Arch. Biol. Sci., Belgrade, 67(2), 561-570, 2015 DOI:10.2298/ABS141027018A

IN VIVO IMMUNOMODULATORY EFFECTS OF THE METHANOLIC LEAF EXTRACT OF *GYMNEMA SYLVESTRE* IN SWISS ALBINO MICE

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Abstract: In the present study we performed a comparative phytochemical analysis of the immunomodulating activities of the methanol leaf extract of *Gymnema sylvestre* (MLEGS) in Swiss albino mice. The phytochemical screening conducted on MLEGS revealed the presence of several phytoconstituents, including saponins, alkaloids, glycosides, phenols, tannins, and flavonoids. Immunomodulatory activities were determined by hemagglutination antibody (HA) titer and delayed-type hypersensitivity (DTH) tests for determining specific and non-specific immune responses. Flow cytometric techniques were *performed* for the estimation of B lymphocytes (CD3 and CD19) and Th2 cytokines (IL-2, IFN-γ and IL-4). The response produced by oral administration of MLEGS elicited a significant reduction *in* a *dose-related* manner in the primary and secondary antibody response and DTH response. The response produced by oral administration of MLEGS caused a significant reduction *in* a *dose-related* manner in the primary and secondary antibody and DTH responses, with maximum reduction observed at 200 mg/kg body wt. The maximal reductions in the production of CD3, CD19, IL-2, IFN-γ and IL-4 were 31.59, 32.12, 29.51, 32.45 and 33.53%, respectively, at 200 mg/kg body weight. This study demonstrates that *G. sylvestre* exerts immunosuppressive effects on the components of the immune system of mice, and points to its significant immunomodulatory potential.

Key words: *Gymnema sylvestre*; methanol leaf extract; immunomodulatory activity; phytochemical analysis

Received October 27, 2014; **Revised** November 12, 2014; **Accepted** January 12, 2015

INTRODUCTION

The protection of our bodies against specific disease and pathogens is associated with the immune system (Agarwal and Singh, 1999). The function and efficiency of the immune system may be influenced by several exogenous and endogenous factors. There are certain agents or compounds known as immunomodulators, which are capable of exerting pharmacological or biological effects on the immune system. The basic strategy underlying immunomodulation is to identify aspects of the host response that can be enhanced or suppressed in such a way as to augment or complement a desired immune response.

Immunomodulators may be synthetic drugs or of herbal origin. Due to the severe side effects related to synthetic drugs, immunomodulation using herbal drugs can provide an alternative to conventional chemotherapy for a variety of diseases, especially when the host defense mechanism has to be activated under the conditions of an impaired immune response (Srikumar et al., 2006). Herbal drugs are easily affordable and less potent than synthetic prescription immunomodulators and are also less likely to cause side effects. Therefore, there is a need to search for plants with immunomodulatory activity to offer a novel approach for the treatment of infectious disease.

Gymnema sylvestre R.Br., commonly known as Gudmar, is a climbing herb belonging to the family *Asclepiadaceae* and commonly found in India, China, Indonesia, Japan, Malaysia, Sri Lanka, Vietnam and South Africa (Bone, 2007). Traditionally, this plant has been used for the treatment of inflammations, hepatosplenomegaly, dyspepsia, constipation, hemorrhoids, helminthiasis, cough, asthma, bronchitis, cardiopathy, jaundice, intermittent fever, piles, amenorrhea, conjunctivitis, leucoderma and urinary disorders (Nadkarnni, 1993). Its roots are used to cure snakebite (Gomes et al., 2010). Triturated leaves of *G. sylvestre* mixed with castor oil are applied to swollen glands and enlargements of internal viscera such as the liver and spleen (Nadkarnni, 1993). The leaves of *G. sylvestre* have been used as a cardiotonic, diuretic, laxative, stimulant, stomachic and uterine tonic, and have shown antiviral, diuretic, anti-allergic, hypoglycemic, hypolipidemic, anti-obesity and dental caries prevention potential (Reddy et al., 2004). *G. sylvestre* is a potent antidiabetic plant used in folk, Ayurvedic and homeopathic medicine. Moreover, *G. sylvestre* has been reported to possess anthelmintic, antimicrobial, antioxidant, anti-hypercholesterolemic, hepatoprotective, anti-allergic, lipid-lowering effects (Ahirwal et al., 2010; Ahirwal et al., 2012; Porchezhian and Dobriyal, 2003). Furthermore, chemical and pharmacological studies of a large group of C-4 gem-dimethylated pentacyclic triterpenoids from *G. sylvestre* have also been performed (Fabio et al., 2013). Previously, the aqueous leaf extract of *G. sylvestre* was tested for its pharmacological activities in order to visualize the movement of neutrophils, chemotaxis and phagocytosis of *C. albicans,* and in nitroblue tetrazolium assays (Malik et al., 2009). In the present study, we examined the immunomodulatory activities of MLEGS by evaluating its effect on the hemagglutinating antibody, delayed-type hypersensitivity (DTH) response, level of intracellular cytokines and its effect on lymphocyte immunophenotyping.

