitro* cytotoxicity assay (Fig. 2.9) was devised by Spielberg (1980) in an attempt to recreate the *in vivo* generation of chemically reactive drug metabolites, by the incorporation of a drug-metabolite-generating system (microsomes and NADPH) and mononuclear leukocytes (MNL) as a readily available target cell. The drug is incubated with a metabolizing system comprising liver microsomes and NADPH, and incubated with peripheral blood mononuclear leukocytes (MNL) taken from patients with and without idiosyncratic adverse reactions. Cytotoxicity can be determined by various methods, the most common being Trypan blue dye exclusion. Metabolism to stable and protein-reactive metabolites within the system can be assessed by using HPLC and determining covalent binding to the microsomal protein, respectively (Fig. 2.9). The degree of bioactivation of the drug to a chemically reactive metabolite is then assessed by the determination of the viability of the leukocytes. The use of a functional assay is complementary to the chemical and immunochemical methods described in the foregoing. The importance of the assay lies in the fact that several variables can be altered independently or in combination to answer different questions. First, cells from patients with and without adverse drug reactions can be used to determine interindividual variation in cellular detoxification processes (Spielberg, 1980).

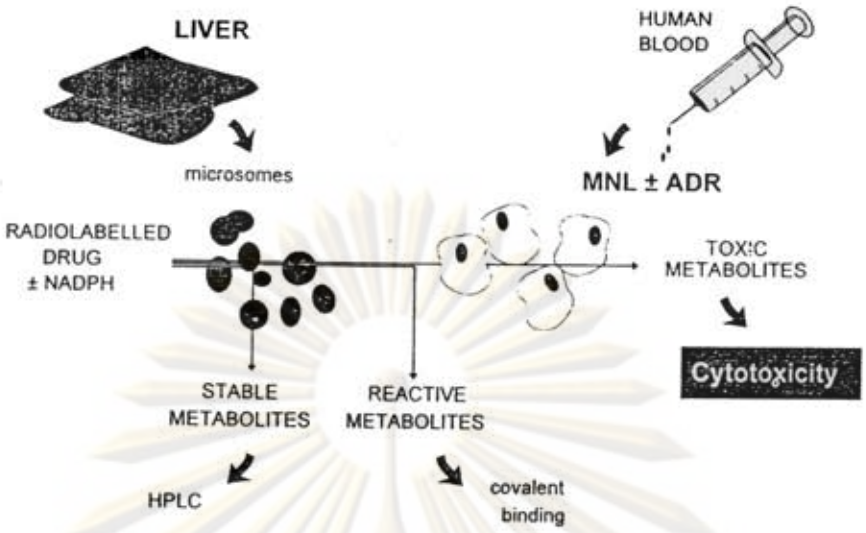


Figure 2.9 A schematic representation of the *in vitro* cytotoxicity assay.

In most of the studies, ether trypan blue dye exclusion or the MTT assay has been used to estimate cell viability. The MTT assay can be used only if a microsomal system is not present in the incubations because microsomes themselves may metabolize MTT. Thus, the MTT assay is suitable only for use when the toxic metabolite itself can be synthesized and then incubated with the cells, as in sulfamethoxazole hydroxylamine (Reider *et al.*, 1989). Trypan blue dye exclusion correlates with other indices of cell damage, including lactate dehydrogenase (LDH) release from the cells and loss of ability to respond to concanavalin a blastogenesis (Spielberg, 1980)

The major problem with the *in vitro* cytotoxicity assay is that it lacks sensitivity in that any changes observed are small (Pirmohamed *et al.*, 1991), and thus, a negative result may not exclude the formation of a toxic metabolite from the coincubated drug. In addition, a chemically reactive metabolite that is not cytotoxic, but may bind irreversibly to protein, may be formed. Therefore, when a radiolabeled compound is available, it may be more useful to combine the determination of both the cytotoxic and protein-reactive metabolites (as well as the stable metabolites) within the same system (Fig. 2.9).

The advantages and disadvantages of using the *in vitro* cytotoxicity assay in elucidating the pathogenesis of idiosyncratic toxicity is illustrated with reference to

several compounds that have been associated with different forms of toxicity (Woolf, 1999).

2.9 Cytotoxic and viability assays

An intensive program to screen plant extracts and other natural materials for anticancer activity began in 1955. Use of *in vitro* assay systems for screening of potential anticancer agents has been common practice almost since the beginnings of cancer chemotherapy. According to the NCI data, many plants extracted posse's cytotoxic activities. Definition of cytotoxicity refers to agents that are toxic to cells *in vitro*. Cytotoxic agents can be further divided into those with cytostatic effect (those that stop cell growth) and cytocidal effect (those that kill cells). The techniques of growing cells as a monolayer have been most frequently applied to the cytotoxicity testing of cancer cell lines. For each compound tested, the IC_{50} (concentration of drug needed to inhibit cell growth by 50%) is generated from the dose-response curves for each cell line. *In vitro* assays are highly useful to evaluate the cytotoxic, mutagenic, and carcinogenic effects of chemical compounds on human cells (Boik, 1996).

2.10 Parameters which vary among different assays: (Freshney, 2000)

2.10.1 Cell culture method

• Seeding density

Seeding density depends on cell size, growth rate, and assay duration. It must be determined individually for each cell type. In a 2-3 day assay, seeding densities are typically in the range of $5\text{-}25\times10^3$ cells per well in 96-well microtitre plates. Time zero values must remain within the linear range of the assay, typically 1.5-2.0 absorbance units.

• Drug solubilization

Stock solution of polar compounds are dissolved in water, buffer, or medium then diluted in complete growth medium to the final test concentration. Non-polar compounds are dissolved in a solvent such as dimethylsulfoxide (DMSO), methanol or ethanol (EtOH) and filter sterilized (0.22 μm pores). A 1:1 mixture of DMSO and EtOH is also a good solvent and it evaporates more slowly than EtOH and chemically sterilizes most test materials. DMSO is toxic to cells at concentrations above 0.1-1.0%. Ethanol is usually growth stimulatory in the 1-2% range. Different cells population exhibit different sensitivities to these organics.

• Drug incubation

It is common procedure to incubate cells with drug solutions immediately after enzyme disaggregation of solid tissue, or harvesting of cell monolayer by trypsinization. There is evidence to suggest that susceptibility of cells to drug is altered by enzyme treatment and does not return to control levels until approximately 12 h after enzyme exposure. It may therefore be expedient to include a pre-incubation recovery period for freshly disaggregated cells to allow for this. Maintenance of pH at 7.4 is essential during the incubation period since alterations in pH will alter cell growth, and alkaline pH particularly will reduce cell viability.

2.10.2 Duration of drug exposure and drug concentration

Assay duration is determined by two factors: (i) the length of time cells need to respond to an experimental treatment; and (ii) the length of time that cells can grow before nutrient depletion sets in. Nutrient depletion typically develops within 3-4 days after plating unless cultures are re-fed. Once it begins a progressive deterioration of cellular health and viability develops rapidly, and becomes a major artifact in data interpretation. Nutrient depletion can be calibrated by comparing the day-by-day growth kinetics of cultures that receive no feeding to cultures that are fed daily. The two curves begin to diverge when depletion sets in. This normally sets the upper limit to assay duration if cultures are not fed. With cytotoxic assays, 36-48 h assay period following a 1-day recovery period is usually adequate to detect the effect of a drug while avoiding the need to re-feed in mid experiment.

2.10.3 End-point used to quantitate drug effect

A variety of methods have been devised for measuring the viability or proliferation of cells *in vitro*. These can be subdivided into four groups:

1) **Reproductive assays** can be used to determine the number of cells in a culture that are capable of forming colonies *in vitro*. In these types of experiments, cells are plated at low densities and the number of colonies is scored after a growth period. These clonogenic assays are the most reliable methods for assessing viable cell number. These methods are very time-consuming and become impractical when many samples have to be analyzed.

2) **Permeability assays** involve staining damaged (leaky) cells with a dye and counting viable cells that exclude the dye. Counts can either be performed manually using a hemocytometer and stained with Trypan blue. This method is quick, inexpensive, and requires only a small fraction of total cells from a cell population. Therefore, this method is generally used to determine the cell concentration (cell

number/ml) in batch cell cultures. This is helpful in ensuring that cell cultures have reached the optimal level of growth and cell density before routine sub-culture, freezing, or any experiment. Or counts can be performed mechanically using for example a flow cytometer and propidium iodide. Alternatively, membrane integrity can be assayed by quantifying the release of substances from cells when membrane integrity is lost, *e. g.*, Lactate dehydrogenase (LDH) or ^{51}Cr

3) **Metabolic activity assays**: MTT reduction – a tetrazolium-based colorimetric assay for cell survival and proliferation In 1983, a quantitative colorimetric assay for mammalian cell survival and cell proliferation was proposed by Mosmann. The assay is dependent on the reduction of yellow-colored tetrazolium salt MTT (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide) by the mitochondrial dehydrogenase of viable cells to form insoluble formazan which precipitates out of solution in the immediate vicinity of the reaction (Fig. 2.10). The assay measure cell respiration and the amount of formazan product is proportional to the number of living cells present in culture and quantitated with an ELISA plate reader. The assay has been shown to be a simple, rapid alternative to counting cells by dye inclusion/exclusion, monitoring the release of ^{51}Cr from lysed cells, or incorporation of [3H]-thymidine into cellular DNA. The MTT assays have been used with a growing number of cell types including primary cultured cells as well as establish cell lines. This colorimetric microplate assay is cost effective because of the number of tests which can be performed at one time without the problem of radio-isotope and contaminated material disposal.

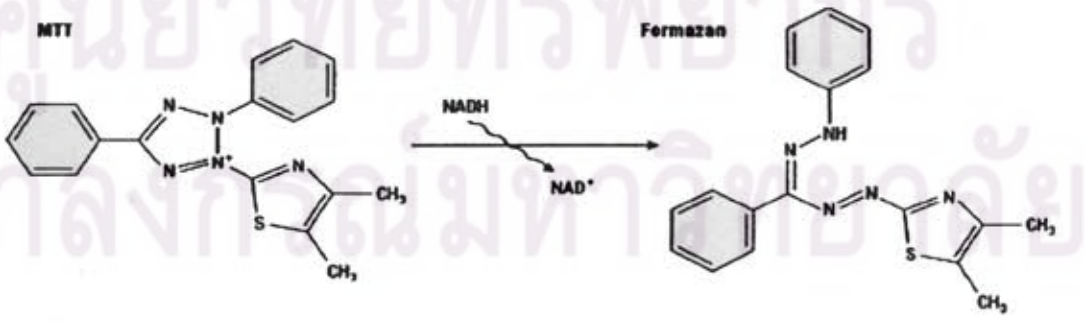


Figure 2.10 Molecular structure of MTT and its corresponding reaction products.

4) **Direct proliferation assays** use DNA synthesis as an indicator of cell growth. These assays are performed using either radioactive or nonradioactive nucleotide analogs. Their incorporation into DNA is then measured.

2.11 Human liver hepatoblastoma cells (Hep-G2)

The liver is known to be the main site of xenobiotic biotransformation due to the ability of this organ to express a plethora of enzymes, both quantitatively and qualitatively. Therefore, cell lines of liver origin are widely used in biomedical research involving xenobiotic metabolism including genotoxicity studies. Of these cell lines, the Hep-G2 cell line is the most versatile one. This cell line retains many of the specialized functions normally lost by primary hepatocytes in culture (Knowles *et al.*, 1980; Aden *et al.* 1979). Hep-G2 has a wide variety of liver-specific metabolic responses to different kind of drug, chemical substances or plant extract (Table 2.7).

Additionally, as reviewed by Knasmuller *et al.* (1998) , Hep-G2 cells express a wide range of phase I and II enzymes. Phase I enzymes such as cytochrome P450 (CYP) 1A1, 1A2, 2B, 2C, 3A and 2E1, arylhydrocarbon hydrolase, nitroreductase, *N*-demethylase, catalase, peroxidase, NAD(P)H:cytochrome *c* reductase, cytochrome P450 reductase and NAD(P)H: Quinone oxidoreductase. Phase II enzymes such as epoxide hydrolase, sulfotransferase, glutatione *S*-transferase (GST), uridine glucuronosyl tranferase and *N*-acetyl transferase.

Table 2.7 Effects of drugs, chemical substances and plant extracts on human liver hepatoblastoma cells (Hep-G2).

Chemicals	Activity	Concentrations	Incubation time	Results	Reference
Tamoxifen	Anti-breast cancer drugs	0.1 - 20 μM	48 h	• Tamoxifen altered cell cycle of transduced Hep-G2 cells, cells decreased G0/G1 cell numbers, diminished proliferation index. • Induced cell death mostly in cells overexpressing CYP3A4 • Without significant effect on cytotoxicity or proliferation of cells engineered to overexpress CYP2E1 or on empty vector transfected cells.	Holownia and Braszko, 2004
Ebselen [2-phenyl-1,2-cenzoisoseleazol-3- (H)-one]	Anti-inflammatory	1 - 50 μM	24 h	• Reduced the suppression of growth Hep-G2 cells caused by hydrogen peroxide. • Displayed a dose-dependent reduction of lactate dehydrogenase leakage and malondialdehyde formation in hydrogen peroxide-treated cells • Able to reduce the reactive oxygen species formation and DNA damaging effect caused by hydrogen peroxide in a dose-dependent manner.	Yang *et al.*, 1999

Table 2.7 Effects of drugs, chemical substances and plant extracts on human liver hepatoblastoma cells (Hep-G2) (continued).

Chemicals	Activity	Concentrations	Incubation time	Results	Refernces
Silymarin	Component of several comercially produced hepatoprotective remedies.	10 - 100 μM	48 h	• Concentration-dependent cytoprotection against the toxic effects of both allyl alcohol and carbon tetrachloride.	Dvorak *et al.*, 2003
Lipiodol (iodinated poppy seed oil)	Chemotherapeutic and radiotherapeutic agents to cancer patients.	2%	72 h	• Lipidol has a selective effect on the uptake of certain cytotoxic agents. • Lipidol -doxorubicin-targeted treatment of Hep-G2 cells may improve the intracellular uptake and hence cytotoxicity of of doxorubicin *in vivo*.	Towu *et al.*, 2004
Apigenin	Antioxidant, carbohydrate metabolism promoter, anti-platelet aggregation and anti-inflammatory agent.	25 and 50 μM	48 h	• Induced programme cell death in term of TNF-α, IFN-γ release and induction of caspases activity. • Induced caspase-3, -7, -10 and caspase-9 activity in a dose-dependent manner.	Khan and Sultana, 2006
Acacetin [5,7-dihydroxy-4'-methoxy-flavone]	Antiperoxidative, anti-inflammatory and antiplasmodial effects	1, 5, 10 and 20 μg/ml	72 h	• Inhibited the proliferation of Hep-G2 by inducting apoptosis and blocking cell cycle progression in the G1 phase. • Increased the expression of p53 and p21/WAF1 protein, contributing to cell cycle arrest.	Hsu *et al.*,2004

Table 2.7 Effects of drugs, chemical substances and plant extracts on human liver hepatoblastoma cells (Hep-G2) (continued).

