

Broussonetia kazinoki Siebold stimulates immune response in ovalbumin-immunized mice

Da Young Jung¹, HyeKyung Ha¹, Hoyoung Lee^{1,2}, Jin-Ah Lee¹, Seung-Il Jeong³,
Young-Jae Choi⁴, Hyeun-Kyoo Shin^{1*}

¹Herbal Medicine EBM Research center, Korea Institute of Oriental Medicine, 483 Exporo, Yuseong-gu, Daejeon, 305-811, Republic of Korea

²Hongcheon Institute of Medicinal Herb, 101 Yeonbong-ri, Hongcheon-eup, Hongcheon, Gangwon, 250-930, Republic of Korea

³Jeonju Biomaterials Institute, Jeonju, 561-360, Republic of Korea

⁴ChunYang Paper Co., Ltd., 16-12, 3 Ga, Pungnam-Dong, Wansan-Gu, Jeonju, Jeonbuk, Republic of Korea

Objective: To evaluate the immune-stimulatory potential of extracts of *Broussonetia kazinoki* Siebold (BK) on specific cellular and humoral immune responses in ovalbumin (OVA)-immunized mice.

Material and Methods: C57BL/6 mice were immunized intraperitoneally with OVA/alum (100 µg/200 µg) on days 1, 8, and 15. BK (100, 300 or 1000 mg/kg) was given to mice orally for 21 days (from day 1 to day 21). At day 22, OVA-, lipopolysaccharide (LPS)- and concanavalin A (Con A)-stimulated splenocyte proliferation and OVA-specific and total antibodies were measured in plasma. Further, the effects of BK on expression of cytokine mRNA in OVA-immunized mice splenocytes were evaluated by RT-PCR analysis.

Results: BK significantly enhanced OVA-, LPS-, and Con A-induced splenocyte proliferation in OVA-immunized mice ($p < 0.01$). BK also significantly enhanced total IgM and OVA-specific IgG1 levels in plasma compared with the OVA control group. Moreover, BK up-regulated significantly the expression of mRNA level of IL-2 and IFN- γ in splenocytes.

Conclusions: BK has immune-stimulating activity in an OVA-immunized mouse model system, enhancing the Th1 immune response. BK showed no cytotoxicity in this system, suggesting that BK may be a safe and effective adjuvant in humans.

Key Words : *Broussonetia kazinoki* Siebold, OVA, splenocyte proliferation, antibody, adjuvant, immune response

Introduction

Adjuvants significantly influence immune responses and can tilt the immune system to favor Th1- or Th2-type responses^{1,2}. The Th1 immune response, mediated by Th1 helper cells, is characterized by production of the cytokines IL-2, TNF- β and IFN- γ , and enhanced synthesis of IgG2a, IgG2b and IgG3.

Moreover, the Th1 immune response is a prerequisite for the production of cytotoxic T lymphocytes (CTLs). In contrast, the Th2 response is characterized by production of the cytokines IL-4, IL-5 and IL-10, and the enhanced production of IgG1 and secretory IgA. Currently, aluminum hydroxide (alum) remains the most widely used adjuvant in human vaccines³. In mice, alum primarily induces increases in IgG1, and

• Received : 11 February 2011

• Revised : 21 April 2011

• Accepted : 22 April 2011

• Correspondence to : Hyeun-Kyoo Shin

Herbal Medicine EBM Research center, Korea Institute of Oriental Medicine, 483 Exporo, Yuseong-gu, Daejeon, 305-811, Republic of Korea

Tel : +82-42-868-9464, Fax : +82-42-864-2120, Email : hkshin@kiom.re.kr

not IgG2a and IgG2b, indicating that alum favors generation of Th2 responses and is but rarely associated with Th1 immune responses⁴. Whereas alum has been found to increase immunoglobulin E (IgE)-mediated antibody responses (allergenicity), alum only weakly stimulates cell-mediated immune responses^{5,6}.

