ISSN: 2231-6078
INTERNATIONAL JOURNAL OF DRUG DISCOVERY AND HERBAL RESEARCH (IJDDHR)
4(4): Oct-Dec: (2014), 761-765

Research Article

Immunorestorative and Anti-Inflammatory Activity of Leaf Aqueous Extract of *Calotropis gigantean* Using Flow Cytometry

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Abstract

In the last five decades, there are number of aqueous extract isolated from the leaves, root and stem of medicinal plants are probably used as a source of therapeutic as well as prophylactic agents. The most promising activities i.e. immunorestorative as well as anti-inflammatory observed in the aqueous extract isolated from the leaves of *Calotropis gigantean* using specific antigen bovine serum albumin (BSA). Cyclophosphamide used as immunosuppressant for these studies and immunized two days prior to immunization with specific antigen i.e. BSA. We evaluated the immunorestorative effect of the aqueous extract on the *in vivo* immune function of the mouse. Swiss mice were treated orally with variable doses of aqueous extract (50 – 300 mg/kg) for 14 days using BSA as an antigen immunized intraperitoneally on day 0 and challenged on day 7. On day 15, we analyzed the antibody titre by Elisa, estimation of Th1 (IFN-gamma, TNF alpha) cytokines from cell culture supernatant and surface markers i.e. CD3/CD4/CD8 population were studied. The results showed that the leaf aqueous extract of *Calotropis gigantean* at the dose of 300 mg/kg, showed decrease in the antibody titre and Th1 cytokine levels (IFN-gamma and TNF alpha) in cell culture supernatant were observed. There was a dose dependent decrease in CD3/CD4/CD8 population in mice was also observed. This suggests that the *Calotropis gigantean* improves the immune system and might be regarded as a biological response modifier.

Key Words: *Calotropis gigantean,* cyclophosphamide, Bovine serum albumin

Introduction

Inflammation is usually associated with pain as a secondary process resulting from the release of analgesic mediators. Through years of ingenious synthesis and structural modifications of natural products, which usually accompany the design and development of new drug substances, many synthetic based anti-inflammatory agents have been prepared and marketed. These have been of immense help in the management of various inflammatory conditions like rheumatism, arthritis and pain. Moreover, in the face of rising costs of synthetic medicines, phytomedicinal treatment of diseases has become the order of the day in all over the world, chiefly because of their ready affordability and availability, especially in the villages where the majority of the people are poor and merely subsisting [1,2]. In this study, we focused on the anti-inflammatory effect of natural plant based leaf aqueous extract on the immunocompromised mice immune system. One of the medicinal plant i.e. *Calotropis gigantean* (calos meaning beautiful and tropos means boat) which is commonly called as giant milkweed or crown flower and belongs to the family Asclepediace [3]. In India, *Calotropis gigantean* also called by different names such as Sanskrit (Mandara, Arka, Ganarupa), Marathi (Rui), Hindi (Aak, madar), Kannada (Ekka), Tamil and Malayalam (Erukku).

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The plants grow very well in India and abroad especially Malaysia, Indonesia, Philippines, Thailand, Vietnam etc in a variety of soils and different environmental conditions and also have immense potential to cure number of diseases and disorders [4, 5]. Generally, this plant is also used in the number of polyherbal preparations. Previously, number of researchers isolated the flavonoids, terpenoids, glycosides, alkaloids and saponins isolated from different parts of the plant i.e. root, stem and leaves of *Caloptropis gigantean* and showed number of medicinal properties like anti-tumor activity [6], anti-microbial [7], anti-bacterial [8], wound healing activity [9], central nervous system activity [10], antidote for scorpion stings as well as insect bites [11], anti-cancer [12] and anthelmintic activity [13]. In this plant, the leaf contains ascorbic acid, ortho-pyrocatechic acid and also contains β-amyrin, stigmasterol, giganteol, calotropin, a triterpenoid flavonoid, flavonoid glycoside, wax, acids and alcohols where as in shoot and leaf extracts possess anti-bacterial activity [14, 15]. Tender fresh leaves have been reported to cure fits and convulsions in nipper [14]. Therefore, efforts are taken to evaluate and validate the evidence regarding safety and practices of Ayurvedic based medicines in immunocompromised patients. Considering this, the present paper which has enlightened the immunorestorative studies (using cyclophosphamide) of aqueous extract of leaf of *Calotropis gigantean* on *in vivo* immune function of mice.

