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# Immunomodulatory Effects of Dietary Safflower Leaf in Chickens

Lee, Sung Hyen · Lillehoj, Hyun S. · Cho, Soo Muk* · Park, Dong Woon
Hong, Yeong Ho · Chun, Hye Kyung* · Lillehoj, Erik P.**

Animal and Natural Resources Institute, Agricultural Research Service, United States Department of Agriculture, Beltsville, MD 20705, USA
National Rural Resources Development Institute, National Institute of Agricultural Science and Technology, Rural Development Administration, Suwon, South Korea*
Department of Pediatrics, University of Maryland School of Medicine, Baltimore, MD 21201, USA**

## 국문초록

홍화 (*Carthamus tinctorius*)는 오래 전부터 각종 감염성 질환이나 암을 치료하는데 효과가 있는 것으로 알려져 왔다. 최근 각종 천연산물에 사람과 동물의 건강을 증진시키는 효과가 있는 것이 밝혀지면서, 홍화의 면역조절 효과에 대한 관심이 높아지고 있으나 이에 대한 과학적 자료가 부족한 실정이다. 따라서, 본 연구는 홍화의 면역 조절 활성을 확인하기 위해 수행되었으며, 면역능 실험을 위해 주로 이용되는 실험용 닭(White Leghorn chickens)에게 홍화잎을 첨가한 실험식이를 3주간 급여한 후 관련 요인들을 분석하였다. 실험 결과, 홍화잎을 섭취한 실험동물에서 비장 면역세포의 증식이 많았고, γδ-TCR+ 세포의 비율이 높아졌으며, 십이지장에서는 IFN-γ, IL-8, IL-10, IL-15 및 NK-lysin 같은 사이토카인의 발현이 높아졌다. 따라서, 홍화잎은 식이와 함께 급여하였을 때 면역 증강 효과가 있는 것으로 밝혀졌으며, 홍화잎을 이용한 각종 제품 개발이 기대된다.

주제어: 홍화잎, 면역조절, 면역세포, 장 면역, 사이토카인

## I. Introduction

Safflower (*Carthamus tinctorius*), belonging to the *Compositae* or *Asteraceae* family, has been cultivated for more than two thousand years and has historically been used as a herbal medicine against infectious diseases and cancers. Due to renewed interest in the use of natural products to enhance human and animal health, safflower has been evaluated as an immunomodulatory agent. Initial safety studies have shown that safflower is nontoxic as a novel pasture species for dairy sheep and cows (Landau et al., 2004; Landau et al., 2005). Furthermore, safflower seed oil inhibited the production of proinflammatory cytokines by endotoxin-stimulated human monocytes (Takii et al., 2003), whereas safflower petals

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**Corresponding Author:** Hyun S. Lillehoj Tel: 301-504-8771; Fax: 301-504-5103
e-mail: Hyun.Lillehoj@ARS.USDA.GOV

were reported to contain polysaccharides that activated macrophages *in vitro* (Ando et al., 2002).

Chickens are commercially important food animals, but also an important source of human infections (Zoete et al., 2006). Chickens have provided excellent *in vitro* and *in vivo* model systems to investigate human immunity (Usuki et al., 2006; Tsurushita et al., 2004; Nishibori et al., 2004; Lee et al., 2007a; Lee et al., 2007b; Lee et al., 2007c). Recent studies from our laboratory have demonstrated that dietary supplementation with *Pediococcus*-based probiotics or a lectin derived from *Fomitella fraxinea* (shiitake mushroom) enhanced innate and adaptive immunities to avian coccidiosis, an intestinal infectious disease caused by the *Eimeria* protozoan parasite (Lee et al., 2007a; Lee et al., 2007b; Dalloul et al., 2006). In general, the effects of natural food and herbal products on host defense against microbial infections and tumors have shown good correlation with their ability to enhance various *in vitro* correlates of innate immunity (Lee et al., 2005; Lee et al., 2007d; Lee et al., 2007e; Kim et al., 2004; Pandey et al., 2005; Park et al., 2004). For example, increased spontaneous splenocyte proliferation following the feeding of some medicinal plants has been attributed to their high phenolic content (Lin & Tang, 2007). Based on these previous studies, the current investigation was conducted to examine the ability of a safflower leaf-supplemented diet to augment innate immunity in chickens.

