In Vitro Analysis of the Immunomodulating Effects of *Allium Hookeri* on Lymphocytes, Macrophages, and Tumour Cells

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The present study investigated the effects of ethanol extracts of *Allium hookeri* (leaf, root, and fermented root) on parameters of innate immunity, tumour cell viability and antioxidant effect *in vitro*. Innate immunity was measured by spleen lymphocyte proliferation, nitric oxide production by chicken macrophage HD11 cells and suppressive effect on tumour cell viability was assessed using chicken RP9 cells. Free radical scavenging capacity as a measure of antioxidant capacity was determined by 0.15 mM of DPPH solution. *In vitro* culture of chicken spleen lymphocytes with ethanol extract of *Allium hookeri* (62.5−500 μg/mL) significantly induced higher proliferation compared with media control. Stimulation of macrophages with ethanol extract of *Allium hookeri* (62.5−500 μg/mL) showed increased Nitric oxide production. Tumor cells growth was significantly inhibited by extracts of *Allium hookeri* at 15.6− 125 μg/mL compared with medium control and all extracts exhibited greater than 80% scavenging activity at 1000 μg/mL compared with ethanol vehicle control. Above all, fermented root extracts showed strongest effects on antioxidant activity compared to leaf and root extracts.

Key words: *Allium hookeri*, innate immunity, lymphocytes, macrophage, poultry, tumour cells

Introduction

Antibiotic growth promoters (AGPs) are commonly used as a dietary supplement to improve growth performance of livestock and poultry (Lillehoj and Lee, 2012; Lee *et al.*, 2015a). However, the use of AGPs in animal agriculture is becoming increasingly restricted, and researchers are attempting to find alternatives to antibiotics for maintaining good growth performance and reducing the negative effects of diseases. In recent years, an increasing number of research trials with the use of feed additives for safe, high-quality meat production in the animal industry has been reported (Lee *et al.*, 2011; Lillehoj and Lee, 2012; Xu *et al.*, 2015a). Many documented in-feed supplements offer alternatives to antibiotics and have been linked to enhanced growth performance and innate immunity. In particular, certain plant-derived phytonutrients have been shown to provide growth-promoting and immune-enhancing effects (Banfield *et al.*, 2002; Van Lunen, 2003; Kim *et al.*, 2015a).

In the case of poultry, a variety of plant-derived feed additives have been reported for their economic benefits in animal production. According to recent reports, supplementation of animal feed with sulforaphane, isolated from broccoli sprout extracts, and essential oils such as oregano, thyme, and rosemary has been shown to enhance intestinal health in broiler chickens (Kristin *et al.*, 2012; Lillehoj and Lee, 2012; Xu *et al.*, 2015b). Similarly, oregano and rosemary oils have been shown to improve meat quality and prevent lipid oxidation in breast and thigh meat (Basmacioglu *et al.*, 2004). Moreover, Hernadez *et al.* (2004) reported that feed containing essential oils (oregano, cinnamon, and labiatae extract) reduces daily feed intake of broilers and improves feed conversion compared with that of control birds.

A few studies have focused on the efficacy of medicinal plants on improving innate immunity, enhancing antioxidant capacity, and inhibiting tumour cell growth (Lee *et al.*, 2007, 2008; Kim *et al.*, 2013). Although a diverse array of me-

dicinal plants have been traditionally used in Asian culture to enhance innate immunity and for treatment of illnesses and cancer, only a few studies have characterised the effects of these medical plants on immunity.

Allium hookeri, a member of the family Alliaceae subgenus Amerallium, is found in Ceylon, Greece, Yunnan, Southern China, Bhutan, Sri Lank, and India and has been used by locals to treat cough and cold and to heal burns and wounds (Sharma *et al*., 2011). The root of *A. hookeri* contains an abundance of organo-sulphur compounds, volatile sulphur compounds, proteins, prostaglandins, fructans, vitamins, and polyphenols as well as *Allicin*, the major flavour compound of garlic (*A. sativum*) (Dziri *et al*., 2012). *Allicin* and organic sulphur compounds are known to reduce cholesterol levels, decrease the risk of heart attack, and exert anti-inflammatory effects (Bae and Bae, 2012; Kim *et al*., 2012). However, few studies have characterised the effects of these medicinal plants on immunity.

