

Brazilian green propolis improves immune function in aged mice

Weina Gao,¹ Jianquan Wu,¹ Jingyu Wei,¹ Lingling Pu,¹ Changjiang Guo,^{1,*} Jijun Yang,¹ Ming Yang² and Haiji Luo²

¹Institute of Health and Environmental Medicine, Tianjin, 300050, China

²By-Health Nutrition and Health Research Center, 510620, China

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Aging weakened innate and adaptive immunity both quantitatively and qualitatively. Some components in propolis could stimulate immune function in young animals or cultured immune cells *in vitro*. Few studies had been carried out in the aged. The present study was to evaluate the effects of Brazilian green propolis supplementation on the immunological parameters in aged mice. Eighty Kunming mice, aged 15–18 months, were randomly assigned to the control and three experimental groups supplemented with different doses (83.3, 157.4 and 352.9 mg/kg.bw respectively) of Brazilian green propolis. The experiment lasted for 4 weeks. Contents of total polyphenol, flavonoid, cinnamic acid and artemillin-C in Brazilian green propolis were analyzed. Splenic NK cytotoxic, T lymphocyte proliferation and antibody generation cells, as well as the phagocytosis of peritoneal macrophages, ear swelling, and serum contents of IgG, IgM, hemolysin and cytokines were measured. After 4 weeks of treatment, the phagocytosis of peritoneal macrophages was enhanced in 157.4 mg/kg and 352.9 mg/kg groups. Ear swelling increased in all propolis treated groups. Antibodies specific to sheep erythrocytes were higher in the groups receiving 157.4 and 352.9 mg/kg.bw than that of control group. IgG level dramatically increased in the groups receiving 83.3 and 157.4 mg/kg.bw in comparison to the control group. These results indicate that administration of Brazilian green propolis have a positive effect on innate and adaptive immunity in aged mice.

Key Words: propolis, immune function, aged mice

Aging is accompanied by a general dysregulation in immune function. This progressive deterioration affects both innate and adaptive immunity. Several studies have demonstrated that aging is associated with decreased phagocytic activity of macrophages, reduced NK cell cytotoxicity, impaired activation and proliferation of T- and B-lymphocytes,^(1–4) and dysregulated production of soluble mediators such as cytokines and chemokines.⁽⁵⁾

Propolis is a chemically complex resinous substance collected by honeybees from leaf buds and cracks in the bark of various plants, comprising plant exudates, secreted substances from bee metabolism, pollen and waxes.⁽⁶⁾ More than 300 chemical compounds have been identified from propolis, including polyphenols (e.g., flavonoids), coumarins, terpenes, amino acids, minerals, and so on.^(7–11) Propolis has been found to have a wide spectrum of biological and pharmaceutical properties and used as a potential immune regulator.⁽¹²⁾ Recently, it was reported that Brazilian green propolis has immune enhancing action both *in vitro* and *in vivo*.^(13,14) However, most experiments *in vivo* were performed on young animals, and limited studies have been carried on aged subjects. In the present study, we investigated the effects of Brazilian green propolis on innate and adaptive immunity in aged mice, so as to provide a practical basis for the application of

Brazilian green propolis in improving innate and adaptive immunity in aged individuals.

Materials and Methods

Chemicals. Concanavalin A (ConA) and cinnamic acid were purchased from Sigma-Aldrich (St. Louis, MO). The artemillin-C standard was purchased from Wako Pure Chemicals (Osaka, Japan). Brazilian green propolis was obtained from Apis Flora Industrial E Comercial Ltda (Sao Paulo, Brazil). All other reagents used were of HPLC-grade, guaranteed or analytical grade.

