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## Immunomodulatory Activity of *Triticum aestivum* and its Effects on Th1/Th2 Cytokines and NFκB P 65 Response

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### ABSTRACT

*Triticum aestivum* which refers to the young grass of the common wheat plant has become extremely popular in treating a variety of health conditions. In this study we have investigated the immunomodulatory activity of *Triticum aestivum* water extract in Swiss albino mice and its effect on Th1/Th2 cytokine production by spleen cells. Female Swiss albino mice were challenged with Sheep Red Blood Cells (SRBC) and treated with either *Triticum aestivum* 540 mg kg<sup>-1</sup> or Prednisolone 5 mg kg<sup>-1</sup> body weight for 15 days. Blood was collected from retroorbital plexus to perform hematological, serological and bone marrow cellularity evaluation. Th1/Th2 cytokines and the p65 subunit of the nuclear factor kappa B (NFκB) were estimated in the splenocytes by ELISA from supernatants of splenocyte culture. Antioxidant activity of *Triticum aestivum* was assessed by using DPPH (2,2'-diphenyl-1-picrylhydrazyl) Assay. Water extract of *Triticum aestivum* was found to increase White Blood Cells (WBC), Red Blood Cells (RBC) and Hemoglobin (Hb) concentration in both normal and myelosuppressed Swiss albino mice. Furthermore, there was significant increase in bone marrow cellularity and hemagglutinin (antibody to SRBC) titer in animals treated with *Triticum aestivum* compared to control group. *Triticum aestivum* water extract upregulated Th1 cytokines (TNF-α, IL-2 and IFN-γ) and Th2 cytokine (IL4). In contrast, IL-1β (a Th1 cytokine) and P65 subunit of NFκB were suppressed in groups treated with *Triticum aestivum*. Moreover, *Triticum aestivum* extract restored Prednisolone suppressed TNF-α and IL-2 cytokines. *Triticum aestivum* appears to have a significant role in immunity and our findings confirm its beneficial role in hemoglobin concentration. Furthermore, the results on Th response suggest a potential role for *Triticum aestivum* in Th1 modulation and thus its potential role, as a candidate drug for inflammatory disorders including cancer management should be explored.

**Key words:** *Triticum aestivum*, immunomodulation, NFκB, cytokines, bone marrow cellularity, haemagglutination

### INTRODUCTION

Naturally occurring anti-inflammatory immunomodulators are extensively investigated for their implication in chronic diseases including cancer. *Triticum aestivum* a major food crop globally, is recognized as a co-adjuvant in cancer treatment (Bonfilia *et al.*, 2009). *Triticum aestivum* refers to the young grass of the common *Triticum aestivum* that is freshly juiced or dried into powder for animal and human consumption. It contains nearly all the nutrients, minerals and vitamins

