Original Article

Safrole-induced expression of proinflammatory responses is associated with phosphorylation of mitogen-activated protein kinase family and the nuclear factor-κB/inhibitor of κB pathway in macrophages

Yung-Lun Ni[a], Huan-Ting Shen[a,b], Min-Wei Lee[c], Kun-Lin Yeh[d], Chen-Yu Chiang[d], Yu-Hsiang Kuan[e,f]*

[a]Department of Pulmonary Medicine, Taichung Tzu Chi Hospital, Buddhist Tzu Chi Medical Hospital, Taichung, Taiwan, [b]Institute of Biochemistry, Microbiology, and Immunology, Chung Shan Medical University, Taichung, Taiwan, [c]Graduate Institute of Microbiology and Public Health, National Chung Hsing University, Taichung, Taiwan, [d]Department of Veterinary Medicine, National Chung Hsing University, Taichung, Taiwan, [e]Department of Pharmacy, Chung Shan Medical University Hospital, Taichung, Taiwan, [f]Department of Pharmacology, School of Medicine, Chung Shan Medical University, Taichung, Taiwan

Abstract

Objective: Safrole, also called shikimol and Sassafras, is the carcinogenic and phenylpropanoid compound extracted from Sassafras tree and anise, betel, and camphor. Moreover, a high concentration of safrole can be occur in the saliva because of betel nut or areca quid chewing which a common habit observed in Southern and Southeastern Asia. Notably, macrophages are crucial phagocytic cells of the immune system. Nonetheless, to date, no evidence has been reported regarding safrole-induced proinflammatory response and the corresponding mechanism in macrophages. **Materials and Methods:** In the present study, the cytokines expression, NO generation, protein phosphorylation, and expression were assessed by enzyme-linked immunosorbent assay, Griess reagent, and Western blot assay, respectively. **Results:** In this study, we determined that safrole induces the generation of nitric oxide and proinflammatory cytokines, including tumor necrosis factor-α, interleukin-1β, and IL-6 in RAW264.7 macrophages in a concentration-dependent manner. Furthermore, inhibitor of κB (IκB) degradation was caused by safrole in a concentration-dependent manner. In addition, the phosphorylation of nuclear factor (NF)-κB and mitogen-activated protein kinase (MAPK) family, including p38 MAPK, extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase, was induced by safrole began to increase at 10 μM and attained a plateau at 100 μM. **Conclusion:** These results indicated that safrole induces the expression of proinflammatory responses in macrophages through the NF-κB/IκB pathway and its upstream factor, MAPK family phosphorylation.

Keywords: *Macrophage, Mitogen-activated protein kinase family, Nuclear factor-κB pathway, Proinflammatory cytokines, Safrole*

Introduction

Safrole, also called shikimol and Sassafras, is a natural phenylpropanoid compound. Safrole is a colorless oily liquid that can be obtained through natural extraction from Sassafras trees (*Sassafras albidum*) and anise (*Illicium anisatum*), betel (*Piper betle* L.), and camphor (*Ocotea pretiosa*) [1-3]. Safrole and its metabolites, including 1-hydroxysafrole, isosafrole, and dihydrosafrole, are carcinogenic and contribute to the development of several cancers, such as oral, esophageal, and hepatocellular cancers [4-7]. Notably, chewing betel nut or areca quid is a common habit observed in Southern and Southeastern Asian countries, including Taiwan, Vietnam, Malaysia, Thailand, and the Philippines [8]. *P. betle* inflorescence is the common component of betel nut or areca quid and contains safrole at a high concentration of approximately 15 mg/g [9]. A previous study determined that chewing betel nut or areca quid with inflorescence of *P. betle* can result in a safrole concentration of 420 μM in saliva [10].

Macrophages are phagocytic cells of the innate immune system that are ubiquitously located in various human tissues [11]. The major function of macrophages is pathogen defense, which is accomplished through phagocytosis, antigen presentation, and secretion of bactericidal substances, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, and nitric oxide (NO), which are significant proinflammatory mediators [12]. Notably, the proinflammatory mediators generated from macrophages promote tumor growth