Analysis of total polyphenols, flavonoid, cinnamic acid, and artemillin-C in propolis. Total polyphenols was determined spectrophotometrically by using the Folin-Ciocalteu method as described previously.⁽¹⁵⁾ Total flavonoid was measured by the aluminium chloride colorimetric assay.⁽¹⁶⁾ Cinnamic acid was detected by a reversed phase (RP)-HPLC procedure reported by Huang *et al.*⁽¹⁷⁾ Artemillin-C was assayed using a HPLC method as described previously.⁽¹⁸⁾

Animals and treatments. Eighty aged Kunming mice (15–18 months), weighing 48–57 g, were obtained from the Laboratory Animal Center of Chinese Academy of Military Medical Sciences (Beijing, China). They were kept in a well-ventilated room at 22–24°C and 50% relative humidity, with 12 h/12 h light-dark cycle. Food and water were provided *ad libitum*. After acclimation for 5 days, they were randomly divided into four groups according to their body weight. Control group received 0.1 ml of plant oil, and other three groups were treated with raw Brazilian propolis at the doses of 83.3, 157.4 and 352.9 mg/kg in 0.1 ml of plant oil respectively, by gavage. The experiment lasted for 4 weeks, and body weight and diet intake were weighed every third day. At the end of the experiment, forty mice were fasted overnight before blood samples were taken from the orbital plexus under diethyl ether anesthetization, and spleen were taken after the mice were sacrificed for measurement of splenic NK cells activity, lymphocyte proliferation and antibody generation cells. Samples of serum were prepared accordingly after centrifugation for determination of serum HC₅₀ value, antibodies (IgG and IgM), and cytokines (IL-1 β , IL-4 and IFN- γ). Meanwhile, forty mice were selected for peritoneal macrophage phagocytosis and ear swelling test. All procedures were performed in accordance with the current Chinese legislation on the care and use of laboratory animals and approved by Department of Scientific Management of the institute.

Phagocytosis by peritoneal macrophages. Peritoneal macrophage cells were isolated as described previously⁽¹⁹⁾ and 0.5 ml of peritoneal macrophage cells suspension was mixed with

*To whom correspondence should be addressed.
E-mail: guo_tj@yahoo.com

Table 1. Effects of Brazilian green propolis on peritoneal phagocytic ability in aged mice

Group	Dose (mg/kg.bw)	Phagocytosis percentage (%)	Phagocytosis index
Control	0	70.71 ± 13.70	1.80 ± 0.48
Brazilian 1	83.3	66.23 ± 6.91	1.61 ± 0.46
Brazilian 2	157.4	83.20 ± 10.25*	2.29 ± 0.46
Brazilian 3	352.9	79.11 ± 9.55	2.53 ± 0.87*

Values are presented as mean ± SD ($n = 10$). * $p < 0.05$, compared with control. Peritoneal phagocytosis at the dose of 157.4 mg/kg and phagocytic index at the dose of 352.9 mg/kg were significantly increased after administration of Brazilian green propolis, and p value were 0.011 and 0.010 respectively.

Table 2. Effects of Brazilian green propolis on NK and T cell function in aged mice

Group	Dose (mg/kg.bw)	NK Cells activity (U/L)	T cell proliferation (OD value)	Ear swelling (mg)
Control	0	154.92 ± 34.94	0.387 ± 0.165	4.9 ± 2.4
Brazilian 1	83.3	135.86 ± 51.90	0.389 ± 0.087	8.9 ± 3.8*
Brazilian 2	157.4	160.53 ± 29.61	0.477 ± 0.047	11.6 ± 5.4*
Brazilian 3	352.9	158.72 ± 37.64	0.484 ± 0.069	18.2 ± 2.5*

Values are presented as mean ± SD ($n = 10$). $p < 0.05$, compared with control. Ear swelling extent was increased significantly in all propolis treatment groups compared with the control group, and p value was 0.031, 0.001 and 0.000 in 83.3 mg/kg, 157.4 mg/kg and 352.9 mg/kg group respectively.

0.5 ml of 1% cock red blood cell suspension. Then, 0.5 ml of mixture was dropped on a glass slide and incubated at 37°C for 20 min. Attached cells were fixed with methanol for 1 min before staining with Giemsa. The slides were washed, air dried and observed under microscope. Phagocytic rate was calculated as the number of macrophages phagocytized chicken red blood cells per one hundred macrophages, and phagocytic index as the average number of chicken red blood cells phagocytized per peritoneal macrophage cell.