The present study was undertaken to explore the immunomodulatory activities of MLEGS on immune system components of Swiss albino mice in order to assess its potential pharmacological value.

MATERIALS AND METHODS

Plant material

The leaves of *G. sylvestre* were collected from Jeevan Herbs Agro Farm, Sagar, MP, India. A herbarium of the source was made and identified, and a voucher specimen number (Bot/H/1314) was submitted at the herbarium of Department of Botany, Dr. H. S. Gour University, Sagar, MP, India.

Preparation of the extracts and phytochemical screening

The leaves of *G. sylvestre* were shade-dried and coarsely ground to powder. The powdered plant material (60 g) was defatted with petroleum ether (500 ml) and then extracted with methanol (500 ml at 40˚C) using a Soxhlet apparatus. The extract was cooled at room temperature, filtered and evaporated to complete dryness. The percentage yield (5.77%) of the extract was calculated and preserved in a sealed vial for further use. Preliminary phytochemical screening of the methanolic extract of *G. sylvestre* revealed the presence of most of the various biologically active phytoconstituents, including alkaloids, glycosides, flavonoids and saponins, as reported previously (Gopinath et al., 2012).

Chemicals and standard drugs

FACS lysing solution, FACS permeabilizing solution, Golgi plug, FITC (Fluorescein isothiocyanate)-labeled anti-CD3 monoclonal antibodies, PE (Pycoerytherin)-labeled anti-CD19, IL-2, IFN-gamma and IL-4 monoclonal antibodies were purchased from BD Biosciences, Bangalore, India. All other reagents used were of analytical grade. Cyclophosphamide and levamisole were used as standard immunosuppressants. Cyclophosphamide and levamisole were dissolved in normal saline water at the concentration of 100 mg/kg body wt and 2.5 mg/kg body wt, respectively, and were administered according to the experimental plan of extract doses.

Experimental animals

The animal use protocol was approved by Dr. Hari Singh Gour University, Sagar, MP, India (Institutional Animal Ethics Committee (Reg. No.- 379/01/ab/CPCSEA)) and was in accordance with Purpose of Control and Supervision on Experimentation on Animals (CPCSEA) and international standards on the care and use of experimental animals (CCAC, 1993). Swiss albino mice (weight 20-25 g) of either sex were used for the study. Animals were housed under standard conditions of temperature (25°C), 12 h/12 h light/dark cycles and fed with standard pellet diet (Lipton India Ltd) and tap water.

Antigen

Fresh blood was collected from sheep killed in the local slaughterhouse. Sheep red blood cells (SRBCs) were collected in Alsever's solution washed three times in large volumes of pyrogen-free 0.9% NaCl, w/v and adjusted to a concentration of 0.5×10^9 cells/ml for immunization.

Hemagglutinating antibody (HA) titer

A method described by Puri et al. (1993) was used for determining the HA titer. The SRBC agglutination test was performed to study the humoral antibody response against antigens. Swiss albino mice (n = 6) were immunized by injecting 200 µl of 5×10^9 SRBC/ml intraperitoneally (i.p.) on day 0. MLEGS was administered to the mice in graded doses (50, 100 and 200 mg/kg body

wt) for seven days. The blood samples were collected from individual animals of all the groups by retro-orbital bleeding on the 7th day (before challenge) for primary antibody titer and on the 14th day (7 days after challenge with 5×10^9 SRBC/ml, i.p.) for secondary antibody titer. Serum was separated from blood. Antibody levels were determined by the hemagglutination technique, and performed by using a 96-well flat-bottomed titer plate. The data obtained were subjected to statistical analysis as described (Makare et al., 2001). The reciprocal of the highest dilution of the test serum agglutination was taken as the antibody titer.