necessary for human health. There are many claims about benefits of *Triticum aestivum* consumption ranging from promotion of general well-being to cancer prevention and heavy-metal detoxification. None of these claims have been substantiated in scientific literature, though there is some evidence in support of its beneficial effects (Ben-Arye *et al.*, 2002; Kothari *et al.*, 2011; Arya and Kumar, 2011). Because of the high chlorophyll content, it reduces the blood transfusion requirement in patients suffering with thalassemia and other diseases where blood transfusion is required (Marawaha *et al.*, 2004; Dey *et al.*, 2006; Mukhopadhyay *et al.*, 2009). It has been reported that *Triticum aestivum* lowers the mutagenic ability of chemical mutagens (Kulkarni *et al.*, 2006; Ferruzzi *et al.*, 2002) and reduces the side effects of chemotherapy (Bar-Sela *et al.*, 2006). In addition, the chlorophyll and fibre content of *Triticum aestivum* have been found to be effective in the treatment of colon cancer (Johan *et al.*, 2005). Inflammation and immunity play a significant role in the pathogenesis and progress of these diseases. T cells play a critical role in the pathogenesis of various diseases through the production of a variety of cytokines. Cytokines such as IL-4, IL-5 and IL-13 are known to influence a wide range of events associated with chronic allergic inflammation in local tissues (Kay, 2000). NF- $\kappa$ B controls many genes involved in inflammation and it is not surprising that NF- $\kappa$ B is found to be chronically active in many inflammatory diseases such as inflammatory bowel disease, arthritis, sepsis, gastritis, asthma, among others. Many natural products (including anti-oxidants) that have been promoted to have anti-cancer and anti-inflammatory activity have also been shown to inhibit NF- $\kappa$ B. In this context, earlier we have shown that the herb *Ocimum sanctum* commonly known as Holy Basil or sacred tulsi increased the production of TNF- $\alpha$ , IL-2, IFN- $\gamma$  and IL-4 significantly and decreased the production of IL-1 $\beta$  and NF- $\kappa$ B in mice (Hemalatha *et al.*, 2011). Further, studies carried out on various other herbs like *J. sinensis*, *P. corylifolia* and Cheong-a-hwan (used widely in Korean traditional medicine) showed an upregulation of OVA-specific Th1 cytokine (IFN- $\gamma$ ) and downregulation of OVA-specific Th2 cytokine (IL-4) in an ovalbumin-induced asthma animal model. These effects indicate that they may be a potential novel therapeutic agent for asthma (Lee and Kim, 2008). There is no information on the effects of *Triticum aestivum* directly on Th1/Th2 cytokines and NF $\kappa$ B p65 responses. However, its beneficial effect on chronic intestinal inflammation has been tested. As *Triticum aestivum* is supposed to have many health beneficial effects on immune related diseases, it is pertinent to understand its immunomodulatory activity. Therefore, the present study was carried out to explore the immunomodulatory activity of *Triticum aestivum* and its effects on T helper (Th1/Th2) cytokines and the transcription factor NF $\kappa$ B activity.

## **MATERIALS AND METHODS**

**Animals:** This animal study was conducted during the period 2009-2010 at National Centre for Laboratory Animal Sciences facility at National Institute of Nutrition (NIN), Hyderabad, India. The animal study protocols were approved by the Scientific Advisory Committee as well as Institutional Animal Ethics Committee of National Institute of Nutrition (NIN). Twenty four (24) female Swiss albino mice weighing 20-25 g were obtained and acclimatized at National Centre for Laboratory Animal Sciences for 1 week and maintained at 24 $\pm$ 2 $^{\circ}$ C, 50-60% relative humidity, with a 12 h light-dark cycle. They were accommodated in individual ventilated cages with stainless steel top grill with food and water spouts and closed bottom. Autoclaved paddy husk was used for bedding with weekly changes. They were fed a casein based (20% protein) pellet control diet.

**Plant material and extract preparation:** *Triticum aestivum* was purchased from herbal stores in Hyderabad and authenticated in Heritage Bio-Natural systems. Dried powder (100 g) was extracted in 300 mL of water for 24 h this was repeated two more times with same volume of water and time period. The fluid fraction of the three extractions was combined, concentrated and dried under vacuum. Accurately weighed quantities of the water extract of *Triticum aestivum* were suspended in 1% gum acacia to prepare a suitable dosage form. The dose levels of the extract were selected on the basis of the human dose and calculated to the rodent dose.

**Animals and treatment:** All the animals (n = 24) were sensitized with 0.5 mL of 20% of fresh sheep red blood cells suspension, injected intraperitoneally on day 0 and then were divided into four groups of six animals each. Animals in group I received the vehicle (2% Gum acacia) orally for a period of 15 days and served as control for other groups. Animals in group II received prednisolone which is used as a standard immunosuppressant at a dose of 5 mg kg<sup>-1</sup> in 2% gum acacia. Group III received *Triticum aestivum* water extract (540 mg kg<sup>-1</sup> in 2% gum acacia) and Group IV received *Triticum aestivum* (540 mg kg<sup>-1</sup>) along with prednisolone (5 mg kg<sup>-1</sup>) in 2% Gum acacia orally for 15 days. Blood samples were collected on 0 and 16th day of the experiment and the total White Blood Cell (WBC) count, Differential Count (DC), Red Blood Cell (RBC) count, haemoglobin concentration and platelet counts were determined using automated blood cell counter (Seimens Adna). Prednisolone was used as a standard immunosuppressant. Sheep Red Blood Cells (SRBC) were used as an antigen at the concentration of 20% for immunization and 2% for challenge.

