

**STUDY OF IMMUNOTOXICOLOGICAL EFFECTS OF
DOK DIN DAENG (*Aeginetia indica* Roxb.)**

MISS WIMOLNUT AUTTACHOAT

**A Thesis Submitted in Partial Fulfillment of the Requirements for
the Degree of Doctor of Philosophy in Environmental Biology
Suranaree University of Technology
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การศึกษาพืชวิทยาและผลต่อระบบภูมิคุ้มกัน
ของดอกดินแดง (*Aeginetia indica* Roxb.)

นางสาววิมลรัฐ อัครโชติ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

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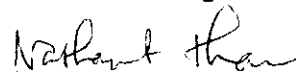
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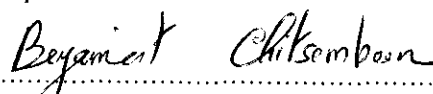
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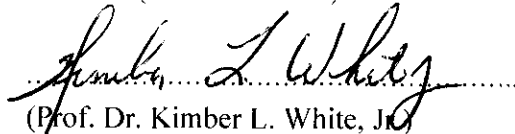
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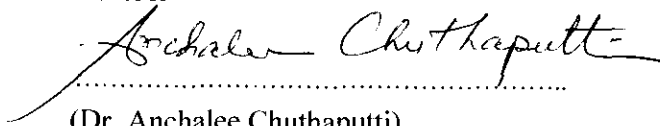
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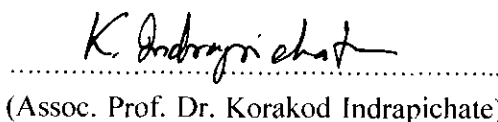
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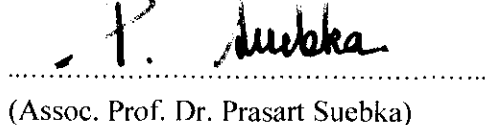
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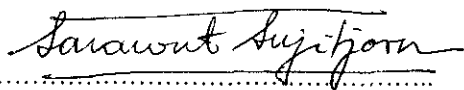


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(STUDY OF IMMUNOTOXICOLOGICAL EFFECTS OF DOK
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การศึกษาพิษต่อระบบภูมิคุ้มกันของดอกดินแดงใช้สารสกัดจากพืชทั้งต้นสกัดด้วยเอทานอล (DDDP) และ น้ำ (WDDDP) และ ส่วนเมล็ดสกัดด้วยบิวทานอล (SDDD) ผลจากการทดลอง *in vitro* เมื่อใช้เซลล์ของหนูเมาส์ C57BL6/j พบว่าสารสกัดจากดอกดินแดง SDDD และ DDDP มีฤทธิ์กระตุ้นระบบภูมิคุ้มกัน สารสกัดจากต้นสามารถกระตุ้นการตอบสนองของ T เซลล์ต่อ concanavalin A และ anti-CD3 แอนติบอดี และกระตุ้นการตอบสนองของ B เซลล์ต่อ lipopolysaccharide ที่ความเข้มข้นระหว่าง 1.25-500 µg/ml หนู B6C3F1 ที่ได้รับสารสกัด DDDP และ WDDDP เป็นเวลา 28 วันไม่เกิดการตายหรือแสดงอาการเป็นพิษ สารสกัด DDDP และ WDDDP สามารถกระตุ้นการทำงานของ T lymphocytes ใน MLR และ CTL ในการทดลอง *in vivo* หรือใน *in vitro* อย่างมีนัยสำคัญทางสถิติ แต่ไม่มีผลกระทบต่อการสร้างแอนติบอดีของ B เซลล์ และ NK เซลล์ นอกจากนั้นเซลล์ม้ามจากหนู B6C3F1 ที่ได้รับสารสกัด DDDP ไม่มีผลเปลี่ยนแปลงการสร้าง IL-2 และ IFN-γ ในการทดลอง *in vitro* อย่างไรก็ตาม หนู B6C3F1 ที่ได้รับสารสกัด WDDDP เกิดการลดค่าร้อยละและจำนวนรวมของ T เซลล์ชนิด CD4⁺CD25⁺ ในม้ามซึ่งมีหน้าที่ควบคุมการกระตุ้นของ T เซลล์อื่น ผลโดยรวมในการศึกษานี้แสดงให้เห็นว่าสารสกัดจากดอกดินแดงมีฤทธิ์กระตุ้นการตอบสนองของระบบภูมิคุ้มกัน

สาขาวิชาชีววิทยา

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ลายมือชื่อนักศึกษา.....

ลายมือชื่ออาจารย์ที่ปรึกษา.....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

**WIMOLNUT AUTTACHOAT : STUDY OF IMMUNOTOXICO-
 LOGICAL EFFECTS OF DOK DIN DAENG (*Aeginetia indica* Roxb.)
 THESIS ADVSIOR: ASST. PROF. BENJAMART CHITSOMBOON,
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This study was focused on the immunotoxicological effects of a whole plant ethanol extracts (DDDP), water extracts (WDDDP) and seed extracts (SDDD) of *Aeginetia indica* Roxb. (DDD). Results from *in vitro* studies in C57BL6/j mice suggest that DDDP and SDDD possess immunostimulatory activity. Enhancement of T cell mitogenic responses to concanavalin A and anti-CD3 and B cell response to lipopolysaccharide were observed at 1.25-500 µg/ml of extracts. Exposure to DDDP and WDDDP in B6C3F1 mice for 28 days did not induce mortality or any overt toxicity. Significant increase in T cell functions including MLR and CTL activity were observed after DDDP and WDDDP treatment in either *in vivo* or *in vitro*. Nevertheless, the antibody forming cell response and NK activity were not affected. In addition, *in vitro* exposure of splenocytes to DDDP did not alter IL-2 and IFN-γ production. Finally, WDDDP exposed mice decreased both the percentage and absolute number of regulatory CD4⁺CD25⁺ T cells in the spleen. Overall, this study demonstrated that the DDD extracts have the potential to stimulate immune response.

School of Biology

Academic Year 2003

Student's Signature.....

Advisor's Signature.....

Co-advisor's Signature.....

Co-advisor's Signature

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LIST OF ABBREVIATIONS

2 ME	2-Mercaptoethanol
%	Percent
α	Alpha
AAGM1	Rabbit Anti-Asialo GM1
AFC	Antibody Plaque Forming Cells
DDD	<i>Aeginetia indica</i> Roxb.
DDDP	Ethanollic Whole Plant Extract
β	Beta
$^{\circ}\text{C}$	Degree Celsius
CD	Cluster of Differentiation
cm	Centimeter
CPM	Counts per Minute
CPS	Cyclophosphamide
Con A	Concanavalin A
CTL	Cytotoxic T cells
DEAE	Diethyl-Aminoethy
EBSS	Earle's Balanced Salt Solution
EDTA	Tripotassium Ethylene Diaminetetracetate
ϵ	Epsilon
ELISA	Enzyme Linked Immunoabsorbant Assay
et al	et alia (and others)

LIST OF ABBREVIATIONS (Continued)

FBS	Fetal Bovine Serum
Fig.	Figure
FITC	Fluorecein Isothiocyanate
fl	Femtoliter
g	Gram
g/dl	Gram per Deciliter
GPC	Guinea Pig Complement
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HeLa	Human cervical carcinoma
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LPS	Lipopolysaccharide
mAB	Monoclonal antibody
Mac3	Mouse antigen for subset of monocytes and macrophage
MC	Modulatory Control
MCH	Mean Corpuscular Hemoglobin
MCHC	Mean Hemoglobin Corpuscular Concentration
MCV	Mean Corpuscular Volume

LIST OF ABBREVIATIONS (Continued)

mg/ml	Milligram per Milliliter
min	Minute
ml	Milliliter
mm ³	Millimeter cubed
MLR	Mixed Leukocytes Response
μm	Micrometer
μg/ml	Microgram per Milliliter
mmho	Milliohm
mM	Milli Molar
MTT	3-4,5-dimethylthiazol-2-yl)-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
pg	Picogram
PI	Propidium Iodide
PE	Phycoerythrin

LIST OF ABBREVIATIONS (Continued)

ppm	Part per Million
rpm	Revolution per Minute
RPMI 1640	Roswell Park Memorial Institute
SDDD	Butanol-saturated water of seed extracts
S.E.	Standard Error
TNF	Tumor Necrosis Factor
WDDDP	Water whole plant extracts
v/v	Volume: Volume
x g	x Gravitational acceleration

CHAPTER I

INTRODUCTION

Scientists are interested in the stimulation of the human's own immune system as an alternative remedy, since many available drugs 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 adverse side effects. At present, numerous medicinal plants have been identified and developed as chemotherapeutic agents and as modulators of the immune system, capable of increasing macrophage activation and enhancing T and B lymphocytes and natural killer cell function.

Several plant-derived immunostimulatory drugs have been used widely in Europe and Asia as traditional medicine for self-medication as well as serving as starting material for various prescription drugs. Examples include the water and alcoholic extracts of *Echinacea purpurea*, *E. angustifolia* and *E. pallida* (herb and root) (Bauer et al., 1998), *Panax ginseng* (Friedl et al., 2001; Wang et al., 2003), *Eleutherococcus senticosus* (Glatthaar-Saalmuler et al., 2001), *Rehmania glutinosa* and etc. (Hong et al., 2002; Arnason, Mata and Romeo, 1995). Among these plants, *Aeginetia indica* Linn. (AIL), a parasitic plant which grows on roots of Japanese pampa grasses or sugar canes has long been used as a tonic and anti-inflammatory medicinal herb in China and Japan (Bando et al., 1988). 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 sarcoma (S-180) tumor cells (Chai et al., 1990) which might be mediated through the CD4⁺ T cells (Chai et al., 1992 and Chai et al., 1994). The polysaccharide fraction used at 100 µg/ml activated B-lymphocytes and thymocytes. *In vitro* studies showed the ability of AIL to induce the production of interleukin-2, interferon γ , and interleukin-6 (Chai et al., 1994). The 55kDa protein from the seed extract, induced various cytokines, including IL-2, IL-6, IL-10, IL-12, and IL-18. There was also induction of IFN- γ , TNF- α , granulocyte macrophage-colony stimulating factor (GM-CSF) and the activation of cytotoxic activities of peripheral blood mononuclear cells (PBMC) in *in vitro* models. (Chai et al., 1994; Okamoto et al., 2000; 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 sesquiterpene, β - steroid, carotenoid, monoterpene, glucoside and flavonoid (Dighe et al., 1977; Endo et al., 1979; Oshima et al., 1984).

In Thailand, *Aeginetia indica* Roxb. (DDD) has several local names such as, Dok din daeng (Trat Province), So-suai (Karen-Mae Hong Son Province), Paak cha khe (North-east), Sop laeng (Song khla Province) or Yaa dok khol (Loei Province) (เต็ม สมิตินันท์, 2544). It has been used in Thai traditional medicine for the treatment of diabetes and dermal swelling when prepared as a whole plant extract (Muller-Oerlinghausen, Ngamwathana, and Kanchanapee, 1971). This plant is a parasitic plant

which grows primarily on the roots of other plants. In Thailand, it likes to grow under the shady areas of bamboo. 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” (นิจศิริ เรื่องรังสี และ พยอม ตันติวัฒน์, 2534).

However, prior to this research, there have been no published studies on the pharmacological, toxicological, or immunological effects of this plant indigenous to Thailand.

Normal chemical constituents found in all plant tissues are principally carbohydrates, fat, oils and protein. Plants may contain other minor compounds such as, phytin, alkaloid, and raffinose etc. Vast arrays of natural organic acid compounds, the products of primary and secondary metabolism, occur in plants that are used in many countries as traditional herbal medicines (Harborne and Baxter, 1993)

It appears that many natural organic compounds in plants have immunostimulating activities, as demonstrated in *in vitro* and experimental animal studies as well as clinical trials. Immunostimulation have been associated with lipophilic compounds (alkylamides) and a polar fraction (cichoric acid and polysaccharides). The other potential immunostimulating compounds are alkaloid (e.g. isopteropodine), terpenoids, flavonoids (e.g. anthocyanin), resin, quinones (e.g. plumbagin), macrocyclic lactones (e.g. bryostatins) and glycoproteins (lectin) (Arnason et al., 1995; Mills et al., 2000).

The most frequent solvents for extracting these organic compounds are alcohol and water extraction. Solvents such as ethanol, methanol, hexane, n-butanol, chloroform and etc., have been used to extract essential oil, polysaccharide, glycoprotein, phenolic, terpenoid, waxes, fats, some resin and portions of wood gum.

By contrast, hot water extractions were used to remove tannin, monosaccharide, flavonoids, polysaccharide 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, 1984; Ross and Brain, 1977)

In addition, plant parts which have usually been used to screen for biological activities in phytomedicine are whole plants, seeds, flowers, roots, and leaves (Weiss, 1998). In the case of *Aeginetia indica*, a parasitic plant, the whole plant has been used in 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 potential immunological effect of DDD. Using aqueous alcohol such as 95% ethanol, water and water saturated with n-butanol (high polarity) for extraction may be useful in obtaining 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 available to provide qualitative and quantitative data on the extract effects on the major components of the immune system including specific and non-specific immunity. The first tier of testing is a screen for general immunotoxicity, and second tier test consists of confirmatory studies and studies to establish the specific mechanism of action. In this research, parameters of the first tier of testing which were evaluated included hematologies, lymphoid organ weights and analysis of cellular subpopulations in spleen by flow cytometry. The second tier was comprised of various functional immune assays such

as lymphoproliferative responses, splenic IgM-antibody plaque-forming cell assay, natural killer cell activity, mixed leukocyte response assay, cytotoxic T lymphocytes assay and cytokine assay. (Vos, De Waal, Loveran, Albers, and Pieters, 1999)

Immunotoxicological *in vitro* studies have proven to be suitable for screening for potential effects on immune function. Therefore, in initial studies DDD extracts were evaluated *in vitro* to study effect of the immune system using C57BL6/j mice, an inbred mouse. This strain of mouse is used as the female parent of a hybrid mouse utilized for *in vivo* toxicological standard test. However, to provide a better understand of the effects of DDD on the immune system and to determine the mechanisms of action of DDD, an hybrid (F1) mouse strain, the B6C3F1 was used. The B6C3F1 is the predominant mouse strain used in conducting immunotoxicological studies.

The exposure period for an immunotoxicology study required to assess chemical-induced alteration in the immune system has been recommended by US National Toxicology Program (NTP) to be a repeat exposure regimen of 14-30 days in young-adult B6C3F1 mice (Dean and Vos, 1986).

In order to provide information on the immunostimulating potential of DDD, *in vivo* experiments were designed evaluating daily intraperitoneal (i.p.) injection of ethanolic whole plant extracts for 28 days, and daily oral administration of Thai traditional water extracts from whole plant of DDD in B6C3F1 mice for 28 days.

As indicated earlier, in Thailand DDD has been used as a traditional plant medicine and as a dessert. No studies have been conducted to examine its toxicity and immunological effects. Since environmental factors have the ability to affect the pharmacological activities of the same kinds of medicinal plants, the pharmacological

activities of the same species of medicinal plants can vary at different geographical locations. In the present study, the immunotoxicological effects of butanol seed extract (SDDD) and ethanol whole plant extract (DDDP) were examined first in *in vitro* studies in C57BL/6j mice with the purpose of screening potential effects of DDD to enhance immune system. The *in vivo* study was conducted in the B6C3F1 mice to compare the relevant results to the *in vitro* study. However, this model was designed to evaluate the immunological effects of ethanol whole plant extract (DDDP), which was the fraction of extract that also contained seed. The latter *in vivo* study was focused on the administration of DDD extract simulating human utilization. The water whole plant extract (WDDDP), traditional whole plant extract, was investigated in B6C3F1 following oral gavage which was the relevant route to the human administration. The mechanism of action of DDDP extract was finally characterized in B6C3F1 mice in *in vitro* studies.

1.1 Objectives of the study

1. To screen for any stimulatory potential effects of SDDD and DDDP extract on various cell types of the immune system *in vitro* study.
2. To study immunotoxicological effects of DDDP extract in B6C3F1 mice following systemic administration by the intraperitoneal route.
3. To study immunotoxicological effects of WDDDP extract in B6C3F1 mice following oral administration.
4. To determine the mechanism of action of immunological effect of DDDP *in vitro*.

1.2 Research hypothesis

1. The water-saturated butanol seed extract of *Aeginetia indica* Roxb. could enhance immune response *in vitro*.
2. The ethanol whole plant extract of *Aeginetia indica* Roxb. could enhance immune response *in vitro* and *in vivo*.
3. The water whole plant extract of *Aeginetia indica* Roxb. could enhance immune response *in vivo*.

1.3 Scope and limitation of the study

Different immunological effects of *Aeginetia indica* Roxb. (DDD) extracts from seeds and whole plants were assessed *in vitro* and *in vivo*. Seed extract was obtained from water-saturated butanol extraction where as dried powder of whole plants were extracted by 95% ethanol and sterile water. The ethanol whole plant extract was selected for the investigation of immunostimulatory activity in B6C3F1 mice. Decoction of whole plant extract of DDD was also prepared and investigated for its immunoenhancing activity in female B6C3F1 mice via oral gavage to mimic the relevant routes of Thai people consumption of this herbal medicine. The immunological parameters evaluated in this study were lymphocytes proliferative responses to specific mitogens and anti-CD3 antibody, plaque forming cell (PFC), mixed lymphocytes response assay (MLR), natural killer cell assay (NK), cytotoxic T lymphocytes assay (CTL), phenotypic surface marker analysis, and cytokine production analysis. The general toxicity parameters including body weight, organ weight, and hematology were investigated.

1.4 Expected results

The outcomes from this study should:

1. Provide information on immunological effects of Thai medicinal plant, *Aeginetia indica* Roxb., which can be used as a basic pharmacological data for the clinical treatment in the future.
2. Provide basic toxicological data of *Aeginetia indica* Roxb. which is beneficial for safety evaluation.
3. Encourage Thai people to realize and appreciate the value of Thai medicinal plant.
4. Enhance the development of Thai traditional medicine practice.
5. Indirectly, enhance local economics in growing high potential medicinal plants.

CHAPTER II

LITERATURE REVIEWS

1. *Aeginetia indica* Roxb.

1.1 Botanical information and distribution

Family: Orobanchaceae

Common name: Dok din daeng (Trat), So-suai (Karen-Mae Hong Son), Paak cha Khe (North-east), Sop laeng (Song Khla) or Yaa dok khol (Loei) (เต็ม สมิตินันท์, 2544).

Botanical description:

This plant is a grass glabrous. Stem is 3-6 cm high (Fig.1 and Fig.2). It is a root parasite which is no ramifications. The scales are narrow and sharp with 10 mm long, which is located at the base of stems, alternating or sub-opposite sometimes. Floral stalks are solitary and terminal, or in very small numbers, alternating or sub-opposite, thin, bare, slightly streaked lengthwise which is high 15-35 cm. Calyx is spathaceous and split in front at almost to the base. It is sharp or spear-shaped with 15-35 mm long which has a pale violet or red-streaked color. Corolla tubular is strangled a little above the base then very strongly bent at an angle in its lower half, the curved axis of the flowers forming a 90 degree angle (or even higher) with the angle of the stalk, violet color, 2-4 cm long; scalloped edge with 5 teeth. Stamens 4, didynamous, inserted a little above the narrowing of the corolla; filets filiform, verrucose; anthers connective,

kidney-shaped, 3-4 mm long, the upper ones without appendages, the 2 lower ones ending in the back like an arched spur, humpbacked, 2-3 mm long. Ovary located above the insertion point of the sepals, petals and stamens with 1 lodge; egg shaped, smoothed out at the top; style long and arched; stigma large, fleshy, peltate-infundibular. Fruit is capsule, included in the persistent calyx, same shape as the ovary; numerous seeds, of a yellowish white color; teguments glassy, flaccid (Gagnopain, 1927).

Ecology and distribution:

This plant is a parasitic plant growing on the roots of other various species of family Graminae, especially on the bamboos, and also on family Zingiberaceas (Gagnopain, 1927).

In Thailand, it likes to grow under bamboo in shady areas (Fig. 3). The flower blossoms between September and October which is the rainy season in Thailand (นิจศิริ เรืองรังสี และ พยอม ตันติวัฒน์, 2534). 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, 1987). The soil analysis and rain precipitation in area from which *Aeginetia indica* Roxb. used in this study is shown in figure 4 and table 1, respectively.

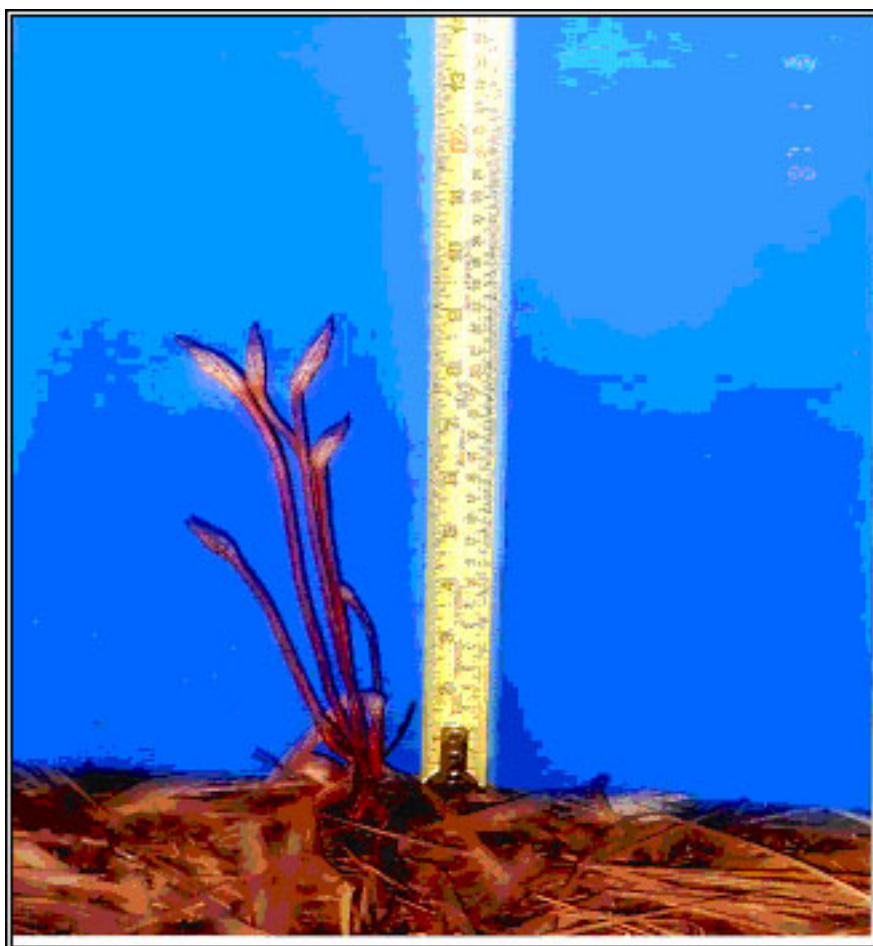


Figure 1 Whole Plant of *Aeginitia indica* Roxb.

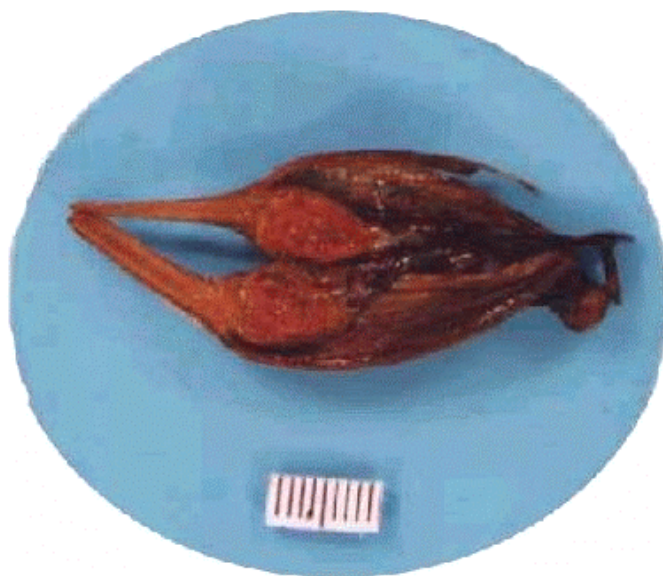


Figure 2 The flower of *Aeginitia indica* Roxb.



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

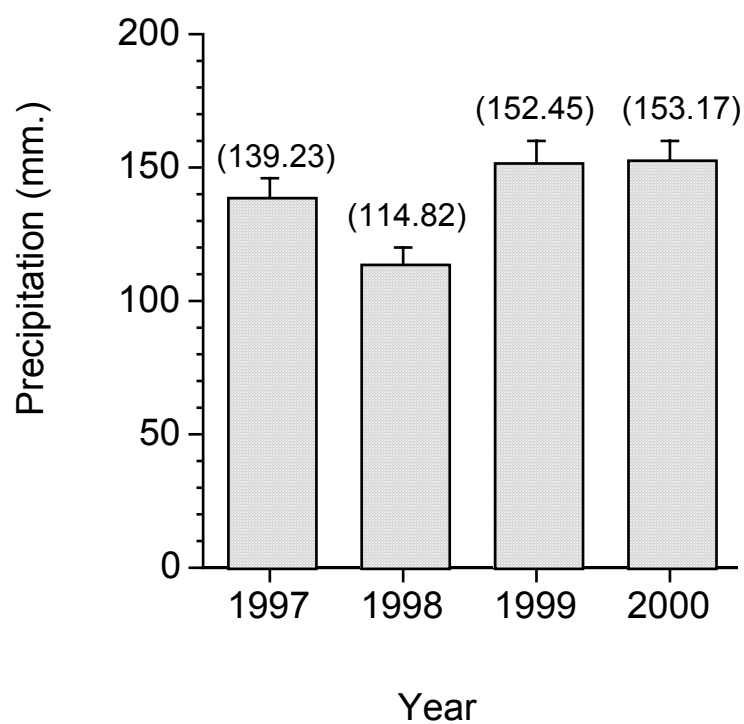


Figure 4 Graph of average precipitation at Aumphur Wang Num Keaw, Nakhon Ratchasima province, in the area of *Aeginetia indica* Roxb. growth. Measured by the Meteorological Department, Aumphur Wang Numkeaw, Nakhon Ratchasima, Thailand.

Table 1 Results of analyzed minerals in soil where *Aeginetia indica* Roxb. grows at Aumphur Wang Num Keaw, Nakhon Ratchasima. This analyzes were evaluated 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		
pH	Concentrate Acid	High Acid	Intermediate Acid	A little bit of Acid	Normal	Base
		4.49				
Salinity	Normal	Very Low	Low	Intermediate	Very High	
	0.03 mmho at room temperature					

1.2 Propagation

Aeginetia indica 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 undifferentiated 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 multicellular 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 haustorium. 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 warm stratified. According to Musselman (1980, quoted in Baskin and Baskin, 1998, p. 461) 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 which were maintained 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 to germinate. 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. These 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 stimulants in root exudates of the principal host species for *Orobanche*. A partially purified germination stimulant from faba bean root exudates possibly may be an unsaturated lactone. Other compounds analyzed from broad bean, tomato and flax were also identified as unsaturated lactones which are composed of a carbonyl group that has a single bond, double bond carbon, alicyclic structure, aliphatic chain, strigol, and its analog. Those compounds were isolated and identified by thin layer chromatography and nuclear magnetic resonance.

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 *Orobanche crenata* seeds from 46% to 26%. (Baskin and Baskin, 1998)

1.3 Chemical constituents

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
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 (นิจศิริ เรืองรังสี และ พยอม ตันติวัฒน์, 2534).

1.4 Ethnopharmacological 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 and Kan, 1990) and liver diseases (Yanfg et al., 1987)

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

1.5 Pharmacological studies:

1.5.1 Antitumor activity

Seed extract (AIL) 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; 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). All of mice in untreated group died of fatal tumor growth in the ascide form within 16 days where as all mice in the AIL-treated group completed recovered from tumor challeng and survived for more than 60 days. Moreover spleen and lymph node cells treated with the AIL extract at 2.5 mg/kg inhibited the growth of Meth A *in vitro* study at the effector to

target ratios of 5:1 and 10:1. However depletion of CD8⁺ T cells did not influence on the survival rate of treated mice that were challenged with Meth A tumor cell. Thus, CD4⁺ T cells were reported as an essential T cells in host resistance against Meth A after AIL treatment *in vivo* (Chai et al., 1992). Also there was antitumor effect of its extract on human salivary adenocarcinoma grown in nude mice (Bando et al., 1980).

1.5.2 Mitogenic activity

The polysaccharide fraction of AIL which was used at 100 µg/ml activated B-lymphocytes and thymocytes (Chai et al., 1994). T enriched lymphocytes proliferated when they were cultured with AIL *in vitro*. Thymocytes showed a slight response when they were stimulated with AIL alone and this response remarkably enhanced thymocytes when they were cultured with AIL in the presence of suboptimal dose of Con A (0.6 µg/ml). According to thymocyte proliferation results *in vitro* study, AIL shown to have a co-stimulating effect with Con A in proliferating thymocytes (Chai et al., 1994).

1.5.3 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- α , 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; Ohe et al., 2001).

2. IMMUNE SYSTEM

The role of the immune system is to confer protection to the host against potential harmful molecules, or pathogen. In performing this function, the immune system engages elements from both the natural (native or innate) and specific immune system. The natural immune system is characterized by its lack of immunological specificity. The natural immunity is comprised of the antigen non specific action of phagocytic cells in the blood and tissues, circulating molecules such as the complement components, and a lymphocytes population known as natural killer cells (NK). NK cells are characterized by their ability to spontaneously kill virally infected cells or a variety of tumors. In contrast, the specific component, adaptive immunity, displays a high degree of specificity as well as the remarkable property of memory. (Bertagnolli, 1993; Goldsby, Kindt, and Osborne, 2000)

An effective immune response involves two major groups of cells: lymphocytes and antigen presenting cells.

2.1 Lymphocytes

Lymphocytes are one of many types of white blood cells produced in the bone marrow by the process of hematopoiesis. Lymphocytes leave the bone marrow, circulate in the blood and lymphatic system, and reside in various lymphoid organs. Because they produce and display antigen binding cell surface receptors, lymphocytes

have the defining immunologic attributes of specificity, diversity, memory, and self/nonself recognition. The two major populations of lymphocytes are B lymphocytes and T lymphocytes (Goldsby et al., 2000)

2.1.1 B lymphocytes

B lymphocytes mature in the bone marrow; when they leave it, each express a unique antigen-binding receptor on its membrane. The B cells receptor is a membrane bound antibody molecule. When a naive B cell, which has not previously encountered antigen, first encounters the antigen that matches its membrane bound antibody, the binding of the antigen to the antibody causes the cell to divide rapidly; its progeny differentiate into memory B cells and effector B cells called plasma cells. Plasma cells do not express membrane bound antibody; instead they produce the antibody in a form that can be secreted. Secreted antibodies are the major effector molecules of humoral immunity (Goldsby et al., 2000).

2.1.2 T lymphocytes

T lymphocytes also arise in the bone marrow, but T cells migrate to the thymus gland to mature. During its maturation within the thymus, the T cell comes to express a unique antigen binding molecule, call the T cell receptor, on its membrane. T cell receptors can recognize only antigen that is bound to cell membrane protein called major histocompatibility complex (MHC) molecules. MHC molecules that function in this recognition event, which is termed “antigen presentation”, are polymorphic (genetically diverse) glycoprotein found on cell membranes (Zinkernagel and Doherty, 1974). In the absence of both antigen and the appropriate MHC molecule, thymocytes

bearing the transgenic receptor fail to develop into mature T cells and instead undergo programmed cell death within the thymus. In the presence of the appropriate MHC allele, however, mature T cells are produced. These events indicate that T cells are positively selected by the correct MHC allele and must recognize it dependently of the antigenic peptide, whereas negative selection eliminates those cells that are specific for self peptides bound to self MHC molecules (Sha et al., 1988; Kisielow et al., 1988).

There are two distinct classes of MHC molecule capable of presenting antigens to T cells. Class I MHC molecule (H-2 K, D and L in mouse) are expressed on almost all nucleated cells, although the level of expression does vary between cell types. On the other hand, MHC class II (H-2E and A in mouse) are expressed only on a subset of cells with specific immunologic function namely dendritic cells, macrophages, B cells, and thymic epithelial cells. The source of antigens presented by MHC class I and class II molecule also differs. MHC class I molecules present antigens derived from cytoplasmic peptides (Townsend and Bodmer, 1989). In contrast, MHC class II molecule present peptide antigens derived predominantly from extracellular source (Broadsky and Guagliardi, 1991).

There are two well-defined subpopulations of T cells; T helper (T_H) and T cytotoxic (T_c) cells. Although a third type of T cell, called a T suppressor (T_s) cells, has been postulated, recent evidence suggest that it may not be distinct from the T_H and T_c subpopulation. T helper and T cytotoxic cells can be distinguished from one another by the presence of either their CD4 or CD8 membrane glycoproteins on their surface. Each co-receptor (CD4 or CD8) recognizes only one class of MHC molecule, and which is involved in T cell differentiation and activation (Anderson, Blue, Morimoto, and Schlossman, 1987). T cells displaying CD4 generally function as T helper cells;

where as those displaying CD8 generally function as T cytotoxic cells (Swain, 1983).

After T helper cells recognizes and interacts with an antigen MHC class II molecule complex, the cell is activated, then it becomes an effector cell that secret various growth factors known collectively as cytokines. Under the influence of T helper derived cytokine, a T cytotoxic cells that recognized antigen-MHC class I molecule complex proliferates and differentiates into an effector cell called cytotoxic T lymphocyte cell (CTL). The CTL has a vital function in monitoring the cells of the body and eliminating any, such as virus infected cells, tumor cells and cells of foreign tissue graft, that display antigen. Recognition of antigen in association with MHC class I molecule results in the death of the presenting cell (Gliffiths and Mueller, 1991).

2.2 Antigen presenting cells

It is essential that activation of T helper cells themselves be carefully regulated, because an inappropriate T helper cells response to self components can have fatal autoimmune consequences. To ensure carefully regulated activation of T helper cells, they can recognize only antigen that is displayed together with class MHC II molecules on the surface of antigen presenting cells (APCs). These specialized cells, which include macrophages, B lymphocytes, and dentritic cells, are distinguished by two properties. First, they express class II MHC molecules on their membrane, and second is they are able to deliver a co-stimulatory signal that is necessary for T helper cells activation (Goldsby et al., 2000). An example of a co-stimulator is the B7 molecule on proffessional antigen-presenting cells that bind to the CD28 molecule on T lymphocytes. The interaction of CD28/CTLA-4 (CD152) with B7 to induce full transcription of IL-2 mRNA is an example of co-stimulator mechanism (Schwartz,

1992).

2.3 CD antigen

CD refers to cluster of differentiation, which was devised to classify the transmembrane proteins identified by various monoclonal antibodies. Each unique transmembrane structure defined by reactivity with a monoclonal antibody is assigned a unique CD number, signifying that the molecule is recognized by one or more, i.e., a “cluster” of, monoclonal antibodies. The availability of panels of monoclonal antibodies reagent possessing defined specificity for a single CD molecule has permitted the routine classification of lymphocyte populations and subpopulations according to surface expression of particular CD molecules (Goldsby et al., 2000).

Examples of some important lymphocyte cell surface protein are CD3, CD4, CD8, CD25. Furthermore, CD28, CD3, CD4 and CD8 are identified as T cell receptor antigen, the CD3 refers to the T cell receptor while CD4 and CD8 are distinguish by the recognition of difference types of MHC. The CD4 generally binds to MHC class II and is defined as the helper T cell subset, in contrast, CD8 commonly binds with MHC class I and is defined as a cytotoxic T cell subset (König, Huang, and Germain, 1992). However, there is evidence that when CD8-alpha chain was attached to the transmembrane and signaling domains of CD4 molecule, this hybrid co-receptor was expressed and was MHC class I restricted in transgenic mice, and instructed the cell to assume as CD4⁺ phenotype (Seong, Chamberlain, and Parnes, 1992). CD25 is known as low-affinity IL-2 receptor which is present on activated T, B, NK cells and macrophages (Shimonkevitz et al., 1987). Another one is CD28 which is widely accepted to cause co-stimulation of T cell proliferation and cytokine production upon

binding CD80 and CD86. It is found predominately on mature CD3⁺ thymocytes, most peripheral T cells and plasma cells (June, Ledbetter, Linsley, and Thompson, 1990).

The method used to provide quantitative data of lymphocytes subpopulation is by labeling cells surface structures with fluorochrome-conjugated antibodies (e.g. FITC-CD3, PE-CD4, FITC-CD8) directed against isolated lymphocyte populations and evaluated with flow cytometry. An instrument used in flow cytometry is called the fluorescence-activated cell sorter (FACS). Fluorescence-activated cell sorting operates by projecting a single stream of cells through a laser beam (e.g. argon 488 nm or helium-neon 633 nm) in such a way that the light scattered from the cells is detected by one or more photomultipliers. The stream enters at the top of the chamber and it breaks up into droplets by a high frequency vibration applied to the flow chamber. The droplets carry the charge briefly applied to the exiting cell stream and are deflected by the electrode plates below the flow chamber. All cells having similar properties are collected into the same tubes. Facscan usually have three or four fluorescence detection channels which collect light at 525 ± 25 nm (FL1-green), 575 ± 25 nm (FL2-orange), 620 ± 25 nm (FL3-red) and 675 ± 25 nm (FL4, long red). Fluorescein isothiocyanate (FITC) and Phycoerythrin (PE) are a good combination for two color immunolabelling provided that the spectral overlap of FITC is subtracted from the PE channel. For multicolor immunolabelling using two colors, FITC (FL1) and PE (FL2) can be used, because they can excite at the same wavelength (488 nm), but the emitted wavelengths of light are different. The information obtained is then processed and displayed as a two or three dimensional graph on the monitor (Battye and Shortman, 1991; Bertagnolli, 1993; Goldsby et al., 2000; Al-Rubeai, Welzenbach, Lloyd, and Emery, 1997).

2.4 Cytokines

The development of an effective immune response involves lymphoid cells, inflammatory cells, and hematopoietic cells. The complex interactions among these cells are mediated by a group of proteins that are collectively designated cytokines to denote their role in cell to cell communication. Cytokines are low molecular weight regulatory proteins or glycoproteins secreted by white blood cells and various other cells in the body in response to a number of stimuli. They also bind to specific receptors on the membrane of the target cells, triggering signal transduction pathway, that ultimately alter gene expression in the target cells. Cytokines regulate the intensity and duration of the immune response by stimulating or inhibiting the activation, proliferation, and/or differentiation of various cells and by regulating the secretion of antibodies or other cytokines. Cytokines are generally secreted after activation of a particular cell, and secretion is short lived, usually ranging from a few hours to a few days (Goldsby et al., 2000).

Because the cytokine network is so complex, cytokines can be grouped into categories based on their immune function. These categories are broad and extensive overlap occurs between them. The first group, identified as mediators of specific immune function, describes the cytokines that promote an antigen-specific response to antigen. This group includes IL-2, IL-4, IL-6, TGF- β , IL-7, IL-10, IL-12 and IL-13. The second group describes the mediators of natural immunity and inflammatory response. These cytokines include TNF, lymphotoxin, IL-1, IL-5, IL-6, IL-8, the type 1 interferons, IFN- α , IFN- β and IFN- γ . Obviously, these first two groups are interdependent, and the functions of these cytokine overlap extensively. A final group of cytokine consists of the cytokine stimulators of hematopoiesis. These cytokine

include the colony stimulating factor, IL-3, GM-CSF, M-CSF, G-CSF and IL-7 (Bertagnoli, 1993).

However secretion of cytokines by antigen-activated T lymphocytes, T helper cells, is necessary for function of both the humoral and cell-mediated immune response. T helper cells can be divided into two group which is T helper 1 cells and T helper 2 cells. The T helper 1 group mainly secretes IL-2, IFN- γ and TNF- β , where as T helper 2 cells secretes IL-4, IL-5, IL-6 and IL-10 (Mosmann and Coffman, 1989).

Due to an importance role of cytokine in the immune system, the ELISPOT assay was developed as assays to determine the number of cells in a population that are producing cytokines specific for a given antigen for which one has a specific antibody to detect the cytokine.

In the ELISPOT assay, the plate is coated with antibody of a population of cells thought the antigen of interest, an anti-cytokine in this example, and then a suspension of a population of cells thought to contain some members synthesizing and secreting the cytokine and some that are non-secreting are layered onto the bottom of the well and incubated. After an appropriate incubation time, cell are removed by washing. A detection antibody coupled to an enzyme and substrate is added. The colored substrate product precipitates and forms a spot only on the areas of the well where cytokine-secreting cells had been deposited. By counting the number of colored spots, it is possible to determine how many cytokine-secreting cells were present in the added cells suspension (Goldsby et al., 2000).

2.5 Organs in immune system

The immune system consists of many different organs and tissues that are found

throughout the body, these organs can be classified functionally into two main groups. The primary lymphoid organs provide appropriate microenvironments for the development and maturation of lymphocytes. The secondary lymphoid organs trap antigen from defined tissues or vascular spaces and are sites where mature lymphocytes can interact effectively with the antigen. The blood vessels and lymphatic systems connect these organs, uniting them into a functional whole immune system.

