

ZERUMBONE, A NATURAL PLANT DIETARY COMPOUND INDUCES EXPRESSION OF INTERLEUKIN-12P70 CYTOKINE IN HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

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ABSTRACT

Objective: Despite possessing many biological activities as antiproliferative, antioxidant, anti-inflammatory, and anticancerous, and zerumbone lacks any evidence for its immunomodulatory activity. This naturally occurring dietary compound needs to be developed as drug to support therapeutic claims in various infections and diseases.

Methods: Hence, in this study, the immunomodulatory effects of zerumbone were investigated by evaluating the effect of this compound toward the lymphocytes proliferation in human peripheral blood mononuclear cells.

Results: Lymphocyte proliferation assay showed that zerumbone was able to activate human lymphocytes at dosage-dependent manner at the highest concentration 40 µl/mL. The production of human interleukin-12p70 cytokine in culture supernatant from activated lymphocytes was upregulated by zerumbone at 24 hrs and gradually decreased at 48 hrs. Hence, the study confirms the immunomodulatory activity of zerumbone which play an important role in boosting up the immune system through cytokine production in dosage dependent manner.

Conclusion: The study concludes that zerumbone could be used as a lead molecule in herbal therapeutic world as an immunomodulatory drug in the treatment of chronic infections and various autoimmune disorders.

Keywords: Zerumbone, Peripheral blood mononuclear cells, Immunomodulation, Cytokine, Lymphocyte proliferation.

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INTRODUCTION

Zerumbone, a naturally occurring nutritional compound is a monocyclic sesquiterpene found in large quantities in rhizome of *Zingiber zerumbet* Smith [1,2] and is present in many natural foods we consume. Its structure possesses three double bonds, two conjugated and one isolated, as well as double conjugated carbonyl group in the 11-membered ring structure [3] and has been found to possess many biomedical properties such as antiproliferative, antioxidant, anti-inflammatory [4-6], anticancer [7-10], and antimicrobial [11-13] activities. It possesses antiproliferative properties toward several cancer cell lines mainly on human cervical cancer (HeLa) cell line [14]. Zerumbone also showed cell proliferation and induction of cytokines in mice thymocytes and splenocytes *in vitro*. It is also reported that zerumbone may be useful in the treatment of Alzheimer's disease [15]. Besides having all these medicinal and therapeutic properties zerumbone is yet to be studied for its immunomodulating activity on human cells such as peripheral blood mononuclear cells (PBMCs) with respect to cytokine study to help fight against various infections and diseases and for the development of the immune system. Cytokine is the major immunoregulator of the body. Modulation of cytokine secretion may offer novel approaches in the treatment of a variety of diseases. Measuring cytokines that are involved in immune regulation has been the focus of researchers for over decades. Interleukin-12p70 (IL-12p70) is a 70-kDa heterodimeric, pro-inflammatory, and multifunctional cytokine produced mostly by monocytes, macrophages, and dendritic cells and acts as a key regulator of cell-mediated immune responses through the differentiation of native CD4+ T-cells into Type 1 helper T cells (Th1). It stimulates the production of interferon-gamma and tumor necrosis factor-alpha. There are many plant-derived materials (aromatic compounds, proteins, lectins, polysaccharides, etc.) have been shown to stimulate the immune system [16]. Essential oil yielding plants such as *Salvia officinalis*, *Syzygium aromaticum*, *Mentha* sps.,

Eucalyptus sps., and *Ocimum basilicum* are found to stimulate immune cell proliferation, cytokines expression both *in vitro* and *in vivo* [17-19]. Zerumbone which is a bioactive compound of essential oil yielding plant of *Z. zerumbet* have been reported to have antioxidative, anticancer, anti-inflammatory [5,6], and antimicrobial properties [12,13] is yet to be investigated with respect to its immunomodulating activity. Keeping this in view, this study has been designed to find out the immunomodulatory role of zerumbone in modulating the cytokine expression and its effect on the proliferation pattern of lymphocytes in human PBMCs. The findings of this study will help us to understand the influence of this active component zerumbone in stimulating the immune response thereby developing the immune system and can be used as a drug. Furthermore, this study can recommend zerumbone to be used in clinical studies for its effectiveness, usefulness, and safety use in herbal therapeutic world.

