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Zerumbone from *Zingiber zerumbet* inhibits innate and adaptive immune responses in Balb/C mice

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ABSTRACT

Zerumbone exhibited various biological properties including *in vitro* immunosuppressive effects. However, its modulatory activity on the immune responses in experimental animal model is largely unknown. This investigation was conducted to explore the effects of daily treatment of zerumbone (25, 50, and 100 mg/kg) isolated from *Zingiber zerumbet* rhizomes for 14 days on various cellular and humoral immune responses in Balb/C mice. For measurement of adaptive immunity, sheep red blood cells (sRBC) were used to immunize the mice on day 0 and orally fed with similar doses of zerumbone for 14 days. The effects of zerumbone on phagocytosis, nitric oxide (NO) release, myeloperoxidase (MPO) activity, proliferation of T and B cells, lymphocyte phenotyping, cytokines release in serum by activated T cells, delayed type hypersensitivity (DTH) and immunoglobulins production (IgG and IgM) were investigated. Zerumbone downregulated the engulfment of *E. coli* by peritoneal macrophages and the release of NO and MPO in a concentration-dependent manner. Zerumbone showed significant and concentration-dependent suppression of T and B lymphocytes proliferation and inhibition of the Th1 and Th2 cytokines release. At higher concentrations of zerumbone, the % expression of $CD4^+$ and $CD8^+$ in splenocytes was significantly inhibited. Zerumbone also concentration-dependently demonstrated strong suppression on sRBC-triggered swelling of mice paw in DTH. Substantial suppression of anti-sRBC immunoglobulins antibody titer was noted in immunized and zerumbone-treated mice in a concentration-dependent manner. The potent suppressive effects of zerumbone on the immune responses suggest that zerumbone can be a potential candidate for development of immunosuppressive agent.

1. Introduction

Immune responses are basically the integral defensive action of the body and any disparity of fundamental responses will lead to various immune disorders. Thus, immune therapy or immunomodulation becomes essential to overcome this anomaly. Precisely, immunomodulation is the notion of modifying the immune response to accomplish the therapeutic benefit. Moreover, immunomodulators in immunotherapy are the proxies, which boost or suppress the host defensive responses and are used together with supplementary therapeutic modalities. Therefore, immunomodulators are used to transform the immune response consistent with the pathological condition. Immunomodulators derived from medicinal herbs have gripped a huge attention from the last few decades as therapeutic drug candidates for their affordability and safety than synthetic ones, as well as for their outstanding contributions for targeted drug actions and delivery mechanisms [1].

Zerumbone is a major constituent of *Zingiber zerumbet* (*Z. zerumbet*) rhizomes. The rhizomes have been popularly used in ethnomedicine to treat numerous inflammatory disorders and as appetizers and food flavorings in various dishes. This cyclic eleven-membered sesquiterpene has been revealed to possess potent biological effects especially anticancer and immunomodulating properties [1,2]. The potent immunosuppressive effect of zerumbone is thought to be due to the presence of its α,β-carbonyl based moiety [3]. Various *in vitro* reports confirmed that zerumbone exerted its inhibitory effects by suppression of several immune responses including cellular and humoral immune responses [2,3]. In our recent investigation, we showed that zerumbone inhibited immune response by downregulating the pro-inflammatory markers generation through inhibition of imperative MAPKs, NF-κB, and PI3K-Akt signaling activation in human macrophages [4]. Although the availing huge *in vitro* reports presented zerumbone as potent therapeutic potential for immune related disorders, the studies were not

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supported enough by prospective *in vivo* reports yet. Hence, in the current investigation, we examined the effects of zerumbone isolated from *Z. zerumbet* rhizomes on innate and adaptive immune responses in Balb/C mice.

2. Materials and methods

2.1. Chemicals and reagents

LPS (derived from *E. coli* 026:B6), concanavalin A (Con A), isothiocynate (FITC)-labeled opsonized *E. coli*, penicillin-streptomycin, Tween 20, RPMI-1640, 3% Brewer's thioglycollate medium lympho-prep™ gradient and fetal bovine serum (FBS) were purchased from Sigma (St Louis, USA). Myeloperoxidase (MPO) was bought from BioVision, Inc. (California, USA). DMEM was acquired from the Thermo Fisher Scientific (Waltham, USA). Sheep red blood cells (sRBC) was procured from Innovative Research Inc. (Novi, MI). Cyclosporin A (Cys A) was acquired from Sigma-Aldrich (Milano, Italy). Colorimetric activity assay kit, AST, ALT and ALP, were procured from Cayman Chemical (Michigan, USA). Mouse anti-sRBC IgM and IgG ELISA kits were procured from the Life Diagnostics, USA. A phagotest kit (Glycotope Technology, Germany), a flow cytometer (BDFACS Canto II equipped with 488 nm argon-ion laser, BD Biosciences), CO_2 incubator (Shell Lab, USA),and a plethysmometer (Ugo Basile, Gemonio, Italy) were available for the study. Xylene, paraffin wax and absolute ethanol was purchased from Sigma-Aldrich (USA).

