รายงานวิจัยฉบับสมบูรณ์

การศึกษาพิษต่อเซลล์เนื้องอกและผลต่อระบบภูมิคุ้มกัน ของสารสกัดจากดอกดินแดง (*Aeginetia indica* Roxb.)

Studies of Cytotoxicity of Tumor Cells and Immunotoxicological Effects of Dok Din Daeng (*Aeginetia indica* Roxb.)

โดย

ผศ. ดร. เบญจมาศ จิตรสมบูรณ์ และ นางสาววิมลณัฐ อัตโชติ

30 กันยายน 2543 ถึง 31 มกราคม 2546

สัญญาเลขที่ BGJ/32/2543

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Abstract

Project code: BGJ/32/2543

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Aeginetia indica Roxb., a parasitic plant which grows on bamboo, has been used in Thai traditional medicine for treatment of diabetes and dermal swelling. However, there are no reports of pharmacological studies and immunological effects of *Aeginetia indica* Roxb.(AIR) in Thailand. The objectives of this study are to explore antitumor activities and the immunological effects of *Aeginetia indica* Roxb. in Thailand. Crude extracts from different parts of the plants, seeds and whole plants, were investigated in the studies by using butanol and ethanol extraction.

Data from *in vitro* toxicological studies of seed and whole plant extracts to normal splenocytes of C57BL6/j mice demonstrated the low toxicity as no toxic effect was found from concentrations ranging from 1.95 to 2,000 μg/ml in the seed extracts, and from 1.25 to 1,000 μg/ml in the whole plant extracts. In addition, at concentration that did not induce toxicity in normal cells, plant extract from AIR has direct cytotoxicity towards tumor cells as evidence of the inhibition of the growth of Human Ovarian cancer cells (HeLa cells) *in vitro* ($p<0.05$) at 1,000 μg/ml.

Results from *in vitro* studies suggests that AIR possess immunostimulatory activity. Enhanced T cell proliferative response to concanavalin A (Con A) was observed in splenocytes exposed to AIR extract from whole plants at concentrations from 1.25 - 500 μg/ml, but no significant effect was observed with the extract from seeds. By contrast, both AIR extracts from whole plants and seeds enhanced the proliferative response to B cell mitogen, Lipopolysaccharide (LPS). Enhanced B cell proliferation was observed with the plant AIR extract at concentrations from 1.25 to 100 μg/ml, whereas higher concentrations suppressed the response. Interestingly, extract from seeds enhanced the B cell responses at all concentrations of AIR evaluated (1.25-2,000 μg/ml). In addition, *in vitro* exposure of splenocytes to seed AIR extract at concentrations of 3.9, 7.28. 31.25 and 500 μg/ml significantly increased T cell proliferative response to anti-CD3ε Ab which mediated through T cell receptor ($p<0.05$). But cultures treated with plant AIR extract only showed slight increased in anti-CD3ε mediated proliferation at a concentration of 100 μg/ml, whereas a significant decrease occurred at 500 and 1,000 μg/ml. Whether the effects of AIR on enhancing the response of T and B lymphocytes is mediated through some specific cytokine pathways requires further clarification. To further investigate the potential of AIR as an immunostimulant *in vivo*, additional studies in evaluating the effects of whole plant and seed extracts of AIR on antibody responses to T-dependent antigens, mixed lymphocytes response, natural killer cell activity and the effects on splenic populations should be further investigated.

Keywords: *Aeginitia indica* Roxb., Dok Din Daeng, immunostimulant, Con A, LPS, anti-CD3ε Ab

บทคัดย่อ

รหัสโตรงการ : BGJ/32/2543

ชื่อโครงการ : การศึกษาพิษต่อเซลล์เนื้องอกและผลต่อระบบภูมิคุ้มกันของสารสกัดจากดอกดินแดง (*Aeginetia indica* Roxb.)

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ดอกดินแดง (*Aeginetia indica* Roxb.) พืชกาฝากที่เจริญอยู่กับต้นไผ่ เป็นสมุนไพรพื้นบ้านไทยที่มีสรรพคุณใช้เป็นยาชงรักษาโรคเบาหวาน และยาต้มเพื่อแก้อาการบวมตามผิวหนังมาแต่โบราณ แต่ยังไม่มีการศึกษาและวิจัยฤทธิ์ทางเภสัชวิทยาของดอกดินแดงในประเทศไทย โครงการวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาพิษต่อเซลล์เนื้องอกและทดสอบผลต่อระบบภูมิคุ้มกันของสารสกัดจากดอกดินแดงในประเทศไทย โดยแยกการสกัดสารจากดอกดินแดงเป็นสองส่วน คือส่วนเมล็ดสกัดด้วยบิวทานอล และส่วนที่เป็นพืชทั้งต้นสกัดด้วยเอธทานอล

ผลการทดลองความเป็นพิษต่อเซลล์ม้ามปรกติ (normal splenocytes) ของหนูสายพันธุ์ C57BL6/j ในห้องปฏิบัติการ (*in vitro*) พบว่าสารสกัดจากดอกดินแดงมีความเป็นพิษต่อเซลล์ต่ำ สารสกัดจากเมล็ดไม่มีพิษต่อเซลล์ในช่วงความเข้มข้นระหว่าง 1.95-2,000 ไมโครกรัมต่อมิลลิตร และสารสกัดจากทั้งต้น ไม่มีพิษต่อเซลล์ในช่วงความเข้มข้นระหว่าง 1.25-1,000 ไมโครกรัมต่อมิลลิตร ในความเข้มข้นที่ไม่เป็นพิษต่อเซลล์ปรกติ สารสกัดมีพิษต่อเซลล์เนื้องอกโดยตรง โดยสารสกัดจากต้นสามารถยับยั้งการเจริญของเซลล์มะเร็ง HeLa (Human ovarian cancer cells) ใน *in vitro* ที่ความเข้มข้น 1,000 ไมโครกรัมต่อมิลลิตร

ผลจากการทดลอง *in vitro* พบว่าสารสกัดจากดอกดินแดงมีฤทธิ์กระตุ้นระบบภูมิคุ้มกัน สารสกัดจากต้นสามารถกระตุ้นการตอบสนองของ T lymphocytes ต่อ Concanavalin A (Con A) ในช่วงความเข้มข้น 1.25 - 500 ไมโครกรัมต่อมิลลิตร และไม่พบการกระตุ้นเมื่อทดสอบด้วยสารสกัดจากเมล็ด แต่สารสกัดของดอกดินแดงทั้งจากต้นและเมล็ดสามารถกระตุ้นการตอบสนองต่อไมโตเจน (mitogen) ของ B เซลล์ Lipopolysaccharide (LPS) โดยสารสกัดจากต้นที่ช่วงความเข้มข้น 1.25-100 ไมโครกรัมต่อมิลลิตร กระตุ้นการเจริญ (proliferation) ของ B เซลล์ แต่ความเข้มข้นที่สูงขึ้นกลับมีฤทธิ์ยับยั้งการตอบสนอง สำหรับสารสกัดจากเมล็ดมีฤทธิ์กระตุ้นการตอบสนองของ B lymphocytes ต่อ LPS ในทุกความเข้มข้นที่ใช้ทดสอบ (1.25 ถึง 2,000ไมโครกรัมต่อมิลลิตร) นอกจากนั้น ผลการศึกษาการกระตุ้นภูมิคุ้มกัน *in vitro* ของสารสกัดจากดอกดินแดงต่อเซลล์ม้ามผ่าน T cell receptor โดยใช้ anti-CD3 Ɛ antibody (Ab) พบว่าสารสกัดจากเมล็ดที่ความเข้มข้น 3.9, 7.28, 31.25 และ 500 ไมโครกรัมต่อมิลลิตร เพิ่มการตอบสนองของ T เซลล์ต่อ anti-CD3 Ɛ Ab อย่างมีนัยสำคัญ ($p<0.05$) แต่สารสกัดส่วนต้นสามารถกระตุ้นการตอบสนองผ่าน anti-CD3 Ɛ Ab เพียงเล็กน้อยที่ความเข้มข้น100 ไมโครกรัมต่อมิลลิตร และมีผลยับยั้งการตอบสนองที่ความเข้มข้น 500 และ 1000 ไมโครกรัมต่อมิลลิตร กลไกการกระตุ้นการตอบสนองของ T และ B lymphocytes โดยดอกดินแดงจะเกี่ยวข้องกับ cytokine pathway ชนิดใดหรือไม่จำเป็นต้องมีการศึกษาต่อไป การศึกษาเพิ่มเติมเกี่ยวกับศักยภาพการออกฤทธิ์กระตุ้นภูมิคุ้มกันของดอกดินแดงในสัตว์ทดลอง (*in vivo*) ควรจะศึกษาผลของสารสกัดทั้งจากเมล็ดและต้นในวิธีการทดสอบต่าง ๆ เพิ่มเติมคือ การศึกษาผลต่อ B เซลล์ในการสร้าง antibody เพื่อตอบสนอง T cell antigen, การตอบสนองของ T เซลล์ใน mixed lymphocyte response ความสามารถในการฆ่าของ natural killer cell (natural killer cell acitivity) และผลต่อกลุ่มประชากรในเซลล์ม้ามของสัตว์ทดลอง

