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Research Article

RELATIVE IMMUNE MODIFIER ACTIVITIES OF *PTEROSPERMUM ACERIFOLIUM*, *MURRAYA KOENIGII* AND *WITHANIA COAGULANS* IN BALB/C MOUSE

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ABSTRACT

Ethanolic extracts prepared from the flowers of *Pterospermum acerifolium* (PA), leaves of *Murraya koenigii* (MK) and fruits of *Withania coagulans* (WC) have been evaluated for their comparative immunomodulatory activities in BALB/c mice. The extracts were fed orally at various log doses viz. 3.0, 10.0 and 30.0 mg/kg of body weight to groups of mice for 14 consecutive days. Picroliv was included as a standard immunostimulant at an optimal immunostimulatory dose (1.0 mg/kg) as positive control. Various pharmacological activities of test samples were analyzed by studying the alteration in the immunological responses of mice post treatment viz. production of Reactive oxygen species (ROS) from peritoneal macrophages and splenic B and T cell lympho-proliferation in presence of mitogens and pro-inflammatory (Th1) and anti-inflammatory (Th2) cytokines. MK did not reveal any noticeable effect while WC significantly up-regulated the ROS production in peritoneal macrophages at higher dose (30.0 mg/kg). PA possessed comparatively potent immunostimulatory activity which was denoted by increased *in vitro* T and B cell proliferation, up-regulated ROS production in the antigen presenting cells (APCs) in a dose dependent manner. The PA extract was therefore further explored at the most active dose (30.0 mg/kg) and an increase in the CD4+ helper (T_H) cells, CD8+ cytotoxic (Tc) cells and CD19+ B cell population with induction of a mixed Th1 (IFN-γ) and Th2 (IL-10) cytokine responses in mice that was more or less comparable to that of known immunomodulatory compound Picroliv was observed. The findings suggest *Pterospermum acerifolium* to contain biologically active molecule/s which are strong immune modifier/s and skew the host helper immune response towards both Th1 and Th2 type with a more biased Th1 response. This plant shows promise as vaccine adjuvant as well as chemotherapy adjunct agent against infectious diseases and may find its use in a variety of immune suppressive disorders. Efforts are underway to localize the immunostimulant activity in single molecule/s.

Keywords: Immunomodulatory; Cytokines; Macrophages; *Pterospermum acerifolium*; *Murraya koenigii*; *Withania coagulans*; BALB/c; lymphocytes.

