Potential Anti-inflammatory Properties of Crude Alcoholic *Extract* of *Ocimum basilicum* L. in Human Peripheral Blood Mononuclear Cells

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Ocimum basilicum **L. (Lamiaceae) is a well-known perennial herb in Indian medicine used to treat various disorders like upper respiratory tract infections and wound healing. Primarily we investigated the anti-inflammatory activity of crude extracts of *Ocimum basilicum* using peripheral blood mononuclear cells (PBMC) of healthy individuals. *Ocimum basilicum* crude methanolic extract showed a good inhibitory effect on the proliferative response of PBMC in mitogenic lymphocyte proliferation assays. Furthermore, gene expression studies on lipopolysaccharide (LPS) induced production of proinflammatory cytokines like Tumor necrosis factor-α (TNF-α), Interleukin-1β (IL-1β) and IL-2 showed down regulation of the markers. It also suppressed the induction of inducible nitric oxide synthase (iNOS) and the subsequent production of nitric oxide (NO) in LPS-stimulated RAW 264.7 macrophages in a time-dependent manner. Our results showed that *Ocimum basilicum* crude methanolic extract inhibits the key proinflammatory cytokines and mediators, which accounts for its anti-inflammatory effect.**

Key words —— *Ocimum basilicum*, pro-inflammatory cytokines, nitric oxide

INTRODUCTION

Ocimum basilicum L. (Lamenaceae) is a well known aromatic and medicinal plant, which attracted great attention in the ancient system of Indian medicine for its use in various ailments such as muscle cramps, pain, insecticidal, anti-ulcer, anti-inflammatory, diabetes and respiratory tract problems.[1–5] Fixed oil of *Ocimum basilicum* was found to possess significant anti-inflammatory activity against carrageenan and different other mediator-induced paw edema in rats.[6] Recent studies demonstrated that the contents of essential oils showed anti-nociceptive effects in mice and antibacterial effects against multidrug resistant clinical isolates.[7, 8] Earlier studies have reported that the fixed oil from *Ocimum basilicum* showed anti-ulcer properties in rat models.[9] So far there has been no *in vitro* study on the immunomodulatory activity of this plant. In this report we have studied the *in vitro* immunomodulatory potential of the crude extracts of *Ocimum basilicum* on mitogen-induced human peripheral blood mononuclear cells (PBMC). We have demonstrated the specific activity of *Ocimum basilicum* on proinflammatory cytokines and mediators.

Proinflammatory cytokines such as TNF-α and IL-1β play an important role in the amplification loop of the immune response, and inhibition of these cytokines is of therapeutic significance.[10–12] In animal models of rheumatoid arthritis, TNF-α inhibition is found to have tremendous value in treating inflammation.[13] Similarly, the proinflammatory mediator nitric oxide (NO) also plays an important role in mediating acute inflammation. NO is a toxic free radical produced by macrophages following tissue injury and the excessive production of NO often results in DNA damage and causes destruction of host tissues. Hence inhibition of this toxic proinflammatory mediator is of therapeutic importance.[14] Upon antigenic stimulation, the T cells are activated to produce IL-2, which is a T cell growth factor. The release of IL-2 in turn activates the T cells to differentiate into either Th1 type or Th2 type cells. Hence, the early inhibition of IL-2 helps in controlling inflammation.

The present study highlights the anti-

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inflammatory and immunomodulatory potential of the plant *Ocimum basilicum* extracts which have been reported in Ayurveda.

MATERIALS AND METHODS

Chemical and Reagents —— Cell culture medium (Roswell Park Memorial Institute (RPMI)-1640) supplemented with glutamine (2 mM), and 10% heat-inactivated fetal calf serum was purchased from GIBCO BRL, Carlsbad, California, U.S.A. [^{3}H] Thymidine was obtained from Amersham Pharmacia Biotech, Amersham, Buckinghamshire, U.K. Trizol reagent for RNA extraction was purchased from SIGMA, St. louis, Missouri, U.S.A. Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (RT) and dNTP and Taq polymerases for polymerase chain reaction (PCR) were obtained from GIBCO BRL, and New England Bio labs (NEB), Hertfordshire, U.K., respectively. Phytohaemagglutinin (PHA) and lipopolysaccharide (LPS) from E. coli serotype 026:B6 were purchased from Sigma. (3-(4,5-Dimethylthiazol-20-yl)-2,5- diphenyltetrazolium bromide (MTT) assay kit was purchased from Promega (Madison, WI, U.S.A.).

