The black seeds of the *Nigella sativa* (NS) plant have an extensive history of medicinal use that dates back thousands of years. In ancient Greece and Egypt, black seed oil was used by physicians to treat an assortment of illnesses including headaches, nasal congestion, toothaches and intestinal worms. (1) NS, a buttercup-like plant that belongs to the Ranunculaceae plant family, is cultivated around the world for its seeds which can be used as a spice or preservative agent. Today however, the seeds are primarily used for the extraction of oil that is used in traditional medicine. NS is thought to promote menstruation and increase milk production in women. (2,3) Contemporary naturopathic medicine around the world has used NS as a diuretic, carminative, treatment for asthma, bronchospasm, respiratory oppression, coughs, back pain, hypertension and obesity. (1,2,4) Until recently, few studies had been conducted to confirm the validity of the proposed medicinal value of NS. In recent years, various studies have been conducted on NS to investigate such properties as anti-microbial, hypotensive, anti-nociceptive, anti-histaminic, immunomodulatory, anti-inflammatory, anti-tumour, and anti-diabetic as well as many other characteristics. (2,5)

Generation of effective immunity requires both innate immunity that recognizes pathogen associated molecular patterns and adaptive immunity that recognizes specific antigens. (6) Innate immunity consists of non-specific cells, including granulocytes, natural killer (NK) cells, and dendritic cells. Adaptive immunity is comprised of a humoral arm mediated by B cells that secret antigen specific antibodies and cellular arm mediated by CD4+ (helper) and CD8+ (cytolytic) T cells. (7) CD4+ T helper cells are responsible for orchestrating an immune response, whereas cytolytic CD8+ T cells are the killer cells that traffic to sites of infection or cancer and lyse infected or tumor cells. Together, these two types of effector T lymphocytes play critical roles in eliminating infections and controlling cancer. One of the precious properties of NS is the immunomodulatory effects of its constituents. Studies begun just over a decade ago suggest that if it is used on an ongoing basis, NS can enhance immune responses in humans. (8) The majority of studies who shown a 55% increase in CD4 to CD8 T cells ratio, and a 30% increase in NK cell function. Recently, a well-designed study analyzed the immunomodulatory effects of the whole extract of NS seeds and their protein components in vitro. (9,10) The composition of the seeds reflects the recommended
optimal dietary intake of n-3 and n-6 fatty acids, i.e.,
it has a ratio of n-3 to n-6 fatty acids of 1 to 4 or 5.\(^{(11)}\)
Dietary supplementation with the NS oil has been found to improve the immune response of healthy elderly subjects, which is mediated by a change in the factors closely associated with T cell activation.\(^{(12)}\)
A variety of experiments have shown that excessive or insufficient production of cytokines may significantly contribute to the pathophysiology of a range of disease responses and are thought to be decisive for pathological or physiological consequences.\(^{(13)}\)
After activation, CD4 T helper cells differentiate into either TH1-type cells, secreting interleukin (IL)-2, IL-6, IL-8, IL-12, interferon-γ (IFN-γ) and tumor necrosis factor α (TNF-α), or TH2 type cells secreting IL-4, IL-5, IL-10, and IL-13. Indeed, the balance between TH1 and TH2 cytokines is critical for the orientation of the inflammatory response toward cell-mediated or humoral-mediated responses. Thus, any factors that can interfere with TH1/TH2 axis might affect the outcome of the response.\(^{(7)}\)

This study aimed to investigate the effects of the NS plant methanolic extract on spontaneous lymphocyte mitogenic activity and mitogen (phytohemagglutinin, PHA) induced-lymphocyte proliferation \textit{in vitro}. This will further enhance our understanding of the immunomodulatory effects of NS for better immunotherapeutic applications.

**METHODS**

**Preparation of Peripheral Blood Mononuclear Cells**
The peripheral blood mononuclear cells (PBMCs) were separated from the whole blood of healthy donors by Ficoll-Hypaque gradient centrifugation (Sigma-Aldrich, Saint Louis, Missouri, USA). The PBMCs were prepared under sterile conditions in RPMI-1640 medium containing 10% fetal calf serum. Their viability, as determined by the trypan blue exclusion test, was more than 98%, and their concentration was finally adjusted to \(2 \times 10^5\) cells/mL.

**Lymphocyte Activation Assay**
One hundred microliters of cell suspension were pipetted into each well of 96-well tissue culture plates; to which 100 \(\mu\)L of media containing different concentrations of methanol extracts (2.5 and 5.0 \(\mu\)g/mL), and 10 \(\mu\)L (20 \(\mu\)g/mL) of media containing a sub-optimal concentration of PHA to stimulate T-cells were added or without PHA. One triplicate series of wells was used as a negative control (without extracts and PHA). The plates were incubated for 48 h at 37 °C in a 5% CO\(_2\) incubator.

**Flow Cytometry Analysis**
All fluorescein isothiocyanate (FITC-A), allophycocyanin (APC) and phycoerythrin (PE-A) conjugated monoclonal antibodies were purchased from BD Biosciences (Franklin Lakes, New Jersey, USA). The isolated PBMC were cultured in triplicate with NS methanolic extract, with or without PHA for 48 h in 37 °C of a 5% CO\(_2\) incubator. Then the numbers of NK cells (CD16, CD56), and helper and induced T cell subsets (CD3, CD4), at 48 h were determined by standard FACScan procedures with monoclonal antibodies according to the manufacturer's protocol. The stained PBMCs were analyzed by a FACSCanto II flow cytometric analyzer (Becton Dickinson, Cookeysville, MD) with FACSDiva software (Becton Dickinson).

**c-DNA Synthesis and Quantitative Real Time-Polymerase Chain Reaction Analysis**
PBMCS were treated with two different concentrations of methanolic extract of NS seed and the procedures of quantitative real time-polymerase chain reaction (RT-PCR) analysis were carried out according to the manufacturer's instructions. Briefly, cDNA was directly prepared from cultured cells by using Cell-to-cDNA synthesis reagent kit (Promega, Fitchburg, Wisconsin, USA) and the levels of IL-6, IL-8 and TNF-α mRNA as well as the reference gene GAPDH were assayed by the gene-specific SYBR Green gene expression assays (QIAGEN, Duesseldorf, Germany). All samples and controls were run in triplicates on an ABI 7500 Fast RT-PCR system. The quantitative RT-PCR data were analyzed by the comparative cycle number threshold method and the fold inductions of samples were compared with the untreated samples.

The GAPDH gene was used as an endogenous control. The amount of gene expression was then calculated as the difference cycle threshold (\(\Delta\)CT) between the CT value of the target gene and GAPDH. \(\Delta\)CT is the difference between the \(\Delta\)CT values of the test sample and the control. Relative expression of target genes was calculated as \(2^{-\Delta\Delta\text{CT}}\).

**Statistical Analysis**
The data were expressed as the mean ± standard