The thymus and bone marrow are the primary lymphoid organs, where maturation of lymphocytes occurred. The lymph nodes, spleen, and various mucosal associated lymphoid tissues (MALT) such as gut-associated lymphoid tissue (GALT) are the secondary lymphoid organs, which trap antigen and provide sites for the mature lymphocytes to interact with that antigen. In addition, there is a tertiary lymphoid tissue, which normally contains fewer lymphoid cells during an inflammatory response. Most prominent of these are cutaneous-associated lymphoid tissues. Once mature lymphocytes have been generated in the primary lymphoid organs, they circulate in the blood and lymphatic system, a network of vessels that collect fluid that has escaped into the tissues from capillaries of the circulatory system and ultimately return it to the blood (Goldsby et al., 2000).

2.5.1 Thymus

The thymus is a flat, bilobed organ situated above the heart. Each lobe is surrounded by a capsule and is divided into lobules, which are separated from each other by strands of connective tissue called trabeculae. Each lobule is organized into two compartments: the outer compartment, or cortex, is densely packed with immature T cells, called thymocytes whereas the inner compartment, or medulla, is sparsely

populated with thymocytes. The thymus is an organ which plays an important role in the maturation of T lymphocytes and is used to study immune function in mice (Goldsby et al., 2000). Thymus is also called the thymus dependent (T) lymphocyte organ, because the recovery of lymphoid and immune functions after exposed to irradiation was shown to be thymus-dependent (Miller, 1962). In the thymus, the immature T cells, or thymocytes, proliferate and differentiate, passing through a series of phenotypic stage that can be identified by distinctive patterns of expression of various cell-surface proteins (Spangrude, Heimfeld, and Weissman, 1988). During their development as thymocytes the cells undergo the gene rearrangements that produce the T- cell receptor, and also undergo the positive and negative selection that shapes the mature T-cell receptor repertoire (Janeway et al., 1999).

2.5.2 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 et al., 1999). In most species, the spleen is the largest aggregate of secondary lymphoid tissue in the body. The total lymphoid content reaches adult level at 8-10 weeks of age in rats and mice. In rodent approximately 50% of spleen cells are B lymphocytes and 30-40% are T lymphocytes. The spleen is the primarily organ used in various assays of immunotoxicological studies to evaluate T and B lymphocyte response of the immune system (Pallardy,

Lebrec, Kerdine, Burleson, and Burleson, 2000).

3. IMMUNOTOXICOLOGICAL STUDIES

An Immunotoxicological study 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 (United States Environmental Protection Agency, 1996).

3.1 Tier testing approaches

Due to the complexity of the immune system and the wide variety of agents that have been shown to modulate the immune system, different approaches for evaluating the immune system have been established to assess effects of chemicals on the immune system of rat and mice. Tier-testing approaches have been used to provide data in immunotoxicological testing. Tier I would focus on evaluating relevant pathological and haematological parameters and Tier II focus on evaluating the various component of immune response.

The first tier approach provides a screening mechanism for identification of

potentially immunotoxic compounds. This tier would include the weight and histology of spleen, thymus, liver, lungs, kidneys and bone marrow, spleen cellularity, analysis of lymphocyte populations in spleen by flow cytometry, haematology and functional assays including the antibody response to sheep red blood cells.

The second-tier assays include enumeration of cell types, including T and B cells, resident peritoneal cells and various stem cells of bone marrow, further functional assays of cell population obtained from animals after exposure to the test compound and host resistance assays. Examples of functional assays that were selected in the US National Toxicology Program (NTP) approach for evaluating cell mediated immunity are spleen lymphocyte response to the T lymphocytes mitogen Con A, MLR, and CTL assay. Furthermore, in the panel of the Dutch National Institute of Public Health and the Environment also included is testing humoral immunity using the mitogen response to LPS. The response of spleen cells to Con A provides an evaluation of the ability of T lymphocytes to undergo blastogenesis and proliferation, the MLR measures the ability to respond to alloantigenic determinants and CTL measures the ability of the spleen cells to recognize allogenic cells and proliferate and differentiate into mature cells which are capable of identifying and lysing the foreign cell. Another assay that is required by NTP for measuring innate or non-specific immunity include natural killer (NK) activity (Vos and Dean, 1986; Vos et al., 1999; Miller and Meredith, 2000).

Additional important fundamental principles of toxicology are dose response relationships and time course of the response. Damage to a target organ is related to the concentration of exposed chemical or metabolite in that organ, and the immune system represents a potential target organ system. Assessment of both toxicology and immune responses will provide a means for preliminary detection of an adverse effect on the

immune system following compound exposure.

3.2 Approaches to immunotoxicity assessment

In designing protocols for immunotoxicity assessment, special attention should be given to the choice of species and age of animals as well as duration, dosage, and route of exposure.

It may be desirable to use the same species and strain for immunology studies that is being used in routine toxicity studies. This allows immune status to be evaluated against the background of other standard toxicological parameters. The rationale for selecting the mouse for immunotoxicity at the NTP in the USA was based on the fact that immune system of the mouse was better characterized than the rat and that functional assays were better defined, and the several challenge models were available in the mouse (Vos and Dean, 1986).

Inbred mice such as C57BL/6j and BALB/c are commonly used in immunology studies and toxicology studies. However NTP (USA) has used the mouse hybrid strain, B6C3F1, rather than other species or strain because this has been used by NTP for many toxicological evaluation including long term cancer studies, other strain have been used for eliciting potential hypersensitivity reaction (Meredith and Miller, 2000).

The exposure interval required for a chemical to produce immune dysfunction depends on the type of immunological injury, chemical threshold, toxicokinetics of the compound, and the functional reserve of the immune parameter affected. In general, a subchronic repeat exposure regimen of 14-30 days is employed in young-adult animals for assessment of chemical induced alterations in immune competence.

Dose selection is critical in immunotoxicity assessment. Ideally, high dose overt

toxicity should be avoided, since severe stress and malnutrition are known to impair immune responses. To establish dose-effect relationship, at least three exposure levels are recommended. However, more recent requirement by the US Federal and Drug Administration (FDA) state that the highest dose should result in a dose producing overt toxicity which is the indicator for chemical absorption into animal. The lowest dose ideally should cause no alteration in immune function (Vos and Dean, 1986).

The route of exposure should, if possible, be the same as the natural route of exposure in man. For the majority of xenobiotics, oral exposure (i.e., feeding or gavage) is preferred (Vos and Dean, 1986). Dosing by gavage affords the most accurate intake of test chemical by the oral route. However, parental administration, intraperitoneal (i.p), may also be used to allow a test compound to be given in the precise amounts that reaches the blood without being changed by passage through the gastrointestinal tract and liver (Hayes, 1994).

3.3 Target organs toxicity

In toxicological studies, toxic substances are generally absorbed into the body, then during distributed throughout by various biological mechanisms, it may be metabolized to products that increase or decrease its toxicity, and finally may be removed from the body by excretion. Due to these processes, toxic substances may cause reversible or irreversible reaction with organs and tissues in the body.

Evaluation of target organs such as kidneys, lungs and liver are necessary to provide preliminary safety of chemicals (Grasso et al., 1991; Hildebrand et al., 1991 quoted in Grasso and Hinton, 2000).

3.3.1 Kidneys

The kidney is an organ that plays an important role in the control and regulation of homeostasis. In this regard the organ has a key role in the regulation of extracellular fluid volume and electrolyte composition. The kidney is also the site of synthesis of hormones and certain vasoactive prostaglandins and kinins that influences systemic metabolic function. Erythropoietin, an important stimulus for erythrocyte formation in the bone marrow, is also synthesized in the kidney. A toxic insult to the kidney may affect some or all of these functions but, in general, it is markers of excretory function such as blood urea nitrogen or creatinin and the presence of glucose, protein or electrolytes in the urine which are commonly monitored as indicators of renal dysfunction. Chemically induced injury to the kidney can occur as results of the direct effect of a chemical or its metabolite on renal cells, or indirectly by altering renal haemodynamics, or by combination of both. The site along the nephron which is damaged is frequently the site of cellular accumulation of the chemical or a metabolite (Lock, 2000). Because of these adverse effects, monitoring changes in absolute kidney weight and relative weight of kidney (percent body weight) whenever possibly is important. The kidney can also be the site of immune disease such as Lupus erythematosus.

3.3.2 Lungs

The ability of a wide range of xenobiotics to cause lung injury following their oral or parenteral administration is now well established. Currently the lung serves as an important site for metabolism of certain xenobiotics as well as endogenous compounds, including phospholipids, serotonin, angiotensin and other vasoactive

substances. This metabolic activity, along with its massive surface area, cellular heterogeneity and the fact that it is exposed to the entire cardiac output, has made the lung a particularly common target organ for toxicity from the bloodborne and airborne xenobiotics. A large number of drugs and chemicals have been identified that produce lung damage. Many of these do by following inhalation. However, there is an interesting group of structurally and chemically diverse xenobiotics that damage the lung following systemic administration. Many of these compounds exhibit a remarkable selectivity for lung tissue, while others damage the lung as well as other organs. Systemically administered xenobiotics may accumulate in lung tissue and thus reach higher concentration than in other tissues. At the same time, a xenobiotic, can damage lung tissue as the result of direct toxicity (e.g. reacting with a lung specific site), or following metabolite activation. Because of these adverse effects, changing of lung weight and relative weight of lung should be investigated. While exposure to chemicals by inhalation is common, anticancer drugs and human exposure to hazardous levels of systemically lung-toxic chemical is relatively rare (Kehrer, 2000).

3.3.3 Liver

The liver has many metabolic roles and is, accordingly, affected by a very large number of xenobiotics. However, the liver also has an immense capacity for self-repair so that the majority of lesions observed in the liver reverse rapidly on cessation of treatment. Nevertheless, the liver's defense can be overwhelmed. One of the major roles of the liver is to protect the body against naturally occurring toxins present in, for example, plants or produced by the body's own intestinal flora. Exposure to small amounts of such material results in changes in the liver which permit efficient removal

of the toxin. As the dose of the compound is increased there may come a point when other functions are comprised and this is defined as the toxic phase response. The liver is also known as the organ that has a major role in detoxifying potential dangerous chemicals, this requires the ability of hepatocytes to take up xenobiotics. Liver also contains numerous isoforms of cytochrome P450, which are important in various metabolic activation mechanisms. The active metabolites formed in the liver will vary in their reactivity and this will, in turn, determine the type of damage which occurs. Because of these adverse effects, potential changes in liver weight should be evaluated (Grasso and Hinton, 2000).

3.4 Immunotoxicology *in vitro*

The immune system is a highly complex entity involving a large number of cell types from varying lineages and is dependent for its normal function on subtle interactions between these cell types requiring both cell-cell contact and communication via soluble mediators and their receptors. Therefore, in order to make any progress in this field, it is necessary to break down immune function into a series of compartments, often related to known pharmaco-toxicological effect of drugs and chemicals on specialized immune responses (Meredith and Miller, 2000).

In vitro models such as, NK assay, blastogenesis of T and B lymphocytes by mitogens, IgM assay and cytokine assay can be performed to understand the function and interaction of immune cells. There are several potential uses of *in vitro* test system in immunotoxicology. First, any appropriate *in vitro* screen can be employed in the process of drug development to assist in screening candidate molecules for efficiency or for potential undesirable side effects. *In vitro* assay can be employed to establish the

mechanism of action, and the cell types adversely affected by the test compound. Finally, there is the use of *in vitro* techniques to assist in regulatory submission. However, the validation process of *in vitro* assay itself is extremely protracted and subjected to multiple interpretation (Meredith and Miller, 2000).

4. IMMUNOMODULATING SUBSTANCES

Immunomodulating substances can be defined as substances, chemicals or drugs that have the ability to either stimulate normal immune response or restore an initially impaired immune competence, and/or inhibit the functioning of immune cells (Descotes, 1986).

4.1 Immunosuppressive agents

Immune suppression refers to decreased immune responsiveness as a consequence of therapeutic intervention with drug or irradiation, or as a consequence of a disease process that adversely affects the immune response (Cruse and Lewis, 1999).

Drugs that inhibit the immune response are called immunosuppressive drugs. These drugs are used mainly in the treatment of graft rejection and autoimmune diseases. Immunosuppressive agents such as, cyclophosphamide, FK506, rapamycin, azathioprine or corticosteroids, antibodies and irradiation are used to cause suppression of immune response (Cruse and Lewis, 1999).

Cyclophosphamide (N,N-bis[2-chloroethyl]-tetrahydro-2H-1,3,2-Oxazaphosphorine-2-amine-2-oxide) is a powerful immunosuppressive drug that is more toxic for B than for T lymphocytes. Therefore, it is a more effective suppressor of humoral antibody synthesis than of cell-mediated immune reactions. Its cytotoxic activity

results from cross-linking DNA stands (mitotic inhibitor) This alkylating action mediates target cell death. It produces dose-related lymphopenia and inhibits lymphocyte proliferation *in vitro*. Cyclophosphamide is beneficial for therapy of various immune disorders including rheumatoid arthritis, systemic lupus erythematosus-associated renal disease and autoimmune hematologic disorder (Cruse and Lewis, 1999).

Another potential way to suppress immunity is using monoclonal antibodies. Administration of anti-T cell monoclonal antibodies results in a rapid depletion of mature T cells through interaction with the CD3 molecule of T cell receptor complex. Anti-asialo-GM1 is also an antibody that inhibits NK cell activity. Anti-Asialo-GM1 (AAGM1) is antiserum that was obtained from bovine brain tissue. Asialo-GM1 is a glycosphingolipid which is expressed at high levels on > 90% of rodent NK cells (Kasai et al., 1980 and Trinchieri, 1989). AAGM1 binds to the surface of NK cells and cause cell lysis and loss of NK function (DeKoning, Hamilton, and Wallace, 1990). However treatment with AAGM1 did not appear to perturb the function of other immune cell (Wilson, MaCay, Butterworth, Munson, and White, 2001).

Other immune suppressive agents (cyclosporine A, FK506 and rapamycin) are fungal metabolites, they block activation of resting T cells by inhibiting the transcription of genes encoding IL-2 and the high affinity IL-2 receptor (Goldsby et al., 2000).

4.2 Immunostimulant and adaptogen

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 defences.

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. If the normally used dose is so small that a direct antimicrobial or antitumoral effect can be excluded, an immune-induced effect most likely results. So in plant or traditional medicine it is necessary to search for the pharmacologically active principles and determine their mechanisms of action through several assays in immunotoxicological studies (Arnason et al., 1995).

5. 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. Agent 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, Whitten, and Ambrogi, 1984).

An example of how dose can affect response, cyclophosphamide at a low dose which is given prior to immunization with a tumor vaccine can stimulate the immune response in both animals and humans (Barbuta, 1998). However, cyclophosphamide is primarily used as an immunosuppressive drug and it has been shown to have several side effects. The stimulatory activity observed with low dose cyclophosphamide may be due to an enhanced sensitivity of regulatory cells (Barbuta, 1998).

The other potential immunostimulating compounds can be subdivided into low and high molecular weight compounds. The first group includes alkaloid (e.g. isopteropodine), terpenoids, resin, flavonoid, 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 et al., 1995).

5.1 Resins

Resins are sticky, water-insoluble substances often exuded by the plant. It is a complex mixture of chemicals which softens on heating. Such resins are often

associated with essential oil (oleoresin), with gums (gum resins) or with oil and gum (oleo-gum resins). Their resin components, which mainly comprise diterpenes known as resin acids, resin alcohols and resin phenols, are soluble in alcohol and ether but are insoluble in water and hexane.

In another context, the term “resin” (or occasionally “rasinoid”) means that part of the plant which is soluble in ether or alcohol, as in kava resin and guaiacum resin. These resins are chemically diverse and can contain resin acids, pyrones, lignans, esters and glycosides among others.

Myrrh (*Commiphora molmol*) is an example of an oleo-gum resin with astringent and antimicrobial properties. The former quality is probably entirely due to the resin and the latter is a combined effect from resin and the essential oil. Tincture of myrrh is a potent antiseptic in the mouth and throat. In modern traditional medicine, it is believed one of its effects is to provoke a local leucocytosis, to effectively stimulate defensive white blood cell responses and so involve the body in eliminating local infections. Application of clinical usage is for improvement in recurrent throat and gum disease.

Resins have also been applied to inflammatory conditions of the upper digestive tract with some benefit. This probably reflects on their astringent property. The oleoresin mastic (*Pistacia lentiscus* var. *chia*) is traditionally used for the relief of dyspepsia and peptic ulcers. Mastic demonstrated a duodenal ulcer healing effect at a 1 g per day in a double-blind, placebo-controlled clinical trial. Cytoprotective and mild antisecretory effect were demonstrated in rat model of ulceration, consistent with astringent activity (Mills, Bone, Corrigan, Duke, and Wright, 2000).

5.2 Flavonoids

Flavonoids are extremely common and widespread in the plant kingdom. They function as plant pigments, being responsible for the colours of flowers and fruits. Being abundant in plants, flavonoids are commonly consumed in the human diet, especially if it is rich in fruits and vegetables.

Many flavonoids are yellow in colour. However, many others are white and the special flavonoid related anthocyanidins are red, blue and purple. Flavonoid consists of a single benzene ring joined to benzo-gamma-pyrone structure. They are formed from three acetate units and phenylpropane unit. The three main types are classified according to the state of oxygenation at carbon 3. These are flavones, flavonols and flavonones. Flavonoid glycosides are generally water soluble.

Examples functions of flavonoids in pharmacological studies, are capillary repair and to protect damaged capillaries, to treat and protect against ischaemic injury, antioxidant including inhibition of lipid peroxidation, wound healing, gastric disorders requiring repair of gastric mucosal barrier, antiplatelet agent, and treatment for inflammation of mucous membranes of the mouth and throat (Mills et al., 2000).

5.3 Polysaccharides

Polysaccharides are polymers based on sugars and uronic acids. They are found in all plants especially as a component of the cell wall. However, some plants significantly accumulate polysaccharides.

Recent studies have determined that the polysaccharide content of most medicinal plants are responsible for their immune stimulating or supporting activities (Arnason et al., 1995). For example, polysaccharides isolated from *Astragalus*

mongholicus, *Acanthopanax senticosus* and *Panax notoginseng*, stimulated macrophages, promoted antibody formation, activated complement, and also increased T lymphocytes proliferation (Li, 1991). Nutrition science has recently recognized expanded functions for complex carbohydrates or polysaccharides 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, 1997; See, 1998). Monosaccharide, oligosaccharide and polysaccharides can be extracted and isolated in water or aqueous ethanol. Herbal extracts prepared in 50% ethanol or stronger will not contain polysaccharides because of their insoluble in ethanol (Harborne, 1984; Mills et al., 2000).

5.4 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 and Baxter, 1993)

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

essential oil. They also have immunomodulation effects on antitumor and antileukaemic activities (Harborne and Baxter, 1993). Essential oils are water-insoluble oily liquids which are usually colourless. They can be isolated by the process of steam distillation (Mills et al., 2000).

Carotenoids which are tetraterpenoids are also found to have immunomodulatory effects. They are usually an extremely wide 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 and Baxter, 1993).

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

5.5 Phenolics

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, 1995). This study demonstrated that this compound group are also involved

in the immunomodulatory effect. Phenolics can be extracted by alcohol solvent and aqueous-alcoholic fraction.

5.6 Glycoproteins

Among the isolated plant constituents available today for immunostimulatory treatment, the “antitumoral” glycoprotein Krestin (*Coriolus versicolor*) is noteworthy. The glycoprotein can also be used as an immunostimulant (Arnason et al., 1995). According to the phytochemical method, protein and polysaccharide are dissolved in alcohol fraction such as ethanol and methanol (Harbone, 1984).

6. PLANT EXTRACTION PROCEDURES

6.1 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 and Baxter, 1993).

The main reason for drying plant before extraction is greater chemical stability and reduce cost (alcohol is often more expensive than the herb). It also prevents contamination of microorganism. The majority of herbs require relatively mild conditions for drying. Commercially, most plants are dried within temperature range of 37-60°C (Werbach and Murray, 2000).

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.

Herbal medicines are usually prepared by liquid extraction because this method has considerable advantages. The main advantage is the easy preparation of formulations for each individual patient. The other advantage of liquids is that, if properly prepared, they involve minimal processing and truly reflect the chemical characteristics of the herb in a compact, convenient form. They also confer considerable dosage flexibility which is especially relevant when prescribing low doses for small children. Liquid are readily absorbed and are convenient to take. In United states health food industry, most extracts utilize alcohol and water mixtures as solvents to remove soluble compound from the herb (Werbach and Murry, 2000).

Historically, ethanol or other solvents such as hexane, chloroform, methanol etc., have been used to extract polysaccharides, glycoprotein, phenolics, terpenoids, essential oil, waxes, fats, some resin and a portion of wood gum. Subsequent hot water extractions were then used to remove tannin, flavonoid, monosaccharide, polysaccharide, starches, and coloured matter. However, the alcohol-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, 1984; Ross and Brain, 1977; Mills et al., 2000).

Disadvantage of liquid preparation by ethanol in clinical practice is that the patient may be allergic to alcohol or in an ex-alcoholic who does not wish to take alcohol in any form. Strict Muslims will also do not take alcohol. In modern phytotherapy, water is often used in the preparation of herbal medicine because of its considerable benefit for delivering oral dose of the herb.

Several water extracts from traditional herbal medicine in various countries have been indicated to have immunostimulant activities. Examples of these are Juzen-Taiho-To (Japanese herbal mixture) modulates cytokine secretion and induces NK, T cells in mouse liver (Mutsumoto, Sakurai, Kiyohara, and Yamada, 2000), *Andrographis paniculata* (India herbal) boost immune function in bacterial and viral infection, Ginger (*Zingibar officinale* Roscoe.) in China, Asia and Australia act as anti-inflammatory and peripheral circulating stimulant, including Paud'arco (*Tabebuaria spp.* Gomes ex DC.) from Brazil has immunostimulant, antitumor and antimicrobial effect (Mills et al., 2000).

Generally, the longer the time allowed for extraction in solvent, the more material 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 apparatus, sonication, supercritical fluid extraction, pressurized fluid extraction, microwave assisted extraction, headspace analysis for volatiles, and distillation etc. (Ross and Brain, 1977). Traditional preparations of extracts in herbal remedies which are economical in preparation are infusion, decoction, maceration, and percolation (Ross and Brain, 1977).

6.1.1 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 hours. The process of Soxhlet extractor is the solvent in the flask is heated to boiling point and the vapour condensed in the reflux condenser. The hot liquid drips onto the plant sample, which is contained in a porous thimble. When the liquid in the extractor chamber reaches the top of the siphon tube it flows back into the heated flask, taking with it any dissolved plant material. The plant material is thus repeatedly extracted and the soluble components are concentrated in the flask. Typical solvents that are used are ethanol, hexane, acetone, methylene chloride, toluene and benzene (Ross and Brain, 1977).

6.1.2 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 material into an organic phase and an aqueous phase. This method uses two organic solvents that will show the different polarities between two phases, such as alcohol (n-butanol) which has a higher polarity than water (Ross and brain, 1977).

6.1.3 Solvent/solvent precipitation

In this extract, plant constituents are dissolved in as suitable solvent is mixed with a less polar miscible solvent causing the selective precipitation of the less soluble plant constituents. This will not give pure material but it is a simple and rapid

purification method. It is used for example, in the isolation of triterpenoid with molluscicidal action of *Phytolacca dodecandra*. The addition of acetone to a methanol extract of phytolacca affords a white solid which is almost entirely glycosidal in nature. This glycoside mixture is quite simply converted into the active molluscicide which is an oleanolic acid derivation (Ross and brain, 1977).

6.1.4 Traditional extraction

Infusion, decoction, maceration, percolation are the simplest process which consists of soaking the herb in the alcohol or water solution for a period of time, and then filtering. Infusion is the process in which the herb remains in hot or cold water for a short time, while decoction is remains in boiled water. The maceration prolongs infusion, often in aqueous alcohol. To precede percolation, it requires maceration and follows by a slow flow of fresh solvent through the ground material.

The major disadvantage of infusion and decoctions is that water is not a good solvent for many of the active components in herbs. This problem is compounded by the relative short extraction time used in their preparation (usually 5-10 minutes) (Ross and brain, 1977; Mills et al., 2000).

6.2 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 (Ross and Brain, 1977).

7. CELL VIABILITY AND CELL PROLIFERATION

DETERMINATION METHODS

Among the literatures, there have several method uses to determine cell viability and cell proliferation as the end point of *in vitro* and *in vivo* immunotoxicology assays. 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 ^3H -Thymidine incorporation assay (United State Environmental Protection Agency, 1996). An alternative method that avoids the radioactive isotope is MTT colorimetric assay (Martin and Claynes, 1993). Another commonly used method to determine viability of cells is trypan blue dye exclusion.

7.1 ^3H -Thymidine incorporation (^3H -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 to determine the amount of proliferation occurring. The ^3H -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).

7.2 3,4, 5-Dimethyl thiazol -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 and lacks the problems associated with radioactive material (Martin and Claynes, 1993).

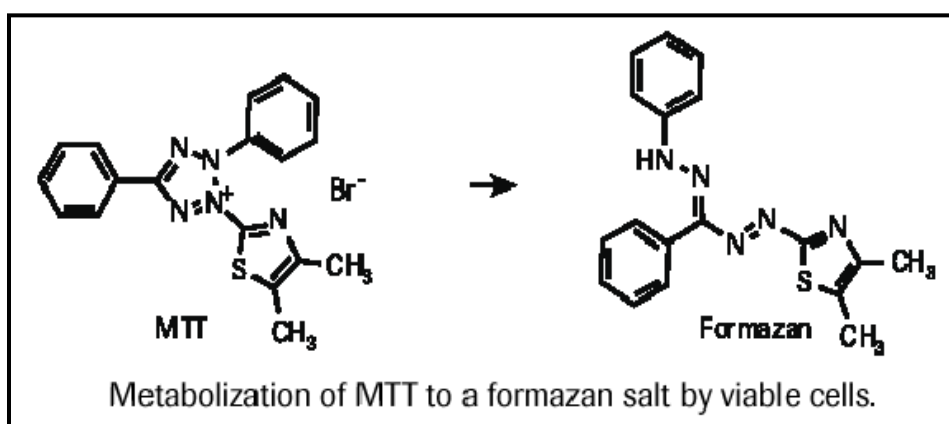


Figure 5 Metabolization of MTT to a formazan salt by mitochondrial enzyme succinate-dehydrogenase by viable cells.

7.3 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 through 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. In conducting this assay one must be aware of time of dye exposure because viable phagocytic cells such as macrophage may take up the dye by pinocytosis (Phillips, 1973).

CHAPTER III

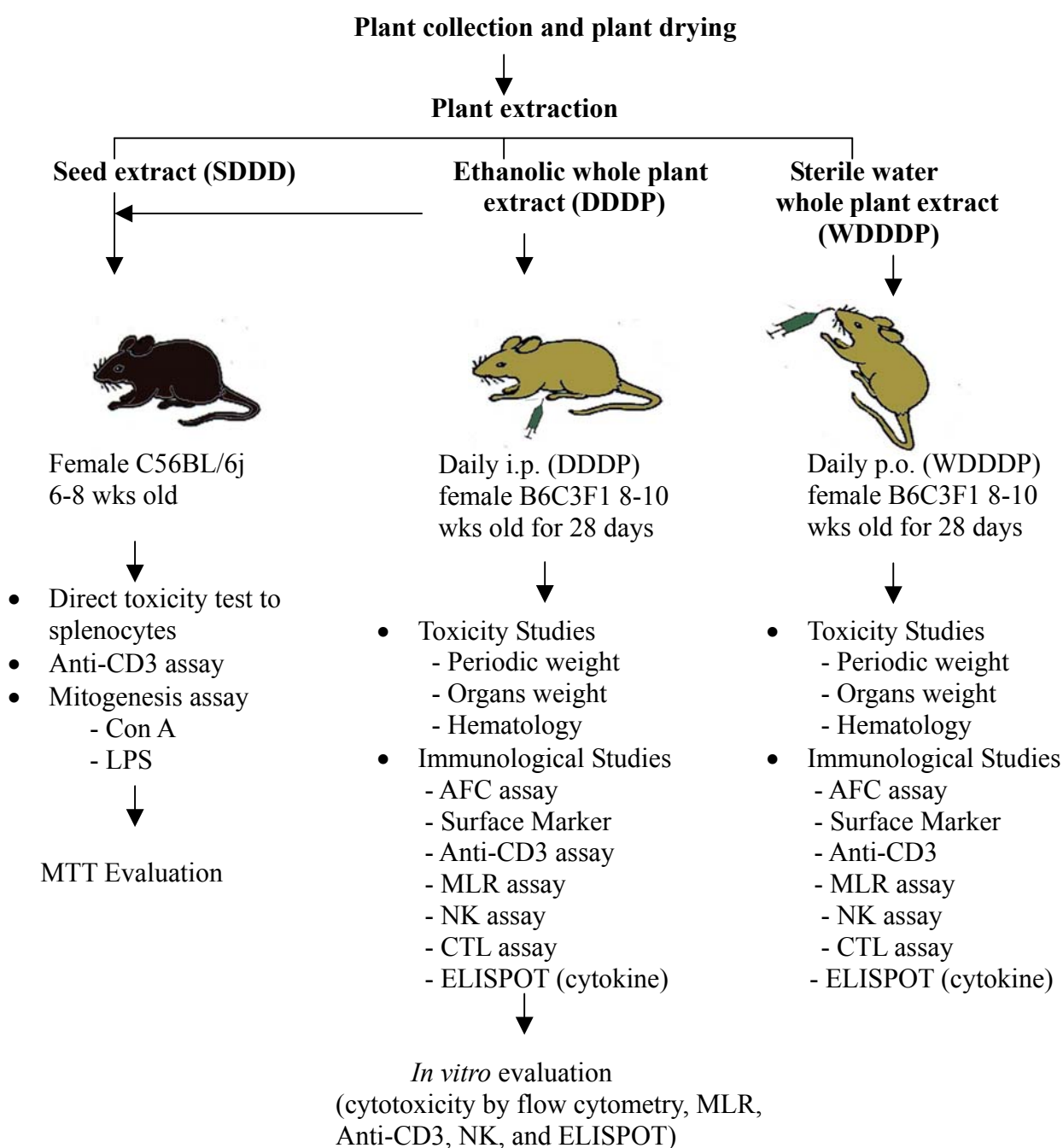
MATERIALS AND METHODS

Because of their potential therapeutic utility, immunotoxicological assessment was undertaken to determine effect of various extracts of DDD (ethanolic whole plant extract, butanolic seed extract, and sterile water whole plant extract). *In vitro* studies were initially conducted to determine direct effect of the extract on immune cells of naïve C57BL/6j mice.

However the normal progression for the development of a pharmaceutical following *in vitro* study is the assesement of toxicological and immunological effects in animal models (*in vivo*). The B6C3F1 mouse is recommended by NTP to use as the model for immunotoxicological assessment purposes. Furthermore the recommended exposed period of 28 days (subchronic study) was utilized.

To determine the effects of the compound on the immune system, ethanolic whole plant extracts (DDDP) was given by i.p. injection for 28 days which permitted quantition of the amount of compound received systemical. However, normally people obtain this herbal medicine from crude whole plant decoction by oral intake, accordingly sterile water whole plant (WDDDP) extract was evaluated following oral gavage for 28 days.

In an attempt to characterize the mechanism of action for the clinical efficacy of DDDP, *in vitro* study was also performed in naive cells from female B6C3F1. An overview of study protocol is presented in Figure 6.

Figure 6 The overview of study protocol

3.1 MATERIALS

3.1.1 Plant

Two batches of fresh plants of *Aeginetia indica* Roxb. (DDD) 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 DDD with the generous help of Dr. J.F., Maxell at Chiang Mai University.

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

3.1.2 Chemicals and instruments

3.1.2.1 Animals

In vitro studies used to screen for direct immuntotoxic effects of DDD were conducted in Thailand and Japan using female C57BL6/j mice 6-8 weeks old. They 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 being used in experiments 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.

In order to provide additional information on overall effect in mice, *in vivo* and *in vitro* studies were performed at Department of Pharmacology and Toxicology,

Virginia Commonwealth University (VCU), Richmond, Virginia. All experiments used female B6C3F1 mice 8-10 weeks old. They were obtained from Taconic Farm (USA.), mice were quarantined for at least one week prior to the experiment, and then they were housed in a temperature-controlled environment at 21-24°C and 40-70% relative humidity with a 12-hour light/dark cycle. Food pellets (NIH-07 mouse/rat diet, Harlan Teklad, USA) and tap water were given *ad libitum*, during all experiments. Mice were determined to be free of hepatitis and Sendai virus by serology testing. All animal procedures were conducted under an animal protocol approved by the VCU Institutional Animal Care and Use Committee (IACUC).

3.1.2.2 Chemicals and instruments

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

Table 3 List of chemicals used in the studies.

Name	Source
⁵¹ Cr (sodium chromate)	PerkinElmer, USA.
Concanavalin A (Con A)	ICN. Co., USA; Phamacia, USA.
Cyclophosphamide(CPS)	Sigma, USA.
[3(4,5-dimethylthiazol-2-yl) ,5-diphenyltetrazolium bromide]-MTT	Wako company, Japan
Dimethyl sulfoxide (DMSO)	Wako company, Japan
Dulbecco's Modified Eagle Media (DMEM) 1X	Gibco, Co., USA.
Ethyl alcohol	Wako company, Japan; AAPER Alcohol and Chemical Co., Kentucky, USA.
Eagle's Minimal Essential Medium (EMEM)1X	Gibco Co.,USA.
Earle's Balanced Salt Solution (EBSS)	Gibco Co., USA.
ELISPOT kit (IL-2, IFN- γ , IL-4)	BD Pharmingen, Sandiego, CA., USA.
Fetal bovine serum (FBS)	Gibco Co., USA. and Hyclone, USA.

Name	Source
Gentamicin	Gibco Co., USA.
Goat anti-mouse Ig-FITC (250 µg/ml)	BD Pharmingen, Sandiego, CA., USA.
Guinea Pig Complement	Gibco Co., USA.
³ H-Thymidine (³ H-TdR)	ICN, CO., USA.
Ham's F12 media	Gibco Co., USA.
Hamster anti-mouse CD3ε- FITC (0.5 mg/ml)	BD Pharmingen, Sandiego, CA., USA.
N-2-hydroxethyl sulfuric acid (HEPES) buffer	Gibco Co., USA.
Isotype hamster IgG ₁ - FITC (0.5 mg/ml)	BD Pharmingen, Sandiego, CA., USA.
Isotype mouse IgG _{2a} - PE (0.2 mg/ml)	BD Pharmingen, Sandiego, CA., USA.
Isotype rat Ig _{1κ} - FITC (0.5 mg/ml)	BD Pharmingen, Sandiego, CA., USA.
Isotype rat IgG _{2aκ} -FITC (0.5 mg/ml)	BD Pharmingen, Sandiego, CA., USA.
Isotype rat IgM _κ - FITC (0.5 mg/ml)	BD Pharmingen, Sandiego, CA., USA.
L-glutamine	Gibco Co., USA.
Lipopolysaccharide (LPS)	Sigma, USA.
2-Mecapto ethanol (2-ME)	Sigma, USA.
Mouse Anti-CD3ε (0.5 mg/ml)	PharMingen, USA.
Mouse anti-mouse NK 1.1-PE (0.2 mg/ml)	BD Pharmingen, Sandiego, CA., USA.
Mitomycin C	Sigma, USA.
n-Butanol	Wako company, Japan and Fisher Scientific Co., USA.
Penicillin and Streptomycin solution	Gibco Co., USA.
Phosphate buffered saline (PBS)	Gibco Co., USA.; Sigma, USA.
Pronase	Calbiochem, USA.
Propidium Iodide	Sigma, USA.
Proteinase K	Gibco Co., USA. and Wako company, Japan
Rabbit anti-asialo GM1 antibody (AAGM1)	WakoBioproduct, Japan
Rat anti-CD16/32 (0.5 mg/ml)	BD Pharmingen, Sandiego, CA., USA.
Rat anti-mouse CD8 _a -FITC (0.5 mg/ml)	BD Pharmingen, Sandiego, CA., USA.
Rat anti-mouse CD25- FITC (0.5 mg/ml)	BD Pharmingen, Sandiego, CA., USA.
Rat anti-mouse Mac3- FITC (0.5 mg/ml)	BD Pharmingen, Sandiego, CA., USA.
Rat anti-mouse CD4 -PE (0.2 mg/ml)	BD Pharmingen, Sandiego, CA., USA.
Rat Ig2 _{aκ} -PE (0.2 mg/ml)	BD Pharmingen, Sandiego, CA., USA.
Roswell Park Memorial Institute (RPMI) 1640	Gibco Co., USA.
Retic-count reagent stain (Thiazole orange)	Becton Dickinson and Company, USA.
Sheep red blood cells (SRBCs)	Lampire, USA.
Sodium Azide	Sigma, USA.
Sodium Bicarbonate	Wako company, Japan; Sigma, USA.
Sodium Pyruvate	Gibco, Co., USA.
Sterile water	Baxter Healthcare Corporation, USA.

Name	Source
Sterile 0.9% Sodium Chloride	Baxter Healthcare Corporation, USA.
Triton X-100	Sigma, USA.
Trypan blue dye	Gibco Co., USA.
Typsin	Gibco Co., USA

Table 4 List of equipments used in the studies.

Name	Source
500 µl Microtest tubes	Bio-rad, USA.
96-well plate flat , round and U bottom	Co-Star (Corning Incorporation, NY, USA.)
Alundum extraction thimbles	Fisher Scientific Co., USA.
Bellco plaque viewer	BellcoGlass, Inc., USA.
Blender	Molinox Co., Germany
Cell strainer	Nunc Co., Denmark
Centrifuge machine	Beckman Coulter Inc.
CO ₂ incubator	BellcoGlass, Inc., USA.
Conical tubes	Nunc Co., Denmark
Coulter counter	Beckman Coulter Inc.
Digital balance	Mettler, Curtin matheson scientific Co., USA.
Disposal pipette	Nunc Co., Denmark.
ELISA plate reader	BIORAD Co., USA; Softmax, USA.
ELISPOT analyzer	BD Bioscience, USA.
Flow cytometer (FACScan)	Beckton Dickinson FACScan
Glassware	Pyrex Co., USA.
Haemocytometer and cover slip	Fisher Scientific Co., USA
Homogenizer	Bosch Co.,USA
Inverted microscope	Olympus optical Co. Japan
K-1000 Hematology analyzer	Roche Diagnostics
Laminar flow hood	Holten, Scientific Promotion Co., England Forma Scientific, USA.
LKB Wallac 1214 rack Beta	Perkin Elmer, Co., USA.
LKB Wallac 1282 CompuGamma	Perkin Elmer, Co., USA.
Light microscope	Nikon Kogaku K.K Co., Japan
Microhematocrit (heparinized)	Fisher Scientific Co., USA.
Microscope slides	Fisher Scientific Co., USA.
Microtainer Brand tube with EDTA	Beckton Dickinson, USA.
Multichannel pipette	Gibco Co., USA.
Pasteur pipette	Fisher Scientific Co., USA.
Petri dish	Nunc Co., Denmark
PHD cell havester	Cambridge Technology, Inc.

Name	Source
Rotary evaporator with vacuum and water bath	Bosch, Co., USA.
Single frost end slide	Fisher Scientific Co., USA.
Snap cap tubes	Nunc Co., Denmark
Soxhlet extraction apparatus	Pyrex Co., USA.
Surgical set	Fisher Scientific Co., USA.
TQ-prep machine	A Beckman Coulter company, USA.

3.2 METHODS

3.2.1 Preparation of crude ethanolic extract of DDD (DDDP)

Whole dried plants 80 g (Fig. 7) were homogenized and ground into powder using an electronic blender. The raw powder (Fig. 8) was put into a thimble (medium porosity, 10-15 μm , sized to fit the soxhlet extractor) (Fig. 9) and then into the soxhlet column. Eight hundred milliter of 95% ethanol was placed into a round-bottomed flask, and connected to the soxhlet extraction apparatus. Several boiling chips were placed into a clean dry receiving flask. The extraction process was performed until the solvent had a clear color (approximately 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 powder of crude extracts was then kept in -20°C freezers. The powder was dissolved in Roswell Park Memorial Institute (RPMI) media (without FBS), filtered through a Millipore filter (pore size = 0.45 μm or 0.22 μm) and was then used as the DDDP extract for *in vitro* study (Fig. 10). For *in vivo* study, DDD's powder was dissolved in 0.9% sodium

chloride, then filtered through a Millipore filter (pore size = 0.45 μm) and used as the DDDP extract.