Broussonetia kazinoki Siebold (Moraceae family) is a plant distributed throughout Korea, China, and Japan⁷. In Chinese folk medicine, the branches, leaves, and fruits of this plant have been used as a diuretic, a tonic, and a suppressor of edema, respectively⁸. The branches, leaves, and fruits of this species have been found to contain many bioactive chemicals, including flavonoids^{9,10}, alkaloids^{11,12,13,14,15} and 1,3-diphenylpropanes¹⁶. Some of these chemicals have exhibited, tyrosinase and antioxidant¹⁷ activities, and inhibit nitric oxide production. However, the adjuvant effects of *B. kazinoki* Sieb (BK) have not yet been determined. We therefore evaluated the immune-stimulatory potential of a BK extract by immunizing mice with ovalbumin (OVA)/ alum, with administration of BK, followed by measurement of plasma-specific antibody responses, lymphocyte proliferation, and cytokine production by splenocytes.

Methods & Materials

1. Materials & Reagents

Roswell Park Memorial Institute (RPMI) 1640 medium, Dulbecco's modified Eagle's medium (DMEM), PBS, penicillin-streptomycin (P&S), and fetal bovine serum (FBS) were purchased from Gibco/BRL (Gaithersburg, MD). Hank's balanced salt solution (HBSS, pH 7.4), dimethyl sulfoxide (DMSO), concanavalin A (Con A), lipopolysaccharide (LPS), RBC lysing buffer, and aluminum hydroxide gel were obtained from Sigma Chemical Co. (St. Louis, MO). OVA was purchased from Thermo Scientific (Waltham, MA) and immunoglobulin G

(IgG), immunoglobulin G1 (IgG1), and immunoglobulin M (IgM) detection ELISA kits were purchased from Bethyl Laboratories (Montgomery, TX). All other chemicals and solvents were of analytical grade.

2. Extraction of *Broussonetia kazinoki* Sieb

The stems of *Broussonetia kazinoki* Sieb (BK) were donated from the Chunyang Paper Co., Ltd. (Jeonju, Korea), in July 2008. The plant material was identified by Professor Hongjun Kim, College of Oriental Medicine, Woosuk University. BK (10 kg) was extracted with H₂O under reflux for 3 h, filtered, and concentrated to yield water extract (130 g).

3. Measurement of nitrite production

RAW 264.7 cells, obtained from the American Tissue Culture Collection (ATCC, Manassas, VA), were maintained in DMEM medium supplemented with 5.5% (v/v) FBS, 1% (w/v) P&S (100 U/mL penicillin, 100 µg/mL streptomycin) at 37°C in a humidified 5% (v/v) CO₂ atmosphere. Cells were plated at a density of 3×10³ cells/well in 96-well plates for proliferation assays (48 hr) and at 2.5×10⁵ cells/well in 48-well plates for NO assays (18 hr). The next day, cells were stimulated with LPS (0.1 µg/mL, positive control) or BK extracts, for the indicated periods.

To measure NO production in culture supernatants, we used the Griess reagent system (Promega Co., Madison, WI). Briefly, 100 µL aliquots of supernatant were withdrawn and incubated at room temperature with 1% (w/v) sulfanilamide (100 µL) for 10 min and with 1% (w/v) α-naphthylamine (100 µL) for 10 min. Absorbance at 540 nm was measured using a Benchmark-Plus ELISA reader (Bio-Rad Laboratories, Hercules, CA) and calibrated against a standard curve.

4. Splenocyte proliferation

Six-week-old male ICR mice, purchased from Orient Bio (Seongnam, Korea), were sacrificed, and

spleens were collected, washed in HBSS, and passed through a cell strainer to obtain a homogeneous cell suspension. Erythrocytes were lysed using an RBC lysis solution. The suspension was centrifuged at 4°C for 3 min, and the pellet was resuspended to a density of 5×10^5 cell/mL in RPMI medium supplemented with P&S and 10% (v/v) FBS. Cells were plated in 96-well plates, cultured for 48 h with BK extract (5-500 $\mu\text{g/mL}$) and incubated with CCK-8 (a tetrazolium salt; Cell Counting Kit-8, Dojindo, Kumamoto, Japan) for 4 hr; absorbance was measured at 450 nm.