Materials and Methods

2.1. Preparation of aqueous extract

Fresh plant leaves of *Calotropis gigantean* were collected from the garden of Vidya Pratishthan's School of Biotechnology (VSBT), Baramati (Pune), Maharashtra. Recently, harvested leaves were properly washed with tap or distilled water and then dried in a shady area. Afterwards, the plant leaves cut into small pieces and weighed properly and then macerated with liquid nitrogen to form the pulverized leaf and this was used in preparation of *Calotropis gigantean* aqueous extract using phosphate buffered saline and proceed with immunopharmacological studies. *Calotropis gigantean* aqueous extract of leaves was centrifuged at 8000 rpm for 7 minutes and the supernatant was retrieved and was used within six hours for various immunopharmacological assays.

2.2. Phytochemical investigation of aqueous extract

The phytochemical screening of *Calotropis gigantean* was carried out using standard methods to detect the primary as well as secondary metabolites in the aqueous extract. During qualitative studies, the phytochemical screening revealed the presence of alkaloids (Wagner's reagent test), flavonoids (Lead acetate test), saponins (Foam test), tannins (ferric chloride test) and glycosides (Borntrager test). In contrast for HPTLC studies, the mobile phase for the aqueous extract of *Calotropis gigantean* was ethyl acetate: n Butanol at the ratio (6:4). The resolution was obtained at 220 nm. The peaks at retardation factor (Rf) value is 0.92 (terpenoids), saponins (0.31 – 0.46, peak area 624 – 1880) an shows the maximum concentration with an area under the curve.

2.3. Cyclophosphamide-induced immunosuppression

Briefly, Swiss mice were distributed into five groups consisting of three animals. The animals were properly maintained as per ethical guidelines (Reg. no. 97/1999). Immune system suppression using cyclophosphamide (250 mg/kg) two days prior to immunization with specific antigen bovine serum albumin (BSA, 1 mg/ml) was given to all mice by a single intraperitoneal injection. Animals were divided into five groups of five animals each: (Group I) control, received normal saline; (Group II) cyclophosphamide, 250 mg/kg body weight; (Group III) 50 mg/kg body weight, (Group IV) 100 mg/kg body weight and (Group V) 300 mg/kg body weight. The leaf of *Calotropis gigantean* was dissolved in phosphate buffered saline and was administered per oral for 14 days. The dose volume was 0.2 ml. On day sixteen, EDTA anti-coagulant blood samples were obatained from retro-orbital plexus of all animals for the estimation of cell surface marker CD3, CD4 and CD8 using flow cytometry and also estimates the antibody titre and Th1 type of cytokines by Elisa. All these studies are repeated three times and these are done according to ethical regulations as well as guidelines on animal research.

2.4. Estimation of antibody (IgG) titre by Elisa

BSA specific IgG antibodies in serum were detected by an indirect ELISA (Sun *et al*, 2004). In brief, Elisa plate wells were coated with 100 µl BSA (1 mg/ml) solution for IgG antibodies in 50 mM carbonate-bicarbonate buffer, pH 9.6) for 24 h at 4 °C. The wells were washed three times with PBS containing 0.05 % Tween 20 containing phosphate buffered saline (PBS) and then blocked with 5 % PBS containing fetal bovine serum at 37 °C for 1 h. After three washings with PBS, 100 µl of diluted serum sample (IgG, 1: 100) or 0.5 % FCS/PBS as control was added to triplicate wells. Elisa plates were then incubated for 1 h at 37 °C, followed by three times of washing. After washing, add 100 µl of rabbit anti-mouse IgG horse radish peroxidase conjugate diluted 1: 1000 with 0.5 % FCS/PBS were added to each plate. Again, the plates were incubated for 1 h at 37 °C. After washing, 100 µl of TMB substrate solution was added and the plate was incubated for 10 min at 37 °C and finally adds 50 µl/well of 2 N H_2SO_4. The optical density (OD) was measured in an ELISA reader at 450 nm.

