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Standardized extract of *Zingiber zerumbet* suppresses LPS-induced pro-inflammatory responses through NF-κB, MAPK and PI3K-Akt signaling pathways in U937 macrophages

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ABSTRACT

Background: *Zingiber zerumbet* rhizome has been used as spices and in traditional medicine to heal various immune-inflammatory related ailments. Although the plant was reported to have potent anti-inflammatory and immunosuppressive properties by several studies, the molecular mechanisms underlying the effects have not been well justified.

Purpose: The study was carried out to investigate the molecular mechanisms underlying the anti-inflammatory properties of the standardized 80% ethanol extract of *Z. zerumbet* through its effect on mitogen-activated protein kinase (MyD88)-dependent nuclear factor-kappa B (NF-κB), mitogen activated protein kinase (MAPK) and phosphatidylinositol 3-kinase/Akt (PI3K-Akt) signaling pathways in lipopolysaccharide (LPS)-induced U937 human macrophages.

Methods: Standardization of the 80% ethanol extract of *Z. zerumbet* was performed by using a validated reversed-phase HPLC method, while LC-MS/MS was used to profile the secondary metabolites. The release of pro-inflammatory markers, tumor necrosis factor (TNF)-α, interleukin (IL)-1β and prostaglandin E_2 (PGE_2) was evaluated by enzyme-linked immunosorbent assay (ELISA), while the Western blot technique was executed to elucidate the expression of mediators linked to MyD88-dependent respective signaling pathways. Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay was carried out to quantify the relative gene expression of cyclooxygenase (COX)-2 and pro-inflammatory mediators at the transcriptional level.

Results: The quantitative and qualitative analyses of *Z. zerumbet* extract showed the presence of several compounds including the major chemical marker zerumbone. *Z. zerumbet* extract suppressed the release of pro-inflammatory mediators, COX-2 protein expression and downregulated the mRNA expression of pro-inflammatory markers. *Z. zerumbet*-treatment also blocked NF-κB activation by preventing the phosphorylation of IKKα/β and NF-κB (p65) as well as the phosphorylation and degradation of IκBα. *Z. zerumbet* extract concentration-dependently inhibited the phosphorylation of respective MAPKs (JNK, ERK, and p38) as well as Akt. Correspondingly, *Z. zerumbet* extract suppressed the upstream signaling adaptor molecules, TLR4 and MyD88 prerequisite for the NF-κB, MAPKs, and PI3K-Akt activation.

Conclusion: The findings suggest that *Z. zerumbet* has impressive role in suppressing inflammation and related immune disorders by inhibition of various pro-inflammatory markers through the imperative MyD88-dependent NF-κB, MAPKs, and PI3K-Akt activation.

Abbreviations: COX-2, cyclooxygenase-2; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; HPLC, high performance liquid chromatography; HRP, horseradish peroxidase; IL-1β, interleukin-1 β; I-κB, I-kappa B kinase; IKK, I-κB kinase; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MyD88, myeloid differentiation primary response gene 88; NF-κB, nuclear factor-kappaB; Pen-strep, penicillin-streptomycin; PGE_2, prostaglandin E_2; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol-3 kinase; qRT-PCR, real-time quantitative reverse transcription polymerase chain reaction; RIPA, radioimmunoprecipitation assay; TBS, tris-buffered saline; TNF-α, tumor necrosis factor-α

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Introduction

Zingiber zerumbet (L.) Roscoe ex Sm. or shampoo ginger is a herbaceous medicinal plant belonging to 'ginger' family Zingiberaceae and found in all tropical places of the world, mainly in Southeast Asia. It is also well-known as Asian ginger, wild ginger, bitter ginger or pinecone ginger. The herb is assumed to be native to Indian subcontinent as well as to the Malaysian peninsula. It has been cultivated for an extensive period in several origins all the way through the Southeast Asia, Pacific, and Oceania. *Z. zerumbet* has vast aspects of medicinal properties based on traditional usages. Among the plant parts, rhizomes are the mostly consumed parts that are utilized by the Asians particularly the Chinese and Indian conventional healers. The rhizomes have been used as spices as well as ethnomedicines in many regions of the Asia to heal various immune-inflammatory related ailments (Haque and Jantan, 2017). It is mainly reported for the management of fever, inflammation, and rheumatoids. It has also been reported for the cure of stomachache, headache, toothache, allergies, asthma, boils, coughs, hemorrhoids, severe sprains, thrush, ear inflammation, gastritis, torment, arthritis, joint pains, and wounds (Haque and Jantan, 2017). Particularly, in Malaysia, the fresh rhizomes are used to heal stomachache, oedema, loss of appetite, as well as sores. The Malays have also used the rhizomes for the management of constipation, vertigo, debility, and gastralgia (Norhayati et al., 1999). Besides, in Indo-Malaysian ethnomedicines, *Z. zerumbet* and *Curcuma xanthorrhiza* are usually utilized ingredients as tonics as well as health supplements (Ruslay et al., 2007).

However, in comparison to the huge ethnomedicinal aspects of the herb, little scientific investigations have been conducted to verify its immunomodulating ethnomedicinal claims. Most of the studies were carried out using the crude rhizome extracts and the active compounds contributing to the biological and pharmacological properties have not been identified. In a report, the water extract of *Z. zerumbet* was found to protect the lungs by attenuating the inflammatory mediators release and gene expressions *in vitro* and *in vivo* (Chaung et al., 2008). Previously, the plant extract was found to possess significant anti-platelet aggregation properties, demonstrating 100% inhibition at 100 µg/ml in human whole blood (Jantan et al., 2008). Zakaria et al. (2011) determined the anti-inflammatory effect of *Z. zerumbet* methanol extract in Sprague-Dawley rats and BALB/c mice (Zakaria et al., 2011). The ethanol and aqueous extracts as well as the essential oils from the rhizomes of Zingiberaceae species including *Z. zerumbet* were reported for their anti-allergic effect on RBL-2H3 cells (Tewtrakul and Subhadhirasakul, 2007). The present investigation was aimed to determine the anti-inflammatory properties of the 80% ethanol extract of *Z. zerumbet* rhizomes on the release and expression of pro-inflammatory markers via MyD88-dependent NF-κB, MAPK, and PI3K-Akt signaling pathways in LPS-activated U937 human macrophages.

Materials and methods

Chemicals and reagents

RPMI-1640 culture medium, PBS, FBS, and Pen-Strep antibiotic were acquired from Gibco (Grand Island, NY, USA). LPS (derived from *Escherichia coli*), PMA, RIPA buffer, MTT reagent and DMSO were attained from Sigma Chemical Co. (St. Louis, USA). 1 × Halt protease and phosphatase inhibitor cocktail was acquired from Pierce (Rockford, USA). Human TNF-α, IL-1β, PGE_2 ELISA kits were obtained from R&D Systems (Minneapolis, USA). Primary antibodies i.e., p-p38 (Thr180/Tyr182), p38, p-ERK1/2 (Thr202/Tyr204), ERK1/2, p-JNK1/2 (Thr183/Tyr185), JNK1/2, p-IκBα (Ser32/36), IκBα, p-IKKα/β (Ser176/180), p-NFκBp65 (Ser536), p-Akt (Ser 473) along with β-actin, and anti-rabbit secondary antibody conjugated to horseradish peroxidase were acquired from Cell Signaling Technology (Beverly, MA). Specific inhibitors were gained from Tocris Biosciences (Bristol, UK). Dexamethasone (purity > 98%) was bought from CCM Duopharma Biotech Bhd (Selangor, Malaysia). All the chemicals and reagents together with biologics and synthetics used in this investigation were endotoxin free.

