



Immunomodulatory efficacy of methanolic extract of *Ajuga macrosperma* in cyclophosphamide induced immunocompromised rat

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ABSTRACT

The present investigation was undertaken to evaluate immunomodulatory potential of *Ajuga macrosperma* against cyclophosphamide (CP) induced immunosuppression in rats. The immunological parameters such as haemagglutination (HA) titre and serum immunoglobulin (Ig) for humoral immunity (HI); lymphocyte stimulation test (LST), phagocytic index test, neutrophil adhesion test (NAT) and delayed type hypersensitivity reaction (DTH) for cellular immunity (CMI) were determined. The cyclophosphamide treatment significantly suppressed both HI and CMI responses, which were significantly improved following treatment with 100 ppm and 200 ppm of methanolic extract of *Ajuga macrosperma* (MEAM) in dose dependent manner. The decreases in HI and CMI related parameters by CP treatment revealed immunosuppression, whereas simultaneous treatment with MEAM ameliorated immune response which indicated immunomodulatory properties of *Ajuga macrosperma*.

Keywords: *Ajuga macrosperma*, Cyclophosphamide, Immunomodulation, Rat

Immunity has great concerns with the health status of the living organism. *Ajuga macrosperma*, a perennial herbal plant, belonging to *Ajuga* genus have various chemical constituents which attribute to its pharmacological actions (Israili and Lyoussi 2009). *A. macrosperma* has been reported to possess antioxidant (Hsieh *et al.* 2016) and immunomodulatory properties (Nisar *et al.* 2014).

Cyclophosphamide causes immunosuppression by elimination of T regulatory cells in simple and tumor-bearing hosts, induction of T cell growth factors such as type I IFNs (Sistigu *et al.* 2011). Levamisole, used as an immunostimulant drug, has both anthelmintic and immunostimulatory potential. In view of lack of reports of investigation on immunomodulatory potential, this study was undertaken to explore the immunomodulatory properties of *Ajuga macrosperma* in rats.

MATERIALS AND METHODS

The present investigation was designed to evaluate the immunomodulatory potential of methanolic extract of *Ajuga macrosperma* (MEAM) following administration of MEAM-1 (100 mg/kg b. wt.) and MEAM-2 (200 mg/kg b. wt.) for 28 days in levamisole (50 mg/kg b. wt. daily) and cyclophosphamide (100 mg/kg b. wt. on 9th and 16th day of the study) treated rats.

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Plant material: The aerial part of *Ajuga macrosperma* collected from the Medicinal Research Development Center, G. B. Pant University of Agriculture and Technology, Pantnagar. The aerial part of the plants, shade dried for 15–20 days. Later the aerial part of *Ajuga macrosperma* were placed in fan equipped incubator at 37°C for 2–4 h and grinded in electric grinder to fine homogenous powder to get powders of *Ajuga macrosperma*. The powder was stored in sealed plastic container in dry place at room temperature till further use. These powders were further subjected to preparation of methanolic extracts for this investigation. The aerial part of *Ajuga macrosperma* (L.), 100 g for powder was taken in a round bottom flask (4 liter capacity) separately and 1000 ml of methanol were added separately and plugged properly and shaken vigorously for few minutes. The flasks were left for 48 to 72 h with intermittent shaking at room temperature. Thereafter, the soaked material in flask was filtered through several layered muslin cloth and centrifuged to separate the supernatant. The supernatant/liquor extract was again filtered with Whatman Filter No. 01 and poured into a thoroughly washed and dried glass tray and left for 7–10 days to dry at 37°C in incubator equipped with fan.

Chemicals: All the chemicals used in this experiment were purchased from Himedia, Mumbai, India and Sigma-Aldrich, St. Louis, USA.

Experimental design: Forty eight rats of either sex 60 to 75 days old, weighing around 175g, were obtained from Experimental Animal House of the College of Veterinary and Animal Sciences, Pantnagar. Rat feed and water were

provided *ad lib.* during the experiment as per standard norms.

The dose of methanolic extract of *Ajuga macrosperma* (MEAM) was selected on the basis of LD₅₀ and that of levamisole and CP was selected on the basis of available literature. After 15 days acclimatization, eight groups of 6 animals each were made and experiment was designed for conducting 28 days sub acute study as mentioned in the Table 1.