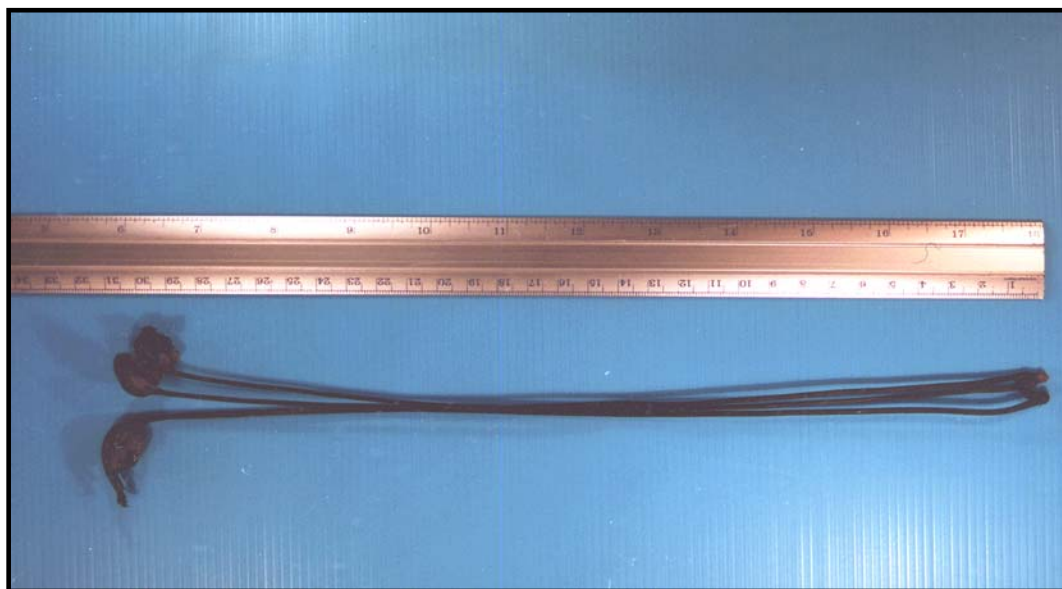


Figure 7 Whole plant of *Aeginetia indica* Roxb. After being dried by hot air oven at 60°C.



Figure 8 Raw powder of whole plant of *Aeginetia indica* Roxb.



Figure 9 Soxhlet extraction apparatus.



Figure 10 Powder of whole plant of *Aeginetia indica* Roxb. extracted by ethanol.

3.2.2 Preparation of aqueous phase of n-butanol extract from DDD's seeds (SDDD).

The extraction procedure was performed as described previously (Chai et al., 1992 ; Morrison and Leive, 1975) with slight modification.

The dried seeds of *Aeginetia indica* Roxb. (DDD) were separated from the plant after being dried in a hot-air oven. Ten milliliters of distilled water was added to 1 g of seed of *Aeginetia indica* Roxb. The plant parts were homogenized in a blender and 10 ml of water-saturated n-butanol were added. The mixture was stirred at 4°C for 15 mins, centrifuged at 35,000 xg for 20 mins, and a water-soluble fraction was collected. The extract was treated with 20 µg/ml proteinase K enzyme at 60°C for 60 mins. Following proteinase K digestion, the precipitated protein was removed by centrifugation in the cold. The extract was dialyzed against phosphate-buffered 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. 11) 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 SDDD extract for *in vitro* studies.



Figure 11 Powder of seed extract from *Aeginitia indica* Roxb. Evaporated from aqueous phase of n-butanol extraction and it was maintained at -20°C .

3.2.3 Preparation of aqueous phase of sterile water extract from the whole plant of DDD (WDDDP).

Preparation was performed according to Thai ethnomedical method using hot water extraction (decoction).

Whole dried plants of DDD, 80 g, were homogenized using an electric blender. The raw powder was macerated with 800 ml of sterile water in a pot for 30 mins at room temperature. This material was heated at $70-80^{\circ}\text{C}$ for 20 mins, and left until it cooled to room temperature before filtration. The extract was filtered through cheese cloth and was used as the WDDDP extract.

3.2.4 Spleen cell collection and single cell suspension preparation

Mice were anesthetized by CO₂ inhalation, and then were sacrificed by cervical dislocation. The spleens were collected and placed in 3 ml Earle's Balance Salt Solution (EBSS) with N-2-hydroxyethyl sulfuric acid (HEPES) buffer. A single cell suspension of spleen was prepared by pressing the spleen between the frosted ends of two microscope slides in a 60x15 mm petri dish under a sterile hood. The slides were washed with buffer using a pasture pipette, and then single cell suspensions were placed into a 5-ml plastic-capped test tube. The splenic cells were then centrifuged at 1,200 rpm for 5-10 minutes and resuspended in Roswell Park Memorial Institute 1640 complete media [with 10% fetal bovine serum (Hyclone)]. The cell's viability was determined by trypan blue dye exclusion and enumerated using a Coulter counter ZBII.

3.2.5 MTT colorimetric assay (*in vitro* studies)

For *in vitro* studies, the proliferation or cell viability was evaluated by the MTT colorimetric method. The assay was performed as described by Martin and Clynes (1993) with slight modification. On the day of evaluation the proliferation or cell viability, MTT was added to obtain the final concentration of 1 µg/ml in each well of 96-well plate. The plates were incubated at 37°C, 5% CO₂ for 4 hours. After incubation, the medium was carefully removed without disturbing the formazan crystals (Fig. 12). One hundred microliter of dimethyl sulfoxide (DMSO) was added to each well and aspirated repeatedly to give a uniform dark purple color (Fig. 13) before reading at 595 nm by an ELISA plate reader.

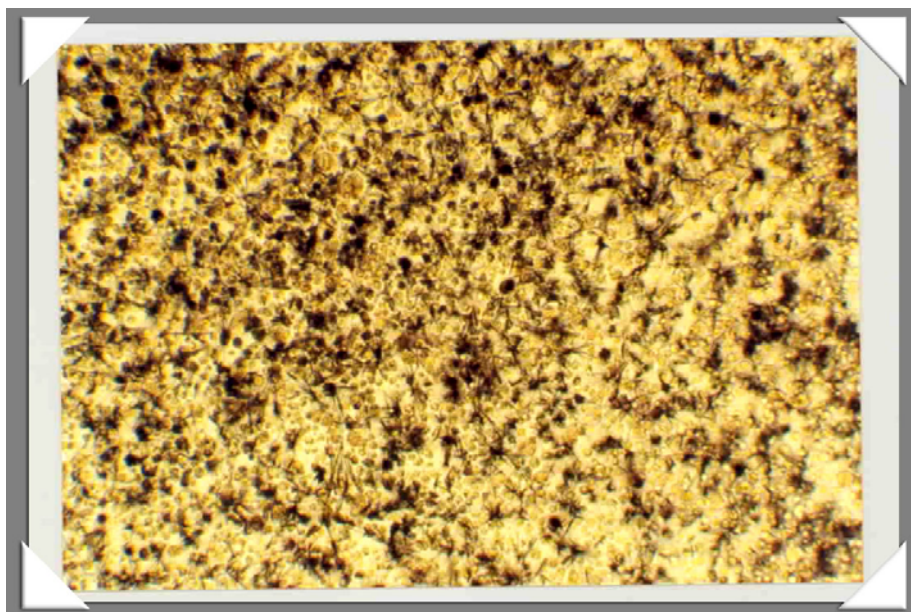


Figure 12 Crystals of formazan product after the addition of MTT for measuring cell proliferation by MTT colorimetric assay.

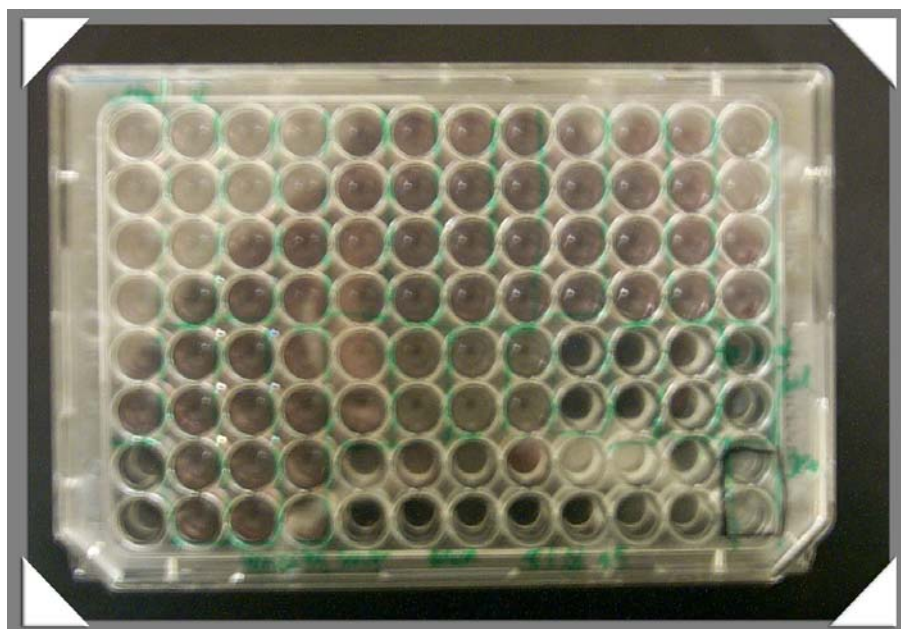


Figure 13 Dark purple color of formazan product that occurred after DMSO was added into each well in the MTT colorimetric assay in a 96-well plate.

3.2.6 Preparation of cancer cell lines

3.2.6.1 Human cervical cancer cells (HeLa)

Human cervical cancer cells (HeLa) is a cell line derived from a human carcinoma of the cervix (Fig.14). HeLa cell, is an adherent cell line which forms a monolayer in a culture flask. Only viable cells can attach to the bottom of the flask. The 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 cryopreservative agent, DMSO. Cells were centrifuged at 350 xg for 5 mins, and the cell's pellets were cultured in Ham's F12 medium supplemented with 10% FCS, L-glutamine, sodium bicarbonate, and penicillin and streptomycin in a 25 cm² culture flask at a 5 ml total volume. Flasks were incubated at 37°C, 5% CO₂. At the log phase (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 cm² culture flask. Flasks were incubated at 37°C, 5% CO₂ 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 1.5×10^5 cells/ml for the cytotoxicity experiment. Furthermore, cells were adjusted to the concentration of 2×10^5 cells/ml and an aliquot of 1 ml per vial was used for making a frozen stock maintained in liquid nitrogen.

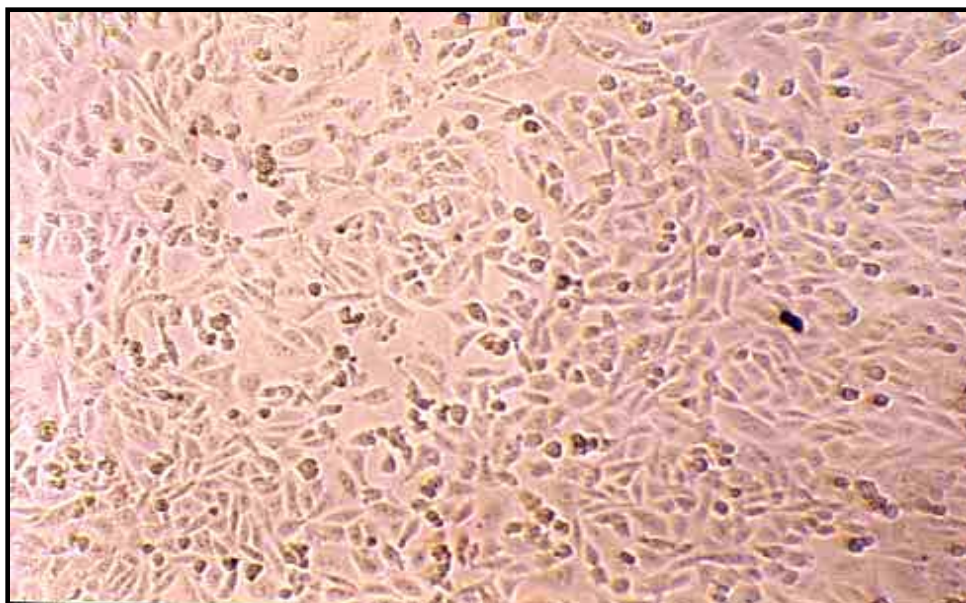


Figure 14 Growth characteristics of normal HeLa cell in Ham's F12 Media viewed under inverted microscope

3.2.6.2 Molony virus induced T cell lymphoma (YAC-1)

Yac-1 cells grow in suspension cultures, they were obtained from ATCC, USA. (TIB-160) as culture flask. To initial culture, cells were removed from the flask and centrifuge at 350xg for 15 mins. The pellets were resuspended with the 10-12 ml of RPMI 1640-10%FBS. The viability of the cell suspension was determined by trypan blue dye exclusion, and were adjusted to a cell density of $2-4 \times 10^5$ viable cells/ml. Cells were cultured in 25 cm² flask at 37°C, 5% CO₂ until cell density was $2-4 \times 10^6$ viable cells/ml (log phase), which was suitable for using in the NK assay.

3.2.6.3 Mastocytoma cells (P815)

The P815 cells are also a type of cancer cell line which grows in suspension cultures. They were obtained from ATCC, USA (TIB-64). Cells were cultured in 25

cm² flask at cell concentration of $2-4 \times 10^5$ cells/ml in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 2mM L-glutamine, 50 µg/ml gentamicin, 1mM sodium pyruvate, and 15mM HEPES. P815 cells were cultured in a CO₂ incubator at 37°C, 5% CO₂. The cell viability for initial cultures was determined by trypan blue dye exclusion. Cells were cultured until log phase growth, at time cell density was $2-4 \times 10^6$ viable cells/ml. This cancer cell line was used in cytotoxic T lymphocyte assay.

3.2.7 *In vitro* experimental protocol by MTT colorimetric evaluation

To screen the immunological effect of various plant extract from DDD, seed (SDDD) and whole plant extract (DDDP) were prepared as described previously. Six to eight week-old female C57BL6/j mice were used.

3.2.7.1 The selection of a suitable concentration of DDDP and SDDD in *in vitro* toxicity studies

In the initial experiment, C57BL6/j 6 mice were used in the toxicity test. Mice were sacrificed by cervical dislocation. Spleens were collected and prepared as described in section 2.4. The spleen cells were diluted to 4×10^6 cells/ml in RPMI media supplemented with 10% heat-inactivated fetal calf serum (FBS) and were maintained at 4°C before being plated on to a flat bottomed 96-well plate in a volume of 100 µl.

Whole plant extract (DDDP) powders and seed extract (SDDD) 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-bottom plate. Before being added to the plate, the extract was sterilized by filtering through a 0.22 µm filter.

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

Splenocytes, 4×10^6 cells/ml, were plated into 96-well plate (flat bottom) in a volume of 100 μ l. Four replicates culture were prepared. Fifty microliter of plant extracts were added into each well to obtain final concentrations of 10,000 to 1.25 μ g/ml or per well. On another plate, 50 μ l of seed extracts were added to each well to obtain final concentrations of 10,000 to 1.95 μ g/ml. The plates were incubated overnight at 37°C, 5% CO₂. Cell viability was determined by using the MTT colorimetric method.

3.2.7.2 The Optimization studies of *in vitro* immunological tests

Typically, most of the immunotoxicological assays use the uptake of radioactive labeled thymidine (³H-TdR) into cellular DNA as the measurement of cell proliferation, however, in these studies the reduction of nonradioactive tetrazolium salt MTT was used as the quantitative colorimetric assay to evaluate the cell proliferation. Therefore, the optimization studies using MTT colorimetric assay in several immunological tests were conducted.

3.2.7.2.1 Anti-CD3 mediated T lymphocyte proliferation assay.

The assay was performed as described by Guo et al., (2001) with slight modification. Mouse AntiCD3 antibody was diluted from a stock solution of 0.5 mg/ml to the final concentration of 1 μ g/ml and 0.5 μ g/ml. One hundred microliter of each concentration was added into a 96-well flat-bottomed plate. 100 μ l of PBS was placed in other wells as background controls. Three triplicate plates were set for each 3 days

of the experiment. The plates were incubated at 4°C for overnight.

On the next day when the assay was performed, mice were sacrificed by cervical dislocation; spleens were collected by aseptic technique, and single cell suspensions were prepared. Four replicates of spleen cells were diluted to 2×10^6 and 4×10^6 cells/ml in RPMI media supplemented with 10% heat-inactivated fetal calf serum (FBS), and were kept at 4°C before being plated onto a 96-well flat-bottomed plate.

Immediately prior to adding the cells, the contents of anti-CD3 antibody coated plates were quickly discarded by flipping and blotting the plates on gauzes under the laminar flow hood. Wells were washed with 200 µl of sterile PBS (1 time) and as much as possible of the remaining content was shaken out. One hundred microliters of various cell dilutions were plated into each concentration of antiCD3- coated plates, 4 replicates were used, then 100 µl of RPMI complete media was added into all wells. The 3 plates were incubated at 37°C, 5% CO₂, and cell proliferation was determined by MTT colorimetric method.

3.2.7.2.2 Lymphocyte proliferation assay : Concanavalin A (Con A) mitogen

The assay was performed as described by Burn et al. (1994) with slight modification. Splenocytes in this assay was prepared as described previously in section 2.4. The spleen cells were diluted to 4×10^6 and 8×10^6 cells/ml in RPMI 1640 supplemented with heat-inactivated 10% FBS, and were maintained at 4°C before being plated into a 96-well plate (flat-bottom).

Con A was weighed and diluted to make various final concentrations of 20,

10, 5 and 2.5 µg/well in EBSS in 96-well flat-bottomed plates. The con A was filtered using a 0.22 µm acrodisc filter.

Twenty five microliters of various concentrations of con A were added into 96-well flat-bottomed plates in four replicates. One hundred microliter of splenocytes from each dilution was added to each concentration of Con A. Then 25 µl of RPMI were added to all wells of the 96-well flat-bottomed plate to make a final volume of 150 µl/well. The plates were incubated for 3 days at 37°C, 5% CO₂. Cell proliferation was determined by MTT colorimetric assay.

3.2.7.2.3 Lymphocyte proliferation assay : Lipopolysaccharide (LPS) mitogen

The spleen cell mitogenicity was evaluated using a modified method from Holsapple et al. (1983). On the day of assay, mice were sacrificed and spleen cell was prepared as described in section 2.4. The spleen cells were diluted to 4x10⁶ and 8x10⁶ cells/ml in RPMI media with 10% FBS, and were kept at 4°C before being plated to a 96-well flat bottom plate.

LPS was diluted from stock (10,000 µg/ml) to make various final concentrations of 1,000, 500, 100, 10 and 5 µg/ml in EBSS, and plated in a 96-well flat-bottom plates. The LPS was filtered through a 0.22 µm acrodisc syringe filter.

Twentyfive microliters of various concentrations of LPS were added to each well in quadruplicates in 96-well flat-bottomed plates. One hundred microliter of each cell dilution was added into each concentration of LPS. Finally a 25 µl of RPMI complete media was added to all wells to make a final volume of 150 µl/well. Plates were incubated at 37°C, 5% CO₂. Cell proliferation was determined by MTT

colorimetric assay.

3.2.7.3 Effect of SDDD and DDDP extracts from DDD on *in vitro* immunological studies

The various *in vitro* immunological assays were performed using the appropriate experimental conditions as determined in the previous optimization studies.

3.2.7.3.1 Effect of SDDD and DDDP extracts on anti-CD3 mediated T lymphocyte proliferation assay.

The four replicates of 1 µg/ml in a volume of 100 µl of mouse anti-CD3 antibody were coated on a 96-well flat-bottom plates and an equal volume of PBS was placed in the other wells as background controls. Plates were incubated at 4°C overnight. On the next day, when the assay was performed, mice were sacrificed, spleens were collected by aseptic technique, and single cell suspensions were prepared. The contents of the anti-CD3 antibody coated plates were discarded in a sterile hood, washed once with PBS immediately prior to the addition of 100 µl of 4×10^6 cells/ml of splenocytes. Then 50 µl of various concentrations of seed extracts were added together in the plates to provide the final concentrations ranging from 2,000-7.81 µg/ml. In addition, the same amount of whole plant extracts was added to each well to provide the final concentrations ranging from 1,000-1.25 µg/ml. The plates were incubated at 37°C, 5% CO₂ and cell proliferation was determined by the MTT colorimetric assay.

3.2.7.3.2 Effects of SDDD and DDDP extracts on lymphoproliferative responses to con A mitogen

Twenty five microliters of con A were added into 96 well plates in four replicates to give 5 µg/well. Then 100 µl of 8×10^6 cells/ml of splenocytes was added. After that 25 µl of various final concentrations of seed extracts ranging from 2,000-1.25 µg/ml, or various final concentrations of whole plant extracts ranging from 1,000-1.25 µg/ml were added to give the final volume of 150 µl/well. Plates were incubated at 37°C and 5% CO₂. After 3 days, cell proliferation was determined by using the MTT colorimetric assay.

3.2.7.3.3 Effects of SDDD and DDDP extracts on lymphoproliferative response to LPS

Twenty five microliter of LPS was added into 96 well plates in four replicates to give final concentration of 10 µg/ml. Then 100 µl of 8×10^6 cells/ml of splenocytes was added. After that 25 µl of seed extracts in various concentrations ranging from 2,000-1.25 µg/ml, or whole plant extracts in various concentrations ranging from 1,000-1.25 µg/ml were added to give the final volume of 150 µl/well. Plates were incubated at 37°C and 5% CO₂. After 3 days, cell proliferation was determined by using the MTT colorimetric assay.

3.2.7.4 Cytotoxicity studies of tumor cells

3.2.7.4.1 Effect of DDDP on Human cervical cancer cell

HeLa cells were thawed from frozen stock and cultured in Ham's F12 medium with 10% FBS. After the log growth phase (2-3 days), cells were diluted to

1.5×10^5 cells/ml and 1 ml of cells was added into a 2 ml petri dish in triplicates. Different concentrations of whole plant extracts were prepared by dissolving the extracts in Ham's F12 media, and sterile-filtered through 0.45 μm acrodisc filter. Then 1 ml of extracts was added into each petri dish containing 1 ml of cancer cells to yield the final concentrations of 10, 100, and 1,000 $\mu\text{g/ml}$ of extract in 2 ml final volume. The cultures were incubated at 37°C , 5% CO_2 overnight. Cell viability was determined by trypan blue exclusion method.

3.2.8 Studies protocol for *in vivo* study in female B6C3F1 mice

In vivo studies were conducted in 8-10 weeks old female B6C3F1 mice. The study was performed to provide information of toxicological and immunological effects in mice exposed to DDDP and WDDDP during a subchronic exposure period (28 days). DDDP was directly administrated to animal by daily i.p. injection to minimize compound metabolism result from passage though the gastrointestinal tract and liver. However, WDDDP was administrated to animal by daily gavage for 28 days, which is the relevant route of human uptake.

Various immunotoxicological assays were selected to provide qualitative and quantitative data on the effects of DDDP and WDDDP on innate immunity, humoral immunity and cell-mediated immunity. Standard toxicology endpoints such as body weights, selected organ weights, and hematological parameters were also evaluated to ensure effects observed were primarily mediated through the immune system.

3.2.8.1 Animal exposure protocol for mice treated with DDDP

The mice were randomized prior to the study, and eight mice were assigned to

each dose group. DDDP was administered in a physiological saline solution with each animal received 0.1 ml/10 g body weight. The doses utilized were 0.25, 2.5, 25, 100 and 250 mg/kg. The vehicle control animals were administered 0.9% sodium chloride with each animal receiving 0.1 ml/10 g body weight.

3.2.8.2 Animal exposure protocol for mice treated with WDDDP

The mice were randomly assign to six experimental groups (n=8), using a computer randomization program. The vehicle control group was given sterile water by oral gavage in a volume of 0.1 ml/10 g body weight. An additional group, use a modulatory control 1 (MC1), was given 50 mg/kg of cyclophosphamide (CPS) on the last four days of exposure period by intraperitoneal (i.p.) injection (0.1 ml/10 g body weight). A 1:10 dilution from stock concentration of 24 mg/ml in PBS of rabbit anti-asialo GM1 antibody (AAGM1) was given to mice by intravenous injection (i.v.) approximately 24 hours prior to sacrifice and was used as a second modulatory control (MC2) in NK assay. The other experimental groups were administrated WDDDP as the stock decoction preparation (100%) or diluted in sterile water to produce concentration of 50%, 25% and 10%. All mice were administrated WDDDP by oral gavage once a day in a volume of 0.1 ml/10 g body weight.

3.2.8.3 Toxicological studies for mice exposed to DDDP and WDDDP

Mice were weighted weekly to observe periodic weights and adjusted doses during the experiments. Approximately, 24 hours after the last days of DDDP or WDDDP administration, mice were anesthetized by CO₂ inhalation, and then sacrificed by cervical dislocation. Animals were necropsied and were observed for possible gross

abnormal changes. The following organs were weighed: thymus, lungs, liver, spleen and kidneys with adrenals.

Hematological parameters were determined on blood collected into EDTA coated tubes by retro-orbital bleeding. Parameters analyzed were the following: number of erythrocytes and leukocytes, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean hemoglobin corpuscular concentration (MCHC). Analysis was conducted using a K-1000 Hematology analyzer. Immature red blood cells (reticulocytes) were enumerated by staining with Retic-count Reagent stain (Thaizole Orange) and were analyzed by the flow cytometry.

3.2.8.4 Immunological studies for mice exposed to DDDP and WDDDP

In addition to general toxicological parameters, DDDP was evaluated for its effects on various components of the immune system including, humoral immunity, cell-mediated immunity, and innate immunity. The effect of humoral immunity was evaluated using the antibody-forming cell (AFC) response to the T-dependent antigen, sheep erythrocytes.

T lymphocytes play a major role in cell-mediated immunity including recognizing and killing specific pathogen, such as bacteria, viruses and parasites. T lymphocytes (T helper) also produce cytokine to activate other immune cells to proliferate. In these studies, T cells activities were evaluated as Anti-CD3 antibody mediated T cells proliferation and MLR assay. Proliferating in the MLR model is mainly CD4⁺ lymphocytes (T helper) in response to class MHC class II determinants on stimulator cells. In contrast, cytotoxic T lymphocytes (CD8⁺) which recognize MHC

class I molecules bound to peptide antigen derived from the pathogen (tumor) or the dysregulated cell to initiate a cytolytic pathway. The activity of cytotoxic T cells was evaluated in the CTL assay.

NK cells, innate immunity cells that lack the immunoglobulin-like T cell receptors, have the ability to kill tumor cells. The killing activity was evaluated as percent cytotoxicity at various effector : target ratios in the NK assay.

Phenotypic analysis of the splenic subpopulations was also determined in these studies to determine if the number of various cells of the immune system were altered following treatment. Cytokine level is one potential mechanism by which cells in immune system can be modulated, detection of alteration in cytokine production was evaluated using the ELISPOT assay.

3.2.8.4.1 Hemolytic plaque assay for detecting single Ig-M forming cells

The antigen-forming cells (AFC) were enumerated using a modified hemolytic plaque assay of Jerne and Nordin (1963) described in detail by Holsapple et al. (1984). Sheep red blood cells (SRBCs) were maintained in Alsever's solution and were prepared for immunizing mice by washing three times with EBSS and then diluting to a concentration of 3.75×10^8 cells/ml. Each animal received SRBCs in a 0.2 ml volume to give the final concentration of 7.5×10^7 /mouse. Mice were immunized by i.v. injection on day 25 of exposure. On day 29, mice were sacrifice, spleen were removed, weighted and placed into a tube with 3 ml EBSS. For the plaque assay, single cell suspensions of splenocytes were diluted in cold EBSS at 1:30 and 1:120 dilutions. The splenocytes were added to prepared tubes contained 0.50 ml of Bacto agar supplemented with 0.05% DEAE dextran and maintained in a water bath at 46-48°C.

When the assay was performed, 25 µl SRBCs were added first, followed by 0.1 ml of test cells, and finally 25 µl of guinea pig complement (GPC) diluted 1:4 from frozen stock in EBSS were added. Immediately after GPC addition, the tube was vortexed and the contents poured into a petri plate and covered with a 45x50 mm coverslip. Petri plates were incubated at 36-38°C for 3 hours to allowed plaque development. The antibody plaque-forming cells (AFC) were enumerated under a Bellco plaque viewer. Experimental results were expressed as specific activity (AFC/10⁶ spleen cells) and total spleen activity (AFC/spleen) by the following formula:

$$\text{AFC}/0.1 \text{ ml test cell} \times 10 \times \text{dilution (1:30 or 1:120)} = \text{AFC/ml in original suspension}$$

$$\text{AFC/ml} \times 3 = \text{AFC/spleen}$$

$$(\text{AFC/ml}) / 10^6 \text{ cells/ml} = \text{AFC}/10^6 \text{ cells}$$

3.2.8.4.2 Anti-CD3 mediated T lymphocyte proliferation assay

The proliferation of splenocytes in the presence of anti-CD3 antibody was performed as described by Guo et al. (2001) with slight modification. One day before the assay, 96-well flat bottom plates were coated with mouse Anti-CD3 antibody, that was diluted with Phosphate Buffered Saline (PBS) to a final concentration of 0.5 µg/ml and 100 µl was added to each well. The background control wells received PBS. Plates were incubated at 4°C overnight.

On day 29, the animals were sacrificed and single cell suspension of splenocytes were prepared as described previously. Then spleen cells were diluted to 2x10⁶ cells/ml in RPMI media supplemented with 10% heat-inactivated FBS.

Prior to the addition of cells, plates were flipped and pressed on to sterile

gauze to removed as much liquid as possible. Plates were then washed with 200 μ l of sterile PBS which was added to all wells. These activities were conducted in a sterile biological hood. One hundred microliters of splenocytes were then added to the wells coated with either anti-CD3 antibody or PBS. Four replicates per sample were used. Final volume of each well consisted of 200 μ l, after 100 μ l of RPMI supplemented 10% FBS was added to the cells. Plates were incubated at 37°C, 5% CO₂ for 3 days, plates were pulsed with 1 μ Ci of ³H-thymidine during the last 18-24 hours of incubation. Cells were harvested using a Cell Harvester (Ph.D.) onto glass filters and counted using a Beta liquid scintillation counter. Cells proliferation was evaluated by ³H-thymidine incorporation, which expressed as count per minute (CPM) per 2x10⁵ cells.

3.2.8.4.3 One-way mixed leukocytes response assay

The mixed leukocyte response (MLR) is an *in vitro* assay which determines the ability of spleen T cells from mice recognize allogeneic cells as “ non-self ” and proliferate in response (Holsapple et al., 1983). The assay was performed as described in detail by LeVier et al. (1994). Spleen single cell suspensions from B6C3F1 mice (responders) were aseptically prepared on the day of assay and were diluted to 1x10⁶ cells/ml in RPMI media supplemented with 10% heat-activated FBS and 2 mercapto ethanol (5x10⁻⁵M, 2 ME).

On the same day, splenocytes of DBA/2 mice (genetically dissimilar stimulators) were aseptically prepared, and diluted to 2x10⁷ cells/ml in EBSS with HEPES. The stimulator cells were treated with mitomycin C (0.5 mg/2x10⁸ total cells) to prevent DBA/2 spleen cell proliferation. Stimulator cells were incubated with mitomycin C for 45 minutes at 4-6% CO₂, 36-38°C. After the mitomycin C treatment,

cells were washed by EBSS with HEPES for 4 times. The stimulator cells were diluted to 4×10^6 cells/ml in RPMI 1640 supplemented with 10% FBS and 2ME. In the assay, 100 μ l of 4×10^6 cells/ml of and 100 μ l of 1×10^6 cells/ml of responder suspension were added to obtained a final optimized ratio of stimulators to responders of 4:1. Responder cell alone with RPMI complete media was used as a background control in this study. Plates were incubated for 5 days at 37°C , 5% CO_2 . On day 4 of incubation, each well was pulsed with 1 μCi of ^3H -thymidine. On day 5, the cells were harvested using a Cell Harvester (Ph.D.) onto glass filters and counted using a Beta liquid scintillation counter. Cells proliferation was measured by ^3H -thymidine incorporation.

3.2.8.4.4 Cytotoxic T lymphocyte assay

Cell- mediated cytotoxicity by cytotoxic T lymphocytes (effector cells) was evaluated using P815 tumor cells as target cells. The assay was conducted into two phases as described previously by Bradley et al. (1994). The first phase was the sensitization phase which allowed the effector cells to recognize the tumor antigens and beginning to proliferate and differentiate into cytotoxic T cells. The second phase was the effector phase of the assay during which cytotoxic T lymphocyte killing of radiolabeled target cells took place.

The sensitization phase was performed on day 29 and spleen cells were prepared as described above. Splenocytes were diluted to 4×10^7 cells/ml in Eagle's Minimal Essential Medium (E-MEM), supplemented with 10%FBS, 25 mM Hepes, 1mM L-glutamine, 50 $\mu\text{g}/\text{ml}$ gentamicin, and 1×10^{-5} 2ME, and then transferred to a 25 cm^2 tissue culture flask. P815 mastocytoma sensitizing cells treated with 50 μg of mitomycin C per 2×10^7 cells for 30 mintues at 37°C , 5% CO_2 . After mitomycin C

treated, cell were washed 4 times with PBS supplemented 5% FBS. Then cells were diluted and added to optimize a final responder: sensitizer ratio of 50:1. The tissue culture flasks were incubated for 5 days at 37°C, 5% CO₂.

Following sensitization, cultured spleen cells were harvested and wash with PBS supplement with 5%FBS 3 times, then resuspended in E-MEM media. The cell viability was measured following incubation with pronase for 10 mins, and cell concentration determined using a Coulter Counter ZBII. The effector phase of the assay was conducted by labeling P815 (target cells) with ⁵¹Cr (500 µCi ⁵¹Cr as sodium chromate per 1.5-2x10⁷ cells) for 60 mins at 37°C. Target cells were wash twice to remove excess chromium with PBS supplemented 5%FBS, followed the third washed by E-MEM media. Then target cells were adjusted to 2x10⁴ cells/ml, and were co-cultured with 100µl of graded numbers of splenic effector cells in round bottom microtiter culture plates at effector: target ratios of 25:1, 12.5:1, 6:1, 3:1, 1.5:1 and 0.75:1. Plates were centrifuged at 50 xg for 5 mins before incubation for 4 hrs at 37°C, 5% CO₂. After incubation, plates were centrifuged at 450 xg for 10 mins to obtain cell-free supernatant from each well for counting in a LKB gamma counter. Target cells in the presence of E-MEM media and 0.1% Triton X- 100 were served as the spontaneous and maximum release, respectively. Results were expressed as the percent of cytotoxicity and calculated by the following formula:

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental CPM} - \text{Spontaneous CPM}}{\text{Maximum release CPM} - \text{Spontaneous CPM}} \times 100$$

3.2.8.4.5 Natural killer cell assay

Natural killer cell activity was determined using the Moloney virus induced T cell lymphoma YAC-1 (ATCC- TIB-160) target cells. The assay was conducted as

described by Smialowicz et al. (1984) with slight modification. Splenocytes were prepared by the same method as described previously, and were diluted to 1×10^7 cell/ml.

The target cells (YAC-1) were labeled with ^{51}Cr (500 μCi ^{51}Cr as sodium chromate per $1.5\text{--}2 \times 10^7$ cells) for 90 min at 37°C with gentle vortexing at 15-20 minute interval. Cells were washed with RPMI supplemented by 10% FBS, and were resuspended in cold RPMI-10%FBS. The target cells viability was assessed by trypan blue dye exclusion and was used when viability was greater than 95%. One hundred microliters of viable labeled target cells at 5×10^4 cells/ml (5×10^3 cells/well) were co-culture with 100 μl of 1×10^7 cell/ml splenic cells (effector cells) in U-bottom microtiter culture plates, and incubated for 4 hours at 37°C , 5% CO_2 . The effector : target ratios utilized were 200:1, 100:1, 50:1, 25:1, 12.5:1 and 6.25:1. After incubation, plates were centrifuged at 300 xg for 10 minutes and a 100 μl of supernatant was removed and counted in LKB gamma counter. The labeled target cells in presence 0.1 ml media and 0.1% triton X-100 served as the spontaneous release and maximum release controls, respectively. Results were expressed as the percent of cytotoxicity and calculated by the following formula:

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental CPM} - \text{Spontaneous CPM}}{\text{Maximum release CPM} - \text{Spontaneous CPM}} \times 100$$

3.2.8.4.6 Phenotypic analysis of spleen cells

Splenocytes were adjusted to 1×10^6 cells/ well for direct fluorescent staining for $\text{NK1.1}^+\text{CD3}^-$ and CD3^+ positive cells. In addition, B cells (Ig^+) and T cell subpopulations ($\text{CD4}^-\text{CD8}^+$, $\text{CD4}^+\text{CD8}^-$, $\text{CD4}^+\text{CD25}^+$) and macrophage (Mac-3^+) were fixed and stained using a culture TQ-Prep to enumerated lymphocyte subsets and

macrophage.

The directed fluorescent staining for NK and T cells was performed by labeling cells with conjugated antibodies specific for the cell phenotypes. The quantification of lymphocyte subsets was performed as previously described with slight modification (LeVier et al., 1995). The surface marker phenotypes of cells were identified by using monoclonal antibodies (mAbs): rat antimouse-CD16/32 (block mouse Fc receptor), mouse anti-mouse NK-1.1 and FITC conjugated hamster anti-mouse CD3 ϵ . The splenocytes were plated in U-bottom 96-well plates, centrifuged 450 xg and washed one time with staining buffer. After gentle mixing the cells with vortex, the appropriate monoclonal antibodies were added to each well. The plates were incubated for 30 minutes at 4°C in the dark. The monoclonal antibodies were removed by centrifugation at 450 xg and the cells were washed one time in staining buffer. The plates were then incubated for 5 minutes at 4°C in the dark with propidium iodide at a dilution of 1:200 of 1 mg/kg stock in staining buffer to determine cell viability. Isotype controls for the specific surface markers included the following: isotype mouse IgG_{2a}, conjugated to PE and isotype hamster IgG₁, conjugated to FITC, respectively. Enumeration of NK and total T lymphocytes population was performed on a FACScan flow cytometry.

In order to enumerate B lymphocytes, T lymphocytes subsets and macrophage, the cells were fixed prior to counting on the flow cytometer as described by a Beckman Coulter company's instruction manual. The various antibodies were diluted 1:80 from stock concentration (as list in Table 3) in staining buffer. A monoclonal antibodies used included the following: goat anti-mouse Ig-FITC; rat anti-mouse CD4 conjugated to PE, rat anti-mouse CD8_a conjugated to FITC, rat

anti-mouse Mac3-FITC; and rat anti-mouse CD25-FITC. An isotype control for each specific surface marker was diluted 1:100 from stock concentration (as list in Table 3) with staining buffer: rat IgG_{2a} κ-PE, isotype rat IgG_{2a} κ-FITC, isotype rat IgG₁ κ-FITC, isotype rat IgM κ-FITC, and isotype hamster IgG group -FITC, this is the isotype for goat anti-mouse. The diluted antibodies and isotype controls were first added into appropriate 12x75 mm tubes respectively, and then 100 µl of diluted (1×10^7 cells/ml) were added into their respective tubes. All tubes were incubated in the refrigerator at 2-8°C for 20 minutes. Next, tubes were loaded onto the TQ-Prep machine to fix the cell by dispensing three ImmunoPrep Reagents. The three reagents were Immunoprep A (formic acid), Immunoprep B (sodium carbonate, sodium chloride, sodium sulfate) and Immunoprep C (paraformaldehyde). These reagents processed the sample by lysing the red blood cells, stabilizing the leukocytes and fixing the cell membrane. Sample were analyzed by using the FACScan Flow Cytometer.

3.2.8.4.7 ELISPOT assay for enumeration of IFN- γ , IL-2 and IL-4 producing cells in splenic lymphocytes.

ELISPOT assay allows the quantitative determination of number of cells in spleen that produce and secrete cytokines. The assay was performed as described by the instruction manual of BD Biosciences Pharmingen (Becton Dickinson and Company) with slight modification. In this approach, a 96 well-polyvinylidene fluoride (PVDF) membrane plate (PharMingen, USA) was coated overnight at 4°C with 100 µl of anti-IFN- γ mAb, anti-IL-4 or anti-IL-2 (Pharmingen, USA). These capture antibodies were diluted 1:200 from stock concentration of 1 mg/ml. Control well were placed with PBS. On the day of the assay, all wells were blocked with 200 µl

of RPMI 1640 supplemented with 10% FBS for 2 hours at room temperature. 100 μ l of splenocytes at concentration of 1×10^7 cells/ml were added and were serially diluted two fold to obtain final concentrations of 1×10^6 , 5×10^5 , 2.5×10^5 and 1.25×10^5 cells/well. Then plates were incubated 16-18 hours at 37°C, 5% CO₂. After incubation, plates were thoroughly washed one time with 0.05% Tween 20 in PBS (Wash Buffer I, Sigma, USA) and incubated with 100 μ l of biotinylated antimouse IFN- γ , IL-4 or IL-2 mAbs (Pharmingen) at 1:250 dilution from stock concentration of 0.5 mg/ml in PBS-10% FBS (dilution buffer). Plates were incubated for 2 hours at room temperature. Plates were washed 3 times with 200 μ l of wash buffer I. Enzyme labeled streptavidin was diluted 1:100 from stock concentration of 0.25 mg/ml for IL-2 and IL-4 and 0.5 mg/ml for IFN- γ in dilution buffer, and added to 100 μ l into each well, incubated at room temperature for 1 hour. After washing the well with wash buffer I for 4 times, following by washing with PBS for 2 times, each well was incubated with 100 μ l of substrate solution in the dark until spots were developed (30 minutes to 1 hour). Finally each well was washed 4 times with deionized water. Once plate was dried, spots were counted by ELISPOT analyzer.

