Original Article
Food Science and Technology

ISSN 0101-2061 (Print)
ISSN 1678-457X (Online)

DOI: https://doi.org/10.1590/fst.96821

Extracts with anti-inflammatory activities from *Acanthopanax trifoliatus* (L.) Merr. by inhibiting LPS-induced expression of iNOS and COX-2

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Abstract

Acanthopanax trifoliatus (L.) Merr. have been used as folk medicine to treat various diseases traditionally and the young leaves and shoots of *A. trifoliatus* are popularly consumed as vegetables and herbal tea in southern China. In the study, we firstly tested the cytotoxicity and NO production of 18 fractions that extracted from the leaves, stems and roots of *A. trifoliatus* to select the bioactive fraction. The increasing evidence suggested that the dichloromethane extract prepared from stems of *A. trifoliatus* (ATSDC) have anti-inflammatory activity. Therefore, the study followed to investigate the effects of ATSDC on the inflammatory response and the molecular mechanisms underpinning this effect in lipopolysaccharide (LPS)-stimulated RAW264.7 cells. The manuscript showed that ATSDC effectively inhibited NO production in LPS-stimulated cells and significantly reduced the production of pro-inflammatory cytokines IL-6, at a dose of 40 μg/mL, whereas TNF-α production tended to decrease under ATSDC treatment. We also confirmed a dose-dependent and significant inhibition of iNOS and COX-2 protein expression. In conclusion, ATSDC exerted strong inhibitory effect on the expression of iNOS and COX-2 protein in LPS-induced RAW 264.7 macrophages and could be potentially used in treatment of inflammatory-related diseases in the future.

Keywords: *Acanthopanax trifoliatus* (L.) Merr.; dichloromethane extract; anti-inflammatory activities; iNOS and COX-2; RAW 264.7 macrophages.

Practical Application:

1. Dichloromethane extract prepared from stems of *Acanthopanax trifoliatus* (L.) Merr. significantly reduced the production of NO, IL-6 and TNF-α in LPS-stimulated RAW264.7 cells.

2. It also exerted strong inhibitory effect on the expression of iNOS and COX-2 protein in LPS-induced RAW 264.7 macrophages.

3. The dichloromethane extract from stems of A. trifoliatus may be able to inhibit the inflammatory diseases.

4. This research also furnished a reliable theoretical and practical basis for the application of *A. trifoliatus* as a natural anti-inflammatory ingredient in health foods or complementary medicines in the future.

1 Introduction

Inflammation is a local response of the immune system to pathogens and damaged cells and is a vital defense mechanism (Nan et al., 2018). Macrophages play an important role in the inflammatory response and overexpress several mediators of inflammation in response to LPS-induced cell injury, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), nitric oxide (NO), inducible nitric oxide synthase (iNOS), and cyclooxygenase (COX)-2 (Zhang et al., 2018; Linghu et al., 2020). NO is mainly synthesized by iNOS which is largely involved in the pathophysiology of many inflammatory diseases (Cheng et al., 2016). COX-2 is another key enzyme in inflammatory responses (Dai et al., 2018). Inhibition of iNOS and COX-2 protein expression and/or enzyme activity have been considered as a potential mechanism of inflammation therapy (Tseng et al., 2018; Lee et al., 2020a). Various agents could serve as an important therapeutic target in the treatment of various inflammation-based pathologies, for example, steroidal hormones. Hence, non-steroidal anti-inflammatory drugs (NSAIDs) are broadly used. However, NSAIDs have severe adverse effects as they damage the upper gastrointestinal tract by inhibiting prostaglandin synthesis (Li et al., 2019). Therefore, the development of new anti-inflammatory agents that are safer and more effective is ongoing (Lee, 2020b).

