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Immunomodulation by Dok Din Daeng (*Aeginetia indica* Roxb.) extracts in female B6C3F1 mice II. Humoral immunity, innate immunity and hematology

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Abstract

In the previous report, we have provided evidence that *Aeginetia indica* Roxbert (DDD) extracts enhance T cell-mediated immune responses. The study reported here was focused on the hematological and immunological effects, including B cells, natural killer (NK) cells, macrophages and neutrophils, of the whole plant extract using water (WDDD) or ethanol (EDDD) as the solvent. The extracts were administered to female B6C3F1 mice by gavage for WDDD (10–100%) and intraperitoneally for EDDD (0.25–250 mg/kg) for 28 days. In addition to hematological evaluation, several quantitative measures and functional assays (e.g., the splenic phenotypic analysis, IgM antibody-forming cell responses, natural killer cell activity, mononuclear phagocyte system [MPS] and neutrophil activity) were employed to examine the effects of DDD extracts on the innate and humoral immunities. The results from this study demonstrated that exposure to WDDD and EDDD produced minimal changes in the activities of B cells and natural killer cells, macrophages and neutrophils. Overall, hematological parameters were not affected by exposure to WDDD or EDDD. Taken together, the enhancing effect of DDD extracts on T cells may be primarily responsible for the successful and long-time use of this traditional herbal medicine in Thailand.

Keywords: *Aeginetia indica* Roxbert; Hematology; Humoral immunity; Natural killer cells; Macrophages; Neutrophils

1. Introduction

Although *Aeginetia indica* Roxbert (DDD) has been used extensively for its beneficial medicinal effects, there have been limited published studies on the hematological and immunological effects of this plant species from Thailand. We have initiated a study in female B6C3F1 mice by examining the modulatory effects of DDD extracts on the immune system. In the previous paper, we have shown that exposure to DDD extracts increases the activities of T cells [1]. The studies reported herein were undertaken to determine if humoral and innate immunities were also stimulated by exposure to DDD extracts, and thus, possibly contribute to its long-term use as a

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medicinal herb. In addition to the effects on the immune system, hematological parameters were also evaluated to provide additional insight into general toxicological properties of the herb not previously reported.

The most frequently used methods for extracting the active compounds in herbs are alcohol and water extractions [2,3]. In our study, both methods were employed to obtain experimental materials. The whole plant extract obtained using aqueous ethanol (95%) as the solvent was designated as EDDD, while WDDD refers to the whole plant extract acquired using hot water as the solvent. Because the Thai people consume this herbal medicine from crude whole plant decoction by the oral route, the female B6C3F1 mice received WDDD by gavage. However, in the studies performed on Japanese *A. indica* Linn., the butanol or alcohol extract was frequently used and administrated by intraperitoneal (i.p.) injection in mice [4–7]. Thus, EDDD was also given to mice intraperitoneally as a comparison in our study. Several quantitative measures and functional assays that are recognized by the Food and Drug Administration (FDA), Environmental Protection Agency (EPA) and National Toxicology Program (NTP) as predictive for identifying compounds with immunomodulatory potential were employed [8]. These included the splenic phenotypic analysis, IgM antibody-forming cell responses, natural killer (NK) cell activity and functional activity of the mononuclear phagocyte system (MPS). In addition, the effects on neutrophils were also evaluated by determining the number of neutrophils and the activity of myeloperoxidase (MPO) which was produced by neutrophils. The results demonstrated that in contrast to the stimulatory effects on T cells, exposure to WDDD and EDDD produced minimal changes in the activities of B cells, natural killer cells, macrophages and neutrophils.

2. Materials and methods

2.1. Preparation of WDDD and EDDD

WDDD and EDDD were prepared as previously described [1].

2.2. Animals and animal exposure

Animal and animal exposure were conducted as previously described [1].

2.3. Toxicological studies

Animal body weights were obtained weekly to determine the effects of DDD extracts on body weight and to adjust dose injection volumes. To evaluate hematological parameters, blood was drawn and collected into EDTA tubes (Becton Dickinson, USA) by retroorbital bleeding. Hematological parameters evaluated included: the number of erythrocytes, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC). Analysis was conducted using a K-1000 Hematology analyzer (Roche Dignostics). The number of reticulocytes was measured using a Retic-count Reagent stain (Thiazole Orange, Becton Dickinson) followed by flow cytometric analysis. The differential white blood cells count was analyzed using a Hemavet series instrument (DrewScientific, Oxford, CT, USA).

2.4. Spleen collection and single cell suspension preparation

Spleen collection and single cell suspension preparation were as previously described [1].

