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RESEARCH ARTICLE

The regulatory effect of flavonoids extracted from *Abutilon theophrasti* leaves on gene expression in LPS-induced ALI mice via the NF-κB and MAPK signaling pathways

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ABSTRACT

Context: ALI is a common disease characterized by acute pulmonary inflammatory disorder. *Abutilon theophrasti* Medik. (Malvaceae), as a Chinese traditional medicine, is used for the treatment of inflammation. Its main constituents are flavonoid compounds.

Objective: This study investigates the regulatory effect of a TFE from *Abutilon theophrasti* leaves on gene expression in LPS-induced ALI mice via the NF-κB and MAPK signaling pathways.

Materials and methods: Kunming mice were intragastrically administered TFE (0.25, 0.5, 1.0 g/kg) for 5 days, and then ALI was induced via intranasal administration 40 μg of LPS in 10 μL PBS after intragastric administration on the 5th day, and PBS and DEX (2 mg/kg) were negative and positive control groups, respectively.

Results: The relative expression of iNOS gene was 0.707, 0.507 and 0.483 for 0.25, 0.5 and 1.0 g/kg TFE, and COX-2 gene expression was also reduced after treatment by three concentrations of TFE with 0.768, 0.545, and 0.478. The mRNA expression levels of p65 were 0.61, 0.43 and 0.27 for 0.25, 0.5 and 1.0 g/kg TFE and IκB levels were increased in a dose-dependent manner with 3.99, 13.69 and 34.36. 0.5 and 1.0 g/kg TFE inhibited the expression of ERK1/2 with 0.59 and 0.38, p38MAPK with 0.62 and 0.54, and JNK with 0.37 and 0.29, and JNK mRNA expression was 0.60 for 0.25 g/kg TFE.

Discussion and conclusion: These results indicate that the regulatory mechanisms of TFE on gene expression in LPS-induced ALI mice include inhibition of the NF-κB and MAPK signaling pathways.

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Anti-inflammatory effect; regulatory mechanism; total flavonoid extract; quantitative real-time PCR; *Abutilon theophrasti*

Introduction

Acute lung injury (ALI) is usually induced by direct or indirect injury factors and is well known for its characteristic alveolar epithelial and capillary endothelial cell damage and diffuse pulmonary interstitial and alveolar edema (Jiang et al. 2016; Tian et al. 2017; Liu et al. 2018). LPS, as a main component of endotoxin, has been extensively used in ALI modeling. *Abutilon theophrasti* Medik. (Malvaceae), is a traditional herbal medicine used in treatments for inflammation, ulcers, swelling, venom, and pain (Fu and Hong 1993; Gu and Jiang 2009; Liu et al. 2010; Su et al. 2010).

It is currently recognized that the NF-κB and MAPK signaling pathways are the principal anti-inflammatory functional mechanistic pathways in LPS-induced ALI and flavonoid compounds are important chemical constituents for the suppression of inflammation by regulating cellular signaling pathways (Li et al. 2014; Yeh et al. 2014; Chu et al. 2016; Liu DD et al. 2016; Tsai et al. 2017). Luteolin (3′,4′,5,7-tetrahydroxy flavonoid) can inhibit the release of pro-inflammatory mediators, NO, inflammatory cytokines, and iNOS and COX-2 expression in PRV-infected RAW264.7 cells following NF-κB signaling pathway activation (Liu CW et al. 2016). It was reported that phloretin mechanistically attenuates the symptoms of inflammation and oxidative stress via blockade of the NF-κB and MAPK pathways in ALI mice (Huang et al. 2016). Five flavonoid components from Nepalese propolis, namely, isoliquiritigenin, chrysin, 3′,4′-dihydroxy-4-methoxydalbergione, 4-methoxydalbergion, and cearoin, exhibited anti-inflammatory activities by regulating the NF-κB pathway (Funakoshi-Tago et al. 2016). Vitexin alleviated inflammatory responses by activating important MAPK signaling pathways, such as p38, ERK1/2 and JNK (Rosa et al. 2016).

