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Full Length Research Paper

Immunosuppressive properties of aqueous extract of *Plumbago zeylanica* in Balb/c mice

Aparanji Poosarla* and Rama Rao Athota

Department of Biochemistry, Andhra University, Visakhapatnam, Andhra Pradesh, India.

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In our investigation, we have screened herbal drug, which consists of a mixture of seven plants, used by the tribal population (in Paderu agency area of Visakhapatnam district of Andhra Pradesh state in India), for the identification of potential immunosuppressive property by testing the aqueous extracts of the plant parts by inducing ovalbumin (OVA) specific IgG antibody responses in a murine system. The aqueous root extract of *Plumbago zeylanica* (PZE) exhibited the significant suppression of OVA-specific IgG antibody response determined by enzyme-linked immunosorbent assay (ELISA). PZE also suppressed the anti-OVA antibody response in dose dependent manner. In addition ethyl acetate fraction of PZE eluted from silica gel (PZE-6) was found to exert a significant suppression of OVA stimulated T cell proliferation. Moreover, PZE is potent in exerting the suppressive effect on the down regulation of anti-OVA antibody and T cell responses and in all the three haplotypes of the mice studied, which indicates that the PZE exerted immunosuppression without linking to genetic variation.

Key words: *Plumbago zeylanica*, immunosuppression, anti-OVA antibody response, enzyme-linked immunosorbent assay.

INTRODUCTION

Ayurveda, the traditional medicinal system of Indian subcontinent is better known for its holistic approach. In this system of medicine, the crude extract, paste and powder prepared from either dried or fresh parts of a single or combinations of plants are being used in the alleviation of the symptoms of different diseases. *Azadirachta indica* (neem), *Ocimum sanctum* (holy basil or tulsi), *Zinziber officinalis* (ginger), *Phyllanthus niruri* (amla), *Curcuma longa* (turmeric), *Piper nigrum* (pepper), etc., are the medicinal plants commonly used in the treatment of many disorders (Sen et al., 1991). The modulation of immune response by medicinal plant products as a possible therapeutic measure has become a subject of scientific investigation. It is being recognized that immunomodulatory therapy by medicinal plants could provide an alternative to the conventional chemotherapy. Most of the drugs cause variety of side effects like gastric irritation and impaired renal function etc. Thus searches for better therapeutic drugs, with minimum side effects to alleviate the symptoms of the disease.

Plumbago zeylanica L (Plumbaginaceae) was selected, as it is easily cultivated in gardens and distributed throughout India (Chopra et al., 1956). A number of naphthoquinones, flavinoids, anthocyanins and β-sitosterol have been reported previously from this plant source (Dinda et al., 1995). The crude extracts of *P. zeylanica* have been used in china and other Asian countries as folk medicine for the treatment of cancer, rheumatoid arthritis and dysmenorrhea (Itoigawa et al., 1991). Chloroform extract showed antibacterial activity (Chakraborty, 1977). The modulatory ability of plumbagin, a natural product isolated from this plant was studied on peritoneal macrophages of Balb/c mice (Abdul, 1995).

MATERIALS AND METHODS

Animals

Female inbred Balb /c (H-2^d), C57BL/6 (H-2^b) and SWR/J (H-2^q) mice of 8 weeks old were procured from laboratory animal service center, National Institute of Nutrition, Hyderabad, India, to study the

*Corresponding author. E-mail: apara_p@yahoo.co.in. Tel: 08912782116.

Table 1. Indian and scientific names of the plant constituents of the herbal drugs.

S/No.	Telugu name	Hindi name	Scientific name	Part used	Family name
1.	Addasaramu	Arusa	*A. vasica*	Root	Acanthaceae
2.	Swarnapathri	Swarnapathri	*Gassia angustifolia*	Leaves	Myrtaceae
3.	Sugandhipala	Ananthmol	*Hemidesmus indicus*	Root	Asclepedaceae
4.	Tippatega	Amrita	*Tinospura cordifolia*	Stem	Minispermaceae
5.	Palasha	Palasha	*B. monosperma*	Seed	Fabaceae
6.	Tellachitramoolam	Chitrak	*P. zeylanica*	Root	Plumbaginaceae
7.	Pilligaddalu	Satavari	*Asparagus racemosus*	Root	Liliaceae

antigen specific antibody response and T cell proliferation. The animals were housed under standard conditions and allowed to be fed with pelleted diet purchased from Brooke Bond Lipton Ltd., Bangalore, India and water *ad libitum*.