Collection of Plants and Preparation of Extracts —— Mature *Ocimum basilicum* whole plants were collected from Tamil Nadu Medicinal Corporation Limited (TAMCOL) medicinal garden at Kolli Hills, South India. The freshly collected plant samples were chopped, shadow dried and coarsely powdered by using a mixer grinder for 5–10 min. One hundred grams of coarse plant powder was extracted using organic solvents in increasing order of polarity (hexane, dichloromethane, ethyl acetate, methanol and water). The extracts were dried under reduced pressure using a rotary flash evaporator. The final yield was 2–4 g for each extract. The dried crude extracts were used for *in vitro* assays, dissolved in dimethyl sulfoxide (DMSO).

Cell Culture —— The murine macrophage cell line, RAW 264.7 (American Type Culture Collection (ATCC), Manassas, Virginia 20108, U.S.A.) was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2 mM glutamine, antibiotics (penicillin 120 U/ml, streptomycin 75 μg/ml, gentamycin 160 μg/ml, and amphotericin B 3 μg/ml) and 10% heat-inactivated fetal bovine serum. All cell cultures were maintained at 37°C in a humidified incubator with 5% CO_2.

PBMC Isolation —— PBMC were isolated as described.[15] Briefly, heparinized venous blood from healthy donors was obtained with their informed consent. Cells were obtained by density gradient centrifugation using ficoll histopaque. The buffy layer containing PBMC was carefully collected, washed twice with RPMI 1640 medium, and then the cells were counted. The cells were then seeded onto 96-well U bottom plates at a density of 0.2×10^6 cells per well.

Mitogen-Induced Lymphocyte Proliferation Assay —— For the cell proliferation study, the PBMC were separated as described. Triplicate cultures in 200 μl of PBMC suspension were cultured in 96-well U bottom micro titer plates at 0.2×10^6 cells/well in RPMI medium containing 10% Fetal Calf Serum (FCS) and 1 μg/ml of PHA. The cells were treated with different concentrations of crude extracts (hexane, dichloromethane, ethyl acetate, methanol and water) and incubated for 3 days. After 72 hr, the cultures were pulsed with [^{3}H]thymidine (0.5 μCi/well) during the last 16 hr of incubation. The cells were then harvested and the radioactivity measured using a scintillation counter (Packard Liquid Scintillation Counter, U.S.A.). The results are expressed as the mean ± scanning electron microscope Standard Error Mean (SEM).

Lactate Dehydrogenase (LDH) Release Assay —— To confirm that the suppressive effect on lymphocyte proliferation was not due to cytotoxicity, the supernatants were also assayed for the enzyme LDH. This stable cytosolic enzyme, which is released upon cell lysis, was measured using the Cyto Tox 96^{TM} assay (Promega).

Determination of NO Production —— The mouse macrophage cell line RAW 264.7 was used to estimate NO production in LPS treated cells. The cells were cultured in DMEM supplemented with 10% FCS and plated in 96-well flat bottom culture plates and incubated at 37°C and 5% CO_2. Different concentrations of *Ocimum basilicum* crude methanolic extract were applied to LPS (1 μg/ml) stimulated RAW 264.7 cells. After 24 hr, nitrite production in the culture supernatants was assayed by the Griess reaction by measuring the total amount of inorganic NO. One hundred μl aliquots were removed from the medium and incubated with an equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthylenediamine hydrochloride in 2.5% H_3PO_4), for 10 min at room temperature, and absorbance was measured at 540 nm in an Enzyme Linked Immuno Sorbent Assay (ELISA) reader. Nitrite concentra-

tion was calculated with reference to a standard curve obtained using sodium nitrite.

Isolation of Total RNA and Semiquantitative Reverse Transcriptase-polymerase Chain Reaction (RT-PCR) —— PBMCs or macrophages were co-incubated with optimized doses of crude methanolic extract for the required time periods and the cells were immediately homogenized using Total RNA Isolation (TRI) reagent (Sigma) and RNA was isolated by phenol-chloroform extraction. The aqueous phase containing RNA was then precipitated by adding an equal volume of isopropyl alcohol. The RNA obtained was then converted to cDNA by reverse transcription using MMLV reverse transcriptase enzyme and subjected to PCR with specific primers. PCR products were run on 1.2% agarose gels, stained with ethidium bromide and photographed. PCR products were consistent with the predicted sizes. PCR products were analyzed on ethidium bromide-stained agarose gels.