Plant collection and preparation of extract

The rhizomes of *Z. zerumbet* were obtained from Landang Kizaherbs, Kampong Batu Gading, Kuala Karu, Temerloh, Pahang, Malaysia. Specimen identification was conducted by Dr. Abdul Latif Mohamad from Faculty of Science and Technology, Universiti Kebangsaan Malaysia (UKM). A voucher of the specimen (No. UKM-HF137) was deposited for further reference at the herbarium of UKM located in Bangi, Malaysia. The extraction of the plant was carried out following the method of Haque et al. (2018a). The rhizomes were dried under shade at 26 ± 2 °C and ground into powder. Extraction was carried out by macerating the powdered material (1750 g) in 80% EtOH at a ratio of 1:6 (sample: solvent) (w/v) for 72 h at room temperature and followed by filtration with Whatman no. 1 filter paper (Whatman plc, Maidstone, UK). The collected filtrates were evaporated *in vacuo* to acquire 248 g of gummy crude extract (14.17%).

Standardization of 80% ethanol extract of Zingiber zerumbet by HPLC

The HPLC analysis was performed on a Waters 2535 Quaternary Gradient Module equipped with PDA detector (Waters 2998). An XBridge™ C-18 analytical column (4.6 mm × 250 mm; particle size, 5 µm) was used for the analysis. The stock solution of *Z. zerumbet* extract in MeOH at 3 mg/ml was prepared and then sonicated for 20 min. The solution was then filtered through a 0.45 µm Millex PTFE membrane filter. Additionally, sample solution for reference standard i.e., zerumbone (purity > 98%) was prepared as 1 mg/ml and then diluted into a series of two-fold dilutions from 125 to 1000 µg/ml. All the solutions were preserved at −20 °C until analysis. Detection was performed at the wavelength ranging from 210 to 350 nm. An injection volume of 20 µl for extract and reference standard was used. The following solvents were used as mobile phase- solvent A (ACN) and B (water). The gradient program used as 65–70% A (10 min) and 70–75% A (6 min) at a constant flow rate of 1.2 ml/min. The detection was measured at a wavelength of 250 nm. The data acquisition was carried out with Empower 3 software. The identification of zerumbone was performed by comparing the retention times (RT) and ultraviolet-visible spectrum (UV–Vis spectra) of the peak along with the peaks of reference standard. Additionally, calibration curve was plotted with four different concentrations of the reference standard solution of zerumbone versus the areas under the peaks. Correspondingly, the standard curve equation obtained from the standard was used for quantification of zerumbone in the *Z. zerumbet* extract.

Validation procedure for HPLC analysis

To validate the method, International Council for Harmonization (ICH) guidelines for the validation of analytical procedures were followed (ICH Harmonised Tripartite Guideline). Determination of precision, linearity, limit of detection (LOD), and limit of quantitation (LOQ) were included during validation. The precision was determined by mainly, repeatability (intra-assay precision) and intermediate precision (inter-assay precision). The linearity was calculated using regression parameter from the calibration curve and correlation coefficient (R^2). LOD and LOQ were determined using the residual standard deviation (RSD) and the slope (S) of the calibration curve and following equations:

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

LC-MS/MS analysis

Chromatographic separation of *Z. zerumbet* extract was performed to profile the secondary metabolites of the extract using an AB Sciex 3200QTrap LC-MS/MS with Flexar FX-15 series UHPLC and a Phenomenex synergi fusion column (100 mm × 2.1 mm; particle size, 3 μm). The following eluents were used: (A) 0.1% formic acid in water and 5 mM ammonium formate and (B) ACN with 0.1% formic acid and 5 mM ammonium formate. The gradient program used was 20–30% B (10 min), 30–40% B (10 min), 40–50% B (10 min), 50–90% B (20 min), 90–90% B (10 min), 90–20% B (10 min) at a persistent flow rate of 250 μl/min, with a sample injection volume of 20 μl. The negative ionization mode along with the following settings was used: source temperature, 500 °C; capillary voltage (IS), 4500 V; Scan range, 100–1500 m/z for full scan and 50–1500 m/z for MS/MS scan. The ACD/Labs advanced chemometrics mass fragmentations predictive software (Toronto, ON, Canada) was used for data acquisition and the resultant peaks of the components were determined comparing with the mass spectral library.

Cell cultures and differentiation induction

U937 cells (ATCC® CRL1593.2™) were cultured and the differentiations of cells into macrophage-like phenotypes were conducted as the method described earlier (Harikrishnan et al., 2018).

Cell viability assay

MTT assay was conducted to confirm the viability of differentiated U937 macrophages as described by Haque et al. (2018a). In brief, the differentiated U937 macrophages (1×10^6 cells/ml) were seeded in 96 well plates with different concentrations of *Z. zerumbet* extract and incubated for 24 h at 37 °C with 5% CO_2. Then, 10 μl of 5 mg/ml MTT reagents were added and the cells were further incubated at 37 °C for 4 h. The formazan crystals were then dissolved in DMSO and the analysis was continued by calculating absorbance values at 570 nm using Tecan's Infinite 200PRO NanoQuant microplate reader.

ELISA immunoassay

The differentiated macrophages were plated into 24 well plates treated with various concentrations of *Z. zerumbet* extract (4.68 to 75 μg/ml) or the positive control, dexamethasone (0.0004 to 4 μg/ml) for 2 h, prior to stimulation by LPS (1 μg/ml) for 24 h to secrete cytokines and PGE_2 and hence, performed the assay as described before (Haque et al., 2018a; Harikrishnan et al., 2018).

Determination of relative gene expression levels by qRT-PCR

The qRT-PCR procedure was carried out to evaluate the modulatory effects of *Z. zerumbet* extract on the expression of pro-inflammatory markers i.e., TNF-α, IL-1β, and COX-2, following the method as reported earlier by Harikrishnan et al. (2018). In brief, 1×10^6 cells/ml of U937 macrophages were pre-treated for 2 h with *Z. zerumbet* extract (4.68 to 75 μg/ml) and later on incubated with LPS (1 μg /ml) for 1 d. The total RNA from treated cells were extracted with innuPREP RNA-mini kit (Analytik Jena, Germany) according to manufacturer's instruction. The quality of RNA was evaluated by computing the 260/280 ratio (>2.0). Total RNA of 1 μg/ml was reverse transcribed by using SensiFAST™ cDNA Synthesis Kit (Bioline USA Inc, Taunton, MA) following manufacturer's instruction. The mRNA quantified by using qRT-PCR (Biorad, Hercules, California, USA) along with SYBR® Green RT-PCR Master Mix (Bioline USA Inc, MA). The cDNA was amplified by using the following primers; TNF-α (Hs_TNF_3_SG QuantiTect Primer QT01079561), COX-2 (Hs_PTGS2_1_SG QuantiTect Primer QT00040586), IL-1β (Hs_IL1B_1_SG QuantiTect Primer QT00021385) and GADPH (Hs_GAPDH_1_SG QuantiTect Primer QT00079247). The PCR reaction mixture comprised of cDNA (2 μl), SYBR® Green Master Mix (10 μl), reverse and forward primers (2 μl) and deionized water (6 μl). The reaction was carried out in the following parameter: 95 °C for 2 sec, 95 °C for 5 min, 60 °C for 10 min and 72 °C for 20 min (36 cycles). The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative fold change between samples. The relative mRNA expression was normalized using GADPH as the housekeeping gene.

Protein extraction and immunoblot analysis

The U937 macrophages were treated with *Z. zerumbet* extract (4.68 to 75 μg/ml) for 2 h preceding to the stimulation with LPS (1 μg/ml) for 30 min (with the exemption of determination of COX-2, 24 h; MyD88 and TLR4, 60 min) and the specific target proteins expression was assessed by Western blotting as reported earlier (Haque et al., 2018a; Haque et al., 2018b; Harikrishnan et al., 2018).

