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# Quercetin Inhibited Murine Leukemia WEHI-3 Cells *In Vivo* and Promoted Immune Response

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**Enhanced flavonoid consumption is closely related with a reduced cancer incidence as shown in epidemiological studies. Quercetin (3,5,7,3′,4′-pentahydroxylflavone) is one of the active components of flavonoids which exist in natural plants, particularly in onions and fruits. It was reported that quercetin induced apoptosis in human cancer cell lines, including human leukemia HL-60 cells, but there is no available information as to its effects on leukemia cells *in vivo*. The purpose of the present studies was to focus on the *in vivo* effects of quercetin on leukemia WEHI-3 cells. The effects of quercetin on WEHI-3 cells injected into BALB/c mice were examined. Quercetin decreased the percentage of Mac-3 and CD11b markers, suggesting that the differentiation of the precursors of macrophages and T cells was inhibited. There was no effect on CD3 levels but increased CD19 levels. Quercetin decreased the weight of the spleen and liver compared with the olive oil treated animals. Quercetin stimulated macrophage phagocytosis of cells isolated from peritoneum. Quercetin also promoted natural killer cell activity. Based on pathological examination, an effect of quercetin was observed in the spleen of mice previously injected with WEHI-3 cells. Apparently, quercetin affects WEHI-3 cells *in vivo*. Copyright © 2009 John Wiley & Sons, Ltd.**

*Keywords:* quercetin; WEHI-3 cells; BALB/c mice; *in vivo*; phagocytosis.

## INTRODUCTION

In Taiwan about 2.1 per 100 000 people die each year of leukemia and it is the 13th most common malignancy based on reports of the 'People Health Bureau of Taiwan'. The treatment of leukemia is not satisfactory and it was reported that an increased consumption of a plant-based diet led to a reduction of colon cancer (Mutoh *et al.*, 2000; Wenzel *et al.*, 2000). Herbal based dietary supplements contain many phytochemicals which might mediate physiological functions related to cancer suppression *in vivo*. Immune responses are involved in many kinds of leukocytes such as T cells, B cells, natural killer cells and macrophages etc. It is well known that CD3 is the marker for T cells, CD11b is the marker for monocytes or NK cells, CD19 is the marker for B cells and Mac-3 is the marker for macrophages. Macrophages perform phagocytosis then digest antigen for helper T cells, then these T cells help B cells form plasma cells to release immunoglobulin (antibody) to bind the antigen. Those antigens will be phagocytosed by macrophages.

Quercetin, one of the active components of flavonoids, possesses various biological activities such as antioxidant, antiinflammation, antiatherosclerosis and antitumor properties (Mutoh *et al.*, 2000; Naderi *et al.*, 2003; Wenzel *et al.*, 2000). One of the antitumor effects of quercetin is to induce cytotoxic effects such as inhibition of cell proliferation and induction of apoptosis in human cancer cell lines. The molecular mechanisms of quercetin induced cytotoxic effects include cell cycle arrest and induction of caspase-mediated apoptosis in breast (Singhal *et al.*, 1995), colon (van Erk *et al.*, 2005), leukemia (Lee *et al.*, 2006; Mertens-Talcott *et al.*, 2003; Shen *et al.*, 2003), lung (Nguyen *et al.*, 2004), liver (Granado-Serrano *et al.*, 2006) and oral (Ong *et al.*, 2004) cancer cells. It was reported that quercetin inhibited the invasive potential of melanoma and prostate cancers (Kandaswami *et al.*, 2005; Zhang *et al.*, 2000; Zhang *et al.*, 2004) and tube formation in human umbilical vascular endothelial cells (Tan *et al.*, 2003). It was also reported that quercetin inhibited lipopolysaccharide- or 12-O-tetradecanoylphorbol-13-acetate-induced inflammation and matrix metalloproteinase-9 (MMP-9) expression in mouse macrophages

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and glioma cells (Chen *et al.*, 2004; Lin *et al.*, 2003). Although various bioactivity studies of quercetin have been carried out the antileukemia capacity of quercetin has not yet been analysed. Therefore, the effects of quercetin on murine leukemia WEHI-3 cells *in vivo* were investigated.