2.5. Splenocyte proliferation assay (*ex vivo*) and estimation of Th1 cytokines

Mice was sacrificed by carbon dioxide anesthesia. The spleens were excised aseptically and lymphocytes isolated. Briefly, single cell suspensions were prepared by teasing the tissue between two glass slides and cells were centrifuged at 1800 rpm for 10 min at 4 °C. Erythrocytes present were lysed with ACK lysis buffer for 8 min. During centrifugation, pellet containing lymphocytes were collected and these cells were washed 2-3 times with PBS and cells were counted with a hemocytometer by the trypan blue dye exclusion technique. To evaluate the effect of leaf aqueous extract on the proliferation of splenic lymphocytes (Gupta *et al*, 2006), spleen cell suspension (2×10^6 cells/ml) were pipetted into flat bottom 96 well tissue culture plates (200 µl/well) in the presence of Con A (5 µg/ml) and LPS (10 µg/ml) cultured at 37 °C for 48 - 72 h and these plates were properly centrifuged at 1400 x g, 5 min and the supernatant was obtained for the estimation of cytokines in cell culture supernatant. Cytokine concentrations in the cell culture supernatant were determined by ELISA kits that were specific against murine cytokines. Levels of Th1 (IFN-gamma and TNF alpha) were measured using ELISA (BD optia, ELISA kit). Assays were performed according to the manufacturer's instructions [16, 17].

2.6. Flow Cytometric analysis in mice

2.6.1. Spleen cells

Flow cytometry analysis of whole blood samples of mice for counting and examine the cells i.e. T cell surface markers suspended in a streaming fluid. To examine the effect of different concentration of *Caloptropis gigantean* aqueous extract in BSA immunized mice on mouse spleen cells and these were analyzed through flow cytometer (FACS Calibur) using 5 µl of mouse anti- mouse CD3, CD8 FITC and CD4 PE lymphoid surface marker monoclonal antibodies to the 40 µl of mouse spleen cells, incubated for 30 minutes at room temperature, and then lysed with 2 ml of FACS lysing solution by centrifuging for 8 minutes at 2600 rpm. After centrifugation, the supernatant was removed or discarded and then properly washed by centrifuging for 7 minutes at 2600 rpm with 1 ml of PBS and then samples were analyzed for 10000 cells on the flow cytometer [18, 19].

2.6.2. Peritoneal macrophages

Swiss mice were injected intraperitoneally with 10 ml of ice cold phosphate buffered saline. The abdominal surface was gently massaged and collects all the peritoneal cells in tubes. The peritoneal cells were washed two to three times in phosphate buffered saline by centrifugation at 1200 rpm, 10 min in cold and finally suspended at 2×10^6 cells/ml in phosphate buffered saline containing 10 % FCS (heat inactivated). 500 µl cell suspensions of BSA immunized mice of variable doses of aqueous extract (50 – 300 mg/kg, body weight) were added in each 6 well plate and then add again exposure of variable doses of aqueous extract. Samples were incubated for 24 h at 37°C in CO_2 incubator and then analyzed the forward and side scatter using flow cytometer.

2.7. Acute toxicity

As per the OECD guidelines, fasting Swiss mice in groups of 10 each were used for each dose. Aqueous extract was administered in graded doses of 50 to 1000 mg/kg i.p. and 100to 3000 mg/kg per oral. The groups of animals administered PBS alone served as control. The animals were observed continuously for any gross behavioural change and mortality for the next 2 weeks.

2.8. Statistical analysis

The difference between the normal control and leaf aqueous extract of *Caloptropis gigantean* treated samples in mice is determined by one way ANOVA test (Bonferroni multiple comparison test).

Results

3.1. Effect of leaf aqueous extract on antibody response

The effect of leaf aqueous extract of *Caloptropis gigantean* on antibody production as shown in (**Fig.1).** In general, the aqueous extract significantly decreased the antibody titer at higher doses as compared to control. The serum antibody titer in BSA-immunized mice was significantly reduced by aqueous extract at 300 mg/kg, where as cyclophosphamide used as standard and significantly reduced the antibody titre as compared to control.

3.2. Effect of leaf aqueous extract on Th1 cytokines from cell culture supernatant

The effect of leaf aqueous extract was observed on day 15 in cell culture supernatant from spleen cells of BSA immunized mice (**Fig.2**). On day 15, spleen cells were collected for the estimation of cytokines in BSA immunized mice. The results showed that the aqueous extract showed the suppression of Th1 (IFN-gamma and TNF alpha) at a dose range of 300 mg/kg as compared to control. Cyclophosphamide used as standard which inhibit the

activity of Th1 (IFN-gamma and TNF alpha) as compared to control.