Inhibition of NF-κB, MAPKs, and PI3K/Akt activation

To inspect the signal transducing pathways involved in the effects of *Z. zerumbet* extract on LPS-stimulated macrophages, the cells were treated with SB202190 (p38 inhibitor), U0126 (an ERK inhibitor), SP600125 (JNK inhibitor), LY294002 (Akt inhibitor), and BAY 11–7082 (NF-κB inhibitor) at 10 μM along with ZZE at 75 μg/ml followed by treatment with LPS (1 μg/ml). Finally, ELISA and Western blot analysis were carried out to study the effects on the production and expression of TNF-α and COX-2 protein (Haque et al., 2018a; Harikrishnan et al., 2018).

Statistical analysis

Every experiment in this study was repeated thrice and the results were interpreted as mean ± SEM. One-way analysis of variance (ANOVA) followed by Dunnett's test was applied for comparison of experimental samples with the control. The variance was measured significant if the P value was less than 0.05 ($P < 0.05$) or below. All statistical designs and calculations were carried out by using of GraphPad Prism 6.0 program (GraphPad Software, San Diego, USA).

Results

Quantitative and qualitative analysis of Zingiber zerumbet extract

The HPLC chromatogram of *Z. zerumbet* extract exhibited one major peak of zerumbone at a respective retention time (RT) of 9.745 min. Identification of the peak was confirmed by comparing it with the chromatogram of reference standard (**Fig. 1**). From the quantitative analysis, the amount of zerumbone found in the extract was 728.19 μg/ml. The calibration curve was established by plotting different concentrations of standard zerumbone ranging from 125 to 1000 μg/ml with a correlation coefficient (R^2) of 0.999. The reproducibility of the method was confirmed by considering the %RSD of intra-day and inter-day precision of RT and corresponding responses (peak area). The % RSD for intra-day and inter-day precision were noted as 0.93 and 1.53% correspondingly with respect to RT while, 0.57 and 5.92% respectively in the case of peak area. Additionally, the LOD and LOQ of *Z. zerumbet* extract were calculated as 0.117 and 0.355 μg/ml, respectively.

LC-MS/MS analysis of *Z. zerumbet* extract tentatively revealed the presence of several secondary metabolites such as ethyl gallate, gallic acid, catechin, kaempferol rhamnoside, kaempferol, kaempferol-3-*O*-(2″, 4″-diacetyl) rhamnoside isomers, kaempferol methylether, kaempferol methylether isomer, kaempferol-3-*O*-(3″,4″-diacetyl) rhamnoside isomers, kaempferol glucoside conjugate, demethoxycurcumin, zerumbone, curcumin, and bisdemethoxycurcumin. The corresponding retention time of the components along with their

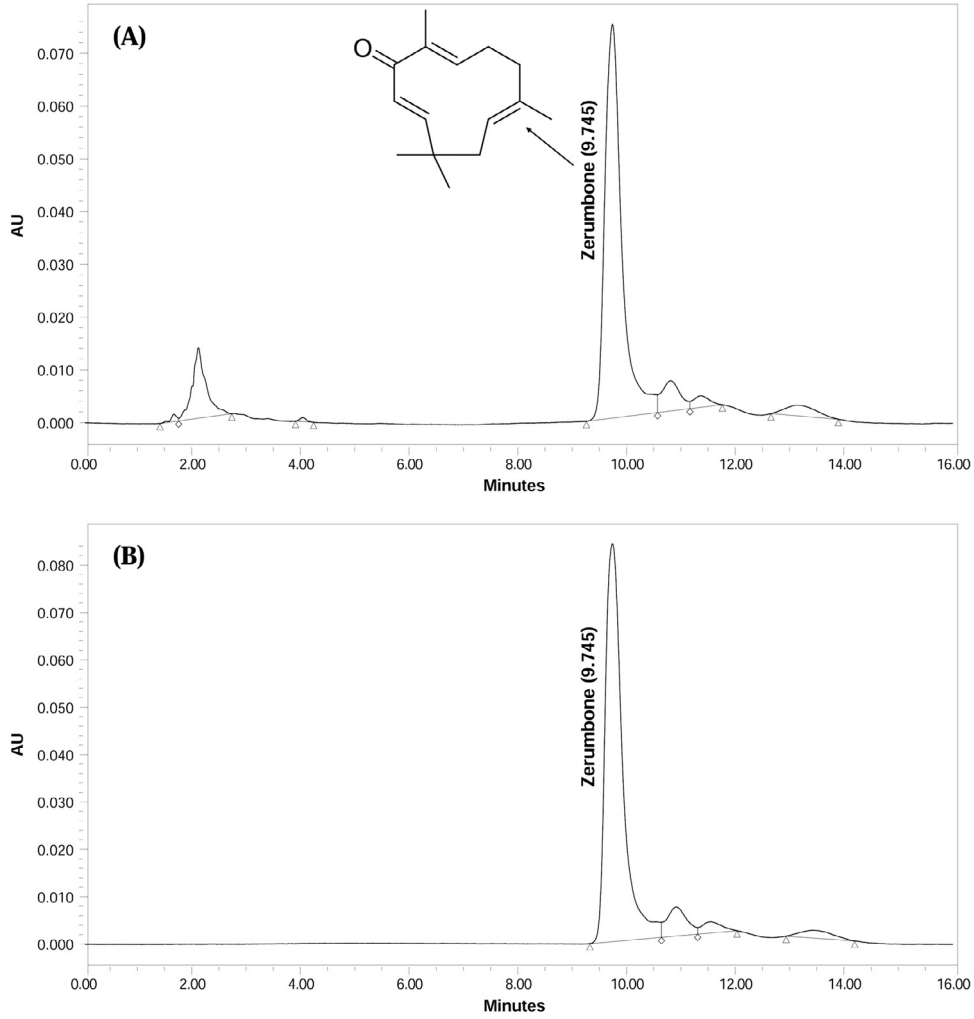

Fig. 1. Representative HPLC chromatogram of (**A**) 80% ethanol extract of *Zingiber zerumbet*, (**B**) Standard zerumbone detected at 250 nm.

Table 1.
Retention times, MS^2 fragments of the major compounds present in *Zingiber zerumbet* extract.

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

* refers to the most intense ion or the base peak.

molecular ion peaks and MS^2 fragmentation ions are presented in Table 1.

PMA induces the morphological and phenotypic changes in U937 cells

To observe the monocyte-derived macrophage properties of U937 cells, 200 nM of PMA was added to induce the cell differentiation. As shown in Fig. 2, there were no morphological changes observed in the

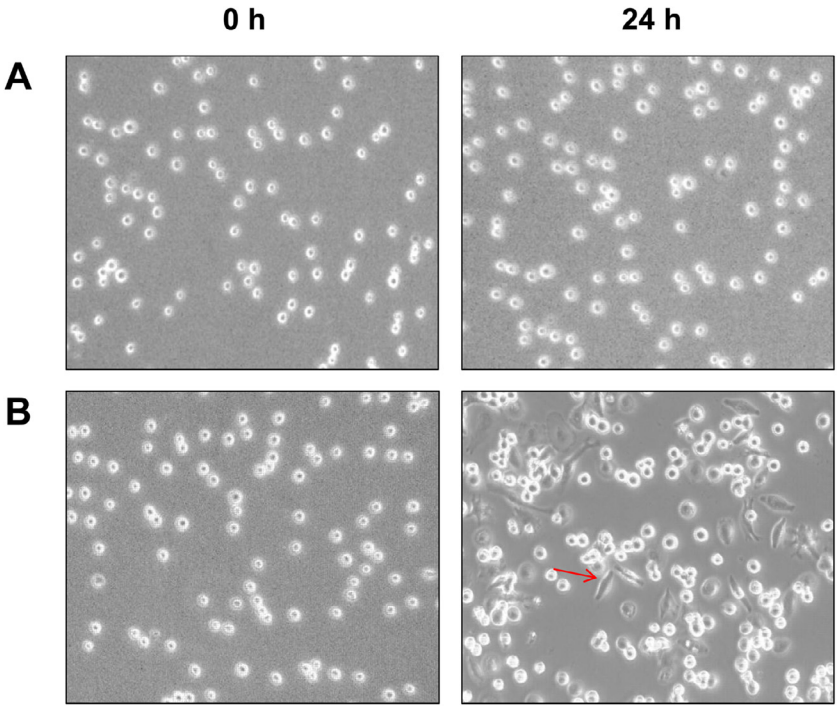

Fig. 2. U937 cells differentiation. The non-induced cells (top row, **A**) and PMA-induced cells (bottom row, **B**). The red arrow indicates the morphology of differentiated cells, which are flat and elongate in shape with pseudopodia formation.