Chemicals	Activity	Concentrations	Incubation time	Results	Refernces
P. peruvuana extracts	Anticancer, antimycobacterial, antileukemic, antipyretic, immunomodulatory and treating diseases.	10, 30 and 50 µg/ml	48 h	• Treatment with cabonyl cyanide *m*-chlorophenyl hydrazone shown to induce cell cycle arrest and apoptosis through mitochondrial dysfunction • The extract possesses potent antihepatoma activity and its effect on apoptosis is associated with mitochondrial dysfunction.	Wu *et al.*, 2004
Impila	Treat stomach complaints, cough, tapeworm infestations and impotence.	10 mg/ml	24 h	• Induced cytotoxicity in Hep-G2 cells *in vitro* involves depletion of cellular glutathione. • Preventing glutathione depletion by supplementing cells with *N*-acetylcysteine reduces cytotoxicity.	Popat *et al.*, 2002
Troglitazone	Antidiabetic agent			• Induced apoptotic cell death characterized by internucleosomal DNA fragmentation and nuclear condensation. • Troglitazone may be one of the factors of liver injury in human.	Yamamoto *et al.*, 2001

CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals and equipments

The chemicals and suppliers used in this study are listed as follows:

Dimethyl Sulfoxide (DMSO)	Merck, Germany
EDTA	Sigma, U.S.A.
95% Ethyl alcohol	Alcohol Refinery, Thailand
Ethidium bromide	Sigma, U.S.A.
Fetal Bovine Serum	Biochrome AG, U.S.A.
Glycine	Sigma, U.S.A.
MTT	Sigma, U.S.A.
Peurarin	Sigma, U.S.A.
Phenol red	Fisher Scientific, U.S.A.
Potassium chloride	Fisher Scientific, U.S.A.
Potassium di-hydrogen phosphate	Merck, Germany
RPMI-1640 medium	Biochrome AG, U.S.A.
Sodium bicarbonate	Sigma, U.S.A.
Sodium chloride	Fisher Scientific, U.S.A.
Sodium di-hydrogen phosphate	Merck, Germany
Trypan blue	The British Drug House, England
Trypsin	Sigma, U.S.A.

The equipments and suppliers used in this study are listed as follows:

Air pump	GAST, U.S.A.
Autoclave	Consolidated, U.S.A.

Automatic micropipettes P20, P200, and P1000	Gilson Medical Electrical S.A., France
Biohazard	Faster, U.S.A.
Haemacytometer	Bright-Line, U.S.A.
Hot-air oven	Contherm, U.S.A.
Inverted microscope	Olympus, U.S.A.
Microtiterplate 96 wells	Nunc, Denmark
Microtiterplate reader / MCC/340	Titertek multiskan®, U.S.A.
Pipette tips 20, 200 and 1000 µl	Gilson Medical Electrical S.A., France
Rotary evaporator	EYELA, Japan
Tissue culture flask (25 cm^2)	Nunc, Denmark
Water bath	EYELA, Japan
Water jacketed CO_2 incubator	Thermo Forma, Japan

3.2 The plant materials

3.2.1 Plant powder preparation (Cherdshewasart *et al.*,2004[a])

The tuberous roots of the wild *P. mirifica* were collected from 28 provinces of Thailand, including Chiang Rai, Chiang Mai, Mae Hong Sorn, Phayao, Nan, Lampang, Phrae, Lamphun, Uttharadith, Sukhothai, Tak, Phitsanulok, Phetchabun, Kamphaeng Phet, Nakorn Sawan, Uthai Thani, Sakon Nakorn, Nong Bua Lam phu, Chaiyaphum, Nakorn Ratchasima, Saraburi, Lop Buri, Kanchanaburi, Phrachin Buri, Ratchaburi, Phetchaburi, Prachuap Khiri Khan and Chumphon (Cherdshewasart *et al.*, 2006, in preparation ; Fig. 3.1).

No.	Provinces
1	Chiang Rai
2	Chiang Mai
3	Mae Hong Son
4	Phayoa
5	Nan
6	Lampang
7	Phrae
8	Lamphun
9	Uttharadith
10	Sukhothai
11	Tak
12	Phitsanulok
13	Phetchabun
14	Kamphaeng Phet
15	Nakhonsawan
16	Uthaithani
17	Sakon Nakhon
18	Nong Bua Lam Phu
19	Chaiyaphum
20	Nakorn Ratchasima
21	Saraburi
22	Lop Buri
23	Kanchanaburi
24	Phrachin Buri
25	Ratchaburi
26	Phetchaburi
27	Prachuapkirikhan
28	Chumphon

Figure 3.1 Sources of plant materials; *P. mirifica* in the experiments (Cherdshewasart *et al.*, 2006).

The tuberous roots of the wild *B. superba* were collected from 25 provinces of Thailand, including Chiang Rai, Chiang Mai, Mae Hong Sorn, Phayao, Nan, Lampang, Phrae, Uttharadith, Tak, Phitsanulok, Phetchabun, Kamphaeng Phet, Nakorn Sawan, Uthai Thani, Sakon Nakorn, Loei, Nong Bua Lam phu, Khon Kaen, Chaiyaphum, Nakorn Ratchasima, Srisaket, Kanchanaburi, Lop Buri, Saraburi, Ratchaburi, Phrachin Buri, Chachoengsao, Chonburi and Chantaburi (Cherdshewasart, unpublished ; Fig. 3.2)

The tuberous roots of the wild *M. collettii* were collected from 4 provinces of Thailand, including Chiang Rai, Chiang Mai, Lumpang, Kanchanaburi (Cherdshewasart, unpublished ; Fig. 3.3).

No.	Provinces
1	Chiang Rai
2	Chiang Mai
3	Mae Hong Son
4	Lampang
5	Uttharadith
6	Tak
7	Phitsanulok
8	Phetchabun
9	Nakhon Sawan
10	Sakon Nakhon
11	Loei
12	Nong Bua Lamphu
13	Khon Kaen
14	Chaiyaphum
15	Nakhonratchasima
16	Srisaket
17	Kanchanaburi
18	Lop Buri
19	Saraburi
20	Ratchaburi
21	Prachinburi
22	Chachoengsoa
23	Chonburi
24	Chantaburi

Figure 3.2 Sources of plant materials; *B. superba* in the experiments.

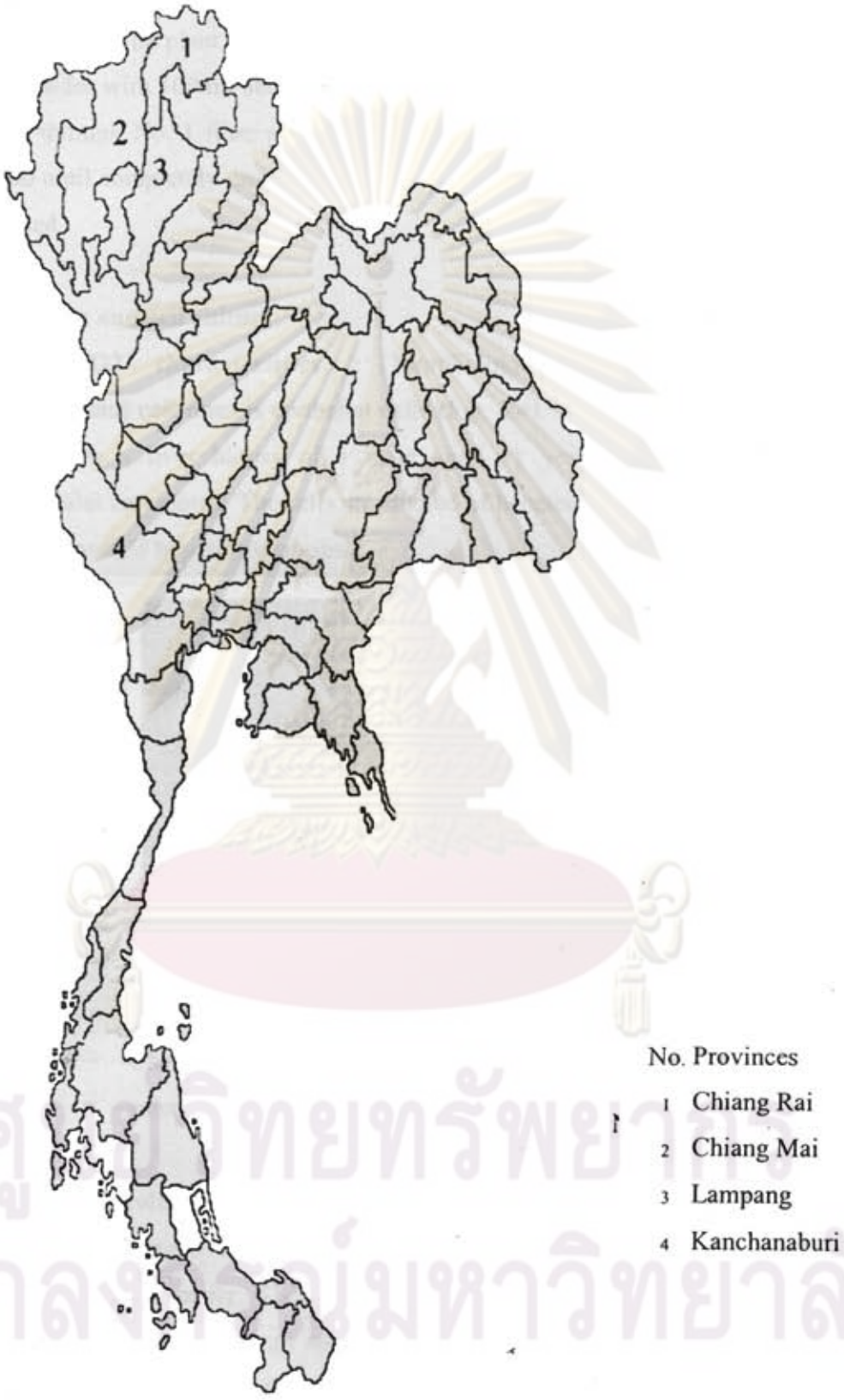


Figure 3.3 Sources of plant materials; *M. collettii* in the experiments.

3.2.2 Plant crude extraction (Cherdshewasart *et al.*, 2004[a])

The plant powder was extracted with 95% ethanol by incubating 10 g of plant powder with 100 ml absolute ethanol for 4 days in dark place. The supernatant was filtered through No. 1 filter paper and subsequently evaporated in the rotary evaporator N-1000 until completely dried. The crude extract was stored in light-protect bottle at 4°C until used.

3.3 Cells and cell culture

Hep-G2 characteristics : Morphological of Hep-G2 (Human liver hepatoblastoma) cell lines is epithelial cells (Fig. 3.4). The Hep G2 cell lines have been isolated from a liver biopsy of a male aged 15 years, with a well differentiated hepatocellular carcinoma. The cells are studied of hepatocyte fuction as an *in vitro* model reflecting possible hepatic metabolism.

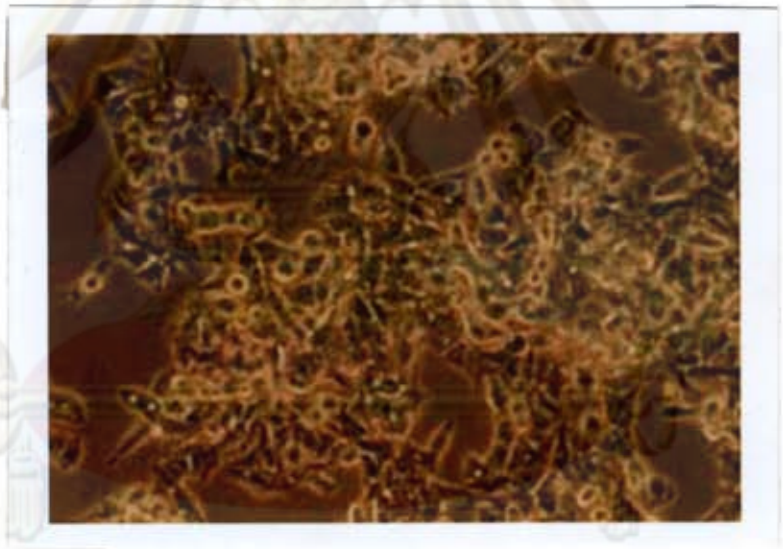


Figure 3.4 Characteristics of human liver hepatoblastoma cells under inverted microscope. (20 x)

Hep-G2 cells were obtained from the American Type Culture Collection; ATCC no. HB8065, storaged at The Institute of Biotechnology and Genetic Engineering, Chulalongkorn University. The cell line was cultured in basal medium consisted of RPMI-1640 medium with 2 g/l sodium bicarbonate. Serum supplemented medium was prepared by adding heat-inactivated fetal bovine serum (FBS) to basal medium. The cells

were routinely propagated in 5% (v/v) serum-supplemented medium at 37 °C in a humidified atmosphere of 5% CO_2 incubator. The cells were routinely subcultured once every 3-4 days to maintain the optimum conditions for the exponential growth.

3.3.1 Subculturing

Hep-G2 cells were subcultured twice a week. Remove RPMI-1640 medium from the flask by the aid of a 10 ml glass pipette. Exponentially growing cells were detached from the surface of the 25 cm^2 T-flask by trypsinization with 2 ml 0.05% Trypsin solution for 3-4 min or until the cells have rounded up from the surface at room temperature or 37 °C in incubator. Trypsinize beyond the time required to detach cells to this degree may damage the cells and reduce plating efficiency. The solution was removed. The cells were re-suspended in 5 ml serum-containing RPMI-1640 medium and dispense into the new culture flasks. The medium was added to the final volume of 10 ml and incubated the flask in the incubator.

3.3.2 Cell suspension preparation for assay

Hep-G2 cells were propagated 3 days prior to the experiment. After trypsinization, the cells were incubated at room temperature or 37 °C in incubator for 3-4 min or until the cells have rounded up from the surface. The solution was removed. The RPMI-1640 medium was added and aspirated gently with the aid of a pipette in order to dissociate into single cells.

3.3.3 Cell count and dilution

The 0.4% Trypan blue dye solution and haemacytometer were applied to determine the viable cell number. Trypan blue is a stain that will only enter across the membranes of non-viable cells. Make a 1:1 dilution of cell suspension with 0.4% Trypan blue and carefully re-suspended with a Pasture pipette. The haemacytometer and coverslip was thoroughly cleaned and wipe with 70% alcohol before use. Hemacytometer chamber was covered with coverslip. Sample was drawn into a Pasteur pipette after mixing thoroughly and placed the tip of the pipette at the junction between the counting chamber and the coverslip to aid capillary action. The cell suspension was drawn to fill the chamber with repeat for the other side of the chamber. The chamber was placed on the stage of the microscope with focus on the counting chamber. Non-viable cells became swollen, larger and dark blue. The viable cells, not stained with Trypan blue, were

counted in 4 of 1-mm square on the corner and on 1-mm square in the middle of hemacytometer (Fig. 3.5).