คำหลัก: *Aeginitia indica* Roxb., ดอกดินแดง การกระตุ้นภูมิคุ้มกัน Con A, LPS, anti-CD3Ɛ Ab

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LIST OF ABBREVIATIONS

%	percent
α	alpha
AIR	*Aeginitia indica* Roxb.
β	beta
°C	degree Celsius
cm	centimeter
CPM	counts per minute
Con A	Concanavalin A
EBSS	Earles Balanced Salt Solution
ε	Epsilon
ELISA	Enzyme Linked Immunoabsorbant Assay
et al	et alia (and others)
Fig.	Figure
g	gram
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HeLa	Human Ovarian Cancer Cells
IFN	Interferon
IL	Interleukin
LPS	Lipopolysaccharide

LIST OF ABBREVIATIONS (Cont.)

mg/ml	milligram per milliliter
min	minute
ml	milliliter
µm	micrometer
µg/ml	microgram per milliliter
mmho	milliohm
mM	milli Molar
MTT	3-4,5-dimethylthaiazol-2yl)-2,5-diphenyl tetrazolium bromide
n	normal
$NaHCO_3$	sodium bicarbonate
NK	natural killer cells
nm	nanometer
no.	number
O.D.	optical density
PBS	phosphate buffer saline
PBMC	peripheral blood mononuclear cells
ppm	parts per million
rpm	revolution per minute
RPMI1640	Roswell Park Memorial Institute

LIST OF ABBREVIATIONS (Cont.)

S.E.	standard error
SOP	Standard Operating Procedure
TNF	tumor necrosis factor
v/v	volume: volume

CHAPTER 1

INTRODUCTION

Scientists are interested in the stimulation of the human's own immunity as an alternative remedy, since many drugs available and radiation therapies for the treatment of numerous serious diseases, such as chronic infections, bone marrow inflammation, cancer and AIDS are quite expensive and have several side effects. At present, various medicinal plants have been explored and developed as chemotherapeutic agents to modulate the immune system, such as to increase macrophage activation, induce T and B lymphocytes and natural killer cell activities.

Several plant-derived immunostimulatory drugs have been used widely in European and Asian traditional medicine for self-medication as well as for prescription. Examples of these are the water and alcoholic extracts of *Echinacea purpurea*, E. *angustifolia* and E.*pallida* (herb and root), *Panax ginseng*, *Eleutherococcus senticosus*, *Rehmania glutinosa* and etc (Arnason, T.J., Mata, R., and Romeo, T.J., 1995). Among these plants, *Aeginetia indica* Linn. (AIL), a parasitic plant on roots of Japanese pampa grasses or sugar cane has long been used as a tonic and anti-inflammatory medicinal herb in China and Japan (Bando *et al.*, 1980). Several studies have reported that AIL has a high potential as a medicinal herb for antitumor activity. The seed extract of AIL has been shown to cure most ddY mice bearing allo-transplantable sacroma (S-180) tumor cells (Chai *et al.*, 1988) which might be mediated through the $CD4^+$ T cells (Chai *et al.*, 1992 and Chai *et al.*, 1994). The polysaccharide fraction which was used at 100 μg/ml activated B-lymphocytes and thymocytes. *In vitro* studies showed the ability of AIL to induce interleukin-2, interferon γ, interleukin-6 (Chai *et al.*, 1994). The 55kDa protein from seed extracts induced Th1 cytokines, such as IL-2, IL-6, IL-10, IL-12, and IL-18. There was also induction of IFN-γ, TNF-∝, granulocyte macrophage-colony stimulating factor (GM-CSF) and activation of killer cell activities of peripheral blood mononuclear cells (PBMC) in the *in vitro* models. (Chai *et al.*, 1994, Okamoto *et al.*, 2000 and Ohe *et al.*, 2001).

The phytochemistry of AIL was studied by chromatographic techniques and various spectroscopic methods, i.e. ion-exchange resins (IR), nuclear magnetic resonance spectroscopy (NMR), ultraviolet-visible spectroscopy (UV), and mass spectroscopy (MS). Results have shown the presence of Susquiterpene, β- Steroid, Carotenoid, Monoterpene, Glucoside and Flavonoid (Dighe *et al.*, 1977; Endo *et al.*, 1979; Oshima *et al.*, 1984).

In Thailand, *Aeginetia indica* Roxb. (AIR) has several local names such as, So-suai (Karen-Mae Hong Son Province), Dok din daeng (Trat Province), Paak cha khe (North-east), Sop laeng (Song khla Province) or Yaa dok khol (Loei Province) (Smitinun,1980). It has been used in Thai traditional medicine for the treatment of diabetes and dermal swelling by whole plant extraction (Muller-Oerlinghausen,B., Ngamwathana,W., and Kanchanapee,P.,1971). This plant is a parasitic plant on the roots of other plants. In Thailand, it likes to grow under bamboo shady areas. The flower blossoms between September and October, which is the rainy season in Thailand. The local people in northern Thailand use the flower for making a dessert called "Dok-din" (Reung-rung see, N., and Tuntiwong, P., 2000). However, there are still no studies of pharmacological functions and immunological effects of this plant in Thailand.

Normal chemical constituents found in all plant tissues are principally carbohydrates, fat, oils and protein. They may contain other minor compounds such as, phytin, alkaloid, and raffinose etc. A vast array of natural organic acid compounds, the products of primary and secondary metabolism, occur in plants that are used in many countries as traditional medicinal plants (Harborne, J.B. and Baxter, H., 1993).

It appears that natural organic compounds in the plants have immunostimulating activities, as demonstrated in many *in vitro* and experimental animal studies as well as clinical trials that are associated with lipophilic compounds (alkylamides) and a polar fraction (cichoric acid and polysaccharides). The other potential immunostimulating compounds are alkaloid (e.g. isopteropodine), terpenoids, quinones (e.g. plumbagin), macrocyclic lactones (e.g. bryostatins) as

well as phenolic compounds (e.g. cichoric acid). and glycoproteins (lectin) (Arnason, T.J., Mata, R., and Romeo, T.J., 1995).

The most frequent solvents for extracting these organic compounds are alcohol and water extraction. Alcohol such as ethanol, methanol, hexane, n-butanol, chloroform and etc., have been used to extract polysaccharide, glycoprotein, phenolic, terpenoid, waxes, fats, some resin and portions of wood gum. By contrast, hot water extractions were used to remove tannin, monosaccharide, gums, starches, and coloured matter. Therefore using less polar solvents such as methyl and ethyl alcohols, or a mixture of these alcohols with water, may insure a more continuous range of polarities to extract immunostimulatory substances from plants (Harbone, J.B., 1984, Ehrman, T., 1994).

In addition, plant parts which are usually used to screen for biological activities and phytomedicine are whole plants, seeds, flowers, roots, and leaves. In the case of *Aeginetia indica*, a parasitic plant, the whole plant has been promoted as traditional medicine and its seed extracts have been proven to have an immunostimulating effect as described previously. In fact, this parasitic plant is mainly composed of stem and flowers which contain seeds. Selection of all plant parts for extraction will help us to better understand the immunological effect of this plant. Using aqueous alcohol such as 95 % ethanol and water saturated with n-butanol (high polarity) to extract may be useful to obtain all substances that have immunostimulatory effects.

Suitable methods have been established to investigate the effects of substances on components of the immune system. Tests are selected to provide qualitative and quantitative data on the chemical effect of components of antibody-mediated and specific and non-specific cell-mediated immunity.

Parameters that have been evaluated include immune system tissue, the weights of certain organs and cellularity, clinical blood chemistry, haematology, humoral and cellular immunity (Buleson, G.R., Dean, J.H. and Munson, E.A, 1995).

