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Original Research Article (Experimental)

Humoral immune and adjuvant responses of mucosally-administered *Tinospora cordifolia* immunomodulatory protein in BALB/c mice

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ABSTRACT

Background: In traditional medicine, guduchi (*Tinospora cordifolia*) is considered as an adaptogen with immunomodulatory prowess. A 25 kDa protein from guduchi stem has been characterized as an immunomodulatory protein (ImP).
Objectives: The aim of this study was to evaluate the intrinsic immunogenicity of guduchi ImP and adjuvant activity using ovalbumin (OVA) as antigen in BALB/c mice.
Materials and Methods: Mice were given guduchi ImP (30 and 60 μg) by intranasal administration to respective groups (n = 6) on days 1, 14 and thereafter weekly till day 42. Immunogenic response was monitored by serum IgG/IgA levels (days 14, 35 and 50). The adjuvant activity was measured by serum anti-OVA IgG/IgA responses to administration of 30 μg OVA with guduchi ImP. The effect of guduchi ImP on the spleen status was examined by splenic weight (day 50).
Results: Guduchi ImP administration displayed a significant increase in anti-guduchi ImP IgG (5–7 fold) and anti-guduchi ImP IgA (3–4 fold) on day 50 vs. control. Guduchi ImP showed a significant increase in anti-OVA IgG (6–7 fold) and anti-OVA IgA (4–5 fold) on day 50 vs. control. The splenic index of guduchi ImP group increased significantly in both the immune and adjuvant response groups; however, the splenic index in the adjuvant response group was markedly higher.
Conclusion: The results indicate that guduchi ImP is a strong immunogen by itself and enhances the immunogenicity of mucosally-administered antigen in BALB/c mice. Based on the results of this animal study, it appears that guduchi ImP shows a potential for future studies in humans.
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1. Introduction

Administration of vaccines is an efficient strategy for the prevention and treatment of infectious diseases. Present immunization protocols with subunit vaccines by mucosal administration rely on effective adjuvants. Cholera toxin (CT) and *Escherichia coli* heat-labile enterotoxins are potent mucosal immunogens which can induce systemic and mucosal responses following administration through mucosal surfaces [1,2]. These immune responses are so potent that they can activate an enhanced immune response to co-administered foreign bystander antigens that are normally poor mucosal immunogens [3,4]. The use of bacterial toxins as mucosal adjuvants in new vaccines has been restricted due to their toxicity [5,6].

In the development of mucosal vaccines, considerable efforts have been focused on the mucosal adjuvant activities of non-toxic proteins based on their potent immunostimulatory activities [6]. Examples include plant lectins [7–9], plant proteins [10], polysaccharides from kiwifruit (*Actinidia eriantha*) [11] and garlic [12]. A number of carbohydrates from plants, bacteria and yeast, as well as synthetic compounds (CpG oligodeoxynucleotides), have emerged as promising vaccine adjuvant candidates [13,14].

Saponins have been studied for their adjuvanticity from various sources like Chinese liquorice (*Glycyrrhiza uralensis* Fisch.) [15], and traditional Chinese medicinal herbs [16]. The most widely used saponin-based adjuvants are isolated from the bark of the soap bark tree (*Quillaja saponaria* Molina), which have been evaluated in numerous clinical trials [17]. Currently, alum remains the only adjuvant approved worldwide for human use [18]; however, alum has no effect on cellular immunity and is faced with increasing

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concerns regarding potential for cumulative aluminum toxicity. Hence, there is a demand for a safe, efficacious adjuvant capable of boosting cellular and humoral immunity.

In Ayurveda (traditional Indian system of medicine), 'rasayana' plants are referred to as specific anti-aging, improving quality-of-life with enhanced intelligence and memory; hence, increased resistance towards diseases suggesting that such plants possess immune-boosting effects [19–21]. Guduchi [*Tinospora cordifolia* (Willd.) Miers; known as guduchi in Sanskrit and amrutha balli in Kannada], a herbaceous vine of Menispermaceae family, has been considered as a 'rasayana' plant; it is widely distributed throughout the Indian subcontinent and China [22].

Aqueous extracts of the stem and root of guduchi have been used therapeutically for their immunomodulation and other medicinal properties [23–25]. Seven immunomodulatory active compounds belonging to different classes have been isolated and characterized from guduchi; the immunomodulatory activity may be attributed to the synergistic effects [26,27]. Further, a polysaccharide (G1-4A) isolated from guduchi has shown promising adjuvant activity [28]. Recently, Gupta et al. [29] have demonstrated that G1-4A inhibits the survival of *Mycobacterium tuberculosis* by modulating host immune responses. The pharmacological evaluation of the extract, fractions and pure molecules from guduchi revealed the ethnomedicinal value for anticancer and immunomodulatory activities [30,31].