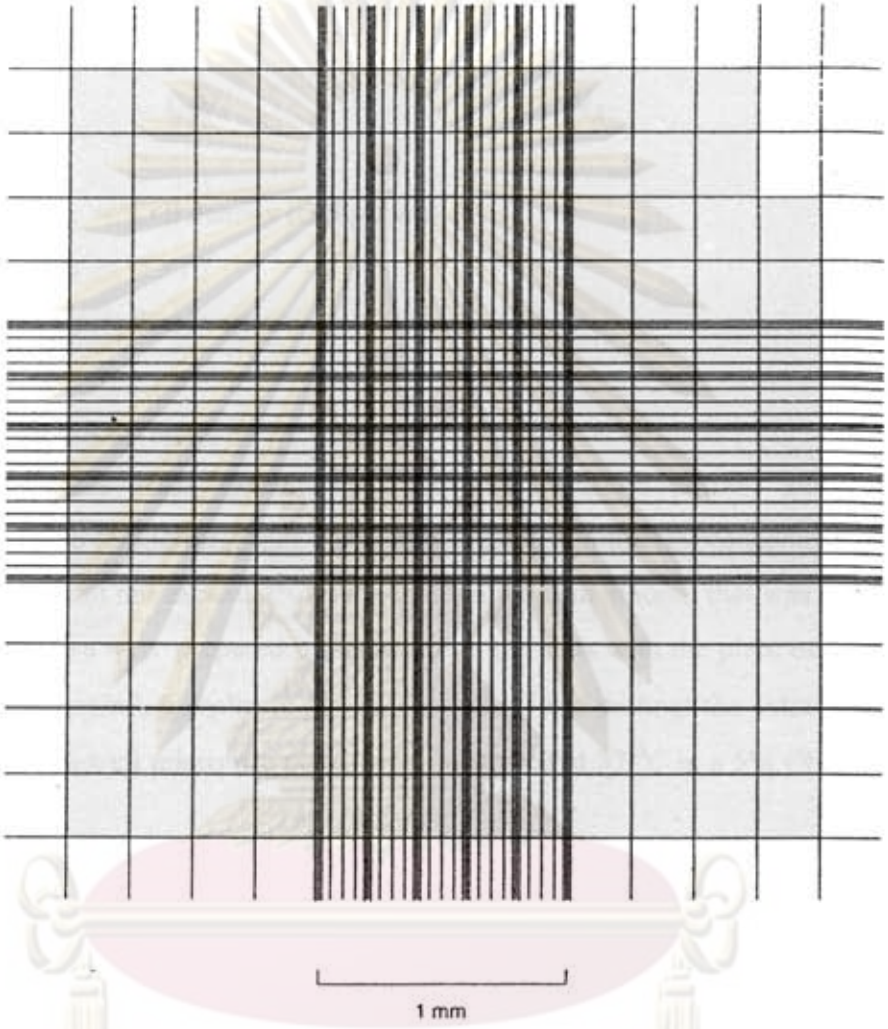


Figure 3.5 Magnified view of the cell counting chamber grid. The central 1-mm square area is divided into 25 smaller aquares, each 1/25 mm square. There are enclosed by triple ruled lines and are further subdivided into 16 squares, each 1/400 mm square.

The cells in each square of the hemocytometer was equivalent to approximately 1 mm, represent a total volume of 0.1 mm^3 and the subsequent cell density per ml was calculated using the following equation:

Cell density (cell per ml) = (total cell count/5) x 2 x10^4

Then, calculate for volume of cell suspension (V_1) (desired cell density = 5 x10^4) by using the following equation:

$$C_1V_1 = C_2V_2$$

C_1 = Cell density (cell per ml)

C_2 = 5 x10^4

V_2 = Final volume

3.4 Cytotoxicity test

The plant crude extract was dissolved in 100% DMSO and adjusted the concentration to 50 mg/ml. Stock solution was diluted to the test concentrations by 100% DMSO which did not exceed 2% of the total volume, an amount that was not toxic to the cells. The assays were prepared by incubationg the cells with the plant extracts (at 1, 10, 100 and 1000 µg/ml, 6 replicate per concentration) and without the extract as a negative control. The 96-well microtitre plates were incubated at 37 °C in a 5% CO_2 incubator for 3 days (72 h).

3.5 Cell proliferation assay

Cell proliferation assay was performed by the MTT (3-(4, 5– dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) colorimetric method (Carmichael et el., 1987 and Twentyman, 1987). After 72 h incubation period, MTT (5 mg/ml) was added at 10 µl/well and incubation was carried out for an additional 3 h at 37 °C in the dark. The solution was then discarded and 150 µl of DMSO and 25 µl of 0.1 M glycine buffer (pH 10.5) were added into each well to dissolve insoluble formazan crystal. Plates were then kept agitation for 5 minutes at room temperature for complete solubilization. The level of colored formazan derivative was analyzed on a microplate reader at a wavelength of 540 nm. The percentage of cell viability was calculated according to the following formula.

$$\text{The \% of cell viability} = \frac{\text{OD of treated cells x 100}}{\text{OD of control cells}}$$

The IC_{50} values were obtained by plotting the percentage of cell viability versus the concentrations.

CHAPTER IV

RESULTS

4.1 Characteristics of the crude plant extracts

The characteristics of the ethanolic crude extract of *P. lobata*, *P. mirifica*, *B. superba* and *M. collettii* are shown in Table 4.1.

Table 4.1 The characteristics of the plant extracts

Plants	Extract characteristic
P. lobata	Sticky light brownish wax-like material. Smell-like ground peanut.
P. mirifica	Sticky light brownish wax-like material. Smell-like ground peanut.
B. superba	Sticky red-brownish wax-like material Smell typical.
M. collettii	Sticky black wax-like crystal. Very strong typical smell.

4.2 Proliferative and anti-proliferative effect on Hep-G2 cells tested with *Pueraria mirifica.*

Extracts of 28 provinces collected *P. mirifica* were tested for *in vitro* cytotoxicity against Hep-G2 cells at the concentration of 1, 10, 100 and 1000 µg/ml. The plant extracts showed varied proliferative and anti-proliferative effect without dose-dependent, on Hep-G2 cells (Table 4.2 ; Fig 4.1).

Table 4.2 The growth response percentage of 28 provinces collected *P. mirifica* extracts compared with Mean of *P. mirifica* population, *P.* lobata extract and the DMSO control on Hep-G2 cell culture ($P < 0.05$).

Provinces	Concentration (µg/ml)			
	1	10	100	1000
Nan	$92.78 \pm 4.22^{++}$	$82.99 \pm 2.32^{**,a,++}$	$97.98 \pm 3.89^{++}$	$160.80 \pm 3.92^{*,b,+}$
Chumphon	$85.85 \pm 4.56^{**,a,++}$	$85.37 \pm 1.85^{++}$	$103.05 \pm 4.43^{++}$	$156.71 \pm 0.61^{*,b,+}$
Kamphaeng Phet	$90.41 \pm 2.01^{**,++}$	$91.55 \pm 4.27^{++}$	$86.99 \pm 3.96^{++}$	$154.23 \pm 13.56^{*,b,+}$
Prachuapkirikhan	$90.47 \pm 3.28^{**,++}$	$94.16 \pm 4.58^{++}$	$107.89 \pm 4.42^{++}$	$150.75 \pm 6.81^{*,b,+}$
Phetchaburi	$78.82 \pm 3.55^{**,a,++}$	$90.43 \pm 2.86^{++}$	112.87 ± 7.23	$143.11 \pm 12.68^{b,+}$
Chiang Rai	$96.32 \pm 2.67^{++}$	$99.51 \pm 6.12^{++}$	$129.53 \pm 7.45^{*,b}$	$140.18 \pm 8.89^{b,+}$
Lamphun	$89.44 \pm 3.49^{**,++}$	$91.83 \pm 5.50^{++}$	112.03 ± 6.11	$136.13 \pm 6.97^{b,+}$
Uthaitani	$94.19 \pm 2.45^{++}$	115.65 ± 3.27^{b}	$95.96 \pm 3.06^{++}$	$132.19 \pm 6.53^{b,+}$
Tak	$90.00 \pm 6.01^{**,++}$	106.95 ± 7.38	$94.76 \pm 3.55^{++}$	$122.36 \pm 9.30^{b,+}$
Nong Bua Lamphu	$110.19 \pm 3.86^{++}$	110.19 ± 2.68	$128.63 \pm 2.89^{*,b}$	$119.45 \pm 7.57^{b,+}$
Uttharadith	$105.94 \pm 1.66^{++}$	$101.08 \pm 5.87^{++}$	$106.17 \pm 7.73^{++}$	$117.21 \pm 23.95^{b,+}$
Nakorn Ratchasima	$117.50 \pm 2.56^{*,b}$	110.97 ± 3.29	$125.63 \pm 5.91^{*,b}$	110.80 ± 4.19
Sukhothai	$95.06 \pm 2.58^{++}$	106.64 ± 2.13	$120.14 \pm 4.06^{*,b}$	110.11 ± 4.37
Phayao	$100.00 \pm 3.53^{++}$	$104.51 \pm 4.62^{++}$	112.26 ± 4.35	109.32 ± 6.41
Saraburi	$96.84 \pm 3.24^{++}$	$101.54 \pm 0.98^{++}$	$108.33 \pm 2.76^{++}$	105.63 ± 5.33
Ratchaburi	$53.45 \pm 1.75^{**,a,++}$	$126.12 \pm 3.15^{*,b}$	$119.22 \pm 4.39^{*,b}$	105.62 ± 4.32
Sakon Nakorn	$125.80 \pm 2.83^{*,b}$	$129.30 \pm 10.66^{*,b}$	$119.51 \pm 2.54^{*,b}$	104.21 ± 5.41
Phrae	$102.16 \pm 2.15^{++}$	117.44 ± 3.41^{b}	$117.44 \pm 1.74^{*,b}$	101.77 ± 5.51
Mae Hong Son	$106.74 \pm 7.22^{++}$	$98.49 \pm 4.43^{++}$	$102.84 \pm 6.10^{++}$	102.75 ± 5.62
Chaiya Phum	$95.14 \pm 2.40^{++}$	$98.07 \pm 2.36^{++}$	$106.79 \pm 2.08^{++}$	99.54 ± 8.14
Lampang	$97.07 \pm 3.54^{++}$	$103.70 \pm 2.06^{++}$	$95.29 \pm 4.06^{++}$	99.46 ± 3.92
Kanchanaburi	$100.54 \pm 2.70^{++}$	$98.53 \pm 3.09^{++}$	$95.99 \pm 2.08^{++}$	94.45 ± 3.71
Prachinburi	$102.38 \pm 3.18^{++}$	$96.71 \pm 2.77^{++}$	$84.45 \pm 5.04^{**,++}$	93.54 ± 8.84
Chiang Mai	$85.96 \pm 2.39^{**,a,++}$	$92.05 \pm 2.03^{++}$	$102.47 \pm 3.40^{++}$	90.90 ± 5.79
Lop Buri	$95.14 \pm 2.94^{++}$	$90.12 \pm 2.39^{++}$	$104.86 \pm 3.86^{++}$	88.50 ± 4.72
Nakhonsawan	109.79 ± 2.97	117.26 ± 4.00^{b}	122.89 ± 7.31^{b}	81.91 ± 3.03
Phitsanulok	$107.52 \pm 2.28^{++}$	110.32 ± 7.06	$81.26 \pm 2.42^{**,a,++}$	$73.77 \pm 1.21^{**}$
Phetchabun	$79.96 \pm 2.17^{**,a,++}$	$87.28 \pm 1.65^{++}$	$104.98 \pm 1.97^{++}$	$70.19 \pm 2.37^{**,a}$
Mean ± S.E.M.	96.94 ± 1.21	101.75 ± 1.17	107.21 ± 1.29	113.18 ± 2.32
P. lobata	122.72 ± 5.13	120.40 ± 3.99	126.69 ± 8.10	88.14 ± 7.15
DMSO	100.00	100.00	100.00	100.00

* ; significant greater than Mean of *P. mirifica* population. ** ; significant less than Mean of *P. mirifica* population. b ; significant greater than the control. a ; significant less than the control. + ; significant greater than *P. lobata*. ++ ; significant less than *P. lobata*.

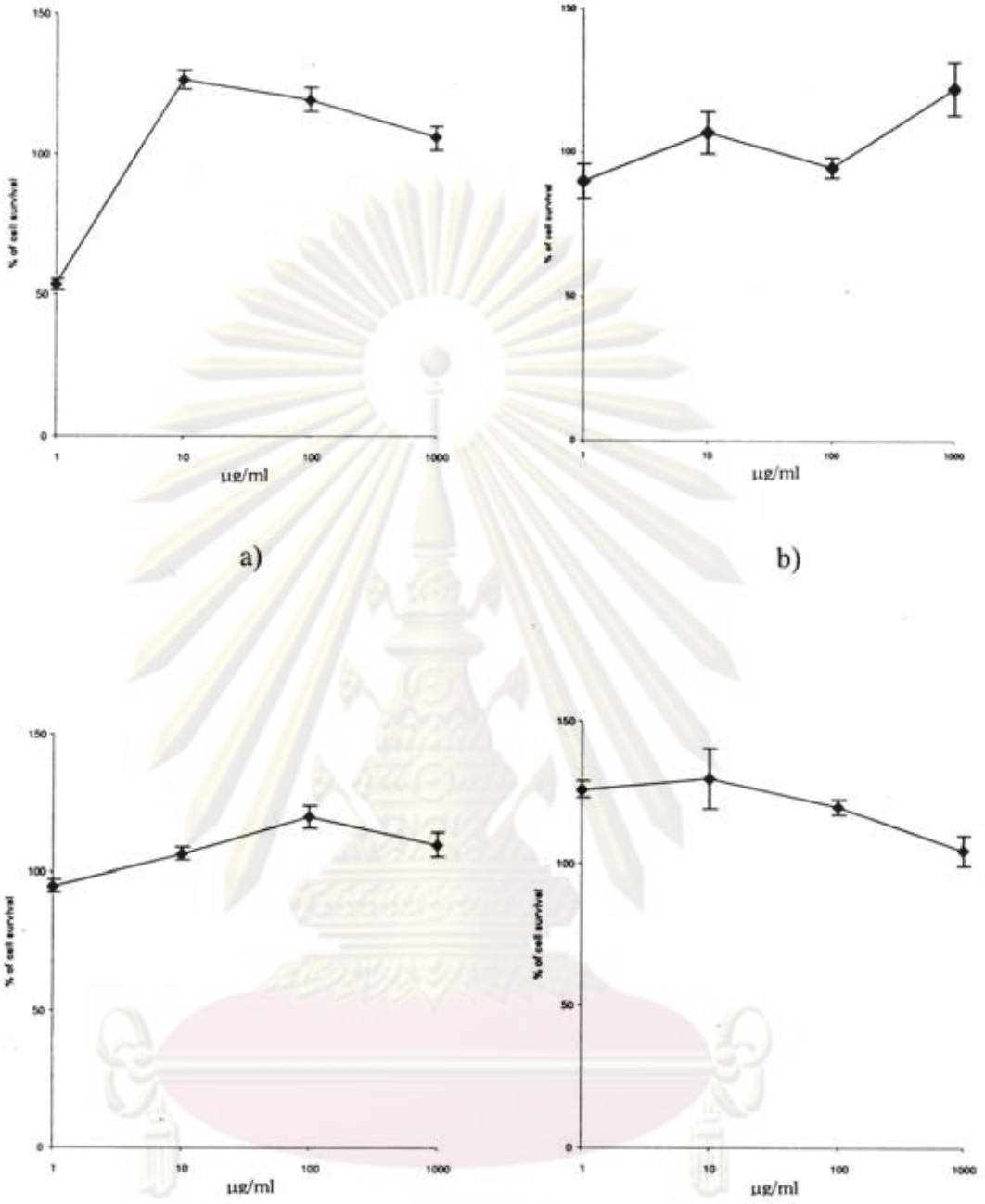


Figure 4.1 The growth response percentage of *P. mirifica* extracts collected from a) Ratchaburi b) Tak c) Sukhothai d) Sakon Nakorn provinces on Hep-G2 cells.