In Thailand AIR has earlier been reported to hae been used in traditional plant medicines and desserts. No studies have been conducted to examine its toxicity and immunological effects. Since environmental factors affect the outcome of pharmacological activities of the same kinds of medicinal plants, the pharmacological activities of the same species of medicinal plants can vary at different locations. Therefore, the antitumor effect of AIR in Thailand could be different from those previous reported in other countries. So the present study is therefore aims to do the following:

1. To collect and extract various parts of *Aeginetia indica* Roxb.: seeds and whole plants.
2. To study the immunotoxicological effects of various parts of *Aeginetia indica* Roxb.: seeds and the whole plant extracts.
3. To study the cytotoxicity of tumor cells of various parts of *Aeginetia indica* Roxb.: seeds, and whole plant extracts.

CHAPTER II

LITERATURE REVIEW

1. *Aeginetia indica* Roxb.

Family: Orobanchaceae

Common name: So-suai (Karen-Mae Hong Son), Dok din daeng (Trat), Paak cha khe (North-east), Sop laeng (Song Khla) or Yaa dok khol (Loei) (Smitinand, T., 2001).

Botanical description: (Fig. 1 and Fig. 2)

Aeginetia indica Roxb. is a fleshy, unbranched, erect parasitic herb without chlorophyll. The stem (peduncle) and the outside of the calyx are a pale light yellowish colour with dense maroon lines or streaks. The calyx inside is a darker maroon in colour. Both sides of the lobes and style are very dark maroon. The tubular, dark purple flower emerges from the ground surface ranging from 15 to 40 cm., with a nodding, and sleeve-like calyx at the terminal end of the stem which contains minute leaf scales. The anthers are whitish, and the stigma is yellow. The fruit is dry and dehiscent. The seeds are minute and pale yellow (Maxwell, J.F., 1989 and Patrick, D.M., 1998).

Ecology and distribution: (Fig 3)

This plant is a parasitic plant on the roots of other plants. In Thailand, it likes to grow under bamboo in shady areas. The flower blossoms between September and October which is the rainy season in Thailand (Reung-rung see, N., and Tuntiwong, P., 2000). It also grows in mixed evergreen-deciduous forests in shady areas which are usually moist and in mostly alluvial areas beside or near streams (Maxwell, J.F., 1989).

Figure 1 Whole Plant of *Aeginitia indica* Roxb.

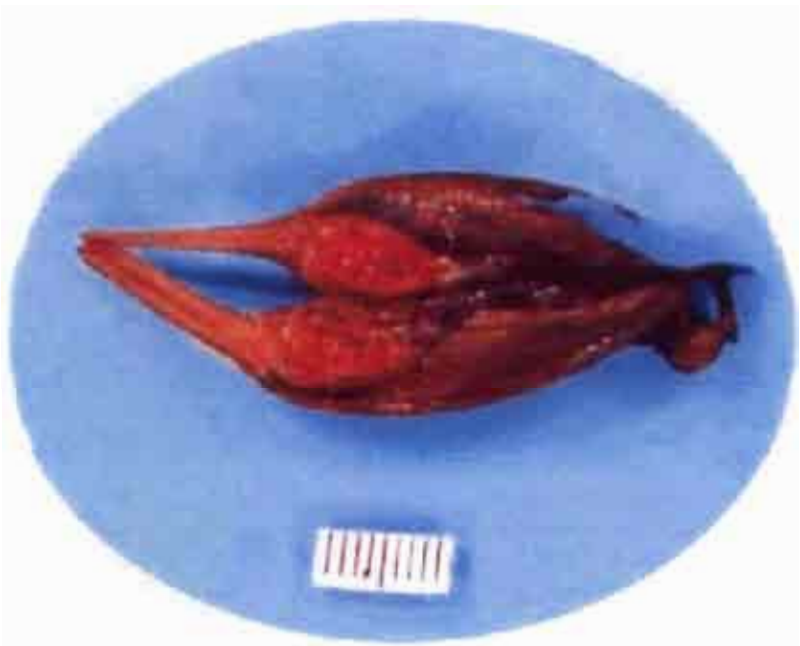

Figure 2 The flower of *Aeginitia indica* Roxb.

Figure 3 Location of plant (*Aeginitia indica* Roxb.) growth at Aumphur Wangnum Keaw, Nakhon Ratchasima province.

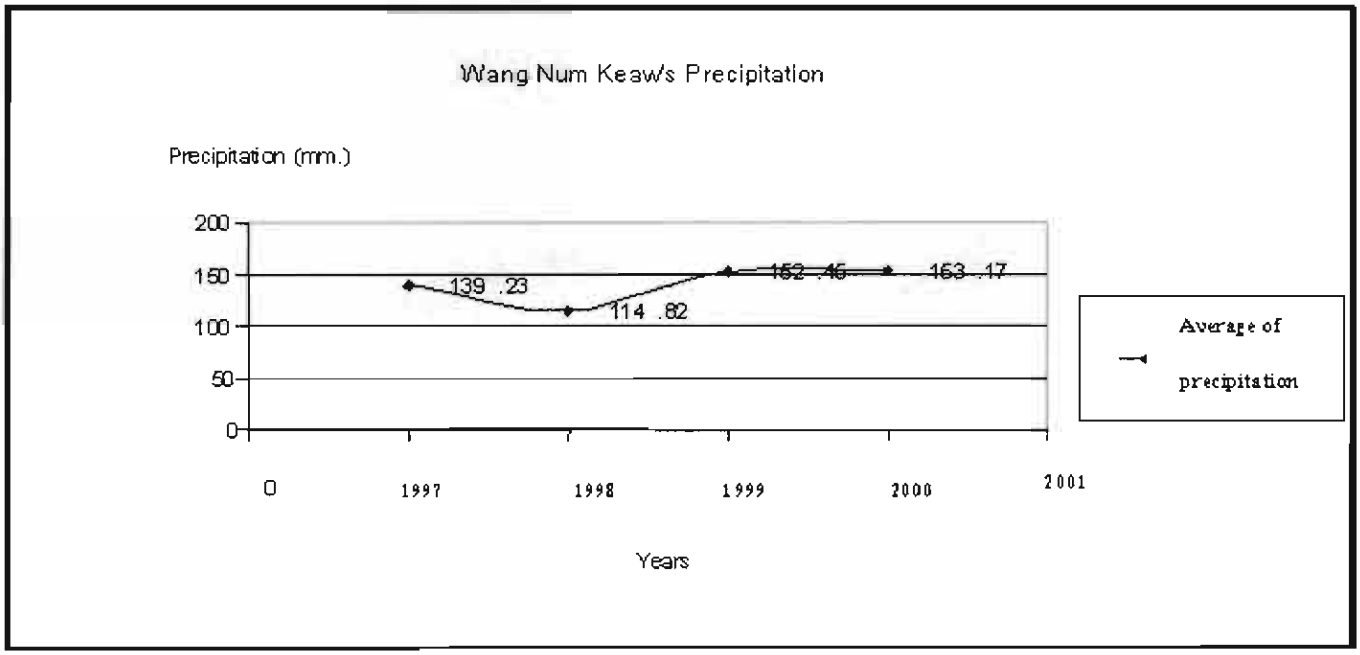

Figure 4 Graph of average precipitation at Aumphur Wang Num Keaw, Nakhon Ratchasima province, in the area of *Aeginetia indica* Roxb. growth. Measured by the Meteorological Department, Aumphur Wang Numkeaw, Nakhon Ratchasima, Thailand.

Table 1 Results of analyzed minerals in soil where *Aeginetia indica* Roxb. grows at Aumphur Wang Num Keaw, Nakhon Ratchasima. Analyzed by the Land Development Department, Nakhon Ratchasima province, Thailand.

Soil Analysis	**Level**					
Type of Mineral	Very low	Low	Intermediate	High	Very High	Over limit
Phosphorus Potassium Calcium Magnesium	1 ppm 24 ppm			4700 ppm		
	Concentrate Acid	High Acid	Intermediate Acid	A little bit of Acid	Normal	Base
pH		4.49				
	Normal	Very Low	Low	Intermediate	Very High	
Salty	0.03 mmho at room temperature					

Propagation: (Baskin C.C, and Baskin J.M, 1998)

Aeginetia indica Roxb. is a parasitic plant which means that it obtains nutrients from another organism called the host. It is classified as a "Holoparasite" which lacks chlorophyll and receives fixed carbon, water, and minerals from the host plant. It has seeds with undiffentiated embryos that consist of only a few cells. Studies on the morphology/anatomy of germination in members of this family indicate that a radicle and cotyledons per seed are never formed. It grows by cell division, emerges through the micropyle, and develops into a muticellular structure called the germ tube. If the host root is present, the germ tube penetrates it and becomes the primary haustorium. It is presumed that chemicals in the exudates of host plant roots stimulate development of haustarium. The part of the germ tube remaining on the outside of the host root enlarges and forms a tubercle, from which the shoot and root-like structures are initiated. It develops a plant body external to the host.