Chemicals and materials

Turkey egg albumin (OVA), goat anti-mouse IgG, tween-20, O-phenylene diamine (OPD), hydrogen peroxide, 96 well microtiter flat bottom Enzyme-Linked Immunosorbent Assay (ELISA) plates (Nunc, Denmark) for determining the humoral immune responses.

Preparation of crude extract

The individual plant parts from the herbal drug, listed in Table 1 were procured from the authorized local medicinal plant distributors and were authenticated with the help of taxonomists of Department of Botany, Andhra University, Visakhapatnam. The plant materials including *P. zeylanica* (PZ) roots were properly cleaned, semi dried in an air circulating oven at temperature not exceeding 30°C.The dried roots powdered to prepare 15% aqueous extract. The extract was prepared by taking 15 g of material in 100 ml of double distilled water and subjected to vigorous grinding to extract the contents of the material. The extract was lyophilized after vacuum filtration. The lyophilized material of *P. zeylanica* was resuspended in Phosphate Buffered Saline (PBS) (0.005 M phosphate, 0.075 M NaCl and pH 7) and designated as *P. zeylanica* extract (PZE). The final concentration was adjusted to 4 mg/ml and the extract was stored at –20°C, until its further use.

Immunization

For studying the humoral immune response, mice were immunized intraperitoneally (IP) with 10 µg OVA and OVA + PZE or specific fractions in a total volume of 0.5 ml of PBS on day 0, 28 and 56. The experimental groups were bled at various intervals of day 14, 21 and 28.

Collection of serum

At the appropriate time intervals, after the immunization, the mice were bled from the tail vein into a glass centrifuge tube and the blood was allowed to clot. The clotted blood was rimmed and the serum was separated after centrifugation at 2000 rpm (remi-8c, India) and frozen stored at -20°C, until it is use for the determination of OVA specific IgG antibody response by ELISA.

Measurement of humoral immune response

Enzyme linked Immunosorbent assay

OVA specific IgG antibody response in the sera of control and test groups were measured by ELISA. Briefly, the 96 well microtitre plates (Nunc, Denmark) were coated with 100 µl of OVA at a concentration of 100 µg/ml in carbonate buffer (pH 9.6) and incubated overnight at 4°C. After incubation, the wells were washed thrice with PBS containing 0.05% tween-20 (PBS-T). The nonspecific binding sites in the wells were blocked with 300 µg/well of 3% skimmed milk powder in PBS for 10 - 12 h at room temperature. After washing the plates, the wells were further incubated with 100 µl, of dilute sera (1:400 in PBS) in triplicates for 1 h at 37°C. The unbound serum constituents were washed off and the levels of total IgG were measured by incubating with 100 µl of goat-antimouse IgG conjugated to horseradish peroxidase (HRPO) at a dilution of 1:1000 for 1 hour at 37°C. Finally, the unbound conjugates were washed with PBS-T and 100 µl of 1.5 M citrate phosphate buffer pH 5.0 containing 4 mg of Orthophenylene diamine (OPD) and 100 µl H_2O_2 were added. The reaction was stopped after 5 min. by adding 50 µl of 8 N H_2SO_4. Colour developed was read at 490 nm using automatic ELISA reader (Bio Rad Model-550). The data expressed was the mean of Optical Density (OD) of the triplicates. Native mice sera or pre-immune sera were taken as the negative control. The OD of the negative control was deducted from the OD given by the sera of mice immunized with OVA or OVA plus PZE.