Delayed-type hypersensitivity (DTH) response

A method described by Doherty (1981) was adopted for phagocytic response assay. MLEGS (50, 100 and 200 mg/kg b wt) p.o. was administered after injecting 200 µl of 5×10^9 SRBC/ml i.p on day 0 to the mice and once daily on consecutive days. Six days later, the thickness of the left hind foot was measured with a spheromicrometer (pitch, 0.01 mm) and this thickness was considered as a control. The mice were then challenged by injecting 20 µl of 5×10^9 SRBC/ml intradermally into the left hind footpad. The foot thickness was measured again after 24 h. The difference between the pre- and post-challenge footpad thickness expressed in mm was taken as a measure of delayed type hypersensitivity (DTH) and the mean values obtained for the treated groups were compared with that of control group.

Flow cytometry studies

For flow cytometric studies, experimental animals were sensitized by injecting 5×10^9 SRBC/ml intraperitoneally (i.p) on day 0. MLEGS administration was carried out daily (once) for 6 consecutive days. After 6 days of MLEGS administration, on day 7, the animals were challenged by injecting the same concentration (5×10^9 SRBC/ml) of SRBC. Blood samples were collected on

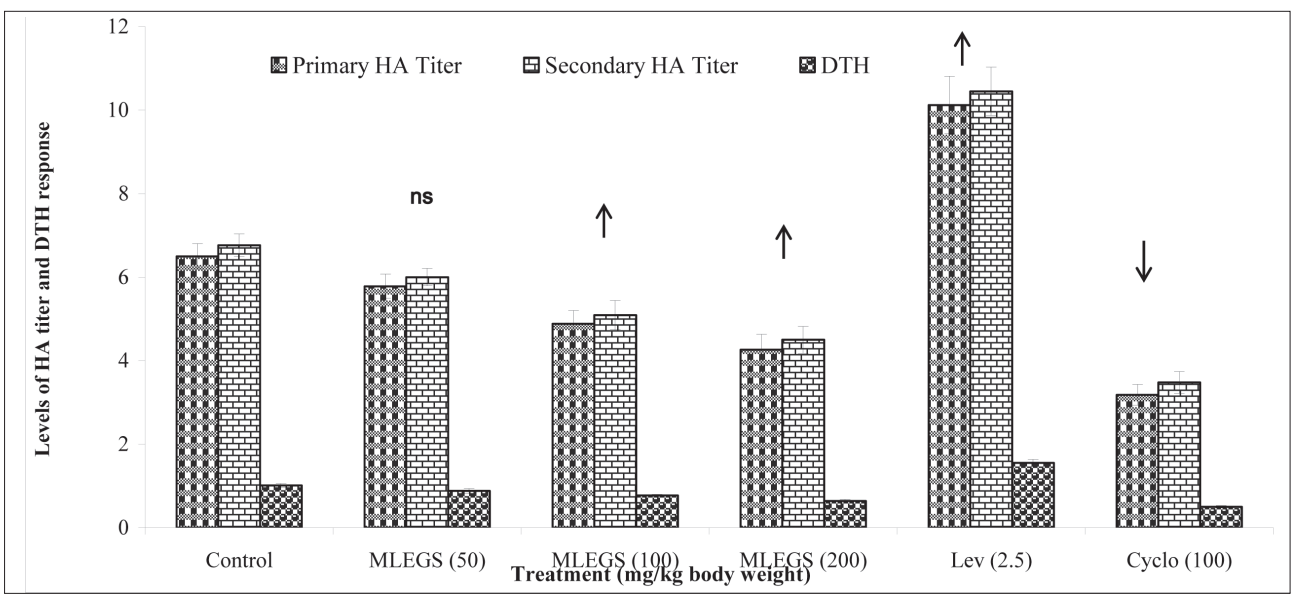

Fig. 1. Effect of methanolic leaf extract of *G. sylvestre* on hemagglutination antibody (HA) titer and the delayed-type hypersensitivity (DTH) response. Ns - non-significant; ↓-% suppression; ↑-% stimulation; MLEGS: Methanolic leaf extract of *Gymnema sylvestre*.

day 8 from all the challenged animals separately by retro-orbital plexus under light ether anesthesia in EDTA-coated tubes for lymphocyte immunophenotyping and intracellular cytokines estimation.