In addition to morphological underdevelopment, seeds of various Orobanchaceae require warm or cold stratification before the germ tube or primary haustorium emerges from the seed. However, if the seed coat is softened by calcium hypochlorite, germination can occur without being its warm stratified. According to Musselman (1980), seeds of this family will not germinate unless afterripening is followed by a period of warm stratification, which is called "conditioning". Information available on Orobanchaceae seeds that require conditioning prior to germination ecology is somewhat similar to that of nonparasitic winter annuals. In fact, it seems reasonable to think of these parasites as winter annuals because annual Orobanchaceae in regions with a Mediterranean climate complete their life cycle during the cool wet season.

Seeds of *Aeginetia indica* require 40-45 days, or longer, to germinate at 20, 15, 30 or 26-28° C, even if they are given chemical or heat shock treatment. Although an 8-day cold stratification treatment broke dormancy in *Aeginetia indica* seeds, dormancy was reinduced in seeds kept at 3-5°C for 36 days or longer. However, little is known about the germination ecology of Orobanchaceae in the temperate region. In fact, it is not known if the underdeveloped embryos have physiological dormancy. It seems logical that physiological dormancy in embryos would be broken during cold stratification in winter and that seeds would germinate in spring. After dormancy-breaking treatments

are complete, the seeds of *Aeginetia indica* germinate in a high percentage when incubated at 25-30°C.

Because haustoria of the Orobanchaceae become attached to roots of the host plant, it makes sense that seeds of these parasites could germinate in darkness. Thus, germination studies are often carried out in darkness; in a few studies comparisons of germination in light and darkness have been made. *Orobanche crenata* seeds germinate in higher percentages in darkness than in light, whereas seeds of *Aeginetia indica* require darkness for germination of old, but not new seeds. When seeds are warm stratified and placed in darkness at the appropriate temperatures, few or no seeds may germinate unless a host root or aqueous solution of root exudates are present for at least 24-48 hours.

Orobanchaceae requires a chemical stimulant from the host plant's root. Considerable effort has been made to identify the chemicals that promote germination in the Orobanchaceae, especially the weedy species of *Orobanche*. However, isolation and identification of these stimulators are quite difficult because of several problems. The problems are the following: the amount of stimulatory compound produced per root appears to be very low; molecules are unstable at high temperatures: root exudates that stimulate germination are mixtures of compounds, and the root of different host species may produce different mixtures of stimulants. In addition, root exudates may contain germination inhibitors, stimulants are produced for varying periods of time by roots of different species, seeds of *Orobanche* have endogenous germination stimulator, and also the amount of stimulant produced by host roots varies with the season in which plants are grown. Moreover, the root of some species produce chemicals that stimulate the germination of *Orobanche* seeds, but the plants producing them are not parasitized. For example, exudates of flax roots stimulated more seeds of *O .ramona* to germinate than those of sorghum or tomato roots. Nevertheless, flax was not parasitized, whereas sorghum and tomato were parasitized.

Attempts have been made to identify the germination stimulant in root exudates of the principle host species for *Orabache*. A partially purified germination stimulant from faba bean root exudates may possibly be unsaturated lactones. Others analyzed from broad bean tomato and flax by thin layer chromatography and nuclear magnetic resonance were identified as unsaturated lactones which have a carbonyl group, single bond and double bond carbon or alycyclic structure, an aliphatic chain, and also strigol, or its analog.

An increase of soil nutrients or the use of nitrogen fertilizers can cause a reduction of some species in Orobanchaceae because they have a low ability to detoxify chemicals. Presence of 8 mM ammonium sulfate during conditioning reduced the germination of *Orbanche crenata* seeds from 46 % to 26%.

Phytochemistry:

Many compounds have been identified in *Aeginetia indica* Linn. Table 2 shows the types and percentages of compounds present in *Aeginetia indica* Linn. from different countries.

Table 2: Compounds obtained from *Aeginetia indica* Linn.

Compounds	Plant part	Plant locality	References
Aeginetia indica Polyene D carotenoid (0.0006 %)	Entire plant	India	Dighe *et al.*, 1977
Aeginetia indica Polyene E carotenoid (0.0001 %)	Entire plant	India	Dighe *et al.*, 1977
Polyene E carotenoid (0.01672 %)	Aerial parts	Taiwan	Oshima *et al.*, 1984
Aeginetia indica Polyene F carotenoid (0.0005 %)	Entire plant	India	Dighe *et al.*, 1977
Aeginetic acid Sesquiterpene (0.032 %)	Entire plant	India	Dighe *et al.*, 1977
Aeginetolide Sesquiterpene (0.00125 %)	Entire plant	India	Dighe *et al.*, 1977
Aeginetoside monoterpene (0.03 %)	Entire plant	Japan	Endo *et al.*, 1979
Apigenin Flavonoid (0.00216 %)	Aerial parts	Taiwan	Oshima *et al.*, 1984
Aucubin, ISO: Sesquiterpene (0.08594 %)	Entire plant	Japan	Endo *et al.*, 1979
Daucosterol steroid (0.00432 %)	Aerial parts	Taiwan	Oshima *et al.*, 1984
Ionone, β-hydroxy: Glucoside Sesquiterpene (0.0247 %)	Entire plant	Japan	Endo *et al.*, 1979
Sitosterol, β: Steroid (0.033 %)	Entire plant	India	Dighe *et al.*, 1977
Steroid (0.00448 %)	Aerial parts	Taiwan	Oshima *et al.*, 1984

In *Aeginetia indica* Roxb., there have also been reports that the colour of its flower occurred because it is composed of a compound in the group of Aucubin, and its colour can turn black by oxidization (Reung-rung see, N., and Tuntiwong, P., 2000).

Traditional medicinal usages:

Aeginetia indica Linn. has traditionally been used as a tonic and an anti-inflammatory medicinal plant in China and Japan. (Chai *et al.*, 1992) It has been used for the treatment of many diseases such as hepatitis (Oshima *et al*, 1984; Lin, C.C.,and Kan, W.S., 1990), and liver diseases (Yanfg et al., 1987)

Aeginetia indica Roxb. has been used in traditional Thai medicine for the treatment of diabetes and dermal swelling (Muller-Oerlinghausen,B., Ngamwathana,W., and Kanchanapee, P.,1971).

Pharmacological studies:

Aeginetia indica Linn.:

1. Antitumor activity

Seed extract cured most ddY mice bearing allo-transplantable sacroma (S-180) tumor cells, and all the cured mice were resistant to subsequent rechallenge with S-180 *in vivo* (Chai *et al.*, 1988 and Kobashi *et al*, 1990). Its extract also mediated potent antitumor immunity for BALB/c mice bearing syngeneic Meth A tumor cells as evidenced by no ascites of tumor growth being detected (Chai *et al.*, 1992). Also there was antitumor effect of its extract on human salivary adenocarcinoma grown in nude mice (Bando *et al.*, 1980).

2. Mitogenic activity

The polysaccharide fraction which was used at 100 μg/ml activated B-lymphocytes and thymocytes (Chai et al., 1994).

3. Immunostimulating activity

The extract of seeds has an ability to induce interleukin-2 (IL-2) and interferon γ (IFN-γ) in human peripheral blood lymphocytes *in vitro*. (Bando *et al.*, 1980) The polysaccharide fraction at 2.5 mg/ml stimulated murine splenocytes to produce IL-2, IFN-γ, tumor necrosis factor (TNF), and IL-6 (Chai *et al.*, 1994). The 55kDa protein from seed extract induced Th1 cytokines, such as IL-2, IL-6, IL-10, IL-12, and IL-18. There was also induction of IFN-γ, TNF-∝, granulocyte macrophage-colony stimulating factor (GM-CSF) and activation of killer cell activities of peripheral blood mononuclear cells (PBMC) in the *in vitro* models. Furthermore, IL-18 played the most significant role for IFN-γ inducing ability and killer cell activation (Okamoto *et al.*, 2000 and Ohe *et al.*, 2001).

2. IMMUNOTOXICOLOGICAL STUDIES

Immunotoxicological studies refers to the ability of a test substance to induce dysfunction or inappropriate suppressive or stimulatory responses in components of the immune system. Immunotoxicity data provides information on health hazards that arise from exposure to chemicals, pharmaceuticals, recombinant biological, or environmental and occupational pollutants.

Immunotoxicological study tests are selected to provide qualitative and quantitative data on the capacity of a chemical to adversely affect components of antibody-mediated and specific and non-specific cell-mediated immunity.

