Original Article

Taxifolin attenuates inflammation via suppressing MAPK signal pathway in vitro and in silico analysis

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A B S T R A C T

Objective: Taxifolin is a natural flavonoid compound that can be isolated from onions, grapes, oranges and grapefruit. It also acts as a medicine food homology with extraordinary antioxidant and anti-inflammatory activity. This study aims to explain the protective effects and potential mechanisms of taxifolin against inflammatory reaction.

Methods: Levels of interleukin (IL)-6, IL-1β and intracellular reactive oxygen species (ROS) were assessed in different time after the treatment of taxifolin in RAW264.7 cells induced by lipopolysaccharide (LPS). Subsequently, the mRNA and protein levels of inducible nitric oxide synthase (iNOS), vascular endothelial growth factor (VEGF), cyclooxygenase (COX)-2, tumor necrosis factor (TNF)-α and the phosphorylation expression levels of the MAPK signal pathway were also evaluated. A silico analysis was used to explain the binding situation for the investigation of taxifolin and MAPK signal pathway. And then MAPK inhibitors were used to reveal the expression level of iNOS, VEGF, COX-2 and TNF-α in RAW264.7 cells.

Results: It was demonstrated that cell inflammatory damage induced by LPS was significantly alleviated after the treatment of taxifolin. Then, the mRNA and protein levels of iNOS, VEGF, COX-2 and TNF-α were reduced and the phosphorylation expression levels of the MAPK signal pathway were down-regulated remarkably as well. In silico analysis, taxifolin could form a relatively stable combination with MAPK signal pathway. And then MAPK inhibitors were used to reveal the expression level of iNOS, VEGF, COX-2 and TNF-α in RAW264.7 cells.

Conclusion: This finding demonstrated that taxifolin improved the inflammatory responses that partly involved in the phosphorylation expression level of MAPK signal pathway in RAW264.7 cells exposed to acute stress.

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1. Introduction

Flavonoids are the plant-derived polyphenol components in most traditional Chinese medicine and possess remarkable anti-inflammation capacity (Nile, Keum, Nile, Jalde, & Patel, 2017; Sudhakaran, Sardesai, & Doseff, 2019). Taxifolin is one of natural plant flavonoids that has been discovered in various plants including grapes, oranges (Wang et al., 2009). As a single compound, taxifolin is an important dietary supplement and used as functional food in general. It exhibits the anti-inflammation and protection effects by scavenging over production of reactive oxygen species (ROS) against oxidative stress (Akinmoladun, Olaniyan, Famusiwa, Josiah, & Olaleye, 2020). Taxifolin can promote the differentiation of osteoblast and prevent the forming of osteoclasts in vitro and in vivo models (Cai et al., 2018). In this study, taxifolin
inhibited the nuclear factor-κB (NF-κB) activation, mitogen-activated protein kinase (MAPK), and decreased Trap, MMP-9, Cathepsin K, C-Fos, Nfatc1, and RANK expression; Taxifolin suppressed the osteoclast activity by improving the bone loss and decreased the levels of interleukin-6 (IL-6), IL-1β, receptor activator of nuclear factor-κB ligand (RANKL) in ovariectomized-induced mice, and the protection effects of taxifolin in LPS-induced bone lysis mouse model have been verified via NF-κB signaling pathway (Zhang, Wang, Yang, Gao, & Tang, 2019). Additionally, Teselkin et al. (2000) founds that taxifolin is more antioxidant effective in rats with tetrachloromethane hepatitis.