5. Animals and Immunization

Six-week-old mice male C57BL/6 mice were purchased from Orient Bio and kept under standard conditions in the Experimental Animal Center of the Korea Institute of Oriental Medicine (KIOM, Daejeon, Korea). Mice were divided into five groups, each containing 10 mice. Mice were injected intraperitoneally with OVA/alum (100/200 μg) at weekly intervals (day 1, 8, and 15). Saline-treated animals were included as normal controls. BK was given orally, at doses of 100, 300 and 1000 mg/kg/day in distilled water, from day 1 to day 21 and vehicle was distilled water. Spleen and plasma were collected at day 22 for splenocyte proliferation assay and measurement of antibodies in plasma. To assess the adjuvant activity of BK, mice were immunized three times at weekly intervals with OVA/Alum and given BK orally.

6. Mitogen- and OVA-stimulated splenocyte proliferation in OVA-immunized mice

Splenocytes from OVA-immunized C57BL/6 mice (5×10^5 cell/mL) were seeded into wells of 96-well plates. To each well was added OVA, LPS, or Con A yielding a final volume of 100 μL , and plates were incubated at 37°C for 48 hr. Cells were incubated with CCK-8 and the absorbance at 450 nm

was measured. The mitogen effect was calculated based on the following formula: $(\%) = 100 \times \text{Abs}_{\text{Extract}} / \text{Abs}_{\text{Control}}$

7. Measurement of total and OVA-specific antibody levels in plasma

Total IgM and IgG and OVA-specific IgG and IgG1 were measured by indirect ELISA assays. Briefly, each well of a 96-well plate (Nunc, Thermo Fisher Scientific, Roskilde, Denmark) was coated by incubation with 100 μL IgM, IgG, and IgG1 capture antibody or 100 μL OVA solution (100 $\mu\text{g/mL}$ in 50 mM carbonate-bicarbonate buffer, pH 9.6) overnight at 4°C. Wells were washed three times with PBS containing 0.05% (v/v) Tween 20 (wash buffer), and blocked with 1% (w/v) BSA/PBS at room temperature for 30 min. After three times washing, 100 μL of diluted plasma samples were added, and the plates were incubated for 1 hr at 37°C and washed five times. 100 μL HRP conjugate (1:10,000 in 1% [w/v] BSA/PBS) were added, and plates were incubated for 1 hr at 37°C and washed five times. 100 μL of substrate solution were added and incubated for 30 min at room temperature. Enzyme reactions were terminated by addition of 100 μL of 2 N H_2SO_4 , and the absorbance of each supernatant at 450 nm was measured in an ELISA reader, with a 570 nm reference channel.

8. RT-PCR for cytokine gene expression

Splenocytes from OVA-immunized mice were seeded into six-well plates. RNA was isolated from each sample, and cDNA was synthesized from 1 μg RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, U.S.A.). PCR amplification reactions were performed using gene-specific primers for the cytokine genes, IL-2 and IFN- γ (Table 1).

9. Statistical analysis

The results were represented as means \pm SEM and

Table 1. Sequences of Primer Used for RT-PCR.

Gene	Primer sequence	Annealing temperature	Cycles	Product size (bp)
β -actin	5'-AATGTAGTTTCATGGATGCC-3' 5'-CCTGATCATGTTGAGACCT-3'	59.5C	20	476
IL-2	5'-CTTGCTAATCACTCCTCACA-3' 5'-AGAAAGTCCACCACAGTTGCT-3'	55C	31	500
IFN- γ	5'-AAATCAGTTGTGCCTCAGTT-3' 5'-CCACTACTGGGAAAAGTCAG-3'	55C	26	250

deemed statistically significant at $p < 0.05$, based on ANOVA or Bonferroni multiple comparison analysis using the Systat[®] 10.0 software (SYSTAT Inc., Evanston, Ill, U.S.A.).

Results

1. Effect of BK on NO production

BK stimulated the proliferation of RAW 264.7 murine macrophage cells 1.6-fold ($163.88 \pm 2.23\%$ of the control). Incubation of RAW 264.7 cells with increasing amounts of water extract of BK (5-100 $\mu\text{g}/\text{mL}$) showed a dose-dependent stimulation of inducible NO synthesis (Fig. 1). The highest concentration tested (100 $\mu\text{g}/\text{mL}$) ($p < 0.01$) exhibited greater

bioactivity than the positive control, 0.1 $\mu\text{g}/\text{mL}$ of LPS.