## II. Materials and methods

### 1. Experimental animals and diets

Fertilized eggs of specific pathogen-free White Leghorn chickens were obtained from SPAFAS (Charles River Laboratories, Preston, CT) and hatched at the Animal and Natural Resources Institute, USDA (Beltsville, MD). One-day-old chickens were randomly assigned to 2 pens (N = 10/pen) of an electrically heated battery. One pen of animals were fed *ad libitum* a standard chicken diet without safflower (negative control group) while the second pen of chickens received the standard diet with 0.5% (w/w) of safflower leaf (SF 0.5 group) for 1 week. One-week-old chickens were separated to cages (N=2/cage) and were fed same diet for 2 weeks. The safflower leaf diet was prepared by thoroughly mixing standard chicken feed and freeze-dried safflower leaf powder supplied by the National Rural Resources Development Institute (Suwon, South Korea). The standard diet was formulated to meet the nutrient requirements for chickens as recommended by the National Research Council (National Research Council, 1994). We measured body weight gains of both experimental groups between days 8-21. All experiments were performed according to the guidelines established by the Beltsville Area Institutional Animal Care and Use Committee.

### 2. Splenocyte proliferation

Spleens were removed at 21 days age and placed in a Petri dish with 10 ml of Hank's balanced salt solution (HBSS) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma, St. Louis, MO). Single cell suspensions were prepared (Kaspers et al., 1994) and lymphocyte proliferation was carried out as described (Okamura et al., 2004). Briefly, splenocytes were adjusted to $1\times10^7$ cells/ml in enriched RPMI-1640 medium without phenol red (Sigma) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin. Splenocytes (100 μl/well) were cultured in 96-well flat bottom plates at 41°C in a humidified incubator (Forma, Marietta, OH) with 5% $CO_2$ for 48 hr. Cell proliferation was determined with 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8, Cell-Counting Kit-8®, Dojindo Molecular Technologies, Gaithersburg, MD) as described (Lee et al., 2007c; Miyamoto et al., 2002). Cell numbers were quantified by optical density (OD)

at 450 nm using a microplate spectrophotometer (BioRad, Richmond, CA).

### 3. Flow cytomeric analysis

Flow cytometric analyses of splenic lymphocytes were performed as described previously (Lillehoj HS, 1994). Single cell suspensions of freshly prepared splenocytes were resuspended in 1.0 ml of flow cytometer buffer (HBSS containing 3% FBS and 0.01% sodium azide). One hundred μl aliquots (approximately $10^6$ cells) were incubated on ice for 40 min with 100 μl of appropriately diluted monoclonal antibodies against chicken CD4, CD8, IgM, αβ-T cell receptor (αβ-TCR), or γδ-TCR as described (Hong et al., 2006a). After washing twice with 2.0 ml of flow buffer, the cells were incubated with fluorescein isothiocyanate- conjugated anti-mouse IgG antibody (Sigma) for 30 min on ice, washed twice, resuspended in 2.0 ml, and analyzed with an Epics model XL flow cytometer (Coulter, Miami, FL). Data were obtained from a total of $10^4$ viable cells.

### 4. Quantification of cytokine and chemokine mRNA levels

Cytokine and chemokine gene expression analysis was carried out by quantitative RT-PCR as described (Hong et al., 2006a; Hong et el., 2006b). The intestinal duodenum was removed from 21 day-old chickens, cut longitudinally, and washed 3 times with ice-cold HBSS containing 100 U/ml of penicillin and 100 μg/ml of streptomycin. The mucosal layer was carefully scraped away using a surgical scalpel, the tissue was washed several times with HBSS containing 0.5 mM EDTA and 5% FBS and incubated for 20 min at 37°C with constant swirling. Cells released into the supernatant were pooled and washed twice with HBSS. Intestinal intraepithelial lymphocytes (IELs) were purified on a discontinuous Percoll density gradient by