It has been shown previously that a major protein of 25 kDa isolated from the dry stem powder of guduchi activates murine thymocytes, splenocytes and macrophages *in vitro* [10]; the acidic protein lacking hemagglutination activity has been named as guduchi immunomodulatory protein (ImP). It appeared interesting to investigate the immunomodulatory aspects of guduchi ImP *in vivo* using ovalbumin (OVA) as a model weak antigen. The results of immunogenic and adjuvant responses of guduchi ImP by mucosal (intranasal) administration in terms of the humoral responses (serum IgG and IgA) and the splenic index in BALB/c mice are studied and presented here.

2. Materials and methods

2.1. Chemicals and reagents

Ovalbumin (OVA; type V, hen egg), concanavalin A (Con A), Q-Sepharose FF anion-exchange resin (bead size: 24–44 μm), goat anti-mouse IgA-alkaline phosphatase (AP) conjugate and goat anti-mouse IgG-AP conjugate were products of Sigma–Aldrich Co., St. Louis, MO, USA. Guduchi dry stem powder (guduchi churna) was a product of Prakruthi Ayurvedic Foundation, Mysuru, India. Flat-bottom 96-well microtiter plates (MICROLON) were purchased from Greiner Bio-One GmbH, Frickenhausen, Germany. All other chemicals and reagents used in this study were of analytical grade.

The guduchi plant, *T cordifolia* (Willd.) Miers, is listed in 'The Plant List' website: http://www.theplantlist.org/tpl/record/tro-20600016 [32]. Guduchi (*T cordifolia*) plants grown in the Agri-Horticulture Department of CSIR-CFTRI (Mysuru) were collected and the stems were air dried under the shade. A specimen of the dried plant was deposited in the plant herbarium (Botany Department, Sri Krishnadevaraya University, Anantapur, Andhra Pradesh, India) for authentication of the plant (voucher no. V-11199).

2.2. Laboratory animals

Eight-week-old female BALB/c mice were procured from the Central Animal Facility of Indian Institute of Science, Bengaluru, India. Following approval from the Institutional Animal Ethics Committee (IAEC approval # 235/12), all experimental procedures involving the handling and caring of animals have been carried out in accordance with the ethical guidelines. Animals were housed and maintained on a standard commercial diet at ambient temperature in a clean environment. The ambient temperature was maintained at 22 ± 3 °C and relative humidity was 55 ± 10% with a 12 h light/dark cycle.

2.3. Preparation of guduchi ImP from guduchi dry stem powder

It has been shown previously that guduchi ImP isolated from dried guduchi stems prepared in our laboratory is identical to guduchi ImP isolated from commercial guduchi stem powder with respect to molecular mass, immunoreactivity by rabbit anti-guduchi ImP antiserum and *in vitro* immunomodulatory properties [10]. In this study, guduchi ImP was prepared from commercial guduchi dry stem powder by the method described by Aranha et al. [10]. Briefly, aqueous extract (20% w/v) of guduchi stem powder was prepared, and subjected to protein precipitation using 80% ammonium sulfate saturation; the ammonium sulfate precipitate was resolubilized in distilled water followed by dialysis using 12–14 kDa cut-off membrane against distilled water at 4 °C and lyophilization. Lyophilized guduchi extract was dissolved in a small volume of 20 mM Tris–HCl buffer, pH 8 (equilibration buffer) and was subjected to anion-exchange chromatography on Q-Sepharose FF as described in Ref. [10]. The fractions corresponding to each step elution of increasing NaCl concentration were pooled, dialyzed using 12–14 kDa cut-off membrane against double distilled water and lyophilized.

2.4. Preparation of protein samples for immunization

Known amount of lyophilized guduchi ImP (or commercial OVA or Con A) was dissolved in autoclaved phosphate-buffered saline (PBS) and filtered using sterile Acrodisc® syringe filters – 0.2 μm Supor® membrane (Pall Life Sciences, Ann Arbor, MI) in a laminar flow hood. The freshly prepared samples were then transferred into sterile Eppendorf tubes in small aliquots, and stored at −20 °C for a maximum period of 45 days and used for all administrations in BALB/c mice.

2.5. Immune response and adjuvant response of guduchi ImP by mucosal route of administration

BALB/c mice were divided into either 4 or 5 groups (n = 6) for studying the immunogenic response or adjuvant response, respectively. The random distribution of animals was done to ensure the same response from animals in each group receiving different administrations; the average weight of the animals in each group was almost similar. All animals were allowed to acclimatize for one week before starting the administration. The study protocol is outlined in Fig. 1.