A function of T lymphocyte is to recognize and proliferate following cytokine stimulation pathways, restimulation of DDDP *in vitro* may allow the previously sensitized T lymphocyte to produce cytokines after being exposed to DDDP *in vivo* for 28 days. To investigate this effect of DDDP, the ELISPOT assay was performed as described above with slight modification.

Briefly, on day 29, splenocytes were prepared and were diluted to 1×10^7 cells/ml, then cells were two fold diluted in the plates coated with IL-2, IL-4 or IFN- γ . DDDP in RPMI media alone was prepared aseptically by filtering through 0.45 μ m, and

was adjusted to final concentration of 1 µg/ml in 200 µl RPMI complete media. Plates were incubated overnight at 37°C, 5% CO₂. Cytokine production was determined as described previously.

3.2.9 *In vitro* direct exposure of DDDP to naïve splenocytes of female B6C3F1 mice

Direct exposure to DDDP *in vitro* was utilized to determine the ability of DDDP to enhance cell-mediated immunity and innate immunity in anti-CD3, MLR assays, and NK assay. To determine if DDDP affects primarily to the immune system, *in vitro* cytotoxicity effects on spleen cells and YAC-1 cells can be evaluated. In this study, viability of splenocytes after exposed to DDDP for approximately 24 hours was determined by flow cytometer analysis, whereas cytotoxicity of ⁵¹Cr labeled YAC-1 was evaluated by measuring the radioactivity from the culture-supernatant of YAC-1 treated with DDDP for 4 hours. As indicated before, a possible pathway of immune enhancement may result from the induction of cytokine. The direct addition of DDDP into cell culture would allow us to better evaluate this mechanism using the ELISPOT assay.

3.2.9.1 Viability test with naïve B6C3F1 mice

Splenocytes were prepared and were adjusted to 1x10⁶ cells/ml. One hundred microlitter of splenocytes was placed into each well of 96 well U bottom plates. DDDP was weighted and adjusted to final concentrations of 0.01 µg/ml to 1,000 µg/ml in RPMI media, and then was filtered through 0.45 µm filter before being added into culture media in a volume of 20 µl per well. Plates were incubated at 37°C, 5% CO₂

approximately 24 hours. Following incubation, plates were centrifuged at 900 xg for 10 mins and supernatants were removed. Staining buffer (0.1% Sodium azide and 1% Bovine serum in PBS) was placed into each well in a volume of 100 µl. After that plates were centrifuged at 450 xg for 4 mins, and supernatant was removed by vacuum aspiration. One hundred microliters of propidium iodide at dilution 1:200 was added in to each well, and plates were incubated for 5 mins on ice in the dark. Finally, cells were removed from each well and cells were placed into 1 ml staining buffer tubes. Cells viability after direct exposed to DDDP *in vitro* was analyzed using a FACScan flow cytometry.

3.2.9.2 Direct cytotoxicity of DDDP to YAC-1

YAC-1 was labeled with sodium chromate (^{51}Cr) as described previously in the NK assay. DDDP was aseptically prepared and co-cultured with YAC-1 in the final concentration ranging from 1 µg/ml to 1,000 µg/ml of 96-well plate (U bottom). The labeled YAC-1 cells in the presence 0.1 ml media and 0.1% Triton X-100 served as the spontaneous release and maximum release controls, respectively. Then plate was incubated for 4 hours at 37°C, 5% CO₂. After incubation, plate was centrifuged to remove supernatant and the amounts of chromium released from dead cells were counted using a Gamma counter.

3.2.9.3 *In vitro* anti-CD3 assay

To measure the direct effect of DDDP in the anti-CD3 assay, the extracts were prepared and added into 96-well flat bottom plates coated with antiCD3 antibody or PBS control. DDDP was diluted with RPMI media, sterile filtered, and was adjusted to

0.01-1,000 µg/ml. One hundred microliter of DDDP at each concentration was added to splenocytes 2×10^5 cells/well. Plates were incubated for 3 days at 37°C, 5% CO₂. The anti-CD3 assay was performed as described in 2.8.4.2.

3.2.9.4 *In vitro* mixed lymphocytes response assay

Direct effects of DDDP extract on T leukocytes proliferation were measured in the *in vitro* MLR assay. Responder cells and stimulator cells were co-culture in 96-well U bottom plate at final ratio of 1:4. Steriled DDDP extracts at concentrations ranging from 0.01 µg/ml to 1,000 µg/ml were added to each well. Plates were incubated for 5 days at 37°C, 5% CO₂. T leukocyte proliferation against allogeneic cells was measured by ³H-TdR incorporation as described in 2.8.4.3.

3.2.9.5 *In vitro* NK assay

NK assay was performed as described in 2.8.4.5 . Briefly, the effector cell was adjusted to 1×10^7 cells/ml, and were co-cultured with ⁵¹Cr labeled YAC-1 at a concentration of 1×10^5 cells/ml to obtained an effector to target ratio of 100:1. Then effector cells were two fold diluted in the plates to yield the effector to target ratios of 50:1 and 25:1 respectively. Next, DDDP extracts at concentrations ranging from 0.001 µg/ml to 300 µg/ml were added into each effector to target ratio to determine the direct effect of DDDP on NK cells activity.

3.2.9.6 *In vitro* ELISPOT assay

In order to determine if DDDP- induced cytokine production, an ELISPOT assay was conducted following directed incubation of splenocytes with DDDP extracts

for either 3 hrs or 22 hrs before measuring cytokine production. Naïve splenocytes from female B6C3F1 were prepared and diluted to 1×10^7 cells/ml. After which, 100 μ l of splenocytes was placed into each well which had been coated with anti-IL-2 antibody, or anti-IFN- γ antibody. Sterile DDDP was then added into each well to yield final concentrations ranging from 0.001 μ g/ml to 100 μ g/ml. Plates were then incubated for 3 hrs or 22 hrs at 37°C, 5% CO₂. Following incubation, plates were evaluated for cytokines production as described in 2.8.4.7.

3.3 STATISTIC ANALYSIS

All results were analyzed for homogeneity of the data by the Barlett's test (Kruskal and Wallis, 1952). Homogeneous data were evaluated by using a one-way analysis of variance (ANOVA), and differences between treatment groups and the vehicle control group were compared by using a Dunnett's two tails Test (Dunnett, 1995). Non-homogenous data was evaluated by using a non-parametric analysis of variance. Significance differences were compared between the experimental groups and the vehicle control groups by using the Wilcoxon rank test (Gross and Clark, 1975). The modulatory controls were compared to the vehicle using a Student's T Test (Sokal and Rohlf, 1981). Jonckheere's test (Holander and Wolfe, 1975) was used as a test for trend across the vehicle and treatment groups. In the results, the data is presented as the mean $\pm$ S.E. of mean.

CHAPTER IV

RESULTS

In vitro results on cytotoxicity and immunological effects of DDDP and SDDD extracts were investigated using the MTT colorimetric assay. The applicable models were conducted in C57BL/6j mice, and the preliminary *in vitro* results were obtained before an *in vivo* assay was performed. The assays were selected to provide information on the direct effect of the test compounds on the immune cells primarily T and B lymphocytes. Lymphoproliferation of T cells were measured after activation with Con A and anti-CD3 antibody, whereas B cells were induced to proliferate after treatment with LPS.

4.1 The Selection of Appropriate Concentration of DDDP and SDDD for *In Vitro* Toxicity Studies

The results of the toxicity studies of both DDDP and SDDD extracts from DDD were shown in Figs.15 and 16. Data from the toxicity tests of seed and whole plant extracts to splenocytes of C57BL6/j mice demonstrated that no toxicity was found for the SDDD extract at concentrations ranging from 1.95 to 2,000 µg/ml (Fig. 15), and at concentrations ranging from 1.25 to 1,000 µg/ml for the DDDP extracts (Fig.16). A toxic effect was only observed at high concentrations of 5,000 to 10,000 µg/ml for the SDDD extract (Fig. 15) and at 2,000-5,000 µg/ml for the DDDP extracts (Fig. 16).

Results from the toxicity studies indicated that the appropriate concentrations of DDD for subsequent *in vitro* immunotoxicological studies is between 1.95 to 2,000 $\mu\text{g/ml}$, and 1.25 to 1,000 $\mu\text{g/ml}$ for the SDDD and DDDP extracts, respectively.

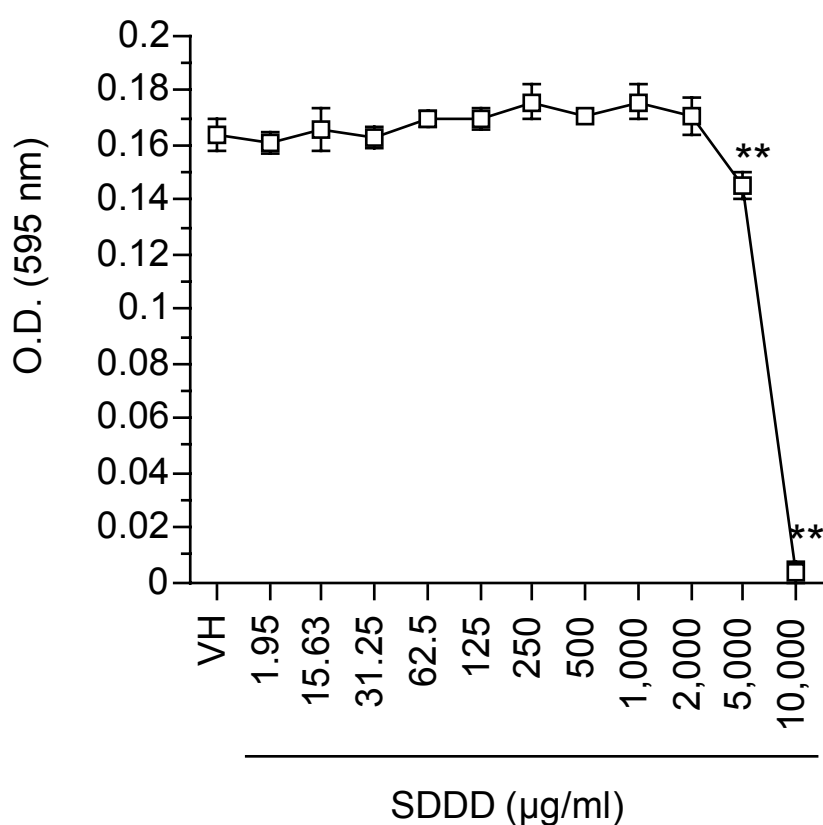


Figure 15 Toxicity test of SDDD extracts of *Aeginetia indica* Roxb.

** represent significant differences at $p \leq 0.01$ as compared to the vehicle control group.

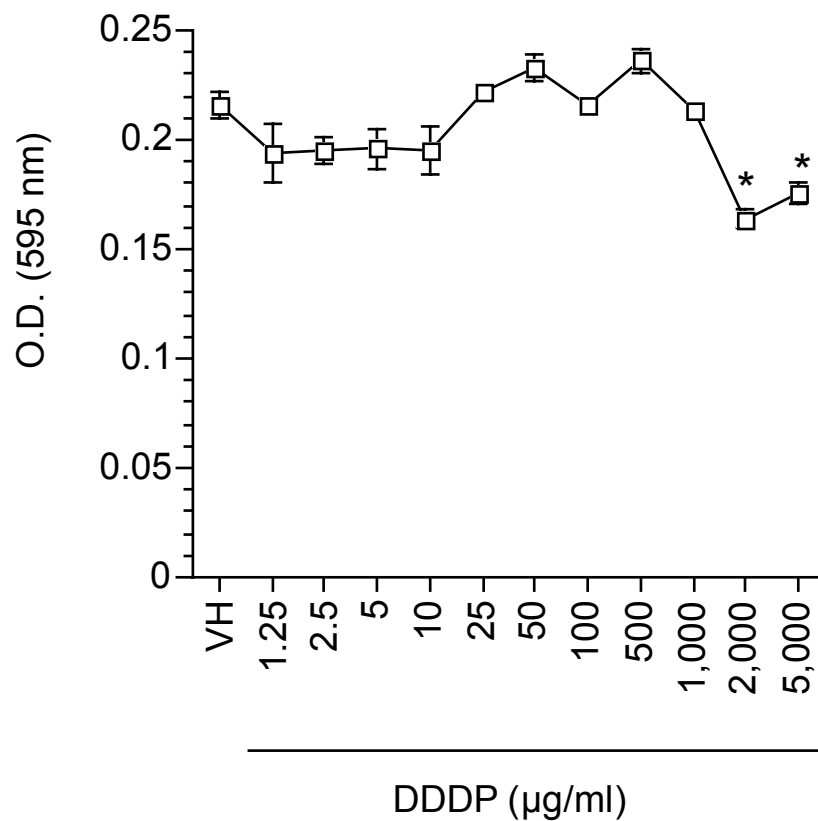


Figure16 Toxicity test of DDDP of *Aeginetia indica* Roxb.* represent significant differences at $p \leq 0.05$ as compared to the vehicle control group.

4.2 The Optimization of *In Vitro* Immunological Assays

4.2.1 AntiCD3 mediated T lymphocyte proliferation assay

The effect of mouse anti-CD3 antibody mediated T lymphocyte proliferation was measured by MTT colorimetric assay. As shown in Figs. 17, 18 and 19, the splenocyte concentration of 4×10^6 cells/ml gave a higher response to mouse anti-CD3 antibody compared to 2×10^6 cells/ml from day 1 to day 3. The $1 \mu\text{g/ml}$ of anti-CD3 antibody induced higher response compared to the concentration at $0.5 \mu\text{g/ml}$ at both cell

concentration (Figs. 17, 18). The results from these studies indicated that optimum conditions for the assay of anti-CD3 mediated T lymphocyte proliferation assay evaluated by MTT colorimetric method is to use splenocytes at a final cell concentration of 4×10^6 cells/ml and mouse anti-CD3 antibody at a final concentration of 1 $\mu\text{g/ml}$.

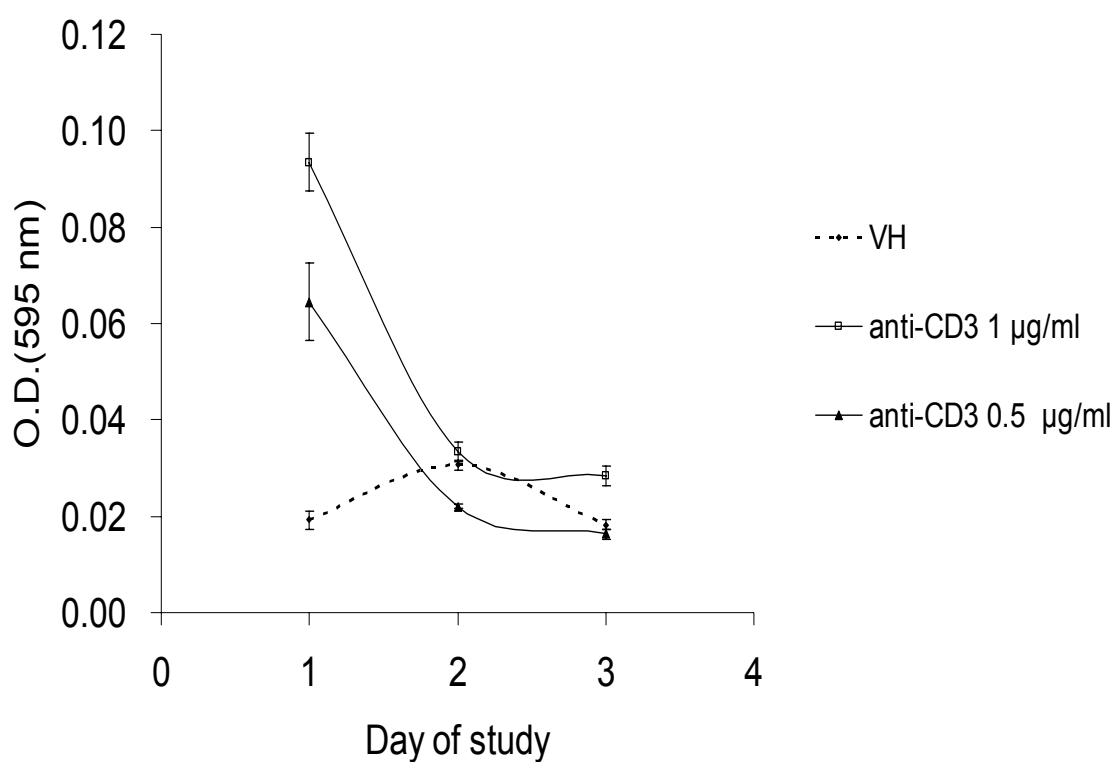


Figure17 Effect of mouse anti-CD3 antibody mediated T cell proliferation on splenocytes at cell concentration of 2×10^6 cells/ml from days 1-3.

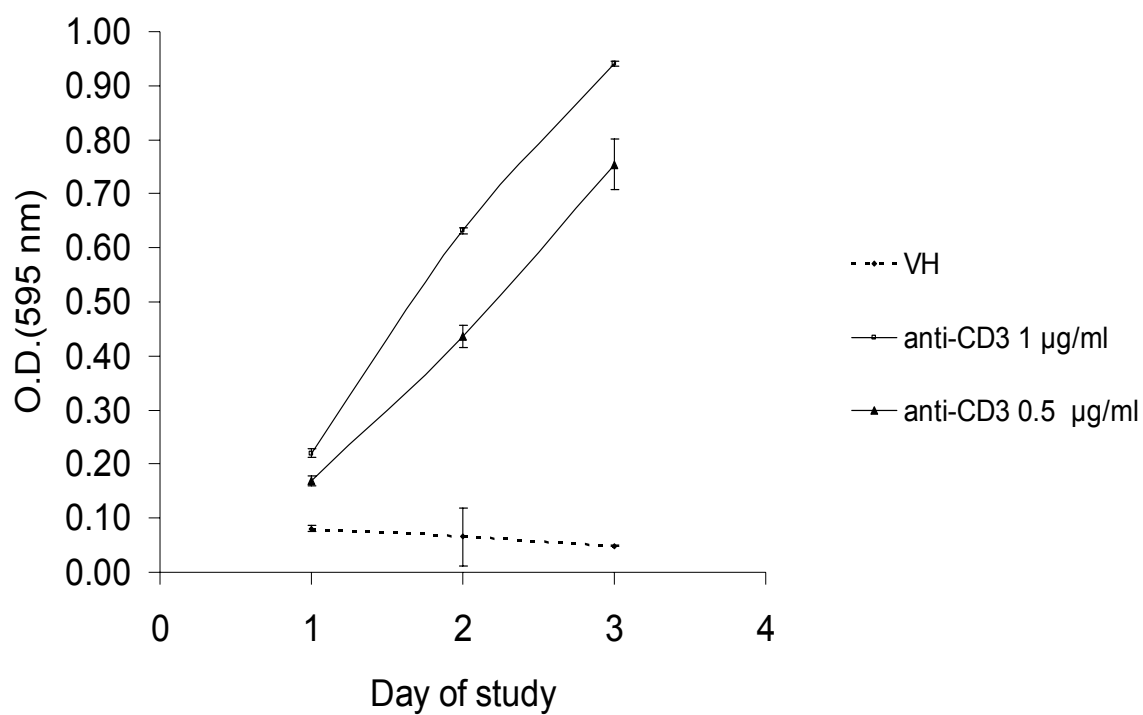


Figure18 Effect of mouse anti-CD3 antibody mediated T lymphocyte proliferation on splenocytes at cell concentration of 4×10^6 cells/ml from days 1-3.

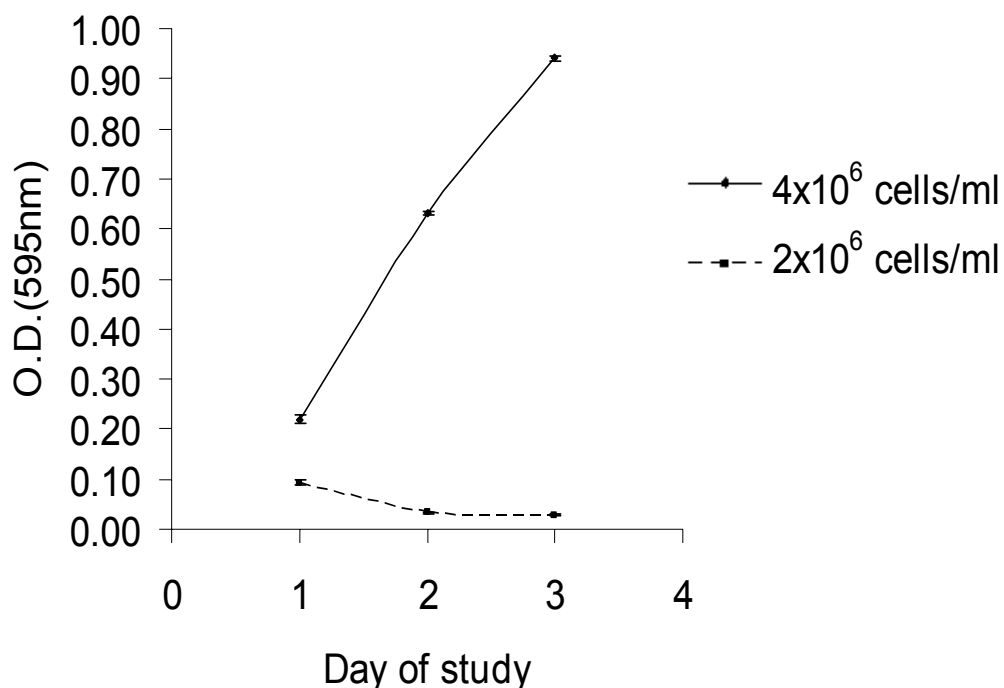


Figure 19 Comparison of the effect of 1 µg/ml of mouse anti-CD3 antibody mediated T cell proliferation between cell concentrations of 2×10^6 cells/ml and 4×10^6 cells/ml on days 1-3.

4.2.2 Lymphocyte proliferation assay : Concanavalin A (Con A) mitogen

The results of proliferative response of splenocytes to con A mitogen measured by MTT colorimetric assay were shown in Figs. 20, 21 and 22. Results indicate that cell concentration of 8×10^6 cells/ml gave a higher response to con A from day 1 to 3 compared to 4×10^6 cells/ml. The studies of titering con A concentrations at 2.5, 5, 10 and 20 µg/ml in the assay indicated that a final concentration of 5 µg/ml gave the highest response in both cultures containing 4 or 8×10^6 cells/ml (Figs. 20, 21), and the optimum response occurred on day 2 or 3 after sensitization with con A (Fig. 22).

Therefore, the results indicated that suitable conditions for con A proliferative response assessed by MTT colorimetric method was to use a concentration of splenocytes at 8×10^6 cells/ml, Con A at final concentration of $5 \mu\text{g/ml}$, and the optimum response occurred around day 2 or 3 after sensitization.

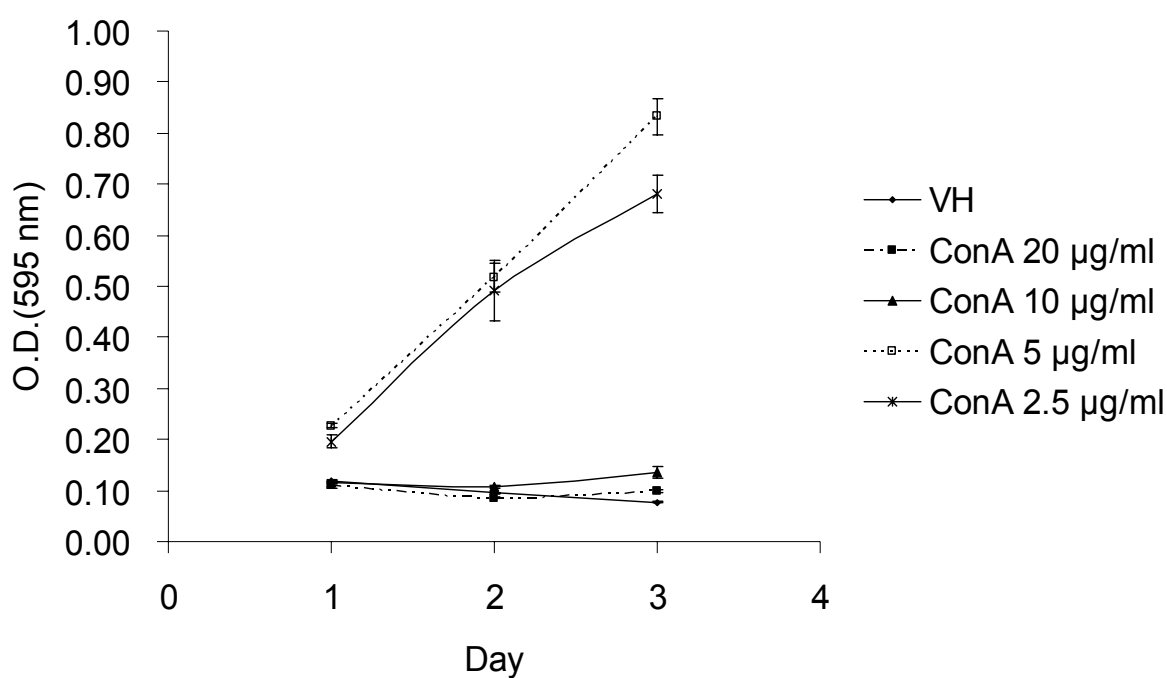


Figure 20 Effect of different concentrations of con A on the proliferative response of splenocytes cultured at 4×10^6 cells/ml from days 1-3.

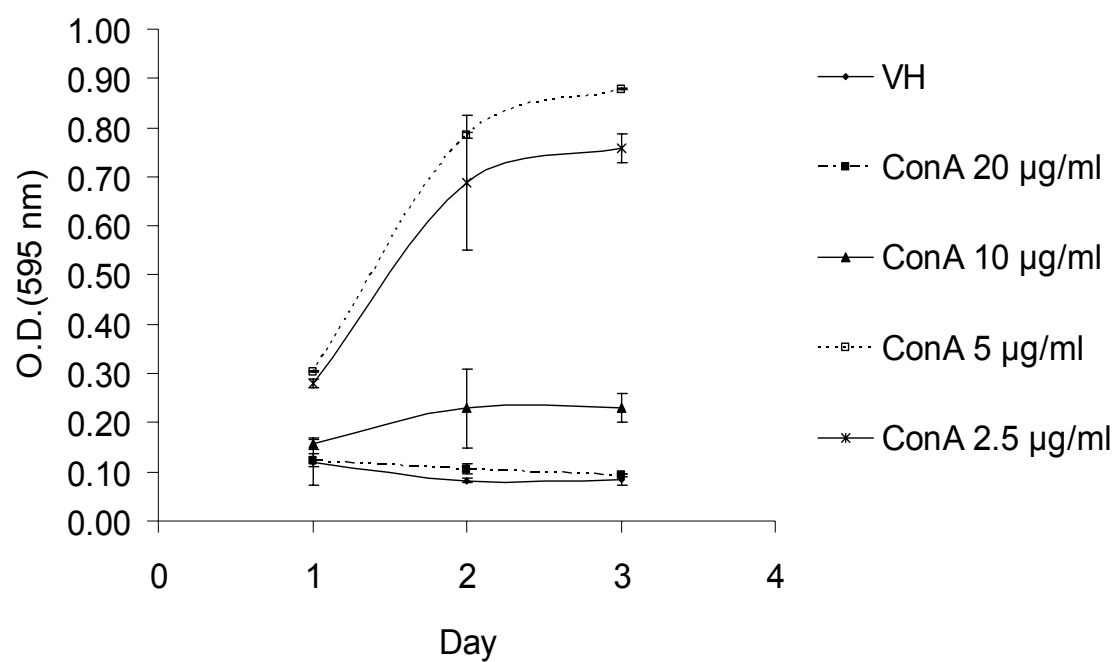


Figure 21 Effects of different concentrations of con A on the proliferative response of splenocytes cultured at 8×10^6 cells/ml from days 1-3.

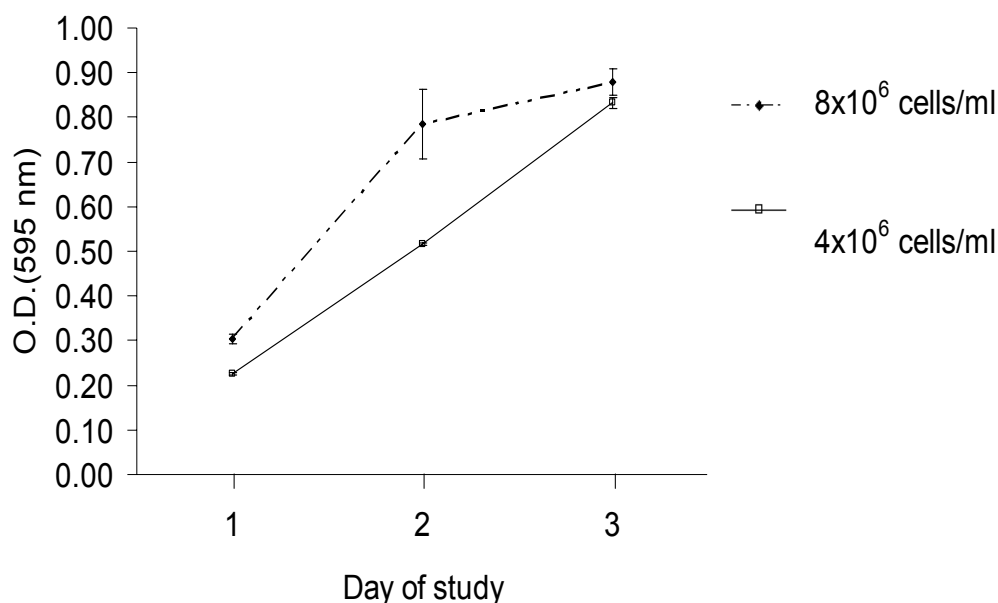


Figure 22 Comparison of the proliferation of cultures containing 4 and 8x10⁶ cells/ml in response to 5 µg/ml of con A on days 1-3.

4.2.3 Lymphocyte proliferation assay : Lipopolysaccharide (LPS) mitogen

The results of proliferative response of splenocytes to LPS mitogen measured by MTT colorimetric assay were shown in Figs. 23, 24 and 25. Results suggested that cell concentration of 8x10⁶ cells/ml gave a higher response to LPS from day 1 to 3 compared to 4x10⁶ cells/ml. The studies of titering LPS concentrations at 5, 10, 100, 500 and 1,000 µg/ml in the assay suggested that a final concentration of 5, 10 or 100 µg/ml gave the highest response depending on the number of cells per culture and the time of incubation. For 4x10⁶ cells/ml of the culture, and the time of incubation the peak response occurred at LPS 10 µg/ml on day 3 following stimulation with LPS or occurred on day 2 in culture containing 8x10⁶ cells/ml and stimulated with LPS 10 µg/ml (Figs. 23, 24 and 25). Therefore, the results indicate that suitable conditions for LPS proliferative response assessed by MTT colorimetric method use a concentration

of splenocytes at 8×10^6 cells/ml, LPS at a final concentration of $10 \mu\text{g/ml}$, and the optimum response occurred around day 2 after sensitization.

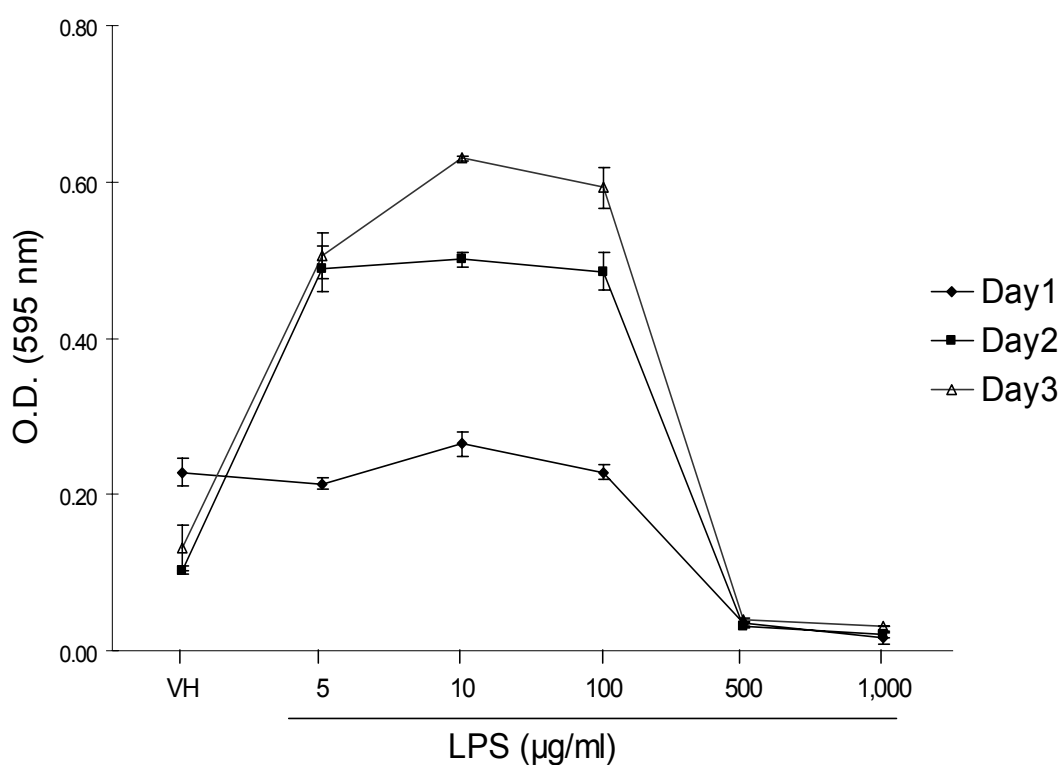


Figure 23 Effects of different concentrations of LPS on the proliferative response of splenocytes cultured at 4×10^6 cells/ml from days 1-3.

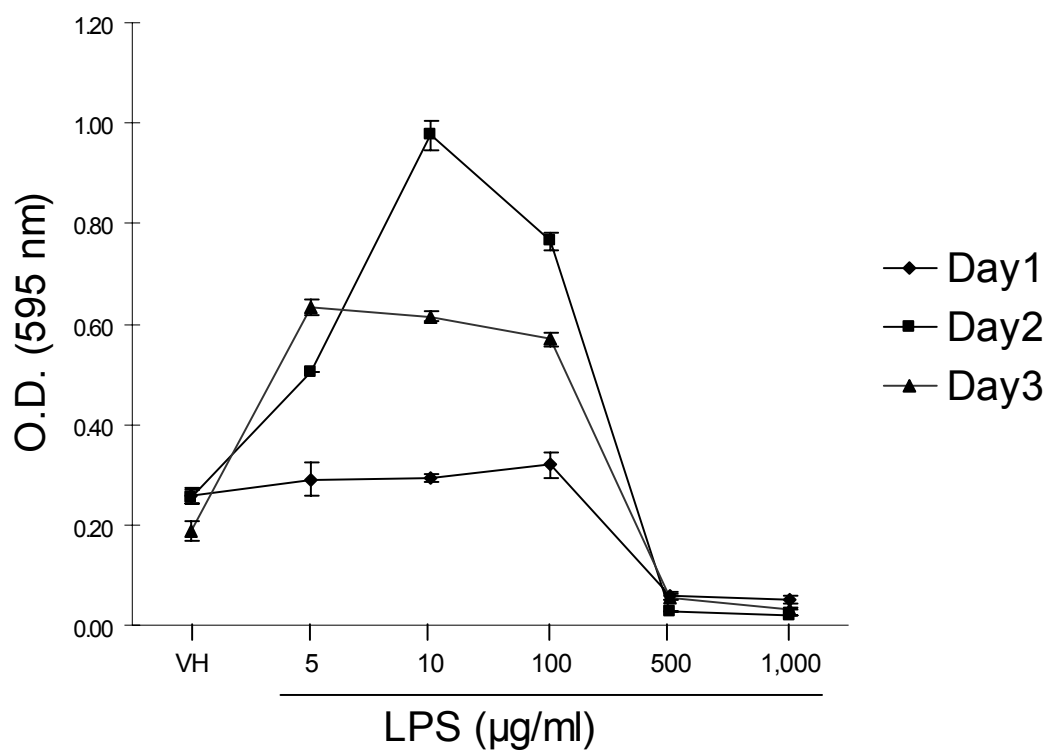


Figure 24 Effects of different concentrations of LPS on the proliferative response of splenocytes cultured at 8×10^6 cells/ml from days 1-3.

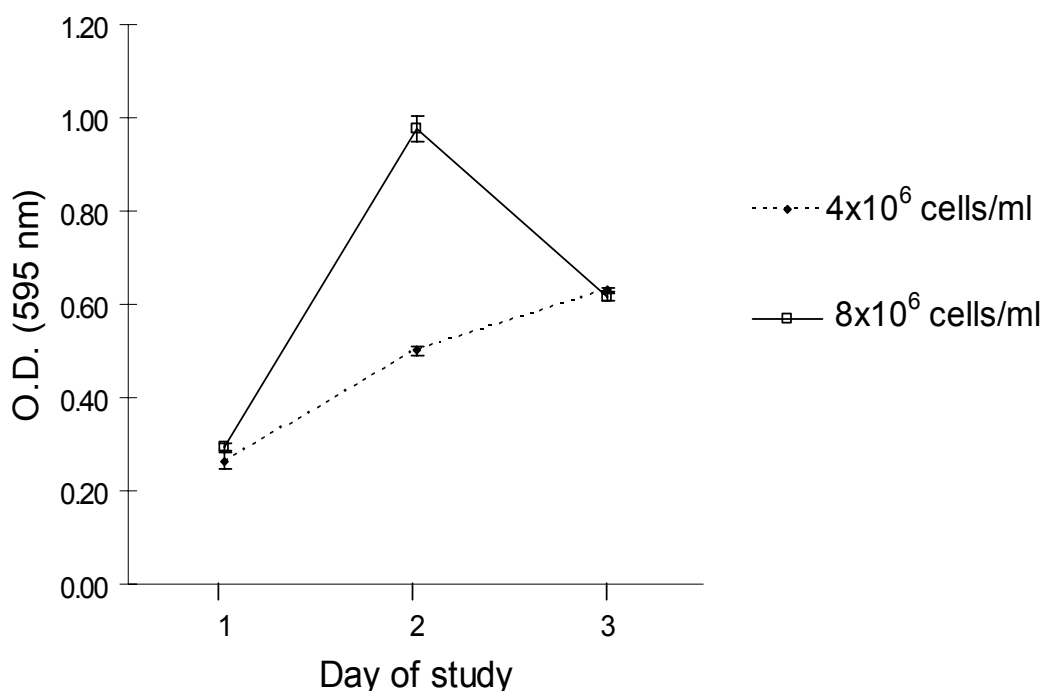


Figure 25 Comparison of the proliferation of cultures containing 4 and 8x10⁶ cells/ml in response to 10 µg/ml of LPS on days 1-3.

4.3 Effect of SDDD and DDDP Extracts from *Aeginetia indica* Roxb. (DDD) on *In Vitro* Immunological Studies

4.3.1 Effect of SDDD extracts and DDDP extracts from DDD on anti-CD3 response of T lymphocyte

Results of the effect of seed extracts (SDDD) on anti-CD3-mediated T lymphocytes response tested on days 1-3 were shown in Figure 26 and 27. Results demonstrated that SDDD extracts of *Aeginetia indica* Roxb. at final concentrations of 500 and 1,000 µg/ml stimulated the proliferation of T lymphocytes significantly ($p \leq 0.05$) compared to the vehicle control group on day 1 (Fig. 27). For day 2

concentrations of extracts at 500, 31.25 and 7.8 $\mu\text{g/ml}$ significantly ($p \leq 0.05$) stimulated T cells more than the vehicle control group (Fig. 27). From the study, the peak response of the vehicle control and most of the treatment groups occurred on day 2 (Fig. 26). The OD 595 nm at the peak response of the vehicle control group on day 2 was 0.864 and was decreased to 0.751 on day 3 (Fig. 26).

Interestingly, the proliferative response on day 2 at the highest SDDD treatment group of 2,000 $\mu\text{g/ml}$ was suppressed when compared to the vehicle control group, but the response on day 3 was significantly higher than the respective controls (Figs. 26 and 27). Therefore, the effect of SDDD at 2,000 $\mu\text{g/ml}$ most likely did not represent a suppression of the response, but only a shift in the time course of the optimum response from day 2 to day 3.

