

Evaluation of Rbc Membrane Stabilization and Immunostimulatory Effect of *Sesbania Grandiflora* Flowers Extract

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Abstract

To investigate of RBC membrane stabilization and immunostimulatory effect, the effect of *Sesbania grandiflora* flower extract.

Heat induced haemolysis method used for a membrane stabilizing activity and measurement of the IgG and IgM serum levels via enzyme-linked immunosorbent assay used for immunostimulatory activity.

The Investigation, it was observed that methanolic and aqueous extract of *Sesbania grandiflora* have significant membrane stabilizing activity. At dose of 200 and 400 mg/kg of methanolic extract showed significant increase in the level of IgM and IgG, after four and seven days of the experiment through modulation of B lymphocyte functions.

These results suggested that methanolic extracts from *Sesbania grandiflora* contained several chemical compounds that possess positive modulator effects on the immune system. In summary, these studies provide important insight into the biological activities of *Sesbania grandiflora*.

Keywords: Immunostimulator, *Sesbania grandiflora*, IgG, IgM, ELISA

Introduction

Sesbania grandiflora (L.) Pers. (Fabaceae), known as Agasti or swamp pea, is an important medicinal plant and native to many Asian countries including India. The bark, leaves, gums, and flowers are considered medicinal. They are used as diuretic, emetic, febrifuge, laxative, and tonic [1]. In *Rasashastra*, it is used for processing of various formulations. The tender leaves, green fruit and flowers are eaten alone as a vegetable or mixed into curries or salads in various parts of South Asia [2]. A tea made from the leaves is believed to have antibiotic, anthelmintic, antitumor and contraceptive properties [3]. The bark is considered as a tonic and an antipyretic, and a remedy for gastric troubles and diabetes. The principal medicinal effects are due to the tree's astringency; hence, it is used against inflammation, venom and other poisons, bacterial infections and a tumour [4].

Sesbania grandiflora L flower and other parts of plants contains proteins, tannins, oleanolic acid, keampferol, grandifloral, cystine, isolucine, asparagine, phenylalamine, valine, nicotinic acid and Vit. C [5]. These chemical constituents are well known for their potential health benefits. *Sesbania grandiflora* have been reported to possess valuable biological activities such as antibacterial and antifungal [6], antioxidant [7], anticonvulsant, anxiolytic [8] hepatoprotective properties [9] and hypolipidemic effect [10].

Our group previously reported Immunomodulatory effects of crude *S. grandiflora* flower extracts as demonstrated by a significant increase in antibody titre level response against several SRBC antigens [11]. In addition, in the present study, we quantified antibody production (ELISA) IgG and IgM levels in sera from

treated animals with the extract against antigens prepared from Sheep RBC.

Materials and Methods

Collection and identification of plant material

The flowers of *Sesbania grandiflora* were collected from Bhopal District, Madhya Pradesh, India. Further taxonomic identification was conducted by the Prof. Madhuri Modak, Botanised, Department of Botany, M.V.M. College, Bhopal, Madhya Pradesh, India. A voucher specimen (Specimen No. BCP/Plant/2011/05) was kept at the Department of Botany.

Preparation of the extract

The flowers of *Sesbania grandiflora* were collected and shade dried. The dried flowers were coarse pulverized and the powder was packed in to soxhlet column and successively extracted with Petroleum ether (PESG), chloroform (CESG), methanol (MESG) and water (AESG) for 24 hrs. The dried extracts were kept in an airtight container in refrigerator below 10°C.

Preliminary phytochemical screening

To identify the essential constituents of the Petroleum ether, chloroform, methanol and aqueous extract of *Sesbania grandiflora* flowers such as alkaloids, terpenes and steroids, saponins, flavonoids, polysaccharides and tannins, a preliminary

phytochemical screening was carried out using various test methods.

Selection and maintenance of animals

Swiss albino mice (DRDO, Gwalior, India) weighing between 18 to 25 g and rats (150 to 200 g) of either sex were used. Animals were housed under standard conditions of temperature, 12 h/12 h light/dark cycle and fed with standard pellet diet and tap water. All the experiments were approved and conducted as per the guidelines of the Institutional Animal Ethical Committee.

