Research Article

Immunomodulation of Zerumbone via Decreasing the Production of Reactive Oxygen Species from Immune Cells

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Abstract

Background and Objective: Zerumbone has been reported to exert anti-inflammatory, anti-ulcer and anti-hyperglycemic effects but the specific mechanism through which zerumbone exerts its anti-inflammatory action through inhibiting reactive oxygen species was not well studied. Hence, this paper studied the zerumbone capacity to inhibit intracellular and extracellular Reactive Oxygen Species (ROS) produced by whole blood cell, polymorphonuclear cells (PMNs) and macrophage cells due to the zymogen and phorbolmyristate acetate (PMA) oxidant effect. Materials and Methods: Zymogen and PMA based chemiluminescence assay were used to determine the immunomodulatory effect of zerumbone at concentrations (100, 10 and 1 μg mL⁻¹) toward production of Reactive Oxygen Species (ROS) from whole blood, PMNs and macrophage. Results: Zerumbone significantly inhibited intracellular and extracellular ROS production by the zymosan/PMA-activated phagocyte cells with IC₅₀ values of (16.3±0.1, 23.7±0.1 and 4.97±0.1 μg mL⁻¹) against whole blood, PMNs and macrophage respectively. Conclusion: The anti-inflammatory activity of zerumbone was so much significant that even strong oxidant (zymogen and PMA) were not able to produce reactive oxygen species when incubated together in phagocytic cells, thus suppress production of ROS. Therefore, it is highly used in herbal medicine as a potent immunomodulatory therapy in various inflammation associated diseases.

Key words: Zerumbone, chemiluminescence assay, polymorphonuclear cells, macrophage, inflammation


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Data Availability: All relevant data are within the paper and its supporting information files.
INTRODUCTION

Inflammation is a complex process considered in the development of various chronic diseases such as cancer, diabetes, gastritis, cardiovascular diseases and atherosclerosis. During the inflammatory process, phagocytes are considered as the first line of defense during natural immune response and produce a sudden burst of reactive oxygen species (ROS) in the site of the inflammation, which perform critical roles in the eradication of a variety of invading agents. Although, ROS molecules are having key roles in mediating innate immune response but its overproduction might cause tissue damage leading to chronic inflammatory-related diseases. Zingiber zerumbet (Smith.) can be found in Southeast Asian countries. In traditional medicine, the rhizomes of this plant are used for the treatment of peptic ulcers, anti-inflammatory, headache, diarrhea and asthma. Zerumbone, a naturally monocyclic sesquiterpene was found as the major bioactive compound from the rhizomes of edible wild Zingiber zerumbet. Even though, the pharmacological studies had shown the validation of zerumbone to be used as anti-inflammatory, anti-cancer, antiulcer and antioxidant, the immunomodulatory activity of zerumbone towards oxidative burst produced by activated immune cells has not been clearly evidenced yet. Therefore, for the first time, zerumbone was remarked for inhibition of ROS activity using chemiluminescence assay, which provide new insight of its mechanism of action related to ROS production from the immune cells.

MATERIALS AND METHODS

Material: Zerumbone, off white colored crystals (10 mg) was gifted from Professor Dr. Rasedee Abdullah, Universiti Putra Malaysia (UPM). It was stored at 2-8°C and just before experiment, it was diluted with dimethyl sulfoxide (DMSO) to make stock solution of 20 mg mL⁻¹. This work was carried out in Dr. Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences, University of Karachi, Pakistan in between June-July, 2018.