At the concentration of 1 μg/ml, there were 2 out of 28 plant samples (7.14% of *P. mirifica* population), including the plant samples collected from Sakon Nakorn (125.80% of cells viability) and Nakorn Ratchasima (117.50% of cells viability) province exhibited significant proliferative effect, $P < 0.05$, than the control (100% of cells viability) on Hep-G2 cells. There were 5 out of 28 plant samples (17.86% of *P. mirifica* population), including the plant samples collected from Ratchaburi (53.45% of cells viability), Phetchaburi (78.82% of cells viability), Phetchabun (79.96% of cells viability), Chumphon (85.85% of cells viability) and Chiang Mai (85.96% of cells viability) provinces exhibited significant anti-proliferative effect than the control (Table 4.2, Fig. 4.2).

At the concentration of 10 μg/ml, there were 5 out of 28 plant samples (17.86% of *P. mirifica* population), including the plant samples collected from Sakon Nakorn (129.30% of cells viability), Ratchaburi (126.12% of cells viability), Phrae (117.44% of cells viability), Nakhonsawan (117.26% of cells viability) and Uthaitani (115.65% of cells viability) provinces exhibited significant proliferative effect as compared with the control. There was 1 out of 28 plant samples (3.57% of *P. mirifica* population), collected from Nan province exhibited significant anti-proliferative effect (82.99% of cells viability) as compared with the control (Table 4.2, Fig. 4.3).

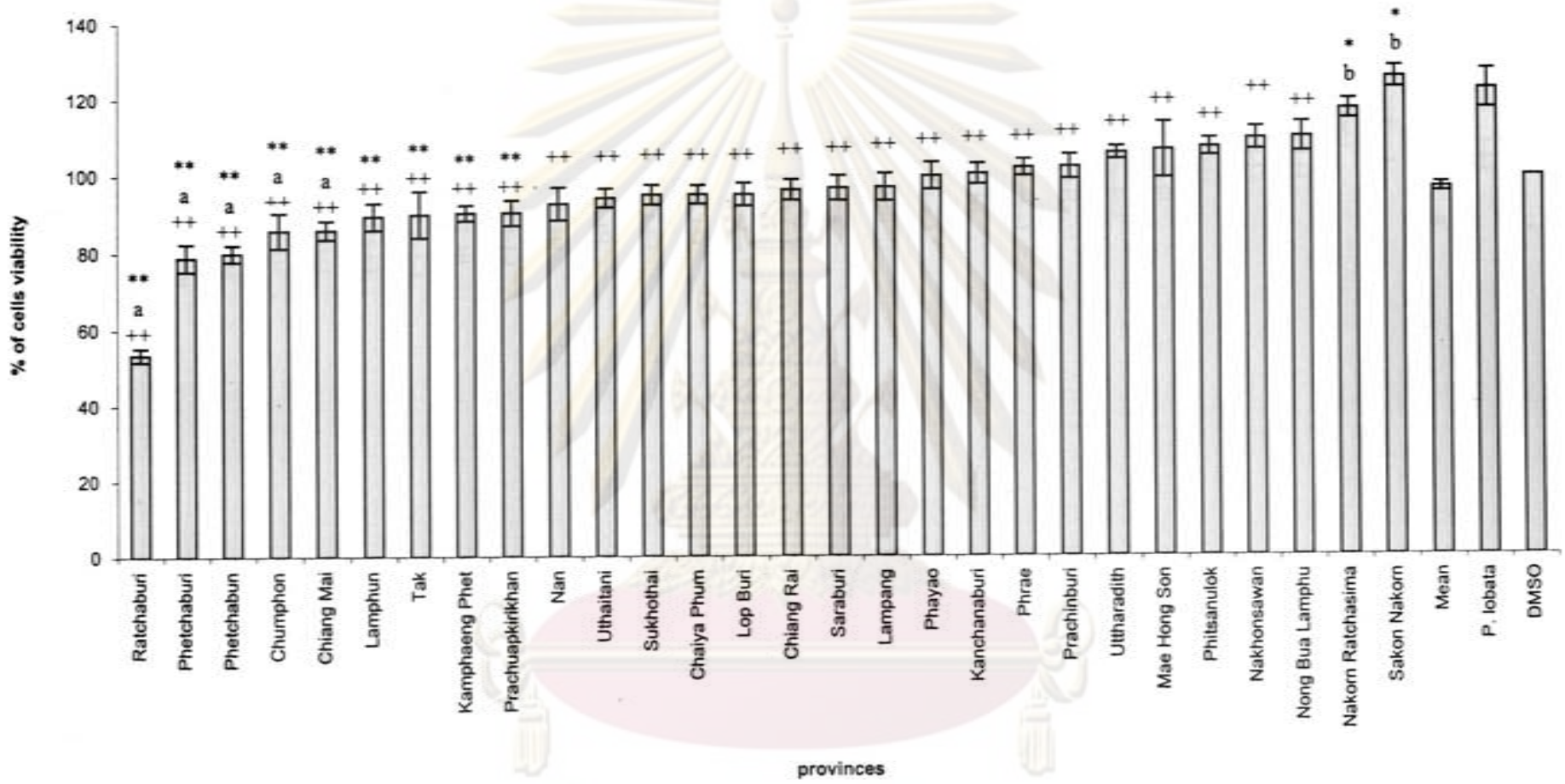


Figure 4.2 Proliferative and anti-proliferative effect of *P. mirifica* extracts, at the concentration of 1 µg/ml, significantly different with the Mean of *P. mirifica* population, *P. lobata* extract and the DMSO control ($P < 0.05$).

*; significant greater than Mean of *P. mirifica* population. ** ; significant less than Mean of *P. mirifica* population. b ; significant greater than DMSO. a ; significant less than DMSO. + ; significant greater than *P. lobata*. ++ ; significant less than *P. lobata*.

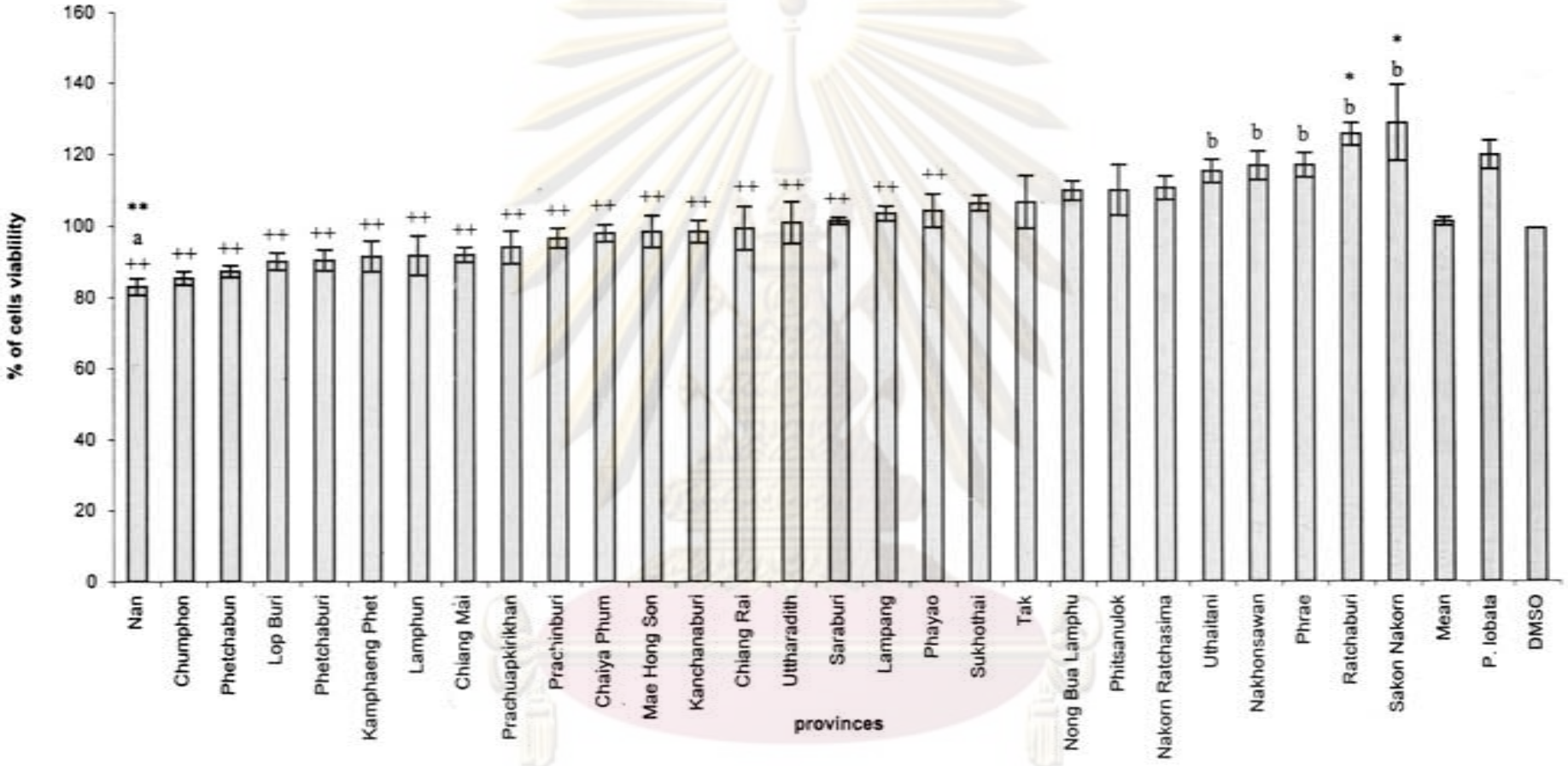


Figure 4.3 Proliferative and anti-proliferative effect of *P. mirifica* extracts, at the concentration of 10 μg/ml, significantly different with the Mean of *P. mirifica* population, *P. lobata* extract and the DMSO control ($P < 0.05$).

*; significant greater than Mean of *P. mirifica* population. ** ; significant less than Mean of *P. mirifica* population. b ; significant greater than DMSO. a ; significant less than DMSO. + ; significant greater than *P. lobata*. ++ ; significant less than *P. lobata*.

At the concentration of 100 μg/ml, there were 8 out of 28 plant samples (28.57% of *P. mirifica* population), including the plant samples collected from Chiang Rai (129.53% of cell viability), Nong Bua Lamphu (128.63% of cell viability), Nakorn Ratchasima (125.63% of cell viability), Nakhonsawan (122.89% of cell viability), Sukhothai (120.14% of cell viability), Sakon Nakorn (119.51% of cell viability), Ratchaburi (119.22% of cell viability) and Phrae (117.44% of cell viability) provinces exhibited significant proliferative effect as compared with the control. There was 1 out of 28 plant samples (3.57% of *P. mirifica* population), collected from Phitsanulok province exhibited significant anti-proliferative effect on Hep-G2 cells (81.26% of cell viability, Fig. 4.4).

At the concentration of 1000 μg/ml, there were 8 out of 28 plant samples (28.57% of *P. mirifica* population), including the plant samples collected from Nan (160.80% of cell viability), Chumphon (156.71% of cell viability), Kamphaeng Phet (154.23% of cell viability), Prachuapkiri Khan (150.75% of cell viability), Phetchaburi (143.11% of cell viability), Chiang Rai (140.18% of cell viability), Lamphun (136.13% of cell viability) and Uthaitani (132.19% of cell viability) provinces exhibited significant proliferative effects as compared with the control. There was 1 out of 28 plant samples (3.57% of *P. mirifica* population), collected from Phetchabun province exhibited significant anti-proliferative effect on Hep-G2 cells (70.19% of cell viability, Fig. 4.5).

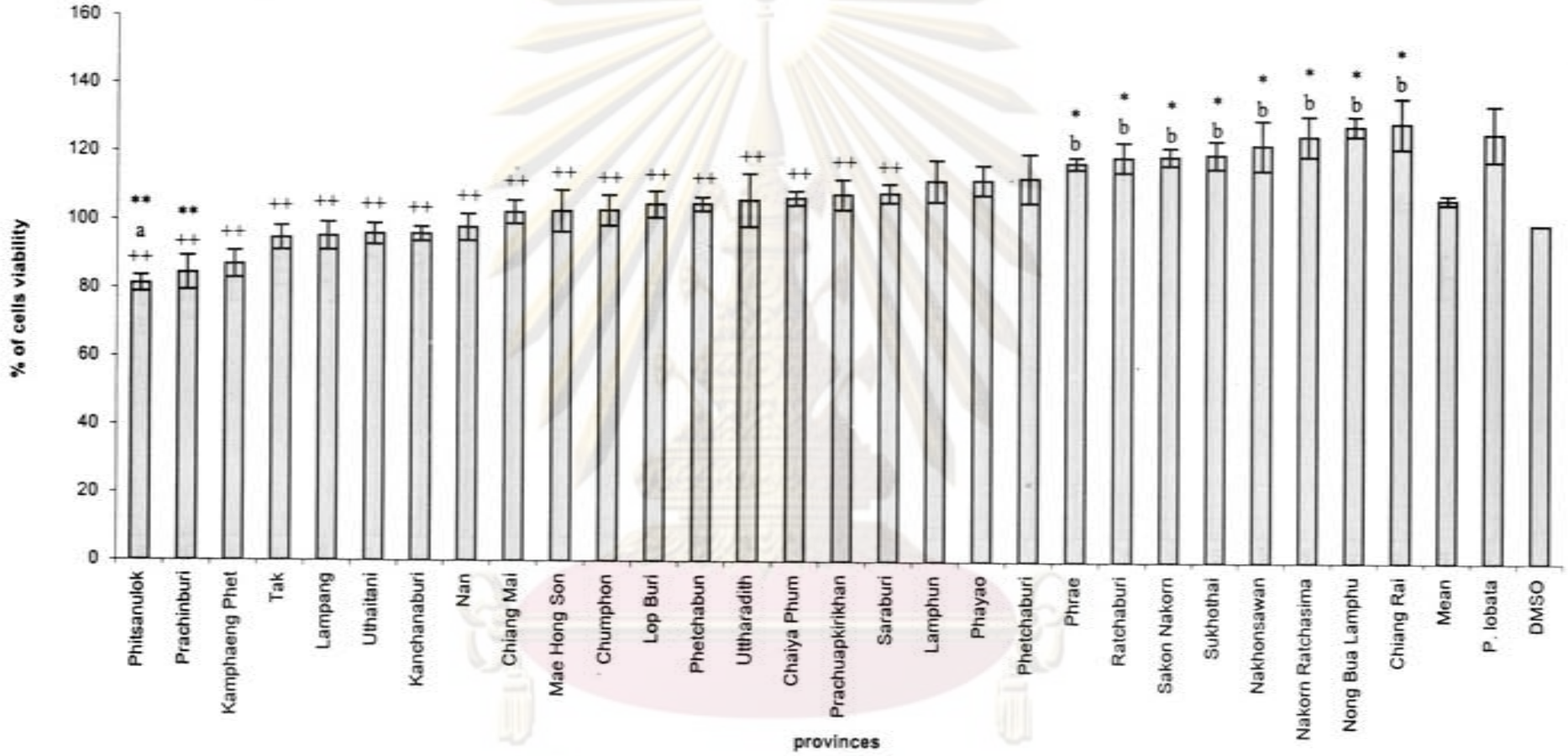


Figure 4.4 Proliferative and anti-proliferative effect of *P. mirifica* extracts, at the concentration of 100 µg/ml, significantly different with the Mean of *P. mirifica* population, *P. lobata* extract and the DMSO control ($P < 0.05$).