Lymphocyte immunophenotyping

A method described by Khan et al. (2006) was adopted for phagocytic response assay. Immunophenotyping focuses on lymphocyte populations involved in acquired immunity and specific molecules present on the cell surface that define the characteristics of lymphocytes, such as state of activation or functional capabilities. Lymphocyte subsets were measured by immunofluorescent antibody staining of whole blood and subsequently analyzed using two-color flow cytometry (Becton & Dickinson, UK). Fluorochrome- conjugated murine monoclonal antibodies directed against receptors CD3 and CD19 were used for the study. FITC-labeled anti-mouse CD3 monoclonal antibody and PE-labeled anti-mouse CD19 monoclonal antibody were added directly to 100 µl of whole blood; only red blood cells were lysed using a whole blood lysing reagent (BD Biosciences). Following the final centrifugation, samples were resuspended in phosphate-buffered saline (pH- 7.4) and analyzed directly on the flow cytometer (LSR, BD Biosciences) using Cell Quest Pro Software (BD Biosciences).

Estimation of intracellular cytokines

The method described by Bani et al. (2005) was adopted for phagocytic response assay. 100 µl of whole blood were taken in falcon tubes and red blood cells were lysed by adding whole blood lysing reagent (BD Biosciences). After washing in phosphate buffer saline (PBS), cells were permeabilized using permeabilizing solution and incubated with anti-mouse IL-2, anti-mouse IFN-gamma and anti-mouse IL-4 for 30 min in the dark. After incubation, cells were given three washes in PBS and after final washing; cells were acquired directly on flow cytometry to measure

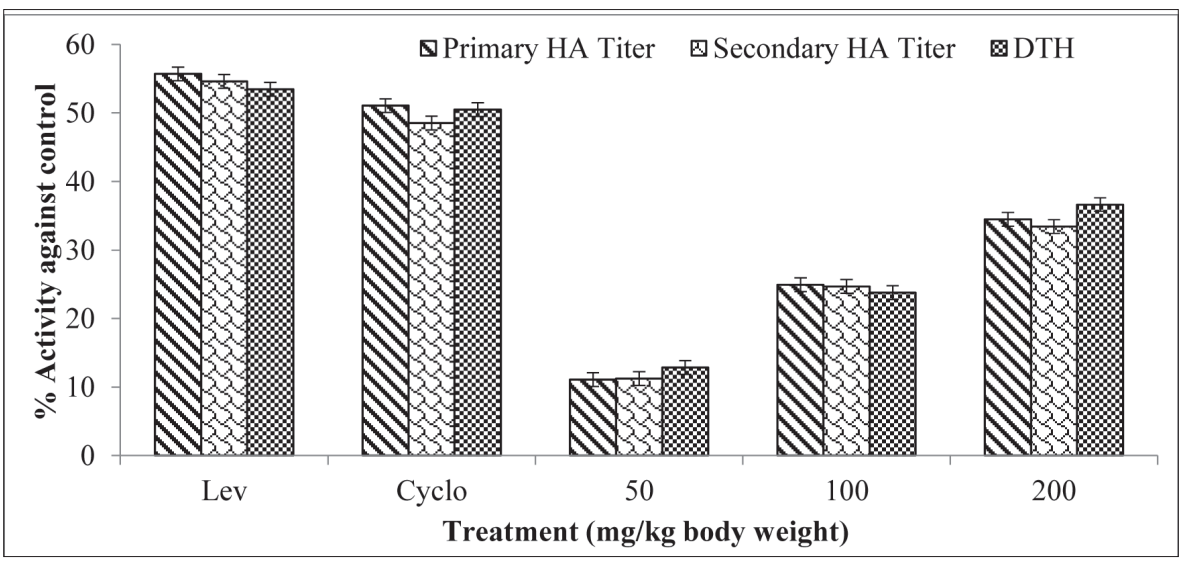

Fig. 2. Effect of methanolic leaf extract of *G. sylvestre* (MLEGS) on percentage activity of hemagglutination antibody (HA) titer and the delayed-type hypersensitivity (DTH) response as compared to control treated animals. All percentage changes were calculated against control values hence the percentage activity for control was considered 0%.