Generally, inflammation is not a primary cause of pathological phenomena but plays a considerable part in development of physiological disorder and diseases. Cytokines play a crucial role in the immune system and the inflammatory response. Among them, excess density of nitric oxide (NO) is believed to be responsible for the synthet by nitric oxide synthase (NOS) and is a proinflammatory factor that plays a vital role in miscellaneous models of inflammatory cells and organisms (Roy, Saqib, Wary, & Baig, 2020; Tejero, Hunt, Santolini, Lehnert, & Stuehr, 2019). Inducible nitric oxide synthase (iNOS) is one of three homologous enzymes that expressed only following induction by inflammatory mediators such as TNF-α, IL-1 or LPS (Fischmann et al., 1999). The effects of nitric oxide in immune regulation can be exerted through multiple mechanisms. Cyclooxygenase-2 (COX-2) is known as the part of regulating prostaglandins (PGs) in many pathological and physiological activities (Benelli, Venè, & Ferrari, 2018; Ristimäki, 2004) and can be induced by an inflammatory stimulus and then produce high protein concentrations of COX-2 (de Vries, 2006). In many inflammatory disorders, COX-2 overexpression is associated with physiological disorder and diseases. Cytokines play a crucial role in the inflammatory cells and organisms (Roy, Saqib, Wary, & Baig, 2020; Tejero, Hunt, Santolini, Lehnert, & Stuehr, 2019). Inducible nitric oxide synthase (iNOS) is one of three homologous enzymes that expressed only following induction by inflammatory mediators such as TNF-α, IL-1 or LPS (Fischmann et al., 1999). The effects of nitric oxide in immune regulation can be exerted through multiple mechanisms. Cyclooxygenase-2 (COX-2) is known as the part of regulating prostaglandins (PGs) in many pathological and physiological activities (Benelli, Venè, & Ferrari, 2018; Ristimäki, 2004) and can be induced by an inflammatory stimulus and then produce high protein concentrations of COX-2 (de Vries, 2006). In many inflammatory disorders, COX-2 overexpression is associated with adverse reactions including pain and fever that can warn patients take care. After the inflammation is cured, COX-2 expression levels are reduced to baseline again. Another important factor vascular endothelial growth factor (VEGF) has connection with inflammatory disorders, and overexpression of VEGF can be detected in chronic and acute inflammation (Ferrara, Gerber, & Lecoutre, 2003; Li et al., 2020). Tumor necrosis factor-α (TNF-α) is one of classic pleiotropic cytokines that induces manifest diseases by intermediating different pathways and leading the expression of other cytokines. Immune cells, mast cells, endothelial cells, neurons, adipose tissue and fibroblasts all can produce TNF-α (Pandi, Jain, Raju, & Khan, 2017).

LPS-induced inflammatory cell model is a commonly used method in vitro. In RAW264.7 cells, the production of NO, COX-2, VEGF and TNF-α can highly express when using LPS as the stimulus and play considerable roles in the development of diseases and disorders (Du et al., 2020; Li et al., 2020). Hence, the present study is to investigate the preventive effects and molecular mechanisms of taxifolin against inflammation in RAW264.7 cells induced by LPS.

2. Materials and methods

2.1. Chemicals

Taxifolin that used for cell treatment were kindly supplied by Professor Fujun Zhou from Tianjin Institute of Pharmaceutical Research Co., Ltd. LPS and filtrated and sterilized phosphate buffer saline (PBS, pH 7.2–7.4) were obtained from Solarbio Science Technology Co., Ltd. (Beijing, China). The RAW264.7 cells (Cat No. CL-0190) were purchased from Procelf Life Science Technology Co., Ltd. (Wuhan, China); Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS) and streptomycin-penicillin mixture (100-fold) were all purchased from Gibco Life Technologies (New York, USA). U0126 (ERK inhibitor), SP600125 (JNK inhibitor) and SB203580 (p38 inhibitor) were purchased from MCE (Shanghai, China distributors). Dexamethasone (DXMS) was obtained from Shanghai Yuanye Bio-Technology Co., Ltd (Shanghai, China).

