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≪**Research Note**≫

Immune Enhancing Properties of Safflower Leaf (*Carthamus tinctorius*) on Chicken Lymphocytes and Macrophages

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Safflower (*Carthamus tinctorius*) has been used as a traditional medicinal plant to enhance natural immunity and treat cancers. However, limited information exists on the mechanisms responsible for its immune enhancing properties. In this study, the immunostimulatory effects of a methanol extract of safflower leaf were assessed by *in vitro* lymphocyte proliferation, tumor cell cytotoxicity, and nitric oxide production by cultured chicken macrophages. A crude methanol extract of safflower leaf stimulated spleen lymphocyte proliferation and nitric oxide production, and inhibited the viability of tumor cells significantly greater than medium controls. Sequential gel filtration chromatographic separation of the methanol extract on Sephadex G-25 and Sephacryl S-200 columns resulted in a partially purified preparation that retained the ability to induce lymphoproliferation, tumor killing, and NO production. These results demonstrate for the first time that safflower leaf contains immunostimulatory and anti-tumor component(s) that may be potentially useful as a dietary immunomodulator for poultry.

Key words: chicken, immunomodulation, lymphoproliferation, safflower leaf, tumor

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Introduction

The immune system of higher vertebrates is composed of a complex network of tissues, cells, and effecter molecules (Lillehoj *et al.,* 2005). Many studies have demonstrated that enhancement of innate and adaptive immunities increase the host's ability to resist infectious diseases and tumor development (Dalloul *et al.,* 2006; Lee *et al.,* 2005). Recent interest in the nutrition-based enhancement of host immunity in humans and animals has spawned new interest in discovering and exploring the potential immune-stimulating properties of naturally occurring dietary substances. For example, the seed oil of safflower (*Carthamus tinctorius* L.) inhibited the production of proinflammatory cytokines by endotoxin (lipopolysaccharide: LPS)-stimulated human monocytes (Takii *et al.,* 2003). Safflower petals contain polysaccharides that activate macrophages *in vitro* (Ando *et al.,* 2002), and in livestock, safflower showed no toxicity as a novel pasture species for sheep and as the sole food for late-pregnancy dairy cows (Landau *et al.,* 2004; Landau *et al.,* 2005). In Korea, safflower seeds have been traditionally used as an herbal medicine to enhance resistance to infectious diseases and treat cancers (Chun, 2006). However, in spite of the well-known medicinal effects of safflower, few studies have been reported describing the effects of safflower on the immune system.

The current investigation was conducted to examine the potential immunoenhancing properties of safflower leaf in chickens, a commercially important food animal. We found that crude and partially purified methanol extracts of safflower leaf enhanced *in vitro* lymphocyte proliferation, inhibited tumor cell viability, and promoted nitric oxide production by a chicken macrophage cell line.

Materials and Methods

Methanol extraction of safflower leaf

Safflower leaf (*Carthamus tinctorius*) (National Rural Resources Development Institute, Rural Development Administration, Suwon, South Korea) was freeze-dried, ground in an electric blender, and stored as a fine powder until use. Methanol extraction was carried out by addi-

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tion of 100 mL of 80% methanol to 30 g of safflower leaf dry powder with vigorous shaking for 48 h at room temperature. The process was repeated three times and the combined extracts were rotary evaporated to dryness (EYELA, Irvine, CA). The residue was freeze-dried, stored at −80℃, dissolved in PBS at 250, 500 and 1,000 μg/mL, and sterilized by membrane filtration through a 0.45 μm filter prior to assay.

Purification of safflower leaf methanol extract

The methanol extract of safflower leaf was dissolved in PBS, applied to a Sephadex G-25 column (1.6×2.5 cm), and resolved by chromatographic filtration using the AKTA-FPLC system (GE Healthcare, Piscataway, NJ) with a HiPrep Sephacryl S-200 column (16×60 cm) in deionized water. Acquisition and treatment of the chromatographic data were carried out using UNICORN software (GE Healthcare) and absorbance at 280 nm was measured for peak detection and quantification. The fraction with 0.1% yield showing the most immunostimulatory activity was freeze- dried and dissolved in PBS at 0.5 or 1.0 μg/mL concentrations for lymphoproliferation, tumor cytotoxicity, and nitric oxide production as described below.

