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ISOLATION OF CONTINENTALIC ACID FROM *ARALIA CACHEMIRICA* AND ITS IMMUNO-BIOLOGICAL EVALUATION

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ABSTRACT

In continuing search for compounds with immunomodulatory activity, plant *Aralia cachemirica* has been found to contain continentalic acid (CA). Isolation of this pharmacologically significant diterpinic acid from *Aralia cachemirica* is novel. Chemical structure of this compound was identified by spectral analysis as (-)-pimara-8(14), 15-dien-19-oic acid, which has been isolated from *A. cordata* and *A. continentalis*. This compound has been evaluated for its effect on immunomodulaion. CA was found to inhibit production of pro-inflammatory cytokines (IL-6, TNF-α) & nitric oxide (NO) in LPS induced macrophage activation, suppressed antibody titres & delayed type hypersensitivity reaction (DTH) when orally administered and increased the phagocytic activity by clearance of *Candida albican* infection from peritoneal cavity in mouse model system.

INTRODUCTION: Immunomodulaion refers to any change in immune response of body involving induction, expression, amplification, or suppression. In some cases we require stimulation of immune response, whereas suppression of immune response is needed in other conditions. Immunosuppressors are required for the treatment of autoimmune diseases and allergies, and in case of organ/tissue transplantation to prevent graft rejection.

Conversely, immunostimulators are known to enhance body's resistance against a number of infections, allergies and cancers serving as immune potentiators in healthy persons and immunotherapeutic agents in case of persons with impaired immunity. Many synthetic and natural compounds are known to possess immunomodulatory activity, but limited efficacy and adverse side effects associated with traditional immunomodulatory drugs drive need to develop new, improved, and target specific immunomodulating agents. According to phylogenic and biogeographic analysis, approximately sixty eight accepted species of genus *Aralia* (Family Araliaceae) are distributed in Asia and North America [1-3]. Several species of genus *Aralia* have pharmacological importance [4-8] and have been used in traditional medicine to treat rheumatic arthritis, nephritis, lumbago, lameness [9-11], gastritis [12] inflammation [13] and diabetes mellitus [14].

Aralia cachemirica is a statuesque clump-forming herbaceous perennial, which is capable of forming upright stems 1 to 3m tall with large bright green tri-pinnate leaves, white flowers and purple-black fruit. In India it is mostly found in North Western Himalayas especially Kashmir, Uttarakhand and Himachal Pradesh [15]. As per detailed literature survey, this plant species has been explored for investigation of essential oil composition [16, 17], isolation of aralosides & acids [18] and hypoglycemic activity [19]. On biological studies of

Continentalic acid, this compound has shown significant analgesic [20], growth inhibition & apoptosis induction [21], antibacterial [22], anti-inflammatory [23], cyclooxygenase inhibitory [24] and cytotoxic & COX-2 inhibitory activities [25].

Herein, we report isolation of continentalic acid (CA) from *Aralia cachemirica* and evaluation of its effect on immunomodulaion by observing its effect on antibody titres, delayed type hypersensitivity reaction (DTH), splenocyte proliferation and nitric oxide & pro-inflammatory cytokines (IL-6 and TNF-α) production from macrophages.

MATERIALS AND METHOD:

Experimental: Melting points were measured with Buchi capillary apparatus. Optical rotation was recorded using Perkin-Elmer 241 polarimeter. TLC was performed on 0.25 mm silica gel 60 F254 plates. Silica gel 60-120 mesh was used for column chromatography. ^{1}H NMR was recorded on Brucker DPX 200 spectrometer. ^{1}H - ^{1}H COSY and ^{13}C NMR were recorded on a Brucker ultrashield- 400 spectrometer.

Chemical shifts are given in δ units, relative to the TMS signal as an internal reference. IR spectra were recorded on a Bruker Vector 22 spectrometer as KBr pellets, with absorption given in cm^{-1}. Mass spectra were recorded on ESI-esquire 3000 Bruker Daltonics instrument and Varian GC-MS/MS 4000 instrument using electron impact method, injector temperature 230^{o}C, column oven 100^{o}C, hold for 5 minutes to 250^{o}C at the rate of 10 deg/min, hold for 10 minutes, helium carrier gas with flow rate 1 ml/min and Varian CP-SIL 8 CB MS column (30m ×0.32mm, 1µm film thickness), temperature 250^{o}C.