Received 19 Sept., 2021
Accepted 20 Oct., 2021
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Natural products are a key source and play an important role in discovering next generation medicines (Wang et al., 2020). *Acanthopanax*, one of the important medicinal genera of Araliaceae, is a plant genus that embraces over 70 plant species that are mainly distributed in China, Korea, Japan, Thailand, and the far-eastern regions of Russia (Yanzhong, 2012; Ganogpichayagrai & Suksaard, 2020; Wang et al., 2020). *Acanthopanax trifoliatus* (L.) Merr. belongs to the *Acanthopanax* genus have been used as both traditional plant food and medicinal plant in the coastal region of South China (Chen et al., 2020). It is commonly used in traditional Chinese medicine with a ginseng-like activity for treating bruising, neuralgia, impotence, and gout in China (Chen et al., 2021). It also exhibits a rather good curative effect for treatment of common cold, jaundice, gastric pain, diarrhea, and ulcer (Sithisarn et al., 2011; Li et al., 2016). Moreover, the young leaves and shoots of *A. trifoliatus* are popularly consumed as vegetables in traditional southern Chinese cuisine that can be stir-fried or cooked in a soup (Peng et al., 2020). In addition, the local people of southern China have been using the leaves of this plant to make tea, which is called "Le Cai", for daily consumption at least 10 years for health management (Roslida et al., 2010; Sithisarn et al., 2011).

Taking into consideration the above facts, present study aimed to evaluate the anti-inflammatory activities of extracts from *A. trifoliatus* through effects on the production of TNF-α, IL-6, and NO, as well as on the expression of iNOS and COX-2 by LPS-induced RAW 264.7 macrophages.

2 Materials and methods

2.1 Plant materials

The roots, stems, and leaves of *A. trifoliatus* were collected in November 2015 in Jiangxi (China). The plant species was confirmed by Professor XiangQian Liu (Hunan Key Laboratory of Traditional Chinese Medicine modernization, Hunan University of Chinese Medicine, Changsha, China), and the voucher specimen (no. 20151125) was deposited at the School of Pharmacy, Hunan University of Chinese Medicine (Changsha, China).

2.2 Preparation of sample

The air-dried stems of *A. trifoliatus* (ATS) were ground into a fine powder. The powder (30 g) was decocted with distilled water on reflux extraction for 2 hour and the other powder (200 g) was decocted with methanol (MeOH, 2 × 2h) on reflux extraction before filtration. The water extract (ATSW) was freeze-dried, while the methanol extract (ATSM) was vacuum dried, the methanol extract was partitioned into H_2O and extracted successively with petroleum ether (PE, 60-90), dichloromethane (CH_2Cl_2), ethyl acetate (EtOAC), *n*-butanol (*n*-BuOH) to obtain a petroleum ether extract (ATSPE), CH_2Cl_2 extract (ATSDC), EtOAc extract (ATSEA), and *n*-BuOH extract (ATSBU).

Using the method mentioned above, the roots of *A. trifoliatus* (ATR) were extracted to obtain ATRW, ATRM, ATRPE, ATRDC, ATREA, and ATRBU. The leaves of *A. trifoliatus* (ATL) were extracted to obtain ATLW, ATLM, ATLPE, ATLDC, ATLEA, and ATLBU. All fractions were stored at 4°C until analyzed. Each fraction was dissolved in dimethyl sulfoxide (DMSO) for functional assays.

2.3 Cell viability

Cell viability assays were performed to determine the cytotoxicity of *A. trifoliatus* using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Amresco, Solon, OH, USA) assay (Park et al., 2015). RAW 264.7 cells [American Type Culture Collection (ATCC); Manassas, VA, USA] were plated at a density of 1×10^5 cells/mL in 96-well plates and cultured at 37 °C and 5% CO_2 in the Dulbecco's Modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY, USA) with 100 μL of 10% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT, USA) for 5 h, then RAW 264.7 cells were incubated 5 μL with various concentrations of samples (10, 20, and 40 μg/mL) for 20 h and the treatment medium then completely replaced with MTT solution, 5μL of MTT solution (5 mg/mL) was then added to each well and the cells were cultured for a further 4 h, The plates were removed from the incubator and the formazan crystals were dissolved by the addition of 100 μL of dimethyl sulfoxide. The absorbance at 570 nm was read on a microplate reader (Spark10M, TECAN, Switzerland) as a measure of cell viability.