Spleen lymphocyte proliferation

All experiments were performed according to the guidelines established by the Beltsville Area Animal Care and Use Committee. The spleens of specific pathogen-free White Leghorn inbred chickens (Hy-vac, Adel, IA) at three weeks of age were removed and placed in a Petri dish with 10 mL of Hanks' balanced salt solution (HBSS) supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma, St. Louis, MO). Single cell preparation and lymphocyte proliferation were carried out as described (Lee *et al.,* 2007a). In brief, isolated splenocytes were adjusted to 1×10^7 cells/mL in enriched RPMI-1640 medium without phenol red (Sigma) supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. Splenocytes (100 μL/well) were cultured in 96-well flat bottom plates with 100 μL of crude safflower leaf methanol extract (250, 500 or 1,000 μg/mL), partially purified fraction (0.5 or 1.0 μg/mL), or 0.25 μg/mL of concanavalin A (Con A, Sigma) as a positive control. The cells were incubated at 41℃ in a humidified incubator (Forma, Marietta, OH) with 5% CO_2 for 48 h and cell proliferation was measured by using [^{3}H]-thymidine (Perkin Elmer Life Science, Boston, MA). The cells were radiolabeled for 4 h with 0.25 μCi/well of [^{3}H]-thymidine (Perkin Elmer Life Science), harvested using a semi-automated cell harvester (Tomtec, Orange, CT), and radioactivity was determined using a liquid scintillation counter (1450 Microbeta Wallac Trilux, Perkin Elmer Life Sciences). These cultures were performed in triplicate, and the radioactivity results were expressed as a stimulation index (SI) defined as the mean cpm of the stimulated culture divided by that of an unstimulated culture (medium control).

Tumor cell viability

RP9 chicken tumor cells, a retrovirus-transformed cell line commonly used to assess chicken NK-cell activity (Lillehoj and Chai,1988), were cultured at 1×10^6 cells/mL (100 μL/well) in 96-well plates with 100 μL/well of crude safflower leaf methanol extract (250, 500 or 1,000 μg/mL) and fraction (0.5 or 1.0 μg/mL) at 41℃ in a humidified incubator supplemented with 5% CO_2 for 48 h. Following incubation, cell viability was measured by using [^{3}H]-thymidine as described above or WST-8 (Cell-Counting Kit-8®, Dojindo Molecular Technologies, Gaithersburg, MD) with 2-[2-methoxy-4-nitrophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfophenyl]-2H-tetrazolium, monosodium salt as previously described (Lee *et al.,* 2007 a). The optical densities were measured at 450 nm using a microplate reader (BioRad, Hercules, CA) for the WST-8 assay. Recombinant human tumor necrosis factor-α (TNF-α, R & D Systems, Minneapolis, MN) was used at 1.0 μg/mL as a positive control.

Nitric oxide (NO) production by macrophages

HD11 macrophages were cultured at 1×10^7 cells/mL (100 μL/well) in 96-well plates with 100 μL/well of crude safflower leaf methanol extract (250, 500 or 1,000 μg/mL), fraction (0.5 or 1.0 μg/mL), and recombinant interferon-γ (1.0 μg/mL) (IFN-γ; ARS, Beltsville) as a positive control (Lillehoj and Choi, 1998) in a humidified incubator at 41℃ and 5% CO_2 for 24 h. Following incubation, 100 μL of cell culture supernatants were transferred to fresh flat-bottom 96-well plates, mixed with 100 μL of Griess reagent (Sigma) and the plates were incubated for 15 min at room temperature. The optical densities were measured at 540 nm using a microplate reader and nitrite concentration (μM) was determined using a standard curve generated with known concentrations of sodium nitrite.

Statistical analysis

Data analyses were performed using GraphPad InStat® Software (San Diego, CA). All data were expressed as means±SEM of triplicate cultures. Analysis of variance (ANOVA) and Tukey mean comparison test ($P<0.05$) were performed to test for differences between the groups and to analyze significant differences among the means.

Results

Effect of Safflower leaf on lymphoproliferation

A crude methanol extract of safflower leaf at 500 μg/mL stimulated splenocyte proliferation at a significantly higher level compared with the medium control (Fig. 1A) ($P<0.05$). When the methanol extract of safflower leaf was partially purified by Sephacryl S-200 column, the purified fraction induced a dose-dependent lymphoproliferation that was significantly greater than the medium control (Fig. 1B) ($P<0.05$). No toxic effect of the crude extract or column fractions on spleen cells was observed at any concentrations tested.

