

Hepatoprotective Effect of Zingerone [4-(4-Hydroxy-3-Methoxyphenyl) Butan-2-One] in Lipopolysaccharide Induced Liver Injury Mouse Model through Downregulation of Inflammatory Mediators

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Abstract

Lipopolysaccharide (LPS) is responsible for causing inflammation leading to septic shock or organ failure. During treatment of infection, high amount of LPS is released in blood circulation due to immediate lysis of bacteria. Since liver helps in clearing LPS from the body, hence it remains the primary target to be stimulated by LPS releasing vigorous amount of inflammatory molecules leading to liver injury. Available anti-inflammatory chemotherapy fails in such situation because it relies predominantly on specific or non specific inhibitors of cyclooxygenase enzyme activity (COX-2) with broad range of hepatic, renal and cardiovascular side effects. There is a need to replace the potential therapies targeting suppression of LPS induced inflammation with those having no or minimal side effects. Active components from dietary medicinal plants like ginger (*Zingiber officinale*) are potential source of non toxic antioxidant as well as anti-inflammatory molecules. In the present study, protective effect of zingerone was evaluated against inflammation induced by LPS in terms of liver histology, serum endotoxin levels, liver function markers (AST, ALT, ALP) and inflammatory mediators (malondialdehyde, reactive nitrogen intermediates, myeloperoxidase). Relative mRNA expression of LPS induced inflammatory markers TLR4, TNF- and iNOS was also evaluated in zingerone treated and untreated groups. Hepatoprotective effect of zingerone was observed leading to significant improvement in liver histology, decreased levels of serum endotoxin levels, liver function markers and MPO, MDA, RNI. It also showed significant down regulation of mRNA expression of TLR4, TNF- and iNOS suggesting potent anti-inflammatory activity of zingerone against *P.aeruginosa* LPS induced liver injury.

Key words: Zingerone, *Pseudomonas aeruginosa*, lipopolysaccharide, Inflammation, Phytochemical, liver injury.

INTRODUCTION

Lipopolysaccharide (LPS), a glycolipid present in the outer membrane of *Pseudomonas aeruginosa*, is responsible for stimulating vigorous inflammatory response. LPS activates immune system and promotes cell proliferation and inflammation (Wagner *et al.*, 2008). LPS induced inflammation plays a very important role in *P. aeruginosa* mediated infections (Le *et al.*, 2012; McIsaac *et al.*, 2012; Davis Jr *et al.*, 2013). *P.aeruginosa*, a gram negative pathogen, is frequently associated with hospital acquired infections. Inflammation can disturb normal cellular function and this can lead to multiple organ dysfunction or lethal septic shock (Marshall *et al.*, 2001; Shen *et al.*, 2004) with a high mortality rate of 40~60% in intensive care units (Davies *et al.*, 1997). Although LPS of *P.aeruginosa* is less cytotoxic than that of *Escherichia coli*, but it induces significant lung inflammation in cystic fibrosis and diffuse panbronchiolitis demonstrating different immunomodulatory properties as compared to LPS of normal microflora of gut (Koyama *et al.*, 2000). Liver plays a central role by virtue of its dual ability. It not only clears LPS but also responds energetically to LPS by

producing various cytokines, hence acting as a primary target organ for LPS induced inflammation [8, 9]. Both *ex vivo* and *in vitro* studies with isolated liver slice models and liver perfusion have shown that tumour necrosis factor alpha (TNF-) and interleukin (IL)-1 are released in response to LPS by Kupffer cells which lead to septic shock (Luster *et al.*, 1994). After binding with LBP (LPS binding protein), LPS-induced cell activation depends on the presence of 3 proteins: CD14, toll-like receptor (TLR) 4 and MD2 comprising the LPS receptor complex (Da Silva Correia *et al.*, 2001) which further activates the NF- B family dependent gene expression. LPS binds with

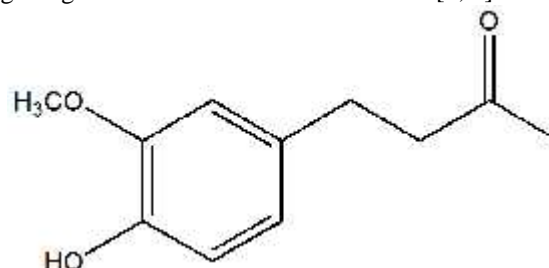


Fig.1: Chemical structure of zingerone.