METHODS

Preparation of sample

Zerumbone ≥98% (high performance liquid chromatography), 10 mg/mL, off white colored powder (10 mg) was purchased from Sigma-Aldrich and was stored at 2-8°C as per manufacturer's instructions. It was diluted with phosphate buffered saline (PBS) to achieve different concentrations (0, 5, 10, 20, 25, and 40 µg/mL).

Isolation of PBMCs

Human peripheral blood from healthy adults were obtained and kept in sterile sodium heparin tubes. To obtain PBMCs, the blood was added with PBS (pH 7.4) and Ficoll-Paque™ PLUS (GE Healthcare). The heparinized blood was then centrifuged for 20 minutes at 2000rpm at a temperature of 15°C to obtain PBMC ring. The ring was extracted using a pipette and transferred to a Falcon tube, and PBS and fetal bovine serum (FBS) were added to the solution to complete 10mL. This solution

was again centrifuged for 20 minutes at 1000 rpm at a temperature of 15°C to obtain leukocyte cloud. The cells from the interphase were extracted and washed 3 times with PBS (10 minutes, 1000 rpm, 15°C). After the last wash, cells cultured in Roswell Park Memorial Institute (RPMI) 1640 medium with 20mm Hepes supplemented with 10% FBS, HiMedia. The number of PBMCs was counted using hemocytometer.

Cell proliferation assays

Cell proliferation assay was performed using MTT assay kit (e-Bioscience) as per the instruction of the manufacturer. PBMCs cultured were resuspended (5×10^6 cells/mL) in RPMI-1640 medium in the presence of lipopolysaccharide (LPS). Then, 100 μ l of different concentration (0, 5, 10, 20, 25, and 40 μ g/mL) of zerumbone was added in RPMI-1640. The culture plates were incubated for 48 hrs in a CO₂ incubator at 5% CO₂ and 37°C temperature. After incubation 10 μ l of [3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide [MTT] 2.5 mg/mL) solution were added to each well, and the plate was wrapped to avoid exposure to light and kept for incubation for 4 hrs. 100 μ l of the solubilizing reagent was added to each well, and the absorbance was recorded at 570 nm using Mindray MR-96A microplate absorbance reader.

Estimation of cytokine (IL-12p70)

Peripheral blood cells were cultured in triplicate at a concentration of 5×10^6 cells/mL. Zerumbone at a concentration of (5 μ g/mL) was added separately in 24 wells cell culture plate. For positive control LPS (10 ng/mL) was used for stimulation of IL-12p70 cytokine. The control group of cells was cultured without addition of zerumbone. The plates were kept for incubation in a CO₂ incubator (5% carbon dioxide) at 37°C for 48 hrs. Separate wells were used for harvesting culture supernatants for cytokine production. The culture supernatants were harvested, centrifuged, and stored at -70°C for estimation of cytokine. Later on, cytokine assay was performed using IL-12p70 sandwich enzyme-linked immunosorbent assay (ELISA) kits (e-Bioscience) as per the manufacturer's instruction, and the absorbance recorded were expressed in picograms per milliliter by interpolation from standard curves.

RESULTS

Lymphocyte proliferation assay

PBMCs count was found to be increased in number with the treatment of different concentrations of zerumbone as compared to the control (without zerumbone). The result showed the increased metabolic activity of cells on PBMCs in response to zerumbone. Zerumbone significantly stimulated the lymphocytes proliferation at all concentrations (0-40 μ g/mL). The highest percentage of cell viability was (166 \pm 5.01)% at 40 μ g/mL after 24 hrs of treatment which when compared with that of the control was much high in number. Similarly, the percentage of cell viability was decreased to some extent to (148 \pm 2.11)% at 40 μ g/mL treating the same after 48 hrs but was found to be higher than the control (Fig. 1).