All the antigens were administered in a constant volume (30 μL per animal) by intranasal route following the standard protocol for mucosal administration of an antigen by intranasal route [7]. In both experimental groups, mice were immunized on days 1, 14, 21, 28, 35 and 42. Mice were slightly anesthetized with low dose of diethyl ether and were held in place. Using a micropipette, 15 μL of antigen or sample solution was placed over one nostril until the liquid delivered through micropipette tip was completely inhaled; this procedure was carried out very gradually and repeated for the other nostril. The animal was constantly monitored for any kind of irritation during the procedure; if any kind of irritation response was observed, then the dosing procedure was stopped for a brief period of 15 s until the animal recovers, and then continued. Once

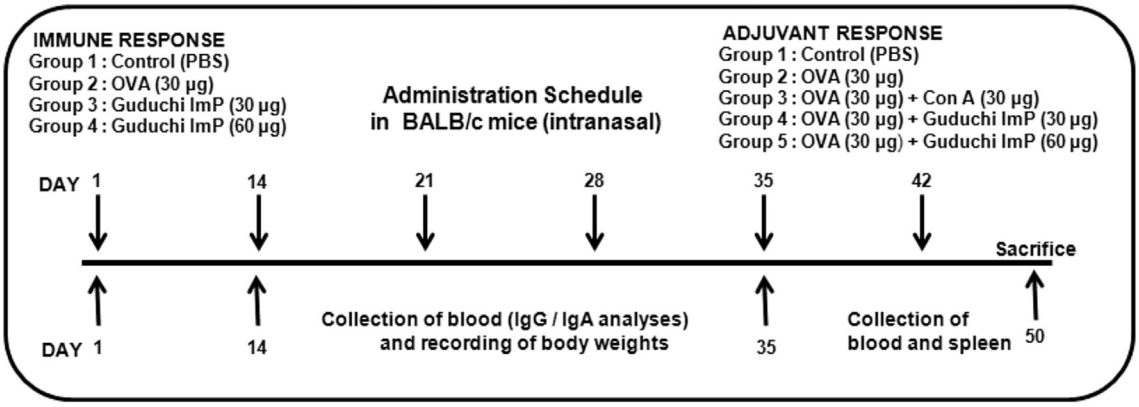

Fig. 1. Schematic representation of antigen and/or guduchi ImP study protocols by intranasal (mucosal) administration in female BALB/c mice. Con A: concanavalin A; ImP: immunomodulatory protein; OVA: ovalbumin; PBS: phosphate-buffered saline.

the animal comes out of the anesthetic effect, it was placed back in the cages.

2.5.1. Immunogenic response of guduchi ImP

BALB/c mice were divided into 4 groups (n = 6) for intranasal administration to examine the immune response (intrinsic immunogenicity) of guduchi ImP. The volume of sample administered for each group was held constant (30 µL per animal). The study protocol is presented in Fig. 1. The first group was the 'control' group wherein only PBS was administered to stress the animals. The second group was administered OVA (30 µg), an immunogenically weak antigen and a model antigen. The third and fourth groups of animals were administered 30 µg guduchi ImP (G1) and 60 µg guduchi ImP (G2), respectively.

2.5.2. Adjuvant response of guduchi ImP for OVA

In order to examine the adjuvant response of guduchi ImP for OVA, BALB/c mice were divided into 5 groups (n = 6). The study protocol is shown in Fig. 1. The volume of sample administered for each group was held constant (30 µL per animal). The first group was an untreated 'control', wherein only PBS was administered to stress the animals. The second group received OVA (30 µg in 30 µL) as the test antigen. The third group received OVA + Con A (30 µg each in a total volume of 30 µL). The fourth and fifth groups were administered guduchi ImP (30 and 60 µg, respectively, along with 30 µg OVA in a final volume of 30 µL).

2.6. Body weights of experimental animals and calculation of splenic index

The body weight of each individual animal in the experimental groups was measured on day 1, 14, 35 and 50 during the experiment; the weights were taken prior to the administration of each sample/vehicle. Monitoring the body weights of animals gives a measure of the effect of administered antigens on their growth or retardation. Spleen of each animal from all experimental groups was collected and weighed after sacrificing the animals on day 50 by an overdose of anesthesia by chloroform. The splenic index was calculated based on spleen weight and animal body weight. The formula for calculating splenic index is: spleen weight (mg) ÷ body weight of the animal (g).

2.7. Collection of blood and separation of serum

Blood was drawn on day 1 before immunization and this represented the pre-immune serum. Blood (60–80 µL) was collected from the experimental animals by retro-orbital vein puncture using heparinized capillary tubes at specific intervals (day 1, 14 and 35). On the final day of the experiment (day 50), animals were terminally anesthetized for collection of blood by cardiac puncture. The collected blood was allowed to stand at room temperature for about 1 h with loose cotton plugging to promote clotting, and then the tubes were transferred to 4 °C for 1 h; the tubes were then centrifuged in the cold at 750×*g* for 10 min. The clear straw-colored serum obtained was stored at −20 °C. The serum samples were used for the analysis of IgA and IgG antibody responses towards the administered test antigens against the 'control'.