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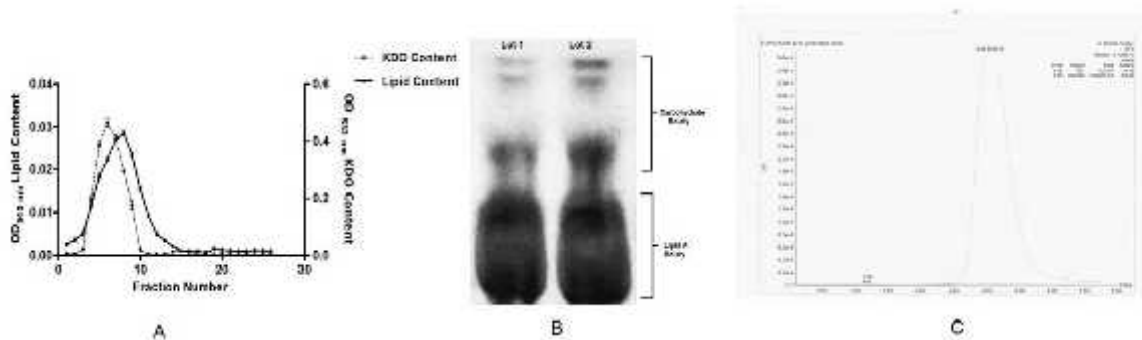


Fig.2: (A) KDO and Lipid content of fraction collected from G-100 column (B) Photograph showing purified LPS in polyacrylamide gel stained with silver stain (C) HPLC profile of purified LPS.

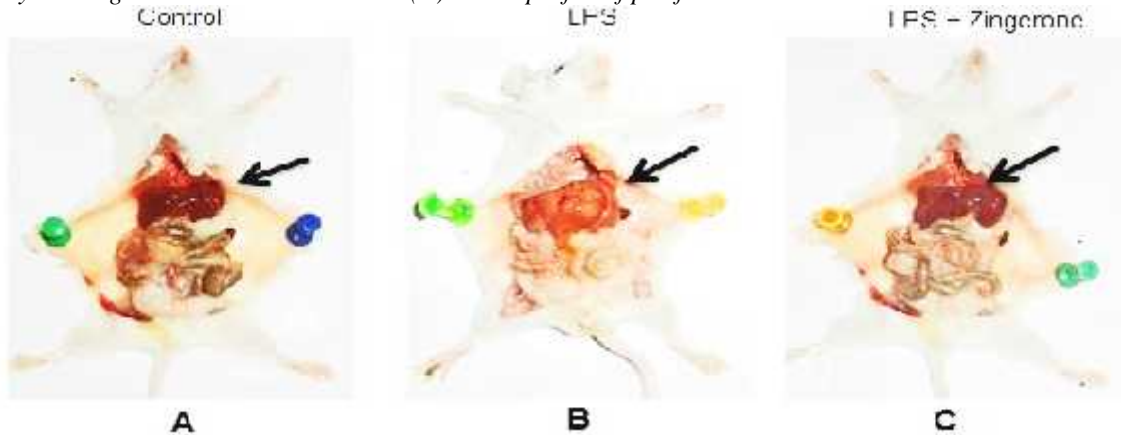


Fig.3: Mice showing liver inflammation (a) control group (b) LPS group (c) zingerone treatment group.

TLR4 receptor which is highly expressed in cells that respond to LPS, such as macrophages, monocytes, hepatocytes and kupffer cells and induce expression of inflammatory genes through TLR4/NF- κ B signaling pathway.

Most of the anti-inflammatory drugs are specific or non specific inhibitors of cyclooxygenase enzyme activity (COX-2) with broad side effects on cardiovascular, gastrointestinal and circulatory system. Therefore, therapy targeting TLR4/NF- κ B signaling with no side effects might provide a modern hope for the suppression of LPS induced inflammation. Herbal medicinal plant like ginger (*Zingiber officinale*) is a natural dietary ingredient and spice with antioxidative, anti-inflammatory and anticarcinogenic activities (Park *et al.*, 1998). Zingerone [4-(4-hydroxy-3-methoxyphenyl) butan-2-one] (Fig.1) is a major component of dry ginger root (Sies *et al.*, 1997) and has been found to downregulate age related activation of proinflammatory enzymes (Kim *et al.*, 2010); protect human lymphocytes from radiation induced genetic damage and apoptosis (Rao *et al.*, 2011). Previously, our study reported direct effect of zingerone on the physiology and biofilm formation of *Pseudomonas aeruginosa* PAO1 (Kumar *et al.*, 2013). To the best of our knowledge few much studies regarding protective effect of zingerone against LPS induced liver injury are available in literature, Keeping this in mind, aim of the present study was to assess hepatoprotective effect of zingerone in LPS induced liver injury mouse model in terms of liver histology, serum endotoxin levels, liver function markers (AST, ALT, ALP), biochemical markers of inflammation; malondialdehyde (MDA), myeloperoxidase (MPO)

nitrogen intermediates (RNI) and mRNA expression of LPS induced inflammatory markers TLR4, TNF- and iNOS.

MATERIALS AND METHODS

Bacterial strain: Standard strain *Pseudomonas aeruginosa* PAO1 was obtained from Dr. Barbara H. Iglewski, Department of Microbiology and Immunology, University of Rochester, New York, USA and was maintained as nutrient agar stubs kept at 4°C.

Drugs and chemicals

Pure zingerone [4-(4-hydroxy-3-methoxyphenyl) butan-2-one] was obtained from Gogia Chemical Industries, India. All other reagents and chemicals used were of analytical grade.

