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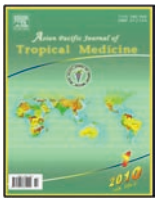

Document heading

Immunomodulating property of *Ocimum sanctum* by regulating the IL-2 production and its mRNA expression using rat´s splenocytes

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ABSTRACT

Objective: To investigate the regulatory effect of aqueous extract of leaves of *Ocimum sanctum* on IL-2 cytokine production in vivo and in vitro, and the effect of leaves extract on general blood picture including T& B lymphocytes. **Methods**: For in vivo studies albino Wistar rats were treated with aqueous crude leaves extract of *Ocimum sanctum* for 20 consecutive days. Spleen cells were harvested and assayed for IL-2 production by using sandwich enzyme-linked immunosorbent assay (ELISA) and mRNA expression methods. For in vitro study aqueous *Ocimum sanctum* leaves extract= in different concentrations (25-500 μg/mL) was added into culture plates containing ConA stimulated splenocytes. To study the overall effect on blood picture, density gradient purified lymphocytes analysis and conventional methodology for total and differential leukocyte count and hemoglobin level were also done. **Results**: It indicated that the rats treated with *Ocimum sanctum* leaves extract had significantly enhanced ($P<0.001$) ability of spleen cells to secrete IL-2. Investigation in vitro also showed regulation of IL-2 production. Blood study exhibited leucocytosis and augmentation of T& B lymphocytes by 25% approximately. 4-5% increase in Hemoglobin value was also noticed. **Conclusion**: Aqueous *Ocimum sanctum* leaves extract may have stimulatory effect on T & B lymphocytes particularly on Th1 subset of lymphocytes as shown by enhancement in IL-2 production.

1. Introduction

Since ancient times, *Ocimum sanctum*(*O. sanctum*), a well known medicinal plant of the Indian subcontinent has been revered and used in Ayurvedic system of medicine. Most of the research done on *O. sanctum*, confirmed dozens of its traditionally known action and therapeutic uses including its remarkable adaptogenic[1,2] and anti stress activity[3-6] as well as its powerful support for the immune system[7-9]. About the immunity, it is well documented that it increases the cell mediated and humoral immunity of the body[10-15]. Now it is well known that herbal medicines act as immunomodulator through the dynamic regulation of information molecules such as cytokines. This may offer an explanation for the effect of this herb on the immune system[16]. But there is little information or documentation regarding the cytokines induction by *O. sanctum*[17]. Our previous studies demonstrated that aqueous *O. sanctum* extract enhanced the synthesis of IFN- γ by rat splenocytes in vivo as well as in vitro studies. Extract was also found as splenocytes proliferative factor[18].

In view of the effect of leaves extract on the enhancement of IFN- γ production and splenocytes proliferation, this study focused on the possible effect of *O. sanctum* on IL-2 production. IL-2 is the principal cytokine produced by native helper T-cells[19, 20]. T-helper cells are divided into Th1 and Th2 cells from the profile of cytokine secretion[21]. It is also known that Th1 cells are able to produce IL-2 & IFN- γ and Th2 cells can produce IL-4 and IL-10 cytokines[22]. IL-2 acts as a growth factor for both Th1 and Th2 lymphocytes[23] and plays a direct role in the induction of B lymphocytes[24].

In the present work we studied the effect of *O. sanctum* leaves aqueous extract on IL-2 cytokine production by spleen cells and on routine blood parameters and T& B lymphocytes in peripheral blood, which are responsible for the overall increase in cell mediated & humoral immunity. Sandwich enzyme-linked immunosorbent assay (ELISA)

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and m-RNA expression for IL-2 production by RT-PCR analysis were performed.

2. Materials and methods

2.1. Chemicals & reagents

RPMI-1640 medium, fetal bovine serum, Concanavalin-A (ConA), 3-(4,5-dimethylthizol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), TRI reagent and histopaque-1077 were purchased from SIGMA, USA. RT-PCR kit & penicillin-streptomycin were from Bangaloregenei, India. Rat IL-2 ELISA kit was obtained from R&D System, USA. Primers used were from Bioserve, India. Nylon wool was purchased from Polysciences. Inc, USA.