2.2. Cell culture and treatment

The culture medium for murine RAW 264.7 cells was DMEM added with 10% FBS and 100 U/ml penicillin and 100 μg/ml streptomycin that cultured in the humidified atmosphere containing 5% CO2 and keeping temperature at 37 °C. After being regular subcultured 2–3 generations, for all experiments, RAW264.7 cells were resuspended and seeded with complete culture medium into 6, 24, 96-well plates at the density of 4 × 10^3, 1 × 10^4, 5 × 10^4 cells/well, respectively. After overnight incubation, prior to LPS treatment, taxifolin at different concentrations (20, 40 and 80 μmol/L), DXMS (5 μmol/L), U0126 (2 μmol/L, 9 h), SB203580 (25 μmol/L, 4 h), SP600125 (10 μmol/L, 4 h) were added to the DMEM medium with 5% FBS, respectively. Then, cells were processed by LPS (200 ng/ml) treatment for another 24 h and the culture medium was replaced with DMEM without FBS. In the end, the cells or cultural supernatants were collected for the next step determination. Additionally, controls received no treatment, and models were treated with LPS only.

2.3. Cell viability analysis

After treating RAW 264.7 cells without or with the tested compounds, the cell viability was determined using MTS assay kit (Bio Vision, USA). In brief, cells were added 20 μL MTS reagent into each well and incubated for 3 h at 37 °C; each concentration was set in parallel with six multiple samples. After that, the 96-well plates were shaking on a shaker briefly and measured the absorbance at 490 nm on a microplate reader (SpectraMax M5, USA), Lastly, the relative cell viability was calculated as Eq. (1):

$$RV = \frac{(A_t - A_b)}{(A_c - A_b)} \times 100\% \quad (1)$$

where A_t is the absorbance of tested compounds, A_b is the absorbance of blank (PBS), A_c is the absorbance of controls.

2.4. Detection of intracellular ROS

After the treatment, the culture medium was aspirated and the cells was incubated with 2’,7’-dichlorodihydrofluorescein diacetate (DCFH-DA) in the dark circumstance for 20 min at 37 °C, according to manufacturer’s guidelines (Beyotime, Shanghai, China). In parallel, Rosup was set as the positive control. Then, using FBS-free DMEM washed the cells three times, gently. After that, cells were resuspended with PBS and the cells fluorescence intensity (λ ex = 488 nm; em = 525 nm) was detected by the fluorescence microplate reader. The intensity of DCF fluorescence will reflect on the level of intracellular ROS, where regarded cells without treatment as the calculation default of 1.

2.5. NO release assay

According to the manufacturer’s instructions, the NO release can be detected by Griess reaction method (Beyotime, Shanghai, China). Each 50 μl cultural supernatant sample was added equal amount of Griess I reagent, mixed absolutely and then 50 μl Griess II reagent was added with thorough mixing, incubated for 10 min at room temperature and protected from light. Set the microplate reader under the option absorbance (540 nm), afterwards, the absorbance of every well was detected. NaNO2 standard solutions were used for correction the absorbance, so the released NO proteins were counted.
2.6. Quantitative sandwich enzyme immunoassay (ELISA)

Culture supernatants were assayed for IL-1β, IL-6, TNF-α, COX-2 and VEGF by ELISA kit (Westang Bio-Tech Co., Ltd, Shanghai, China) following the manufacturer’s instructions. The absorbance at 450 nm was measured by the microplate reader.

2.7. Reverse transcription and quantitative real-time PCR

After treatment, total RNA was extracted using TRIzol™ reagent (Invitrogen, Carlsbad, California, USA) and the expression levels of TLR4, MyD88, IL-1β, IL-6, iNOS, TNF-α, COX-2 and VEGF were analyzed by qRT-PCR and the procedure was according to the protocol of the manufactures. RNA samples (2 μg) were reverse transcribed using Transcriptor First Strand cDNA Synthesis Kit and the cDNA was amplified by using FastStart Universal SYBR Green Master (RoX) (Roche, Basel, Switzerland). The sequences of these primers were listed in Table 1. Magnification conditions were 95 °C for 2 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Relative gene expression levels were calculated by the second derivative maximum analysis by using the 2−ΔΔ Ct equation with results standardized to endogenous reference mRNA levels of β-actin. The equation was as Eq. (2):

\[ 2^{-\Delta\Delta Ct} = 2^{-(Ct_{1} - Ct_{2}) - (Ct_{3} - Ct_{4})} \]  

