International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 8, Issue 7, 2016

Original Article

# IMMUNOMODULATORY ACTIVITY OF *INDIGOFERA TINCTORIA* LEAF EXTRACT ON *IN VITRO* MACROPHAGE RESPONSES AND LYMPHOCYTE PROLIFERATION

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*Received: 17 Nov 2015 Revised and Accepted: 17 May 2016*

**ABSTRACT**

**Objective:** In this study the effects of ethanolic and aqueous extracts of *Indigofera tinctoria* were investigated on the functions of peritoneal macrophages and peripheral blood lymphocytes isolated from Wistar rats through *in vitro* studies.

**Methods:** Macrophage functions were determined through Nitric oxide (NO) estimation, Arginase production, lysosomal, phagocytic activities and analysis of proinflammatory cytokines (TNF-α and IL-1β) production. The lymphocytes proliferation was analyzed by MTT assay.

**Results:** The results indicated that both ethanolic and aqueous extracts of *I. tinctoria* were enhanced nitric oxide (NO) production at 50 µg/ml whereas it suppressed the production of arginase at 100 µg/ml by the macrophage. Moreover lysosomal and phagocytic activity of the macrophage was enhanced by the ethanolic and aqueous extracts at a dose of 100 µg/ml. The extracts also enhanced the production of pro-inflammatory cytokines by the macrophages. With the lymphocytes, the proliferation was significantly increased by ethanolic and aqueous extracts in the $EC_{50}$ value of 32.27 µg/ml and 49.06 µg/ml after 48 h incubation.

**Conclusion:** Collectively the results demonstrated that both extracts of *I. tinctoria* enhanced innate as well as adaptive immune response and proved the immunostimulating potential of the plant *I. tinctoria*.

**Keywords:** *Indigofera tinctoria*, Immunomodulation, Macrophage, Lymphocyte proliferation

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## INTRODUCTION

The immune system is a complicated network of cells and tissues working together to distinguish self-particles from non-self invaders such as microorganisms [1]. Plant-derived compounds, bacterial products, synthetic drugs and marine compounds have been used as immunomodulation agents. However, plant-derived compounds also have an important role as compared to many of the other immunomodulators because of their broad spectrum of therapeutic properties and low toxicity [2]. The key components of an immune system are lymphocytes and macrophages. Modulation of lymphocyte and macrophage activities plays a crucial role in many disease conditions [3]. Conversely, modulation by drugs/exogenous substances can also be used to counter many of these pathologic states.

Adaptive immunity mainly works through lymphocytes. The importance of lymphocyte function in the body defense against infection, self-tolerance, tumor surveillance and controlling metabolic disorders (in non-immune tissues) is well-documented [4]. In the innate immune system activated macrophages play a vital role in responses by way of phagocytosis, generation of toxic oxygen and nitrogen radicals, formation/release of nitric oxide (NO) and secretion of various endogenous mediators including pro-inflammatory cytokines and prostaglandins (PG) [5]. Further, macrophages are involved in adaptive immunity by their own activation by cytokines and other co-stimulatory molecules in cell-mediated immune response [6, 7].

Plant-derived compounds have exhibited numerous beneficial therapeutic applications, and there has been increasing attention as to mechanisms involved in these processes of modulation of host immune responses [8]. *Indigofera tinctoria* is a shrub that belongs to the family *Fabaceae* and its various parts have been traditionally used in Indian and Chinese medicinal system for the treatments of various ailments. For example, its roots and leaves have been used to treat epilepsy and hydrophobia [9]. Aerial parts have been evaluated for use as antiproliferative agent's activity against lung cancer (10). Aqueous extracts have been shown to impart antioxidant effects *in vitro* (11). Analyzes have suggested that indirubin and indigotin are the active compounds in the leaves of *I. tinctoria* [12, 13].

Even with this growing background on the immunomodulatory potentials of *I. tinctoria* more specific effects on the key immune cell types, i.e., macrophages and lymphocytes remain somewhat unknown. Accordingly the present work was undertaken to study the effects of ethanolic and aqueous extracts from *I. tinctoria* leaves on Wistar albino rat peritoneal macrophage and peripheral blood lymphocyte functions following *in vitro I. tinctoria* extract exposures.