the fluorescence intensity of population. A fluorescence trigger was set on the FITC parameter to analyze the different numbers of cytokines. Fluorescence compensation, data analysis and data presentation were performed using Cell Quest Pro software. Data transformation for compensated data was done using default values provided by the software. Percentage activity was calculated using the following formula: Percent activity (%) = ICmc – ICmt / ICmc × 100, where ICmc is the mean values of intracellular cytokines of untreated (control) blood samples and ICmt is the mean values of intracellular cytokines of treated blood samples.

Statistical analysis

The data obtained from animal experiments were expressed as mean standard error (±S.E.M.). Statistical differences between the treatments and control were evaluated using ANOVA and the Bonferroni test. Significance of data was expressed as ***$p<0.001$; **$p<0.01$; *$p<0.05$.

RESULTS

Effect of MLEGS on hemagglutinating antibody (HA) titer

The HA titer was used to assess humoral immune response. Humoral antibody response to SRBC challenge was found to be significantly improved by the MLEGS when analyzed by measuring the agglutination titer against SRBC antigen at various serum dilutions. The mean value of HA titer of the extract was compared to the vehicle control. The MLEGS showed a dose-dependent decrease in the primary and secondary antibody response. The most significant results were obtained at the concentration of 200 mg/kg body wt, at which the primary and secondary hemagglutination titers were 4.26±0.37 and 4.50±0.32, respectively. All the results were compared with the standards and control (Figs. 1 and 2). Standard drugs (cyclophosphamide and levamisole) showed secondary hemagglutination titers by 3.48±0.26 and 10.45±0.58, respectively (Fig. 1). These results revealed the significant immunosuppressive effect of MLEGS at the humoral immunity level.

Effect of MLEGS on delayed-type hypersensitivity (DTH) response

The cell-mediated immune response of MLEGS was assessed by DTH reaction, i.e. footpad reaction. DTH response to SRBC was calculated as the measure of paw edema thickness (mm) for 50, 100 and 200 mg/kg body weight of each animal after treatment with MLEGS and compared with the control. In the case of the DTH test, the MLEGS showed a dose-dependent decrease in DTH response (Figs. 1 and 2). The maximum effect was observed at 200 mg/kg body weight, which was found to be 0.64±0.04. In the case of the standard drugs, levamisole (2.5 mg/kg body weight) showed a thickness of 1.55±0.09, while cyclophosphamide (100 mg/kg body weight) showed a thickness of 0.50±0.03 (Fig. 1). These results revealed that the MLEGS showed significant immunosuppressive activity at the cellular immunity level. The inhibitory effect of MLEGS on DTH response showed reduced paw thickness as compared to the control group, confirming its stimulatory effect on T cells.

Effect of MLEGS on lymphocyte immunophenotyping

The MLEGS showed a dose-related decrease in the percentage of CD3 and CD19; the maximum fall was seen at 200 mg/kg body weight by 31.59

(31.00±1.77) and 32.12% (33.06±2.12) for CD3 and CD19, respectively, when compared to the sensitized control (Table 1). In the case of cyclophosphamide, a 43.79 and 46.29% decrease in the percentage of CD3 and CD19 was observed, respectively (Table 1).

Effect of MLEGS on changes in intracellular cytokines level

In the assay of estimation of intracellular cytokines, MLEGS inhibited the percentage of IL-2, IFN- γ and IL-4 in a dose-dependent manner. The maximum decrease in percentage was seen at 200 mg/kg body wt, which was 29.51 (3.08±0.09), 32.45 (2.56±0.08) and 33.53% (2.18±0.09) for IL-2, IFN-γ and IL-4, respectively, when compared with the sensitized control. In case of cyclophosphamide, a 41.87, 47.75 and 41.46% decrease in the percentage of IL-2, IFN-γ and IL-4, was seen, respectively (Table 1).

