



## Original Article

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

Yung-Lun Ni<sup>a</sup>, Huan-Ting Shen<sup>a,b</sup>, Min-Wei Lee<sup>c</sup>, Kun-Lin Yeh<sup>d</sup>, Chen-Yu Chiang<sup>d</sup>, Yu-Hsiang Kuan<sup>e,f\*</sup>

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

<sup>a</sup>Department of Veterinary Medicine, National Chung Hsing University, Taichung, Taiwan, <sup>e</sup>Department of Pharmacy, Chung Shan Medical University Hospital, Taichung, Taiwan, <sup>f</sup>Department of Pharmacology, School of Medicine, Chung Shan Medical University, Taichung, Taiwan

<sup>a</sup>Department of Veterinary Medicine, National Chung Hsing University, Taichung, Taiwan, <sup>e</sup>Department of Pharmacy, Chung Shan Medical University Hospital, Taichung, Taiwan, <sup>f</sup>Department of Pharmacology, School of Medicine, Chung Shan Medical University, Taichung, Taiwan

Submission : 16-Apr-2020  
Revision : 20-May-2020  
Acceptance : 06-Jun-2020  
Web Publication : 06-Aug-2020

## 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- $\alpha$ , interleukin-1 $\beta$ , and IL-6 in RAW264.7 macrophages in a concentration-dependent manner. Furthermore, inhibitor of  $\kappa$ B (I $\kappa$ B) degradation was caused by safrole in a concentration-dependent manner. In addition, the phosphorylation of nuclear factor (NF)- $\kappa$ B 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  $\mu$ M and attained a plateau at 100  $\mu$ M. **Conclusion:** These results indicated that safrole induces the expression of proinflammatory responses in macrophages through the NF- $\kappa$ B/I $\kappa$ B pathway and its upstream factor, MAPK family phosphorylation.

**KEYWORDS:** Macrophage, Mitogen-activated protein kinase family, Nuclear factor- $\kappa$ B 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  $\mu$ M 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- $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-1 $\beta$ , 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](mailto:kuanyh@csmu.edu.tw)

This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

For reprints contact: [WKHLRPMedknow\\_reprints@wolterskluwer.com](mailto:WKHLRPMedknow_reprints@wolterskluwer.com)

**How to cite this article:** Ni YL, Shen HT, Lee MW, Yeh KL, Chiang CY, Kuan YH. Safrole-induced expression of proinflammatory responses is associated with phosphorylation of mitogen-activated protein kinase family and the nuclear factor- $\kappa$ B/inhibitor of  $\kappa$ B pathway in macrophages. Tzu Chi Med J 2020; 32(4): 344-50.

Access this article online	
<b>Quick Response Code:</b> 	<b>Website:</b> <a href="http://www.tcmjmed.com">www.tcmjmed.com</a>
	<b>DOI:</b> 10.4103/tcmj.tcmj_78_20

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)- $\kappa$ B pathways [13]. Notably, a previous study demonstrated macrophage phagocytosis was induced by saffrole in mice and cell models [14,15]. In the recent study, we have purposed toxic effects, which including cytotoxicity, genotoxicity, and apoptosis, induced by saffrole 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 saffrole in macrophages. The present study attempted to assess the potency of saffrole as a macrophage stimulator and explored the possible involvement of the MAPK family and NF- $\kappa$ B 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- $\kappa$ B p65 were purchased from Santa Cruz Biotechnology (St Louis, MO, USA). Antibodies of inducible NO synthase (iNOS), the inhibitor of  $\kappa$ B (IkB),  $\beta$ -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- $\alpha$ , IL-1  $\beta$ , and IL-6 were obtained from Biolegend (CA, USA). Saffrole, dimethyl sulfoxide (DMSO), phosphate-buffered saline, and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Saffrole was dissolved with DMSO and tested using concentrations of 1, 10, 100, and 300  $\mu$ M. 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%  $\text{NaHCO}_3$  maintained at 37°C in a humidified atmosphere of 95% air and 5%  $\text{CO}_2$ . 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- $\alpha$ , IL-1  $\beta$ , and IL-6 were measured using ELISA assay kits. Briefly,  $5 \times 10^4$  cells were incubated with or without saffrole 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 saffrole 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, IkB, and  $\beta$ -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  $\pm$  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.