2. Effect of BK on splenocyte proliferation

BK stimulated the proliferation of splenocytes. Maximal splenocyte proliferation was observed at a concentration of BK 10 $\mu\text{g}/\text{mL}$ ($142.03 \pm 3.52\%$, $p < 0.01$, Fig. 2).

3. Effect of BK on mitogen- and OVA-stimulated splenocyte proliferation in OVA-immunized mice

BK significantly enhanced splenocyte proliferation ($p < 0.001$) induced by the B cell mitogen LPS and by the T cell mitogen Con A, compared with OVA

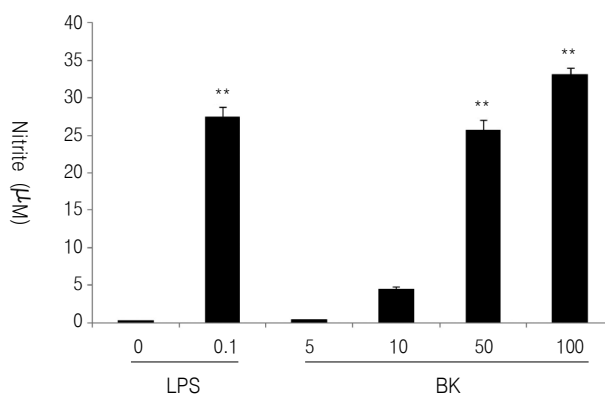


Fig. 1. Effect of *Broussonetia kazinoki* water extract (BK) on nitric oxide (NO) production in RAW 264.7 cells. The cells (2.5×10^5 cells/well in 48-well plate) were incubated for 18 hr in DMEM containing 5.5% FBS and were treated for 18 hr with BK extract followed treat with and with LPS (0.1 $\mu\text{g}/\text{mL}$). NO produced was measured by the Griess reagent method. Each data value is presented as mean \pm SEM (n=3). The units of concentrations of LPS and BK are $\mu\text{g}/\text{mL}$. Significant differences with control (LPS 0) were designated as $**p < 0.01$.

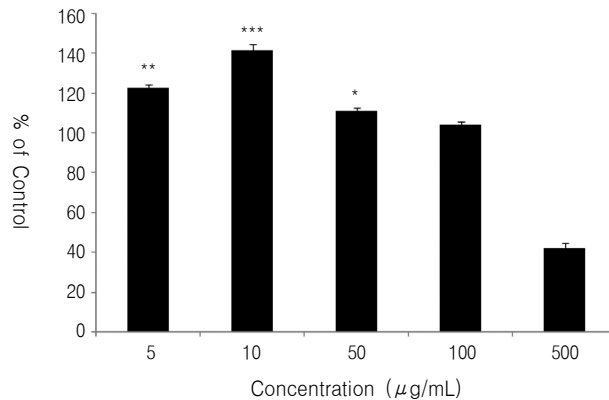


Fig. 2. Effect of extract of BK on splenocyte proliferation. A spleen was removed from specific pathogen-free ICR mice to prepared single cell suspension. The splenocyte responses to BK were determined by ELISA. All values represent the mean \pm SEM (n=3). Significant differences among the different concentrations in each extract were designated as ***p<0.001, **p<0.01 and *p<0.05.

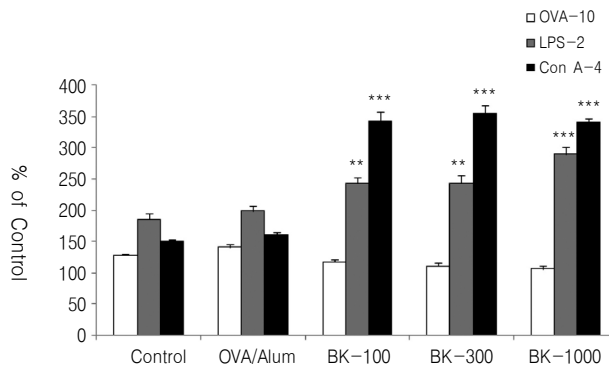


Fig. 3. Effect of BK on OVA-, LPS- and Con A-stimulated splenocyte proliferation *in vivo*. C57BL/6 mice were immunized once on the intraperitoneal injection with OVA/alum (100/200 µg) at weekly intervals and oral administration with BK of 100, 300, 1000 mg/kg/day. Splenocytes were prepared 1 weeks after the last immunization and cultured with OVA (10 µg/mL), LPS (2 µg/mL) and Con A (4 µg/mL) for 48 hr. Splenocyte proliferation was measured by the CCK-8 method as described in the text, and shown as % of control. The values are presented as mean \pm SEM (n=5). Significant differences with OVA/alum groups were designated as **p<0.01 and ***p<0.001.

immunized-, BK-untreated mice (Fig. 3). However, no significant difference was observed among mice given the three BK doses (100, 300, and 1000 mg/kg).

