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Extract of *Hedyotis diffusa* Willd Influences Murine Leukemia WEHI-3 Cells *In Vivo* as well as Promoting T- and B-Cell Proliferation in Leukemic Mice

CHIN-CHUNG LIN[1,2], CHAO-LIN KUO[3], MAU-HVA LEE[12], SHU-CHUN HSU[4], AN-CHENG HUANG[5], NOU-YING TANG[6], JING-PIN LIN[6], JAI-SING YANG[7], CHI-CHENG LU[8], JO-HUA CHIANG[8], FU-SHIN CHUEH[9] and JING-GUNG CHUNG[10,11]

Departments of [1]Chinese Medicine, and [12]Internal Medicine, Fong-Yuan Hospital, Department of Health, Executive Yuan, Taichung 420, Taiwan, R.O.C.;
[2]School of Medicine and Nursing, HungKuang University, Taichung 433, Taiwan, R.O.C.;
Schools of [3]Chinese Pharmaceutical Sciences and Chinese Medicine Resources,
[6]Chinese Medicine, Departments of [4]Nutrition, [7]Pharmacology,
[10]Biological Science and Technology, China Medical University, Taichung 404, Taiwan, R.O.C.;
[5]Department of Nursing, St. Mary's Medicine Nursing and Management College, Yilan 266, Taiwan, R.O.C.;
[8]Department of Life Sciences, National Chung Hsing University, Taichung 402, Taiwan, R.O.C.;
Departments of [9]Health and Nutrition Biotechnology and
[11]Biotechnology, Asia University, Taichung 413, Taiwan, R.O.C.

Abstract. *Medicinal plants and herbs are widely used in the treatment of various types of cancer in Taiwan, China and many other countries. Hedyotis diffusa Willd (HDW) has been known as a traditional Chinese medicine for a long time, and possesses various bioactivities and anticancer activity. There is no available information on the effects of HDW extracts in leukemic mice and on immune responses in vivo. In this study, we established murine WEHI-3 leukemia in BALB/c mice and hypothesized that an aqueous HDW extract might have antileukemia effects on leukemic animals in vivo. The major characteristic of leukemic mice was an enlarged spleen after intraperitoneal injection with WEHI-3 cells. HDW extract reduced the weights of spleen and liver, but had no significant effect on body weight in WEHI-3 leukemic mice. HDW extract increased the percentage of CD11b cell surface marker (monocytes), but it reduced the percentage of CD3 (T-cell) and CD19 (B-cell) markers. However, HDW extract did not affect the level of Mac-3 and there was no influence on phagocytosis by macrophages from peripheral blood mononuclear cells and the peritoneal cavity in leukemic mice. The isolated splenocytes from HDW extract-treated leukemic mice demonstrated an increase of T- and B-cell proliferation in vivo. Based on these results, HDW extract would appear to have antileukemia activity in WEHI-3 cell-induced leukemia in vivo.*

Correspondence to: Jing-Gung Chung, Ph.D., Department of Biological Science and Technology, China Medical University, No. 91, Hsueh-Shih Road, Taichung 40402, Taiwan, R.O.C. Tel: +886 422053366 ext. 2161, Fax: +886 422053764. e-mail: jgchung@mail.cmu.edu.tw and Chao-Lin Kuo, Ph.D., School of Chinese Pharmaceutical Sciences and Chinese Medicine Resources, China Medical University. No 91, Hsueh-Shih Road, Taichung 40402, Taiwan. Tel: +886 422053366 ext. 5202, Fax: +886 422070439, e-mail: clkuo@mail.cmu.edu.tw

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Human leukemia is one of the major cause of deaths and is also a highly aggressive disease (1). Approximately 3,250 people under the age of 20 years are diagnosed with leukemia each year, of whom about 2,400 cases are acute lymphoblastic leukemia in the United States. (2). In Taiwan, about 4.0 per 100,000 population die of leukemia based on the reports of the Department of Health, R.O.C. (Taiwan). Therapies for leukemia are based on intensive chemotherapy and/or hematopoietic stem cell transplantation, radiotherapy and chemotherapy (3, 4). However, the cure rate and side-effects of these treatments are still unsatisfactory. Herbal-based dietary supplements contain numerous phytochemicals which can be used as cancer suppressors, such as paclitaxel (5).