Previous studies have shown that TFE from *A. theophrasti* leaves exhibits potentially protective effects on inflammatory reactions in LPS-induced RAW 264.7 cells and ALI mice by activation of the NF-κB and MAPK signaling pathways (Tian et al. 2018, 2019). However, no evidence has been reported for the direct effect of TFE from *A. theophrasti* leaves on gene expression *in vivo*. In this paper, we investigated the regulatory effects of TFE on gene expression in LPS-induced ALI mice via NF-κB and MAPK signaling pathways and tried to reveal its possible regulatory mechanism.

Materials and methods

Chemicals and reagents

Escherichia coli lipopolysaccharide (LPS) was obtained from Sigma-Aldrich (St. Louis, MO, USA), and dexamethasone

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sodium phosphate (No.100016-201216, purity >99%) was obtained from National Institutes for Food and Drug Control. PrimeScript™ RT reagent Kit and DL2000 DNA Marker were purchased from TaKaRa (Dalian, China). TransStart® Top Green qPCR SuperMix and Trizol were obtained from TransGen Biotech (Beijing, China). All other analytical grade reagents were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

Animals

Animal experiments were carried out under the institutional guide for the Care and Use of Laboratory Animals in Shenyang Agricultural University according to the National Institutes of Health Guide for Care and Use of Laboratory Animals. Male Kunming mice (6–8 weeks) were purchased from Liaoning Changsheng Biotechnology Co Ltd. (Benxi, Liaoning, China) and fed in a specific pathogen-free animal house with a temperature of 24 ± 1 °C, 60%–65% humidity and free to access water and food.

Experimental protocol

To induce the ALI model, mice ($n = 60$, 10/group) were randomly divided into six groups: the control group, the LPS group, LPS + TFE (0.25, 0.5, 1.0 g/kg) groups and the LPS + DEX (2 mg/kg) group. Mice were intragastrically administered normal saline, TFE (0.25, 0.5, 1.0 g/kg) or DEX (2 mg/kg) for five days, and intranasally instilled with 40 μg of LPS in 10 μL PBS to induce lung injury 30 min after intragastric administration on the fifth day.

Plant materials and TFE preparation

A. theophrasti leaves were collected from KaiTong, Jilin province of China, in September of 2014 and authenticated by Botanist Prof. Shaofan Du, Shenyang Agricultural University, Shenyang, China. The voucher specimens KT/JL/CH/ATM/09/14 have been deposited 505 Herbarium of Animal pharmacy in College of Animal Husbandry and Veterinary, Shenyang Agricultural University.

The extraction and preparation of TFE from *A. theophrasti* leaves were carried out according to Tian et al. (2018), and the TFE residue was stored at 4 °C for further study.

Quantitative real-time PCR assay

Total RNA was isolated from the lungs tissue by using a Trizol reagent (Vazyme Biotech Co., Ltd., USA) according to the manufacturer's instruction. Reverse transcription was then conducted, and cDNAs were subjected to real-time PCR by using the following primers: iNOS forward, GACGAGACGGATAGGCAGAG and reverse, CACATGCAAGGAAGGGAACT; COX-2 forward, ACCAGCAGTTCCAGTATCAGA and reverse, CAGGAGGAT GGAGTTGTTGTAG; IκB forward, GCCATCCCAGGCAGTAT CTA and reverse, TTCCAAGACCAGACCTCCAG; p65 forward, GACCTGGAGCAAGCCATTAG and reverse, CACTGTCACC TGGAAGCAGA; ERK1/2 forward, GCGGCTGAAGGAGTTGAT and reverse, CAGGTAGGAGCAGGACCAGA; p38 forward, CTATGGCTCGGTGTGTGCT and reverse, GACGCAA CTCTCGGTAGGTC; JNK forward, TGTTCCCCGATGTGCTT-TT and reverse, CGTTGATGTATGGGTGCTG; β-actin forward, GTGCTATGTTGC-TCTAGACTTCG and reverse, ATGCC ACAGGATTCCATACC. After normalization to the β-actin (internal control) level, fold changes in the expression levels of target mRNAs (iNOS, COX-2, IκB, p65, ERK1/2, p38 and JNK) were computed, and the results were evaluated by using the $2^{-\triangle\triangle CT}$ method.

