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Immunomodulatory Activity and Th1/Th2 Cytokine Response of *Ocimum sanctum* in Myelosuppressed Swiss Albino Mice

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ABSTRACT

Ocimum sanctum (*O. sanctum*) has gained special attention due to its biological properties, however, little is known about its immunomodulatory effects. The purpose of this study was to investigate effect of *O. sanctum* on inflammation and immune response and its effect on Th1/Th2 cytokine production by spleen cells of myelosuppressed mice model. Female *Swiss albino* mice were challenged with SRBC and then were grouped and treated with either *O. sanctum* methanolic extract 850 mg kg^{-1} or Prednisolone 5 mg kg^{-1} body weight for 15 days. Blood was collected on 16th day from retro orbital plexus to perform hematological and immunological tests. Bone marrow cellularity was determined and supernatants of splenocytes cultures were analyzed for Th1/Th2 cytokines by ELISA. Antioxidant activity of *O. sanctum* was evaluated by DPPH (2,2'-diphenyl-1-picrylhydrazyl) assay. Treatment with *O. sanctum* showed significant increase in bone marrow cellularity ($p<0.01$), total WBC count ($p<0.01$) and hemoglobin concentration ($p<0.01$). In addition, there was a significant increase in SRBC antibody titer (1:1024) compared to the control group (1:256). *O. sanctum* increased the production of TNF-α, IL-2, IFN-γ and IL-4 ($p<0.05$) significantly and decreased the production of IL-1β and NF-kB. The methanolic extracts of *O. sanctum* showed free radical scavenging activity at 140 μg mL^{-1} concentration as IC_{50}. This study documented improved haemoglobin concentration with *O. sanctum* treatment. These results support the use of this herb for wound healing and infection. The results also suggest potential use of *O. sanctum* as adjuvant in cancer therapy, myelotoxicity and in nutritional anemia.

Key words: *Ocimum sanctum*, immunomodulation, NF-kB, cytokines, bone marrow cellularity, myelosuppression, prednisolone

INTRODUCTION

Ocimum sanctum commonly known as Holy Basil or sacred tulasi, belongs to the family *Lamiaceae* and is a fragrant bushy plant found in the semitropical and tropical parts of India. From the ancient times different parts of *O. sanctum* are traditionally used in Ayurveda and Siddha systems of medicine for treating infections, skin diseases, hepatic disorders, cold, cough and malaria fever. Many studies have shown a number of beneficial effects of *O. sanctum* such as anti-inflammatory (Singh *et al.,* 1996), humoral immune response modulation and promotion of wound healing (Mediratta *et al.,* 1988; Shetty *et al.,* 2008). Mediratta *et al.* (2002) have shown the effect

of *O. sanctum* seed oil on some immunological parameters in both stressed and non-stressed animals. Oil extracted from *O. sanctum* showed good antibacterial activity against *Staphylococcus aureus, Bacillus pumilus* and *Pseudomonas aeruginosa*, showed improved wound healing and increased production of TNF-α (Singh and Majumdar, 1999; Goel *et al.*, 2010a). In addition to its antibacterial, antiinflammation and wound healing properties, *O. sanctum* had also been studied for its antihypersensitivity and antioxidant properties (Mediratta *et al.*, 1988; Shetty *et al.*, 2006; Uma Devi *et al.*, 2000).

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-κB controls many genes involved in inflammation and it is not surprising that NF-κB 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-κB. Though *O. santum* is known for its wound healing and anti-inflammatory properties, except a few studies on humoral immune response such as SRBC antibody titers or paw edema; studies describing the effect of *O. sanctum* on Th1/Th2 modulation or cytokines responses are lacking. Therefore,the present study was carried out to investigate the effect of *O. sanctum* methanolic extract on Th1/Th2 cytokine production, NFkB p^{65}activity, heamopitic activity and antibody response to Sheep Red Blood Cells (SRBC) in myelosuppressed mice model. In addition, its antioxidant property was also studied.

MATERIALS AND METHODS

Plant material-Preparation and extract: Dried and powdered leaves of *O. sanctum* were procured from herbal stores at Hyderabad and authenticated in Heritage Bio-Natural systems pvt. Ltd. The study was conducted from 2009 to 2010. Hundred grams of dried and powdered leaves of *O. sanctum* were soaked in methanol for 3 consecutive days at an interval of 24 h. The fluid fraction of the three extractions was combined, concentrated and dried under vacuum. Accurately weighed quantities of the methanol extract of *O. sanctum* were suspended in 2% 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 for rodent dose.