The aim of this study was to examine the potential immune-enhancing properties of ethanol extracts of leaf, root, and fermented root of *A. hookeri* on the innate immune function of poultry. Cell proliferation and nitric oxide (NO) production assays were conducted to assess the effects of plant extracts on innate immunity. Additionally, avian tumour cells were used to further characterise the inhibitory effects of *A. hookeri*, and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assays were used to investigate the free radical scavenging activity of *A. hookeri* extracts.

Materials and Methods

Preparation of Samples

A. hookeri was obtained from the Agricultural Development & Technology Centre (Sunchang, South Korea). The voucher specimen (RDAAH15) is preserved at the National Institute of Agricultural Sciences (Jeonju, Korea). Fermented *A. hookeri* (patent# 10-2014-0154145) was obtained from the Centre for Healthcare Technology Development of Chonbuk National University, South Korea.

All samples (leaf, root, and fermented root) were subjected to freeze drying (PVTFD 10R; Ilsin Lab, Yangju, Korea), ground in a 40-mesh grinder (FM909T; Hanil, Wonju, Korea), and freeze dried. The dried powder was stored at $-80℃$ until use.

Ethanol Extraction

Ethanol extraction was carried out by adding 100 mL of 80% ethanol to 10-g samples of *A. hookeri* leaf, root, or fermented root and incubating at $18℃$ for 48 h with vigorous shaking. The mixtures were then filtered using Whatmann filter paper No. 2 and concentrated using a rotary evaporator (IKA, NC, USA). The remaining residues were freeze-dried and stored at $-80℃$. Before testing, the samples were dissolved in enriched RPMI-1064 medium without phenol red (Sigma, St. Louis, MO, USA) supplemented with 1μg/mL 5-fluorocytosine and 100 U/mL penicillin. The samples were then sterilised by membrane filtration through a 0.2-μm filter (Nalgene, Rochester, NY, USA) before use.

Proliferation of Splenic Lymphocytes

All experimental protocols were approved by the Animal Care Committee of the Beltsville Agricultural Research Center. At 4 weeks of age, specific pathogen-free Ross/Ross broilers (Longenecker's Hatchery, Elizabethtown, PA, USA) were euthanatised by cervical dislocation. Spleens were collected from 3 birds (raised on regular feed without any additives) and placed in petri dishes with 10 mL Hank's balanced salt solution supplemented with 100 U/mL penicillin and 100μg/mL streptomycin (Sigma). Collected spleens were isolated from each spleen individually and pooled before the start of the *in vitro* experiment. Single lymphocytes were prepared as previously described (Lee *et al*., 2008). Briefly, the spleens were gently passed through a cell strainer, and the resulting single cells were separated using Histopaque-1077 (Sigma) density gradient medium by centrifugation. Isolated spleen cells were adjusted to 1×10^{6} cells/mL in enriched RPMI-1604 medium without phenol red (Sigma) supplemented with 10% foetal bovine serum, 100 U/mL penicillin, and 100μg/mL streptomycin. Splenic cells (100μL/well) were cultured in 96-well flat-bottom plates with 100μL/well of *A. hookeri* leaf, root, or fermented root ethanol extract (500, 250, 125, and 62.5 μg/mL). The positive control was concanavalin A (Con A; 20μg/mL; Sigma), and medium alone was used as a negative control. All extracts were added to 3 well /group and the cells were cultured at $41℃$ in a humidified incubator (Forma, Marietta, OH, USA) with 5% carbon dioxide/95% air for 48 h. Cell proliferation was determined with 2-(2methoxy-4-nitrophenyl) -3-(4-nitrophenyl) -5-(2, 4-disulfophenyl) -2H-tetrazoilum (WST-8, Cell-Counting Kit-8; Dojindo Molecular Technologies, Gaithersburg, MD, USA) as previously described (Lee *et al*., 2007). The results were expressed as optical density measured at 450 nm using a microplate reader (ELx800^{TM}; BioTek, Winooski, VT, USA).

Inhibition of Tumour Cell Growth

Retrovirus-transformed chicken RP9 cells were used to test the inhibitory effects of *A. hookeri* extract on tumour cell growth. RP9 cells were cultured at 1×10^{6} cells/mL (100 μL/well) in 96-well plates with 100μL/well of plant extracts (125, 62.5, 31.3, and 15.6 μg/mL). Recombinant chicken NK lysin, which was produced as previously described (Hong *et al*., 2006), was used as a positive control (5 μg/mL), and media alone was used as a negative control. After incubation for 48 h, the optical density was measured at 450 nm for the WST-8 assay.