Analytical HPLC analysis was conducted using Agilent 1100 series. LC-ESI-MS was performed using Bruker Daltonics esquire 3000 ion trap mass spectrometer with an electrospray interface connected to Agilent 1100 series LC. LC conditions employed in both cases were as follows: C_8 column reversed-phase (E-Merck, 4.0mm×250mm, 5µm particle size) maintained at 30^{o}C, quaternary pump, photodiode array detector, water-acetonitrile (15:85, v/v) isocratic mobile phase at a flow rate of 0.8 ml min^{-1} using automatic sample injection module.

Plant material: The aerial parts of plant *Aralia Cachemirica* were collected from khillanmarg area of Kashmir (J&K), India after proper identification and authentication of plant by department of Bioscience and Biotechnology, Banasthali Vidyapeeth, Rajasthan, India, in the month of May 2008 (no. BV 168).

Extraction and Isolation: Crushed and air-dried leaves, stems and twigs (680gms) of *Aralia cachemirica* were extracted with methanol through percolator for 72 hours. The suspension was filtered and the filtrate was concentrated under reduced pressure to yield 87.95 gm of dry methanol extract. The extract was chromatographed on silica gel column eluting with n-hexane- ethyl acetate gradient. Fractions of similar composition as determined by TLC analysis were pooled. Further purification of these fractions on silica gel column with the same elution solvent and crystallization in hexane yielded pure colorless needles of (-)-pimara-8(14), 15-dien-19-oic acid (360 mg).

Reagents: Medium RPMI 1640 (Himedia, Bombay, India), 96 V-wells microtitration plates and micro tissue culture plates (96 U-wells) from Tarsons, trypan blue (Micro Labs, Bombay), fetal calf serum (FCS), lipopolysaccharides (LPS, Escherichia coli 055 B5), dimethylsulphoxide (DMSO), Hank's balanced salt solution (HBSS), HEPES, 2-mercaptoethanol, penicillin, streptomycin, and Betamethasone (BMS) were purchased from Sigma Chemicals, USA. L-Glutamine, sulfanilamide and naphthylenediamine hydrochloride were likewise purchased from Sigma. Assay kits for all cytokines IL-6 and TNF-α were purchased from BD, USA.

Preparation of Test Material: For *in vivo* studies in Balb/c mice, CA was suspended in 1% (w/v) gum acacia, while for *in vitro* studies a stock solution of test materials in 10% dimethylsulphoxide was prepared. In the cell culture supernatant <0.1%did not interfere with the test system.

Experimental Animals: The study was conducted on male Balb/c mice (18-22 g). The ethical committee of the Indian Institute of Integrative Medicine, Jammu (CSIR, India) instituted for animal handling approved all the protocols. The animals were bred and maintained under standard laboratory conditions: temperature

(25±2°C) and a photoperiod of 12 h. Commercial pellet diet and water were given *ad libitum*.

Immunization Schedule: SRBC collected in Alsever's solution, were washed three times in large volumes of pyrogen-free 0.9% normal saline and adjusted to a concentration of 5×10^9 cells/mL for immunization and challenge. The animals were divided into five groups of six animals each. Group I (control) received 1% gum acacia; Group II received Betamethasone- BMS, a standard immunosuppressor (0.05 mg/kg b.wt); Group III received CA (25 mg/kg b.wt); (Group IV) received CA (50 mg/kg b.wt) and Group V received CA (100 mg/kg b.wt). CA was dissolved in 1% gum acacia and was administered orally for 14 days. The dose volume was 0.2 mL.

