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Biological Safety and B Cells Activation Effects of *Stephania delavayi* Diels.

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Abstract

Stephania delavayi **Diels. has been used as an immune activator or an anti-inflammatory drug in China. We examined the immune modulation effect and 7-days repeated-dose toxicity to validate its biological safety and efficiency. Mice were repeatedly administrated with 50 mg/kg *S. delavayi* Diels. daily by I.P for 7 days. *S. delavayi* Diels. induced B cell activation but had no effect on other immune cells such as T cell, natural killer (NK) cell, and macrophage (Mφ). *S. delavayi* Diels.-treated group exhibited no statistical significance from the control group in physical conditions; body weight, complete blood count (CBC), serum biochemical indexes etc. There was no difference between the control group and *S. delavayi* Diels.-treated group in gross findings such as histopathological alteration. In conclusion, *S. delavayi* Diels. is safe above the dose of immune modulation.**

Keywords: *Stephania delavayi* Diels., Biological safety, Mice, Immunemodulation

Stephania genus is a herb which belong to in Menispermaceae family Dicotylendons classes and includes several species having various pharmacological effects. *Stephania cepharantha* Hayata (*S. cepharantha* Hayata) suppressed arachidonic acid-induced inflammation[1] and *Stephania delavayi* Diels. (*S. delavayi* Diels.) is and has been used for relieving pain and curing acute gastroenteritis in China[2]. *S. cepharantha* Hayata was fractionated into several molecules, but *S. delavayi* Diels. was not. Nevertheless, *S. delavayi* Diels. is a component of Immunsan® which is an immune stimulator and an element of PC-SPES which is a drug for prostate cancer treatement[3,4]. Although it has been used for a long time, biological safety and the mode of action are not clear. Thus, we confirmed immunity-related action mechanism and 7-days repeated-dose toxicity.

Immune response consists of humoral immune response and cell-mediated immune response; humoral immune response is governed by B cells and cell-mediated is done by T cells[5]. B cells play a role of making antibodies against antigens and then they take charges of adaptive immunity. In traditional medicine, there are many prescriptions for modulating the immune system such as Gamipaemo-tang[6] and Cocheongryong-tang[7]. Recently researches for elucidating the action mechanism and confirming biological safety are increased.

We confirmed the biological safety of *S. delavayi* Diels. through the appearance judge, the change of body weight, CRC, serum biological indexes, histopathological signs etc.

S. delavayi Diels. Reduces Lipopolysaccaride (LPS)-stimulated B Cell Proliferation and Induces CD19 Activation

In *S. delavayi* Diels.-treated mice, the immune response was suppressed by treating LPS as a B-cell mitogen in a time-dependent manner (Figure 1A). *S. delavayi* Diels. impaired LPS-induced B cell stimulation. LPS-induced B cell proliferation was enhanced at 48 hr than at 24 hr ($P<0.05$ vs. corresponding vehicle). Neither a T cell stimulator concanavalin A (ConA) nor a poke weed mitogen (PWM) helping T and B cell stimulation, affects the proliferation of lymphocytes in the presence of *S. delavayi* Diels. (Figure 1B).