2.8. Analysis of serum IgA and IgG by ELISA

ELISA was performed for the detection of guduchi ImP-specific IgG and IgA as well as OVA-specific IgG and IgA in BALB/c mice sera as described for the immunogenicity and adjuvant activity of garlic lectins and fructans [8,12]. Microtiter plate wells were coated with 100 µL per well of 0.1 mg/mL antigen (OVA or guduchi ImP) in 0.1 M carbonate-bicarbonate buffer, pH 9.6, and incubated at 4 °C overnight. Mouse serum was diluted in PBS containing 0.05% Tween-20 and 1% BSA (1:50 dilution for IgG analysis; 1:20 dilution for IgA analysis). Goat anti-mouse IgG-AP conjugate (100 µL/well of 1:1000 dilution in PBS containing 0.05% Tween-20, 1% BSA and 0.5% gelatin) and goat anti-mouse IgA-AP conjugate (100 µL/well of 1:5000 dilution in PBS containing 0.05% Tween-20, 1% BSA and 0.5% gelatin) were used as the source of secondary antibodies.

2.9. Statistical analysis

Data were expressed as the arithmetic mean and standard deviation (S.D.). Differences between paired groups were determined using the Student's *t*-test; $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Purification and characterization of guduchi ImP

Among the Q-Sepharose chromatographic eluates obtained with step-elution, the 0.2 M NaCl eluate contained guduchi ImP as

judged by the lymphocyte proliferation assay using murine thymocytes/splenocytes (data not shown). Guduchi ImP was purified from the dried stem powder of guduchi in a yield of ~150 mg per 100 g; it appeared as a 25 kDa protein band on SDS gels under both reducing and non-reducing conditions. The lymphoproliferative properties of guduchi ImP was confirmed by a 3-fold mitogenic activity towards murine splenocytes and a 6-fold mitogenic activity towards murine thymocytes compared to untreated cells (data not shown). These immunostimulatory characteristics of the purified protein *in vitro* were found to be in accordance with the earlier observations of Aranha et al. [10] on the characterization of guduchi ImP.

3.2. Guduchi ImP administered BALB/c mice show significant increase in body weight

The body weights of the animals in the control and treated groups (immune response) were carefully monitored to assess their health status, and are shown in Table 1. The body weights of all the four groups in the immune response experiment were found to be increased ($p < 0.05$) on day 50 compared to the body weights of their respective group on day 1. Between the groups in the immune response study, significant increase ($p < 0.05$) in body weight was seen in guduchi ImP-administered animals compared to the body weight of the control group on day 35.

Similarly, in the adjuvant response experiment (Table 2), the group that had received OVA with 60 μg guduchi ImP registered an increase in body weight compared to the control group on day 35. On day 50, there was a significant increase ($p < 0.05$) in the body weights in all the 5 treatment groups compared with the respective group on day 1. Among the 5 groups of animals on day 50, the group that had received OVA plus 60 μg guduchi ImP showed a significant increase ($p < 0.05$) in the body weight compared to the control group on day 50.

3.3. Guduchi ImP shows improved IgG and IgA responses upon intranasal administration

Antibodies are produced during humoral immune response; IgG and IgA levels are considered as biomarkers for humoral immune response status. The humoral immune response (serum IgG) to the administration of OVA or guduchi ImP (either 30 or 60 μg protein) is shown in Fig. 2 (panel a). The IgG level observed in the case of pre-immune serum for all test groups was found to be approximately similar to the Control group (data not shown). In this set of experiment, serum IgG response to OVA on days 14, 35, and 50 are very similar and there is no significant increase in anti-OVA IgG response compared to the serum IgG of control group. However, the guduchi ImP treatment groups showed a significant increase in IgG response to guduchi ImP. The serum IgG response in the case of 30 μg guduchi ImP administration showed 3-fold increase on day 35, and 5-fold increase on day 50 compared to the control group ($p < 0.05$). However, the 60 μg guduchi ImP administered group registered a more significant increase in serum IgG on days 14 (1.6-fold), 35 (~5-fold; $p < 0.05$), and 50 (~7-fold; $p < 0.01$).