Anti-SRBC Antibody Titre: Mice were immunized by injecting 0.2 ml of 10% of fresh SRBC suspension intra peritoneally on day 0. Blood samples were collected in micro centrifuge tubes from individual animals by retro-orbital plexus on day 7 for primary antibody titre and day 14 for secondary antibody titre. Serum was separated and antibody levels were determined by the haemagglutination technique [26]. Briefly, equal volumes of individual serum samples of each group were pooled. Two fold dilutions of pooled serum samples were made in 25µL volumes of normal saline in a micro titration plate to which were added 25µL of 1% suspension of SRBC in saline. After mixing, the plates were incubated at room temperature for 1 h and examined for haemagglutination under the microscope. The reciprocal of the highest dilution of the test serum giving agglutination was taken as the antibody titre.

Delayed Type Hypersensitivity (DTH): CA was administered 2 h after SRBC injection and once daily on consecutive days; six days later, the thickness of the left hind footpad was measured with a spheromicrometer (pitch, 0.01 mm) and was considered as the 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 and 48 h [27].

Spleenocyte Proliferation Assay: Spleen collected under aseptic conditions in HBSS, was minced using a pair of scissors and passed through a fine steel mesh to obtain a homogeneous cell suspension and the erythrocytes were lysed with ammonium chloride (0.8%, w/v). After centrifugation (380 × g at 4°C for 10 min), the pelleted cells were washed three times with PBS and resuspended in complete medium [RPMI 1640 supplemented with 12 mM HEPES (pH 7.1), 0.05 mM 2-mercaptoethanol, 100 IU/mL penicillin, 100 µg/mL streptomycin and 10% FCS].

The cell number was counted with a haemocytometer by the trypan blue dye exclusion technique [28]. Cell viability exceeded 95%**.** To evaluate the effect of CA on the proliferation of splenic lymphocytes, the spleen cell suspension (1×10^7 cell/mL) was pipetted into 96-well plates (200 µL/well) and cultured at 37 °C for 72 h in a humid saturated atmosphere containing 5% CO2 in the presence of Con-A (5µg/mL) and LPS (10µg/mL). After 72 h, 20 µL of MTT solution (5mg/ mL) was added to each well and incubated for 4 h. The plates were centrifuged (1400 × g, 5 min) and the untransformed MTT was removed carefully by pipetting. To each well, 100µL of a DMSO working solution (192µL DMSO with 8µL 1 M HCl) was added and the absorbance was evaluated in an ELISA reader at 570 nm after 15 min.

Collection of Peritoneal Macrophages, Nitrite and Cytokines Assay: A volume of 10 ml of RPMI 1640 was injected into the peritoneal cavity of challenged mice on day 28. After 5 min, the medium was taken out and centrifuged at 1800 ×g for 10 min. at 4^0C. The cell pellet was re suspended in RPMI 1640 medium. Macrophages (3×10^6) were seeded in 24-well culture plate in a CO_2 incubator for 3 hrs.

At the end of incubation period, non-adherent cells were removed and plates were further incubated for 48 h in the presence of LPS (1µg/ml). Supernatants were collected after centrifugation and kept at -80^0C for the measurement of cytokines. For the NO_2^- assay (nitrite content), 100µl of culture media was incubated with 150µl of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine in 2.5% phosphoric acid solution) at room temperature for 10 min in 96-well micro plate [29]**.**

Absorbance at 540nm was read using an ELISA plate reader. Standard calibration curves were prepared using sodium nitrite as standard.

Cytokine Production from Macrophages: Measurement of cytokines (IL-6 and TNF-α) was carried out using commercial kits (BD OptEIA set) as per the manufacturer's instructions.

Statistical Analysis: Data are expressed as mean ± S.E. and statistical analysis was carried out using one-way ANOVA followed by Bonferroni correction multiple comparison test.

RESULTS AND DISCUSSION:

Chemistry: The air-dried and crushed leaves, stems and twigs were extracted with methanol and column chromatography of methanol extract yielded pure Continentalic acid, m.p. 159-160^{o}C**,** $[\alpha]_D$ **-**119.6 (c 0.2, $CHCl_3$). It was identified as (-)-pimara-8(14), 15-dien-19-oic acid (continentalic acid, **Figure 1**) by comparison of its spectral data with literature data [22-25, 30]. ESI of the molecule under negative ion electrospray mode produced peak at *m/z* 300.8 $[M-H]^-$ and GC mass spectrum showed molecular ion peak at m/z 302.1 giving possible molecular formula $C_{20}H_{30}O_2$.

