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Enhancement of the immune responses to vaccination against foot-and-mouth disease in mice by oral administration of an extract made from *Rhizoma Atractylodis Macrocephalae* (RAM)

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ABSTRACT

This study was designed to evaluate the effects of oral administration of a water extract made from the *Rhizoma Atractylodis Macrocephalae* (RAM) on the immune responses in mice immunized with FMDV type O vaccine. Thirty-five ICR mice were randomly divided into five groups with seven animals in each group, and orally administered daily for 4 days at a dose equivalent to 0, 0.0625, 0.125, 0.25 or 0.5 g of dried RAM, respectively. After that, the animals were subcutaneously immunized twice with FMDV vaccine at 2-week intervals. Blood samples were collected 3 weeks after boosting for measurement of FMDV-specific IgG titers and the IgG subclasses, lymphocyte proliferation as well as production IL-5 and IFN-γ. Results indicated that serum FMDV-specific IgG titers and the IgG subclass responses were significantly enhanced in mice orally administered RAM at the dose of 0.25 or 0.5 g when compared with the control group ($P < 0.05$). Splenocyte proliferation in response to Con A and LPS and production of IL-5 and IFN-γ by splenocytes were also significantly enhanced ($P < 0.05$). Considering the immunomodulatory effect and safety of RAM demonstrated in this study, this herb deserves further investigation to evaluate its potential improvement of FMD vaccination in other animals such as pigs, goats and cattle.

1. Introduction

Foot-and-mouth disease (FMD) is a highly contagious disease that affects cloven-hoofed animals such as cattle, swine and sheep [1]. Because FMD virus (FMDV) can rapidly spread between susceptible animals, it is listed by the World Organization for Animal Health (OIE) one of the most important animal diseases in the world [2]. Vaccination is a common practice against FMD in many countries. However, failure to elicit effective immune responses by vaccination has been frequently reported [3–5]. For examples, Xie et al. [6] observed only 20.9% of piglets had produced immune responses with antibody titer high enough for protection following vaccination against FMD in Ningxia Province of China. Hao et al. [7] analyzed 91 serum samples of the pigs having received vaccination against FMD and found that only 31.9% of the samples had antibody titer required for immune protection immunity. Therefore, there is a need to improve currently available vaccines in order to effectively protect humans and animals from infection.

Strategies to improve the immune response to vaccination have included the use of higher vaccine dose or increasing number of doses, use of different route of administration (e.g., intradermal versus intramuscular administration), accelerating dosing schedule and use of adjuvants such as antigen delivery systems and various immunomodulators [8–16] There is growing evidence that medicinal herbs and the ingredients enhance the immune response to vaccines direct against infectious agents [17,18]. Medicinal herbs have a variety of effects on immune responses and co-administration with herbal extracts has been shown to increase the antibody response and to enhance the proliferative response of T cells [19,20]. The efficacy of herbal extracts as adjuvant to vaccines has been an object of several clinical trials conducted in both humans and animals [21–24].

Atractylodis macrocephalae Koidz. is a plant in family of Compositae with a plenty of natural resource in Zhejiang, Jiangsu and Anhui Provinces in China. The *Rhizoma Atractylodis Macrocephalae* Koidz. (RAM) as a traditional Chinese medicine has been utilized for at least 2000 years. The annually consumed RAM for medical purpose is estimated at 7000 tons in this country [25]. The RAM is often cooked together with other herbs and the soup is used for oral administration in the treatment of diseases such as diarrhea and infections in humans and animals. For examples, Zhou [26] has reported effective treatment of infections in the upper

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respiratory tract in 125 cases with *Shen Ling Bai Zhu San*, which contains *Rhizoma Atractylodis Macrocephalae*, *Radix Codonopsis Pilosulae*, *Poria*, *Radix Glycyrrhizae*, *Rhizoma Dioscorea*, *Semen Dolichoris Album*, *Semen Nelumbinis*, *Semen Coicis*, *Fructus Amomi* and *Radix Platycodi*.

Our previous study has shown that oral administration of a water extract made from the *Rhizoma Atractylodis Macrocephalae* Koidz. has significantly increased immune responses elicited by a model antigen ovalbumin in mice. Based on that observation, this study was designed to investigate the effects of oral administration of RAM on the immune responses in mice immunized with a commercial FMDV vaccine by measuring serum specific antibody responses, lymphocyte proliferation and cytokine production.