This plan of work was undertaken after approval by the Institutional Ethical Committee (IAEC) vide approval number IAEC/VPT/CVASC/ 222 dated 07.01.2016.

Assessment of humoral and cell mediated immune response: The humoral immunity in rat was estimated by HA titers and immunoglobulin (Ig). The cell-mediated immune response of the rat was estimated by measuring delayed type hypersensitivity reaction (DTH), lymphocyte stimulation test (LST), phagocytic index test and neutrophil adhesion test.

Haemagglutination antibody (HA) titer: The microtitre HA technique as described by Puri *et al.* (1994) was employed to determine the serum antibody titer. Antibody titers post 7th day at 14 and 21 days interval were referred as primary humoral immune response after 14th day and secondary humoral immune response after 21th day, respectively. Level of total serum immunoglobulins was estimated by using zinc sulphate turbidity test method given by Mc Evan *et al.* 1969.

Lymphocyte stimulation test (LST): The evaluation of activity of T-lymphocytes was carried out by the method described by Rai-el-Balha *et al.* (1985). The lymphocytes were separated from the blood employing the method of Boyum (1968). Cell viability was examined using trypan blue dye exclusion test Boyse *et al.* (1968). The evaluation of the activity of lymphocytes was carried out by the method as explained by Rai-el-Balha *et al.* (1985).

Delayed type hypersensitivity (DTH): Delayed type hypersensitivity reaction to sheep red blood cells (SRBC) was carried out by procedure as described by Phanuphak *et al.* (1974).

Carbon clearance test: The method as described by Cheng *et al.* (2005) was employed to determine the phagocytic index using Indian ink at 28th day of study to all the animals.

Determination of neutrophil adhesion: The neutrophil adhesion test was performed according to the method of (Wilkinson 1978).

Histopathological examination: After the completion of experimental period, all the rats from all the groups were euthanized humanely. All the rats were subjected to detailed post mortem examination and the gross lesions duly recorded in spleen (Lillie 1965).

Statistical analysis: The data was analyzed for significant difference by employing ANOVA at 5% level of significance (Snedcor and Cochran 1967).

RESULTS AND DISCUSSION

Antibody titers obtained on 14th day after immunization (on 7th day) and on 21st day after challenge (on 14th day) with SRBCs were considered as primary and secondary humoral immune response, respectively, as depicted in Table 1. The reduction in HA titers in CP treated rats indicated suppression of humoral immune response which was ameliorated by administration of MEAM in a dose dependent manner indicating reversal of suppressive effect of CP on humoral immune response. However, levamisole treatment showed poor humoral immune response as HA titres in CP + levamisole treated group IV showed significantly ($P < 0.05$) lowered HA titre as compared to MEAM plus CYP and control groups. Amelioration in HA titre might be attributed to its phytoconstituents like withanolides, iridoidic acids, and neo clerodane diterpenoids which have strong immunomodulatory potential as they stimulate protein synthesis, bone marrow and NK cells (Castro *et al.* 2015). Decline in HA titre to SRBC might be due to destruction of antigens by the reticuloendothelial system (Ghosh and Chauhan 1991). This finding is corroborated with the finding of (Egba *et al.* 2014).

Total immunoglobulin (Ig) was significantly ($P < 0.05$) reduced by 55% in CP treated group II in comparison to control group I (Table 1). But simultaneous feeding of MEAM @ 100 and 200 mg/kg body weight, the immunoglobulin concentration increased by 130 to 260 percent in dose dependent manner. The level of serum antibodies against the antigen is considered as the index of humoral immune response in the body (Gatne *et al.* 2006). Immunoglobulin level in MEAM treated rats were even better than levamisole treated group III. The increased in Ig level due to MEAM might be due to increased proliferation of B lymphocytes or increased production of cytokines as its phytoconstituents like withanolides, iridoidic acids, and neo clerodane diterpenoids which have strong immuno-