cells without PMA induction during the incubation time. The morphology observed for non-induced U937 cells were small and round, grew in suspension form, and did not adhere to the surface of the culture flask. However, the morphological changes were observed in those cells induced with PMA for 24 h.

Effects of zingiber zerumbet extract on cell viability

The differentiated macrophages were treated with various concentrations of ZZE (4.68 to 300 μg/ml) to ascertain the safe doses used for the experiments. The viability of cells was noted near about 100% before the LPS and samples treatment. Once treated, as shown in Fig. 3, doses at 75 μg/ml and lower revealed high cell viability (more than or equal to 90%). Hence, the safe doses determined in this study were ranging from 75 μg/ml or below.

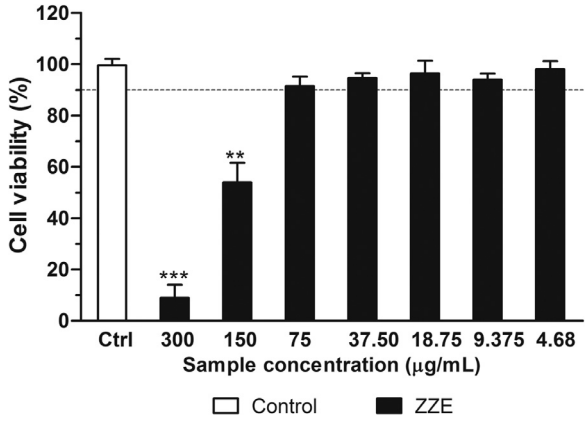

Fig. 3. The cytotoxicity of *Zingiber zerumbet* extract (ZZE) in differentiated U937 macrophages. All the values are stated as mean ± SEM (n = 3). ***P < 0.001 and **P < 0.01 control (Ctrl) versus ZZE treated group. ZZE = 80% ethanol extract of *Z. zerumbet*.

Zingiber zerumbet extract suppressed the pro-inflammatory cytokines release and mRNA expression in LPS-activated U937 macrophages

The release of TNF-α and IL-1β were quantified by ELISA. *Z. zerumbet* extract was found to attenuate the TNF-α and IL-1β release in a concentration-dependent manner (Fig. 4A and B). Alternatively, *Z. zerumbet* extract could not show any significant effect in the absence of LPS treatment and the effects were relatively alike untreated cells (control). The IC_{50} values of *Z. zerumbet* extract were noted as 4.61 and 4.72 μg/ml for TNF-α and IL-1β, correspondingly, while the positive control, dexamethasone (DEX) showed IC_{50} values of 0.176 and 0.002 μg/ml. The suppressive effect of *Z. zerumbet* extract on TNF-α release at the concentration of 37.5 and 75 μg/ml were found comparable with the effect displayed by standard drug DEX (4 μg/ml). In case of inhibition of IL-1β, the effect of *Z. zerumbet* extract at 75 μg/ml was comparable with the effect produced by DEX as well.

In line with the ELISA results, from the qRT-PCR analysis it was found that *Z. zerumbet* extract at doses of 4.68, 18.75, and 75 μg/ml successively inhibited the TNF-α and IL-1β mRNA expression in a concentration-dependent manner (Fig. 4C and D). As shown in the Figure, *Z. zerumbet* extract showed highly significant (P < 0.001) inhibition at 18.75 and 75 μg/ml (23.14 and 30.77 folds, respectively) for TNF-α, while standard drug DEX showed significant inhibition by 8.22 folds at 4 μg/ml. The outcomes revealed that the inhibitions of TNF-α and IL-1β at the protein level were interlinked with the inhibition at the mRNA level in LPS-activated macrophages. However, *Z. zerumbet* extract without LPS treatment could not show any significant effect on the mRNA expression of TNF-α and IL1-β, and the effects found were similar to untreated control. Therefore, treatments with *Z. zerumbet* extract alone had been excluded from further studies.

Zingiber zerumbet extract suppressed the PGE_2 release and COX-2 expression

The investigation further exhibited that LPS induction for 24 h enhanced the PGE_2 release as well as COX-2 protein expression in macrophages, which were found to be attenuated upon *Z. zerumbet* extract

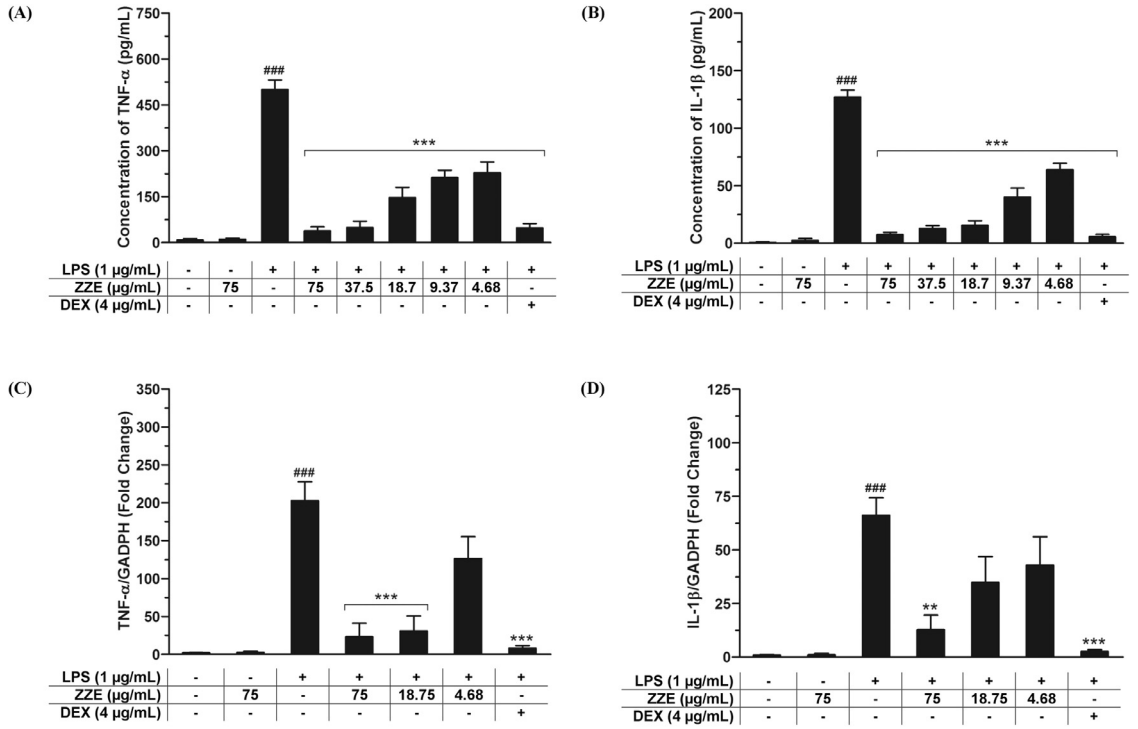

Fig. 4. Effects of *Zingiber zerumbet* extract (ZZE) on the pro-inflammatory cytokines release. (**A** and **B**) Effects of ZZE on TNF-α and IL-1β production. (**C** and **D**) Effects of ZZE on the TNF-α and IL-1β mRNA expression. Data are presented as mean ± SEM ($n = 3$). $^{###}P < 0.001$ denotes the significant difference from the control. $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$ denote significance to the LPS alone versus ZZE or DEX pretreated. LPS = Lipopolysaccharide; ZZE = 80% ethanol extract of *Z. zerumbet*; DEX = Dexamethasone.