## Methods

**Determination of the organ weight and bone marrow cellularity:** Animals were weighed after last dose of drug treatment and were sacrificed. Weight of vital organs such as liver, spleen, thymus and kidney were recorded and expressed as relative organ weights. Bone marrow was collected from femur in medium containing 2% Fetal calf serum. The number of bone marrow cells was determined using a haemocytometer and expressed as total live cells per femur.

**T cell dependent hemagglutinin antibody (HA) response:** All the four groups were sensitized with 0.5 mL of 20% of fresh sheep red blood cell suspension injected intraperitoneally on day 0. Blood samples were collected in microcentrifuge tubes from individual animal from retro-orbital plexus on the 16th day and serum was separated. Antibody levels were determined by haemagglutination technique (Hay *et al.*, 2002).

**Estimation of cytokines (IL-1 $\beta$ , IL-2, IL-4 IFN- $\gamma$ , TNF- $\alpha$ ) in Con-A stimulated Splenocytes:** Spleen was teased and dispersed and passed through a sterilized stainless sieve (200 mesh) to obtain a single-cell suspension. The cells thus obtained were washed twice with RPMI1640 medium and were stimulated with Concanavalin A in 24-well flat bottomed plates, by incubating for 24 h in 5% CO<sub>2</sub>. Cells were then centrifuged for 5 min at 1500 rpm and the cytokines were estimated in the cell supernatant by mouse multiplex ELISA Kit as recommended by the manufacturer. All the tests were performed in duplicates.

**Estimation of NF $\kappa$ B in the nuclear fraction of splenocytes:** Spleen cells 1.0 $\times$ 10<sup>7</sup> cells/mL were suspended in RPMI 1640, centrifuged at 300x g for 5 min at 4°C and the supernatant was discarded. The pellet was washed in 5 mL phosphate buffer and resuspended in 50  $\mu$ L of complete

nuclear extraction buffer. After centrifugation the P65 subunit of the NFκB was estimated in the supernatant by ELISA (Trans AM) Kit as recommended by the manufacturer. All the tests were performed in duplicates.

**DPPH radical assay:** DPPH assay was carried out by measuring absorbance at 517 nm using ethanol as blank. One milliliter of 0.3 mM DPPH diluted in ethanol was used as control. Inhibition of DPPH radical was calculated using the equation:

$$I (\%) = 100 \times (A_0 - A_s) / A_0$$

where  $A_0$  is the absorbance of the control (containing all reagents except the test compound) and  $A_s$  is the absorbance of the tested sample. The IC50 value represented the concentration of the test extract that caused 50% inhibition.

**Statistical analysis:** Statistical analysis was performed by using Graphpad prism 5.0 software for windows. Means were compared using Tukeys multiple comparison post-hoc test and a p-value of <0.05 was considered significant.

## RESULTS

Animals treated with prednisolone showed significant lowering of the hemoglobin concentration, red blood cell counts and total white blood cell counts compared to the control group. Differential white blood cell counts showed a relative lowering of the lymphocyte percentage and increase in neutrophils (data not shown). As expected, the bone marrow cellularity and antibody response to SRBC were impaired with prednisolone compared to the control group. However, spleen weight was comparable.

**Effect of *Triticum aestivum* on hematological parameters and bone marrow cellularity:** Treatment of normal animals with *Triticum aestivum* for 15 days increased red blood cell counts and hemoglobin concentration levels significantly compared to the control group. *Triticum aestivum* prevented the adverse effects of prednisolone and increased RBC count (7.87 millions/cmm) and hemoglobin (14.7 g dL<sup>-1</sup>) concentration significantly when compared to animals treated with