INTRODUCTION

India is rich in traditional medicinal plants for skirmishing diseases which are the source of newer chemical therapeutic entities. Some of these plants have been explored for their pharmacological characteristics, while many still remain untouched. In the proposed study, three plants viz. *Pterospermum acerifolium* flowers (PA) (Sterculiaceae), *Murraya koenigii* leaves (MK) (Rutaceae) and *Withania coagulans* fruits (WC) (Solanaceae) with diverse range of pharmacological activities have been selected to exploit their comparative immunomodulatory efficacies in BALB/c murine model. Topical application of PA flowers finds its traditional use in cancer, small pox and hemicranias[1, 2]. PA commonly known as Kanak Champa, Karnikara, Muchukunda and Matsakanda, is a wild Indian shrub found particularly in sub-Himalayan tract, outer Himalayan valley and hills up to 4000 ft[3]. Ayurvedic text depicts its traditional use in homeostasis, inflammation, ear pain, blood trouble, small pox, leucorrhoea, leprosy, ulcer, cancer, and hemicranias, as laxative, anthelmintic, antimicrobial and hepato-protective[1, 2, 4-9]. Preliminary phytochemical analysis of the leaf extract of PA by Santanu Sannigrahi[10] revealed the presence of several compounds like steroids, flavonoids, alkaloids and glycosides. We earlier reported immunosuppressive activity in the seed extract of PA plant[11]; however, flower part remained unexplored. *Withania coagulans*, another species of medicinally important genera *Withania* commonly known as Indian cheese maker is prevalent in dry parts of India[12]. Its various parts have been ascribed to possess diverse biological activities[13] including anti-hyperglycemic activity[14]. WC is known to contain withanolides (withaferin-A), phenolic tannin, flavonoids and flavonols[15]. In spite of innumerable reports on the various pharmacological properties of WC, the immunomodulatory activity in the fruit ethanolic extract has not been investigated. The other plant *Murraya koenigii*, commonly known as curry-leaf tree also falls under traditional medicinal plants. This plant is found almost everywhere in the Indian subcontinent, excluding the higher levels of the Himalayas[16] and leaves of this (MK) plant find wide use in kitchen as a spice and condiment in tropical countries including India. The leaves, bark and the root are used intensively in indigenous medicine from ancient time, as a tonic for stomachache, stimulant and carminative[17]. MK leaves mixed with fat separated butter are used for the treatment of amoebiasis, diabetes and hepatitis in Ayurveda[18-21]. Thus from the literature it becomes apparent that in spite of extensive traditional use of these three plants, their effect on the immune response of host especially in the vegetative parts has not been seriously investigated which may contain different chemical constituents extractable in different solvents. The current investigation is an attempt taken to justify the traditional use and pharmacological claim about these three plants MK, WC and PA and identify the best among them. The ethanol extract of the flower part of PA in the present investigation demonstrated noticeable immunostimulant characteristics compared to MK and WC in BALB/c mice at various doses in a dose dependent manner and therefore studied in more details.

MATERIALS AND METHODS

Plant Material

The flowers of PA, MK leaves and WC fruits were collected from Lucknow, India, in March 2008 and identified by Dr. A. K. Mangal, Central Council for Research in Ayurveda and Siddha. Specimens of the above three plants (Specimen Number 4740 for PA, 4763 for MK & 4554 for WC) have been preserved in the investigators Laboratory.

Extraction

The air-dried and powdered PA flowers (500 g), MK leaves (500 g) and WC fruits (500 g) were extracted exhaustively with 95% ethanol (4 x 1L) at room temperature (RT). Each extraction was carried out for 16 h. The combined percolates were filtered and solvent was evaporated under vacuum using rotavapour at 45 °C which afforded ethanolic extract (80 g, 16% for PA; 55 g, 11% for MK and 60 g, 12% for WC).

Laboratory chemicals

Medium RPMI-1640 (Sigma, USA) containing Phenol Red was used in all the studies. The medium was fortified with 1% antibiotic-antimycotic cocktail (Sigma, USA) and 10% Foetal bovine serum (GIBCO, USA). HEPES was purchased from Sigma Chemical Co. (St. Louis, MO). All antibodies were purchased from Becton and Dickinson (BD) (San Diego, CA, USA). All other chemicals used were of analytical grade available locally.

Animals

Inbred BALB/c mice of either sex weighing between 18 to 20 g were randomly distributed in groups as per experimental protocols (n=5). The animals were housed under standard conditions of temperature (23 ± 1°C), relative humidity (55 ± 10%), and 12 h/12 h light/ dark cycles at National Laboratory Animal Centre (NLAC), Central Drug Research Institute (CDRI), Lucknow, India and fed with standard pellet diet and water *ad libitum*. All the animal handling and experimental protocols including number of mice employed in the study were duly approved by the Institutional Animal Ethics Committee (IAEC) bearing approval no. 38/08/PARA/IAEC dated 08.02.2008.

Experimental protocol

The extracts (PA, MK and WC) were prepared fresh as suspensions in distilled water and fed orally to various groups of mice for 14 consecutive days at three log doses (3, 10 and 30 mg/kg). The positive control group received standard immunostimulatory compound Picroliv at 1.0 mg/kg while the untreated control mice were fed with the vehicle under identical conditions. A total of 15 mice were used for each extract (for three separate doses), another group of 5 mice were used for Picroliv treatment while the remaining 5 mice received only vehicle as untreated control (total 55 mice). The experiment was repeated with active sample only at an optimal immunostimulatory dose.