*; significant greater than Mean of *P. mirifica* population. ** ; significant less than Mean of *P. mirifica* population. b ; significant greater than DMSO. a ; significant less than DMSO. + ; significant greater than *P. lobata*. ++ ; significant less than *P. lobata*.

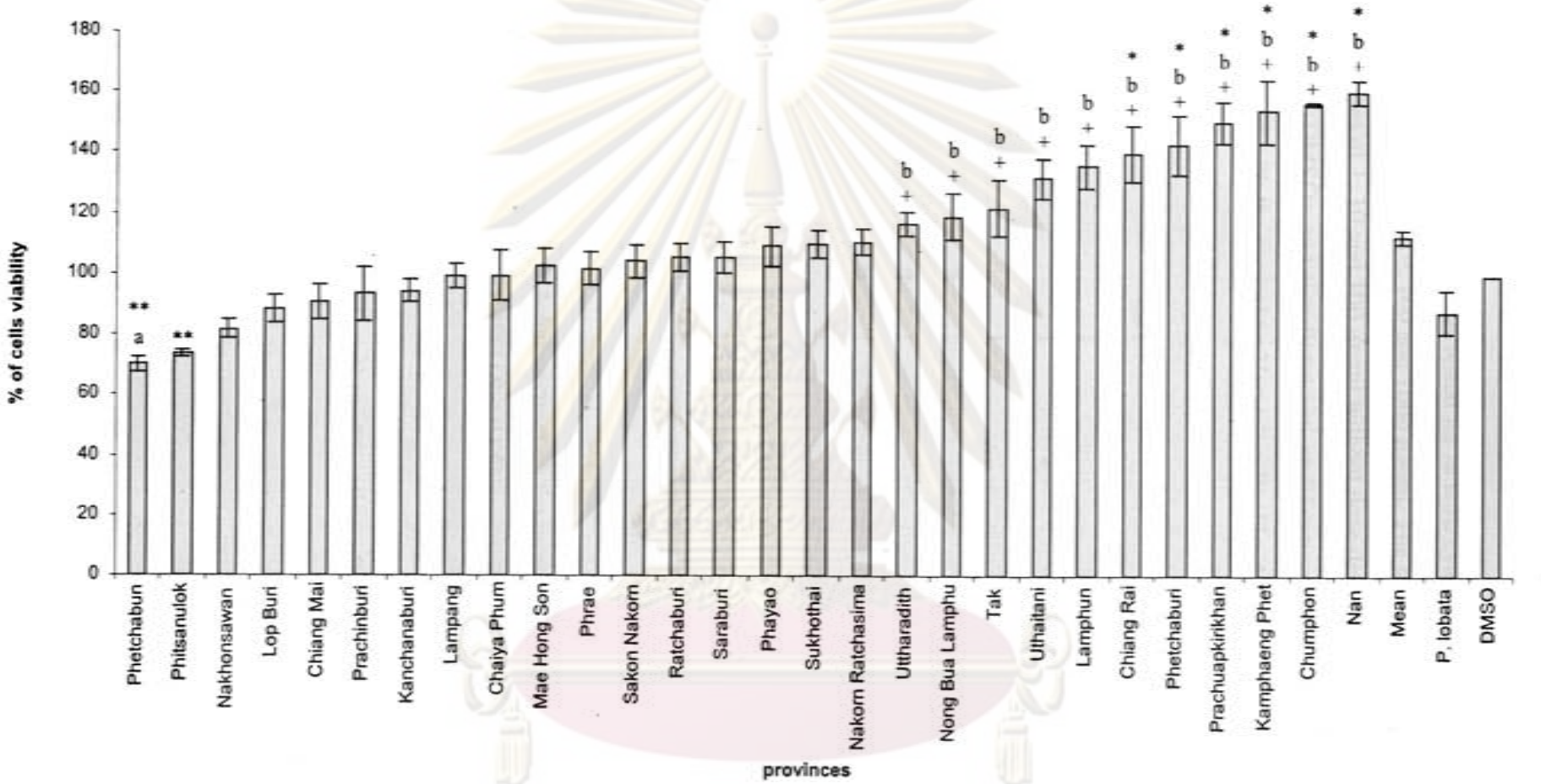


Figure 4.5 Proliferative and anti-proliferative effect of *P. mirifica* extracts, at the concentration of 1000 µg/ml, significantly different with the Mean of *P. mirifica* population, *P. lobata* extract and the DMSO control ($P < 0.05$).

* ; significant greater than Mean of *P. mirifica* population. ** ; significant less than Mean of *P. mirifica* population. b ; significant greater than DMSO. a ; significant less than DMSO. + ; significant greater than *P. lobata* extract. - ; significant less than *P. lobata* extract.

4.3 Proliferative and anti-proliferative effect on Hep-G2 cells tested with *P. lobata*.

P. lobata extract was tested for *in vitro* cytotoxicity against Hep-G2 cells at the concentration of 1, 10, 100 and 1000 μg/ml. The plant extracts showed proliferative at the doses of 1, 10 and 100 μg/ml without dose-dependent, but not significant than the control (DMSO), and significant anti-proliferative effect at the dose of 1000 μg/ml as compared with the control on Hep-G2 cells (Fig. 4.6).

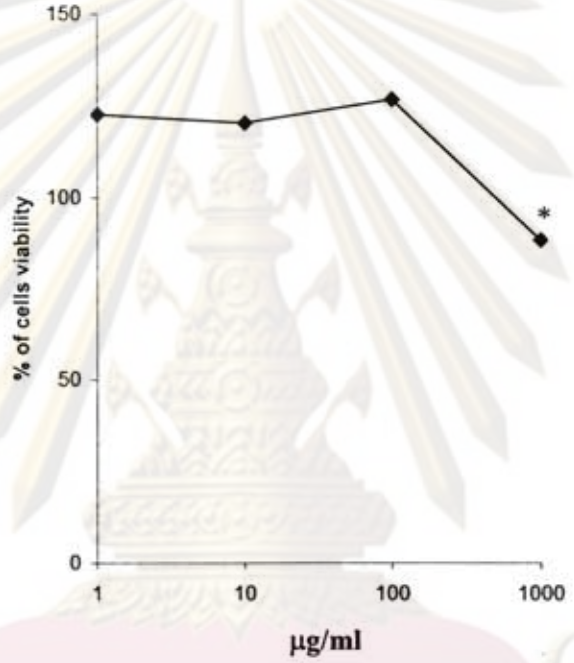


Figure 4.6 Effect of *P. lobata* extract on the growth of Hep-G2 cell culture.
* ; significant less than DMSO ($P < 0.05$).

4.4 Proliferative and anti-proliferative effect on Hep-G2 cells tested with puerarin.

Puerarin, one of the glycoside isoflavone was tested for *in vitro* cytotoxicity against Hep-G2 cells at the concentration of 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11} and 10^{-12} M. At the concentration 10^{-6} M, puerarin exhibited no proliferative effect (103.73% of cells viability). The percent of cells viability slowly decreased as the concentration of puerarin increased (97.76, 94.49, 87.80, 78.06, 77.36 and 72.57% of cells viability, respectively), with significant proliferation at the concentration of 10^{-10}, 10^{-11} and 10^{-12}

M as compared with the control. Therefore, puerarin showed dose-dependent cytotoxicity on Hep-G2 cells (Fig. 4.7).

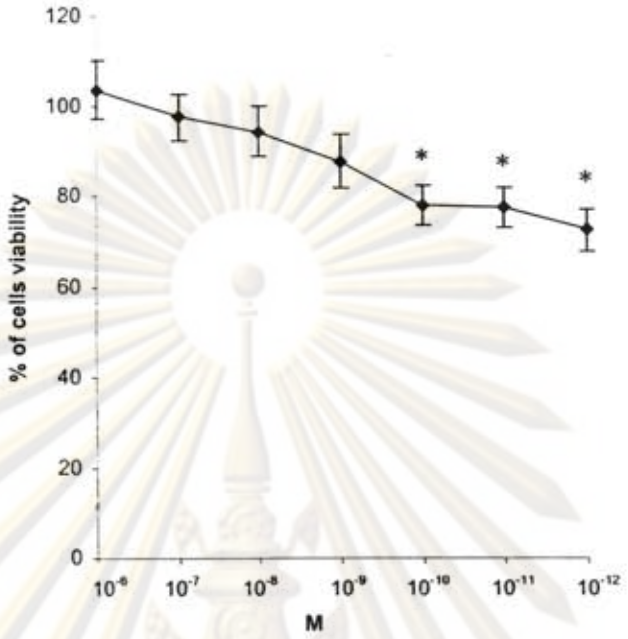


Figure 4.7 Effect of puerarin on the growth of Hep-G2 cell culture.
* ; significant less than DMSO ($P < 0.05$).

4.5 Proliferative and anti-proliferative effect on Hep-G2 cells tested with *B. superba*.

The wild *B. superba* collected from 24 provinces of Thailand showed varied proliferative and anti-proliferative effect without dose-dependent, on Hep-G2 cells (Table 4.3 ; Fig. 4.8).

Table 4.3 The growth response percentage of 24 provinces collected *B. superba* extracts compared with Mean of *B. superba* population and the DMSO control on Hep-G2 cell culture ($P < 0.05$).

Provinces	Concentration (µg/ml)			
	1	10	100	1000
Chiang Rai	82.77 ± 1.41^{a}	$69.82 \pm 3.05^{**,a}$	$112.84 \pm 3.23^{*}$	$402.02 \pm 7.47^{*,b}$
Loei	111.18 ± 3.20	84.27 ± 4.45	82.87 ± 2.75^{a}	$290.17 \pm 8.61^{*,b}$
Chonburi	101.08 ± 3.99	114.39 ± 4.92	84.96 ± 2.16^{a}	$262.83 \pm 10.42^{*,b}$
Tak	$70.51 \pm 4.76^{**,a}$	$68.35 \pm 4.21^{**,a}$	$109.06 \pm 4.35^{*}$	123.67 ± 1.86^{b}
Sakorn Nakorn	93.13 ± 2.81	91.63 ± 13.35	85.57 ± 2.81^{a}	122.80 ± 5.14^{b}
Nakorn Ratchasima	99.64 ± 2.82	104.71 ± 1.98	85.04 ± 1.66^{a}	121.01 ± 3.10^{b}
Mae Hong Sorn	109.29 ± 4.33	101.37 ± 3.95	71.99 ± 2.63^{a}	116.38 ± 2.53
Chaiya Phum	94.66 ± 2.88	106.19 ± 1.61	$110.82 \pm 1.28^{*}$	108.47 ± 7.37
Saraburi	92.73 ± 4.96	81.12 ± 3.85^{a}	84.39 ± 3.63^{a}	104.33 ± 3.04
Chantaburi	82.76 ± 4.31^{a}	75.00 ± 2.01^{a}	82.62 ± 2.50^{a}	101.65 ± 1.24
Phetchabun	103.57 ± 2.49	97.52 ± 3.13	91.36 ± 4.80	92.86 ± 5.34
Kanchanaburi	$130.61 \pm 5.08^{*,b}$	$135.65 \pm 6.33^{*}$	97.45 ± 4.11	90.76 ± 4.61
Nong Bua Lamphu	95.28 ± 4.39	93.32 ± 2.53	88.56 ± 4.10	88.51 ± 4.63
Lampang	87.38 ± 2.41	97.15 ± 2.77	101.00 ± 3.09	73.01 ± 3.69^{a}
Uttharadith	95.45 ± 2.05	105.72 ± 5.11	85.54 ± 2.09^{a}	70.69 ± 2.37^{a}
Lop Buri	83.95 ± 2.64	81.45 ± 1.72	$60.90 \pm 1.61^{**,a}$	67.68 ± 2.02^{a}
Pitsanulok	75.93 ± 4.92^{a}	85.72 ± 1.85	76.94 ± 3.10^{a}	65.76 ± 2.29^{a}
Chachoengsoa	87.39 ± 4.84	98.41 ± 4.70	91.29 ± 4.45	65.58 ± 1.49^{a}
Chiang Mai	108.48 ± 5.04	99.88 ± 6.34	78.38 ± 4.92^{a}	65.24 ± 2.62^{a}
Nakornsawan	94.15 ± 6.39	96.67 ± 3.58	95.69 ± 5.16	59.19 ± 1.26^{a}
Ratchaburi	104.69 ± 9.67	106.07 ± 9.36^{b}	83.92 ± 9.31^{a}	52.91 ± 17.62^{a}
Phrachinburi	107.40 ± 2.32	115.70 ± 5.05	$107.04 \pm 4.31^{*}$	52.13 ± 2.46^{a}
Srisaket	93.09 ± 3.15	94.55 ± 4.71	89.55 ± 6.11	49.55 ± 1.65^{a}
Khon Kaen	89.77 ± 5.19	88.03 ± 2.08	$67.44 \pm 2.50^{**,a}$	43.48 ± 1.10^{a}
Mean + S.E.M.	94.97 ± 1.32	95.86 ± 1.45	87.51 ± 1.19	108.35 ± 5.34
DMSO	100.00	100.00	100.00	100.00

* ; significant greater than Mean of *B. superba* population. ** ; significant less than Mean of *B. superba* population. b ; significant greater than the control. a ; significant less than the control.