Statistical analysis

Data were expressed as standard error of the means (S.E.M) of and statistical analysis was carried out employing one-way analysis of variance (ANOVA) followed by Tukey Kramer multiple comparisons test for membrane stabilizing and Dunnett test, which compares the test groups with the control group for ELISA.

Determination of Membrane Stabilizing Activity

Membrane stabilization activity was measured through rat erythrocytes hemolytic assay methods [14,15]. Blood was collected by heparinised syringe through retro orbital plexus puncture of rats. The blood was centrifuged for 10 mins at 3000 rpm to separate RBC, which was settled down at the lower portion of the centrifuge tube. The assay mixture was prepared by using 2ml of hyposaline, 1 ml of sodium phosphate (0.15M) buffer at pH 7.4. Test samples (PESG 50, PESG 100, CESH 50, CESH 100, MESG50, MESG100, AESG50, and AESG100) were prepared using varying concentration (50 and 100 µg/ml) in isosaline and 0.5ml of 10 % (v/v) erythrocyte suspension in isosaline was added to make volume of 4.5ml. Control was prepared as the above solution except the drug and standard solution was prepared by standard Indomethacin with concentration 10 µl/ml. The assay mixtures were incubated at 56±1 C for 30mins. The solutions were cooled adequately and centrifuged at 3000 rpm for 20 min. to settle down rupture RBC. The absorption readings of release haemoglobin of these assay mixtures were taken at 560 nm. The percentage of membrane stabilizing activity was calculated based on this following expression.

Equation for determination of membranestabilizingon activity

$$\% \text{ Membrane Stabilizing Activity} = 100 \frac{\text{OD Control} - \text{OD Test}}{\text{OD Control}}$$

Acute toxicity study in mice

The two extracts (MESG and AESG) were tested for acute toxicity studies as performed according to the Organization of Economic Co-operation and Development (OECD) guideline for testing of

chemicals (OECD, 423). Three healthy female albino mice weighing 25-30g, maintained under controlled conditions were administered a single oral dose of 2,000 mg/kg body weight. If mortality was observed in two or three animals among three animals then the dose administered was assigned as a toxic dose. If mortality was observed in one animal, then same dose was repeated again to confirm the toxic dose [16].

Enzyme-linked immunosorbent assay (ELISA)

Experimental Design

Animals were divided into 5 groups of six animals each.

Group I: Animals received orally 1ml of 1% NaCMC (Positive control)

Group II: Animals received orally 50 mg/kg Levamisole (Standard)

Group III and IV: Animals received methanolic extracts of *Sesbania grandiflora* 200 and 400 mg/kg, oral (Test groups)

Group V-VI: Animals received aqueous extracts of *Sesbania grandiflora* 250 and 500 mg/kg, oral (Test groups)

Preparation of SRBC membrane antigens for ELISA

Briefly, sheep blood was collected in Alsever's solution and cells were isolated by natural process at 1000 revolutions per minute for fifteen min. Once removal of plasma and therefore the Buffy coat, the cells were washed with 5 volumes of 0.9% NaCl 3 times. The washed pellets were suspended in approximately two volumes of 0.05 M Tris-HCl with 0.1 mM EDTA (pH 7.6), were mixed completely, and were centrifuged at 25000 revolutions per minute for thirty min. This method was recurrent as necessary until the supernatant became clear. The pellet suspension was more filtered through 3 layers of gauze, centrifuged once more and resuspended in 0.1% sodium dodecyl sulfate (SDS) with 0.02% sodium azide (three times pellet volume). The solubilized membrane antigens were dialyzed against 0.1% SDS in PBS and hold on at -20°C [17].

