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Synthesis and biological evaluation of myricetin-pentadienone hybrids as potential anti-inflammatory agents *in vitro* and in vivo

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ABSTRACT

Some important pro-inflammatory cytokines such as interleukin-6, tumor necrosis factor-α and nitric oxide are thought to play key roles in the destruction of cartilage and bone tissue in joints affected by rheumatoid arthritis. In the present study, a series of new myricetin-pentadienone hybrids were designed and synthesized. Majority of them effectively inhibited the expressions liposaccharide-induced secretion of IL-6, TNF-α and NO in RAW264.7. The most prominent compound **5o** could significantly decrease production of above inflammatory factors with IC_{50} values of 5.22 μM, 8.22 μM and 9.31 μM, respectively. Preliminary mechanism studies indicated that it could inhibit the expression of thioredoxin reductase, resulting in inhibiting of cell signaling pathway nuclear factor (N-κB) and mitogen-activated protein kinases. Significantly, compound **5o** was found to effectively inhibit Freund's complete adjuvant induced rat adjuvant arthritis in vivo.

1. Introduction

Rheumatoid arthritis (RA) is defined as the chronic inflammation of one or more joints, with presence of pain, erythema, swelling, an increase in the temperature in the affected areas [1,2]. RA is a complex process involving numerous inflammatory mediators [3]. Although RA changes have been extensively investigated, there is still a still lack of effective drugs. During RA, there are many pro-inflammatory cytokines such as IL-1, IL-6, TNF-α and chemokines release to the synovial space [4]. Therefore, it is urgent to find effective drugs for RA by inhibiting the production and release of pro-inflammatory cytokines.

The thioredoxin system, which consists of thioredoxin (Trx), thioredoxin reductase (TrxR) and nicotinamide adenine dinucleotide phosphate (NADPH), plays an essential role in oxidative stress, nitrosative stress, redox regulation and regulating intracellular signaling pathways [5–7]. Trx, a small redox active protein, is one of the defense proteins induced in response to various oxidative stress conditions [8,9]. The reduction of oxidized Trx by NADPH is catalyzed by TrxR [10,11]. In addition to its effective antioxidant effect, Trx system also has anti-inflammatory properties [12,13], mainly because it can suppress neutrophil chemotaxis to inflammatory sites and inhibit the expression and activation of macrophage migration-inhibitory factors [14]. On the one hand, stimulated by LPS, then activation of nuclear factor (NF-κB) enters nucleus to induce the release of pro-inflammatory cytokines [15]. In nucleus, reduced TRX-1 potentiates NF-κB binding to DNA, and consequently, enhances transcription of inflammatory mediators [16]. On the other hand, when H_2O_2 stimulates macrophage, oxidized Trx can activate mitogen-activated protein kinases (MAPK) which enter the nucleus to induce cell apoptosis [17].

Curcumin has attracted much attention regarding its anti-inflammatory activity [18]. But, due to low bioavailability, the potential utility is limited by its instability and poor solubility. Thus, on basis of improving stability and solubility, a number of analogs/derivatives have been designed and synthesized. Among those compounds, diarylpentadienone derivatives with good biological profile have been discovered [19,20].

Myricetin, a polyphenolic flavonoid, is present in a wide variety of fruits with anti-cancer activity, its derivatives inhibiting of intestinal tumor igenesis through the NF-κB/IL-6 pathway has been reported [21,22]. However, substantial limitation of availability, due to low stability in body has been observed [23,24]. It is presumed that the polyphenolic moiety may affect this property. To overcome this limitation, methylated-myricetin derivatives should be avoid it.

Based on the above-mentioned, we have introduced methylated-myricetin moiety into the diarylpentadienone skeleton, a series of myricetin-pentadienone hybrids were synthesized at first. The preliminary activity results showed some compounds could inhibit expression of TrxR. It is becoming increasingly clear that TrxR plays a key

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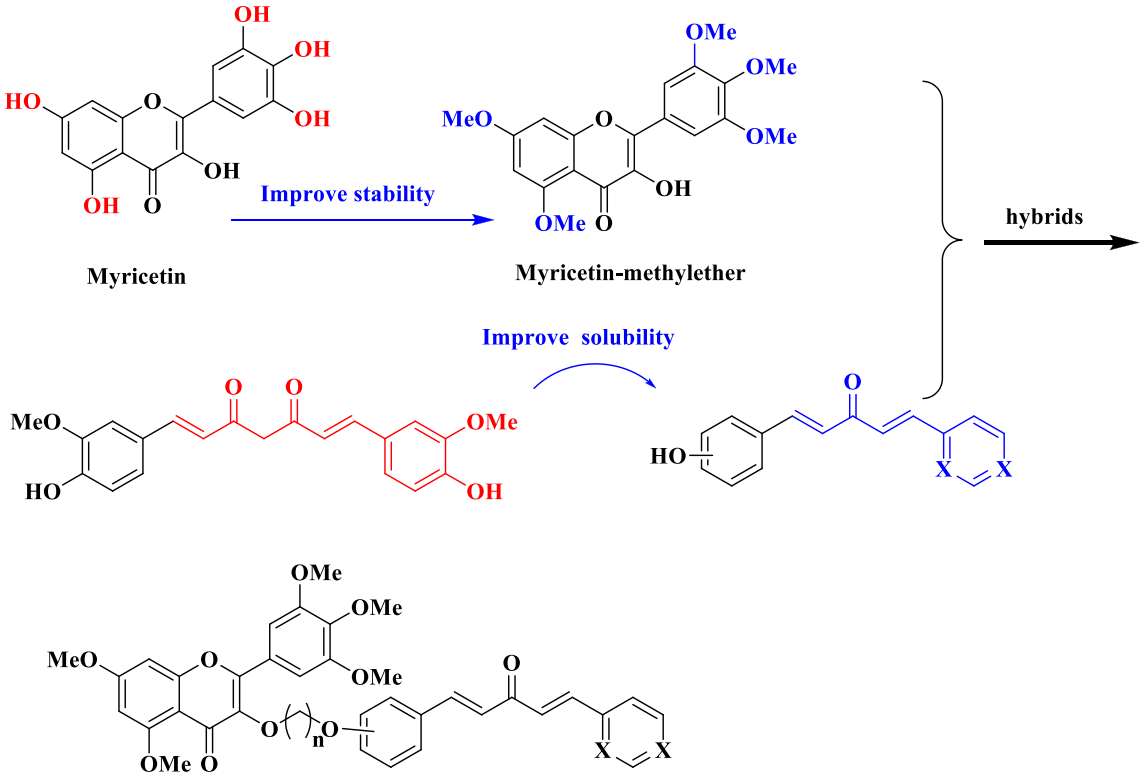


Fig. 1. Idea of synthesis.

role in inflammation, and accumulating evidences indicate that TrxR may be a promising anti-inflammatory target. Herein, in continuation to extend our research of inflammation, more compounds with methylated-myricetin-pentadienone moieties (Fig. 1) were designed and screened for their anti-inflammatory activities in this study.

2. Results and discussion

2.1. Chemistry

Acetone and benzaldehyde in presence of 5% NaOH through Claisen-Schmidt condensation, *α, β*-unsaturated ketone (compound **1**) with good yield was obtained. The key intermediate pentadienone (compound **2**) was synthesized with the reaction of aldehyde ketone condensation. During the above reaction, the pH was adjusted to 5 by 5% HCl. Myricetin used as the raw material, 3-hydroxy-4H-chromen-4-one (compound **3**) was synthesized by removing glycosides. Then, compound **3** and 1,3 (or 4) dibrombutane with K_2CO_3 dissolved in DMF, 3-(3-bromopropoxy/butoxy)−5,7-dimethoxy-2-(3,4,5-trimethoxyphenyl)-4H-chromen-4-one (compound **4**) was prepared. Compounds **5a–5w** (Fig. 2) have been fully characterized as *Supporting information*.

2.2. Assessment of toxicity

In order to ensure the safety of the title compounds and avoid their false positive anti-inflammatory activity, MTT was used to detect cytotoxicity against RAW264.7 cells. As shown in Fig. 3, most of the compounds showed low toxicity at 20 μM. Among then, compounds **5o**, **5p** and **5q** showed lower toxicity. Therefore, these compounds are valuable for further evaluation. Though the interference of drugs over the mitochondrial activity can be interpreted as loss of viability. Still, there is an extensive use of this assay as a viability assay [25].