2.2. Preparation of herbal drug extract

Leaves of *O. sanctum* were collected from the garden of College of Veterinary Sciences, DUVASU, Mathura. The leaves and seeds were authenticated from the National Botanical Research Institute, Lucknow. Leaves were dried in shade and pulverized. 100 mg powder was extracted with 500 mL Triple distilled water (TDW) at room temperature for 3 days with a few drops of chloroform to avoid fungal growth. The extract was filtered, lyophilized and stored.

2.3. Animals

Wistar rats (100-120 mg), purchased from IVRI, Bareilly, India were kept at (24±2) ℃. Food and water were available ad libitum. The care of the animals was in accordance to the Guidelines for the care and use of Animals in Scientific Research, prepared by Indian National Science Academy, New Delhi[25]. The rats were randomly divided into two groups with 10 rats in each group. *O. sanctum* aqueous leaves extract which was found to be non-toxic and effective stimulator of haemopoetic system in preliminary studies was administrated (P.O.) at dose of 250 mg/kg by blunt 20 gauge needle syringe once a day in a volume of 100 μL for 20 days in experimental group; while in control group equal volume of water (placebo) was given. The experimental protocol was approved by the Institutional Animal Ethics Committee.

2.4. Effect on IL-2 production

2.4.1. Preparation of splenocytes

Spleen cell suspensions from each animal group were prepared as advocated by Xie *et al*[26] in RPMI-1640 medium and depleted of red blood cells by incubating it with 0.15 M ammonium chloride in 0.01 M tris buffer for 15 mins. Cells were washed twice in RPMI-1640 medium by centrifugation at 1 200 rpm for 10 mins. The pallet thus obtained was resuspended in RPMI-1640 medium supplemented with 2 mM L-glutamine, 100 units/mL of penicillin, 100 μg/mL streptomycin, 5×10^{-5} M β mercaptoethanol, 25 mM $NaHCO_3$ and 10% fetal bovine serum. Viable spleen cells were counted in hemocytometer by trypan blue exclusion. Triplicate cultures were performed in 96 wells flat bottomed tissue culture plates in a final volume of 200 μL/well containing 4×10^5 cells. ConA was added at 5 μg/mL. In study in vitro, plant extract at different concentrations (25-500 μg/mL) were added into splenocytes of control animals in the presence of ConA. Then the cells were incubated for 48 hrs at 37 ℃, 5% CO_2. Cell culture supernatants were removed and stored at -70℃ for IL-2 cytokine assay.

2.4.2. IL-2 assay

Il-2 cytokine was quantitated by sandwich ELISA as per protocol of R & D Systems, USA. In brief, ELISA plates were coated with goat anti rat IL-2 antibodies overnight at 4 ℃. Additional binding sites were blocked with 1% BSA in PBS. Both standard cytokine and samples (culture supernatants) were added into the ELISA plate. The plate was incubated for 2 hrs at room temperature and washed. Afterwards, biotinylated goat anti rat IL-2 was added, the plate was incubated for another 2 hrs and washed. Strepavidin-HRP conjugate was further added for 20 mins sin dark and washed. Finally the substrate, TMB was added for 20 mins in dark. The reaction was stopped by 2N H_2SO_4 and optical density of each well was determined at dual wavelength 450 nm-570 nm by an ELISA reader. The concentrations of IL-2 in the samples were determined with the help of standard curve of IL-2.

2.5. Effect on IL-2 gene expression

The level of IL-2 mRNA in the spleen cells was estimated using RT-PCR analysis after 24 hrs culture in the presence of 5 μg/mL ConA.

2.5.1. Extraction of total RNA

10^7 spleen cells from control and *O. sanctum* treated animals after 24 hrs cultures were lysed with 1 mL TRI reagent and the mixture was forced to pass through pipettes to release RNA from the cells. The homogenate was then vigorously mixed with 0.2 mL chloroform. After sitting at room temperature for 15 mins, the mixture was spun at 12 000 g for 15 mins at 4 ℃ to separate the aqueous phase from organic phase. The aqueous phase was transferred into a new tube and RNA was precipated with 0.5 mL of isopropanol and centrifuged at 12 000 g for 10 mins at 4 ℃. The RNA pellet was washed by 75% ethanol and finally suspended in 20 μL of RNAse free water.