Animals: 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 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±20°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 weakly changes. They were fed a casein based (20% protein) pellet control diet.

Animals treatment: Twenty four female *Swiss albino* mice were sensitized with 0.5 mL of 20% of fresh SRBC suspension, which was injected on day 0. The animals then were divided into four groups of six animals each. Animals in Group I received the vehicle (2% Gum acacia) and were treated as a control group. Animals in Group II received prednisolone (5 mg/Kg/b.wt.) in 2% Gum acacia. Group III animals received *O. sanctum* methanolic extract (850 mg/Kg/b.wt.) in 2% Gum

acacia and Group IV animals received prednisolone (5 mg/Kg/b.wt.) along with *O. sanctum* methanolic extract (850 mg/Kg/b.wt.) in 2% Gum acacia, orally, for a period of 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, and SRBC as an antigen at the concentration of 20% for immunization and 2% for challenge.

Determination of the bone marrow cellularity: Bone marrow was collected from femur in medium containing 2% Fetal Calf Serum (FCS). 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 animals in four groups were sensitized with 0.5 mL of 20% fresh SRBC suspension injected intraperitonially on day 0. Blood samples were collected in microcentrifuge tubes from retro-orbital plexus of each animal on 16th day of extract administration. The serum was separated and pooled the sera each group. Antibody titres were determined by haemagglutination technique. Two fold dilutions of pooled sera were made in 25 µL volumes of PBS in "U" bottomed microtitration plate and to this was added 25 µL of 2% SRBC in saline. After mixing, the plates were incubated at 37°C for 1 h and examined for haemagglutination under microscope. The reciprocal of the highest dilution of the test serum giving agglutination was taken as the antibody titre.

Estimation of cytokines (IL-1β, IL-2, IL-4, IFN-γ, TNF-α) 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. Cells were washed twice with RPMI1640 medium. 1×10^{6} cells mL^{-1} was dispensed into the 24-well flat bottomed plates in the absence and presence of 0.5 µg mL^{-1} Concanavalin A. These cells were incubated in 5% CO_2 humidified incubator for 24 h. Cells were centrifuged for 5 mins 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 NFkB in the nuclear fraction of splenocytes: Preparation of nuclear extract of splenocytes was performed as per instructions given in Trans AM Flexi kit. The NFkB was estimated by ELISA (Trans AM Flexi) Kit. All the tests were performed in duplicates.

DPPH radical assay: Different aliquots of standard and test were mixed with Tris HCl buffer and made the volume to 1 mL. To this added 1 mL of 0.3mM DPPH (2,2'-diphenyl-1-picrylhydrazyl) in methanol and made the final volume to 2 mL. These mixtures were shaked well and kept in dark for 30 min. The absorbance was measured at 517 nm using ethanol as blank. One milliliter of 0.3 mM DPPH was diluted in ethanol, which was used as a control. Inhibition of DPPH radical was calculated using the equation: IC_{50} (%) = $100 \times (A_0\text{-}A_s)/A_0$, where A_0 is absorbance of the control, A_s is absorbance of the sample and IC_{50} is concentration of the test extract that caused 50% inhibition.

Statistical analysis: Data are presented as mean±Standard Error (SE). Analysis of variance (ANOVA) was used to estimate the main effects and interactions. P values <0.05 were considered significant. Duncan's test was used to identify the groups that are homogenous with respect to mean. Analysis was performed using SPSS (version 11.5, SPSS Inc, Chicago, IL).

RESULTS

Effect of *O. sanctum* methanolic extract on the haematological parameters, T cell dependent antibody response and bone marrow cellularity : There was a significant increase in haemoglobin concentration ($p<0.01$), RBC and WBC count in *O. sanctum* treated mice compared to controls. However, there was no change in lymphocyte population with *O. sanctum,* though, there was a significant ($p<0.01$) decrease in lymphocyte population in Prednisolone treated mice compared to the control (Table 1). *O. sanctum* treated mice showed significant ($p<0.01$) increase in bone marrow cellularity compared to the control mice. Furthermore, *O. sanctum* prevented the suppressive effect of prednisolone on bone marrow cellularity (Table 2). *O. sanctum* treatment increased the production of SRBC antibody titre (1:1024) compared to the control mice and prevented the suppressive effect of prednisolone on antibody response to SRBC (Fig. 1).