(2)

In above-mentioned calculation equation, the gene expression level in the control samples (without treatment) was set as default (numerical values were 1). Ct1 showed the cycles of tested gene in the control samples when its magnification curve achieved to the threshold value of fluorescence signal, Ct2 indicated the cycles of tested gene in treatment groups when its magnification curve achieved to the threshold value of fluorescence signal; Ct1 showed the cycles of tested gene in the parallel group samples when its magnification curve achieved to the threshold value of fluorescence signal. Ct1 and Ct2 were the cycles of β-actin in the parallel group samples where its magnification curve achieved to the threshold value of fluorescence signal.

2.8. Western blot analysis

The protein was extracted from cultured cells and separated by SDS-PAGE and transferred to PVDF membranes. The membranes were incubated with different primary antibodies at 4 °C overnight and then incubated with secondary antibodies for 2 h at room temperature. Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (affinity, S0002, 1:5000) was used to detect proteins by ultra-sensitive chemiluminescence (ECL) Star reagents (Beyotime, Shanghai, China). The images were visualized by the FluorChem R (ProteinSimple, Silicon Valley, San Francisco, USA) and analyzed by the NIH imaging program (ImageJ). Primary antibodies used were: β-actin (affinity, AB_2839420, 1:1000), ERK (GeneTex, GTX17942, 1:500), phospho-ERK1/2 (GeneTex, GTX24819, 1:200), JNK (GeneTex, GTX52360, 1:500), phospho-JNK (abcam, ab124956, 1:400), p38 (GeneTex, GTX110720, 1:500) and phospho-p38 (GeneTex, GTX133460, 1:500).

2.9. Molecular docking simulation study

2.9.1. Protein preparation

The docking crystal structure of ERK (PDB ID: 2QJG), JNK (PDB ID: 2P33), p38α (PDB ID: 2BAL) were all retrieved from protein data bank (https://www.rcsb.org). The structures with added hydrogen and deleted water molecules were prepared by the protein preparation wizard of Schrödinger Maestro software, after removing the ligands then the grids of binding sites were generated by setting various volumes of grid boxes using receptor grid generation module.

2.9.2. Ligands preparation

The 3D structure of taxifolin and all the primary ligands in the protein crystals were downloaded from PubChem (https://pubchem.ncbi.nlm.nih.gov/). Then uploading all 3D structures of ligands to Maestro and the ligprep work module was operated for adding hydrogen and deleting water molecules and produced the different conformation of each ligand. The stable conformer of each ligand with minimum potential energy was further processed.

2.9.3. Protein ligand docking

Each ligand was docked into the circled binding site of certain protein grid by running standard docking procedure (SP) in the docking module, and retained the lowest binding pose of every docking step. Finally, the outcomes of docking were analyzed using glide XP visualizer, the active site interaction scores along with binding energies were selected to rank multiple binding conformations.

2.10. Statistical analysis

Results were expressed as means ± standard error of mean (SEM). The independent samples t-test was used for comparisons. A P value of <0.05 was considered statistically significant.