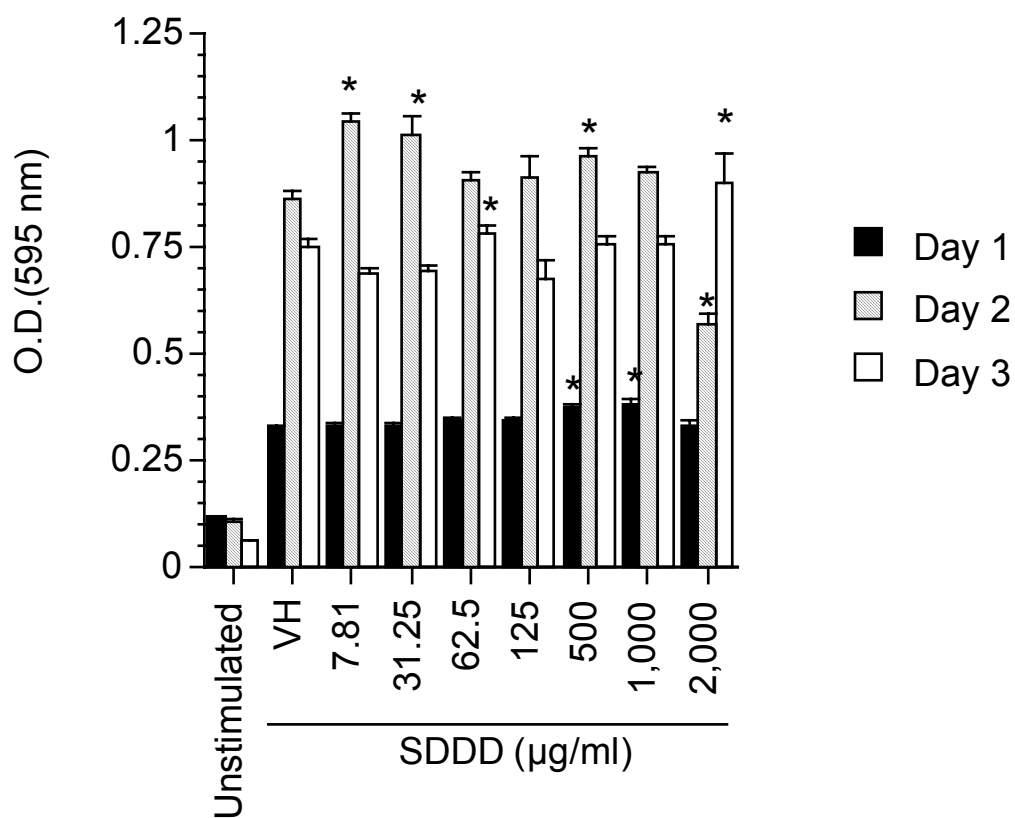


Figure 26 Effect of SDDD extract on anti-CD3 antibody mediated T lymphocyte proliferation on days 1-3. * represent significant differences at $p \leq 0.05$ compared to the vehicle control group.

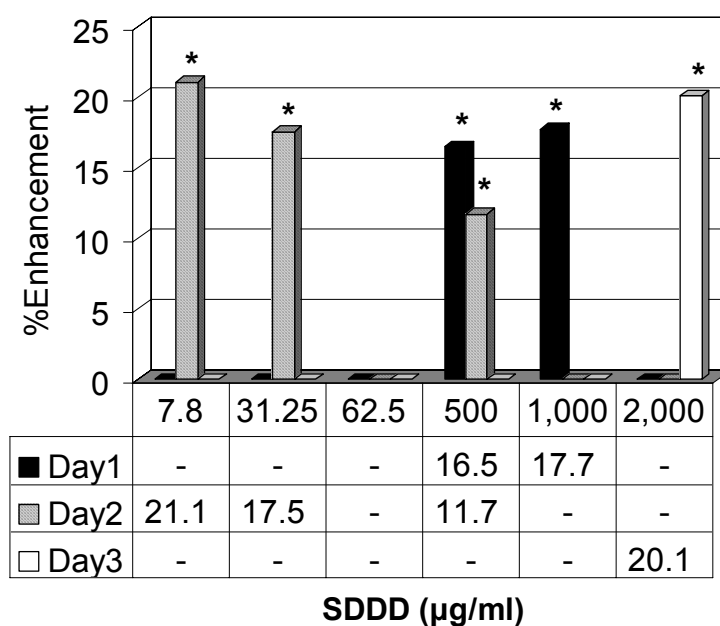


Figure 27 Percentage enhancement of splenocyte proliferative response upon stimulation with anti-CD3 antibody in the presence of various concentrations of SDDD extracts (control group: 0.00%). * represent significant differences at $p \leq 0.05$ compared to the vehicle control group.

Presented in the figures 28 and 29 are the results of the anti-CD3 antibody mediated T lymphocyte proliferation test in the presence of DDDP extracts on days 1-3. The results show that DDDP extracts at a final concentration of 100 µg/ml stimulated T lymphocytes to proliferate more than the vehicle control group on day 1 and day 3. Five microgram per milliliter (5 µg/ml) of DDDP extract also significantly stimulated the T cell response to anti-CD3 antibody on day 1 at $P \leq 0.01$ (Fig. 29). At 1,000 and 500 µg/ml concentrations of extracts, there were significantly ($P \leq 0.01$) suppression of

T lymphocytes proliferation from day 1 until day 3 (Fig. 28) and the effect was not due to direct cytotoxicity to cells (Fig. 16). Furthermore, 1.25 and 2.5 $\mu\text{g/ml}$ of extracts suppressed the response of cells on day 1 ($P \leq 0.01$), but on day 2 there were no suppression of either DDDP concentrations and, in fact, at concentration of 1.25 $\mu\text{g/ml}$, there was a significant stimulation of the response at $p \leq 0.05$ (Figs. 28 and 29).

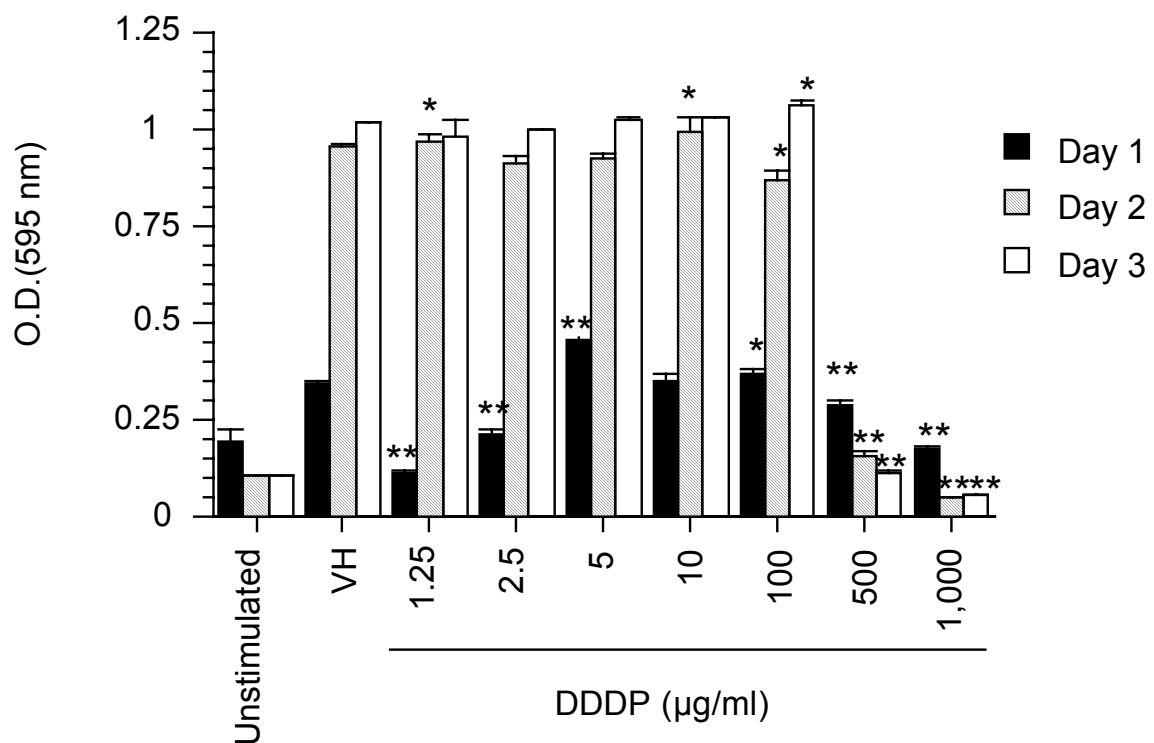


Figure 28 Effect of DDDP extract on anti-CD3 antibody mediated T lymphocyte proliferation on days 1-3. *,**represent significant differences from compare to the vehicle control group at $p \leq 0.05$, and $p \leq 0.01$, respectively .

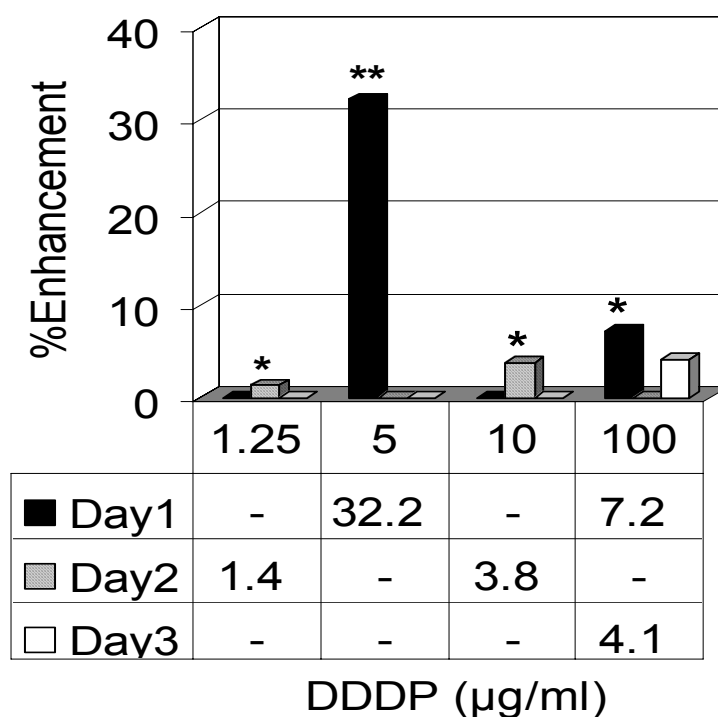


Figure 29 Percentage of enhancement of splenocyte proliferative response upon stimulation with anti-CD3 antibody in the presence of various concentrations of DDDP extracts (control group: 0.00%). *,**represent significant differences from compared to the vehicle control group at $p \leq 0.05$, and $p \leq 0.01$, respectively .

4.3.2 Effect of SDDD and DDDP extracts from DDD on lymphoproliferative response to concanavalin A (Con A) mitogen

The results in Figs. 31-32 showed the effect of DDDP extracts on spleen cell response to con A mitogen on days 1-3. As shown, DDDP extracts at concentrations of 1.25 to 500 µg/ml stimulated T lymphocyte proliferative response to con A significantly at $p \leq 0.05$ and $p \leq 0.01$ from day 1 to day 3. However, at the highest concentration of DDDP extracts tested, 1,000 µg/ml, there was an inhibition of cell

proliferation and the suppression of the response was not due to direct cytotoxicity of the DDDP extracts on the cells as suggested by the result in Figure 16.

The results of the SDDD extracts on spleen cell mitogenic response to con A indicate that seed extracts did not have as strong effect in activating T lymphocyte as did the DDDP extracts. The only enhancement of the response that differed significantly from vehicle control ($p \leq 0.05$) was observed at the concentration of 10 $\mu\text{g/ml}$, and on day 3 after sensitization (Fig. 30).

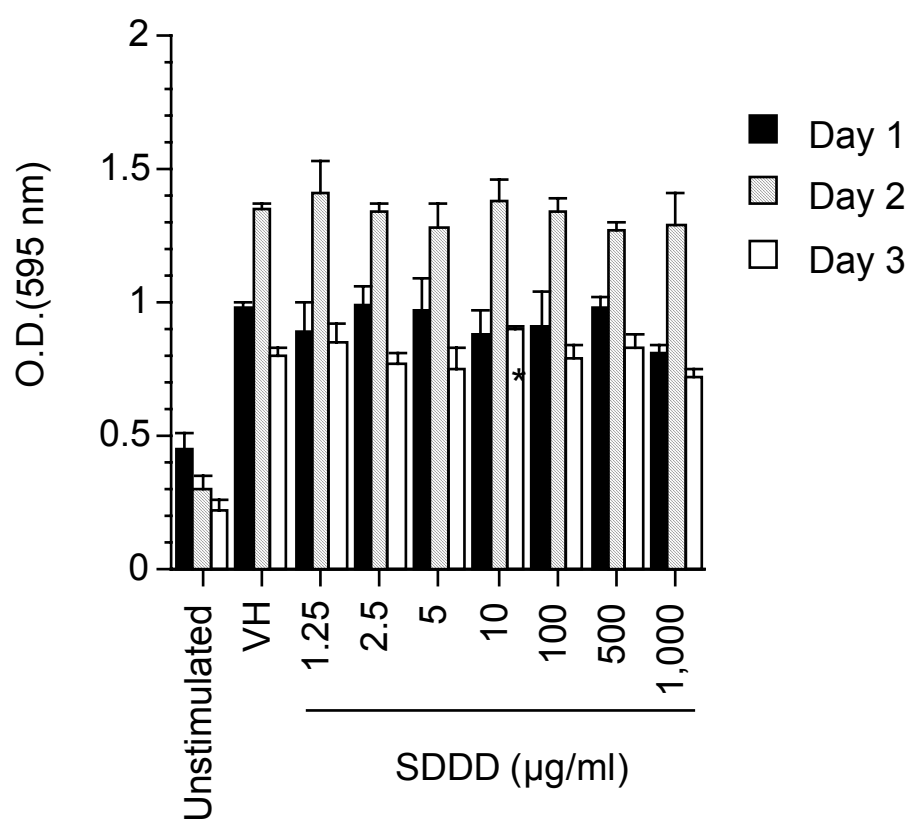


Figure 30 Effect of SDDD extract on lymphoproliferative response to con A mitogen on days 1-3. * represent significant differences at $p \leq 0.05$ compared to the control group.

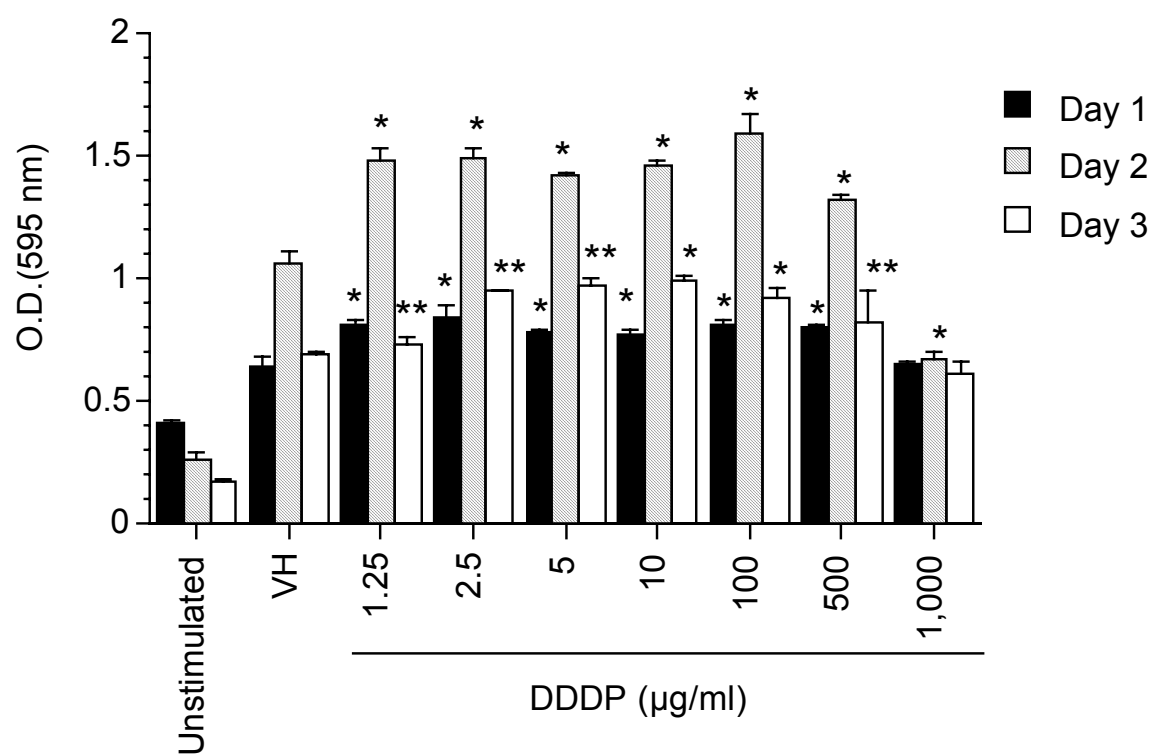


Figure 31 Effect of DDDP extract on lymphoproliferative response to con A mitogen on days 1-3. *,** represent significant differences compared to the vehicle control group at $p \leq 0.05$ and $p \leq 0.01$, respectively.

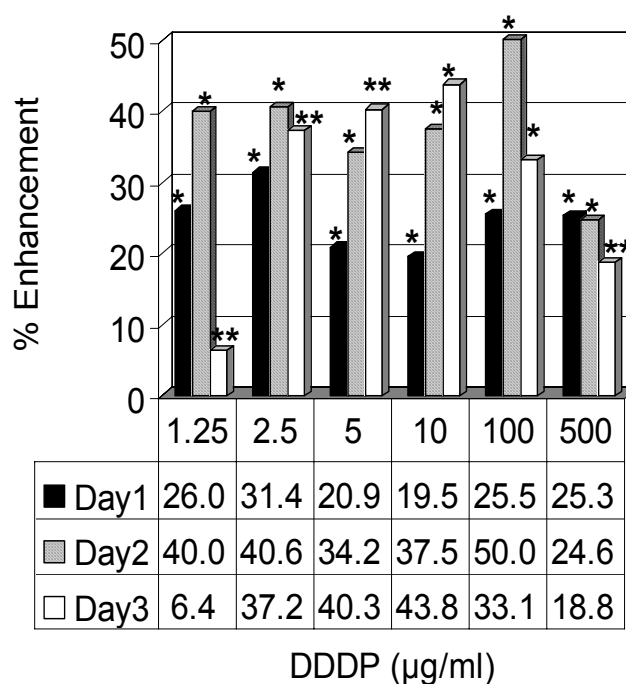


Figure 32 Percentage of enhancement of splenocyte proliferative response upon stimulation with con A in the presence of various concentrations of DDDP extracts (control group: 0.00%). *,** represent significant differences compared to the vehicle control group at $p \leq 0.05$ and $p \leq 0.01$, respectively.

4.3.3 Effect of SDDD and DDDP extracts from DDD on lymphoproliferative response to Lipopolysaccharide (LPS)

The effects of SDDD extracts on lymphocyte response to LPS mitogen on day 1 until day 3 are presented in Figs. 33 and 34. On day 1, the data showed that SDDD at a concentration of 2.5 µg/ml stimulated B lymphocyte response to LPS significantly at $P \leq 0.05$. On day 2 which was the peak response, seed extracts at concentrations of 1.25,

10, 100, 1,000 and 2,000 $\mu\text{g/ml}$ significantly ($p \leq 0.05$ and $p \leq 0.01$) stimulated the B lymphocyte response to LPS to a greater degree than the vehicle control group.

The data on day 3 indicated that concentrations of seed extracts (SDDD) at 1,000-2,000 $\mu\text{g/ml}$ enhanced B lymphocyte response against LPS significantly higher than controls at $P \leq 0.01$. At 1.25 and 100 $\mu\text{g/ml}$ of SDDD extracts, there were suppression of the B lymphocyte response to LPS on day 1, but there was no suppression on day 2. In fact, on day 2, which was the peak response day, both concentrations of SDDD extracts stimulated responses higher than vehicle controls significantly at $p \leq 0.05$ and $p \leq 0.01$, respectively (Fig. 33).

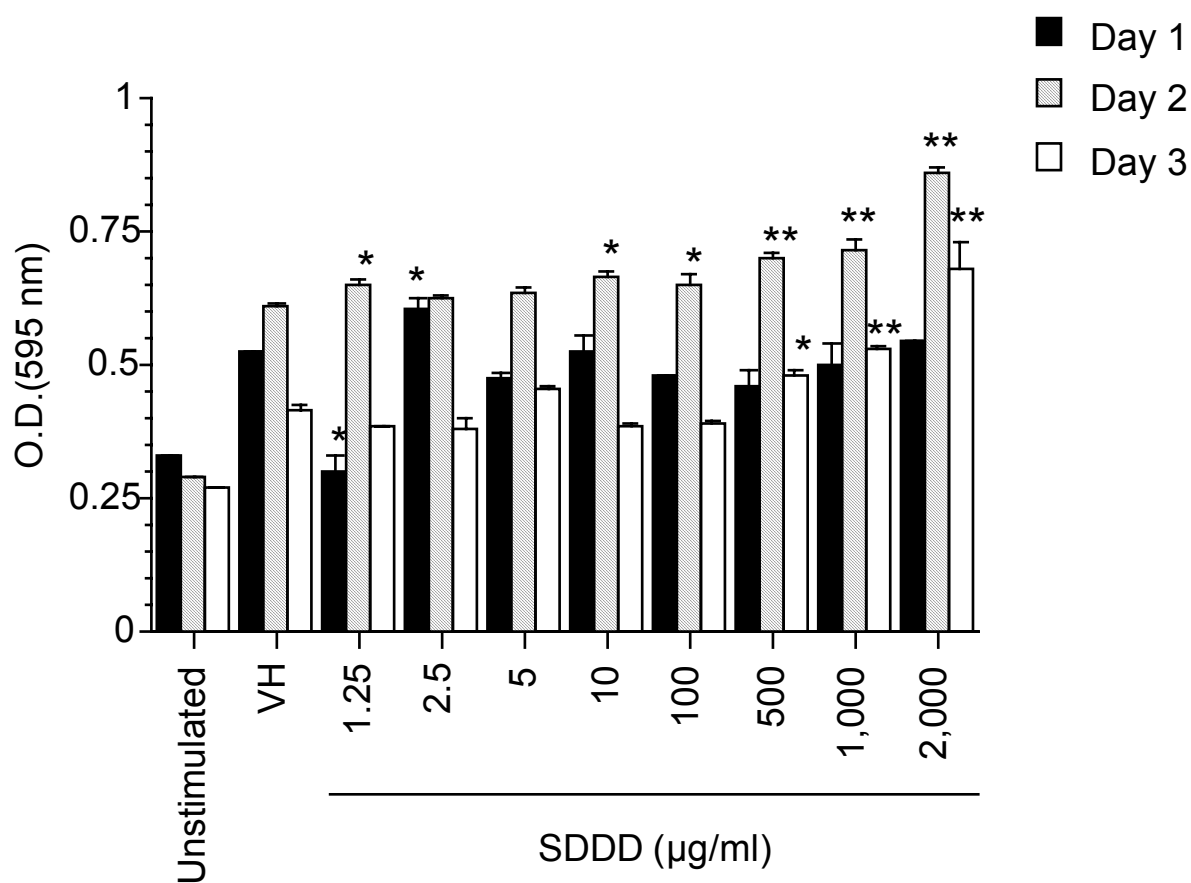


Figure 33 Effect of SDDD extracts on lymphoproliferative response to LPS on days 1-3. *,** represent significant differences compare to the control group at $p \leq 0.05$ and $p \leq 0.01$, respectively.

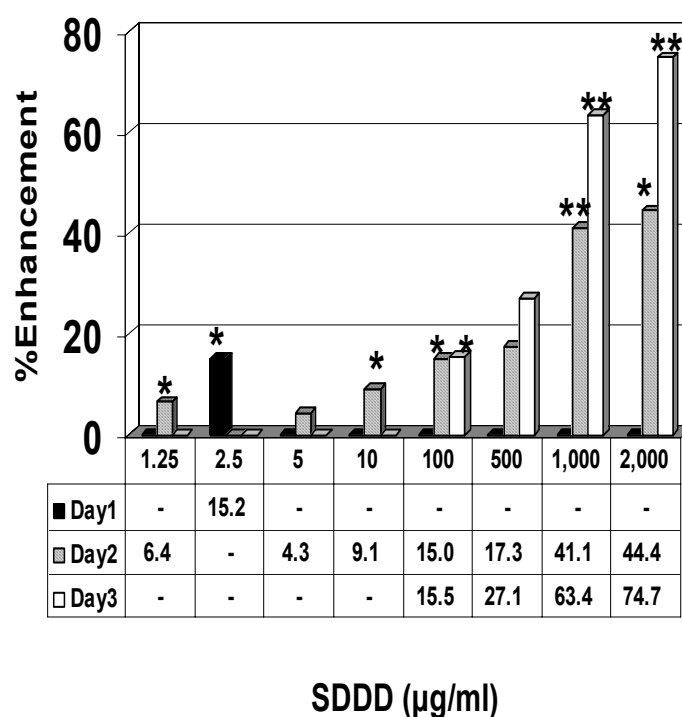


Figure 34 Percentage of enhancement of splenocyte proliferative response upon stimulation with LPS in the presence of various concentrations of SDDD extracts. (control group: 0.00%). *,** represent significant differences compare to the control group at $p \leq 0.05$ and $p \leq 0.01$, respectively.

The effect of DDDP extracts on spleen cell mitogenic response to LPS on day 1 until day 3 were presented in Fig. 35 and Fig. 36. Results demonstrated that DDDP extracts at concentrations of 1.25-100 µg/ml significantly stimulated B lymphocyte responses to a greater degree than vehicle controls at $p \leq 0.05$ and $p \leq 0.01$ on days 1-3, with the exception on day 1, in which the concentration of DDDP extracts at 1.25 µg/ml did not significantly stimulate B lymphocytes. As seen previously, at 500 and 1,000

$\mu\text{g/ml}$, there was a significant suppression of the response ($p \leq 0.01$) (Fig. 35), and the effects do not appear to be due to direct cytotoxicity to cells (Fig.16).

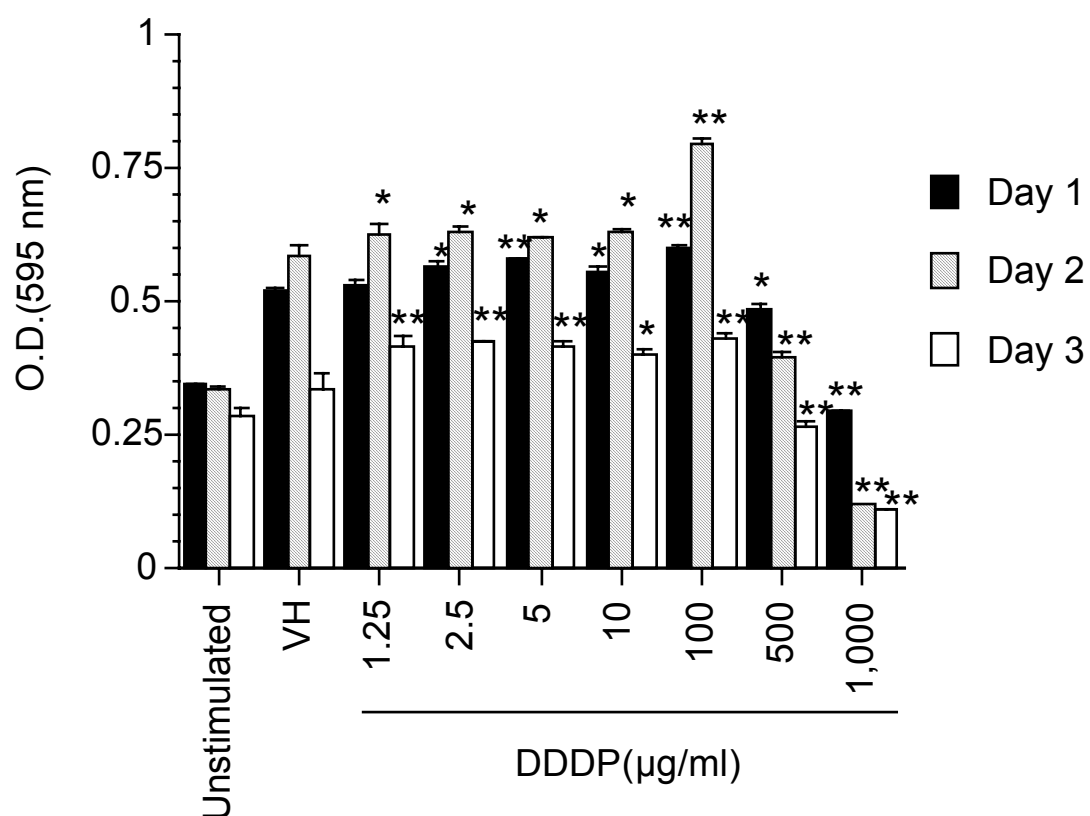


Figure 35 Effect of DDDP on lymphoproliferative response to LPS on days 1-3.

*, ** represent significant differences as compared to the vehicle control group at $p \leq 0.05$ and $p \leq 0.01$, respectively.

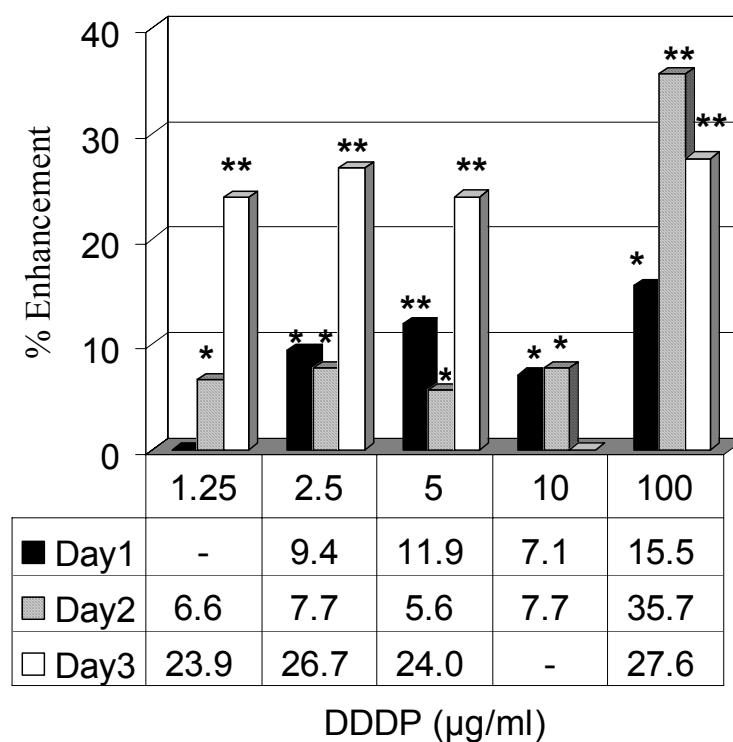


Figure 36 Percentage of enhancement of splenocyte proliferative response upon the stimulation with LPS in the presence of various concentrations of DDDP extracts (control group: 0.00%). *, ** represent significant differences as compared to the vehicle control group at $p \leq 0.05$ and $p \leq 0.01$, respectively.

4.3.4 Effect of DDDP on Human cervical cancer cells (HeLa)

The result of the cytotoxicity of DDDP extracts from *Aeginetia indica* Roxb. on HeLa cells are presented in Table 5. At 1,000 µg/ml of DDDP have a significant cytotoxic effect on the HeLa cancer cells ($p \leq 0.05$). However, this highest concentration also suppressed splenocytes proliferation as demonstrated in the anti-CD3 and mitogenicity assays. Taken together these data demonstrated a general anti-proliferative effect of DDDP at a concentration of 1,000 µg/ml.

Table 5 Results of the cytotoxic effect of DDDP extracts from *Aeginetia indica* Roxb. on HeLa cells

Experimental groups	Cell concentration ($\times 10^4$ cells/ml) Mean $\pm$ S.E.
VH	26.5 $\pm$ 2.16
10 μ g/ml	23.8 $\pm$ 1.92
100 μ g/ml	23.3 $\pm$ 2.20
1,000 μ g/ml	12.3 $\pm$ 1.58*

*represent a significant difference from the vehicle control group at $p \leq 0.05$.

(replication number = 3).

4.4 Toxicity Results and Immunological Effects in Female B6C3F1 Mice Treated with DDDP

As shown in the *in vitro* study, both the SDDD and DDDP extracts from *Aeginetia indica* Roxb. were able to enhance immune responses as evaluated by proliferation responses (4.3.1-4.3.3) to Con A and anti-CD3 of T cells, and LPS of B cells. Those *in vitro* studies demonstrated that the plant extract had greater immunoenhancing ability than the seed extract. Furthermore, in Thai ethnomedical preparation, the Thai people utilize the entire plant including, stems, leaves, seeds. The extraction product of the whole plant is referred to as DDDP. Accordingly, DDDP was selected for evaluation of its effects on innate immunity, humoral immunity and cell-mediated immunity in *in vivo* models. To properly interpret data of immunological results, the basic toxicological parameters were also evaluated in the same studies. Several toxicological parameters such as body weights, selected organ weights, and hematological

parameters were evaluated. *In vivo* toxicological studies were initially conducted in female B6C3F1 mice using i.p injection to insure the quantitation of DDDP administrated and allows distributed of DDDP into the blood without significant chemical modification which can occur in the gastrointestinal tract.

4.4.1 Toxicity results of mice administrated DDDP by i.p. injection

4.4.1.1 Results of body weights and organ weights on B6C3F1 mice exposed to DDDP

Shown in Table 6 is the lack of effects of DDDP on body weight over the 28-day treatment period. No statistically significant differences were observed between the vehicle controls and all of the DDDP treatment groups at the time periods evaluated. These results represented the combined body weight data from three studies that were all 28 days in duration. However, periodic weight was significantly ($P \leq 0.01$) increase on the day 29 at the three lower dose (0.25, 2.5 and 25 mg/kg) of DDDP exposure. In addition, there were no DDDP-related deaths following i.p. injection of DDDP during the 28-days exposure period or when the exposure was continued for 31 days, as a result of inclement weather (Table 7).

Shown in Table 8 were the effects of DDDP on liver, spleen, lung, thymus and kidney organ weights. While no statistically significant effects were observed at any dose level for the lung, thymus or kidney, a slight increase was observed in the absolute liver and spleen weights of the animals treated with the 250 mg/kg dose of DDDP. However, this increase did not reach the level of statistical significance ($p \leq 0.05$) when the data were evaluated as relative organ weight (% Body Weight).

Table 6 Body weight (g) and body weight changes (g) in female B6C3F1 mice exposed to DDDP for 28 days

Parameter	Vehicle ^a (24)	DDDP ^b (mg/kg)					H/NH	Trend Analysis ^c
		0.25 (24)	2.5 (24)	25 (24)	100 (16)	250 (8)		
Day 1	21.6 ± 0.3	21.8 ± 0.3	21.5 ± 0.4	21.7 ± 0.3	21.2 ± 0.2	22.9 ± 0.6	NH	NS
Day 8	22.2 ± 0.3	22.1 ± 0.3	22.1 ± 0.3	22.3 ± 0.2	21.9 ± 0.2	23.2 ± 0.5	H	NS
Day 15	22.5 ± 0.3	23.0 ± 0.3	23.0 ± 0.3	23.0 ± 0.2	22.6 ± 0.2	23.4 ± 0.4	H	p≤0.05
Day 22	23.1 ± 0.3	23.6 ± 0.3	23.5 ± 0.4	23.4 ± 0.2	23.1 ± 0.3	23.7 ± 0.4	H	NS
Day 29	23.9 ± 0.4	24.6 ± 0.4	24.2 ± 0.4	24.7 ± 0.3	23.9 ± 0.3	24.7 ± 0.5	H	NS
Day 8–Day 1	0.65 ± 0.16	0.30 ± 0.15	0.63 ± 0.17	0.63 ± 0.18	0.65 ± 0.15	0.24 ± 0.26	H	NS
Day 15–Day 1	0.96 ± 0.17	1.08 ± 0.20	1.22 ± 0.25	1.32 ± 0.23	1.40 ± 0.18	0.47 ± 0.32	H	NS
Day 22–Day 1	1.52 ± 0.22	1.82 ± 0.24	2.01 ± 0.20	1.74 ± 0.27	1.90 ± 0.22	0.83 ± 0.37	H	NS
Day 29–Day 1	2.31 ± 0.14	2.88 ± 0.30 ^{**}	2.74 ± 0.19 ^{**}	3.00 ± 0.24 ^{**}	2.66 ± 0.26	1.84 ± 0.39	NH	p≤0.05

Mean in the same row differ significantly from the vehicle control group indicate as * p≤0.05, ** p≤0.01

Values represent the mean ± S.E.; The number of animals indicated in parentheses (n = 8-24)

H= homogeneous data, NH = non-homogeneous data, and NS = not significant

^a Vehicle control group (0.9% sodium chloride)

^b Treatment group is DDDP (0.25 mg/kg to 250 mg/kg)

^c The Jonckheere's test was used to test for dose-related trends among the vehicle and treatment groups

Table 7 Body weight (g) and body weight changes (g) in female B6C3F1 mice exposed to DDDP for 31 days

Parameter	Vehicle ^a	DDDP ^b (mg/kg)				H/NH	Trend Analysis ^c
		0.25	2.5	25	250		
Day 1	23.3 ± 0.5	23.1 ± 0.5	23.1 ± 0.6	23.3 ± 0.7	23.4 ± 0.6	H	NS
Day 8	22.9 ± 0.6	22.9 ± 0.5	23.4 ± 0.5	23.4 ± 0.7	23.8 ± 0.7	H	NS
Day 15	23.7 ± 0.6	23.3 ± 0.5	23.7 ± 0.4	24.1 ± 0.7	24.5 ± 0.8	H	NS
Day 22	24.1 ± 0.8	24.5 ± 0.6	24.4 ± 0.5	24.9 ± 0.8	25.7 ± 0.7	H	NS
Day 29	25.1 ± 0.7	25.5 ± 0.6	25.9 ± 0.7	26.1 ± 0.9	26.7 ± 1.0	H	NS
Day 32	25.0 ± 0.8	25.6 ± 0.5	26.1 ± 0.6	26.4 ± 0.9	27.0 ± 1.0	H	p≤0.05
Day 8–Day 1	-0.4 ± 0.3	-0.3 ± 0.2	0.3 ± 0.2	0.0 ± 0.2	0.3 ± 0.3	H	p≤0.05
Day 15–Day 1	0.4 ± 0.2	0.2 ± 0.2	0.6 ± 0.5	0.8 ± 0.2	1.1 ± 0.3	H	p≤0.05
Day 22–Day 1	0.8 ± 0.4	1.4 ± 0.2	1.3 ± 0.5	1.6 ± 0.1	2.3 ± 0.2*	NH	p≤0.01
Day 29–Day 1	1.8 ± 0.4	2.4 ± 0.2	2.8 ± 0.5	2.7 ± 0.4	3.3 ± 0.6	H	p≤0.05
Day 32–Day 1	1.7 ± 0.4	2.5 ± 0.2	3.0 ± 0.5	3.0 ± 0.4	3.6 ± 0.6*	H	p≤0.01

Mean in the same row differ significantly from the vehicle control group indicate as * p≤0.05

Values represent the mean ± S.E.; n = 8

H= homogeneous data, NH = non-homogeneous data, and NS = not significant

^a Vehicle control group (0.9% sodium chloride)

^b Treatment group is DDDP (0.25 mg/kg to 250 mg/kg)

^c The Jonckheere's test was used to test for dose-related trends among the vehicle and treatment groups

Table 8 Body weight (g) and organ weights (mg) in female B6C3F1 mice exposed to DDDP for 28 days

Parameter	Vehicle ^a	DDDP ^b (mg/kg)				H/NH	Trend Analysis ^c
		0.25	2.5	25	250		
Body Weight (g)	25.2 ± 0.8	25.6 ± 0.5	26.1 ± 0.6	26.4 ± 0.9	27.1 ± 1.0	H	p≤0.05
Liver (mg)	1181 ± 46	1153 ± 17	1334 ± 68	1194 ± 36	1350 ± 46*	NH	p≤0.01
% Body Weight	4.7 ± 0.2	4.5 ± 0.1	5.1 ± 0.2	4.6 ± 0.2	5.0 ± 0.1	H	p≤0.05
Spleen (mg)	63 ± 5	73 ± 6	78 ± 5	70 ± 6	89 ± 6**	H	p≤0.01
% Body Weight	0.251 ± 0.023	0.286 ± 0.023	0.298 ± 0.019	0.265 ± 0.021	0.329 ± 0.023	H	NS
Lungs (mg)	202 ± 10	205 ± 15	197 ± 4	210 ± 8	200 ± 10	NH	NS
% Body Weight	0.80 ± 0.02	0.80 ± 0.05	0.76 ± 0.01	0.80 ± 0.03	0.74 ± 0.03	NH	NS
Thymus (mg)	63 ± 8	65 ± 5	64 ± 3	76 ± 6	68 ± 6	H	NS
% Body Weight	0.246 ± 0.028	0.256 ± 0.018	0.250 ± 0.014	0.295 ± 0.029	0.248 ± 0.018	H	NS
Kidney (mg)	351 ± 12	361 ± 11	382 ± 13	377 ± 14	393 ± 16	H	p≤0.05
% Body Weight	1.40 ± 0.03	1.41 ± 0.03	1.46 ± 0.04	1.44 ± 0.08	1.45 ± 0.02	NH	NS

Mean in the same row differ significantly from the vehicle control group indicate as * p≤0.05, ** p≤0.01

Values represent the mean ± S.E., n = 8.