2.5.2. cDNA synthesis

cDNA was synthesized according to the protocol of RT-PCR kit. 5 μL volume of total RNA was added into 10 μL RNAse free water and then mixed with 0.2 μg of oligo (dt)18 primer in 1 μL . The mixture was heated at 65 ℃ for 10 mins, followed by 2 mins cooling at room temperature and then placed on ice. A reaction mixture containing 4 dNTPs, DTT, RT buffer, RNAsin and M-MuLV reverse transcriptase was subsequently mixed with RNA. The reaction was carried out at 37 ℃ for 1 hr to synthesize the cDNA and then incubated at 95 ℃ for 2 mins to denature the RNA-cDNA hybrids and quickly placed on ice.

2.5.3. PCR

3 μL cDNA was mixed with PCR buffer, 4 dNTPs, Taq DNA polymerase and 100 μg of each forward and reverse primer. The sequences of primers advocated by Asa Melhus[27] were as following:

Il-2: 5′-AGC TGT TGC TGG ACT TAC AGG-3′, 5′-AAT TCC ACC ACA GTT GCT GG-3′;

β - actin: 5′- TGG AGA AGA GCT ATG AGC TGC-3′, 5′-TCC ACA CAG AGT ACT TGC GC-3′.

β-actin was taken as internal control. The PCR conditions were denaturated at 94 ℃ for 1 min, annealed at 55 ℃ for 1 min and extended at 72 ℃ for 2 mins. After 35 cycles of amplification, the final extension was done at 72 ℃ for 10 mins. The PCR products were subjected to gel electrophoresis on 1.5% agarose containing ethidium bromide. The amplicions were compared with a DNA molecular size marker (100 bp ladder) under UV light. The images were analyzed densitometrically. For each cDNA sample, the densitometric units of the amplified c-DNA fragments were counted for semi quantitative evaluation.

2.6. Effect on hematological parameters

Blood was collected from the orbital plexes and various parameters including total leukocyte count (heamocytometer), differential count (leishman´s stain) and hemoglobin level were recorded by conventional procedures.san extension of TYPHIdot™ which already well evaluated[5, 6]. These methods were based on the recombinant protein produced from the gene sequence of the 50 kD OMP.

2.7. Effect on T & B lymphocytes

3 mL of blood was also taken from each animal of both groups and after layering the blood over histopaque 1077 (sigma), total lymphocytes were separated and counted. T and B lymphocytes were separated on sterilized nylon wool columns (Poly sciences Inc, Washington) made in 10 mL syringe according to their protocol. After incubating the lymphocytes on nylon wool columns for one hour at 37 ℃, nonadherent T-cells were collected in RPMI-1640 while the plastic adherent B-cell were eluted by pressing the column with piston (2-3 times). The T and B-cell were separately counted by heamocytometer.

2.8. Statistical analysis

All values were expressed as mean±standard error of mean (SEM). Analysis was done using graph pad prism version 5.0. The parameters were evaluated with paired *t*-test. A *P*-value of 0.05 or less was taken as statistically significant.

3. Results

3.1. Effect on IL-2 production

Both studies in vivo and in vitro showed that *O. sanctum* extract enhanced the production of IL-2. Test in vivo showed concentration of IL-2 in control group was (569.22±120.97) pg/mL without any change, while the concentration in *O. sanctum* treated group was (667.71±137.50) pg/mL with change percentage as 17.3%. The *O. sanctum* extract appeared significant effect ($P<0.001$). The IL-2 production was also increased significantly with *O. sanctum* concentrations at 100 μg/mL, 250 μg/mL & 500 μg/mL ($P<0.001$, Table 1), and the increase was dose-dependent.