Table 1: Effect of *Triticum aestivum* on haematological parameters

Parameters	Vehicle control	Prednisone	<i>Triticum aestivum</i>	Prednisone + <i>Triticum aestivum</i>
WBC (10E3/mm <sup>3</sup> ) 3.2-12.7	4.72±0.47 <sup>a</sup> (4.74, 5.18)	2.97±0.52 <sup>b</sup> (2.48, 3.52)	6.35±0.26 <sup>c</sup> (6.39, 6.59)	6.23±0.40 <sup>c</sup> (6.01, 6.69)
RBC (10E6/mm <sup>3</sup> ) 7.0-10.1	8.45±0.11 <sup>a</sup> (8.34, 8.56)	5.81±0.88 <sup>b</sup> (5.15, 6.81)	10.55±0.80 <sup>c</sup> (9.78, 11.37)	7.87±0.41 <sup>c</sup> (7.91, 8.26)
Lymphocytes (%) 60.2-95.0	64.29±6.53 <sup>a</sup> (59.16, 62.07)	49.04±2.67 <sup>b</sup> (46.83, 48.29)	87.3±0.26 <sup>c</sup> (87.2, 87.6)	55±4.67 <sup>a</sup> (56.8, 58.5)
Hb (g dL <sup>-1</sup> ) 11.8-14.9	13.53±0.31 <sup>a</sup> (13.2, 13.8)	11.33±0.39 <sup>b</sup> (10.97, 11.74)	14.83±0.85 <sup>c</sup> (14.5, 15.8)	14.7±0.44 <sup>c</sup> (14.9, 15)

WBC : a vs. b = p<0.01; a vs. c = 0.01; b vs. c = 0.001, RBC : a vs. b = p<0.01; a vs. c = 0.05; b vs. c = 0.001, Lymphocytes: a vs. b = p<0.01; a vs. c = 0.001; b vs. c = 0.001, Hemoglobin (Hb): a vs. b = p<0.001; a vs. c = 0.001; b vs. c = 0.001, Values are Mean±SME, Tukeys multiple comparison post-hoc test: Different alphabets indicate significant differences and values with same superscript are not significant

Prednisolone alone (5.81 millions/cmm, 11.33 g dL<sup>-1</sup>, respectively). In addition, there was significant increase in bone marrow cellularity (1670×10<sup>4</sup> cells/ femur) in *Triticum aestivum* treated animals compared to control group (912×10<sup>4</sup> cells/ femur) (Table 1, Fig. 1a).

**T cell dependent antibody response to SRBC with *Triticum aestivum*:** The hemagglutinin (antibody response to SRBC) was significantly increased (1:1024) in the animals treated with the *Triticum aestivum* extract compared to the control group (1:256) and furthermore, *Triticum aestivum* extract prevented the adverse effect of prednisolone on hemagglutinin response (1:512) (Fig. 1b).

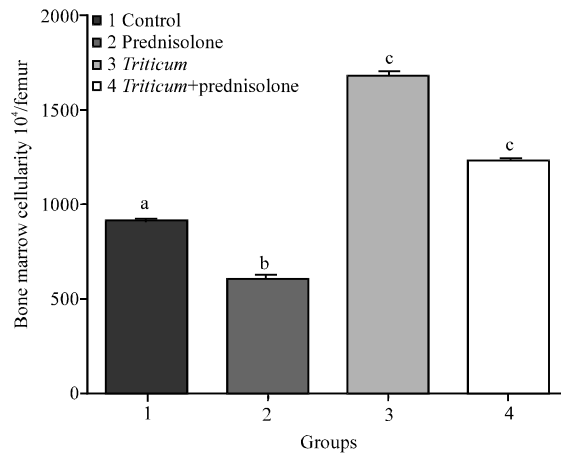


Fig. 1a: Effect of *Triticum aestivum* on bone marrow cellularity. Significances: a vs. b p<0.01; a vs. c p<0.001; b vs. c p<0.001

