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Evaluation of Immunomodulatory Potential of *Amaranthus Dubius* Leaf on Rodent Models

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ABSTRACT

Amaranthus dubius is known for its various remedies against many ill conditions. Present study has been carried out to delayed type hypersensitivity reaction also stimulated by *Amaranthus dubius* significantly indicates that the extract could stimulate the haemopoetic system. The mechanism of action could be unfolded only after detailed investigations whereby the extract modulates the immune system however; the extract contains compounds which had immunomodulatory activity. CMI responses are critical to defence against infectious organisms, infection of foreign grafts, tumor immunity and delayed-type hypersensitivity reactions. Therefore, increase in DTH reaction in rat in response to T cell dependent antigen revealed the stimulatory effect by pretreatment of levamisole on T cell. DTH is a part of the process of graft rejection, tumour immunity, and, most important, immunity to many intracellular infectious microorganisms, especially those causing chronic diseases.

Keywords: *Amaranthus dubius,* Immunomodulatory Potential, Antigen, Hypersensitivity

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INTRODUCTION

Immunology is the study of the methods by which the body defends itself from infectious agents and other foreign substances in its environment[1]. An infectious organism that causes a disease is called a pathogen and the individual (person or animal) that is infected by a pathogen is called the host. There are thousands of components to the immune system and it would appear that the immune system is far more complicated than necessary for achieving what is, on the surface, a simple task of eliminating a pathogenic organism or abnormal 'self' cells[2].

However there are a number of reasons for this complexity, including the desirability of eliminating pathogens without causing damage to the host. Getting rid of a pathogen or dead host cells is theoretically easy, but eliminating these without damaging the host is much more complicated. As a consequence of this dynamic complexity, the immune system is able to generate a tremendous variety of cells and molecules capable of specifically recognizing and eliminating an apparently limitless variety of foreign invaders, in addition to the recognition and destruction of abnormal cells[3]. Once a foreign protein, microorganism (e.g., bacterium, fungus or virus) or abnormal cell is recognized, the immune system enlists the participation of a variety of cells and molecules to mount an appropriate effector response to eliminate or neutralize them[4]. Later exposure to the same foreign organism induces a memory response, characterized by a heightened immune reactivity, which serves to eliminate the microbial pathogen, prevent disease and protect against the development of some tumour cells.

Amaranthus dubius Linn. (Family: Amaranthaceae) is commonly known as spiny amaranth, prickly amaranth or thorny amaranth. It is widely distributed throughout the tropics and warm temperate regions of Asia as a weed in cultivated as well as fallow lands[5]. In India, it is commonly known as "Kate Wali Chaulai (Kanatabhajii)" and used as vegetable and cultivated throughout India. It is widely used in folk lore medicinal system of India. The whole plant and parts of the plant contains medicinally active constituents such as alkaloids, flavonoids, glycosides, phenolic acids, steroids, amino acids, terpenoids, lipids, saponins, betalains, b-sitosterol, stigmasterol, linoleicacid, rutin, catechuic tannins and carotenoids[6]. Extracts of *A. spinosus* have been reported to show diuretic, antidiabetic, antipyretic, anti-snake venom, antileprotic, anti-gonorrheal antioxidant, anti-cholesterolemic, antipyretic, anti-inflammatory, spermatogenic, antitumor, antifertility, anti-malarial, hepatoprotective activities[7-10] and also effects on hematology and biochemical changes in the epididymis[11].

Thus, we reported in present study; phytochemical and evaluation of immunomodulatory potential of *Amaranthus dubius* leaf on rodent models.

MATERIALS & METHODS

Plant Material

A. dubius as a whole plant was collected from in and around places of Bhopal district in Madhya Pradesh state and identified in Department of Botany of Safia College.

Extraction preparation

Whole plant was air dried under shaded conditions and coarsely powdered. Hundred gram of powder was refluxed with 600 ml of water at 100oC for 24 hours. The extract was filtered through double layer 100 µm nylon wire mesh and concentrated at 50oC to obtain crude aqueous extract.