4. Effect of BK on IgM, IgG, and OVA-specific antibody levels in plasma

The level of total IgG, which was enhanced by

OVA/Alum, was not affected by BK (Fig. 4). In contrast, BK enhanced the plasma level of total IgM over that observed in OVA immunized mice (Fig. 4-A). In addition, BK enhanced plasma OVA-specific IgG1 but did not alter the OVA-specific IgG (Fig. 4-B).

5. Effect of BK on cytokine mRNA levels in splenocytes from OVA-immunized mice

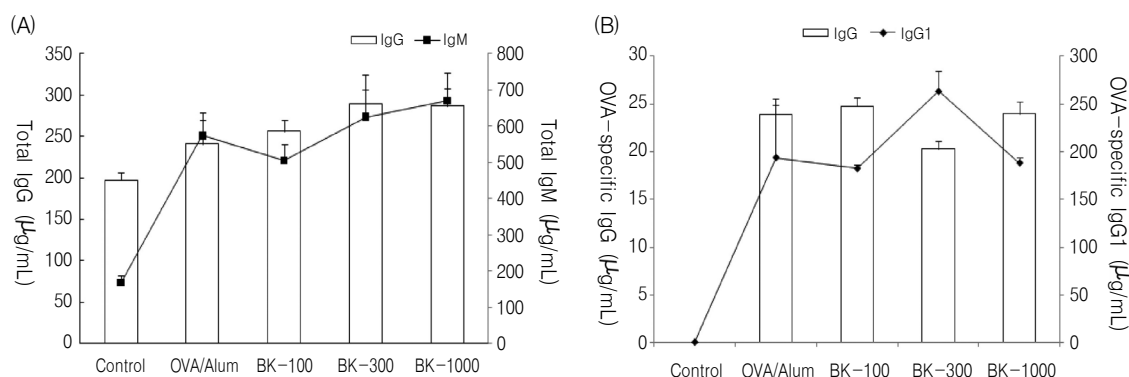


Fig. 4. Effects of BK on total IgM, IgG (A) and OVA-specific IgG, IgG1 (B) level in plasma. Total IgM, IgG and OVA-specific IgG, IgG1 level in plasma was assayed by ELISA. Each value represents a mean \pm SEM (n=5). No significant differences with OVA/alum groups were designated as $p > 0.05$.

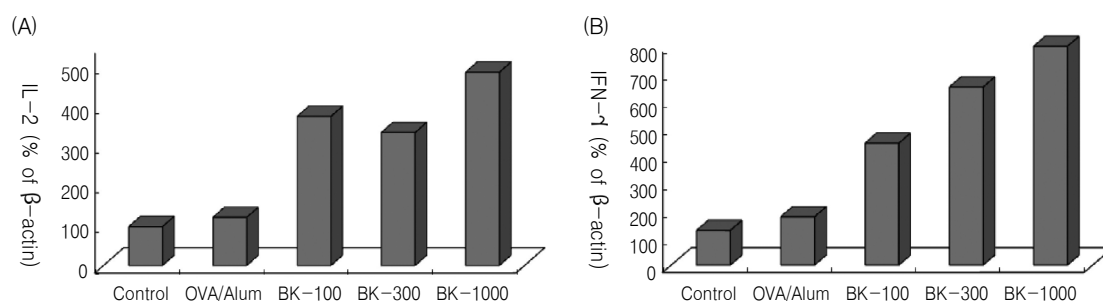


Fig. 5. Effect of BK on the cytokines (A; IL-2, B: IFN- γ) mRNA gene expression in splenocytes from OVA-immunized mice.