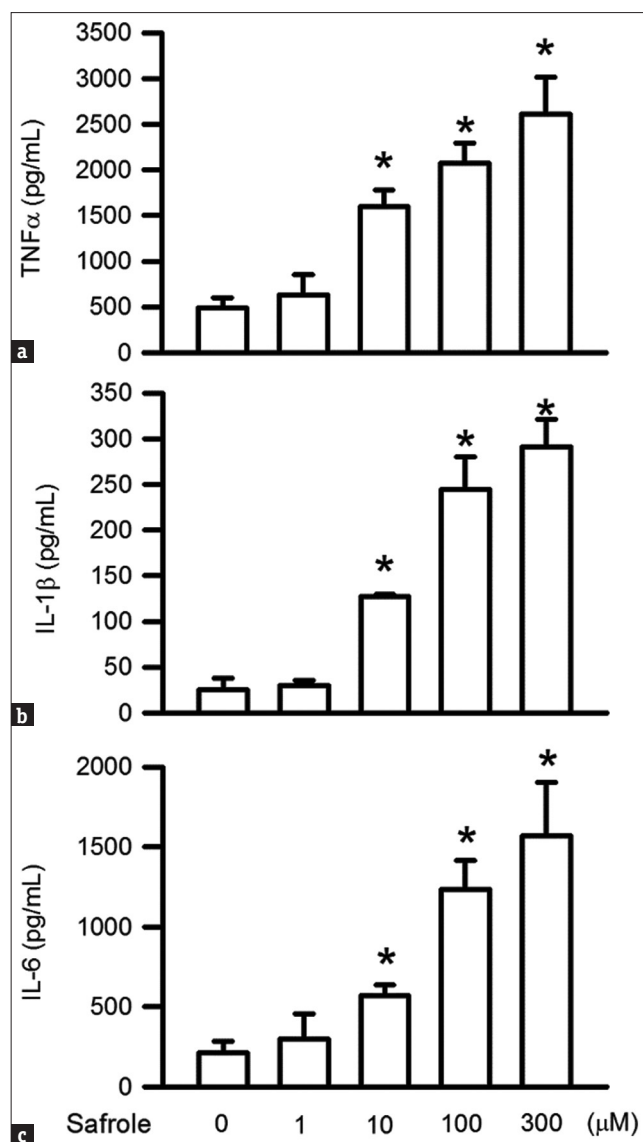
## RESULTS

### Effects of saffrole on proinflammatory cytokine generation

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

### Effects of saffrole 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

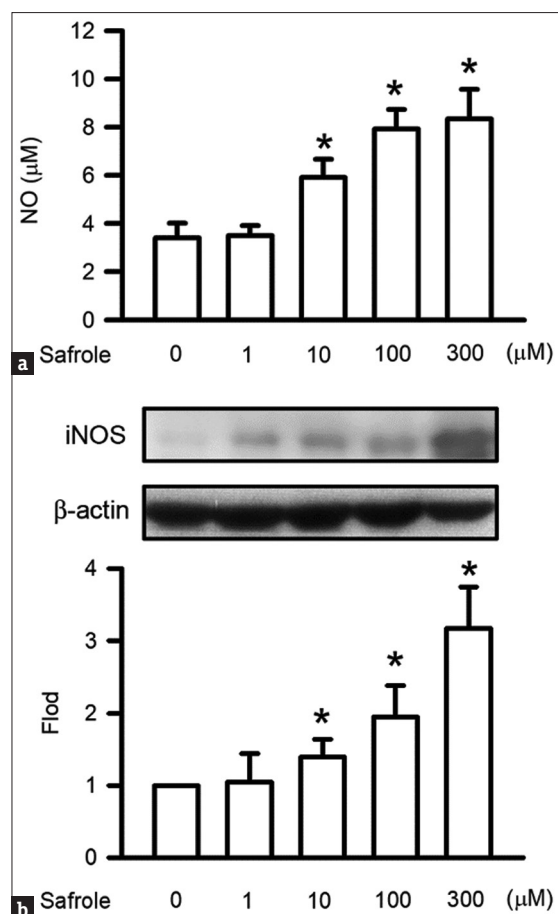


**Figure 1:** Saffrole was demonstrated to induce generation of proinflammatory cytokines, including tumor necrosis factor- $\alpha$  (a), interleukin-1  $\beta$  (b), and interleukin-6 (c) in RAW264.7 macrophages. The generation of proinflammatory cytokines was measured by ELISA assay kit after treated with saffrole for 12 h at 0, 1, 10, 100, or 300  $\mu$ M. RAW264.7 cells treated with saffrole at 0  $\mu$ M served as the control group. Results are expressed as means  $\pm$  standard deviation ( $n = 4$ ). \* $P < 0.05$  considers significant as compared with control group

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

#### Effects of saffrole on nuclear factor- $\kappa$ B p65 phosphorylation

The secretion of proinflammatory cytokines and NO is regulated by NF- $\kappa$ B p65 activation and phosphorylation.