## MATERIALS AND METHODS

### Animals

Wistar rats (male, 180-200 g) were purchased from Tamil Nadu Veterinary and Animal Science University (Chennai, India). All animals were maintained in a pathogen-free animal house (University of Madras) under normal conditions (21±2 °C, 12-h light: dark cycle) and provided *ad libitum* access to standard rodent chow and filtered water. The Institutional Animal Ethics Committee (IAEC) of the University of Madras approved this study.

### Preparation of plant extracts

*Indigofera tinctoria* plant material was collected from Tamil Nadu; samples were placed in a repository and authenticated by Dr. P. Jeyaraman, Director of Plant Anatomy Research Institute, Tambaram, Chennai, Tamilnadu, India. Collected plant leaves~1( kg) were washed, shade-dried and then homogenized. For the latter, an aqueous extract (10 %) was prepared in double-distilled water with stirring at 4 °C for 24 h. The extract was filtered, lyophilized and stored at-20 °C for further analysis. The ethanolic extract was prepared using a Soxhlet apparatus; isolated materials were dried in a rotary evaporator under reduced pressure. Average yields for the aqueous and ethanolic extracts were 12-14 and 15-18% respectively.

Working solutions of each extract were prepared in the range of 5, 10, 25, 50, 75 and 100 µg/ml by dissolving the aqueous material in

double-distilled water and the ethanolic extract in 10% dimethyl sulfoxide (DMSO; SRL Chemicals, Mumbai, India).

**Macrophage isolation from peritoneal fluid**

Peritoneal macrophages (PEM) were isolated from peritoneal rat cavities using ice-cold sterile phosphate-buffered saline (pH 7.4) (14). Rats were euthanized by cervical dislocation their abdominal cavities were visualized and then 5 ml ice-cold PBS was introduced into the cavity. After gentle massaging the fluid was withdrawn and placed in a centrifuge tube held on ice. The process was repeated five times for each rat and fluids were pooled. After centrifugation of the pooled fluid (3000 rpm, 10 min, 4 ℃) each cell pellet was suspended in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (100 U/ml) (all chemical supplies from Sigma, Chennai) and allowed to adhere for 3 hr at 37 °C in 5% $CO_2$ humidified incubator. Thereafter, non-adherent cells were washed away with medium and the adherent cells then collected for use in the various assays outlined below.

**Analysis of cell viability**

The effects of *I. tinctoria* extracts on PEM viability were evaluated using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyl-tetrazolium bromide) assay [15]. In brief, PEM were placed into wells of a 96-well plate (5 x $10^6$ cells/well) and then received *I. tinctoria* ethanolic and aqueous extracts (5-100 µg/ml); control wells received medium only. After 48 h incubation at 37 °C, a solution of MTT (20 µl; 5 mg/ml in PBS) was added to the wells and the cells cultured a further 4 h. Thereafter, 100 µl DMSO was added to each well to dissolve the formazan particles that had formed in live cells. The plates were then analyzed by measures of absorbance at 540 nm in a plate reader (BioTek, Winooski, VT). Viability was expressed as a percentage (%) compared to the positive control (absence of any extract).

**Determination of nitric oxide (NO) production**

PEM NO production was determined using a Griess reaction [16]. In brief, PEM were placed into wells of a 96-well plate (5 x $10^6$ cells/ well) and then received *I. tinctoria* ethanolic and aqueous extracts (5-100 µg/ml); control wells received medium only. After 48 h incubation at 37 °C the cell-free culture medium was removed from each well. This material (100 µl) was then combined with an equal volume of Griess solution (1% sulfanilamide, 0.1% naphthyl ethylenediamine in 5% phosphoric acid) and incubated at room temperature for 10 min. Thereafter, the absorbance was measured at 540 nm in the plate reader and concentrations of NO calculated by extrapolation from a standard curve prepared in parallel using sodium nitrite standards.