2.4 NO and inflammatory cytokine assays

The RAW 264.7 cells were seeded into a 96 well plate at a density of 2.5×10^4 cells/well and allowed to adhere for 4 h. Then, cells were pretreated with various concentrations of samples (10, 20, and 40 μg/mL) for 1 h and stimulated with 5 μL of lipopolysaccharide (LPS) at 0.5 μg/mL for 24 h. To measure NO secretion, the cell supernatant was harvested and reacted with the Griess reagent [0.1% N-(1-naphathyl)-ethylenediamine, 1% sulfanilamide in 5% phosphoric acid] for 10 min at room temperature in the dark. The absorbance at 540 nm was detected and the concentration was calculated using a nitrite standard solution.

The levels of IL-6 and TNF-α were measured using a commercial BD OptEIA™ ELISA kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocols.

2.5 Western blot analysis

Total protein (30 μg) was separated on a 10% SDS polyacrylamide gel and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). Each membrane was then incubated for 1 h in 5% skim milk in TBS-T buffer (0.1 M Tris-HCl, pH 7.4, 0.9% NaCl, 0.1% Tween-20) to block non-specific binding and was then incubated with primary antibodies that recognized iNOS (Cat. no. ADI-905-431, 1:1,000; obtained from Enzo Life Sciences, Farmingdale, NY, USA), COX-2 (Cat. no. sc-1747, 1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), β-actin (Cat. no. MA5-15379, 1:2,000; Cell Signaling Technology, Danvers, MA, USA), Each protein was detected using a chemiluminescence detection system according to the instructions of the manufacturer (ECL; Amersham, Berkshire, UK)

2.6 Statistical analysis

The results are expressed as the means ± SEM of the sample size determinations. Statistical significance was determined using a two-tailed Student's t-test for independent means. The test results are reported as two-tailed *P*-values, where $P < 0.05$ was considered to indicate a statistically significant difference.

3 Results

3.1 Effect of 18 fractions extracted from ATR, ATS, and ATL on the viability of RAW264.7 macrophages

The cytotoxic effect of 18 fractions extracted from ATR, ATS, ATL on LPS-stimulated RAW264.7 macrophages was determined at concentration of 20 μg/mL using the MTT assay. As shown in Figure 1A, the results of this analysis demonstrated that 18 fractions produced no significant change in cell viability compared to the untreated control group**.**

3.2 Effect of 18 fractions extracted from ATR, ATS, and ATL on NO production of RAW264.7 macrophages

NO is a signaling molecule which plays an important role in the inflammatory response. To examine whether 18 fractions extracted from ATR, ATS, ATL treatment could modulate NO production, we measured the NO secretion in LPS-induced RAW 264.7 cells after 18 fractions treatment separately at concentration of 20 μg/mL, using a Griess reagent assay. As shown in Figure 1B, LPS treatment significantly induced NO production compared to that in the untreated control, while cells pretreated with ATSDC demonstrated a significant inhibition of NO production.

Then, the study determined the viability and NO production of ATSDC at different concentrations (10, 20, and 40 μg/mL) before the next inflammatory cytokine assays. As shown in Figure 2AB, there was no significant change in cell survival at concentrations (10, 20, and 40 μg/mL) of ATSDC, which greatly reduced the production of NO in dose-dependent manner.

3.3 Effect of ATSDC on the expression of IL-6 and TNF-α in RAW264.7 macrophages

To determine whether the ability of ATSDC to inhibit inflammatory signaling corresponded to a reduction in the secretion of pro-inflammatory cytokines, we investigated cytokine secretion in LPS-activated macrophages using ELISA kits. As shown in Figure 2CD, at a dose of 40 μg/mL, ATSDC treatment dramatically decreased the production of the pro-inflammatory IL-6 and TNF-α.