3. Results

3.1. Cytotoxicity of taxifolin in RAW 264.7 cells

Taxifolin (2R,3R)-3,3',4',5,7-pentahydroxyflavanone) is a flavonoid compound containing an ortho phenolic hydroxyl group (Fig. 1A). The effect of taxifolin on the viability of RAW264.7 cells was assessed by using an MTS assay kit. Taxifolin was dissolved in DMSO, so the DMSO vehicle as the control group was chose to compare the differences among these groups containing DMSO, and the final highest concentration of DMSO in the medium was controlled at 0.4%, which suggesting that DMSO has no cytotoxicity in RAW 264.7 cells (Fig. 1B). What’s more, there was no obvious cytotoxicity that observed in this cells model when the taxifolin concentration was lower than 80 μmol/L. Afterwards, to determine the effect of taxifolin on LPS-induced cell viability, cells were treated with taxifolin (20, 40, 80 μmol/L), obviously, the cell viability

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Table 1

| Genes   | Primer sequences                      |
|---------|---------------------------------------|
| TLR4    | AGCTTCTCGAATTTTTGACGACCTTC            |
|         | TCAGAGGTCTGTAAGGCCATGC                |
| MyD88   | ACGTCTGCTGTCATCCATCGGCA               |
|         | GCTGAGTCGCAAACTCGTCTGG                |
| IL-1β   | TGAGCTTCCAGGAGTCTGACGA                |
|         | GCCATCTGAGATGAGATGTCG                 |
| IL-6    | CTGCAAGTCATCGATCTGGTC                 |
|         | GAGAGCAGGGAGGATCTGACAGG               |
| iNOS    | CAGCAGTAGTTGCTCTCTCT                 |
|         | GAGCAGCAGGGAGGATCTGACAGG             |
| TTN-α   | GGCTGTGCTATCTGGAGGCTCTT               |
|         | GCCATAGAACGAGTGGAGGAC                 |
| COX-2   | AGTTGACCTGGAGGATCTGTC                 |
|         | GCCATAGAACGAGTGGAGGAC                 |
| VEGF    | CTGCAGTGGAGGATCTGTC                  |
|         | GCTGAGGTCTGTAAGGCCATGC               |
| β-actin | TCTCAGGAGGCTGACAGTC                 |
|         | GCTGAGGTCTGTAAGGCCATGC               |
was significantly reduced to 83.67% by using 200 ng/mL LPS. Not surprisingly, the decreased cell survival rates were attenuated after the treatment of taxifolin in a dose-dependent manner for 24 h (as shown in Fig. 1C). In summary, the treatment with taxifolin effectively prevented cell injury.

3.2 Taxifolin moderated LPS-mediated pro-inflammation cytokine expression

We further explored the effect of taxifolin on LPS-mediated pro-inflammation cytokine expression levels. Our results demonstrated that after 24 h exposed of 200 ng/mL LPS, the mRNA levels of TLR4 and MyD88 were more than 5.51- and 4.82-fold, respectively, compared to that in cells without treatment, while mRNA levels of TLR4 and MyD88 were markedly decreased to 89% and 92% (20 μmol/L), 56% and 77% (40 μmol/L), 8% and 73% (80 μmol/L) in cells treated with taxifolin separately, compared with cells treated with LPS (Fig. 2A and B).

What’s more, as indicated in Fig. 3A, the intracellular ROS fluorescence intensity after the stimulation of LPS was significantly different from that of the control cell group (2.78-fold). Taxifolin 20, 40 and 80 μmol/L dose groups had the reduction of ROS intensity with a dose-dependent manner. Additionally, IL-1β, IL-6 protein and mRNA levels were significantly accumulated when cells were treated with LPS. Compared to model group, the concentration of IL-1β decreased and it can be reduced to 84%, 82% and 74% at 24 h, respectively (Fig. 3B); The expression level of IL-1β mRNA decreased with increasing dose of taxifolin treatment (Fig. 3D). As shown in Fig. 3C, compared to model group, after 24 h of treatment, a significant difference existed in all taxifolin dose groups; And IL-6 mRNA level decreased with a dose-dependent manner of taxifolin (Fig. 3E). These results suggested that after administration, taxifolin exhibits remarkably effective anti-inflammatory value in activated RAW264.7 cells.