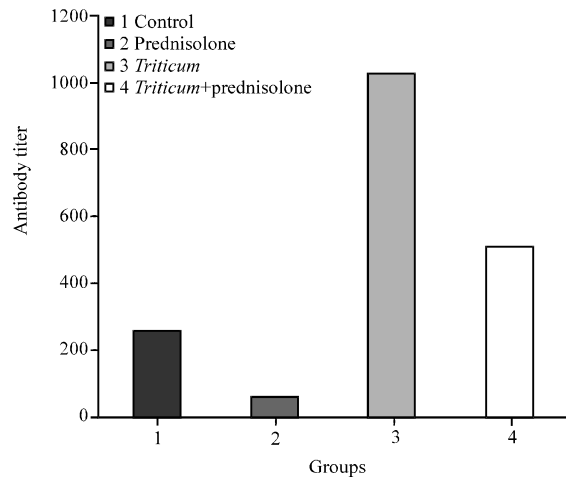


Fig. 1b: Effect of *Triticum aestivum* on hemagglutinin antibody response. The hemagglutinin (antibody response to SRBC) -- control group (1:256), Prednisolone treated group (1:64), *Triticum aestivum* treated group (1:1024) and prednisolone + *Triticum aestivum* treated group (1:512)

**Antioxidant activity of *Triticum aestivum*:** The investigated *Triticum aestivum* water extract showed substantial antioxidant activity. Scavenging of DPPH radical was concentration dependent, however the activity occurred at much higher concentration with *Triticum aestivum* extract (IC<sub>50</sub>: 140  $\mu\text{g mL}^{-1}$ ) compared to that of Ascorbic acid (IC<sub>50</sub>: 14  $\mu\text{g mL}^{-1}$ ) (Fig. 2a, b).

**Effect of *Triticum aestivum* on Th1 cytokines (IL-1 $\beta$ , IL-2, IFN- $\gamma$  and TNF- $\alpha$ ) and Th2 cytokine (IL-4) production from Splenocytes:** The levels of IL-1 $\beta$ , IL-2, IL-4 and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interferon gamma (IFN- $\gamma$ ) were quantified by ELISA from supernatants of splenocytes cultured with Concanavalin A. The Th1 cytokines IL 2 (85.43  $\text{pg mL}^{-1}$ ), TNF- $\alpha$  (25.33  $\text{pg mL}^{-1}$ ) and IFN- $\gamma$  (77.63  $\text{pg mL}^{-1}$ ) were significantly increased by *Triticum aestivum* (group III) compared with control (group I) (IL 2-7.40  $\text{pg mL}^{-1}$ , TNF- $\alpha$ -9.46  $\text{pg mL}^{-1}$ , IFN- $\gamma$ -11.76  $\text{pg mL}^{-1}$ ). Prednisolone (group II) had no effect on IFN- $\gamma$  production but suppressed the production of IL 2 (2.49  $\text{pg mL}^{-1}$ ) and TNF- $\alpha$  (0.55  $\text{pg mL}^{-1}$ ). However, the suppressive effect of prednisolone on IL-2 and TNF- $\alpha$  production was prevented when *Triticum aestivum* was given along with prednisolone (group IV). The Th2 cytokine, IL-4 production was also increased with *Triticum aestivum* treatment (4.75  $\text{pg mL}^{-1}$ ) compared to control (1.42  $\text{pg mL}^{-1}$ ); however, *Triticum aestivum* did not prevent the suppressive effect of prednisolone on IL-4 (0.63  $\text{pg mL}^{-1}$ ). Surprisingly, IL-1 $\beta$  was significantly decreased with *Triticum aestivum* (5.28  $\text{pg mL}^{-1}$ ) treatment compared to the control group (10.56  $\text{pg mL}^{-1}$ ) (Fig. 3). Prednisolone (1.11  $\text{pg mL}^{-1}$ ) and prednisolone with *Triticum aestivum* (0.77  $\text{pg mL}^{-1}$ ) caused more aggressive suppression of IL-1 $\beta$  production (Fig. 3).

**Effect of *Triticum aestivum* on the production of P65 (Rel A) of NF $\kappa$ B in the nuclear fraction of Splenocytes in Con-A stimulated Splenocytes:** To study the effect of *Triticum aestivum* on NF $\kappa$ B, splenocytes isolated from the mice treated with water extract of *Triticum aestivum* for 15 days were stimulated by con-A. The p 65 was impaired with *Triticum aestivum* water extract treatment. Both prednisolone and *Triticum aestivum* significantly decreased p 65 (rel A) concentration (2.49 and 1.23  $\text{mg mL}^{-1}$ , respectively) compared to the control (5.13  $\text{mg mL}^{-1}$ ). Even in group IV mice treated with both prednisolone and *Triticum aestivum* there was significant decrease in p 65 (rel A) concentration (1.49  $\text{mg mL}^{-1}$ ) (Fig. 4).