Cytokine production and estimation of human PBMCs

Production of human IL-12p70 cytokine was measured using sandwich ELISA according to the manufacturer's instructions (eBioscience). All the experiments were carried out in 96 well microtitre plates. Both the culture supernatants of the sample and the control were added to the microtitre plates. The plate was wrapped properly and incubated at room temperature for 4 hrs. The plate was washed three times with wash buffer with the proper aspiration of the wells and was tapped carefully onto an absorbent pad to remove excess buffer content from the wells. After washing substrate solution 3, 3', 5, 5'-tetramethylbenzidine was added and the plate was incubated at room temperature for 10 minutes. Stop solution (1 NH₂SO₄) was added to the wells to stop the enzyme substrate reaction and the reading was taken immediately using Mindray MR-96 A microplate absorbance reader at 450 nm. The level of IL-12p70 after co-incubation of zerumbone with human PBMCs showed that the secretion of cytokine increased at different concentrations of zerumbone. There was significant ($p < 0.05$) dose-dependent upregulation or immunostimulation of IL-12p70

cytokine after 24 and 48 hrs of incubation as compared to the control (0 μ g/mL). The secretion of IL-12p70 after 24 hrs of incubation was up-regulated by zerumbone from (1.198 pg/mL) in the control group to (2.367 pg/mL) at the highest concentration (40 μ g/mL). A similar result was also found after treating the sample with zerumbone after 48 hrs of incubation. The cytokine concentration was found to increase along with the increasing concentration of zerumbone from (0.98 pg/mL) in the control group to (2.485 pg/mL) at the maximum concentration (40 μ g/mL) (Fig. 2).

DISCUSSION

Utilization of natural products with therapeutic properties is as old as human civilization; therefore, natural products are becoming increasingly important as sources of pharmacotherapeutics. For a long period of time natural compounds from medicinal plants and their products were the main sources of drugs and are claimed to have different therapeutic properties [19]. Zerumbone, a sesquiterpene is the principal bioactive compound of *Z. zerumbet* and is widely studied for its medicinal and therapeutic properties. It possesses anticancer, anti-inflammatory, antimalarial, and many other biological activities [20]. The previous studies reported zerumbone to be the major active component of *Z. zerumbet* rhizome [20,21]. In view of the relatively high

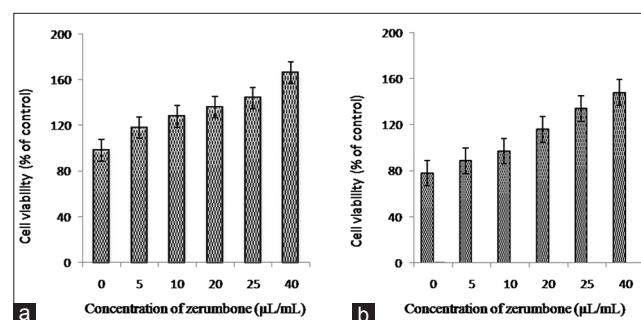


Fig. 1: Effect of zerumbone on the proliferation of peripheral blood mononuclear cells. All data are presented as the mean \pm standard deviation of five measurements. Peripheral blood mononuclear cells (5×10^6 cells/mL) were cultured *in vitro* with lipopolysaccharide (1 μ L/mL) along with different doses of zerumbone (0-40 μ L/mL). (a) Peripheral blood mononuclear cells viability after 24 hrs of incubation. (b) Peripheral blood mononuclear cells viability after 48 hrs of incubation. The peripheral blood mononuclear cells viability was measured by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide method

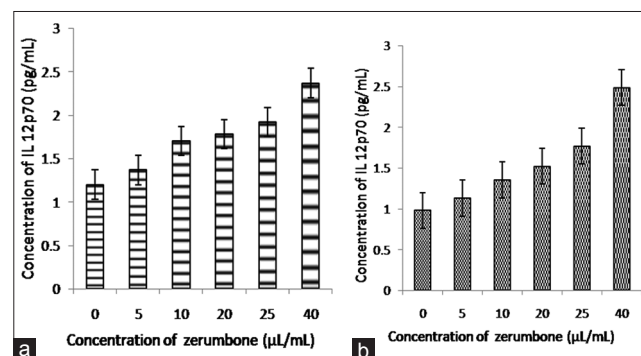


Fig. 2: Effects of different concentrations of zerumbone on expression pattern of interleukin-12p70 cytokine after different hours of incubation (a) interleukin-12p70 cytokine at various concentrations of zerumbone after 24 hrs of incubation. (b) Interleukin-12p70 cytokine at various concentrations of zerumbone after 48 hrs of incubation. All data are presented as the mean \pm standard deviation of five measurements

