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# Wogonin, a Natural and Biologically-active Flavonoid, Influences a Murine WEHI-3 Leukemia Model *in Vivo* Through Enhancing Populations of T- and B-Cells

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# Wogonin, a Natural and Biologically-active Flavonoid, Influences a Murine WEHI-3 Leukemia Model *in Vivo* Through Enhancing Populations of T- and B-Cells

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**Abstract.** *Wogonin, a natural and biologically-active flavonoid found in plants, has been reported to exhibit anticancer effects on several cancer cell types. However, there is no available information regarding the responses to wogonin in leukemia mouse models. At concentrations of 10-200 μM, wogonin reduced the percentage of viable WEHI-3 cells in a concentration-dependent manner. In an in vivo study, WEHI-3 cells were intraperitoneally injected into normal BALB/c mice for establishing leukemic BALB/c mice to determine the anti-leukemia activity of wogonin. Wogonin increased the survival rate and the body weight of leukemic mice when compared to vehicle (olive oil)-treated groups. Furthermore, the results also revealed that wogonin increased the percentage of cluster of differentiation-3 CD3 (T-cell marker) and CD19 (B-cell marker) but reduced that of Mac-3 (macrophages) and CD11b (monocytes) cell surface markers in treated mice as compared with the untreated leukemia group. Based on these observations, wogonin might exhibit anti-leukemia effects on murine WEHI-3 cell line-induced leukemia in vivo.*

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*Key Words:* Wogonin, flavonoid, BALB/c mice, murine WEHI-3 leukemia model.

Leukemia and lymphomas account for about half of all childhood cancers (1). Leukemia is the second most malignant disease in children (2) and is the most frequent type of cancer in children less than 14 years of age (3). So far, the treatments of patients with leukemia include radiotherapy, chemotherapy, or a combination of radiotherapy with chemotherapy, but treatments are still unsatisfactory. Reports have shown that increased consumption of a plant-based diet can reduce the risk of cancer development (4-6).

Wogonin (5,7-dihydroxy-8-methoxyflavone), a naturally-occurring flavonoid from the root of the *Scutellaria baicalensis* Georgi, has been used for treating allergic and inflammatory diseases (7, 8). Numerous studies have reported that wogonin induces apoptosis in many human cancer cell types such as osteosarcoma (9), leukemia (10), breast cancer (11) and glioma (12). Furthermore, reports have shown that wogonin induces cell differentiation, apoptosis and cell-cycle arrest (13-15) and also suppresses the growth of human cancer xenografts *in vivo* (11, 16). It was reported that wogonin improves functional outcomes and reduces activation of Toll-like receptor-4 (TLR4)/ Nuclear Factor-Kappa B (NF-κB) signaling in experimental traumatic brain injury (17). Several studies also showed that

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wogonin has no or little toxicity towards normal cells and had no obvious toxicity in animals (11, 13, 18-20). More interestingly, in early clinical trials, *Scutellaria* extracts have been successfully tested in patients with advanced breast cancer (21, 22).

In the present study, we investigated whether wogonin can promote the survival rate of leukemic BALB/c mice *in vivo*.

## Materials and Methods

*Materials and reagents*. Wogonin, dimethyl sulfoxide (DMSO), propidium iodide (PI), RNase A and Triton X-100 were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Fetal bovine serum (FBS), RPMI-1640 medium, L-glutamine and penicillin-streptomycin were obtained from Gibco Life Technologies (Carlsbad, CA, USA).

*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). The WEHI-3 cells were immediately placed in plastic culture flasks (75 $cm^2$) in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin under a humidified atmosphere with 5% $CO_2$ at 37˚C. Cells were then cultivated for two complete cycles in an incubator.

*Viability determination*. $2\times10^5$ WEHI-3 cells/well were placed into each well of 24-well plates for 24 and 48 h. Then wogonin (dissolved in DMSO) was individually added to the wells at final concentrations of 0, 10, 25, 50, 100 and 200 μM, and 0.1% of DMSO as a control group in culture medium for incubation for 24 and 48 h. At the end of incubation, cells from each well were harvested for the determination of viability by using a flow cytometric method as described previously (23).