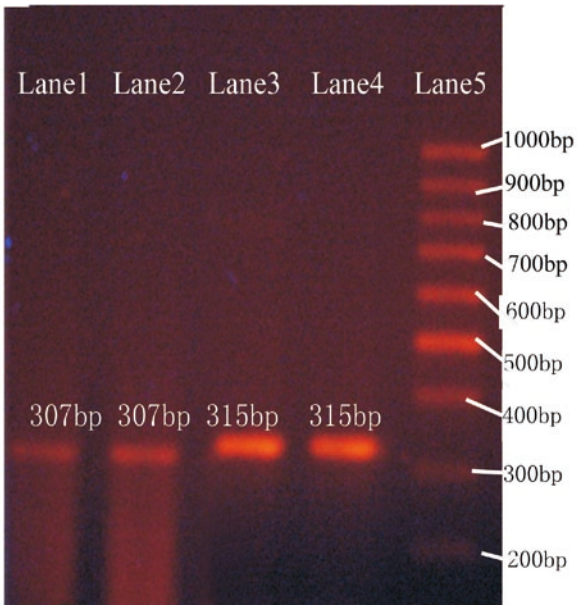
3.2. Effect on IL -2 gene expression

IL-2 production was also measured by IL-2 m-RNA expression. RT-PCR analysis of IL-2 m-RNA expression showed that the length of IL-2 and β-actin were 307 and 315, respectively. The raw volume of peak of IL-2 and β-actin were 44 662.44 and 134 234.42, respectively in control group without any significant change; while the raw volume of peak of IL-2 and β-actin were 103 883.91 and 137 091.44, respectively, with change percentage as 132.6% and 127%, respectively in treat group (Figure1).

Table1
In vitro effect of *O. sanctum* aqueous leaves extract on IL-2 production from splenocytes.

Groups	Concentration of IL-2 (pg/mL, mean±SEM)	change in IL-2 concentration(%)
ConA control	569.22±120.97	--
ConA+ *O.sanctum* (25 μg/mL)	572.93±122.01	0.65
ConA+ *O.sanctum* (50 μg/mL)	588.64±126.53*	3.41*
ConA+ *O.sanctum* (125 μg/mL)	619.42±129.56**	8.8**
ConA+ *O.sanctum* (250 μg/mL)	655.25±135.54**	15.1**
ConA+ *O.sanctum* (500 μg/mL)	632.78±132.04**	11.2**

* $P<0.05$, ** $P<0.01$.



Lane1: IL-2 (control); Lane2: IL-2 (*O. sanctum*); Lane3: β-actin (control); Lane4: β-actin (*O. sanctum*); Lane5: 100 bp ladder.

Figure 1. m-RNA expression of IL-2 cytokine& β-actin (control protein) in control and *O. sanctum* treated rats´ splenocytes by RT-PCR.

3.3. Effects on hematological parameters

Pretreatment for 21 days with *O. sanctum* extract produced a striking leukocytosis (Figure 2) with a marked lymphocytosis (Figure 3). Elevated level of Hb was also recorded in *O.sanctum* fed animals (Figure 4).

3.4. Effect on T & B lymphocytes

The *O. sanctum* leaves produced a stimulatory effect on T-B lymphocytes as augmentation indication of in the number of total lymphocytes, T as well as B lymphocytes (Figure 5).

4. Discussion

Our observation in this study demonstrated that *O.sanctum* leaves have immunomodulating effect by stimulating IL-2

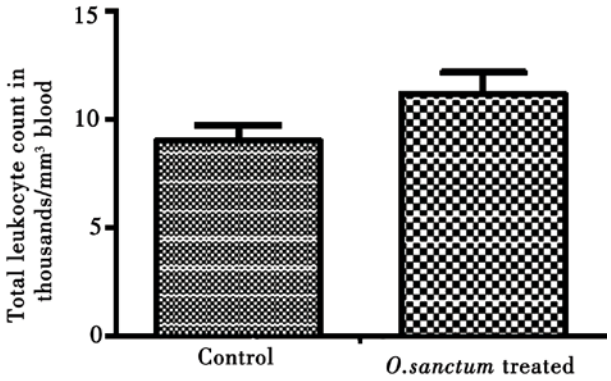


Figure 2. Effect of *O. sanctum* aqueous leaves extract on total leukocyte count in blood.

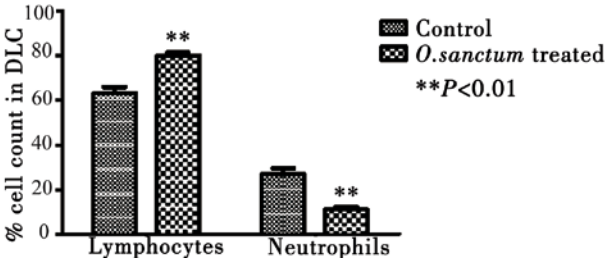


Figure 3. Effect of *O. sanctum* aqueous leaves extract on lymphocytes & Neutrophils percentage in DLC of blood.