It is well documented that many herbs have been shown to contain phytochemicals with potent pharmacological activities (6). *Hedyotis diffusa* Willd (HDW) (Rubiaceae), a traditional herbal medicine, has been used for treating many types of cancer and tumor in Taiwan and China for a long time (7-11) and is widely applied in the treatment of inflammation, such as appendicitis, urethritis, and bronchitis, due to its antibacterial activity (12-14). It was reported that the triterpenes and polysaccharide from HDW inhibited the proliferation of tumor cells (9, 15). Moreover, and extract of this herb induced apoptosis in human breast cancer cells (8). Several reports have shown that *Oldenlandiae diffusae* Herba inhibited mutagenesis and tumor growth *in vivo* (16), DNA binding and metabolism of aflatoxin B1 (17, 18) and benzo(a)pyrene bioactivated by Aroclor 1254 (polychlorinated biphenyl)-induced rat liver supernatant (19). In addition, *Oldenlandiae diffusae* increased the production of NO and tumor necrosis factor (TNF)-α by interferon (IFN)-γ-primed macrophages (20), and it was used to treat malignant tumors and to stimulate the reticuloendothelial system (21). Two anthraquinones (2-hydroxy-3-methylanthraquinone and 1-methoxy-2-hydroxyanthraquinone), isolated from a water extract of HDW, inhibited activity of Src tyrosine kinase and pp60src, and arrested the growth of breast cancer and hepatoma cells (22). Recently, it was reported that the methylanthraquinone from HDW induced apoptosis in MCF-7 cells *via* Ca^{2+}/calpain/caspase-4 pathway (8). However, there is no available information on the effect of HDW extract on immune response in a leukemic mouse model *in vivo*. Therefore, the purpose of present study was to investigate the effect of HDW extracts on immune response in leukemia in BALB/c mice *in vivo*.

Materials and Methods

Materials and reagents. RPMI-1640 medium, fetal bovine serum (FBS), penicillin-streptomycin and L-glutamine were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA).

Preparations of HDW extract. The extract of HDW was provided by Dr. Chin-Chung Lin (Department of Chinese Medicine, Fong-Yuan Hospital, Taichung, Taiwan, ROC). The HDW plants were dried and ground into a fine powder. For aqueous extraction, 5 kg of the powdered samples were mixed with 2,000 ml of boiling distilled deionized water (DDW). Filtrate was collected twice by filter paper and DDW was evaporated to dryness with a reduced pressure concentrator for approximately 6 h to obtain dry extracts. The extraction yield of the aqueous extracts of HDW was 3.71%. For *in vivo* studies, dry HDW extracts were dissolved in DDW to concentrations of 16 and 32 mg/kg before being filtered through a 0.22 mm filter (23).

WEHI-3 murine leukemia cells. The WEHI-3 murine myelomonocytic leukemia cell line was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). Cells were immediately grown in 75-cm^2 culture plastic flasks containing RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin grown at 37˚C under a humidified 5% CO_2 atmosphere (24-26). The cells were cultivated for two complete cycles in an incubator.

Establishment of leukemic mice and treatment with crude extract of HDW. Male BALB/c mice, approximately 22-28 g in weight and at 4-6 weeks of age, were obtained from the National Laboratory Animal Center (Taipei, Taiwan, ROC). Forty mice were randomly divided into four groups and then each group received different treatments. Among them, thirty mice were intraperitoneally (*i.p.*) injected with WEHI-3 cells (1×10^5/0.1 ml/mouse) for 2 weeks and then all animal were randomly separated into 3 groups as a model of leukemia. Group I mice served as normal mice (10 animals, untreated control). Group II mice were *i.p.* injected with WEHI-3 cells as leukemia group (10 animals). Group III animals were treated with crude extracts of HDW (16 mg/kg) in DDW (10 animals) after *i.p.* injection of WEHI-3 cells. Group IV animals were treated with crude extracts of HDW (32 mg/kg) in DDW (10 animals) after *i.p.* injection of WEHI-3 cells. The crude extract of HDW was administered by oral gavage to each animal from the treatment groups with the above dose daily for up to 2 weeks prior to their being weighed (23, 27). These mice were maintained at 25˚C on a 12-h light/dark cycle in the animal center of the China Medical University followed the Institutional Animal Care and Use Committee (IACUC) as previously described (23, 27). The animals were anesthetized by CO_2 and sacrificed for further studies.

Spleen and liver tissues analysis. All animals from each group after exposure to extracts of HDW were weighed and spleen and liver samples were isolated and weighed individually (23, 28).