2. Materials and methods

2.1. Animals

Female ICR mice were purchased from Shanghai Laboratory Animal Center (SLAC) Co. Ltd. (Shanghai, China), and housed in polypropylene cages with sawdust bedding in hygienically controlled environment. The average body weight was 24 ± 1.2 g. Feed and water were supplied *ad libitum*. All procedures related to the animals and their care throughout this study conformed to the internationally accepted principles as found in the Guidelines for Keeping Experimental Animals issued by the government of China.

2.2. Rhizoma Atractylodis Macrocephalae (RAM)

Dried RAM were purchased from Hu Qing Yu Tang Co. Ltd, Hangzhou, China. Drug source was in Panan County of Zhejiang Province of China where the best RAM is produced. The rhizome is harvested in winter when the active principles are believed to reach the highest. After cleaning and drying, RAM is cut into pieces for medical use. The qualified RAM has light fragrance with sweet taste. The drug (50 g) was ground into powder and saturated in 500 ml of water. After that, the soup was harvested by passing through a filter paper and condensed at a concentration equivalent to 4 g of dried RAM per ml by a R502B rotary evaporator (Shenko Tech Co. Ltd., Shanghai, China). The herbal solution was kept at 4 °C before use.

2.3. Oral administration of RAM extract and immunization

Thirty-five ICR mice were randomly divided into five groups with seven mice in each. The animals were subcutaneously injected twice with 200 μl of FMDV type O vaccine (Lanzhou Veterinary Research Institute) with 2-week intervals. One day before each immunization, the mice had already been orally administered daily for 4 days with 0.25 ml of 0.89% saline solution containing RAM of 0, 0.0625, 0.125, 0.25 or 0.5 g (equivalent to 0, 2.6, 5.2, 10.4 or 20.8 g/kg body weight). Blood samples were collected 2 weeks after the boost for detection of IgG titers and the IgG subclasses. Splenocytes were collected for determination of cell proliferation and production of IFN-γ and IL-5.

2.4. FMDV type O specific IgG titer and the IgG subclasses

Serum samples were analyzed for measurement of FMDV-specific IgG titer and the isotypes by an indirect double antibody sandwich enzyme-linked immunosorbent assay as described by Song et al. [27]. The wells of polyvinyl 96-well microtitre plates were coated with 50 μl rabbit anti-FMDV serotype O antibody (LVRI, China) diluted in 0.05 M carbonate/bicarbonate buffer (1:800), pH 9.6 and incubated overnight at 4 °C. After five washes with phosphate buffer saline containing 0.05% Tween-20 (PBST), the wells were blocked with 5% skimmed milk and incubated at 37 °C for 2 h. Thereafter, 50 μl FMDV type O antigen (LVRI) (1:3 dilution) was added and incubated at 4 °C for 2 h. After five washes, 50 μl of serum (diluted serially for IgG titer analysis or diluted 1:50 in PBS 5% skimmed milk for isotype analysis) was added to each well and incubated at 37 °C for 1 h. Plates were then washed five times in PBST. For IgG titer detection, 50 μl of goat anti-mouse IgG (1:500) (Kirkegaard, Perry Lab., Maryland, USA) was added to the wells and incubated at 37 °C for 1 h. Plates were washed again with PBST. Fifty microliters of 3,3′,5,5′-tetramethyl benzidine solution (100 μg/ml of 0.1 M citrate–phosphate, pH 5.0) was added to each well and incubated for 15 min at 37 °C. The reaction was stopped by adding 50 μl of 2 M H_2SO_4 to each well. The optical density of the plate was read by an automatic ELISA plate reader at 450 nm. Values above the cut-off background level (mean value of sera from unimmunized mice multiplied by a factor of 2.1) were considered positive. Titers were depicted as reciprocal end-dilutions. For subclasses, 50 μl of biotin-conjugated goat anti-mouse IgG1, IgG2a, IgG2b or IgG3 (1:600) (Santa Cruz Biotechnology Inc., California, USA) was added to corresponding plate and then incubated for 1 h at 37 °C. After washing, 50 μl of horseradish peroxidase conjugated anti-biotin (BD Biosciences, Pharmingen, USA) diluted 1:4000 in PBST was added to each well and incubated for 1 h at 37 °C. Incubations, washing and development were as described above for detection of FMDV-specific IgG titer. The optical density of the plate was read at 450 nm.