pre-treatment at 4.68, 18.75, and 75 μg/ml in a concentration-dependent manner. As well, the effect of *Z. zerumbet* extract at pre-translational level was observed in LPS-activated U937 macrophages. In the investigation, the treatment with LPS was found to enhance the COX-2 mRNA by 281.9 folds. As shown in Fig. 5, it was observed that *Z. zerumbet* extract consecutively downregulated the COX-2 mRNA expression in LPS-activated macrophages. *Z. zerumbet* extract showed significant suppression of COX-2 mRNA expression at 18.75 μg/ml ($P < 0.01$) and 75 μg/ml ($P < 0.001$) by 73.36 and 31.08 folds, respectively.

Zingiber zerumbet extract blocked NF-κB activation in LPS-activated U937 macrophages

We further inspected the activity of *Z. zerumbet* extract on NF-κB signaling activation. As illustrated in Fig. 6, treatment with *Z. zerumbet* extract was able to modulate the NF-κB signal transduction by inhibiting the phosphorylation of IKKα/β, IκBα, and p65 of NF-κB, which were upregulated by LPS treatment. *Z. zerumbet* extract showed most significant ($P < 0.001$) inhibition at the doses of 75 μg/ml and 18.75 μg/ml, while all the treated doses showed highly significant inhibition in the case of IκBα phosphorylation. The degradation of IκBα was also observed to be prevented by *Z. zerumbet* extract pretreatment. The findings support the theory that *Z. zerumbet* extract influenced NF-κB activation signaling pathway by modulating IκB phosphorylation and degradation in LPS activated U937 macrophages. Significant inhibition ($P < 0.01$) of p65 phosphorylation had also been noted at low dose (4.68 μg/ml) of *Z. zerumbet* extract pre-treatment.

Zingiber zerumbet extract attenuated MAPKs and PI3K-Akt phosphorylation in LPS-activated U937 macrophages

Besides NF-κB signaling pathway, to scrutinize whether *Z. zerumbet* extract mediate the NF-κB instigation via the MAPKs signaling pathway, the LPS-activated MAPKs proteins, JNK, ERK and p38 MAPK were examined. As illustrated in Fig. 7, the inhibition upon all treated doses of *Z. zerumbet* extract was highly significant ($P < 0.001$) in case of MAPKs phosphorylation while, doses of *Z. zerumbet* extract at 18.75 and 75 ug/ml were noted highly significant ($P < 0.001$) in the case of phosphorylation of Akt. This result postulates that the 2 h pre-treatment of *Z. zerumbet* extract was effectively suppress the MAPKs and Akt phosphorylation.

Effects of NF-κB, MAPKs, and PI3k/Akt inhibitors

In order to further authenticate that the suppression of inflammatory signaling molecules by *Z. zerumbet* extract related to the NF-κB, MAPKs and Akt signaling pathway downregulation, we studied the effect of specific NF-κB, MAPKs and Akt inhibitors on TNF-α release and COX-2 expression. The treatment of LPS-stimulated cells with BAY 11–7082, LY294002, SP600125, U0126, and SB202190 showed notable suppressive effect on TNF-α release and COX-2 protein expression (Fig. 8). Taken together, our results propose that *Z. zerumbet* extract attenuates TNF-α and COX-2 expression by downregulating LPS triggered p38, ERK, JNK MAPKs, Akt and NF-κB signal transducing pathway.

Zingiber zerumbet extract suppressed MyD88 and TLR4 expression in LPS-activated U937 macrophages

In ensuing to the outcomes of the above investigation, it was further designed to observe the effects of *Z. zerumbet* extract on MyD88 and TLR4 to confirm whether these upstream signaling adaptor molecules play roles in altering the activation of NF-κB, MAPKs, and PI3K-Akt signaling in LPS-activated U937 macrophages. As illustrated in Fig. 9, *Z. zerumbet* extract concentration-dependently suppressed the expression of MyD88 and TLR4 adaptor molecules that was in consistent with the inhibitory effects of *Z. zerumbet* extract on the NF-κB, MAPK, and

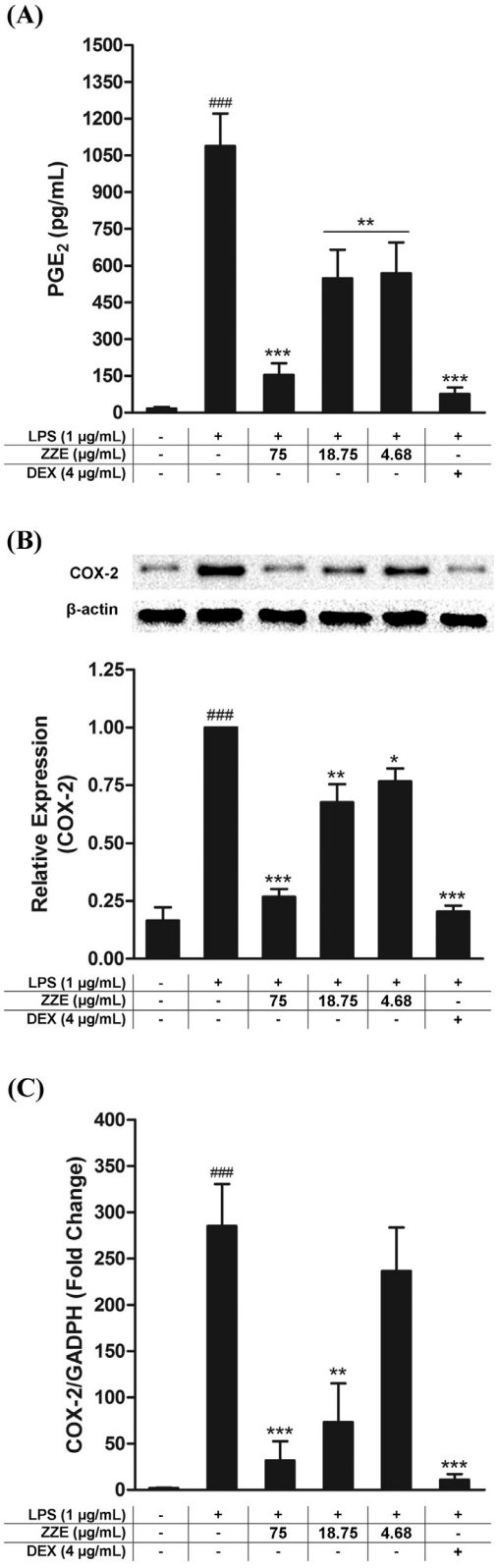

Fig. 5. The expression of PGE_2 and COX-2. (**A** and **B**) Effects of ZZE on the PGE_2 release and COX-2 protein expression. (**C**) Effects of ZZE on the COX-2 mRNA expression. Data are presented as mean ± SEM ($n = 3$). $^{###}P < 0.001$ denotes the significant difference from the control. $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$ denote significance to the LPS alone versus ZZE pre-treated.