***Address for correspondence:**
Prof. Yu-Hsiang Kuan,
Department of Pharmacology, School of Medicine, Chung Shan Medical University, 110, Section 1, Jianguo North Road, Taichung, Taiwan.
E-mail: kuanyh@csmu.edu.tw

and metastasis in the tumor microenvironment [13]. The mitogen-activated protein kinase (MAPK) family, including p38 MAPK, extracellular signal-regulated protein kinase (ERK)-1/2, and c-Jun N-terminal kinase (JNK), mediates crucial signaling responses to generate proinflammatory mediators via the proinflammatory transcription factor and nuclear factor (NF)-κB pathways [13]. Notably, a previous study demonstrated macrophage phagocytosis was induced by safrole in mice and cell models [14,15]. In the recent study, we have purposed toxic effects, which including cytotoxicity, genotoxicity, and apoptosis, induced by safrole via intracellular reactive oxygen species generation and Akt phosphorylation in macrophages [16]. However, there is no evidence to purpose that the mechanism of the proinflammatory effects induced by safrole in macrophages. The present study attempted to assess the potency of safrole as a macrophage stimulator and explored the possible involvement of the MAPK family and NF-κB pathway in proinflammatory responses.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and antibiotic–antimycotic solution were purchased from Thermo Fisher Scientific (Grand Island, NY, USA). Antibodies against phosphorylated and nonphosphorylated forms of ERK, p38 MAPK, JNK, and NF-κB p65 were purchased from Santa Cruz Biotechnology (St Louis, MO, USA). Antibodies of inducible NO synthase (iNOS), the inhibitor of κB (IκB), β-actin, and secondary antibodies were obtained from Santa Cruz Biotechnology (St Louis, MO, USA). Enhanced chemiluminescence reagents were purchased from Millipore Corp. (Bedford, MA, USA). Enzyme-linked immunosorbent assay (ELISA) assay kits for TNF-α, IL-1 β, and IL-6 were obtained from Biolegend (CA, USA). Safrole, dimethyl sulfoxide (DMSO), phosphate-buffered saline, and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Safrole was dissolved with DMSO and tested using concentrations of 1, 10, 100, and 300 μM. The final concentration of DMSO in all experiments was not more than 0.5% (v/v).

Cell culture

The RAW264.7 mouse macrophage cell line (Bioresource Collection and Research Center, Number: 60001) was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). RAW264.7 cells were cultured in DMEM supplemented with 10% FBS, 1% antibiotic–antimycotic solution, 25 mM HEPES, 1 mM sodium pyruvate, and 0.2% NaHCO$_3$ maintained at 37°C in a humidified atmosphere of 95% air and 5% CO$_2$. After 1 day of culture, the medium was changed to serum-free DMEM for further experiments [17].

Measurement of proinflammatory cytokines

The protein concentrations of TNF-α, IL-1 β, and IL-6 were measured using ELISA assay kits. Briefly, 5×10^4 cells were incubated with or without safrole at the indicated concentration for 12 h. The cytokine level in the culture medium was measured using ELISA kits according to the manufacturer's instructions [12].

Determination of NO generation

The NO content in the culture medium was determined through a method previously described [12]. Briefly, RAW264.7 cells were treated with or without safrole at the indicated concentration for 12 h. An equal volume of Griess reagent was added to the culture medium at room temperature for 30 min. The optical density was measured at 540 nm using a microplate reader.

Western blotting

After treatment, the RAW264.7 cells were harvested and lysed in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% deoxycholate, 0.2% aprotinin, and 1 mM phenylmethylsulfonyl fluoride at 4°C for 30 min. The protein content in the supernatant was measured using the Bradford assay after centrifugation. Equal protein samples were separated using SDS gel electrophoresis and electrotransferred onto polyvinylidene difluoride membranes in a wet electrophoretic transfer cell. After blocking with skim milk, the membranes were incubated with antibodies, including P-p65, P-p38 MAPK, P-ERK, P-JNK, p65, p38 MAPK, ERK, JNK, iNOS, IκB, and β-actin. The membranes were washed and reprobed with appropriate horseradish peroxidase-conjugated secondary antibodies. The membranes were visualized using enhanced chemiluminescence Western blotting reagents and developed using the Fusion Solo (Vilber, Paris, France) [18].

Statistical analysis

The data from threat least three independent experiments were expressed as means ± standard deviation. Statistical analysis was performed using a one-way analysis of variance followed by Bonferroni's test for multigroup comparisons, and statistical significance ($P < 0.05$) was evaluated using SPSS 11.0.

RESULTS

Effects of safrole on proinflammatory cytokine generation

The generation of proinflammatory cytokines, including TNFα, IL-1β, and IL-6, was induced through macrophage activation. The levels of TNFα, IL-1β, and IL-6 were assessed using the ELISA assay kits. The level of TNFα generated from 490.6 ± 109.6 (control) to 1602.3 ± 176.0, 2075.9 ± 217.7, and 2610.3 ± 403.6 pg/mL by 10, 100, and 300 μM safrole, respectively. The level of IL-1β generated from 25.2 ± 12.4 (control) to 127.1 ± 2.5, 244.4 ± 35.5, and 291.6 ± 29.5 pg/mL by 10, 100, and 300 μM safrole, respectively. The level of IL-6 generated from 213.4 ± 72.6 (control) to 568.6 ± 69.2, 1236.3 ± 177.9, and 1570.8 ± 333.9 pg/mL by 10, 100, and 300 μM safrole, respectively. Safrole was demonstrated to significantly induce TNFα, IL-1β, and IL-6 generation in a concentration-dependent manner, with the significant induction starting at 10 μM [$P < 0.05$, Figure 1].