**Figure 2:** Saffrole was demonstrated to induce NO generation (a) and inducible nitric oxide synthase expression (b) in RAW264.7 macrophages. RAW264.7 cells incubated with saffrole at 0, 1, 10, 100, or 300  $\mu$ M for 12 h. RAW264.7 cells treated with saffrole at 0  $\mu$ M 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  $\pm$  standard deviation ( $n = 4$ ). \* $P < 0.05$  considers significant as compared with control group

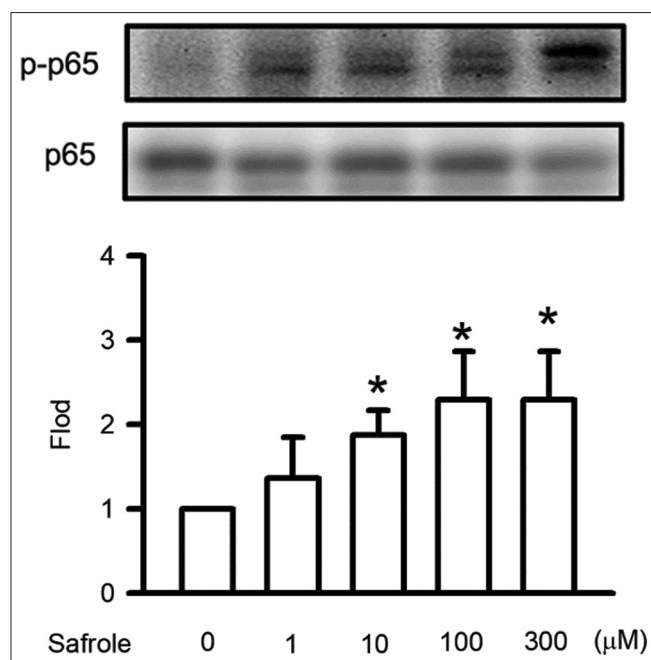
Phosphorylation of NF- $\kappa$ B p65 was estimated using the Western blot assay. The phosphorylation of NF- $\kappa$ B p65 induced from  $1.0 \pm 0.0$  (control) to  $1.8 \pm 0.3$ ,  $2.3 \pm 0.6$ , and  $2.3 \pm 0.6$   $\mu$ M by 10, 100, and 300  $\mu$ M saffrole, respectively. Notably, saffrole induced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

#### Effects of saffrole on inhibitor of $\kappa$ B degradation

Phosphorylation of NF- $\kappa$ B p65 is induced by I $\kappa$ B degradation. Degradation of I $\kappa$ B was measured using the Western blot assay. The degradation of NF- $\kappa$ B p65 induced from  $1.0 \pm 0.0$  (control) to  $0.6 \pm 1.6$ ,  $0.4 \pm 0.1$ , and  $0.3 \pm 0.1$   $\mu$ M by 10, 100, and 300  $\mu$ M saffrole, respectively. Degradation of I $\kappa$ B was induced by saffrole and the significant induction starting at 10  $\mu$ M [ $P < 0.05$ , Figure 4].