The humoral immune response (serum IgA) to the administration of OVA or guduchi ImP as the antigens is shown in Fig. 2 (panel b). There was no significant increase in IgA antibodies to OVA on days 14, 35 and 50, and the levels were similar to the serum IgA levels of the control group. However, the guduchi ImP groups display a significant increase in serum IgA. The 30 μg guduchi ImP group of animals showed a significant increase ($p < 0.05$) on days 14 (1.6-fold), 35 (2.6-fold), and 50 (3-fold) compared to control group. The 60 μg guduchi ImP dose group also displayed a significant

Table 1
Body weights of BALB/c mice on selected days during the evaluation of immune response of guduchi ImP. Body weight is given in g (mean ± S.D.).

Group	Day 1	Day 14	Day 35	Day 50
Control	28.59 ± 0.85	28.80 ± 1.09	29.92 ± 1.45	31.98 ± 1.63*
OVA	28.81 ± 1.20	28.92 ± 1.51	29.95 ± 1.75	31.65 ± 1.55*
ImP G1	28.79 ± 1.58	28.85 ± 1.65	30.87 ± 1.80†	32.45 ± 1.89*
ImP G2	28.68 ± 1.37	28.97 ± 1.11	30.98 ± 1.70†	32.88 ± 1.90*

n = 6 per group; OVA, ovalbumin (30 μg); ImP G1, guduchi immunomodulatory protein 30 μg; ImP G2, guduchi immunomodulatory protein 60 μg; *$p < 0.05$ vs. respective groups on Day 1; †$p < 0.05$ vs. Control group on Day 35.

Table 2
Body weights of BALB/c mice on selected days during the evaluation of the adjuvant response of guduchi ImP. Body weight is shown in g (mean ± S.D.).

Group	Day 1	Day 14	Day 35	Day 50
Control	28.25 ± 1.07	28.80 ± 1.32	29.50 ± 1.65	31.50 ± 1.40*
OVA	28.35 ± 1.10	28.85 ± 1.27	29.62 ± 1.70	31.80 ± 1.72*
OVA + Con A	28.23 ± 1.35	28.90 ± 1.33	29.80 ± 1.43	32.02 ± 1.35*
OVA + ImP G1	28.06 ± 1.25	28.92 ± 1.39	29.85 ± 1.20	32.22 ± 1.80*
OVA + ImP G2	28.00 ± 1.22	28.95 ± 1.42	30.55 ± 1.55†	32.98 ± 1.73*†

n = 6 per group; OVA, ovalbumin (30 μg); Con A, concanavalin A (30 μg); ImP G1, guduchi immunomodulatory protein 30 μg; ImP G2, guduchi immunomodulatory protein 60 μg. *$p < 0.05$ vs. respective groups on Day 1. †$p < 0.05$ vs. the Control group on the respective day (Day 35 or Day 50).

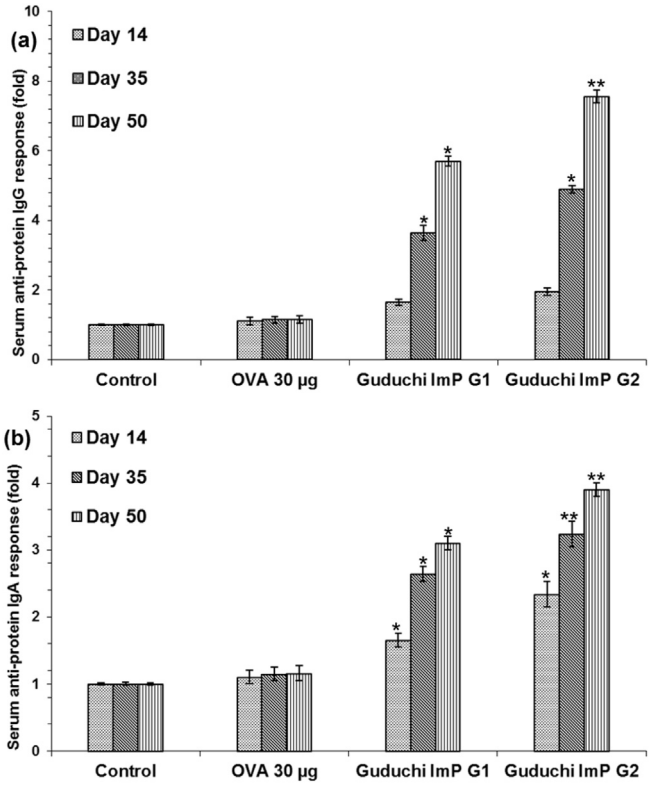

Fig. 2. Anti-protein IgG (panel a) and IgA (panel b) responses against guduchi ImP or OVA measured in BALB/c mice serum following intranasal administration. IgG and IgA responses were measured by ELISA using either OVA or guduchi ImP as the coating antigen (10 μg). Guduchi ImP G1: 30 μg dose; guduchi ImP G2: 60 μg dose. The IgG and IgA responses are denoted as fold-increase over control taking the absorbance of the control as 1. Secondary antibody conjugate dilution: 1:1000 of goat anti-mouse IgG-AP or 1:5000 of goat anti-mouse IgA-AP. Panel (a): anti-protein IgG response in BALB/c mice serum; 1:50 serum dilution was used for IgG detection. Panel (b): anti-protein IgA response in BALB/c mice serum; 1:20 serum dilution was used for IgA detection. Values shown are mean ± S.D. (n = 3). *p* value: *≤0.05 or **<0.01 vs. Control.

increase in serum IgA response on days 14 (2.4-fold; $p < 0.05$), 35 (3-fold; $p < 0.01$), and 50 (~4-fold; $p < 0.01$).