Table 1. Experimental design

Group	Treatment	Duration
I	1 ml distilled water, p. o.	28 days
II	CP @ 100 mg/kg b. wt., p.o. on 9 th and 16 th day	28 days
III	Levamisole @ 50 mg/kg b. wt., sc daily for 28 days	28 days
IV	CP @ 100 mg/kg b. wt. on 9 th and 16 th day + Levamisole @ 50 mg/kg b. wt, p.o. daily for 28 days	28 days
V	MEAM @ 100 mg/kg b. wt., p.o. daily for 28 days	28 days
VI	MEAM @ 200 mg/kg b. wt., p.o. daily for 28 days	28 days
VII	CP @ 100 mg/kg b wt. on 9 th and 16 th day + MEAM @ 100 mg/kg b wt., p.o. daily for 28 day	28 days
VIII	CP @ 100 mg/kg b wt, po on 9 th and 16 th day + MEAM @ 200 mg/kg b wt., p.o. daily for 28 days	28 days

Table 1. Effect on humoral immunity hemagglutination antibody (HA) titre and total immunoglobulin (Ig:g/L) level following oral administration of methanolic extract of *Ajuga macrosperma* for 28 days in immunocompromised rat

Group	Treatment	Dose and route	Total Ig (g/L)	HA titre (log ₂)	
				Primary immune	Secondary immune
I	Control	1 ml of distilled water, po	3.137±0.145	6.333±0.236	8.000±0.408
II	CYP	100 mg/kg b wt, po	1.4130.246 ^a	0.667±0.236 ^a	2.000±0.000 ^a
III	Levamisole	50 mg/kg b wt, sc	4.506±0.473 ^{ab}	6.333±0.816 ^b	7.000±0.408 ^b
IV	CYP+	100 mg/kg b wt, po and	2.589±0.110 ^c	2.000±0.816 ^{ac}	4.333±0.624 ^a
	Levamisole	50 mg/kg b wt, sc respectively			
V	MEAM-1	100 mg/kg b wt, po	4.732±0.111 ^{abd}	7.000±0.236 ^{bd}	7.000±0.236 ^b
VI	MEAM-2	200 mg/kg b wt, po	5.052±0.185 ^{abd}	8.000±1.080 ^{bd}	8.000±0.707 ^{bd}
VII	CYP+MEAM-1	Both 100 mg/kg b wt, po	3.262±0.399 ^{bcef}	6.333±0.624 ^{bd}	7.000±0.236 ^b
VIII	CYP+MEAM-2	100 mg/kg b wt, po and 200 mg/kg b wt, po respectively	4.013±0.225 ^{bd}	7.000±0.624 ^{bd}	8.000±0.408 ^{bd}

Values are mean±S.E. (n=6); CYP, CP; MEAM, methanolic extract of *Ajuga macrosperma*. CYP was administered on 9th and 16th days only a significant (P<0.05) difference as compared to Group I within same column; ^bsignificant (P<0.05) difference as compared to Group II within same column; ^csignificant (P<0.05) difference as compared to group III within same column; ^dsignificant (P<0.05) difference as compared to group IV within same column e significant (P<0.05) difference as compared to group V within same column; ^fsignificant (P<0.05) difference as compared to group VI within same column.

modulatory potential by their ability to stimulate protein synthesis, activate bone marrow and NK cells functions (Castro *et al.* 2015).

A significant (P<0.05) increase in foot pad skin thickness in MEAM+ CP treated rats as compared to CP alone in this study reflected the restoration of cellular immunity (Table 2). This might be attributed to the reduction in T cell count due to inhibition of cell proliferation (Cooper *et al.* 2011) of expansion of primal and activated antigen specific T cell (Dhasarathan *et al.* 2011). Immunostimulation of cellular response by MEAM might have occurred due to stimulation and proliferation of T lymphocytes. This finding is corroborated with finding of Egba *et al.* (2014).