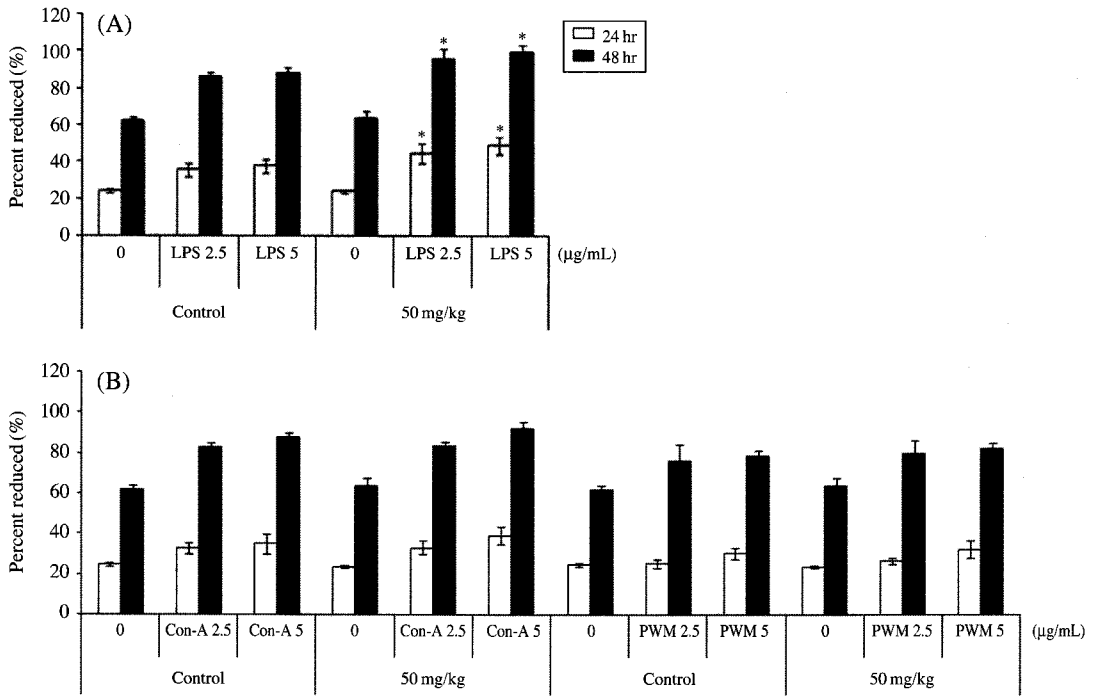

Figure 1. The pharmacological action of *S. delavayi* Diels. in response to B- and T-cell proliferation. (A) The pharmacological action of *S. delavayi* Diels. in response to LPS-induced B-cell proliferation in mice. *S. delavayi* Diels. reduces LPS-stimulated B cell proliferation in time-dependent manner. 0=No LPS. Each value is the mean±S.E.M of 3 observations. $P<0.05$ vs. corresponding vhicle (B) The pharmacological action of *S. delavayi* Diels. in response to Con-A-induced T-cell proliferation and PWM-induced cell proliferation of B cell and T cell in mice. A T cell stimulator, ConA, did not change of immune response induced by *S. delavayi* Diels. Similarly co-stimulator of B cell and T cell, PWM, did not influence immune response induced by *S. delavayi* Diels. 0=No Con-A., No PWM. Each value is the mean±S.E.M of 3 observations.

To confirm *S. delavayi* Diels.'s immunemodulative effects, we examined the expression levels of the cell surface molecules including CD3 (T cell-related molecule), CD4 (Class II MHC-restricted T cell-related molecule), CD8 (Class I MHC-restriced T cell-related molecule), CD19 (B cell-related molecule), CD11B (monocytes/macrophage-related molecule), and NK 1.1 (NK cell-related molecule). *S. delavayi* Diels. increased CD19 activation from 38.62% to 43.57% ($P<0.05$) but not the other molecules (Figure 2 and Table 1). This is similar to the result from the LPS-induced B cell proliferation.

S. delavayi Diels. Is Safe in 7-days Repeated-Dose Treatment in Mice

After 7-days repeated-dose i.p. injection once a day, the biological safety of *S. delavayi* Diels. was certified. Among the results of CBC, leukocytes-related components were slightly upregulated within the normal range; white blood cells, neutrophils, lymphocytes, monocytes, eosinophils, and basophils (Table 1).

For the confirmation of safety of liver and kidney, we compared the serum biochemical indexes including glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), blood urea nitrogen (BUN), ammonia (NH_3), directbilirubin (TBIL), and albumin (ALB) (Table 2) and observed the histopathological changes (Figure 3). The serum biochemical indexes of the control and *S. delavayi* Diels. treated groups had no difference, and the histopathological change in liver, kidney, spleen etc. was not observed. These results mean that *S. delavayi* Diels. is a safe candidate drug to use as an immune modulator.