3.4. Guduchi ImP shows immunoadjuvant humoral response towards OVA, a weak antigen

In order to investigate the humoral adjuvant effect of guduchi ImP, OVA alone or OVA with guduchi ImP was administered to mice through the mucosal (intranasal) route. OVA was used as an experimental weak antigen, and the serum IgG and IgA responses against OVA were measured to evaluate the immunoadjuvant activity of guduchi ImP. Anti-OVA serum IgG response at different periods of the administration protocol (days 14, 35 and 50) are shown in Fig. 3 (panel a). The IgG response to OVA alone barely increased above that from the control group; however, in the presence of Con A as a positive control, the serum IgG response showed a significant increase (2.8-fold; $p < 0.05$) on day 50 compared with the OVA alone group. The serum IgG response to OVA on days 35 and 50 displayed a significant increase in both the 30 μg guduchi ImP (G1) and 60 μg guduchi ImP (G2) groups compared with the OVA alone group. The 30 μg treatment group showed 3.6-fold increase ($p < 0.05$) on day 35 and ~6-fold increase ($p < 0.01$) on day 50. In the case of the 60 μg guduchi ImP group, the anti-OVA serum IgG increased by 4.2-fold ($p < 0.05$) on day 35 and 7.2-fold ($p < 0.01$) on day 50.

The humoral response (serum IgA) to OVA alone or in the presence of 30 μg guduchi ImP (G1) and 60 μg guduchi ImP (G2) is shown in Fig. 3 (panel b). The serum IgA response to the administration of OVA alone does not show any increase in comparison to the control group; however, Con A at 30 μg produces a 3-fold increase ($p < 0.05$) in anti-OVA IgA response on day 50. The 30 μg guduchi ImP group showed 3.2-fold increase ($p < 0.05$) on day 35 and 3.8-fold increase ($p < 0.05$) on day 50. Similarly, in the presence of 60 μg guduchi ImP, a 3-fold and 3.6-fold increase were seen on day 14 and day 35, respectively; the anti-OVA serum IgA response significantly increased by 5-fold ($p < 0.01$) on day 50.

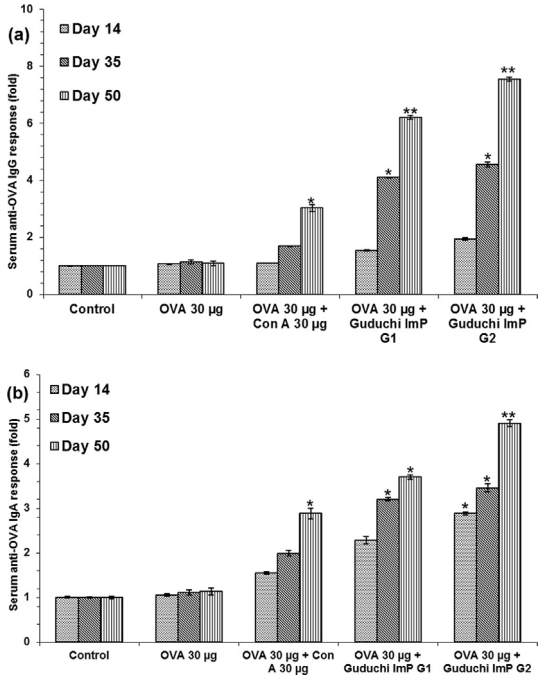

Fig. 3. Serum anti-OVA IgG (panel a) and anti-OVA IgA (panel b) responses against OVA alone, or OVA with either Con A or guduchi ImP at different days following intranasal administration in BALB/c mice. IgG and IgA responses were measured by ELISA using OVA as the coating antigen (10 μg). Guduchi ImP G1: 30 μg dose; guduchi ImP G2: 60 μg dose. The IgG and IgA responses are denoted as fold-increase over control taking the absorbance of the control as 1. Secondary antibody conjugates: goat anti-mouse IgG-AP (1:1000 dilution) or goat anti-mouse IgA-AP (1:5000 dilution). Panel (a): anti-OVA IgG response in BALB/c mice serum; 1:50 serum dilution was used for anti-OVA IgG detection; panel (b): anti-OVA IgA response in BALB/c mice serum; 1:20 serum dilution was used for anti-OVA IgA detection. Each value is represented as mean ± S.D. (n = 3). Level of significance: $^{*}p < 0.05$ vs. Control, $^{**}p < 0.01$ vs. Control.