Effects of safrole on nitric oxide generation and inducible nitric oxide synthase expression

Generation of NO plays a crucial role in macrophage activation via the upregulation of iNOS expression. The level of NO was estimated using the Griess reaction. The
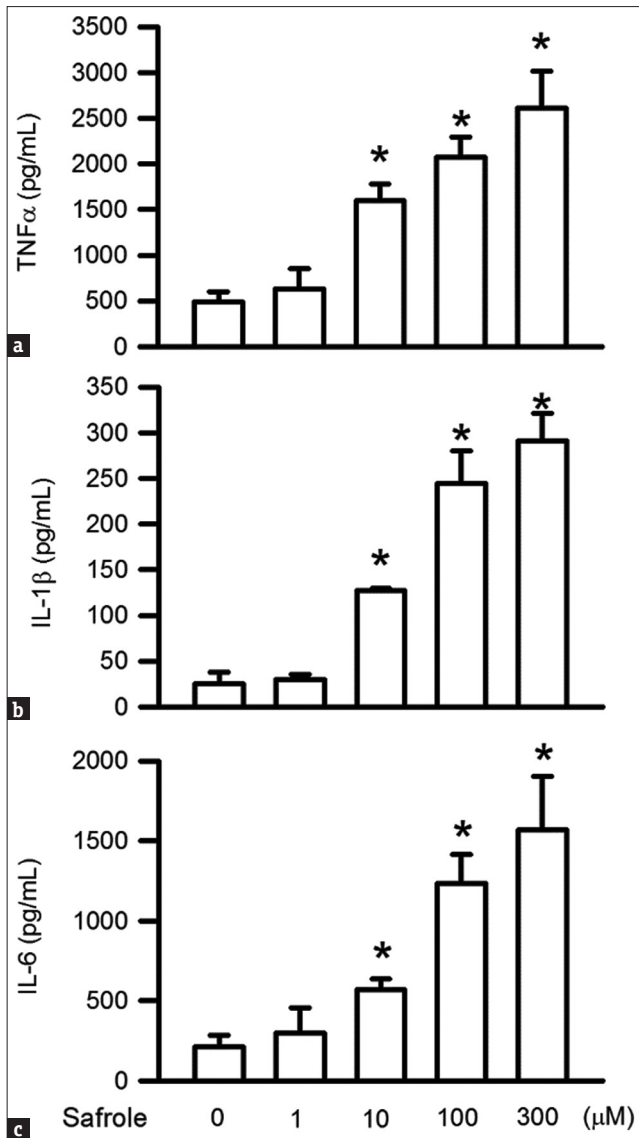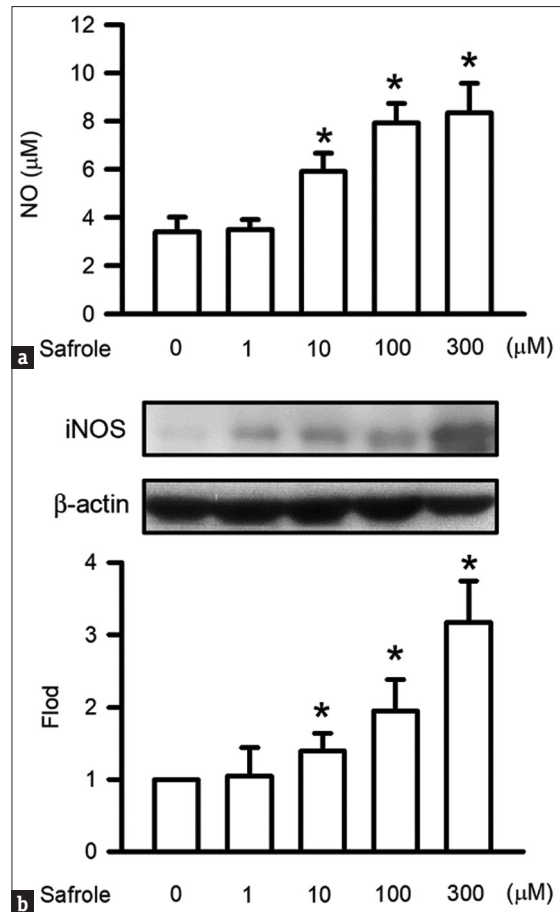
Figure 1: Safrole was demonstrated to induce generation of proinflammatory cytokines, including tumor necrosis factor-α (a), interleukin-1 β (b), and interleukin-6 (c) in RAW264.7 macrophages. The generation of proinflammatory cytokines was measured by ELISA assay kit after treated with safrole for 12 h at 0, 1, 10, 100, or 300 μM. RAW264.7 cells treated with safrole at 0 μM served as the control group. Results are expressed as means ± standard deviation ($n = 4$). *$P < 0.05$ considers significant as compared with control group

