

Inactivated herpes simplex virus-1 vaccine formulated in aqueous and alcoholic extracts of propolis boosts cellular and IgG responses

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ARTICLE INFO

Article type:

Short Communication

Article history:

Received: Sep 25, 2023

Accepted: Feb 21, 2024

Keywords:

Adjuvant

HSV-1

Propolis

Th1

Vaccine potency

ABSTRACT

Objective(s): In this study, the adjuvant activity of aqueous and alcoholic extracts of propolis was examined on the inactivated herpes simplex virus-1 (HSV-1).

Materials and Methods: BALB/C mice were administered with inactivated (HSV-1; the KOS strain) plus alcoholic and aqueous extracts, followed by assessment of the cellular and humoral immune responses.

Results: Alcoholic and aqueous extracts, as an adjuvant, revealed a significant increase in lymphocyte proliferation and cytotoxic T lymphocyte (CTL) responses versus the HSV-1 group. In addition, HSV-1 plus alcoholic extract showed a remarkable increase in IFN- γ cytokine and IFN- γ /IL-4 ratio. On the other hand, both alcoholic and aqueous extracts in the HSV-1 vaccine suppressed the IL-4 cytokine response as compared with the HSV-1 vaccine. In addition, HSV-1 plus alcoholic extract showed a significant increment in IgG₁, IgG_{2a}, and IgG_{2b} isotypes as compared with the HSV-1 vaccine.

Conclusion: Propolis extracts seem to modulate the immune response against inactivated HSV-1 model and can be used as a suitable vaccine adjuvant or a component of a complex adjuvant against infectious diseases.

► Please cite this article as:

Mojarab S, Karimi P, Shahbazzadeh D, Moghbeli M, Pooshang Bagheri K, Beig Parikhani A, Dehghan R, Zafari E, Moravej A, Pouriayeali MH, Mirtalebi SF, Pakjoo M, Yazdani Sh, Abdollahpour-Alitappeh M, Mahdavi M. Inactivated herpes simplex virus-1 vaccine formulated in aqueous and alcoholic extracts of propolis boosts cellular and IgG responses. Iran J Basic Med Sci 2024; 27:

Introduction

Although vaccines can decrease the incidence of infectious diseases, some vaccines lack sufficient efficacy, highlighting the need for improving the efficacy of such vaccines. Adjuvants are substances combined with candidate antigens to improve the vaccine efficiency. There are natural compounds with immunomodulation properties such as propolis (1, 2). Propolis is a resin-brown substance that is collected by worker bees (*Apis mellifera meda*) and was shown to have boosting effects on Th1 and

humoral responses in several vaccine models (3-6). In the present study, inactivated HSV-1 was formulated in aqueous and alcoholic extracts of propolis derived from the Iranian Honey bee, as an adjuvant, and then the immune responses of the newly formulated vaccine were assessed in mice.

Materials and Methods

Propolis, kindly gifted by Dr. Pouria Ghasemi from the Pasteur Institute of Iran, was homogenized either in distilled water or ethanol (95-96%) for about 2 weeks (7). The vaccine

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contained 5 µg of inactivated HSV-1 in 200 µl of phosphate-buffered saline (PBS) buffer with or without 5 mg of the aqueous extract of propolis. Six- to 8-week-old BALB/C mice were divided into six groups containing 6 mice in each one. Groups 1 through 6 received 5 µg inactivated HSV-1+5 mg of alcoholic extract, 5 µg inactivated HSV-1+5 mg of aqueous extract, 5 µg inactivated HSV-1, 5 mg of alcoholic extract, 5 mg of aqueous extract, and PBS as control groups, respectively.