Extraction and Purification of LPS: Hot water phenol extraction procedure of Westphal and Jann (Westphal *et al.*, 1965) as modified by Morrison and Leive (Morrison *et al.*, 1975) was used to extract LPS from *P. aeruginosa* PAO1. Crude LPS was purified by sephadex G-100 column using tris-HCl buffer (pH 8.4) and the fractions giving high KDO, carbohydrate and lipid content were ultracentrifuged at 1,00,000 x g. Gel like pellet obtained was lyophilized and stored at 4°C. Extraction was done in two lots under similar conditions and finally Lot-1 and Lot-2 were pooled together.

Characterization of *P.aeruginosa* LPS

KDO Estimation: KDO (2-keto-3-deoxyoctanate) content was estimated according to the method of Waravdekar and Saslaw (Waravdekar *et al.*, 1959). Briefly, H₂SO₄, periodic acid and thiobarbituric acid (TBA) was added to the sample and O.D. was measured at 532 nm.

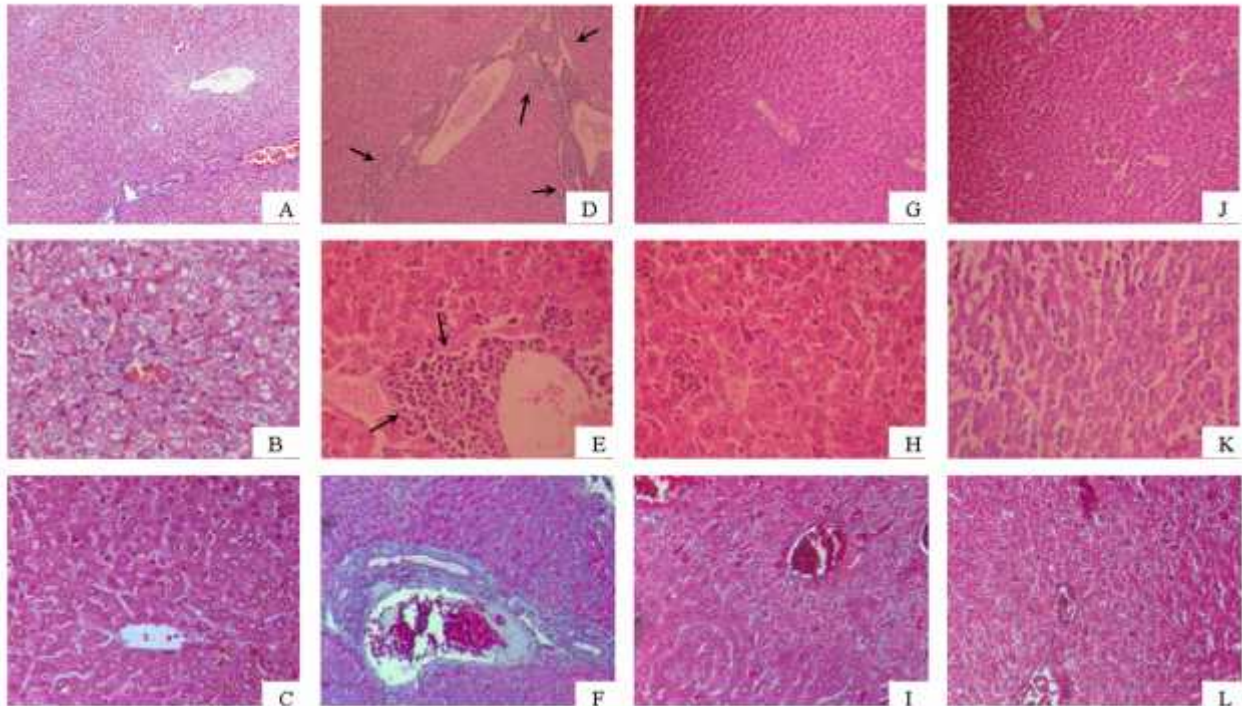


Fig.4: Liver tissue in LPS treated animals showed high infiltration of neutrophilic granulocytes, necrosis of hepatocyte and hepatic portal inflammatory bridge formation (marker of high inflammation) along with hepatic portal haemorrhage and liver tissue fibrosis was observed (Fig.4 D, E, F). Pre-treatment (Fig.4 G, H, I) as well as post treatment (Fig.4 J, K, L) of zingerone significantly protected mice from LPS induced liver injury and liver tissue appeared to be normal as was observed in control groups (normal saline, zingerone alone (Fig.4 A, B, C).

Table1: Hepatoprotective effect of Zingerone on liver function markers (AST, ALT and ALP) in the serum of LPS induced BALB/c mice (*P < 0.05).