Statistical analysis

Data analyses were performed by SPSS 17.0 (SPSS 17.0 for WINDOWS; SPSS Inc., Chicago, IL) and the experiment results were expressed as means ± standard deviation. In all statistical analyses, p values < 0.05 were regarded as statistically significant and p values < 0.01 as very significant.

Results and discussion

Effects of TFE on iNOS and COX-2 expression levels following LPS-induced ALI

In the toxicity test, mice were given a dose of 5000 mg/kg.b.w, and there was no death in the mice, which proved that *A. theophrasti* leaves flavonoid extract was nontoxic and safe. According to the results of previous studies (Tian et al. 2018), the main flavonoid compositions in TFE were rutin, quercetin7-*O*-β-glucoside, kaempferol 3-*O*-α-rhamnopyranosyl(1 → 6)-β-glucopyranoside, luteolin and apigenin 7-*O*-β-diglucoside, and the content of rutin was the highest, which means that rutin may be the major substance basis of anti-inflammatory effect in TFE. Therefore, the anti-inflammation mechanism of TFE will be discussed and revealed on gene regulation in combination with rutin and other monomeric flavonoid compounds.

The genes expression levels of COX-2 and iNOS were very significantly ($p \leq 0.01$) concentration-dependently decreased in the 50, 100 and 200 μg/mL of TFE treated groups compared with the LPS model group in RAW 264.7 cells (Tian et al. 2018). In this study, TFE dose-dependently inhibited both iNOS and COX-2 mRNA expression in LPS-induced mice, and the results indicated that the underlying mechanism of TFE inhibition of the inflammatory mediators, iNOS and COX-2, occurred at the transcription level.

The mRNA expression levels of iNOS (Figure 1) and COX-2 (Figure 2) were regulated by TFE in LPS-induced ALI. The relative expression level of iNOS gene was 0.707, 0.507 and 0.483, and significantly inhibited ($p < 0.01$) by TFE (0.25, 0.5 and 1.0 g/kg) and Dex (2 mg/kg) with 0.411 compared with the LPS group in a dose-dependent manner (Figure 1). Additionally, compared

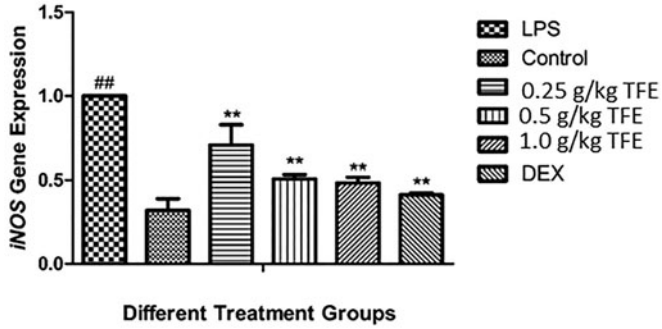

Figure 1. Effects of TFE on LPS induced iNOS gene expression in lung tissues of LPS-induced ALI. (#compared with the control, *compared with LPS, $^{*}p < 0.05$, $^{**/\#\#}p < 0.01$).

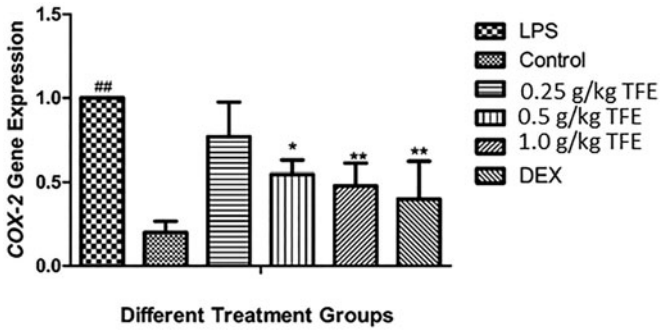

Figure 2. Effects of TFE on LPS induced COX-2 gene expression in lung tissues of LPS-induced ALI. (#compared with the control, *compared with LPS, $^{*}p < 0.05$, $^{**/\#\#}p < 0.01$).

