**In vitro Effects of Methanol Extracts of Korean Medicinal Fruits (Persimmon, Raspberry, Tomato) on Chicken Lymphocytes, Macrophages, and Tumor Cells**

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A variety of fruits have traditionally been used in Asian cultures to enhance resistance to diseases and treat cancers. However, limited information exists on the underlying mechanisms responsible for these effects. The present investigation was conducted to examine the ability of three Korean indigenous fruits (persimmon, raspberry and tomato) to stimulate lymphocyte proliferation and macrophage nitric oxide production as parameters of innate immunity, and to inhibit tumor cell growth. *In vitro* co-culture of chicken spleen lymphocytes with methanol extracts of persimmon (*Diospyros kaki*) or tomato (*Lycopersicon esculentum*) induced greater cell proliferation compared with cells treated with the vehicle control. Stimulation of chicken macrophages with extracts of persimmon or raspberry (*Rubus crataegifolius*), but not tomato, stimulated robust nitric oxide production to levels similar to that produced by interferon-γ. All fruit extracts uniformly inhibited the growth of chicken tumor cells *in vitro*. These results provide a rational basis for future studies investigating the effects of medicinal fruits on innate immunity and carcinogenesis in humans and animals.

**Key words:** fruit, immunomodulation, macrophage, splenocyte, tumor


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**Introduction**

Various fruits have been used in Asian cultures to enhance resistance to diseases, including infections and cancers. For example, persimmon (*Diospyros kaki*), raspberry (*Rubus crataegifolius*) and tomato (*Lycopersicon esculentum*) have traditionally been used as health-promoting medicinal foods in Korean society. While the active ingredients and mechanisms of action have not been completely characterized, multiple reports have provided a scientific basis for their effects. For example, extracts of persimmon peels showed protective effects against high glucose-induced oxidative stress in porcine renal epithelial LLC-PK\(_1\) cells (Yokozawa *et al.*, 2007) and altered lipid profiles and anti-oxidative activity in *in vitro* and *in vivo* studies (Lee *et al.*, 2006). Additionally, persimmon reduced body weight gain in rats fed a high-fat diet (Ahn *et al.*, 2002) and inhibited development of dermatitis and blocked spontaneous increase in serum IgE levels in NC/Nga atopic dermatitis-model mice (Kotani *et al.*, 2000). Lyophilized raspberry showed a strong chemopreventive effect on esophageal tumorigenesis (Kresty *et al.*, 2001) and modulated N-nitrosomethylbenzylamine metabolism in the esophagus and liver of Fischer 344 rats (Reen *et al.*, 2006). Tomato consumption has been positively correlated with reduced incidence of respiratory infections in humans (Fawzi *et al.*, 2000). Similarly, inhabitants of the Mediterranean region, in general, showed lower incidence of several important chronic diseases, including coronary heart disease and cancers, compared with other geographic regions, which was attributed to the higher than average consumption of lycopene and other tomato products (Weisburger, 2002). These examples of dietary enhancement of disease resistance provide a rational basis to further explore the immunostimulating properties of food substances in human and veterinary clinical medicine. In this regard, recent progress has been reported in the commercial application of plant phytonutrients to enhance...
innate immunity against infectious diseases and tumors (Masihi, 2000; Sampedro et al., 2004; Okamura et al., 2004; Lee et al., 2005; Usuki et al., 2006). The present investigation was conducted to examine the effects of organic extracts of persimmon, raspberry, and tomato on various parameters of chicken innate immunity and tumor cell growth. The chicken model was used since avians are economically important food animals with well-characterized innate immune systems (Tsurushita et al., 2004; Nishibori et al., 2004; Usuki et al., 2006; ) as well as to further extend our previous studies on phytonutrients and in this species (Lee et al., 2005; Lee et al., 2006; Lee et al., 2007c; Lee et al., 2008a; Lee et al., 2008b).

**Materials and Methods**

**Preparation of methanol extracts**

Methanol extracts of persimmon, raspberry, and tomato were obtained from the National Rural Resources Development Institute of the Rural Development Administration (Suwon, Korea). Extraction was carried out by adding 100 ml of 80% methanol to 30 g of fresh fruit samples with vigorous shaking for 48 hr at room temperature. The process was repeated three times, the extracts were pooled and dried using a rotary evaporator (Eyela, Irvine, CA), and the residues were freeze-dried and stored at −80°C. Prior to in vitro testing, the residues were dissolved in PBS at 125, 250 and 500 μg/ml and sterilized by membrane filtration through a 0.22 μm filter (Nalgene, Rochester, NY).