*Male BALB/c mice*. Forty male BALB/c mice at the age of eight weeks, around 22-25 g in weight, were obtained from the Laboratory Animal Center, College of Medicine, National Taiwan University (Taipei, Taiwan, ROC) and kept in the Animal Center of China Medical University. The whole animal study was carried out following the institutional guidelines (Affidavit of Approval of Animal Use Protocol) which was approved by the Institutional Animal Care and Use Committee (IACUC) of China Medical University (Taichung, Taiwan, ROC).

*Establishment of leukemic mice and wogonin treatment*. A total of fourty BALB/c mice were used for the experiments. Thirty BALB/c mice were individually intraperitoneally (*i.p.*) injected with $1\times10^5$ WEHI-3 cells. After 2 weeks, they were randomly separated into three groups as a model of leukemia. Another ten mice were used as control without WEHI-3 cell injection. Group I mice were normal mice (10 animals) and were treated with normal diet only. Group II WEHI-3-injected mice were treated with olive oil (vehicle) as control (10 animals). Group III WEHI-3-injected mice were treated with wogonin (30 mg/kg) in olive oil (10 animals). Group IV WEHI-3-injected mice were treated with wogonin (10 mg/kg) in olive oil (10 animals). Wogonin was administered by oral gavage to the treatment groups at the above doses daily for two weeks before mice were weighed and sacrificed by euthanasia with $CO_2$ (23).

*Immunofluorescence staining for surface markers from leukemic mice*. After all animals were treated for two weeks, in order to measure the surface markers, blood samples of 1 ml from all experimental mice were collected before mice were sacrificed. Each collected red blood cell sample from each animal was lysed with 1×Pharm Lyse™ lysing buffer (BD Biosciences Pharmingen Inc., San Diego, CA, USA). All samples were centrifuged for 15 min at 1500×*g* at 4˚C to isolate white blood cells then all isolated cells were stained by the R-Phycoerythrin (PE)-labeled anti-mouse Mac-3 antibodies, Fluorescein isothiocyanate (FITC)-labeled anti-mouse CD11b, FITC-labeled anti-mouse CD3 and PE-labeled anti-mouse CD19 (BD Biosciences Pharmingen Inc.) for 30 min before being analyzed for cell markers by flow cytometry as previously described (23).

## Results

*Wogonin reduces the percentage of viable WEHI-3 cells*. In order to examine whether or not wogonin induced cytotoxic effects on mouse leukemia cells, the WEHI-3 cells were treated with different concentrations of wogonin for 24 and 48 h before all cells were measured for the percentage of viable cells by flow cytometric assay. The results are shown in Figure 1, indicating that wogonin reduced the percentage of viable WEHI-3 cells in a dose-dependent manner.

*Wogonin affects the growth of leukemic mice*. In this experiment, thirty mice were used as a leukemia model and 10 mice were not *i.p.* injected with WEHI-3 cells, as a normal group (Group I). Thirty male BALB/c mice were *i.p.* injected with WEHI-3 cells before being randomly separated into three groups. Group II mice were treated with olive oil alone. Group III mice were treated with wogonin (30 mg/kg) in olive oil. Group IV mice were treated with wogonin (10 mg/kg) in olive oil. Animals were treated for two weeks and then were examined and measured for survival rate and body weight in all groups. The results shown in Figure 2A indicate that wogonin at both doses significantly increased the survival rate. Figure 2B shows that both doses of wogonin increased the body weight when compared to the olive oil-treated leukemic group.

*Wogonin affected surface markers on whole blood cells from WEHI-3-leukemic BALB/c mice*. For investigating whether wogonin affects the level of cell surface markers from leukemic mice, leukocytes from wogonin-treated and untreated (control) groups were isolated and levels of Mac-3, CD19, CD3, and CD11b were measured by a flow cytometric assay and results are shown in Figure 3. The data from each treatment indicate that wogonin significantly reduced the levels of Mac-3 (Figure 3A) and CD11b (Figure 3B) but increased the levels of CD3 (Figure 3C) and CD19 (Figure 3D) when compared to the control leukemic group.