2.2. Preparation of plant extract and isolation of zerumbone

Z. zerumbet rhizomes were collected from Kuala Krau, Pahang, Malaysia and was identified by a botanist at the Faculty of Science and Technology, Universiti Kebangsaan Malaysia (UKM). A voucher specimen was deposited at the Herbarium of UKM (No. UKM-HF137). Isolation of zerumbone was performed following the technique of Haque et al. [4]. Briefly, after being cut into small pieces, the rhizomes were air-dried under shed at room temperature and ground into a coarse powder. The extract was prepared by soaking the powdered sample (1.75 kg) with 80% ethanol (3 × 3 L) for 3 days at 25 °C. Then, by using a rotary evaporator, the extract obtained was freeze-dried yielding a crude *Z. zerumbet* extract (248.0 g) (14.17%). Ten g of the extract was then subjected to repeated column chromatography (40–63 μm, 3 × 60 cm) with n-hexane - EtOAc (10:0–7:3 ratios, v/v) as eluents. The eluates were then evaporated, and crystallization from n-hexane - EtOAc yielded zerumbone (87.4 mg). The identity and purity of zerumbone (Fig. 1) were assured considering its physicochemical features, HRESI-MS and NMR data [4]. The possible presence of endotoxin in zerumbone was determined by Limulus Amebocyte Lysate (LAL) assay kit (Cambrex Bioscience, Walkersville, MD) according to manufacturer's instructions. Zerumbone was found to be free of endotoxin contamination. Any endotoxin contamination throughout the study was avoided by using endotoxin free chemicals and reagents, buffer and sterile water. Zerumbone was administered orally to the experimental animals after resuspending in 5% Tween 20 solution.

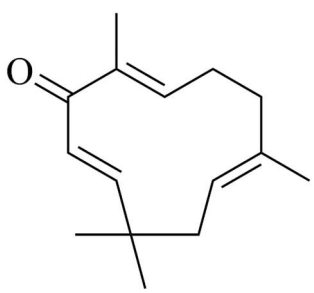

Fig. 1. The chemical structure of zerumbone.

2.3. Experimental animals

Male Balb/C mice of inbred strains (18–22 g; 7–8 weeks old) were obtained from Faculty of Science and Technology, UKM. Approval for animal study was obtained from the Animal Ethics Committee of UKM (No. FF/2014/IBRAHIM/22-JAN.-2014-MAY-2015). The experimental mice were kept in plastic enclosures (4 mice/cage) under controlled environmental conditions (60% humidity, 23 ± 1 °C) with 12 h dark/light schedule. The mice were adapted for a week preceding to the study. Food pellets and water *ad libitum* were given liberally during the whole study.

2.4. Oral toxicity assay of zerumbone

Sub chronic oral toxicity test for zerumbone was performed following the OECD Guidelines 423 [5]. Six groups, each containing six mice were arbitrarily distributed. The mice were treated as follows; Group I (negative control) served normal food and vehicle control (Group II) was administered with 5% Tween 20 solution. Every mouse of Group IV to VII (treatment groups) was subjected to 14 days of a single daily dose of zerumbone at 25, 50, 100 or 200 mg/kg. The positive control group was orally administered (oral gavage) with an immunosuppressant, Cys A at a dose of 50 mg/kg for 14 days. All animals were weighed daily and their scarce changes of normal activities and physiological anomalies were also noted. The animals were then scarified through cervical dislocation and weight of internal organs, including liver, heart and kidney were measured. The determination of AST, ALT, ALP, and creatinine levels were carried out on collected serum samples. Biochemical analyses were assessed colorimetrically and the organs were subjected to histological analysis.

2.5. Experimental designs

Three different experimental designs were planned for the study. In the first design, the animals were distributed at random into 6 groups (6 mice/group) as follows. Group I (received normal diet, as negative control); Group II (administered 5% of Tween 20 solution, as vehicle control by oral gavage); Group III (treated with 50 mg/kg Cys A, as positive control); Groups IV to VI (received zerumbone at doses of 25, 50, 100 mg/kg). All mice were administered with zerumbone or Cys A once a day for 14 days by oral gavage. The safe doses of zerumbone were chosen based on the toxicity study while the dose used for Cys A was decided on literature evidence [6,7]. On day 15, the mice were humanely killed to collect blood samples for determination of NO production, phagocytosis and MPO level. In the second design, the animals were immunized intraperitoneally with sRBC at 5.0×10^8 cells/mL on day 0. The immunization and challenging of treated mice were executed according to the protocol reported by Ilangkovan et al. [7]. The mice in Groups IV to VI were administered with zerumbone at aforementioned doses for 14 days. On day 15, the animals were sacrificed, and the spleen was collected for the analysis of T and B lymphocytes proliferation and T lymphocyte phenotyping. Cytokines serum levels by activated T cells were also assessed. In the third design, the animals were immunized with sRBC on day 0 and fed with zerumbone for 14 days. At the end of treatment, the mice were evaluated for delayed type hypersensitivity (DTH) and the serum was collected for determination of immunoglobulins.

2.6. Isolation of peritoneal macrophages

Fourteen days after the treatment with zerumbone, all the experimental animals were humanely killed. For isolation of peritoneal macrophages, the method reported by Ahmad et al. was employed with minor modification [8]. Briefly, the mice were administered with RPMI medium (10 mL) in the peritoneal cavity, followed by gentle lavaging. After removing the medium from peritoneal cavity it was centrifuged

(400 ×*g*) at 4 °C for 10 min. The pellet containing the cells were re-suspended in DMEM with 10% (v/v) FBS, seeded in a 24-well plate and incubated at 37 °C for 3 h. Upon completion of incubation period, the non-adherent cells were discarded. Finally, the peritoneal cells (adherent) at a concentration of 5×10^5 cells/mL were adjusted by trypan blue exclusion technique.