**Arginase activity assay**

The PEM arginase activity was evaluated by analyzed the content of urea [17]. In brief, PEM were placed into wells of a 96-well plate (5 x $10^6$ cells/ well) and then received *I. tinctoria* ethanolic and aqueous extracts (5-100 µg/ml); control wells received medium only. After 48 h incubation at 37 °C culture media were removed, and the remaining adherent cells were lysed by addition of 100 µl of a solution of 0.1% Triton X-100 (an incubation for 10 min at room temperature). Thereafter, 100 µl of Tris-HCl (25 mM) and 50 µl $MgCl_2$ (10 mM) solutions were added to the lysate in each well and the plates were incubated at 56 °C for 10 min. Finally, 50 µl of a solution of L-arginine (0.5 M, pH-9.7; Sigma) was combined with 50 µl of the cell lysate that had been transferred to a well in a new 90-well plate, and the mixture was incubated for 60 min at 37 °C. The reaction was stopped by addition of 400 µl ml of a mixture of concentrated sulfuric and phosphoric acid in water (1:3:7, v/v/v) to each well. The concentration of urea in the solution was then evaluated by measures at 570 nm in the plate reader. One unit of enzyme activity (1 U) was designated as the amount of arginase that had catalyzed the formation of 1 M urea/minute (i.e., over the 60-min reaction period). All data were then reported as U/ml lysate.

**Pinocytic activity assay**

PEM pinocytic activity was analyzed by neutral red uptake, according to the methods of Plytycz *et al.,* [18]. In brief, PEM were placed into wells of a 96-well plate (5 x $10^6$ cells/well) and then received *I. tinctoria* ethanolic and aqueous extracts (5-100 µg/ml); control wells received medium only. After 48 h incubation at 37 °C culture media were removed and 0.1% neutral red solution was added to each well (100 µl/well). The plates were then incubated for 4 h at 37 °C. After this period, the medium was removed and the macrophages were washed twice with PBS; the cells were then treated with 100 µl cell lysing solution (10 mM Tris buffer, pH 8.0)/well and the plates were incubated for another 2 h at 37 °C. The absorbance at 540 nm was then measured in each well using the plate reader. Pinocytic activity was expressed in terms of absolute OD values (reflecting dye uptake).

**Lysosomal activity assay**

Phosphatase-enhancing activity in macrophages was determined according to the method of Suzuki *et al.,* [19]. In brief, PEM were cultured (5 x $10^6$ cells/well of a 96-well plate) with different concentrations of ethanolic and aqueous extracts of *I. tinctoria* (5-100 µg/ml) for 48 h at 37 °C. Thereafter, the macrophages received 25 µl 0.1% Triton X-100, 150 µl of a *p*-nitrophenyl phosphate (pNPP, 10 nM) and 50 µl of citrate buffer (0.1 M, pH 5.0) then the plates were incubated for 1 hr at 37 °C. At that point, 50 µl borate buffer (0.2 M, pH 9.8) was added to each well and after 10 min phosphatase activity (i.e., levels of pNP formed) by the macrophages was assessed using absorbance measured at 405 nm in the plate reader. By comparing the relative $OD_{405}$ values between the control and treated cultures the percentage (%) change in lysosomal activity in the cells was calculated.

***In vitro* phagocytic assay (nitroblue tetrazolium (NBT) reduction test)**

NBT reduction assay was carried out according to the method of Rainard [20]. In brief, PEM (5 x $10^6$ cells/well of a 96-well plate) were cultured with different concentrations of ethanolic and aqueous extracts of *I. tinctoria* (5-100 µg/ml) for 48 h at 37 °C. Thereafter 20 µl yeast suspension (5 × $10^7$ cells/ml in PBS) and 20 µl nitroblue tetrazolium (1.5 mg/ml) (NBT (Hi-Media, Mumbai) in PBS were added to each well. Wells that received PBS+DMSO (adjusted to 0.1% (v/v)) were used as controls. Cells were then incubated for 60 min at 37 °C; the supernatant was then removed and the adherent macrophages rinsed with RPMI 1640. The cells were air-dried before 120 µl of 2M KOH and 140 µl DMSO were added to each well. The absorbance of the turquoise blue solution was measured at 570 nm ($OD_{570}$) in the plate reader. Percentage NBT reduction (reflecting phagocytic activity) was calculated as following equation:

$$\text{Phagocytic index} = (OD_{sample} - OD_{control})/OD_{control} \text{ X } 100.$$

**Pro-inflammatory cytokine assay**

In brief, PEM were placed into wells of a 96-well plate (5 x $10^6$ cells/ well) and then received *I. tinctoria* ethanolic and aqueous extracts (5-100 µg/ml); control wells received medium only. After 48 h incubation at 37 °C, the culture medium was isolated, and levels of pro-inflammatory cytokines TNF-α and IL-1β in the supernatants were analyzed using commercially available ELISA kits (Santa Cruz Biotechnology, Texas, USA) according to manufacturer instructions. The level of sensitivity of the kits was 80 pg TNF-α/ml and 25 pg IL-1β/ml.