2.5. Hemolytic plaque assay for detecting IgM antibody-forming cells

The primary IgM antibody-forming cell response to sheep red blood cells (sRBC) was measured using a modified hemolytic plaque assay of Jerne and Nordin [9]. Mice were sensitized with 7.5×10^7 sRBC (intravenously, i.v.) 4 days prior to sacrifice. Spleen cell suspensions were prepared as described above, and an aliquot of cells was added to a test tube containing guinea pig complement, sRBC and warm agar (46–48 °C). The mixture was plated in a petri dish, covered with a microscope cover slip and incubated at 37 °C for 3 h. Cell counts were performed, and the developed plaques were counted using a Bellco plaque viewer. The data were expressed as antibody forming cells (AFC)/10^6 spleen cells and AFC ($\times 10^3$)/spleen.

2.6. Natural killer (NK) cell activity

The activity of NK cells was assayed as described by Reynolds and Herbman [10] with modification. Single cell suspensions were adjusted to six concentrations: 1×10, 5×10, 2.5×10, 1.25×10, 0.625×10 and 0.31×10 cells/ml. The target cells (5×10^4/ml), ^{51}Cr-YAC-1 cells, were added to each well of a 96-well plate in a volume of 0.1 ml. The effector cells at a volume of 0.1 ml were added to each of two replicate wells of target cells at each effector concentration to obtain effector/target ratios of 200:1, 100:1, 50:1, 25:1, 12.5:1 and 6.25:1. The spontaneous release and the maximum release were determined by adding 0.1 ml of medium and Triton X-100 (0.1%) to each of 12 replicate wells containing the target cells, respectively. Following a 4-h incubation, the plates were centrifuged, and 0.1 ml of the supernatant was removed from each well and counted using a.

2.7. Flow cytometric analysis

The percentages of B cells (Ig^+), NK cells ($NK1.1^+CD3^-$) and macrophages ($Mac3^+$) in the spleen were measured using flow cytometric analysis as previously described [1]. Irrelevant, isotype matched antibodies were used as the control. The antibodies utilized were obtained from BD Pharmingen (San Diego, CA, USA) and diluted 1:80 with staining buffer, which included goat anti-mouse Ig–FITC, mouse anti-mouse NK1.1–PE, rat anti-mouse CD3–FITC and anti-mouse Mac3–FITC.

2.8. Evaluation of the mononuclear phagocyte system (MPS)

The MPS activity was evaluated in mice by measuring the vascular clearance of i.v. injected ^{51}Cr-labled sheep erythrocytes (sRBCs) and their subsequent phagocytosis by macrophage in the liver, spleen, lungs and thymus. This assay was performed as describe by Karrow et al. [11]. On day 29 of study, mice were injected (i.v.) with ^{51}Cr-labled sRBCs (5 μl/g body weight or approximately 5×10^9 sRBCs/ml). Blood samples were obtained from the tail vein over the next 30 min to determine the vascular clearance ($T_{1/2}$) of the labeled sRBCs. After 60 min, the animals were sacrificed, exanguinated by decapitation, and their liver, spleen, lungs and thymus were removed to measure organ radioactivity as an indicator of tissue macrophage function. The positive control for this study was Maleic vinyl ether (MVE, 50 mg/kg, Hercules, Wilmington, DE, USA). It was administered to mice by i.v injection 1 day before the day of the assay.

2.9. Measurement of neutrophil activity

Measurement of myeloperoxidase activity was performed as described with modification [12–14]. After exposure to WDDD for 28 days, mice were anesthetized by i.p. injection of a mixture of ketamine and xylazine in saline. Then, mice were treated intranasally with lipopolysaccharide (LPS, 0.3 mg/kg) for 24 h and *N*–Formyl–Met–Leu–Phe (fMLP, 2 mg/kg, Sigma) for 2 h to recruit neutrophils into the lung before sacrifice. The whole lungs were removed and homogenized in a buffer containing 100 mM sodium acetate, 0.5% hexadecyltrimethylammonium bromide (HETAB) and 5 mM EDTA. The homogenates were sonicated and centrifuged at $13{,}000 \times g$ for 2 min. The supernatants were collected and serially diluted from 1:2 to 1:2,048 with assay buffer in one lane of a 96-well plate. The final volume was 75 μl per well. Seventy-five microliters of TMB substrate was added into each well. The plates were read within 2 min at 450 nm after adding 50 μl of the stop solution (2N H_2SO_4). The result was expressed as midpoint tier at an optical density of 0.5.