3.4 Effect of ATSDC on iNOS and COX-2 protein expression

Two other common mediators of inflammation are iNOS and COX-2. To evaluate whether ATSDC influence iNOS and COX-2 expression, we performed Western blot analysis. LPS-stimulated cells exhibited a significant increase in iNOS and COX-2 expression, when compared to the untreated control. Treatment with ATSDC greatly down-regulated the production of iNOS and COX-2 stimulated by LPS in a concentration-dependent manner, as shown in Figure 3.

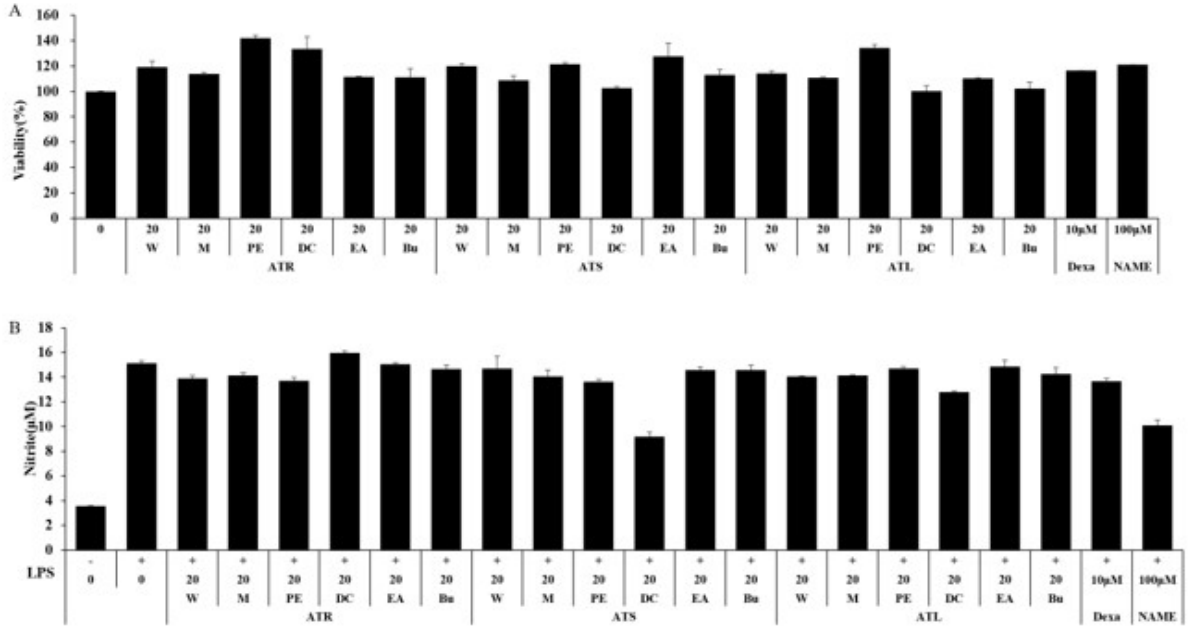

Figure 1. Effect of 18 different fractions on viability and NO production of LPS-induced RAW264.7 macrophages. Cells were pretreated for 1h with 18 different fractions (20 μg/mL) and then treated with LPS (0.5 μg/mL) for 24 h. (**A**) Cell viability was measured using an MTT assay; (**B**) NO production was measured using a Griess reagent assay; **Dexamethasone (Dexa) and** L-NG-monomethyl arginine (L-NMMA) were used as a positive control. Data were denoted as the mean ± SEM (n = 3).