Whole-blood samples and immunofluorescence staining for surface markers. At the end of the treatment of each group, blood samples of 1 ml from each animal were collected from the heart before mice were sacrificed. Each blood sample was immediately exposed to 1× Pharm Lyse™ lysing buffer (BD Biosciences, San Jose, CA, USA) for lysing of the red blood cells and then was centrifuged at 4˚C for 15 min at 1500 rpm. The isolated white blood cells were stained by the fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD3, phycoerythrin (PE)-conjugated anti-mouse Mac-3, PE-conjugated anti-mouse CD19 and FITC-conjugated anti-mouse CD11b antibodies (BD Pharmingen Inc, San Diego, CA, USA) before being analyzed to determine the levels of cell markers by flow cytometry (FACS Calibur™ ; Becton Dickinson, Franklin Lakes, NJ, USA) as previously described (23, 29).

Quantification of phagocytic activity of macrophages. The measurement of phagocytosis by peripheral blood mononuclear cells (PBMCs) and macrophages from the peritoneal cavity of mice after treatment with or without HDW was performed by using the PHAGOTEST kit (Glycotope Biotechnology GmbH, Heidelberg, Germany) as previously described (25, 30). Approximately 1×10^5 leukocytes in 100 μl whole blood or macrophages from each group were incubated for 1 h at 37˚C with FITC-labelled *Escherichia coli* (20 μl). The reaction was stopped by the addition of quenching solution (100 μl) according to the manufacturer's instruction. After the completion of phagocytosis by monocytes/macrophages, DNA was stained according to the manufacturer's protocol. Cells were analyzed by flow cytometery as previously described (25, 30). Fluorescence data were collected on 10,000 cells and analyzed using the BD CELLQUEST Pro software.

Assessment of T- and B-cell proliferation. Splenocytes (1×10^5 cells/well) were isolated from the spleens of each mouse from each treatment and 100 μl of RPMI-1640 medium with FBS was added.