Parameters that are evaluated include immune system tissue and the weight of organs and cellularity, clinical blood chemistry, haematology, humoral immunity, and cellular immunity. . (Buleson, G.R., Dean, J.H. and Munson, E.A, 1995)

In vitro and *in vivo* test systems are used to determine the functional state and the efficiency of the cellular and humoral immunity. Non-specific immunity are also appropriate for the screening tests of plant constituents with immunomodulatory activities. Examples of such test systems are: anti-CD3 mediated T cell proliferation, Hemolytic plaque assay, and Spleen cell mitogenecity assay etc. (Arnason, T.J., Mata, R., and Romeo, T.J., 1995)

2.1 Spleen

The spleen is a fist-sized organ just behind the stomach that collects antigens from the blood. It also collects and disposes of senescent red blood cells. The bulk of the spleen is composed of red pulp, which is the site of red blood cell disposal. The lymphocyte surround the arterioles entering the organ, forming areas of white pulp, the inner region of which is divided into a periteriolar lymphoid sheath (PALS), containing mainly T Cells and flanking B-Cell corona (Janeway, C.A, Jr., *et al.*, 1999). This organ is mainly used in various assays of immunotoxicological studies to evaluate T and B lymphocyte response of the immune system.

2.2 Humoral immunity

The main role of B cells in adaptive immunity is to secrete antibodies. An antibody plaque-forming cell assay is used to test the effect of substances on the humoral response. (United States Environmental Protection Agency, 1996)

2.2.1 Hemolytic plaque assay for detecting single IgM-forming cells

Following exposure of animals to a T-dependent antigen such as sheep erythrocytes (SRBC), antibody producing cells (B lymphocytes) can be recovered from the spleens and enumerated by counting plaques, which are zones of hemolysis in a background of intact red blood cells. At the center of each plaque is the mature B lymphocyte. The hemolysis results from the interaction of complement with the antigen-antibody complex on the surface of the red blood cell (SOP/PFC/006, Medical College of Virginia, 2000).

2.3 Cellular immunity

T and B lymphocytes are important cells of immune responses to kill antigens and regulate functions of the immune system. Non-specific proliferation of lymphocytes against mitogens such as concanavalin A (Con A) and lipopolysaccharide (LPS) is a potential assay to evaluate the responses of T and B lymphocyte to substances on cell mediated immunity. Specific cell mediated immunity can be studied by assessment through the anti-CD3 mediated T-cell proliferation assay which demonstrates the effect of exposed substances to T lymphocyte response. (Fudenberg, H.H, Whitten, H.D. and Ambrogi, F., 1984; United States Environmental Protection Agency, 1996)

3. IMMUNOMODULATING SUBSTANCES

Immunomodulating substances are substances, chemicals or drugs that have been found to either enhance or inhibit the functioning of immune cells. (United States Environmental Protection Agency, 1996)

3.1 Immunosuppressive drugss

The compounds that inhibit the immune response are called immunosuppressive drugs. These drugs are used mainly in the treatment of graft rejection and autoimmune diseases (Janeway, C.A, Jr., *et al.*, 1999).

3.2 Immunostimulant and adaptogen drugs

The terms of "immunostimulants" and "adaptogens" both describe drugs capable of increasing the resistance of an organism against a stressor of variable origin. Both types of drugs achieve this enhancement primarily by non-specific mechanisms of actions. Immunostimulants generally stimulate, in a non-antigen dependent manner, the function and efficiency of the non-specific immune system in order to counteract microbial infections or immunosuppressive states.

Adaptogens are believed to reinforce the non-specific power of resistance of the body against physical, chemical or biological noxious agents. Enhanced power of resistance can manifest itself by a variety of phenomena including: prolonged maintenance of body temperature following cold temperature stress, improved cognitive abilities, increase in locomotors and explorative activities, improvement in emotional behaviour; prevention of stomach ulcers by aspirin and

improvement of general immune defense.

With respect to the mechanism of action, immunostimulants influence primarily the humoral and cellular immune system; whereas, adaptogens are thought to influence the immune and the endocrine systems. Immunostimulants are effective prophylactically as well as therapeutically, while adaptogens are of use primarily in the prevention of a stressful situation.

Since the term immunostimulation is not found in the older literature, other criteria must be applied for the selection of plants. Good candidates are plants described for their antibacterial, antiviral, antifungal (anti-infectious) or antitumoral activities. Another quantity of the adminstered drug needed for anti-infectious or antitumor activity. If the normally used dose is so small that a direct antimicrobial or antitumoral effect can be excluded, an immune-induced effect most likely appears. So plant or traditional medicine is necessary to search for the active principles and clarify their mechanisms of action through several assays in immunotoxicological studies (Arnason, T.J., Mata, R.,and Romeo, T.J., 1995).

4. TOPICAL IMMUNOSTIMULATING AGENTS

Immunostimulating agents are believed to act by directly sensitizing or enhancing direct cells that participate in the immune system, i.e., T lymphocytes, B lymphocytes, NK, Macrophages, etc. Moreover, the specific site of action within the immune system may be difficult to ascertain. It may modulate, alter, or may have a direct effect on the cells participating in the reaction. In the effector phase of the humoral immune response, qualitative changes in antibody, alteration in the activation of the classic and alternative complement pathways, or change in the release as well as action of pharmacological mediators may occur (Fudenberg, H.H, Whitten, H.D. and Ambrogi, F., 1984). Phytochemicals are now being widely used and explored more for multi-disciplinary purposes because of their effect as immunostimulating agents. The potential immunostimulating compounds can be subdivided into low and high molecular weight compounds. The first group includes alkaloid (e.g. isopteropodine), terpenoids, quinones (e.g. plumbagin), macrocyclic lactones (e.g. bryostatins) as well as phenolic compounds (e.g. cichoric acid). The second group are polysaccharide and glycoproteins (lectin) (Arnason, T.J., Mata, R., and Romeo, T.J., 1995).

4.1 Polysaccharide

Recent studies have determined that the polysaccharide content of most medicinal plants are responsible for their immune stimulating or supporting activities (Arnason, T.J., Mata, R., and Romeo, T.J., 1995). For example, polysaccharide or saponin isolated from *Astragalus mongholicus*, *Acanthopanax senticosus* and *Panax notoginseng*, stimulated macrophages, promoted antibody formation, activated complement, and also increased T lymphocytes proliferation (Li, X.Y., 1991). Nutrition science has recently recognized expanded functions for complex carbohydrates or polysaccharide in addition to providing a source of cell energy-glucose metabolism and regulation. Also recent studies have confirmed that saccharides play an important role in supporting immune response (Ganapini, K., 1997 and See *et al.*, 1998). Monosaccharide, oligosaccharide and polysaccharide can be extracted and isolated in water or aqueous ethanol (Harborne, B.J., 1984).

4.2 Terpenoids

This group of organic compounds can be divided into many groups such as Monoterpenoids, Iridoids, Sesquiterpenoids, Triterpenoid saponin, Steroid saponin, and Carotenoids etc. (Harborne, B.J. and Baxter, H., 1993)

Aucubin (Aucubiside, Rhinnanthin) is one of the most common iridoids, occurring in many families of the dicotaledon, e.g., *Aucuba japonica* (Cornaceae) and some *Rhinnanthus* spp. (Scrophulariaceae). It is reported that it acts as an immunomodulator on antitumor activities (Harborne, B.J. and Baxter, H., 1993). These organic compounds are water-soluble, therefore, they can be isolated by ethanol and water extraction. (Harborne, B.J., 1984).

Other examples of biological active compounds which are immunostimulants in this group are Scandoside methyl ester and Secologanin (Loniceroside). Both of these are in a set of

sesquiterpeniods, which is the main compound occurring in plant essential oil. They also have immunomodulation effects on antitumor and antileukaemic activities (Harborne, B.J. and Baxter, H., 1993).

Caroteniods which are tetraterpenoids are also found to have immunomodulatory effects. They are usually an extremely widely distributed group of lipid –soluble pigment that appear in yellow flowered composites and orange or red fruits. An example of this compound is Guaiszulene (*S*-Guaiazulene, Azulon, Eucazulen, Kessazulen or Vaumigan). It is in blue oil which is produced during the steam distillation of chamomile (*Matricaria chamomilla*;- Compositae) which has been reported by others to have anti-inflammatory activity. (Harborne, B.J. and Baxter, H., 1993)

Steroidal and triterpenoidal glucosides from plants have been reported to be extracted and isolated by ethanol and water extraction (Harbone, B.J., 1984).