Assay of splenic NK cytotoxicity, T lymphocyte proliferation and antibody generation cells. Splenocyte preparation was based on the method reported by Han *et al.*⁽²⁰⁾ NK cell-mediated cytotoxic activity was determined by using a colorimetric assay based on the measurement of lactic acid dehydrogenase (LDH) activity released from the cytosol of lysed Yac-1 target cells into the supernatant.⁽²¹⁾ A commercial LDH activity determination kit was obtained from Nanjing Jiancheng Bioengineering Institute, China. The splenic T lymphocyte proliferation was assayed according to an improved MTT assay described previously by Pagliarone and Hernandez.^(22,23) Antibody generation cells were measured by a spectrophotometric method as reported by Li *et al.*⁽²⁴⁾

Measurement of serum IgG, IgM and cytokines. Serum contents of IgG and IgM were assayed by an immunoturbidimetric method, using a commercial kit (Shanghai North and Biochemical Reagent Co., Ltd.). The contents of IL-1 β , IL-4 and IFN- γ in serum were measured by enzyme linked immunosorbent assay (ELISA), using commercial kits from BD Biosciences (R&D Company, VA).

Determination of hemolysin to SRBC. The assay described by Wen *et al.*⁽²⁵⁾ was followed to analyze serum hemolysin to SRBC. The absorbance for each tube was recorded and the 50% hemolytic dose (HC₅₀) calculated by the following formula. Sample HC₅₀ = sample absorbance/50% hemolytic absorbance × dilution factor.

Ear swelling test. The ear swelling was measured according to the method described by Escandell *et al.*⁽²⁶⁾ After being challenged for 48 h, a piece of ear tissue (8 mm in diameter) was taken, and weighed with an analytical balance. The swelling was calculated as the increased weight of right ear (challenged side) over the left one (blank side).

Statistics analysis. Data were expressed as mean ± SD, and significant differences among treatments were determined by analysis of variance (ANOVA) followed by LSD test. Statistic significances were accepted at $p < 0.05$.

Results

Contents of total polyphenols, flavonoids, cinnamic acid and artepillin-C in Brazilian green propolis. The contents of total polyphenols, flavonoids, cinnamic acid and artepillin-C in Brazilian propolis were 189.12 mg/g, 98.46 mg/g, 1.95 mg/g and 23 mg/g, respectively.

Body weight and food intake. No significant difference was observed in the average body weight and food intake between the experimental and control groups during the experimental period (data was not shown).

Phagocytosis of peritoneal macrophages. Administration of Brazilian green propolis could promote peritoneal phagocytosis at the dose of 157.4 mg/kg, and increased phagocytic index at the dose of 352.9 mg/kg significantly in comparison to the control group (Table 1).

Splenic NK cell activity and T cell proliferation. No effect was observed on splenic NK cell activity after the treatment of Brazilian green propolis (Table 2). Splenic T cell proliferation was slightly increased at the doses of 157.4 and 352.9 mg/kg, but without statistical significance (Table 2).

Ear swelling extent. Ear swelling extent was increased in all propolis treatment groups compared with the control group, in a dose dependent manner (Table 2).

Serum contents of IgG, IgM, hemolysin and cytokines, and number of antibody generation cells. Serum IgG level was also dramatically increased in mice receiving 83.3 and 157.4 mg/kg.bw of Brazilian green propolis compared to the control mice, while no significant difference was noted in serum IgM level among different groups. Serum hemolysin was found to be higher in mice receiving 157.4 and 352.9 mg/kg.bw of Brazilian green propolis when compared to the control mice (Table 3). No significant increase in serum IL-1 β , IFN- γ and IL-4 levels was found after the administration of Brazilian green propolis (Table 4). Only a slight increase was observed in the number of antibody generation cells in mice receiving 157.4 mg/