#### Effects of saffrole on phosphorylation of the mitogen-activated protein kinase family

Degradation of I $\kappa$ B and phosphorylation of NF- $\kappa$ B p65 are stimulated by activation and phosphorylation of the MAPK family, including p38 MAPK, ERK, and JNK. Phosphorylation of the MAPK family was measured

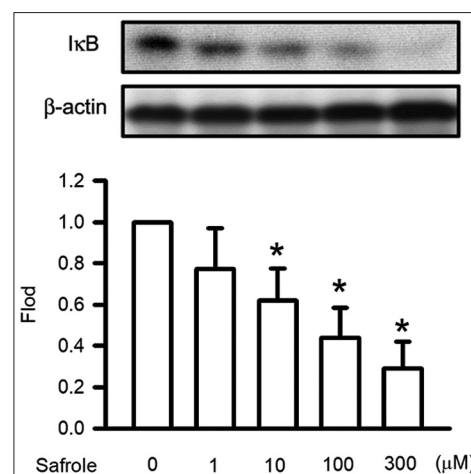


**Figure 3:** Safrole was demonstrated to induce p65 phosphorylation in RAW264.7 macrophages. RAW264.7 cells incubated with safrole at 0, 1, 10, 100, or 300  $\mu\text{M}$  for 12 h. RAW264.7 cells treated with safrole at 0  $\mu\text{M}$  served as the control group. The p65 phosphorylation were measured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

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

Degradation of I $\kappa$ B and phosphorylation of NF- $\kappa$ B 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  $1.0 \pm 0.0$  (control) to  $1.6 \pm 0.4$ ,  $2.3 \pm 0.4$ , and  $2.0 \pm 0.4$   $\mu\text{M}$  by 10, 100, and 300  $\mu\text{M}$  safrole, respectively. The phosphorylation of ERK induced from  $1.0 \pm 0.0$  (control) to  $1.7 \pm 0.3$ ,  $2.5 \pm 0.4$ , and  $2.4 \pm 0.3$   $\mu\text{M}$  by 10, 100, and 300  $\mu\text{M}$  safrole, respectively. The phosphorylation of JNK induced from  $1.0 \pm 0.0$  (control) to  $1.6 \pm 0.4$ ,  $2.3 \pm 0.4$ , and  $2.0 \pm 0.4$   $\mu\text{M}$  by 10, 100, and 300  $\mu\text{M}$  safrole, respectively. Our results indicated that the phosphorylation of the MAPK family was induced by safrole and the significant induction starting at 10  $\mu\text{M}$  [ $P < 0.05$ , Figure 5].



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

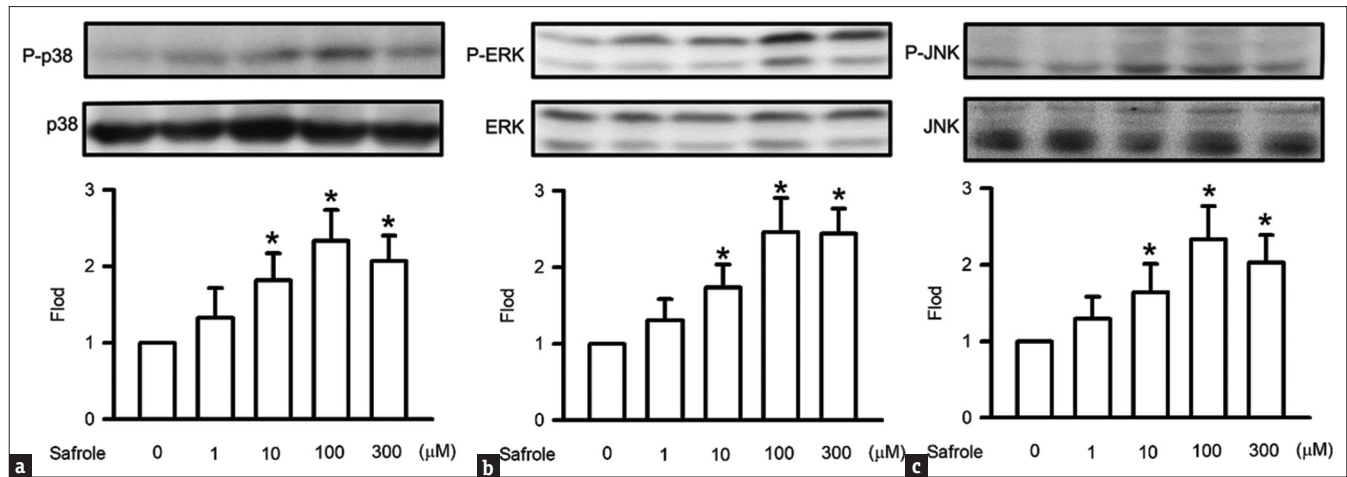
#### Inhibitory effects of mitogen-activated protein kinase and nuclear factor- $\kappa\text{B}$ inhibitors on generation of nitric oxide and cytokines induced by safrole