T cell proliferation assay

For testing the effect of PZE fractions on OVA induced T cell proliferation, mice were subcutaneously injected on day 0, into the hind footpad with OVA in presence of Freund' complete adjuvant and booster dose was given on day 8 with Freund' incomplete adjuvant. On day 12 mice were sacrificed and their spleens were collected. Test groups received PZE fractions in olive oil alone. The T cells were isolated according to the procedure of (Francis et al., 1990). Briefly, the spleens were teased to get single cell suspension by incubating with 1 ml of 0.9% NH_4Cl for 1 min at 37°C, followed by arresting the activity of NH_4Cl by adding RPMI twice the volume of the incubation mixture. The cells were washed and suspended in RPMI and incubated in a Petri dish coated with goat anti-mouse immunoglobulins at 1:1000 dilutions in PBS to remove the B cell population. The unbound cells were carefully separated and resuspended in fetal calf serum. The viability of cells was checked with tryphan blue exclusion (Kalten Bach et al., 1958). These purified cells were employed in Ovalbumin induced T cell proliferation assay.

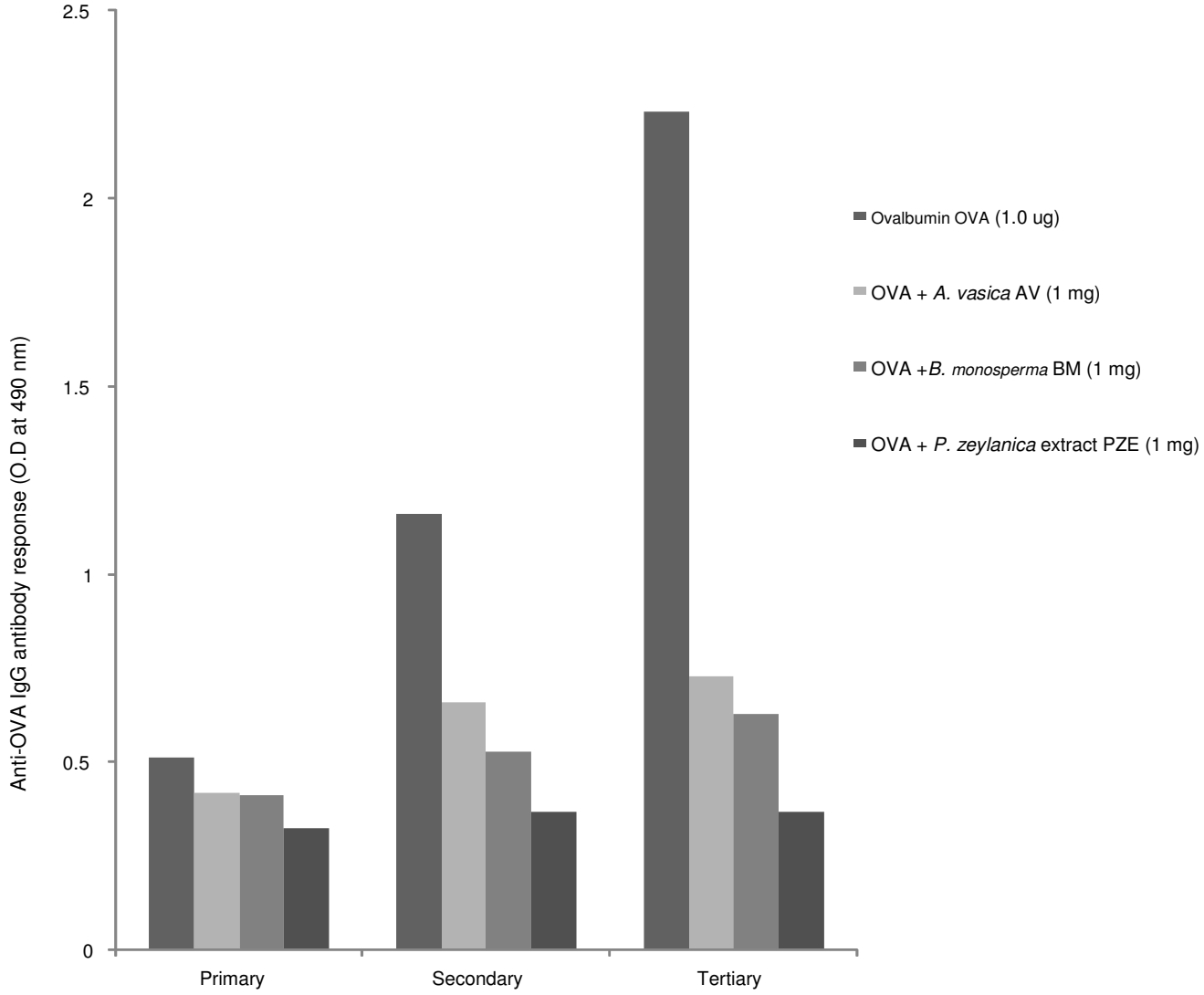

Figure 1. Screening of different plant extracts for OVA (ovalbumin) specific antibody response. Balb/c mice were injected with 2 mg of each of aqueous extracts of *A. vasica* (AV), *B. monosperma* (BM) and *P. zeylanica* (PZE), on days 0, 28 and 56. Control groups received either OVA or PBS alone. All experimental groups were bled from the tail vein 14 days after primary and 7 days after secondary and tertiary immunizations. Anti-OVA (Anti-ovalbumin) IgG antibody responses in the sera were measured by ELISA with a dilution factor of the sera of 1:400. The results presented are the mean of the triplicates of each serum dilution.