On day 15, animals were euthanized humanely to determine *in vitro* proliferation of T and B lymphocytes in presence of mitogens and production of reactive oxygen species (ROS) by the peritoneal macrophages. Since PA revealed promising immunostimulant activity in these two preliminary immune parameters, this plant extract was further explored at its optimal immunostimulatory dose of 30 mg/kg to dissect its efficacy profile in augmenting the expansion of sub-clones of T cells (T_H and T_C cells), CD19+ B cells along with the production of cytokines (Th1/Th2) using flow cytometry on Fluorescent Activated Cell Sorter (FACS, FACSCalibur, Becton Dickinson, USA). The two other plants extracts viz. MK and WC were not investigated further due to negligible immunomodulatory action in mice.

Production of ROS by peritoneal macrophages

ROS in peritoneal macrophage cells were determined through a fluorometric assay using 2', 7'-dichlorofluorescin diacetate (DCF-DA) (Sigma, USA) on FACS as per the protocol of Zurgil et al[22] with minor modification as described earlier[23]. Briefly, freshly harvested macrophages of both treated and untreated groups were adjusted to a concentration of 1×10^6 cells/ml in phosphate buffer saline (PBS), washed thrice with PBS and transferred to FACS tubes (1×10^6 cells/tube). For probe loading, cells were incubated with the DCF-DA for 15 min at 37 °C at a final concentration of 1 µM, washed twice in PBS and ROS level in individual living cells was determined by measuring the fluorescence intensity on FACSCalibur. Data were analyzed by CellQuest Software (Becton Dickinson, USA) and mean ROS values were evaluated for cell population in individual animal.

Cellular immune response by lymphocyte transformation test (LTT)

Single cell suspension of lymphocytes from the spleens of test sample treated and untreated mice were washed and suspended ($5x10^6$ cells/ml) in RPMI-1640 medium containing 10% heat inactivated fetal bovine serum and plated in triplicates in NUNC 96 well culture plate as described earlier[24]. Cell culture was stimulated with LPS and Con A as B and T cell mitogens respectively and the plate was incubated for 48 h at 37 °C in presence of 5% CO_2 in air. The cells were pulsed with [3H]-thymidine (1µCi/ well), incubated further for 18 h and cells were harvested and counted on a β-counter to assess incorporation of radioactivity in the form of counts per minute (cpm). The results have been expressed as stimulation indices (S.I.) as a ratio of stimulated and unstimulated cells.

CD4+/CD8+ T cell sub-sets and CD19+ B lymphocyte population

Cell surface staining was carried out to assess lymphocyte population by FACS using fluorochrome conjugated monoclonal antibodies (BD San Diego, CA, USA) directed against different CD antigens viz. CD4 (Fluorescent Iso Thio Cynate or FITC), CD8 (Phycoerythrin or PE) and CD19 (FITC) following manufacturer's protocol as described earlier[25]. Single cell suspension of splenocyte (1×10^6 cells) were initially blocked with Mouse Seroblock FcR at RT for 10 min, washed and labeled with rat anti-mouse CD4+ (FITC) for another 10 min at RT and finally incubated with rat anti-mouse CD8+ (PE) for another 10 min. The cells in another tube were labeled with monoclonal antibody to CD19 in the same way and the remaining third tube served as control with no labeling. Cell pellet was suspended in sheath fluid and analyzed on FACS using CellQuest analysis software (Becton- Dickinson, San Diego, CA) after gating the forward- and side-scatter settings to exclude debris. For each determination 10,000 cells were analyzed and the results are expressed as percentage of each cell population.