2.10. Statistical analysis

Statistical analysis was conducted as previously described [1].

3. Results

3.1. Effect of DDD extract on hematology parameters

Mice were exposed to WDDD (p.o.) and EDDD (i.p.) daily for 28 days as described. The effects of WDDD on the hematological parameters are shown

in Table 1. When compared to the vehicle control group, there were no significant differences in the number of erythrocytes, the concentration of hemoglobin, MCH, MCHC and platelet counts. A slight increase of MCV was observed in mice administrated with 100% WDDD. However, the hematocrit and reticulocyte values were slightly decreased in mice exposed to 10% WDDD and 25% WDDD, respectively. The effects of EDDD on the hematological parameters are also shown in Table 1 as a comparison. There were no significant differences in the number of erythrocytes, the concentration of hemoglobin, hematocrit, MCH, MCHC, the percentage of reticulocytes or platelet numbers between animals treated with EDDD and the vehicle control animals. However, exposure to EDDD produced slight, albeit statistically significant, increases in MCV in all the treatment groups (Table 1).

The number and percentage of lymphocytes, neutrophils, monocytes, eosinophils and basophils in the blood were determined as described. Exposure to WDDD for 28 days had no effect on the number of these cells no matter whether the data were expressed as an absolute value or a percent value (Table 2).

3.2. Effect of DDD extract on splenocyte phenotype

Phenotypic analyses of total B cells, NK cells and macrophages were conducted using flow cytometric analysis. Exposure to WDDD by gavage or EDDD (i.p.) for 28 days had no effects on the

Table 1
Hematology in female B6C3F1 mice exposed to WDDD and EDDD for 28 days

Exposure	Parameter							
	Erythrocytes (10^6/mm^3)	Hemoglobin (g/dl)	Hematocrit (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)	Platelets (10^3/μl)	Reticulocytes (%)
WDDD[a]								
Vehicle[a]	9.5 ± 0.7	15.95 ± 0.93	46.99 ± 0.37	49.67 ± 0.15	16.9 ± 0.1	34.0 ± 0.2	567.6 ± 93.0	4.21 ± 0.23
WDDD 10%	8.9 ± 0.2	15.46 ± 0.16	44.31 ± 1.07*	49.72 ± 0.11	17.4 ± 0.4	35.0 ± 0.8	473.5 ± 59.0	3.79 ± 0.20
WDDD 25%	9.3 ± 0.1	15.54 ± 0.29	46.09 ± 0.60	49.60 ± 0.10	16.7 ± 0.2	33.7 ± 0.3	448.1 ± 85.0	3.45 ± 0.12*
WDDD 50%	9.4 ± 0.1	15.47 ± 0.29	45.89 ± 0.47	49.91 ± 0.21	16.5 ± 0.4	33.1 ± 0.7	562.4 ± 39.0	3.96 ± 0.07
WDDD 100%	9.2 ± 0.1	15.58 ± 0.10	46.14 ± 0.18	50.36 ± 0.26*	17.0 ± 0.1	33.8 ± 0.2	637.8 ± 101.1	3.81 ± 0.19
H/NH	NH	NH	NH	H	NH	NH	H	H
Trend analysis[b]	NS	$p \leq 0.05$	NS	NS	NS	NS	NS	NS
EDDD[c]								
Vehicle[c]	8.3 ± 0.5	14.65 ± 0.48	41.79 ± 2.46	50.11 ± 0.08	18.0 ± 1.1	35.9 ± 2.3	545.3 ± 86.2	6.60 ± 0.43
EDDD 0.25 mg/kg	8.2 ± 0.4	13.79 ± 0.57	41.70 ± 1.90	50.86 ± 0.14**	16.9 ± 0.3	33.1 ± 0.6	458.9 ± 52.1	6.94 ± 0.34
EDDD 2.5 mg/kg	9.1 ± 0.1	15.55 ± 0.13	46.06 ± 0.37	50.88 ± 0.16**	17.2 ± 0.1	33.8 ± 0.1	648.5 ± 104.0	6.80 ± 0.61
EDDD 25 mg/kg	8.2 ± 0.6	12.80 ± 1.15	41.58 ± 2.67	50.56 ± 0.37*	15.7 ± 1.5	31.1 ± 2.9	334.1 ± 94.5	6.96 ± 0.55
EDDD 250 mg/kg	7.5 ± 0.5	13.48 ± 0.77	38.08 ± 2.48	50.83 ± 0.30*	18.1 ± 0.6	35.7 ± 1.1	448.3 ± 82.9	6.92 ± 0.40
H/NH	NH	NH	NH	NH	NH	NH	H	H
Trend analysis[b]	NS	NS	NS	$p \leq 0.05$	NS	NS	NS	NS