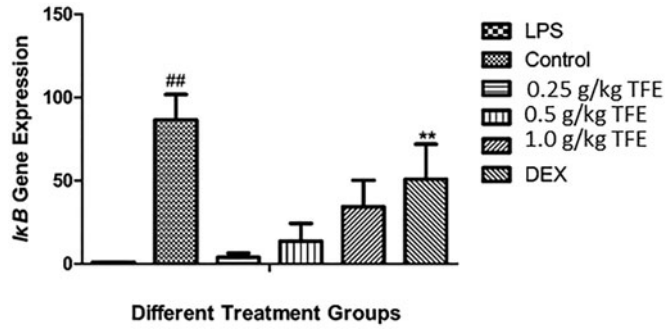

Figure 3. Effects of TFE on LPS induced IκB gene expression in lung tissues of LPS-induced ALI. (#compared with the control, *compared with LPS, $^{**/\#\#}p < 0.01$).

with that in the LPS group, the relative COX-2 gene expression was also reduced after treatment with 50, 100 and 200 μg/mL of TFE with 0.768, 0.545, and 0.478 or Dex with 0.399. Specifically, significant differences were observed between the high-dose and middle-dose TFE groups and the LPS groups ($p < 0.01$, and $p < 0.05$, respectively); however, no significant differences were observed between the low-dose TFE and LPS groups ($p > 0.05$, Figure 2).

iNOS and COX-2 proteins levels were decreased in a concentration dependent manner after treated by 0.25, 0.5 and 1.0 g/kg of TFE compared with LPS group in ALI mice (Tian et al. 2019). The results of the real-time fluorescence quantitative PCR experiments indicated that the TFE treatment reduced iNOS and COX-2 mRNA levels. This means that TFE can down regulate iNOS and COX-2 mRNA expression levels, which are consistent with previous studies. These results indicate that TFE may exhibit anti-inflammatory activity by regulating the transcription levels of the inflammatory mediators, NO and COX-2.

Effect of TFE on LPS-induced NF-κB signaling pathway activation

The effect of TFE on the mRNA expression and the proteins phosphorylation levels of IκB and p65 were evaluated with LPS-induced RAW 264.7 cells, and TFE can very significantly ($p \leq 0.01$) suppress mRNA expression of the p65 gene, and increases IκB gene expression and significantly reduces the expression of p-IκB and p-p65 by treatment with 50, 100 and 200 μg/mL compared with the LPS treatment group (Tian et al. 2018). In addition, the phosphorylation levels of p65 and IκB proteins were very significantly ($p < 0.01$) or significantly($p < 0.05$) inhibited by 1.0 or 0.5 g/kg TFE in LPS induced ALI mice (Tian et al. 2019).

IκB and p65 mRNA expression levels were measured by real-time fluorescence quantitative PCR to investigate the regulatory effects on the NF-κB signaling pathway. Pretreatment with TFE (0.25, 0.5 and 1.0 g/kg) increased the IκB mRNA expression levels (Figure 3) and reduced the p65 mRNA expression levels (Figure 4).

We found that p65 mRNA expression was significantly reduced ($p < 0.01$) following TFE treatment (0.25, 0.5 and 1.0 g/kg). Moreover, the IκB gene expression levels improved in a concentration-dependent manner after LPS-induction compared with the LPS model group, although no significant differences between the three dose groups and the LPS group were observed ($p > 0.05$). These results indicate that TFE possibly exerted its anti-inflammatory effect by upregulating the IκB mRNA