Intracellular Th1 and Th2 cytokines

The measurement of intracellular cytokines in the splenocytes was done as per manufacturer's instructions using antibodies and reagents from BD (San Diego, CA, USA). Briefly, splenocytes (2×10^6/ml) were incubated with Brefeldin A (10 µg/ml) in dark for 6 h in CO_2 incubator at 37 °C, re-incubated with mouse Seroblock FcR for another 10 min. and washed in PBS to further add FITC-rat anti mouse CD4 antibody. Leucoperm A and Leucoperm B (Serotec, UK) were added at RT for 15 min each and cells were dispensed in two tubes each containing 1×10^6 cells/100 µl PBS. PE-rat anti mouse monoclonal antibodies to cytokines IL-10 and IFN-γ were added individually to two separate tubes, cells were washed and finally suspended in 250 µl of PBS containing 0.5% Para formaldehyde for FACS readings as described earlier[23].

Statistical Analyses

Data have been expressed as the mean ± standard error (S.E.) and statistical analyses were carried out by employing the Student's t-test and analysis of variance by one way ANOVA (Dunnett's Multiple Comparison Test). A conventional p <0.05 (*) was taken as evidence of low significant difference and p <0.01 (**) was considered as highly significant while p >0.05 was not significant.

RESULTS

PA is most efficient in inducing ROS generation by APCs

The extracts derived from PA and WC plants induced ROS generation in the peritoneal macrophages in a dose dependent fashion. However, the values were statistically significant at 10 and 30 mg/kg in case of PA (p <0.01) and at only 30 mg/kg (p <0.01) in case of WC and the degree of ROS induction by the two extracts at 30 mg/kg were comparable. MK did not affect the ROS content at any of the three doses tried (p >0.05). The standard immunostimulatory compound Picroliv (positive control) also represented significant increase in ROS production (p <0.01) though at the tested low dose of 1 mg/kg (Fig.1).

PA extract stimulates proliferation of lymphocytes *in vitro*

The PA extract significantly augmented the B lymphocyte (p <0.01) (Fig.2) and T lymphocyte (p <0.05 to p <0.01) (Fig.3) proliferation *in vitro* at all the three doses in a dose dependent manner with highest stimulation exhibited at the highest dose of 30 mg/kg. The extracts from the two other plants (MK and WC) did not demonstrate cellular proliferation at any dose when compared to that of untreated controls. Picroliv on the other hand significantly (p <0.05) induced both B and T cell proliferation though to a lesser extent than PA (Fig.2 and 3). Nevertheless, the dose of Picroliv was much low.

Effect on T-cell sub-population and B cells (Flow cytometric measurement)

At the most effective immunostimulatory dose of 30 mg/kg, PA extract noticeably up-regulated both the helper (CD4+) and cytotoxic (CD8+) T cell population (p <0.01). The CD19+ B cell expansion was also evident, however, the increase was not found to be statistically significant (p >0.05) when these were compared with those of the control group. The picroliv treated group represented significant proliferation of CD4, CD8 and CD19 positive cells (p <0.05) (Fig.4).

The extract of PA induces production of Th1 and Th2 cytokines

The population of CD4+ T cells producing both Th1 (IFN-y) and Th2 (IL-10) type of cytokines was assessed by FACS. Both pro-inflammatory (IFN-y) and anti-inflammatory (IL-10) classes of cytokines were up-regulated intracellularly in the spleen cells of PA and Picroliv treated mice. Nevetheless, the increase of IL-10 production was not significant (p <0.05) in case of PA treated animals unlike picroliv treated ones (Fig.4). Thus PA ethanolic extract induces both Th1 and Th2 helper T cell response with major bias towards Th1 in contrast to picroliv which elicits both the immune arms.