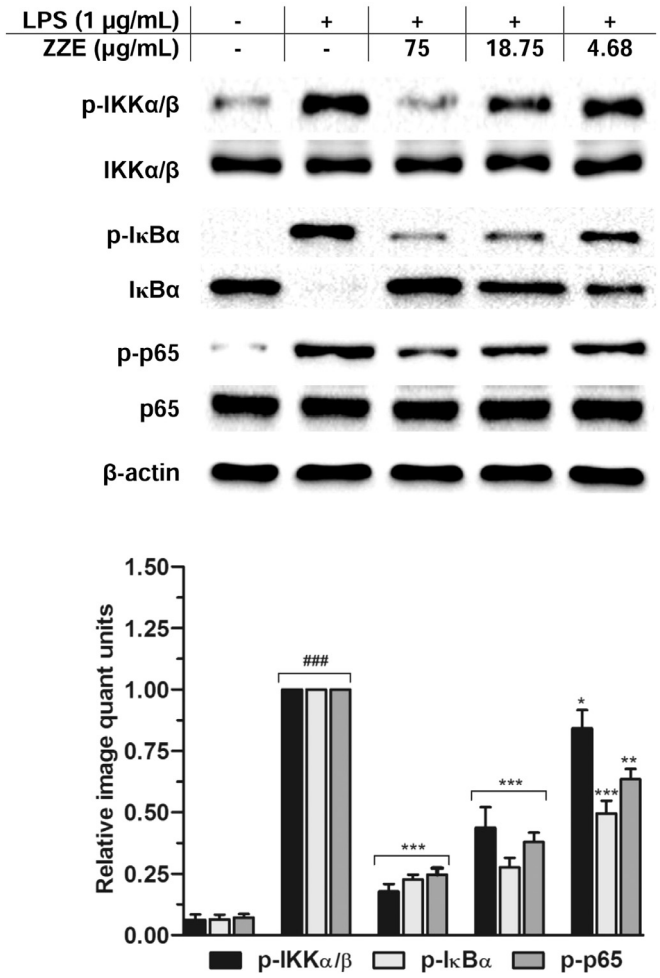

Fig. 6. Effects of *Zingiber zerumbet* extract (ZZE) on the NF-κB signaling pathway. Respective Western blots presenting the effects of ZZE on the phosphorylation of IKKα/β, IκBα, and p65 of NF-κB. Data are expressed as mean ± SEM ($n = 3$). $^{###}P < 0.001$ denotes the significant difference from the control. $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$ denote significance to the LPS alone versus ZZE pre-treated.

PI3K-Akt signaling pathways in LPS-induced macrophages. Notably, *Z. zerumbet* extract showed most significant inhibition ($P < 0.001$) in case of MyD88 at all treated doses.

Discussion

Immune system, simply termed as a complex defense mechanism of the body is comprised of two major types of immunity i.e., innate and adaptive. The proper functioning of this intricate system relies on the balance of both immunities and coordination of various immune cells. Macrophages are the blood-circulating monocytes and are the major immune cells of innate immune system. The U937 monocytes are the most frequently used cells in *in vitro* model of human macrophage function since primary human tissue macrophages cannot be replicated (Chanput et al., 2015). While transformed into a macrophage lineage, these cells display properties similar to the human macrophages (Sharp, 2013). Macrophages activation is imperative in innate or non-specific immune response for the instigation and transmission of numerous defensive signals and reactions against infectious pathogenic agents (Twigg, 2004). LPS is known as a classic element and pro-inflammatory stimuli, which generally bonds to toll-like receptor 4 (TLR-4) in macrophages and recruits the adaptor molecule MyD88 that ultimately triggers and promotes the signal transduction pathway kinases

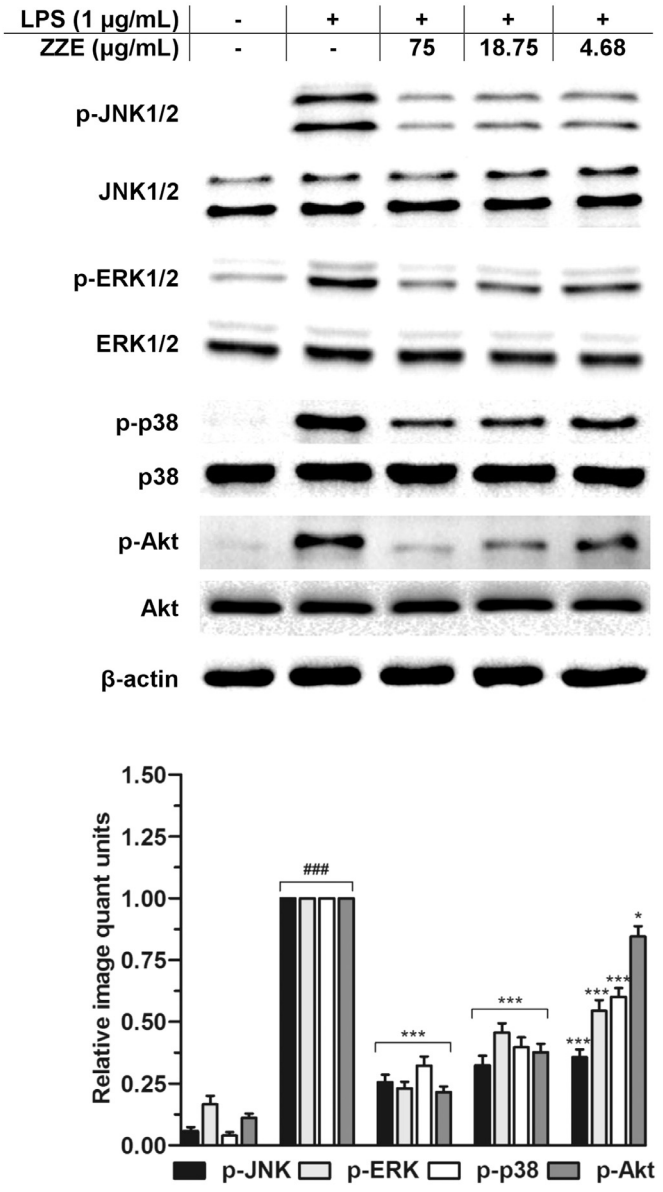

Fig. 7. Effects of *Zingiber zerumbet* extract (ZZE) on MAPK and PI3K-Akt signaling pathways. Respective Western blots presenting the effects of ZZE on the JNK, ERK, and p38 MAP kinases as well as on phosphorylation of Akt. Data are expressed as mean ± SEM. ($n = 3$). $^{###}P < 0.001$ denotes the significant difference from the control. $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$ denote significance to the LPS alone versus ZZE pre-treated.

to induce immunological responses followed by the imperative NF-κB, MAPK, and PI3K-Akt signaling pathways. Respectively, NF-κB enhances the gene expression to release various pro-inflammatory mediators including cytokines and enzymes.