The five cytokine mRNAs, IL-2, IL-4, IL-10, TNF- α and IFN- γ , were detected in the splenocytes from the mice untreated and treated BK. The expression levels of IL-2 and IFN- γ were up-regulated in splenocytes of the mice by administration of BK, dose-dependent manner (Fig. 5). However, IL-4, IL-10 and TNF- α expression levels were not changed significantly (data not shown).

Discussion

In general, immune activation involves the proliferation of macrophages and splenocytes. Moreover,

low levels of NO have been implicated in lymphocyte activation and proliferation. NO donors such as sodium nitroprusside, and to a lesser degree gaseous NO, have been shown to increase lymphocyte uptake of glucose (an early event during lymphocyte activation), to stimulate TNF- α production and nuclear transcription factor κ B (NF- κ B) binding activity, and to enhance the activity of the tyrosine kinase p56lck, which has been implicated in lymphocyte signaling events. All the above changes are achieved via a cGMP-independent mechanism, although NO activation of guanylate cyclase may be necessary for optimal lymphocyte proliferation. L-arginine depletion and NOS inhibitors have also

been shown to impair PHA-stimulated proliferation, whereas dietary L-arginine supplementation in humans has been found to increase lymphocyte mitogenic responses to Con A and PHA. L-arginine has also been shown to enhance natural killer cell and lymphokine-activated killer cell activity both *in vitro* and *in vivo*¹⁸⁾. We found that BK enhanced the proliferation of the mouse macrophage cell line RAW 264.7, and enhanced NO production by these cells, indicating that BK may promote immune responses via a NO-associated mechanism.

Cell-mediated immunity, mediated by T lymphocytes, is important in combating intracellular microbial infections. Helper T cells induce B lymphocytes to secrete antibodies, whereas cytotoxic T lymphocytes assist phagocytes to destroy ingested microbes and kill intracellular microbes. Humoral immunity, mediated by antibodies produced by B lymphocytes, functions by neutralizing and eliminating extracellular microbes and microbial toxins¹⁹⁾. We found that BK promoted immune responses in mice, as well as promoting splenocyte proliferation *in vitro*, suggesting that the adjuvant activity of BK may be associated with enhancement of splenocyte proliferation.

In addition to showing that BK has an adjuvant effect in OVA-immunized mice, we found that BK enhanced lymphocyte responses to Con A, LPS and OVA in immunized mice. These results indicate that BK acts by increasing the activation potential of T and B lymphocytes²⁰⁾. We therefore assayed whether BK could enhance cellular immune responses in immunized mice. Our finding that BK could enhance the level of OVA-specific IgG1 indicated that BK could strongly modulate humoral and cell-mediated immune responses by increasing lymphocyte proliferation and activating effector T cells²¹⁾. To determine whether BK acted primarily through the Th1 or Th2 pathway, we assessed the effects of BK on the levels of mRNA encoding the Th1/Th2 cytokines IL-2, IL-4, IL-10, TNF- α and IFN- γ in splenocytes of OVA-immunized mice. We found that BK significantly

enhanced the levels of mRNAs encoding the Th1 cytokines IL-2 and IFN- γ .

In conclusion, BK significantly enhanced both cellular and humoral immune responses in mice immunized with OVA, providing evidence for an adjuvant activity of BK in up-regulating the Th1 immune responses.

Acknowledgements

This research was supported by grants from Korea Institute of Oriental Medicine (#K09061) and Small and Medium Business Administration (SMBA), Korea

References

1. Livingston PO, Adluri S, Helling F, Yao TJ, Kensil CR, Newman MJ, et al. Phase 1 trial of immunological adjuvant QS-21 with a GM2 ganglioside-keyhole limpet haemocyanin conjugate vaccine in patients with malignant melanoma. *Vaccine*. 1994;12(14):1275-1280.
2. Cox JC, Coulter AR. Adjuvants-a classification and review of their modes of action. *Vaccine*. 1997;15(3):48-56.
3. Pascual DM, Morales RD, Gil ED, Munoz LM, Lopez JE, Casanueva, OLJ. Adjuvants present regulatory challenges. *Vaccine*. 2006;24(S2):S88-S89.
4. HogenEsch H. Mechanisms of stimulation of the immune response by aluminum adjuvants. *Vaccine*. 2002;20(S3):S34-S39.
5. Relyveld EH, Bizzini B, Gupta RK. Rational approaches to reduce reactions man to vaccines containing tetanus and diphtheria toxoids. *Vaccine*. 1998;16(9-10):1016-1023.
6. Schirmbeck R, Melber K, Kuhrber A, Janowicz ZA. Immunization with soluble hepatitis B virus surface protein elicits murine H-2 class I-restricted