Discussion

S. delavayi Diels. has been used for pain relieving and curinig gastroenteritis for a long time in China, but its biological safety and mode of action is not clear. Thereafter the purpose of this study was to examine the immunomodulatory effect and safety after 7-days repeated-dose exposure of *S. delavayi* Diels. using mice. In the results, 7-days repeated-dose *S. delavayi*

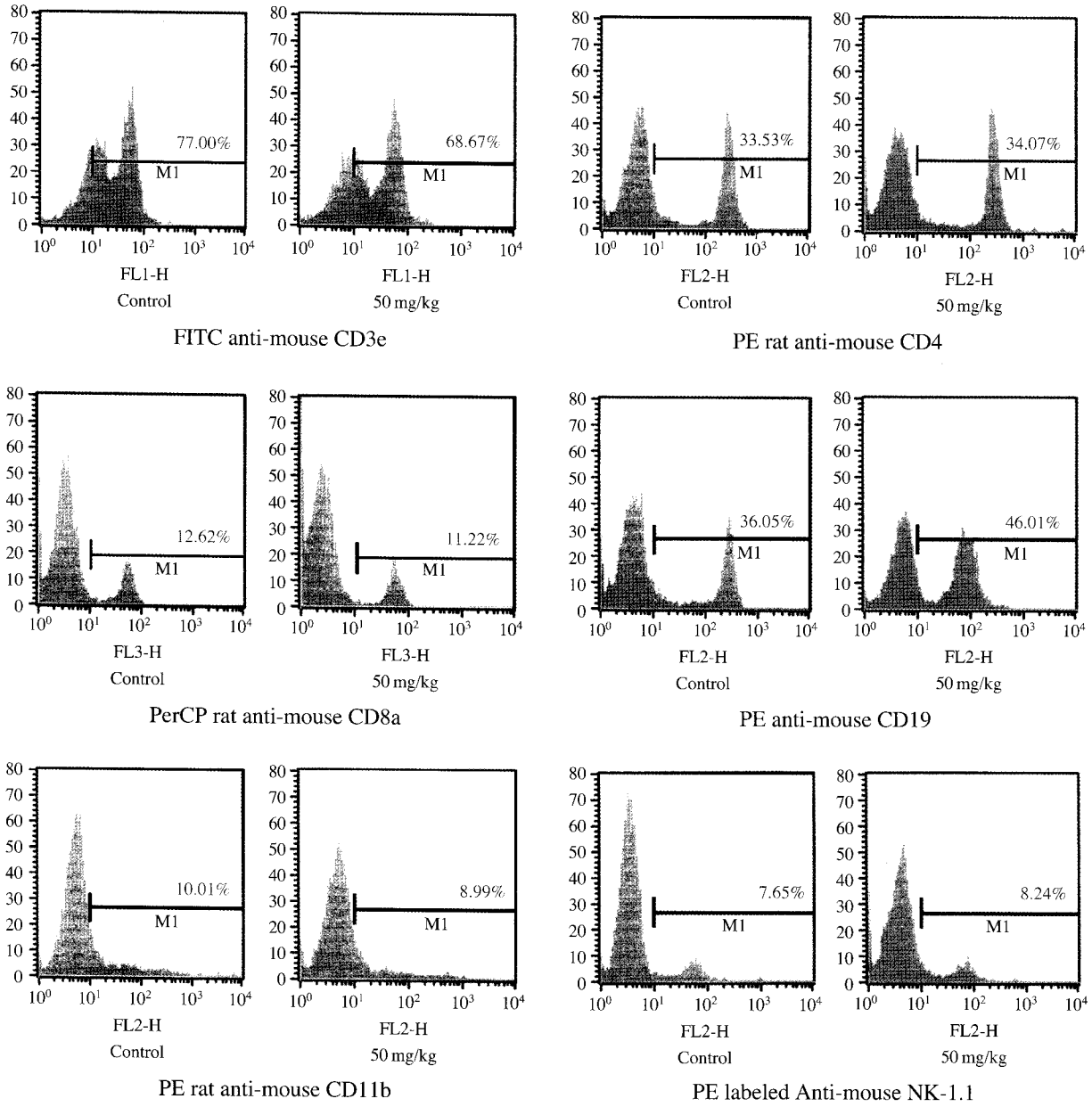

Figure 2. Effects of *S. delavayi* Diels. on the expression of surface molecules by FACS analysis. Exception CD19 (B cell-related molecule), the other molecules were not changed. CD19 was increased from 38.62±2.99% to 43.57±3.16%. Each value is the mean±S.E.M of 3 observations. $P<0.05$ vs. corresponding vehicle.