Groups	AST(IU/L)	ALT(IU/L)	ALP(IU/L)
Control	57.71 ± 3.55	57.77 ± 3.57	107.18 ± 7.14
Zingerone	53.99 ± 1.92	53.86 ± 2.46	95.23 ± 7.04
LPS	172.12 ± 4.91	193.14 ± 4.49	214.26 ± 8.40
Pre treatment	120.91 ± 7.94*	127.76 ± 10.23*	161.96 ± 9.51*
Post treatment	116.82 ± 6.36*	125.26 ± 5.73*	170.83 ± 7.75*

Lipid Estimation: Total lipid content was estimated according to the method of Frings and Dunn (Frings *et al.*, 1970). Concentrated H₂SO₄ was added to lyophilized LPS preparation and mixed with vanillin reagent. Absorbance was measured at 540 nm.

SDS-PAGE and modified silver staining: SDS-PAGE was performed according to the method of Laemmli (Laemmli, 1970). LPS preparations were boiled for 5 min at 100°C in 0.05 M tris-HCl buffer (pH 6.8) containing 2% (wt/vol) SDS, 10% (wt/vol) sucrose, 0.01% bromphenol blue, and fractionated on SDS-polyacrylamide gel containing 4% and 12.5% acrylamide in the stacking and separating gels, respectively. SDS-PAGE-fractionated LPS preparations were stained by silver staining method of Tsai *et al.* (Tsai *et al.*, 1997) modified by Fomsgaard *et al.* (Fomsgaard *et al.*, 1990).

Liquid chromatography of purified LPS: Liquid chromatographic separations were carried out using Waters Alliance 2795 system equipped with PDA detector (USA). The method was optimized at 0.15 ml/min flow rate and 210 nm wavelength UV detection. Separation was carried out with Xtera C-18 column (2.1 X 100mm 5µm) (Waters, USA). Mixture of water and acetonitrile (90:10) was used as mobile phase.

Evaluation of protective effect of zingerone in mouse model for LPS induced liver injury

Experimental design: Animal experiments were strictly carried out in accordance with the guidelines provided by the Council for the Purpose of Control and Safety of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. Animal

Table 2: List of primer sequence for TLR4, TNF- and iNOS genes.

GENES	LEFT PRIMER	RIGHT PRIMER	PCR Product Size (bp)
TLR4	5'-GCTTTCACCTCTGCCTTCAC-3'	5'-TGCCGTTTCTTGTCTTCTCCT-3'	395
TNF- α	5'-TATGGCTCAGGGTCCAAC-3'	5'-AAGCAAAAGAGGAGGCAACA-3'	495
iNOS	5'-AGACCTCAACAGAGCCCTCA-3'	5'-GAACCTCCAGGCACACAGTT-3'	263
GAPDH	5'-AACTTTGGCATTGTGGAAGG-3'	5'-GGATGCAGGGATGATGTTCT-3'	132

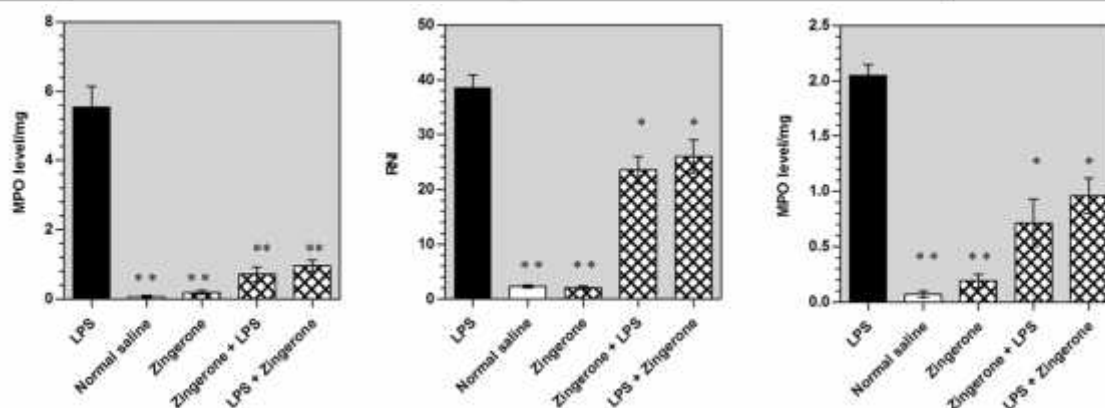


Figure 5

ethical clearance was taken from Panjab University Ethical Committee (PUEC) for conducting these experiments.

BALB/c mice of either sex (8–10 week-old; 30–32 g) were procured from Central Animal House Panjab University Chandigarh. Animals were allowed free access to food and water at all times and were maintained in a controlled temperature (20–25°C) and humid (50±5%) environment. Group of 6 mice in duplicate were used. For inducing liver injury, dose of LPS (1mg/kg body wt.) was standardized in BALB/c mouse

model (data not shown). Zingerone dose selected was 100 mg/kg approximately corresponds to 1/10th of LD₅₀ (Rao *et al.*, 2009). To evaluate the protective effect zingerone was selected which showed no interference with normal liver function (AST, ALT, ALP) in BALB/c mice. All mice except controls received an intraperitoneal (1mg/kg body wt) injection of LPS on each day from day 1 to 6. The experiment was carried out in two different modes. In mode A, the mice were pre-treated with a single dose of zingerone intraperitoneally (100mg/kg body wt.) 30 min before LPS administration. In mode B, the mice were administrated with a single dose of zingerone 30 min after LPS treatment. Zingerone, LPS and saline treated groups served as control groups. All mice were sacrificed on 7th day, liver was removed aseptically and analyzed for histopathological and biochemical markers of inflammation.