T cell proliferation and cell cultures were carried out in sterile microtitre plates (Nunc, Denmark) with 96 flat bottom wells. All the reagents used were filter sterilized using 0.22 nunc millipore filters prior to adding into the wells. For each individual assay, the cultures were done in triplicate, with each well containing 2 into 105 cells in 250 µl RPMI medium. Cells were stimulated with OVA in absence or presence of different doses of PZE. Cultures were incubated for 72 h at 37°C in a humified 5% CO_2 modulator chamber. After 18 h, the cultures were incubated with tritiated thymidine (0.5 µci/ well). After incubation period, cells were harvested on glass fibre filters, using Nunc cell harvester and the thymidine incorporation was determined by liquid scintillation counter by adding 5 ml of scintillation fluid and 500 mg of 1,4 –bis{5-phenyl-2-oxazole}-benzene: 2,2'- P- phenylene-bis {5-phenyl- oxazole} per litre. The results were expressed as counts per minutes (cpm) and plotted against culture blanks, concentrations of ovalbumin and ovalbumin plus PZE-6.

RESULTS

In order to identify the potent plant sources for the detection of immunosuppressive compounds, the individual plant sources were used to get aqueous extracts lyophilized and dissolved in PBS and administered into Balb/c mice by an IP route in presence or absence of ovalbumin (OVA). The list of the plants and their parts used in the herbal drug are given in the Table 1. Out of these, three plants namely *P. zeylanica, Adathoda vasica* and *Butea monosperma* exhibited significant suppression of OVA-specific IgG antibody responses. The results in the Figure 1 revealed that among these three plant sources, *P. zeylanica* extract (PZE) showed potent suppression of primary, secondary and tertiary OVA-specific IgG