Experimental Animals

Normal healthy male Wistar rats (Rattus norvegicus) weighing 200-240g were used in the present investigation. The animals were maintained as per the guidelines for care and use of Animals for Scientific Research proposed by Indian National Science Academy, 2000 at Department of Zoology, in group of three animals in polypropylene rat cages under 12:12 hrs. Light-dark schedule and fed with rat pellet diet and water was provided *ad libitum*.

Experimental Design

The animals were divided into four group viz. Group I, Group II, Group III and Group IV, consisting 10 animals in each group. The aqueous extract of A. spinosus was administered orally at 125, 250, 500 and 1000 mg/Kg body wt. /day respectively for 60 days. Control animals were administered with distilled water. Following completion of respective treatment schedule, all the animals were withdrawn from the treatment for a further period up to 90 days. Five animals from each group were sacrificed the next day following the 60th day of treatment and remaining five after 90 days of treatment withdrawal.

Acute Toxicity and Lethality:

Investigation on acute toxicity of the extract with estimation of the median lethal dose (LD_{50}) was carried out using Lorke's method (1983). Thirteen experimental animals (mice with weight range of 20g-30g) were used for the test. In the investigation, three groups of mice containing three mice each were administered 10-, 100- and 1000g/kg respectively of the aqueous extract intraperitoneally (*ip*). They were observed closely for 24 hr for lethality or any other behavioral response. Based on the result, further increased doses of 1500-, 2000-, 3000- and 5000 mg/kg were administered ip to four other mice respectively. They were also observed for 24 hr for any death or behavioural changes.

Experimental Design

A total of fifty (50) Wistar albino rats used in Delayed Type Hypersensitivity Reaction and Humoural Antibody titre (twenty five (25) rats for each parameter) were grouped as follows:

Group A: Rats were treated with SRBC and normal saline and it served as control

Group B: Rats were treated with SRBC and 50 mg/kg body weight of extract

Group C: Rats were treated with SRBC and 100 mg/kg body weight of extract

Group D: Rats were treated with SRBC and 250 mg/kg body weight of extract

Group E: Rats were treated with SRBC and 25 mg/kg body weight of levamisol

Immunomodulatory Activity of Extracts

Preparation of Antigen:

Fresh sheep blood was obtained from the animal farm of the Faculty of Veterinary Medicine, Sheep red blood cells (SRBCs) were washed three times in continous volume of pyrogen-free sterile normal saline by centrifugation at 3000×g for 10 min on each occasion. The washed SRBCs were adjusted to a concentration of 109cells/ml for immunization and challenge.

Delayed Type Hypersensitivity (DTH) Reaction

Delayed type hypersensitivity (DTH) reaction was investigated using the method of Navedet al. (2005). DTH reaction was induced in rats using SRBCs as antigen. Animals were sensitized by subcutaneous injection of 0.02 ml of 109cells/ml SRBCs (day 0) in the plantar region of right hind foot paw and challenged on day 5 by subcutaneous injection of the same amount of antigen into the left hind paw. The oedema produced by antigenic challenge in the left hind paw was measured as the difference in the paw thickness before challenge; 24 hr and 48 hr after the challenge. The paw thickness was measured with a pocket-sized screw guage[12]. The extracts were administered three days prior to sensitization and continued daily till the challenge.

Humoural Antibody (HA) Synthesis

Rats were immunized by an intraperitoneal injection (i.p) of 0.1ml of 109 SRBC ml^{-1} on day 0 and challenged by similar ip injection of the same amount of 0.1 ml on day 5. Primary antibody titre was determined on day 5 (before the challenge) and secondary titre on day 10 (Sharma et al., 1996) by haemagglutination technique[13]. The aqueous extract (50, 100 and 250 $mgKg^{-1}$) was administered 3 days prior to immunization and continued daily for 5 days after challenge. Blood samples were obtained by retro-orbital puncture and collected in a test tube and allowed to clot. For each sample, a 25µL serum was obtained after centrifugation and serially diluted two-fold in 96-U well microtitre plates using pyrogen-free sterile normal saline as control. The diluted sera were challenged with 25µL of 1% (v/v) SRBC in the plates and then incubated at 370C for 1hr. The highest dilution giving rise to visible haemagglutination was taken and antibody titre expressed in graded manner, the minimum dilution (1/2) being ranked as 1 (calculated as Log2 of the dilution factor). The mean ranks of different treatment groups were compared for statistical significance.