4.3 Phenolics

Topical phenolics are anti-oxidant agents, which are composed of many sub-classes such as anthocyanins, minor flavonoids and isoflavonoids etc. Antioxidants are agents that neutralize the negative by-products of metabolism called free radicals, which can damage DNA molecules and lead to cancer. They also counteract environmental carcinogens, protect against cardiovascular disease, fight sun damage to skin and may thwart the effects of Alzheimer's and other age-related diseases. Recently, anthocyanins, which are the blue pigment of blueberries have been reported to enhance resistance to free radical formation against free radicals within red blood cells *in vivo* (Barharbor,M.E., 1995). This showed that this compound group are also involved in the immunomodulatory effect. Phenolics can be extracted by alcohol solvent and aqueous-alcoholic fraction.

4.4 Glycoproteins

Among the isolated plant constituents available today for immunostimulatory treatment, the anitumoral" glycoprotein Krestin (*Coriolus versicolar*) is noteworthy. The glycoprotein can also be used as an immunostimulant (Arnason, T.J., Mata, R., and Romeo, T.J., 1995). According to the phytochemical method, protein and polysaccharide are dissolved in alcohol fraction such as ethanol and methanol (Harbone, B.J., 1984).

5. PLANT EXTRACTION PROCESS

5.1 Plant Extraction and Isolation of Compounds

Extraction of organic constituents from biological material is intrinsically dependent on the type and state of the material being extracted. The first consideration is the state of the tissue, or other biological material, being extracted. Parts of the plant that have been shown to have immunological effects are usually whole plants, seeds, leaves, roots and flowers (Harborne, B.J. and Baxter, H., 1993).

The general objective for extraction of biological material is to maximize the release of compounds from cells. The two tactics are to rupture the cells in order to remove the content and to optimize the surface area of the material with an extraction solvent which increases the amount of leaching from cells. Both of these ends can be achieved by grinding tissues into the smallest possible particles. A wide representation of constituents present is most desirable for bioactivity screening. Historically, ethanol or other alcohol such as n-butanol, chloroform, methanol etc., has been used to extract polysaccharide, glycoprotein, phenolics, terpenoids, waxes, fats, some resin and a portion of wood gum. Subsequent hot water extractions were then used to remove tannin, monosaccharide, gums, starches, and coloured matter. However, the alcoho-soluble and water-soluble principles may not overlap. Use of somewhat less polar solvents, particularly methyl and ethyl alcohols, or a mixture of these alcohols with water may insure a more continuous range of polarities (Harbone, B.J 1984, Ehrman, T., 1994).

Generally, the longer the time allowed for extraction in solvent, the more will be extracted.

Reflux is a reaction of solvent to keep a material dissolved at a constant temperature by boiling a solvent, condensing it, and returning it to the vessel. There are several methods that are used to extract compounds from solids (e.g. plants, soil, sediment, fish, and earthworms). They are Soxhlet, Sonication, Supercrinical fluid extraction, pressurized fluid extraction, microwave-assisted extraction, headspace analysis for volatiles, and distillation etc. (United States Environmental Protection Agency, 2001).

5.1.1 Soxhlet extraction

Soxhlet extraction is a standard and reliable method that involves multiple extractions by distilling and condensing vapor of solvent. Solvent is re-used for multiple extractions. The solvent is near the boiling point during extraction. This process occurs continuously up to at least 14-16 hrs. Typical solvents that are used are ethanol, hexane, acetone, methylene chloride, toluene and benzene. (United States Environmental Protection Agency, 2001)

5.1.2 Liquid-Liquid extraction

There is one commonly used method to separate a hydrophobic into a non-polar solvent. The process needs to use equipment such as a separator funnel to separate into an organic phase and an aqueous phase. This method may have to use two organic solvents that show the different polarities between two phases, such as alcohol (n-butanol) which has a higher polarity than water (United States Environmental Protection Agency, 2001).

5.2 Filtering and concentrating the samples

The final step after extracting the plant material is to recover a solution of the extracted compounds. The initial solution is recovered by filtration to remove all of the unextractable matter, including cellular matrices and other constituents which are insoluble in the extraction solvent. Large particles of the insoluble material are easily removed by filtering through cotton or glass wool plugs in the stand funnel. This filter is typically vacuum-filtered through paper, glass or other types of filters. Filtration can be repeated in order to maximize recovery.

A concentrated sample is usually obtained by drying the plant extract into powder by removing the extract solvent. Most commonly, when preparing a limited numbers of extracts, organic solvents are removed in a rotary evaporator (Ehrman, T., 1994; United States Environmental Protection Agency, 2001).

6. CELL VIABILITY AND CELL PROLIFERATION DETERMINATION METHOD

The end point of *in vitro* and *in vivo* immunotoxicology assays is to determine the cell viability and cell proliferation. Compounds are shown to be either toxic to cells, or to enhance or suppress cell proliferation. A conventional way of measuring cell proliferation is using radioactive ^{3}H-Thymidine incorporation assay (United State Environmental Protection Agency, 1996). An alternative method that avoids the radioactive isotope is MTT colorimetric assay (Martin, A., and Claynes, M.,1993). Another commonly used method to determine viability of cells is trypan blue dye exclusion.

6.1 ^{3}H-THYMIDINE INCORPORATION (^{3}H-TdR)

The incorporation of radiolabel in cells is the measurement of blastogenesis, and is expressed as counts per minute (CPM) using the Beta-counter machine. The ^{3}H-TdR technique is proven to have high sensitivity but the problems of the processing steps required for liquid scintillation counting as well as the safety in handling and waste disposal are major drawbacks (United States Environmental Protection Agency, 1996).

6.2 3-4, 5-DIMETHYLTHIAZOL-2YL)-2,5-DIPHENYL TETRAZOLIUM BROMIDE (MTT) COLORIMETRIC ASSAY

This assay measures the activity of various enzyme dehydrogenases in mitochondria. The tetrazolium salt MTT is cleaved by mitochondrial enzyme succinate-dehydrogenase into a blue formazan color product which only occurs in living cells (Fig. 5). The assay is useful for measuring cell survival and proliferation (Martin, A., and Claynes, M., 1993).

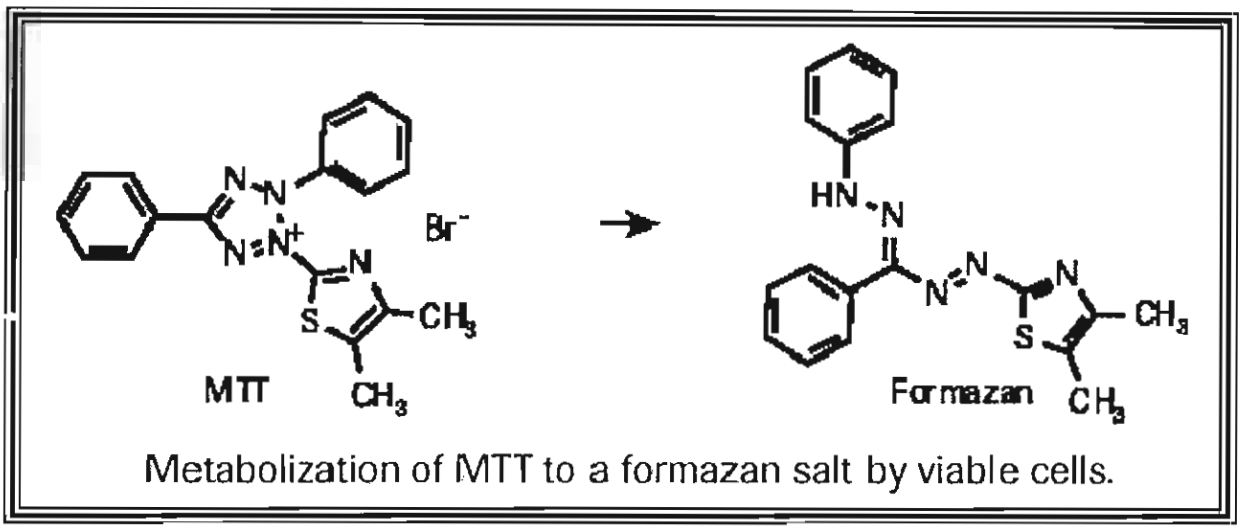

Figure 5 Metabolization of MTT to a formazan salt by mitochondrial enzyme succinate-dehydrogenase by viable cells.

6.3 TRYPAN BLUE DYE EXCLUSION

Trypan blue exclusion is a well known method that has been used for a long time to measure and determine cell viability. It is based on the principle that live cells possess intact cell membranes that exclude trypan blue. Only the dead cells will allow color to pass though cell membrane and stain blue. Therefore, a viable cell will have a clear cytoplasm, whereas a nonviable cell will have a blue cytoplasm under a microscope. (Phillips, H.I, 1973).

