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RESEARCH ARTICLE

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Immunosuppressive effects of the standardized extract of *Zingiber zerumbet* on innate immune responses in Wistar rats

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Zingiber zerumbet rhizome has been used in traditional medicine mainly for the treatment of various immune-inflammatory related ailments and has been shown to exhibit a wide spectrum of biological effects especially antioxidant and anti-inflammatory activities. The present study was aimed to investigate the immunosuppressive effects of the standardized 80% ethanol extract of *Z. zerumbet* at 100, 200, and 400 mg/kg on the innate immune responses in male Wistar rats. The immune parameters determined were chemotaxis of neutrophils, Mac-1 expression, engulfment of *Escherichia coli* by neutrophils, reactive oxygen species production, and plasma lysozyme and ceruloplasmin levels. Zerumbone was qualitatively and quantitatively determined in the extract by using a validated reversed-phase HPLC, whereas liquid chromatography tandem-mass spectrometry (LC -MS/MS) was used to profile the secondary metabolites. *Z. zerumbet* significantly inhibited the migration of neutrophils, expressions of CD11b/CD18 integrin, phagocytic activity, and production of reactive oxygen species in a dose-dependent manner. The extract also dose-dependently inhibited the expressions of lysozyme and ceruloplasmin in the rat plasma. *Z. zerumbet* extract possessed strong inhibitory effects on the innate immune responses and has potential to be developed into an effective immunosuppressive agent.

KEYWORDS

immunosuppression, innate immunity, neutrophil migration, phagocytosis, reactive oxygen species, *Zingiber zerumbet*

1 | INTRODUCTION

The immune system is an intricate and complex network of homeostatic process, comprises of balanced multicellular and physiological mechanisms in order to fight against pathogens. The dynamic interplay between antigens and a network of immunologically competent cells determines the effectiveness and efficiency of the immune system in host defense. Impaired immune response occurs when there is a failure to maintain an adequate redox regulation of the immune response (Gostner, Becker, Fuchs, & Sucher, 2013). An overactive immune system may lead to diseases including chronic inflammatory disorders, autoimmunity, systemic vasodilation, and sepsis whereas immunosuppression leads to infectious diseases and immunodeficiency (Galgani, Di Giacomo, Matarese, & La Cava, 2009). Modulation of the immune system is necessary for the treatment and management of various diseases due to defect or imbalance of the immune systems.

Immunomodulators have been used clinically to restore immune imbalance by modulating, stimulating, or suppressing components of the innate or adaptive immunity to decrease the risk of chronic infections (Arshad, Jantan, Bukhari, & Haque, 2017). New and safer drugs are required as alternatives to the several chemical immunomodulators such as nonsteroidal anti-inflammatory drugs, corticosteroids, cyclosporin A (Cys A), which have serious side effects (Jantan, Ahmad, & Bukhari, 2015). Several medicinal plants have been used traditionally to treat certain immunological disorders as well as many plants have been reported to possess potent immunomodulatory

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properties such as *Viscum album*, *Panax ginseng*, *Asparagus racemos*, *Tinospora crispa*, *Tinospora cardifolia*, *Ocimum sanctum*, and *Azadirachta indica* (Haque, Jantan, & Bukhari, 2017a).

Rhizomes of *Zingiber zerumbet* (L.) Roscoe ex Sm. have been widely used as spices as well as traditional health supplement or remedy to treat various types of disorders related to the immune system such as inflammation, rheumatoid arthritis, allergies, bacterial infections, asthma, and edema. The plant has also been used to treat vertigo, debility, peptic ulcer, coughs, hemorrhoids, loss of appetite, sores, and wounds (Yob et al., 2011). Some of the strong pharmacological activities exerted by *Z. zerumbet* rhizomes were antioxidant, anti-inflammatory, immunomodulation, antimicrobial, anthelmintic, larvicidal, antihyperglycemic, and antihyperlipidemic properties (Haque & Jantan, 2017; Singh et al., 2012). Among the bioactive metabolites present in *Z. zerumbet*, zerumbone has been comprehensively studied for numerous pharmacological perspectives (Haque, Jantan, & Bukhari, 2017b). Our previous study indicated that *Z. zerumbet* and zerumbone suppressed the activation of inflammatory mediators in lipopolysaccharide (LPS)-induced U937 macrophages through the MyD88-dependent NF-κB/MAPK/PI3K-Akt signaling pathways (Haque, Jantan, & Harikrishnan, 2018a, 2018b). However, there is little scientific information on the immunomodulatory effects of *Z. zerumbet* on the humoral and cellular components of the immune system. Thus, this study was carried out to investigate the immunomodulatory effects of the standardized extract of *Z. zerumbet* rhizomes on selected parameters of innate cellular and humoral immune responses in Wistar rats, which include the four major stages of phagocytosis process (chemotaxis, CD11b/CD18 integrin expression, phagocytosis, and reactive oxygen species (ROS) production) of neutrophils as well as serum ceruloplasmin and lysozyme levels.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Eighty percent ethanol, acetonitrile and methanol HPLC grade, hematoxylin, and xylene were obtained from British Drug Houses (BDH) (Merck KGaA, Darmstadt, Germany). Hank's balance salt solution and LPS were purchased from Innovative Research, Inc. (Sarasota, Florida, USA). Millex polytetrafluoroethylene (PTFE) membrane (Millipore), paraffin waxes, phosphate-buffered saline (PBS) tablet, lymphoprep™ gradient, trypan blue reagent, fetal bovine serum (FBS), penicillin–streptomycin solution, Roswell Park Memorial Institute (RPMI)-1640 was obtained from Sigma-Aldrich Corp (St Louis, Missouri, USA). Standard compound, zerumbone (99% purity), was purchased from ChromaDex (California, USA). Cys A (99% purity) was purchased from Sigma-Aldrich (Milano, Italy). Pharm lyse lysing solution, fluorochrome conjugated antibodies such as APC-CD11b (0.2 mg/ml), FITC-CD18 (0.5 mg/ml), APC-IgG_1 (0.5 mg/ml), FITC-IgG_1 (0.5 mg/ml) were all acquired from BD Bioscience Pharmingen (Franklin Lakes, USA). CytoSelect 24-well cell migration assay kit, which was used in cell migration assay, was purchased from Cell Biolabs, Inc. (San Diego, California, USA). Phagotest and phagoburst kits were obtained from Glycotope Biotechnology (Berlin, Germany). Rat lysozyme C (LYZ) ELISA kit and rat ceruloplasmin (ferroxidase) CP ELISA kit were acquired from Cusabio Biotech Co., Ltd. (Wuhan, China).