Inhibitory activity of Safflower leaf against tumor cells

Crude methanol extract of safflower leaf significantly

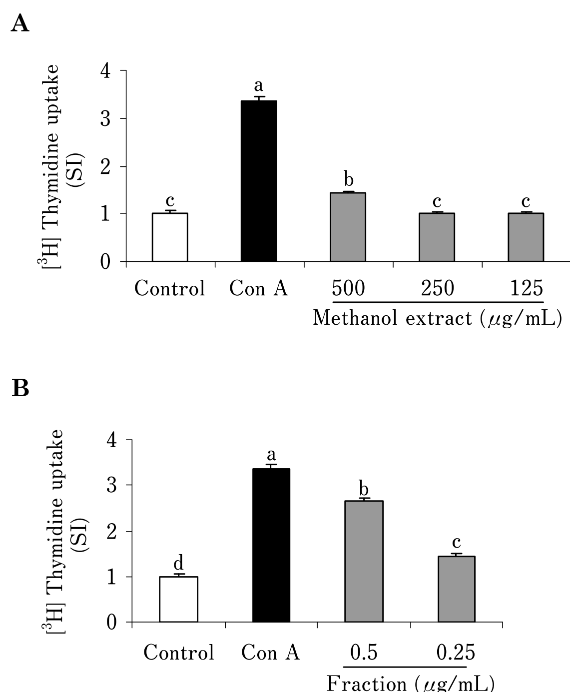

Fig. 1. Effects of crude methanol extract and purified fractions of safflower leaf on spleen lymphocyte proliferation. (A) Methanol extract and (B) purified fraction were tested at the indicated concentrations. Cell proliferation was measured by [^{3}H]-thymidine uptake and expressed as a stimulation index (SI) which was defined as the mean cpm of the stimulated culture divided by that of an unstimulated culture (medium control) as described in the Materials and Methods. Each bar represents the mean (SI)$\pm$SEM values obtained from triplicate culture wells. Within each graph, bars not sharing the indicated letters are significantly different ($P<0.05$) according to the Tukey test.

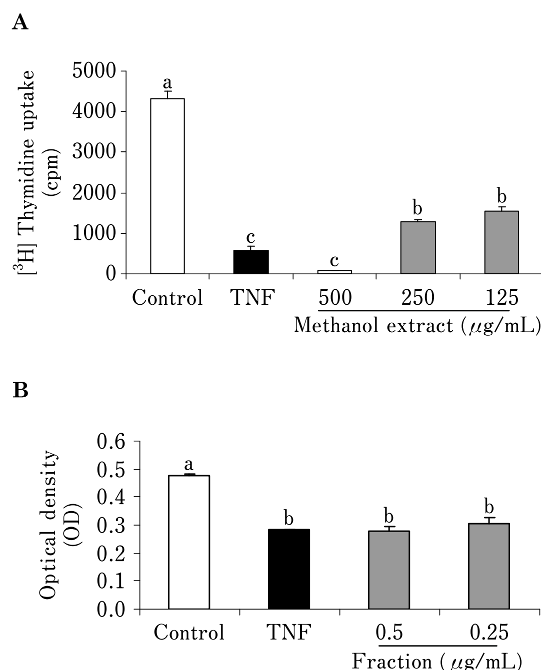

Fig. 2. Effects of crude methanol extract and purified fractions of safflower leaf on RP9 tumor cell viability. (A) Methanol extract and (B) partially purified fraction were tested at the indicated concentrations. Cell viability was measured by [^{3}H]-thymidine uptake (A) or the WST-8 assay (B) as described in the Materials and Methods. Each bar represents the mean$\pm$SEM values obtained from triplicate culture wells. Within each graph, bars not sharing the indicated letters are significantly different ($P<0.05$) according to the Tukey test.

decreased the viability of RP9 tumor cells at all concentrations tested compared with the medium control (Fig. 2A) ($P<0.05$). Its effect on RP9 tumor cells at 500μg/mL was similar to that of human recombinant TNF-α. The partially purified fraction retained the cytotoxic effect on RP9 tumor cells at 0.25μg/mL (Fig. 2B).

Effect of Safflower leaf on macrophage NO production

The crude methanol extract of safflower leaf stimulated NO production by HD11 macrophages at a significantly greater level compared with the medium control when tested at 250 and 500μg/mL (Fig. 3A) ($P<0.05$). Similar to the lymphoproliferation assay, NO inducing activity was contained in purified fraction at 0.25μg/mL following filtration using Sephacryl S-200 column and comparable to that of recombinant chicken IFN-γ (Fig. 3B).