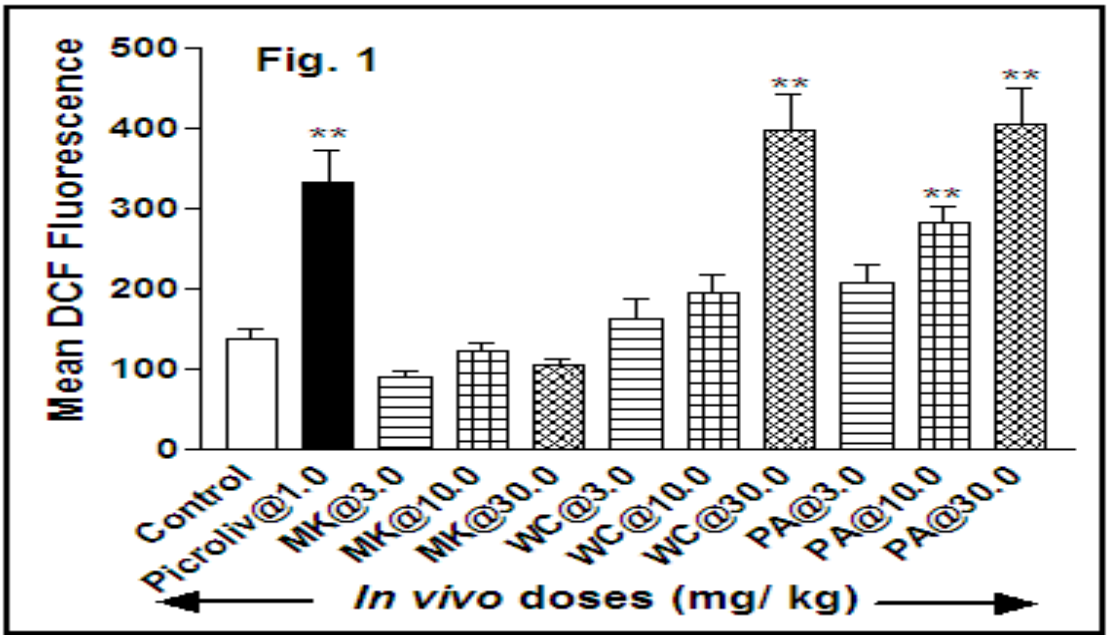

Fig. 1: Macrophages were collected from the peritoneal cavity of PA, MK, WC extract (3, 10 and 30 mg/kg) and Picroliv (1 mg/kg) treated and untreated/ control mice, washed with incomplete RPMI medium and adjusted to a concentration of 1×10^6 cells/ml. The macrophages were washed and re-suspended in PBS. ROS levels were determined by using DCF-DA fluorescent dye read on FACS. Bars represent mean ± SE.