CHAPTER III

MATERIALS AND METHODS

1. MATERIALS

1.1 PLANT

Two batches of fresh plants of *Aeginetia indica* Roxb. (AIR) were collected from Aumphur Wang Num Keaw (Nakhon Ratchasima) in July and December 2001. From comparison with the specimen kept in the CMU Herbarium, Faculty of Science, Chiang Mai University, Chiang Mai, the plants were confirmed as AIR with the generous help of Dr. Maxell, J.F at Chiang Mai University.

The whole plants of AIR were dried in an electric hot air oven at 60 °C (Fig. 6) for about 3 days or until dried. Dried plants were kept in a non-defrost-freezer at -70 °C.

1.2 CHEMICALS AND INSTRUMENTS

The chemicals and instruments employed in the present study are summarized in Tables 3 and 4.

1.3 ANIMALS

Female C57BL6/j mice 6-8 weeks old, were obtained from the following laboratories

(1) National Laboratory Animal Center, Salaya, Nakhon Pathom, Thailand.

(2) Animal Center at University of Tsukuba, Tsukuba City, Ibaraki, Japan

These animals were acclimatized for a week before the experiment and then they were housed individually in stainless steel hanging cages. They were kept in a temperature-controlled room (25± 2°C) under a 12 hour light-dark cycle. Food pellets and tap water were given *ad libitum*, throughout the experiments.

Table 3 List of chemicals used in the studies.

Name	Source
Ethyl alcohol	Wako company, Japan
n-Butanol	Wako company, Japan
Earle's Balanced Salt Solution	Gibco Co., USA
Fetal calf bovine serum	Gibco Co., USA. and Hyclone, USA.
HEPES buffer	Gibco Co., USA.
[3(4,5-dimethylthiazol-2-yl) ,5-diphenyltetrazolium bromide]-MTT	Wako company, Japan
Roswell Park Memorial Institute (RPMI) 1640	Gibco Co., USA.
Phosphate buffer saline (PBS)	Gibco Co., USA.
Dimethyl sulfoxide (DMSO)	Wako company, Japan
Mouse Anti-CD3ϵ	PharMingen, USA.
Concanavalin A (Con A)	ICN. Co., USA.
Lipopolysaccharide (LPS)	Sigma, USA.
Ham's F12 media	Gibco Co., USA.
Trypan blue dye	Gibco Co., USA.
Typsin	Gibco Co., USA
Protinase K	Gibco Co., USA. and Wako company, Japan
L-glutamine	Gibco Co., USA.
Penicillin and Streptomycin solution	Gibco Co., USA.
Sodium Bicarbonate	Wako company, Japan
Gentamicin	Gibco Co., USA.

Table 4 List of equipments used in the studies.

Name	Source
Blender	Molinex Co., Germany
Digital balance	Mettler, Curtin matheson scientific Co., USA.
Soxhlet extraction apparatus	Pyrex Co., USA.
Alundum extraction thimbles	Fisher Scientific Co., USA.
Rotary evaporator with vacuum and water bath	Bosch, Co,USA.
Laminar flow hood	Holten, Scientific Promotion Co., England
Glassware	Pyrex Co., USA.
Homogenizer	Bosch Co.,USA
Light microscope	Nikon Kogaku K.K Co., Japan
Inverted microscope	Olympus optical Co. Japan
Microscope slides	Fisher Scientific Co., USA.
Petri dish	Nunc Co., Denmark
Single frost end slide	Fisher Scientific Co., USA
96-well plate flat bottom	Nunc Co., Denmark
Pasteur pipette	Fisher Scientific Co., USA.
Disposal pipette	Nunc Co., Denmark.
ELISA plate reader	BIORAD Co., USA.
Surgical set	Fisher Scientific Co., USA
Snap cap tubes	Nunc Co., Denmark
Conical tubes	Nunc Co., Denmark
Cell stainer	Nunc Co., Denmark
Multichannel pipette	Gibco Co., USA.
Hemocytometer and cover slip	Fisher Scientific Co., USA

2. METHODS

2.1 PREPARATION OF CRUDE ETHANOLIC EXTRACT OF AIR (Modified from Ehrman, T., 1994)

Whole dried plants 10 g (Fig. 6) were homogenized and ground into powder by electronic motor. The raw powder (Fig. 7) was put into a thimble (medium porosity, 10-15 μm, sized to fit the soxhlet extractor) (Fig. 8) and then into the soxhlet column. 500 ml of 95% ethanol was filled into a round-bottomed flask, and connected to the soxhlet extraction apparatus. Several boiling chips were placed into a clean dry receiving flask. This extraction process was performed until the solvent had a clear color (about 2-3 days). The reflux rate was periodically checked and adjusted so that the heating rate was four to five exchanges per hour in the soxhlet thimble. When the extraction was complete, the thimble was removed and the sample was carefully transferred to a beaker. Any residual solvent was removed by filtering through cotton wool. The sample was washed thoroughly with 95% ethanol and then all of the filtrates were collected. The flask with the extract solution was placed on the rotary evaporator and the solvent was removed under vacuum. The powders of crude extracts were then kept in -20°C freezers. The powder was dissolved in Roswell Park Memorial Institute (RPMI) media (without FCS), filtered through a Millipore filter (pore size = 0.45 μm or 0.22 μm) and was then used as the AIR extract (Fig. 9).

Figure 6 Whole plant of *Aeginetia indica* Roxb. after being dried by hot air oven at 60° C.

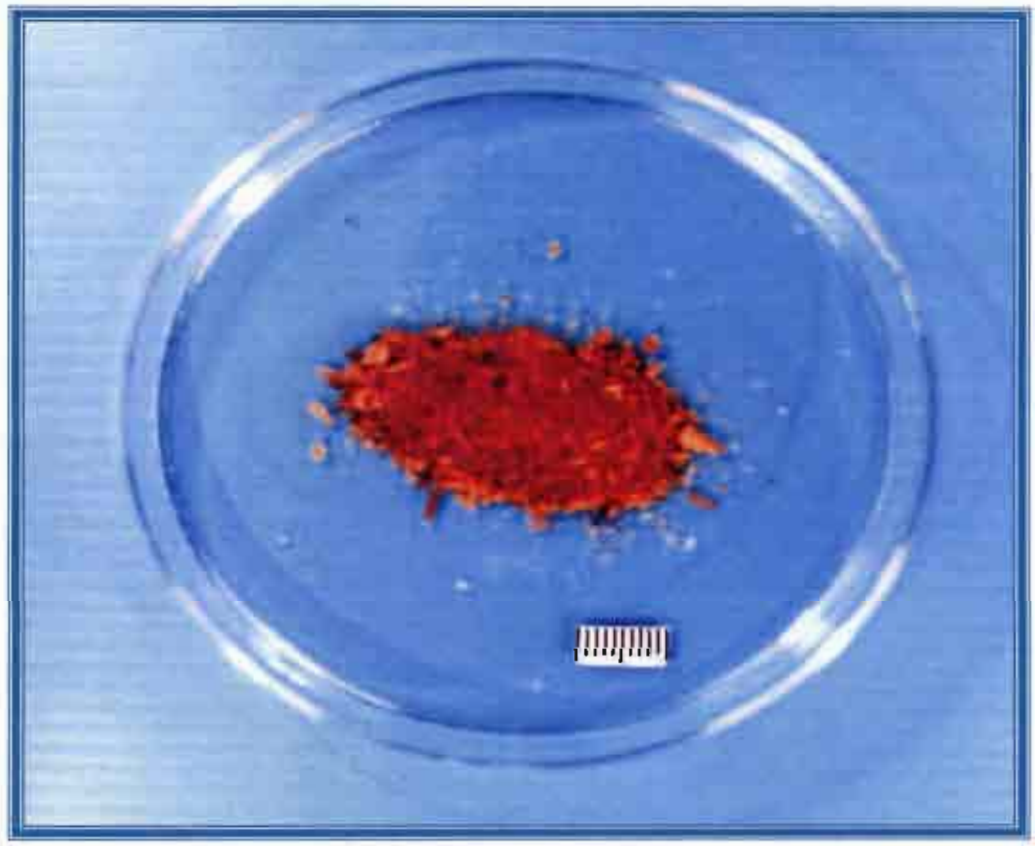

Figure 7 Raw powder of whole plant of *Aeginetia indica* Roxb.

Figure 8 Soxhlet extraction apparatus.

Figure 9 Powder of whole plant from *Aeginitia indica* Roxb. by ethanol extraction.