Statistical Analysis —— All data are expressed as the mean ± SEM. The statistical significance was evaluated by one-way analysis of variance (ANOVA) using SPSS version 10.0 (SPSS, Cary, NC, U.S.A.). When there was a significant difference, Tukey's multiple comparisons were performed by fixing the significance level at $p < 0.05$.

RESULTS AND DISCUSSION

Medicinal plants and their products have been used for many centuries to treat different kinds of acute and chronic inflammatory diseases like wound healing, edema and rheumatoid arthritis.[15] Recently, several studies have reported *in vitro* effect on lymphocyte proliferation in the presence of mitogenic cells, and the specific antigens.[16] With this literary background, crude extracts from *Ocimum basilicum* were investigated for anti-inflammatory properties by evaluating the inhibitory effects on a variety of key mediators that regulate immune responses *in vitro*. The dried powder was sequentially extracted with hexane, dichloromethane, ethyl acetate, methanol and water, and the extracts were tested for their inhibitory effect on mitogen-induced lymphocyte proliferation.[16] DMSO was used for reconstitution of the concentrated extracts. The concentration of DMSO was below 0.02%.

PBMC resembling primary culture is considered to be the best *in vitro* model for studying the immunomodulatory properties of plant drugs. Therefore, using thymidine incorporation assay, the ability to evaluate and determine the optimum concentration of crude extracts, varying doses were co-incubated with PHA induced PBMC for 72 hr and the inhibitory effect was studied. Significant inhibition of 78% of crude methanolic extract was found at 30 μg/ml [Fig. 1(B)]. However, no signif-

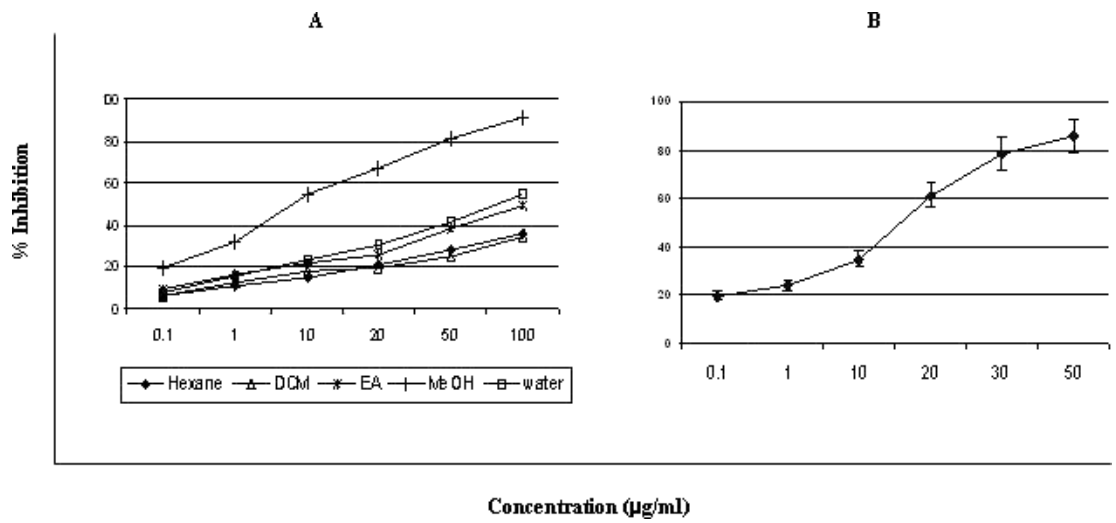

Fig. 1. (A) Analysis of the Lymphocyte Proliferation Assay for Crude *Ocimum basilicum* Extracts on Mitogen-induced Human PBMC at 72 hr. (B) Dose-response Analysis of Methanol Crude Extract in Mitogen-stimulated PBMC

Dose-response study was performed for *Ocimum basilicum* crude methanolic extract on mitogen-induced PBMC with different concentrations (0.1, 1, 10, 20, 30 and 50 μg/ml). The optimum dose was found to be 30 μg/ml. The results are expressed in terms of percentage inhibition with respect to stimulated control.