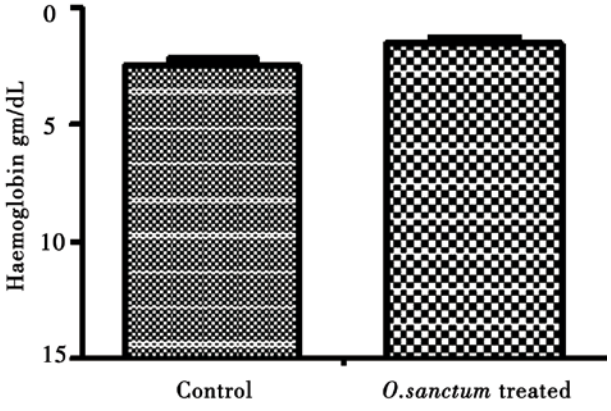


Figure 4. Effect of *O. sanctum* aqueous leaves extract on hemoglobin concentration in blood.

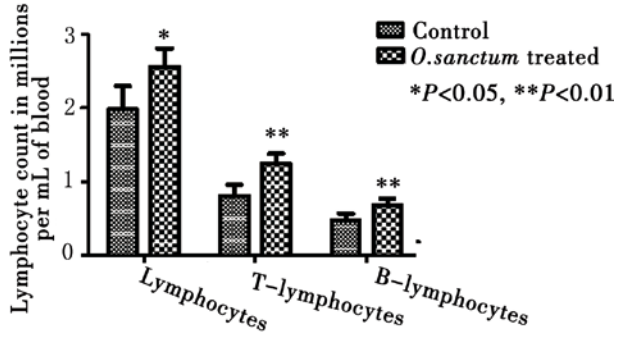


Figure 5. Effect of *O. sanctum* aqueous leaves extract on total lymphocytes and T&B lymphocytes concentrations in blood.

cytokine production. Both secretion and m-RNA expression of IL-2 were increased in the spleen cells taken from *O. sanctum* leaves extract fed rats. Experiments in vitro also exhibited increase in IL-2 synthesis. IL-2 is a well known cytokine that is produced by naive $CD4^+$ T cells and under the influence of IL-2 secretion CD^+ T cells get differentiated into Th1 cells[24, 28].

Moreover, our previous study also indicated that *O. sanctum* leaves extract modulate IFN-γ production[18]. IFN-γ is a defining cytokine of the subset Th1. Thus it may be possible that leaves extract may also have potential to stimulate Th1 which in turn produce IL-2 and IFN-γ cytokines that promote differentiation of fully cytotoxic TC cells from $CD8^+$ precursor. IL-2 stimulates the growth, differentiation and survival of antigen selected cytotoxic T-cells via the activation and expression of specific gene[29-30]. This pattern of cytokine induction makes the Th1 subset essentially suit to viral infection and intracellular pathogenesis. Recently, ultra low dose of IL-2 therapy in patients with AIDS, promotes Th-1 cytokine profile i.e. IFN-γ and enhances immune functions[31-32]. Higher cost and the administration of IL-2 may have intense adverse localized effect. While augmentation of IL-2 level by *O. sanctum* leaves extract as seen in this study may exclude above stated side effects. Thus it can be beneficially used as an alternative medicine approaches in immune suppressed conditions like AIDS without any toxic effects.

Our study also demonstrated that the *O. sanctum* improves overall body constitution by increasing Hb, leukocytosis and lymphocytosis. Several researches have confirmed repeatedly that *O. sanctum* increases both specific and nonspecific immunity[10,11,12,33]. Thus a rise in white blood cell count with significant increase in lymphocyte count accounts for its immunostimulating activity.

In our study we used crude extract which may be proved more potent and effective than any single purified compound because ingredients/components present in crude extract may show synergistic activity. Many works reported that the use of whole plants, instead of isolated chemicals may offer a safer clinical strategy in the treatment of many diseases[34-36].

It is concluded that at the present time when we are facing with the limitations of modern medical science in responding to the diseases, phototherapy appears to be a potential part of the solution. The researches in vivo and in vitro demonstrated that *O. sanctum* modulate the secretion of cytokines and show promising therapeutic value. Such scientific validation will facilitate the medicinal efficacy of Indian herbal medicinal system.

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