RESULTS AND DISCUSSION

Effect of Extract on Humoural Immunity

Fig. 1 below reveals the effect of the extract on humoural immune response which was assessed using humoural antibody titre. The result indicates that there was augmentation of the humoural immune response to SRBCs by the extract and levamisol which was evidenced by the increase in the antibody titre. It also reveals that as the number of pretreatment days increased, the primary and secondary antibody increased in the rat. Both the primary and secondary antibody titre induced a significant rise ($p < 0.05$) in the antibody titre when compared to the control A. Administration of the extract produced dose dependent increase in humoural antibody titre. It is also obvious from the figure that the effect of 250mg/kg of extract was more than that of levamisol solution.

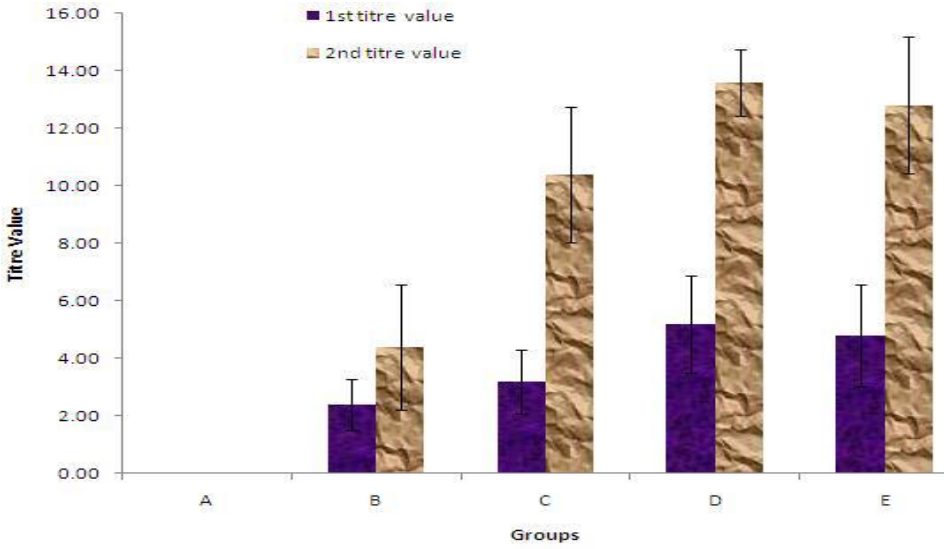

Fig. 1: Effect of *A. dubius* on primary and secondary antibody titre.

The values are expressed as (Mean ± SD) n=5 per group $p < 0.05$
Group A = Rats treated with SRBC and normal saline and it served as the control
Group B = Rats treated with SRBC and 50 mg/kg body weight of extract
Group C = Rats treated with SRBC and 100 mg/kg body weight of extract
Group D = Rats treated with SRBC and 250 mg/kg body weight of extract
Group E = Rats treated with SRBC and 500 mg/kg body weight of extract

Effect of extract on Cell Mediated Immunity

Fig. 2 shows the induction of DTH reaction induced in the hind foot paw of rats using SRBC and DTH response checked by increased footpad thickness using vernier calliper. The challenged rat elicited a significant increase ($p < 0.05$) in thickness of footpad 24 hr and 48 hr after challenge. Administration of 50, 100 and 250 mg/kg of extract produced a dose and time dependent increase in footpad swelling of the rats.

At 24 hr after challenge, it is evident that the extract doses of 50, 100 and 250 mg/kg and 25 mg/kg body weight of levamisol significantly boosted ($p < 0.05$) DTH reactions observed as 1.412, 1.504, 1.816 and 1.827 mm difference respectively in thickness of footpad before challenge and 24 hr after challenge. In group A serving as the control, there was a non-insignificant increase ($p < 0.05$) with a difference of 0.614 mm in the thickness of footpad before challenge and 24 hr after challenge.

At 48 hr after challenge, Fig. 2 also shows an additional increase in footpad swelling of rats in groups B, C D and E observed as 1.908, 1.918, 2.304 and 2.326 mm respectively. This reveals that at 48 hr after challenge, the extract and levamisol still had stimulatory effect on T lymphocyte and accessory cell types required for expression of DTH reaction.