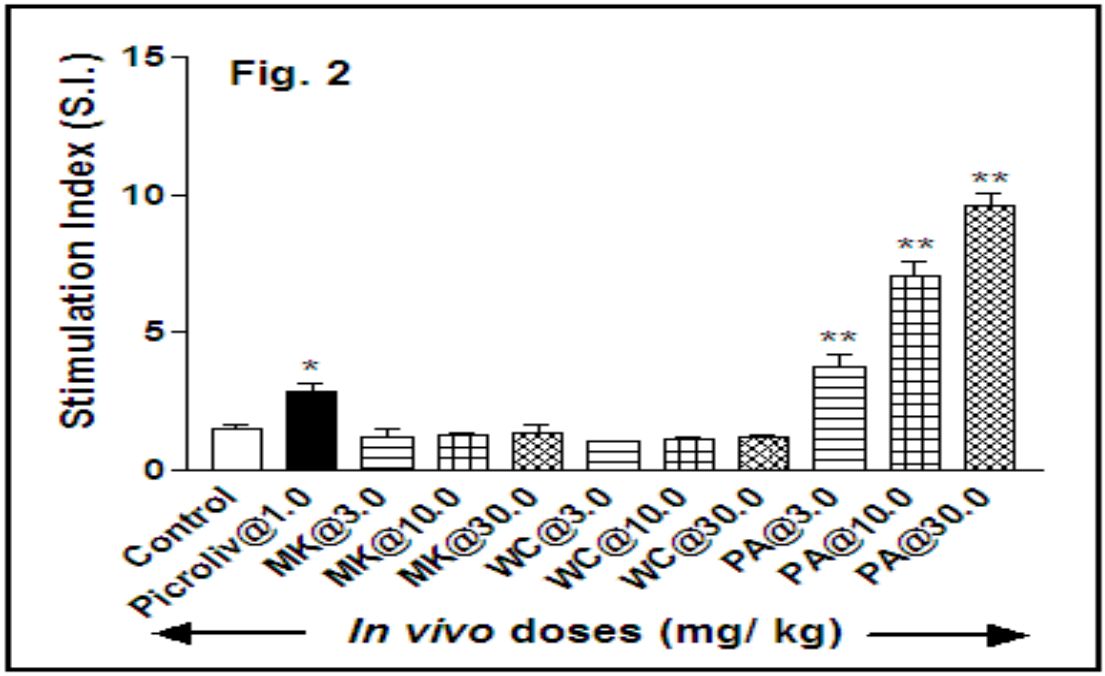

Fig. 2: Effect of PA, MK, WC ethanolic extracts (3, 10 and 30 mg/kg) and Picroliv (1 mg/kg) on B-lymphocyte proliferation of treated animals. B-cell mitogen Lipopolysaccharide (LPS) was added *in vitro* to the splenic lymphocytes from the treated and untreated animals at an optimal concentration of 2.5 µg/ml and the results are expressed as stimulation indices (S.I.). Bars represent mean ± SE values.