Figure 2: Safrole was demonstrated to induce NO generation (a) and inducible nitric oxide synthase expression (b) in RAW264.7 macrophages. RAW264.7 cells incubated with safrole at 0, 1, 10, 100, or 300 μM for 12 h. RAW264.7 cells treated with safrole at 0 μM served as the control group. The nitric oxide generation and inducible nitric oxide synthase expression were measured by Griess reaction and Western blot assay, respectively. Results are expressed as means ± standard deviation ($n = 4$). *$P < 0.05$ considers significant as compared with control group

level of NO generated from 3.4 ± 0.6 (control) to 5.9 ± 0.7, 7.9 ± 0.8, and 8.3 ± 1.2 μM by 10, 100, and 300 μM safrole, respectively. Safrole significantly induced NO generation in a concentration-dependent manner, with the significant induction starting at 10 μM [$P < 0.05$, Figure 2]. The expression of iNOS was estimated using the Western blot assay. The expression of iNOS induced from 1.0 ± 0.0 (control) to 1.4 ± 0.2, 2.0 ± 0.5, and 3.2 ± 0.7 μM by 10, 100, and 300 μM safrole, respectively. Notably, safrole significantly induced iNOS expression in a concentration-dependent manner, with the significant induction starting at 10 μM [$P < 0.05$, Figure 2].

Effects of safrole on nuclear factor-κB p65 phosphorylation

The secretion of proinflammatory cytokines and NO is regulated by NF-κB p65 activation and phosphorylation.

Phosphorylation of NF-κB p65 was estimated using the Western blot assay. The phosphorylation of NF-κB p65 induced from 1.0 ± 0.0 (control) to 1.8 ± 0.3, 2.3 ± 0.6, and 2.3 ± 0.6 μM by 10, 100, and 300 μM safrole, respectively. Notably, safrole induced NF-κB p65 phosphorylation and the significant induction starting at 10 μM [$P < 0.05$, Figure 3].

Effects of safrole on inhibitor of κB degradation

Phosphorylation of NF-κB p65 is induced by IκB degradation. Degradation of IκB was measured using the Western blot assay. The degradation of NF-κB p65 induced from 1.0 ± 0.0 (control) to 0.6 ± 1.6, 0.4 ± 0.1, and 0.3 ± 0.1 μM by 10, 100, and 300 μM safrole, respectively. Degradation of IκB was induced by safrole and the significant induction starting at 10 μM [$P < 0.05$, Figure 4].

Effects of safrole on phosphorylation of the mitogen-activated protein kinase family

Degradation of IκB and phosphorylation of NF-κB p65 are stimulated by activation and phosphorylation of the MAPK family, including p38 MAPK, ERK, and JNK. Phosphorylation of the MAPK family was measured