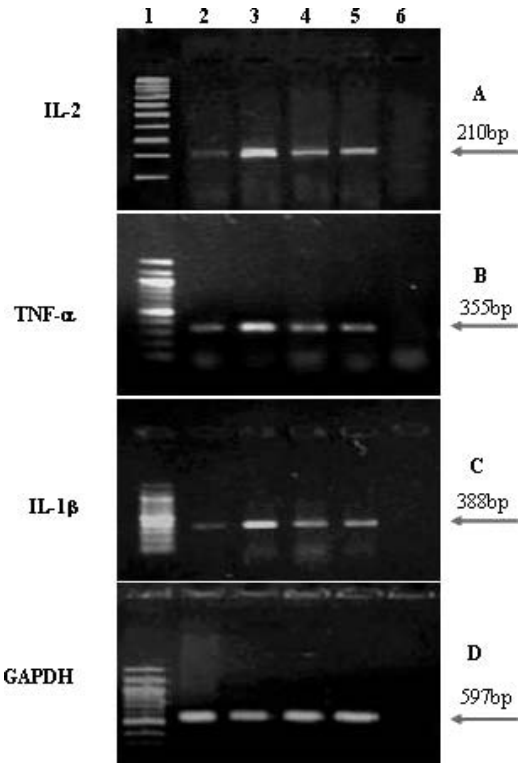

Fig. 2. Analysis of *Ocimum basilicum* Crude Extract on LPS-induced Proinflammatory Cytokine Expression in PBMC

(A) IL-2, (B) TNF-α, (C) IL-1β and (D) GAPDH. PBMCs were induced with LPS (1 μg/ml) at 12 hr and the inhibitory effect of crude methanolic extract on cytokine expression was studied by RT-PCR analysis. Lane 1: 100 bp Marker, Lane 2: Cell control, Lane 3: Cells+LPS, Lane 4: Dexamethosone, Lane 5: Crude methanolic extract (30 μg/ml), Lane 6: PCR negative control.

icant inhibition of proliferation was observed in the other crude extracts even at higher concentrations [Fig. 1(A)]. Thus, the inhibitory effects of *Ocimum basilicum* crude methanol extract on PHA activated PBMC proliferation could be suggestive of suppression of T cell proliferation. Because of its pivotal role in immune regulation,[17, 18] T cell activation provides a target for pharmacological modulation aimed at achieving clinically useful immunosuppression.[19] Further, this inhibition was not due to its cellular toxicity which was confirmed by MTT assay at 72 hr (data not shown). Based on its effect on mitogen-induced lymphocyte proliferation, methanolic crude extract was subjected to RT-PCR to assess the expression level of proinflammatory cytokines. The optimum dose selected was 30 μg/ml.

RT-PCR analysis was performed to study the effect of *Ocimum basilicum* crude methanolic extract on IL-2 mRNA expression in PBMC. Observation showed that *Ocimum basilicum* methanolic extract inhibited IL-2 mRNA expression in PBMC stimulated with 1 μg/ml of LPS [Fig. 2(A)] at the 12 hr time point and this decrease was in correlation with the previously observed inhibition of PBMC proliferation by the crude methanolic extract [Fig. 1(B)]. The expression level was normalized with the internal control Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [Fig. 2(D)]. IL-2 is the major T cell growth factor that is required for driving T cells into the proliferation cycle.[20] The inhibition of IL-2 would have positively culminated in the inhibition of T cell proliferation.

TNF-α and IL-1β play important roles in the amplification loop of the inflammation response as they can stimulate themselves in autocrine and paracrine fashion.[10, 11] Both TNF-α and IL-1β activate the inflammatory cells and induce the production of other inflammatory mediators that in turn modulate important cellular events including gene expression, DNA damage and cellular proliferation contributing to various inflammatory disorders. Therefore, cellular manipulation of the production of TNF-α and IL-1β is of importance for regulating the inflammatory response.[21] In this regard, we assayed the ability of the crude methanolic extract to decrease the LPS-induced TNF-α and IL-1β production in human PBMC at the 12 hr time point [Fig. 2(B) and 2(C)]. As seen in Fig. 2(A) and 2(B), the crude methanolic extract showed considerable inhibition of the proinflammatory cytokines TNF-α and IL-1β. This inhibition observed in proinflammatory gene expression, under LPS-induced conditions, supports the anti-inflammatory potential of the crude extract.