3.3. Effect of leaf aqueous extract on T (CD3, CD4 and CD8) cell population and peritoneal macrophages activation

The effect of leaf aqueous extract of *Caloptropis gigantean* on T cell surface markers i.e. CD3, CD4 and CD8 population and activation of peritoneal macrophages as shown in **Fig.3 (A, B).** In general, the aqueous extract significantly inhibited the population at higher doses as compared to control. On day 15, EDTA blood and peritoneal cells was collected for the estimation of surface markers and peritoneal macrophage activation in BSA-immunized mice. The results showed that the aqueous extract inhibited the population of surface markers (i.e. CD3/CD4/CD8 population) and peritoneal macrophages at a dose range of 300 mg/kg. Cyclophosphamide used as standard and inhibited the population of T cell surface markers and peritoneal macrophages.

3.4. Safety studies

Swiss mice used for testing the safety of aqueous extract remained clinically healthy during the entire period of experiment. Neither local reactions nor alterations in the mouse behaviour were observed.

Discussion

Natural products are a source of many traditional medicines and even some synthetic herbal medicines. In some parts of the world the herbal medicines are commonly used to treat a number of diseases. The search for the alternate systems of medicine such as Unani, Siddha, Ayurveda comprise of the natural products having potential anti-inflammatory, antibiotic and other activities has gained an importance considering the harmful side effects which the modern synthetic medicine has. Now a day, the importance of medicinal plants has been increasing both for pharmaceutical industry and traditional users. Most of the countries believed or rely on traditional medicines either it is developing or under developing country [20, 21].

Most of the modern medicines are synthesized or produced indirectly from several medicinal plants e.g. aspirin. Medicinal plants are directly used as medicines by a majority of cultures around the world, e.g. Indian as well as Chinese medicine. Now a day's world population moves towards the plant based medicines for the treatment of protozoan, bacterial as well as viral diseases. The number of medicinal plants has been screened or analyzed for their anti-inflammatory activity, but only couple of plants reached to the clinical trial studies. In contrast, the number of researchers focused on the primary as well as secondary metabolites isolated from the root, bark and leaf of the plant for the specific treatment related to inflammation and arthritis. In spite of the vast diversity of medicinal plants, there are only a few reports on some of their anti-inflammatory activity, in contrast, there are many reports regarding anti-bacterial, anti-fungal, and anti-malarial activities [18, 19, 22].

In this study, the aqueous extract isolated from the leaves of *Caloptropis gigantean*, were screened for anti-inflammatory activities on the following bioassays: IgG antibody titre; T cell surface markers i.e. CD3, CD4 and CD8, activation of peritoneal macrophages and also determined the Th1 (IFN-gamma, TNF alpha) type of cytokines in serum. The results showed that the aqueous extract derived from *Caloptropis gigantean* showed inhibitory activity on the antibody titre, cell surface markers (i.e. CD3, CD4, CD8), peritoneal macrophages and also decreases the level of Th1 (IFN-gamma, TNF alpha) level of cytokines in serum. These studies showed that the effect of aqueous extract of *Caloptropis gigantean* on antibody titre to BSA was examined; it was found that oral administration of leaf aqueous extract into mice markedly augmented the antibody response to BSA. The result indicated an inhibitory effect of aqueous extract on the ability of mice to produce antibodies against a T dependent antigen. The data showed that aqueous extract had potent anti-inflammatory activity *in vivo*.

Cell mediated immunity, mediated by T lymphocytes which played an important role to fight against intracellular infections. The capacity to elicit an effective T cell immunity can be shown by the Th1 cytokine estimation. The results indicated that *Caloptropis gigantean* could significantly inhibit the potential of T cells in BSA-immunized mice. The results of cell mediated response and status of IFN-gamma, TNF alpha and phagocytic activity after immunization with T-dependent antigen suggest that the activity of *Caloptropis gigantean* could be mediated through the anti-inflammatory effect on T lymphocytes and macrophages [23, 24]. Macrophages reside within the peritoneal cavity of mice and these were originated from specific white blood cells called monocytes which are present in the blood. Monocytes and macrophages are phagocytes, acting in either or innate as well as cell-mediated immunity of vertebrate animals. The results showed that there is significant decrease in level of macrophages at higher doses as compared to control.

