

*Full Length Research Paper*

# Alteration of lymphocyte subpopulations in mice fed lutein from marigold extract

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Lutein, a member of the xanthophyll carotenoids, has a wide variety of health benefits. These include prevention of age-related macular degeneration or cataracts and protection of ultraviolet -induced skin damage. Lutein also possesses anti-tumor and immune modulatory activities in BALB/c mice. However, in such animal model, the effects of lutein on lymphocyte subpopulations have not yet been assessed and were therefore determined in this study. Female BALB/c mice were fed orally with lutein from marigold extract (10 and 20 mg/kg body weight daily) or vehicle for 4 weeks. Total white blood cell counts and splenic lymphocyte subpopulations were examined on weeks 2 and 4. Lutein from marigold extract did not significantly influence the total numbers of white blood cells during the experimental period. Mice fed marigold lutein had comparable numbers of CD21/35+ (mature B), CD3+ (total T) and CD4+ (T helper) lymphocytes to controls as seen throughout a 4-week period. However, the percentage of CD8+ (T cytotoxic) cells were significantly elevated ( $p < 0.05$ ) in mice fed marigold lutein 20 mg/kg (week 4) compared to the controls. Together, our results demonstrated that lutein from marigold extract influenced the population of cytotoxic T lymphocytes in BALB/c mice. This finding suggests the enhancement of cell-mediated immunity and may support, partly, anti-tumor activity of such lutein.

**Key words:** Lutein, marigold, lymphocyte subpopulation.

## INTRODUCTION

Lutein, a member of the xanthophyll carotenoids, is commonly present in a variety of leafy vegetables, in orange colored fruits and vegetables and in egg yolks (Wang et al., 2006; Tsui and Cheryan, 2007). Lutein possesses various biological activities, including prevention of age-related macular degeneration (Ribaya-Mercado and Blumberg, 2004) or cataracts (Arnal et al., 2009) and protection of ultraviolet (UV) -induced skin damage (Lee et al., 2004). Accumulating evidence also indicated a special role of the xanthophyll lutein in regulating the immune functions.

Mice supplemented with lutein showed an increased phytohemagglutinin (PHA) - stimulated lymphocyte proliferation (Chew et al., 1996). Lutein also enhanced antibody production in response to T-dependent antigen in spleen cells *in vitro* and in mice *in vivo* (Jyonouchi et al., 1994). In addition, the immunostimulatory activity of dietary lutein was demonstrated in domestic cats (Kim et al., 2000b) and dogs (Kim et al., 2000a). Cats fed lutein showed increased concanavalin A (ConA) and pokeweed mitogen (PWM) - induced peripheral blood mononuclear lymphocyte proliferation, delayed-type hypersensitivity response, numbers of CD4+ and CD21+ lymphocytes and immunoglobulin G (IgG) production (Kim et al., 2000b).

Similarly, enhanced PHA, ConA and PWM - stimulated peripheral blood mononuclear lymphocyte proliferation, delayed-type hypersensitivity response, numbers of

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**Table 1.** Body weight and spleen and thymus indices of mice fed marigold lutein daily for 2 and 4 weeks.

Period (Week)	Treatment group <sup>3</sup>	Body weight <sup>1</sup> (g)	Spleen index <sup>1,2</sup>	Thymus index <sup>1,2</sup>
2	Control	21.47 ± 0.40	0.39 ± 0.04	0.20 ± 0.01
	CLT10	22.22 ± 0.33	0.45 ± 0.01	0.26 ± 0.03
	CLT20	23.23 ± 0.28*	0.42 ± 0.01	0.21 ± 0.01
4	Control	22.92 ± 0.33	0.40 ± 0.01	0.16 ± 0.01
	CLT10	23.58 ± 0.24	0.41 ± 0.02	0.16 ± 0.01
	CLT20	23.63 ± 0.21	0.41 ± 0.01	0.18 ± 0.01

<sup>1</sup>Values are mean ± S.E.M. (n = 5). \**p* < 0.05 compared to the control. <sup>2</sup>Spleen and thymus indices are expressed as 100 X spleen or thymus weights/BW. <sup>3</sup>CLT10, CLT20; lutein from marigold extract 10 and 20 mg/kg BW treated groups.