## MATERIALS AND METHODS

**Materials and reagents.** Quercetin and olive oil were obtained from Sigma Co. (St Louis, MO, USA). RPMI 1640, fetal bovine serum, penicillin–streptomycin and glutamine were obtained from Gibco BRL (Grand Island, NY, USA).

**BALB/c mice.** Fifty male BALB/c mice 8 weeks of age (approximately 22–28 g) were purchased from the Laboratory Animal Center, National Taiwan University, College of Medicine (Taipei, Taiwan).

**Murine leukemia WEHI-3 cells.** The murine myelomonocytic leukemia cell line (WEHI-3) was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The cells were plated onto 75 $cm^2$ cell culture flasks and grown in RPMI 1640 medium containing 1% penicillin-streptomycin (100 U/mL penicillin and 100 μg/mL streptomycin), 1% glutamine and 10% fetal bovine serum, at 37 °C under a humidified 5% $CO_2$ atmosphere.

***In vivo* studies.** Fifty BALB/c mice were divided into five groups (10 animals per group) and maintained at 25 °C on a 12 h light/dark cycle. Quercetin (2 and 4 mg/kg) was administered by gauge in isotonic saline (1 mL of saline administered) and control animals received 1% olive oil only. Group I was the control and Group II was treated with olive oil only. Group III was injected i.p. with WEHI-3 cells only. Group IV was injected i.p.with WEHI-3 cells and then treated with quercetin (2 mg/kg) in olive oil. Group V was injected i.p. with WEHI-3 cells and then treated with quercetin (4 mg/kg) in olive oil. Mice were treated daily for 3 weeks before being weighed and killed (Su *et al.*, 2008).

**Blood collection and immunofluorescence staining.** After 3 weeks, 1 mL of blood was collected from all animals. The blood sample was treated immediately with ammonium chloride to lyse the red blood cells and then centrifuged at 1500 rpm (1000 × **g**) at 4 °C for 15 min. Isolated white blood cells were examined for cell surface markers of T cells (CD3), B cells (CD19), monocytes and macrophages (CD11b and Mac-3) (WEHI-3 is a myelomonocytic leukemia cell line) using staining with anti-CD3, CD11b, CD19 and Mac-3 antibodies (BD Pharmingen, San Diego, CA, USA) then were stained with the second fluorescent antibody to determine the cell marker levels by flow cytometry (FACS Calibur™, Becton Dickinson, NJ, USA) as described (Loken *et al.*, 2000).

**Liver and spleen tissues.** All mice were weighed before blood was drawn. The liver and spleen were isolated and also weighed for each animal (Su *et al.*, 2008).

**Phagocytic activity of macrophages.** Macrophage phagocytosis was measured by using the Phagotest kit (Orpegen Pharma, Heidelberg, Germany). The cells were isolated from PBMC and the peritoneum of the control and experimental animals. Isolated cells from each group were individually incubated for 4 h at 37 °C with opsonized fluorescein isothiocyanate (FITC)-labeled *E. coli* (20 μL) as per the manufacturer's instruction. An ice-cold quenching solution (100 μL) was added to stop the reaction. After the completion of phagocytosis, the monocytes/macrophages were fixed, and cell cycle analysis for viable cells (dead cells were stained) was performed according to the manufacturer's instructions. The cell preparations were analysed by a flow cytometer (FACSCalibur, Becton Dickinson). The fluorescence data were collected on 10 000 cells and analysed using Cellquest software (Stachowska *et al.*, 2007).

**Histopathology.** Spleen samples from each group were fixed in 4% formaldehyde and embedded in paraffin. Sections of 5 mm were stained with hematoxylin and eosin according to standard procedures (Yang *et al.*, 2006).