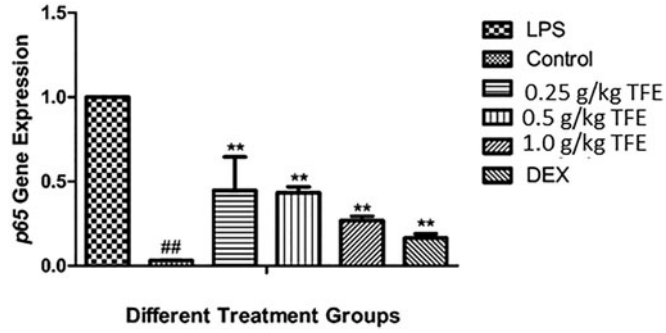

Figure 4. Effects of TFE on the p65 gene expression in NF-κB signaling pathway in lung tissues of LPS-induced ALI. (#compared with the control, *compared with LPS, $^{*/\#\#}p < 0.01$).

expression levels and down regulating the p65 mRNA expression levels in the NF-κB signaling pathway in LPS-activated ALI mice.

The p65 mRNA expression level was 0.61, 0.43, 0.27 and 0.16 after pretreatment with 0.25, 0.5 and 1.0 g/kg TFE and Dex, and decreased significantly ($p < 0.01$); on the contrary, the relative expression level of IκB gene was 3.99, 13.69 and 34.36, and increased in a dose-dependent manner, although no significant differences were noted ($p > 0.05$) compared with the LPS model group. In the NF-κB signaling pathway, the regulatory effect of TFE on p65 mRNA expression levels was more remarkable than on IκB levels.

Effect of TFE on LPS-induced MAPK signaling pathway activation

MAPK is another important signaling pathway that modulates the inflammatory process. The mRNA expression levels of ERK1/2, p38 and JNK were all significantly suppressed by 50, 100 and 200 μg/mL of TFE compared with LPS alone treatment group, and their phosphorylation proteins levels were also significantly inhibited by TFE in LPS-stimulated RAW 264.7 cells (Tian et al. 2018). It revealed further that TFE could inhibit LPS-induced phosphorylation of three major proteins in MAPK signaling pathway with a dose-dependent manner in ALI mice and the phosphorylation levels of ERK1/2 were very significantly ($p < 0.01$) inhibited by 1.0 g/kg TFE. In addition, P-p38MAPK and P-JNK expressions were significantly ($p < 0.05$) decreased by 1.0 g/kg TFE (Tian et al. 2018).

Therefore, an anti-inflammatory mechanism of TFE was revealed further by analyzing the mRNA expression levels of

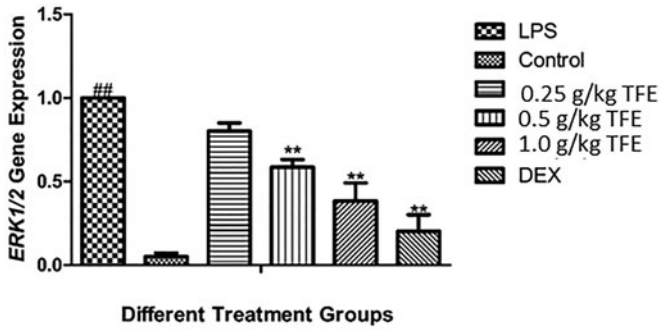

Figure 5. Effects of TFE on the ERK1/2 gene expression in MAPK signaling pathway in lung tissues of LPS-induced ALI. (#compared with the control, *compared with LPS, $^{**/\#\#}p < 0.01$).

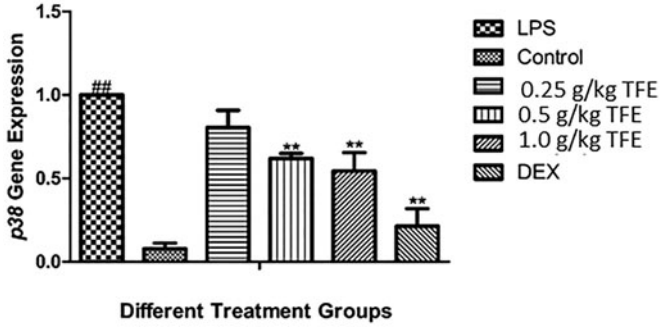

Figure 6. Effects of TFE on the p38α gene expression in MAPK signaling pathway in lung tissues of LPS-induced ALI. (#compared with the control, *compared with LPS, $^{**/\#\#}p < 0.01$). (#compared with the control, *compared with LPS, $^{**/\#\#}p < 0.01$).