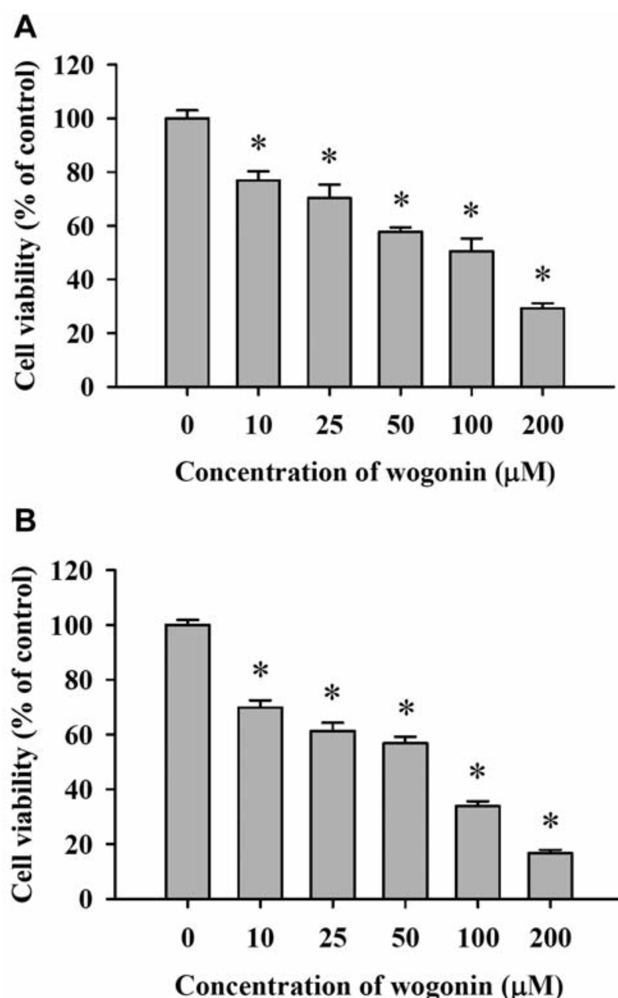


Figure 1. *Wogonin reduced the percentage of viable WEHI-3 cells. Cells in 24-well plates were treated with 0, 10, 25, 50, 100 and 200 μM of wogonin for 24 (A) and 48 (B) h. Cells were harvested for measuring the percentage of viability by flow cytometric assay as described in the Materials and Methods. Significantly different from the control at *p<0.05.*