Table 3. Effects of Brazilian green propolis on humoral immunity in aged mice

Group	Dose (mg/kg.bw)	IgG (g/L)	IgM (g/L)	Hemolysin	Number of antibody generation cells
Control	0	23.03 ± 8.30	0.22 ± 0.04	1.35 ± 0.03	1.83 ± 0.66
Brazilian 1	83.3	34.48 ± 5.94*	0.21 ± 0.02	1.40 ± 0.03	1.75 ± 0.76
Brazilian 2	157.4	32.35 ± 9.08*	0.22 ± 0.06	1.56 ± 0.22*	2.00 ± 0.78
Brazilian 3	352.9	31.53 ± 10.28	0.20 ± 0.03	1.48 ± 0.03*	1.56 ± 0.65

Values are presented as mean ± SD ($n = 10$). * $p < 0.05$, compared with control. Serum IgG level was dramatically increased in mice receiving 83.3 and 157.4 mg/kg.bw of Brazilian green propolis compared to the control mice, and p value was 0.013 and 0.041 respectively. Serum hemolysin was found to be higher in mice receiving 157.4 and 352.9 mg/kg.bw of Brazilian green propolis when compared to the control mice, and p value was 0.002 and 0.046 respectively.

Table 4. Effects of Brazilian green propolis on serum cytokines in aged mice

Group	Dose (mg/kg.bw)	IL-1 β (pg/ml)	IFN- γ (pg/ml)	IL-4 (pg/ml)
Control	0	10.07 ± 1.80	206.62 ± 28.63	148.61 ± 19.22
Brazilian 1	83.3	10.20 ± 1.33	236.70 ± 33.06	152.29 ± 17.72
Brazilian 2	157.4	13.93 ± 5.90	242.95 ± 57.89	141.37 ± 15.90
Brazilian 3	352.9	13.44 ± 3.24	220.52 ± 23.88	161.15 ± 7.32

Data are expressed as mean ± SD ($n = 10$). No significant increase in serum IL-1 β , IFN- γ and IL-4 levels was found after the administration of Brazilian green propolis.

kg.bw of Brazilian green propolis (Table 3).

Discussion

Macrophages are an important component in innate immunity. They function primarily as phagocytic cells and are capable of phagocytosing pathogenic organisms. In addition, macrophages are potent cytokines producer and play a crucial role in a variety of processes, including antigen presentation and wound healing.⁽²⁷⁾ The results of the present study suggested that the peritoneal macrophages were activated by Brazilian green propolis administration, as indicated by increased phagocytic ability. Several studies had demonstrated that some of ingredients in propolis, such as caffeic acid phenethyl ester, cinnamic acids, and artemillin C, could activate macrophages *in vitro* and *in vivo*.⁽²⁸⁻³¹⁾

The extent of ear swelling after challenging is closely associated with intact cellular immunity and has been shown to be a sensitive, quantitative and reproducible measurement of cell-mediated immunity.^(32,33) The results of this study indicated that the cellular immunity of aged mice was activated by Brazilian green propolis. The increased ear swelling could be attributed to the changes in some components of the cellular immune system that occurred at the same time. In the present study, activated peritoneal macrophages may contribute significantly to the increase in ear swelling.

Aging is associated with a decline in B cell function and humoral response is substantially impaired.^(34,35) The number of mature B cells and IgG level decrease significantly in the aged.⁽³⁶⁾ The specific antibody response also decreases with aging.⁽³⁷⁾ In the present study, Brazilian green propolis increased serum IgG level and SRBC specific antibody production in aged mice, suggesting improved IgG production and enhanced specific antibody response. Similar results have been reported in laying hens, turbot and chickens.⁽³⁸⁻⁴²⁾