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

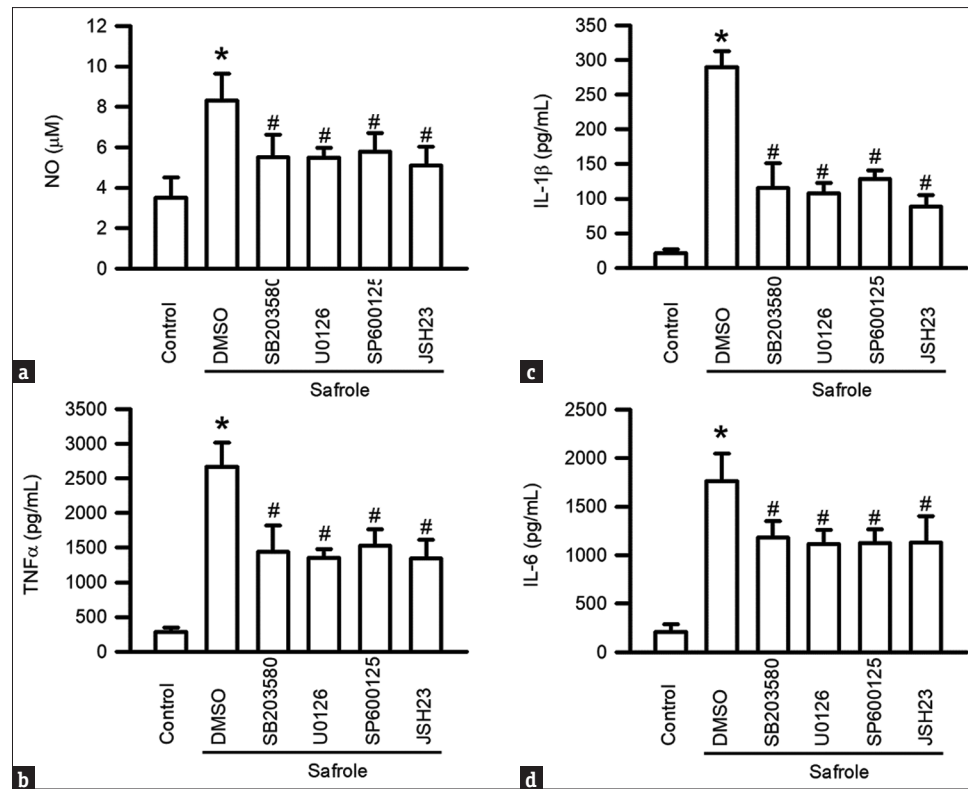
#### DISCUSSION

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  $\mu\text{M}$  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  $\mu$ M for 30 min. RAW264.7 cells treated with safrole at 0  $\mu$ M 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  $\pm$  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- $\alpha$  (a), interleukin-1  $\beta$  (b), and interleukin-6 (c) were induced by safrole in RAW264.7 macrophages. The cells incubated with SB203580 at 30  $\mu$ M, U0126 at 1  $\mu$ M, SP600125 at 30  $\mu$ M, and JSH23 at 30  $\mu$ M for 1 h, and then the cells treated with safrole at 300  $\mu$ M for 12 h. The nitric oxide generation was measured by Griess reaction. The secretion of tumor necrosis factor- $\alpha$ , interleukin-1  $\beta$ , and interleukin-6 were detected by by Enzyme-linked immunosorbent assay kits. Results are expressed as means  $\pm$  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  $\mu$ M for 12 h (the survival rate of the control group and 300  $\mu$ M safrole were  $100.0 \pm 0.0$  and  $99.9 \pm 1.8\%$ , 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 TNF $\alpha$ , IL-1 $\beta$ , and IL-6, that assist in pathogen defense [12]. In addition, the expression of proinflammatory cytokines results in inflammatory