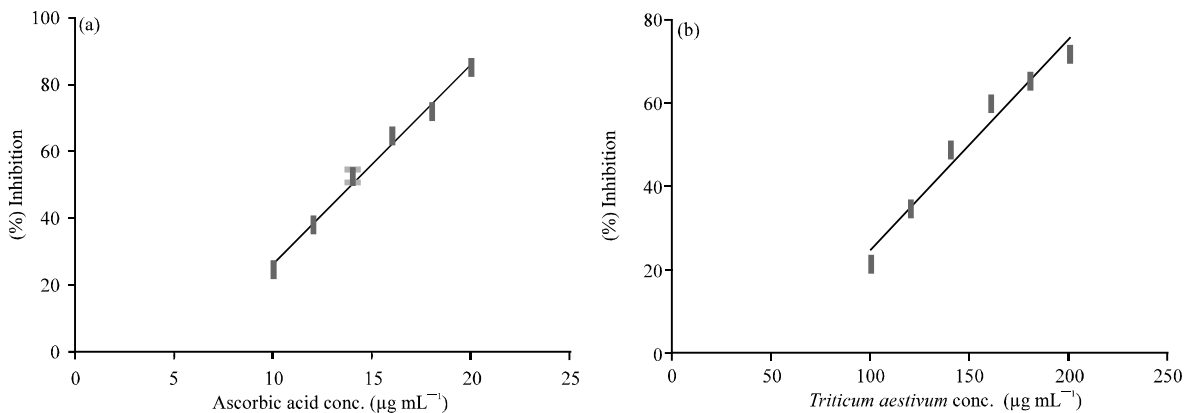


Fig. 2(a-b): Antioxidant potential of (a) *Ascorbic acid* and (b) *Triticum aestivum*