***In vitro* lymphocyte proliferation**

Lymphocyte proliferation was evaluated using the colorimetric MTT assay [21]. In brief, peripheral blood lymphocytes (PBL) were isolated from the Wistar rat blood using Ficoll-Hypaque density gradient centrifugation [22]. The lymphocytes were collected from the interface and thoroughly washed twice with RPMI 1640. After the viability of the cells was checked (using trypan-blue dye exclusion) and the cells (5 × $10^6$ cells/ml) were placed into 96-well plates and then received different concentrations of the ethanolic and aqueous extracts of *I. tinctoria* (5-100 µg/ml). Positive control wells received phytohemagglutinin (PHA, Sigma); negative control wells (to assess spontaneous/background proliferation) received medium only. All cells were then cultured for 48 h in a 5% $CO_2$ humidified incubator. At the end of this period, MTT (20 µl (5 mg/ml

in PBS)) solution was added to the wells and the cells cultured a further 4 h. Thereafter, 100 µl DMSO was added to each well to dissolve the formazan particles. The plates were then analyzed by measures of absorbance at 540 nm in the plate reader after subtracting for background proliferation (negative control cells) results were expressed as a percentage of proliferation (%) compared to the positive (PHA) control cells.

**Statistical analysis**

All data are presented as means±SD. The statistical analysis of the experimental results was performed using a one-way analysis of variance (ANOVA) followed by a Dunnett's test or Tukey's multiple comparisons tests wherever applicable. All analyses were done using Prism statistical software (GraphPad, San Diego, CA). A p-value<0.05 was considered significant.

**RESULTS**

**Effect of extracts on peritoneal macrophage viability**

The toxicity of each extract against the PEM was evaluated using an MTT assay. At the highest test dose (100 µg/ml) the ethanolic extracts resulted in 97% viability; aqueous extract at this dose did not affect cell viability after 48h (fig. 1).

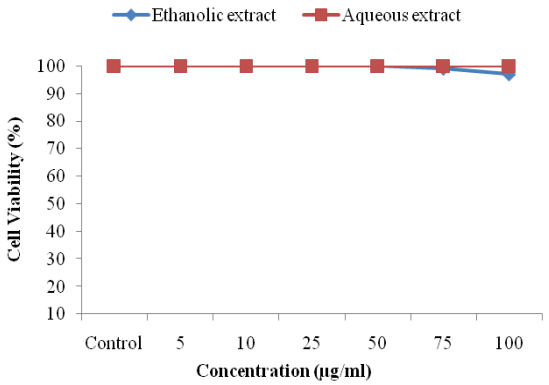


**Fig. 1: Peritoneal macrophage viability treated with various concentrations of ethanolic and aqueous extracts of *I. tinctoria* for 48 h. Values shown are mean±SD (triplicate samples per dose)**

**Extract effects on nitric oxide (NO) production**

Nitric oxide (NO) formation as an indicator of macrophage activation was evaluated in the treated PEM (fig. 2). The results indicated that NO production by PEM incubated with either extract for 48 h was significantly increased in a dose-related manner up to 50 µg/ml. At this dose maximal enhanced NO production was noted, i.e., 70±5 and 66±3 µmol/ml for the ethanolic and aqueous extracts respectively. Above this dose, there was a trend to lower in NO formation. Control PEM NO production was 20±3.0 µmol/ml.

**Extract effects on arginase activity**

The effect of the extracts on PEM arginase activity was evaluated by monitoring cell formation of urea (fig. 3). The results showed both extracts could cause decreases in arginase activity with increasing concentrations, with maximal inhibition occurring with the 50 µg/ml levels. Use of 5, 10, 25, 50, 75, and 100 µg/ml ethanolic extract resulted in arginase activity levels of 7.65±0.03, 7.57±0.08, 6.00±0.02, 5.71±0.07, 6.01±0.08 and 7.73±0.08 U/ml lysate respectively. With dose-matched treatments of the aqueous extracts, the levels were respectively 8.00±0.02, 7.85±0.05, 7.02±0.03, 6.81±0.08, 6.95±0.09 and 7.07±0.03 U/ml lysate. Control cells had an activity level of 9.714±0.12 U/ml lysate.