DISCUSSION

The immunomodulatory activities of MLEGS, an important plant of the indigenous system of Indian medicine, were explored. Our results support the traditional claim made about *G. sylvestre* for medicinal purposes. In addition, the presence of various phytochemicals in the MLEGS reveals its biological and pharmaceutical importance. These phytochemicals were found to possess a wide range of biological and therapeutic potential, including immunomodulatory effects *in vivo* (Kumar et al., 2012). Recently Yadav et al. (2012) reported the immunomodulatory potential of ethanolic leaf extracts of *Spilanthes acmella* in neutrophil adhesion, HA titer and DTH response models in experimental animal. In addition, Shukla et al. (2009) also reported the immunomodulatory activity of ethanolic seed extracts of *Caesalpinia bonducella in vivo*. The *Caesalpinia*

Table 1. Effect of methanolic leaf extract of *G. sylvestre* on lymphocyte immunophenotyping and intracellular cytokine estimation by flow cytometry

Group	Dose (mg/kg body wt.)	CD3	CD19	IL-2	IFN-gamma	IL-4
		Mean ± S.E	Mean ± S.E	Mean ± S.E	Mean ± S.E	Mean ± S.E
Normal Control	-	31.56±0.99	36.41±1.00	2.76±0.08	2.19±0.15	1.97±0.11
Sensitized control		45.32±1.91	48.71±1.87	4.37±0.10	3.79±0.13	3.28±0.12
MLEGS	50	37.12±1.99ns	42.11±2.00ns	4.00±0.09ns	3.27±0.09ns	2.98±0.14ns
		18.09%↓	13.54%↓	8.46%↓	13.72%↓	9.14%↓
MLEGS	100	34.18±1.86**	37.13±1.99**	3.68±0.07ns	2.88±0.07*	2.75±0.09ns
		24.58%↓	23.77%↓	15.78%↓	24.01%↓	16.15%↓
MLEGS	200	31.00±1.77***	33.06±2.12***	3.08±0.09**	2.56±0.08***	2.18±0.09***
		31.59%↓	32.12%↓	29.51%↓	32.45%↓	33.53%↓
Levamisole	2.5	67.89±2.75***	70.15±2.89***	6.29±0.45***	5.49±0.38***	4.69±0.21***
		49.80%↑	44.01%↑	43.93%↑	44.85%↑	42.98%↑
Cyclophosphamide	100	25.47±1.62***	26.16±1.67***	2.54±0.06***	1.98±0.08***	1.92±0.08***
		43.79%↓	46.29%↓	41.87%↓	47.75%↓	41.46%↓

ns- Non-significant, ↓-% suppression, ↑-% stimulation; MLEGS: Methanolic leaf extract of *Gymnema sylvestre*.

bonducella ethanolic seed extract possessed various phytochemicals, including alkaloids, glycosides, flavonoids and saponins (Wagner, 1983). Interestingly, similar phytochemicals were also present in the MLEGS. These findings confirm that the active phytochemicals present in the MLEGS might be responsible for the immunomodulatory effect observed in this study as also evident in the work of Shukla et al. (2009).