CD5+, CD4+ and CD8+ lymphocytes and IgG production were also observed in dogs supplemented with lutein (Kim et al., 2000a). Of significance, lutein has also been reported to inhibit the growth of mammary tumors in BALB/c mice (Park et al., 1998). In such animal model, however, the effect of lutein from marigold extract on leukocyte subpopulations has not yet been determined. Lymphocytes are well-recognized as the master cells of the immune system and play a significant role in host defense against infections and tumors. Therefore, the effects of lutein on subpopulations of the lymphocytes were determined in this study.

## MATERIALS AND METHODS

### Animals and experimental design

Healthy female BALB/c mice (6 to 8 weeks old; 20.0 ± 0.1 g) were obtained from the National Laboratory Animal Center, Mahidol University, Thailand. Animals were housed in 25 x 32 cm cages constantly maintained at 25±1°C with a 12 h dark: light cycle and had free access to sterile water and standard mouse diet. All animal procedures were approved by the Animal Research Ethics Committee, Naresuan University. Mice were randomly divided into three groups of five each and orally administered with 10 or 20 mg/kg body weight (BW) of lutein from marigold extract (97% xanthophyll; P.R. China) or vehicle once daily for a period of 4 weeks. Body weights were recorded weekly.

On weeks 2 and 4 after the initial lutein administration, 5 mice in each group were killed by intraperitoneal injection with an overdose of pentobarbital sodium (Nembutal<sup>®</sup>, Ceva Sante Animale, France; 40 to 50 mg/kg BW). Blood were immediately collected by cardiac puncture, subsequently spleens and thymus were aseptically removed and weighed.

### Preparation of splenic single cells

Spleens were placed in PCM buffer prepared with sterile phosphate buffer saline (PBS) pH 7.4, 7×10<sup>-4</sup> M CaCl<sub>2</sub>, 5×10<sup>-4</sup> M MgCl<sub>2</sub> and 5% (v/v) fetal bovine serum (FBS; Gibco, South America). Single cell suspension was prepared by passing the PCM buffer through the spleen. Red blood cells were removed using 0.17 M NH<sub>4</sub>Cl and the resulting cell suspensions washed twice with PCM buffer. Cell pellets were finally resuspended with complete RPMI-1640 medium (PAA laboratories GmbH, Austria) containing 5% FBS (Gibco), 0.01 M hydroxyethyl piperazineethanesulfonic acid (HEPES) (Hyclone, Utah), 5×10<sup>-5</sup> M β-mercaptoethanol (BIO-RAD), 2 mM L-glutamine

(Hyclone), 100 U/ml penicillin and 100 µg/ml streptomycin (PAA). Viability of splenic cells was determined by trypan blue dye exclusion (Abuharfeil et al., 2001).

### Determination of total white blood cells (WBC)

Blood samples in 1% ethylenediaminetetraacetic acid (EDTA) were subjected to red blood cell lysis using RBC lysis buffer. White blood cells were counted using a hemocytometer and the total WBC expressed as cells/µL.

### Determination of splenic lymphocyte subpopulations

A total of 4 × 10<sup>5</sup> splenic single cells were stained for 30 min on ice with fluorescently conjugated monoclonal antibody specific for mouse lymphocyte surface markers as follows: Fluorescein isothiocyanate conjugated anti-CD3 (total T cells), phycoerythrin (PE) conjugated anti-CD4 (T helper cells), PE conjugated anti-CD8 (T cytotoxic cells) and PE conjugated anti-CD21/35 (mature B cells). These fluorescently conjugated monoclonal antibodies were all purchased from eBioscience (San Diego, CA). Stained cells were then washed twice with PBS pH 7.4 containing 2% heat-inactivated fetal bovine serum and fixed with 1% paraformaldehyde. The percentages of lymphocyte subpopulations were subjected to flow cytometric analysis using a FACScalibur and CellQuest Pro software (BD Biosciences).