2.7. Phagocytic assay

The phagocytic activity by peritoneal macrophages was examined by employing phagocytic assay kit as reported previously [7]. Briefly 100 μL of isolated peritoneal macrophages of zerumbone-treated (25, 50, 100 mg/kg) animals were incubated with 20 μL of fluorescent-labeled *Escherichia coli* (*E. coli*) in shaking water bath (37 °C) for 10 min. The negative control tube was remained in ice. At the end of incubation, 100 μL of quenching solution was added, vortexed and centrifuged for 5 min (4 °C) at 250 ×*g*. Finally, 200 μL of DNA staining solution was supplemented into each tube followed by vortexing. The engulfment of bacteria labeled with fluorescein isothiocyanate (%) by macrophages was examined by flow cytometer and was determined as % phagocytizing macrophages.

2.8. Determination of nitric oxide production

The isolation of peritoneal macrophages and measurement of NO production by peritoneal macrophages of zerumbone-treated and non-treated mice was achieved by following the method described by Ilangkovan et al. [7]. The Griess reagent assay was employed in which measuring the accumulated nitrite levels, the NO production by peritoneal macrophages was calculated. Briefly, after adding to a culture plate, the peritoneal macrophages (5×10^5 cells/mL) were activated with LPS at 1 μg/mL and incubated for 48 h at 37 °C. Subsequently, 100 μL of Griess reagent was supplemented to 100 μL supernatant and incubated at 25 °C for 10 min. Finally, the absorbance was noted at 540 nm wavelength with a microplate reader. In order to analyse the amount of nitrite, a sodium nitrite standard curve was employed.

2.9. Measurement of myeloperoxidase activity

The MPO activity was determined by employing a commercially available MPO activity assay kit, following the protocol provided by the manufacturer. Concisely, liver tissues collected from the experimental animals treated/un-treated with zerumbone at different doses were homogenised as well as centrifuged at 13000 *g* for 10 min (4 °C). Afterwards, 50 μL of samples supernatant was mixed with buffer (40 μL) for MPO assay in the 96-well plate. In addition, to start the reaction, 10 μL MPO substrate solution was added per well and incubated for 60 min (25 °C). In the last step, to stop the reaction, stop solution (2 μL) was added and after this Ellman's reagent (50 μL) was added into each well. A microplate reader was used to determine MPO activity by recording absorbance at 412 nm.

2.10. Preparation of spleen cell suspension

The cell suspension of spleen was prepared to explore the effects of zerumbone on lymphocyte proliferation and effector cells expression. From the sRBC-immunized experimental animals, the spleen was aseptically removed and the splenocyte suspension was prepared using the method of Arshad et al. with minor modification [9]. Concisely, 70% alcohol swabs were applied to clean the abdominal cavity of experimental animals and an incision was made to remove the spleen. The spleen was put into cold PBS and before cutting into small pieces, it was positioned onto 70 μm cell strainer. Finally, the small pieces of spleen were pushed through the strainer by using a syringe plunger. The cells were then washed with enough PBS at 300 ×*g* for 5 min. Afterward removing the upper layer, the cell pellet was re-suspended in Pharm Lyse lysing solution (2 mL) and kept for 3 min at room temp and again centrifuged at 250 ×*g* (5 min). Finally, the cells were suspended in complete medium (RPMI-1640) and cell viability was assessed by trypan blue exclusion technique.

2.11. T and B lymphocyte proliferation assay

The T and B-lymphocyte proliferative responses were conducted on a liquid scintillation counter with ^{3}H-thymidine following the method of Arshad et al. [9]. Briefly, 200 μL of prepared spleen cell suspension (4×10^5 cells/mL) was added into 96-well plates. Afterwards, mitogens like LPS (5 μg/mL) and Con A (10 μg/mL) were supplemented in the respective wells in order to stimulate the splenocytes and incubated for 48 h at 37 °C. ^{3}H-thymidine (0.5 μCi/well) was added in all wells and kept for incubation for 24 h. Lastly, by using a Nunc cell harvester the cells were harvested and 5 mL of scintillation fluid was added to determine the thymidine incorporation. The data was represented as the stimulation index (SI), by applying the following formula:

SI
= average cpm with stimulation
/average cpm without stimulation (medium only)

2.12. T lymphocyte phenotyping assay

T cell subsets from spleen lymphocytes were analyzed according to the protocol reported previously [10]. Spleen cell suspension with a concentration of 1×10^6 cells/mL was prepared. Briefly, 50 μL of prepared cell suspension was mixed with APC-H7-conjugated anti-mouse $CD4^+$ antibodies (for identifying $CD4^+$ T cells) (10 μL) and APC-conjugated anti-mouse CD8 antibodies (for identifying $CD8^+$ T-cells) (10 μL) and incubated on ice for 30 min. Upon completion of incubation period, the cell suspension and antibodies were washed with 2 mL of PBS (250 ×*g* for 5 min). After centrifugation, the upper layer was removed while suspending the pellet in 300 μL of PBS. The analysis was carried out with multicolour flow cytometry employing CellQuest Pro Software and the outcomes were presented in percentage (%) of $CD4^+$ and $CD8^+$ expression.