**NK activity.** Approximately $1 \times 10^5$ leukocytes from mice spleen in 1 mL of medium were cultured in each well of a 96-well. About $2.5 \times 10^7$ cells of YAC-1 (NK target cells) in 15 mL tubes with serum-free RPMI-1640 medium and the PKH-67/Dil.C buffer was added to the cells then mixed thoroughly for 2 min at 25 °C, then 2 mL PBS was added for 1 min. Then 4 mL RPMI-1640 was added for 10 min incubation, then centrifuged at 1200 rpm and 25 °C. About $2.5 \times 10^6$ cells of YAC-1 were placed onto 96-well plates for 100 μL before the addition of the leukocytes to the well for 12 h and determination of the NK cell activation by flow cytometry as described previously (Yang *et al.*, 2006).

**Statistical analysis.** Data were expressed as mean ± SD and differences between the control and experimental groups were analysed by Student's *t*-test. A value of $p < 0.05$ was used as the level of significance.

## RESULTS

### Injection of WEHI-3 cells induces leukemia tumors in mice

Representative whole body images of the control and quercetin treated BALB/c mice after injection with WEHI-3 cells for 3 weeks and the presence of leukemia tumors are shown in Fig. 1A. The spleen and liver size was decreased compared with the WEHI-3 cells only treated mice.

### The effects of quercetin on the weights of spleen and liver from BALB/c mice after injection with WEHI-3 cells

Spleen and liver tissues were isolated from animals and were weighed individually. Representative results are presented in Fig. 1B and C. The results indicate that quercetin affected the weights of the spleen and liver.



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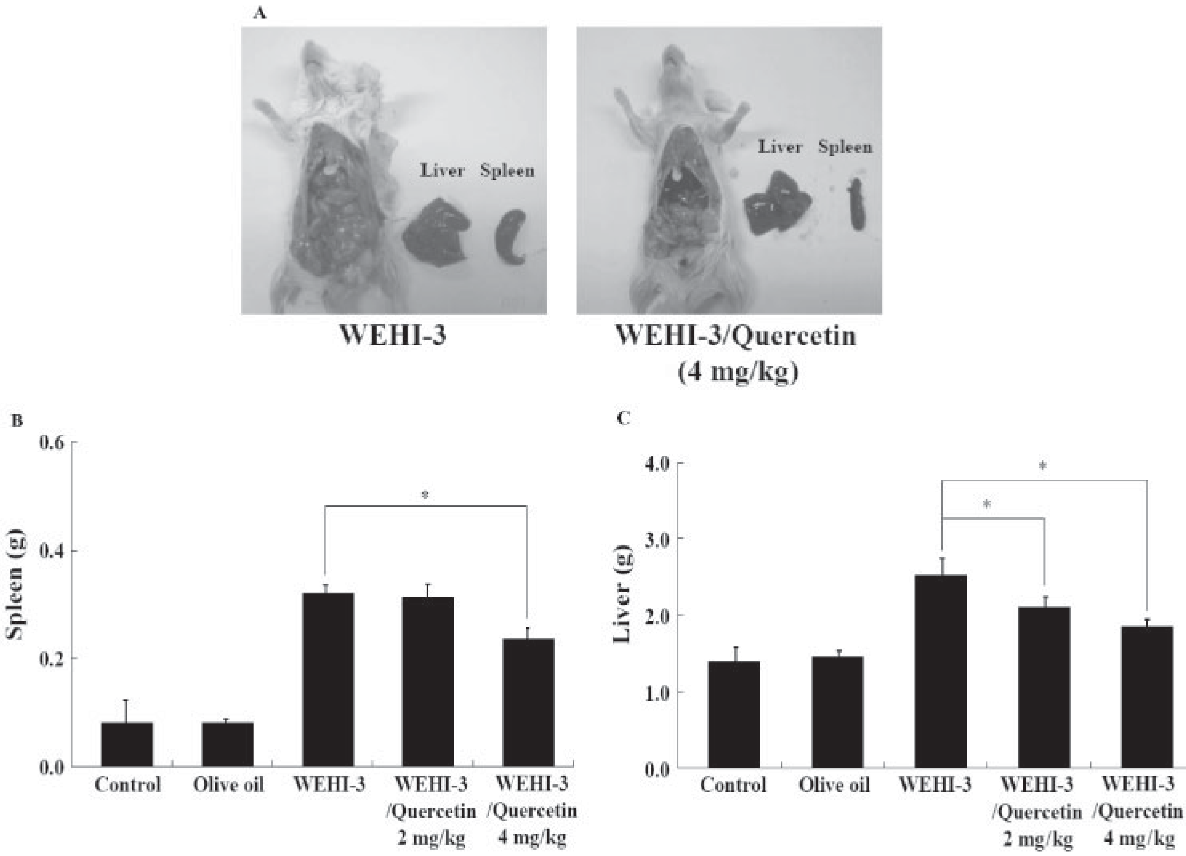