Effect of *O. sanctum* methanolic extract on Th1 and Th2 cytokine profile and NFkB P^{65} activity: *O. sanctum* treatment significantly increased the production of Th1 cytokines ($p<0.01$) IL-2, TNF-α and IFN-γ and Th2 cytokine ($p<0.05$) IL-4, compared to control mice. *O. sanctum* and

Table 1: Effect of *O. sanctum* on haematological parameters

Parameters	Control (n = 06)	Prednisolone (n = 06)	*O. sanctum* (n = 06)	Prednisolone + *O. sanctum* (n = 06)
WBC ($10E^3$/mm^3)	3.81±0.3* ^{a}(2.82, 4.81)	2.7±0.19 ^{a}(2.1, 3.3)	7.9±1.08 ^{b}(3.26, 12.53)	5.01±0.95 ^{a}(2.58, 7.45)
RBC ($10E^6$/mm^3)	8.92±0.14* a(8.33, 9.51)	6.78±0.2 ^{c}(6.15, 7.42)	9.71±0.1 ^{b}(9.28, 10.14)	8.94±0.18 ^{a}(8.49, 9.39)
Hb (g/dl)	14.17±0.18* ^{a}(13.41, 14.93)	11.18±0.43 ^{c}(9.8, 12.55)	15.27±0.13 ^{b}(14.69, 15.84)	14.05±0.28 ^{a}(13.32, 14.78)
Lymphocytes (%)	73.07±3.78* ^{a}(56.79, 89.34)	58.65±1.72 ^{b}(53.19, 64.11)	80.13±0.66 ^{a}(77.31, 82.96)	40.54±3.33 ^{c}(31.28, 49.8)

All values are Mean±SE; 95% CIs in parentheses; $p<0.01$. Means bearing similar superscripts in each row do not differ significantly.

Table 2: Effect of *O. sanctum* on Bone Marrow Cellularity

Treatment groups	Bone marrow cellularity
Control (n = 06)	1370±150*^{a} (-535.93, 3275.93)
Prednisolone (n = 06)	700±220^b (-2095.37, 3495.37)
Ocimum (n = 06)	2340±220^c (-455.37, 5135.37)
Prednisolone + *O. sanctum* (n = 06)	1540±80.83 ^{a}(1192.22, 1887.78)