**Extract effects on pinocytic activity**

PEM pinocytic activity was determined by measures of neutral red dye uptake. As shown in fig. 4 the level of dye uptake by the cells increased with increasing concentrations of either extract in a seemingly dose-related manner. At some of the doses, significant differences were observed between the treated and control PEM. Specifically, while control cells had average $OD_{540}$ values of 0.190±0.007, cells treated with the maximal level of the ethanolic and aqueous extracts had values of respectively 0.593±0.013 and 0.512±0.015.

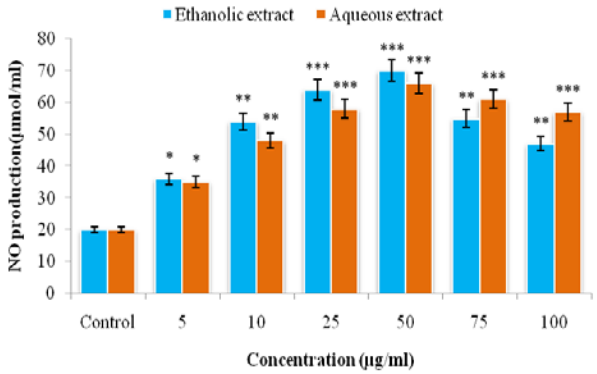


**Fig. 2: Nitric oxide (NO) production of peritoneal macrophage treated with various concentrations of ethanolic and aqueous extracts of *I. tinctoria* for 48 h. Values shown are mean±SD (triplicate samples per dose). Value significantly different from corresponding control at **p*<0.05, ***p*<0.01, *** *p*<0.001**

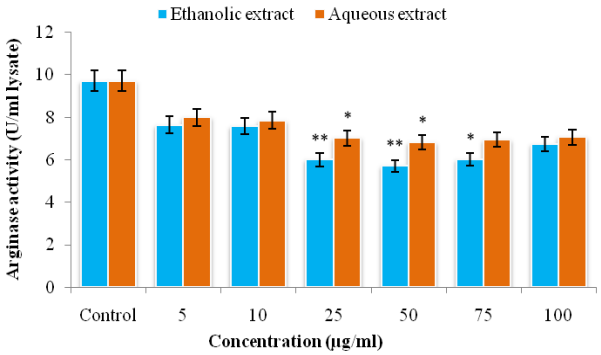


**Fig. 3: Arginase activity of peritoneal macrophage treated with various concentration of ethanolic and aqueous extracts of *I. tinctoria* leaves on for 48 h. Values shown are mean±SD (triplicate samples per dose) Value significantly different from corresponding control at **p*<0.05, ***p*<0.01, *** *p*<0.001**

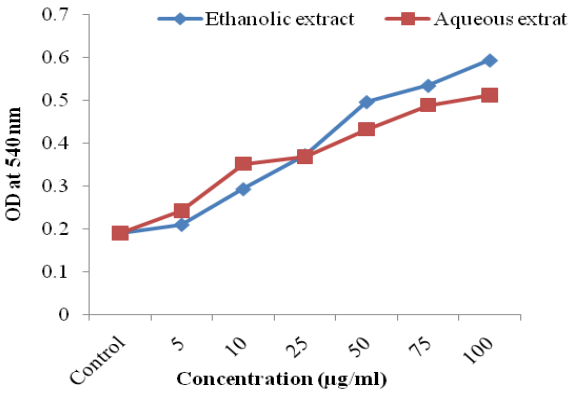


**Fig. 4: Pinocytic activity of peritoneal macrophage treated with various concentration of ethanolic and aqueous extracts of *I. tinctoria* leaves on for 48 h. Values shown are mean±SD (triplicate samples per dose)**

**Extract effects on lysosomal activity**

The intracellular lysosomal activity of PEM was determined by measures of acid phosphatase activity. Results are shown in fig. 5 illustrate how the ethanolic and aqueous extracts caused enhanced intracellular lysosomal activity significantly so as the dose levels increased. Control cells had an activity level of 2.39±0.1%. Use of doses of 5, 10, 25, 50, 75 and 100 µg/ml ethanolic extract resulted in values of 11.4±0.8, 32.4±0.1, 39.0±0.4, 60.0±0.2, 63.8±0.2 and 72.4±0.1% respectively. With dose-matched treatments of the aqueous extracts, the increments were respectively 2.8±0.4, 5.7±0.4, 20.0±0.2, 28.6±0.1, 42.8±0.4 and 58.2±0.1 %.