NO production by LPS-stimulated RAW 264.7 macrophages is reflected in the accumulation of nitrite in the cell culture medium. RAW 264.7 cells were treated with various concentrations of *Ocimum basilicum* crude methanol extract in the presence of LPS (1 μg/ml). After 24 hr, nitrite production in the culture supernatants was assayed by the Griess reaction by measuring of the total amount of inorganic NO. A significant decrease in NO production was observed on treatment with crude methanolic extract at 12 hr [Fig. 3(A)] and could have resulted from the suppression of inducible nitric oxide synthase (iNOS) gene induction, which was studied by RT-PCR as shown in Fig. 3(B). NO plays a major role in inflammation and is chiefly produced by iNOS. It is involved in the regulation of many cell functions and in the expression of several diseases

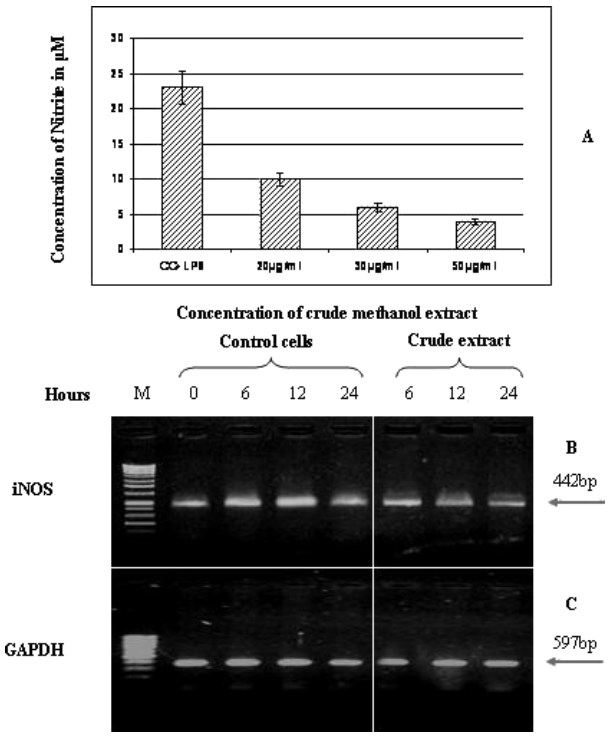

Fig. 3. Analysis of *Ocimum basilicum* crude extract on Nitric Oxide production in RAW cells.

(A) Effect of *Ocimum basilicum* crude methanol extract on NO production in RAW cell line. RAW cells were induced with 1 µg/ml of LPS and the effect of *Ocimum basilicum* crude extract on NO production was evaluated after 24 hr incubation. NO produced in culture supernatants was measured using $NaNO_2$ as standard. (B) Analysis of iNOS transcripts on LPS stimulated macrophages at 12 hr. (C) GAPDH expression profiles. RAW cells were induced with LPS (1 µg/ml) for various lengths of time (6, 12, 24 hr) and the inhibitory effect of crude methanol extract on iNOS expression was evaluated.

conditions such as atherosclerosis and arthritis.[22, 23] Excessive production of NO is indicated in both acute and chronic inflammations.[24] Therefore, the use of exogenous modulators becomes necessary for avoiding NO overproduction. Diverse plant-derived materials have been investigated as inhibitors of NO production, and the development of effective inhibitors for NO production in inflammatory cells is eagerly anticipated for the treatment of diseases mediated by NO.[25] In this study, the crude methanolic extract of *Ocimum basilicum* showed significant down regulation of NO production, which is in accordance with the inhibition seen in iNOS expression at 12 hr. As iNOS is the enzyme responsible for the production of NO, this data correlates well with the observations seen in macrophages.

The crude methanolic extract of the plant *Ocimum basilicum* thus exerts anti-inflammatory properties in human PBMC through down regulation of certain key proinflammatory cytokines and mediators under LPS-induced conditions. Hence, further analysis of the effect of the crude extract on other mediators of inflammation and also isolation of the principal bioactive component from the crude methanolic extract and its validation will help in the molecular profiling of the anti-inflammatory properties of the plant.

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