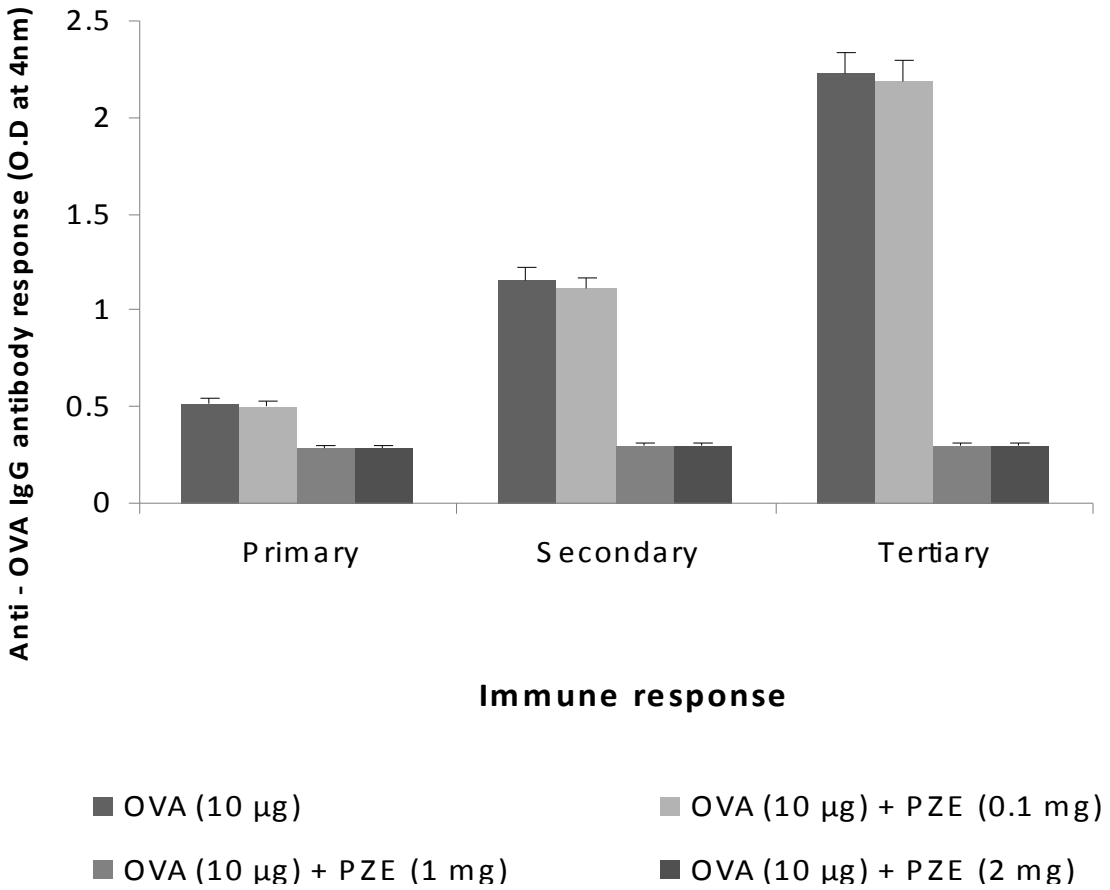

Figure 2. The dose effect of *P. zeylanica* extract (PZE) was checked by an IP injections of Ovalbumin OVA (10 µg) mixed with different concentrations of 0.1, 1.0 and 2.0 mg of PZE in different groups of BALB/c mice on days 0, 28 and 56. Control groups received either OVA or PBS alone. All experimental groups were bled from the tail vein 14 days after primary and 7 days after secondary and tertiary immunizations. Anti-OVA IgG antibody responses in the sera were measured by ELISA with dilution factor of 1: 400. The p value of groups received 1.0 and 2.0 mg of PZE is < 0.05.

antibody response in mice. Mice were immunized with different concentrations 0.1, 1.0 and 2.0 mg of PZE (lyophilized aqueous extract) along with OVA on days 0, 28 and 56 and were bled 14 days after primary and 7 days after secondary and tertiary immunizations. The serum was tested for the anti-OVA IgG antibody responses as determined by ELISA. The results in the Figure 2 demonstrate that a significant dose dependent suppression was observed with 1.0 and 2.0 mg of PZE. In order to study the effect of PZE in different haplotypes (that is Balb/c (H-2^d), C57Bl/6 (H-2^b) and SWR/J (H-2^q)), mice were immunized with 10 µg of OVA in presence or absence of 1.0 mg of PZE on days 0, 28 and 56. The results in the Figure 3 demonstrate that PZE is potent in exerting the suppressive effect on the down regulation of anti-OVA antibody responses in all the three haplotypes of the mice studied, which indicates that the PZE exerted immunosuppression irrespective of genetic variation. The effect of shallow gradient PZE fractions eluted from silica gel column using 5 - 30% ethyl acetate in benzene was studied on *in vitro* OVA induced T cell proliferation. The results in the Figure 4 indicated that PZE-6 (30%) was found to inhibit the OVA induced T cell proliferation.

DISCUSSION

India with its wealth and variety of medicinal plants and the knowledge about their medicinal uses that has been accumulated over generations offers a great number of popular remedies for the treatment of diseases including Rheumatoid Arthritis (RA), cancer and other autoimmune diseases etc. Many of these are in common use even today. About 2500 plants of therapeutic value are mentioned in the Ayurvedic and Unani systems of medicine. Out of these, quite a large number of plants have been used as immunosuppressive compounds. The present study, establishes the immunosuppressive activity of the *P. zeylanica*. In murine model the aqueous