The activated macrophages generally produce the pro-inflammatory markers like TNF-α, IL1β, PGE_2, COX-2, NO, iNOS that aid in the process of inflammatory damage. Nevertheless, the excessive release of these markers plays as causative agent for chronic inflammation, which results in autoinflammatory and autoimmune diseases. Hence, natural products that are able to suppress the excessive release of these pro-inflammatory markers could be alternative immunosuppressive and anti-inflammatory therapeutic candidates. From the present investigation, the levels of the pro-inflammatory markers i.e., TNF-α, IL-1β, PGE_2, COX-2 production and expression were found to enhance in the culture supernatants upon macrophages activation via stimulation with LPS. It was observed that the treatment with *Z. zerumbet* extract

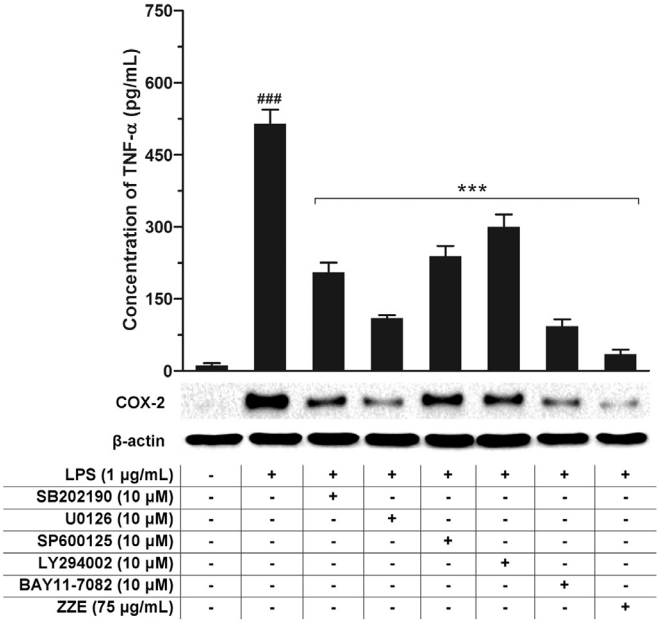

Fig. 8. The effects of MAPKs, Akt, and NF-κB inhibitors. The effects of SB202190 (p38 inhibitor), U0126 (ERK inhibitor), SP600125 (JNK inhibitor), LY294002 (Akt inhibitor), and BAY 11-7082 (NF-κB inhibitor) on the TNF-α release and COX-2 protein expression in LPS-activated U937 macrophages. Data are presented as mean ± SEM ($n = 3$). $^{###}P < 0.001$ indicates significant difference from the unstimulated control group; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ LPS treatment versus inhibitors pre-treatment.

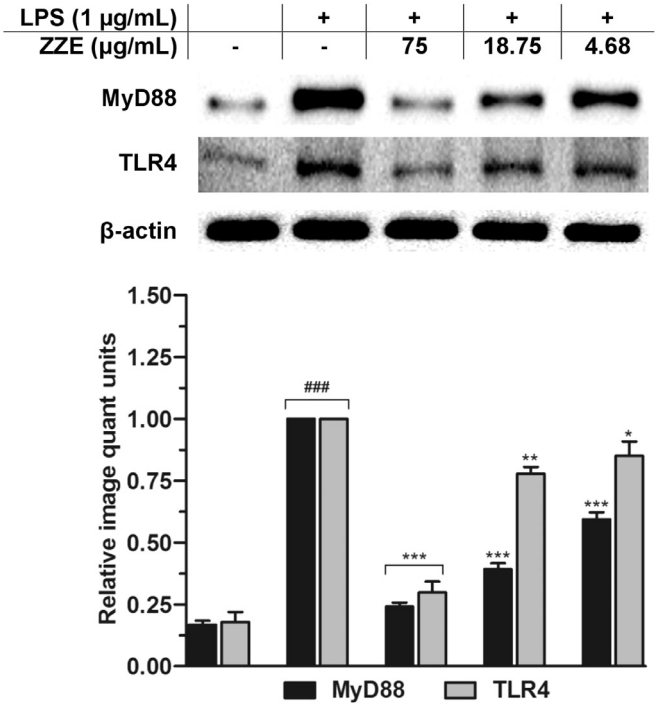

Fig. 9. Effects of *Zingiber zerumbet* extract (ZZE) on MyD88 and TLR4 expression. Respective Western blots presenting the effects of ZZE on MyD88 and TLR4 expression in LPS-activated U937 macrophages. Data are expressed as mean ± SEM ($n = 3$). $^{###}P < 0.001$ denotes the significant difference from the control. $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$ denote significance to the LPS alone versus ZZE pre-treated.

revealed concentration-dependent suppression of these markers production and mRNA expression where the suppressive effects were found comparable to that of the anti-inflammatory agent DEX. And interestingly, the inhibitory effect of *Z. zerumbet* extract on TNF-α from the investigation was in line with the investigation lead by Chaung et al. (2008) where the aqueous extract of *Z. zerumbet* extract inhibited the production of TNF-α concentration-dependently in the mouse macrophages (Chaung et al., 2008). It is to be noted that from our experiment NO could not be detected at quantifiable level in the LPS-primed U937 macrophages which is in agreement with earlier report postulated by Tham et al. (2010). The probable cause for this occurrence may be due to the fact that deficiency of tetrahydrobiopterin (BH_4) in U937 cells which is a vital cofactor for NO production (Bertholet et al., 1999; Tham et al., 2010). However, *Z. zerumbet* extract without LPS treatment could not show any significant effect on the release and mRNA expression of the respective markers and the effects found were similar to untreated control. Therefore, treatments with *Z. zerumbet* extract alone had been excluded from further studies.

As consequence of the initial investigations, the inhibitory effects of *Z. zerumbet* extract were investigated on the NF-κB signaling pathway, the most domineering pathway during inflammatory conditions. NF-κB is becoming as a major drug target to heal a large extent of diseases due to its fundamental role in various pathological conditions (Lawrence, 2009; Muriel, 2009; Roman-Blas and Jimenez, 2006). In the main, NF-κB is the decisive pathway for production and expression of major pro-inflammatory markers in LPS-induced macrophages (Haque et al., 2018a; Haque et al., 2018b; Zhou et al., 2018). Normally, the actions of NF-κB are strictly governed in a cell by several inhibitors of NF-κB namely, I kappa B (IκB) proteins (e.g., IκBα). These IκB proteins are regulated discretely by means of phosphorylation and proteolysis (Haque et al., 2018b). In the latent cells, the NF-κB dimer (p65/p50) typically resides in the cytoplasm where it binds to the inhibitory IκB protein (Lu et al., 2017). In response to the pro-inflammatory stimuli like LPS, the IκB protein promptly phosphorylates and degrades by the proteosomal pathway (Harikrishnan et al., 2018; Pasparakis, 2009). Henceforth, the free NF-κB discharges and translocates into the cell's nucleus where it binds to the specific binding region in the promoter locates of targeted genes to excite transcription of the genes for mediating copious immune mediators together with respective pro-inflammatory markers (Appleby et al., 1994; Lin et al., 1996). Adding too, the previous investigations had been proved that deterring the excessive gene activity of NF-κB in cell nuclei can downregulate the release of enzymes and pro-inflammatory markers involved during inflammatory processes (Moon et al., 2007). Thus, the suppression of the persistent or overactivation of NF-κB pathway might prime to develop potential anti-inflammatory and immunosuppressive drug candidates to treat various inflammatory and autoimmune diseases such as arthritis, eczema, sclerosis, lupus, psoriasis, inflammatory bowel diseases and so on. The present investigation revealed that pre-treatment with *Z. zerumbet* extract for 2 h considerably inhibited the phosphorylation of IKKα/β and p65 as well as inhibited the IκBα phosphorylation and degradation.

Besides NF-κB, the MAPKs and PI3K-Akt signaling are considered to perform a critical role during several biomolecular progressions. Phosphorylation of MAPKs (JNK1/2, ERK1/2, and p38) and Akt implement key role during the macrophage activation by prompting the regulation of NF-κB signaling events (Haque et al., 2018a; Haque et al., 2018b). Therefore, the present investigation further focused to determine the effects of the *Z. zerumbet* extract on the phosphorylation of LPS-activated MAPKs and Akt. The consequences shown that 30 min induction with LPS prompted the respective MAPKs i.e., JNK1/2, ERK1/2, and p38 phosphorylation, which were expressively blocked by 2 h of *Z. zerumbet* extract pre-treatment in a concentration-dependent manner without prying the total level of respective protein kinases. The inhibition upon all treated doses of *Z. zerumbet* extract was highly significant in case of the MAPKs phosphorylation. Additionally, the 30 min LPS stimulation gave significant augmentation of Akt phosphorylation levels in the U937 macrophages. Treatment with the *Z. zerumbet* extract for 2 h inhibited the phosphorylated Akt in a concentration-dependent manner without meddling the total level. These findings correlate our aforementioned findings where *Z. zerumbet* extract suppressed NF-κB pathways followed by the inhibition of the release and mRNA expression of respective pro-inflammatory markers. Analogously, the findings furthermore reconfirm the outcomes from the study with selective inhibitors where the pretreatment with the respective inhibitors inhibits significantly the activation of NF-κB, MAPKs and Akt signaling pathways.