Measurement of IgM and IgG responses by ELISA

Mice were separated into experimental groups (n = 6). Positive control and treated groups were intraperitoneally immunized with sheep red blood cells (SRBC) (0.1 mL, 25% suspension in saline). The mouse blood sample was obtained at the time of immunization (day 0) and 4, 7 and 14 days after the single treatment with extracts. Anti-SRBC circulating antibody titers of mice were determined by ELISA. SRBC antigen, in aliquots of a five µg/mL suspension in PBS, pH 7.2 (125 µL/well), was incubated during the night at 4°C in high-binding micro plates. Before every subsequent step, plates were washed 3 times with PBS-T. Unbound sites were blocked with 3% powdered milk powder in PBS (200 µL/well) for two h at room temperature (RT). Serum from experimental animals diluted 1:8 in PBS-T was added to the wells (125 µL/well) followed, once one h incubation at RT, by peroxidase conjugated anti-mouse IgG (1:1000) and anti-mouse IgM (1:2000) diluted in PBS-T (125 µL/well). Once another hour at RT, OPD substrate

was added (200 $\mu\text{L}/\text{well}$) and therefore the plates were incubated within the dark for twenty min at RT. The reaction was stopped with two M H_2SO_4 (75 $\mu\text{L}/\text{well}$) and therefore the color every well was evaluated at 492 nm on ELISA reader [18].

Results

Phytochemical screening

Table 1: Result of preliminary phytochemical screening of *Sesbania grandiflora* flowers

Tests	Petroleum ether	Chloroform	Methanol	Water
Alkaloids	-	-	+	+
Saponins	-	-	+++	+
Flavonoid	-	-	+++	+
Phenolic Compound	-	-	+++	+
Amino Acid	-	-	++	+
Polysaccharides	-	-	+	+
Fats and Oils	+	+	-	-

-, Absent, +: Trace amounts ++: Present, +++: High

Membrane Stabilization Activity of *Sesbania grandiflora* flower extract

This study revealed the affectivity of *Sesbania grandiflora* different extracts compared to the standard Indomethacin. The efficacy of the *Sesbania grandiflora* against the hyposaline-induced lyses of erythrocytes was comparable to that of the standard. The membrane stabilization activity of methanolic and aqueous extracts of *Sesbania grandiflora* was shown significantly against the standard Indomethacin respectively. The percentage membrane

The preliminary phytochemical screening of *Sesbania grandiflora* flowers exposed the presence of alkaloids, flavonoids, saponins, phenolics and polysaccharides as essential phytochemical constituents of the methanolic and aqueous flower extract. Petroleum ether, chloroform extracts of *Sesbania grandiflora* showed the presence of phytosterol and fixed oils. (Table 1).

stabilization activity of MESG (50.60%), AESG (30.17%), compared to the standard Indomethacin (61.98%) was observed. The both extracts of *Sesbania grandiflora* had shown the significant activity ($P < 0.05$) as compared to the standard which was determined by the one way ANOVA (Analysis of variance) followed by Tukey-Kramer multiple comparison test. (Table 2)

Table 2 Membrane Stabilization Activity of *Sesbania grandiflora* flower extract

S. No	Test sample	Conc. ($\mu\text{g}/\text{mL}$)	Optical Density (O.D)	Membrane stabilization (%)
1	Control		0.905 \pm 0.009	--
2	PESG 50	50	0.811 \pm 0.014	10.39
3	PESG100	100	0.797 \pm 0.02	11.93
4	CESG 50	50	0.803 \pm 0.03	11.27
5	CESG 100	100	0.798 \pm 0.02	11.82
6	MESG 50	50	0.468 \pm 0.018 ^b	48.28
7	MESG 100	100	0.447 \pm 0.015 ^b	50.60
8	AESG 50	50	0.677 \pm 0.018 ^b	25.19
9	AESG 100	100	0.632 \pm 0.03 ^b	30.17
10	Indomethacin	10	0.344 \pm 0.005 ^a	61.98

Each value represents the mean \pm SD of six experiments. Statistical analysis was done using ANOVA followed by Tukey-Kramer multiple comparisons test.

^a $P < 0.01$ was considered statistically more significant compared with control

^b $P < 0.05$ was considered statistically more significant compared with control

Acute toxicity study

The LD₅₀ of methanolic and aqueous extract of *Sesbania grandiflora* flowers was determined. No mortality was observed at 2000 and 5000mg/kg respectively.