Induction of NO Production in Macrophages

Induction of NO production was performed using the chicken macrophage cell line HD11. HD11 cells were cultured in triplicate in 96-well plates at a concentration of 1×10^{6} cells/well with 100μL/mL plant extracts (500, 250, 125, 62.5, and 31.25 μg/mL), 5.0 μg/mL lipopolysaccharide (LPS) as a positive control (Lillehoj and Li, 2004), or medium alone as a negative control in a humidified incubator at $41℃$ with 5% CO_2 for 24 h. After incubation, 100μL/mL cell culture supernatant was transferred to flash flat-bottom 96-well plates, mixed with 100μL/mL Griess reagent (Sigma),

and incubated for 15 min at room temperature. Absorbance was measured at 540 nm on a microplate reader, and the NO concentration was determined using a standard curve generated with known concentrations of sodium nitrite (Kaspers *et al.*, 1994).

Free Radical Scavenging Activity

Free radical scavenging activity of plant extracts (1000, 500, 250, 125, and 62.5 μg/mL) was measured using DPPH. For this assay, 100 μg/mL L-ascorbic acid (as a positive control) and ethanol vehicle alone (as a negative control) were mixed with 0.15 mM DPPH solution in ethanol. The reaction mixture was shaken intensely at room temperature for 30 min, and samples were analysed in triplicate. Decreased optical density was measured with a microplate reader at 517 nm, and the percent inhibition was calculated using the following formula: ([control−sample] / control)×100%.

Statistical Analysis

All experiments were carried out in triplicate and repeated three times. Statistical analysis was performed using SPSS software (SPSS 22.0 for Windows, Chicago, IL, USA), and all data were expressed as the mean±SD of triplicate cultures. Analysis of variance (ANOVA) and *t*-tests were used to evaluate differences between the mean value of negative control-treated and extracted samples. Differences with *p* values of less than 0.05 were considered statistically significant.

Results

Effects of A. hookeri on Splenic Lymphocyte Proliferation

The concentration response of *A. hookeri* extracts (62.5−500 μg/mL) on the proliferation of spleen cells for 48 h is illustrated in Fig. 1. Compared with the medium control, all *A. hookeri* extracts (62.5−500 μg/mL) significantly stimulated splenocyte proliferation in a concentration-dependent manner. Among the three samples, leaf extract showed higher stimulatory activity than the other extracts (root and fermented root) at 500 μg/mL. The root and fermented root extracts showed similar stimulatory effects at all doses, and at 500 μg/mL level, the stimulatory effect was similar to that of Con A. No toxic effects of plant extracts on spleen cells were observed at any of the concentrations tested (Fig. 1).

Inhibitory Activity on Tumour Cells

The inhibitory effects of *A. hookeri* extracts on chicken tumour cells are shown in Fig. 2. Compared with the medium control, all *A. hookeri* extracts significantly decreased RP9 tumour cell growth in a concentration-dependent manner. The leaf extract showed the highest inhibitory activity at 15.6−125 μg/mL, with the strongest effect at 62.5 μg/mL (Fig. 2).

Nitric Oxide Production

The stimulatory effects of *A. hookeri* on the production of NO by HD11 macrophages are shown in Fig. 3. Compared with the medium control, all *A. hookeri* extracts stimulated NO production by macrophages in a concentration-independent manner. The highest NO production was observed for leaf extracts at 62.5−500 μg/mL. The root and fermented root extracts showed similar capabilities (Fig. 3).