Mean values in the same row significantly different from vehicle control group are indicated as: *$p \leq 0.05$, **$p \leq 0.01$.
Values represent the mean ± S.E., $n = 8$.
H = homogeneous data; NH = non-homogeneous data; NS = not significant.
MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration.
[a] Vehicle control group is sterile water. Treatment group is WDDD (10–100%).
[b] The Jonckheere's Test was used to test for dose-related trends among the vehicle and exposed groups.
[c] Vehicle control group is 0.9% sodium chloride. Treatment group is EDDD (0.25–2.50 mg/kg).

Table 2
Leukocyte cell differential blood counts in female B6C3F1 mice exposed to WDDD for 28 days

Parameter	Vehicle[a]	WDDD[b]				H/NH
		10%	25%	50%	100%	
Leukocytes (10^3/mm^3)	5.8 ± 0.48	5.8 ± 0.49	6.4 ± 0.85	5.8 ± 0.35	6.1 ± 0.44	H
% Lymphocytes	8.3 ± 2.08	79 ± 2.90	76 ± 2.66	78 ± 2.47	75 ± 1.97	H
% Neutrophilss	9 ± 1.46	12 ± 2.07	14 ± 1.46	12 ± 1.58	14 ± 1.51	H
% Monocytes	6 ± 0.40	7 ± 0.19	7 ± 0.58	7 ± 0.41	7 ± 0.44	H
% Eosinophils	2 ± 0.38	1 ± 0.58	2 ± 0.64	2 ± 0.57	3 ± 0.52	H
% Basophils	0 ± 0.18	0 ± 0.26	1 ± 0.31	1 ± 0.33	1 ± 0.19	H
				× 10^3/mm^3		
Total lymphocytes	4786 ± 298.80	4566 ± 303.72	4839 ± 703.54	4480 ± 160.47	4544 ± 316.31	H
Total neutrophils	566 ± 144.91	745 ± 184.06	924 ± 132.62	723 ± 128.28	881 ± 127.12	H
Total mocytes	356 ± 41.06	390 ± 37.32	448 ± 66.78	389 ± 40.15	413 ± 25.27	H
Total Eosinophils	92 ± 30.09	90 ± 42.68	126 ± 36.64	135 ± 41.88	171 ± 30.67	H
Total Basophils	30 ± 8.23	30 ± 18.51	46 ± 16.36	60 ± 19.46	51 ± 10.60	H

Values represent the mean ± S.E., $n=8$.
H = homogeneous data; NH = non-homogeneous data; NS = not significant.
[a] Vehicle control group (sterile water).
[b] Treatment groups (WDDD from concentrations of 10–100%).

percent values of total B cells (Ig^+), NK cells ($NK1.1^+CD3^-$) and macrophages ($Mac3^+$; Table 3). When the data were expressed as absolute values, exposure to EDDD produced a slight increase in the number of macrophages at the 0.25 mg/kg dose level (Table 4).

3.3. Effect of DDD extract on neutrophil activity (MPO)

Myeloperoxidase is one of the antimicrobial enzymes produced by neutrophils. Changes in the MPO activity indicate the functional alteration of neutrophils [12,13]. In this study, MPO activity was presented as the midpoint tier. Exposure to WDDD for 28 days did not produce significant differences between WDDD treatment groups and the vehicle control group (Fig. 1), although a decreasing trend was apparent.

3.4. Effect of DDD extract on natural killer (NK) cell activity

To determine the effect of DDD extracts on innate immunity, natural killer cell activity was evaluated in B6C3F1 mice following a 28-day administration of WDDD and EDDD (Fig. 2). The assay was performed at six different effector/target ratios using YAC-1 cells as the target. Over-

Table 3
Percent values of splenocyte differentials in female B6C3F1 mice exposed to WDDD and EDDD