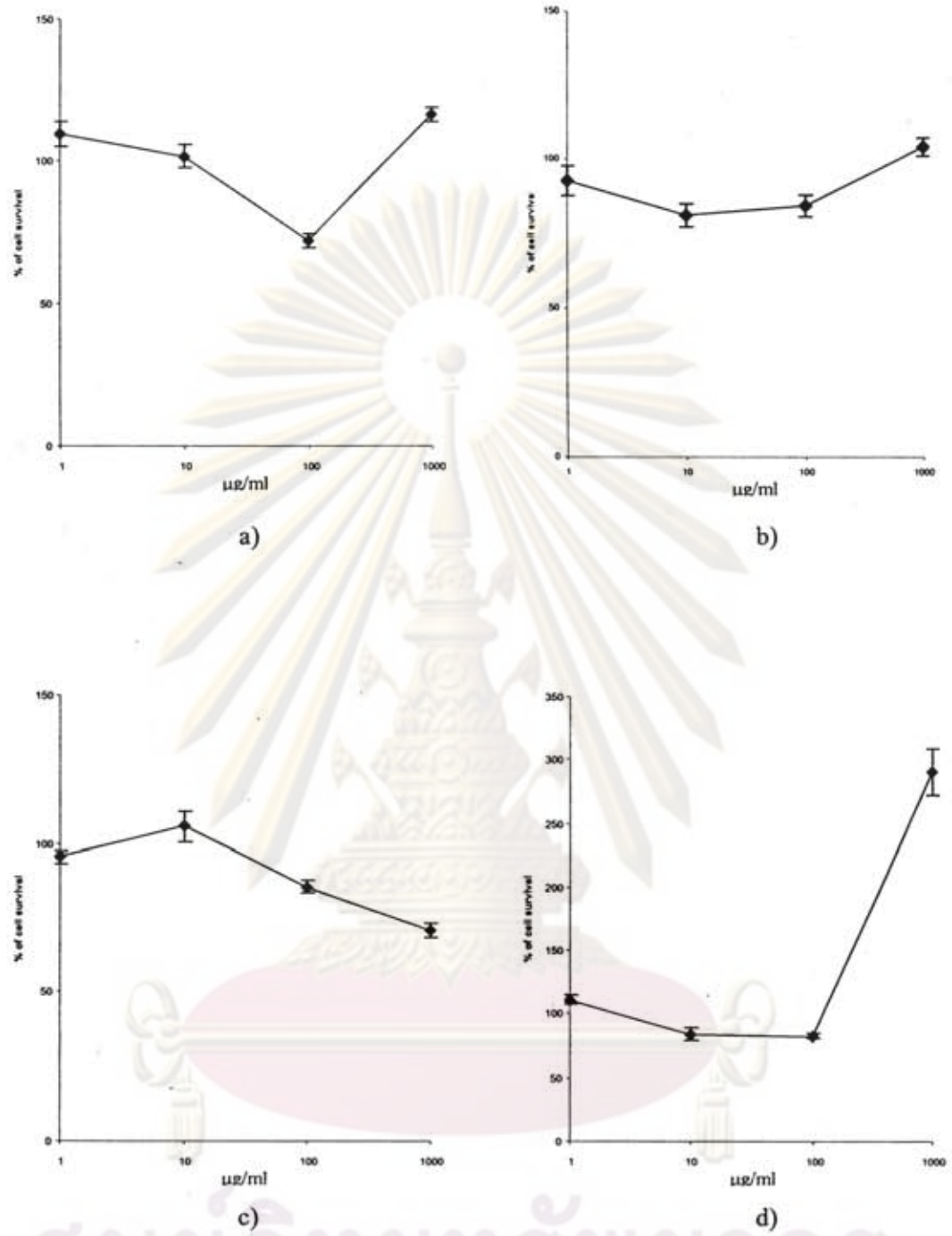


Figure 4.8 The growth response percentage of *B. superba* extracts collected from a) Mae Hong Son b) Saraburi c) Uttharadith d) Loei provinces on Hep-G2 cells.

At the concentration of 1 μg/ml, there was only 1 out of 24 plant sample (4.17% of *B. superba* population), collected from Kanchanaburi province exhibited significant proliferative effect (130.61% of cells viability) as compared with the control. There were 4 out of 24 plant samples (16.67% of *B. superba* population), including the plant samples collected from Tak (70.51% of cells viability), Pitsanulok (75.93% of cells viability), Chantaburi (82.76% of cells viability) and Chiang Rai (82.77% of cells viability) provinces exhibited significant anti-proliferative effect as compared with the control (Table 4.3, Fig. 4.9).

At the concentration of 10 μg/ml, there was only 1 out of 24 plant sample (4.17% of *B. superba* population), collected from Ratchaburi province exhibited significant proliferative effect (106.07% of cells viability) as compared with the control. There were 4 out of 24 plant samples (16.67% of *B. superba* population), including the plant samples collected from Tak (68.35% of cells viability), Chiagn Rai (69.82% of cells viability), Chantaburi (75.00% of cells viability) and Saraburi (81.12% of cells viability) provinces exhibited significant anti-proliferative effect as compared with the control (Table 4.3, Fig. 4.10).

At the concentration of 100 μg/ml, there was no plant sample exhibited significant proliferative effect as compared with the control. There were 13 out of 24 plant samples (51.17% of *B. superba* population) exhibited significant anti-proliferative effect as compared with the control, including the plant samples collected from Lop Buri (60.90% of cells viability), Khon Kaen (67.44% of cells viability), Mae Hong Son (71.99% of cells viability), Pitsanulok (76.94% of cells viability), Chiang Mai (78.38% of cells viability), Chantaburi (82.62%), Loei (82.87%), Ratchaburi (83.92%), Saraburi (84.39), Chonburi (84.96% of cells viability), Nakorn Ratchasima (85.04% of cells viability), Uttharadith (85.54% of cells viability) and Sakornnakorn (85.57% of cells viability) provinces (Table 4.3, Fig. 4.11).

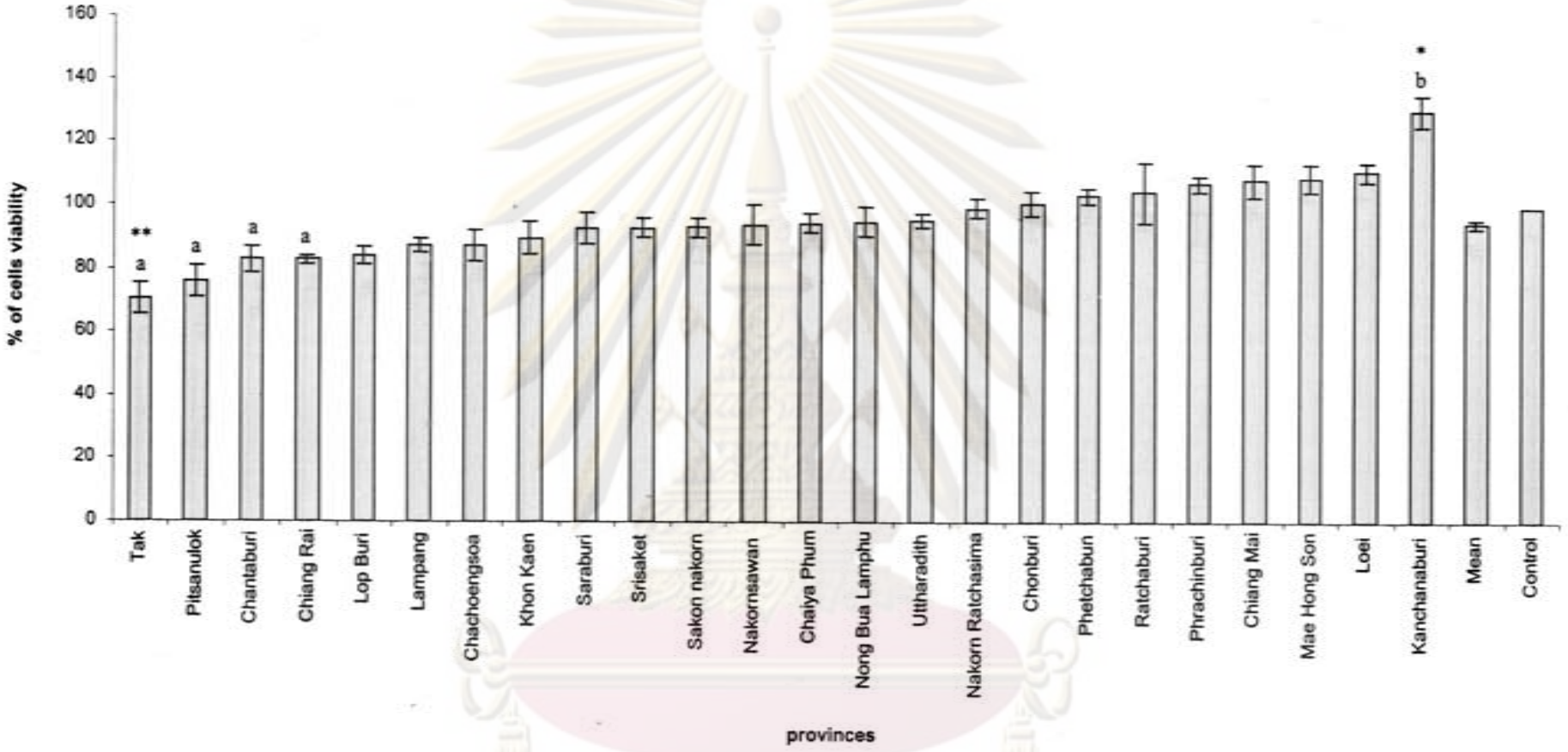


Figure 4.9 Proliferative and anti-proliferative effect of *B. superba* extracts, at the concentration of 1 μg/ml, significantly different with the Mean of *B. superba* population and the DMSO control ($P < 0.05$).

* ; significant greater than Mean of *B. superba* population. ** ; significant less than Mean of *B. superba* population.

b ; significant greater than the control. a ; significant less than the control.

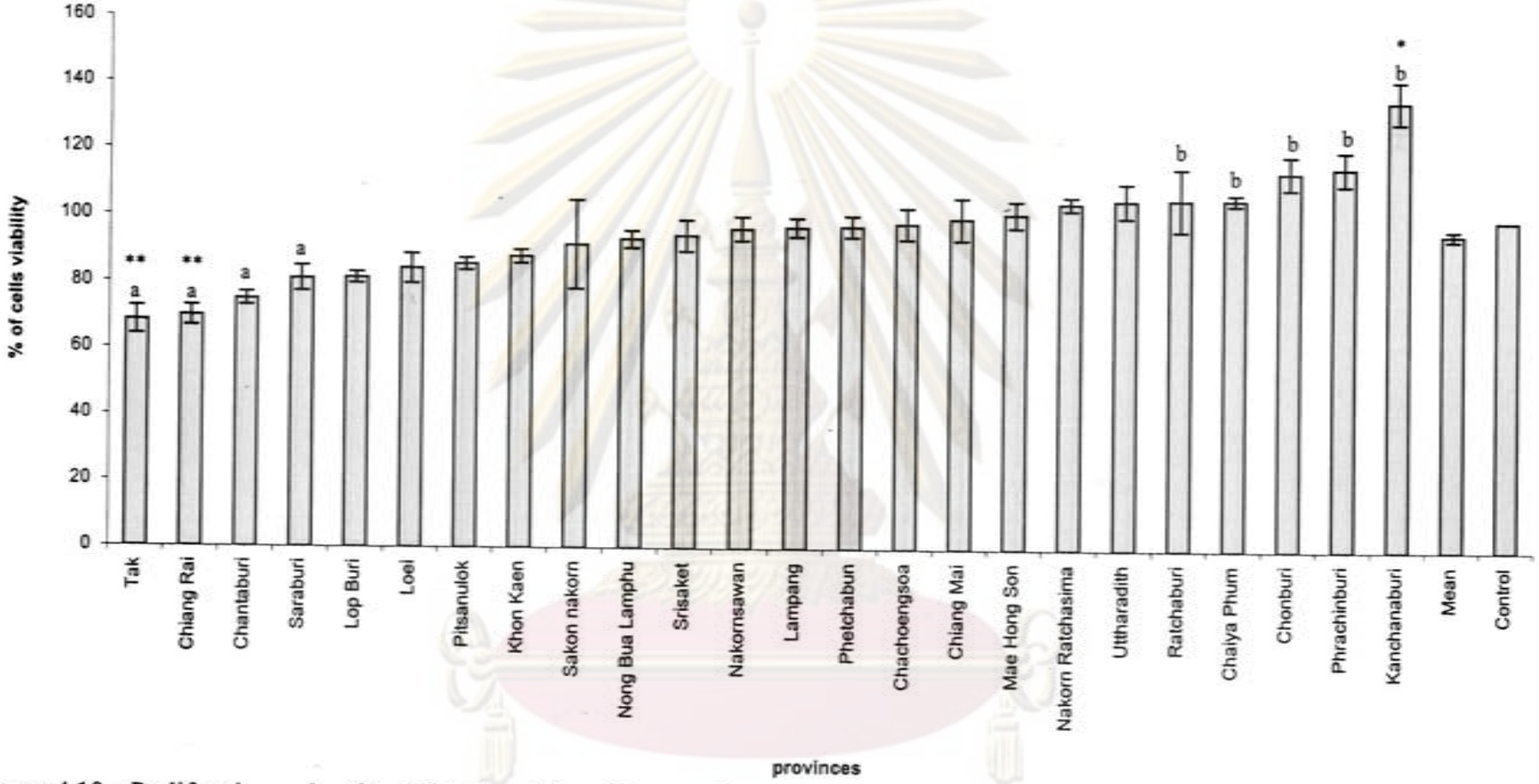


Figure 4.10 Proliferative and anti-proliferative effect of *B. superba* extracts, at the concentration of 10 µg/ml, significantly different with the Mean of *B. superba* population and the DMSO control ($P < 0.05$).

* ; significant greater than Mean of *B. superba* population. ** ; significant less than Mean of *B. superba* population.

b ; significant greater than the control. a ; significant less than the control.