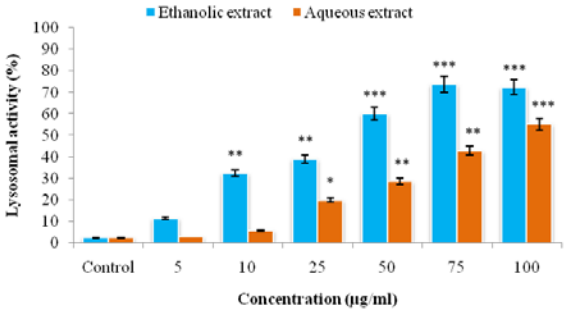


**Fig. 5: Lysosomal activity of peritoneal macrophage treated with various concentration of ethanolic and aqueous extracts of *I. tinctoria* leaves on for 48 h. Values shown are mean±SD (triplicate samples per dose). Value significantly different from corresponding control at **p*<0.05, ***p*<0.01, *** *p*<0.001**

**Extract effects on phagocytosis**

PEM phagocytic activity was determined by measures of NBT reduction. After 60 min incubation with yeast cells and NBT the effects of the ethanol and aqueous extracts of *I. tinctoria* on phagocytic indices of the cells were evaluated. The results showed that following exposure to the ethanol and aqueous extracts PEM phagocytic activity was increased significantly so as the dose levels increased (fig. 6). Control cells had an index of 52±2 %. Use of doses of 5, 10, 25, 50, 75, and 100 µg/ml ethanolic extract resulted in indices of respectively 55±3, 63±4, 70±4, 72±3, 78±1 and 80±3 %. In studies with the aqueous extract the resulting dose-matched values were 50±2, 59±1, 65±6, 68±3, 72±4 and 72±2 %.

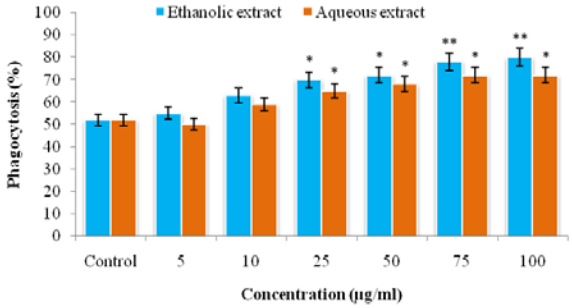


**Fig. 6: *In vitro* phagocytic assay on nitroblue tetrazolium (NBT) reduction test of peritoneal macrophage treated with various concentration of ethanolic and aqueous extracts of *I. tinctoria* leaves on for 48 h. Values shown are mean±SD (triplicate samples per dose). Value significantly different from corresponding control at **p*<0.05, ***p*<0.01, *** *p*<0.001**

**Extract effects on production of pro-inflammatory cytokines**

Ethanolic and aqueous extracts of *I. tinctoria* affected the production of pro-inflammatory cytokines TNF-α and IL-1β by PEM, significantly so as the dose levels increased (fig. 7A & B). Compared to the control (spontaneous formation) the lowest (5 µg/ml) dose of each extract caused insignificant nominal increases in TNF-α and IL-1β release. By comparison 100 µg/ml doses resulted in TNF-α formation/release levels of 6.5±0.30 (ethanolic) and 5.2±0.28 ng/ml (aqueous) and IL-1β formation/release levels of 660±10 (ethanolic) and 460±12 pg/ml (aqueous).