2.2 PREPARATION OF AQUEOUS PHASE OF n-BUTANOL EXTRACT FROM AIR SEEDS

The extraction procedure was performed as described previously (Chai et al., 1992 and Morrison and Leive, 1975) with slight modification. The dried seeds of *Aeginitia indica* Roxb. (AIR) were separated from the plant after being dried in a hot-air oven. 10 ml of distilled water was added to 1 g of seed of *Aeginetia indica* Roxb. The plant parts were homogenized in a motor and 10 ml of water-saturated butanol-1 were added. The mixture was stirred at 4° C for 15 mins, centrifuged at 35,000g for 20 mins, and a water-soluble fraction was collected. The extract was treated with 20 μg/ml protinase K enzyme (Gibco BRL) at 60 °C for 60 mins. Following protinase K digestion, the precipitated protein was removed by centrifugation in the cold. The extract was dialyzed against phosphate-buffer saline (PBS-0.1 M sodium phosphate, pH 7.2) overnight. The extract solution was evaporated by a rotary evaporator. The powder of extracts (Fig. 10) was kept in -20°C freezers. The powder was dissolved in a suitable media (RPMI media without FCS), passed through a millipore filter (pore size = 0.45 μm or 0.22 μm), and was then used as the AIR extract.

Figure 10 Powder of seed extract from *Aeginitia indica* Roxb. evaporated from aqueous phase of n-butanol extraction and it was kept at -20° C.

2.3 SPLEEN CELL COLLECTION AND SINGLE CELL SUSPENSION PREPARATION

Mice were sacrificed by cervical dislocation. The spleens were removed and placed in about 3 ml Earle's Balance Salt Solution (EBSS) with HEPES buffer. A single cell suspension of spleen was prepared by pressing the spleen between two frosted ends of microscope slides(SOP/PFC/006, Medical College of Virginia, 2000). The slides were washed with buffer and pipetted into a 60 x 15 mm petridish. The suspensions were filtered though a cell strainer (pore size 40 μm and then put into a 5 ml plastic capped test tube. The splenocytes were centrifuged at 1,200 rpm for 5 minutes and then resuspended in fresh RPMI complete media (RPMI+10% FBS). The cells viability was determined by trypan blue dye exclusion.

2.4 MTT COLORIMETRIC ASSAY

The assay was slightly modified from the method of Martin,A., and Clynes, M.(1993) for evaluation of the proliferation or cell viability. The assay was performed by adding 1 μg/ml of MTT final concentration into each well of 96-well plates. The plates were incubated at 37°C, 5% CO_2 for 4 hours. After incubation, the medium was carefully removed without disturbing the formazan crystals (Fig. 11). 100 μl of Dimethyl sulfoxide (DMSO) was added to each well and aspirated repeatedly to give a uniform dark purple color (Fig. 12) before reading at 595 nm by an ELISA plate reader.

Figure 11 Crystals of formazan product after the addition of MTT for measuring cell proliferation by MTT colorimetric assay.

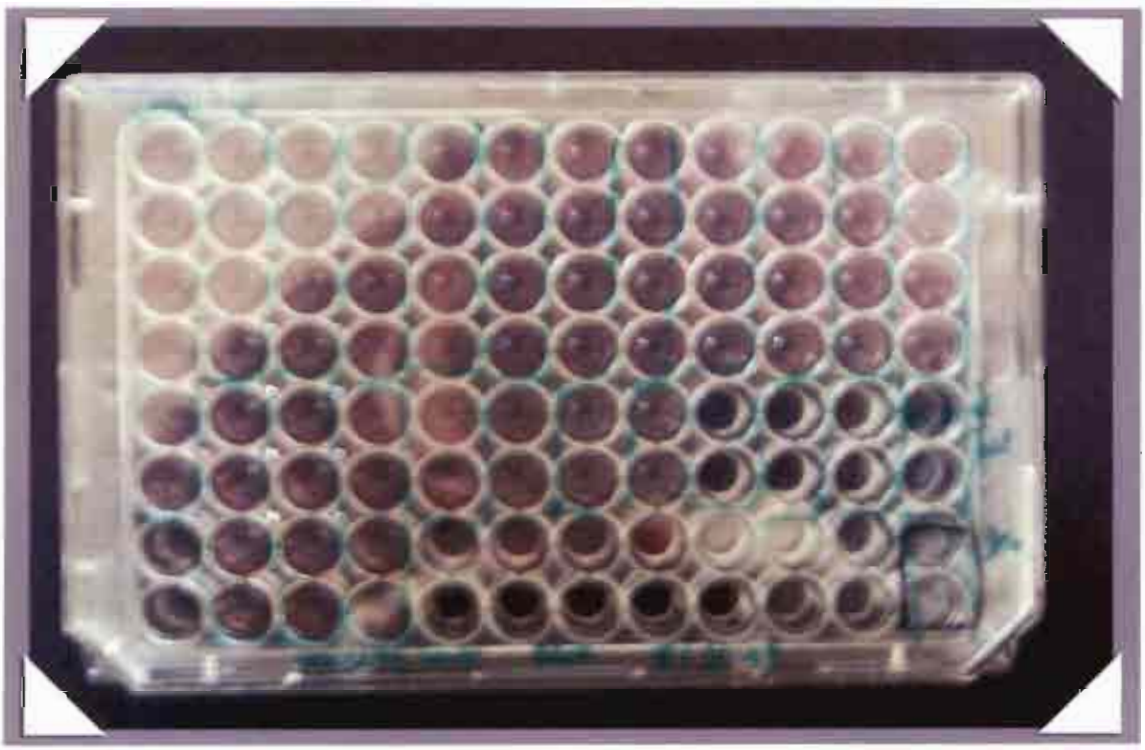

Figure 12 Dark purple color of formazan product that occurred after DMSO was added into each well in the MTT colorimetric assay in a 96-well plate.

2.5 PREPARATION OF HUMAN OVARIAN CANCER CELL (HeLa)

Human ovarian cancer cells (HeLa cells-Fig. 13) is the human ovary cancer cell line. The growth characteristics of HeLa is anchorage and it grows as a monolayer in a culture flask. Only viable cells can attach to the bottom of the flask. HeLa cells were obtained from Riken Gene Bank,

Japan. Cells were thawed from frozen stock in liquid nitrogen and washed with PBS twice to remove the toxic cryopreservative agent, DMSO. The cells were centrifuged at 1,200 rpm for 5 mins, and the cells pellets were cultured in Ham's F12 medium supplemented with 10% FCS, L-glutamine, sodium bicarbonate, and penicillin and streptomycin in a 25 cm^2culture flask at a 5 ml total volume. Flasks were incubated at 37°C, 5% CO_2. At the log phase growth (2-3 days) cells were removed from the culture flasks by adding 0.5 ml of trypsin (0.5% trypsin with 0.2% EDTA w/v) into a 25 cm^2 culture flask. Flasks were incubated at 37°C, 5% CO_2 for 5 mins and the cells were washed twice with PBS. A single cell suspension was prepared by resuspending the cells with a Pasteur pipette. Cells were diluted to make the final concentration of $1.5x10^5$ cells/ml for the cytotoxicity experiment. Further- more, cells were prepared to the concentration of $2x10^5$ cell/ml and aliquot 1 ml per vial for making a frozen stock in liquid nitrogen. .

Figure 13 Growth characteristics of normal HeLa cell in Ham's F12 Media viewed under inverted microscope

2.6 EXPERIMENT PROTOCOL

2.6.1 The selection of a suitable concentration of whole plant extracts and seed extracts of AIR in *in vitro* toxicity studies

In the first experiment, C57BL6/j 6 mice were used in the toxicity test. Mice were sacrificed by cervical dislocation. Spleens were collected by aseptic technique and single cell suspensions were prepared. The spleen cells were cultured and diluted to $4x10^6$ cells/ml in RPMI media supplemented with 10% heat-inactivated fetal calf serum (FBS) and were kept at 4°C before being plated on a flat bottomed 96-well plate.

Whole plant extract powders were weighed and diluted in RPMI media alone to a final concentration of 10,000 µg/ml to 1.25 µg/ml in a 96-well flat-bottomed plate. Before being added to the plate, the extract was sterilized and filtered though a 0.22 µm filter.

All *In vitro* toxicity studies were done under a sterile hood and the experiment protocols were as follows:

$4x10^6$ cells/ml of splenocytes were plated to 96-well plate (flat bottom) for 4 replicates. 50 µl of plant extracts were added into each well to obtain final concentrations of 10,000 to 1.25 µg/ml or per well. On another plate, 50 µl of seed extracts were added to each well to obtain final concentrations of 10,000 to 1.95 µg/ml. The plates were incubated overnight at 37°C, 5% CO_2. Cell viability was determined by using the MTT colorimetric method.