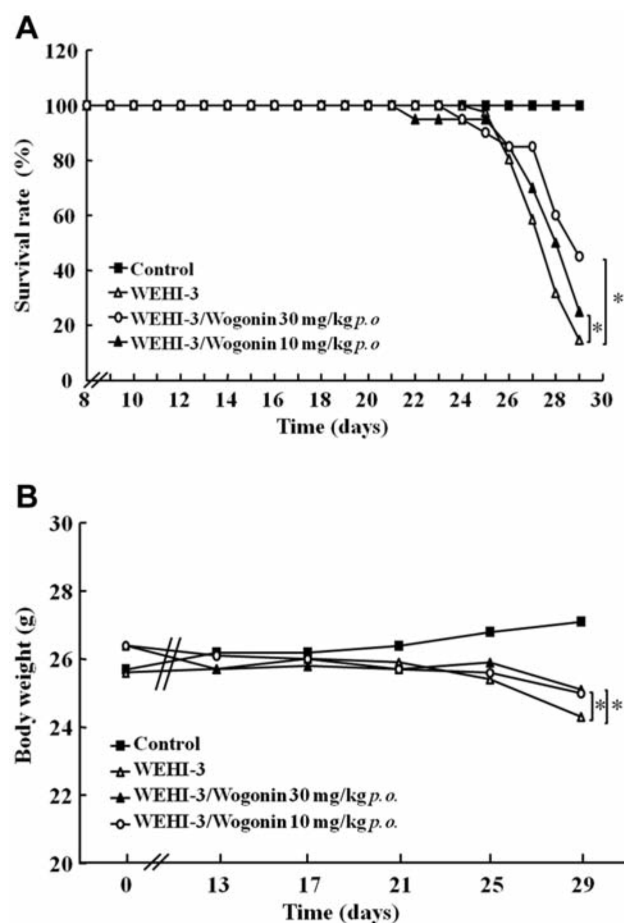


Figure 2. *Wogonin affects the survival rate and growth of leukemic BALB/c mice. All mice except the normal group (Group I) were intraperitoneally injected with WEHI-3 cells then divided into three groups: group II was orally-treated with olive oil-alone; group III was treated with wogonin at 30 mg/kg and group IV was treated with wogonin at 10 mg/kg for two weeks. Survival rates were calculated (A) and body weights were measured (B). Significantly different at *p<0.05.*

## Discussion

Many studies have shown that wogonin induces cytotoxic effects on various cancer cells through cell-cycle arrest and apoptosis including breast cancer cells (24), malignant T-cells (13) and osteosarcoma (9). However, there are no cytotoxic effects on normal cells even at concentrations up to 100 μM (25, 26). Thus, wogonin may be a potential anticancer drug. Our previous studies also showed that reactive oxygen species play an important role in wogonin-induced apoptosis of bone osteosarcoma cells by AKT-modulated, BAX and BCL-2-related intrinsic apoptotic pathways (9). However, there is no available information to show the effect of wogonin on the growth of leukemic mice *in vivo*. Herein, we investigated the effect of wogonin on the growth and immune-associated cell markers in WEHI-3 cell-generated leukemic mice *in vivo*.

Our results indicate that wogonin reduced the percentage of total viable WEHI-3 cells and this effect was concentration-dependent (Figure 1). This is in agreement with a report from Lee *et al*., which indicated that wogonin induced cytotoxicity in human promyelo-leukemic cells (27). Results from Figure 2A indicate that wogonin at both doses significantly promoted the survival rate of leukemic mice; however, Figure 2B demonstrates that wogonin did not significantly affect the weights of animals, nor the weights of the liver and spleen (data not shown) in leukemic mice when compared to leukemic mice not treated with wogonin.