3.3 Taxifolin moderated generation of NO, VEGF, TNF-α and COX-2

We investigated whether taxifolin can down-regulate the expression levels of NO, VEGF, TNF-α and COX-2. As interpreted in Fig. 4A, NO secretion level was induced markedly higher to 75.8-fold by the treatment of LPS for 24 h, compared to that in cells without treatment. Taxifolin (20, 40, 80 μmol/L) could reduce the varying degrees of NO expression that the reduction was about 66%, 71% and 77%, respectively. As shown in Fig. 4B–D, after the treatment of 200 ng/mL LPS, VEGF, TNF-α and COX-2 production level increased approximately 1.8-, 1.3- and 1.6-fold, respectively. Meanwhile, compared to cells treated with LPS alone, after treated with taxifolin, VEGF, TNF-α and COX-2 secretion level was obviously reduced in an increasing dose-dependent manner. Moreover, iNOS, VEGF, TNF-α and COX-2 mRNA expression level also lessened significantly with the increasing dose of taxifolin (as shown in Fig. 4E–H). From above results, it was not difficult to infer that taxifolin could remit inflammation response by reducing the production of these important inflammatory cytokines.

3.4 Molecular docking between taxifolin and MAPK signal pathway

Next, we chose molecular docking analysis to illustrate the potent binding pattern between taxifolin and Mitogen-activated

Fig. 1. Taxifolin alleviated LPS-induced cell injury. (A) Molecular structure of taxifolin. (B) Cell viability after treatment with taxifolin at different concentrations for 24 h. (C) Cell viability after treatment with LPS or LPS + taxifolin for 24 h. The data were representative of six independent experiments and were presented as the means ± SEM. *P < 0.05, **P < 0.01 vs control; *P < 0.05 vs model.

Fig. 2. Effect of taxifolin on TLR4 activation. (A) Relative TLR4 mRNA expression level. (B) Relative MyD88 mRNA expression level. Cells were treated with LPS (24 h) or LPS + Taxifolin (24 h) and gene expression of TLR4 and MyD88 was quantified by real-time RT-PCR. The data were representative of three independent experiments and were presented as the means ± SEM. **P < 0.01 vs control; *P < 0.05, **P < 0.01 vs model.
Fig. 3. Taxifolin inhibited LPS-induced ROS production and expression levels of IL-1β, IL-6. (A) Intracellular ROS fluorescence intensity. (B) Concentrations of IL-1β in culture medium. (C) Concentrations of IL-6 in culture medium. (D) Relative IL-1β mRNA expression level. (E) Relative IL-6 mRNA expression level. RAW264.7 cells were treated with LPS (24 h) or LPS (24 h) + taxifolin (24 h). The expression of intracellular ROS was detected by ROS assay kit, the concentration of IL-1β and IL-6 in cell supernatant was detected by ELISA and gene expression of IL-1β and IL-6 was quantified by real-time RT-PCR. The data were representative of three independent experiments and were presented as the means ± SEM. **P < 0.01 vs control; *P < 0.05, ***P < 0.01 vs model.