Histopathological examination: Liver tissue were fixed in 10% buffered normal saline and dehydrated in 30-100% gradient ethanol. Paraffin wax blocks were prepared and each 5 μ thin sections were stained with hematoxylin-eosin and Masson's trichrome stain. Liver sections were examined for inflammatory response and liver tissue fibrosis.

Serum Endotoxin test: LAL Endotoxin Assay Kit (GenScript USA Inc.) was used for the detection of endotoxin level in serum samples. Briefly, 0.1 ml plasma was incubated with 0.1 ml Limulus ameobocyte lysate (LAL) at 37°C. Absorbance was measured at 545 nm spectrophotometrically. The serum endotoxin levels were calculated against a standard curve of pure endotoxin of *E. coli* as per manufacturer's instructions.

Liver function tests: Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) enzyme activities in serum were determined using ERBA test kits (ERBA Diagnostics, Mannheim, Germany).

Biochemical analysis of liver homogenate for inflammatory mediators

Preparation of tissue homogenate: Liver tissue was harvested, washed in ice cold physiological saline and homogenized in phosphate buffer saline using glass homogenizer to obtain 10% homogenate. The tissue homogenate was centrifuged at 12,000 X g for 10 minutes at 4°C and the supernatant was collected.

Malondialdehyde (MDA) estimation: Induction of pathology was evaluated on the basis of Malondialdehyde, the index of lipid peroxidation as per previous method of Anjaneyulu and Chopra (Anjaneyulu *et al.*, 2004). Briefly, tissue homogenate was added to tris HCl followed by the addition of ice-cold trichloroacetic acid. Supernatant was taken and mixed with thiobarbituric acid. Tubes were covered and kept in a boiling water bath for 10 min. After cooling, absorbance was read at 532 nm. The level of lipid peroxide was expressed as nmoles of MDA formed/mg of protein.

Reactive nitrogen intermediates (RNI) estimation : Nitrite was estimated in the liver tissue of mice following the

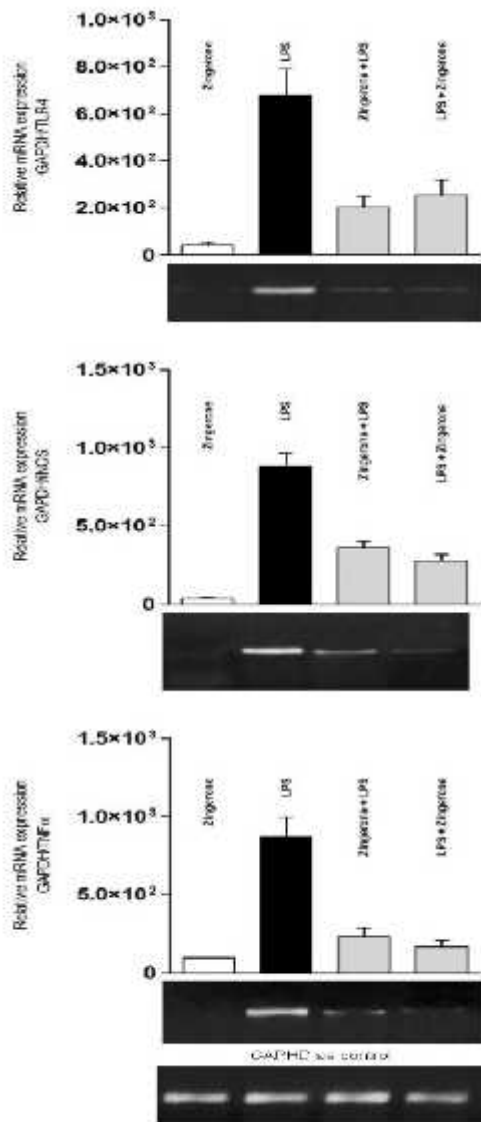


Fig.6: Relative mRNA expression of TLR4, TNF- α and iNOS (GAPDH as control) in pre and post treated groups after LPS administration as compared to LPS and zingerone control groups.

method of Rockett *et al.*, (Rockett *et al.*, 1994) briefly, samples were mixed with Griess reagent (Sigma Aldrich Chemicals Ltd., St Louis, MO, USA) followed by addition of trichloroacetic acid and incubated for two minutes at room temperature. After centrifugation, the optical density of supernatant was read at 540 nm.

Myeloperoxidase (MPO) estimation: MPO activity was quantified using the myeloperoxidase assay by the method of Hang *et al.*, (Hang *et al.*, 1999). Briefly, tissue was homogenized in potassium phosphate buffer with hexadecyl trimethyl ammonium bromide and EDTA. The homogenate was sonicated and centrifuged. Supernatant was mixed with o-dianisidine and absorbance was read at 490 nm at 0 min, 1 min, 2 min at room temperature to determine change in absorbance per minute. It has been calculated by using the formula: MPO activity (U/mg) = X/ weight of the tissue taken, where X = 10 X change in absorbance per min/volume of supernatant taken in the final concentration.