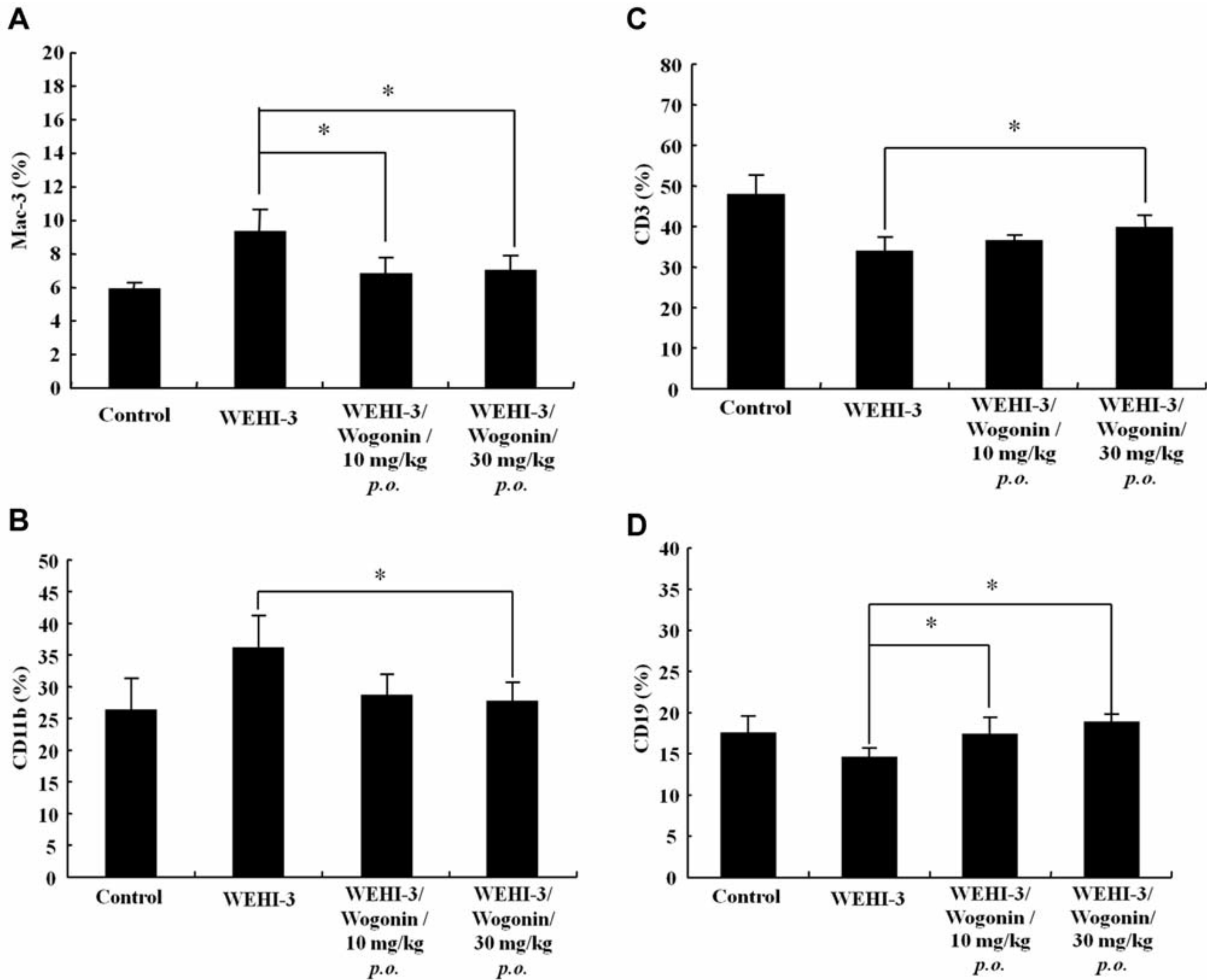


Figure 3. *Wogonin affects the level of cell surface markers in white blood cells from leukemic BALB/c mice. All mice except the normal group were intraperitoneally injected with WEHI-3 cells, followed by oral treatment with or without wogonin for two weeks. Blood was collected from each animal and was analyzed for cell markers by flow cytometry as described in the Materials and Methods. A: Mac-3; B: CD11b; C: CD3 and D: CD19 The data are expressed as the mean±S.D. of four experiments (n=10). Significantly different at *p<0.05.*

It was reported that B-cell development and humoral immune responses are controlled by signaling thresholds that are differentially regulated by the CD22 and CD19 cell surface receptors *in vivo*. The differential regulation of tyrosine phosphorylation by CD19 and CD22 may provide a molecular mechanism for adjusting B Cell Receptor (BCR) signaling thresholds (28). Furthermore, it is well-known that CD19 is an activated B-cell surface marker (29), and B-cell differentiation also requires the interaction of various cytokines that are secreted from macrophages or T-cells (4). Herein, our results indicate that wogonin promoted the population of $CD19^+$ cells (Figure 3D). This finding indicates that wogonin may promote the B-cell population. Figure 3C shows that wogonin at the low concentration applied promoted the population of $CD3^+$ cells but at higher concentration did not significantly induce increased T-cell population. Based on the results from Figure 3, we conclude that wogonin reduced the Mac-$3^+$ population at both concentrations but only low concentrations showed a significantly reduced $CD11b^+$ population.

Based on these observations, we may suggest that wogonin promotes an immune response through increasing B- and T-cells populations in WEHI-3-generated leukemic BALB/c mice *in vivo*. This is the first finding showing that oral treatment with wogonin increased the growth survival rate of leukemic mice. Wogonin may act as a potent immunological adjuvant *in vivo* in leukemia.

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