Determination of intracellular ROS production by the isolated human polymorphs neutrophils (PMNs): About 10 mL of heparinized blood was collected aseptically from a healthy volunteer in compliance with Independent Ethics Committee of ICCBS, University of Karachi with informed consent ICCBS/IEC-028-HB-2017/PROTOCOL/1.0. The PMNs were isolated by Ficoll-hypaque density gradient centrifugation method. Briefly, on a 50 mL Falcon centrifuge tube, whole blood, lymphocytes separation medium (LSM) and Hank’s Balance Salt Solution (Ca²⁺ and Mg²⁺ free) (HBSS⁻⁻) were mixed in equal volumes. Neutrophils were purified from Red Blood Cells (RBCs) contamination using distilled water for 1 min for the lysis of RBCs; 1 mL of HBSS⁻⁻ (2x) was then added to stop lysis. A neutrophil count was done on a hemocytometer using the trypan blue exclusion method and cells concentration were adjusted to 1×10⁶ cells mL⁻¹ using Hank’s Balance Salt Solution containing Ca²⁺ and Mg²⁺ (HBSS++).

Determination of extracellular ROS production by the isolated mice macrophages: In compliance with Animal Care and Use Committee of ICCBS, one healthy albino mice (18-25 g) was immunized with 1 mL of fetal bovine serum (FBS) intraperitoneal using sterile 1 mL syringe and the mice was kept for 72 h and sacrificed by cervical dislocation. About 10 mL of 10% RPMI medium was injected into the peritoneal cavity after sterilizing whole animal body by dipping into 70% ethanol. Peritoneal cavity was massaged for 2 min and the abdominal skin was cut from the lower side and retracted to expose the peritoneal cavity. The injected RPMI containing macrophage was collected with a help of a sterile syringe from the peritoneum and centrifuged at 400 rpm for 20 min at 4°C. After discarding the supernatant, the pellet was washed with incomplete RPMI medium at 300 rpm for 10 min at 4°C, then pellet was re-suspended in 1 mL incomplete RPMI medium/HBSS++. Trypan blue exclusion method was used to check cell viability and count cell number.

Chemiluminescence technique: Chemiluminescence test was performed as described previously with modifications by Helfand et al. Briefly, 25 μL of three different concentrations of zerumbone (1, 10 and 100 μg mL⁻¹) and ibuprofen as a drug control were mixed separately with 25 μL diluted human whole blood (diluted in sterile HBSS++ (1:20)), isolated PMNs (1x10⁶ cells mL⁻¹) and isolated mice macrophages. (2x10⁶ cells mL⁻¹). For positive control, wells received only the HBSS++ but not extracts. After 20 min incubation of plates at 37°C (Thermostated chamber of Luminometer), 25 μL of serum opsonized zymosan as activator and 25 μL of luminol dye were added to wells containing whole human blood and isolated PMNs. Similarly, 25 μL of PMA as activator and 25 μL
of lucigenin dye were added to those wells containing isolated mice macrophages, such that final volume of all wells become 100 µL. Relative Luminescence Unit (RLU) of each well were obtained. The following formula was used to calculate percentage inhibition for each concentration of zerumbone:

\[
\text{% production of ROS} = \frac{\text{Average reading of test compound}}{\text{Average reading of positive control}} \times 100
\]

**Statistical analysis:** All results were presented as mean±standard deviation and statistical significance was performed by one-way analysis of variance (ANOVA) with Tukey’s *post-hoc* test using GraphPad prism 6.0 statistical software with significant differences set at *p*<0.001.

**RESULTS**

**Effect of zerumbone on intracellular ROS production by the whole human blood and isolated polymorphs neutrophils (PMNs):** The potential of zerumbone to modulate intracellular ROS released by zymogen induced human whole blood and isolated PMNs were assayed by determining oxidative burst in the presence or absence of zerumbone (Fig. 1). It was found that zymogen highly produced intracellular ROS from the induced human whole blood and PMNs cells. Upon addition of zerumbone at three different concentrations (100, 10 and 1 µg mL⁻¹), the percentage of ROS production was found to be 1.7, 63.3 and 67.9 and 4.4, 79.1 and 98 for whole blood and PMNs respectively. Zerumbone significantly (*p*<0.001) decreased the production of intracellular ROS from these cells in a concentration dependent manner having IC₅₀ values of 16.3±0.1 and 23.7±0.1 µg mL⁻¹, against whole human blood and PMNs cells, respectively.