Brazilian green propolis characterized by prenylated phenylpropanoids and caffeoylquinic acids.⁽⁴³⁾ Artemillin C and p-coumaric are major constituents of a propolis sample from Sao Paulo state (southeast Brazil).⁽⁴⁴⁾ In the present study, the analysis of composition indicated that Brazilian green propolis used in this study was rich in polyphenols, flavonoids, cinnamic acid and artemillin-C. Ansorge *et al.*⁽⁴⁵⁾ demonstrated that propolis had a direct regulatory effect on basic functional properties of immune cells which may be mediated by the Erk2 MAP-kinase signal

pathway. Water extract of propolis was effective in stimulating NO release and surface molecule expression in murine macrophage cells.⁽²⁰⁾ The flavonoids could be an important component in activating innate and adaptive immune cells as reported by Yuan *et al.*⁽⁴²⁾ Phenolic compounds, such as cinnamic acid had been demonstrated to be effective in stimulating cytokine IL-1 β production in murine macrophages.⁽⁴⁶⁾ A study conducted by Fischer *et al.*⁽⁴⁷⁾ showed that the polyphenol compounds extracted from Brazilian green propolis could promote specific antibody secretion in mice. Artemillin-C, a low-molecular-weight phenolic compound, was effective in T helper cells expansion and activation, as well as macrophage activation as demonstrated previously by Cheung *et al.* and Kimoto *et al.*^(31,48) Therefore, we consider a possibility that artemillin-C may be one of the most important ingredients in Brazilian green propolis in activating macrophage phagocytosis.

Conclusions

In conclusion, Brazilian green propolis is effective in improving immune function in aged mice, especially at a dose of 157.4 mg/kg.bw. The peritoneal macrophage function was activated and serum IgG, hemolysin, as well as ear swelling were increased in response to the administration of Brazilian green propolis.

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Conflict of Interest

No potential conflicts of interest were disclosed.