As these signaling events are the resultant of upstream signaling events like TLR4 and MyD88 activation, we further focused to investigate whether *Z. zerumbet* extract suppressed these adopter molecules. These adopter molecules are the critical upstream signalling molecules, which play vital role in mediating both innate and adaptive immunity. Upon activation, TLR4 mediates several signaling events via conscription of MyD88 (Haque et al., 2018b). Our study exposed that pre-treatment with *Z. zerumbet* extract prevented the activation of TLR4 and MyD88, which is found in consistent with the effects of our above investigation. On this viewpoint, from this mechanistic investigation, it is recommended that *Z. zerumbet* extract might mitigate the activation of NF-κB, MAPKs and PI3K-Akt signaling through suppression of MyD88-dependent signaling events and hereafter, possessed the potent immunosuppressive properties, and can be preventive and develop into a potential immunosuppressive therapeutic candidate for the treatment of various inflammatory and other related immune disorders.

In our previous study we also reported that zerumbone isolated from *Z. zerumbet* extract suppressed these MyD88-dependent signaling events in macrophages (Haque et al., 2018a). In comparison, our recent investigation revealed that the *Z. zerumbet* extract was more potent, varying in the effects in suppressing these respective pathways. As from the quantitative and qualitative analysis, it was revealed that the 80% ethanol extract of *Z. zerumbet* rhizomes was rich in terpenoids, flavonoids, and kaempferol glycosides, hence, it let to conclude that the significant effects of the plant might be due to the synergism of these several bioactive metabolites.

Conclusion

In summary, the findings demonstrated that standardized extract *Z. zerumbet* rhizomes inhibited the pro-inflammatory markers generation and mRNA transcription followed by the attenuation of MyD88-dependent NF-κB, MAPKs, and PI3K-Akt signaling pathways in LPS-induced U937 macrophages (**Fig. 10**). The results suggest that *Z. zerumbet* extract can be developed into potent inhibitors of inflammatory-mediated signaling pathways in human macrophages. As the extract found rich in several active chemical markers, future research should focus on extensive phytochemical studies to isolate and identify the secondary metabolites followed by appropriate screening for identifying bioactive compounds as novel immunomodulators. More mechanistic investigations taking into account additional signaling pathways and molecular markers on the analogous and/or the other cellular models like human PBMC-derived macrophages or THP-1 cell line is recommended. Additionally, for further confirmation, investigation on the precise effects of *Z. zerumbet* extract is necessary to elucidate more detailed anti-inflammatory mechanism on the TLR4 and MyD88 signaling molecules specially, on the dimerization of TLR4 that might subsequently block the inflammatory signaling cascade molecular mechanisms responsible for the anti-inflammatory effects of *Z. zerumbet* extract. Besides, prospective pharmacokinetic investigations followed by extensive *in vivo* studies and clinical trials have to be tracked to headway the plant into potent immunosuppressive drug candidate to treat inflammatory and other associated immune diseases.

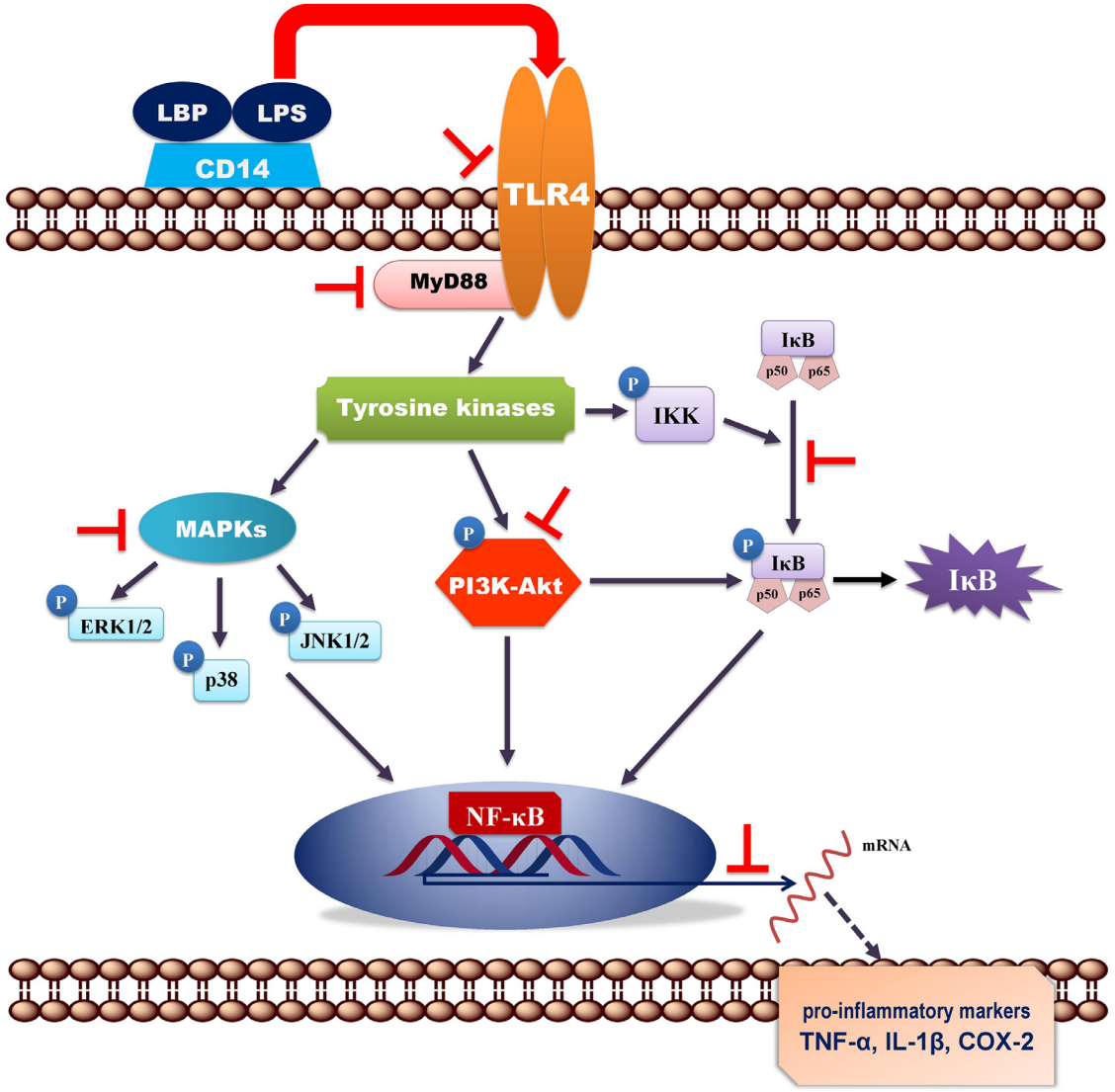

Fig. 10. Graphic representation of the proposed mechanisms of *Zingiber zerumbet* extract in the suppression () of LPS-induced pro-inflammatory responses in human macrophages.

Acknowledgments

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Conflict of interest

The authors have no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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