Diels. treatment impairs B cell-related immune reaction. Although the response tended to be reduced compared to the control animals, splenic lymphocytes did not show any significant response to ConA (a T cell stimulator) and PWM (a costimulator of T cells and B cells) after prolonged *S. delavayi* Diels. treatment. This suggests that the treatment of *S. delavayi* Diels. deos not impair T cell function. The lymphocyte proliferative response in the presence of 7-days repeated-dose exposure of *S. delavayi* Diels. was suppressed by treating 2.5 μg/mL and 5 μg/mL LPS, a B cell stimulator. This suggests that the main target residues for *S. delavayi* Diels.-induced suppression in LPS-responsive populations, presumably, are B lymphocytes in the spleen. Therefore, repeated-dose exposure of *S. delavayi* Diels. enhances immune stimulation through the B cell-mediated mode of action. Seven-days repeated-dose *S. delavayi* Diels. exposure prove to be safe to

Table 1. Hematological values of mice I.P injected daily with *S. delavayi* Diels. for 7 days.

		Control	50 mg/kg
Leukocytes	WBC (k/μL)	8.44±4.33$^{NS)}$	11.19±5.13
	NE (k/μL)	2.22±1.34$^{NS)}$	3.13±1.41
	LY (k/μL)	5.29±2.53$^{NS)}$	6.73±3.23
	MO (k/μL)	0.47±0.31$^{NS)}$	0.62±0.25
	EO (k/μL)	0.34±0.25$^{NS)}$	0.55±0.36
	BA (k/μL)	0.11±0.05$^{NS)}$	0.16±0.11
Erythrocytes	RBC (M/μL)	7.62±0.54$^{NS)}$	7.75±0.67
	Hb (M/dL)	12.52±0.55$^{NS)}$	12.18±0.59
	HCT (%)	43.8±3.62$^{NS)}$	44.64±4.67
	MCV (fL)	57.56±3.68$^{NS)}$	57.61±3.51
	MCH (pg)	4.07±0.86$^{NS)}$	4.55±1.14
	MCHC (g/dL)	7.11±1.51$^{NS)}$	7.89±1.78
	RDW (%)	15.78±0.65$^{NS)}$	16.05±0.34
Thrombocyte	PLT (k/μL)	532.4±209.9$^{NS)}$	378.4±180.1
	MPV (fL)	4.66±0.18$^{NS)}$	4.8±0.17

Values are average±SD of 10 mice.
NS : Values within the row are not significant at $P<0.05$ by Tukey's multiple range test.
WBC : White blood cell, NE : Neutrophil, LY : Lymphocyte, MO : Monocyte, EO : Eosinophil, BA : Basophil, RBC : Red blood cell, Hb : Hemoglobin, HCT : Hematocrit, MCV : Mean Corpuscular Volume, MCH : Mean Corpuscular Hemoglobin, MCHC : Mean Corpuscular Hemoglobin Concentration, RDW : Red cell Distribution Width, PLT : Platelet, MPV : Mean Platelet Volume

Table 2. Hematological enzyme of mice treated with 50 mg /kg *S. delavayi* Diels. or with PBS for 7 days.