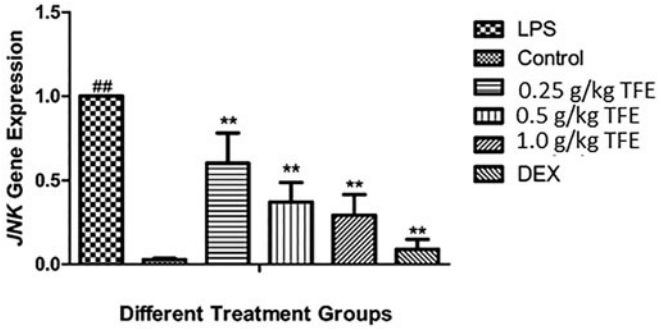

Figure 7. Effects of TFE on the JNK gene expression in MAPK signaling pathway in lung tissues of LPS-induced ALI. (#compared with the control, *compared with LPS, $^{**/\#\#}p < 0.01$).

ERK1/2 (Figure 5), p38MAPK (Figure 6), and JNK (Figure 7) at 6 h after LPS treatment. We found that 0.5 and 1.0 g/kg TFE significantly inhibited ($p < 0.01$) the mRNA expression of ERK1/2 with 0.59 and 0.38, p38MAPK with 0.62 and 0.54, and JNK with 0.37 and 0.29, and comparing to the LPS group, JNK mRNA expression was 0.60, and significantly ($p < 0.01$) reduced after pretreatment with 0.25 g/kg TFE (Figure 7). However, there was no significant differences for ERK1/2 with 0.80 (Figure 5) and p38MAPK with 0.80 ($p > 0.05$, Figure 6). The regulatory mechanism of TFE on the MAPK signaling pathway was elucidated by evaluating the mRNA expression of p38α, JNK and ERK1/2 in LPS-induced ALI mice.

The results showed that TFE could inhibit the transcriptional enhancement of IκB, p65, ERK1/2, p38MAPK and JNK genes in NF-κB and MAPK signaling pathways in a dose-dependent manner in LPS-induced ALI mice. Base on the above experimental results, TFE can significantly inhibit inflammatory responses by regulating transcription of important genes in the NF-κB and MAPK signaling pathways.

Conclusions

The present study provides evidence that TFE from *A. theophrasti* leaves can exhibit therapeutic effects against LPS-induced ALI by diminishing the mRNA expression of the pro-inflammatory mediators, iNOS and COX-2, as well as upregulating IκB mRNA expression levels and downregulating p65, p38α, JNK and ERK1/2 mRNA expression levels in the NF-κB and MAPK signaling pathways in LPS-activated ALI mice. These results strongly suggest that TFE is a natural and healthy anti-inflammatory agent that can regulate inflammatory responses.

Disclosure statement

No potential conflict of interest was reported by the authors.

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References

Chu CJ, Yao S, Chen JL, Wei XC, Xia L, Chen DF, Zhang J. 2016. *Eupatorium lindleyanum* D.C. flavonoids fraction attenuates lipopolysaccharide-induced acute lung injury in mice. Int Immunopharmacol. 39: 23–33.

Fu CD, Hong YF. 1993. Research on chemical composition of *Abutilon theophrasti* Medic. Foreign Med Sci. 15:4–7.

Funakoshi-Tago M, Ohsawa K, Ishikawa T, Nakamura F, Ueda F, Narukawa Y, Kiuchi F, Tamura H, Tago K, Kasahara T. 2016. Inhibitory effects of flavonoids extracted from Nepalese propolis on the LPS signaling pathway. Int Immunopharmacol. 11:550–560.

Gu GY, Jiang Y. 2009. Research on chemical composition and pharmacological action of *Abutilon indicum* and *Abutilon*. Modern Pharm Clin. 24: 338–340.