2.5. Splenocyte proliferation assay

Spleen collected from the FMDV-immunized ICR mice under aseptic conditions, in Hank's balanced salt solution (HBSS, Sigma), was minced and passed through a fine steel mesh to obtain a homogeneous cell suspension. After centrifugation ($380 \times g$ at 4 °C for 10 min), the pelleted cells were washed three times in HBSS and resuspended in complete medium (RPMI 1640 supplemented with 0.05 mM 2-mercaptoethanol, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% heat inactivated FCS). Cell numbers were counted with a haemocytometer by trypan blue dye exclusion technique. Cell viability exceeded 95%. Splenocyte proliferation was assayed as described previously [24]. The stimulation index (SI) was calculated based on the following formula: SI = the absorbance value for mitogen cultures divided by the absorbance value for non-stimulated cultures.

2.6. IFN-γ and IL-5 production by splenocytes in vitro

Single cell suspensions were adjusted to a concentration of 2.5×10^6 cells/ml in a complete medium. To a 96-well flat-bottom microtiter plate (Nunc), 100 μl of the cell suspension and equal volume of Con A solution (Final concentration 5 μg/ml) were added. The plates were incubated at 37 °C in a 5% CO_2 atmosphere for 48 h. After that, the culture supernatants were collected for cytokine assay. The concentrations of IFN-γ and IL-5 were determined by a commercial capture ELISA kit (R&D Systems Inc., Minneapolis, USA). Concentrations of cytokines were calculated from interpolation of the cytokine standard curve.

2.7. Statistical analysis

Data analysis was performed with SPSS software (SPSS, Version 11.5, SPSS Inc., Chicago, IL). ANOVA with Bonferroni post-hoc test was used for multiple comparisons between groups. Values were expressed as the mean ± standard deviation (S.D.). *P*-values of less than 0.05 were considered statistically significant.

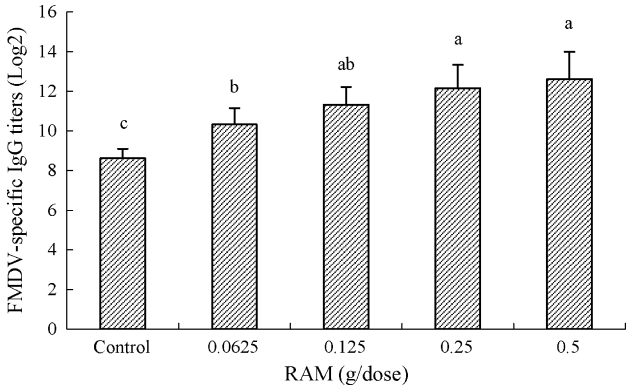

Fig. 1. FMDV-specific IgG titers. Mice were orally administered RAM solution (equivalent to 0, 0.0625, 0.125, 0.25 or 0.5 g of RAM/mouse/day) for 4 days, and then immunized s.c. twice with 200 μl of FMDV vaccine at 2-week intervals. Blood samples were collected 2 weeks after boosting for measurement of FMDV-specific IgG titers by an indirect ELISA. Values above the cut-off background level (the mean from unimmunized mice as negative control multiplied by a factor of 2.1) were considered positive. Titers were depicted as reciprocal end-dilutions. The values are presented as mean ± S.D. ($n = 7$). Bars with different letters are statistically different ($P < 0.05$).

3. Results

3.1. FMDV-specific IgG and the IgG isotypes

FMDV-specific IgG titers were significantly higher in mice orally administered RAM once a day for 4 days at a dose of 0.0625, 0.125, 0.25 and 0.5 g than in mice administered saline solution only ($P < 0.05$) (Fig. 1). The IgG titers tended to increase with increasing dose of RAM administered.

Fig. 2. indicated that all IgG isotypes tended to be higher in mice administered RAM than in the group, but significantly increased IgG isotypes were found in mice administered RAM at doses of 0.25 and 0.5 g ($P < 0.05$).