**Figure 1.** Representative pictures from dissected BALB/c mice after injection with WEHI-3 cells and/or treated with quercetin for 3 weeks. BALB/c mice were injected with WEHI-3 cells ($1 \times 10^5$ cells/100 μL) in PBS for 3 weeks and/or co-treated with quercetin, blood was collected and animals (A) were killed for examinations of spleen (B) and liver (C) tissues and were individually photographed and weighed ($n$ = 10).

After exposure to 2 and 4 mg/kg quercetin, the spleen weights in the control and quercetin treated groups were decreased by 2% and 19%, respectively, the liver weights were decreased by 14% and 28%, respectively. These effects were dose-dependent.

### Effects of quercetin on whole blood cell surface markers of BALB/c mice after injection with WEHI-3 cells

The percentage changes in cell markers of white blood cells from BALB/c mice after treatment with quercetin in olive oil or olive oil treatment are shown in Fig. 2A, B, C and D. Quercetin reduced the levels of Mac-3 (Fig. 2C) and CD11b (Fig. 2D) but increased the levels of CD19 (Fig. 2B) and had little, if any, effect on CD3 (Fig. 2A).

### Effects of quercetin on macrophage activity of BALB/c mice after injection with WEHI-3 cells

The percentage changes of macrophages with phagocytosed green fluorescent particles from PBMC and peritoneum of the control and quercetin treated groups are presented in Fig. 3. Quercetin did not induce significant differences in macrophage activity in cells isolated from PBMC (Fig. 3A). However, quercetin stimulated macrophage activity in cells from the peritoneum (Fig. 3B). These effects were dose-dependent manners.

### Effects of quercetin on activity of natural killer cells from BALB/c mice after injection with WEHI-3 cells

The YAC-1 target cells were killed by NK cells isolated from the spleen of mice after being treated with quercetin in a target cell ratio of 25:1, both doses of quercetin showed a significant difference between the control and tested agents treatment in a target cell ratio of 25:1 (Fig. 4).

### Morphological effects of quercetin on spleen of BALB/c mice after injection with WEHI-3 cells

There was a marked expansion in the red pulp, but the white pulp showed little change in spleen tissue (Fig. 5). Neoplastic cells contained large irregular nuclei accompanied by clumped chromatin and prominent nucleoli, abundant clear and light eosinophilic cytoplasm. Often mitotic figures were also noted.