### Statistical analysis

All values expressed are mean ± S.E.M. Data were analyzed by one-way analysis of variance (ANOVA) using statistical package for the social sciences (SPSS) program. Differences among treatment means within a sampling period were compared using Tukey's post hoc test. Differences with *p* < 0.05 were considered as statistically significant.

## RESULTS AND DISCUSSION

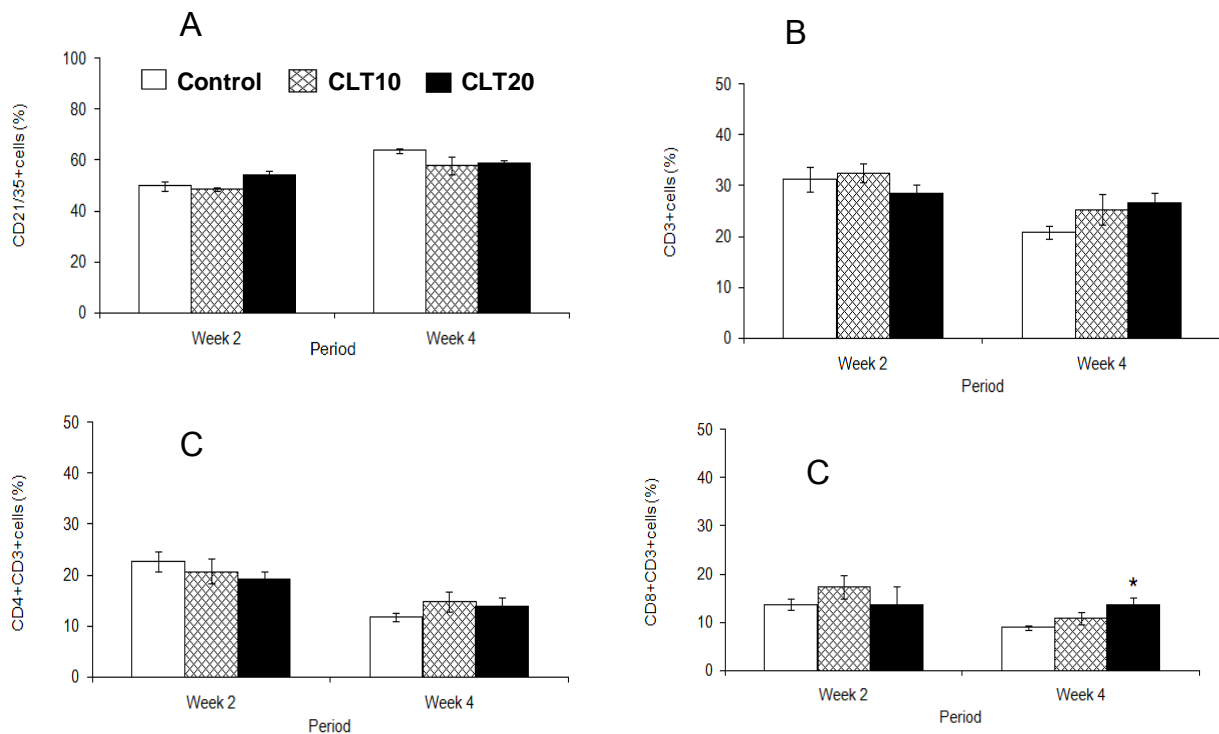
### Effects of lutein from marigold extract on body weight and lymphoid organ indices

Daily oral administration of lutein from marigold extract did not result in any mortality. The body weight and the spleen and thymus indices did not differ among lutein-treated and control animals throughout the study period (Table 1), suggesting that marigold lutein is not harmful to the lymphoid organs of the immune system.