2.3. Anti-inflammatory screening

IL-6, TNF-α and NO have been shown contribute to the initiation and progression of an inflammation-cause RA [26]. We use an enzyme-linked immunosorbent assay (ELISA) and Griess Reagent assay to screen for changes in LPS-induced IL-6, TNF-α and NO release by the synthesized compounds in RAW264.7 cells. Macrophages were pre-treated with 20 μM compounds for 1 h and then treated with LPS (0.5 μg/mL) for 24 h. The cell conditioned medium was collected, the IL-6, TNF-α and NO in the media was detected by ELISA and Griess Reagent assay. The screening results indicated that most of the title compounds reduced the LPS-induced IL-6, TNF-α and NO secretion at a dosage of 20 μM (Fig. 4). It is noteworthy that compounds **5o**, **5p** and **5q** exhibited stronger inhibiting of IL-6, TNF-α and NO production compared with the refererce Indomethacin. Therefore, these compounds were used for further evaluation.

2.4. Antioxidant screening

H_2O_2 (100 μM) reduced cell viability in these experiments to 45–60% of that in the vehicle control group[27,28]. In order to evaluate the antioxidant activity of synthetic compounds, RAW 264.7 cells were pre-incubated with compounds **5a–5w** (10, 1, 0.1 μM) for 1 h and treated with H_2O_2 (100 μM) for 24 h. MTT was used to detect cytotoxicity. The screening results indicated that most of the compounds reduced the H_2O_2-induced cytotoxicity at a dosage of 10 μM (Fig. 5). Of these, compounds **5o**, **5p** and **5q** showed potential activity.

2.5. Inhibiting of LPS-induced cytokine release and H_2O_2-induced oxidation reaction

Our next objective was to determine whether compounds **5o**, **5p** and **5q** inhibitory effect on inflammatory cytokine release with dose-

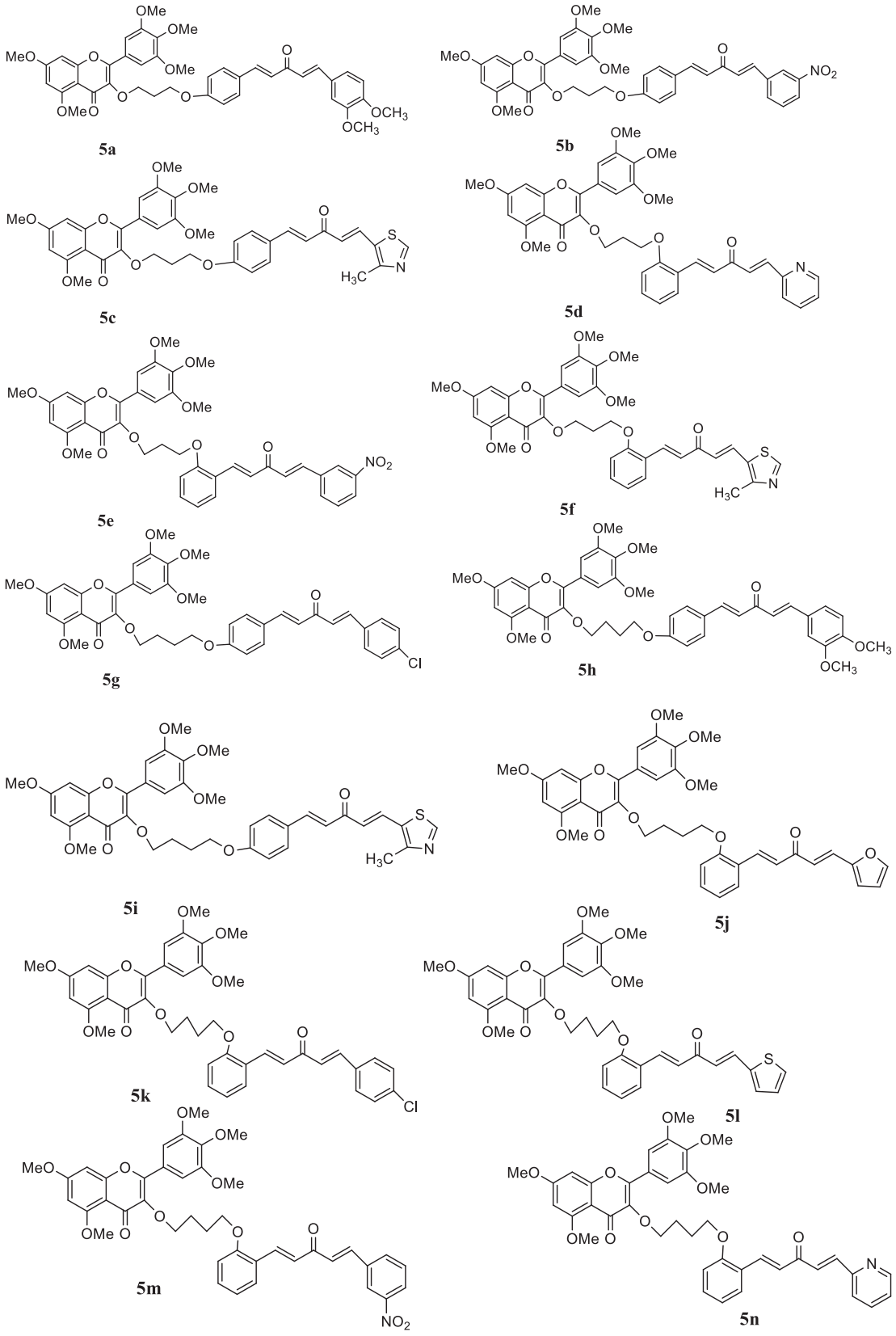


Fig. 2. Structures of **5a–5w.**

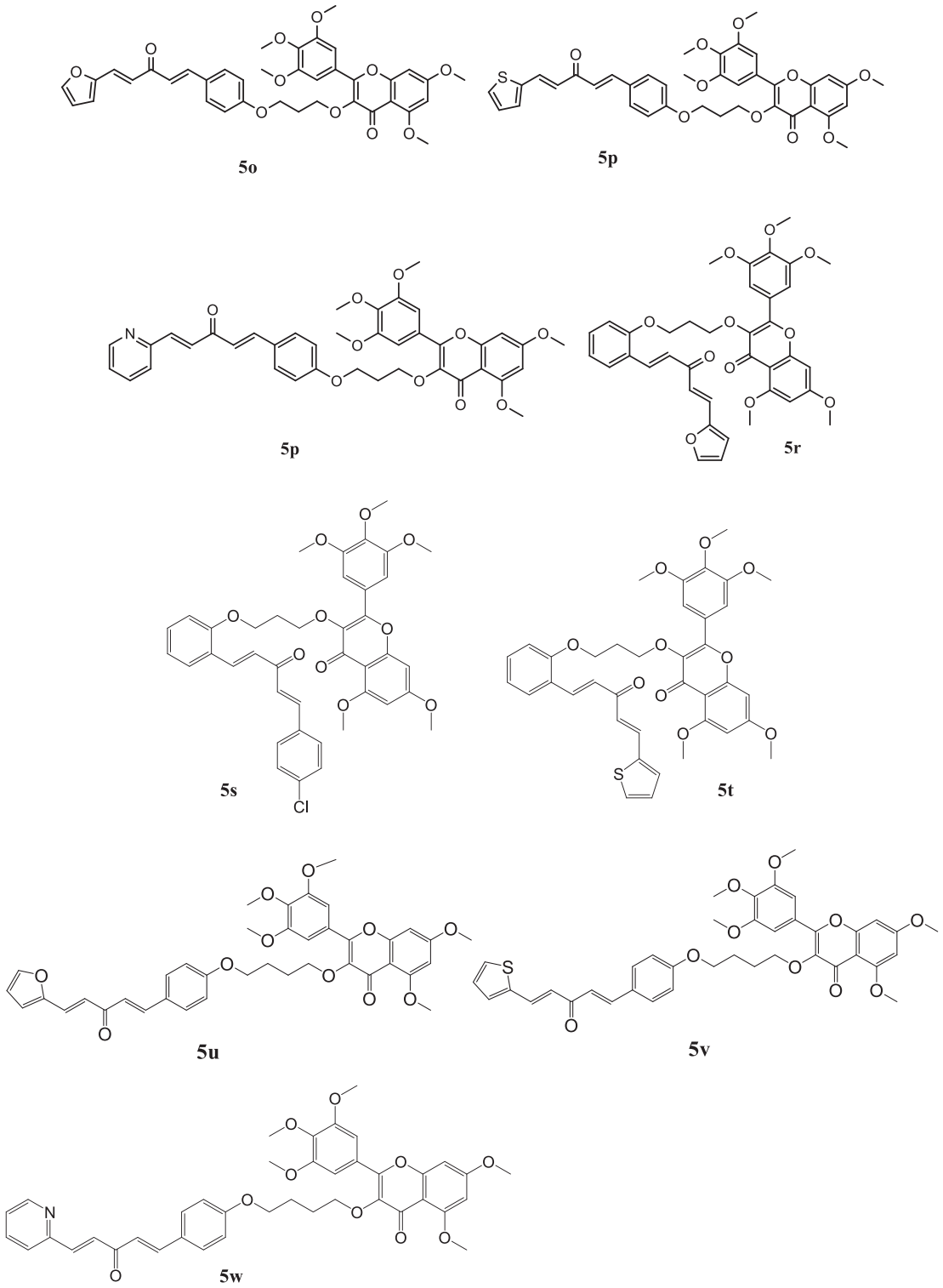


Fig. 2. (*continued*)