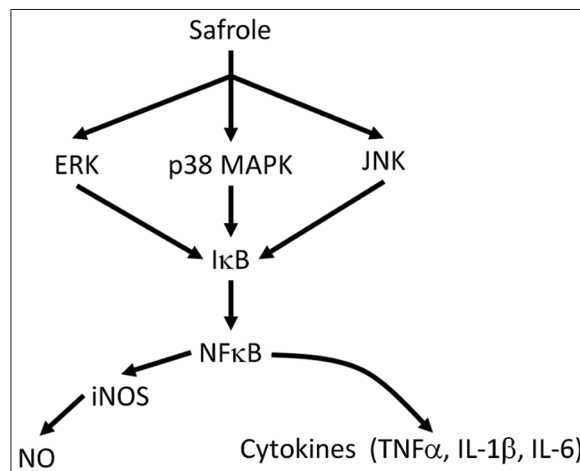
responses that precede tumor development [19]. The primary producers of  $\text{TNF}\alpha$ ,  $\text{IL-1}\beta$ , and  $\text{IL-6}$  are macrophages, which are highly sensitive to them. Therefore, these proinflammatory cytokines play the role of master regulator of inflammatory cytokine production 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 saffrole 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 saffrole could result in excessive inflammation and peripheral tissue destruction.

The proinflammatory transcription factor, NF- $\kappa$ B, modulates multiple signaling mechanism pathways involved in the generation of  $\text{TNF}\alpha$ ,  $\text{IL-1}\beta$ , and  $\text{IL-6}$  and expression of iNOS. NF- $\kappa$ B, 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- $\kappa$ B1), and p52 and its precursor p100 (also called NF- $\kappa$ B2) [23]. The most abundant subunit of NF- $\kappa$ B 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- $\kappa$ B p65 is in the inactive form as a latent complex binding to I $\kappa$ B within the cytoplasm [24]. After activation, NF- $\kappa$ B p65 dissociates because of the degradation of I $\kappa$ B and translocates from the cytoplasm to the nucleus [25]. Notably, NF- $\kappa$ B expression is induced by saffrole in human buccal mucosa fibroblasts and human umbilical vein endothelial cells [26,27]. According to our data, phosphorylation of NF- $\kappa$ B p65 and degradation of I $\kappa$ B were significantly increased by saffrole at the concentration of 10  $\mu\text{M}$ . We also found significantly increased generation of proinflammatory cytokines and mediators by saffrole at the concentration of 10  $\mu\text{M}$ . These findings indicate that the generation of proinflammatory cytokines and mediators was induced by saffrole via the NF- $\kappa$ B pathway, including NF- $\kappa$ B phosphorylation and I $\kappa$ B degradation.

Notably, the degradation of I $\kappa$ B 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- $\kappa$ B expression induced by saffrole. These results indicate that saffrole-induced NF- $\kappa$ B 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 saffrole at the concentration of 10  $\mu\text{M}$ . The level of MPAP phosphorylation induced by saffrole at the concentration of 100 and 300  $\mu\text{M}$  was higher than 10  $\mu\text{M}$ . However, there are similar effects of MAPK phosphorylation induced by saffrole at the concentration of 100 and 300  $\mu\text{M}$ . These results suggested that the phosphorylation level of MAPK began to increase at 10  $\mu\text{M}$  and attained a plateau at 100  $\mu\text{M}$ . This reactive trend would be also found in phosphorylation of NF- $\kappa$ B p65. These results suggest that NF- $\kappa$ B phosphorylation was induced by saffrole 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 saffrole 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 saffrole on RAW264.7 macrophages. We demonstrated for the first time that proinflammatory cytokines, including  $\text{TNF}\alpha$ ,  $\text{IL-1}\beta$ ,  $\text{IL-6}$ , and NO, were induced by saffrole. Furthermore, we determined that saffrole induced NF- $\kappa$ B phosphorylation and I $\kappa$ B degradation, leading to the generation of proinflammatory cytokines and NO. In addition, we observed that phosphorylation of ERK, JNK, and p38 MAPK induced by saffrole exhibited similar concentration trends as NF- $\kappa$ B phosphorylation and I $\kappa$ B degradation did. These results suggested that the expression of proinflammatory cytokines and NO in macrophages treated with saffrole occurs via the NF- $\kappa$ B/I $\kappa$ B 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

This manuscript was edited by Wallace Academic Editing.