H= homogeneous data, NH = non-homogeneous data, and NS = not significant

^a Vehicle control group (0.9% sodium chloride)

^b Treatment group is DDDP (0.25 mg/kg to 250 mg/kg)

^c The Jonckheere's test was used to test for dose-related trends among the vehicle and treatment groups

Table 9 Hematology in female B6C3F1 mice exposed to DDDP for 28 days

Parameter	Vehicle ^a	DDDP ^b (mg/kg)				H/NH	Trend Analysis ^c
		0.25	2.5	25	250		
Erythrocytes (10 ⁶ /mm ³)	8.3 ± 0.5	8.2 ± 0.4	9.1 ± 0.1	8.2 ± 0.6	7.5 ± 0.5	NH	NS
Hemoglobin (g/dl)	14.65 ± 0.48	13.79 ± 0.57	15.55 ± 0.13	12.80 ± 1.15	13.48 ± 0.77	NH	NS
Hematocrit (%)	41.79 ± 2.46	41.70 ± 1.90	46.06 ± 0.37	41.58 ± 2.67	38.08 ± 2.48	NH	NS
MCV (fl)	50.11 ± 0.08	50.86 ± 0.14**	50.88 ± 0.16**	50.56 ± 0.37*	50.83 ± 0.30*	NH	p≤0.05
MCH (pg)	18.0 ± 1.1	16.9 ± 0.3	17.2 ± 0.1	15.7 ± 1.5	18.1 ± 0.6	NH	NS
MCHC(g/dl)	35.9 ± 2.3	33.1 ± 0.6	33.8 ± 0.1	31.1 ± 2.9	35.7 ± 1.1	NH	NS
Reticulocytes (%)	6.60 ± 0.43	6.94 ± 0.34	6.80 ± 0.61	6.96 ± 0.55	6.92 ± 0.40	H	NS
Platelets (10 ³ /μl)	545.3 ± 86.2	458.9 ± 52.1	648.5 ± 104.0	334.1 ± 94.5	448.3 ± 82.9	H	NS
Leukocytes (10 ³ /mm ³)	6.2 ± 0.8	5.4 ± 0.5	5.7 ± 0.5	3.3 ± 0.5**	4.8 ± 0.6	H	p≤0.05

Mean in the same row differ significantly from vehicle control group indicate as * p≤0.05, ** p≤0.01

Values represents the mean ± SE, n = 8

H= homogeneous data, NH= non-homogeneous data, NS= not significant

MCV = mean corpuscular volume; MCH = mean cell hemoglobin; MCHC = mean corpuscular hemoglobin concentration

^a Vehicle control group (0.9% sodium chloride)

^b Treatment groups is DDDP (0.25 mg/kg to 250 mg/kg)

^cThe Jonckheere's Test was used to test for dose-related trends among the vehicle and treatment groups.

4.4.1.2 Hematology analysis results in mice exposed to DDDP

Hematological parameters are presented in Table 9. There were no significant differences in erythrocytes, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCHC), reticulocytes, or platelet numbers between animals treated with DDDP and the vehicle control animals. The slight, albeit statistical significance, increases observed in MCV are not considered to be biological relevant. Treatment with DDDP resulted in a dose-related decrease in leukocytes numbers, with the greatest decrease reaching the level of statistical significance ($p \leq 0.01$) at the 25 mg/kg dose level. Overall, DDDP had minimal effects on the general toxicological parameters evaluated.

4.4.2 Immunological effect of mice exposed to DDDP for 28 days

4.4.2.1 Antibody-forming cell response to the T-dependent antigen, sheep erythrocytes

The induction of B cells to proliferate by LPS is the results of LPS binding to the LPS receptor, CD14, on the B cell which results in a calcium dependent-mediated proliferation response (Cruse and Lewis, 1999). This proliferation is not mediated by the B-cell antigen receptor. Hence, the antibody-forming cell (AFC) response to the T-dependent antigen SRBC (plaque assay) is a better indication for humoral immunity of DDDP, because this response requires the coordinated interaction of several different immune cells including macrophages (or antigen presenting cells APCs), T cells, and B cells. Therefore xenobiotic-induced effect on the function of any of these cells may impact the ability of B cell to produce antigen-specific antibody.

The effect of DDDP on the humoral-mediated response is shown in Fig. 37. Treatment with DDDP did not significantly alter the plaque response when evaluated as either specific activity (AFC/ 10^6 spleen cells) or total spleen activity (AFC/spleen). While a slight decrease in the humoral immune response was observed at the 250 mg/kg dose level, the decrease did not reach the level of statistical significance ($p \leq 0.05$). The results demonstrated that daily exposure to DDDP for 28 days did not significantly alter the humoral immune response.

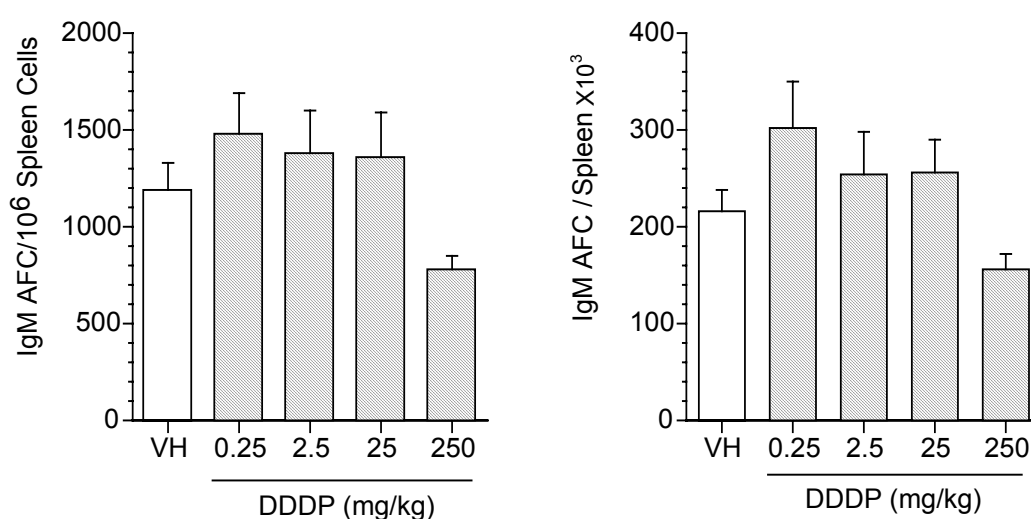


Figure 37 Spleen IgM antibody-forming cells (AFC) response from female B6C3F1 mice treated with DDDP for 28 days. The results are expressed as the mean $\pm$ S.E. of the specific activity (IgM AFC/ 10^6 spleen cells) and total spleen activity (IgM AFC/spleen weight $\times 10^3$), $n = 8$.

4.4.2.2 Mixed leukocyte response

Unlike the lacking effect on the humoral immune response, daily treatment

with DDDP produced a significant increase in the MLR cell-mediated response, with the animals from the three lowest dose levels responding to a significantly greater degree than the vehicle (VH) control animals (Fig. 38). The percent increase was approximately 43%, 40% and 28% for mice treated with DDDP at 0.25 mg/kg, 2.5 mg/kg, and 25 mg/kg, respectively. Mice exposed to DDDP 100 mg/kg also showed a slight increase of 8%; however, this did not reach the level of statistical significance ($p \leq 0.05$).

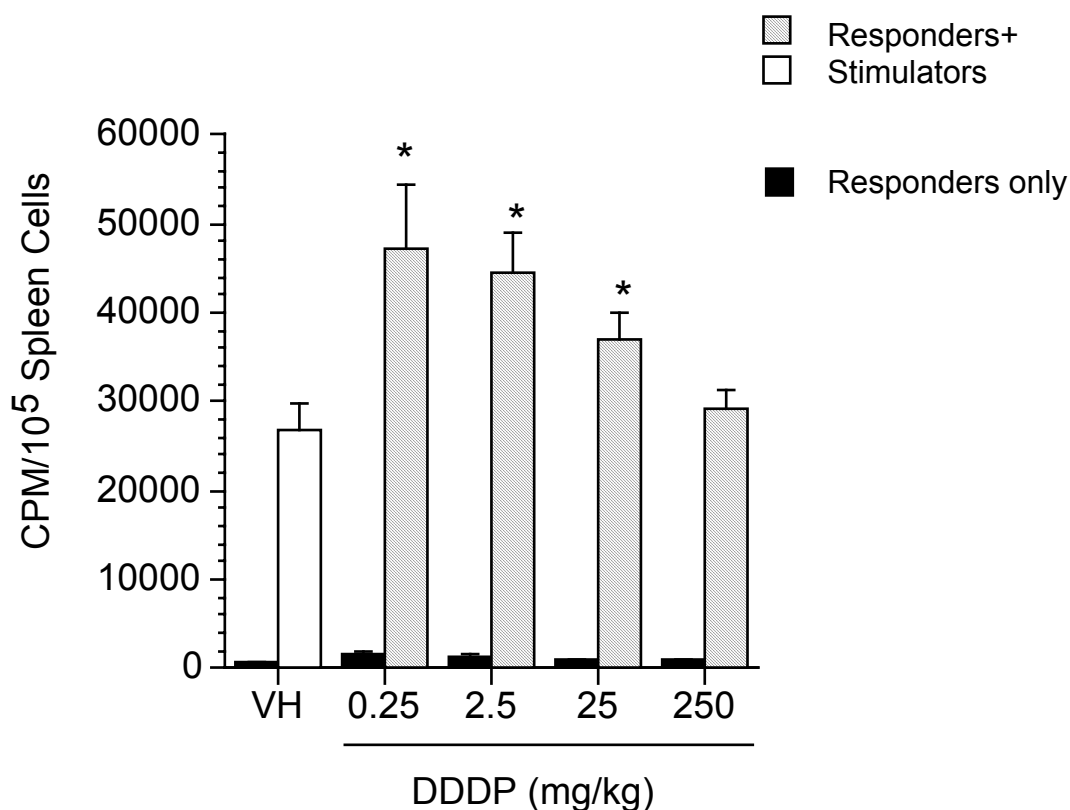


Figure 38 T lymphocyte responses against allogeneic stimulation in MLR assay. The results are expressed as mean $\pm$ S.E. of T lymphocyte proliferation as CPM/ 1×10^5 cells ($n = 8$). * Significant at $p \leq 0.05$.

4.4.2.3 Proliferative response following stimulation with anti-CD3 antibody

Shown in Figure 39 is the result of the anti-CD3-mediated stimulation of T cells from animals treated daily with DDDP for 28 days. While not reaching the level of statistical significance ($p \leq 0.05$), slight increase was observed in the proliferative response at all dose levels of DDDP compared to the vehicle controls. These include a 15% increase at the 0.25 mg/kg dose level, and 19%, 15% and 11% at the 2.5, 25, and 250 mg/kg dose levels, respectively.

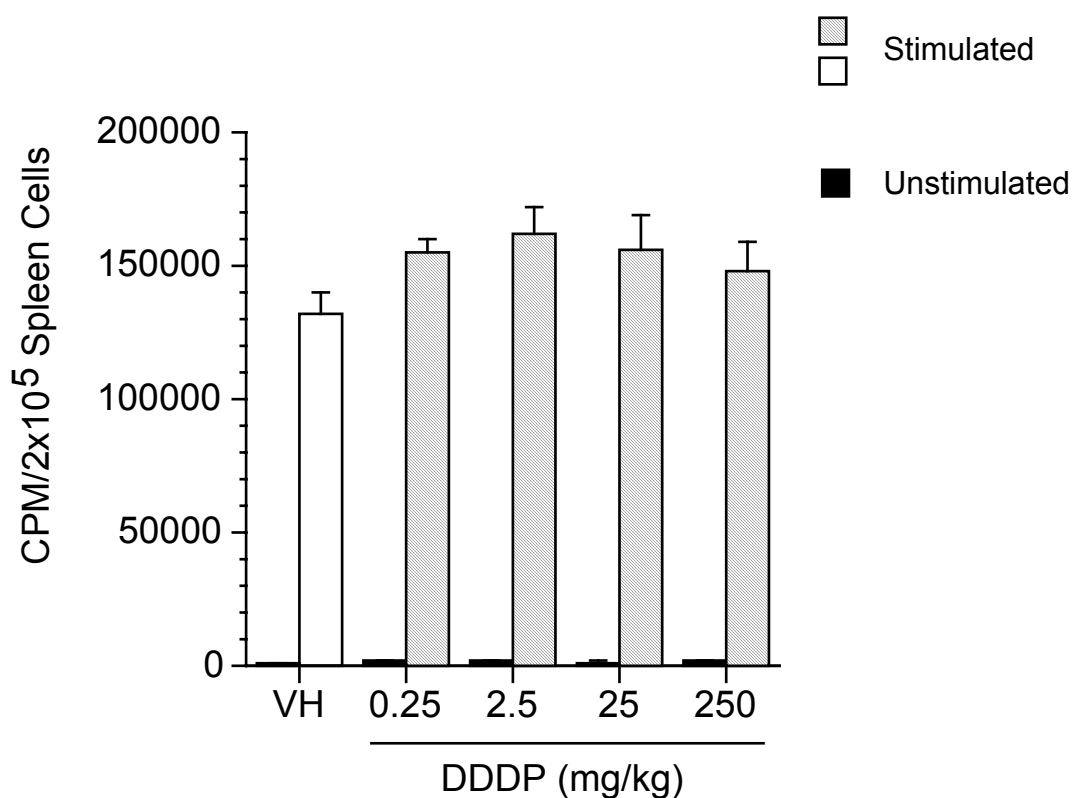


Figure 39 Anti-CD3 antibody mediated T lymphocyte proliferation response in female B6C3F1 mice treated with DDDP for 28 days. The results are expressed as mean $\pm$ S.E., $n = 8$.

4.4.2.4 Natural killer cell activity

To determine if treatment with DDDP for 28 days modulate innate immunity, the effects of DDDP on natural killer (NK) cell activity was evaluated following daily i.p injection for 28 days. Treatment with DDDP did not affect NK activity as shown in Table 10. Noteworthy, the spleen weight of the highest DDDP treated group, 250 mg/kg was increased.

Table 10 Natural killer cell activity in female B6C3F1 mice exposed to DDDP for 28 days

Parameter	Vehicle ^a	DDDP ^b (mg/kg)				H/NH	Trend Analysis ^c
		0.25	2.5	25	250		
Effector:Target Ratio							
6.25:1	2.8 ± 0.3	2.6 ± 1.0	2.1 ± 0.4	3.5 ± 0.3	2.8 ± 0.4	NH	NS
12.5:1	3.3 ± 0.6	3.9 ± 1.2	4.0 ± 0.5	4.7 ± 0.3	3.9 ± 0.5	NH	NS
25:1	5.0 ± 0.8	5.7 ± 0.9	4.1 ± 0.9	6.2 ± 0.5	5.6 ± 0.7	H	NS
50:1	9.4 ± 2.9	8.1 ± 1.3	5.3 ± 1.3	9.5 ± 0.7	8.3 ± 1.1	NH	NS
100:1	11.8 ± 2.0	12.7 ± 1.8	9.3 ± 1.8	16.0 ± 1.8	12.8 ± 1.9	H	NS
200:1	18.7 ± 2.9	21.3 ± 2.8	15.9 ± 3.7	25.2 ± 2.2	20.6 ± 2.9	H	NS
Spleen Weight (mg)	69 ± 5	87 ± 6	76 ± 5	86 ± 5	89 ± 4*	H	p≤0.05

Spontaneous release over the 4-hour incubation period was 11.6% of maximum release.

Mean in the same row differ significantly from vehicle control group indicate as * p≤0.05

Values represents the mean ± SE, n = 8

H= homogeneous data, NH= non-homogeneous data, NS= not significant

^a Vehicle control group (0.9% sodium chloride)

^b Treatment groups is DDDP (0.25 mg/kg to 250 mg/kg)

^cThe Jonckheere's Test was used to test for dose-related trends among the vehicle and treatment groups.

4.4.2.5 Cytotoxic T lymphocyte (CTL) activity

The proliferation and the killing activity of CTL represent an effector mechanism for resistance to viral infections and surveillance against neoplastically transformed cells. The cytolytic activity of CTL is assessed in a 4-hours ^{51}Cr -release assay after a 5-day sensitization to allogeneic-inactivated tumor cells expressing MHC class I molecules. Results shown in Figure 40 summarize the effect of DDDP on CTL activity. Animals treated with DDDP (25mg/kg) induced significantly greater cytolytic activity as compared to vehicle control animals at effector to target ratios of 6:1, 3:1, 1.5:1, and 0.75:1 ($p \leq 0.01$ and $p \leq 0.05$). The cytotoxic activity observed was approximately 50%, 55%, 59% and 55%, respectively. At the effector : target (E:T) ratio 25:1, enhanced in CTL function was also demonstrated which was approximately 34% greater than the vehicle control group. However this increase did not reach the level of statistical significance. Mice treated with DDDP 2.5 mg/kg or less did not differ significantly from the vehicle control group. Overall, exposed to DDDP at all doses level increase the killing activity of CTL as compared to the vehicle control group. A dose dependent responses was observed in the (E:T) killing activity of animal administrated the highest dose of DDDP (250 mg/kg). However the observed result was slightly decreased as compared to the animal treated with DDDP at 25 mg/kg.

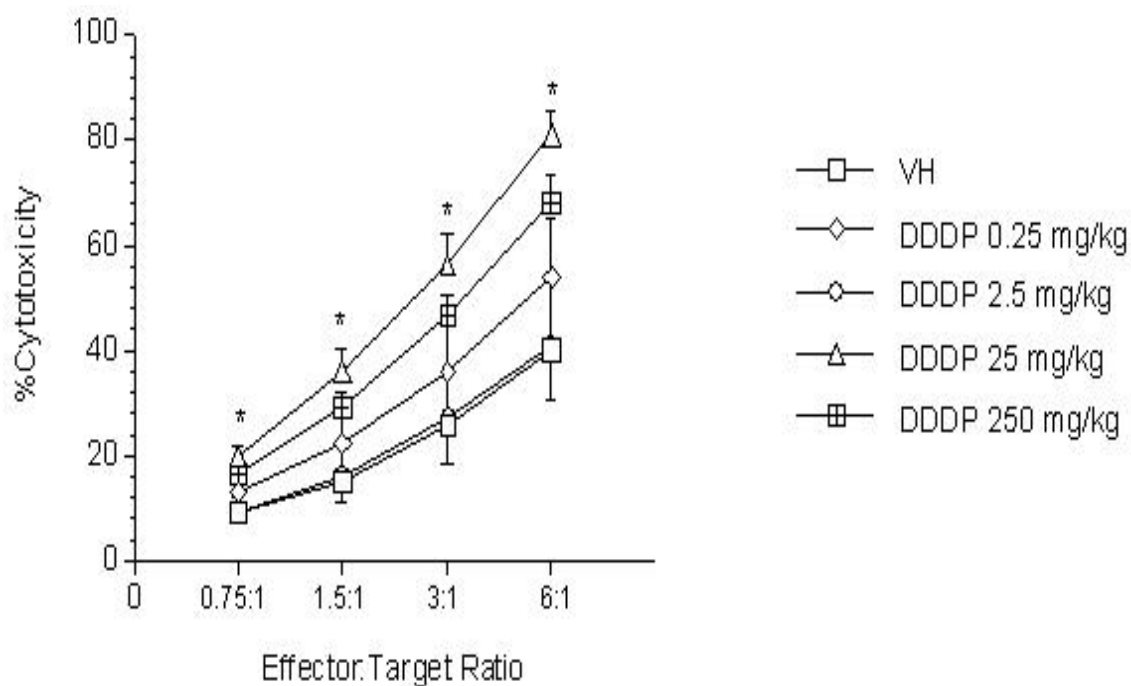


Figure 40 Spleen cytotoxic T cell activity in female B6C3F1 mice treated with DDDP for 28 days. The results are expressed as the mean $\pm$ S.E. of % cytotoxicity of T lymphocytes (n = 8). Spontaneous release over the 4-hour incubation period was 12.18% of maximum release. *, ** indicate significant difference between treatment groups and vehicle control group at $p \leq 0.05$ and $p \leq 0.01$, respectively.

4.4.2.6 Splenic phenotype analysis

In order to ascertain the mechanism by which DDDP enhanced the T-cell proliferative responses, phenotypic analysis of the splenocytes was undertaken. To determine if DDDP produced an increase in T-cell number or decrease the number of regulatory T cells ($CD4^+CD25^+$), two mechanisms that could have resulted in an enhanced cell-mediated immune response, the initial evaluation following 31 days of exposure focused on enumerating total B cells, total T cells, T cell subsets including regulatory T cells, macrophage and NK cells. These results are present in Table 11 as the percentage values and as absolute values in Table 12. As can be seen in Table 11, there were no statistically significant differences in any of these splenic subpopulations of the DDDP-treated animals as compared to the vehicle controls. When the data were evaluated as absolute numbers, Table 12, there was a slight increase in spleen cell number of the animals treated with the low dose of DDDP (18.2 vs. 15.3×10^7). While this increase did not reach the level of statistical significance, it contributed to the increase in the absolute number observed in the low dose (0.25 mg/kg) animals where the total number of T cells ($CD3^+$), cytotoxic T cell ($CD4^+CD8^+$) and macrophages were significantly ($p \leq 0.05$) elevated. An increase in T-cell number alone can not account for the enhanced MLR response, since the 2.5 and 25 mg/kg dose groups did not have increased number of T cells when evaluated either as percent or absolute numbers. In the second study, $CD4^+$, $CD8^+$ and immature $CD4^+CD8^+$ double positive cells were also enumerated. As observed in the first study when evaluated as percent values, there were no significant differences between vehicle- and DDDP-treated animals for the percentage of total T cells or any of the T-cell subsets (Table 13). While changes were observed in the absolute number

of CD3⁺, CD4⁺ and CD8⁺ cells (Table 14), these appear to be directly related to the low vehicle spleen cell number which was observed in the second study (Table 14). These results indicate that the increase in T-cell proliferation was not due to an alteration in splenic subpopulations.

Table 11 Percent values of splenocyte with differential surface marker in female B6C3F1 mice exposed to DDDP for 31 days

Exposure	Ig ⁺ ^c	CD3 ⁺ ^d	CD4 ⁺ CD8 ⁻ ^e	CD4 ⁻ CD8 ⁺ ^f	CD4 ⁺ CD25 ⁺ ^g	NK1.1 ⁺ CD3 ⁻ ^h	MAC-3 ⁺ ⁱ
Vehicle ^a	43.3 ± 2.6	29.8 ± 1.1	18.5 ± 1.2	13.0 ± 0.4	7.1 ± 0.7	3.9 ± 0.1	1.2 ± 0.2
DDDP ^b							
0.25 mg/kg	42.6 ± 1.7	30.0 ± 1.0	17.3 ± 0.8	13.8 ± 0.4	7.2 ± 0.6	3.9 ± 0.1	1.7 ± 0.2
2.5 mg/kg	41.8 ± 2.6	27.7 ± 1.3	20.2 ± 0.4	13.1 ± 0.4	6.0 ± 0.2	3.9 ± 0.1	1.2 ± 0.2
25 mg/kg	41.7 ± 2.7	27.1 ± 1.1	17.3 ± 1.5	12.1 ± 0.4	7.2 ± 0.5	3.7 ± 0.3	1.2 ± 0.2
250 mg/kg	42.6 ± 1.1	26.4 ± 1.1	20.0 ± 1.5	13.7 ± 0.8	5.9 ± 0.4	3.4 ± 0.2	1.3 ± 0.2
H/NH	H	H	NH	NH	H	NH	H
Trend Analysis ^j	NS	p≤0.01	NS	NS	NS	p≤0.05	NS

Values represent the mean ± S.E.; n = 8.

H= homogeneous data, NH = non-homogeneous data, and NS = not significant

^a Vehicle control group (0.9% sodium chloride); ^b Treatment group is DDDP (0.25 mg/kg to 250 mg/kg)

^c Total B cells; ^d Total T cells; ^e T helper cell; ^f Suppressor/cytotoxic T cell; ^g Regulatory T cell

^h Natural killer Cell; ⁱ Macrophage

^j The Jonckheere's test was used to test for dose-related trends among the vehicle and treatment groups.

Table 12 Absolute values of splenocyte with differential surface marker in female B6C3F1 mice exposed to DDDP for 31 days

Exposure	Spleen Cells x 10 ⁷	Ig ⁺ ^c	CD3 ⁺ ^d	CD4 ⁺ CD8 ⁻ ^e	CD4 ⁻ CD8 ⁺ ^{fe}	CD4 ⁺ CD25 ⁺ ^g	NK1.1 ⁺ CD3 ⁻ ^h	MAC-3 ⁺ ⁱ
Vehicle ^a	15.3 ± 0.7	66.7 ± 5.7	45.3 ± 2.0	27.9 ± 1.3	19.7 ± 0.9	11.1 ± 1.5	6.0 ± 0.4	1.8 ± 0.3
DDDP ^b								
0.25 mg/kg	18.2 ± 0.8	77.5 ± 5.1	54.4 ± 2.7*	31.2 ± 1.3	24.9 ± 0.8**	13.2 ± 1.3	7.1 ± 0.4	3.2 ± 0.3*
2.5 mg/kg	16.7 ± 0.4	69.7 ± 4.8	46.1 ± 2.2	33.7 ± 2.5	21.8 ± 0.5	9.9 ± 0.4	6.5 ± 0.2	2.0 ± 0.4
25 mg/kg	14.3 ± 1.1	60.0 ± 6.9	38.7 ± 2.9	25.4 ± 3.7	17.2 ± 1.1	10.5 ± 1.3	5.3 ± 0.5	1.8 ± 0.4
250 mg/kg	16.7 ± 1.0	71.0 ± 4.7	43.5 ± 1.8	33.2 ± 3.0	21.9 ± 2.2	9.9 ± 1.2	5.6 ± 0.3	2.1 ± 0.3
H/NH	H	H	H	NH	NH	NH	H	H
Trend Analysis ^j	NS	NS	p≤0.05	NS	NS	p≤0.05	p≤0.05	NS

Mean in the same column differ significantly from vehicle control group indicate as * p≤0.05

Values represents the mean ± SE, n = 8

H= homogeneous data, NH = non-homogeneous data, and NS = not significant

^a Vehicle control group (0.9% sodium chloride); ^b Treatment group is DDDP (0.25 mg/kg to 250 mg/kg)

^c Total B cells; ^d Total T cells; ^e T helper cell; ^f Suppressor/cytotoxic T cell; ^g Regulatory T cell

^h Natural killer Cell; ⁱ Macrophage

^j The Jonckheere's test was used to test for dose-related trends among the vehicle and treatment groups.

Table 13 Percent values of splenocyte with differential surface marker in female B6C3F1 mice exposed to DDDP for 28 days

Exposure	Ig ⁺ ^c	CD3 ⁺ ^d	CD4 ⁺ CD8 ⁻ ^e	CD4-CD8 ⁺ ^f	CD4 ⁺ CD8 ⁺ ^g	NK1.1 ⁺ CD3 ⁻ ^h
Vehicle ^a	56.5 ± 0.6	45.3 ± 1.4	25.2 ± 1.4	13.9 ± 1.2	0.4 ± 0.1	3.0 ± 0.2
DDDP ^b						
0.25 mg/kg	56.2 ± 0.9	42.1 ± 1.5	23.6 ± 1.3	13.6 ± 0.8	0.5 ± 0.1	3.2 ± 0.2
2.5 mg/kg	53.6 ± 0.7	45.6 ± 1.6	24.2 ± 1.3	13.7 ± 0.7	0.7 ± 0.2	3.0 ± 0.2
25 mg/kg	51.1 ± 1.1**	49.5 ± 1.6	27.0 ± 0.9	14.7 ± 0.9	0.8 ± 0.2	2.5 ± 0.2
100 mg/kg	54.9 ± 1.1	46.1 ± 1.0	26.9 ± 1.0	14.8 ± 0.5	0.4 ± 0.1	2.7 ± 0.2
H/NH	H	H	H	H	NH	H
Trend Analysis ⁱ	p≤0.01	NS	p≤0.05	NS	NS	p≤0.05

Mean in the same column differ significantly from vehicle control group indicate as ** p≤0.01

Values represents the mean ± SE, n = 8

H= homogeneous data, NH = non-homogeneous data, and NS = not significant

^a Vehicle control group (0.9% sodium chloride); ^b Treatment group is DDDP (0.25 mg/kg to 100 mg/kg);

^c Total B cells; ^d Total T cells; ^e T helper cell; ^f Suppressor/Cytotoxic T cell;

^g Double positive T cells (Immature T cells); ^h Natural killer cell

ⁱ The Jonckheere's test was used to test for dose-related trends among the vehicle and treatment groups.

Table 14 Absolute values of splenocyte with differential surface marker in female B6C3F1 mice exposed to DDDP for 28 days

Exposure	Spleen Cells x 10 ⁷	Ig ⁺ c	CD3 ⁺ d	CD4 ⁺ CD8 ⁻ e	CD4 ⁻ CD8 ⁺ f	CD4 ⁺ CD8 ⁺ g	NK1.1 ⁺ CD3 ⁻ h
Vehicle ^a	13.4 ± 0.4	75.9 ± 2.1	60.9 ± 2.7	33.9 ± 2.2	18.7 ± 1.7	0.5 ± 0.1	4.1 ± 0.3
DDDP ^b							
0.25 mg/kg	15.9 ± 0.7	89.5 ± 4.6	67.2 ± 4.3	37.5 ± 2.7	21.6 ± 1.7	0.8 ± 0.2	5.1 ± 0.4
2.5 mg/kg	17.2 ± 0.9**	92.2 ± 5.1	78.7 ± 5.9*	41.2 ± 2.3	23.2 ± 0.9	1.1 ± 0.2	5.1 ± 0.4
25 mg/kg	16.5 ± 1.1*	84.9 ± 6.9	81.2 ± 5.0**	44.4 ± 2.9*	23.7 ± 1.0*	1.2 ± 0.2	4.1 ± 0.4
100 mg/kg	16.9 ± 0.7*	93.0 ± 4.4	77.8 ± 3.0*	45.3 ± 2.1**	24.8 ± 0.8**	0.6 ± 0.1	4.6 ± 0.3
H/NH	H	H	H	H	H	H	H
Trend Analysis ⁱ	p≤0.01	p≤0.05	p≤0.01	p≤0.01	p≤0.01	NS	NS

Mean in the same column differ significantly from vehicle control group indicate as * p≤0.05, ** p≤0.01

Values represents the mean ± SE, n = 8

H= homogeneous data, NH = non-homogeneous data, and NS = not significant

^a Vehicle control group (0.9% sodium chloride); ^b Treatment group is DDDP (0.25 mg/kg to 100 mg/kg)

^c Total B cells; ^d Total T cells; ^e T helper cell; ^f Suppressor/cytotoxic T cell

^g Double positive T cell (Immature T cells); ^h Natural Killer Cell

ⁱ The Jonckheere's test was used to test for dose-related trends among the vehicle and treatment groups.

4.4.2.7 Cytokine production

Another possible mechanism by which DDDP may enhance cell mediated responses may result from increased cytokine secretions. The differentiation of T and B lymphocytes is mainly regulated by cytokine production from T helper cell. T helper 1 (Th1) cells primarily produce IL-2 and IFN- γ , whereas T helper 2 (Th2) cells produce IL-4, IL-5, IL-10 and IL-13. It is widely accepted that IL-2 and IFN- γ have a major effect on T lymphocytes proliferation. In contrast, IL-4 is considered to be a cytokine key in B lymphocytes proliferation. In this experimental study, the ELISPOT assay was performed to assess involvement of IL-2, IFN- γ and IL-4 in the ability of DDDP to enhance T lymphocytes activity. Mice were first exposed to DDDP *in vivo*, and splenocytes were then restimulation with DDDP *in vitro*, which provided a secondary signal to T-lymphocytes previously sensitized in *in vivo*.

As illustrated in Table 15, mice that were treated with DDDP (0.25-25 mg/kg) had a very slight increase in the number of IL-2 and IL-4 secreting cells as compared to the vehicle control group. However this effect did not reach the level of statistical significance. For the T cell cytokine mediator, IFN- γ , the response observed at the 250 mg/kg dose was less than that of vehicle animals. Based on the dose response pattern observed in the modulation in the MLR, CTL and anti-CD3 assay at the highest dose, 250 mg/kg, modulation of cytokines by DDDP may contribute in part to the response observed.

Described in Table 15, no difference was observed in IL-4 and IFN- γ production comparable to the vehicle control group.

Table 15 Assessment of cytokine productions (IL-2, IL-4 and IFN- γ) in female B6C3F1 mice exposed to DDDP for 28 days

Exposure	(Spots/well)		
	IL-2 ^c	IFN- γ ^c	IL-4 ^c
Vehicle ^a	7.0 $\pm$ 1.71	151.0 $\pm$ 20.84	10.0 $\pm$ 3.3
DDDP ^b (mg/kg)			
0.25	10.0 $\pm$ 3.58	145.0 $\pm$ 22.00	14.0 $\pm$ 2.27
2.5	10.0 $\pm$ 3.46	127.0 $\pm$ 31.39	12.0 $\pm$ 3.32
25	10.0 $\pm$ 1.44	153.0 $\pm$ 18.75	14.0 $\pm$ 1.75
250	7.0 $\pm$ 2.03	103.0 $\pm$ 16.37	12.0 $\pm$ 3.38
H/NH	H	H	H

Values represents the mean $\pm$ SE, n = 8

H= homogeneous data, NH = non-homogeneous data

^a Vehicle control group (0.9% sodium chloride)

^b Treatment group is DDDP (0.25 mg/kg to 250 mg/kg)

^c Spots/ $\times 10^6$ cells/well

4.5 Toxicity Results and Immunological Effect in Female B6C3F1 Mice Treated with WDDDP

As indicated previously, the tradition clinical approach to consuming the water extract of DDD is by drink. Accordingly, studies were undertaken using the oral route exposure.

4.5.1 Toxicity results of mice administrated WDDDP by oral gavage

The parameter assessed in toxicity studies include: body weights, organ weights and hematology. All of tests previously discussed were used for evaluating effect on immune system following traditional usage of DDD-decoction (WDDDP).

4.5.1.1 Body weights and organ weights analysis of mice exposed to WDDDP

In these short term repeated exposure studies, body weights were monitored on day 8, 15, 22 and 28. Daily administration of WDDDP did not result in any mortality or any signs of toxicity as demonstrated by mean body weights. Shown in Table 16 are the results from the combined body weight from four studies. Furthermore, no toxic effects were observed in mice exposed with WDDDP for 32 days, when a study was extended due to inclement weather (Table 17).

Organ weight measurements were evaluated as both absolute organ weights and organ to body weight ratio (relative weight) for the liver, spleen, lung, thymus and kidneys (Table 18). No significant difference was observed between the vehicle controls and WDDDP treated animal for any organ evaluated with the exception of absolute lung weight which was significantly enhanced at the low dose level (10%). However, the increase was not observed when evaluated as relative lung weight, the more appropriate measurement.

Table 16 Body weight (g) and body weight changes (g) in female B6C3F1 mice exposed to WDDDP for 28 days

Parameter	Vehicle ^a	DDDP ^b (mg/kg)				CPS ^b 50 mg/kg	H/NH	Trend Analysis ^c
		10%	25%	50%	100%			
Day 1	23.6 ± 0.3	23.6 ± 0.3	23.5 ± 0.3	23.4 ± 0.3	23.3 ± 0.3	23.4 ± 0.4	H	NS
Day 8	23.2 ± 0.2	23.6 ± 0.3	23.0 ± 0.2	23.3 ± 0.3	23.0 ± 0.3	23.7 ± 0.4	H	NS
Day 15	23.7 ± 0.3	23.9 ± 0.3	23.7 ± 0.3	23.7 ± 0.4	23.5 ± 0.3	24.2 ± 0.5	H	NS
Day 22	24.5 ± 0.3	25.1 ± 0.4	24.3 ± 0.2	24.4 ± 0.4	24.3 ± 0.3	25.2 ± 0.5	H	NS
Day 29	25.1 ± 0.3	25.8 ± 0.4	25.2 ± 0.3	25.3 ± 0.4	25.0 ± 0.3	25.1 ± 0.5	H	NS
Day 8-Day 1	-0.40 ± 0.18	-0.04 ± 0.19	-0.44 ± 0.17	-0.16 ± 0.21	-0.28 ± 0.18	-0.29 ± 0.21	H	NS
Day 15-Day 1	0.12 ± 0.22	0.30 ± 0.18	0.21 ± 0.23	0.24 ± 0.24	0.23 ± 0.22	0.84 ± 0.27	H	NS
Day 22-Day 1	0.85 ± 0.24	1.48 ± 0.21	0.85 ± 0.25	0.96 ± 0.27	0.96 ± 0.29	1.83 ± 0.31*	NH	NS
Day 29-Day 1	1.51 ± 0.20	2.15 ± 0.22	1.75 ± 0.25	1.84 ± 0.27	1.70 ± 0.27	1.77 ± 0.31	H	NS

Mean in the same row differ significantly from vehicle control group indicate as * $p \leq 0.05$

Values represents the mean ± SE, n = 32

H= homogeneous data, NH = non-homogeneous data, and NS = not significant

^a Vehicle control group (sterile water)

^b Treatment group is WDDDP from concentration 10% to 100%, and the modulatory control group is CPS (cyclophosphamide 50 mg/kg)

^c The Jonckheere's test was used to test for dose-related trends among the vehicle and treatment groups.

Table 17 Body weight (g) and body weight changes (g) in female B6C3F1 mice exposed to WDDDP for 32 days

Parameter	Vehicle ^a	WDDDP ^b				CPS ^b 50 mg/kg	H/NH	Trend Analysis ^c
		10%	25%	50%	100%			
Day 1	26.1 ± 0.7	26.1 ± 0.8	26.2 ± 0.7	26.1 ± 0.7	26.2 ± 0.6	26.3 ± 0.6	H	NS
Day 8	24.5 ± 0.6	24.7 ± 0.7	24.7 ± 0.4	24.5 ± 0.5	24.5 ± 0.5	26.1 ± 0.9	H	NS
Day 15	26.0 ± 0.8	25.9 ± 0.7	26.1 ± 0.5	25.5 ± 0.7	25.7 ± 0.5	27.3 ± 0.9	H	NS
Day 22	26.1 ± 0.6	26.8 ± 0.5	26.9 ± 0.4	26.1 ± 0.7	26.3 ± 0.6	28.6 ± 0.9*	H	NS
Day 29	26.8 ± 0.9	27.5 ± 0.7	28.3 ± 0.5	26.4 ± 0.7	27.0 ± 0.6	28.5 ± 0.9	H	NS
Day 32	27.8 ± 1.0	29.0 ± 0.8	29.5 ± 0.7	27.5 ± 0.8	27.1 ± 0.5	29.0 ± 1.0	H	NS
Day 8-Day 1	-1.6 ± 0.6	-1.4 ± 0.3	-1.5 ± 0.5	-1.6 ± 0.4	-1.7 ± 0.6	-0.2 ± 0.3	H	NS
Day 15-Day 1	-0.1 ± 0.6	-0.2 ± 0.2	-0.2 ± 0.5	-0.7 ± 0.3	-0.5 ± 0.4	1.0 ± 0.5	H	NS
Day 22-day 1	-0.1 ± 0.6	0.7 ± 0.3	0.7 ± 0.6	-0.1 ± 0.3	0.1 ± 0.6	2.3 ± 0.4*	H	NS
Day 29-Day 1	0.7 ± 0.8	1.4 ± 0.3	2.1 ± 0.6	0.3 ± 0.5	0.8 ± 0.4	2.2 ± 0.4	H	NS
Day 32-Day 1	1.7 ± 0.7	2.9 ± 0.4	3.3 ± 0.6	1.4 ± 0.5	0.9 ± 0.6	2.7 ± 0.6	H	NS

Mean in the same row differ significantly from vehicle control group indicate as * $p \leq 0.05$

Values represents the mean ± SE, n = 8

H= homogeneous data, NH = non-homogeneous data, and NS = not significant

^a Vehicle control group (sterile water)

^b Treatment group is WDDDP from concentration 10% to 100%, and the modulatory control group is

CPS (cyclophosphamide 50 mg/kg)

^c The Jonckheere's test was used to test for dose-related trends among the vehicle and treatment groups.

Table 18 Body weight (g) and organ weights (mg) in female B6C3F1 mice exposed to WDDDP for 32 days

Parameter	Vehicle ^a	WDDDP ^b				CPS ^b 50 mg/kg	H/NH	Trend Analysis ^c
		10%	25%	50%	100%			
Body Weight (g)	27.8 ± 1.0	29.0 ± 0.8	29.3 ± 0.7	27.7 ± 0.8	27.2 ± 0.6	29.2 ± 1.1	H	NS
Liver (mg)	1413 ± 75	1620 ± 59	1588 ± 63	1421 ± 61	1417 ± 45	1508 ± 43	H	NS
% Body Weight	5.1 ± 0.2	5.6 ± 0.3	5.4 ± 0.2	5.2 ± 0.3	5.2 ± 0.1	5.2 ± 0.3	H	NS
Spleen (mg)	82 ± 6	73 ± 4	79 ± 5	78 ± 5	69 ± 4	53 ± 3**	H	NS
% Body Weight	0.293 ± 0.015	0.256 ± 0.020	0.271 ± 0.017	0.286 ± 0.027	0.255 ± 0.015	0.183 ± 0.014**	H	NS
Lungs (mg)	183 ± 6	228 ± 10*	223 ± 13	205 ± 8	203 ± 8	222 ± 18	H	NS
% Body Weight	0.66 ± 0.04	0.79 ± 0.05	0.76 ± 0.04	0.74 ± 0.04	0.75 ± 0.04	0.77 ± 0.07	H	NS
Thymus (mg)	57 ± 5	61 ± 3	57 ± 3	54 ± 5	46 ± 4	30 ± 4**	H	p≤0.05
% Body Weight	0.210 ± 0.023	0.214 ± 0.018	0.195 ± 0.013	0.198 ± 0.021	0.170 ± 0.016	0.108 ± 0.017**	H	p≤0.05
Kidney (mg)	404 ± 17	431 ± 8	439 ± 13	405 ± 15	409 ± 7	420 ± 13	H	NS
% Body Weight	1.46 ± 0.06	1.49 ± 0.05	1.50 ± 0.05	1.48 ± 0.08	1.51 ± 0.04	1.46 ± 0.07	H	NS

Mean in the same row differ significantly from vehicle control group indicate as * p≤0.05, ** p≤0.01

Values represents the mean ± SE, n = 8

H= homogeneous data, NH = non-homogeneous data, and NS = not significant

^a Vehicle control group (sterile water)

^b Treatment group is WDDDP from concentration 10% to 100%, and the modulatory control group is
CPS (cyclophosphamide 50 mg/kg)

^c The Jonckheere's test was used to test for dose-related trends among the vehicle and treatment groups.