dependent pattern. We pretreated RAW264.7 cells with compounds **5o**, **5p**, **5q** at concentrations of 20, 10, 5 and 2.5 μM for 1 h, and subsequently incubated the cells with 0.5 μg/mL LPS for 24 h. The release of inflammatory cytokines into the culture medium was measured by ELISA and Griess Reagent assay. Compounds **5o**, **5p** and **5q** showed well-defined a dose-dependent inhibitory activity on IL-6, TNF-α and NO releases (Fig. 6A–C). When the concentration is 2.5–20 μM, the IC_{50} values of IL-6, TNF-α and NO were observed for compound **5o** (5.22 μM, 8.22 μM, 9.31 μM, respectively), compound **5p** (7.68 μM, 11.88 μM, 14.88 μM, respectively), compound **5q** (10.53 μM, 15.57 μM, 11.04 μM, respectively). Moreover, the IC_{50} values of these tested compounds could be very useful for our next study. On the other hand, RAW 264.7 cells were pre-incubated with all compounds (10, 1, 0.1, 0.01, 001 μM) for 1 h and treated with H_2O_2 (150 μM) for 24 h. MTT results showed that when the concentration of the compound was 10 nM, it still had protective effect compared with the H_2O_2-induced

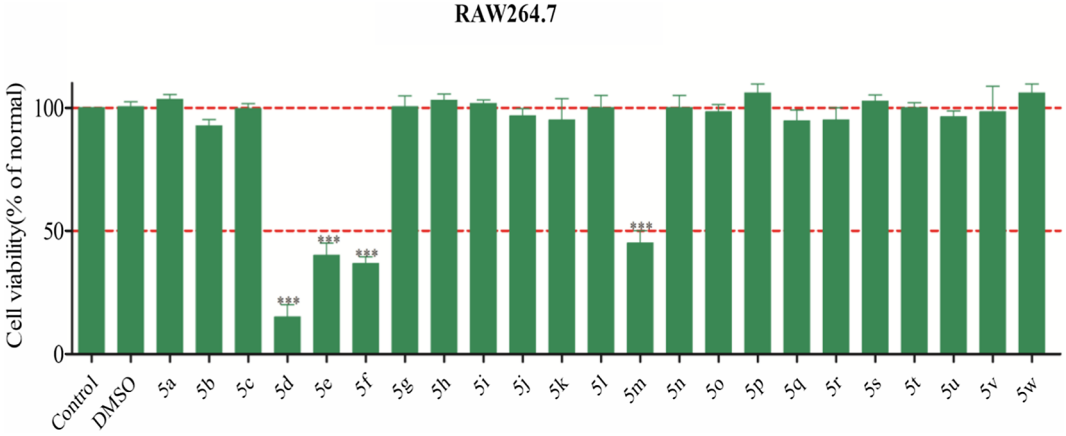


Fig. 3. The cytotoxic evaluation of compounds **5a**–**5w** against RAW264.7 cells.[a] ([a]The cell viability was evaluated by MTT assay. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compare with control group.)

Group (Fig. 6D). It's not hard to find out that compound **5o** is the best one. Therefore, we choose it as the title compound for further mechanism study.

2.6. Mechanism exploration of compound *5o*

2.6.1. Morphological changes of the cells

RAW264.7 cells were seeded in 6 cm culture dishes overnight and pretreated with compound **5o**, at concentrations of 20, 10, 5, 2.5 μM for 1 h before with LPS (0.5 μg/mL) for 24 h. RAW264.7 cells were round and bright in normal. When LPS stimulates cells, a large number of cell pseudopods appear, and a large number of vacuoles appear on the cell membrane. Title compound **5o** ameliorated the degree of cell deformation compared with the LPS group. Morphologically, the inflammatory state of cells was reversed (Fig. 7).

2.6.2. Inhibits TrxR activity and intracellular ROS

The Trx system plays an important role in the balance of redox in cells to prevent excessive ROS accumulation [29]. The inhibiting expression of TrxR and Trx may disturb the redox balance, leading to intracellular ROS unbalance [30–32]. Previously, we reported that curcumin analogue hybrids could exhibit anticancer activity and was found to have efficacy in potential cytokines inhibitory activity through inhibiting of TrxR. Compared with the control group, TrxR activity in the LPS-treated group decreased. We found an interesting phenomenon as shown in Fig. 8, title compound **5o** did not increase the activity of TrxR, and there was no statistical difference between the different concentrations ($p > 0.05$). The results showed that compound **5o** had no effect on TrxR activity (Fig. 8A). Further research findings, the LPS-treated group cells excessive ROS accumulation, while compound **5o** reduce ROS in a dose-dependent manner (Fig. 8B). Therefore, these results deserve further study.

2.6.3. Suppresses LPS-induced inflammatory response

COX-2 and iNOS are important pro-inflammatory proteins, which are associated with a variety of inflammatory diseases [33]. Because COX-2 and iNOS proteins are at the downstream of cellular signaling pathways, when LPS stimulates cells, activation of the cellular pathway results in high expression of COX-2 and iNOS proteins. Therefore, the detection of COX-2 and iNOS expressions is necessary. As shown in Fig. 9, the expressions of COX-2 and iNOS increased significantly when LPS (0.5 μg/mL) stimulated for 24 h. Title compound **5o** could reduce the expressions of COX-2 and iNOS in a concentration-dependent manner. These results demonstrated that compound **5o** could inhibit COX-2 and iNOS in macrophages and LPS-induced inflammation.

2.6.4. Inhibition LPS-induced TrxR/NF-κB signaling pathways activation

The thioredoxin system contains Trx, TrxR, NAPDH. Trx and TrxR are over expressed in various cancer and inflammation tissues [34–36]. Trx is a 12 K_D protein responsible for cell redox homeostasis [37]. In the nucleus, Trx in the reduced state has the ability to enhance the binding of transcription factors to DNA [38]. The subunits p50 and p65 of NF-κB are usually isolated in the cytoplasm by tight association with IκB-α protein [39]. After LPS stimuli cell activation of the toll-like-receptor 4 (TLR4), IκB-α phosphorylation and proteasomal-degradation allows the transcription factor NF-κB subunits to translate to the nucleus [40]. In the nucleus, transcription factor NF-κB undergo several post-translational modifications, which leads to their binding to DNA and transcription of several pro-inflammation proteins and inflammation factors, such as inflammatory mediators and TLRs. TrxR is the only enzyme in the cell which reduces oxidized Trx [41]. So, the expression of TrxR is essential for the regulation of inflammation. As shown in Fig. 10, the LPS (0.5 μg/mL) stimulation could markedly augment TrxR expression. However, compound **5o** concentration dependently suppressed LPS-induced TrxR expression (Fig. 10A). Further experiments, showed compound **5o** could inhibit the activation of NF-κB pathway by effects of IκB and p65 phosphorylation and degradation (Fig. 10B and C). This results demonstrated that compound **5o** prevented LPS-induced inflammatory response in macrophages.

2.6.5. Inhibition H_2O_2-induced TrxR/MAPK signaling pathways activation

Reductive Trx binds with ASK and inhibits its activity, which also makes ASK1 ubiquitin degradation. While oxidized Trx cannot bind to ASK1, it directly or indirectly activates ASK1 mediated apoptotic pathway [42]. Reactive oxygen radicals can dissociate Trx from ASK1 and activate ASK1. TrxR is the only enzyme that can reduce oxidation state Trx in cells [43]. The TrxR expression is necessary for cell antioxidant stress and apoptosis inhibition. TrxR activity was inhibited by H_2O_2 stimulation, resulting in high expression of Trx oxidation. These changes led to dissociation of ASK1 from Trx (reduced)-ASK1 complexes, resulting in activation of the MAPK pathway, and the transcription factor AP-1 enters the nucleus, causing them to bind to DNA and transcription [44]. From Fig. 11, we found compound **5o** could inhibit the TrxR, P38 and JNK phosphorylation.