Fig. 4. Taxifolin inhibited LPS-induced iNOS, VEGF, TNF-α and COX-2 activation. (A) NO density in culture medium. (B) Concentrations of VEGF in culture medium. (C) Concentrations of TNF-α in culture medium. (D) Concentrations of COX-2 in culture medium. (E) Relative iNOS mRNA expression level. (F) Relative VEGF mRNA expression level. (G) Relative TNF-α mRNA expression level. (H) Relative COX-2 mRNA expression level. RAW264.7 cells were treated with LPS (24 h) or LPS + Taxifolin (24 h). The expression of NO was detected by NO assay kit, the concentration of VEGF, TNF-α and COX-2 in cell supernatant was detected by ELISA and gene expression of iNOS, VEGF, TNF-α and COX-2 was quantified by real-time RT-PCR. The data were representative of three independent experiments and were presented as the means ± SEM. #P < 0.05, ##P < 0.01 vs control; *P < 0.05, **P < 0.01 vs model.
protein kinases (MAPKs) signal pathway (ERK, JNK, p38α). As exhibited in Fig. 5 and Table 2, we found that each of three proteins were docked with both the molecules, taxifolin as well as their own ligand. In compare to taxifolin, the original ligand (19A) in the ERK crystal structure showed more confirmations but taxifolin was observed to has more efficient docking score alongside with glide energy, moreover, taxifolin showed the same hydrogen bonds involvement as its original ligand (Fig. 5A and B). Two identical hydrogen bonds (Met 149 and Asn 152) were present in the hydrogen bonds formed by both ligands, taxifolin and J07, with the JNK crystal protein (Fig. 5C and D), but DXMS did not combine well with JNK crystal protein (Supplementary data 1). And two hydrogen bonds (His 107 and Met 109) formed by taxifolin with p38α crystal protein, consistent with the hydrogen bond formed by PQA and DXMS (Fig. 5E and F and Supplementary materials). These data indi-

![Fig. 5. Binding-interaction analysis of different ligands with crystal structure of ERK (PDB ID: 2OJG), JNK (PDB ID: 2P33) and p38α (PDB ID: 2BAL). (A) Analysis of taxifolin with crystal structure of ERK. (B) Analysis of 19A (the original ligand) with crystal structure of ERK. (C) Analysis of taxifolin with crystal structure of JNK. (D) Analysis of J07 (the original ligand) with crystal structure of JNK. (E) Analysis of taxifolin with crystal structure of p38α. (F) Analysis of PQA (the original ligand) with crystal structure of p38α.](image)

| Protein names | Protein codes | Ligands | Docking scores | Glide energy | H-bonds       |
|---------------|---------------|---------|----------------|--------------|---------------|
| ERK           | 2OJG          | taxifolin | −9.00          | −49.98       | 4 (Lys 52, Gln 103, Asp 104, Met 106) |
|               |               | 19A      | −9.56          | −53.59       | 4 (Lys 52, Gln 103, Asp 104, Met 106) |
| JNK           | 2P33          | taxifolin | −4.56          | −43.15       | 3 (Glu 147, Met 149, Asn 152) |
|               |               | J07      | −10.81         | −55.22       | 3 (Met 149, Met 149, Asn 152) |
| p38α          | 2BAL          | taxifolin | −8.26          | −49.71       | 3 (Ala 51, His 107, Met 109) |
|               |               | PQA      | −9.54          | −54.60       | 3 (Ser 32, His 107, Met 109) |
Phosphorylation (Davies & Tournier, 2012). Therefore, we used human body. SP600125 is an anthrapyrazolone inhibitor of JNK1/2, JNK1, JNK2 and JNK3, among which JNK1/2 is widely expressed in 80% and 36% in the administration with taxifolin (20, 40, 80 μmol/L) and U0126 (2 μmol/L), as well as the phosphorylation levels of JNK1/2 and P38 compared with those in LPS-treated cells, revealing that taxifolin, like MAPK pathway inhibitors, can reduce the accumulation of inflammatory response through the MAPK pathway.