**Table 1.** Oligonucleotide primers used in this study

| RNA target | Primer sequences | PCR product size (bp) | Accession no. |
|---|---|---|---|
| GAPDH | | | |
| Forward | 5'-GGTGGTGCTAAGCGTGTTAT-3' | 264 | K01458 |
| Reverse | 5'-ACCTCTGTCATCTCTCCACA-3' | | |
| IFN-γ | | | |
| Forward | 5'-AGCTGACGGTGGACCTATTATT-3' | 259 | Y07922 |
| Reverse | 5'-GGCTTTGCGCTGGATTC-3' | | |
| IL-8 | | | |
| Forward | 5'-GGCTTGCTAGGGGAAATGA-3' | 200 | AJ009800 |
| Reverse | 5'-AGCTGACTCTGACTAGGAAACTGT-3' | | |
| IL-10 | | | |
| Forward | 5'-CGGGAGCTGAGGGTGAA-3' | 272 | AJ621614 |
| Reverse | 5'-GTGAAGAAGCGGTGACAGC-3' | | |
| IL-15 | | | |
| Forward | 5'-TCTGTTCTTCTGTTCTGAGTGATG-3' | 243 | AF139097 |
| Reverse | 5'-AGTGATTTGCTTCTGTCTTTGGTA-3' | | |
| IL-17 | | | |
| Forward | 5'-CTCCGATCCCTTATTCTCCTC-3' | 292 | AJ493595 |
| Reverse | 5'-AAGCGGTTGTGGTCCTCAT-3' | | |
| IL-18 | | | |
| Forward | 5'-GGAATGCGATGCCTTTTG-3' | 264 | AJ277865 |
| Reverse | 5'-ATTTTCCCATGCTCTTTCTCA-3' | | |
| NK-lysin | | | |
| Forward | 5'-GATGGTTCAGCTGCGTGGGATGC-3' | 217 | DO186291 |
| Reverse | 5'-CTGCCGGAGCTTCTTCAACA-3' | | |

centrifugation at 600×g for 25 min at 24°C and total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA). Five micrograms of total RNA were treated with 1.0 U of DNase I and 1.0 μl of 10X reaction buffer (Sigma), incubated for 15 min at room temperature, 1.0 μl of stop solution was added to inactivate DNase I, and the mixture was heated at 70°C for 10 minutes. RNA was reverse- transcribed using the StrataScript first-strand synthesis system (Stratagene, La Jolla, CA) according to the manufacturer's recommendations. Briefly, 5.0 μg of RNA were combined with 10X first strand buffer, 1.0 μl of oligo(dT) primer (5.0 μg/μl), 0.8 μl of dNTP mix (25mM of each dNTP), and RNase-free water to a total volume of 19 μl. The mixture was incubated at 65°C for 5 min, cooled to room temperature, then 50 U of StrataScript reverse transcriptase were added, the mixture was incubated at 42°C for 1 hr, and the reaction was stopped by heating at 70°C for 5 min. Quantitative RT-PCR oligonucleotide primers for chicken cytokines and GAPDH control are listed in Table 1. Amplification and detection were carried out using equivalent amounts of total RNA from IELs using the Mx3000P system and Brilliant SYBR Green QPCR master mix (Stratagene). Standard curves were generated using $\log_{10}$ diluted standard RNA and levels of individual transcripts were normalized to those of GAPDH analyzed by the Q-gene program (Muller et al., 2002). Each analysis was performed in triplicate. To normalize individual replicates, the logarithmic-scaled raw data unit cycle threshold ($C_t$) was transformed into linear unit of normalized expressions and calculating means and SEM for the references and individual targets, followed by determination of mean normalized expression (MNE) using the Q-gene program (Hong et al., 2006a; Hong et el., 2006b; Hong et al., 2006c; Hong et al., 2006d).

### 5. Statistical analyses

All samples were analyzed in triplicate and data were expressed as mean ± SEM values. The data were analyzed by the Mann-Whitney test for overall comparison using InStat® software (Graphpad, San Diego, CA). Differences were considered significant at the level of $P < 0.05$.

## III. Results

### 1. Body weight gain

Initially, we determined whether or not chickens fed a safflower leaf-supplemented diet exhibited abnormal growth performance compared to animals given a conventional diet by measuring body weight gains of both experimental groups between days 8-21. As shown in Fig. 1, there was no significant difference in body weight gain between the control and safflower-fed SF 0.5 groups.