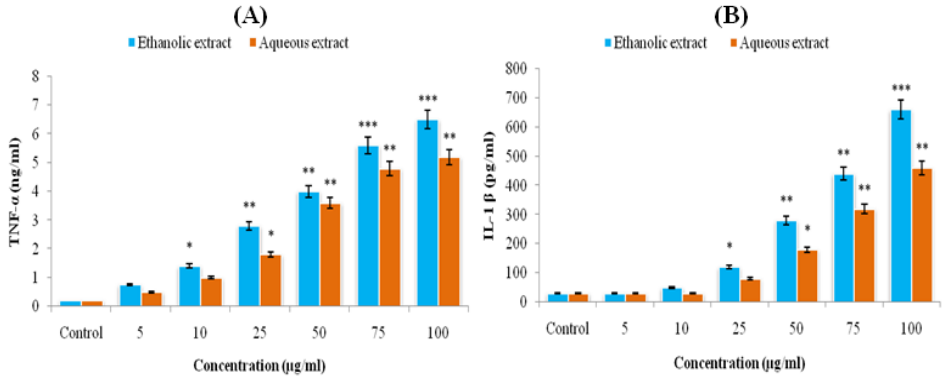


**Fig. 7: Pro inflammatory cytokine production of peritoneal macrophage treated with various concentration of ethanolic and aqueous extracts of *I. tinctoria* for 48 h (A) TNF-α (B) IL-1 β. Values shown are mean±SD (triplicate samples per dose). Value significantly different from corresponding control at **p*<0.05, ***p*<0.01, *** *p*<0.001**

**Extract effects on lymphocyte proliferation**

The proliferation index of rat peripheral blood lymphocytes exposed to each extract was evaluated (fig. 8). Both the ethanolic and aqueous extracts of *I. tinctoria* enhanced lymphocyte proliferation. The ethanolic extracts enhanced proliferation (relative to spontaneous levels by the cells) to 38.2±0.3, 44.6±0.4, 50.8±0.5, 65.2±0.7, 70.9±0.6 and 83.2±0.1 % at respectively by 5, 10, 25, 50, 75 and 100 µg/ml doses. Aqueous extracts at these same doses enhanced the proliferation levels to 15.1±0.5, 20.7±0.2, 36.7±0.1, 46.4±0.9, 51.9±0.7 and 53.4±0.9 % respectively. There was no significant spontaneous proliferation among the control cells.

**DISCUSSION**

The modulation of cell immune response by molecules from the medicinal plants is an interesting area for inflammation, autoimmunity and anticancer therapy [23, 24]. Numerous plants are used in traditional medicinal system showed to stimulate or inhibit the immune responses [25]. Medicinal plants and their products are most useful in health and disease treatment through the involvement of various biological activities like anti-oxidant and anti-ulcerogenic activities [26]. Moreover several medicinal plants possess immunomodulatory activities as they impart anti-inflammatory, anti-microbial, and anti-tumor effects under varying

experimental conditions. Investigating the effects of plant substances that enhance or suppress macrophage and lymphocyte response gives evidence to study the immunomodulation and drug discovery [27].

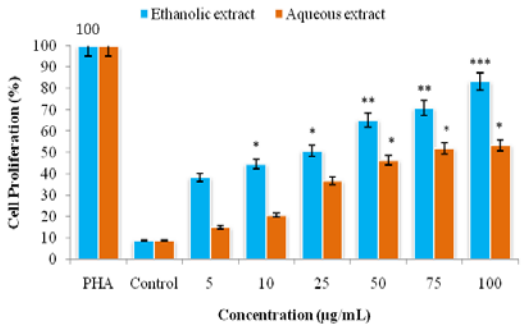


**Fig. 8: Proliferation of peripheral blood lyphocytes (MTT assay) treated with various concentration of ethanolic and aqueous extracts of *I. tinctoria* leaves on for 48 h. Values shown are mean±SD (triplicate samples per dose). Value significantly different from corresponding control at **p*<0.05, ***p*<0.01, *** *p*<0.001**

Nitric oxide is a toxic molecule produced by macrophage, which is toxic to disease-causing pathogens and also acts as signaling molecules in many biological functions such as immunomodulatory and cytotoxic activity against tumor cells. It acts as potent pre-oxidant molecules, which is able to induce oxidative damage against cellular targets [28]. Our results are correlated with Elmowalid *et al.,* [29] who reported that an aqueous extract of *Nigella saliva* seed enhanced NO production (to 102 µmol/ml at a dose of 10 µg/ml). Here, the ethanolic and aqueous extracts of *I. tinctoria* increased NO production by the PEM up to 70 and 66 µmol/ml respectively at a test dose of 50 µg/ml. Further, morin hydrate (a flavonoid) isolated from plant leaves of *Psidium gujava* increased macrophage NO production [2]. The results here suggested that the *I. tinctoria* plant extracts might impact on the pathogen killing capacity of macrophages.

Arginase is an essential regulator of NO production since both arginase and inducible NO synthase (iNOS) use L-arginine as a substrate. Importantly, iNOS synthesizes NO and arginase synthesizes ornithine that reduces production of NO by macrophages [30]. Interestingly the results here indicated that the ethanolic and aqueous extracts of *I. tinctoria* considerably decreased arginase activity and enhanced NO production by the peritoneal macrophages. These results were along the lines of those seen in another study wherein a polysaccharide from *Sipunculus nudus* decreased arginase activity and increased NO production by peritoneal macrophages [31].