Experimental mice were immunized subcutaneously on days 0, 14, and 28. Two weeks after the third immunization, 3×10^5 of spleen cells were dispensed into 96-well flat-bottom culture plates and stimulated with 1 µg/well of inactivated HSV-1 virus for 72 hr. Afterward, blood samples were collected from the mice 2 weeks after the third injection through the retro-orbital puncturing of mice for antibody assay. The lymphocyte proliferation was then assessed using a BrdU kit (Roche, Germany). Interferon-gamma (IFN-γ) and interleukin 4 (IL-4) cytokines were measured on the spleen cell culture supernatant using commercial Enzyme-linked immunosorbent assay (ELISA) kits (Quantikine, R&D Systems, USA) according to the manufacturer's instructions. After 36 hr of antigen stimulation, the culture supernatant was collected and used for Granzyme B (Gr-B) assay using an ELISA kit (eBiosystem, USA). In order to evaluate the immunoglobulin G (IgG) response, HSV-1 inactivated viruses were coated on 96-well ELISA plates (Greiner, Germany) and incubated for 24 hr at 4 °C. The wells were washed with PBS containing 0.05% Tween 20 (washing buffer) and blocked with 5% skimmed milk in washing buffer for 60 min at 37 °C (blocking buffer). Subsequently, 100 µl of 1/50 to 1/6400 dilutions of sera were added into each well and incubated for 90 min at 37 °C. The wells were washed before incubation for 90 min with 100 µl of 1/10000 dilution HRP-conjugated anti-mouse antibody (Sigma, USA). Following five-time washing, the wells were incubated in the dark for 30 min with 100 µl of the TMB substrate, the reaction was stopped with 2N H₂SO₄, and the color density was read with an ELISA plate reader at 450 nm (BioTek, USA). Meanwhile, specific IgG₁, IgG_{2a}, IgG_{2b}, and IgM subclasses were evaluated using an ISO-2 kit according to our optimized ELISA (Sigma, USA) (8). Lymphocyte proliferation assay, cytokines, and Gr-B assay were performed in duplicate, and antibody ELISA was performed in triplicate. The Mann-Whitney U test was used in all statistical analyses. *P*-values less than 0.05 were considered statistically significant in all circumstances.

Results

Mice immunized with the HSV-1 vaccine alone or with propolis alcoholic and aqueous extracts showed a significant increase in lymphocyte proliferation as compared with the control groups. At the same time, HSV-1 with propolis alcoholic extract showed a significant increment as compared with the HSV-1 group ($P=0.0152$)(Figure 1).

IFN-γ cytokine response in all vaccine groups showed a significant rise as compared with the control groups ($P<0.0002$). Additionally, the vaccine with alcoholic extract showed a remarkable increment as compared with the vaccine plus aqueous extract and HSV-1 vaccine ($P<0.0010$) (Figure 2A). Results from the IL-4 cytokine response in HSV-1 plus alcoholic and/or aqueous extracts as well as the HSV-1 vaccine showed a significant rise as compared with

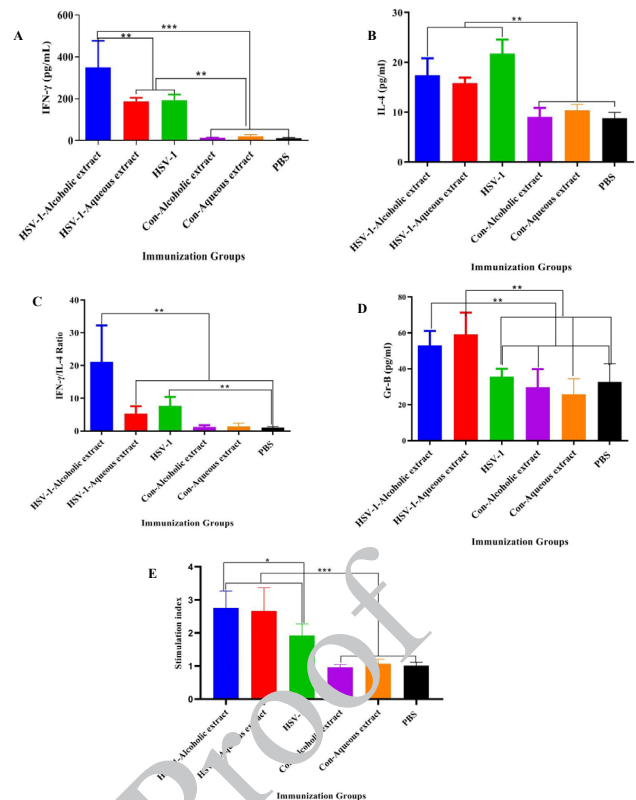


Figure 1. Results from lymphocyte proliferation of experimental mice against herpes simplex virus-1 (HSV-1) vaccine. Alcoholic and aqueous extracts of propolis in the vaccine formulation showed a significant increase against the HSV-1 vaccine ($P<0.028$)

the control groups (Figure 2B). Furthermore, the IFN-γ/IL-4 ratio in HSV-1 plus alcoholic extract showed a remarkable increase as compared with all other experimental groups (Figure 2C). Results from Gr-B release as a criterion of cytotoxic T lymphocytes (CTL) activity in the vaccine with alcoholic extract with or without aqueous extracts led to a significant rise as compared with the control groups and the HSV-1 vaccine group (Figure 3).