2.2 | Preparation of plant extracts

Rhizomes of *Z. zerumbet* were collected from Kuala Krau, Temerloh, Pahang, Malaysia. The plant was identified by Dr. Abdul Latif Mohamad, Faculty of Science and Technology, Universiti Kebangsaan Malaysia (UKM), and a voucher specimen was deposited at the Herbarium of UKM (No. UKM-HF137). The rhizomes were cut into small pieces, air-dried at room temperature under the shed, followed by grinding into a coarse powder. The powdered sample (1.75 kg) was extracted with 80% ethanol (3 × 3 L) for 3 days at 25°C. The filtrates were concentrated by using a rotary evaporator at 37–40°C, followed by freeze-drying to produce a crude *Z. zerumbet* extract (248.0 g; Haque et al., 2018a). The crude extract was then stored at 4°C until further use. For the in vivo experiment, the extract was prepared as an oral emulsion by using 3.33% Tween 20.

2.3 | Standardization of 80% ethanol extract of *Z. zerumbet* by HPLC

The 80% ethanol extract of *Z. zerumbet* stock solution was prepared in methanol at a concentration of 3 mg/ml, and solution for the reference standard, zerumbone, was prepared separately at a concentration of 1 mg/ml. It was further diluted into a series of two-fold dilutions (125–1,000 μg/ml). All of these samples and standard solutions were sonicated for 15 min, and then they were filtered through a 0.45-μm Millex PTFE membrane (Maidstone, Kent, UK). Standardized of the extract was performed on basis of the reference standard zerumbone by using a reverse phase, Xbridge® C-18 column (4.6 × 250 mm, 5 μm), and detector PDA (Waters 2998) at a wavelength of 250 nm. The diluted solutions of the extract and the reference standard were analyzed separately using a gradient elution technique with water (Solvent A) and acetonitrile (Solvent B) as mobile phase at a flow rate of 1.2 ml/min. The following procedure was used; the initial composition was 65% Solvent B followed by increasing Solvent B to 70% in 10 min. Solvent B was kept increasing to 75% until 16 min. The identification of compounds in the sample was carried out by comparing the retention times and UV–Vis spectra of the sample peaks with the standard peak. Chromatograms to show verification of zerumbone peak in the *Z. zerumbet* extract by spiking technique are shown in Figure S1. The calibration curve was plotted for the standard compound by using four concentrations of prepared standard dilution versus the areas under the peaks. The standard curve equations obtained was used to quantify the compounds in the extract.

2.4 | Validation procedure for HPLC analysis

International conference on harmonisation (ICH) guideline was followed to validate the HPLC method and analytical procedure used in this study (ICH, 2005). The validation involved accuracy, precision, linearity, limit of detection (LOD) and limit of quantification (LOQ).

Accuracy was obtained by spiking the known reference standard at certain amount to the blank sample over nine determinations (three concentrations/three replicates). Although precision was acquired from intraassay and interday precisions, intraassay precision was performed by the same method over a short interval of time whereas interday precision was performed on different days. Four dilutions of the reference standard (zerumbone), prepared in triplicate, were used to construct calibration curve by plotting corresponding peak areas against concentration of the standards. The correlation coefficient (R^2) obtained from the standard curve equation depicted the linearity value. The residual standard deviation (RSD) and slope (S) of the calibration curve were used to calculate the LOD and LOQ by using the following equations:

$$\text{LOD} = 3.3 \times (\text{RSD}/S) \text{ and } \text{LOQ} = 10 \times (\text{RSD}/S).$$

2.5 | LC–MS/MS analysis

The active and secondary metabolites in the 80% ethanol extract of *Z. zerumbet* were profiled using LC–MS/MS. A 100 × 2.1 mm Phenomenex synergi fusion column (particle size, 3 μm; Phenomenex, CA) equipped in AB Sciex 3200QTrap LC–MS/MS with Flexar FX-15 series UHPLC (PerkinElmer, Shelton, CT, USA) was used. The condition used was 4,500 V as capillary voltage (IS), 100–1,500 *m/z* (full scan), and 50–1,500 *m/z* (MS/MS scan) along with 500°C of source temperature. The eluents were used as (A) 0.1% formic acid in water and 5-mm ammonium formate and (B) acetonitrile with 0.1% formic acid and 5-mm ammonium formate. The gradient program was started with 20–30% B for 10 min and increasing to 40% B for another 10 min. The eluent B kept increasing to 50% for 10 min before reaching to 90% B for the next 20 min. Ninety percent B was maintained again for 10 min followed with decreasing to 20% B for another 10 min. A constant flow rate of 250 μl/min with an injection volume of 20 μl was maintained as well as negative ionization mode was used for the analysis. LC–MS/MS full chromatogram of *Z. zerumbet* is shown in Figure S2. The detected component peaks were compared with mass spectral library by using ACD Labs (Toronto, ON, Canada) advanced chemometrics mass fragmentations predictive software.