**Spleen lymphocyte proliferation**

All experiments were approved by the Beltsville Agricultural Research Center Animal Care and Use Committee. Specific Pathogen-Free White Leghorn SC inbred chickens (Hy-Vac, Adel, IA) were maintained at the Beltsville Agricultural Research Center Animal Facility and were fed ad libitum with a standard diet that was formulated to meet the recommended nutrient requirements (National Research Council, 1994). At 3 weeks of age, the animals were euthanized by cervical dislocation and spleens were placed in 10 ml of Hanks’ balanced salt solution supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma, St. Louis, MO). Single cell suspensions were prepared as described (Okamura et al., 2004; Lee et al., 2008a), adjusted to 1 × 10⁶ 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, and 100 μl/well were cultured in 96-well flat bottom plates at 41°C and 5% CO₂ for 48 hr with the fruit extracts or positive (5.0 μg/ml of concanavalin A, Sigma) or negative (PBS) controls. Following incubation, the cells were radiolabeled for 4 hr with 0.25 μCi/well of [³H]-thymidine (Perkin Elmer Life Science, Boston, MA), harvested using a semi-automated cell harvester (Tomtec, Orange, CT), and the incorporated radioactivity was determined by liquid scintillation counting (1450 Microbeta Wallac Trilux, Perkin Elmer Life Sciences). All cultures were performed in triplicate and the results for each treatment group were expressed as the stimulation index (SI) (SI = mean cpm of the fruit extract-treated cultures− mean cpm of PBS-treated cultures) (Lee et al., 2007c).

**Nitric oxide (NO) production**

HD11 chicken macrophages were cultured in triplicate at 1 × 10⁶ cells/ml in 96-well plates (100 μl/well) at 41°C and 5% CO₂ for 24 hr with fruit extracts or positive (1.0 μg/ml of recombinant chicken interferon-γ (IFN-γ)) (Song et al., 1997) or negative (PBS) controls (Lillehoj et al., 2004). Culture supernatants (100 μl) were transferred to clean 96-well plates, mixed with 100 μl of Griess reagent (Sigma), incubated for 15 min at room temperature, optical density at 540 nm (OD₅₄₀) was measured, and the nitrite concentration was determined using a standard curve generated with known concentrations of sodium nitrite.

**Inhibition of tumor cell growth**

RP9 chicken B-lymphoma cells (Sharma and Okazaki, 1981) were cultured in triplicate at 1 × 10⁶ cells/ml in 96-well plates (100 μl/well) at 41°C and 5% CO₂ for 48 hr with fruit extracts or positive (lipopolysaccharide-induced TNF-α factor [LITAF]) (Hong et al., 2006) or negative (PBS) controls. Tumor cell viability was measured using the WST-8 reagent (Cell-Counting Kit-8®, Dojindo Molecular Technologies, Gaithersburg, MD). OD₅₇₀ values were measured as an indicator of cell numbers as described (Miyamoto et al., 2002).

**Statistical analyses**

Statistical analyses were performed using SPSS software (SPSS 15.0 K for Windows, Chicago, IL). All data were expressed as mean ± SEM values. The ANOVA test was used to test for differences between the different treatment groups. To analyze differences between the mean values, the Duncan’s multiple range test was used. Differences were considered statistically significant at P < 0.05 (Sokal and Rohlf, 1969).

**Results**

**Spleen lymphocyte proliferation**

Persimmon and tomato extracts at 500 μg/ml significantly enhanced splenocyte proliferation compared with the PBS control (Fig. 1A). The proliferation induced by the persimmon extract was 73.3% of that produced by 5.0 μg/ml of concanavalin A. Increased proliferation also was observed at 250 μg/ml of persimmon (Fig. 1B) and at 125 μg/ml of tomato extracts (Fig. 1C). In contrast, methanol extracts of raspberry did not stimulate splenocyte proliferation.

**NO production**

Persimmon and raspberry extracts significantly increased NO production by HD11 macrophages at 500 μg/ml compared with PBS-treated cells (Fig. 2A). Increased NO production also was observed at 250 μg/ml of persimmon (Fig. 2B) and at 250 and 125 μg/ml of raspberry extracts (Figs. 2B-C). In contrast, methanol extracts of tomato did not stimulate NO production.
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Fig. 1. Effect of methanol extracts of persimmon, raspberry and tomato on splenocyte proliferation. Chicken splenocytes (1×10^6 cells/well) were cultured for 48 hr in 96-well plates with 100 μl/well of the indicated fruit extracts at (A) 500 μg/ml, (B) 250 μg/ml, or (C) 125 μg/ml, 5.0 μg/ml of concanavalin A (positive control), or PBS (negative control). Following incubation, cell proliferation was determined by [3H]-thymidine incorporation as described in the Material and Methods and expressed as the stimulation index (SI) (SI=mean cpm of the fruit extract-treated cultures ÷ mean cpm of PBS-treated cultures). Each bar represents the mean±SEM. Bars not sharing the indicated letters are significantly different (P<0.05) according to the Duncan’s multiple range test.

Tumor cell growth

Extracts of persimmon, raspberry, and tomato significantly reduced the growth of RP9 tumor cells compared with the PBS control at all concentrations tested (Figs. 3 A-C). The levels of growth inhibition observed in all cases were comparable to that produced by the LITAF positive control.
Fig. 3. Effects of methanol extracts of persimmon, raspberry and tomato on tumor cell growth. Chicken RP9 tumor cells (1 × 10^5 cells/well) were cultured for 48 hr with 100 μl of the indicated fruit extracts at (A) 500 μg/ml, (B) 250 μg/ml, and (C) 125 μg/ml, 1.0 μg/ml of LITAF (positive control) or PBS (negative control). Tumor cell viability was determined with WST-8 reagent as described in the Materials and Methods. Each bar represents the mean ± SEM. Bars not sharing the indicated letters are significantly different (*P < 0.05) according to the Duncan’s multiple range test.