## Financial support and sponsorship

The authors would like to thank the Ministry of Science and Technology of the Republic of China, Taiwan (grant No. MOST 106-232-B-040-022-MY3, 105-2320-B-040-022, and 104-2320-B-040-006) and the Buddhist Tzu Chi Medical Foundation (project No. TTRCD107-04).

## Conflicts of interest

There are no conflicts of interest.

## REFERENCES

1. Takeshita T, Tao F, Kojima N, Kanaly RA. Triple quadrupole mass spectrometry comparative dna adductomics of hep g2 cells following exposure to safrole. *Toxicol Lett* 2019;300:92-104.
2. Al-Malahmeh AJ, Alajlouni AM, Ning J, Wesseling S, Vervoort J, Rietjens IM. Determination and risk assessment of naturally occurring genotoxic and carcinogenic alkenylbenzenes in nutmeg-based plant food supplements. *J Appl Toxicol* 2017;37:1254-64.
3. Yang AH, Zhang L, Zhi DX, Liu WL, Gao X, He X. Identification and analysis of the reactive metabolites related to the hepatotoxicity of safrole. *Xenobiotica* 2018;48:1164-72.
4. Tsou HH, Ko HT, Chen CT, Wang TW, Lee CH, Liu TY, et al. Betel quid containing safrole enhances metabolic activation of tobacco specific 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). *Environ Pollut* 2019;251:13-21.
5. Liu T, Zhang L, Joo D, Sun SC. NF- $\kappa$ B signaling in inflammation. *Signal Transduct Target Ther* 2017. pii: 17023.
6. Lee JM, Liu TY, Wu DC, Tang HC, Leh J, Wu MT, et al. Safrole-dna adducts in tissues from esophageal cancer patients: Clues to areca-related esophageal carcinogenesis. *Mutat Res* 2005;565:121-8.
7. Chung YT, Chen CL, Wu CC, Chan SA, Chi CW, Liu TY. Safrole-DNA adduct in hepatocellular carcinoma associated with betel quid chewing. *Toxicol Lett* 2008;183:21-7.
8. Khawaja MR, Mazahir S, Majeed A, Malik F, Merchant KA, Maqsood M, et al. Chewing of betel, areca and tobacco: Perceptions and knowledge regarding their role in head and neck cancers in an urban squatter settlement in Pakistan. *Asian. Pac J Cancer Prev* 2006;7:95-100.
9. Hwang LS, Wang CK, Sheu MJ, Kao LS. Phenolic compounds of *Piper betle* flower as flavoring and neuronal activity modulating agents. In: Ho CT, Lee CY, Huang MT, editors. *Phenolic Compounds in Food and Their Effects on Health. I: Analysis, Occurrence and Chemistry*. Washington, DC: American Chemical Society; 1992. p. 200-13.
10. Wang CK, Hwang LS. Phenolic compounds of betel quid chewing juice. *Food Sci* 1993;20:458-71.
11. Varol C, Mildner A, Jung S. Macrophages: Development and tissue specialization. *Annu Rev Immunol* 2015;33:643-75.
12. Huang FM, Chang YC, Lee SS, Yang ML, Kuan YH. Expression of pro-inflammatory cytokines and mediators induced by Bisphenol A via ERK-NF $\kappa$ B and JAK1/2-STAT3 pathways in macrophages. *Environ Toxicol* 2019;34:486-94.
13. Hu Y, Zhou Q, Liu T, Liu Z. Coixol suppresses NF- $\kappa$ B, MAPK pathways and NLRP3 inflammasome activation in lipopolysaccharide-induced RAW 264.7 cells. *Molecules* 2020;25. Pii: E894.
14. Wu L, Zhang X, Zhang B, Shi H, Yuan X, Sun Y, et al. Exosomes derived from gastric cancer cells activate NF- $\kappa$ B pathway in macrophages to promote cancer progression. *Tumour Biol* 2016;7:12169-80.