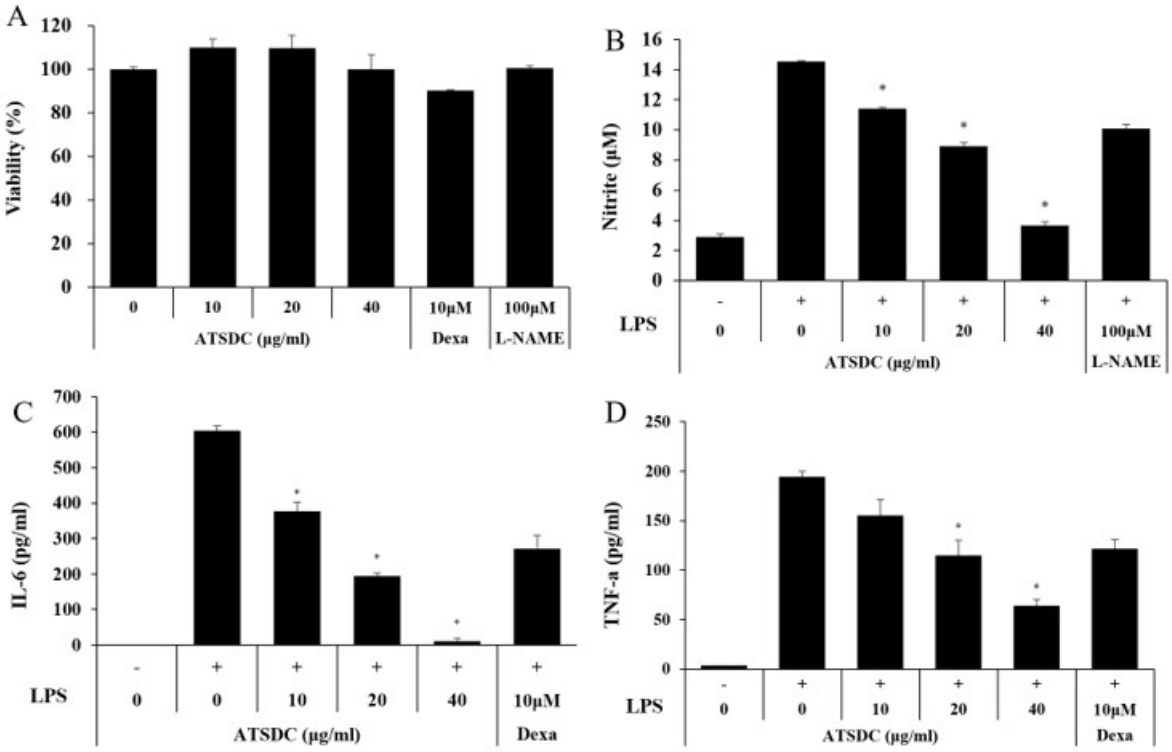

Figure 2. Effect of ATSDC on IL-6 and TNF-α production in LPS-induced RAW 264.7 macrophages. Cells were pretreated for 1h with ATSDC (10, 20, and 40 μg/mL) and then treated with LPS (0.5 μg/mL) for 24 h. (**A**) Cell viability was measured using an MTT assay; (**B**) NO production was measured using a Griess reagent assay; **Dexamethasone (Dexa) and** L-NG-monomethyl arginine (L-NMMA) were used as a positive control. (**C**) IL-6 and (**D**) TNF-α contents in the culture medium were determined by ELISA kits. Dexametasone (Dexa) was used as positive control. Data were denoted as the mean ± SEM (n = 3). $^{*}P < 0.05$, relative to control group.