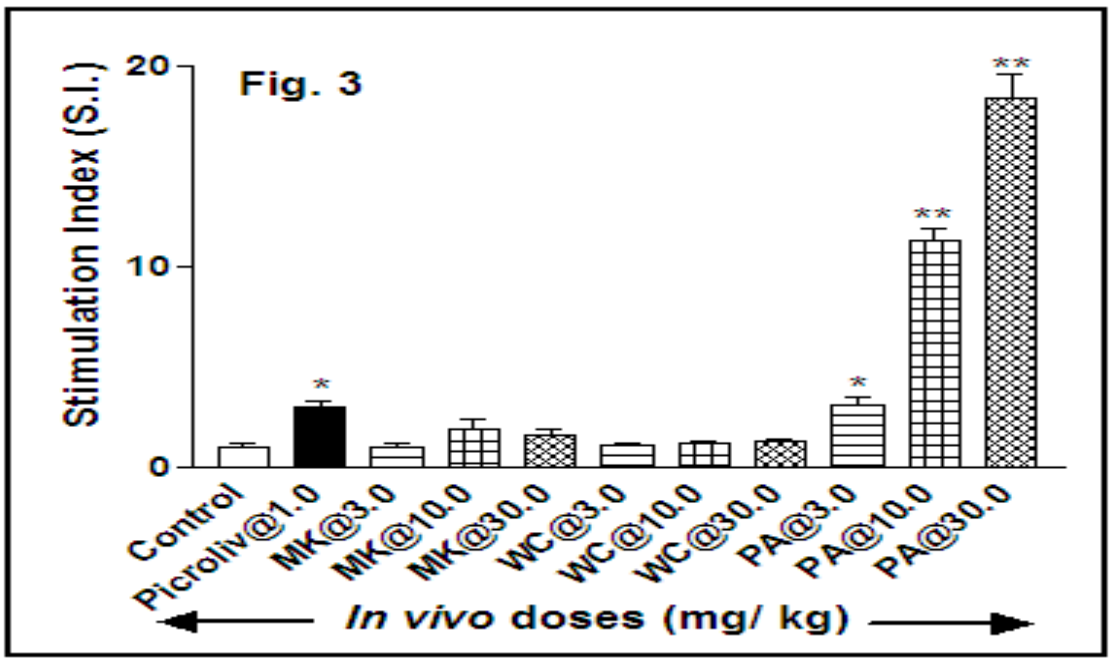

Fig. 3: Effect of PA, MK, WC ethanolic extracts (3, 10 and 30 mg/kg) and Picroliv (1 mg/kg) on T-lymphocyte proliferation of treated animals. T-cell mitogen Concanavaline A (Con A) was added *in vitro* to the splenic lymphocytes of treated and untreated animals at an optimal concentration of 2.5 µg/ml and the results are expressed as stimulation indices (S.I.). Bars represent mean ± SE values.

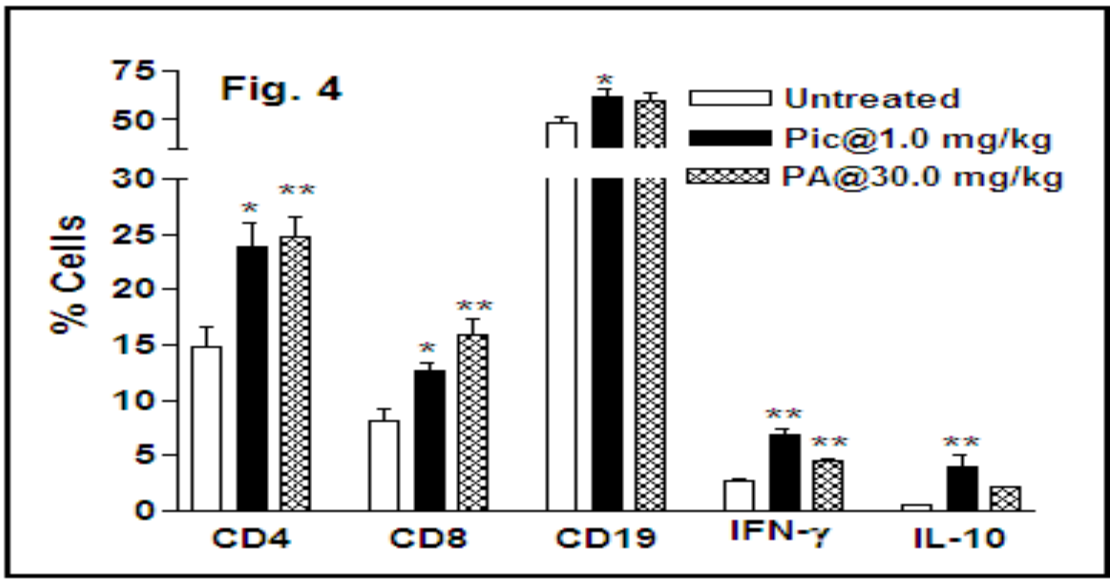

Fig. 4: Flow cytometric detection of splenic CD4+, CD8+ T cell sub-populations, CD19+ B-cell population and production of intracellular cytokines IFN-γ and IL-10 in mice fed with PA ethanolic extract (30 mg/kg) and Picroliv (1 mg/kg). Bars represent mean ± SE values.

DISCUSSION

Side effects and expenses associated with present allopathic drugs have necessitated the need to search alternative chemical entities from natural sources which are anticipated to possess minimal allergic or other side effects. The need of a better and safe immune modifier drug impelled us to look for pharmacological activities in some of the Indian medicinal plants. Search for immunomodulatory agents is highly desired in various diseased conditions especially those which affect the immune machinery of the host either due to disease pathogens or other medical interventions. We selected three medicinally pertinent plants *Withania coagulans*, *Murraya koenigii* and *Pterospermum acerifolium*. Leaves of *Murraya koenigii* are commonly used as flavoring agent in Indian curry preparations since ancient times. The Indian *M. koenigii* and Chinese *M. paniculata* are the two species available and both have some common medicinal properties. We earlier reported remarkable immunosuppressive activity in the extracts prepared from the seeds of *Pterospermum acerifolium* (PA) [11].