Exposure	Parameters[a]		
	Ig+	NK1.1+CD3$^-$	Mac3+
WDDD[b]			
Vehicle[b]	56.0 ± 1.8	3.9 ± 0.2	3.3 ± 0.7
WDDD 10%	55.7 ± 1.5	4.1 ± 0.3	5.0 ± 0.5
WDDD 25%	51.2 ± 2.0	3.6 ± 0.1	2.4 ± 0.4
WDDD 50%	54.9 ± 1.6	3.2 ± 0.3	1.9 ± 0.6
WDDD 100%	54.4 ± 2.6	3.4 ± 0.4	3.0 ± 0.3
H/NH	H	NH	H
Trend analysis[c]	NS	$p \leq 0.05$	NS
EDDD[d]			
Vehicle[d]	43.3 ± 2.6	3.9 ± 0.1	1.2 ± 0.2
EDDD 0.25 mg/kg	42.6 ± 1.7	3.9 ± 0.1	1.7 ± 0.2
EDDD 2.5 mg/kg	41.8 ± 2.6	3.9 ± 0.1	1.2 ± 0.2
EDDD 25 mg/kg	41.7 ± 2.7	3.7 ± 0.3	1.2 ± 0.2
EDDD 250 mg/kg	42.6 ± 1.1	3.4 ± 0.2	1.3 ± 0.2
H/NH	H	NH	NH
Trend analysis[c]	NS	$p \leq 0.05$	NS

Values represent the mean ± S.E., $n=8$.
H = homogeneous data; NH = non-homogeneous data; NS = not significant.
[a] Ig+ = Total B lymphocyre; NK1.1+CD3 – = Natural killer cell; Mac-3+ = Macrophage.
[b] Vehicle control group is stirile water, Treatment group is WDDD (10–100%).
[c] The Jonckheere's test was used to test for dose-related trends among the vehicle and exposed groups.
[d] Vehicle control group is 0.9% sodium chloride. Treatment group is EDDD (0.25–250 mg/kg).

Table 4
Absolute values of splenocyte differentials in female B6C3F1 mice exposed to WDDD and EDDD

Exposure	Parameters[a]			
	Spleen cell ($\times 10^3$)	Ig+	NK1.1+ CD3−	Mac3+
WDDD[b]				
Vehicle[b]	17.0 ± 0.8	81.9 ± 11.2	7.3 ± 0.5	6.3 ± 0.7
WDDD 10%	16.0 ± 1.0	89.7 ± 6.8	7.6 ± 0.8	7.0 ± 1.4
WDDD 25%	16.9 ± 1.0	85.1 ± 5.7	7.2 ± 0.7	6.8 ± 0.9
WDDD 50%	16.0 ± 0.7	87.2 ± 4.1	6.0 ± 0.4	4.7 ± 0.6
WDDD 100%	18.5 ± 0.7	100.7 ± 6.8	7.5 ± 0.4	6.3 ± 0.7
H/NH	H	NH	NH	H
Trend analysis[c]	NS	NS	NS	NS
EDDD[d]				
Vehicle[d]	15.3 ± 0.7	66.7 ± 5.7	6.0 ± 0.4	1.8 ± 0.3
EDDD 0.25 mg/kg	18.2 ± 0.8	77.5 ± 5.1	7.1 ± 0.4	3.2 ± 0.3*
EDDD 2.5 mg/kg	16.7 ± 0.4	69.7 ± 4.8	6.5 ± 0.2	2.0 ± 0.4
EDDD 25 mg/kg	14.3 ± 1.1	60.0 ± 6.9	5.3 ± 0.5	1.8 ± 0.4
EDDD 250 mg/kg	16.7 ± 1.0	71.0 ± 4.7	5.6 ± 0.3	2.1 ± 0.3
H/NH	H	H	NH	H
Trend analysis[c]	NS	NS	$p \leq 0.05$	NS

Values represent the mean ± S.E., $n = 8$.
H = homogeneous data; NH = non-homogeneous data; NS = not significant.
[a] Ig+ = Total B lymphocyte; NK1.1+CD3− = Natural killer cell; Mac-3+ = Macrophage.
[b] Vehicle control group is sterile water, Treatment group is WDDD (10–100%).
[c] The Jonckheere's test was used to test for dose-related trends among the vehicle and exposed groups.
[d] Vehicle control group is 0.9% sodium chloride. Treatment group is EDDD (0.25–250 mg/kg).

all, WDDD or EDDD treatment did not significantly affect NK cell activity.

3.5. Effect of DDD extract on antibody-forming cell (AFC) response to the T-dependent antigen, sheep erythrocytes

To evaluate the effect of DDD extracts on the humoral component of the immune response, the AFC response to sheep erythrocytes was conducted. The results are presented as AFC/10^6 spleen cells (specific activity) and AFC ($\times 10^3$)/spleen (total spleen activity). As shown in Fig. 3, daily gavage with WDDD or injection of EDDD (i.p.) for 28 days did not significantly affect AFC response to sheep erythrocytes, no matter whether the data were expressed as AFC/10^6 spleen cells (Fig. 3A and B) or AFC ($\times 10^3$)/spleen (data not shown).