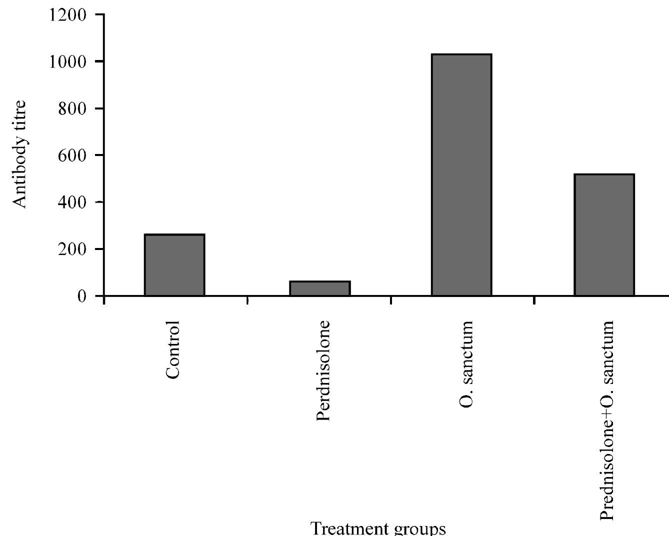

Fig. 1: Effect of *O. sanctum* on heamagglutinin antibody response

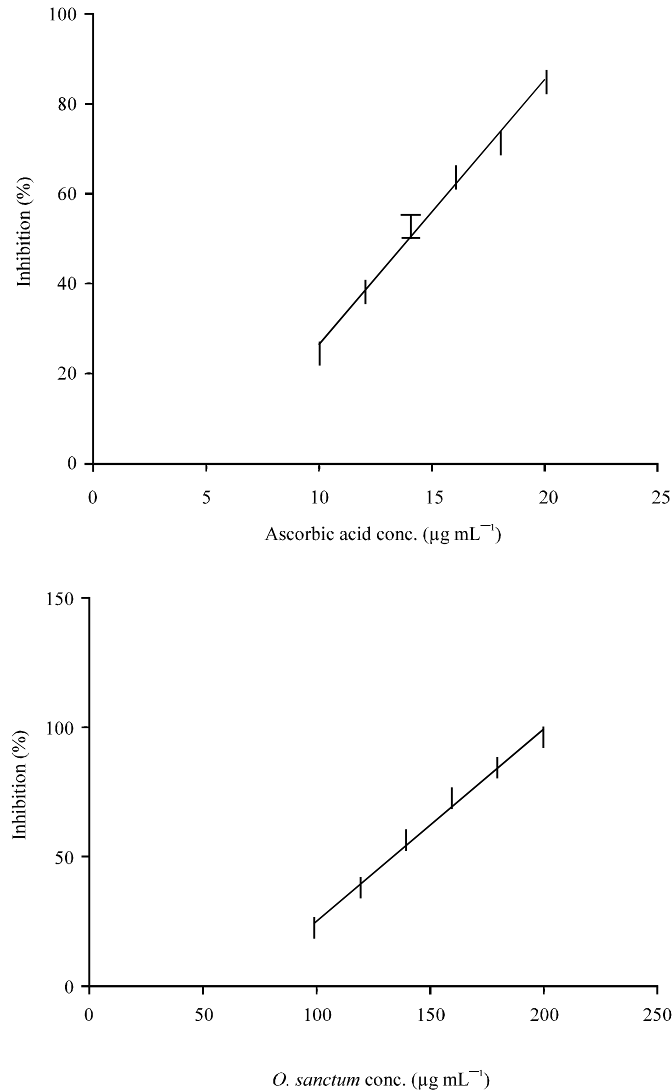

Fig. 2: Effect of Antioxidant potential of *O. sanctum*

prednisolone suppressed the production of IL-1β significantly ($p<0.01$) compared to controls. Significant ($p<0.05$) increase in TNF-α levels was observed in mice treated with prednisolone compared to control group (Fig. 3a-e). There was a significant decrease ($p<0.01$) in NFkB P^{65} activity with *O. sanctum* treatment and combined treatment with Prednisolone compared to control group. However, prednisolone enhanced significantly ($p<0.01$) NFkB P^{65} activity when given alone compared to controls (Fig. 4).

Antioxidant activity of *O. sanctum* methanolic extract: Free radical activity of *O. sanctum* methanolic extract and ascorbic acid activities are depicted in Fig. 2. Though *O. sanctum* free radical activity was not comparable to ascorbic acid, it showed a modest free radical scavenging activity at 140 µg mL^{-1} as compared to ascorbic acid which showed at 14 µg mL^{-1}.