- CD8⁺ cytotoxic T lymphocyte responses *in vivo*. J Immunol. 1994;152(3):1110-1119.
- Bae KH. Medical plants of Korea. Seoul:Kyohak publishing Co. 2000:65.
 - Ryu JH, Ahn H, Lee HJ. Inhibition of nitric oxide production on LPS-activated macrophages by kazinol B from *Broussonetia kazinoki*. Fitoterapia. 2003;74(4):350-354.
 - Ko HH, Yen MH, Wu RR, Won SJ, Lin CN. Cytotoxic isoprenylated flavans of *Broussonetia kazinoki*. J Nat Prod. 1999;62(1):164-166.
 - Zhang PC, Wang S, Wu Y, Chen RY, Yu DQ. Five new diprenylated flavonols from the leaves of *Broussonetia kazinoki*. J Nat Prod. 2001;64(9):1206-1209.
 - Shibano M, Kitagawa S, Nakamura S, Akazawa N, Kusano G. Studies on the constituents of *Broussonetia* species. II Six new pyrrolidine alkaloids, broussonetine A, B, E, F and broussonetinine A and B, as inhibitors of glycosidases from *Broussonetia kazinoki* Sieb. Chem Pharm Bull. 1997;45(4):700-705.
 - Shibano M, Nakamura S, Akazawa N, Kusano. Studies on the constituents of *Broussonetia* species. III. Two new pyrrolidine alkaloids, broussonetinines G and H, as inhibitors of glycosidase from *Broussonetia kazinoki* Sieb. Chem Pharm Bull. 1998;46(6):1048-1050.
 - Shibano M, Tsukamoto D, Fujimoto R, Masui Y, Sugimoto H, Kusano G. Studies on the constituents of *Broussonetia* species. VII. Four new pyrrolidine alkaloids, broussonetines M, O, P, and Q, as inhibitors of glycosidase from *Broussonetia kazinoki* Sieb. Chem Pharm Bull. 2000;48(9):1281-1285.
 - Tsukamoto D, Shibano M, Kusano G. Studies on the constituents of *Broussonetia* Species X. Six new alkaloids from *Broussonetia kazinoki* Sieb. Chem Pharm Bull. 2001a;49(11):1487-1491.
 - Tsukamoto D, Shibano M, Okamoto R, Kusano G. Studies on the constituents of *Broussonetia* species VIII. Four new pyrrolidine alkaloids, broussonetines R, S, T, and V and a new pyrroline alkaloid, roussonetine U, from *Broussonetia kazinoki* Sieb. Chem Pharm Bull. 2001b;49(4):492-496.
 - Baek YS, Ryu YB, Curtis MJ, Ha TJ, Rengasamy R, Yang MS, et al. Tyrosinase inhibitory effects of 1,3-diphenylpropanes from *Broussonetia kazinoki*. Bioorg Med Chem. 2009;17(1):35-41.
 - Lee HJ, Park JH, Jang DI, Ryu JH. Antioxidant components from *Broussonetia kazinoki*. Yakhak Hoechi. 1997;41(4):439-443.
 - Roswitha F, Thomas M, Brigitte K, Paul GS. Stimulation nitric oxide synthesis by the aqueous Extract of *Panax ginseng* root in RAW 264.7 cells. Br J Pharmacol. 2001;134(8):1663-1670.
 - Abbas AK, Lichtman AH. Basic immunology, functions and disorders of the immune system. 2nd ed. Philadelphia:Saunders. 2004.
 - Tizard IR. Veterinary immunology an introduction, 6th ed. Philadelphia:Saunders. 2000.
 - Constant SL, Bottomly K. Induction of Th1 and Th2 CD4⁺ T cell responses the alternative approaches. Annu Rev Immunol. 1997;15:297-322.