2.6 | Experimental animals

All animal studies were conducted by following an approved protocol from UKM Animal Ethics Committee (FF/2016/IBRAHIM/28-SEPT./780-SEPT.-2016-MAC.-2018). Inbred male Wistar rats ranging from 6 to 8 weeks old (200 to 250 g) were obtained from the Laboratory Animal Resource Unit, Faculty of Medicine, UKM. The rats were quarantined for at least 1 week before the experiment. The rats were randomly divided, and three rats were housed per cage in plastic cages. The animals were acclimatized at 22 ± 3°C, 40–60% humidity, and a 12-hr light/dark cycle. They were provided with standard laboratory pellet diet and water ad libitum.

2.7 | Acute oral toxicity test

Acute oral toxicity test was conducted according to the Organization of Economic Co-operation and Development protocol (Guideline 423; Jonsson et al., 2013). The animals were randomly divided into six groups (each containing six rats). Three groups were orally administered with the extract at 100-, 200-, and 400-mg/kg body weight (BW), and one group received the extract at the limit dose of 2,000-mg/kg BW daily for 14 days, whereas the negative control group (received a regular diet) and the vehicle control group (received 3.33% Tween 20) did not receive any treatment. The animals were weighed daily, and changes in their general behavior and appearance of any toxicity symptoms were observed. At the end of study, all rats were humanely killed through cervical dislocation. The liver was observed for any obvious pathological changes and gross necropsy. Part of the liver was fixed in 10% formalin solution overnight and after tissue processing, (paraffin)-embedding, and sectioning into 5-μm thickness; pathological changes were examined by using hematoxylin and eosin under light microscope (40×; Jonsson et al., 2013).

2.8 | Experimental design for immune study

The rats were randomly divided into six groups with six rats each (Table 1). The first group served as nonsensitized control group whereas the second group was vehicle control group, which was orally fed with 3.33% Tween 20. The animals in third group were orally administered with an immunosuppressive drug, Cys A (10 mg/kg) whereas groups 4–6 served as treated groups and administered with the extract (100-, 200-, and 400-mg/kg BW) daily for 15 days, p.o. The doses of Cys A used in this study were based on a previous study by Huyan, Lin, Gao, Chen, and Fan (2011). On the 14th day, 500-μl blood was collected from lateral tail vein of each rat in order to carry out phagotest assay, ROS production assay and CD11b/CD18 integrin expression assay. Whereas on the 15th day, blood was obtained for isolation of neutrophils and plasma for determination of cell migration and levels of lysozyme and ceruloplasmin.

2.9 | Isolation of neutrophils from whole rat blood

Isolation of neutrophils from whole rat blood was conducted by following Histopaque gradient technique as described by Ilangkovan, Jantan, Mesaik, and Bukhari (2015). Briefly, whole rat blood was collected from treated rats into a 10-ml falcon tube and layered on Lymphoprep™ solution with 1:1 ratio. The tube containing the two-step gradients was centrifuged at 400 *g*, 4°C for 45 min. The supernatant formed was removed and pre-warmed pharm lyse lysing solution was added in the tube. After incubation, the tube was centrifuged at 250 *g*, 4°C for 5 min. Supernatant formed was discarded while the pellet formed was resuspended in PBS. The cell suspension was then determined for neutrophil count using hemocytometer and inverted light microscopy at 400× to evaluate neutrophil purity (>90% pure). Cells with fine granules and multilobed nuclei were counted as neutrophils. Then, the cell suspension was diluted with PBS to adjust approximately to 1×10^6 cells/ml. The cells were checked for viability by using the trypan blue exclusion assay.

TABLE 1 Retention times, MS^2 fragments of the tentative chemical markers present in *Zingiber zerumbet*

No.	Retention time (min)	Molecular ion peak $(M-H)^-$	MS^2 fragmentation ions	Tentative compounds identified
1	2.317	197[a]	182, 169, 125, 69	Ethyl gallate
2	2.650	169	169, 125[a], 95, 69	Gallic acid
3	4.334	289[a]	271, 261, 245, 201, 125	Catechin
4	5.063	430	432, 327, 284[a], 256, 227, 212, 163	Kaempferol rhamnoside
5	5.680	285[a]	267, 257, 229, 227, 187, 169, 143	Kaempferol
6	6.130	514	516, 474, 456, 396, 285[a], 256, 228, 151	Kaempferol-3-*O*-(2″, 4″-diacetyl) rhamnoside isomer
7	9.060	472	474, 414, 291, 287[a], 256, 233, 228, 152	Kaempferol-3-*O*-(2″- or 3″-acetyl) rhamnoside isomer
8	9.331	298	299, 284, 256[a], 228, 211, 183, 171, 132	Kaempferol methylether
9	10.848	298	300, 256[a], 228, 212, 184, 163	Kaempferol methylether isomer
10	12.533	514	516, 474, 456, 414, 299, 291, 287[a], 268, 256, 228, 212, 152	Kaempferol-3-*O*-(3″,4″-diacetyl) rhamnoside isomer
11	13.431	514	516, 474, 456, 414, 291, 286[a], 261, 256, 240, 228, 212, 59	Kaempferol-3-*O*-(3″,4″-diacetyl) rhamnoside isomer2
12	16.409	557	556, 497, 455, 289, 284[a], 255, 227	Kaempferol glucoside conjugate
13	16.410	556	557, 498, 456, 289, 284[a], 256, 228	Kaempferol glucoside conjugate
14	20.510	337	337, 322, 217, 202, 198, 173, 158, 134, 119[a]	Desmethoxycurcumin
15	20.622	367	367[a], 352, 247, 217, 202, 173, 149, 134, 119	Curcumin
16	21.016	307[a]	289, 187, 161, 143, 119	Bisdesmethoxycurcumin
17	21.914	217	217, 201, 173, 168, 158[a], 133, 69	Zerumbone
18	22.477	369[a]	354, 325, 218, 203, 173, 149, 134	Curcumin isomer