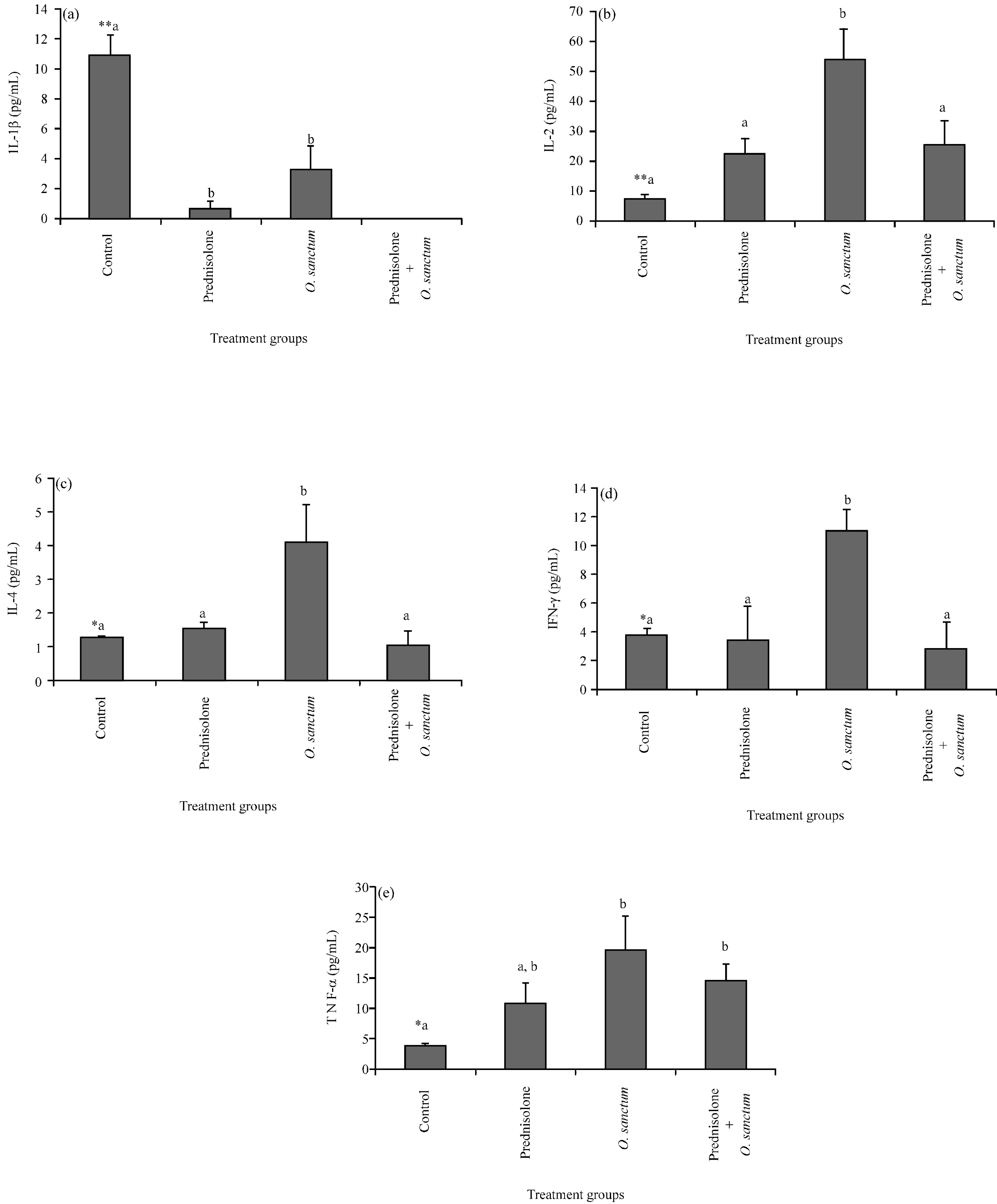

Fig. 3a-e: Effect of *O. sanctum* on Th1 and Th2 cytokine response All values are Mean±SE; 95% CIs in parentheses; p *<0.05 **<0.01 Means bearing similar superscripts in each bar do not differ significantly

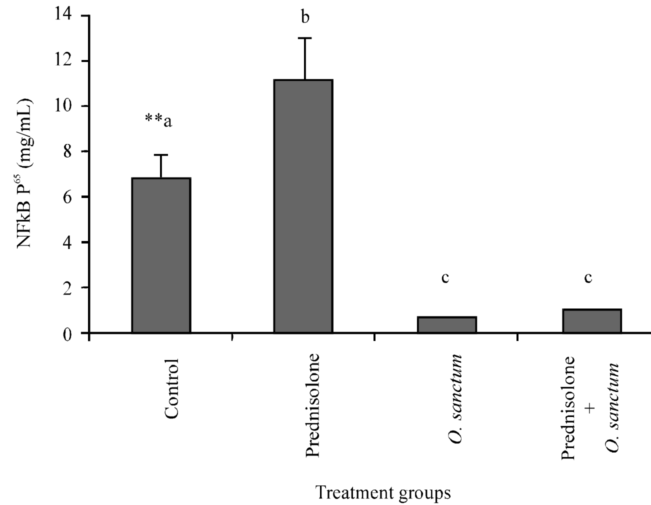

Fig. 4: Effect of *O. sanctum* on the production of p65 (Rel A) of NF-κB All values are Mean±SE; 95% CIs in parentheses; p *<0.05 **<0.01 Means bearing similar superscripts in each bar do not differ significantly