As shown in Table 2, a significant (P<0.01) improvement were recorded in stimulation index by 97% and 206% simultaneous feeding of Group VII (MEAM-1+CP) and Group VIII (MEAM-2+CP), which was better more than the levamisole treated group IV. The lymphocyte stimulation index in CP treated group was significantly (P<0.05) lower than control which could be attributed to the depletion of lymphocytes in the circulation and lymph nodes and lymphocytopenia. MEAM+CP treated groups showed significant (P<0.05) increase in T lymphocyte proliferation as compared to CP treated group which indicated lympho-proliferative properties of MEAM in a dose dependent manner. Enhancement of LST by MEAM could be because of its ability to increase stimulation, proliferation and maturation of lymphocytes as its phytoconstituents like withanolides, iridoidic acids and neo clerodane diterpenoids which have strong immunomodulatory potential as they stimulate protein synthesis and activate function of bone marrow and NK cells (Castro *et al.* 2015).

A significant (P<0.05) reduction in phagocytic index by 84% was observed in groups II as compared to group I

(Table 2). A significant (P<0.05) increase in phagocytic index was found in groups IV, VII and VIII by 262, 262, 300% respectively, as compared to group II. Thus, CP treatment suppressed the phagocytic index of macrophages which was reversed by levamisole and MEAM. MEAM-2 +CP showed better increase in phagocytic activity as compared to levamisole+CP treated group indicating MEAM is better immunostimulant.

Phagocytic index was calculated by the clearance of colloidal carbon particles from the circulatory system using an exponential equation. Macrophages are the major phagocytic cells, which removes the foreign particles from the systemic circulation. CP plus MEAM treatment ameliorated the anti-phagocytic effect of CP in a dose dependent manner as compared to CP group II. It is clear from the finding that MEAM increases the activity of reticulo-endothelial system. It has reported that oral administration of aqueous and ethanolic extracts of *Ocimum basilicum* increases the activity of reticulo-endothelial system (Dashputre *et al.* 2010).

Groups IV, VII and VIII showed significant (P<0.05) increase in percent neutrophil adhesion by 60, 93 and 129% respectively, as compared to group II which indicated reversal of CP induced effect on neutrophil adhesion by levamisole and MEAM. Thus, there was a significant reduction in neutrophil adhesion in CP treated groups whereas levamisole and MEAM improved neutrophil adhesion indicating activation of neutrophils by MEAM in a dose dependent manner.

Neutrophils play a major role in the first line of defense mechanism involving chemotaxis and phagocytosis (Raj and Gothandam 2015). It was observed that CP plus MEAM treated groups showed significantly (P<0.05) higher percent neutrophil adhesion in a dose dependent manner than CP

Table 2. Effect on parameters of cellular immunity following oral administration of methanolic extract of *Ajuga macrosperma* for 28 days in immunocompromised rats

Group	Treatment	Dose and route	Percent Neutrophil adhesion	Phagocytic index	Stimulation index	Foot pad thickness (mm)	
						12 hours	24 hours
I.	Control	1 ml of distilled water, po	11.120±0.780	0.050±0.0008	0.920±0.012	0.533±0.006	0.567±0.024
II.	CYP	100 mg/kg b wt, po	5.570±0.290 ^a	0.008±0.0008 ^a	0.470±0.176	0.280±0.004 ^a	0.267±0.006 ^a
III.	Levamisole	50 mg/kg b wt, sc	16.570±1.159 ^{ab}	0.051±0.0012 ^b	0.790±0.102	0.512±0.011 ^b	0.541±0.008
IV.	CYP+Levamisole	100 mg/kg b wt, po and 50 mg/kg b wt, sc respectively	8.840±0.506 ^c	0.029±0.0004 ^{ab}	0.490±0.122	0.260±0.004 ^{ac}	0.233±0.004 ^{ac}
V.	MEAM-1	100 mg/kg b wt, po	13.940±1.115 ^{bd}	0.047±0.0020 ^{bd}	0.990±0.045	0.633±0.008 ^{bd}	0.667±0.008 ^{bcd}
VI.	MEAM-2	200 mg/kg b wt, po	15.620±1.200 ^{abd}	0.051±0.0004 ^{bd}	1.750±0.131 ^{abcde}	0.833±0.009 ^{abcde}	0.933±0.024 ^{abcde}
VII.	CYP+MEAM-1	Both 100 mg/kg b wt, po	10.720±1.127 ^{bef}	0.029±0.0004 ^{abcef}	0.929±0.029 ^f	0.587±0.024 ^{bdf}	0.602±0.009 ^{bdf}
VIII.	CYP+MEAM-2	100 mg/kg b wt, po and 200 mg/kg b wt, po respectively	12.750±0.727 ^b	0.032±0.0008 ^{abcef}	1.440±0.171 ^{bcd}	0.733±0.009 ^{abcdeg}	0.833±0.008 ^{abcdeg}