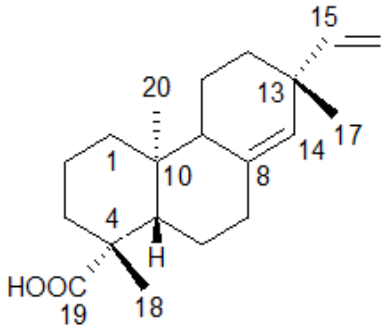

FIG. 1: CHEMICAL STRUCTURE OF CONTINENTALIC ACID (CA)

IR spectra exhibited bands at 1692.6 cm^{-1} (COOH), 998.9, 918.9 cm^{-1} (monosubstituted double bond), 864.4, 848.2 cm^{-1} (trisubstituted double bond). ^{1}H NMR (200 MHZ**,** $CDCl_3$) gave characteristic proton signals at δ: 0.65, 1.00 and 1.26 (3 × 3H, s, three tertiary methyl groups), 4.86-4.96 (2H, m, two vinyl protons) two doublet of doublet peaks overlapped in this case and could not be distinguished even after expansion, 5.14 (1H, s, one olefinic proton of trisubstituted double bond), 5.71 (1H, dd, *J* = 10.8, 16.8 Hz). Additional confirmation of structure of continentalic acid was obtained from ^{1}H - ^{1}H COSY and finally from GC-MS/MS fragmentation pattern (**Figure 2**), which was exactly similar to that of Continentalic acid isolated from *Aralia continentalis* [23].

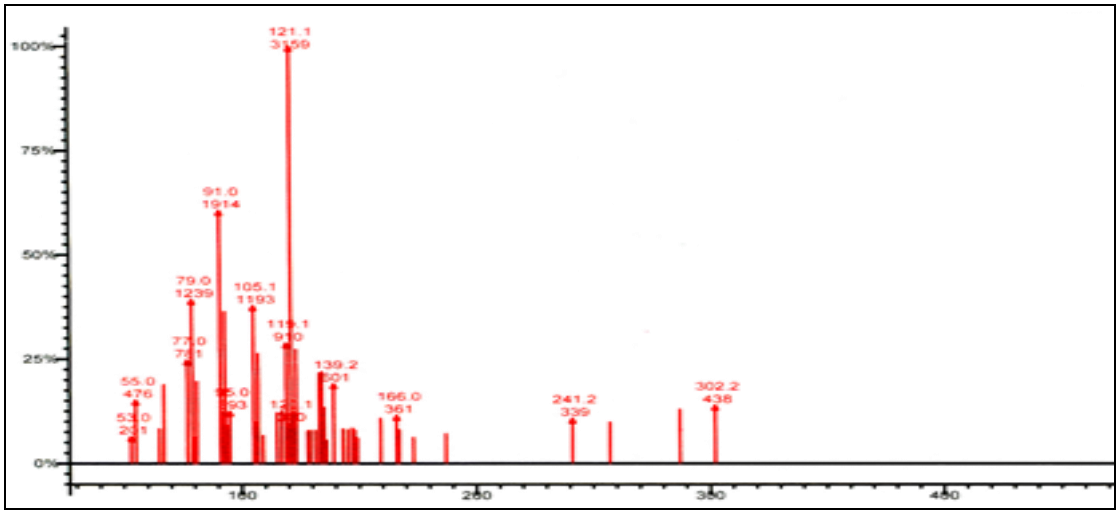

FIG. 2: GC MASS SPECTRUM OF CA

Continentalic acid showed a single spot on TLC at Rf 0.65 using elution solvent n-Hexane-EtOAC (9:1). GC chromatogram of continentalic acid gave a single peak (Rt 25.48). HPLC analysis of continentalic acid gave a single peak (Rt 6.79) and further analysis by LC-ESI-MS under negative ion electrospray mode assigned m/z 300.8 $[M-H]^-$ to this peak without any fragment ions produced. ^{13}C NMR (400MHz, CDCl3) δ: 39.8 (C-1),19.6 (C-2), 38.5 (C-3), 44.0 (C-4), 56.1 (C-5), 24.6 (C-6), 35.8 (C-7), 137.9 (C-8), 50.5 (C-9), 38.2 (C-10), 19.6 (C-11), 36.4 (C-12), 39.2 (C-13), 128.0 (C-14), 147.1 (C-15), 113.4 (C-16), 29.7 (C-17), 29.2 (C-18), 183.7(C-19), 14.1 (C-20).