Table 19 Hematology values of female B6C3F1 mice exposed to WDDDP for 28 days

Parameter	Vehicle ^a	WDDDP ^b				CPS 50 mg/kg	H/NH	Trend Analysis ^c
		10%	25%	50%	100%			
Erythrocytes($10^6/\text{mm}^3$)	9.5 ± 0.7	8.9 ± 0.2	9.3 ± 0.1	9.4 ± 0.1	9.2 ± 0.1	7.9 ± 0.1	NH	NS
Hemoglobin (g/dl)	15.95 ± 0.93	15.46 ± 0.16	15.54 ± 0.29	15.47 ± 0.29	15.58 ± 0.10	13.81 ± 0.20**	NH	p≤0.05
Hematocrit (%)	46.99 ± 0.37	44.31 ± 1.07*	46.09 ± 0.60	46.89 ± 0.47	46.14 ± 0.18	39.93 ± 0.69**	NH	NS
MCV (fl)	49.67 ± 0.15	49.72 ± 0.11	49.60 ± 0.10	49.91 ± 0.21	50.36 ± 0.26*	50.38 ± 0.17**	H	NS
MCH (pg)	16.9 ± 0.1	17.4 ± 0.4	16.7 ± 0.2	16.5 ± 0.4	17.0 ± 0.1	17.4 ± 0.2	NH	NS
MCHC (g/dl)	34.0 ± 0.2	35.0 ± 0.8	33.7 ± 0.3	33.1 ± 0.7	33.8 ± 0.2	34.6 ± 0.4	NH	NS
Reticulocyte (%)	4.21 ± 0.23	3.79 ± 0.20	3.45 ± 0.12 *	3.96 ± 0.07	3.81 ± 0.19	1.60 ± 0.20**	H	NS
Platelets ($10^3/\mu\text{l}$)	567.6 ± 92.97	473.5 ± 59.02	448.1 ± 85.03	562.4 ± 39.89	637.8 ± 101.11	549.8 ± 135.06	H	NS
Leukocytes ($10^3/\text{mm}^3$)	4.8 ± 0.5	4.3 ± 0.5	5.2 ± 0.6	7.3 ± 0.6*	5.4 ± 0.7	3.1 ± 0.4	H	p≤0.05

Mean in the same row differ significantly from vehicle control group indicate as * p≤0.05, ** p≤0.01

Values represents the mean ± SE, n = 8

H= homogeneous data, NH = non-homogeneous data, and NS = not significant

MCV = mean corpuscular volume; MCH = mean cell hemoglobin; MCHC = mean corpuscular hemoglobin concentration.

^a Vehicle control group (Sterile water)

^b Treatment group is WDDDP from concentration 10% to 100%, and the modulatory control group is CPS (cyclophosphamide 50 mg/kg)

^cThe Jonckheere's Test was used to test for dose-related trends among the vehicle and exposed groups.

4.5.1.2 Hematology analysis in mice exposed WDDDP

Hematological parameters for assessment of peripheral blood and hematopoietic tissue are presented in Table 19. Compared to the vehicle control group, there were no significant difference in erythrocytes, hemoglobin, MCH, MCHC and platelet counts. A slight increase of MCV was observed in mice administered 100% WDDDP. In addition, the hematocrit and reticulocytes values were slightly decreased in mice exposed to 10% WDDDP and 25% WDDDP, respectively. These changes were not observed at the highest dose levels. Overall treatment with WDDDP did not affect erythrocyte parameters as shown in Table 19. A dose related increase in leukocyte number was observed with WDDDP administration, the increase reached the level of statistical significance for the 50% dose level (Fig. 41) CPS, an alkylating agent which depresses bone marrow function, produced a significant decrease ($p \leq 0.01$) in hemoglobin, hematocrit and reticulocytes in mice treated with 50 mg/kg by i.p. injection (Table 19).

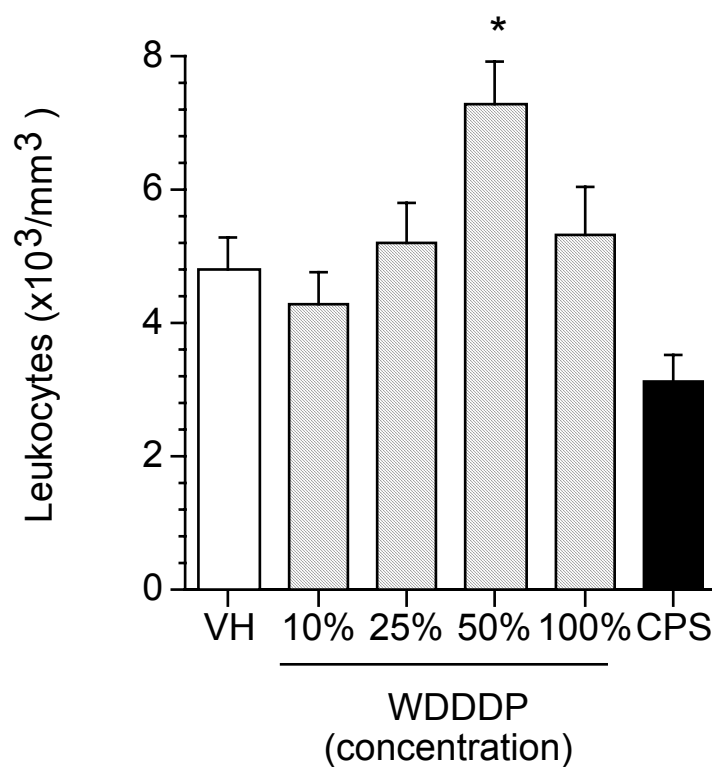


Figure 41 Leukocytes number of mice treated with WDDDP (10% to 100%) for 28 days. The results are represented as mean $\pm$ S.E, n = 8. The modulatory control group was CPS (cyclophosphamide 50 mg/kg). * indicated significant difference between treatment groups and vehicle control group at $p \leq 0.05$.

4.5.2 Immunological effects in female B6C3F1 mice administrated WDDDP by oral gavage

Immunological evaluating was aimed to determine effect of WDDDP on humoral immunity, innate immunity and cell-mediated immunity. To provide in depth mechanism, splenic phenotype analysis and cytokine assay (ELISPOT) were performed in this study.

4.5.2.1 Antibody-Forming cells (AFC) response to the T-dependent antigen, sheep erythrocytes

To evaluate the effect of WDDDP on humoral component of the immune response, the AFC response to sheep erythrocytes (Plaque assay) was conducted. The results from this study are presented as AFC/ 10^6 spleen cells (specific activity) and AFC/spleen weight $\times 10^3$ (total spleen activity). As shown in Figure 42, daily oral gavage with WDDDP for 28 days did not affect humoral mediated immunity. Treatment with CPS produced the anticipated reduction in plaques in the AFC assay.

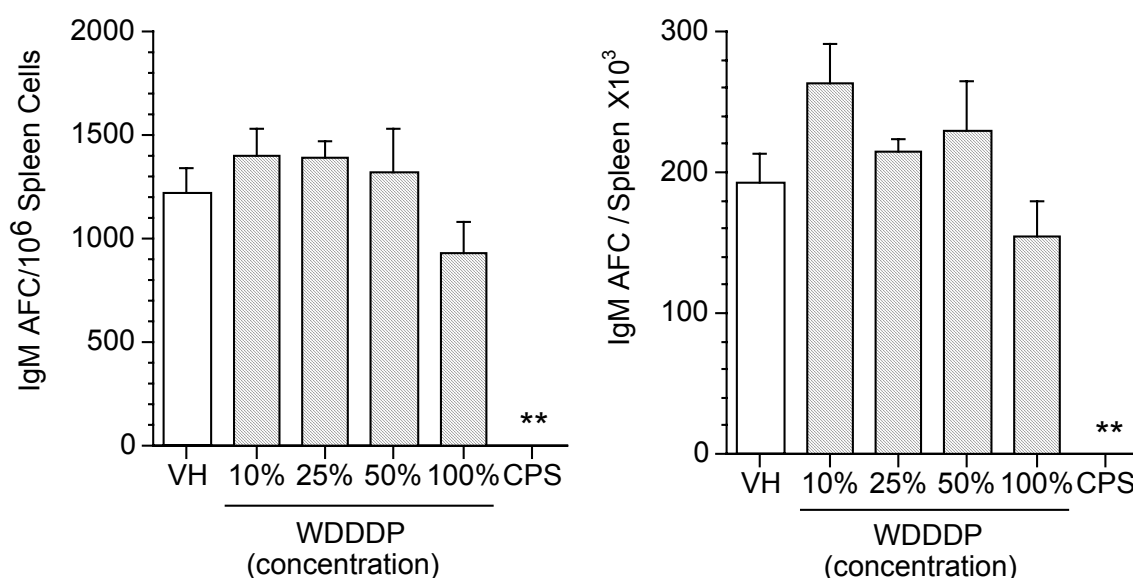


Figure 42 Spleen IgM antibody-forming cells (AFC) from female B6C3F1 mice treated with WDDDP for 28 days. The results are expressed as the mean $\pm$ S.E. of the specific activity (IgM AFC/10⁶ spleen cells) and total spleen activity (IgM AFC/spleenweight x 10³). The modulatory control group was CPS (cyclophosphamide 50 mg/kg).

* * significant at $p \leq 0.01$.

4.5.2.2 Mixed leukocyte response

In order to evaluate the effects of the WDDDP decoction on cell-mediated immunity (CMI), three parameters of CMI were utilized. These included T cell proliferation following stimulation with anti-CD3 antibody, mixed leukocytes response to allogeneic DBA/2 spleen cells and the cytotoxic T lymphocyte response to the P815 mastocytoma target cell.

In response to stimulation by allogeneic spleen cells, a stimulatory dose-response relationship was observed in T lymphocyte proliferation in mice treated with WDDDP. The maximum proliferation of T lymphocytes activated by DBA/2 splenic MHC molecules was observed at the highest dose of WDDDP (100%), which represented an increase of 46% compared to the vehicle control. Furthermore the enhancing effect was 30% greater than vehicle control group in the 50% WDDDP concentration dose group. Althoughs, the enhancement did not reach the level of statistical significance when using non-parametric analysis. When evaluated using ANOVA following by the Dunnett's T test, the response of the high (100%) dose group was found to significantly different than the vehicle controls.

As anticipated, mice treated with CPS showed a significant decrease in MLR when compared to the vehicle control mice. The stimulatory effect observed in mice administrated WDDDP is illustrated in Figure 43.

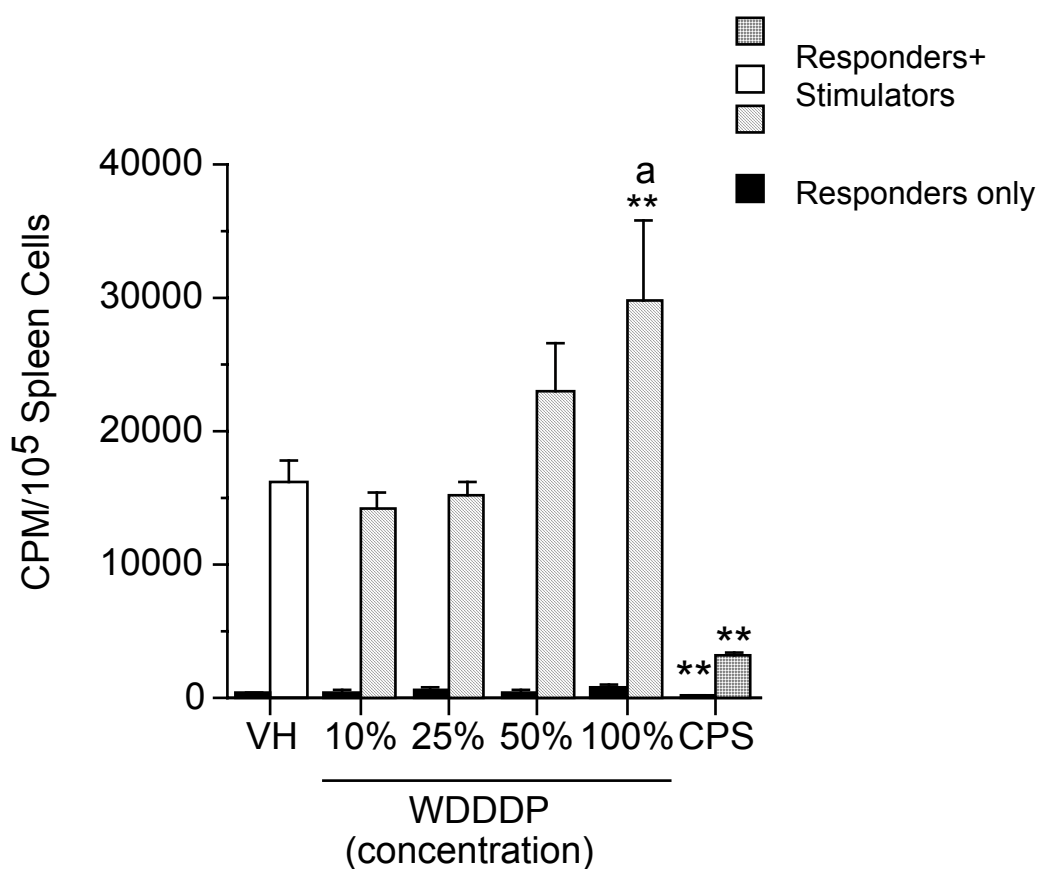


Figure 43 T leukocytes proliferation against allogeneic stimulation in MLR assay. The results are expressed as mean $\pm$ S.E. of T lymphocytes proliferation as CPM/1x10⁵ cells (n = 8). The cyclophosphamide (CPS)-modulatory control mice were treated on day 25-28 with 50 mg/kg CPS. * * indicated significant difference between treatment groups and vehicle control group at $p \leq 0.01$. * * **a** represent significant difference between WDDDP-treatment groups and vehicle control group at $p \leq 0.01$ by Dunnett's T test.

4.5.2.3 Proliferative response following stimulation with anti-CD3 antibody

Administration of WDDDP and stimulation with anti-CD3 antibody resulted in an increased proliferative response of the T lymphocytes at the highest dose levels. Anti-CD3 antibody binds to the T-cell receptor complex and activates the T cells to proliferate. A significant enhancement of T lymphocyte proliferation was observed in mice treated with 50% WDDDP, which was increase 31% over the vehicle control response (Figure 44). In mice consuming 100% WDDDP level an increase of 20% was also observed, however the increase did not reach level of statistical significance. CPS administrated at a dose level of 50 mg/kg, significantly decrease the anti-CD3 antibody mediated response when compared to the vehicle control group.

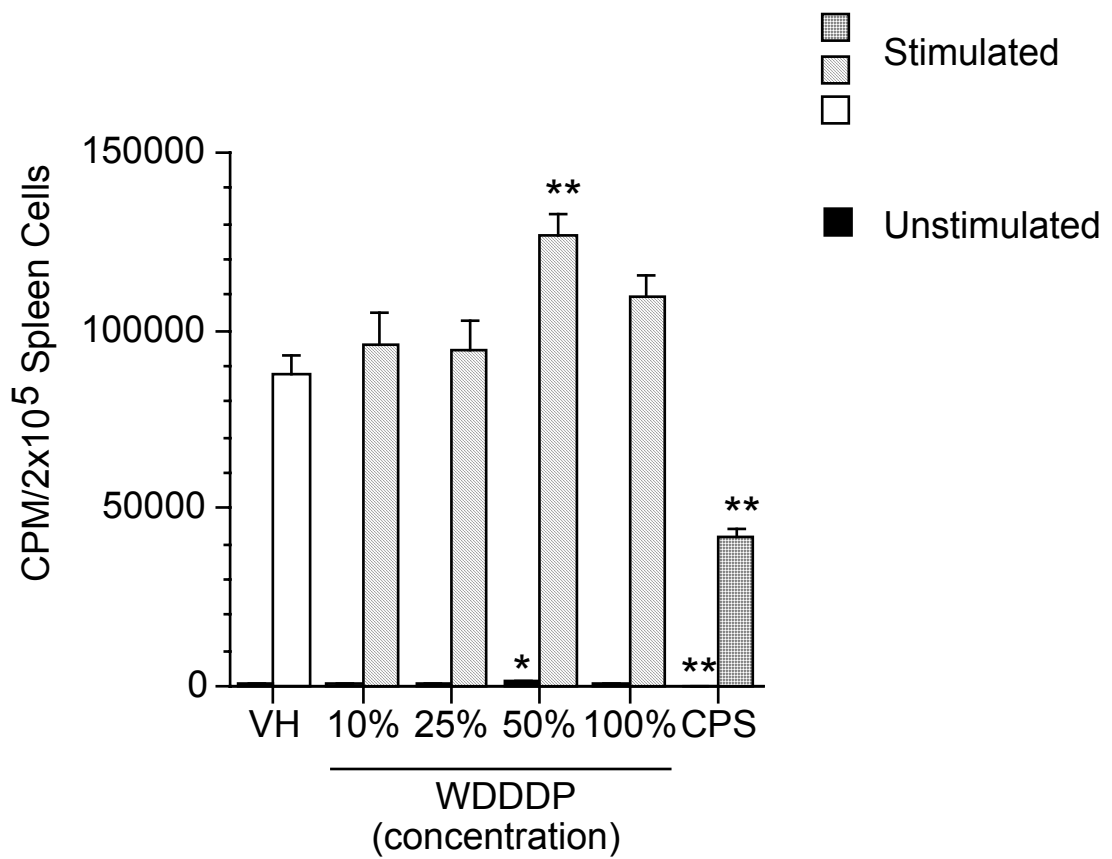


Figure 44 Anti-CD3 mediated T lymphocytes response in female B6C3F1 mice treated with WDDDP for 28 days. The results are expressed as the mean $\pm$ S.E. of T lymphocyte proliferation as CPM/2x10⁵ cells (n = 8). The cyclophosphamide (CPS)-modulatory control mice were treated on day 25-28 with 50 mg/kg CPS.

* *,* indicated significant difference between treatment groups and vehicle control group at $p \leq 0.05$ and $p \leq 0.01$, respectively.

4.5.2.4 Natural killer cell activity result

To determine the effect on innate immunity, natural killer cell activity was evaluated in B6C3F1 mice following 28 days administrated of WDDDP. Overall, WDDDP treatment did not significantly affect splenic NK cell activity. However in mice treated with AAGM1 antibody, NK activity was significantly inhibited at all effector to target ratios (Table 20).

Table 20 Natural killer cell activity in female B6C3F1 mice exposed to WDDDP for 28 days

Parameter	Vehicle ^a	WDDDP ^b				AAGM1 ^b 1:10	H/NH	Trend Analysis ^c
		10%	25%	50%	100%			
Effector:Target Ratio								
6.25:1	5.0 ± 0.6	4.8 ± 0.6	4.3 ± 0.5	4.4 ± 0.8	4.3 ± 0.6	2.8 ± 0.5*	H	NS
12.5:1	5.6 ± 0.7	5.2 ± 0.7	5.7 ± 0.6	5.6 ± 0.6	5.4 ± 0.7	2.5 ± 0.4**	H	NS
25:1	6.5 ± 0.7	6.7 ± 1.1	6.6 ± 0.7	4.8 ± 0.9	6.9 ± 0.7	2.7 ± 0.6**	H	NS
50:1	9.1 ± 1.0	8.4 ± 1.2	8.4 ± 1.3	8.2 ± 1.2	10.0 ± 0.9	3.4 ± 0.4**	NH	NS
100:1	13.7 ± 1.4	12.4 ± 1.9	11.0 ± 1.4	11.3 ± 1.6	14.3 ± 1.6	4.0 ± 0.5**	NH	NS
200:1	21.5 ± 1.8	21.4 ± 2.8	20.8 ± 2.7	20.4 ± 2.8	26.0 ± 2.2	5.6 ± 0.4**	NH	NS
Spleen Weight (mg)	74 ± 3	86 ± 2	81 ± 4	75 ± 4	77 ± 2	99 ± 3**	H	NS

Spontaneous release over 4-hour incubation period was 13.2% of maximum release.

Mean in the same row differ significantly from vehicle control group indicate as * p≤0.05, ** p≤0.01

Values represents the mean ± S.E., n = 8

H= homogeneous data, NH = non-homogeneous data, and NS = not significant

^a Vehicle control group (Sterile water)

^b Treatment group is WDDDP from concentration 10% to 100%, and the modulatory control group is anti-asialo GM1 (AAGM1 1:10 dilution)

^cThe Jonckheere's Test was used to test for dose-related trends among the vehicle and exposed groups.

4.5.2.5 Cytotoxic T lymphocytes (CTL)

The cytotoxic T cell response is an extension of the MLR response, in that T cells must further mature to cytotoxic cells capable of killing P815 target cells. As can be seen in Table 21, treatment with WDDDP for 28 days resulted in enhanced cytotoxic T cell activity at all dose levels for each of the effector to target ratios evaluated. In all cases, the response of the WDDDP treated mice was greater than that of the vehicle control animals. This is clearly illustrated in Table 21 with the 100% WDDDP decoction, the preparation consumed by those using the herbal medicine. Due to the variability associated with this experiment, the increased did not reach the level of statistical significance ($p \leq 0.05$). However, as can be seen WDDDP did enhance cytotoxic T-cell activity with animal treated with WDDDP having higher response than the vehicle controls.

Table 21 Cytotoxic T cell activity in female B6C3F1 mice exposed to WDDDP for 28 days

Parameter	Vehicle ^a	WDDDP ^b				CPS ^b 50 mg/kg	H/NH	Trend Analysis ^c
		10%	25%	50%	100%			
Effector:Target Ratio								
0.75:1	12.4 ± 3.3	16.6 ± 0.4	19.5 ± 5.5	21.1 ± 5.3	19.4 ± 4.6	1.3 ± 0.5**	H	NS
1.5:1	20.2 ± 5.5	25.2 ± 5.9	32.0 ± 8.9	34.6 ± 8.5	31.6 ± 0.7	0.7 ± 0.4**	H	NS
3:1	32.5 ± 8.4	40.5 ± 8.6	47.0 ± 11.0	50.9 ± 12.0	47.5 ± 8.5	0.6 ± 0.4**	H	NS
6:1	49.1 ± 11.0	56.8 ± 11.2	61.2 ± 11.1	66.0 ± 14.2	66.2 ± 8.8	0.7 ± 0.4**	H	NS
12.5:1	66.4 ± 9.6	72.6 ± 12.7	76.8 ± 10.1	77.3 ± 15.5	82.4 ± 6.5	1.1 ± 0.1**	H	NS
25:1	81.5 ± 8.4	82.7 ± 13.7	87.6 ± 6.3	82.6 ± 15.0	95.6 ± 3.7	2.2 ± 0.4**	NH	NS
Spleen Weight (mg)	74 ± 3	86 ± 2	81 ± 4	75 ± 4	77 ± 2	56 ± 3*	H	NS

Spontaneous release over 4-hour incubation period was 12.18 % of maximum release.

Mean in the same row differ significantly from vehicle control group indicate as * p≤0.05, ** p≤0.01

Values represents the mean ± S.E., n = 8

H= homogeneous data, NH = non-homogeneous data, and NS = not significant

^a Vehicle control group (Sterile water)

^b Treatment group is WDDDP from concentration 10% to 100%, and the modulatory control group is CPS (Cyclophosphamide 50 mg/kg)

^cThe Jonckheere's Test was used to test for dose-related trends among the vehicle and exposed groups.

4.5.2.6 Splenic phenotype analysis

A possible mechanism by which WDDDP enhanced the various cell-mediated parameters observed was by altering T-cell numbers or subpopulations. Therefore, phenotypic analysis of total B cells, total T cells, T cells subsets, macrophage and NK was conducted and the cell types enumerated.

The percent values, and the absolute values of these results are present in Table 22 and 23 respectively. Orally administrated WDDDP for 32 days did not affect the total number of spleen cells (Table 23). Furthermore, no change was observed in the percent or absolute values of splenic B cells (Ig^+), total T cells (CD3^+), T helper cell ($\text{CD4}^+\text{CD8}^+$), immature T cells ($\text{CD4}^+\text{CD8}^+$), NK cells ($\text{NK1.1}^+\text{CD3}^-$), and macrophage (Mac-3^+). However, mice administrated WDDDP at the 25%, 50% and 100% dose levels showed significant suppression of T regulatory cells ($\text{CD4}^+\text{CD25}^+$) by 29%, 52% and 43% respectively, as illustrated in Table 22. In addition, the absolute number of the regulatory cell population was suppressed approximately 30%, 52% and 43% in mice exposed to WDDDP 25%, 50% and 100% dose level, respectively (Table 23).

Similar result was also observed in the absolute values of T suppressor/cytotoxic cell ($\text{CD4}^-\text{CD8}^+$), which was significantly depressed as compared to the vehicle control group. A 23% and 22% suppression observed in mice treated with WDDDP 50% and 100%, respectively. The 13% suppression observed with the low dose of WDDDP did not reach the level of statistical significance. As anticipated, the potent immunosuppressive agent, CPS, produced statistically significant changes in various splenic subpopulation.

Table 22 Percent values of splenocyte with differential surface marker in female B6C3F1 mice exposed to WDDDP for 32 days

Exposure	Parameters							
	Ig ⁺ ^c	CD3 ⁺ ^d	CD4 ⁺ CD8 ⁻ ^e	CD4 ⁻ CD8 ⁺ ^f	CD4 ⁺ CD8 ⁺ ^g	CD4 ⁺ CD25 ⁺ ^h	NK1.1 ⁺ CD3 ⁻ ⁱ	Mac-3 ⁺ ⁱ
Vehicle ^a	56.0 ± 1.8	31.0 ± 1.3	21.6 ± 1.3	13.1 ± 0.8	0.3 ± 0.0	4.2 ± 0.3	3.9 ± 0.2	3.3 ± 0.7
WDDDP ^b								
10%	55.7 ± 1.5	32.7 ± 0.6	22.0 ± 0.6	13.0 ± 0.3	0.3 ± 0.1	3.9 ± 0.4	4.1 ± 0.3	5.0 ± 0.5
25%	51.2 ± 2.0	29.8 ± 1.2	18.8 ± 1.1	11.3 ± 0.8	0.4 ± 0.1	3.0 ± 0.2**	3.6 ± 0.1	2.4 ± 0.4
50%	54.9 ± 1.6	29.1 ± 0.8	18.4 ± 0.8	10.7 ± 0.4	0.3 ± 0.1	2.0 ± 0.2**	3.2 ± 0.3	1.9 ± 0.6
100%	54.4 ± 2.6	28.7 ± 0.7	19.7 ± 0.4	10.6 ± 0.4	0.3 ± 0.0	2.4 ± 0.2**	3.4 ± 0.4	3.0 ± 0.3
CPS (50 mg/kg)	39.6 ± 1.7**	40.2 ± 1.9**	29.0 ± 2.3**	21.5 ± 1.8**	0.5 ± 0.1	3.8 ± 0.2	1.8 ± 0.2**	7.6 ± 0.7**
H/NH	H	H	NH	NH	H	H	NH	H
Trend Analysis ^k	NS	p≤0.05	p≤0.05	p≤0.01	p≤0.05	p≤0.01	p≤0.05	NS

Mean in the same column differ significantly from vehicle control group indicate as * p≤0.05, ** p≤0.01

Values represents the mean ± SE, n = 5-8

H= homogeneous data, NH = non-homogeneous data, and NS = not significant

^a Vehicle control group (sterile water)

^b Treatment group is WDDDP from 10% to 100%, the modulatory control is CPS (cyclophosphamide).

^c Total B cells; ^d Total T cells; ^e T helper cell; ^f Suppressor/Cytotoxic T cell; ^g Double positive T cell ; ^h Regulatory T cells

ⁱ Natural killer cells; ^j Macrophage

^k The Jonckheere's test was used to test for dose-related trends among the vehicle and treatment groups

Table 23 Absolute values of splenocyte with differential surface marker in female B6C3F1 mice exposed to WDDDP 32 days

Exposure	Parameters								
	Spleen Cells (x10 ⁷)	Ig ⁺ ^c	CD3 ⁺ ^d	CD4 ⁺ CD8 ⁻ ^e	CD4 ⁻ CD8 ⁺ ^f	CD4 ⁺ CD8 ⁺ ^g	CD4 ⁺ CD25 ⁺ ^h	NK1.1 ⁺ CD3 ⁺ ⁱ	Mac-3 ⁺ ^j
Vehicle ^a	17.8 ± 1.1	100.0 ± 7.8	54.5 ± 2.5	38.0 ± 2.6	23.0 ± 1.5	0.6 ± 0.1	2.3 ± 0.1	6.8 ± 0.4	6.1 ± 1.7
WDDDP ^b									
10%	17.2 ± 0.6	95.4 ± 3.2	56.2 ± 2.6	37.6 ± 1.5	22.2 ± 1.0	0.6 ± 0.1	2.2 ± 0.2	7.0 ± 0.6	8.6 ± 1.0
25%	18.3 ± 0.9	80.6 ± 10.8	54.1 ± 2.2	34.0 ± 1.8	20.2 ± 1.2	0.7 ± 0.1	1.6 ± 0.1*	6.6 ± 0.4	4.5 ± 0.1
50%	16.5 ± 0.4	90.4 ± 1.7	48.1 ± 1.8	30.3 ± 1.3	17.6 ± 0.6*	0.6 ± 0.1	1.1 ± 0.1*	5.4 ± 0.6	3.1 ± 0.1
100%	16.9 ± 1.2	91.3 ± 6.8	48.2 ± 3.2	33.3 ± 2.7	17.9 ± 1.3*	0.5 ± 0.1	1.3 ± 0.1**	5.8 ± 0.7	5.0 ± 0.5
CPS (50 mg/kg)	7.9 ± 0.3	31.4 ± 0.2**	31.5 ± 1.6*	22.9 ± 2.0**	17.0 ± 1.6*	0.4 ± 0.1	1.5 ± 0.1**	1.4 ± 0.2**	5.8 ± 0.3
H/NH	NH	NH	H	H	H	H	H	NH	NH
Trend Analysis ^k	NS	NS	p≤0.01	p≤0.01	p≤0.01	NS	p≤0.01	p≤0.05	NS

Mean in the same column differ significantly from vehicle control group indicate as * p≤0.05, ** p≤0.01

Values represents the mean ± SE, n = 5- 8

H= homogeneous data, NH = non-homogeneous data, and NS = not significant

^a Vehicle control group (sterile water)

^b Treatment group is WDDDP from 10% to 100%, the modulatory control is CPS (cyclophosphamide).

^c Total B cells; ^d Total T cells; ^e T helper cell; ^f Suppressor/Cytotoxic T cell; ^g Double positive T cell ; ^h T regulatory cells

ⁱ Natural killer cells; ^j Macrophage

^k The Jonckheere's test was used to test for dose-related trends among the vehicle and treatment groups

4.5.2.7 Cytokine production

The cytokine secretion of T helper 1 (IL-2 and IFN- γ) and T helper 2 (IL-4) were evaluated in this studies using the ELISPOT assay. The detection of cytokine production was presented as spot numbers of cytokine secreting cell. There was no observable difference in IL-2, IL-4 and IFN- γ levels in mice treated with all concentration of WDDDP (10% to 100%) for 28 days. However, IFN- γ production, one of the key cytokine needed for T lymphocytes activation, were observed to be slightly increased as compared to the vehicle control group in mice-treated WDDDP as shown in Table 24. However these effects did not reach the level of statistical significance. Slight increases in IL-2 were observed at the lower doses levels of WDDDP.

Table 24 Cytokine productions (IL-2, IL-4, and IFN- γ) evaluated through ELISPOT assay in female B6C3F1 mice exposed to WDDDP for 28 days

Exposure	VH	WDDDP			
		10%	25%	50%	100%
Cytokine	Spots/wells				
IL-2 ^c	45.0 $\pm$ 9.73 (8)	55.0 $\pm$ 12.51 (8)	51.0 $\pm$ 16.41 (8)	64.0 $\pm$ 26.48 (8)	45.0 $\pm$ 8.71 (8)
IFN- γ ^d	232.0 $\pm$ 73.01 115.11 (6)	498.0 $\pm$ 173.77 (6)	360.0 $\pm$ 79.23 (7)	368.0 $\pm$ 134.21 (7)	279.0 $\pm$ (7)
IL-4 ^c	34.0 $\pm$ 8.96 (8)	46.0 $\pm$ 9.45 (8)	44.0 $\pm$ 11.77 (8)	28.0 $\pm$ 6.96 (8)	41.0 $\pm$ 11.8 (7)
H/NH	H	H	H	H	H

Values represent the mean $\pm$ S.E., The number of animals indicated in paratheses.

H = homogeneous data, NH = non-homogeneous data

^a Vehicle control group (sterile water)

^b Treatment group is WDDDP from 10% to 100%, the modulatory control was CPS (cyclophosphamide)

^c Spots/ $\times 10^6$ cells/well

^d Spots/ 5×10^5 cells/well

4.6 *In Vitro* Effect of DDDP to Splenocytes of B6C3F1 Mice

The assessment of the *in vitro* effects was undertaken in this study in an attempt to understand the mechanism of action of DDDP in *in vivo*. However, to quantify the amount of extract added into the cultures, the DDDP ethanol extract was used in *in vitro* study.

4.6.1 Toxicity study of DDDP to normal splenocytes of female B6C3F1 mice exposed to DDDP (*In vitro*)

To select the appropriated concentration of DDDP for the subsequent *in vitro* studies, the toxic effect of DDDP to normal splenocytes of B6C3F1 mice was

investigated.

Results were represented as % viability from flow cytometry analysis. As shown in Table 25 cell viability of the normal splenocytes in the cultures was not significantly different from the vehicle controls, until the concentration of DDDP equal to or greater than 600 µg/ml.

Table 25 Cell viability test of normal splenocytes of female B6C3F1 mice exposed to DDDP for 24 hours *in vitro*.

Exposure	% Viability
Vehicle ^a	82.10 ± 0.51
DDDP ^b	
0.001 µg/ml	81.29 ± 0.34
0.003 µg/ml	81.99 ± 0.37
0.01 µg/ml	81.38 ± 0.34
0.03 µg/ml	80.50 ± 0.80
0.1 µg/ml	81.27 ± 0.49
0.3 µg/ml	81.31 ± 0.38
1 µg/ml	79.88 ± 0.19
3 µg/ml	79.64 ± 0.97
10 µg/ml	80.49 ± 0.43
30 µg/ml	81.44 ± 0.66
100 µg/ml	81.72 ± 0.46
300 µg/ml	79.58 ± 1.35
600 µg/ml	74.80 ± 1.13**
1,000 µg/ml	66.68 ± 1.49**
H/NH	H

Mean in the same column differ significantly from vehicle control group indicate as

** p≤0.01

Values represents mean ± S.E., n = 8.

H = homogeneous data, NH = non-homogeneous data

^a Vehicle control group (RPMI media alone)

^b Treatment groups are DDDP from 0.001 - 1,000 µg/ml

4.6.2 Direct cytotoxicity effect of DDDP to YAC-1 tumor cell line

To obtain the suitable DDDP concentrations that should be used in the NK assay, the direct cytotoxicity effect on YAC-1 cells was conducted. Results indicated that the DDDP extract at the concentrations ranging from 1 µg/ml to 300 µg/ml were not significantly toxic to YAC-1 cell compared to the vehicle control group, as shown in Table 26.

Table 26 *In vitro* direct cytotoxicity study of YAC-1 tumor cell line exposed to DDDP for 4 hours.

Exposure	⁵¹ Cr released (CPM)
Vehicle ^a	4888.00 ± 98.17
DDDP ^b	
1 µg/ml	5469.12 ± 137.35
3 µg/ml	5132.25 ± 93.31
10 µg/ml	4898.63 ± 103.83
30 µg/ml	4972.00 ± 91.46
100 µg/ml	4920.00 ± 70.49
300 µg/ml	5887.00 ± 411.88
1,000 µg/ml	11188.13 ± 666.68**
H/NH	H

Mean in the same column differed significantly from vehicle control group indicates as ** p≤0.01

Values represents the mean ± S.E., n = 8.

H = homogeneous data, NH = non-homogeneous data

^a Vehicle control group (RPMI media alone)

^b Treatment groups are DDDP from 1 - 1,000 µg/ml

4.7 Immunological Study of DDDP to Normal Splenocytes of B6C3F1 mice (*in vitro*)

4.7.1 Effect of DDP on natural killer cell (NK) activity *in vitro*.

To determine if DDDP directly affected NK activity, DDDP was added directly into NK cultures and incubated for 4-hours. As shown in Table 27, the most consistent finding was that at dose level of 30 µg/ml and greater, a decrease in NK activity was observed at the 100:1 and 25:1 effector to target ratios. Slight increase and decrease were observed at some lower dose levels, but they were only observed in one effector to target ratio.

Table 27 Natural killer cells activity in female B6C3F1 mice exposed to DDDP for 4 hours (*In vitro*)

Exposure	Effector: Target ratio		
	100:1	50:1	25:1
Vehicle	19.26 ± 0.32	14.29 ± 0.20	8.49 ± 0.10
DDDP			
0.001 µg/ml	20.11 ± 0.36	15.58 ± 0.50	8.44 ± 0.33
0.003 µg/ml	19.11 ± 0.67	15.63 ± 0.37	8.00 ± 0.34
0.01 µg/ml	18.58 ± 0.75	15.41 ± 0.34	8.05 ± 0.24
0.03 µg/ml	18.74 ± 0.46	15.65 ± 0.65	8.10 ± 0.24
0.1 µg/ml	19.54 ± 0.38	14.53 ± 0.26	7.44 ± 0.15*
1 µg/ml	17.29 ± 0.54	26.63 ± 1.90**	7.75 ± 0.22
10 µg/ml	16.99 ± 0.47	15.04 ± 0.43	7.49 ± 0.18*
30 µg/ml	15.58 ± 0.11**	14.56 ± 0.38	7.46 ± 0.21*
100 µg/ml	15.93 ± 0.40**	13.95 ± 0.56	6.83 ± 0.18*
300 µg/ml	18.13 ± 1.13**	14.25 ± 0.77	6.08 ± 0.27*
H/NH	H	H	H

Spontaneous release over the 4-hours incubation period was 7.1% of maximum release.

Mean in the same column differ significantly from vehicle control group indicates as

* $p \leq 0.05$, ** $p \leq 0.01$

Values represents the mean ± S.E., n = 8.

H = homogeneous data, NH = non-homogeneous data

^a Vehicle control group (RPMI media alone)

^b Treatment group is DDDP from 0.01 - 300 µg/ml

4.7.2 The *in vitro* effect of DDDP effect mixed leukocyte response

Result of the direct effect of DDDP in MLR assay was demonstrated in Figure 45. The DDDP extract had the potential to activate T leukocytes proliferation in *in vitro* assay. The significant enhancement was observed at 1 µg/ml treatment, and its stimulation was 30% greater than the vehicle control group. Another slight increase

of T leukocytes proliferation was observed at 0.3 $\mu\text{g/ml}$ treatment group with approximately 23% greater than the vehicle control group. The *in vitro* results agreed with the MLR result conduct in *in vivo*. The stimulation of T leukocytes responses were observed at the low doses of DDDP treatment, whereas the responses were decreased at the high doses levels. However, the suppression of T leukocytes proliferation response to allogeneic cells in MLR at DDDP 100 $\mu\text{g/ml}$ until the 300 $\mu\text{g/ml}$ concentration were not due to the direct cytotoxicity of DDDP as illustrated in Table 25.