Reverse transcription-polymerase chain reaction (RT-PCR): Nucleotide sequence for genes was taken from NCBI data base. For each gene, primers were designed using Primer 3 online tool. Primer sequences used for PCR amplification of cDNA are mentioned in Table 2. Liver tissue was homogenized with Trizol Reagent (Invitrogen). Homogenate was centrifuged at 3000 g at 4 $^{\circ}$ C for 10 min. Supernatant was mixed with chloroform and centrifuged again at 15,000 g at 4 $^{\circ}$ C for 15 min. The second supernatant (RNA) was mixed with isopropanol and centrifuged at 15,000 g at 4 $^{\circ}$ C for 10 min. After precipitating the samples with 75% ethanol, the total amount of RNA was determined using the spectrophotometric analyzer (Nano Drop 100-Thermo Scientific USA). RNA was reverse-transcribed into cDNA using a First-Strand cDNA Synthesis kit (Fermentas) with oligo (dT) primer. The cDNA was amplified with specific primers for TLR-4, TNF- α , iNOS and GAPDH as a control. The primers were designed by using Primer 3.0 software tool (Table 2). The amplification buffer consisted of a Taq DNA polymerase and 10 mM dNTP Mix (Fermentas). An oligonucleotide primer (0.5 mM) and the cDNA samples were added to the buffer, and the sample (20 μ l) was incubated using a MJ Minin PCR Thermal Cycler (BioRad). Thermal cycles were run 34 times. The cycles lasted for 30 S at 94 $^{\circ}$ C, for 60 s at 68 $^{\circ}$ C for iNOS, TLR4 and TNF- α . For GAPDH the cycles lasted for 30 S at 94 $^{\circ}$ C, for 60 S at 55 $^{\circ}$ C and the final incubation was at 72 $^{\circ}$ C for 5 min. Amplified PCR products were separated electrophoretically on a 1.0 % agarose gel, and bands were visualized with ethidium bromide under ultraviolet transillumination. Densitometry of PCR product was performed by Gel Doc Multi-Analyst (BioRad).

Statistical analysis: All experiments were performed in duplicate and repeated on different days. The effect of zingerone treatment on LPS induced inflammation in different groups as compared to control was evaluated using one-way ANOVA test. P values were calculated and P < 0.05 was considered significant. Data was analyzed using Graph Prism 5.0 software. Values were expressed as mean + S.E.M.

RESULTS

Extraction purification and characterization of LPS: LPS was extracted by phenol extraction method and it was further purified by enzymatic digestion, ultracentrifugation and G-100 column chromatography. Fractions (4.0 to 10.0) giving high lipid and KDO content (Fig.2 A) were pooled together. High lipid-A and o-polysaccharide moiety was observed on SDS PAGE (Fig.2 B), suggesting that PAO1 is a smooth strain possessing complete LPS molecule. HPLC analysis revealed high purity of extracted LPS of *P.aeruginosa* (Fig. 2 C).

Evaluation of protective effect of zingerone

Zingerone protected liver tissue injury: Macroscopically liver of LPS induced animals was inflamed Fig 3 (B) as compared to liver of normal saline (Fig.3 A) and zingerone treated animals (Fig. 3 C).

Liver Histology: Histological analysis of liver tissue in LPS induced animals showed high infiltration of

neutrophilic granulocytes, necrosis of hepatocyte and hepatic portal inflammatory bridge formation (potential marker of high inflammation) along with hepatic portal haemorrhage and liver tissue fibrosis (Fig.4 D, E, F). Pre-treatment (Fig.4 G, H, I) as well as post treatment (Fig.4 J, K, L) of zingerone significantly protected mice from LPS induced liver injury. Liver tissue appeared normal as compared to that of control groups (Fig.4 A, B, C) indicating protection by zingerone treatment against LPS induced liver injury.

Zingerone reduced serum endotoxin levels: Low serum endotoxin level in normal saline group ($0.085 + 0.02$ EU/ml) and zingerone control ($0.076 + 0.03$ EU/ml) were observed. The endotoxin level in the LPS administered groups was significantly higher ($0.402 + 0.04$ EU/ml) but pre and post treatment of zingerone showed significant reduction in serum endotoxin levels ($0.274 + 0.05$ and $0.262 + 0.07$).

Zingerone reduced elevated AST, ALT and ALP levels: Normal saline and zingerone control groups showed normal serum AST, ALT and ALP level (Table. 1). Significant increase in serum levels of liver function markers was observed in LPS induced groups. Pre and post zingerone treatment could significantly decrease the liver function markers.