3.5. Guduchi ImP administration increases the splenic index of BALB/c mice

Splenic index was represented as the spleen weight (in mg) divided by the body weight of the animal (in g); it was calculated after sacrificing the animals on day 50. The splenic index of OVA-administered mice in both immune response and adjuvant response experiments did not differ much from the splenic index of control group (Fig. 4). However, guduchi ImP-administered mice show significant increase ($p < 0.05$) in the splenic index in both the experimental setup compared to the control group, with the higher dose of guduchi ImP (60 μg) showing higher splenic index than the lower dose (30 μg). Further, the splenic indices of mice in the adjuvant response experiment are significantly higher ($p < 0.05$) compared to the splenic indices of mice in the immune response experiment as analyzed by Duncan's multiple range test (Fig. 4).

4. Discussion

In Ayurvedic medicine, guduchi is considered as one of the most divine herb for a wide range of pharmaceutical activities [25,27,33]. It has a rich history in the Indian sub-continent where it has been used and investigated since thousands of years. The rasayanas (adaptogens) prepared from guduchi are noteworthy and promising cure for various disorders [19]. In recent decades, evidence-based research has been carried out to demonstrate the immunomodulatory properties of guduchi and application of its isolated constituents [10,26,27,34–36]. Immunomodulation by various bioactive phytoagents is gaining importance in the management of certain infections, autoimmune diseases, graft rejection and neoplastic disorders due to their proven properties with minimal side-effects as compared to allopathic medicines [21,29].

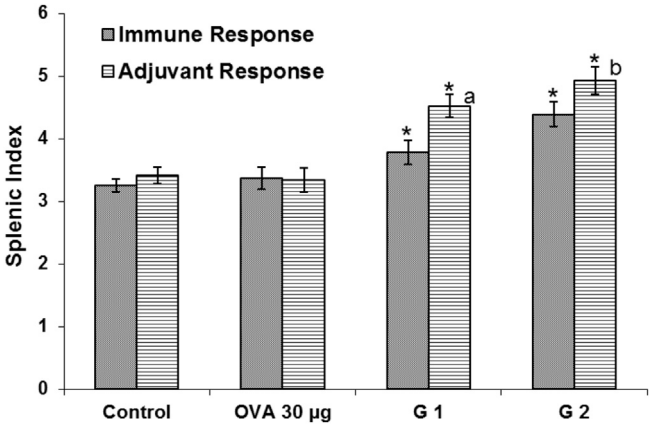

Fig. 4. Splenic index of BALB/c mice on day 50 following intranasal administration of guduchi ImP in immune response and adjuvant response groups (n = 6). Immune response group: G1, guduchi ImP 30 μg; G2, guduchi ImP 60 μg. Adjuvant response group: G1, OVA 30 μg + guduchi ImP 30 μg; G2, OVA 30 μg + guduchi ImP 60 μg. Each value is represented as mean ± S.D. (n = 6). $^{*}p < 0.05$ compared to Control as analyzed by Student's *t*-test. Within the G1 and G2 groups, the adjuvant response histogram bars are significantly different (indicated by the letters 'a' and 'b') compared to the immune response histogram bars as analyzed by Duncan's multiple range test (DMRT); a and b: $p < 0.05$.

Guduchi stem contains an abundant protein of 25 kDa which has been isolated and characterized as having immunomodulatory properties *in vitro* [10]. In the present investigation, the humoral immune and adjuvant responses of guduchi ImP were investigated in BALB/c mice by mucosal route (intranasal) of administration. OVA is a well-known model antigen with weak immunogenic property [37]. Mucosal routes for vaccine delivery are non-invasive; so, administration is relatively simple and inexpensive [6]. Furthermore, the potential to induce mucosal and systemic immune responses after mucosal vaccine delivery allows the possibility of effective immunization against many diseases [38]. The serum IgG response to guduchi ImP in comparison to the response from OVA provides a measure of the systemic immune response (intrinsic immunogenicity) of guduchi ImP upon mucosal administration.