3.5. Taxifolin down-regulated phosphorylation level of MAPK signal pathway

We further investigated whether taxifolin alleviating inflammation is associated with the phosphorylation of MAPK. U0126 is currently-one of the most widely used specific MAPK pathway inhibitors. As an inhibitor of mitogen-activated protein kinase (MEK) acting on the upstream of ERK1/2, U0126 can bind to MEK without competition, inhibit its enzyme activity, and block the phosphorylation of downstream ERK1/2 (Su et al., 2014). SB203580 is a pyridine imidazole P38 MAPK inhibitor that are able to bind with the threonine side chain in the ATP binding pocket of p38α MAPK (Beardmore et al., 2005). There are three types of JNK: JNK1, JNK2 and JNK3, among which JNK1/2 is widely expressed in human body. SP600125 is an anthrapyrazolone inhibitor of JNK1/2 phosphorylation (Davies & Tournier, 2012). Therefore, we used above inhibitors to act as positive controls. As illustrated in Fig. 6, Western blot results demonstrated that after LPS treatment the relative expression levels of p-ERK/ERK, p-JNK1/2/JNK1/2, p-P38/P38 proteins increased by 18.64-, 29.95-, 26.02-folds comparing to those in control group. Pleasantly, the phosphorylation level of MAPK (Beardmore et al., 2005). There are three types of JNK: JNK1, JNK2 and JNK3, among which JNK1/2 is widely expressed in human body. SP600125 is an anthrapyrazolone inhibitor of JNK1/2 phosphorylation (Davies & Tournier, 2012). Therefore, we used above inhibitors to act as positive controls. As illustrated in Fig. 6, Western blot results demonstrated that after LPS treatment the relative expression levels of p-ERK/ERK, p-JNK1/2/JNK1/2, p-P38/P38 proteins increased by 18.64-, 29.95-, 26.02-folds comparing to those in control group. Pleasantly, the phosphorylation level of MAPK...
the expressions of NO, VEGF, TNF-α and COX-2 could be induced by LPS in RAW264.7 cells that play integral role in fighting against inflammation (Du et al., 2020; Li et al., 2020). One previous study shows that taxifolin improves the oxidative stress response by measuring xanthine/xanthine oxidase (XO) and 2,2-diphenyl bitter hydrazide radical (DPPH) levels while also reducing pro-inflammatory cytokines (iNOS, IL-1β, IL-6 and GM-CSF), the activity of NF-κB and activator protein-1 (AP-1) (Rhee et al., 2008). In our study, Figs. 2–4 exhibited that taxifolin did remarkably reduce the levels of oxidation- and inflammation-specific genes by using LPS-induced RAW264.7 cell model. All data suggest that taxifolin can ameliorate the inflammatory injury to RAW264.7 cells by modulating different mediators of oxidative stress and inflammatory responses, suggesting that taxifolin has great potential for future research in the treatment of inflammatory diseases.

The MAPK signaling pathway is closely associated with the progression of inflammation. The p38 MAPK family members consist of p38α, p38β, p38γ and p38δ, which share approximately 60% identical in their amino acid sequence (Yong, Koh, & Moon, 2009). And Victoria with her colleagues found that p38α MAPK is a major subtype involved in immune and inflammatory responses (Beardmore et al., 2005). Further, taxifolin’s mitigatory effect on the phosphorylation levels of ERK, JNK and p38 pathways were showed in Figs. 3 and 5. However, through the current experimental results with MAPK pathway inhibitors, we found that taxifolin did not rely completely on the MAPK pathway to suppress the gene expression of iNOS, VEGF, COX-2 and TNF-α (as shown in Fig. 7). Taken together, the results indicate that the downregulation of phosphorylated ERK, JNK and p38 levels in the MAPK signaling pathway is a possible mechanism for taxifolin. And of course, taxifolin also exerts anti-inflammatory effects on LPS-activated inflammatory response through other signaling pathways.

5. Conclusion

This study investigated the anti-inflammation of taxifolin, as well as the reduction of phosphorylation of MAPK signaling pathway. We found taxifolin decreased the expression of pro-inflammatory cytokines and the phosphorylation of MAPK signaling pathway. Whereas, taxifolin downregulated the expression of iNOS, VEGF, COX-2 and TNF-α partly relying on the MAPK signaling pathway. The scientific explanation of the theory of taxifolin will certainly further promote the reasonable, effective application in daily diet and health protection. Therefore, a healthy consumption of onions, grapes and others including taxifolin may be favor of the reduction of inflammation in the human body.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chmed.2021.03.002.

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