2.6.6. Effect on histopathological changes of AA-induced rat ankle joints

Oxidative stress is closely related to rheumatoid arthritis. The effects on pathological changes of AA-induced rat ankle joints was detected to confirm the activity of compound **5o** in *vivo*. Photomicrographs of knee joints sections stained with H&E showed AA group synovial hyperplasia, serious cartilage destruction and acute inflammatory cells infiltration compared with the normal group. H&E

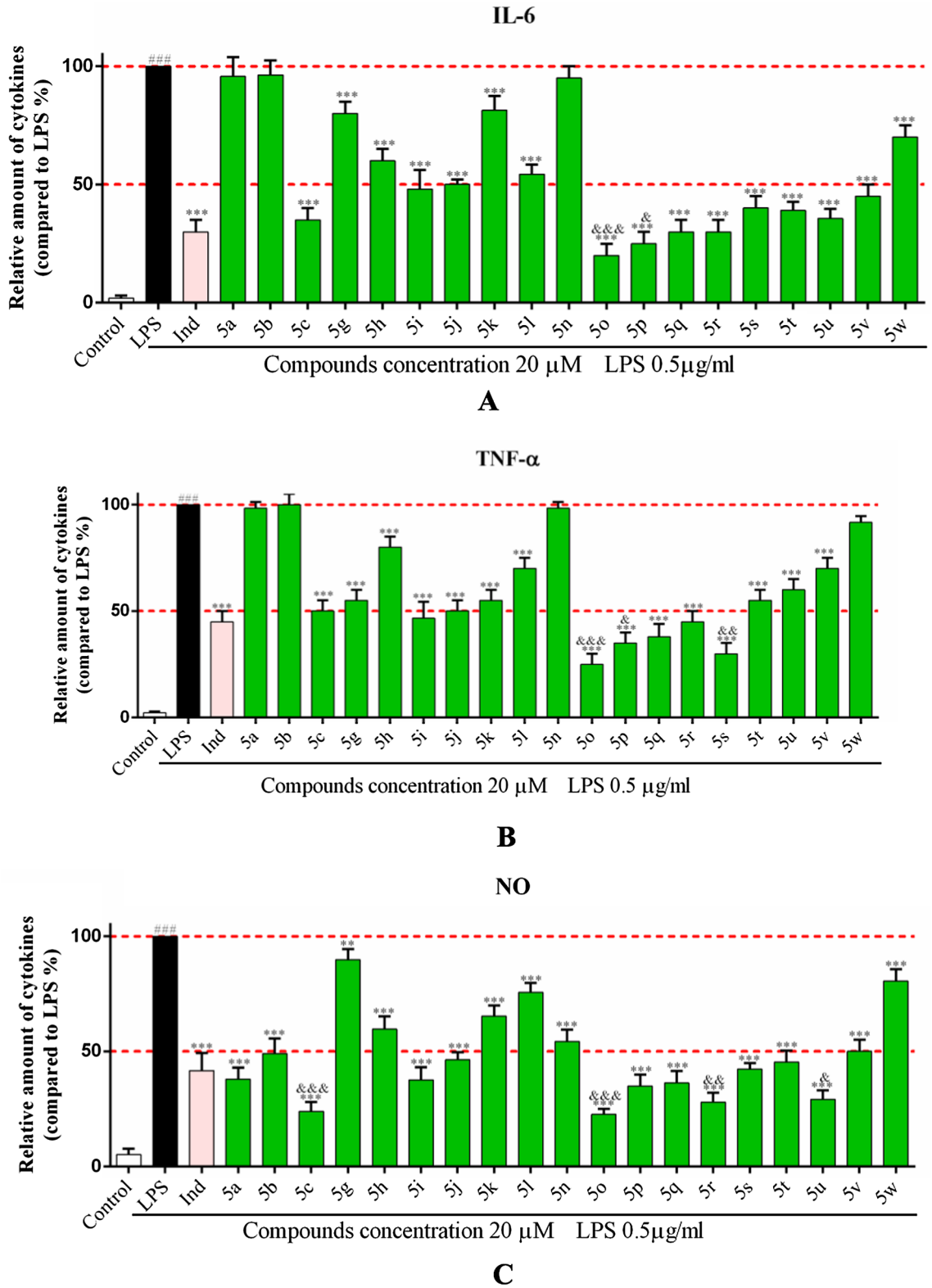


Fig. 4. Inhibiting of IL-6, TNF-α and NO productions by compounds **5a**–**5w**. (**A**) Effects of all compounds on IL-6 secretion. (**B**) Effects of all compounds on TNF-α secretion. (**C**) Effects of all compounds on NO secretion. Ind: positive Indomethacin $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ *vs* LPS group. $^{\#\#\#}p < 0.001$ *vs* control group. $^{\&\&\&}p < 0.001$, $^{\&\&}p < 0.01$, $^{\&}p < 0.05$ *vs* Ind group.

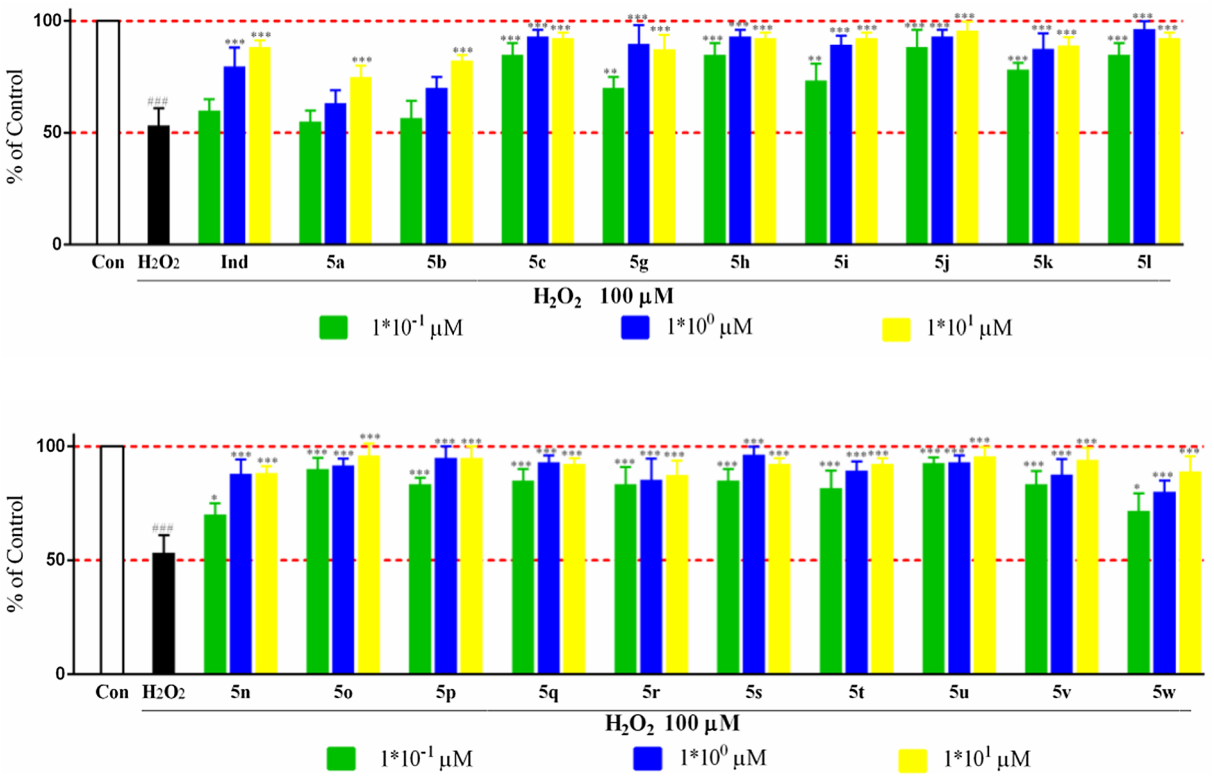


Fig. 5. Compounds **5a**–**5w** inhibited H_2O_2-induced oxidant response.[a] ([a]The cell viability was evaluated by MTT assay. $^{\#\#\#}p < 0.001$ *vs* control group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compare with H_2O_2 group.)

showed AA rats treated with Aspirin (50 mg/kg) exhibited moderatesynovial hyperplasia and serious inflammatory cells infiltration. AA rats treated with compound **5o** (45 mg/kg) exhibited moderatesynovial hyperplasia and serious cartilage damage, but compound **5o** (90 mg/kg) only mild synovial hyperplasia with intact cartilage although slight inflammatory cells infiltration remained. In conclusion, compound **5o** could significantly inhibit synovial hyperplasia and cartilage destruction in a dose-dependent manner and had a certain therapeutic effect on adjuvant arthritis in rats (Fig. 12).