3.6. Effect of DDD extract on the activity of the mononuclear phagocyte system (MPS)

The assay for the mononuclear phagocyte system was used to evaluate the ability of the fixed macrophages to phagocytize radiolabeled particles. These macrophages line the liver endothelium, the spleen, the lungs and the thymus. The kidneys were used to monitor spontaneous release of the chromium from the labeled RBCs. The results are expressed as vascular clearance (half-life), and the organ distribution of radiolabeled sheep erythrocytes (Table 5). There was no significant difference between the WDDD treatment groups and the vehicle control group in the vascular half-life of the radiolabeled sheep erythrocytes or the distribution of radiolabeled sheep erythrocytes among the liver, spleen, lungs, thymus and kidneys. As expected, MVE, the positive control, significantly inhibited the phagocytic function in the liver, spleen and thymus compare to the vehicle control (Table 5).

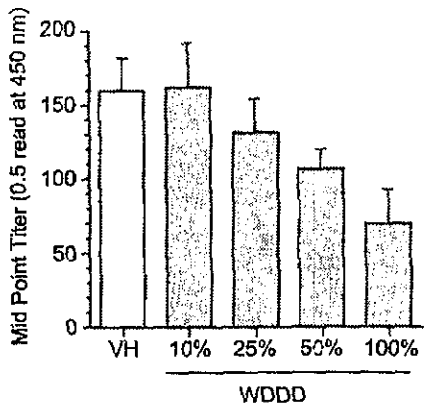

Fig. 1. Neutrophil activity measured by MPO assay in B6C3F1 mice exposed to WDDD for 28 days. The results are expressed as the mean ± S.E. ($n = 8$). Statistics were performed as described.

4. Discussion

For many generations, DDD has been used in Thailand to treat various diseases [15]. However, prior to this work, there have been no published studies on the pharmacological and immunological effects of this plant species from Thailand. In our previous paper, DDD extracts have been shown to enhance the activities of T cells [1]. In the current paper, we reported the effects of WDDD and EDDD on the hematological parameters and other components of the immune system, the innate immunity and humoral immunity.

Numerous medical plants have been identified and developed as chemotherapeutic agents and as modulators of the immune system, capable of increasing macrophage activation and enhancing the functions of T and B lymphocytes and natural killer cells. Several plant-derived immunostimulatory drugs have been

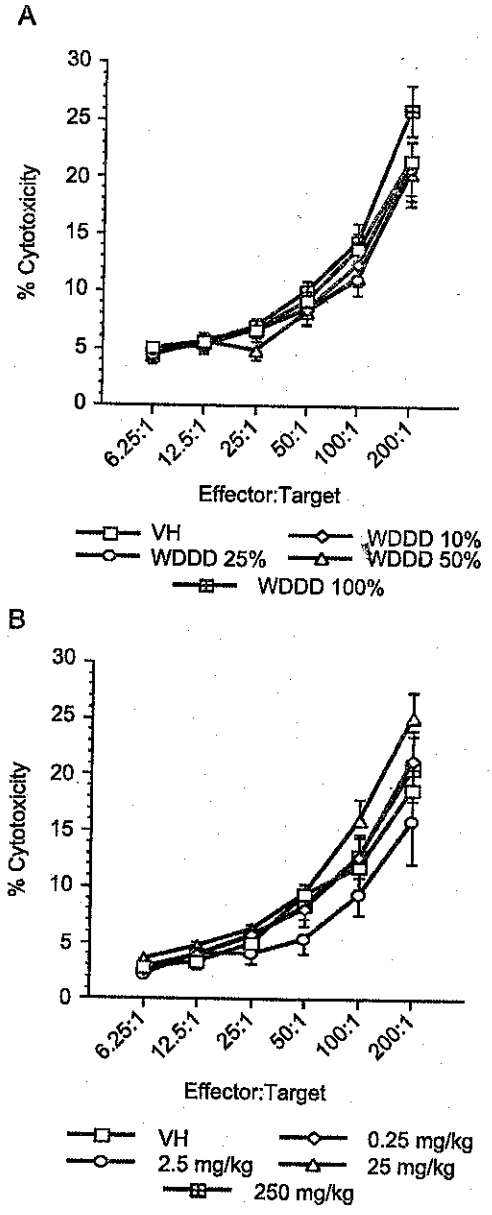

Fig. 2. Effect of DDD extracts on NK cell activity. (A) WDDD; (B) EDDD. Mice were exposed to WDDD or EDDD, and the activity of NK cells was determined as described. Values represent the mean ± S.E. derived from eight animals. Statistics were performed as described.