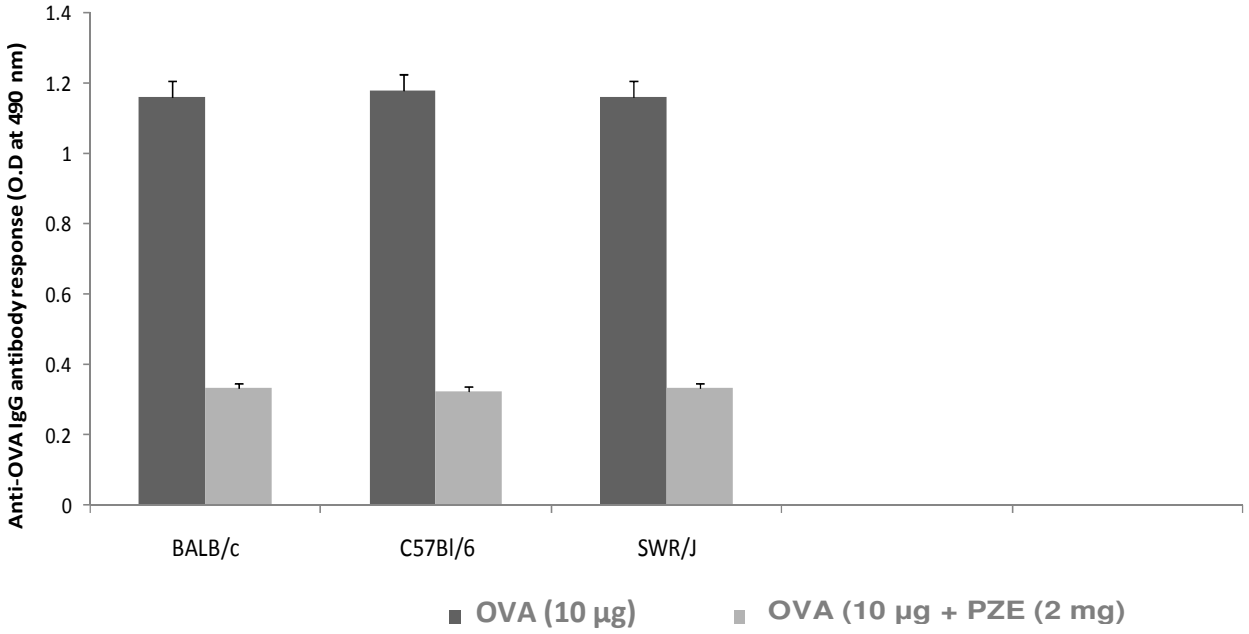

Figure 3. Effect of *P. zeylanica* (PZE) on the induction of anti-ovalbumin (anti-OVA) IgG antibody response in different haplot types of miceMice {BALB/c (H-2^d), C57Bl/6 (H-2^b), SWR/j (H-2^q)} with different genetic backgrounds were IP with 10 µg of OVA in absence or presence of 2 mg PZE on days 0, 28 and 56. Mice were bled on day 14 after primary and 7 days after secondary and tertiary immunizations and anti-OVA IgG antibody levels were determined by ELISA.