Zingerone suppressed release of LPS induced inflammatory mediators

Malondialdehyde (MDA) : Liver homogenate of LPS induced animals showed very high lipid peroxidation. MDA content was $74.39 + 3.90$ nmoles/mg in the liver homogenate (Fig.5 A). Zingerone + LPS group ($32.89 + 8.65$ nmoles/mg) and LPS + Zingerone ($36.65 + 5.88$ nmoles/mg) group showed significantly ($p < 0.05$) low level of MDA content in liver as compared to LPS induced animals. Statistically there was no difference ($p > 0.05$) between Zingerone + LPS group and LPS + Zingerone treated groups. Saline treated ($2.70 + 0.59$ nmoles/mg) and only zingerone treated ($8.10 + 2.37$ nmoles/mg) group showed significantly less ($p < 0.001$) amount of MDA in the liver homogenates (Fig.5 -A).

Reactive nitrogen intermediates (RNI): RNI (Reactive nitrogen intermediate) level was also estimated and result showed very high NO content ($38.7 + 3.11$ nmoles/mg) in the liver homogenate of LPS induced animals (Fig.5-B). Zingerone + LPS group ($23.25 + 3.53$ nmoles/mg) and LPS + Zingerone group ($26.0 + 4.24$ nmoles/mg) showed significant reduction ($p < 0.05$) in RNI content in liver than that of only LPS induced animals. Negligible amount of RNI was observed in liver homogenate of saline treated ($2.35 + 0.35$ nmoles/mg) and zingerone treated ($2.1 + 0.42$ nmoles/mg) groups (Fig. 5 B).

Myeloperoxidase assay (MPO): Neutrophil infiltration marker (MPO) was also estimated in liver homogenates. LPS induced animals had very high MPO content ($5.55 + 0.60$ U/mg) (Fig. 5 C). While pre zingerone ($0.71 + 0.30$ U/mg) and post zingerone treatment ($0.96 + 0.22$ U/mg) showed significantly ($p < 0.05$) low level of MPO in liver. Saline treated and zingerone treated groups showed negligible amount of MPO in the liver homogenate (Fig. 5 C).

Zingerone down regulated relative mRNA expression of inflammatory markers (TLR4, TNF- and iNOS): LPS administration caused potential increase in TLR4/NF- B dependent expression of genes. TLR4, TNF- and iNOS mRNA expression levels were significantly enhanced as compared to control groups. Zingerone treatment significantly down regulated the mRNA expression levels of all the genes. Zingerone treated mice showed > 2 fold decrease in TLR4 and TNF- mRNA expression as compared to liver of LPS administered mice ($p < 0.05$). Relative expression of mRNA of iNOS was also down regulated by > 1.5 fold. Results showed that pre as well as post zingerone treatment significantly reduced the expression of these inflammatory markers.

DISCUSSION

LPS is mainly responsible for toxicity and enhanced production of inflammatory mediators during *P.aeruginosa* infection via TLR4 mediated signalling pathway (Raetz *et al.*, 1991). Hydrophobic Lipid A component of LPS allows it to anchor in the outer membrane of *P. aeruginosa*. During infection and even during antibiotic treatment liver becomes the primary target organ for LPS induced stimulation leading to liver injury. LPS-induced liver injury has been used as an experimental model to analyze the mechanism of endotoxin-induced liver inflammation using *E.coli* LPS (Galanos *et al.*, 1979; Kato *et al.*, 1995) Latest studies have demonstrated that LPS of pathogenic organism has different immunostimulatory potential as compared to LPS of normal microflora of gut (Koyama *et al.*, 2000). LC-MS (Liquid chromatographic-massspectrometry) spectra also revealed distinct chemical constituents of *E.coli* and *P.aeruginosa* LPS supporting their unique immunostimulatory activity (Kato *et al.*, 1995). However, immunostimulatory mechanism of *P. aeruginosa* LPS is still not well understood. Smooth strains producing high LPS content are known to be responsible for the induction of infection with high inflammation leading to systemic shock, liver, kidney, lung or multiple organ failure (Priebe *et al.*, 2004). In the present study, lipid characterization and profiling with SDS-polyacrylamide gel electrophoresis of purified LPS preparation revealed high lipid A component with carbohydrate moiety suggesting complete smooth type LPS molecule. LPS induced liver injury mouse model was established using purified LPS of *P. aeruginosa* PAO1.

Anti-inflammatory drugs have serious side effects on liver, kidney and cardiovascular system. Some of the phytochemicals are known to have potential anti-inflammatory activity without any side effects. Recently, herbal medicines have received great attention as an alternate conventional therapy against liver toxicity. Some studies have shown that dry ginger extract and active components from ginger i.e. gingerols and shogaols significantly reduced liver inflammation and inhibit LPS induced liver injury (Ahn *et al.*, 2009; Choi *et al.*, 2013). In our study, LPS induced mice showed severe liver injury with high infiltration of neutrophilic granulocytes, indistinct boundaries between nucleus and cytoplasm of

