SHORT COMMUNICATION

Anti-inflammatory activity of the water extract of *Semiliquidambar cathayensis* leaf

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\textbf{ABSTRACT}

In this study, the anti-inflammatory effect of the water extract of *Semiliquidambar cathayensis* leaf (WESCL) was evaluated for the first time. WESCL exhibited anti-inflammatory activity by significantly reducing the cell metamorphosis, the production of nitric oxide (NO), and reactive oxygen species (ROS) in LPS-stimulated RAW 264.7 macrophages while showing no cytotoxic effect to the cells. Furthermore, an \textit{in vivo} study revealed that WESCL could alleviate the disease development of osteoarthritis (OA) and decrease the level of interleukin-6 (IL-6) in mice. Chemical composition analysis indicated that WESCL contained high amounts of phenolic compounds, flavonoids, and triterpenoid saponins, with the total content being 30.6 mg gallic acid equivalent (GAE)/g, 38.2 mg quercetin equivalent (QE)/g, 100.5 mg oleanolic acid equivalent (OAE)/g, respectively. The present study successfully identified WESCL as a naturally-occurring anti-inflammatory agent, supporting its potent application for the treatment of inflammation-related diseases.

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1. Introduction

*Semiliquidambar cathayensis* Chang (Hamamelidaceae family) is a species only distributed in the South of China and is currently listed in the catalog of national protection level II. Its roots and barks were widely used as folk traditional medications for the treatment of inflammation-related diseases, such as rheumatoid arthritis, lumbar muscle degeneration, postpartum flaccidity disease, hemiplegia, and hepatitis (Liang et al. 2015). The high medicinal demands for the roots and barks of this plant by locals cause this plant to get endemic. However, despite being widely used as folk medications, as far as we know, the biological effects of this plant were largely unexplored, lacking scientific data to support its folk medicinal applications. It was reported that *S. cathayensis* had blood-activating and stasis-dissolving effects in a rat model (Liang et al. 2015), and Sun et al. (2014) found that *S. cathayensis* had an inhibitory effect against hepatitis B virus. Moreover, some earlier studies investigated the phytochemical composition of *S. cathayensis*, revealing that it mainly contained triterpenoids (Zhou et al. 2002), flavonoids and polyphenolic compounds (Qiu et al. 2020).

Inflammation is a kind of natural response and protection against a variety of harmful stimuli such as lipopolysaccharide (LPS) (Zhang et al. 2022). If prompt treatment is not given, inflammation may then lead to cancer development. Currently, there are many anti-inflammatory drugs, such as indomethacin, ibuprofen, aspirin and so on. But these drugs always cause some adverse reactions in the body, especially damage to the kidneys (Sun et al. 2019). Therefore, searching for novel anti-inflammatory drugs, especially from plant resources, should be of great significance. Traditional Chinese Medicines (TCM) are well known as rich sources of biologically interesting natural products, and are widely used to treat various inflammations with multi-component, multi-target, multi-function and multi-pathway characters (Sun et al. 2019; Guo et al. 2020).

As a part of our continuous screening program for biologically interesting components from natural resources, the plant *S. cathayensis* was selected due to its anti-inflammatory applications by folk medicinal practitioners. Because of the shortage of this plant resource, the leaf of *S. cathayensis* instead of its root and bark was investigated. Herein, the anti-inflammatory effect of WESCL was reported together with its chemical composition involving the total content of phenolic compounds, flavonoids, and triterpenoid saponins. The results presented here might benefit the development of naturally-occurring anti-inflammatory agents from *S. cathayensis* leaf, and help the conservation of this rare plant.

2. Results and discussions

2.1. Effect of WESCL on cell viability

As shown in Figure S1 (supplemental material), after being treated with different concentrations of WESCL (0.2 and 2 mg/mL), LPS (1.5 μg/mL) and WESCL&LPS, the cell viability exhibited no significant difference among the treated groups and the normal control group (NC) (p > 0.05), indicating that LPS at 1.5 μg/mL and the WESCL within the concentrations of 2 mg/mL, had no side effect on the cell viability. Interestingly,
the treatment of WESCL mildly promoted cell proliferation when compared to the NC group, despite no significant difference between them, supporting the application potency of WESCL. Therefore, these concentrations were used for further anti-inflammatory tests. Similarly, LPS at 1.0 (Sun et al. 2019) and 1.5 μg/mL (Durgha et al. 2016) were used to stimulate the inflammation of RAW264.7 cells without compromising the cell viability.

### 2.2. Effect of WESCL on the cell morphology

Cell morphology is an important characteristic of cells. Therefore, examining the changes in cell morphology of LPS induced macrophages can provide direct insight into the cells’ response to anti-inflammatory agents. Figure S2 (supplemental material) shows that the normal control cells had a round shape, while the treatment of LPS significantly challenged the morphology of the cells, forming more pseudopods, irregular shapes and cellular vacuoles than those of the control group. This morphology was a characteristic of LPS-stimulated cells, similar to those reported previously (Liu et al. 2017; Sun et al. 2019). However, after being treated with WESCL, especially at 2 mg/mL, some metamorphic cells were reverted to normal shape, significantly reducing the production of pseudopods, irregular shapes and vacuoles among LPS-stimulated cells. Similar phenomena were also observed in previous studies: for example, silymarin, a polyphenolic flavonoid isolated from milk thistle (Silybum marianum), significantly inhibited LPS-induced morphological changes in the mouse RAW264.7 macrophage cell line (Kim et al. 2015); xanthan gum was found to prevent the morphology alteration associated with LPS induced RAW264.7 cells (Liu et al. 2017).