Furthermore, it has been demonstrated as well as confirmed that the Th1 type of cytokine i.e. IFN-gamma and TNF alpha are significant as well as crucial B cell that switches the triggering as well as enhancement of antigen-specific IgG2a secreting B cells and most of the viral infections induce an antibody-mediated response characterized by a predominance of IgG2a. In this survey, our data represents that mice immunized with BSA, aqueous extract had a significant decrease in the Th1 type of cytokine IFN-gamma and TNF alpha in serum [23, 24]. We speculated that aqueous extract might mediate their function *in vivo* in mice by reducing the induction of proinflammatory cytokines, which resulted in inhibiting CD4+ and CD8+ T-cell response. The inhibitory effect on CD4+ and CD8+ T cells, thereby confirming its general effect on the cell-mediated immune response.

Conclusion

Oral administration of aqueous extract of *Caloptropis gigantean* leaves displayed an immunorestorative (induced by cyclophosphamide) as well as anti-inflammatory effect on antibody titre, T cell surface markers (i.e. CD3, CD4 and CD8) and peritoneal macrophages activation in mice. Moreover, leaf aqueous extract at 300 mg/kg showed anti-inflammatory effect in mice and thus support the traditional as well as medicinal use of *Caloptropis gigantean* as immunoprotective and could be a promising alternative for cancer therapy.

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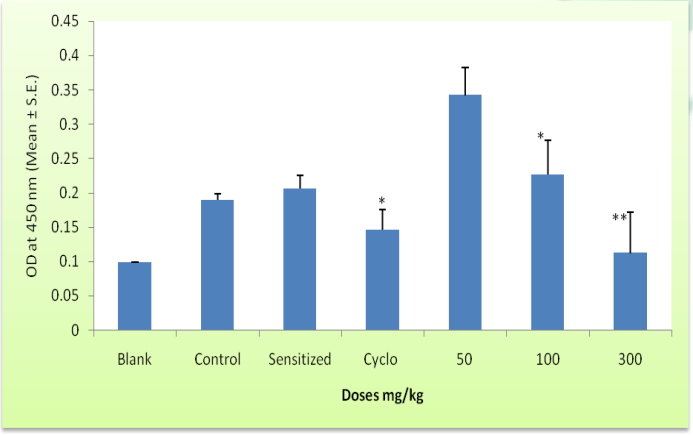

Fig.1. ELISA assay. On day 15, blood samples were obtained from retro-orbital plexus for the estimation of antibody titre. The results are presented as Mean ± S.E. Experiment repeatedly three times. *P* values: **P < 0.05, **P < 0.01, ***P < 0.001* as compared to control.

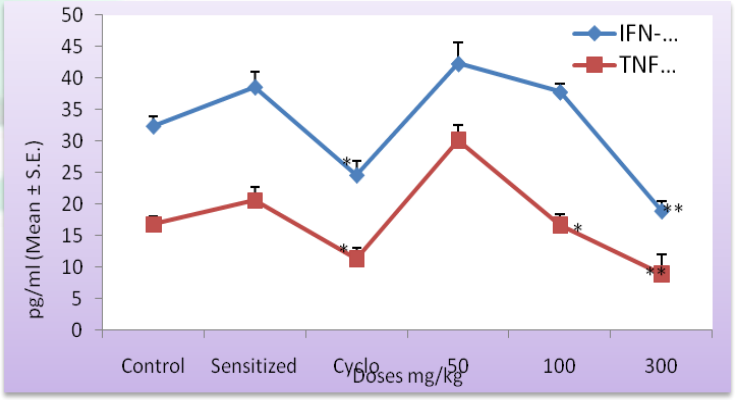

Fig.2. Effect of variable doses of *Caloptropis gigantean* on Th1 (IFN-gamma and TNF alpha) cytokines in spleen cell culture supernatant. Groups received variable doses of leaf aqueous extract (50,100 and 300 mg/kg) from day 0 to 14. Spleen cells were collected and cultured for 48 h in presence of exposure of variable doses of aqueous extract (50 – 300 mg/kg). The plates were centrifuged and the cell culture supernatant was collected for the estimation of Th1 (IFN-gamma, TNF alpha) cytokines. Each bar represents the group mean (n=5). Value for the concentration of cytokine expressed in pg/ml. Experiment repeatedly three times. *P* values: **P < 0.05, **P < 0.01* and ****P < 0.001* when compared to the value of control