[a]Base peak.

2.10 | Cell migration assay

The inhibitory effects on the migration of isolated neutrophils were determined by using 24-well cytoselect migration assay kit as per manufacturer's instructions. The concentration of neutrophils isolated from rat whole blood was adjusted to 3×10^6 cells/ml by using serum-free media. A total of 500-µl RPMI with 10% FBS (chemoattractant) was added into the lower chamber of kit whereas 150 µl of cell suspension with a final concentration of 1.5×10^6 cells/ml was added in the upper chamber (inserts) and incubated at 37°C for 2.5 h. Afterwards, media inside the upper chamber was carefully aspirated out, and the inserts were transferred into clean wells containing 200 µl of cell detachment solution. After incubation, the media solution (400 µl) from lower chamber was transferred to the well containing 200 µl of cell detachment solution. A standard curve using cell suspension (with different concentrations) was prepared. Afterwards, 60 µl of Cyquant dye lysis buffer was added, and the mixture was kept in dark for 20 min. Finally, 200 µl of the mixture was transferred into another fluorescent 96-well plate and were analyzed by using fluorescent plate reader. Number of cells migrated was calculated and analyzed by referring to the standard curve prepared.

2.11 | CD11b/CD18 integrin expression assay

The CD11b/CD18 integrin expression on neutrophils was examined by following the previously described method of Podolnikova, Kushchayeva, Wu, Faust, and Ugarova (2016) with slight modification. One hundred microliter of whole blood was incubated with 10 µl of LPS (3.5 µg/ml) for 30 min at 37°C in CO_2 incubator. After incubation, blood samples were directly transferred into ice. Conjugated antibodies, APC-CD11b (2.5 µl) and FITC-CD18 (10 µl), were added into sample tubes, whereas, five APC-IgG_x2081; (5 µl) and FITC-IgG_x2081; (5 µl) were added in negative control tube and kept on ice for 1 hr. Later, 1 ml of prewarmed pharm lyse lysing solution (1X) was added and incubated in dark for 20 min at 37°C. Finally, after washing, the pellet was resuspended in 500 µl of PBS before reading the results. Percentages of neutrophils expressing CD11b/CD18 integrin were determined by a flow cytometer.

2.12 | Phagocytic assay

Phagotest assay kit was used to conduct the phagocytic assay, following standard protocol provided by the manufacturer (Glycotope Biotechnology) with slight modification. This assay allows the quantitative determination of leukocyte phagocytosis (ingestion of bacteria). It specifically measures the percentage of phagocytes that have ingested bacteria and their activity (number of bacteria per cell). One hundred microliter of blood samples collected from experimental animals were incubated with 10 µl of precooled *Escherichia coli* bacteria in shaking water bath at 37°C except for negative control tube. After the specified time, the samples were simultaneously shifted back into an ice bath, and 100 µl of quenching solution was added and vortexed vigorously. Later, 3 ml of washing solution was added and centrifuged at 250 *g*, 4°C for 5 min. The supernatant

was discarded while the pellet was resuspended in 2 ml of lysing solution and kept for 20 min at room temperature. Samples were spun down at 250 *g*, 4°C for 5 min. After washing, finally, 200 µl of DNA staining solution was added into each tube, mixed well, and analyzed by using a flow cytometer. Phagocytic activity was expressed in percentage of *E. coli* that had been phagocytized by neutrophil cells.

2.13 | ROS production assay

The assay procedure performed in this study followed the manufacturer's protocol provided by Glycotope Biotechnology. This assay allows the quantitative determination of ROS produced by leukocytes. It uses fluorogenic substrate, dihydrorhodamine 123 to determine the percentage of phagocytic cells producing reactive oxidants (conversion of dihydrorhodamine 123 to R 123) and their enzymatic activity (amount of R 123 per cell). Heparinized rat whole blood collected from tail vein was kept at 0°C for 10 min, and then a total of 100-µl blood sample was incubated with 20 µl of precooled *E. coli*. After incubation, 20 µl of substrate solution was pipetted into each sample, and the mixture was incubated in shaking water bath (37°C) for 10 min. Later, 2 ml of prewarmed lysing solution was added, and the mixture was kept at room temperature for 20 min. Finally, samples were centrifuged with 200-µl DNA staining solution and kept for 10 min while protected from light. Results were evaluated by using flow cytometer within 30 min after the incubation time.