Values in the Table are mean±S.E. (n=6); CYP, CP; MEAM, methanolic extract of *Ajuga macrosperma*. CYP was administered on 9th and 16th days only a significant (P<0.05) difference as compared to Group I within same column; ^asignificant (P<0.05) difference as compared to Group II within same column; ^bsignificant (P<0.05) difference as compared to Group III within same column; ^csignificant (P<0.05) difference as compared to Group IV within same column; ^dsignificant (P<0.05) difference as compared to Group V within same column; ^esignificant (P<0.05) difference as compared to Group VI within same column.

treated rats. It is thus evident from the present finding that methanolic extract of *Ajuga macrosperma* increased adhesion tendency of neutrophils towards nylon fibres in a dose dependent manner.

The lymphoid organ spleen on histopathological examination group II rats (Fig. 1) showed severe depletion of lymphoid cells, reticular cell hyperplasia, and depletion of red pulp and presence of hemosidrin pigment. Lesions were similar and of varying intensity in other groups. Lesions in spleen were severest in group II followed by groups VII, IV and VIII rats (Fig. 2) indicating the

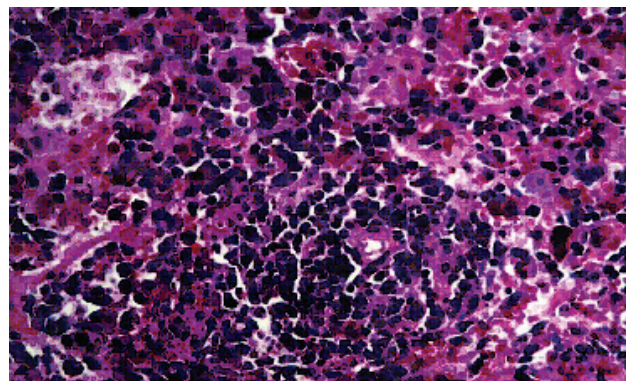


Fig. 1. Photomicrograph of spleen of group II (CP) showing severe depletion of lymphoid cells, depletion of red pulp and presence of hemosidrin pigment. (H&E, 400x).

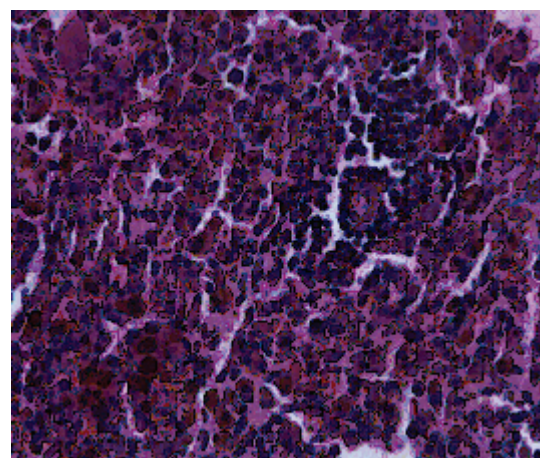


Fig. 2. Photomicrograph of spleen of group IV (CP + MEAM-1) showing depletion of lymphoid cells, reticular cell hyperplasia and depletion of red pulp. (H&E, 400x).

ameliorative effect of MEAM in spleen.

It is concluded from this 28 days study in rats that oral administration of CP @ 100 mg/kg b wt at 9th and 16th day caused immunotoxic, suppressed humoral and cellular immune response. These adverse effects of CP were ameliorated by the oral treatment with methanolic extract of aerial parts of *Ajuga macrosperma* (MEAM) @ 100 and 200 mg/kg b wt for 28 days, even better than levamisole (50 mg/kg b wt, sc), as evident by immunological parameters as also evidenced by the microscopic pathological examination.

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