3. Conclusions

In summary, to better treat rheumatoid arthritis, based on rational design, a series of myricetin-pentadienone hybrids were designed and synthesized. Compared with previous studies, myricetin derivatives were usually used as anti-tumor activity, most of which had strong cell cytotoxicity. In this study, new myricetin derivatives were designed and discovered, presenting low cell toxicity and high anti-inflammatory activity. Preliminary inflammatory screening showed that most of the compounds had good IL-6, TNF-α and NO inhibitory activity. Among them, some compounds (**5o**, **5p** and **5q**), presenting low toxicity were selected for further assessment of their IL-6, TNF-α and NO inhibitory activities. Specifically, title compound **5o** could inhibit IL-6 (5.22 μM), TNF-α (8.22 μM) and NO (9.31 μM) secretion in a dose-dependent manner. The preliminary mechanism indicated that title compound could target TrxR proteins, reduce cellular inflammation and increase cellular antioxidant stress. One side, this compound inhibited the high expression of TrxR and inhibited Trx binding to NF-κB. On the other hand, it could regulate expression of TrxR and reduction of TXR in the oxidized state to the reduced state. The further study showed that this compound effectively reduced AA-induced rat ankle joints in *vivo* (Fig. 13).

4. Experimental section

4.1. Chemistry

The reactions were monitored by thin layer chromatography (TLC). Melting points were determined on a XT4MP apparatus (Taike Corp., Beijing, China), and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Brucker AM-500 (500 MHz) spectrometer with the solvent of DMSO-d_6 and $CDCl_3$. All reagents were purchased from standard commercial suppliers.

4.2. General procedure for compounds *5a–5w*

To a *N, N*-dimethylformamide (30 mL) solution of 3-(3-bromopropoxy)-5,7-dimethoxy-2-(3,4,5-trimethoxyphenyl)-4H-chromen-4-one (3.0 mmol), (*1E,4E*)-1-(3,4-dimethoxyphenyl)-5-(4-hydroxyphenyl) penta-1,4-dien-3-one (3.0 mmol) and potassium carbonate (3.0 mmol) was added, stirred for 20 min, then refluxed for 8 h. Until the reaction was complete, 200 mL ice water was added, twenty hours later, the crude residue was purified by chromatography on SiO_2 (chloroform: ethanol = 4:1, v/v) to give the title compound **5a** as yellow solids. Compounds **5b** ~ **5w** were synthesized by the same method. Synthesis of them as *Supporting information*.

4.3. Cell culture

Mouse peritoneal macrophages were purchased from BeNa Culture Collection Company. RAW264.7 cells were cultured in DMEM medium (Hyclone, USA) supplemented with 10% fetal bovine serum (Biological Industries, Israel), 100 μg/mL streptomycin and100 U/mL penicillin (Beyotime). Cells were cultured at 37 °C with 5% CO_2 in a humidified atmosphere.

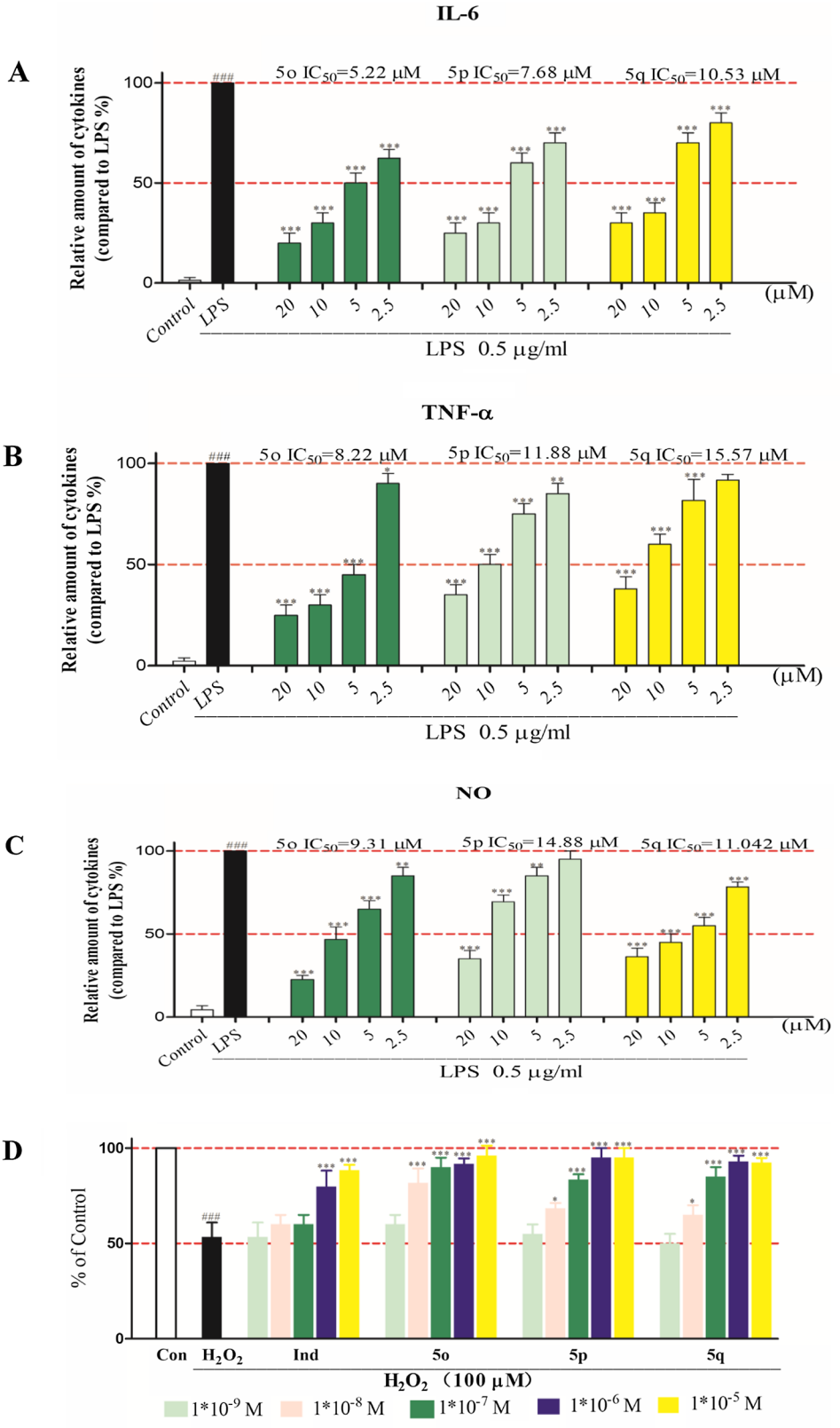


Fig. 6. Compounds **5o–5p** inhibiting of LPS-induced cytokine release and H_2O_2-induced oxidant in a dose-dependent manner. (A) IL-6. (B) TNF-α. (C) NO. (D) antioxidant. $^{###}p < 0.001$ *vs* control group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compare with LPS group or H_2O_2 group.