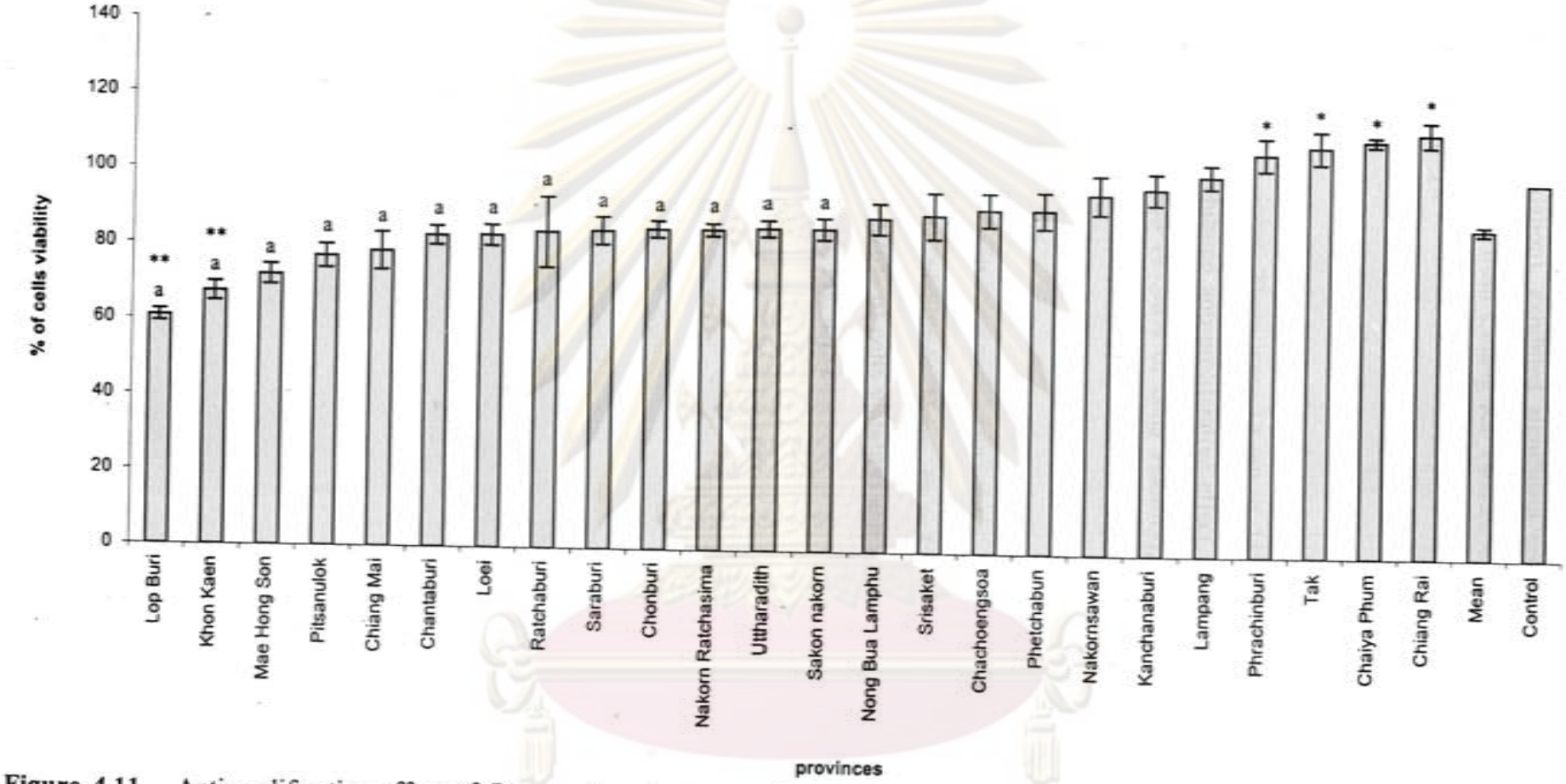


Figure 4.11 Anti-proliferative effect of *B. superba* extracts, at the concentration of 100 µg/ml, significantly different with the Mean of *B. superba* population and the DMSO control ($P < 0.05$).

* ; significant greater than Mean of *B. superba* population. ** ; significant less than Mean of *B. superba* population.

b ; significant greater than the control. a ; significant less than the control.

At the concentration of 1000 μg/ml, there were 10 plant samples (41.67% of *B. superba* population), exhibited significant proliferative effect, including the plant samples collected from Chiang Rai (402.02% of cells viability), Loei (290.17% of cells viability), Chonburi (262.83% of cells viability), Tak (123.67% of cells viability), Sakon Nakorn (122.80% of cells viability), Nakorn Ratchasima (121.01% of cells viability), Mae Hong Son (116.38% of cells viability), Chaiyaphum (108.47% of cells viability), Saraburi (104.33% of cells viability) and Chantaburi (101.65% of cells viability) provinces. There were 14 out of 24 plant samples (58.33% of *B. superba* population) exhibited anti-proliferative effect, including the plant samples collected from Khon Kaen (43.48% of cells viability), Srisaket (49.55% of cells viability), Phrachinburi (52.13% of cells viability), Ratchaburi (52.91% of cells viability), Nakornsawan (59.19% of cells viability), Chiang Mai (65.24% of cells viability), Chachoengsoa (65.58% of cells viability), Pitsanulok (65.76% of cells viability), Lop Buri (67.68% of cells viability), Uttharadith (70.69% of cells viability), Lampang (73.01% of cells viability), Nong Bua Lamphu (88.51% of cells viability), Kanchanaburi (90.76% of cells viability) and Phetchabun (92.86% of cells viability) provinces (Table 4.3, Fig. 4.12).

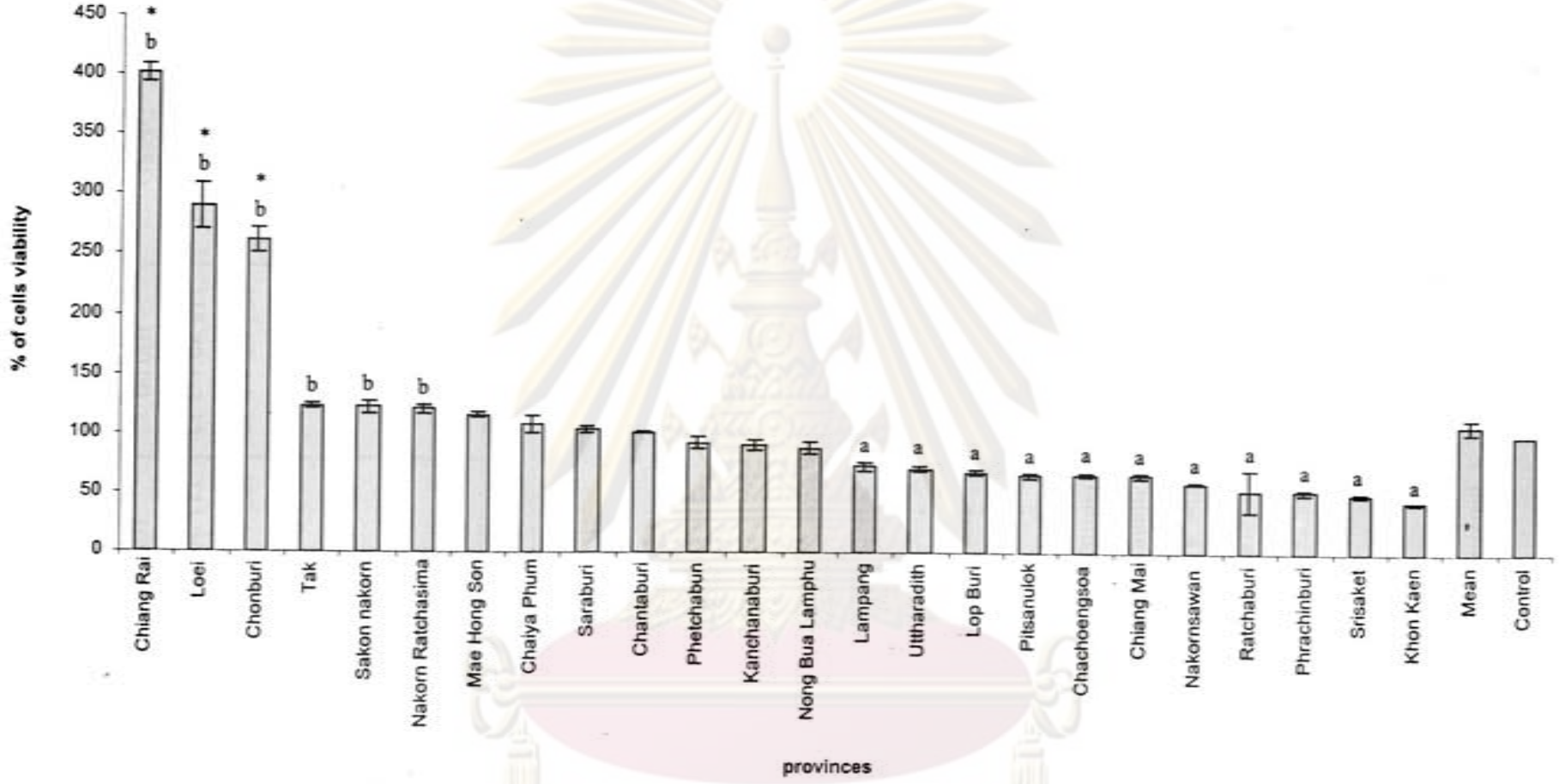


Figure 4.12 Proliferative and anti-proliferative effect of *B. superba* extracts, at the concentration of 1000 µg/ml, significantly different with the Mean of *B. superba* population and the DMSO control ($P<0.05$).

* ; significant greater than Mean of *B. superba* population. ** ; significant less than Mean of *B. superba* population.

b ; significant greater than the control. a ; significant less than the control.

4.6 Proliferative and anti-proliferative effect on Hep-G2 cells tested with *M. collettii*.

M. collettii collected from 4 provinces in Thailand showed variation of proliferative and anti-proliferative effect on Hep-G2 cells (Table 4.4). At the concentration of 1 μg/ml the plant extract collected from Kanchanaburi (122.78% of cells viability) and Chiang Rai (124.18% % of cells viability) provinces exhibited significant proliferative effect as compared with the control (Fig. 4.13). At the concentration of 10 μg/ml, there was no significant difference between the tested plant samples as compared with the control (Fig. 4.14). At the concentration of 100 μg/ml the plant sample collected from Chiang Rai province exhibited significant proliferative effect (134.64% of cells viability) and the plant sample collected from Chiang Mai province exhibited significant anti-proliferative effect (78.54% of cells viability) as compared with the control (Fig. 4.15). At the concentration of 1000 μg/ml, the plant sample collected from Kanchanaburi (383.10 % of cells viability) and Chiang Rai (178.83% % of cells viability) provinces exhibited significantly proliferative effect and the plant sample collected from Chiang Mai province exhibited significant anti-proliferative effect (60.26% of cells viability) as compared with the control (Fig. 4.16).

Table 4.4 The growth response percentage of *M. collettii* extract on Hep-G2 cells culture compare with the DMSO control ($P < 0.05$).

Provinces	Concentration (μg/ml)			
	1	10	100	1000
Kanchanaburi	122.78 ± 2.02^{b}	125.68 ± 3.03	111.33 ± 5.41	$383.10 \pm 12.44^{*,b}$
Chiang Rai	110.56 ± 2.49	105.87 ± 1.53	134.64 ± 3.92^{b}	178.83 ± 10.46^{b}
Lampang	124.18 ± 7.59^{b}	107.76 ± 10.11	118.22 ± 11.39	80.59 ± 1.81
Chiang Mai	106.05 ± 9.38	105.46 ± 11.08	$78.54 \pm 3.80^{**,a}$	$60.26 \pm 3.01^{**,a}$
Mean ± S.E.M.	115.89 ± 3.33	111.20 ± 3.99	110.68 ± 5.32	175.69 ± 26.94
DMSO	100.00	100.00	100.00	100.00

* ; significant greater than Mean of *M. collettii* population. ** ; significant less than Mean of *M. collettii* population. b ; significant greater than the control. a ; significant less than the DMSO.

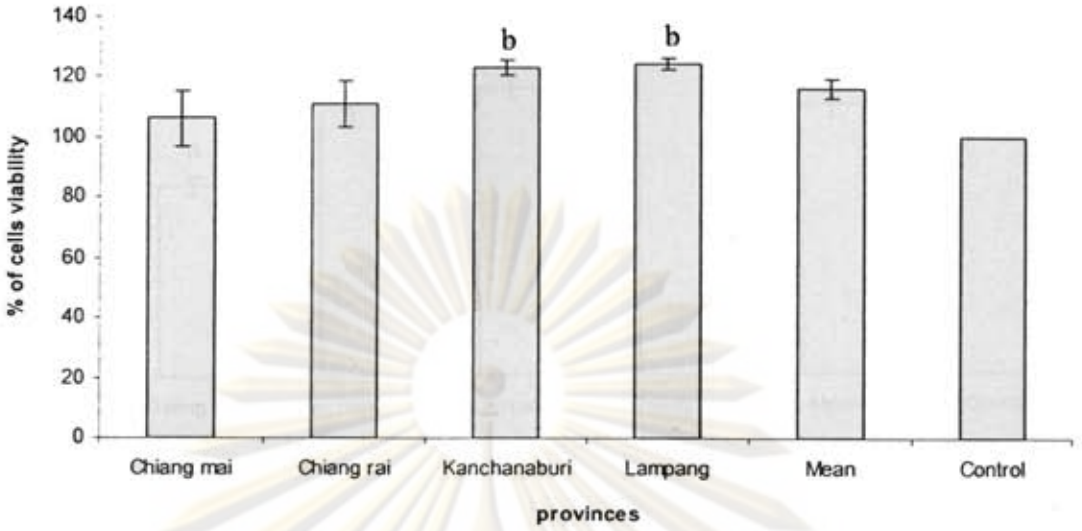


Figure 4.11 Proliferative and anti-proliferative effect of *M. collettii* extracts on Hep-G2 cells, at the concentration of 1 μg/ml.

b ; significant greater than the DMSO(P<0.05).

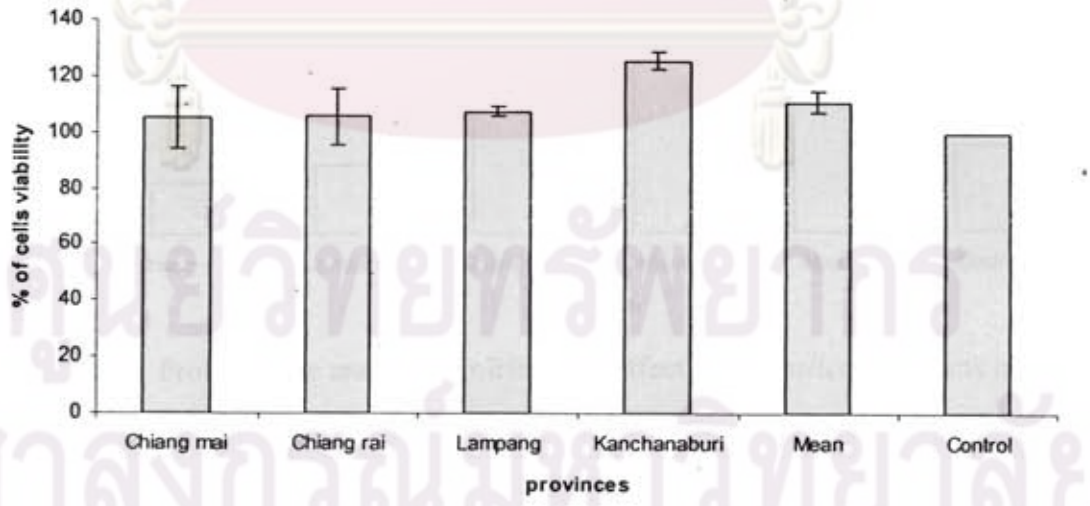


Figure 4.12 Proliferative and anti-proliferative effect of *M. collettii* extracts on Hep-G2 cells, at the concentration of 10 μg/ml.