Detection of IgM and IgG levels in sera from mice treated with the *Sesbania grandiflora* extract against SRBC in ELISA

Both doses of methanolic extracts (200 and 400 mg/kg) and 500 mg/kg of aqueous extract of *Sesbania grandiflora* significantly increase the IgM titer value ($p < 0.01$, $p < 0.01$ and $p < 0.05$, respectively) after four days immunization and treatment. A same summary observed at the dose of 400 mg/kg ($p < 0.01$) of methanolic extracts, after 7 days. Methanolic extracts of 200 and 400 mg/kg also significantly ($p < 0.01$ and $p < 0.001$, respectively) increased IgG titers in mice treated after seven days when compared with immunized only mice (positive control). A similar

profile was obtained after 14 days, but the differences were significant ($p < 0.05$ and $p < 0.01$, respectively). No significant effect on antibody (IgG titers), was observed after treatments with aqueous extracts in immunized animals for any of the doses or periods evaluated. The results are shown in Figure.1.

Discussion

The therapeutic potential of several plant species and the necessity for scientific validation of the use of popular plants in medicine have prompted increased interest in the field. A large number of plant species and their components have been shown to be potent immunomodulators acting as anti-inflammatory, anti-stress, and anticancer agents [17].

The initial chemical studies of the *Sesbania grandiflora* extracts have demonstrated the occurrence of flavonoids, tannins, glycosides, and saponins and these constituents are well established for their anticancer, antioxidant, anti-inflammatory and immunomodulatory activity [18].

The immune system is the complex defence system in our body. The immune system consists of the innate (nonspecific) and

adaptive (Specific) system by which immune system can protect the host from antigens. In the innate immune system is our primary defence system that protects hosts from primary threats from antigens. The erythrocyte membranes resemble lysosomal membranes that were affected by exposure of soluble antigens. Therefore, effects of extract of stabilizing the erythrocytes membrane is the important parameters for the detection of its anti-inflammatory and immunomodulatory activity [13,19]. There is a correlation between the anti-inflammatory and immunomodulatory drugs because those drugs are acting as an anti-inflammatory are acting through the innate immune system [20]. From this *in vitro* membrane stabilization, we will determine the immune system modulating activity of different successive extracts of *Sesbania grandiflora*. In the traditional system of medicine the *Sesbania grandiflora* in herbal formulation and alone were used as an anti-inflammatory and antipyretic activity. The present study was designed in a manner that compare the membrane stabilization activity of the *Sesbania grandiflora* with standard drug indomethacin. From the experiment, it concluded that methanolic and aqueous extracts of *Sesbania grandiflora* flowers