3.2. Proliferation of splenocytes isolated from FMDV-immunized mice

The effects of RAM on splenocyte proliferative responses to Con A and LPS are shown in Fig. 3. SI for the proliferation in response to Con A and LPS were numerically increased in mice administered 0.0625 and 0.125 g of RAM but significantly increased in mice administered 0.25 and 0.5 g of RAM when compared with the control ($P < 0.05$).

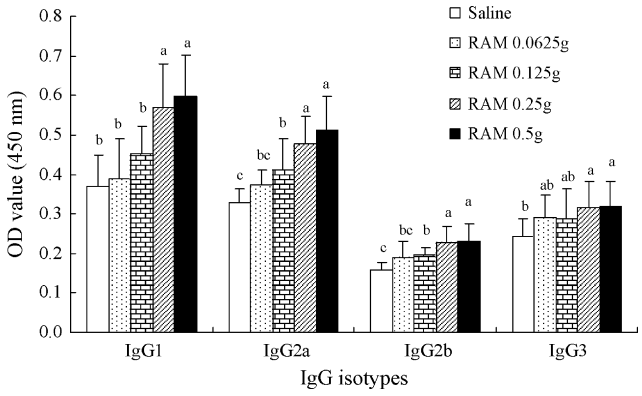

Fig. 2. FMDV-specific IgG isotypes. Mice were orally administered RAM solution (equivalent to 0, 0.0625, 0.125, 0.25 or 0.5 g of RAM/mouse/day) for 4 days, and then immunized s.c. twice with 200 μl of FMDV vaccine at 2-week intervals. Blood samples were collected 2 weeks after boosting for measurement of FMDV-specific IgG isotypes by an indirect ELISA. The values are presented as mean ± S.D. ($n = 7$). Bars with different letters are statistically different ($P < 0.05$).

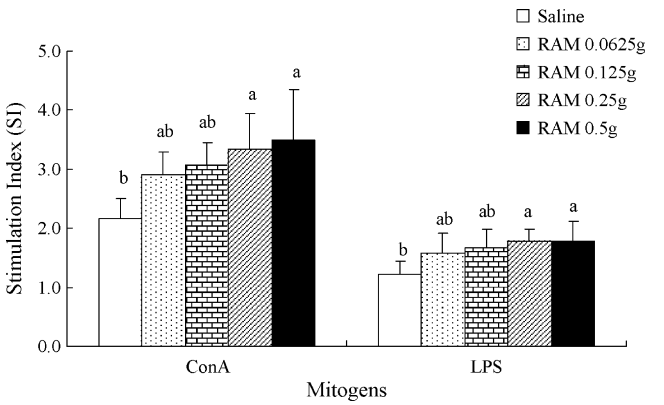

Fig. 3. Splenocyte proliferative responses to Con A and LPS. Mice were orally administered RAM solution (equivalent to 0, 0.0625, 0.125, 0.25 or 0.5 g of RAM/mouse/day) for 4 days, and then immunized s.c. twice with 200 μl of FMDV vaccine at 2-week intervals. Splenocytes were prepared 2 weeks after the last immunization and cultured with Con A (5 μg/ml) or LPS (5 μg/ml) or RPMI 1640. Splenocyte proliferation was measured by the MTT method as described in the text, and shown as a stimulation index (SI). The values are represented mean ± S.D. ($n = 7$) Bars with different letters are statistically different ($P < 0.05$).

3.3. Effects of oral administration of RAM extract on IL-5 and IFN-γ production by mouse splenocytes

Cytokines secreted in the supernatants are shown in Fig. 4. IFN-γ and IL-5 production were significantly higher in the cultures of cells isolated from the mice administered RAM (0.2 g) than from the mice administered saline solution only ($P < 0.05$).

3.4. Effects of oral administration of RAM on the body weight of mice

No abnormal behavior and side effects were found in mice throughout the experiment. There was no significant difference for the body weight between the mice administered RAM (0.0625, 0.125, 0.25 or 0.5 g) and the control mice administered saline solution as indicated in Table 1 ($P > 0.05$).

4. Discussion

Enhanced immune responses to vaccination against FMD have been demonstrated in mice by oral administration of an extract made from *Rhizoma Atractylodis Macrocephalae*. After oral administration for 4 days of RAM, immunization of a commercial FMDV vaccine induced significantly higher serum specific IgG and the IgG isotype responses, splenocyte proliferation in response to Con A and LPS as well as production of IL-5 and IFN-γ by splenocytes than in mice administered saline solution alone.