**Effect of zerumbone on extracellular ROS production by the isolated mice macrophages:** The potential of zerumbone to modulate extracellular ROS released by isolated mice macrophages was also assayed by determining oxidative burst in the presence or absence of zerumbone (Fig. 2). The PMA highly triggered the production of extracellular ROS from the mice macrophages. Upon addition of zerumbone at three different concentrations 100, 10 and 1 µg mL⁻¹, the percentage of ROS production was found to be 0.5, 23.6 and 99.1 in isolated macrophages respectively. Zerumbone significantly (*p*<0.001) decreased the production of extracellular ROS from mice macrophages at 10 and 100 µg mL⁻¹ and having IC₅₀ value of 4.97±0.1 µg mL⁻¹.

**DISCUSSION**

In this study, zerumbone exhibited a dose-dependent suppression of oxidative burst levels in whole blood. Similarly, zerumbone showed a significant oxidative burst inhibition in human PMNs (Fig. 1). Furthermore, oxidative burst inhibitory activity was observed with zerumbone in macrophages also (Fig. 2). This study has also showed that zerumbone can modulate the ROS expression in activated phagocytes. The inhibited level of these molecules might be related to antioxidant and anti-inflammatory capacity provided by sesquiterpene compound (zerumbone).

Many natural products from medicinal plants have been utilized as a therapeutic agent to treat various diseases;
therefore, researchers are immensely focusing in the area of ethnopharmacology\(^7\). Zerumbone is a major sesquiterpene compound that can be found in the \textit{Z. zerumbet} and is reported for its therapeutic efficacy and mechanism of action\(^8,9,10,11\). Nowadays, immunomodulation drug against infections has become area of interest for researchers, leading them to examine for natural compound-derived plants with potent inhibitors of ROS production by immune cells in inflammatory processes. Previous reports showed that zerumbone can modulate the immune cell through different mechanism\(^12,13\). As a follow-up to this studies, for the first time, the present study intend to explore on the modulation effects of zerumbone that may be considered as a potential therapeutic for inflammation due to ROS production in zymosan/PMA-induced immune cells. Stimulation of the phagocytes ROS secretion through zymosan/ PMA is always used to screen the immunomodulatory efficiency of a compound. Thus, excess production of ROS by the activated macrophages causes chronic inflammatory reactions\(^6\). Luminol and lucigenin chemiluminescence, probes are used in measuring intracellular and extracellular ROS, respectively\(^14,15,16\). Furthermore, the inhibited level of ROS might be explained by capacity of compound to inhibit the receptor complement and protein kinase C (PKC), resulting in the inhibition of a NADPH oxidase enzyme\(^17,18,19\). The result in this study is in agreement with outcomes reported by Singh et al.\(^20\) who proved that zerumbone has a scavenging activity toward the free radical molecules. Thus, these outcomes suggested that zerumbone can act as strong natural therapeutic agent in controlling the inflammation.

CONCLUSION

Zerumbone, which act as immuno-modulators of ROS production in mitogen-activated immune cells, may be suggested as a potent anti-inflammatory agent that can normalized the conditions created by inflammation and considered it as a natural therapy in the treatment of inflammatory-related conditions. This study has also supported the folklore use of \textit{Zingiber zerumbet} in the traditional herbal medicine.

SIGNIFICANCE STATEMENT

This study signifies the anti-inflammatory property of zerumbone by showing its immuno-modulatory efficiency on activated immune cells. This study gives new insight in the anti-inflammatory activity of zerumbone by giving the IC\(_{50}\) concentration required to decrease both intracellular and extracellular ROS production from activated PMNs and macrophages, respectively. Thus this study will help the traditional practitioners to adjust the dose of zerumbone for the therapy against inflammatory diseases with less side effects.

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REFERENCES