Figure 3: Safrole was demonstrated to induce p65 phosphorylation in RAW264.7 macrophages. RAW264.7 cells incubated with safrole at 0, 1, 10, 100, or 300 μM for 12 h. RAW264.7 cells treated with safrole at 0 μM served as the control group. The p65 phosphorylation were measured by Western blot assay. Results are expressed as means ± standard deviation ($n = 3$). *$P < 0.05$ considers significant as compared with control group
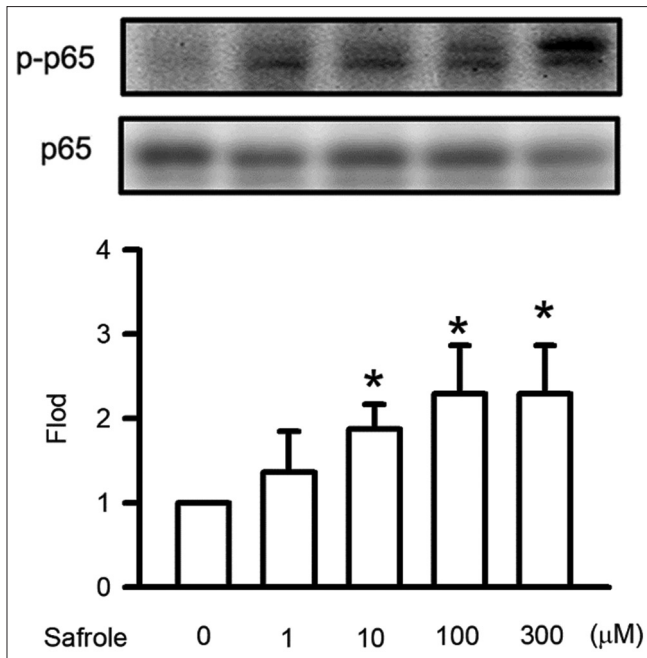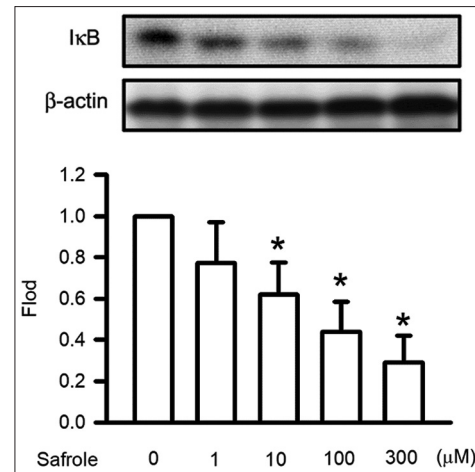
using the Western blot assay. The phosphorylation of p38 induced from 1.0 ± 0.0 (control) to 1.6 ± 0.4, 2.3 ± 0.4, and 2.0 ± 0.4 μM by 10, 100, and 300 μM safrole, respectively. The phosphorylation of ERK induced from 1.0 ± 0.0 (control) to 1.7 ± 0.3, 2.5 ± 0.4, and 2.4 ± 0.3 μM by 10, 100, and 300 μM safrole, respectively. The phosphorylation of JNK induced from 1.0 ± 0.0 (control) to 1.6 ± 0.4, 2.3 ± 0.4, and 2.0 ± 0.4 μM by 10, 100, and 300 μM safrole, respectively. Our results indicated that the phosphorylation of the MAPK family was induced by safrole and the significant induction starting at 10 μM [$P < 0.05$, Figure 5].

Effects of safrole on phosphorylation of the mitogen-activated protein kinase family

Degradation of IκB and phosphorylation of NF-κB p65 are stimulated by activation and phosphorylation of the MAPK family, including p38 MAPK, ERK, and JNK. Phosphorylation of the MAPK family was measured using the Western blot assay. The phosphorylation of p38 induced from 1.0 ± 0.0 (control) to 1.6 ± 0.4, 2.3 ± 0.4, and 2.0 ± 0.4 μM by 10, 100, and 300 μM safrole, respectively. The phosphorylation of ERK induced from 1.0 ± 0.0 (control) to 1.7 ± 0.3, 2.5 ± 0.4, and 2.4 ± 0.3 μM by 10, 100, and 300 μM safrole, respectively. The phosphorylation of JNK induced from 1.0 ± 0.0 (control) to 1.6 ± 0.4, 2.3 ± 0.4, and 2.0 ± 0.4 μM by 10, 100, and 300 μM safrole, respectively. Our results indicated that the phosphorylation of the MAPK family was induced by safrole and the significant induction starting at 10 μM [$P < 0.05$, Figure 5].
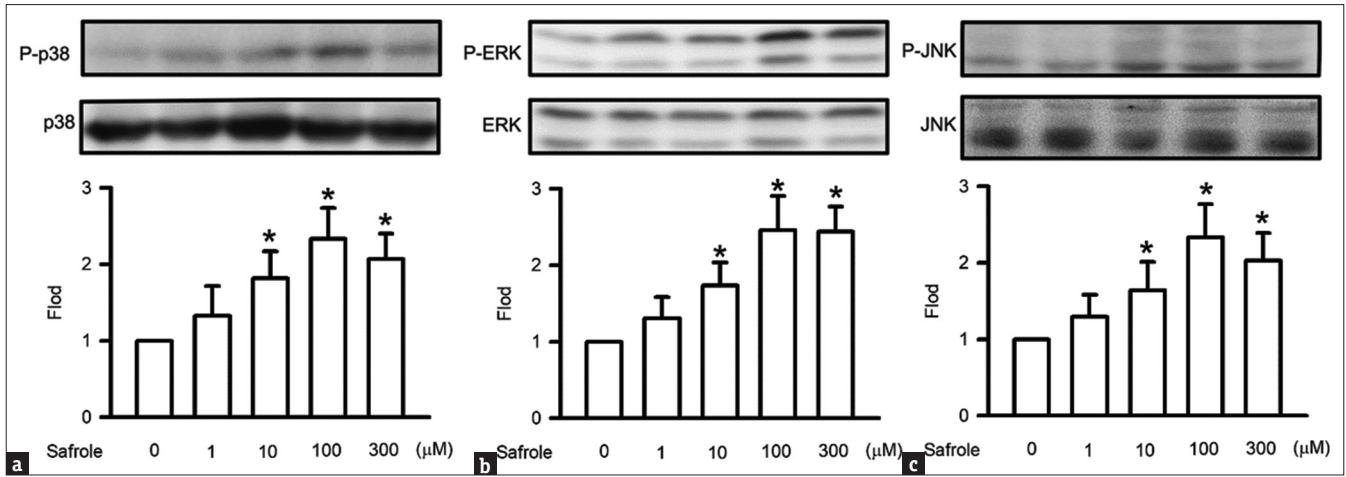