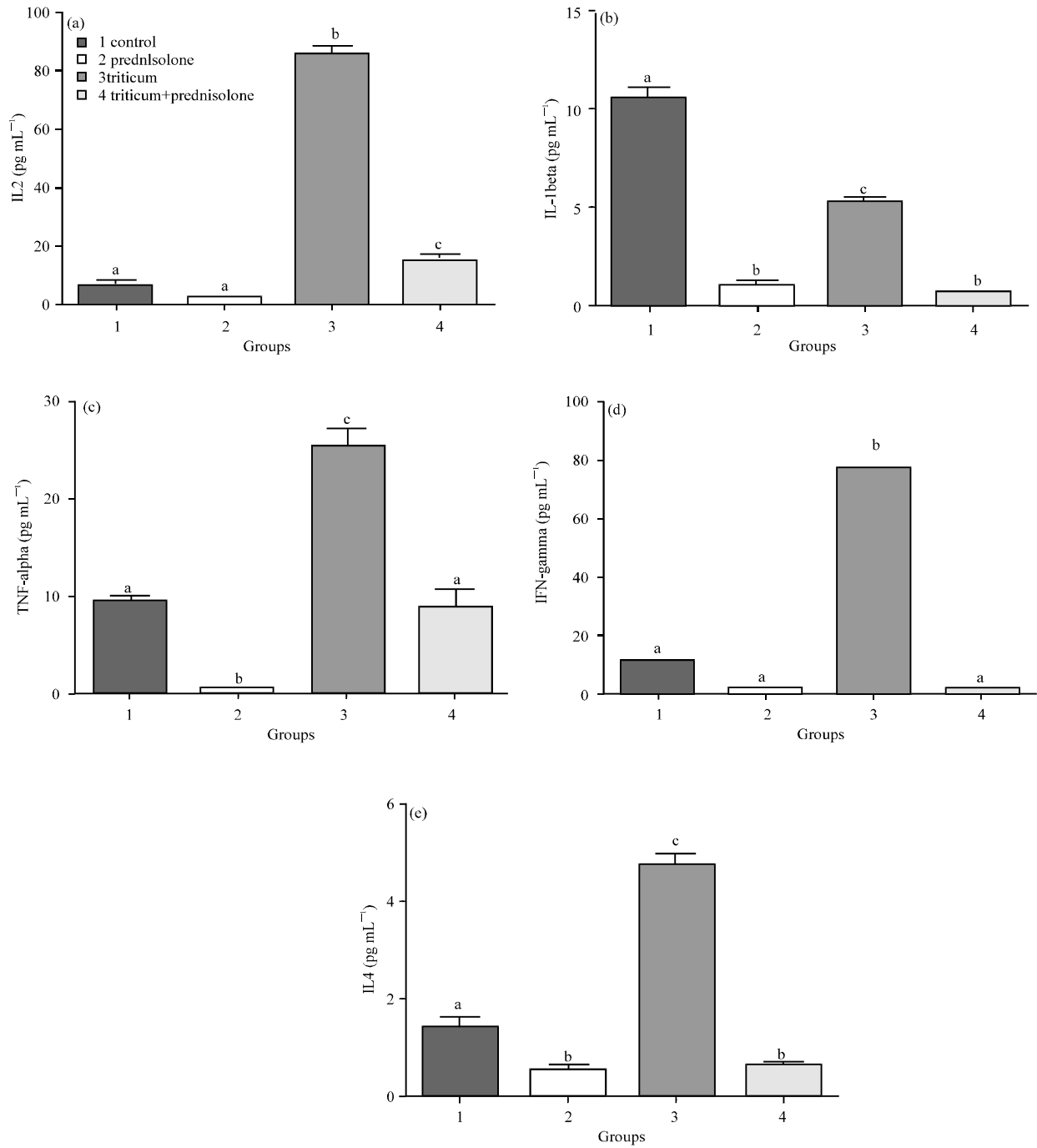


Fig. 3 (a-e): Effect of *Triticum aestivum* on Th1 and Th2 cytokine response. Values are Mean with SME, Tukeys multiple comparison post-hoc test: Different alphabets indicate significant differences and bars with same superscript are not significant. Fig. 3a, vs. c p<0.001, a vs. d p<0.05 Fig. 3b, a vs. b p<0.001, a vs. c p<0.001, a vs. d p<0.001, Fig. 3c, a vs. b p<0.01, a vs. c p<0.001 Fig. 3d, a vs. c p<0.001, Fig. 3e, a vs. d p<0.05, a vs. c p<0.001, a vs. d p<0.05



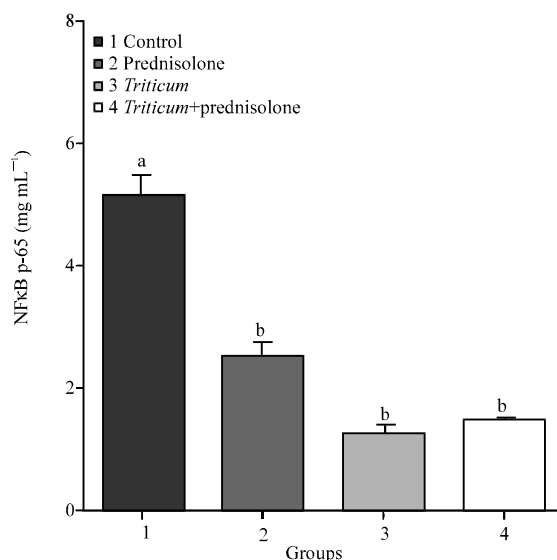


Fig. 4: Effect of *Triticum aestivum* on the production of p65 (Rel A) of NF-κB. Significance: a vs. b =  $p < 0.010$ , Values are Mean $\pm$ SME, Tukeys multiple comparison post-hoc test: Different alphabets indicate significant differences and bars with same superscript are not significant