Discussion

Safflower petals have been shown to activate macrophages (Ando *et al.,* 2002) and safflower seed oil inhibits the production of proinflammatory cytokines by monocytes (Takii *et al.,* 2003). However, much less is known about the biological effects of safflower leaf.

The chicken is an economically important food animal and its immune responses have been relatively well defined (Lee *et al.,* 2007b; Lee *et al.,* 2007c). In this study, we showed that safflower leaf, which is used as a traditional medicinal plant to enhance immunity against infectious diseases and tumors in humans, contains one or more components that stimulate chicken immunity as assessed by splenic lymphoproliferation, tumor killing and NO production. Previous studies have demonstrated that the effects of natural food and herbal products on host defense against microbes and tumors were correlated with their ability to influence lymphocyte proliferation (Lin *et al.,* 2005; Lee *et al.,* 2007a). The results of this study corroborate and extend these prior investigations and suggest that safflower leaf could be used as a dietary supplement to enhance immunity in commercially important veterinary animals. Our results also showed that safflower leaf contains potent anti-tumor activity, as demonstrated by the strong inhibitory effect on RP9 tumor cell viability at a

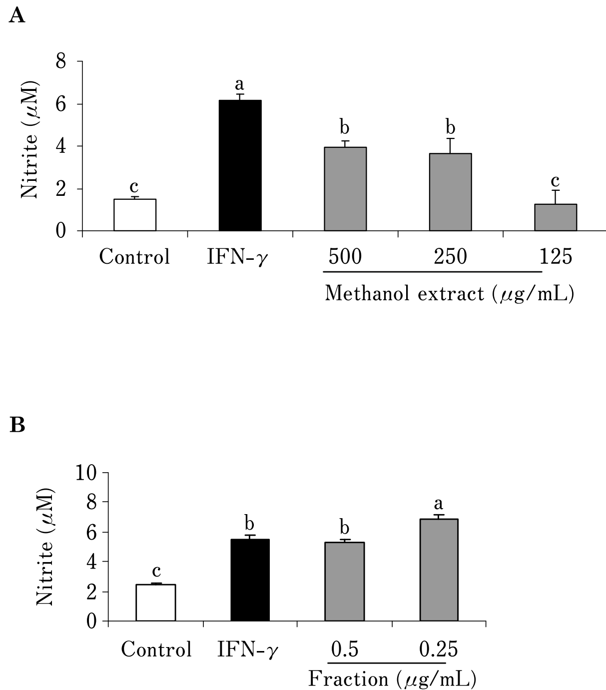

Fig. 3. Effects of crude methanol extract and purified fraction of safflower leaf on NO production by HD11 macrophages. (A) Methanol extract and (B) fraction were tested at the indicated concentrations. NO levels were measured as described in the Materials and Methods. Each bar represents the mean±SEM values obtained from triplicate culture wells. Within each graph, bars not sharing the indicated letters are significantly different ($P<0.05$) according to the Tukey test.

level comparable to that of TNF-α, one of the most potent anti-tumor molecules known (Tabata *et al.,* 1999). These results support those of a previous study demonstrating that components purified from safflower petals possess pharmacologic activity against cancer (Ando *et al.,* 2002).

Macrophages play an important role in host defense against infectious agents and tumors, in part, through the elaboration of effecter molecules such as NO (Lillehoj and Li, 2004; Santoni *et al.,* 1999), and many natural products stimulate immunity through activation of macrophages (Sugawara *et al.,* 1984; Suzuki *et al.,* 1994). A previous study showed that polysaccharides purified from dried safflower petals stimulated the production of inflammatory cytokines by peritoneal macrophages (Wakabayashi *et al.,* 1997). In this study, the partially purified fraction of safflower leaf induced significantly higher NO production at 0.25 and 0.50 μg/mL compared with the medium control and the level of NO production was comparable to that induced by interferon-γ. Interestingly, the lower concentration of purified fraction stimulated greater NO than the higher concentration, possibly due to the presence of a counteracting inhibitor in this partially purified fraction. In general, however, the more purified fractions of safflower leaf induced more effective immune responses and antitumor activity than the crude methanol extract.

In summary, extracts of safflower leaf showed high immunomodulating activity through the ability to enhance splenocyte proliferation and macrophage activation, and directly decrease the viability of a tumor cell line. These results provide a rationale basis for further purification, identification, and analysis of the immunostimulating components of this medicinal plant.

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