Results from specific total IgG antibody showed a significant total IgG increment of mice immunized with the HSV-1 vaccine plus alcoholic extract as compared with the alcoholic extract control and PBS group at the dilutions of 1/50 to 1/1600 (Figure 4).

Results from specific IgG₁ isotype in the vaccine with alcoholic extract with or without aqueous extracts and HSV-1 vaccine revealed a remarkable rise as compared with the control groups (Figure 5A). The IgG_{2a} isotype in the vaccine with alcoholic extracts showed a significant increase as compared with the alcoholic extract control and PBS group, while HSV-1 plus aqueous extract and HSV-1 vaccine revealed a remarkable increase versus PBS (Figure 5B).

Results from specific IgG_{2b} isotype in the vaccine with alcoholic extracts showed an increase as compared with the HSV-1 plus aqueous, alcoholic extract control, and PBS group. Mice immunized with the HSV-1 vaccine plus alcoholic extract showed a significant increment in the IgG_{2b} response versus the HSV-1 plus aqueous extract group ($P=0.0371$)(Figure 5C).

Results from the specific IgM isotype in the vaccine with alcoholic extract with or without aqueous extracts and HSV-

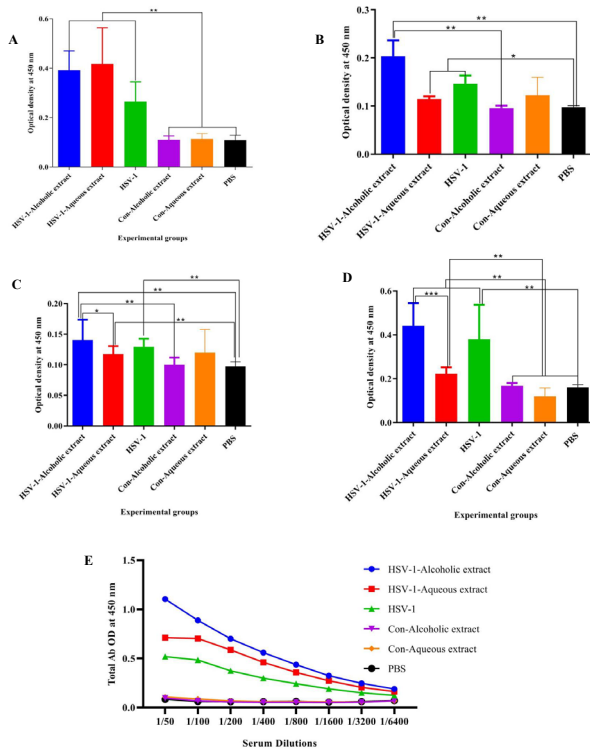


Figure 2. Results from (A) IFN- γ , (B) IL-4 cytokines, and (C) IFN- γ /IL-4 ratio in the experimental groups (A) Immunization with herpes simplex virus-1 (HSV-1) vaccine plus alcoholic extract showed a significant rise in IFN- γ cytokine versus HSV plus aqueous extract and HSV-1 vaccine ($P=0.014$ and $P=0.016$, respectively). (B) Immunization with HSV-1 vaccine formulated in alcoholic and/or aqueous extracts suppressed IL-4 cytokine response versus the HSV-1 vaccine ($P=0.01$ and $P=0.063$, respectively). (C) Results from the IFN- γ /IL-4 ratio in the HSV-1 plus alcoholic extract group showed a remarkable increase versus all other experimental groups ($P<0.0221$)

1 vaccine revealed an outstanding increase as compared with the control groups. Additionally, mice immunized with the HSV-1 vaccine plus alcoholic extract showed a significant increment in the IgM response versus the HSV-1 plus aqueous extract group (Figure 5D).

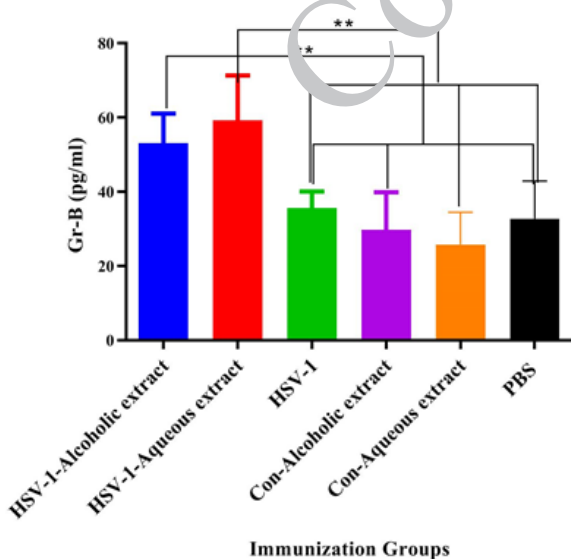


Figure 3. Results from cytotoxic T lymphocyte (CTL) activity in the experimental groups (A) Immunization with the vaccine plus alcoholic and vaccine plus aqueous extracts showed a significant increase compared with the *Herpes Simplex Virus-1* (HSV-1) vaccine group ($P=0.0005$ and $P=0.0006$, respectively)