	Control	50 mg/kg
GOT (U/I)	79.29±25.93$^{NS)}$	69.13±32.47
GPT (U/I)	34.14±15.49$^{NS)}$	27.5±13.1
BUN (mg/dL)	18.29±4.21$^{NS)}$	15.65±2.6
NH_3(μg/dL)	97.29±30.94$^{NS)}$	82.25±13.32
TBIL (mg/dL)	0.27±0.05$^{NS)}$	0.25±0.08
ALB (g/dL)	2.26±0.13$^{NS)}$	2.41±0.17

1) Values are mean±S.D., N=10.
2) NS : Values within the row are not significant at $P<0.05$ by Tukey's multiple range test.
GOT : glutamic oxaloacetic transaminase, GPT : glutamic pyruvic transaminase, BUN : blood urea nitrogen, TBIL : directbilirubin, ALB : albumin

use this agent as an immune modulator for bio-organisms.

Methods

Animals

Ten female and 10 male ICR mice were purchased from OrientBio (Sungnam, Korea) and acclimated for 7 days. All animals were housed in a temperature and relative humidity-controlled environment (22±3°C, 12-hr light/dark cycle) during acclimation and experiment and fed *ad libitum* with Purina diet (Purina Korea, Korea) and water.

Five female and 5 male mice per each group were treated with phosphate buffer saline (PBS) and with 50 mg/kg *S. delavayi* Diels. i.p. injection once a day for 7 days respectively. The body weights of them were checked every 3 days. After 1 days of final injection, mice were weighed body weight, judged the appearance, anesthetised with isofluran, collected whole blood through intracardic route, and sacrificed using isofluran. Liver, kidney and spleen were collected for histopathological examination. Three spleen samples among 10 mice per each group were used for splenocytes collection.

Biological Toxicity Analysis

Using the collected whole blood, CBC was measured with Hemavet950 (Drew Scientific Group, USA). After sacrificing mice, all organs were checked with the unaided eyes for analysis pathological changes and fixed hearts, lungs, livers, kidneys, spleens, urinary bladders, testises and ovaries with 10% neutral formalin. Fixed samples were embedded with paraffin using Tissue-Tek VIP (Sakura, Japan) and sliced 3 μm thickness. Histopathological study was performed on all slices stained with H&E and by light microscopy.

Lymphocyte Proliferation and Cell Surface Molecules Analysis

Lymphocytes proliferation analysis was performed by the method of Ahmed *et al.*[8]. Collected splenocytes were seeded on 96-well plate with 5×10^5 cells/well/100 μL using with 100 U/μL penicillin-sterptomycin and 10% fetal bovine serum RPMI 1640 (growth media), added with LPS (0-5 μg/mL), ConA (0-5 μg /mL), or PWM (0-5 μg/mL), and fitted to 200 μL/well at final volume with growth media. Cells were incubated in a CO_2 incubator (5% at 37°C) under humidified conditions for 24 hr or 48 hr. Alamar Blue® (Alamar, Sacramento, CA) assay was performed according to the manufacturer's instructions and the results were determined by Multi-Detection Microplate-Reader (Bio-TEK®, USA). The mean specific absorbance OD of triplicates of respective groups was calculated.

For cell surface molecules analysis, collected splenocytes were fitted 1×10^8 cells/mL using growth media into tubes for FACS analysis and 10 μL antibodies; FITC anti-mouse CD3e (for T cell-related molecule), PE rat anti-mouse CD4 (for Class II MHC-restricted T cell-related molecule), PerCP rat anti-mouse CD8a (for Class I MHC-restricted T cell-related molecule), PE anti-mouse CD19 (for B cell-related molecule), PE rat