## DISCUSSION

Quercetin is a component of flavonoids which has been reported to have anticancer activity such as: (1) apoptosis in different human cancer cell lines (Granado-Serrano *et al.*, 2006; Lee *et al.*, 2006; Mutoh *et al.*, 2000; Nguyen *et al.*, 2004; Singhal *et al.*, 1995; Vijayababu *et al.*, 2006; Zhang *et al.*, 2000); (2) suppression of cytokine and



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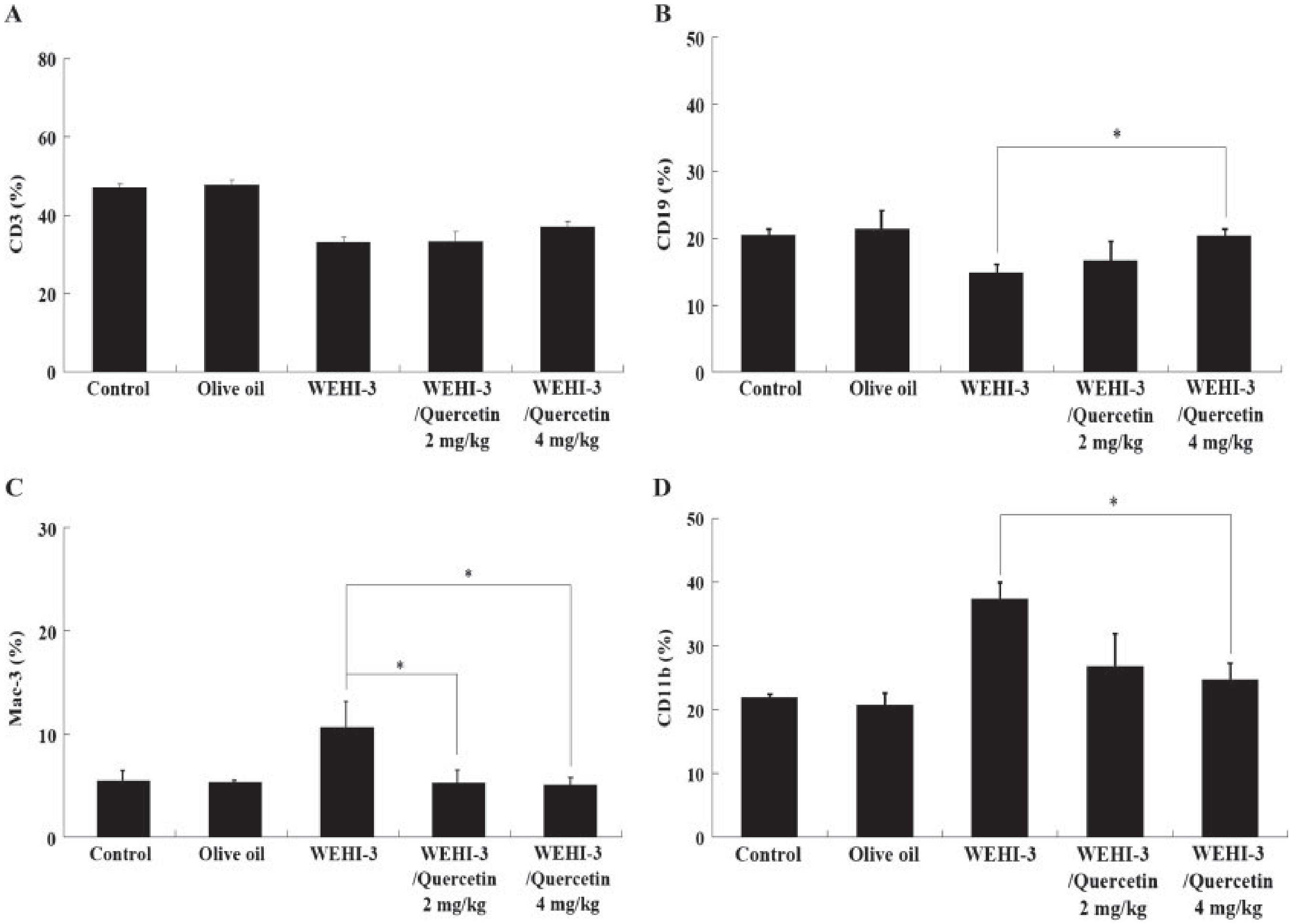