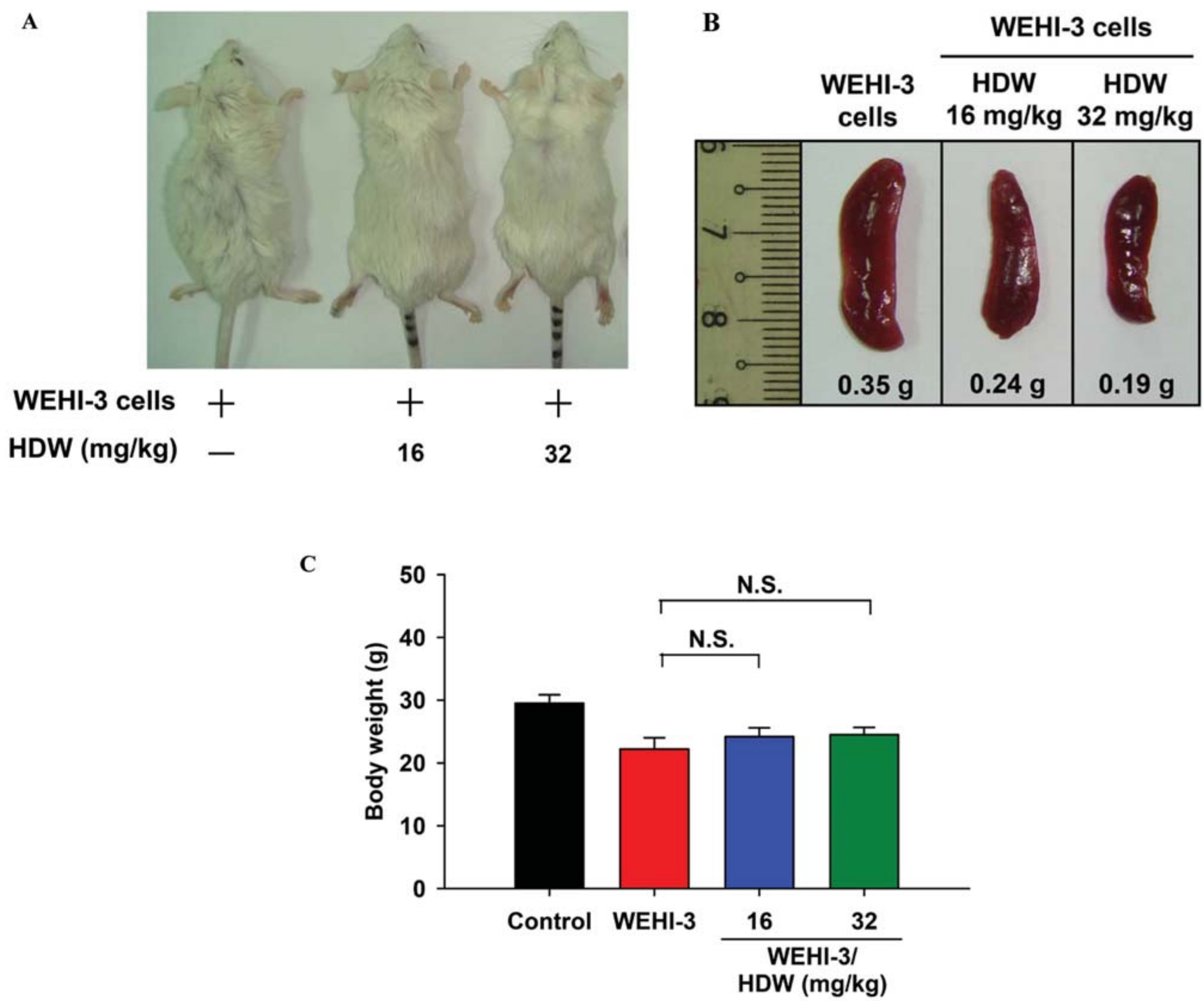

Figure 1. *Representative animals and spleens in leukemic BALB/c mice after exposure to crude extracts of HDW. BALB/c mice, after 2 weeks' intraperitoneal injection with WEHI-3 cells, were then orally treated with HDW for 16 and 32 mg/kg for 2 weeks. Animals were sacrificed then photographed, and representative mice (A) and representative spleens (B) are shown. All individual animals were weighed and the average weight of all animals from each group (C) was recorded. Each point is the mean±S.D. of three experiments (n=10). N.S., Not significant.*

Splenocytes were placed in 96-well plates and stimulated with concanavalin A (Con A, 1 μg/ml; Sigma-Aldrich Corp., St. Louis, MO, USA) for T- cell and lipopolysaccharide (LPS, 1 μg/ml; Sigma-Aldrich Corp.) for B-cells and maintained for 3 and 5 days' incubation, respectively. The cells were collected by centrifugation at 1,500 rpm for 5 min, and T- and B-cell proliferation determined using the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA) as previously described (28, 31).

Quantification of natural killer (NK) cell cytotoxic activity. Splenocytes (1×10^7 cells/well) in 1 ml of RPMI-1640 medium from each group were cultured in each well of 24-well culture plates for 24 h. YAC-1 cells (2.5×10^5) from the Food Industry Research and Development Institute were cultured in 15-ml tubes with serum-free RPMI-1640 medium and PKH 67/Dilent C buffer (Sigma-Aldrich Corp.) was added to the cells, mixed thoroughly for 2 min at 25˚C then 2 ml phosphate buffered saline (PBS) was added for 1 min. About 4 ml of RPMI-1640 medium were added for a 10 min incubation which was followed by centrifugation at 1200 rpm at 25˚C. YAC-1 cells in 100 μl were placed on 96-well plates before the addition of the splenocytes to the wells for 12 h and determination of NK cell cytotoxic activity with a propidium iodide (Sigma-Aldrich Corp.) exclusion method by flow cytometry as previously described (28, 31).

Statistics analysis. Results were expressed as the mean±S.D. and the difference between HDW-untreated and treated leukemia groups was analyzed by one-way ANOVA followed by Dunnett's test. A *p*-value of less than 0.05 was taken as significant.

Results

Effects of HDW extract on BALB/c mice after intraperitoneal injection with WEHI-3 cells. Mice were sacrificed then photographed and representative animals are presented in Figure 1A. Representative spleens are presented in Figure 1B, which indicate that HDW reduced the size of the spleen and increasing the dose of HDW led to greater reduction in the spleen size. All individual animals were weighed and the average of all animals from each group are presented in Figure 1C, which indicates that oral treatment of HDW did not affect the body weight of mice with leukemia.

HDW extract affected spleen and liver weights of leukemic BALB/c mice. After animals were treated with 16 or 32 mg/kg of HDW, they were sacrificed and spleen and liver tissues were isolated and weighed individually, and the results are presented in Figure 2A and B. The high dose of HDW (32 mg/kg) significantly reduced the weights of both spleen and liver when compared with the untreated leukemia group.