Free Radical Scavenging Capacity

The free radical scavenging capacity of *A. hookeri*, as determined by DPPH assay, is shown in Fig. 4. The results showed that all extracts exhibited greater than 80% scavenging activity at 1000 μg/mL compared with the ethanol vehicle control. A similar trend in free radical scavenging ability was observed in leaf and root extracts (125−1000
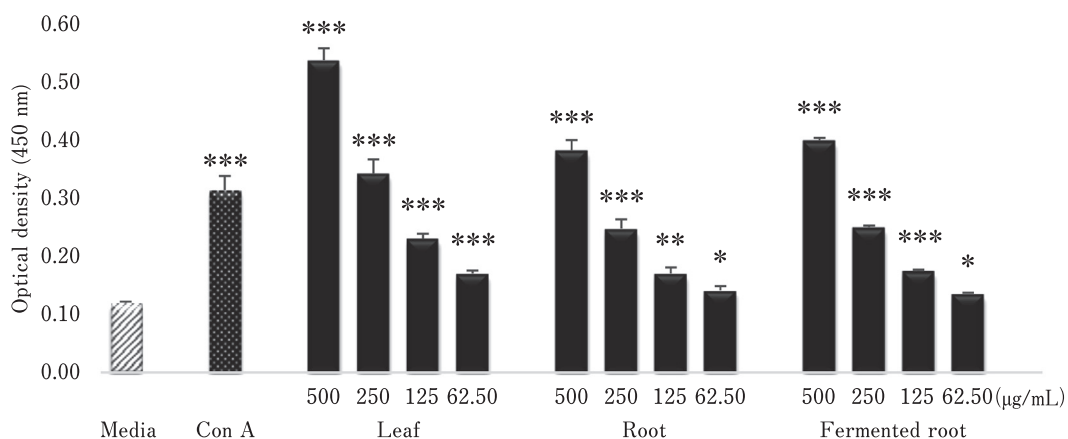

Fig. 1. **Effects of ethanol extracts of *A. hookeri* leaf, root, and fermented root on splenocyte proliferation.** Chicken spleen cells were incubated with the indicated concentrations of each extract, Con A (20 μg/mL), or medium alone as a negative control. After 48 h, cell viability was measured by CCK-8 assay. Each bar represents the mean±SD (*n*= 3). Each value was compared by t-test with the control (media alone). Significant differences are indicated as * *P*＜0.05, ** *P*＜0.01, and *** *P* ＜0.001.
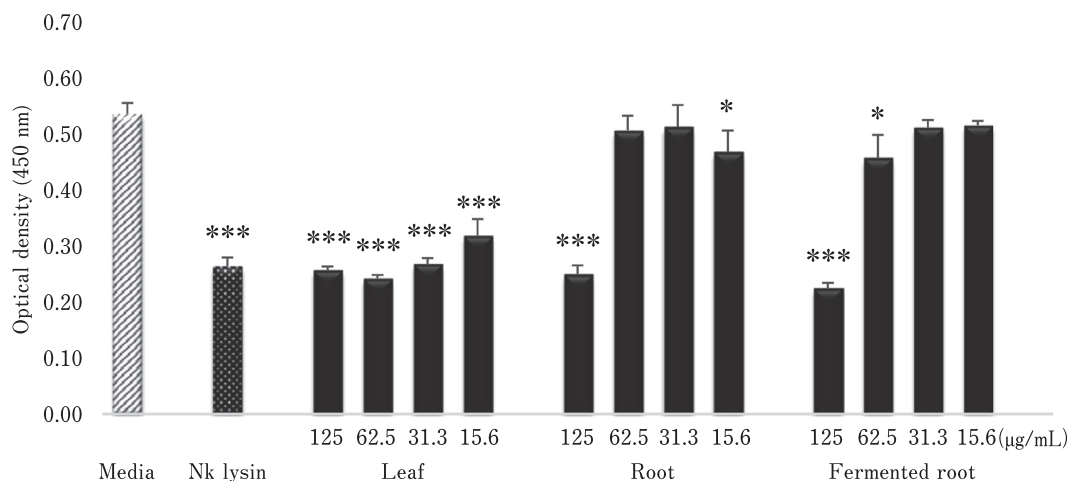
Fig. 2. **Inhibitory effects of *A. hookeri* leaf, root, and fermented root on the viability of RP9 tumour cells.** RP9 cells were incubated with the indicated concentration of each extract, recombinant chicken NK-lysin (5 μg/mL), or medium alone as a negative control. After 48 h, cell viability was measured by CCK-8 assay. Each bar represents the mean ±SD (*n*=3). Each value was compared by t-test with the control (media alone). Significant differences are indicated as * *P*<0.05 and *** *P*<0.001.
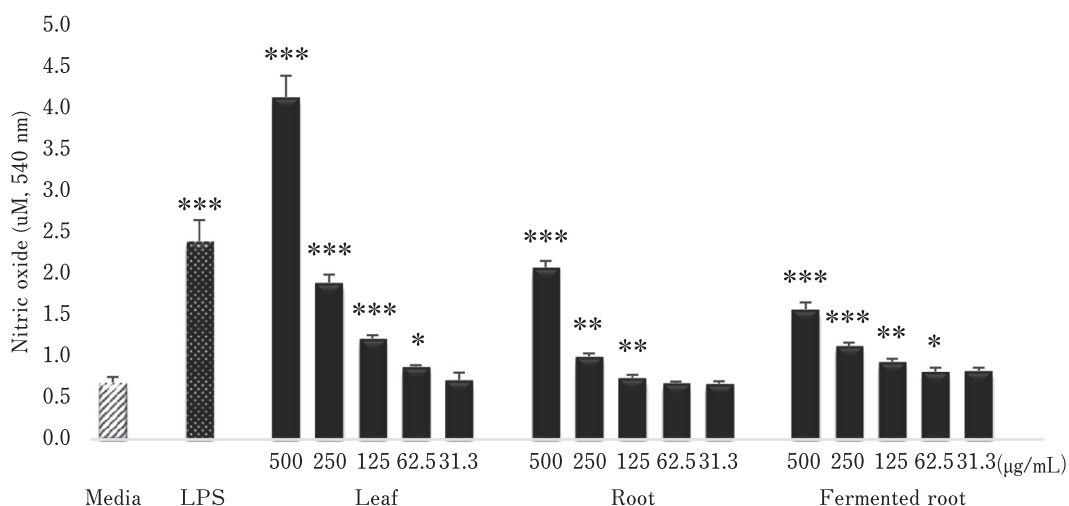