15. Yu FS, Yang JS, Yu CS, Chiang JH, Lu CC, Chung HK, et al. Safrole suppresses murine myelomonocytic leukemia wehi-3 cells *in vivo*, and stimulates macrophage phagocytosis and natural killer cell cytotoxicity in leukemic mice. *Environ Toxicol* 2013;28:601-8.
16. Fan MJ, Lin SY, Yu CC, Tang NY, Ho HC, Chung HK, et al. Safrole-modulated immune response is mediated through enhancing the cd11b surface marker and stimulating the phagocytosis by macrophages in balb/c mice. *Hum Exp Toxicol* 2012;31:898-904.
17. Chien KJ, Yang ML, Tsai PK, Su CH, Chen CH, Horng CT, et al. Safrole induced cytotoxicity, DNA damage, and apoptosis in macrophages via reactive oxygen species generation and AKT phosphorylation. *Environ Toxicol Pharmacol* 2018;64:94-100.
18. Huang FM, Chang YC, Lee SS, Ho YC, Yang ML, Lin HW, et al. Bisphenol a exhibits cytotoxic or genotoxic potential via oxidative stress-associated mitochondrial apoptotic pathway in murine macrophages. *Food Chem Toxicol* 2018;122:215-24.
19. Tsai PK, Wu SW, Chiang CY, Lee MW, Chen HY, Chen WY, et al. Evaluation of cytotoxicity, apoptosis, and genotoxicity induced by indium chloride in macrophages through mitochondrial dysfunction and reactive oxygen species generation. *Ecotoxicol Environ Saf* 2020;193:110348.
20. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. *Cell* 2010;140:883-99.
21. Parameswaran N, Patial S. Tumor necrosis factor- $\alpha$  signaling in macrophages. *Crit Rev Eukaryot Gene Expr* 2010;20:87-103.
22. Wang Q, He Z, Huang M, Liu T, Wang Y, Xu H, et al. Vascular NICHE IL-6 induces alternative macrophage activation in glioblastoma through HIF-2 $\alpha$ . *Nat Commun* 2018;9:559.
23. O'shea JM, Perkins ND. Regulation of the rela (p65) transactivation domain. *Biochem Soc Trans* 2008;36:603-8.
24. Suzuki J, Ogawa M, Muto S, Itai A, Isobe M, Hirata Y, et al. Novel I $\kappa$ B kinase inhibitors for treatment of nuclear factor- $\kappa$ B-related diseases. *Expert Opin Investig Drugs* 2011;20:395-405.
25. Liu TY, Chung YT, Wang PF, Chi CW, Hsieh LL. Safrole-DNA adducts in human peripheral blood-an association with areca quid chewing and cyp2e1 polymorphisms. *Mutat Res* 2004;559:59-66.
26. Su L, Zhao J, Zhao BX, Zhang SL, Miao JY. Safrole oxide induces human umbilical vein endothelial cell transdifferentiation to 5-hydroxytryptaminergic neuron-like cells through tropomyosin receptor kinase A/cyclooxygenase 2/nuclear factor-kappa B/interleukin 8 signaling. *Int J Biochem Cell Biol* 2011;43:1512-22.
27. Ni WF, Tsai CH, Yang SF, Chang YC. Elevated expression of nf-kappab in oral submucous fibrosis-evidence for nf-kappab induction by safrole in human buccal mucosal fibroblasts. *Oral Oncol* 2007;43:557-62.
28. Hinz M, Scheidereit C. The I $\kappa$ B kinase complex in NF- $\kappa$ B regulation and beyond. *EMBO Rep* 2018;15:46-61.
29. Takekawa M, Kubota Y, Nakamura T, Ichikawa K. Regulation of stress-activated map kinase pathways during cell fate decisions. *Nagoya J Med Sci* 2011;73:1-4.
30. Ren J, Su D, Li L, Cai H, Zhang M, Zhai J, et al. Anti-inflammatory effects of aureusidin in lps-stimulated raw264.7 macrophages via suppressing NF- $\kappa$ B and activating ros- and mapks-dependent Nrf2/HO-1 signaling pathways. *Toxicol Appl Pharmacol* 2020;387:114846.