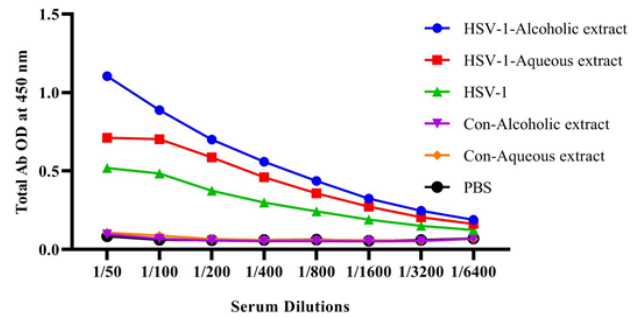


Figure 4. Results from specific total IgG antibody Immunization with the *Herpes Simplex Virus-1* (HSV-1) vaccine plus alcoholic extract showed a significant increase versus the HSV-1 vaccine at dilutions of 1/50 to 1/800 ($P=0.0255$). In addition, immunization with HSV-1 plus aqueous extract at dilutions of 1/50 to 1/200 showed a significant increment versus the HSV-1 vaccine group ($P<0.0100$). Furthermore, the HSV-1 vaccine plus alcoholic extract revealed a remarkable increase versus the HSV-1 plus aqueous extract at dilutions of 1/50 and 1/100 ($P<0.0384$)

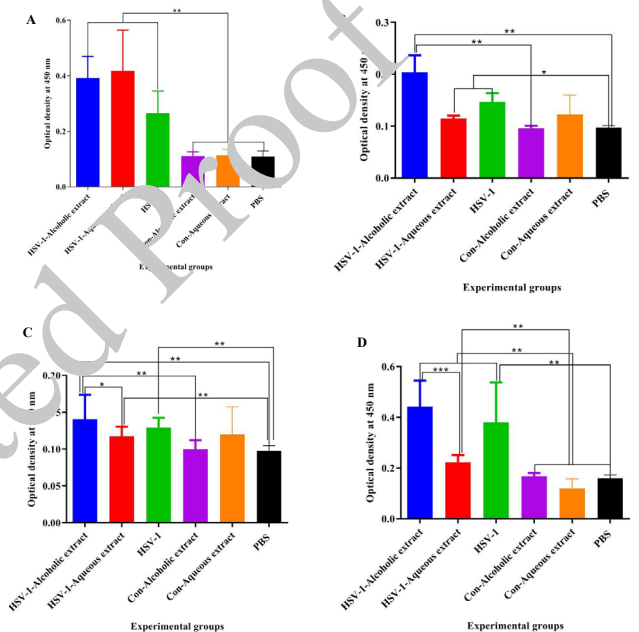


Figure 5. Results from specific (A) IgG₁, (B) IgG_{2a}, (C) IgG_{2b}, and (D) IgM isotype antibodies in the experimental mice (A) Immunization with the herpes simplex virus-1 (HSV-1) vaccine in alcoholic and/or aqueous extracts formulation showed a significant increase in IgG₁ isotype versus the HSV-1 vaccine group ($P=0.005$ and $P=0.0078$, respectively). (B) Results from the specific IgG_{2a} isotype revealed that immunization with HSV-1 plus alcoholic extract showed a significant increase versus HSV-1 plus aqueous extract and HSV-1 vaccine group ($P=0.0001$ and $P=0.0002$, respectively). (C) Results from the specific IgG_{2b} isotype revealed that immunization with HSV-1 plus alcoholic extract showed a significant rise versus the HSV-1 plus aqueous extract group ($P=0.0371$). (D) Immunization with HSV-1 vaccine plus alcoholic extract showed a remarkable increase in IgM response versus the HSV-1 plus aqueous extract group ($P=0.0001$)

Discussion

Studies showed that propolis could modulate immune responses in several aspects, such as Th1 and Th17 polarization, CTL improvement, and induction of IgG response (5, 9, 10). In this study, lymphocyte proliferation as a criterion of cellular immune response showed that both alcoholic and aqueous extracts of propolis improved T-cell responses. In a study carried out by Conti *et al.*, propolis could stimulate lymphocyte proliferation responses through the modulation of dendritic cells (10). Of note, some bodies of evidence showed the potency of propolis extracts in triggering T cell proliferation (7, 11, 12), which is in parallel to our findings, demonstrating the ability of both alcoholic and aqueous extracts of propolis to stimulate

cellular immune responses. It is clear that cellular immunity is critical in controlling viral infections (13) and this is an advantage for propolis in the vaccine formulation.