The most important function of macrophage is pinocytic activity; it's enhancing the phagocytic activity of macrophages. Our result suggested the both ethanolic and aqueous extract of *I. tinctoria* leaves increase the pinocytic activity and indicated the strong phagocytic activity of macrophages. Similarly, Cheng *et al.,* [32] reported that the polysaccharides isolated from the *Glycyrrhiza uralensis* fish enhanced the pinocytic activity of peritoneal macrophages in a dose-dependent manner.

Acid phosphatase has the capacity to generate Reactive Oxygen species (ROS). In macrophage, the enhancement of phosphatase activity leads to enhance the ROS production and increased the bacterial killing activity of macrophages [33]. Our results suggested that the both ethanolic and aqueous extracts of *I. tinctoria* enhanced the intracellular lysosomal enzyme activity up to 72.38±0.1% and 58.24±0.1% at 100µg/ml of concentration. Our study correlated with Manosroe *et al.,* [34] reported that *Clausena excavate* acetone extract enhances the intracellular lysosomal activity of macrophages to 60% at 112.5 µg/ml.

Phagocytosis cells like neutrophil, monocytes and macrophages are key components of the innate immune response. However, Macrophages play a dominant role in phagocytosis which carried out the intracellular killing of antigen and other apoptotic cells. So, the modulation of macrophage activity seems to be the foremost role in regulation of innate immunity [35] Elmowalid *et al.,* [29] studied on aqueous extract of Nigella seed enhance the phagocytic activity to 98% at 40 µg/ml concentration. Manosroi *et al.,* [36] quoted that methanolic extract of *Poulteria cambodiana* enhance the phagocytic activity to 80% at 1.00 mg/ml concentration. *Clausena excavate* acetone extract increased the phagocytosis process of macrophages to 72% at 62.5 µg/ml concentration. Similarly, both ethanolic and aqueous extracts of *I. tinctoria* enhance the phagocyte activity to 80 % and 72 % at 100 µg/ml concentration.

Cytokines have immense power on the immune response through the activation, regulation, and differentiation of different cells against disease-causing microorganisms [31]. The proinflammatory cytokines like TNF-α deserve a vital role in the immune and inflammatory response. Similarly, the IL-1β is proinflammatory cytokines play a crucial role in fever during acute phase protein response [37]. Our results indicated that *I. tinctoria* plant extracts were probably enhanced the production of proinflammatory cytokines TNF-α and IL-1β by macrophages. In contrast, the aqueous extract of *Tournefortia sarmentosa* enhances the proinflammatory cytokine production by macrophages through *in vitro* [38].

Lymphocytes are the principal cells of the adaptive immune system involved in health and diseases. The previous studies also reported that host defense against pathogens and tumors were directly correlated with lymphocyte proliferation [39, 40]. In our findings, both ethanolic and aqueous extracts were enhanced the lymphocyte proliferation to 83.25% and 53.45% in 100 µg/ml concentration. In accordance with the previous study, methanolic extract of *Pouteria camodiana* enhances the proliferation of lymphocytes to 82% at 100 µg/ml concentration [36]. This result proved the nontoxicity of *I. tinctoria* extracts to the lymphocytes.

## CONCLUSION

The present findings showed that both the ethanolic and aqueous extracts of *I. tinctoria* did not cause direct toxic effects on isolated immune cells (at the doses tested) and suggested that they could potentially enhance innate as well as adaptive immune responses. This activity may be mediated through changes in lymphocyte proliferation and/or in the regulation of macrophage activation (NO production, lysosomal activity, phagocytosis, of pro-inflammatory cytokines release, arginase activity). These strong immuno-modulatory activities of *I. tinctoria* noted here *in vitro* help to explain some of the medicinal benefits attributed to the plant in traditional medicine and could be used to provide a future basis for the development of this plant as an immunoregulating substance.

## ACKNOWLEDGEMENT

The authors gratefully acknowledge UGC-UPE–PHASE II New Delhi (No. 2013/PFEP/C3/280) for financial support.

## CONFLICT OF INTERESTS

The authors declare that we have no conflicts of interest.

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