**Table 2.** Total white blood cells in mice fed marigold lutein daily for 2 and 4 weeks.

Period (Week)	Treatment group <sup>2</sup>	Total WBC (X 10 <sup>2</sup> cells/ $\mu$ L) <sup>1</sup>
2	Control	11.40 $\pm$ 1.70
	CLT10	13.20 $\pm$ 0.40
	CLT20	10.10 $\pm$ 1.00
4	Control	7.00 $\pm$ 1.60
	CLT10	11.10 $\pm$ 2.90
	CLT20	12.40 $\pm$ 2.10

<sup>1</sup>Values are mean  $\pm$  S.E.M. (n=5). <sup>2</sup>CLT10, CLT20; lutein from marigold extract 10 and 20 mg/kg BW treated groups.



**Figure 1.** Percentages of (A) CD21/35+ (mature B), (B) CD3+ (total T) (C) CD4+ (T helper) and (D) CD8+ (T cytotoxic) cells in mice fed lutein from marigold extract on weeks 2 and 4. Values are means  $\pm$  S.E.M. (n=5). \**p* < 0.05 compared to the control. CLT10, CLT20; lutein from marigold extract 10 and 20 mg/kg BW treated groups.

### Effect of lutein from marigold extract on total white blood cell counts

Table 2 illustrates the total numbers of WBC in mice fed marigold lutein daily for 2 and 4 weeks. Counts of the total WBC in mice fed marigold lutein either 10 or 20 mg/kg were in the normal range (Zhang et al., 2011) and not significantly different from the control animals during all periods.

### Effect of lutein from marigold extract on lymphocyte subpopulations

Lymphocytes are well-documented as the master immune cells that play a significant role in defense

against infections and cancers. The number of lymphocytes in particular the two main lymphocyte T subsets (T helper and T cytotoxic) have become recognized as an important meaningful parameter for evaluating immunomodulatory activity (Dhur et al., 1991). Changes in the percentages of lymphocyte subpopulations upon oral administration of marigold lutein were therefore determined. This study employed the BALB/c mice as this animal model has been accepted as an appropriated model for studying the immune response of carotenoids (Lee et al., 1999).

The percentages of CD21/35+ (mature B), CD3+ (total T) and CD4+ (T helper) lymphocytes were similar among treatment groups throughout the study period (Figure 1A to C). However, on week 4, the percentage of CD8+ (T

cytotoxic) cells were significantly elevated ( $p < 0.05$ ) in mice fed marigold lutein 20 mg/kg BW compared to the controls as demonstrated in Figure 1D.

Lutein from marigold extract influenced the percentages of lymphocyte subsets has previously been reported in cats (Kim et al., 2000b) and dogs (Kim, 2000a). In dogs, dietary lutein increased the percentages of total T cells, T helper and T cytotoxic lymphocytes (Kim, 2000a) whereas the increased percentages of T helper and B lymphocytes were observed in cats (Kim et al., 2000b).

Our study, which was performed in BALB/c mice, also demonstrated the changes of lymphocyte subpopulations upon oral administration of lutein. These alterations differed in patterns and degrees of immune responses from those previously reported in cats (Kim et al., 2000b) and dogs (Kim et al., 2000a). It is possible that the animal models as well as the dose and time of lutein administration would contribute to such differences. Nevertheless, our findings suggested that lutein from marigold extract influenced the cell-mediated arm of the immune system and this may support, in part, the role of marigold lutein in inhibiting tumor growth in BALB/c mice reported previously (Park et al., 1998).

## Conclusion

This study demonstrated that lutein from marigold extract increased the population of cytotoxic T lymphocytes in BALB/c mice, indicating immune stimulation of the cell-mediated arm.

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