**Figure 2.** The effects of quercetin on cell markers of white blood cells from BALB/c mice. BALB/c mice were injected with WEHI-3 cells ($1 \times 10^5$ cells/100 μL) in PBS for 3 weeks and treated without or with quercetin for 3 weeks. Blood was collected from individual animals and analysed for cell markers (A) CD3, (B) CD19, (C) Mac-3 and (D) CD11b by flow cytometry as described in Materials and Methods. Each point is mean ± SD. * $p < 0.05$ ($n = 10$).

growth factor-induced invasiveness (Huang *et al.*, 1999; Lee *et al.*, 2004) and; (3) inhibition of metastasis of melanoma and prostate cancer (Piantelli *et al.*, 2006; Vijayababu *et al.*, 2006). It was also reported that quercetin induced apoptosis in human leukemia HL-60 cells *in vitro* (Hibasami *et al.*, 2005). However, the role of quercetin on leukemia cells *in vivo* has not been determined. Primary experiments also demonstrated that quercetin induced cytotoxicity in human leukemia HL-60 cells and murine leukemia WEHI-3 cells therefore raising the possibility that quercetin could affect mouse WEHI-3 leukemia cells *in vivo*. The present study showed that quercetin effectively suppressed leukemia WEHI-3 cells which had been injected into BALB/c mice *in vivo*. The study used WEHI-3 cells for i.p. injection into BALB/c mice because leukemic animals have been used to test for anticancer agents and also it is a low cost, easy method that takes a short time to develop leukemia *in vivo*.

The data indicated that quercetin significantly decreased the average size and weight of the liver and spleen of BALB/c mice injected with leukemia cells. Quercetin also decreased the percentage of Mac-3 and CD11b cells, promoted CD19 cells but did not alter CD3 markers in the blood. The stimulating effects of quercetin on the CD19 marker indicated it may promote B cell numbers. Quercetin increased macrophage phagocytosis from isolated PBMC and peritoneum and promoted natural killer cell activity. This is in agreement with regard to agents that promote immune responses are designed to increase the phagocytosis of macrophages and the activities of natural killer cells. The results demonstrated that quercetin inhibited leukemia-related spleen growth. A notable characteristic of the leukemia model used in this study is the elevation of peripheral monocytes and granulocytes with immature morphology, as well as enlarged and infiltrated spleens compared with the normal counterpart (He and Na, 2001). Based on these observations it is indicated that quercetin also promoted immune responses in BALB/c mice *in vivo*.

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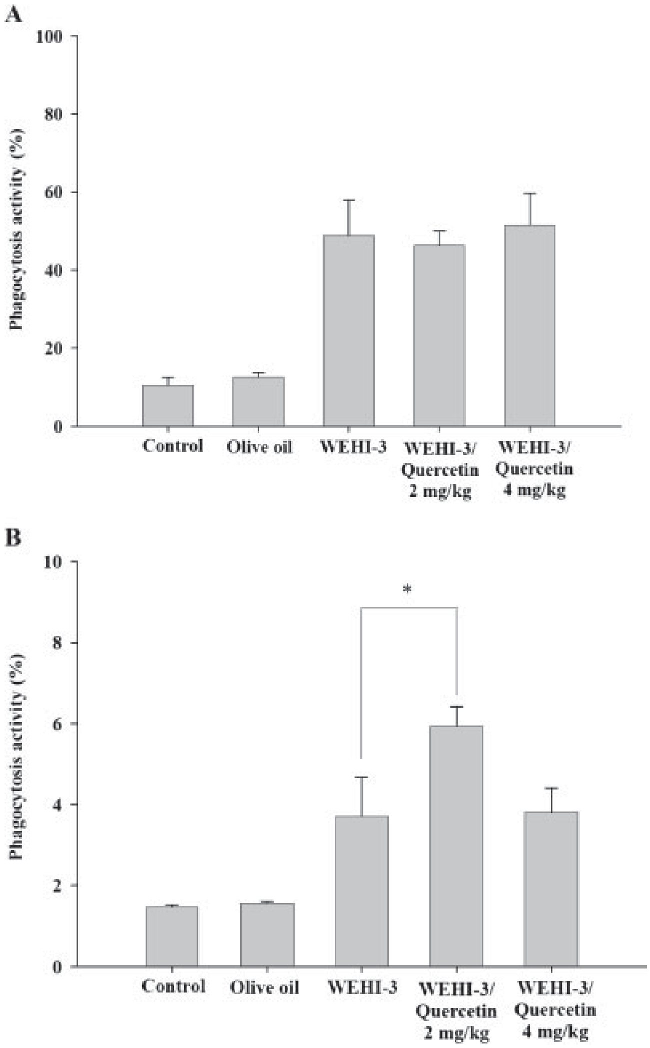