2.13. Th1/Th2 cytokines immunoassay

The inhibitory effect of zerumbone on Th1/Th2 cytokines in sensitized Balb/c mice was assessed by using ELISA kits and the experiment was conducted by using manufacturer's protocol. After collecting the whole blood from zerumbone-treated/untreated mice, it was exposed to centrifugation at 2000 ×*g* (20 min) and serum was separated. Briefly, the standard and serum samples (50 μL) were incorporated into pre-coated (antibodies specific for particular Th1/Th2 cytokines) 96 well plate and incubated for 2 h at 37 °C. The completion of incubation time was followed by washing steps and thereafter, 100 μL of cytokine specific conjugate was placed into the wells. Subsequent to 2 h incubation, substrate solution (100 μL) was added and lastly, stop solution was pipetted into the wells. A microplate reader was employed to measure the absorbance at 450 nm.

2.14. Mouse anti-sRBC IgM and IgG detection in serum

ELISA kits were used to measure the serum levels of IgM and IgG in mice and the assay was conducted according to procedure provided in

Table 1
Evaluation of liver and kidney toxicity of zerumbone on biochemical parameters of serum in Balb/C mice.

Group	Treatment	Dose mg/kg	ALT (U/L)	ALP (U/L)	AST (U/L)	Creatinine (μmol/L)
I	Control	–	59.8 ± 2.1	150.3 ± 2.1	78.1 ± 2.1	22.0 ± 0.4
II	Vehicle	–	58.0 ± 1.8	149.8 ± 1.6	76.8 ± 2.5	22.8 ± 1.5
III	Cyclosporin A	50	67.7 ± 1.2*	193.5 ± 1.2*	131.1 ± 1.0*	26.5 ± 0.7*
IV	Zerumbone	25	53.3 ± 1.2	144.1 ± 2.1	74.3 ± 1.1	20.8 ± 1.2
V	Zerumbone	50	48.2 ± 3.2	146.1 ± 2.1	68.3 ± 3.2	21.1 ± 2.1
VI	Zerumbone	100	52.1 ± 2.1	145.6 ± 4.0	72.1 ± 2.0	23.1 ± 2.2

Notes: Mean ± SEM with n = 6. Statistical tests used were ANOVA followed by post-Dunnett's test.
* $P < 0.05$ verses control group.

the manufacturer's manual. Balb/C mice (male), distributed into six groups with six mice each were intraperitoneally immunized with 200 μL of sRBC (5×10^9 cells/mL) on day 0 of experiment [11]. After being immunized, all the experimental animals were orally administered with zerumbone for 14 days. On day 8 and 14, blood samples were taken from treated and untreated mice to examine the level of IgG and IgM antibody, respectively. Serum was isolated by centrifuging at 3500 ×*g* for 10 min at 4 °C. One hundred μL of standards and samples were added into each well of pre coated 96-well ELISA plate and incubated for 45 min at 25 °C. Subsequently, addition of 100 μL of enzyme-conjugated reagent ensued and incubated again for 45 min. Finally, 100 μL of TMB reagent was supplemented into each well and then the reaction was stopped by adding 100 μL of stop solution. A microplate reader was used to measure the absorption at 450 nm.

2.15. Delayed type hypersensitivity reaction

The delayed type hypersensitivity reaction (DTHR) was assessed employing the method reported previously [7]. A digital plethysmometer was used to measure paw thickness. The experimental animals were distributed into six groups with six mice per group and were immunized by intra-peritoneally injecting with 200 μL sRBC (5×10^9 cells/mL) on day 0. Post immunization, the experimental animals were orally administered with zerumbone for a period of 14 days. On day 14, the hind footpad thickness was measured using digital Plethysmometer. Subsequently, the left hind foot pads of all animals were challenged by 20 μL of sRBC (5×10^9 cells/mL) injection intradermally while the right hind foot pad thickness was taken as control. After 24 h, the thickness of foot pad was analyzed yet again and the variance among left hind paw and right hind paw thickness was denoted to as foot pad reaction.

2.16. Statistical analysis

One-way analysis of variance (ANOVA) followed by Dunnett's test was employed to compare treated samples with a single control, employing the GraphPad Prism-6 (GraphPad Software, Inc., USA). The results were interpreted as mean ± SEM of 6 values. The difference was deliberated significant if the probability value was < 0.05 ($P < 0.05$) or below.

3. Results and discussion

3.1. Subchronic oral toxicity of zerumbone

Animals administered with three different concentrations of zerumbone (25, 50, 100 mg/kg) revealed no considerable deviations in body weight and weights of the vital organs when compared to control group. The given concentrations of zerumbone did not adversely affect the vital organs, spleen and liver. Nonetheless, relatively adverse effects were noted in the group administered with zerumbone at a concentration of 200 mg/kg. There were loss of appetite, lowered body