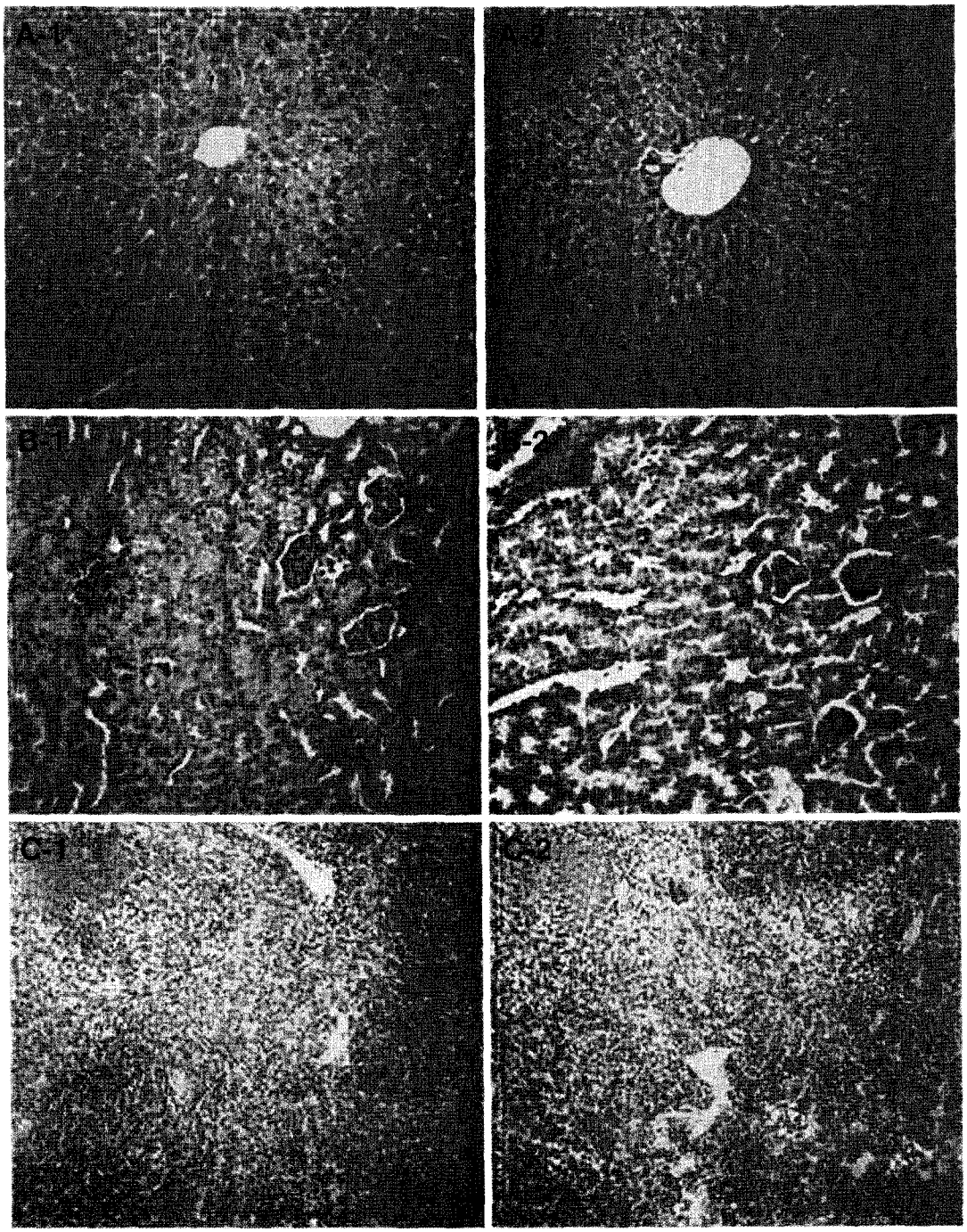

Figure 3. Representative images of liver, kidney and spleen in control and in treated with 50 mg/kg *S. delavayi* Diels. There was no changes in all organs between in control group and in treated group with 50 mg/kg *S. delavayi* Diels. 100 (A-1) Liver in control (A-2) Liver in with 50 mg/kg *S. delavayi* Diels. (B-1) Kidney in control (B-2) Kidney in with 50 mg/kg *S. delavayi* Diels. (C-1) Spleen in control (C-2) Spleen in with 50 mg/kg *S. delavayi* Diels.

anti-mouse CD11B (for monocytes/macrophage-related molecule), and PE labeled anti-mouse NK 1.1 (for NK cell-related molecule). And well mixed and reacted for 2 hr in 4°C. They were twice washed with 1 mL PBS buffer and measured using a BD Model FACscan (Becton Dickinson Inc., USA).

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