References

- Bernstein E, Kaye D, Abrutyn E, Gross P, Dorfman M, Murasko DM. Immune response to influenza vaccination in a large healthy elderly population. *Vaccine* 1999; **17**: 82–94.
- Frasca D, Blomberg BB. Effects of aging on B cell function. *Curr Opin Immunol* 2009; **21**: 425–430.
- Linton PJ, Dorshkind K. Age-related changes in lymphocyte development and function. *Nat Immunol* 2004; **5**: 133–139.
- Pawelec G, Derhovanessian E, Larbi A, Strindhall J, Wikby A. Cytomegalovirus and human immunosenescence. *Rev Med Virol* 2009; **19**: 47–56.
- Weiskopf D, Weinberger B, Grubeck-Loebenstein B. The aging of the immune system. *Transpl Int* 2009; **22**: 1041–1050.
- Benkovic V, Horvat Knezevic A, Brozovic G, et al. Enhanced antitumor activity of irinotecan combined with propolis and its polyphenolic compounds on Ehrlich ascites tumor in mice. *Biomed Pharmacother* 2007; **61**: 292–297.
- Marcucci MC, Ferreres F, Garcia-Viguera C, et al. Phenolic compounds from Brazilian propolis with pharmacological activities. *J Ethnopharmacol* 2001; **74**: 105–112.
- Falcão SI, Vale N, Gomes P, et al. Phenolic profiling of Portuguese propolis by LC-MS spectrometry: uncommon propolis rich in flavonoid glycosides. *Phytochem Anal* 2013; **24**: 309–318.
- Shi H, Yang H, Zhang X, Yu LL. Identification and quantification of phytochemical composition and anti-inflammatory and radical scavenging properties of methanolic extracts of Chinese propolis. *J Agric Food Chem* 2012; **60**: 12403–12410.
- Chen CN, Hsiao CJ, Lee SS, et al. Chemical modification and anticancer effect of prenylated flavanones from Taiwanese propolis. *Nat Prod Res* 2012; **26**: 116–124.
- Bonvehí JS, Bermejo FJ. Element content of propolis collected from different areas of South Spain. *Environ Monit Assess* 2013; **185**: 6035–6047.
- Sforcin JM. Propolis and the immune system: a review. *J Ethnopharmacol* 2007; **113**: 1–14.
- Khalil ML. Biological activity of bee propolis in health and disease. *Asian Pac J Cancer Prev* 2006; **7**: 22–31.
- Nassar SA, Mohamed AH, Soufy H, Nasr SM, Mahran KM. Immunostimulant effect of Egyptian propolis in rabbits. *ScientificWorldJournal* 2012; **2012**: 901516.
- Ainsworth EA, Gillespie KM. Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent. *Nat Protoc* 2007; **2**: 875–877.
- Zhishen J, Mengcheng T, Jianming W. The determination of flavonoid contents in mulberry and their scavenging effect on superoxide radicals. *Food Chem* 1999; **64**: 555–559.
- Huang WY, Zhang HC, Liu WX, Li CY. Survey of antioxidant capacity and phenolic composition of blueberry, blackberry, and strawberry in Nanjing. *J Zhejiang Univ Sci B* 2012; **13**: 94–102.
- Matsuda AH, de Almeida-Muradian LB. Validated method for the quantification of artemillin-C in Brazilian propolis. *Phytochem Anal* 2008; **19**: 179–183.
- Sengupta M, Sharma GD, Chakraborty B. Effect of aqueous extract of *Tinospora cordifolia* on functions of peritoneal macrophages isolated from CCl₄ intoxicated male albino mice. *BMC Complement Altern Med* 2011; **11**: 102.
- Han S, Sung KH, Yim D, et al. Activation of murine macrophage cell line RAW 264.7 by Korean propolis. *Arch Pharm Res* 2002; **25**: 895–902.
- Lv LH, Yu JD, Li GL, et al. Functional distinction of rat liver natural killer cells from spleen natural killer cells under normal and acidic conditions *in vitro*. *Hepatobiliary Pancreat Dis Int* 2012; **11**: 285–293.
- Pagliarone AC, Missima F, Orsatti CL, Bachiaga TF, Sforcin JM. Propolis effect on Th1/Th2 cytokines production by acutely stressed mice. *J Ethnopharmacol* 2009; **125**: 230–233.
- Hernandez J, Goycoolea FM, Quintero J, et al. Sonoran propolis: chemical composition and antiproliferative activity on cancer cell lines. *Planta Med* 2007; **73**: 1469–1474.
- Simpson MA, Gozzo JJ. Spectrophotometric determination of lymphocyte mediated sheep red blood cell hemolysis *in vitro*. *J Immunol Methods* 1978; **21**: 159–165.
- Wen XM, Zhang YL, Liu XM, Guo SX, Wang H. Immune responses in mice to arecoline mediated by lymphocyte muscarinic acetylcholine receptor. *Cell Biol Int* 2006; **30**: 1048–1053.
- Escandell JM, Recio MC, Máñez S, et al. Dihydrocucurbitacin B inhibits delayed type hypersensitivity reactions by suppressing lymphocyte proliferation. *J Pharmacol Exp Ther* 2007; **322**: 1261–1268.