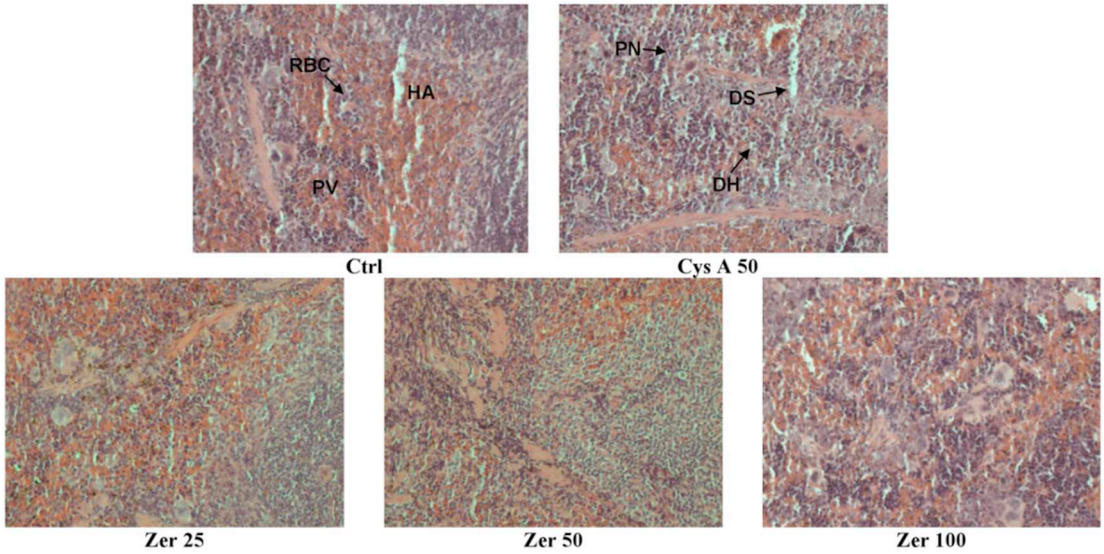

Fig. 2. The photomicrographs of stained liver segments from the control animal, Ctrl = Nonsensitized control, Zer 25 = Zerumbone 25 mg/kg, Zer 50 = Zerumbone 50 mg/kg, Zer 100 = Zerumbone 100 mg/kg, Cys A50 = Cyclosporin A 50 mg/kg, HA = Hepatic artery, DH = Dilated hepatocytes, DS = Dilated sinusoids, PV = Portal vein, RBC = Red blood cells, PN = Pyknotic nucleus (H & E staining, original magnification ×40).

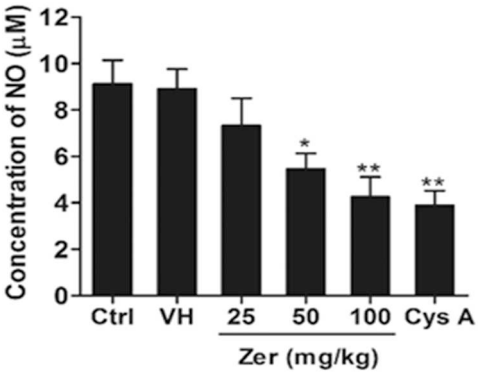

Fig. 3. Effect of zerumbone on nitric oxide production. Mean ± SEM with n = 6. Statistical tests used were ANOVA followed by post-Dunnett's test, *P < 0.05, **P < 0.01 verses control group; Ctrl = Control, Cys A = Cyclosporine A, Zer α = Zerumbone.

temperature, changes in general behavior activities, color of skin, hairs, teeth and eyes. Besides, significant reduction in body weight and vital organ weights were observed in experimental mice that were administered with Cys A, indicating toxicity in the experimental mice [12]. The toxic effects of treated doses of zerumbone on mice were also determined by biochemical and histopathological analyses. Zerumbone administered daily at 25, 50 and 100 mg/kg per day for 14 days consecutively did not demonstrate any significant alteration of serum levels of ALT, ALP, AST, and creatinine as shown in Table 1. However, there were marked increases (P < 0.05) in the levels of the biomarkers in serum of Cys A-treated animals in comparison to the control group.

Macroscopic inspection revealed that no evident changes were found in the kidney, liver, heart, spleen and lung from both treated groups and control group. Analogous outcomes were noted in the liver which showed no apparent changes in the cell assembly between the treated groups and control group at the mentioned doses. The microscopic analysis showed that the organs of zerumbone-treated animals did not display any changes in cell assembly; the hepatocytes were round, polygonal along with clear spherical nucleus (Fig. 2). Therefore, the results indicated that zerumbone at 25, 50 and 100 mg/kg used in this study was entirely safe.

3.2. Effect of zerumbone on nitric oxide production

NO is considered as one of the important mediators of immune inflammatory response [13,14]. In this investigation, isolated peritoneal macrophage from zerumbone-treated mice at 25, 50 and 100 mg/kg showed substantial inhibition of LPS-primed NO production (Fig. 3). Notably, at 100 mg/kg the inhibition (P < 0.01) was found comparable to that of Cys A (50 mg/kg). The result supports previous *in vitro* reports where zerumbone from *Z. zerumbet* was found as a potent downregulator of NO generation [2,15]. Numerous studies have suggested that the release of NO is interlinked with the activation of NF-κB and MAPK signal transduction as well as altered protein kinase C (PKC)-alpha. Therefore, the inhibitory effect exerted by zerumbone treatment might be due to downregulation of NF-κB and MAPKs as well as PKC-alpha signaling activation. Moreover, the aptitude of zerumbone in inhibiting NO release recommends the therapeutic effectiveness of this bioactive metabolite for the aid of inflammation associated with over secretion of NO [16].

Table 2
Effects of zerumbone on phagocytic activity (%).