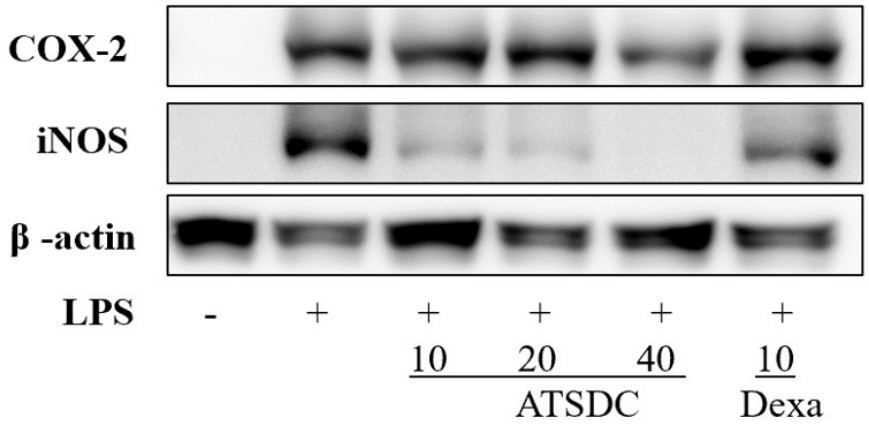

Figure 3. Effect of ATSDC on LPS-induced iNOS and COX-2 protein expression in RAW264.7 cells. Cells were pretreated for 1h with ATSDC (10, 20, and 40 μg/mL) and then treated with LPS (0.5 μg/mL) for 24 h. iNOS and COX-2 and β-actin were detected by Western blot analysis.

4 Discussion

The current findings showed that both iNOS and COX-2 pathway were remarkable inhibited by the dichloromethane extract prepared from stems of *A. trifoliatus*. In addition, we also found that ATSDC inhibited NO, TNF-α, and IL-6 production in LPS-stimulated RAW264.7 cells.

Inflammation, a basic defense mechanism, protects organs from endogenous and exogenous stimuli, such as bacterial

infection (Hou et al., 2018). However, excessive inflammation causes many diseases, such as inflammatory arthritis, asthma, and atherosclerosis (Lee et al., 2020a). LPS, a main component of outer cell membrane of Gram-negative bacteria, has been recognized as one of the most potent microbial initiators of inflammation (Li et al., 2019; Ko et al., 2019). Upon stimulation with LPS, RAW 264.7 macrophages cells could trigger the secretion of numerous inflammatory cytokines and mediators such as NO, TNF-α, IL-6 and so on (Luo et al., 2020). NO is a vital cellular signaling and defensing molecule involved in inflammation. It can be catalyzed via iNOS (Wang et al., 2019). The over-production of NO has been linked to various inflammatory disorders (Lee et al., 2019). In addition, the production of NO is also regulated by the enzyme of COX-2 (Wu et al., 2020). Accumulated data indicate that COX-2 is involved in many inflammatory processes and induced in various disease, such as carcinomas and liver inflammation, suggesting that COX-2 plays a key role in inflammation (Cheng et al., 2016). In our study, we firstly tested the cytotoxicity and NO production of 18 fractions that extracted from the leaves, stems, roots of *A. trifoliatus* to select the bioactive fraction. Fraction of ATSDC shows great cell viability and significant inhibitory of NO production in dose-dependent manner compared to the other tested extracts. It demonstrated that the inhibitory effect of NO production of the stems of *A. trifoliatus* may better than the effect of leaves and roots of *A. trifoliatus*.

Pro-inflammatory cytokines such as TNF-α, and IL-6 can induce cell and tissue damage and also activate macrophages in various inflammation-associated diseases (Olajide et al., 2020). TNF-α induces synergy in NO production in LPS-stimulated macrophages, causing inflammatory responses such as fever, vasodilatation, and edema (Zelová & Hošek, 2013). In addition, IL-6 is a multifunctional cytokine that plays a role in inflammation response through the stimulation of acute phase responses, hematopoiesis, and immune reactions (Zbakh et al., 2020). Therefore, targeting the inflammatory mediators and cytokines is a useful strategy in anti-inflammatory therapy (Lee et al., 2019). In the present study, we found that ATSDC significantly inhibited the LPS-induced production of NO by suppressing the expression of iNOS and COX-2 proteins in a dose-dependent manner, at a dose that was not cytotoxic. We also found that the pro-inflammatory cytokines, TNF-α and IL-6, were clearly inhibited by pre-treatment with ATSDC in LPS-stimulated RAW264.7 cells.