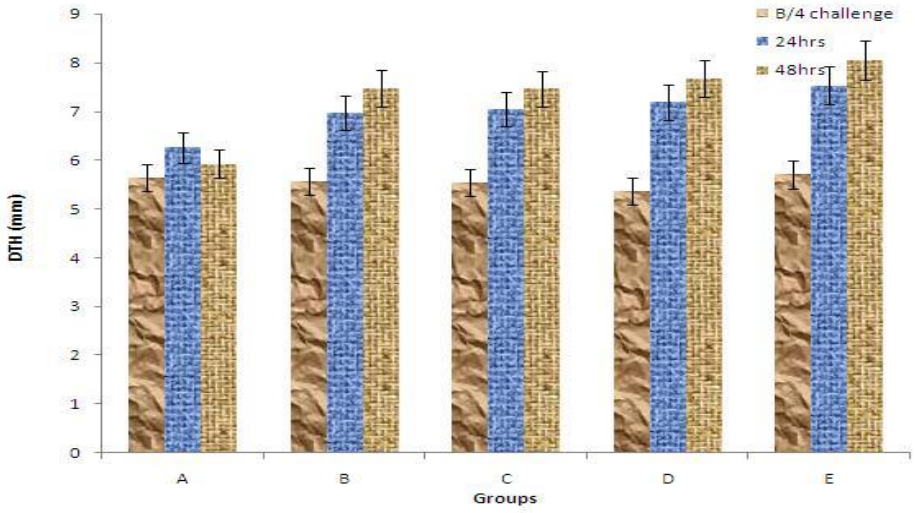

Fig. 2: Effect of *A. dubius* treatment on cell mediated immune response by DTH induced footpad oedema.

The values are expressed as (Mean ± SD) n=5 per group $p < 0.05$.
Group A = Rats treated with SRBC and normal saline and it served as control
Group B = Rats treated with SRBC and 50 mg/kg body weight of extract
Group C = Rats treated with SRBC and 100 mg/kg body weight of extract
Group D = Rats treated with SRBC and 250 mg/kg body weight of extract
Group E = Rats treated with SRBC and 500 mg/kg body weight of extract

IV. DISCUSSION

Immunomodulation is a procedure which can alter the immune system of an organism by interfering with its functions; if it results in an enhancement of immune reactions it is named as an immune-stimulative drug which primarily implies stimulation of specific and nonspecific system, *i.e*. granulocytes, macrophages, complement, certain T-lymphocytes and different effector substances. Immuno-suppression implies mainly to reduce resistance against infections and stress and it may occur on account of environmental or chemotherapeutic factor. The results obtained in the present study indicate that aqueous extract of *A. dubius* is a potent immune-stimulant, stimulating specific and non-specific immune mechanisms. To evaluate the effect of aqueous extract of *A. dubius* on humoural response, its influence was tested on sheep erythrocyte specific Ab titre in rats. From the results obtained in the present study and shown in Fig. 1 above, there was increase in humoural immune response to SRBCs as evidenced by increase in antibody titre. This indicates that aqueous extract of *A. dubius* is a potent immunostimulant, with the ability to stimulate specific and non-specific immune mechanisms. This suggests that the extract might have the ability of enhancing the interaction of B cells with the antigen and subsequent proliferation and differentiation of B-lymphocytes into antibody-secreting plasma cells. The antibody produced by the activated plasma cell then inactivates the antigen by complement fixation where proteins attach to antigen surface and causes holes to form leading to cell lysis; by neutralization where the Ab binds to specific site preventing antigen attachment; by agglutination which involves clumping together of the Ag inactivating them; by precipitation whereby insolubility is forced and the antigen settles out of solution. Moreover, the extract might be able to activate lymphoid dendritic cell which stimulate lower amounts of IL-12 and higher amounts of IFN-α which primarily elicit Th2 development. Activated Th2 cell stimulate Ab production by secreting IL-4 which proliferates B-cell to plasma cell. Activated Th2 also releases cytokines such as IL-2, IL-4 and IL-10 which activates mast cells leading to the production of serotonin and histamine, mediators of inflammatory response.