## DISCUSSION

Induction of Th1-promoting cytokines with adjuvants is generally used to enhance anti-tumor immunity to reduce or prevent tumor growth. In many carcinomas, low levels of TNF- $\alpha$  exposure promotes increased tumor growth, invasion and metastasis. Nevertheless, a high dose of TNF- $\alpha$  has a cytotoxic effect on certain tumor cells and induces massive necrosis as well as apoptosis, high dose of TNF- $\alpha$  stimulates IL-1 $\beta$  which is now known to promote undesirable side effects such as *in-vivo* angiogenesis and invasiveness of different tumor cells (Voronov *et al.*, 2003; Larmoniera *et al.*, 2007; Szlosarek *et al.*, 2001; Balkwill, 2009). Therefore, high concentration of TNF- $\alpha$  with low level of IL1  $\beta$  would have a better outcome of cancer treatment. In the present study the group that received *Triticum aestivum* water extract had high concentration of IL2 and TNF- $\alpha$  on a background of low IL1  $\beta$ . A similar response; that is, Th1 upregulation had been observed with other herbs such as *J. sinensis*, *P. corylifolia*, Cheong-a-hwan (used widely in Korean traditional medicine) but downregulation of IL1  $\beta$  was observed only with *Triticum aestivum* as observed with *Ocimum sanctum* in our earlier study (Lee and Kim, 2008; Hemalatha *et al.*, 2011). In addition, a selective stimulation of Th1 immunity and reduction in tumor size had been reported with *Withania somnifera* (Malik *et al.*, 2009).

The p 65 that is downstream in the classical pathway of NF $\kappa$ B activation was impaired with *Triticum aestivum* water extract treatment. p 50/p 105, p 52/p 100, p 65 (rel A), rel B and c-rel are the five subunits of NF $\kappa$ B that exist in unstimulated cells as homo or heterodimers bound to I $\kappa$ B family proteins (Hayden and Ghosh, 2004). Of the 5 subunits, the p 65 (Rel A) subunit protects the cells from apoptosis during TNF $\alpha$  signaling (Beg *et al.*, 1995). In the present study the *Triticum aestivum* suppressed the expression of the p65 (rel A) subunit of NF $\kappa$ B.

The transcription factor NF $\kappa$ B controls the expression of genes involved in immune responses, apoptosis and cell proliferation. Inhibitors of P65 subunit of NF- $\kappa$ B hold great promise for treating a variety of diseases including cancer. It has been shown that a negative feedback mechanism

resulting from high concentrated of TNF- $\alpha$  suppresses the NF $\kappa$ B activation and increases the signals for cell death (Catalina *et al.*, 2006). Therapeutic gains are possible if natural or synthetically derived inhibitors of NF- $\kappa$ B are given in combination with TNF-  $\alpha$  (Catalina *et al.*, 2006). In the present study, *Triticum aestivum* inhibited the NF- $\kappa$ B and IL-1 $\beta$  and induced TNF- $\alpha$ . Put together these results suggest a potential role for *Triticum aestivum* in Th1 modulation.

Apart from the effect of *Triticum aestivum* on T helper modulation, the present study provides evidence on the beneficial effects of *Triticum aestivum* on bone marrow cellularity, hemopoietic activity and hemoglobin concentration. Moreover, the *Triticum aestivum* extract increased the T-cell dependent antibody response to SRBC which was similar to the observations made with *Withania somnifera* and *Ocimum sanctum* herbs (Hemalatha *et al.*, 2011; Leemol and Girija, 2000; Mediratta *et al.*, 2002). Prednisolone is a widely used anti-inflammatory agent especially in patients with rheumatoid arthritis, however, long term use of this drug decreases wound healing and increases infections (Elenkov and Chrousos, 2002; Petrovsky, 2001; Elenkov, 2004). Though *Triticum aestivum* prevented the adverse effect of prednisolone and restored the total WBC count and bone marrow cellularity, it had no effect on the adverse effect of prednisolone induced Th1, Th2 heamagglutinin responses.

## CONCLUSION

These data suggest that *Triticum aestivum* can prevent prednisolone induced myelosuppression and the data on the modulation of Th1 cytokine responses suggest a potential role in the treatment of immuno-inflammatory disorders like asthma, arthritis and also cancer. Further exploratory studies are needed to delineate the mechanisms underlying the effects of *Triticum aestivum*. Studies are planned to explore the role of *Triticum aestivum* extract on tumors induced in animal models.

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