HDW extract affected whole-blood cell surface markers in WEHI-3 leukemic BALB/c mice. To investigate whether HDW affected the levels of cell surface marker, leukocytes from HDW-treated or untreated animals were isolated and levels of CD3, CD19, CD11b and Mac-3 were determined and the results for cell markers are presented in Figure 3A, B, C and D, respectively. The results indicated that HDW reduced the levels of CD3 (Figure 3A) (16 mg/kg/day: 16.6%; 32 mg/kg/day: 13.8%) and CD19 (Figure 3B) (16 mg/kg/day: 11.0%; 32 mg/kg/day: 8.2%), but it increased the levels of CD11b (Figure 3C) (16 mg/kg/day: 63.6%; 32 mg/kg/day: 69.3%). However, HDW did not significantly affect the level of Mac-3 surface marker when compared to the untreated WEHI-3 leukemia group (Figure 3D). These results indicated that HDW might reduce the levels of CD3 (T-cells) and CD19 (B-cells), and was able to increase the level of CD11b (monocytes), but it did not affect Mac-3 (macrophages) levels in leukemic mice.

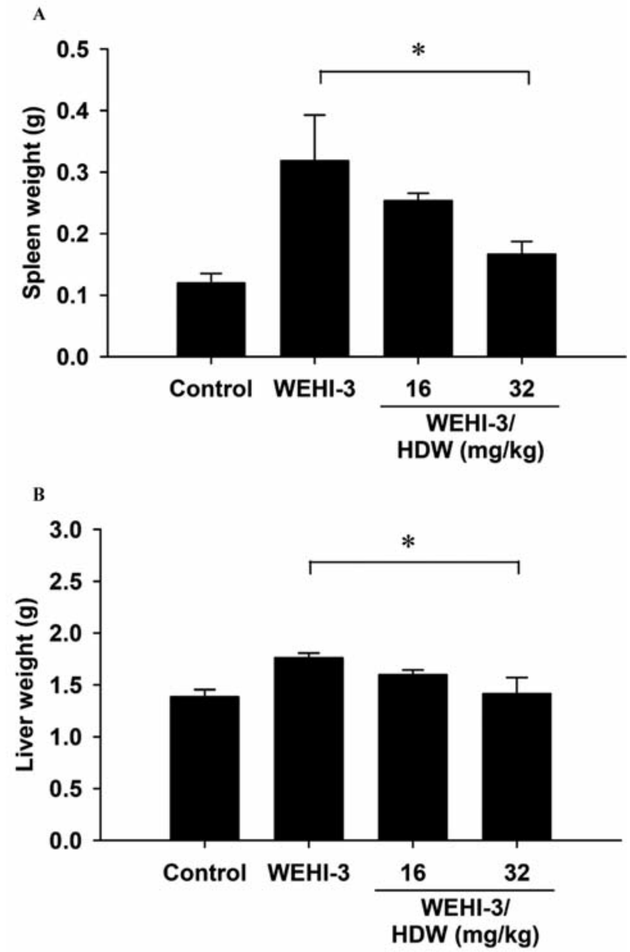

Figure 2. *Treatment with HDW extract treatment affected the weights of spleen and liver from BALB/c leukemic mice. Animals intraperitoneally injected with WEHI-3 cells were orally treated with HDW extracts at 16 and 32 mg/kg for 14 days, and then spleen (A) and liver (B) tissues were isolated and weighed as described in the Materials and Methods. Data represent the mean±S.D. of at least three independent experiments (n=10). A p-value less than 0.05 was considered significant by one-way ANOVA followed by Dunnett's test.*

Effect of HDW extract on phagocytotic activity of PBMCs and macrophages from the peritoneal cavity in leukemic BALB/c mice. Macrophages were isolated from each group of leukemic BALB/c mice after exposure to 16 and 32 mg/kg/day of HDW by oral administration for 14 days. The percentage of phagocytosis of PBMCs and peritoneal macrophages are shown in Figure 4A and B, which indicate that HDW did not affect phagocytosis by macrophages in leukemic BALB/c mice at either dose.

HDW extract affected T- and B-cell proliferation in WEHI-3 leukemic BALB/c mice. After mice were intraperitoneally injected with WEHI-3 cells for 2 weeks, mice were orally treated with HDW (16 and 32 mg/kg/mouse) for 2 weeks. Splenocytes were isolated from leukemic BALB/c mice for T- and B-cell proliferation examinations. As can be seen in Figure 5A and B, the results indicate that HDW promoted T- and B-cell proliferation regardless of Con A or LPS stimulation.