The ability of the extract to induce primary Ab titre shows that it may stimulate IgM which is the major Ab present in the body during primary immune response. IgM is effective in activating the complement components which facilitate the phagocytosis of pathogens by macrophages. Furthermore, the result also showed that the extract significantly increased ($p < 0.05$) secondary Ab titre suggesting that on re-exposure to the same Ag, the extract was able to accelerate secondary response and enhance the production of IgG. IgG and IgM are the major immunoglobulins which are involved in the complement activation, opsonization, and neutralization of toxins. The stimulation of immunoglobulin by the extract suggests that it activates IgA and lactoferrin the major immunoprotein present in breast milk and which confers immunity to the neonate. The augmentation of the humoural responsiveness to SRBC in rats as consequence of both pre and post immunization treatment with levamisol corresponds with the report of Lai et al., (2002) who reported levamisol improved immunitary defences by its interferon-like activity and by restoring Th cell activity; evoking maturation of thermocytes; increasing the metabolic activity of blood monocytes and their affinity for the Fc fragment of IgG. Cell-mediated immunity (CMI) involves effector mechanisms carried out by T lymphocytes and their products (lymphokines). DTH is a Type IV hypersensitivity reaction that develops when antigen activates sensitized TDTH cells. DTH response was checked by increased footpad thickness using verniercaliper. The animals treated with levamisol and extracts showed a significant ($p < 0.05$) change in DTH response as compared to control animals. As can be evident from (Fig. 2), a significant ($P < 0.05$) increase in DTH response was observed at the doses: 50, 100 and 250 mg/kg dose of Senna extract and levamisol 25 mg/kg. Therefore, increase in DTH reaction in rats in response to T cell dependent antigen revealed the stimulatory effect of aqueous extract of Senna on T cells. The result shown above suggests that during the first stage of the DTH response known as sensitization stage, the extract might have activated Th1 cells and also expanded clonally APCs with class II MHC. Then the protein of the antigen (in this case SRBC) will be presented at the external wall of the APC in a changed MHC class II, to chemotactically attract naive proT-lymphocyte known as Th0. The Ag-peptide will be taken from the MHC of the APC and bound to T-cell receptor activating the Th0 cell converting it to Th1 cell. Th1 cell then commences the effector phase of the DTH response. Moreover, the activated Th1 which may have been enhanced by the extract, proliferates and releases cytokines (IL-2, IL-12, IL-18, IFN-γ, TNF-α) which increases vascular permeability, induces vasodilation, activates and accumulates macrophages and NK cells and all these promotes increased phagocytic activity and increased concentration of lytic enzymes. The probable stimulation of Th1 cell by the extract also suggest that the NK cell which was subsequently stimulated used a protein named perfrorin to destroy the SRBC by inoculating the protein into the antigen (in this case SRBC) cell membrane. This perforin molecule then assembles in the membrane to form a pore, through which other molecules can flow into the target. Moreover, it also produces IFN which are powerful activators of macrophages. Treatment with the aqueous extract of Senna enhanced DTH reaction, which is reflected from the increased footpad thickness compared to control group suggesting heightened infiltration of macrophages to the inflammatory site. The probable mechanism of action of the extract might be by activating myeloid dendritic cells which are known to also produce a large amount of IL-12 and preferentially induce Th1 development. The extract might be immunogenic by activating complement which recruit and stimulate cellular elements of the immune system, such as histamine, basophils, macrophages, within 24-72 hr initiating inflammatory reactions and phagocytosis of pathogens which lead to increase in the rat paw oedema as depicted in Fig 2 above. Increase in the DTH response indicates that drug has a stimulatory effect on lymphocytes and necessary cell types required for the expression reaction. As shown in fig. 2 above, DTH response, which is direct co-related to cell-mediated immunity, was significantly increased ($p < 0.05$) with aqueous extract of Senna extract as compared to untreated control. There are some plants like Aesculusindica, Argyreiaspeciosa which are reported for acting on delayed type of hypersensitivity. The increase in rat paw oedema of rats treated with levamisol as shown in the Fig above corresponds with former reports of Yadavet al., (2011) showing the stimulatory effect of levamisol on lymphocytes and other cell types. Lai et al., (2002) reported that levamisol improved DTH in immunodepressed individuals to restore Th and Ts cell activity and also increases their activity.

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