This finding led us to check other parts of this plant (flowers) for immunomodulatory efficacy. The current findings indicated strong immune stimulatory activity in flowers of PA. The ethanolic extract of PA flowers when fed orally to BALB/c mice caused considerable proliferation of both T and B lymphocytes and activated peritoneal macrophages. Apart from PA two other traditional medicinal plants;

MK (ethanolic extract of leaves) and WC (ethanolic extract of fruits) were also investigated for immunomodulatory action. Since the MK & WC extracts in preliminary evaluation did not induce significant ROS production (except WC at 30 mg/kg) or proliferation of splenic B and T lymphocytes at all the doses tried, these two preparations were not followed further. It is difficult to say whether these two plants lacked immunomodulatory properties or it was because of absence of active chemical moieties in the part of the plant used for evaluation or the nature of chemical constituents present due to the type of solvent used in extraction process[26] as in case of anti-inflammatory properties of *Calotropis procera* where differences in anti-inflammatory response was reported with different extracts of the same plant part[27]. Shah and Juvekar[28] reported no effect of methanolic extract of MK leaves on macrophage function including oxidant activity while the same authors reported macrophage activation, nitric oxide up-regulation and augmentation in the antibody level when aqueous extract of the same part of plant (leaf) was investigated[29]. The seed part of PA extracted with ethanol or hexane solvent revealed immunosuppressive activity in mice in our earlier report[11]. On contrary, flower portion extracted with ethanol when used in the present investigation demonstrated considerable immunostimulatory action inducing ROS production, T and B cell proliferation, and augmented expression of T and B cell CD antigens with production of both Th1 and Th2 cytokines. These findings therefore indicate the presence of diverse active constituents in both flowers and seeds which may be isolated with different solvent/s. Macrophages play an important role in the defense mechanism against host infection and the killing of tumor cells. Phagocytosis by activated macrophages is accompanied by the production of O^{2-} and H_2O_2 as well as the release of lysosomal enzymes such as acid phosphatase; these products are involved in killing and digesting microbial pathogens[30, 31]. The helper and cytotoxic T cells were also increased after treatment with PA flower extract. It is to be noted that in folklore medicine, PA leaf and bark extracts find their use as an anti-inflammatory agent. The current findings indicate stimulation of oxidative burst in APCs indicating the lack of anti-inflammatory action as also evidenced by increasing concentration of pro-inflammatory cytokine IFN-y. CD4+ T cells recognize antigens presented by the major histocompatibility complex class II (MHC II) proteins and mediate both cellular and humoral immune responses through Th1 and Th2 cytokines respectively while CD8+ T cells recognize antigens presented by MHC I molecules and mediate cellular immune responses through cytotoxic T cells. In the present study, significant up regulation of the CD4+ and CD8+ positive cell population after PA treatment was observed. These results showed that PA targets both the Th1 and Th2 cells and thus might be useful for treating pathological disorders where both pro-inflammatory and anti-inflammatory immune arms are compromised.

CONCLUSION

The ethanolic extract of *Pterospermum acerifolium* flowers possessed remarkable immunostimulatory properties while the ethanolic leaf extract of *Murraya koenigii* and ethanolic fruit extract of *Withania coagulans* did not demonstrate any significant immunostimulatory activity. PA has both immune stimulatory (flowers) and suppressive (Seeds) constituents. The part of the plant and the solvent used for extraction therefore appears crucial for getting the desired bioactivity. The findings reveal the presence of biologically active molecule/s in PA flowers having immunomodulatory properties which may find their use in immunosuppressed conditions or as adjuvant to candidate vaccine molecules and therefore, molecular targets of PA and the single molecule/s responsible for the above activity may further be investigated. Efforts are underway to purify the extract further in a bid to isolate chemical constituents and locate the biological activity in a single molecule through bioactivity guided fractionation and bio-evaluation.

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