Fig. 3. **Effects of *A. hookeri* leaf, root, and fermented root extracts on nitric oxide production.** HD11 macrophage cells were incubated with the indicated concentration of each extract, LPS (5 μg/mL), or medium alone as a negative control. After 24 h, cell viability was measured by CCK-8 assay. Each bar represents the mean±SD (*n*=3). Each value was compared by t-test with the control (media alone). Significant differences are indicated as * *P*<0.05, ** *P*<0.01, and *** *P* <0.001.

μg/mL). Generally, fermented root extracts showed higher DPPH radical scavenging capacity than other extracts at the same concentration, and the activities were identical at 500 and 1000 μg/mL (Fig. 4).
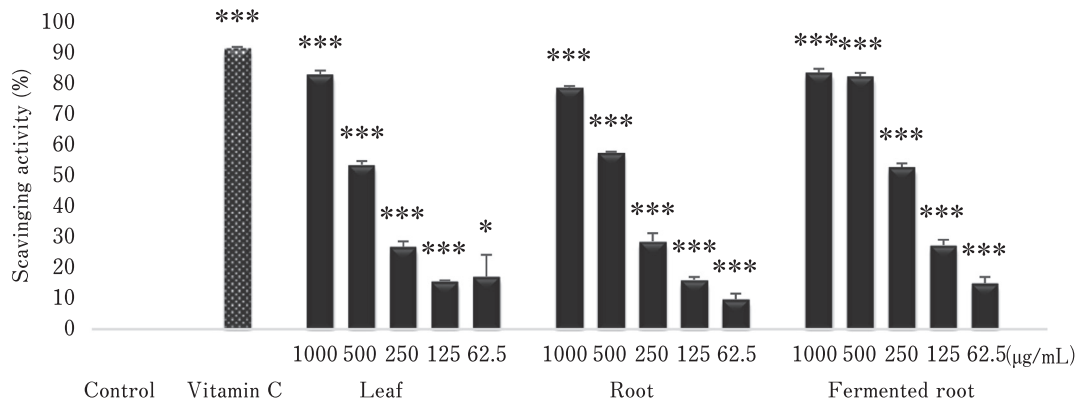
Fig. 4. **Antioxidant effects of *A. hookeri* leaf, root, and fermented root extracts.** 2, 2-Diphenyl-1-picrylhydrazyl (DPPH; 0.15 mM) in ethanol was mixed with the indicated concentration of each plant extract, 100 μg/mL vitamin C as a positive control, or ethanol only as a blank control. The OD at 517 nm was measured, and the scavenging ability was calculated. Each bar represents the mean ± SD ($n=3$). Each value was compared by t-test with the control (ethanol alone). Significant differences are indicated as $*P<0.05$ and $***P<0.001$.

Discussion

The current investigation was carried out to assess the potential immune-enhancing properties of leaf, root, and fermented root extracts derived from *A. hookeri* on innate immunity and tumour cell viability in chickens. The immune-stimulating effects of *A. hookeri* have been reported in mice (Kim *et al*., 2015b; Lee *et al*., 2015b). Supplementation of standard diet with fermented and nonfermented *A. hookeri* powder has various effects, including regulation of blood lipid levels and antidiabetic activities, in type 2 diabetic mice (Kim *et al*., 2015b; Lee *et al*., 2015b). Additionally, an *in vitro* study characterised the anti-inflammatory and antioxidant effects *of A. hookeri* in rat macrophages (Kim *et al*., 2012). However, until now, no reports exist describing the effects of *A. hookeri* in chickens. The chicken is an economically important animal for both meat and egg production; therefore, a better understanding of the host immune function of birds is necessary.