Biological Activity: In order to evaluate the immunomodulatory activity, we carried out a number of assays in different immune response cells. We investigated the possible immunomodulatory effect of CA using different immunological parameters. Betamethasone (BMS), a known immunosuppressive standard drug was used as positive control (0.05 mg/kg, p.o.). The preliminary experiments showed that the CA extracted from plant *Aralia cachemirica* was found to induce significant immuno-suppressive T-cell response. Further test models were used for determining the possible immunosuppressive activity against LPS induced macrophage activation and pro-inflammatory cytokine production.

Measurement of Anti-SRBC Antibody Titre: Anti-SRBC antibody (IgM and IgG) titres were measured in mice serum of different groups, collected retro-orbitally on 7 and 14 days after immunization and treatment. Results given in **Table 1** indicate that CA (25–100 mg/kg, p.o.) produced a dose-related inhibition in the primary and secondary antibody synthesis. The maximum inhibition was observed at 100 mg/kg in the primary and secondary antibody titre. Administration of BMS (0.05 mg/kg, p.o.) used as a positive control resulted in a significant decrease in the humoral antibody titre compared with the control animals (Table 1).

Measurement of DTH reaction: DTH reaction to SRBC is given in Table 1 in which data are expressed in terms of the swelling of the footpad. After administration of the CA (25-100 mg/kg, p.o.), a significant dose related decrease in footpad thickness was found at 24 and 48 h as compared with the control group.

TABLE 1: EFFECT OF CA ON ANTIBODY (IGG AND IGM) TITRE AND DELAYED TYPE HYPERSENSITIVITY

Treatment	Dose (mg/kg)	Antibody Response (Mean ± S.E.Log-2 titres)				DTH response {Paw oedema (mm)} (Mean ± S.E.)	
		Day 7 Primary response		Day 14 Secondary response		24 hr	48 hr
		IgG	IgM	IgG	IgM		
Control	-	7.2 ± 0.41	6.2 ± 0.41	8.2± 0.24	7.4± 0.44	2.47 ± 0.02	2.51±0.02
BMS	0.05	5.2±0.22**	4.2±0.32**	5.2±0.22**	4.5± 0.46*	0.75±0.03**	0.49±0.07**
CA	25	7.2± 0.24	8.2± 0.44 *	7.2± 0.24*	8.8± 0.44*	1.75 ± 0.01*	1.51±0.03**
CA	50	6.2 ± 0.21	5.6 ± 0.35*	6.2± 0.21*	6.8± 0.33*	1.13±0.022*	0.88±0.03**
CA	100	4.8±0.31**	4.0±0.33**	4.8±0.31**	5.4±0.45**	0.88±0.023**	0.68±0.04**

P*<0.05, P**<0.01

Effect of CA on spleenocyte proliferation: To confirm the effect of the CA on the cellular immune response, the proliferation of spleenocytes in response to Con A (5 µg/mL) and LPS (10 µg/mL) was evaluated. The results indicated that the proliferation in CA treated groups at 25, 50 and 100 mg/kg was suppressed in a dose-dependent manner compared with the control group, maximum inhibition observed at 100 mg/kg (**Figure 3**).