cost and severe side effects of allopathic medicines, researchers are now focusing on examining natural plant-derived products for modern therapeutics by positively modulating the immune system against infections. Numerous experiments reported the immunomodulatory activity of sesquiterpenes [22,23] that induces immunomodulatory effect through apoptosis of nuclear factor- κ B inactivation. The previous studies also reported that zerumbone was able to activate mice thymocytes, splenocytes, and PBMCs at dosage dependent pattern [24]. In this study, for the first time, we explored the immunomodulatory effects of zerumbone in human PBMCs. IL-12p70 has been shown to play a critical role and is associated with various autoimmune and inflammatory disorders such as osteoarthritis and rheumatoid arthritis and has been shown to be directly involved in the induction of various diseases including multiple sclerosis, inflammatory bowel disease, diabetes mellitus and glomerulonephritis [25-28]. Immunomodulators derived from medicinal plants are critically being considered as an alternative approach to conventional treatment against various diseases. Estimation of the immune cells through lymphocyte proliferation is always used to screen the potential immunomodulatory effect of a compound. Our study exhibited an impressive therapeutic role of zerumbone in the stimulation and proliferation of human lymphocytes in dose-dependent concentrations which indicates the immunomodulatory activity of this bioactive compound. In the LPS stimulated proliferation assay, the lymphocytes number were found to be increased along with the increasing concentrations of zerumbone after 48 hrs of incubation. The percentage of cell viability was the highest at the maximal dose, i.e., 40 μ g/mL which is (166 \pm 5.01)% and (148 \pm 2.11)% after 24 hrs and 48 hrs of treatment. Human PBMCs proliferation was much higher in LPS treated sample along with zerumbone than LPS alone. The proliferative activities of the compound exert stimulatory activity to the immune system. Cytokines have been known to play a key role in regulating the proliferation and differentiation of lymphocytes [24], hence the production of human IL-12p70 suggests the immunostimulatory effect of zerumbone on lymphocytes. After performing the cytokine estimation assay using ELISA, the result showed that zerumbone had significantly induced higher production of human IL-2p70 with the value of 2.367 pg/mL after 24 hrs and 2.485 pg/mL after 48 hrs of incubation at the highest concentration. This mitogenic effect of zerumbone which induced the human IL-12p70 cytokine is a human PBMCs stimulating factor has an immense potential in the treatment of various diseases and IL-12 immunotherapy could be of importance in the treatment of diseases where a Th1 response is desirable. Thus, this study suggested that zerumbone can act as a novel therapeutic agent in the development and maintaining of the immune system. The results indicated that zerumbone, a natural immunomodulator can obtain continuous immune activation and is urgently required in the field of therapeutics and can be used as immunomodulatory agent which can react toward the immune cell cytokine production in dose-dependent manner. Although it has showed promising results as an immunomodulator, further studies of this compound will help to elucidate the exact mechanism of action involved in this bioactive compound. There are some mechanisms of action of an herb which are still unclear and remain to be elucidated, require further study as potential therapeutic agents for immunological applications [29]. Once the exact mechanism of this compound is elucidated, this monocyclic sesquiterpene (zerumbone) which is also a plant secondary metabolite could be used as a novel drug in treating various chronic diseases leading from infections to cancer particularly in boosting up the immune system. These experimental observations suggest that zerumbone can safely support and augment conventional therapy for treating infections and various autoimmune disorders.

CONCLUSION

In conclusion, our study demonstrated that zerumbone can act as an immunostimulant on human lymphocytes. It also does not show any effect on the cell viability which implies its potential use in cancer chemotherapy where immune cells are suppressed. This study suggests zerumbone as potent immunomodulator that can be used

as an immunomodulatory drug and in pharmaceuticals as a therapy for patients in the future.

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