Cytokine IL-4, as a Th2 (T helper type 2), and IFN- γ , as a Th-1 response (14), indicated that alcoholic extract in the HSV-1 vaccine formulation improved the IFN- γ cytokine response. Meanwhile, both alcoholic and aqueous extracts in the HSV-1 vaccine suppressed IL-4 cytokine release. These findings showed the potency of alcoholic and aqueous extracts in the induction of the Th1 (T helper type 1) response either by increasing IFN- γ cytokine responses or through suppression of IL-4 cytokine responses. However, the alcoholic extract showed a more robust Th1 response.

In a study, the ethanolic extract of propolis and Alum, as an adjuvant, plus the inactivated Suid herpesvirus type 1 (*SuHV-1*) vaccine showed a Th1 response (15). In a study performed by Bezerra *et al.*, a recombinant protein rCP01850 plus Brazilian red propolis showed a Th1 response and protection against *Corynebacterium pseudotuberculosis* challenge in mice (16). All of these studies confirmed the immunomodulatory effect of propolis extracts in the induction of the Th1 immune response as demonstrated in this study. Additionally, the IFN- γ /IL-4 ratio, as another parameter of confirming Th1 response, showed the highest level in the HSV-1 vaccine plus the alcoholic extract group which showed a Th1 response.

Results from CTL activity showed a significant rise in the HSV-1 vaccine plus alcoholic and aqueous extracts versus the HSV-1 vaccine. A study conducted by Onur *et al.* on breast cancer-bearing mice showed that propolis induced CTL activity of mice as well as improving the anti-tumor CTL response when used in combination with acidophilus milk, suggesting that propolis extracts are useful as an adjuvant in the vaccine formulation to induce cellular immune for cancer immunotherapy (17). It is well-known that Th1 and CTL responses are important in controlling tumor growth (18). The specific IgG response in the HSV-1 vaccine plus both alcoholic and aqueous extracts was able to boost humoral immune responses. Several studies confirmed the boosting activity of propolis extracts on the antibody response in vaccine models (16, 19). Improvement of humoral immune responses by propolis extracts is an important property, because of the important role of humoral immune response in the neutralization of viruses and clearance of the body (20).

Results from antibody isotypes showed that alcoholic extract of propolis increased IgG₁, IgG_{2a}, and IgG_{2b} while not showing a positive effect on IgM response isotype responses in comparison with the HSV-1 group. Nevertheless, the aqueous extract revealed a boosting effect only on the IgG₁ response in comparison with the HSV-1 group. This finding showed that the bioactivity of these extracts on the humoral immune response is different and alcoholic extract is more efficient than aqueous extract in the induction of multi-isotype humoral immune responses. Induction of multi-isotype humoral immune response is very important because each specific isotype of antibody has a special function in the humoral immune response, and stimulation of antibody response with different isotypes means higher bioactivity of humoral immune responses.

Conclusion

Alcoholic and aqueous extracts of propolis boosted

cellular and humoral immune response versus HSV-1 vaccine which may candidate these components as valuable vaccine adjuvants.

Acknowledgment

The results presented in this paper were part of a student thesis. This study was supported by a grant from the Pasteur Institute of Iran. The authors are grateful to the Department of Virology of Pasteur Institute of Iran and their staff for technical support.

Authors' Contributions

S M, P K, D S, K PB, A BP, R D, SE Z, A M, M P, M AA, and M M performed animal handling, vaccine immunization, serum sample preparation, cytokines assay, igg Elisa, and data acquisition. S M, D S, M M, K PB, and M M performed virus propagation and inactivation and alcoholic and aqueous extraction of propolis. P K, S M, A BP, R D, SE Z, A M, M P, M AA, and M M helped with vaccine formulation, statistical analysis, and drafting the manuscript. D S, M M, K PB, and M M provided hypothesis of the study and study planning, interpreted the data, and critically reviewed the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

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Corrected Proof