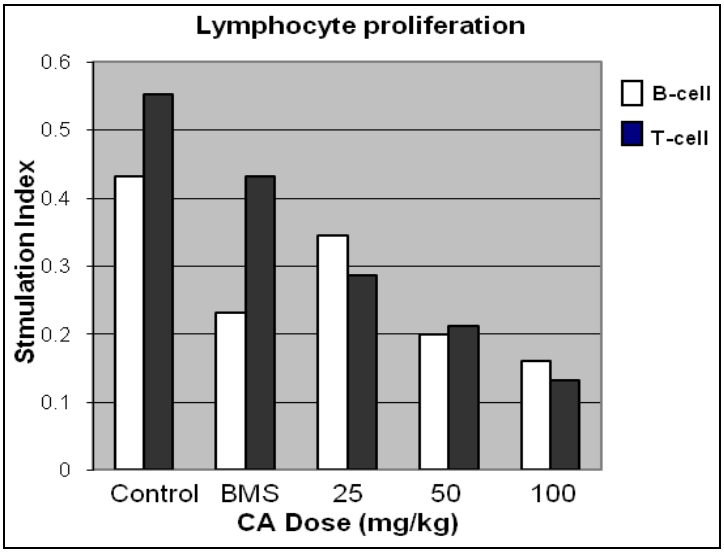

FIG. 3: EFFECT OF CA ON SPLENOCYTE CELL PROLIFERATION *EX VIVO*

Values shown as stimulation index (SI) as measured by MTT assay are means ± S.E. (n=10); one-way ANOVA followed by Bonferroni multiple comparison test

Effect of CA on nitrite level: The effect of CA (25, 50 and 100 mg/kg) on nitric oxide production is shown in **Figure 4**. The nitrite concentration was determined by extrapolation from a sodium nitrite standard curve and the results are expressed in µM. The maximum inhibition in nitrite production was observed at 100 mg/kg compared with the control group. Increasing doses of CA registered a significant decrease in the production of nitrite content.

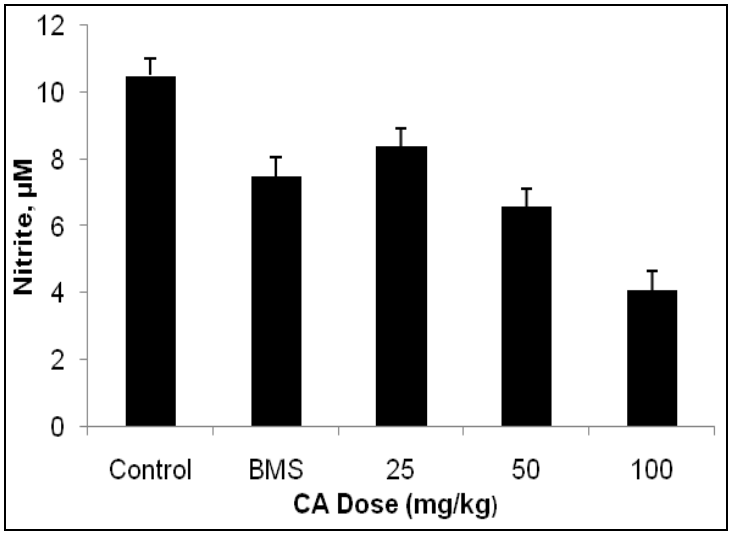

FIG. 4 INFLUENCE OF CA ON THE FORMATION OF NITRITE CONTENTS IN MOUSE PERITONEAL MACROPHAGE *EX VIVO*
Values are Mean ± S.E. (n=10); one-way ANOVA followed by Bonferroni multiple comparison test.

Effect of CA on IL-6 and TNF-α production from macrophages: Effect of CA on IL-6 and TNF-α production was observed in peritoneal macrophages. Results shown indicate that CA inhibited IL-6 and TNF-α release in a dose-related manner. At a dose of 100 mg/kg, inhibition in IL-6 (**Figure 5**) and TNF-α (**Figure 6**) production was found to be the most significant.