The mouse model has been used to study the immunity of a host against FMDV infections [28,29,34]. Salguero et al. have reported that the mice immunized with conventional inactivated FMDV vaccine can be protected against challenge with a lethal dose of FMDV

Table 1
Effects of oral administration of RAM on the mean body weight (mean ± S.D. gram) of mice ($n = 7$).

Gram of RAM/mouse	Before immunization*	Before booster*	2 weeks after booster*
0	23.86 ± 1.11	26.26 ± 1.18	27.21 ± 1.41
0.0625	24.30 ± 1.35	26.29 ± 1.64	26.96 ± 1.52
0.125	23.89 ± 0.81	26.97 ± 0.83	28.40 ± 0.81
0.25	24.40 ± 1.32	28.06 ± 2.07	28.74 ± 1.48
0.5	23.49 ± 1.41	26.14 ± 1.71	26.94 ± 1.57

* No statistical difference between groups at the same time ($P < 0.05$).

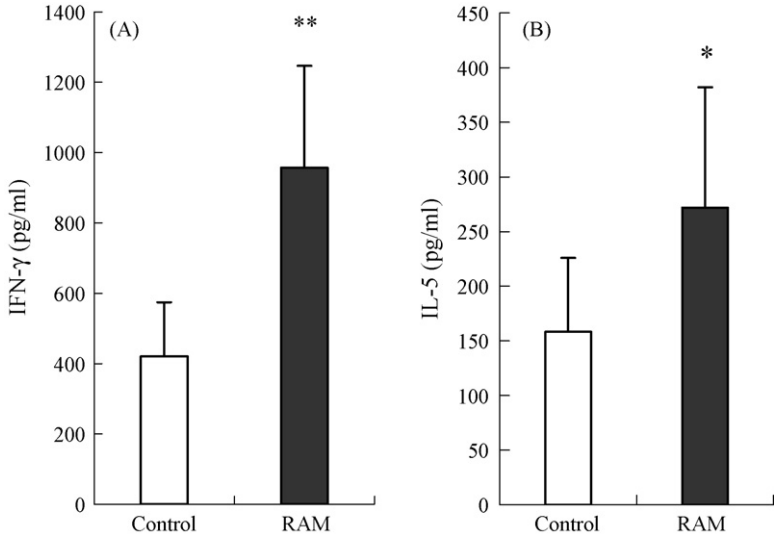

Fig. 4. IFN-γ (A) and IL-5 (B) production by ICR mouse splenocytes stimulated with Con A. Mice were orally administered RAM solution (equivalent to 0.25 g of RAM/mouse/day) for 4 days, and then immunized s.c. twice with 200 μl of FMDV vaccine at 2-week intervals. Splenocytes were prepared 2 weeks after the last immunization and cultured with Con A (5 μg/ml) for 48 h. The supernatants were harvested for determination of IL-5 and IFN-γ by a capture ELISA. The values are presented as mean ± S.D. ($n = 7$). Bars designated as * are statistically different from the control ($P < 0.05$).

[28]. Wong et al. have observed that a FMD DNA vaccine inducing immune response in mice can also elicit protection in swine against FMD infection [29]. Humoral immune response against FMD infection has been well documented [30,31] and the contribution of antibodies to the major immune defense against FMDV is clear [32,33]. Studies in animal models [32,34] have shown that specific antibodies play an important role in the immune defense against FMD. However, poor humoral immune response to vaccination of some commercial FMD vaccines has been reported previously in both experimental animals and pigs [34,24]. Fig. 1 showed that oral administration of RAM significantly increased serum FMDV-specific IgG titers to a commercial FMD vaccine. This result is similar to that previously found when OVA was used as antigen in a mouse model.

Oral administration of RAM significantly increased IgG1, IgG2a, IgG2b and IgG3 as indicated in Fig. 2. In mice, Th2 lymphocyte cytokines such as IL-4, IL-5 and IL-10 augment the production of IgG1, while the IL-2, TNF-β and IFN-γ produced by Th1 lymphocytes improve the production of IgG2a [35,36]. In terms of the body response against infectious diseases, the Th1 response primarily targets intracellular pathogens such as viruses and certain bacteria, while the Th2 response is mainly targeted to extra cellular pathogens, such as most bacteria and certain parasites [35,36]. Enhanced production of all IgG subclasses may be explained by increased release of both IL-5 and IFN-γ as shown in Fig. 4. All these suggested that both Th1 and Th2 immune responses were activated. Thus, when the purpose of a vaccination is to activate both Th1 and Th2 immune response, the oral administration of RAM before immunization is indicated.