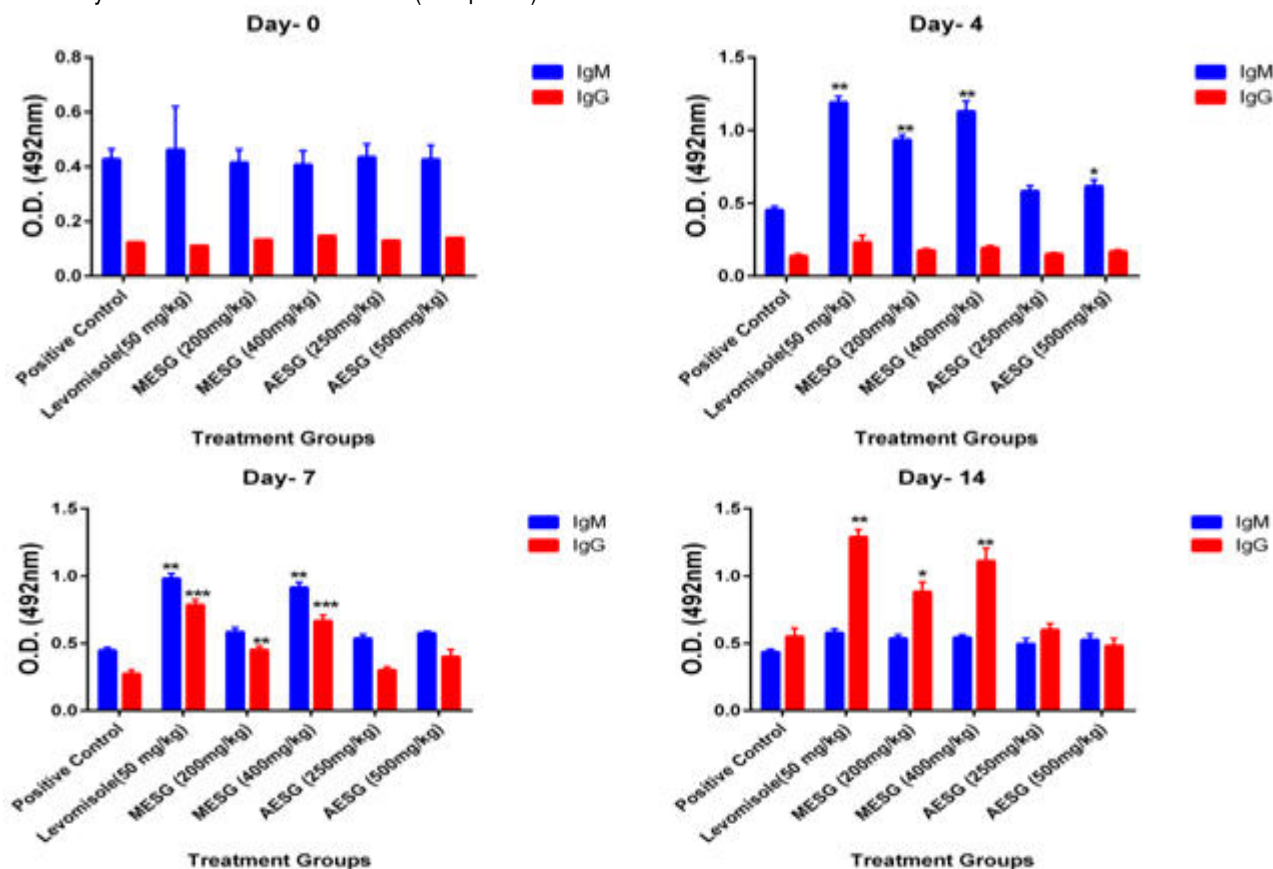


Figure. 1 Effect of *Sesbania grandiflora* methanolic and aqueous extracts on anti-sheep red blood cells (SRBC) antibody IgM and IgG titers on days 0, 4, 7 and 14 after mice immunization.

Data are means \pm SEM of six animals. Statistical analysis was carried out employing the ANOVA followed by Dunnett test *: $P < 0.05$, **: $P < 0.01$ ***: $P < 0.001$ compared with the positive control group. shown us the most significant ($P < 0.05$) membrane stabilization activity against standard indomethacin.

The humoral immune response can be measured as an increase level of total antibodies or of specific antibodies against a non-pathogenic antigen such as SRBCs. The level of a specific antibody in the serum can also be used as a measure of the functional status of humoral immune response-antigen recognition, activation, and expression [23,24]. The integrity of the antibody mediated (humoral) immune response depends upon a competent population of B- lymphocytes, which have the functional capacity to develop into antibody producing plasma cells. Interpretation of the antibody response to SRBCs is complicated by the fact the blood cells are antigenically complex. The animal can make antibodies against foreign blood cells. These pre existing or natural antibodies are not derived from prior contact with foreign RBC, but result from exposure to similar or identical epitopes that commonly occurs in nature [25]. Because of the antigenic complexity of SRBCs, it is not known against which particular antigens the antibodies are being raised. Therefore, ELISA method was used to detect specific antibodies produced against certain antigens. In this study, the methanolic extracts of *Sesbania grandiflora* flowers, 200, and 400 mg/kg significantly increase or activate the secretion of immunoglobins such as IgM and IgG.

Conclusion

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