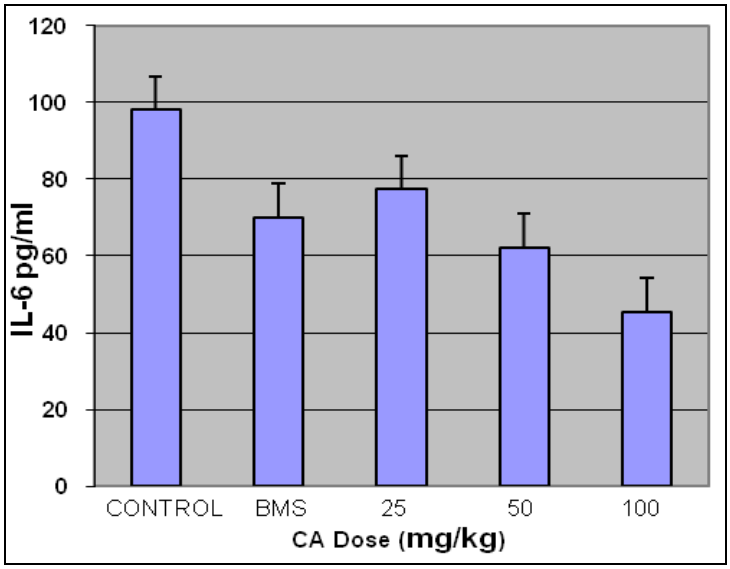

FIG. 5 INFLUENCE OF CA ON THE IL-6 EXPRESSION ON MOUSE PERITONEAL MACROPHAGES *EX- VIVO.*
Values are Mean ± S.E. (n=6); one-way ANOVA followed by Bonferroni multiple comparison test.

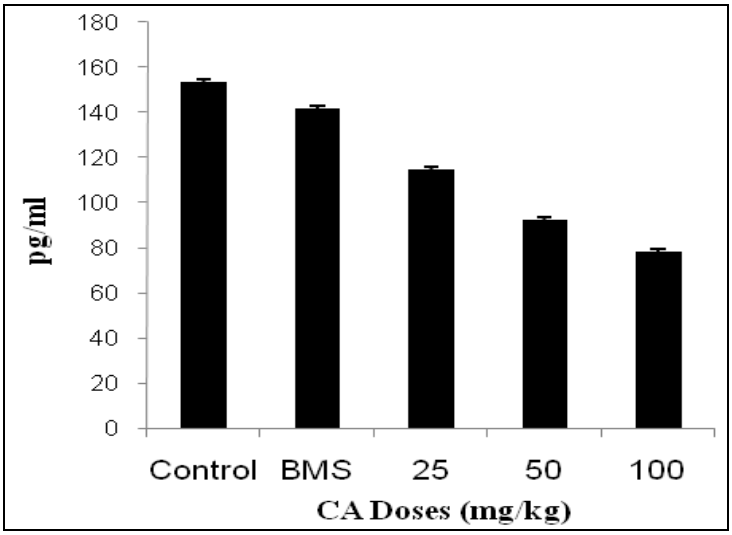

FIG. 6: INFLUENCE OF CA ON TNF-α.
Values are means ± S.E. triplicate wells, one-way ANOVA followed by Bonferroni multiple comparison test.

CONCLUSION: The aim of our study was to evaluate immunomodulatory effect of continentalic acid (CA) isolated from plant *Aralia cachemirica*. The results mentioned above have demonstrated that CA possesses a potent immunosuppressive action. The inhibition of the humoral response against SRBCs in Balb/c mice by CA was evidenced by the decrease in HA titre. A DTH reaction is an expression of cell-mediated immunity and plays a role in many inflammatory disorders.

Treatment with CA inhibited the DTH reaction, as reflected by the decreased footpad thickness compared to the control group. In our present investigation, we found that CA could inhibit Con-A and LPS induced spleenocyte proliferation, indicating its role in inhibiting T-cell activation. In further studies, CA was found to interfere with macrophage activation induced with LPS. This finding indicates that CA inhibits LPS induced macrophage activation by inhibiting the pro-inflammatory cytokines release.

In view of the pivotal role played by macrophages in coordinating the processing and presentation of antigen to B-cells, CA was evaluated for its effect on NO production from macrophages. In this study, we found that CA could significantly inhibit the NO and pro-inflammatory cytokines (IL-6 and TNF-α) production from macrophages. These findings open new insights into immunosuppressive potential of continentalic acid for future use in immunotherapy.

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