Figure 4: Safrole was demonstrated to induce inhibitor of κB degradation in RAW264.7 macrophages. RAW264.7 cells incubated with safrole at 0, 1, 10, 100, or 300 μM for 12 h. RAW264.7 cells treated with safrole at 0 μM served as the control group. The inhibitor of κB degradation was measured by Western blot assay. Results are expressed as means ± standard deviation ($n = 3$). *$P < 0.05$ considers significant as compared with control group

Inhibitory effects of mitogen-activated protein kinase and nuclear factor-κB inhibitors on generation of nitric oxide and cytokines induced by safrole

To demonstrate the effects of MAPK family and NF-κB pathway participated in the generation of NO and cytokines induced by safrole. We used the p38 MAPK, ERK, JNK, and NF-κB p65 inhibitors including SB203580, U0126, SP600125, and JSH23 pretreated with the RAW264.7 cells for 1 h and then stimulated by safrole at 300 μM for 12 h. As shown in Figure 6, the level of NO generated from 3.5 ± 1.0 (control) to 8.3 ± 1.3 μM by 300 μM safrole. Pretreated with SB203580, U0126, SP600125, and JSH23 reduced the level of NO to 5.5 ± 1.1, 5.5 ± 0.5, 5.8 ± 0.9, and 5.1 ± 0.9 μM, respectively. The level of TNFα generated from 287.4 ± 64.2 (control) to 2668.6 ± 348.0 pg/mL μM by 300 μM safrole. Pretreated with SB203580, U0126, SP600125, and JSH23 reduced the level of TNFα to 1438.5 ± 348.0, 1349.2 ± 127.5, 1528.9 ± 236.4, and 134.8 ± 277.3 pg/mL, respectively. The level of IL-1β generated from 21.1 ± 5.4 (control) to 290.0 ± 22.8 pg/mL μM by 300 μM safrole. Pretreated with SB203580, U0126, SP600125, and JSH23 reduced the level of IL-1β to 115.8 ± 35.0, 107.3 ± 15.6, 128.1 ± 13.0, and 89.0 ± 16.5 pg/mL, respectively. The level of IL-6 generated from 210.4 ± 74.9 (control) to 1766.5 ± 281.9 pg/mL μM by 300 μM safrole. Pretreated with SB203580, U0126, SP600125, and JSH23 reduced the level of IL-6 to 1316.3 ± 70.5, 1116.1 ± 144.3, 1124.7 ± 143.5, and 1131.2 ± 270.0 pg/mL, respectively. Our results indicated the safrole-induced generation of NO and cytokines were reduced by MAPK and NF-κB p65 inhibitors [$P < 0.05$, Figure 6].

DISCUSSION

Macrophages are the predominant cells of the innate immune system in human beings and provide a nonspecific, first-line defense system [11]. After RAW264.7 cells treated with safrole at the concentration of 10 μM for