In the recent years, much attention has been focused on using traditional herbal medicines containing bioactive compounds as alternatives to the existing anti-inflammatory drugs that have many side effects (Hou et al., 2018; Zamani et al., 2019; Olajide et al., 2020; Lee et al., 2020a). To our knowledge, phytochemical investigations of *A. trifoliatus* lead to the identification of a series of chemical components including chlorogenic acid, isochlorogenic acids, sesquiterpenoids, stilbenoids, polysaccharides, flavonoids and triterpenoids (Yook et al., 1999; Kiem et al., 2004; Phuong et al., 2006; Sithisarn et al., 2011; Li et al., 2016; Peng et al., 2020). Biological studies also reported that *A. trifoliatus* extracts showed numerous biological activities including antioxidative, anti-inflammatory, anti-cancer, and neuroprotective effects (Sithisarn & Jarikasem, 2009; Sithisarn et al., 2013; Wang et al., 2014; Chen et al., 2019). However, the stems of *A. trifoliatus* has not been reported to have effects against inflammation in LPS-induced RAW264.7 macrophages. Hence, ATSDC could be further investigated to isolate and identify bioactive compounds that may be a potential therapeutic agent for the treatment of inflammation-related diseases.

5 Conclusion

In conclusion, the present study showed ATSDC treatment suppressed the NO production, likely through decreased the protein expression of iNOS and COX-2, and inhibited inflammatory cytokines such as TNF-α, and IL-6 in LPS-induced RAW264.7 cells. These results suggested that ATSDC possess effective anti-inflammatory activities and implies that the stem bark of *A. trifoliatus* may be able to inhibit the inflammatory diseases. In addition, the root bark of *Acanthopanax* plants, for example, Acanthopanax cortex, which is non-renewable resources have been traditionally used to treat many diseases (Chinese Pharmacopoeia Commission, 2015). In this research, ATS that are renewable and sustainable resources have significant anti-inflammatory activities and could be a new source of anti-inflammatory drugs. Moreover, the leaves of *A. trifoliatus* that are popularly consumed as vegetables and herbal teas with high nutritional value and health care function in Guangdong province that has been prevalently planted for years (Roslida et al., 2010; Sithisarn et al., 2011; Peng et al., 2020). Hence, this research also furnished a reliable theoretical and practical basis for the application of *A. trifoliatus* as a natural anti-inflammatory ingredient in health foods or complementary medicines in the future.

Abbreviations

A. trifoliatus, *Acanthopanax trifoliatus* (Linn.) Merr.; ATR, the roots of *A. trifoliatus*; ATS, the stems of *A. trifoliatus*; ATL, the leaves of *A. trifoliatus*; ATRM, the methanol extract from ATR; ATRW, the water extract from ATR; ATRPE, the petroleum ether extract from ATR; ATRDC, the dichloromethane extract from ATR; ATREA, the ethyl acetate extract from ATR; ATRBU, the n-butanol extract from ATR; ATSM, the methanol extract from ATS; ATSW, the water extract from ATS; ATSPE, the petroleum ether extract from ATS; ATSDC, the dichloromethane extract from ATS; ATSEA, the ethyl acetate extract from ATS; ATSBU, the n-butanol extract from ATS; ATLM, the methanol extract from ATL; ATLW, the water extract from ATL; ATLPE, the petroleum ether extract from ATL; ATLDC, the dichloromethane extract from ATL; ATLEA, the ethyl acetate extract from ATL; ATLBU, the n-butanol extract from ATL; DMEM, Dulbecco's Modified Eagle's medium; L-NMMA, L-NG-monomethyl arginine; FBS, Fetal bovine serum; LPS, Lipopolysaccharide; NO, Nitric oxide; TNF-α, Tumor necrosis factor-α; IL-6, Interleukin-6; iNOS, Inducible nitric oxide synthase; COX-2, Cyclooxygenase 2; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

The authors are grateful to the colleagues who contributed to this study. This work is financially supported by the Natural Science Foundation of Hunan Province, China (grant numbers 2019JJ40223); First-class Discipline Project on Chinese Pharmacology of Hunan University of Chinese Medicine (school file number: "science" [2018] No. 3) and The Key Discipline of Biological Engineering of Hunan University of Chinese Medicine [2018] No. 3.

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