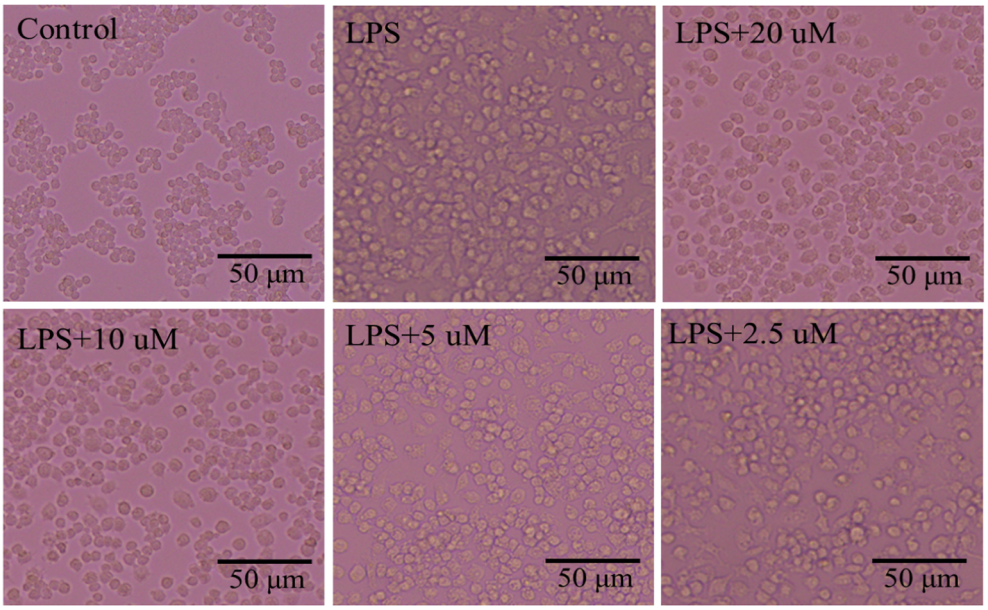


Fig. 7. Morphology of RAW264.7 cells using inverted phase-contrast microscope (magnification × 10). RAWW264.7 cells treated with title compound **5o** (10, 5, 2.5 and 1.25 μM) for 24 h. The concentration of LPS is 0.5 μg/mL.

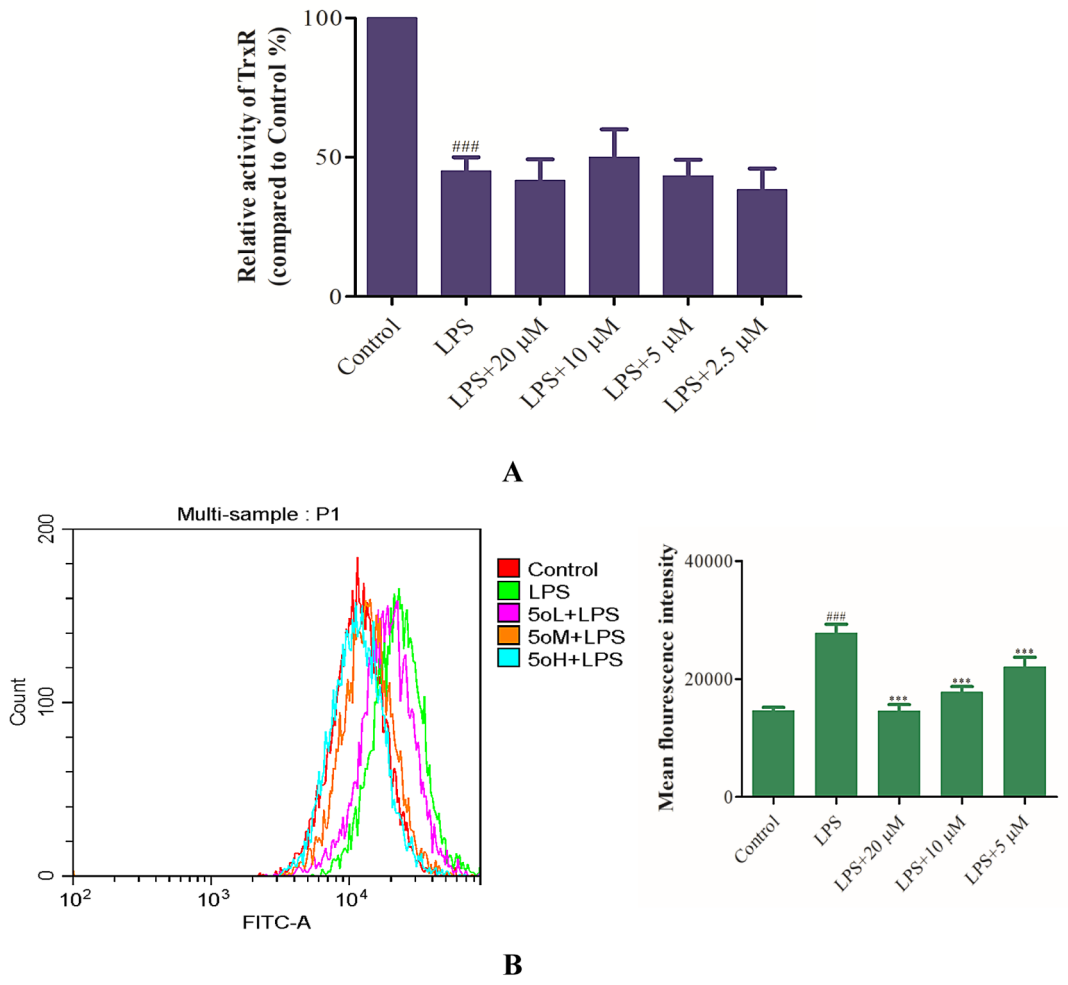


Fig. 8. Physiological significance of targeting TrxR by compound **5o**. (A) Inhibition of TrxR activity in RAW264.7 cells by compound **5o**; (B) Treatment with LPS induced the accumulation of toxic ROS in RAW264.7 cells. The compound **5o** concentration is 5 μM, 10 μM, 20 μM. $###p < 0.001$ *vs* control group. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared with LPS group.

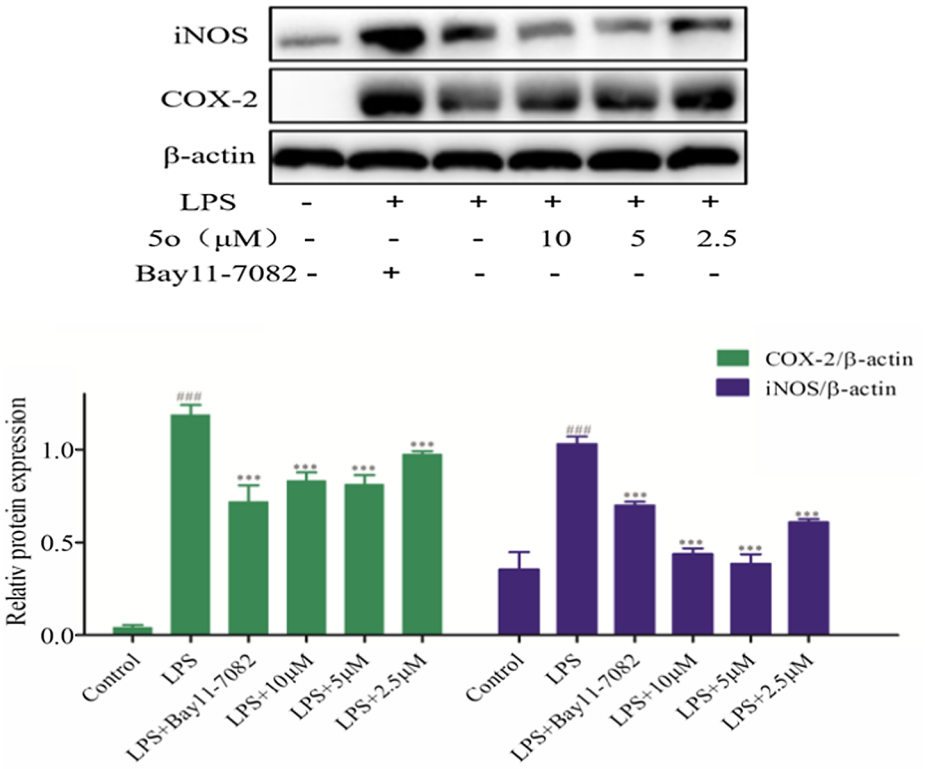


Fig. 9. Compound **5o** inhibited LPS-induced iNOS and COX-2 proteins expression in RAW 264.7 cells. Pretreatment with compound **5o** (2.5–10 μM) 1 h before cells were stimulated with LPS (0.5 μg/mL) for 24 h. COX-2, iNOS and β-actin were detected by Western blot. Bay11-7082 (5 μM) is the NF-κB inhibitor. $^{###}p < 0.001$ compared with control cells, $^{***}p < 0.001$ compared with LPS-stimulated cells; Three separate experiments were shown by blots.