2.14 | Determination of plasma lysozyme and ceruloplasmin levels

The plasma levels of lysozyme and ceruloplasmin produced in treated and nontreated rats were analyzed by using rat lysozyme C (LYZ) ELISA kit and rat ceruloplasmin (Ferroxidase; CP) ELISA kit, respectively. All the reagents were prepared as described in the manual provided. Plasma was separated from rat whole blood by centrifugation at 2,000 *g*, 4°C for 20 min and was diluted with sample diluent (1:1,000). The experiment was performed by using the manufacturer's guidelines, and the results were read on a photometric plate reader at 450 nm.

2.15 | Statistical analysis

All data were subjected to one-way analysis of variance followed by post-Dunnett's test by using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). Results were presented in mean ± standard error of the mean for six rats per group with $p < 0.05$, $p < 0.01$, and $p < 0.001$ were considered statistically significant.

3 | RESULTS

3.1 | Quantification of chemical marker

In the HPLC chromatogram of 80% ethanol extract of *Z. zerumbet*, zerumbone was identified as a major peak at the RT of 9.745 min. The identification was confirmed by comparing the peak with the chromatogram of reference standard, zerumbone (Figure 1). The quantitative analysis further revealed 242.73 mg/g of zerumbone in the *Z. zerumbet* extract.

3.2 | Validation of the RP-HPLC method

The calibration curve of zerumbone (125–1,000 µg/ml) showed high accuracy, with R^2 of 0.999. The precision and repeatability of the HPLC method were validated by considering the %RSD of intra-day and inter-day assay precision of retention times and corresponding peak area. The %RSD for intraday assay and interday assay precision was analyzed as 0.93 and 1.53% disparately in respect to the retention time while 0.57 and 5.92% correspondingly in the case of peak area. Moreover, the HPLC method was able to detect zerumbone as lowest as 0.117 µg/ml (LOD) and quantify it at least 0.355 µg/ml (LOQ).

3.3 | LC–MS/MS profiling

The phytochemical analysis and characterization with LC–MS/MS results showed various tentative secondary metabolites including zerumbone, gallic acid, ethyl gallate, catechin, curcumin, kaempferol, kaempferol rhamnoside, kaempferol-3-*O*-(2″,4″-diacetyl) rhamnoside isomers, kaempferol methylether isomer, kaempferol-3-*O*-(3″,4″-diacetyl) rhamnoside isomers, kaempferol methylether, kaempferol glucoside conjugate, bisdesmethoxycurcumin, and desmethoxycurcumin in *Z. zerumbet* extract. The resultant retention times of the components along with their molecular ion peaks and MS^2 fragmentation ions are shown in Table 1.

3.4 | Acute toxicity test

Acute toxicity test for the determination of safe doses of *Z. zerumbet* extract was conducted by following the Organization of Economic Co-operation and Development Guideline 423. Results showed that the experimental animals orally fed with the extract at three different doses (100, 200, and 400 mg/kg), and vehicle solution (3.33% Tween 20) did not show any signs of toxicity such as shivering, salivation, diarrhea, lacrimation, hair erection, and convulsion throughout the study. The BW as well as relative organ index of all experimental animals (data not shown) did not reveal any toxicity symptoms. The BW of rats in all groups was increased from first day to 14th day, but it was not significantly different when compared with the control group. The increment of BW indicated that the rats were healthy since decrease in the BW is one of the indicators of adverse effects. This was further confirmed by the gross necropsy findings where no visible lesion was observed in liver of treated groups. The histopathology of the normal group and treated groups showed a normal distribution of hepatocytes with clear visible nuclei, a portal triad and central vein. The centrilobular tissue of the hepatic lobule was clearly visualized inside and surrounding the terminal hepatic venule of the liver. Besides, there was no dilatation of central vein in all treatment groups when compared with control group. In addition, no spotty necrosis evident of liver toxicity progression was seen in the treated groups.