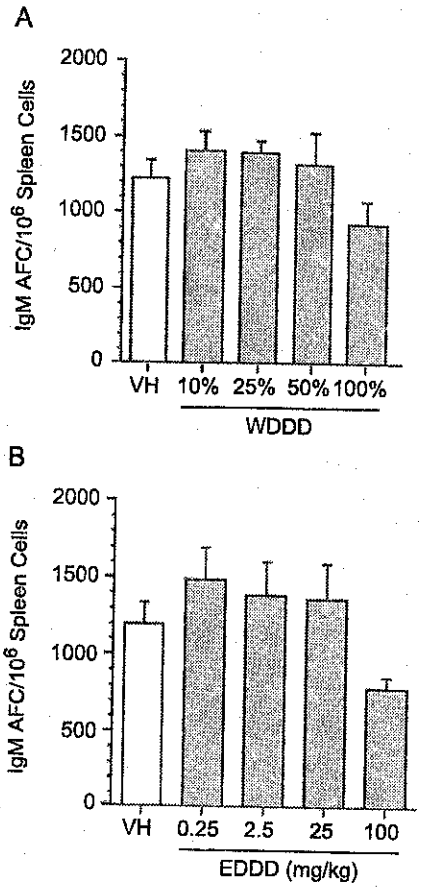

Fig. 3. Effect of DDD extracts on IgM antibody-forming cell (AFC) response. (A) WDDD; (B) EDDD. Mice were exposed to WDDD or EDDD, and the number of IgM antibody-forming cells to sRBC in splenocytes was determined as described. Values represent the mean ± S.E. derived from eight animals. Statistics were performed as described.

Table 5
Functional activity of the mononuclear phagocytic system in female B6C3F1 mice exposed to WDDD for 28 days

Parameter	Vehicle[a]	WDDD[b]				MVE[c]	H/NH	Trend analysis[d]
		10%	25%	50%	100%	50 mg/kg		
Body weight (g)	23.1 ± 0.5	22.8 ± 0.2	22.9 ± 0.6	23.0 ± 0.4	23.0 ± 0.3	22.2 ± 0.5	H	NS
Half-life	12.3 ± 0.7	10.3 ± 1.5	14.1 ± 3.4	9.1 ± 1.0	9.8 ± 0.9	42.8 ± 27.0	NH	$p \leq 0.05$
Liver								
weight (mg)	884 ± 35	876 ± 24	898 ± 40	807 ± 25	852 ± 20	949 ± 34	H	NS
% Uptake	30.1 ± 2.2	27.5 ± 2.3	32.1 ± 4.6	29.4 ± 1.3	30.6 ± 2.1	5.6 ± 3.7**	NH	NS
cpm/mg	164 ± 9	150 ± 12	174.1 ± 23	176 ± 7	173 ± 11	29 ± 20**	NH	NS
Spleen								
weight (mg)	74 ± 3	73 ± 2	71 ± 3	68 ± 2	77 ± 2	88 ± 5*	H	NS
% Uptake	12.9 ± 0.9	12.6 ± 1.3	11.3 ± 6.0	12.2 ± 0.4	11.7 ± 0.6	7.4 ± 2.1*	NH	$p \leq 0.05$
cpm/mg	840. ± 48	818 ± 76	776 ± 161	866 ± 34	736 ± 38	404 ± 116**	NH	NS
Lung								
weight (mg)	146 ± 9	151 ± 10	157 ± 11	154 ± 7	146 ± 9	148 ± 4	H	NS
% Uptake	0.7 ± 0.2	0.7 ± 0.1	2.3 ± 1.8	0.7 ± 0.0	0.6 ± 0.1	1.7 ± 0.5	NH	NS
cpm/mg	22 ± 4	22 ± 3	78 ± 60	23 ± 1	20 ± 3	52 ± 15	NH	NS
Thymus								
weight (mg)	45 ± 4.131	48 ± 5.836	38 ± 4.615	48 ± 3.615	50 ± 7.618	35 ± 2.295*	H	NS
% Uptake	0.02 ± 0.002	0.04 ± 0.012	0.04 ± 0.022	0.02 ± 0.005	0.24 ± 0.180	0.07 ± 0.018	NH	NS
cpm/mg	2 ± 0.193	4 ± 0.688	7 ± 4.831	2 ± 0.409	16 ± 9.936	8 ± 2.182	NH	NS
Kidney								
weight (mg)	289 ± 12	259 ± 14	285 ± 10	276 ± 8	273 ± 5	274 ± 10	H	NS
% Uptake	1.6 ± 0.3	1.5 ± 0.2	1.8 ± 0.1	1.4 ± 0.1	1.3 ± 0.3	0.5 ± 0.1**	NH	NS
cpm/mg	26 ± 4	28 ± 3	30 ± 3	24 ± 2	23 ± 4	9 ± 1**	NH	NS