Modulation of the immune response, through stimulation or suppression, helps in maintaining homeostasis. Agents that activate host defense mechanisms in the presence of an impaired immune responsiveness can provide supportive therapy to conventional chemotherapy (Wagner, 1983). A wide range of immunosuppressive drugs has now been adopted to control unwanted immune responses, particularly those causing autoimmune disease and transplant rejection. The clinical application of immunosuppressants has significantly improved patient survival with first-year survival up to 90% for renal transplant (Puri et al., 1993). However, immunosuppressants cause a number of serious adverse effects among which nephrotoxicity, hepatotoxicity, induction of diabetes, induction of hypertension and neurotoxicity are the most notorious for cyclosporine and tacrolimus (Waldmann, 2003). Consequently, there continues to be a high demand for new immunosuppressants. Gupta et al. (2009) also evaluated the immunomodulatory activities of an aqueous leaf extract of *G. sylvestre* and observed that it showed predominantly significant *in vitro* immunomodulatory activity on human neutrophils in all parameters assessed. Another study conducted on the leaves of *G. sylvestre* revealed they significantly increased the phagocytic function of human neutrophils when compared to a control, indicating a possible immunostimulating effect. Malik et al. (2009) observed that the aqueous leaf extract of *G. sylvestre* showed remarkable immunostimulatory activity at 10, 25, 50, 100, and 1000 μg/mL on human neutrophils under *in vitro* conditions. The findings obtained in the present study are in strong agreement with previously reported studies of immunomodulatory effects, confirming that MLEGS derived from *G. sylvestre* possessed significant immunostimulatory activities.

Our research focused on the effect of MLEGS on the modulation of immune response, and the results revealed that MLEGS showed immunosuppression at the level of humoral and cell mediated responses. Similarly, it significantly inhibited the percentage of lymphocytes (CD3 and CD19) and intracellular cytokines (IL-2, IFN-γ and IL-4). This may be due to the concentration of active phytochemicals present in this particular plant extract, such as tannins, alkaloids, glycosides, flavonoids and saponins, confirming the immunosuppressive efficacy of *G. sylvestre*. The results of this assay confirm that *G. sylvestre* could serve as a source of potent pharmaceutical drugs with effective immunosuppressive properties.

Reduction in paw edema in later hours may be due to the rapid actions of enzymes and mediators that increase phagocytosis. Our results confirmed the potential of saponins and similar types of compounds to reduce paw edema, probably through increased release of serine proteases and immunohormones, including cytokines, by neighboring cells (Shukla et al., 2011). These metabolites and activated macrophages eliminate the causative agents; hence, the edema is gradually reduced (Shukla et al., 2011).

In addition, augmentation of the humoral immune response to SRBCs by plant extracts, observed as an increase in the antibody titer in rats, indicated the enhanced responsiveness of T and B lymphocyte subsets, which are involved

in antibody synthesis (Benacerraf, 1978). The high values of HA titer obtained in the case of MLEGS indicated that immunostimulation was achieved through humoral immunity. Similarly, the ethanolic leaves extract of *Cleome gynandra* also exhibited significant immunosuppression activities in a dose-dependent manner (Gaur et al., 2009). In another case, some pharmacological activities of the aqueous extract of fruit of *Lagenaria siceraria* showed preferential suppression of different components of cell-mediated immunity (Sankari et al., 2010). Bafna and Mishra (2006) reported that animals treated with different doses of the methanol extract of *Curculigo orchioides* showed an increase in the hemagglutination titer in a dose-dependent manner and such results reveal the significance of the present study. Similar findings with an alcoholic extract of *Isatis cappadocica* (Rezaeipoor et al., 2000), and aqueous and alcoholic extracts of *Echinacea purpurea* (Frier et al., 2003) have been reported with an increased titer of IgM. The aqueous extract of *Clausena excavate* has also been found to show similar results with regard to DTH response and HA titer (Manosroi et al., 2005).

CONCLUSION

The present study provides support for the use of *G. sylvestre* in traditional medicine in the treatment of autoimmune diseases. MLEGS significantly decreased the primary and secondary antibody titer and delayed type hypersensitivity and inhibited the increase in CD3 and CD19 lymphocytes and cytokines, IL-2, IFN-γ and IL-4. These findings indicate that MLEGS possesses significant immunosuppressive activity and recommend further studies aimed at developing an affective immunosuppressive drug with no adverse side effects.

Acknowledgments: The authors are grateful to the Head, Department of Botany, Dr. H.S. Gour University, Sagar (M.P.) India, Regional Research Lab (Jammu) India for providing the laboratory facilities, and to the University Grant Commission (UGC), New Delhi, India for providing financial assistance.

Authors' contribution: LA, SS, MKD, VB conceived and designed the experiments. LA, SA, MKD, VB performed the experiments. AM, SS analyzed the data. SS and LA wrote the paper.

Conflict of interest disclosure: Authors declare that there is no conflict of interest.

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