**Figure 3.** The effects of quercetin on macrophage phagocytosis from BALB/c mice. BALB/c mice were injected with WEHI-3 cells ($1 \times 10^5$ cells/100 μL) in PBS for 3 weeks and treated without or with quercetin for 3 weeks. Cells were collected from (A) PBMC and (B) peritoneum of animals and analysed for macrophage phagocytosis by flow cytometry as described in Materials and Methods. Each point is mean ± SD. * $p < 0.05$ ($n = 10$).

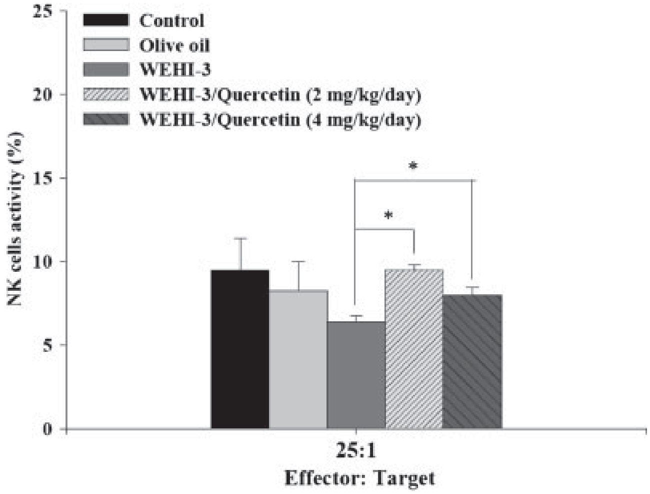


**Figure 4.** The effects of quercetin on NK cell activity from BALB/c mice. BALB/c mice were injected with WEHI-3 cells ($1 \times 10^5$ cells/100 μL) in PBS for 3 weeks and treated without or with quercetin for 3 weeks. Blood was collected from peritoneum of animal and analysed for NK cell activity by flow cytometry as described in Materials and Methods. Each point is mean ± SD. * $p < 0.05$ ($n = 10$).

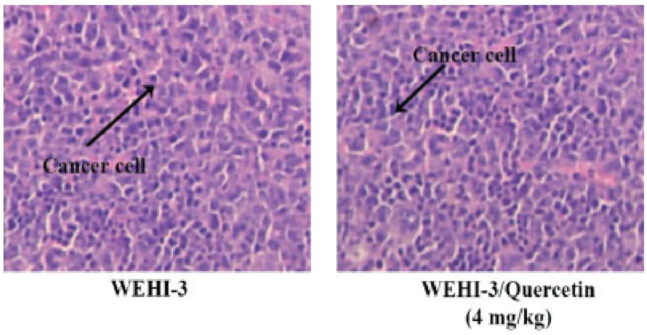


**Figure 5.** The effects of quercetin on the histopathology of the spleen from BALB/c mice. BALB/c mice were injected with WEHI-3 cells after treatment with quercetin. Spleens from each animal of each group were excised for histopathological examination as described in Materials and Methods.

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