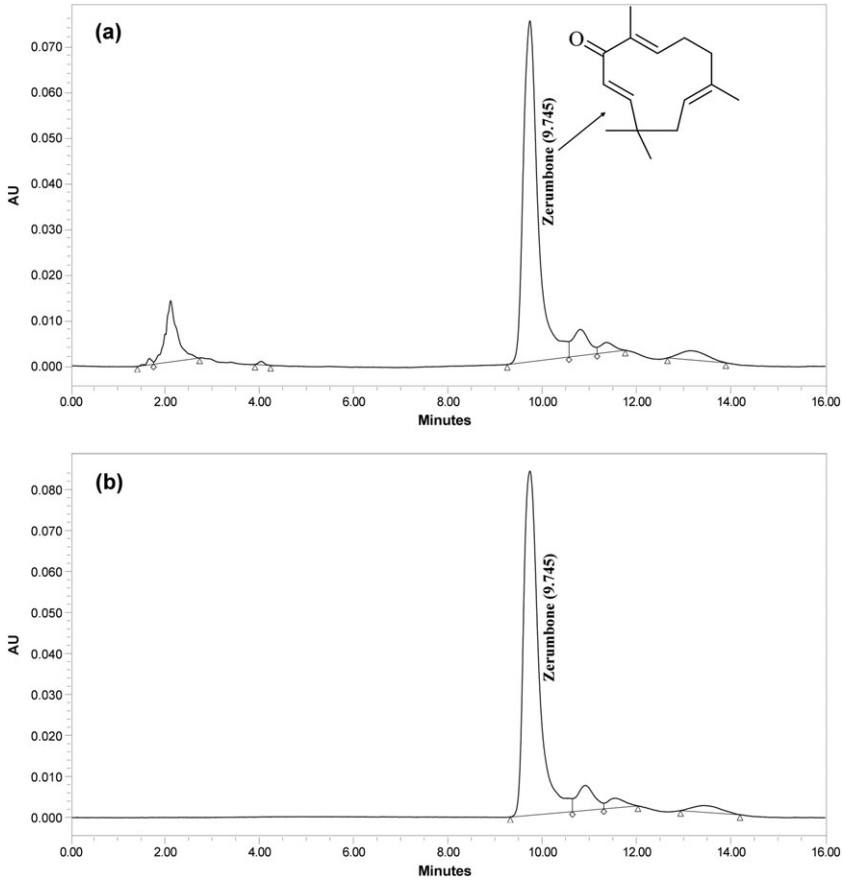

FIGURE 1 Reversed phase HPLC chromatograms of (a) 80% EtOH extract of *Zingiber zerumbet*, (b) standard zerumbone detected at the wavelength of 250 nm

Therefore, our finding revealed that the *Z. zerumbet* extract was safe at the treated doses (Figure 2).

3.5 | Cell migration assay

Effect of *Z. zerumbet* extract on the migration of neutrophils isolated from rat whole blood is shown in Figure 3. It was noticed that the extract was capable of reducing the migration of neutrophils toward the chemoattractant dose-dependently as well as showed a strong inhibitory activity at all doses when compared with the control group. The lowest dose of extract (100 mg/kg) exhibited a comparable inhibitory effect with the positive control Cys A. In addition, it was observed that *Z. zerumbet* extract was more potent than the positive control at the high doses of extract (200 and 400 mg/kg).

3.6 | CD11b/CD18 integrin expression assay

Expressions of CD11b/CD18 integrins by neutrophils isolated from rat whole blood were determined by flow cytometer. Figure S3 shows flow cytometric evaluation of the effect of *Z. zerumbet* in the

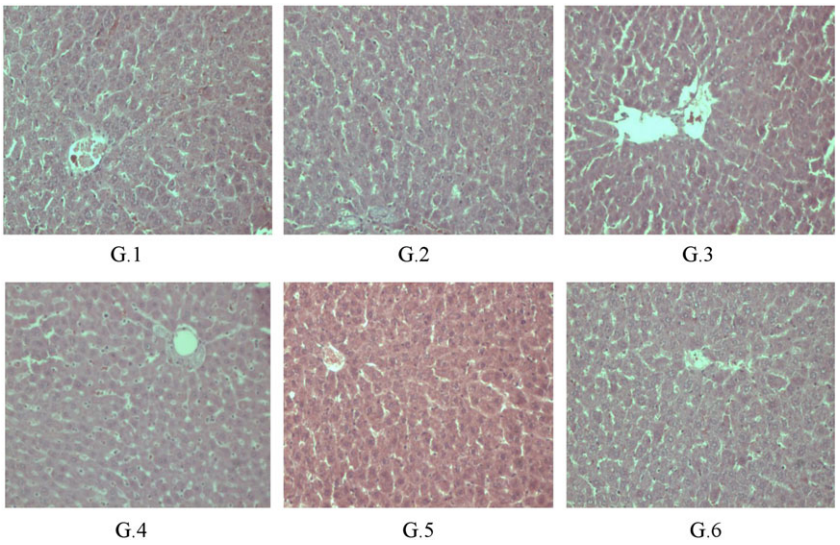

FIGURE 2 Photomicrographs of liver sections from control rats (G.1); 3.33% Tween 20 (G.2); 2,000-mg/kg ZZE (G.3); 100-mg/kg ZZE (G.4); 200-mg/kg ZZE (G.5); and 400-mg/kg ZZE (G.6)-treated. ZZE: *Zingiber zerumbet* extract. (H and E staining, original magnification 40×) [Colour figure can be viewed at wileyonlinelibrary.com]

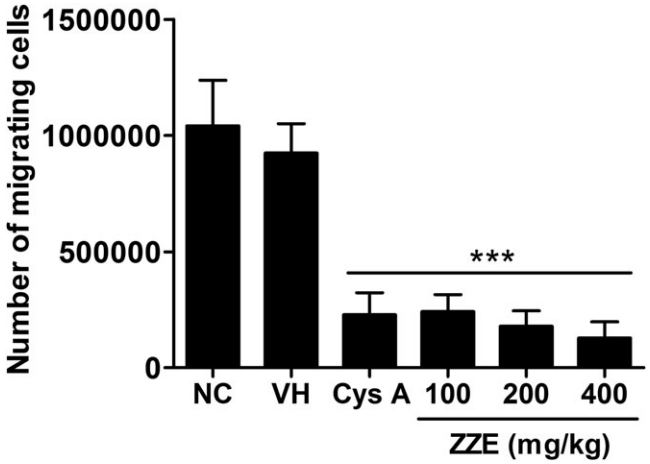

FIGURE 3 Inhibitory effects of *Zingiber zerumbet* (100, 200, and 400 mg/kg) on neutrophil migration. Mean ± SEM, with $n = 6$ in each group, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$; statistical analysis employed was ANOVA, followed by post-Dunnett's test. NC: nonsensitized control; VH: vehicle; Cys A: cyclosporin A; ZZE: *Zingiber zerumbet* extract

expression of CD11b/CD18 integrins by neutrophils isolated from rat whole blood. Results showed that *Z. zerumbet* extract dose-dependently inhibited the integrin expression (Table 2). As compared with the control group, at a dose of 100 mg/kg, the extract did not show any significant change in integrin expression. However, higher doses of the extract were significantly different compared to the control group.