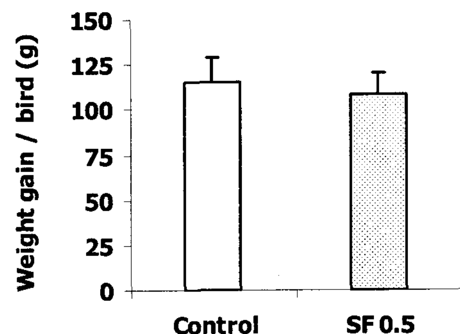
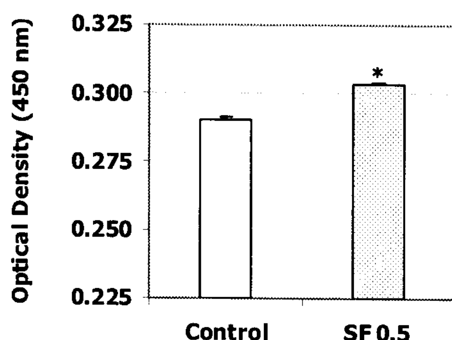



Fig. 1. Body weight gains of chickens fed a safflower-supplemented diet; Chickens were fed a control diet without safflower leaf or 0.5% safflower leaf-supplemented diet for 3 weeks and body weights were measured at 8 and 21 days of age. Each bar represents the mean ± SEM values (N=10).

### 2. Spleen lymphocyte proliferation and T and B cell subsets

As shown in Fig. 2, splenocyte spontaneous proliferation was significantly greater in the safflower leaf-supplemented group compared with




* $p < 0.05$.

Fig. 2. Spleen lymphocyte proliferation in chickens fed a safflower-supplemented diet; Chickens fed a control diet without safflower leaf or 0.5% safflower leaf- supplemented diet for 3 weeks and spontaneous splenocyte proliferation was determined at 21 days of age. Each bar represents the mean ± SEM values of triplicate samples from 3 different chickens.

Table 2. Spleen lymphocytes subpopulations in chickens fed a safflower-supplemented diet

| Group | CD4 | CD8 | IgM | γδ-TCR | αβ-TCR |
|---|---|---|---|---|---|
| Control | 46.6 | 47.2 | 12.3 | 7.5 | 67.2 |
| SF 0.5 | 50.6 | 41.3* | 11.8 | 12.2* | 65.1 |

Chickens were fed non-supplemented (control) or 0.5% safflower leaf-supplemented diets (SF 0.5) for 3 week post-hatch and spleen cells were examined for lymphocyte subpopulations by flow cytometry. Data were expressed as mean percentages ± SEM (N = 3). * $p < 0.05$.

the control group. Table 2 shows the T and B splenic lymphocyte subpopulations in chickens fed control and 0.5% safflower-supplemented diets. There were no significant differences in the percentages of $CD4^+$, $\alpha\beta$-$TCR^+$, or $IgM^+$ cells between the control and SF 0.5 groups. By contrast, the percentage of $CD8^+$ cells was decreased and the percentage of $\gamma\delta$-$TCR^+$ cells was increased in the SF 0.5 group compared to controls.