Group	Treatment	Dose (mg/kg)	Phagocytic activity (%)
I	Control	–	83.6 ± 3.5
II	Vehicle	–	82.7 ± 2.3
III	Cyclosporin A	50	36.9 ± 3.2**
IV	Zerumbone	25	72.0 ± 2.3*
V	Zerumbone	50	66.5 ± 2.9**
VI	Zerumbone	100	59.1 ± 2.4**

Notes: Mean ± SEM with n = 6. Statistical tests used were ANOVA followed by post-Dunnett's test.
* P < 0.05.
** P < 0.01 verses control group.

3.3. Phagocytic activity

The phagocytic property as evaluated by employing flow cytometer revealed that treatment with zerumbone in experimental mice significantly suppressed the peritoneal macrophages engulfment of *E. coli* (Table 2). The inhibition was dose-dependent and maximum suppressive effects were witnessed at higher concentrations. The structure of zerumbone which contain α, β carbonyl based moiety might act as the foremost contributors for inhibiting *E. coli* engulfment by peritoneal macrophages.

3.4. Effect of zerumbone on myeloperoxidase activity

Zerumbone was tested for the MPO activity *in vivo* and the outcome revealed that zerumbone exhibited significant concentration-dependent downregulation of plasma MPO levels. As revealed in Fig. 4, suppression of MPO activity was highest at the maximum concentration of 100 mg/kg (72.94%) as compared to the Cys A which showed inhibition of 81.12%. The downregulation of MPO has been reported to be advantageous for management of a wide array of immune syndromes as it plays important role in cellular homeostasis, and impaired levels cause instigation and development of serious and chronic inflammatory disorders. The significant inhibition of MPO could be attributed to the inhibition of Mac-1, as the downregulation of Mac-1 suppresses the sequential phagocytosis process and releae of toxic radicals and enzymes [17].

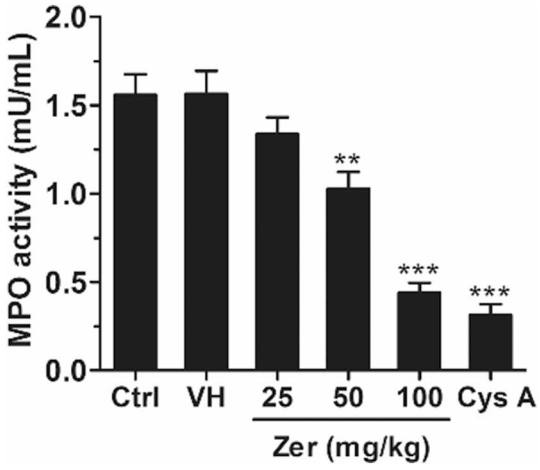

Fig. 4. Effect of zerumbone on myeloperoxidase activity. Mean ± SEM with n = 6. Statistical tests used were ANOVA followed by post-Dunnett's test, **P < 0.01,***P < 0.001 verses control group. Ctrl = Control, Cys A = Cyclosporine A, Zer = Zerumbone.

3.5. Effect of zerumbone on T and B lymphocyte proliferation

LPS and Con A which are respectively B-cell and T-cell mitogens, were found to stimulate the splenocyte proliferation in sRBC-immunized animals (Table 3). The treatment with zerumbone suppressed T and B lymphocytes proliferation significantly and dose-dependently. As shown in Table 3, LPS- and Con A-triggered lympocyte proliferations were significantly suppressed by zerumbone at 50 and 100 mg/kg, which were comparable with the effect of Cys A. The importance of T cells in mediation of immune functions by modifying the cellular immune system has been widely reported. Various evidences have displayed the interlink of the mechanism of release of cytokines with T cell and B cell production [7]. Thus, the downregulation of T and B lymphocytes by zerumbone might be associated with the cytokines intervened mechanism [18].

3.6. Effect of zerumbone on T lymphocyte phenotyping

T lymphocyte comprises two essential subpopulations namely T-helper cells ($CD4^+$) and cytotoxic T-suppressor cells ($CD8^+$), which are different functionally and phenotypically and may able to modify the immune system by regulating the release of different cytokines [19]. The mechanism of zerumbone as an immunosuppressive agent was further evaluated by determining its effect on the population of these cell surface markers in splenocytes by using flow cytometry. Splenocytes were isolated from spleen of sRBC-immunized treated and untreated mice. As illustrated in Table 3, zerumbone significantly ($P < 0.05$) and concentration-dependently attenuated $CD4^+$ and $CD8^+$ T-lympocytes. Zerumbone at 100 mg/kg showed inhibitory effect comparable to that of Cys A (50 mg/kg). Consequently, the reduced level (%) of $CD4^+$ and $CD8^+$ in the sRBC-immunized mice indicated that Th cells and CTLs were both attenuated by zerumbone.