Huang WC, Lai CL, Liang YT, Hung HC, Liu HC, Liou CJ. 2016. Phloretin attenuates LPS-induced acute lung injury in mice via modulation of the NF-κB and MAPK pathways. Int Immunopharmacol. 40:98–105.

Jiang WJ, Luo F, Lu QF, Liu JY, Li PJ, Wang XF, Fu YL, Hao K, Yan TH, Ding XS. 2016. The protective effect of trillin LPS-induced acute lung injury by the regulations of inflammation and oxidative state. Chem Biol Interact. 243:127–134.

Li C, He J, Gao Y, Xing Y, Hou J, Tian J. 2014. Preventive effect of total flavones of choerospondias axillaries on ischemia/reperfusion-induced myocardial infarction-related MAPK signaling pathway. Cardiovasc Toxicol. 14(2):145–152.

Liu DD, Cao G, Han LK, Ye YL, SiMa YH, Ge WH. 2016. Flavonoids from *Radix Tetrastigmae* inhibit TLR4/MD-2 mediated JNK and NF-κB pathway with anti-inflammatory properties. Cytokine. 8:29–36.

Liu CW, Lin HW, Yang DJ, Chen SY, Tseng JK, Chang TJ, Chang YY. 2016. Luteolin inhibits viral-induced inflammatory response in RAW264.7 cells via suppression of STAT1/3 dependent NF-κB and activation of HO-1. Free Radic Biol Med. 95:180–189.

Liu H, Ni SF, Kang JH, Luo RF, Wu YF, Cui YT, Li ZX. 2010. The pharmaceutical research situation of plants of *Abutilon*. Northwest Pharm J. 25: 68–69.

Liu L, Xu D, Liu P, Liu FY, Dai LQ, Yan H, Wen FQ. 2018. Effects of calcium gluconate on lipopolysaccharide-induced acute lung injury in mice. Biochem Biophys Res Commun. 503(4):2931–2935.

Rosa SIG, Rios-Santos F, Balogun SO, Martins DTDO. 2016. Vitexin reduces neutrophil migration to inflammatory focus bydown-regulating pro-

inflammatory mediators via inhibition of p38, ERK1/2 and JNK pathway. Phytomedicine. 23(1):9–17.

Su LJ, Yang LH, Zhang XM, Zhang WP. 2010. Antiinflammatory analgesic active site research of the stems and leaves of *Abutilon theophrasti* Medic. Chin Sci Tech Med. 17:314.

Tian CL, Zhang P, Yang CX, Gao X, Wang H, Guo YR, Liu MC. 2018. Extraction process, component analysis, and in vitro antioxidant, antibacterial, and anti-inflammatory activities of total flavonoid extracts from *Abutilon theophrasti* Medic. Leaves. Mediat Inflamm. 2:1–17.

Tian CL, Zhang P, Yang J, Zhang ZH, Wang H, Guo YR, Liu MC. 2019. The protective effect of the flavonoid fraction of *Abutilon theophrasti* Medic. leaves on LPS-induced acute lung injury in mice via the NF-κB and MAPK signalling pathways. Biomed Pharmacother. 109:1024–1031.

Tian L, Li WM, Wang T. 2017. Therapeutic effects of silibinin on LPS-induced acute lung injury by inhibiting NLRP3 and NF-κB signaling pathways. Microb Pathogen. 108:104–108.

Tsai Y-F, Chu T-C, Chang W-Y, Wu Y-C, Chang F-R, Yang S-C, Wu T-Y, Hsu Y-M, Chen C-Y, Chang S-H, Hwang T-L. 2017. 6-Hydroxy-5,7-dimethoxy-flavone suppresses the neutrophil respiratory burst via selective PDE4 inhibition to ameliorate acute lung injury. Free Radic Biol Med. 106:379–392.

Yeh C-H, Yang J-J, Yang M-L, Li Y-C, Kuan Y-H. 2014. Rutin decreases lipopolysaccharide-induced acute lung injury via inhibition of oxidative stress and the MAPK- NF-κB pathway. Free Radic Biol Med. 69: 249–257.