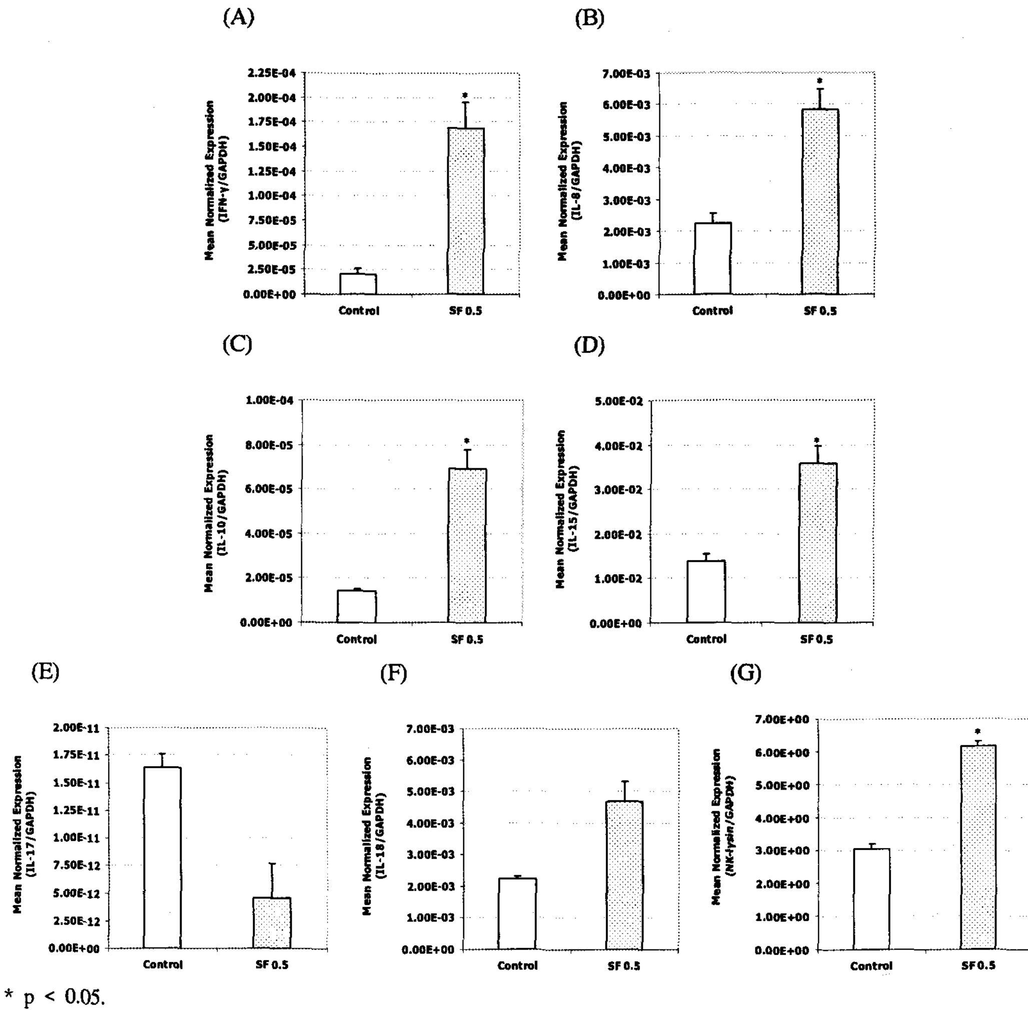
### 3. Cytokine and chemokine transcript levels

IFN-γ transcript levels in intestinal IELs were significantly increased (8.1-fold) in the SF 0.5 group compared with the control group (Fig. 3A). Similarly, mRNAs for IL-8, IL-10, IL-15, and NK-lysin in SF 0.5 animals were significantly increased compared with those in control animals (2.6-, 4.8-, 2.6-, 2.1-, and 2.0-fold, respectively) as shown in Fig. 3. Although IL-17 transcripts were decreased and IL-18 transcripts were increased in safflower-fed animals, the differences between the 2 groups for both cytokines did not achieve statistical significance.

## IV. Discussion

On the basis of the known medicinal properties of safflower, and the limited studies that have examined its potential as an immune system stimulator (Takii et al., 2003; Ando et al., 2002), this investigation was conducted to examine the effects of dietary safflower leaf, given as a freeze-dried powder in standard chicken feed, on avian innate immune responses. Our results demonstrated that safflower leaf showed no general toxic effect, as manifested by normal body weight gain. Dietary safflower leaf, however, increased splenocyte proliferation, enhanced the percentage of $\gamma\delta$-$TCR^+$ cells, decreased the fraction of $CD8^+$ T cells, and augmented the levels of mRNAs for IFN-γ, IL-8, IL-10, IL-15, and NK-lysin in intestinal lymphocytes.

Alterations of splenic lymphocyte proliferation and surface marker subpopulations following dietary safflower leaf supplementation were investigated to better define the nature of the immune response affected by this treatment. Our observation that safflower decreased the percentage of $CD8^+$ T cells corroborates that of Yun et al. (2003), who demonstrated that dietary oat β-glucan had the same effect, concomitant with enhanced disease resistance against bacterial or parasitic infections. We also detected increased




* $p < 0.05$.