Values represent the mean ± S.E., $n = 8$.
Mean values significantly different from vehicle control group are indicated as: $*p \leq 0.05$, $**p \leq 0.01$.
H = homogeneous data; NH = non-homogeneous data; NS = not significant.
[a] Vehicle control group (sterile water).
[b] Treatment groups (WDDD from concentrations of 10–100%).
[c] The modulatory control is Makeic vinylether (MVE). It was administered i.v. on day 28.
[d] The Jonckheere's test was used to test for dose-related trends among the vehicle and exposed groups.

used widely in Europe and Asia as traditional medicine for self-medication as well as serving as starting materials for various prescription drugs. Examples include the water and alcoholic extracts of *Echinacea purpurea* [16], *Echinacea angustifolia* and *Echinacea pallida* (herb and root), *Panax ginseng* [17,18] *Eleutherococcus senticosus* [19] and *Rehmania glutinosa*, etc. [20,21]. For *A. indica* Roxbert, there have been no published studies on its hematological and immunological effects. Our current study, together with our previous report [1], represents a series of papers focused on the immunomodulatory effects of DDD. These findings indicate that DDD extracts can increase the function of T cells but not that of B cells, NK cells, macrophage and neutrophils in female B6C3F1 mice.

There are several factors that might contribute to the negative findings on the humoral and innate immunities. In addition to the whole plant, various parts of the plant have been screened for biological activities and phytomedicine use; these include the seeds, flowers, roots and leaves [22]. In the studies performed on *A. indica* Linn., the same plant species from Japan, the butanolic seed extracts were used and have been shown to have anti-tumor activities in mice [4–7]. It is possible that the seeds have more active ingredients than the whole plant extracts.

The NK assays used in our study were designed to examine the basal activity of NK cells. In pathological situations such as tumor development, the immune system is activated and soluble factors such as cytokines are secreted to enhance the innate immunity. Lymphokine-activated killer cells have been shown to be successful in cancer immunotherapy [23]. There is evidence that some chemicals affect the activity of activated NK cells but not that of resting NK cells

[24]. Future studies will consider using IL-2 augmented NK cell assay [24] or poly I:C-augmented NK cell assay [25] to evaluate the effects of DDD extracts.

The B6C3F1 mouse is a hybrid of male C3H/HeN and female C57BL/6 mice. Following immune activation, C57BL/6 mice preferentially produce Th1 cytokines [26,27]. It is possible that the DDD extracts do have effects on humoral immunity but were not able to be detected in B6C3F1 mice. It will be desirable to determine the effects of DDD extracts in a Th2 strain of mouse such as the BALB/c strain [26,27].

There was a slight increase in the spleen cell number of the animals treated with the low dose of EDDD (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 of macrophages observed in the low dose group (0.25 mg/kg). However, the biological relevance of this increase, if there is one, at the lowest dose level but not at higher dose levels is currently unknown. As demonstrated in the MPS assay, exposure to WDDD had no effect on the phagocytic activity of tissue macrophages including those of the spleen. Additionally, we do not believe that the slight decreases in hematocrit and reticulocyte values observed in the intermediate dose groups of WDDD-exposed mice were biologically meaningful because they were not seen in mice exposed to EDDD.

As for the mean corpuscular volume (MCV), while a significant increase was observed in both the WDDD and EDDD exposed mice, we do not believe it is biologically significant. The low variability associated with automatically hematological evaluation often produces results that are statistically significant but not biologically relevant. This appears to be the case here. Furthermore, alterations in other hematological parameters that would be associated with hematoiogical dysfunction such as hematocrit and erythrocyte counts were not observed.

In conclusion, our study provided the first report on the immunomodulatory effects of DDD. Although no significant changes in the activities of B cells, NK cells, macrophages and neutrophils were identified, this report complemented our first report on the immunostimulatory effect of DDD extracts on T cells [1]. Thus, the enhancing effect of DDD extracts on T cells most likely contributes to the successful and long-time use of this traditional herbal medicine in Thailand. Our findings demonstrate that *A. indica* Roxb. can be added to the ever growing list of herbal medicines that have the potential to enhance immune responses.

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