3.7. Measurement of Th1/Th2 cytokines

Cytokines are the low molecular weight glycoproteins or polypeptides which are considered as the important mediators of immune and inflammatory responses [20–22]. T helper 1 (Th1) and Th2 cells are predominantly the subset of helper T cells. The homeostasis between Th1 cytokines and Th2 cytokines is imperative in regulation of immune system [24]. The IFN-γ, IL-2, TNF-α are the cytokines released by Th1 cells while Th2 cells release IL-4 as well as IL-6. The release of cytokines in the sera of immunized mice was analyzed using ELISA to confirm that Th1/Th2 cytokines were linked to immunosuppressive effects of zerumbone. As illustrated in Fig. 5, zerumbone significantly and concentration-dependently downregulated the release of respective cytokines. Notably, the suppression of TNF-α at 100 mg/kg was more effective as compared to the Cys A at 50 mg/kg. Interestingly, the result was found consistent with our report on *in vitro* immunosuppressive effect of zerumbone on TNF-α in human macrophages [23]. Moreover, zerumbone was also found to inhibit the release of IL-2, IL-4 and IL-6, which are in line with previous findings [2,24]. The outcomes gained from this investigation signify that zerumbone downregulated the release of TNF-α, IFN-γ, IL-6, IL-4, IL-2. The significant suppression of cytokines by helper T cells can be attributed to the inhibition of IL-12 which is considered as one of the major mechanism that affects the cytokine production in $CD4^+$ cells.

3.8. Effect of zerumbone on serum level of IgG and IgM antibodies

The serum levels of immunoglobulins were determined using collected blood on day 0, 4 7 and 14 after the immunization with sRBC. The anti-sRBC IgG and IgM antibody titer was found to be inhibited in immunized and zerumbone-treated experimental animals (Table 4). The aptitude of zerumbone to decrease primary and secondary antibody release in serum level could be because of its ability to impede T and B lymphocyte subsets activation as well as abridged receptiveness of macrophages. In addition, the capability of zerumbone in inhibiting COX-2 might also be one of the causes for prospective downregulation of the bioactive metabolite on the production of IgM and IgG antibodies. The downregulation of COX-2 had been stated to blight the expression of vital plasma cell transcription factors as well as B lymphocytes differentiation [25].

3.9. Delayed type hypersensitivity reaction

DTHR due to sRBC was determined by measuring paw edema thickness (mm) at 24 h after administration with different doses of zerumbone (25, 50, 100 mg/kg) and compared with the sensitized control (immunized with sRBC). Immunization with sRBC upregulated the thickness of paw within the 18 to 24 h. The immunized-treated groups that were administered with zerumbone at various concentrations were found to exhibit significant inhibition in paw oedema. Table 4 shows that DTHR induced by sRBC was downregulation by the zerumbone-treated groups at higher doses (50 and 100 mg/kg). DTHR occurs after the second contact with the T cell dependent antigen (sRBC), where T helper cells caused the release of cytokines and different immune cells responsible for inflammation [26]. The significant immunosuppressive activity at the cellular immunity level could be due to inhibition on the resultant release of cytokines and T lymphocytes activation. There is also a possibility that zerumbone might have affected the release of histamine and arachidonic acid metabolism pathway involved in the progression of DTHR [27].

Table 3
$CD4^+$ and $CD8^+$ expression in spleen cells (%) and T- and B-lymphocytes proliferation (SI) in zerumbone-treated mice.

Treatment	Dose (mg/kg)	T-cell subset detection (%)		Lymphocyte proliferation (SI)	
		$CD4^+$	$CD8^+$	T cell	B cell
Control	–	50.5 ± 2.1	29.6 ± 2.0	3.4 ± 2.1	1.8 ± 0.9
Vehicle	–	49.7 ± 0.8	30.1 ± 1.1	3.2 ± 2.1	1.8 ± 2.2
Cyclosporine A	50	41.1 ± 3.1*	18.3 ± 0.5*	2.1 ± 2.1*	0.9 ± 2.0*
Zerumbone	25	50.4 ± 1.1	28.3 ± 0.6	2.8 ± 1.0*	1.7 ± 1.7
Zerumbone	50	41.6 ± 4.2*	26.1 ± 0.3*	2.6 ± 0.1*	1.5 ± 1.7*
Zerumbone	100	39.2 ± 3.8*	25.7 ± 3.0*	2.2 ± 1.0*	1.1 ± 2.0*

Notes: Mean ± SEM with n = 6. Statistical tests used were ANOVA followed by post-Dunnett's test.
* $P < 0.05$ verses control group.