3.7 | Phagocytic assay

Effect of *Z. zerumbet* extract on engulfment of *E. coli* by pretreated neutrophils isolated from rat whole blood is represented in Table 2. Figure S4 shows flow cytometric evaluation of the effect of *Z. zerumbet* on phagocytic activity of neutrophils. Results depicted that the extract was capable of suppressing the engulfment of *E. coli* at all three doses (100, 200, and 400 mg/kg) dose-dependently. On the other hand, the suppressive effects at all the doses were significantly different when compared with the control group. However, moderate inhibition of bacteria engulfment by neutrophils was observed at 100 and 200 mg/kg while 400-mg/kg dose exhibited a high engulfment inhibitory activity.

3.8 | ROS production assay

Production of ROS by neutrophils isolated from rat whole blood was measured by using flow cytometer. Figure S5 shows flow cytometric evaluation of the effect of *Z. zerumbet* on ROS production in neutrophils. The results showed that the inhibition of ROS production was increased dose-dependently as well as significantly different when compared with the control group (Table 2). The lowest dose of *Z. zerumbet* extract-treated neutrophils (100 mg/kg) exhibited a comparable effect with the positive control group. While at higher doses (200 and 400 mg/kg), the extract showed a high ability in inhibiting the ROS production and were more potent than the positive control group.

3.9 | Determination of lysozyme and ceruloplasmin production

The effects of *Z. zerumbet* extract on the expression of lysozyme and ceruloplasmin are shown in Figure 4. As illustrated, both lysozyme and ceruloplasmin levels in all *Z. zerumbet* extract-treated groups were highly and dose-dependently suppressed when compared with the control group. The 200-mg/kg dose group produced a comparable lysozyme level with the positive group. Whereas at 400 mg/kg, the extract was slightly more potent than the Cys A in inhibiting the production of lysozyme. Correspondingly, the inhibition of ceruloplasmin production level was highly significant at a dose of 400 mg/kg whereas 200-mg/kg dose exhibited a comparable ceruloplasmin level with the positive control group. The extract group at 100 mg/kg showed less inhibitory effects on ceruloplasmin level as compared with the control group.

4 | DISCUSSION

The homeostatic interplay between immunological components ensures the effectiveness and efficiency of the immune system. Over the years, medicinal plants have been considered as potent and safe

TABLE 2 Percentage of CD11b/CD18 integrin expressed, phagocytic activity, and reactive oxygen species production in neutrophils isolated from rat whole blood (mean ± SEM, with $n = 6$)

Group	Dose (mg/kg)	CD11b/CD18 expression (%)	Phagocytic activity (%)	Reactive oxygen species production (%)
Nonsensitized control	Tween 20	92.01 ± 1.32	86.71 ± 1.71	81.00 ± 1.76
Vehicle control		87.94 ± 2.70	81.36 ± 3.47	78.12 ± 3.75
Cyclosporin A	10	77.09 ± 4.65*	73.79 ± 2.20*	62.78 ± 5.41*
ZZE Dose 1	100	78.81 ± 4.40	72.84 ± 2.41**	61.07 ± 3.94*
ZZE Dose 2	200	75.50 ± 4.62*	71.27 ± 3.47**	58.82 ± 3.83**
ZZE Dose 3	400	74.94 ± 3.62*	67.44 ± 3.22***	55.12 ± 6.44**

Note. ZZE: *Zingiber zerumbet* extract. Statistical analysis employed was ANOVA, followed by post-Dunnett's test.
$^{*}p < 0.05$. $^{**}p < 0.01$. $^{***}p < 0.001$.

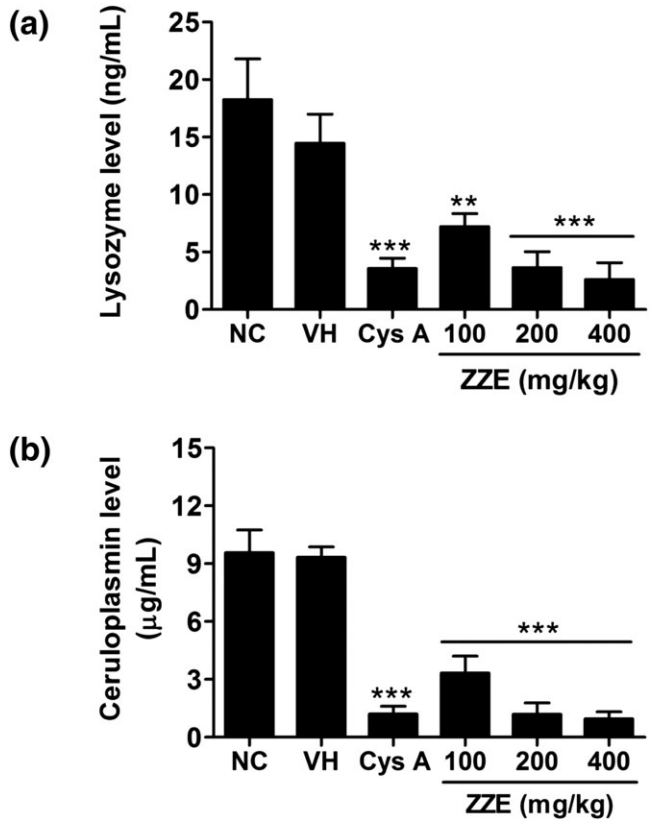

FIGURE 4 The effects of *Zingiber zerumbet* (100, 200, and 400 mg/kg) on lysozyme and ceruloplasmin levels. Mean ± SEM, with $n = 6$ in each group, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$; statistical analysis employed was ANOVA, followed by post-Dunnett's test. NC: nonsensitized control; VH: vehicle; Cys A: cyclosporin A; ZZE: *Zingiber zerumbet* extract