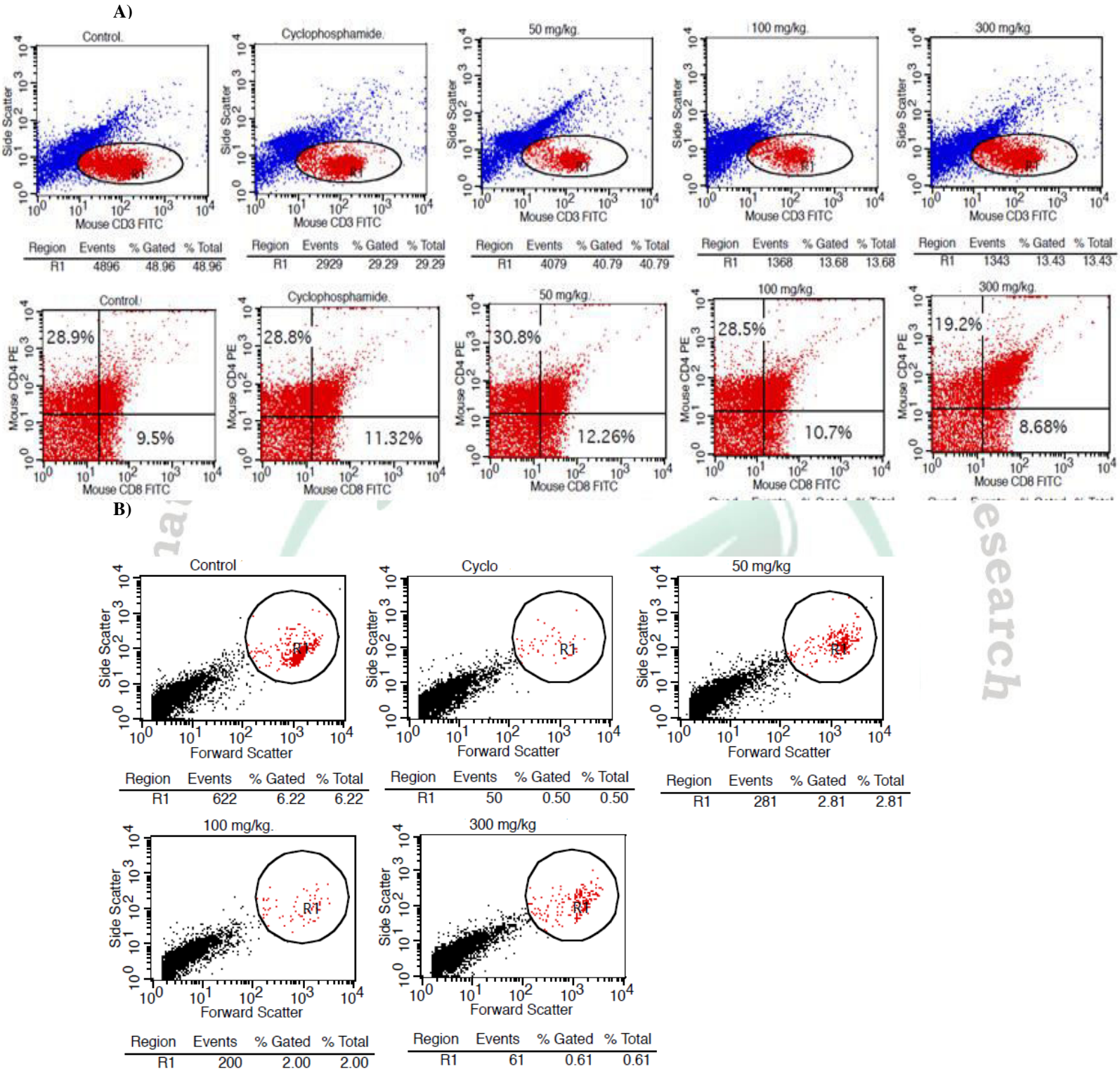

Fig.3. Flow cytometric analysis of *Caloptropis gigantean* on T (CD3, CD4 and CD8) cell population and peritoneal macrophages. EDTA whole blood and mouse peritoneal cells were collected on day 15. A) Staining of EDTA whole blood with FITC conjugated monoclonal antibody i.e. CD3 and CD8 and PE conjugated monoclonal antibody i.e. CD4. B) In contrast, mouse peritoneal cells (2 x 10^6 cells/ml) dissolved in phosphate buffered saline containing 10 % FCS (heat inactivated). 500 µl cell suspensions containing 2 x 10^6 cells/ml of immunized mice of variable doses of aqueous extract (50 – 300 mg/kg, body weight) were added in each 6 well plate and then add again exposure of aqueous extract. Samples were incubated for 24 h at 37°C in CO2 incubator and then analyzed the forward and side scatter using flow cytometer. Experiment repeatedly three times.