DISCUSSION

In the present study, inflammation and immunomodulation activity of *O. sanctum,* a popular traditional plant in medicine was investigated with special reference to Th1 and Th2 phenotype response and NFkB activity. *Ocimum sanctum* is an extensively used medicinal plant in the Ayurvedic system of medicine and is known for its beneficial effects on human health. *Ocimum sanctum* treatment resulted in increased haemoglobin concentration in line with that observed by other investigators (Goel *et al.*, 2010b). The production of SRBC antigen-specific antibodies represents a major defense mechanism to assess T-cell-dependent antibody responses. Similar to earlier reports, we documented enhanced SRBC agglutinin titers with *O. sanctum* treatment (Mediratta *et al.*, 1988). The mechanism for the enhanced SRBC antibody response has not been defined in this study. However, heightened IL2 response observed in this study could have contributed to enhance SRBC antibody response (Callard *et al.*, 1991). These results provide evidence supporting *O. sanctum*'s role as anti-bacterial and anti-viral compound (Gupta and Charan, 2005). Furthermore, *O. sanctum* reduced the adverse effects of predinosolone and increased bone marrow cellularity, haemopoetic activity considerably as reported earlier with Ashwagandha (Ziauddin *et al.*, 1996).

Transcription factor NFkB controls the expression of genes involved in immune responses, apoptosis and cell proliferation. P50/P105, P52/P100 (NFkB2), P65 (Rel A), Rel B and C-Rel are the five subunits of NFkB, that exist in unstimulated cells as homo or hetero dimer bound to IkB family proteins (Matthew and Ghosh, 2004). P65 or Rel A exists in a wide variety of cell types and is a key molecule in the classical pathway. The classical pathway is typically triggered by ligand binding to tumor necrosis factor type 1/2 receptors (TNFR1/2), T-Cell Receptors (TCR), B-Cell Receptors (BCR), or the Toll-Like Receptor (TLR) - interleukin-1 receptor (IL- 1R) super family members. This pathway terminates in the increased transcription of target genes encoding chemokines, cytokines, and adhesion molecules, perpetuating inflammatory responses and promoting cell survival and thus protects cells from apoptosis during TNFα signaling (Beg *et al.*, 1995). However, overexpression of

NF-κB, together with COX-2 and LOX5 is frequent in cancer. The anti-inflammatory property of *O. sanctum* has been atributed to decreased CoX-2 and LOX-5 enzymes activity (Singh *et al.*, 1996), but suppression of NF-κB classical pathway as observed in the present study could be yet another mechanism by which *O. sanctum* might act as anti- inflammatory agent. *O. sanctum* contains Ursolic acid and Carnosol components which have been shown to down regulate NFκB and also possess anticancer activity (Lo *et al.*, 2002). *O. sanctum* contains a number of compounds such as carnosol, ursolic acid, rosmarinic acid, apigenin, eugenol, cirsilineol and cirsimaritin, all of which have shown to have potent redox/anti-oxidant properties as well as COX-2 inhibitory effects (Kelm *et al.*, 2000). Thus apart from anti-inflammatory role, *O. sanctum* could be a potential anti cancer agent.

Furthermore, *O. sanctum* reduced NF-κB activity even in prednisolone treated animals wherein prednisolone per se has increased NF-κB activity.

In the present study we observed upregulation of IL-2, IFN-γ and TNF-α with *O. sanctum*. Similarly to that observed elsewhere (Goel *et al.*, 2010b). Additionally, the present study also demonstrated down regulation of IL-1 β, which had not been reported earlier. Studies on cancer immunity have demonstrated enhanced anti-tumor immunity and reduction of tumor growth with specific adjuvants (Dredge *et al.*, 2002). Many cancer vaccines, particularly in combination with immune adjuvants, elicit strong cellular immune responses leading to the production of Th1 type cytokines such as IL-2, IFN-γ, TNF- α (Dalgleish, 2000). However, one major adverse effect of most of these adjuvants is increased IL-1β production, which is now known to contribute to invivo angiogenesis and invasiveness of different tumor cells (Voronov *et al.*, 2007). The interesting aspect of the present study is, upregulation of IFN-γ and TNF-α on a backdrop of low IL-1β. *O. sanctum* suppressed IL-1β production, whereas it increased IL-2 , IFN-γ and TNF-α (Mondal, 2010).

Taken together, these results suggest a potential adjuvant role of *O. sanctum* in cancer therapy. The present study also suggests that *O. sanctum* might modulate inflammation and immune response by modulating NFkB activity. However, more studies are required to delineate the role of *O. sanctum* on inflammation and mechanism there of.

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