therapeutic candidates to modulate the immune system (Mohamed, Jantan, & Haque, 2017). *Z. zerumbet* is one of the potential medicinal plants as it has strong pharmacological activities in treating various types of immunological diseases traditionally and scientifically. Mainly, the pharmacological activity of medicinal plant depends on its chemical constituents, chemical nature, and structure of its bioactive chemical marker contents. The extract of *Z. zerumbet* rhizomes was standardized by using its bioactive chemical marker, zerumbone. It can be anticipated that the immunomodulatory activity exhibited by the plant extract in this study may be due to the pharmacological activities of zerumbone as being the principal bioactive compound of *Z. erumbet* (Haque et al., 2017b; Haque & Jantan, 2017; Koga, Beltrame, & Pereira, 2016).

Neutrophils are the professional phagocytic cells and are the first one to respond to any foreign particle attacking the body. Neutrophil-derived cytokines and phagocytosis have proven to be crucial in various inflammatory and immune system disorders (Arshad, Jantan, Bukhari, & Fauzi, 2018). In this study, *Z. zerumbet* extract exhibited strong suppressant effects on the FBS-induced chemotaxis (ex vivo) of neutrophils as compared with the control, Cys A. This provides evidence that *Z. zerumbet* extract has potential to be used as a safer natural-based immunosuppressant supplement.

Besides, CD11b/CD18 integrin is responsible in adhesion between neutrophils and opsonized pathogens. Overexpression of this integrin could lead to certain inflammatory disease like ulcerative colitis and inflammatory bowel diseases (Palmen, Dijkstra, Van der Ende, Pena, & Van Rees, 1995; Wolf et al., 2018). The significant inhibition of integrin liberation demonstrated in this study might be due to the inhibition of Ca^{2+} changes in the cytosolic of neutrophils. Rises of global Ca^{2+} concentration will activate the calpain, which enhances the expression of Mac-1. The effect of *Z. zerumbet* extract in reducing the phagocytosis process was further confirmed by the phagocytic assay in which *Z. zerumbet* extract suppressed the engulfment activity of neutrophils toward the opsonized pathogens. This may be attributed to the net effect of extract in suppressing the previous two major stages of phagocytosis process. Besides, the extension of the neutrophils pseudopod around the successful attached particles might also slow down, which lead to ineffectiveness of phagosome closure and pseudopod retraction (Dewitt & Hallett, 2002).

Upon phagocytosis, the increase in glucose and oxygen consumption leads to the production of oxygen-containing compounds (respiratory burst), which kill the phagocytosed bacteria. Nicotinamide adenine dinucleotide phosphate (NADPH) is formed upon the metabolism of glucose, and this NADPH is oxidized by the activated NADPH oxidase resulting in the superoxide anion production. Subsequently, superoxide dismutase converts superoxide anion to H_2O_2 and singlet oxygen. The superoxide anion can also react with H_2O_2 causing the formation of hydroxyl radical and more singlet oxygen. All of these reactions ultimately result in the production of the toxic oxygen compounds like superoxide anion (O^{2-}), H_2O_2, singlet oxygen ($^1O_2$), and hydroxyl radical (OH•; Dewitt & Hallett, 2002). The strong inhibitory activity of ROS production from the extract-treated neutrophils may be due to potential antioxidant ability of zerumbone, a marker compound of *Z. zerumbet* extract (Lim, 2016). The suppression of ROS generation could be due to the reduced NADPH-oxidase expression or blocking the electron transfer from NADPH to molecular oxygen, which could result in H_2O_2 production (Maraldi, 2013; Osto & Cosentino, 2010).

Ceruloplasmin and lysozyme being part of the innate immune system play an important role in cellular and humoral immune responses (Swaminathan, Ravi, Kumar, Kumar, & Chandra, 2011). Inappropriate reaction and deposition of lysozyme on tissues and organs could lead to lysozyme amyloidosis (Pleyer, Flesche, & Saeed, 2015). The inhibition of these enzymes can be attributed to the inhibition of proinflammatory cytokines like TNF-α and IFN-γ, which causes the phagocytic cells to release these enzymes (Iqbal et al., 2013). Therefore, the inhibition of ceruloplasmin by *Z. zerumbet* extract might be due to the inhibition of aforementioned proinflammatory cytokines.

5 | CONCLUSION

In conclusion, the extract has potential as an immunomodulating agent that could be beneficial in the treatment of immune-related diseases. The results also provide support for the traditional use of *Z. zerumbet* to heal various immune-inflammatory related diseases. However, extensive molecular study needs to be carried out in order to explain the specific mechanisms involved in the immunomodulatory activities.

Besides, further investigation on the identified bioactive chemical marker in the plant extract should be assessed to recognize that the immunosuppressive effects exerted by this extract was due to specific chemical marker or resulted from the synergistic effects from several bioactive compounds.

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CONFLICT OF INTEREST

The authors declare no conflict of interest occurred while conducting this study.

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