Effect of HDW extract on NK cell cytotoxic activity of splenocytes in WEHI-3 leukemic BALB/c mice. To investigate whether HDW is able to act on NK cell cytotoxic activity, splenocytes from the leukemic mice with or without HDW treatment were isolated and NK cell cytotoxic activity was determined. The results shown in Figure 6 indicate that the

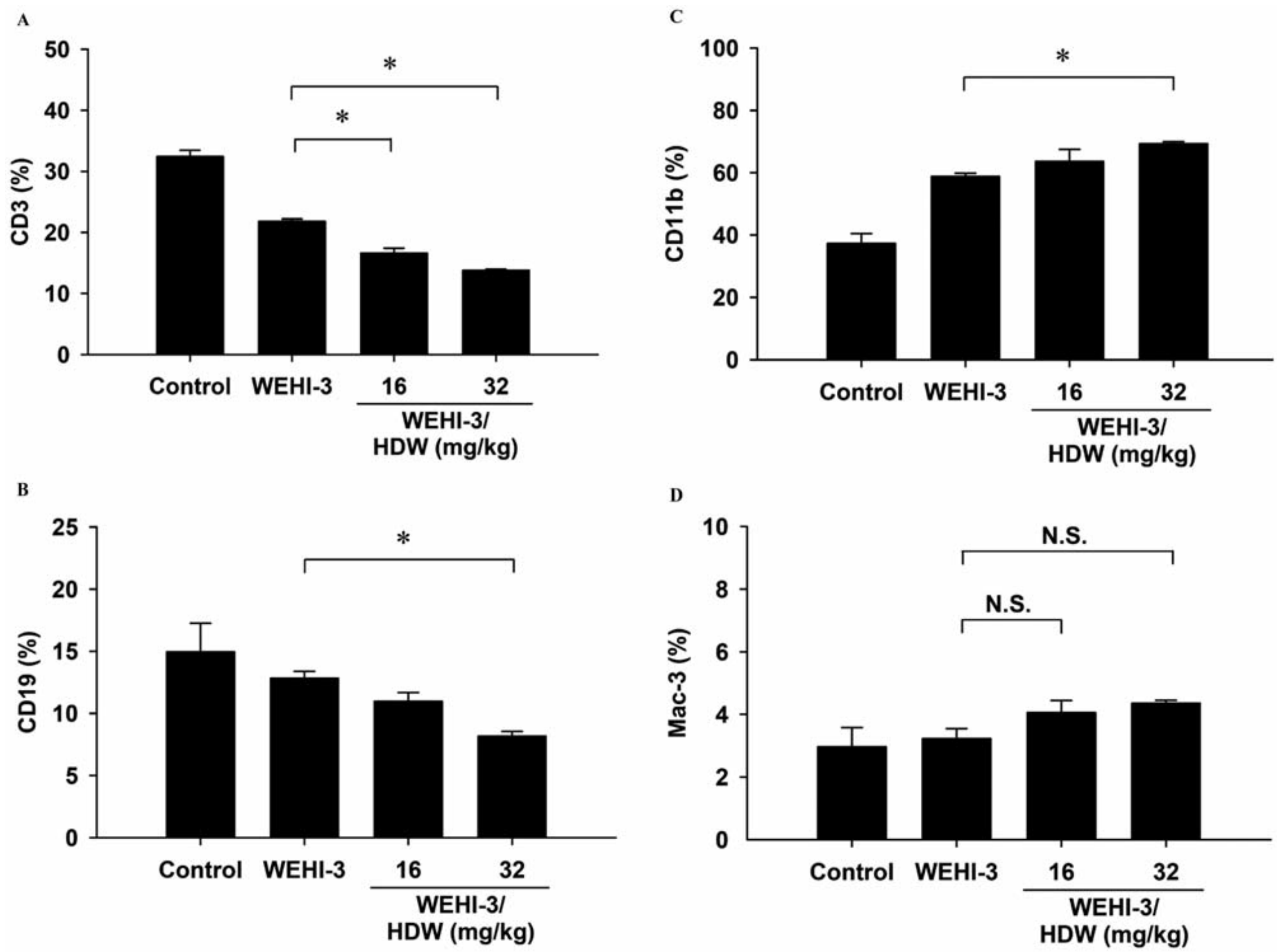

Figure 3. *HDW extract affected the cell markers of white blood cells (WBC) of leukemic BALB/c mice. The blood samples (1 ml) from each animal of each group were collected and immediately exposed to 1× Pharm LyseTM lysing buffer for lysing of the red blood cells. The WBC were then isolated and stained by FITC-conjugated anti-mouse CD3 (A), PE-conjugated anti-mouse CD19 (B), FITC-conjugated anti-mouse CD11b (C) and PE-conjugated anti-mouse Mac-3 (D) antibodies before being analyzed to determine the cell marker levels by flow cytometry as described in the Materials and Methods. Each point is the mean±SD of independent experiments. *p<0.05 (n=10) significant by one-way ANOVA followed by Dunnett's test.*

YAC-1 target cells were not killed by NK cells from the leukemic mice after exposure to HDW extracts at 16 or 32 mg/kg/day at target cell ratios of 25:1 and 50:1, and in fact reduced cytotoxicity at 32 mg/kg.