Figure 5: Safrole was demonstrated to induce phosphorylation of mitogen-activated protein kinase family, including p38 mitogen-activated protein kinase (a), extracellular signal-regulated kinase (b), and c-Jun N-terminal kinase (c) in RAW264.7 macrophages. RAW264.7 cells incubated with safrole at 0, 1, 10, 100, or 300 μM for 30 min. RAW264.7 cells treated with safrole at 0 μM served as the control group. Phosphorylation of p38 mitogen-activated protein kinase phosphorylation, extracellular signal-regulated kinase, and c-Jun N-terminal kinase was measured by Western blot assay. Results are expressed as means ± standard deviation ($n = 3$). *$P < 0.05$ considers significant as compared with control group
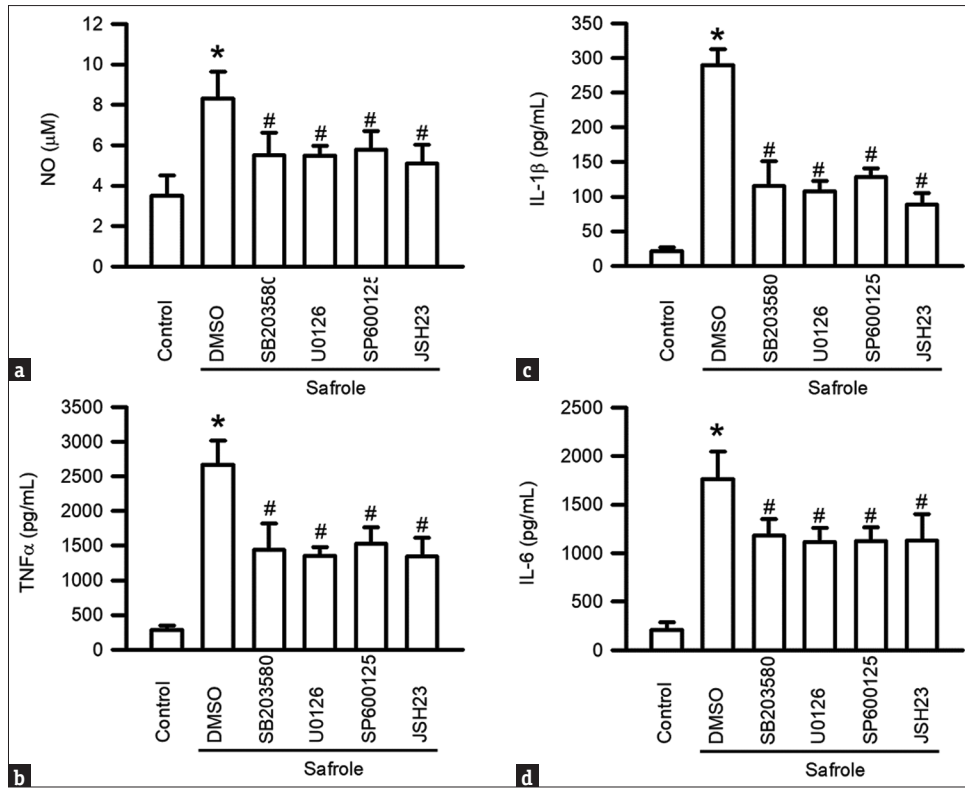
Figure 6: Inhibitory effects of SB203580, U0126, SP600125, and JSH23 on the generation of nitric oxide (a), tumor necrosis factor-α (a), interleukin-1 β (b), and interleukin-6 (c) were induced by safrole in RAW264.7 macrophages. The cells incubated with SB203580 at 30 μM, U0126 at 1 μM, SP600125 at 30 μM, and JSH23 at 30 μM for 1 h, and then the cells treated with safrole at 300 μM for 12 h. The nitric oxide generation was measured by Griess reaction. The secretion of tumor necrosis factor-α, interleukin-1 β, and interleukin-6 were detected by by Enzyme-linked immunosorbent assay kits. Results are expressed as means ± standard deviation ($n = 3$). *$P < 0.05$ considers significant as compared with control group. #$P < 0.05$ considers significant as compared with safrole group

24 h significantly induce cytotoxicity, DNA damage, and apoptosis [16]. There were no cytotoxic effects on RAW264.7 cells treated with safrole at 300 μM for 12 h (the survival rate of the control group and 300 μM safrrole were 100.0 ± 0.0 and 99.9 ± 1.8%, respectively). For avoidance of the toxic effects, the proinflammatory effect of RAW264.7 cells

incubated with safrole at the indicated concentration was performed all experiments for 12 h in the present study. Invading pathogens activate macrophages, which then secrete proinflammatory cytokines, including TNFα, IL-1β, and IL-6, that assist in pathogen defense [12]. In addition, the expression of proinflammatory cytokines results in inflammatory

responses that precede tumor development [19]. The primary producers of TNFα, IL-1β, and IL-6 are macrophages, which are highly sensitive to them. Therefore, these proinflammatory cytokines play the role of master regulator of inflammatory cytokine production and are implicated in several inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel disease, juvenile chronic arthritis, atherosclerosis, and sepsis [20-22]. Furthermore, macrophage functions, such as adhesion, phagocytosis, proliferation, and production of bactericidal substances, are promoted by these proinflammatory cytokines [20-22]. In addition, the generation of NO is a characteristic feature of macrophage activation and participation in the defense against foreign pathogens [11,12]. The amount of NO produced by iNOS after stimulation has been implicated in host-defense immunology and inflammatory responses [11]. Therefore, NO is a crucial bactericidal and proinflammatory mediator in macrophage activation [11]. To the best of our knowledge, the present study was the first to determine that safrole induces the secretion of proinflammatory cytokines and mediators in a concentration-dependent manner in macrophages. These results suggest that macrophage overexpression of proinflammatory cytokines and mediators induced by safrole could result in excessive inflammation and peripheral tissue destruction.