The increased production of IgG and the IgG subclasses (as indicated in Figs. 1 and 2) may be due to an activation of specific lymphocytes by RAM. Fig. 3 shows that both Con A- and LPS-induced proliferations were significantly enhanced, suggesting that T as well as B cells were activated [37]. In order to induce antibody production, triggered B lymphocytes are required for clonal expansion. The enhanced lymphocyte responses to Con A or LPS stimulation paralleled the increased serum IgG responses detected in the mice administered RAM. Similar results have been found in other studies. Chang et al. [38] reported that oral administration of an extract made from *Rhizoma Atractylodis Macrocephalae* for 10 days significantly enhanced lymphocyte transformation in response to PHA stimulation in mice. Hu et al. [39] have found an increased body weight and potentiated lymphocyte proliferation in response to Con A and LPS stimulation in rats fed a diet supplemented with the powder made from *Rhizoma Atractylodis Macrocephalae*.

Chemical analysis has indicated that RAM is full of volatile oil and polysacchrides [40]. Volatile oil fraction has been found to contain selina-4(15)-7(11)-dien-8-one, atractylenolides I, II, III, IV, 8-β-ethoxy-atractylenolide, 3-β-acetoxyatractylon, 3-β-hydroxyatroctylon and hiessol, etc. [40]. Both volatile oil and polysaccharide fractions have been reported to have immunomodulatory properties. Guan et al. have observed a significantly increased expression of Fc receptors on the peritoneal macrophages in mice orally administered for 7 days the oil fractions at a dose of 15 g/kg body weight [41]. Mao et al. have found that incubation of polysaccharide fraction with mouse spleen lymphocytes can significantly enhance the proliferative response to Con A or PHA and increase IL-2 production [42]. Therefore, the immunomodulatory effects found in our study may be attributed to both volatile oil and polysaccharide fractions in RAM.

In addition to the immunomodulatory effects, RAM has anti-inflammatory activity as well. RAM together with other herbs has been used to treat infections in the upper respiratory tract in humans [26]. Dong et al. have found that xylene-induced acute inflammatory edema of the ear in mice has been significantly reduced after oral administration of the compounds such as atractylenolide isolated from the rhizome [43]. Imbalance of Th1/Th2 lymphocytes can be one of the reasons for some inflammatory diseases [44,45]. In this study, RAM has been found to stimulate both IFN-γ (Th1-like cytokine) and IL-5 (Th2-like cytokine), suggesting that can RAM promote balanced Th1/Th2 responses. This may constitute the anti-inflammatory mechanism of RAM.

A conventional approach to improvement of the efficacy of vaccination is to add adjuvant to vaccines. Adjuvant used for this purpose should be safe enough to induce minimal adverse effects to prove acceptable for use in healthy individuals. Many natural products have been reported having immunomodulatory properties while their active principles are usually unclear. Thus, purification of the herbal extracts is usually difficult, and irritation will take place when unpurified herbal extracts are co-injected with immunizing antigens. As traditional medicinal herbs are usually administered by oral route, oral use of immunomodulators can avoid the side effects

found in parenteral administration. For example, oral administration of crude saponins made from the bark of the *Quillaja* tree has been proven to have immunopotentiating activity with moderated toxicity, however, they are toxic when administered parenterally [46,47]. In our study, no abnormal behavior and side effects were found in mice throughout the experiment, and there was no significant difference for the body weight between the mice administered RAM and the control mice administered saline solution as indicated in Table 1, suggesting that oral administration of RAM is safe.

In summary, the oral administration for 4 days of RAM significantly increased the IgG and the IgG subclass responses, splenocyte proliferation as well as production of IL-5 and IFN-γ in mice immunized with a commercial FMD vaccine. Considering the immunomodulatory effect and safety of RAM demonstrated in this study, this herb deserves further investigation to evaluate its potential improvement of FMD vaccination in other animals such as pigs, goats and cattle.

Acknowledgements

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