A. hookeri is a wild herb that has been used to treat cancer and inflammatory diseases in India and Myanmar due to its richness in phytonutrients. The plant is a rich source of ascorbic acid, polyphenols, flavonoids, and organic natural sulphur (methyl sulfonyl methane) (Singh and Singh, 2014). Organosulphur compounds have been shown to have diverse biological beneficial effects, such as antioxidant effects and anti-inflammatory properties (Vazquez-Prieto and Miatello, 2010). Therefore, the antioxidant effects of *A. hookeri* in the current study may be related to the presence of organosulphur compounds.

In the present study, we demonstrated for the first time that the ethanol extracts of *A. hookeri* (leaf, root and fermented root) activated innate immunity and inhibited the growth of

tumour cells in poultry. *A. hookeri* extracts increased the proliferation of spleen lymphocytes and inhibited the growth of tumour cells in a concentration-dependent manner. *A. hookeri* also induced nitric oxide secretion by chicken macrophages. The immune system is made of a complex network of cells, such as lymphocytes and macrophages, that work together to defend the body against foreign substances, such as bacteria or viruses (Kim *et al*., 2015a). Among the various types of immune cells, macrophages play an important role in host defence through regulation of lymphocyte activation and proliferation. In addition, macrophages play an essential role in the activation of T and B lymphocytes by antigen and allogenic cells (Elhelu, 1983). Nathan and Xie (1994) reported that lipopolysaccharide (LPS) -stimulated macrophages and pro-inflammatory cytokines, such as interferon-c (IFN-c) and tumour necrosis factor α (TNF-α), produce large amounts of NO, an important signalling molecule. Previous studies have indicated that NO regulates cell proliferation and inhibits angiogenesis, tumour growth, and metastasis (Napoli *et al*., 2013). Moreover, NO has been reported to have tumouricidal activity by induction of apoptosis in a concentration-dependent manner (Nicotera *et al*., 1995). In this report, we showed that *A. hookeri* extracts exhibited strong antitumour activity, which may be related to NO production by macrophages. Previous study by Kim *et al*. (2012) reported the anti-inflammatory effect of *A. hookeri* in LPS-induced mouse macrophage cells. In that test, the treatment with methanol extracts of roots significantly inhibited LPS-induced nitric oxide formation in dose-dependent manner and also decreased TNF-a and IL-6 production. This contrast in results can be explained that first, no LPS was used for the stimulation of chicken macrophage cells in the present study. LPS stimulates immune responses by inter-

acting with the membrane receptor CD14 to induce the generation of cytokines such as tumour necrosis factor (TNF) -α, interleukin (IL)-1, and IL-6 (Meng and Lowell, 1997). Secondly, discrepant results can be explained by source of macrophages (chicken and mouse).

Many studies have reported the beneficial effects of fermentation on improving the activity of plant phenolic compounds. Schubert *et al.* (1999) reported high antioxidant activity and potent anti-inflammatory activity of fermented pomegranate juice. Oh *et al*. (2012) reported the effects of fermented Oyaksungisan on LPS-stimulated macrophages. They compared the anti-inflammatory activity of nonfermented and fermented Oyaksunkisan and showed that the anti-inflammatory activity increased with fermentation. Consistent with this, in the present study, we found that the fermented root extract of *A. hookeri* had the strongest effects on antioxidant activity compared with the nonfermented leaf and root and showed identical antioxidant activity at 500 and 1000 μg/mL.

The results obtained indicate that, the ethanol extracts of *A. hookeri* (leaf, root, and fermented root) significantly enhanced the proliferation of spleen lymphocytes, inhibited tumour cell viability, and stimulated NO production by HD11 macrophages compared to medium control. The leaf extracts effectively increased the proliferation of spleen lymphocytes and NO secretion by chicken macrophages and inhibited the growth of tumour cells, whereas fermented root extracts showed the strongest effects on antioxidant activity. These results suggest that *A. hookeri* had immune-stimulating properties that could be beneficial for poultry health.

Acknowledgments

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