4.4. Detection of toxicity

RAW264.7 cells were seeded into 96-well plates at a density of 1×10^4 cells/well and permitted to continue to grow about 24 h. All compounds (20 μM) pretreatment RAW264.7 cells for 24 h. Then 20 μL MTT (5 mg/mL) was added into each well and were incubated for 4 h at 37 °C. After 4 h of incubation, the culture media were removed, 150 μL DMSO were added to each well and shaking the 96-well in a horizontal shaker about 15 min. At last, the absorbance at 492 nm was measured by a microplate reader (MQX200, Bio-Tek, USA).

4.5. Determination of IL-6, TNF-α and NO

RAW264.7 cells were seeded into 48-well plates at a density of 6×10^4 cells/well and permitted to continue to grow about 24 h. RAW264.7 cells were pretreated with compounds (20 μM) for 1 h, incubated with LPS (0.5 μg/mL Sigma, USA) for 24 h. Collect cell supernatant for detection. The levels of TNF-α and IL-6 in the culture medium were measured by ELISA kit (eBioScience, San Diego, CA). NO production was measured using Griess Reagent assay (Beyotime, China), according to the manufacturer's instructions.

4.6. Oxidative stress screening

RAW264.7 cells were seeded into 96-well plates at a density of 2×10^4 cells/well and permitted to continue to grow about 24 h·H_2O_2 (150 μM) reduced cell viability in these experiments to 45–60% of that in the vehicle control group. RAW 264.7 cells were pre-incubated with all compounds (10, 1, 0.1 μM) for 1 h and treated with H_2O_2 (150 μM) for 24 h. MTT was used to detect cytotoxicity.

4.7. Morphological changes of the cells

RAW264.7 cells were seeded in 6 cm culture dishes overnight and pretreated with compound **5o**, at concentrations of 20, 10, 5, 2.5 μM for 1 h before with LPS (0.5 μg/mL) for 24 h. Morphological changes were observed in these cells under the microscope (OLYMPUS, Japan).

4.8. Detection of intracellular ROS

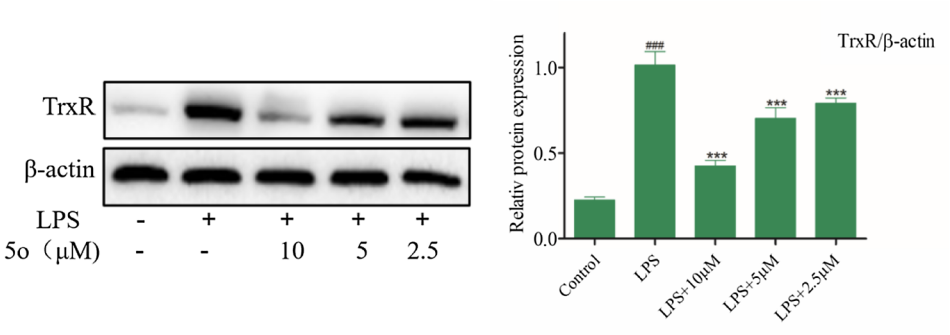
Detection of intracellular reactive oxygen species (ROS) with DCFH-DA fluorescent probe. RAW264.7 cells were seeded into 6-well plates at a density of 1.5×10^6 cells/well grow about 24 h. cells were pre-incubated with all compounds (20, 10, 5 μM) for 1 h and treated with LPS (0.5 μg/mL) for 6 h. The cells were treated with DCFH-DA (BestBio, China) for 30 min at 37 °C in the dark. According to the manufacturer's instructions, use flow cytometry detecting the intracellular reactive oxygen species and analyze by flowjo software.

4.9. Determination of TrxR activity in RAW264.7 cells lysates

TrxR activity was measured using the TrxR Assay kit (BestBio, China). Firstly, we should add 150 μL cell lysate to the prepared cells. Collect supernatant for detection. Secondly, mixed all additives rapidly with supernatant according to the protocol. Finally, we measured the absorbance at 412 nm at 10 s (a1) and 310 s (a2) respectively. BCA assay kit (Beyotime, China) was used determine the concentration of proteins (Cpr). The activity of TrxR was calculated by formula: TrxR (U/mg prot) = 147 × (ΔA sample − ΔA blank) ÷ Cpr.

4.10. Western blotting

Cells were seeded into 6 cm culture dishes with 3×10^6 cells and maintained overnight. RAW264.7 cells were pretreated with compound **5o** (10, 5, 2.5 μM) for 1 h before were stimulated LPS (0.5 μg/mL) for 0.5 h. Add 300 μL RIPA cell lysis buffer (Beyotime, China) to the prepared cells and collect supernatant for detection. Total proteins were separated by 12% SDS-PAGE. The resulting blots were blocked with 5% milk (about 2 h) and incubated with anti-p65 antibody (1:1000; abcam, USA), anti-p-p65 antibody (1:1000; abcam, USA), COX-2 (1:1000;



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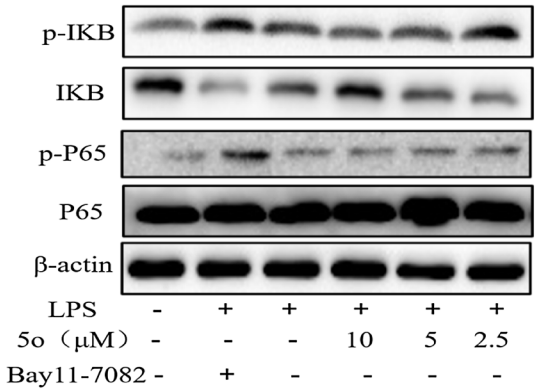

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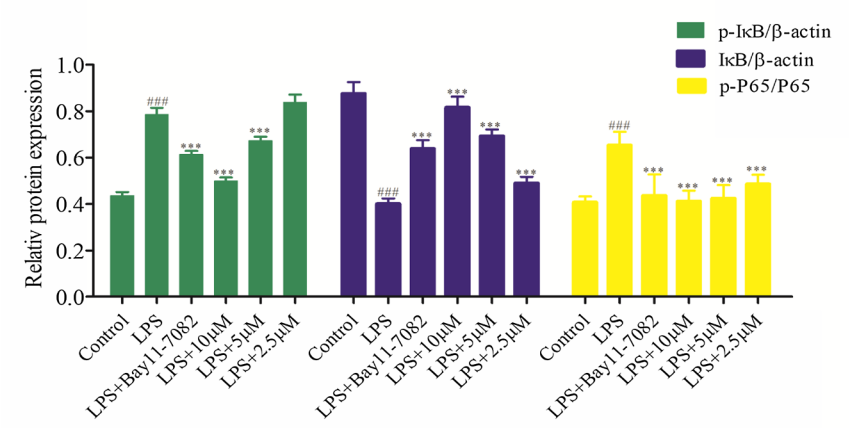


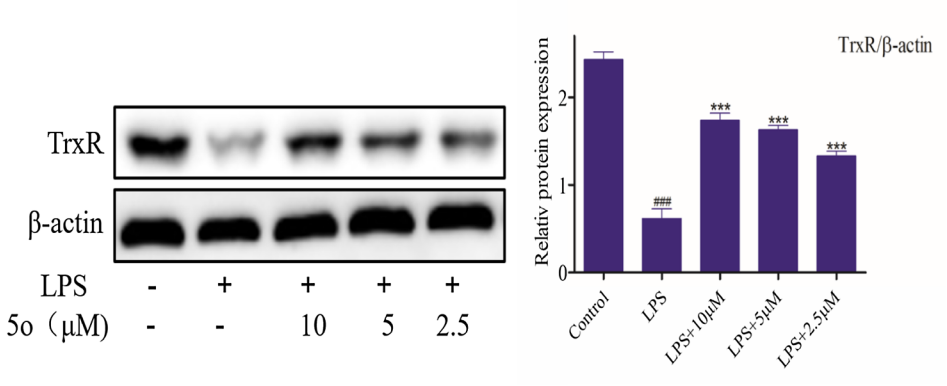
C

Fig. 10. Compound **5o** inhibited LPS-induced TrxR/NF-κB active in RAW 264.7 cells by Western blot analysis. (A) Detection of TrxR level by Western blotting; (B, C) NF-κB protein expression level in RAW264.7 cells were measured. RAW 264.7 cells were treatment with compound 5o (2.5–10 μM) 1 h and were stimulated with LPS (0.5 μg/mL) for 24 h. TrxR, p-IκB, IκB, p-*P*65, *P*65 and β-actin were detected by Western blot. Bay11-7082 (5 μM) is the NF-κB inhibitor. ###$p < 0.001$ compared with Control cells, ***$p < 0.001$ compared with LPS-stimulated cells; Three separate experiments were shown by blots.