The proinflammatory transcription factor, NF-κB, modulates multiple signaling mechanism pathways involved in the generation of TNFα, IL-1β, and IL-6 and expression of iNOS. NF-κB, the inducible and proinflammatory transcription factor family, is composed of five proteins, namely p65 or RelA, RelB, c-Rel, p50 and its precursor p105 (also called NF-κB1), and p52 and its precursor p100 (also called NF-κB2) [23]. The most abundant subunit of NF-κB is p65, which regulates various cell functions through the highly potent transcriptional activation domain on C-terminal ends in the nucleus [23]. In a resting condition, NF-κB p65 is in the inactive form as a latent complex binding to IκB within the cytoplasm [24]. After activation, NF-κB p65 dissociates because of the degradation of IκB and translocates from the cytoplasm to the nucleus [25]. Notably, NF-κB expression is induced by safrole in human buccal mucosa fibroblasts and human umbilical vein endothelial cells [26,27]. According to our data, phosphorylation of NF-κB p65 and degradation of IκB were significantly increased by safrole at the concentration of 10 μM. We also found significantly increased generation of proinflammatory cytokines and mediators by safrole at the concentration of 10 μM. These findings indicate that the generation of proinflammatory cytokines and mediators was induced by safrole via the NF-κB pathway, including NF-κB phosphorylation and IκB degradation.

Notably, the degradation of IκB is triggered by the phosphorylation of the MAPK family [28]. In mammals, the three subclasses of the MAPK family include ERK, JNK, and p38 MAPK. Activation of the MAPK family is induced by phosphorylation on the threonine and tyrosine residues within a conserved Thr-X-Tyr motif in the activation loop. The MAPK family is involved in transmitting signals from various extracellular ligands and converting them into a wide range of intracellular responses [29]. The MAPK family plays a crucial role in proinflammatory responses [30]. After human buccal mucosal fibroblasts were treated with PD98059, the inhibitor of ERK activation reduced the NF-κB expression induced by safrole. These results indicate that safrole-induced NF-κB expression was mediated by ERK activation in fibroblasts [27]. Herein, the results indicated that phosphorylation of ERK, JNK, and p38 MAPK was markedly induced by safrole at the concentration of 10 μM. The level of MPAK phosphorylation induced by safrole at the concentration of 100 and 300 μM was higher than 10 μM. However, there are similar effects of MAPK phosphorylation induced by safrole at the concentration of 100 and 300 μM. These results suggested that the phosphorylation level of MAPK began to increase at 10 μM and attained a plateau at 100 μM. This reactive trend would be also found in phosphorylation of NF-κB p65. These results suggest that NF-κB phosphorylation was induced by safrole through phosphorylation of ERK, JNK, and p38 MAPK.

In the previous study, we have demonstrated that induction of cytotoxicity, DNA damage, and apoptosis in macrophage treatment with safrole for 24 h was through the generation of ROS and inhibition of antioxidative enzymes possibly via Akt phosphorylation. To the best of our knowledge, this study is the first systemic attempt to evaluate the proinflammatory responses and mechanisms of safrole on RAW264.7 macrophages. We demonstrated for the first time that proinflammatory cytokines, including TNFα, IL-1β, IL-6, and NO, were induced by safrole. Furthermore, we determined that safrole induced NF-κB phosphorylation and IκB degradation, leading to the generation of proinflammatory cytokines and NO. In addition, we observed that phosphorylation of ERK, JNK, and p38 MAPK induced by safrole exhibited similar concentration trends as NF-κB phosphorylation and IκB degradation did. These results suggested that the expression of proinflammatory cytokines and NO in macrophages treated with safrole occurs via the NF-κB/IκB pathway and its upstream factor, MAPK family phosphorylation [Figure 7].
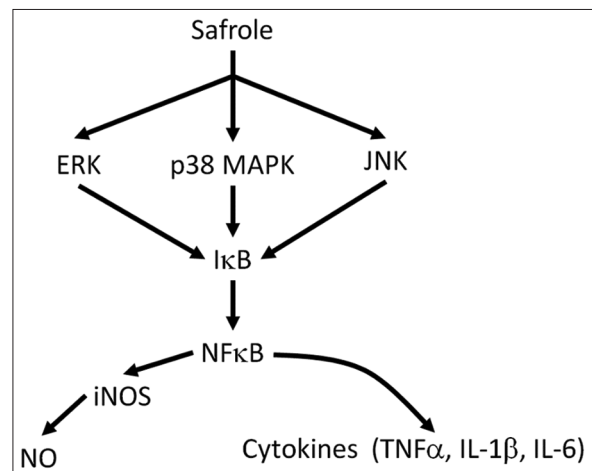

Figure 7: Scheme of the mechanisms in the protective effect of nerolidol on lipopolysaccharide-induced acute lung injury

Acknowledgments

Conflicts of interest

There are no conflicts of interest.

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