In this study, it was observed that guduchi ImP shows an effective immunogenic response for the production of its own serum IgG antibodies; the increase in serum IgG is 5-fold and 7-fold on day 50 at guduchi ImP doses of 30 and 60 μg, respectively, in comparison to the OVA group. This indicates that guduchi ImP has intrinsic immunogenicity without the need for an adjuvant; such antibodies generated without the use of an adjuvant may be akin to 'natural antibodies' reported for several dietary proteins such as avidin, alliinase, bromelain, lactoferrin and several plant lectins (banana, garlic, peanut, soybean and wheat germ) [39]. Humans and other vertebrates contain 'natural antibodies' which are present in serum prior to viral or bacterial infections; the broad reactivity pattern of the natural antibodies may help to protect against a variety of pathogens not previously encountered [40].

IgA is the primary immunoglobulin produced at the mucosal surface [41,42]. Secretory IgA in mucosal secretions provides protection against bacterial and viral pathogens, and neutralizes microbial toxins [43]. In the present study, administration of guduchi ImP by mucosal route has elicited the production of significant serum IgA; nearly 3-fold and 4-fold increases in serum IgA on day 50 were seen at 30 μg and 60 μg doses of guduchi ImP, respectively. Since many of the Ayurvedic medicines containing guduchi are taken orally, it is likely that they produce remarkable humoral immune response in humans thereby providing immunity to a variety of infections and immune disorders.

In both the experiments (immunogenic and adjuvant response), the control group treated with PBS show basal levels of IgG and IgA already present in the serum. Similarly, OVA (a weak antigen) does not kindle the immune system for antibody production by itself [37]; hence, the IgG and IgA levels in OVA-treated groups show only a marginal increase compared to those seen in the case of control groups (PBS-treated groups); however, this increase is not significant.

Adjuvants (components of current vaccines) increase the immunogenicity of co-administered antigens such as purified or recombinant proteins, which are *per se* less immunogenic than whole or split, killed or attenuated pathogens used in earlier vaccines [44]. Formulation of vaccines with an adjuvant effectively enhances systemic and local antibody responses leading to a superior protection against infections [45]. Alum, the widely used adjuvant, still has certain limitations which include local reactions, augmentation of IgE responses, ineffectiveness for some antigens and inability to augment cell-mediated immune responses, especially cytotoxic T-cell responses [46]. Guduchi has been used as an adjuvant in various immunological treatments [24,31,36]. In this study, guduchi ImP augmented the serum anti-OVA IgG by 6-fold (30 μg dose) and 7-fold (60 μg dose), respectively, on day 50. Similarly, there was a 4-fold and 5-fold increase in serum anti-OVA IgA at guduchi ImP dose of 30 μg and 60 μg, respectively, on day 50. The adjuvant immune responses of guduchi ImP for OVA in the present study indicates that guduchi ImP is a potential adjuvant derived from an herb widely used in the preparation of several Ayurvedic medicines.

In the present investigation, mice show significant growth at the end of the experiment (50 days in both immune and adjuvant response experiments) as seen in the control groups treated with PBS or OVA alone. When the body weights of animals were examined regularly for all the groups, it was observed that there was significant difference in the body weights of animals of different treatment groups. After sacrificing the animals on 50th day, it was found that even spleen weight of guduchi ImP-treated animals was increased significantly. This observation may be due to a combination of (i) an increase in body weight due to aging, and (ii) the effect of guduchi ImP. Therefore, it can be concluded from this study that guduchi ImP not only displays immunogenic and adjuvant property but also has a slight growth (anabolic) effect as seen from the body weights on day 35 and 50 compared to the control group.

Increase in the splenic index of animals treated with guduchi ImP indicates that there is an increase in the spleen weight of guduchi ImP administered mice on day 50. Further, between the two groups of BALB/c mice, the adjuvant response group showed a significant increase in the splenic index compared to that of the immune response group. These observations are in accordance with other investigations demonstrating the up-regulated response of splenocytes following intranasal administration of the combined adjuvant formulation [47]. It has been demonstrated that the activation of spleen cells by ArtinM [a mannose-specific lectin from jack bean (*Artocarpus integrifolia*) seeds] may account for its immunomodulatory properties [48].

5. Conclusion

The results of the present study demonstrate that purified guduchi ImP at 30 μg and 60 μg doses showed humoral immune response (serum IgG and IgA increase) in BALB/c mice upon intranasal administration, thus indicating its intrinsic immunogenicity. In addition, guduchi ImP displayed humoral adjuvant response for OVA (a model weak antigen) as seen by increased serum anti-OVA IgG and IgA on days 35 and 50. The splenic index of guduchi ImP administered mice was significantly higher compared to the control; further, the splenic index of the adjuvant response group was significantly higher than that of the immune response group. Overall, it can be concluded that intranasal administration of guduchi ImP provides natural antibodies (serum IgG and IgA), and acts as an adjuvant for a weak antigen like OVA. Therefore, guduchi ImP is an immune system booster and has the potential to be used as a mucosal adjuvant for experimental antigens in further studies on humans.

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Conflict of interest

None.

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