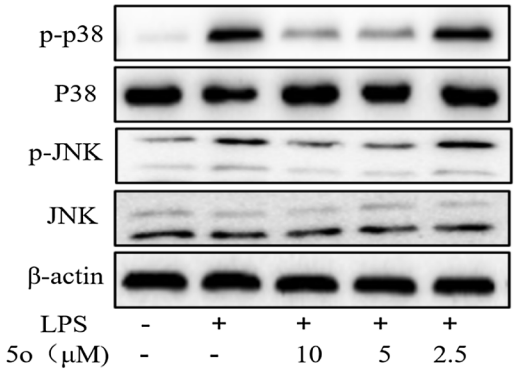
abcam, USA), iNOS (1:1000; abcam, USA), p-IκB (1:1000; CST, USA), IκB (1:1000; CST, USA), P38 (1:1000; proteintech, China), p-P38 (1:500; proteintech, China), JNK (1:500; proteintech, China), p-JNK (1:500; proteintech, China), TrxR (1:500; proteintech, China) overnight at 4 °C. At last, the blots were detected with the chemiluminescence (ECL) system.

4.11. *In vivo experiments*

Thirty-five male Sprague-Dawley (SD) rats weighing 160–180 g were obtained from Animal Department of Anhui Medical University. About one week adaptive environment, SD rats were randomly divided into five groups (7 rats per group): normal, AA (adjuvant arthritis), aspirin (50 mg/kg) and compound **5o** (45, 90 mg/kg). All experiments and animal care procedures were approved by the Animal Resource Center of Anhui Medical University. Compound **5o** and aspirin in 0.5% carboxymethyl cellulose sodium (CMC-Na) solution at concentrations of 9, 18 mg/mL and 10 mg/mL, were administered intragastrically one milliliter per mouse, starting from day 14–28, once a day. Animals were anaesthetized and executed 28 days later. The right ankle joint was fixed with 4% polyformaldehyde



A



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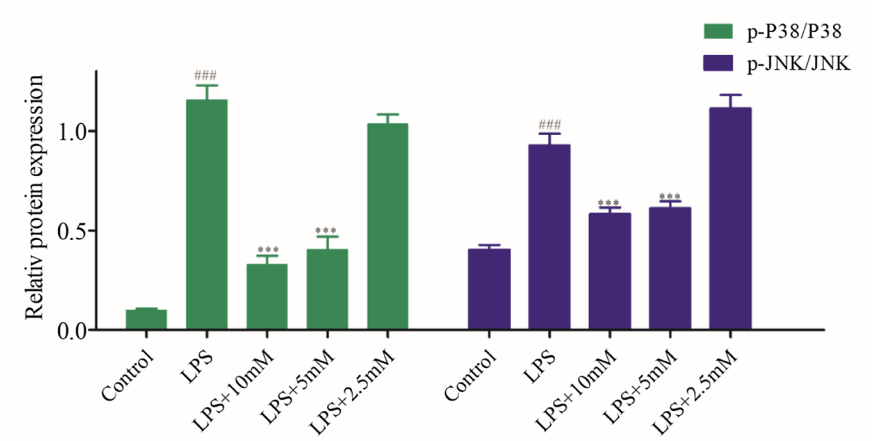

C

Fig. 11. Compound **5o** inhibit H_2O_2-induced TrxR/MAPK active in RAW 264.7 cells by Western blot. (A) Detection of TrxR level by Western blotting; (B, C) MAPK protein expression level in RAW264.7 cells were measured. After pretreatment with compound **5o** (2.5–10 μM) 1 h, RAW 264.7 cells were stimulated with H_2O_2 (150 μM) for 24 h. P38, p-P38, JNK, p-JNK and β-actin were detected by Western blot. $^{###}p < 0.001$ compared with control cells, $^{***}p < 0.001$ compared with LPS-stimulated cells; Three separate experiments were shown by blots.

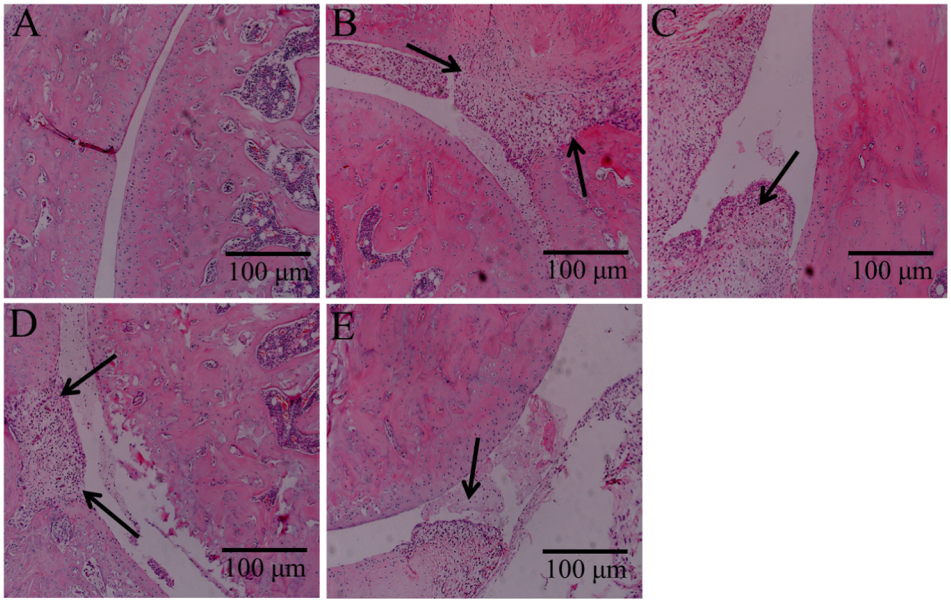

Fig. 12. Effect of compound **5o** on pathological changes of AA-induced rat ankle joints by HE staining (magnification 100 ×). (A) Normal; (B) AA; (C) Aspirin 50 mg/kg; (D) Compound **5o** 45 mg/kg; (E) Compound **5o** 90 mg/kg. AA, adjuvant arthritis; Aspirin used as the positive control.

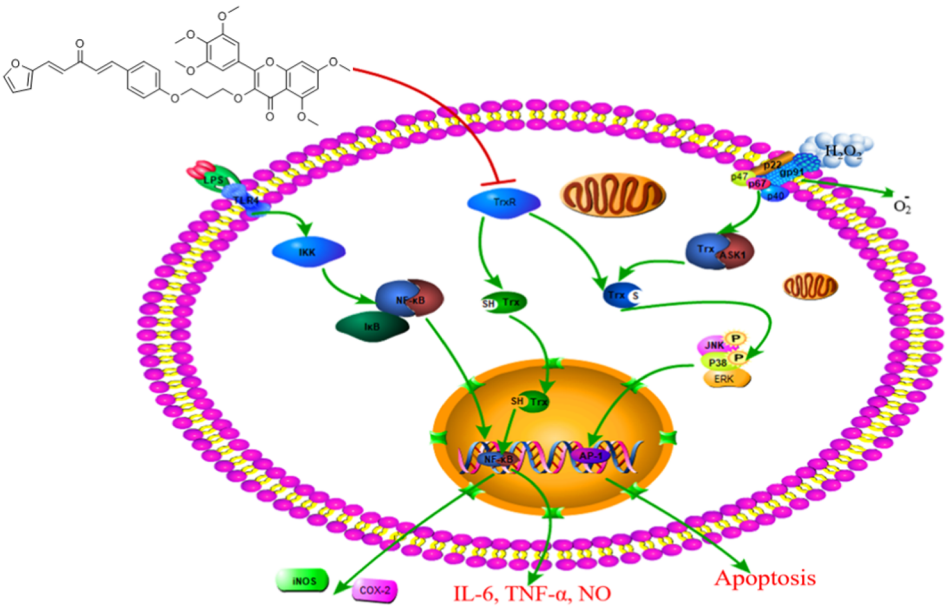

Fig. 13. The possible molecular mechanisms involved of compound **5o** against TrxR protein.

buffer and decalcified with dilute nitric acid. The ankle joint tissue was paraffin embedded and sliced for histopathological analysis.

4.12. Statistical analysis

All data are expressed as mean (SD). Analysis of variance (ANOVA) was used to determine statistical data. The value of $p < 0.05$ was considered to be statistically significant. Graphpad Prism 5 soft and SPSS 17.0 were used for mapping and data analysis. Repeat all the experimental data at least three times.

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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.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2020.103597.

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