Fig. 3. Cytokine and chemokine mRNA levels in chickens fed a safflower-supplemented diet; Chickens fed a control diet without safflower leaf or 0.5% safflower leaf-supplemented diet for 3 weeks. The levels of mRNAs encoding the indicated molecules in intestinal IELs were determined by quantitative RT-PCR at 21 days of age, and normalized to GAPDH mRNA. Each bar represents the mean ± SEM values of triplicate samples from 3 different chickens.

levels of $\gamma\delta$-TCR$^+$ cells in safflower-fed animals. $\gamma\delta$-TCR$^+$ cells secrete important immunoregulatory cytokines, are found to occur predominately in some pathological situations, and have been suggested to play a critical role in mediating a cytotoxic effect against intracellular parasites (Haas et al., 1993; Lillehoj HS, 1989; Trout & Lillehoj, 1996). By contrast, the population of IgM$^+$ cells, previously proposed as critical for immune surveillance during innate immunity (Vollmers & Brandlein, 2005), was not changed in the SF 0.5 group compared with controls.

With the advent of the chicken genome project, a number of chicken cytokine and chemokine genes have been discovered (Lillehoj HS, 1994; Hong et el., 2006b; Hong et al., 2006c; Hong et al., 2006d; Min & Lillehoj, 2002; Min & Lillehoj, 2004; Swaggerty et al.,

2004). Cloning of these genes has led to the development of a large array of reagents for investigating avian innate and acquired immune responses at the molecular and cellular levels. Several experimental strategies to enhance protective immunity to avian pathogens using some of these cloned cytokines and chemokines have been reported (Trout & Lillehoj, 1996; Song et al., 2000; Lillehoj et al., 2004; Ding et al., 2004). An illustrative example is IFN-γ. IFN-γ is a pro- inflammatory cytokine that serves as a common marker of cellular immunity. Elevated levels of IFN-γ are associated with immune responses to parasitic infections (Hong et el., 2006b; Lillehoj & Trout, 1996; Lillehoj & Choi, 1998; Yun et al., 2000), and early cellular immune responses characterized by IFN-γ production are critical for protection against experimental coccidiosis due to *Eimeria* infection (Lillehoj et al., 2004; Choi et al., 1999; Min et a., 2003). Min et al. (2001) showed that coccidiosis was significantly reduced in chickens given an *Eimeria* subunit vaccine in conjunction with recombinant IFN-γ compared with animals given the vaccine alone.

An adjuvant effect against experimental coccidiosis also was observed in chickens vaccinated with recombinant forms of IL-8, IL-15, or IL-17 (Ding et al., 2004; Min et al., 2001). In the case of IL-15, the immunostimulatory effect was correlated with the ability of the cytokine to induce the proliferation of T lymphocytes and NK cells (Lillehoj et al., 2001; Choi & Lillehoj, 2000). On the other hand, IL-10 is an immunosuppressive cytokine and IL-10-producing dendritic cells stimulate regulatory T cells (Furset & Sioud, 2007), so the net effect of safflower on chicken immunity depends on both stimulatory and suppressive activities. Our results also indicated that the safflower diet promoted the expression of NK-lysin, a cationic peptide produced by natural killer (NK) cells. NK-lysin exhibits anti-bacterial activity against *Escherichia coli* and *Bacillus megaterium* (Andersson et al., 1996). In chickens, the levels of NK-lysin mRNA are highest in intestinal IELs, indicating that this molecule is essential for the control of enteric infections. Recombinant chicken NK-lysin expressed in COS7 cells exhibited anti-tumor cell activity against LSCC-RP9, a retrovirus-transformed B cell line (Hong et al., 2006c). Thus, up-regulation of chicken NK-lysin expression by safflower leaf may play an important role during anti-microbial and anti-tumor defenses.

In conclusion, we report that chickens fed a 0.5% safflower-supplemented diet exhibited elevated spontaneous spleen lymphoproliferation, decreased $CD8^+$ and increased $\gamma\delta$-$TCR^+$ splenic lymphocytes, and higher levels of transcripts encoding IFN-γ, IL-8, IL-10, IL-15, and NK-lysin compared with animals fed a non-supplemented control diet. Thus, the effect of safflower on chicken immunity is likely to involve a complex network of effecter cells and molecules, the end result of which is determined by a balance between their stimulatory and suppressive properties. Further investigations of the protective immune mechanisms stimulated by safflower are needed to determine whether or not dietary supplementation of chickens may potentially be used against economically important avian diseases.

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