- Kovacs EJ, Palmer JL, Fortin CF, Fülöp T Jr, Goldstein DR, Linton PJ. Aging and innate immunity in the mouse: impact of intrinsic and extrinsic factors. *Trends Immunol* 2009; **30**: 319–324.
- Orsatti CL, Missima F, Pagliarone AC, et al. Propolis immunomodulatory action *in vivo* on Toll-like receptors 2 and 4 expression and on pro-inflammatory cytokines production in mice. *Phytother Res* 2010; **24**: 1141–1146.
- de Castro PA, Savoldi M, Bonatto D, et al. Molecular characterization of propolis induced cell death in *Saccharomyces cerevisiae*. *Eukaryot Cell* 2011; **10**: 398–411.
- Chan GC, Cheung KW, Sze DM. The immunomodulatory and anticancer properties of propolis. *Clin Rev Allergy Immunol* 2013; **44**: 262–273.
- Kimoto T, Arai S, Kohguchi M, et al. Apoptosis and suppression of tumor growth by artemillin C extracted from Brazilian propolis. *Cancer Detect Prev* 1998; **22**: 506–515.
- Kobayashi K, Kaneda K, Kasama T. Immunopathogenesis of delayed-type hypersensitivity. *Microsc Res Tech* 2001; **53**: 241–245.
- Feng YH, Zhou WL, Wu QL, Li XY, Zhao WM, Zou JP. Low dose of resveratrol enhanced immune response of mice. *Acta Pharmacol Sin* 2002; **23**: 893–897.
- Johnson SA, Cambier JC. Ageing, autoimmunity and arthritis: senescence of the B cell compartment—implications for humoral immunity. *Arthritis Res Ther* 2004; **6**: 131–139.
- Frasca D, Riley RL, Blomberg BB. Humoral immune response and B-cell functions including immunoglobulin class switch are downregulated in aged mice and humans. *Semin Immunol* 2005; **17**: 378–384.
- Frasca D, Landin AM, Lechner SC, et al. Aging downregulates the transcription factor E2A, activation-induced cytidine deaminase, and Ig class switch in human B cells. *J Immunol* 2008; **180**: 5283–5290.
- Rajczyk K, Vargha P, Beregi E. Relationship between immunoglobulin levels and specific antibody titers in the elderly. *Z Gerontol* 1986; **19**: 158–161.
- Cetin E, Silici S, Cetin N, Güçlü BK. Effects of diets containing different concentrations of propolis on hematological and immunological variables in laying hens. *Poult Sci* 2010; **89**: 1703–1708.
- Freitas JA, Vanat N, Pinheiro JW, Balarin MR, Sforcin JM, Venancio EJ. The effects of propolis on antibody production by laying hens. *Poult Sci* 2011; **90**: 1227–1233.
- Fan Y, Hu Y, Wang D, et al. Epimedium polysaccharide and propolis flavone can synergistically stimulate lymphocyte proliferation *in vitro* and enhance the immune responses to ND vaccine in chickens. *Int J Biol Macromol* 2010; **47**: 87–92.
- Zheng Z, Yingeng W, Qingyin W, et al. Study on the immune enhancement of different immunoadjuvants used in the pentavalent vaccine for turbot. *Fish Shellfish Immunol* 2012; **32**: 391–395.
- Yuan J, Liu J, Hu Y, et al. The immunological activity of propolis flavonoids liposome on the immune response against ND vaccine. *Int J Biol Macromol* 2012; **51**: 400–405.
- Righi AA, Negri G, Salatino A. Comparative chemistry of propolis from eight brazilian localities. *Evid Based Complement Alternat Med* 2013; **2013**: 267878.
- Kumazawa S, Yoneda M, Shibata I, Kanaeda J, Hamasaka T, Nakayama T. Direct evidence for the plant origin of Brazilian propolis by the observation of honeybee behavior and phytochemical analysis. *Chem Pharm Bull (Tokyo)* 2003; **51**: 740–742.
- Ansorge S, Reinhold D, Lendeckel U. Propolis and some of its constituents down-regulate DNA synthesis and inflammatory cytokine production but induce TGF- β 1 production of human immune cells. *Z Naturforsch C* 2003; **58**: 580–589.
- Bachiaga TF, Orsatti CL, Pagliarone AC, Sforcin JM. The effects of propolis and its isolated compounds on cytokine production by murine macrophages. *Phytother Res* 2012; **26**: 1308–1313.
- Fischer G, Paulino N, Marcucci MC, et al. Green propolis phenolic compounds act as vaccine adjuvants, improving humoral and cellular responses in mice inoculated with inactivated vaccines. *Mem Inst Oswaldo Cruz* 2010; **105**: 908–913.
- Cheung KW, Sze DM, Chan WK, Deng RX, Tu W, Chan GC. Brazilian green propolis and its constituent, artemillin C inhibits allogeneic activated human CD4 T cells expansion and activation. *J Ethnopharmacol* 2011; **138**: 463–471.