Discussion

Several reports have shown that crude extracts of HDW have biological activities, including antitumor activity (7-11). Up to now, three major classes of compounds, the triterpenes, polysaccharide and anthraquinones, have been reported as bioactive compounds from this herb (12, 32). In this study, we established leukemic mice through the injection of WEHI-3 cells, and then mice were treated for two weeks with HDW extracts. Results from *in vivo* experiments indicated that HDW extract was able to promote immune responses in leukemic mice *in vivo*. HDW extract also promoted T- and B-cell proliferation (Figure 5).

HDW extract increased the T- and B-cell proliferation index by two- or three-fold, respectively when compared with the untreated leukemic mice (Figure 5A and B). Based on these observations, HDW extracts not only appeared to increase the humoral immune response, but also the cellular immune responses after the leukemic mice were treated with HDW extract by oral administration.

Much evidence has shown that NK cell cytotoxic activity and phagocytosis by macrophages both played a major role in the immune responses after animals were exposed to antigens (33-35). In order to investigate whether or not HDW promoted immune responses through the increase of immune

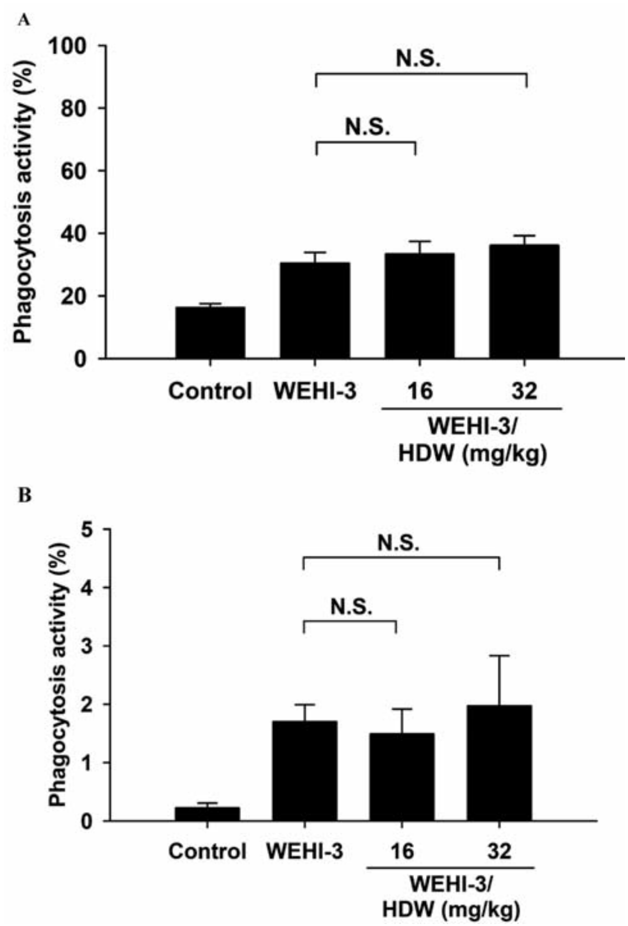

Figure 4. *Effects of HDW extract on the phagocytotic activity by macrophages in leukemic BALB/c mice. Macrophages were isolated from PBMCs (A) and the peritoneal cavity (B) of each group of leukemic mice after daily exposure to 16 and 32 mg/kg of HDW extracts by oral administration for 14 days. The percentage of phagocytosis with green fluorescent particles (FITC-E. coli.) was determined by flow cytometric analysis as described in the Materials and Methods. Each point is the mean±S.D (n=10). N.S., Not significant when compared with untreated leukemic mice.*