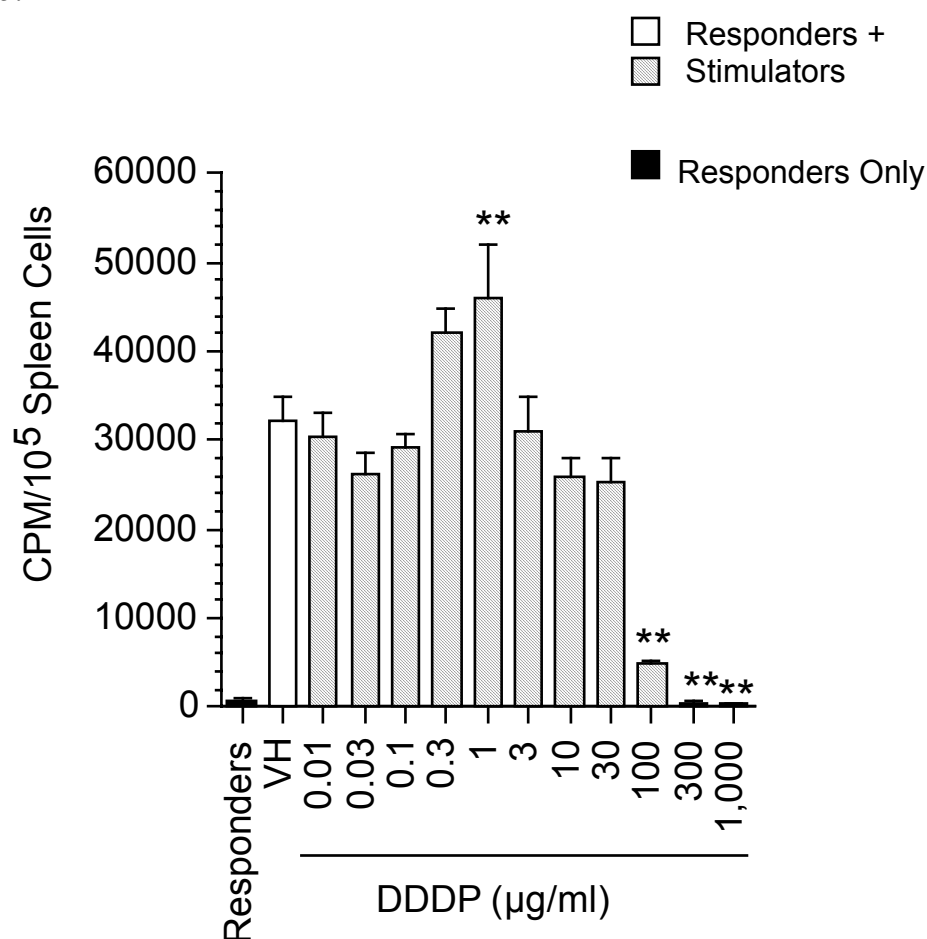


Figure 45 T leukocytes response against allogeneic stimulation in *in vitro* MLR assay. The results are expressed as mean $\pm$ S.E., $n = 6-8$. * * indicated significant difference between treatment groups and vehicle control group at $p \leq 0.01$.

4.7.3 Effect of DDDP on anti-CD3 assay *in vitro* .

The DDDP extract at 0.03 and 0.3 µg/ml significantly stimulated the anti-CD3-mediated response ($p \leq 0.01$). The percents of increase were 22% and 23%, respectively. (Fig. 46). The results demonstrated that the low doses of DDDP effectively activated T lymphocytes proliferation while the high dose significantly decreased the response. However, the suppression observed at the concentrations of 100 µg/ml and 300 µg/ml of DDDP extract did not represent a direct toxicity to the normal spleen cells as indicated in Table 25.

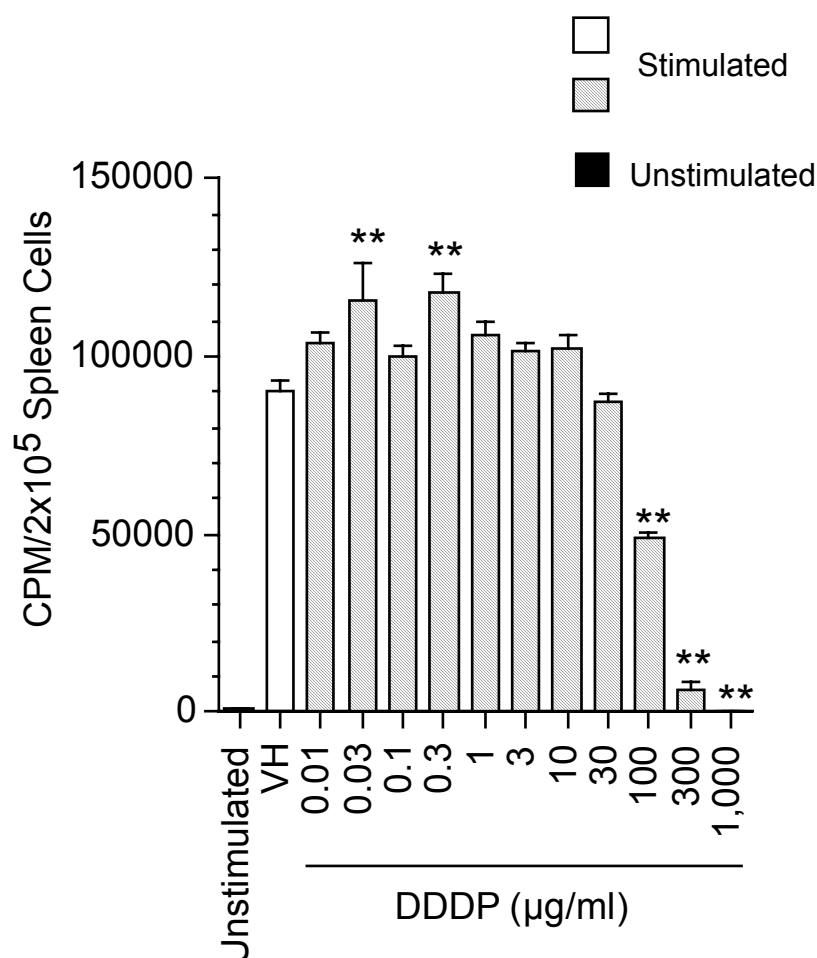


Figure 46 In vitro effects of DDDP on T lymphocytes proliferation against anti-CD3 antibody stimulation. Results are expressed as mean $\pm$ S.E., $n = 8$. * * indicated significant difference between treatment groups and vehicle control group at $p \leq 0.01$.

4.7.4 Effect of DDDP on cytokine production in ELISPOT assay

As seen, the direct addition of the DDDP extracts into splenocyte cultures did not alter the IL-2 and IFN- γ production. (Table 28-29). As was observed in the *in vivo* studies a slight enhancement by DDDP treatment was observed compared to the vehicle controls. However, the increase did not reach the level of statistical

significance.

Table 28 Cytokine production (IL-2, IFN- γ) in female B6C3F1 mice exposed to DDDP *in vitro* for 3 hours.

Exposure	Cytokine (Spots/well)	
	IL-2 ^c	IFN- γ ^c
Vehicle	9.0 $\pm$ 2.01	14.0 $\pm$ 1.84
DDDP		
0.001 μ g/ml	19.0 $\pm$ 2.60	18.0 $\pm$ 2.16
0.003 μ g/ml	13.0 $\pm$ 2.75	18.0 $\pm$ 2.32
0.01 μ g/ml	15.0 $\pm$ 3.28	13.0 $\pm$ 1.47
0.03 μ g/ml	12.0 $\pm$ 2.72	12.0 $\pm$ 3.68
0.1 μ g/ml	5.0 $\pm$ 2.49	8.0 $\pm$ 2.78
0.3 μ g/ml	9.0 $\pm$ 3.38	12.0 $\pm$ 4.40
1 μ g/ml	11.0 $\pm$ 6.14	12.0 $\pm$ 3.20
3 μ g/ml	14.0 $\pm$ 2.34	23.0 $\pm$ 3.77
10 μ g/ml	16.0 $\pm$ 7.61	46.0 $\pm$ 2.28*
30 μ g/ml	4.0 $\pm$ 0.40	25.0 $\pm$ 3.12
100 μ g/ml	4.0 $\pm$ 1.32	18.0 $\pm$ 4.13
H/NH	H	H

Mean in the same column differ significantly from vehicle control group are indicated as * $p \leq 0.05$

Values represents the mean $\pm$ S.E., n = 4.

H = homogeneous data, NH = non-homogeneous data

^a Vehicle control group (RPMI media alone)

^b Treatment group is DDDP from 0.001 - 100 μ g/ml

^c Spots/ $\times 10^6$ cells/well

Table 29 Cytokine production (IL-2, IFN- γ) in female B6C3F1 mice exposed to DDDP *in vitro* for 22 hours.

Exposure	Cytokine (Spots/well)	
	IL-2 ^c	IFN- γ ^c
Vehicle	15.0 $\pm$ 2.87	39.0 $\pm$ 1.60
DDDP		
0.001 μ g/ml	12.0 $\pm$ 1.38	46.0 $\pm$ 2.31
0.003 μ g/ml	18.0 $\pm$ 2.86	34.0 $\pm$ 2.72
0.01 μ g/ml	14.0 $\pm$ 1.08	34.0 $\pm$ 1.75
0.03 μ g/ml	12.0 $\pm$ 2.72	28.0 $\pm$ 4.85
0.1 μ g/ml	25.0 $\pm$ 3.97	41.0 $\pm$ 7.77
0.3 μ g/ml	25.0 $\pm$ 6.28	47.0 $\pm$ 8.50
1 μ g/ml	18.0 $\pm$ 1.70	28.0 $\pm$ 2.40
3 μ g/ml	20.0 $\pm$ 2.86	34.0 $\pm$ 1.87
10 μ g/ml	16.0 $\pm$ 3.47	40.0 $\pm$ 2.56
30 μ g/ml	16.0 $\pm$ 2.10	31.0 $\pm$ 4.17
100 μ g/ml	18.0 $\pm$ 3.12	35.0 $\pm$ 6.34
H/NH	H	H

Mean in the same column differ significantly from vehicle control group are indicated as * $p \leq 0.05$

Values represents the mean $\pm$ S.E., n = 4.

H = homogeneous data, NH = non-homogeneous data

^a Vehicle control group (RPMI media alone)

^b Treatment group is DDDP from 0.001 - 100 μ g/ml

^c Spots/ $\times 10^6$ cells/well

CHAPTER V

DISCUSSION

The normal progression for the development of a pharmaceutical found to be efficacious is first preclinical studies in animals followed by clinical trials which, with the regulatory's agencies's approval, culminates in the drug being used in the appropriate general population. However, for many traditional herbal drugs the process has been reversed.

For many generations herbals drugs have been used to treat diseases in humans and in just recent years have preclinical animal studies and clinical trial been undertaken for a selective few herbal medicines. This has also been the case for the medicinal herb *Aeginetia indica* Roxb. (DDD). For many generations, DDD has been used in numerous provinces of Thailand to treat various diseases. However, prior to this work there have not been any published findings on preclinical or controlled clinical studies with DDD from Thailand.

The importance of considering medicinal plants for clinical practice as immunomodulators needs to be comprehensive and systematic. Evaluations of both *in vitro* and *in vivo* assessment of humoral and cellular immune responses are required. The screening assays for plant constituents should be easy enough to be performed and at the same time efficient enough to allow the determination of the effects on immune functions.

Several *in vitro* and *in vivo* assays such as, anti-CD3 antibody mediated T cell

proliferation assay, hemolytic plaque assay, spleen cell mitogenicity assay, natural killer cells assay, mixed leukocytes response assay, cytotoxic T lymphocytes (CTL) assay, splenic population analysis and cytokine evaluation etc., are potential assays to be used as screening tools to survey plants that modulate humoral and cellular immunity. Standard toxicological investigations such as, body weights, organ weights and hematology are necessary toxicological parameter which also need to be concomitantly evaluated to help interpret the immunotoxicological effects of drugs.

T lymphocytes and B lymphocytes are important cells which respond to antigens and regulate immune functions. Non-specific lymphocyte proliferation against mitogens such as Con A and LPS is one potential assay to evaluate T and B lymphocyte responses to substances on humoral and cell mediated immunity. The anti-CD3 antibody mediated T-cell proliferation assay, mixed leukocytes response assay, and cytotoxic T lymphocytes demonstrate the effect of exposed substances to T lymphocyte responses. The hemolytic plaque assay (antibody plaque-forming cell response or AFC) is important for detecting immunotoxicity on humoral immunity especially the B cell or plasma cell activity. Lymphocyte cytolytic activity by the innate immunity can be detected using the NK assay. CTL activity has proven to be a valuable tool in immunotoxicity testing for assessing activation, proliferation, and differentiation phase of cell-mediated immune response by the T lymphocytes that have a major role to kill the tumor cells. (Fudenberg, Whitten, and Ambrogi, 1984; Pallardy, Lebrech, Kerdine, Burleson, and Burneson, 2000; United States Environmental Protection Agency, 1996).

The spleen is an organ that produces lymphocytes and stores erythrocytes. It is similar to the lymph node except that it is larger and filled with blood, therefore, it

serves as a reservoir for blood, it filters and purifies the blood and lymph fluid that flows through it. It also contains mainly T Cells and B-Cells (Janeway, C.A, Jr., et al., 1999). This organ is mainly used in the various assays of immunotoxicological studies to evaluate T and B lymphocyte responses in the immune system.

A systemic approach was followed in determining if DDD had potential as an immunoenhance drug which may have accounted for its continued use as a part of traditional Thai medicine. Initial *in vitro* studies were undertaken to determine which cells of the immune system were affected. These *in vitro* studies also provide guidance as to whenever the whole plant or plant parts, i.e. seed where the more effective and thus worthy of additional study.

The *in vivo* models were used to assess the effects of DDD in the whole animal. In these *in vivo* studies, first parenteral route, i.p., of drug administration was used to insure quantitative drug exposure occurred and finally the more appropriate approach, oral gavage, was used to simulate the clinical administration of the traditional Thai preparation of herbal drug. Additional *in vitro* studies were conducted in an attempt to elucidate the mechanism of action of DDD.

In the first *in vitro* study, splenocytes were prepared from C57BL6/j mice and were tested for the immunotoxicological effects through various assays, such as anti-CD3 antibody mediated T-cell proliferation, non-specific lymphocytes proliferation against mitogens such as Con A and LPS, and cytotoxicity to HeLa cancer cells. Cell viability and cell proliferation in this study were evaluated by using MTT colorimetric assay. These *in vitro* studies suggest that extracts from seeds (SDDD) and ethanolic whole plants (DDDP) of *Aeginetia indica* Roxb. (DDD) from Thailand have immunostimulatory effects. Both SDDD and DDDP extracts stimulated spleen cells

of C57BL/6j mice through anti-CD3 antibody mediated T lymphocyte proliferation. They also strongly stimulated the lymphoproliferative response to the B cell mitogen, LPS. In addition, the DDDP extract, but not the SDDD extract of DDD, also stimulated the lymphoproliferative response to the T cell mitogen, Con A. Moreover, DDDP extract also showed the effect of inhibiting HeLa cancer cells at high concentrations although the same dose was observed to suppress splenic cells proliferation in mitogenesis assays. Thus the data from the direct cytotoxicity to the tumor cells was not effective enough to assume that DDDP had the anti-tumor effect.

However, at high concentrations of DDDP extracts, there was suppression in the proliferative response of splenocytes to mitogens which did not induce direct cytotoxicity to cells.

Therefore, the screening experiments in *in vitro* in C57BL/6j mice indicated that T and B lymphocytes are among the cells of the immune system which are stimulated by the DDD extracts, and the whole plant extract was able to produce greater enhancement than the seed extract.

Based on the result of the *in vitro* studies, the *in vivo* experiments were conducted to determine immunostimulating effects occurred in female B6C3F1 mice. Based on the recommendation of US National Toxicology Program (NTP), B6C3F1 mice selected as a suitable model for *in vivo* studies to evaluate various immunotoxicological assays.

As noted earlier, the DDDP extract could enhance T lymphocytes and B lymphocytes in *in vitro* assay, further studies were focused on mice which were administered the DDDP extract by intraperitoneal injection (i.p) as a route that allowed quantitation of drug administered and that avoided gastrointestinal and liver

biotransformation which may affect the compound. The DDDP ethanol extract was selected for the first *in vivo* investigation since it was shown active *in vitro*. The approaches in immunotoxicity testing were performed to provide qualitative and quantitative data on the effects of ethanolic plant extract (DDDP) on innate immunity, humoral immunity and cell-mediated immunity. All tests which evaluated cells proliferation used a ^3H -thymidine incorporation which has proven more sensitive than MTT colorimetric assay. Toxicological parameter such as body weight, organ weights and hematology were evaluated for identifying potential toxic effects from this DDDP extracts.

These studies demonstrated that, following 28 days of i.p. administration in female B6C3F1 mice to DDDP, produced a minimal toxicity. Only at the high dose (250 mg/kg) were slight effects observed on spleen and liver absolute organ weights. However when evaluated as relative weight (percent body weight) effects were not statistically significant from the saline controls. A slight effect on white blood cell count number was also observed following DDDP treatment. A significant decrease in leukocyte was only apparent at the 25 mg/kg dose. In evaluation of the effects of DDDP on the immune system, treatment with the DDDP extract did not affect innate immunity, as evaluated in the NK cells assay and no effect on humoral immunity was observed when evaluated using the antibody-forming cells response to the T-dependent antigen sheep erythrocytes. In contrast, cell-mediated immunity was influenced by the DDDP extract which resulted in significant enhancement of T lymphocytes proliferation in the mixed leukocytes response assay and increase cytotoxic T lymphocyte assay. Consistent with the effect in the *in vitro* study, described above, was the enhancing of T lymphocytes functions. The dose-response

relationship of T lymphocytes proliferation was clearly observed in DDDP-treated mice with low dose activation and the high dose suppression. Both *in vitro* and *in vivo* results demonstrated that at the low dose level, DDDP was able to activate T cells while at the high dose, it was shown to suppress this response. The stimulatory effect on T lymphocyte was not observed to the same degree in the anti-CD3 assay as was seen in the MLR and CTL assay. Due to the greater requirements of T lymphocytes to proliferate and differentiate in MLR and CTL assay, the anti-CD3 tends to be less sensitive than these assays.

The B cells stimulation by LPS *in vitro* did not predict the results observed in B lymphocytes producing IgM antibody in the AFC assay. *In vitro* LPS stimulates a B lymphocytes to proliferate. The polyclonal B-cell activation occurs through a calcium-independent manner, and LPS is a potent B cell stimulator. This is in contrast to the AFC assay which is a valuable assay to detect B lymphocyte function for producing antibody via specific antigen receptors (T-dependent antigens).

Characterization of the mechanism of action in cell-mediated immunity by DDDP did not appear to be related to the splenic subpopulation or cytokine level change. The mechanism by which DDDP extracts enhance T cell responses is currently unknown. However, the results in this study demonstrated the significant increase in cell-mediated immunity response (MLR, CTL). It is possible that the DDDP extracts do have effect on cytokine production. However, the cytokine signal may be so low, that it is able to stimulate function but not be detected in the ELISPOT assay.

In practice, the herbal therapeutic obtained from *Aeginetia indica* Roxb. (DDD), is prepared by whole plant decoction which is consumed by Thai people by drinking the extract. Therefore oral exposure (oral gavage) was selected to provide information

using a route of relevant to human exposure. The whole plant extract of DDD was prepared closely to the traditional process used in Thailand for preparing the decoction for human use in treating disease. The major difference was that sterile water was used for laboratory studies to prevent contamination of microorganism in the preparation process. In Thailand potable water would have been used.

In the current study, the toxicological data demonstrated that daily oral (p.o) administration sterile water whole plant (WDDDP) extract for 28-32 days did not induce major significant changes in toxicological parameters as compared to the control group. Slight changes in hematological parameter were not dose dependent in nature, with the exception of a slight increase in leukocyte number. With regard to the effects of WDDDP on the immune system, the data demonstrated that humoral immunity as evaluated by the antibody response to the T-dependent antigen SRBCs, and innate immunity evaluated as natural killer cell activity were not affected following administration of WDDDP. In contrast, in all of cell mediated parameters evaluated, it was demonstrated that administration of WDDDP was capable of enhancing cell-mediated immunity.

In the first cell mediated immune parameter evaluated, anti-CD3 mediated T cell proliferation, enhanced responses were observed at the two highest doses. T cell lymphoproliferation is normally mediated through the T-cell receptor complex (TCR), when a specific antigen peptide is present to the T-cell in context of either MHC class I and class II molecules on antigen presenting cells. In the anti-CD3 assay, a polyclonal T cell response is obtained when the antibody directed against the epsilon (ϵ) subunit of the CD3 component (145-2C11 in mice) of the TCR binds activating the receptor (Pallardy et al., 2000). Because of the direct activation of the TCR, the anti-

CD3 antibody mediated proliferation tends to be less sensitive than the MLR assay, which requires recognition of genetically dissimilar MHC molecules of the stimulator cells, followed by T cell lymphoproliferation. Treatment with WDDDP produced a dose response related increase in the MLR, with the greatest effect being observed at the two highest concentrations, the dose level which would be ingested by Thai patient.

Similarly, the CTL response was also enhanced at all does levels as compared to the vehicle treated animals. While not reaching the level of statistical significance, at all dose levels and effectors to target ratios evaluated, the dose response of the WDDDP treated mice was greater than control responses with the greatest effect being observed at the two highest dose levels. Proliferation in the MLR assay is mainly the response of CD4⁺ lymphocytes in response to MHC class II determinations, where as CD8⁺ cells responding to the MHC class I determinates predominate in the CTL assay (König et al., 1992). An enhanced response was observed in the CTL assay, even though there was a significant decrease in the total number of CD4⁺CD8⁺ suppressor/cytotoxic T-cells following WDDDP treatment.

In the latter experimental studies, *in vitro* studies in B6C3F1 mice were performed in order to determine if the mechanism of action of DDDP on the immune system. As demonstrated in the response at the high doses, DDDP suppressed T lymphocytes proliferation in the anti-CD3, MLR and CTL assays. This response could not be attributed to the direct affect of DDDP on cells viability when the concentration of DDDP was lower than 1,000 µg/ml. The confirmatory result observed in that DDDP directly activated T lymphocytes was demonstrated in the *in vitro* MLR and anti-CD3 assay. Hence the cell-mediated stimulation of mice administrated with DDDP *in vivo*

was not due to the hormone manipulation or another indirect mechanism.

The mechanism by which WDDDP enhances cell mediated immunity remains to be elucidated. However, the results presented demonstrated a decrease in the number of CD4⁺CD25⁺ T regulatory cells. This regulatory T cell acts as an inhibitor of IL-2 cytokine mediated responses (Kuniyasu et al., 2000; Stephen et al., 2001). A decrease in the number of IL-2 inhibitory cells could possible result in an increase in IL-2 and other T helper 1 (TH1) cytokines. This is potentially one of the mechanisms of action of the water soluble extract of DDD. However the cytokine production observed in WDDDP-treated mice did not demonstrate any relationship to this regulatory T cell response. Thus, the WDDDP extract may activate cytokine levels to level above the threshold that could stimulates T lymphocytes responses. However these levels of cytokine productions may not be detected in the ELISPOT assay. This potential mechanism of action is futhur supported by the finding that *Aeginetia indica* Linn., a species related to DDD that grows in Japan has been reported to stimulate CD4⁺T cells to produce increased amounts of various interleukins including IL-2, IL-6, IL-10, IL-12 and IL-18 (Chai et al., 1992; Chai et al., 1994; Okamoto et al., 2000; Ohe et al., 2001). There is evidence that elimination of CD4⁺CD25⁺ T cells using anti-CD25 mAb *in vivo* can induce a potent tumor-specific CTL response (Shimizu et al., 1999; Onizuka et al., 1999; Steitz et al., 2001; Tanaka et al., 2002).

The effects from these studies suggests DDD extracts are potential immuno stimulants. Further studies are needed to elucidate the mechanisms of each extract from DDD on the immune system to develop ways for effectively treating or preventing immune diseases.

CHAPTER VI

CONCLUSION

In the present study, the immunological effect of seed (SDDD), ethanolic whole plant (DDDP) and sterile water whole plant (WDDDP) extract of *Aeginetia indica* Roxb. (DDD) were investigated using various *in vitro* and *in vivo* immunotoxicology assays. In addition, the cytotoxicity of DDDP extract on tumor cells was also investigated.

In order to avoid using radioactive materials, the MTT colormetric assay was modified and was used to measure the cell proliferation in the first *in vitro* study. The optimization of the tests suggested that the appropriate condition for the anti-CD3 antibody mediated T cell proliferation assay was to use cell concentrations of 4×10^6 cells/ml and 1 $\mu\text{g/ml}$ of anti-CD3 mouse antibodies. For the lymphoproliferative response to con A, the optimum concentration of con A was 5 $\mu\text{g/ml}$, and the splenocyte concentration of 8×10^6 cells/ml provided the highest response. Moreover, the 10 $\mu\text{g/ml}$ of LPS and the cell concentration of 8×10^6 cells/ml provide the highest stimulation of B cells in the optimization assay.

The toxicity tests of SDDD and DDDP extract were conducted to select the appropriate concentrations of both extracts used in various *in vitro* immunological studies using MTT colorimetric assay. Data from the toxicity tests *in vitro* in female C57BL/6j mice indicated that no toxic effect was found from concentrations ranging from 1.95 to 2,000 $\mu\text{g/ml}$ in the SDDD extract, and from 1.25 to 1,000 $\mu\text{g/ml}$ in the

DDDP extracts.

The screening effects of SDDD and DDDP extracts were investigated by using MTT colorimetric assay. The immunological studies of SDDD and DDDP extracts on anti-CD3 antibody mediated T cell proliferation assay revealed that SDDD extract at final concentrations of ranging from 7.8, 500 and 1,000 $\mu\text{g/ml}$ significantly ($p \leq 0.05$) stimulated T lymphocyte proliferation higher than controls on day 1 and day 2. On day 3 the 2,000 $\mu\text{g/ml}$ concentration of extract stimulated T lymphocytes significantly at $p \leq 0.05$. Furthermore, the 100 $\mu\text{g/ml}$ DDDP extract stimulated proliferation of T lymphocytes more than the control group on days 1-3. In contrast, at higher concentrations of 500 and 1,000 $\mu\text{g/ml}$, the DDDP extract significantly ($p \leq 0.01$) suppressed T lymphocyte proliferation from day 1 until day 3.

The DDDP extracts at concentrations ranging from 1.25 to 500 $\mu\text{g/ml}$ also stimulated the lymphoproliferative response to con A significantly ($p \leq 0.05$ and $p \leq 0.01$) from day 1 to day 3. In contrast, the seed extract did not stimulate the lymphoproliferative response to con A.

The strongly enhanced B lymphocyte response to LPS was found in SDDD extract at a concentration of 2.5 $\mu\text{g/ml}$ and was significantly higher than controls on day 1 ($P \leq 0.05$). For day 2, the SDDD extract at concentrations ranging from 1.25 to 2,000 $\mu\text{g/ml}$ stimulated B lymphoproliferative responses to LPS significantly ($p \leq 0.05$ and $p \leq 0.01$). In addition, the concentrations of DDDP extract ranging from 1.25 to 100 $\mu\text{g/ml}$ also stimulated lymphoproliferative responses to LPS significantly at $P \leq 0.05$ and $p \leq 0.01$ on days 1-3. However, at high concentrations, 500 and 1,000 $\mu\text{g/ml}$, the whole plant suppressed the B lymphocyte proliferative response to LPS significantly ($p \leq 0.01$).

In addition, the inhibition of HeLa cancer cells was observed at DDDP extract concentration of 1,000 µg/ml as determined by the trypan blue exclusion method.

The preliminary results from the first *in vitro* study in C57BL/6j mice demonstrate that SDDD and DDDP from DDD have immunostimulant potential. In order to confirm DDD potential as an immunostimulant, *in vivo* studies evaluating the effects of DDDP on various immunological assays, such as plaque-forming cell response, natural killer cell activity, mixed leukocytes response, anti-CD3 mediated T lymphocytes response assay, cytotoxic T lymphocyte assay, cytokine assay and the effects on the ratio of splenic populations were investigated in female B6C3F1 mice.

Results demonstrated that following 28 days of i.p. administration in female B6C3F1 mice produced minimal toxic effects. The slight effects on spleen and liver absolute organ weights were observed only at the high dose (250 mg/kg). These effects were not statistically significant from the saline controls when the data expressed as relative weight (percent body weight), the more appropriate measurement of organ weight. The other toxicological parameters effected, decrease in white blood cell count, was only significantly decreased at the 25 mg/kg dose. The present finding in the NK cells assay and antibody-forming cells response to the T-dependent antigen sheep erythrocytes, the DDDP extracts did not affect on innate immunity and humoral immunity. Clearly, the enhancing in cell-mediated immunity was influenced by the DDDP extract which resulted in the great enhancing of T lymphocyte activity in the mixed leukocytes response assay and the cytotoxic T lymphocyte assay. The stimulative doses level occurred between 0.25 mg/kg and 25 mg/kg of DDDP. The suppression of T lymphocytes was observed in the high dose treatment groups at 100 and 250 mg/kg. The current results indicated a dose-response

relationship of T lymphocytes proliferation in DDDP-treated mice in which the low doses produced activation and the high dose suppression. The induction of T lymphocytes proliferation was not as great as was observed in the anti-CD3 assay as much as illustrated in MLR and CTL assays. Only slight increase was presented at the mice that were given 0.25 to 25 mg/kg DDDP.

The results of the phenotypic analysis of the splenic subpopulations of DDDP-treated mice demonstrated that there was not an effect to the number of T cells subsets. The other splenic subpopulations including B cells, NK cell, and macrophage also were not affected in mice exposed DDDP extracts. DDDP exposure did not alter cytokine levels that were measured by ELISPOT assay. The mechanism of DDDP to activate T cells response is remaining unknown.

Investigation of the effect of WDDDP, water extract of DDD was performed in female B6C3F1 mice. This extract was simulation of the Thai traditional preparation. The results indicated that WDDDP did not induce any sign of toxicity to the mice, except the slight increase in lungs weight was observed in mice treated with 10% WDDDP without the significant increase in lungs weight to body weight ratio. The slight suppression also observed in hematocrit and reticulocyte value, but this effect was not a dose-response. The approximate two percents increase was observed in MCV values at the 100% of WDDDP dose level, thus this was not considered to be biological relevant. The significant increase of white blood cells was illustrated in mice administrated 50% WDDDP, which was probably resulted the immunostimulating. In addition, mice administrated WDDDP did not affect innate and humoral immunity which was represented in the NK assay and AFC response.

Furthermore, this current research demonstrated the potential effect of WDDDP to

enhance T lymphocytes proliferation in anti-CD3, MLR and CTL assay. The great enhancement was observed in anti-CD3 assay in mice treated WDDDP 50% and 100% dose levels, the percents enhancement were 20% and 30%, respectively. The maximum increase T lymphocytes proliferation at approximate 46% was indicated in MLR assay with only statistical significance following a Dunnett's T test. The slight stimulation of cytolytic activity of T lymphocytes were presented in the CTL assay at the two highest of WDDDP dose groups.

The possible mechanism involved in this effect may be the results of the regulatory T cells suppression which was statistical significance comparable to the control group at $p \leq 0.01$. However, mice administrated WDDDP did not produce any effect to the total B cells, total T cells, T helper cell, immature T cell, NK and macrophage. WDDDP also did not affect the cytokine productions which were evaluated as spots of cytokine secreting cell in ELISPOT assay.

Following incubation of DDDP with naïve splenocytes from B6C3F1 mice *in vitro*, results was demonstrated that the suppression of T lymphocytes proliferation in anti-CD3, MLR and CTL assay did not directly affect the cell viability except at the 600-1,000 $\mu\text{g/ml}$ of DDDP. The data presented in *in vitro* NK assay was repeated in the *in vivo* results, DDDP did not alter innate immunity.

To assess the mechanism of DDDP activated cell-mediated immunity, untreated or treated DDDP were co-cultured with splenic cells in anti-CD3 and MLR assay in the *in vitro* study. Indeed, naïve splenice cells treated with DDDP enhanced T lymphocytes proliferation greater than the untreated group at the low doses of DDDP. The dose response relationship was clearly supported in the *in vivo* results in the anti-CD3 and MLR assays. The low doses of DDDP enhanced T lymphocytes where the

high doses were suppressed. Moreover, directly adding DDDP extract *in vitro* did not establish a relationship between IL-2 and IFN- γ production with the cell-mediated stimulation.

In conclusion, this current research provided the first report on the immunomodulatory effects of DDD. This immunostimulatory effect of DDD most likely contributes to the successful and long time use of this traditional herbal medicine in Thailand. These findings demonstrate that *Aeginetia indica* Roxb. can be added to the ever growing list of herbal medicine that have the potential to enhance immune response.

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APPENDICES

APPENDIX A

IN VITRO ASSAY IN C57BL/6j MICE

RPMI 1640 (1X CULTURE MEDIA):

Commercial RPMI-1640 10X	
with L-glutamine without NaHCO ₃ (GIBCO)	100.00 ml
L-glutamine 200mM (GIBCO)	10.00 ml
7.5%NaHCO ₃ (GIBCO)	50.00 ml
FBS ^a (GIBCO)	10.00 ml
HEPES buffer 1 M (GIBCO)	15.00 ml
(N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)	
Streptomycin (GIBCO)	100.00 µg
Sodium Penicillin G (GIBCO)	100.00 unit
Sterile ultra purified water (laboratory supply) added to	1,000.00 ml

PROCEDURE:

The pH of the solution was adjusted to 7.0-7.4 and sterilized by the filtration method using a millipore sterile filter which has a pore size of 0.2 µm. After the solution was filtered, it was checked for sterility and kept at 2-8°C.

^aFBS was heat activated at 56°C for 20 minutes. Heat inactivated FBS was added on the day of assay to make RPMI complete medium.

EARLES BALANCED SALT SOLUTION (EBSS with HEPES):

Commercial EBSS 10X (GIBCO)	100.00 ml
(Earle's Balanced Salt Solution)	
1M HEPES buffer	15.00 ml
(N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)	
Sterile ultra purified water added to	1,000.00 ml
Gentamicin 50 mg/ml (GIBCO)	1.00 ml

PROCEDURE:

This solution was mixed together under a sterile hood and stored at 4°C.

PHOSPHATE BUFFERED SALINE:

Commercial PBS 10X (GIBCO)	100.00 ml
(Phosphate buffered saline)	
Sterile ultra purified water added to	1,000.00 ml

PROCEDURE:

This solution was mixed together under a sterile hood and stored at 4°C.

HAM' F 12 CULTURE MEDIUM:

Commercial Ham's F12 medium with L-glutamine without NaHCO ₃ (GIBCO)	10.6 g
L-glutamine	0.292 g
7.5%NaHCO ₃	2.00 g
FBS ^a (inactivated fetal bovine serum, Hyclone)	10.00 ml
HEPES buffer 1 M (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)	15.00 ml
Streptomycin	100.00 µg
Sodium Penicillin G	100.00 unit
Sterile Ultra purified water added to	1,000.00 ml

PROCEDURE:

The pH of the solution was adjusted to 7.0-7.4 and sterilized by the filtration method using a millipore sterile filter which has a pore size of 0.2 µm. After the solution was filtered, it was checked for sterility and kept at 2-8°C.

^aFBS was added on the day of assay for complete medium.

APPENDIX B

IN VITRO and *IN VIVO* ASSAY IN B6C3F1 MICE

RPMI 1640 1X CULTURE MEDIUM (with 2ME):

Commercial RPMI-1640 1X with L-glutamine without NaHCO ₃ (GIBCO)	825.00 ml
(Roswell Park Memorial Institute)	
L-glutamine 200mM	10.00 ml
7.5% NaHCO ₃	50.00 ml
FBS (inactivated fetal bovine serum, Hyclone)	100.00 ml
HEPES buffer 1 M	15.00 ml
(N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)	
Gentamicin	1 ml
2-ME ^a (1,000 X)	1ml

PROCEDURE:

This solution was mixed together under a sterile hood and stored at 4°C.

^a 2-ME at concentration 1,000 X was prepared by adding 0.177 ml of 2 ME to 5 ml of EBSS with HEPES to yield a 10,000 X solution. The previous solution then was diluted at 1:10 ratio into EBSS with HEPES that was given a 1,000 X solution. The final concentration of 2ME (1,000 X) as added into the RPMI media on the day of the MLR, anti-CD3 and CTL assay.

EARLES BALANCED SALT SOLUTION (EBSS without HEPES):

Commercial EBSS 10X (GIBCO)	100.00 ml
(Earle's Balanced Salt Solution)	
1M HEPES buffer	15.00 ml
(N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)	
Commercial sterile water (Baxter) added to	1,000.00 ml

PROCEDURE:

This solution was mixed together, and then the pH of the solution was adjusted to 7.0-7.4, after that it was stored at 2-8°C. This solution was used in anti-plaque forming cell response assay.

DULBECCO MODIFIED EAGLE MEDIA (DMEM 1X):

Commercial DMEM 1X (GIBCO)	859 ml
(Dulbecco's Modified Eagle Media)	
HEPES buffer 1M	15 ml
L-glutamine	10 ml
Gentamicin	1 ml
FBS	100 ml
Sodium Pyruvate 100 mM(GIBCO)	10 ml

PROCEDURE:

This solution was mixed together, and stored at 2-8°C.

EAGLE'S MINIMAL ESSENTIAL MEDIA (with 0.15% NaHCO₃):

Commercial EMEM 10X with Earle's salt (GIBCO)	100 ml
(Eagle's Minimal Essential Media)	
Comercial sterile water (Baxter) added to	748 ml
HEPES buffer 1M	15 ml
L-glutamine 200 mM	10 ml
Gentamicin	1 ml
FBS	100 ml
NaHCO ₃	25 ml
2-ME ^a (1,000 X)	1 ml

PROCEDURE:

This solution was mixed together, and stored at 2-8°C.

^a 2-ME is added on the day of the assay.

PHOSPHATE BUFFERED SALINE:

Powder of commercial PBS ^a (Sigma)	1 packet
(Phosphate buffered saline)	
Comercial Sterile water	1,000.00 ml

PROCEDURE:

This powder was dissolved under a sterile hood and stored at 4°C. This was yield 0.01 M phosphate buffered saline at pH 7.4. The solution was filtered through 0.2 µm filter.

^a One packet of 0.01 M phosphate buffer saline composed of 0.138 M NaCl and 0.027 M KCl.

CYCLOPHOSPHAMIDE:

Commercial powder of cyclophosphamide (CPS-Sigma contain 5 g/vial)	5 mg
Sterile filtered PBS	1 ml

PROCEDURE:

The desired amount of CPS was weighted into a glass beaker. Sterile filtered PBS (0.45 µm) was added to achieve the desired concentration. The solution was stirred approximately 30 minutes or until it homogeneous. The solution was maintained at - 20°C. The concentration of 5 mg/ml was defined as 50 mg/kg when injected mice at the ratio of 0.1ml solution /10g mice.

ANTI ASIALO GM1 (RABBIT):

Commercial solution of Anti Asialo GM1 (AAGM1-Wako Bioproduct)	24 mg
Commercial sterile water	1 ml

PROCEDURE:

Reconstitute the material in whole bottle with 1 ml of sterile distilled water. Then it was diluted to 1:10 dilution with sterile physiological saline for animal injection. This solution was maintained at 4°C.

MITOMYCIN C^a :

Comercial powder of mitomycin C (Sigma-2mg/bottle)	1 bottle
EBSS with HEPES	0.4 ml

PROCEDURE:

Reconstitute the material in whole bottle with 0.4 ml of EBSS with HEPES as the insert directs until all of the powder was dissolved into the solution.

^a Mitomycin C is a carcinogen, glove protection should be done during preparation.

PHOSPHATE BUFFERED SALINE (with 0.05% Tween 20)^a:

Comercial powder of PBS+0.05% Tween 20(Sigma)	1 packet
Deionized water (Biotech)	1,000 ml

PROCEDURE:

This powder was dissolved and pH was adjusted to 7.2-7.4. The solution was stored at 2-8°C. The expiration of this solution was three days after preparation date.

^a This solution was used in ELISPOT assay as a wash buffered I. One packet contained 138 mM NaCl, 2.7 mM KCl and Tween 20 0.05% w/v.

PHOSPHATE BUFFERED SALINE (with 10% FBS)^a:

Sterile filtered PBS	990 ml
FBS ^a	10 ml

PROCEDURE:

This powder was dissolved under the sterile hood and stored at 2-8°C. The expiration of this solution was three days after preparation date.

^aThis solution was used in ELISPOT assay as a diluent solution and no need for sterilization. However, when this solution was used in CTL assay, it required sterilization.

AEC STOCK SOLUTION (ELISPOT):

Comercial AEC ^a (Sigma)	100 mg
(3-amino-9-ethylcarbazole)	
DMF (Sigma)	10 ml
(N,N-dimethylformamide)	

PROCEDURE:

This powder was dissolved under the sterile hood and stored at room temperature.

^a Mask protection was required when weighting out AEC.

PREPARATION OF 0.1 M ACETATE SOLUTION (ELISPOT):

Acetic acid 0.2 M (Fisher)	148 ml
Sodium Acetate 0.2 M (Fisher)	352 ml
Deionized water (ITI laboratory supply)	500 ml

PROCEDURE:

This solution was mixed together under the sterile hood and stored at room temperature.

SUBSTRATE SOLUTION (ELISPOT):

AEC solution	333.3 μ l
Acetate solution	10 ml
Hydrogen peroxide (30% H ₂ O ₂ , Fisher)	5 μ l

PROCEDURE:

The AEC solution was added into the acetate solution, and then filtered through 0.45 μ m filter. After that the hydrogen peroxide solution was immediately added. This solution was stored at room temperature during experimentation performed.

STAINING BUFFER:

Sterile filtered PBS	1,000 ml
Bovine serum albumin (Sigma)	10 g
Sodium azide (Sigma)	1 g

PROCEDURE:

This powder was dissolved under the sterile hood and stored at 2-8°C.

PROPIDIUM IODIDE (PI) STOCK:

Propidium iodide (Sigma)	10 mg
Staining Buffer	10 ml

PROCEDURE:

This powder was dissolved under the sterile hood and stored at 2-8°C. This solution was diluted at ratio 1:200 for flow cytometric analysis

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