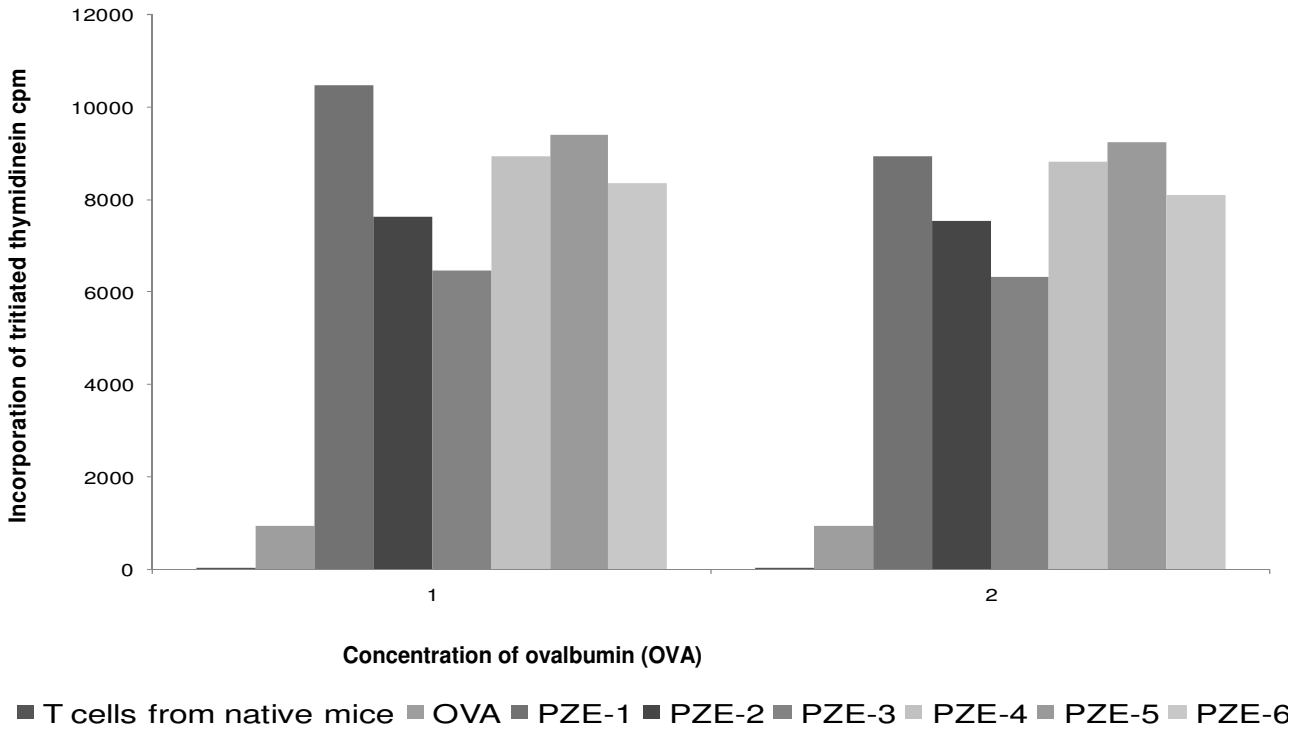

Figure 4. Effect of *P. zeylanica* extract (PZE) fraction on of ovalbumin (OVA) specific T cell proliferation in BALB/c mice. Balb/c mice were immunized with 10 µg of OVA in one of the footpads and sacrificed on day 12 to isolate splenic T lymphocytes. OVA primed T cells at a density of 2 into105 cell/well and incubate with either 1 or 2 µg of OVA in presence or absence of (1.0 µg/well) PZE fractions (PZE-1, 2, 3, 4, 5 and 6) for 72 h. Cultures were pulsed with tritiated thymidine on day 4 and the incorporation of radioactivity in the harvested cells was measured by liquid scintillation counter per minute (cpm). Cells from the normal mice served as culture blanks. The p value of groups received PZE-6 (10 µg) is < 0.05.

extract of *P. zeylanica* was found to be down regulating the ovalbumin specific humoral and cell mediated immune responses. Previous work on medicinal plants reported that the alcoholic extract of leaves from *Nyctanthes arboristis* exhibits anti-inflammatory activity (Saxena, 1984). The crude extracts prepared from the roots, leaves and fruits of *Withania somnifera* exhibited immunomodulatory activity (srinivasulu amara et al., 1999). The immunomodulatory effect of the methanolic extract obtained from dried leaves of Bidens pilosa L. (Pereira et al., 1999). A number of immunosuppressive compounds are known to inhibit the T cell proliferation induced by antigens. However they differ in their mechanism of action. For example, Cs A and FK506 inhibit the T cell proliferation induced by antigen. Seselin purified from *P. zeylanica* on phytohemagglutinin (PHA)-stimulated cell proliferation was studied in human peripheral blood mononuclear cells (Tsai et al., 2008). Our results indicated that the ethyl acetate fraction of PZE (PZE-6) not only inhibited Ovalbumin induced IgG antibody response but also selectively inhibited the antigen (OVA) induced T cell proliferation. It was also revealed the presence of active immunosuppressive compounds in the PZE-6 fraction of *P. zeylanica.*

Conclusion

P. zeylanica extract suppressed T cell responses in Balb/c mice, will be further studied to propose the possible immunosuppressive mechanism. PZE would be effectively used in different immunopathological conditions as a good anti-inflammatory and anti-arthritic agent. It may also have therapeutic applications in transplantation biology where it can be used as immunosuppressive source.

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