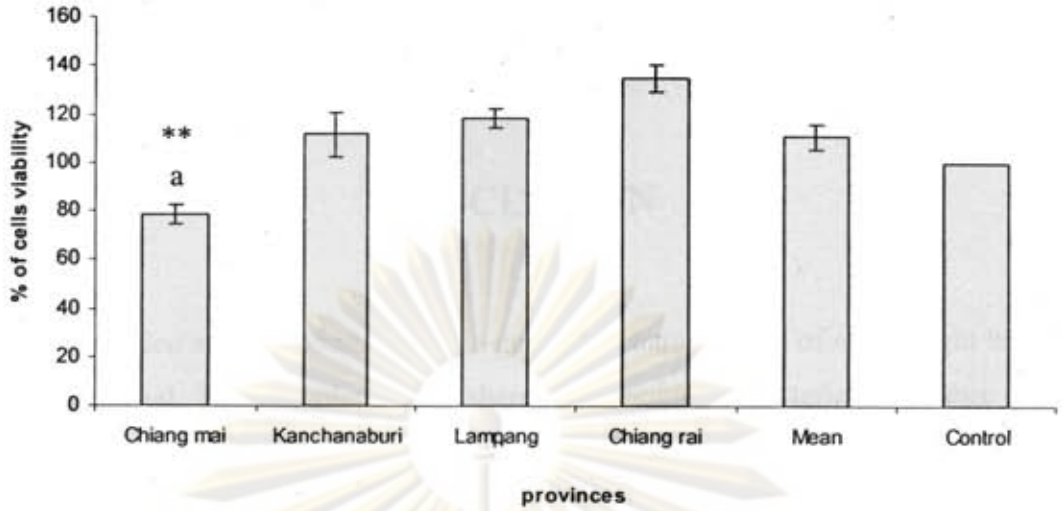


Figure 4.13 Proliferative and anti-proliferative effect of *M. collettii* extracts on Hep-G2 cells, at the concentration of 100 μg/ml (P<0.05).

** ; significant less than the Mean of *M. collettii* population.

b ; significant greater than the DMSO.

a ; significant less than the DMSO.

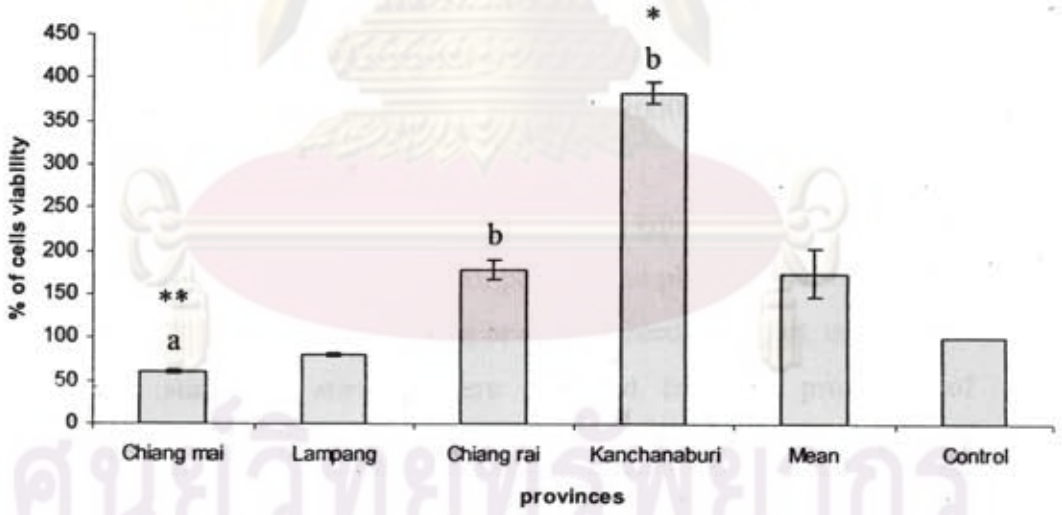


Figure 4.14 Proliferative and anti-proliferative effect of *M. collettii* extracts on Hep-G2 cells, at the concentration of 1000 μg/ml (P<0.05).

* ; significant greater than the Mean of *M. collettii* population.

** ; significant less than the Mean of *M. collettii* population.

b ; significant greater than the DMSO.

a ; significant less than the DMSO.

CHAPTER V

DISCUSSION

P. mirifica and *P. lobata* shared a common characteristic of sticky light brownish wax-like material. The two plants also shared a common characteristic of tuber forming with white meat. There were 2 main shapes of *P. lobata* tuber that are round and oval while *P. mirifica* exhibits various tuber shape (Cherdshewasart, unpublished). This may be the reason why the two crude extracts look very similar. Even though puerarin is the major constituent of *P. lobata* (Guerra *et al.*, 2000). and *P. mirifica* (Cherdshewasart et al., 2006[a]) tuberous roots, the bioactivity has to be compared between the 2 plants. *B. superba* extract is red-brownish color with typical smell. The fresh tuber releases red sap once damage or cut and turns into dark brownish color once dried. Under ethanolic extraction, the dark brownish color is still remained. The chemical contents are different from *P. mirifica* and *P. lobata*, that are flavonoid, flavonoid glycoside, 3-hydroxy-9-methoxypterocarpan (Medicarpin), 7-hydroxy-4'-methoxy-isoflavone (Formononetin), 7,4'-dimethoxyisoflavone, 5,4'-dihydroxy-7-metoxyisoflavone (Prunetin) and 7-hydroxy-6,4'-dimethoxyisoflavone (Roengsamran *et al.*, 2000, Ngamrojvanish *et al.*, 2006, inpreparation)

M. collettii extract is black color with strong typical smell. Normally only the live stem and root harbour black bark. But all parts of the plant, including root, stem, flower, pod and seed will turn black after cutting or dried (Cherdshewasart, unpublished).

In this study, *P. mirifica* were collected from 28 provinces of Thailand (Cherdshewasart *et al.*, 2006[a], in preparation). In fact these plant population may remain in another provinces and they are awaiting for us to be explored. Our effort has been limited by research found which lead us to lesser capability to obtain more plant samples. Any how, the collected plants samples are widely distributed in every part of Thailand (Fig 4.1). More important, the forests in every part of Thailand are under rapid invasion by farmers and land developers and results in a rapid diminish of the native plant habitats. Therefore, some of the exist plant samples in this study may not exist at the present.

From the proliferative and anti-proliferative assay with Hep-G2 cell, there are 11 and 5 of *P. mirifica* population exhibited more proliferative and anti-proliferative,

respectively than the control. The plant sample collected from Nan province at the concentration of 1000 μg/ml and Uthaitani province at the concentration of 10 μg/ml exhibits highest and lowest proliferative effect, respectively. The plant sample collected from Ratchaburi province at the concentration of 1 μg/ml and Chiang Mai at the concentration of 1 μg/ml exhibits highest and lowest antiproliferative effect, respectively. The results demonstrate that the plant population exhibits diversity in terms of bioactivity, especially cytotoxicity in this study. The test of the same group of plant samples also exhibits a varied degree of estrogenic effect by using the test of DPPH (Sutijit, 2003), MCF-7 (Trisap, 2003), rat uterotrophic and cornification (Cherdshewasart, 2006[b]) and Ames test (Pulcharoen, 2006, inpreparation). The plant bioassays are submitted to correlation analysis with isoflavone content (Cherdshewasart, 2006[a]). It is found that genistein content is most likely related with antioxidant activity and estrogenic activity using the test of MCF-7 but not tested by uterotrophic, mutagenic/antimutagenic and cytotoxicity assays.

Most important, both Hep-G2 and MCF-7 cell contains ERα. The response of the plant extracts to MCF-7 and Hep-G2 cells is found not to be correlated.

P. lobata extract shows higher proliferative effect on Hep-G2 cells than these of the mean of *P. mirifica* populations at the concentration of 1, 10 and 100 μg/ml but less than these of the mean of *P. mirifica* populations at the concentration of 1000 μg/ml. (Table 4.2). Though *P. lobata* contain high amount of isoflavone but there is no any report for the presence of miroestrol and deoxymiroestrol detected. However, the difference in the active phytochemicals component may influence on this effect. Furthermore, there are 2 reports confirm that *P. lobata* exhibits no uterotrophic effect on the tests with ovariectomized rats and biphasic response to MCf-7 cells (Cherdshewasart *et al.*, 2004).

Puerarin is a glycoside isoflavone. This chemical shows low estrogenic effect on the test using MCF-7 cells (Chansakaow *et al.*, 2000). But in this study using Hep-G2 instead, we found that puerarin does not show proliferative effect but dose-dependent anti-proliferative effect was observed. Therefore, it can be conclude that puerarin does not influence on proliferative effect.

B. superba extracts showed varied degree of proliferative and anti-proliferative effects (Table 4.3). Since there is a great difference between the plant sample with highest and lowest proliferative/antiproliferative Chaing Rai (1000 μg/ml) / Chantaburi (1000

μg/ml) and Khon Kaen (1000 μg/ml) / Phetchabun (1000 μg/ml), respectively. Though there is no established data on active chemical analysis of the plant populations, clear variation of active chemicals within the plant populations was observed. The variation in leaf morphology and color of the bud is noticed in the plant populations (Cherdshewasart, unpublished), attempts are being made in the RAPD analysis (Chanchao, unpublished). Noticeably, that only one sample, from Lampang province is analysed with MCF-7 cells growth, only anti-proliferation effect has been found at medium and high dose (Cherdshewasart et al., 2004). Statistic analysis of MCF-7 test of the plant population (Trisap, 2004) with Hep-G2 test correlation is not found between the 2 tests. Active chemicals are isolated from the plant tubers, including flavonoid and flavonoid glycoside (Roengsamran *et al.*, 2000), 3-hydroxy-9-methoxypterocarpan (Medicarpin), 7-hydroxy-4'-methoxy-isoflavone (Formononetin), 7,4'-dimethoxyisoflavone, 5,4'-dihydroxy-7-metoxyisoflavone (Prunetin) and 7-hydroxy-6,4'-dimethoxyisoflavone (Ngamrojvanich *et al.*, 2006, inpreparation). No data of cytotoxicity of first two components has been reported. However, the second chemical group show cytotoxicity in the test with BC and KB cancer cell (Ngamrojvinich *et al.*, 2006, inpreparation). Nevertheless, no pure chemical isolatedfrom all plants in this study has been tested. Therefore, we can not conclude that there are any relationship between chemical component and activity tested or not.

M. collettii collected from Kanchanaburi province shows strong proliferative effect on Hep-G2 from the dose of 1, 10 and 1000 μg/ml. At the highest dose, it shows 383.10% of cell growth as compare with the control. The plant sample from Chiang Rai province shows proliferative effect in lower degree than the sample collected from Kanchanaburi. It is noticed that the same plant sample was tested with MCF-7 and HeLa cells and resulted in high degree of cytotoxicity (Cherdshewasart 2004[a], 2004[b]). The plant sample collected from Lampang province showed proliferative effect only at low dose. Wheras, the plant sample collected from Chiang Mai showed strong anti-proliferatve effect at the dose of 100 and 1000 μg/ml. Thus, the only one extract of *M. collettii* showed cytotoxic to Hep-G2 cells. Even there are only 4 collected samples available for the test but the results shows a great variation of the bioactivity, especially on cytotoxicity. At least 3 chemicals were found in the plant sample collected from Chiang Rai province and all there chemicals were proved to be cytotoxic to many cancer cell culture (Petsom *et al.*, 2006, in preparation).

Among all the test plant samples, *B.superba* population shows the strongest proliferative and anti-proliferative to Hep-G2 cells There should be at least 2 awareness from this study. Firstly the anti-proliferative may refer to cytotoxic. Consumption of plant products with cytotoxic to Hep-G2 cells may induce human hepatic injury. Secondly, consumption of plant products with proliferative effect to Hep-G2 may .promote the malignancy of the tumor cells. Very interestingly, proliferative effect on Hep-G2 itself may refer to rejuvenating effect of the plant extracts. Therefore, it is possible that this activity can inducible function on injured hepatic issue which will lead to normal hepatocyte. This knowledge will confirm the reason why Thai people in the past comsumed Kwao Krua plants, especially *P. mirifica*, based on rejuvenating effect (Suntara, 1931). Also the results from experiment will be the scientific based information to explain and support these belief.

Compare to the previous study using MCF-7 cells for cytotoxicity test (Trisap, 2003) and Hep-G2 cells in this study, no correlation of cytotoxicity effect from *P. lobata*, *P. mirifica*, *B. superba* and *M. collettii* had been observed. The difference of results may be caused from the fact that MCF-7 cells contain ERα without metabolism activation function. On the contrasy, Hep-G2 cells contain ERα with metabolism activation function and promptly function after activation. Thus, the difference of the results may highly be influenced from the drug metabolizing enzymes in the hepatocytes.

Perspectives

The study has shown diversity of bioactivity in term of proliferative and antiproliferative effect to Hep-G2 cells. Some plant samples are interesting for development into products for special purposes, eg., treatment of cancer cells by plant sample containing strong cytotoxicity or manufacture into de-aging or rejuvenating products by plants containing strong proliferative effects. More detail studies are still required for the Kwao Krua plants. When there are demands to manufacture the plant materials into commercialized products, more and more data is still needed to ensure for the efficacy and safety of such products.

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APPENDICES

RPMI-1640 medium

RPMI-1640 powder medium	10.43 g
$NaHCO_3$	2 g
Sterile water	1 L

Weight and mix all ingredients in sterile water. Filtrate with 0.22 μm nitrocellulose membrane. Dispense the filtrate into bottles. All bottled mediums are stored in 4 °C until use.

0.05% Trypan ble dye (in HEPES-Buffer Saline)

HEPES-buffer saline

NaCl	8 g
KCl	0.4 g
Na_2HPO_4	0.1 g
Dextrose	1 g
HEPES	2.38 g
Distilled water	1 L

All ingredients were mixed in 1 L volumetric flask and stirred with magnetic stirre until all ingredients were completely disslved. Then 0.05 g of trypsin powder was added. The solution was stirred until trypsin was completely dissolved. The adjust pH to 7.0 (by add 7.5% $NaHCO_3$ and/or 1% HCl). The solution was filtrated (through 0.22 μm nitrocellulose membrane) and dispensed into bottles. The bottles trypsin was stored in 37 °C incubator for 24 hr. for sterillity test.

Trypan blue dye

Trypan blue	1.6 g
NaCl	3.24 g
KH_2PO_4	0.24 g

Distilled water	400 ml

All ingredients were mixed altogether, heat and stirred with magnetic stirre until completely dissolved. Adjust pH to 7.2-7.3 (by add 7.5% $NaHCO_3$ and/or 1% HCl). Then dispensed into light protecting bottles.

Phosphate buffer solution

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.2 g
Distilled water	1 L

All ingredients were mixed and dispensed into bottles. All bottles were autoclaved for 15 minute.

MTT solution

MTT: 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide	0.5 mg
0.89% Normal saline	1 ml

Add MTT 0.5 mg into 1 ml of 0.89% normal saline. All ingredients were mixed and sterilized by filter. Then dispensed into light protecting bottles and freshly prepared for every experiment.

BIOGRAPHY

MissWanrawee Sangkapong was born on January 20th, 1982 in Bangkok, Thailand. She was graduated with Bachelor degree of Science, King's Monkut of Institute of Ladkrabang in 2002. She has enrolled in the Graduate school, Chulalongkorn University for Master Degree of Science in Biotechnology during 2003-2006.