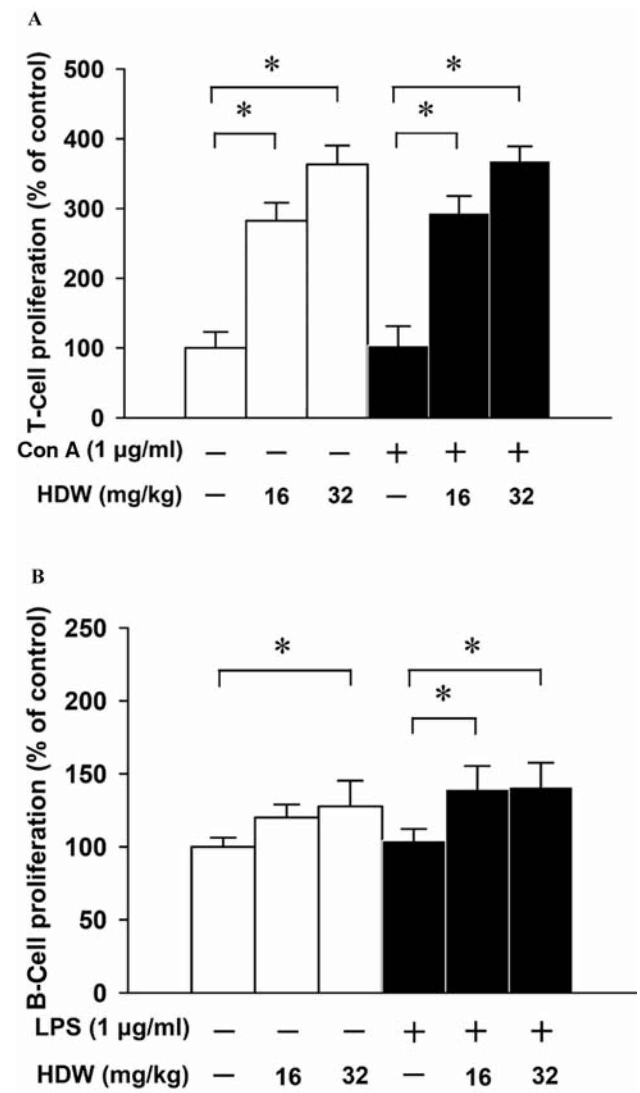

Figure 5. *The effect of HDW extract on T- and B-cell proliferation of Con-A and LPS-stimulated splenocytes from leukemic BALB/c mice. The mice were intraperitoneally injected with WEHI-3 cells for 2 weeks and then orally treated with or without HDW extracts (16 and 32 mg/kg/mouse) for 2 weeks. Splenocytes were isolated from leukemic BALB/c mice for T- (A) and B- (B) cell proliferation examinations as described in the Materials and Methods. Each point is mean the ±S.D (n=10). *p<0.05, significant when compared with the untreated WEHI-3 leukemic mice (one-way ANOVA followed by Dunnett's test).*

cells, we examined the levels of cell surface markers from leukemic mice after dietary treatment with HDW extract. Results showed that the percentages of CD3- and CD19-positive cells significantly increased in HDW-treated leukemic mice, but that the population of CD11b- and Mac-3- positive cells significantly decreased in examined leukemic mice *in vivo*.

It was reported that B-cell differentiation requires the interaction of various cytokines which are secreted from macrophages or T-cells (36) and the CD19 antigen present on cell surface membranes of non-activated B lymphocytes (37). The results indicated that HDW extract inhibited leukemia-related spleen growth and promoted immune responses (Figure 5). In our study, the notable characteristic of the leukemia model is the elevation of peripheral monocytes and granulocytes with immature morphology as well as enlarged and infiltrated spleens compared with normal counterpart (38). In the present study, we found that HDW extract promoted immune responses in BALB/c leukemic mice thus acting as a potent immunological adjuvant.

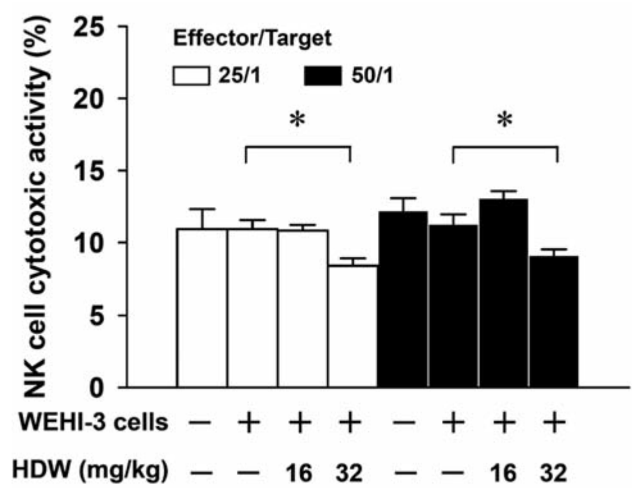

Figure 6. *Effects of HDW on the cytotoxic activity of natural killer (NK) cells in leukemic BALB/c mice. The percentage of YAC-1 target cells killed by NK cells from the mice after treatment with HDW extract by oral administration at 16 and 32 mg/kg/day at target cells ratio of 25:1 and 50:1 was determined Each point is the mean±S.D.*p<0.05 was considered significant when compared with the untreated WEHI-3 leukemic mice (n=10) (one-way ANOVA followed by Dunnett's test).*

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