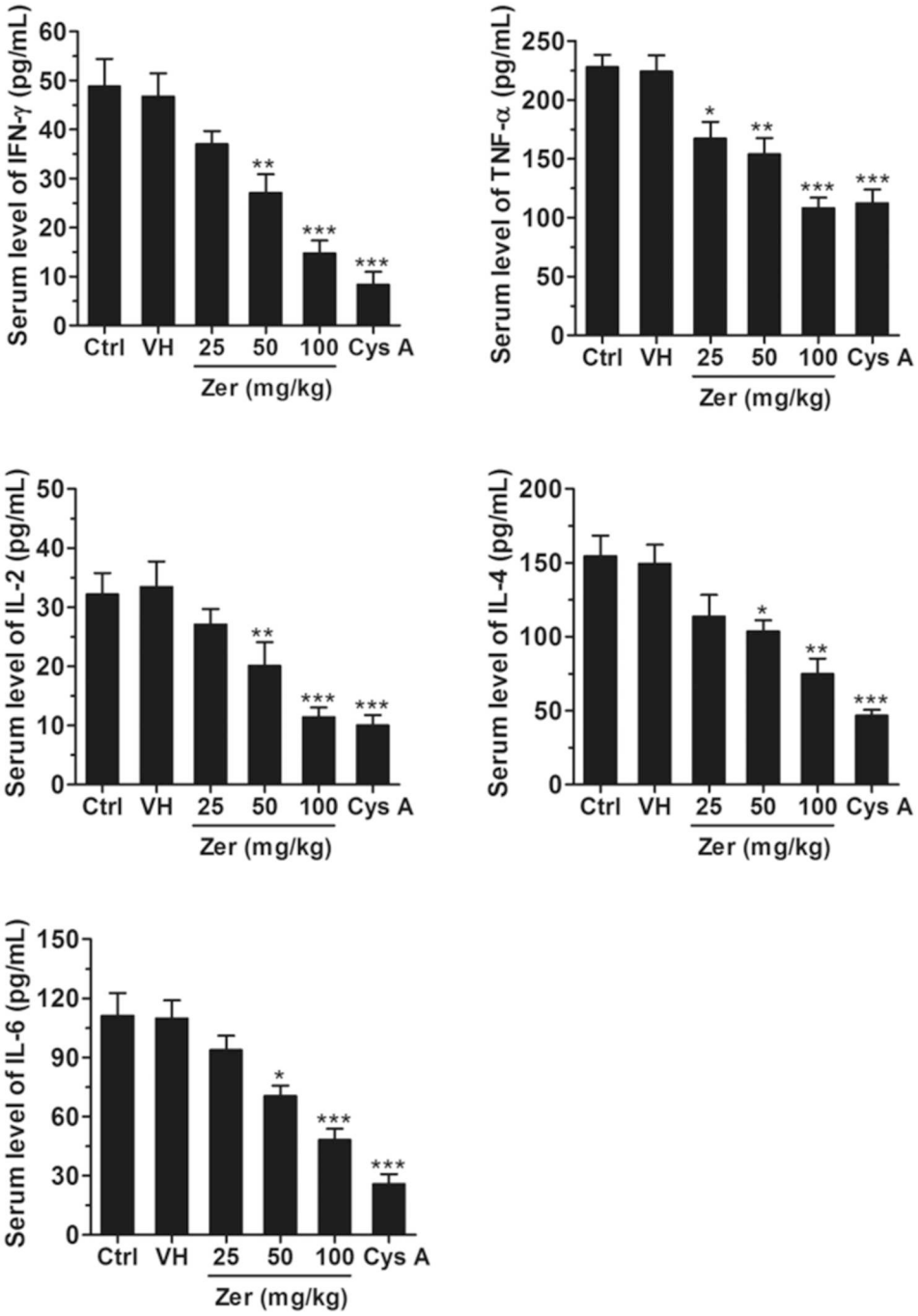

Fig. 5. Effects of zerumbone on Th1/Th2 cytokines release. Mean ± SEM with n = 6. *P < 0.05,**P < 0.01,***P < 0.001; statistical tests used were ANOVA followed by post-Dunnett's test. Ctrl = Control, Cys A = Cyclosporine A, Zer = Zerumbone. Results are expressed as mean ± SEM, with n = 6. Statistical tests employed were ANOVA followed by post-Dunnett's test, *P < 0.05,**P < 0.01,***P < 0.001 verses control group.

4. Conclusions

Zerumbone exhibited potent inhibitory effects on both innate and adaptive immune responses in male Balb/C mice. Zerumbone downregulated phagocytosis as well as NO and MPO release in the experimental animals significantly and dose-dependently. Zerumbone treatment caused a substantial and concentration-dependent downregulation of T and B lymphocytes proliferation and attenuation of the Th1/Th2 cytokines release. Besides, zerumbone possessed a significant diminution in the % expression of $CD4^+$ and $CD8^+$ in splenocytes. DTHR due to sRBC was significantly downregulated by zerumbone at high doses. Additionally, a significant decrease in the release of serum immunoglobulins (IgM and IgG) level was witnessed. The effects of zerumbone in Balb/C mice are supported by the *in vitro* effects where zerumbone possessed potent immunosuppressive effects by interfering the MyD88 dependent signaling activation in macrophages

Table 4
Effects of zerumbone on delayed type hypersensitivity reaction (DTHR) and serum levels of IgM and IgG in mice.

Treatment	Dose (mg/kg)	Paw thickness (mm)		Production of immunoglobulins (ng/mL)	
		24 h oedema (mm)	Inhibition (%)	IgM	IgG
Non-immunized mice	–	0.22 ± 0.05	–	5.8 ± 1.8	4.2 ± 1.3
Control	–	0.38 ± 0.13	–	145.7 ± 2.0	347.0 ± 3.9
Vehicle	–	0.38 ± 0.12	–	146.2 ± 1.8	352.2 ± 1.5
Cyclosporine A	50	0.23 ± 0.14	83.3*	69.3 ± 1.1**	193.1 ± 0.9**
Zerumbone	25	0.31 ± 0.09	43.8	151.1 ± 1.1*	341.1 ± 4.1
Zerumbone	50	0.30 ± 0.11	50.0*	137.9 ± 0.9*	335.2 ± 2.9*
Zerumbone	100	0.28 ± 0.16	62.5*	121.5 ± 3.1**	321.1 ± 3.1*

Notes: Mean ± SEM with n = 6. Statistical tests used were ANOVA followed by post-Dunnett's test.
* $P < 0.05$.
** $P < 0.01$ verses control group.

[4]. Moreover, the findings from the toxicology investigation evidently displayed that zerumbone was lack of any adverse effects on the principal organs of tested mice, recommending the potential development of zerumbone as a potent immunosuppressive agent.

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