liver cells, hepatic portal inflammatory bridge formation, haemorrhage and hepatocyte necrosis. Biochemical analysis of liver homogenate indicated elevated levels of MDA, MPO and RNI. Lipid peroxidation is one of the best marker of tissue injury induced by LPS which causes the oxidative degradation of lipids and is also indicative of inflammatory injury and tissue damage. Elevated MDA levels observed, indicate oxidative damage via free radical damage of lipids in cell membranes resulting in cell death and tissue injury. Further products of lipid peroxidation may lead to alteration in the biological membrane structure resulting in serious cellular injury. Pathological changes may also be related to reactive nitrogen intermediates (RNI) which are potential source of free radical damage. Since neutrophils are major effector cells in the liver tissue damage and are important source of free radicals (Sener *et al.*, 2003), myeloperoxidase activity has been used as a marker of local and systemic inflammation (De Francischi *et al.*, 2009) relating to not only host response but also to tissue destruction. Enhanced MPO activity observed may have contributed to hepatocyte necrosis, elevated serum AST, ALT and ALP levels favouring liver tissue necrosis and hepatic inflammation.

In the present study, zingerone treatment could prevent tissue destruction since zingerone treated liver tissues showed no hepatic portal bridge formation and less neutrophilic granulocyte infiltration. Hepatocyte necrosis and haemorrhage was also found to be reduced in liver tissue. This was further supported by low levels MDA, RNI and MPO in zingerone treated animals were found to be quite low. Anti-inflammatory activity of zingerone could be attributed to phenolic nature of zingerone which might have lead to scavenging of free radicals (Kim *et al.*, 2010) and protection of liver cells from oxidative damage. Methoxy group with phenolic hydroxyl group in zingerone facilitate proton release and long chain ethyl methyl ketone group which play an important role in bulk stabilization of the molecule (Rao *et al.*, 2011). This may lead to cell penetration and scavenging of free radicals. Decreased inflammatory mediators also brought significant reduction in the serum AST, ALT and ALP levels indicating improved functioning of liver.

P.aeruginosa LPS significantly enhanced mRNA expression of TLR4 receptor. Due to increased number of TLR4 receptors on the liver cell surface, excessive binding of LPS to the cells and more potent induction of inflammatory response takes place. Zingerone significantly reduced the mRNA expression of TLR4 receptor resulting in less binding with LPS with cells leading to decreased inflammatory response. Role of LPS in activating TLR4 in liver injury has been demonstrated in TLR4 mutant C3H/HeJ mice. Mice (C3H/HeJ) with mutations in toll-like receptor 4 (TLR4) were hyporesponsive to endotoxin and showed less liver injury histologically, lower alanine amino transferase levels, and lower TNF- α mRNA expression than wild-type controls (Uesugi *et al.*, 2001) indicating importance of TLR4. TNF- α is major cytokine which play a crucial role in LPS-induced liver injury leading to hepatotoxicity (Tukov *et al.*,

2007). In the present study, LPS enhanced tremendous increase in TNF- α level in liver tissue indicating that its production is mainly responsible for liver injury. However, Zingerone treated liver cells showed significantly lower levels of TNF- α thereby reducing the levels of hepatotoxicity, tissue inflammation, necrosis and injury. Studies have shown that iNOS expression is enhanced by LPS which leads to generation of nitric oxide radicals causing acute tissue injury (Shen *et al.*, 2007). These radicals disrupted cell membrane and cause necrosis of tissue. Hyper expression of iNOS clearly indicated that oxidative damage to the liver is contributed by iNOS. Zingerone treatment significantly suppressed the mRNA expression of iNOS gene suggesting its potent antioxidant activity. Zingerone has been reported to inhibit monocyte chemoattractant protein-1 release from adipocytes; suppress inflammatory action of macrophages (Woo *et al.*, 2007) and inhibit NF- κ B activation and proinflammatory mediator release (Kim *et al.*, 2010). Hence hepatoprotective effect of zingerone in terms of down regulation of the mRNA expression of TLR4, TNF- α and iNOS may be attributed to Interference with the NF- κ B activation,

Anti-inflammatory effect of zingerone may be pointed to multiple targeted action including free radical scavenging effect due to either hydroxyl group on aromatic ring and/or presence of double bond between C-4 and C-3 in the structure of zingerone (Kabuto *et al.*, 2005), Reduction in binding of LPS to LPS receptor (TLR4) and interference and down regulation of inflammatory signalling cascade (NF- κ B activation) may be another mechanism attributed to zingerone. The present study provides an insight on the impact of zingerone in suppressing inflammatory mediator production, reducing oxidative damage to liver tissue hence protecting liver from LPS induced injury. Results showed for the first time suggested that zingerone could inhibit LPS-induced liver injury protecting liver from potent inflammatory damage.

CONCLUSION

Zingerone is a nontoxic and inexpensive dietary natural compound with potent anti-inflammatory and pharmacological activities having no side effect. It showed hepatoprotective effect against LPS induced liver injury via scavenging of free radicals and down regulating production of inflammatory mediators. This study opens different areas to venture zingerone as potential anti-inflammatory molecule against LPS induced inflammation in *P. aeruginosa* infections.

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