Discussion

In vitro cell culture systems have provided a wealth of information on the biological effects of phytochemicals and phytonutrients derived from fruits and vegetables, and on the mechanisms through which diets high in these foods reduce the risk of chronic diseases (Steinmetz et al., 1996; Lee et al., 2007c). Previous studies have demonstrated the stimulatory effects of natural foods and traditional medicinal plants on lymphocyte proliferation (Lee et al., 2005; Pandey et al., 2005; Sun et al., 2006). However, little is known about the biological effects of most long-established medicinal Korean fruits, particularly those evaluated in this study, and no reports have described their effects on innate immunity in terms of the parameters examined in this paper. Our results provided clear evidence that methanol extracts of persimmon, raspberry, and/or tomato enhanced in vitro splenocyte proliferation and macrophage NO production, and reduced tumor cell growth, compared with PBS-treated cells. In general, our results corroborate previous studies that documented the beneficial properties of these fruits and others. For example, feeding of persimmon leaves to rats on a high-fat diet suppressed body weight gain and lowered plasma and hepatic lipid concentrations compared with animals given the high lipid diet alone (Lee et al., 2006). Extracts of persimmon leaves also were shown to inhibit the development of dermatitis in NC/Nga mice (Kotani et al., 2000). Finally, treatment of experimental mice with oligonol, a compound produced by the oligomerization of polyphenols such as proanthocyanidin from persimmon, grape, and apple, decreased the severity of infection-induced inflammation (Tomobe et al., 2007).

Macrophages play a significant role in host defense against infectious agents and tumors mediated, in part, by the elaboration of soluble inflammatory molecules such as NO (Stuehr and Nathan, 1989). In this study, raspberry extracts significantly increased NO production by HD11 macrophages compared with the vehicle control and this effect was comparable to that of IFN-γ, a potent cytokine involved in the differentiation, maturation and proliferation of hematopoietic cells that enhances nonspecific immunity to tumors, as well as to microbial, viral and parasitic pathogens (Lillegard and Choi, 1998; Lee et al., 2008b). Interestingly, tomato extract did not stimulate NO production in spite of previously reported studies demonstrating that dietary supplementation with tomato-derived lycopene or carotenoids reduced the activity of 3-hydroxy-3-methyl glutaryl coenzyme A (HMGCbA) reductase, the rate limiting enzyme in cholesterol synthesis (Fuhrman et al., 1997; Gorinstein et al., 2000). Nevertheless, given the involvement of NO during innate immune response, macrophage-mediated killing, and direct growth inhibition of microorganisms and tumor cells as formerly documented (Engel et al., 2002; Stuehr et al., 1989), our results clearly imply an important role for dietary fruits in the enhancement of innate immunity.

Host immune function is critically essential in the response to tumorigenesis (Lee et al., 2005). In our previous study, we demonstrated that traditional Asian medicinal plants such as dandelion and safflower inhibited tumor cell growth in vitro (Lee et al., 2005; Lee et al., 2007c; Lee et al., 2008b). This effect can now be extended to medicinal fruits. The anti-tumor activities of these foods were comparable to that mediated by LITAF, a transcription factor originally cloned from the LPS-stimulated THP-1
human macrophage cell line. Treatment of cells with LITAF induces the expression of TNF-α, one of the most potent anti-tumor molecules known (Hong et al., 2006). The ability of persimmon and tomato to inhibit the growth of tumor cells may be related to the stimulation of cancer-specific cytotoxic T cell stimulation (Takii et al., 2003), while the high anti-tumor activity of raspberry may be explained by macrophages NO production (Lee et al., 2007c; Stuehr et al., 1989).

In conclusion, we demonstrate for the first time that the methanol extracts of three common Korean medicinal fruits are capable of stimulating in vitro parameters of innate immunity, as well as mediating the suppression of tumor cell growth. Further studies to elucidate the nature of the immunomodulating activities of these and other phytonutrients from fruits and vegetables will facilitate the development of novel dietary strategies against infectious diseases and tumors in both humans and domestic food animals.

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References


Lee SH, Lillehoj HS, Park DW, Hong YH and Lin JJ. Effect of Pediococcus and Saccharomyces-based probiotic (MitoMax®) on coccidiosis in broiler chickens. Comparative Immunology, Microbiology and Infectious Diseases, 30: 261–268. 2007b.


Okamura M, Lillehoj HS, Raybourne RB, Baru US and Hechert RA. Cell-mediated immune responses to a killed Salmonella enteritidis vaccine: Lymphocyte proliferation, T-cell changes and interleukin-6 (IL-6), IL-1, IL-2 and IFN-γ production. Comparative Immunology, Microbiology and Infectious Diseases, 27: 255–272, 2004.