### 2.3. Effect of WESCL on the generation of NO

NO (Nitric Oxide) could change the permeability of the cell membrane and increase the release of inflammatory factors involving tumor necrosis factor-α (TNF-α) and Interleukin-6 (IL-6), resulting in the development of inflammation (Kim et al. 2020). Therefore, the present study evaluated the anti-inflammatory effects of WESCL by testing NO production in LPS-stimulated macrophages. As shown in Figure S3 (supplemental material), stimulation with LPS resulted in a significant increase in NO production relative to that in the normal controls (NC, p < 0.05). However, the NO production in LPS-stimulated RAW264.7 cells was significantly inhibited when treated with WESCL. The inhibitory effect was more pronounced at a higher concentration of 2 mg/mL. When compared to the positive drug Dexamethasone (Dex, IC_{50} < 0.1 mg/mL), WESCL (IC_{50} = 2.12 mg/mL) was less potent. iNOS is the key enzyme that promotes the generation of NO and could be induced by LPS stimulation (Kim et al. 2020). When stimulated with LPS, iNOS gene expression was increased and excessive NO was released (Bian and Murad 2007). Therefore, WESCL might reduce LPS-stimulated NO overproduction via potent inhibition of iNOS gene overexpression, or by suppressing iNOS enzymatic activity.
2.4. Effect of WESCL on the production of ROS in LPS-stimulated RAW264.7 cell

LPS could lead to the damage of mitochondria, resulting in the overproduction of intracellular reactive oxygen species (ROS), which exerts oxidative stress in cells and exacerbates the progress of inflammation (Cao et al. 2017). In this study, ROS was detected by a Dichlorodihydrofluorescein diacetate (DCFH-DA) probe which can emit fluorescence upon oxidation by ROS. As shown in Figure S4A (supplemental material), the LPS stimulation significantly increased the production of ROS when compared with the untreated normal control group (NC). For the group treated with a low concentration of WESCL (0.2 mg/mL), the fluorescence intensity exhibited some degrees of decrease relative to the only LPS group, but there was no significant difference between them (p > 0.05). However, when treated with a high concentration of WESCL (2 mg/mL), the production of ROS was significantly inhibited, where its fluorescence intensity was even weaker than that of the Dex-treated group despite showing no significant difference (p > 0.05). In good agreement with the results revealed in Figure S4A, the images obtained from the fluorescence microscope also supported that the WESCL could inhibit the accumulation of ROS. As shown in Figure S4B (supplemental material), the number of fluorescence emitting cells in the WESCL-treated group was less than that in only the LPS group, and a higher concentration of WESCL resulted in a fewer number of fluorescent cells, being consistent with the observations in Figure S4A. ROS function as intracellular signaling molecules and are often considered as important indicators of body inflammation (Ihsan et al. 2018). Meanwhile, NO and ROS are produced concomitantly in response to pathogen and stress challenges, and can act co-operatively in mediating the defense responses (Wu and Wu 2008). In addition, it has been reported that the iNOS/NO pathway plays a crucial role in inducing ROS accumulation (Liu et al. 2005). Therefore, we suspected that the inhibitory effects on NO production by WESCL might be due in part to its ROS scavenging ability. The present study found that WESCL could inhibit the LPS-stimulated production of both ROS and NO in a dose-dependent manner, supporting the potent application of WESCL as an anti-inflammatory agent.

2.5. In vivo study by using a mouse model of osteoarthritis (OA)

During inflammatory development, synovium hyperplasia and hypertrophy, cartilage degradation and pannus formation have critical roles in OA pathogenesis (Huang et al. 2019). As shown in Figure S5A (supplemental material), when compared with the NC group, synovium hypertrophy (marked by blue arrows) and hyperplasia around the lateral condyle (marked by blue arc) were present in the OA group, along with the cartilage erosion (marked by black arrows). These symptoms were less severe in the WESCL group, but there was no significant difference between them. Furthermore, the pannus (marked by red arrows) formed inside the intercondylar groove of the mouse due to inflammation, and its formation was much more pronounced in the OA group than that in the WESCL group, suggesting the treatment of WESCL could alleviate the disease development of OA. Meanwhile, the levels of Interleukin-6 (IL-6) in the serum of mice were investigated two weeks after the injection of monosodium iodoacetate (MIA). IL-6 is a pleiotropic cytokine, which elicits a broad spectrum of inflammatory
events and has shown significant involvement in the pathogenesis of OA (Nees et al. 2020). As displayed in Figure S5B, the level of IL-6 in the group treated with WESCL was lower than that in the OA group, while exhibiting no significant difference between them \((p > 0.05)\). These results allowed us to assume that other inflammatory factors in addition to IL-6 might be targeted by WESCL, but more studies should be carried out in future to demonstrate the action mechanism of WESCL. It was reported that all TNF-\(\alpha\), IL-1\(\beta\), IL-6, IL-17 and IL-18 were generally recognized as the most important inflammatory factors controlling the progression of OA (Liao et al. 2020).

**2.6. Chemical composition of WESCL**

Only a few previous studies investigated the phytochemical composition of *S. cathayensis*, however, mainly focusing on its barks and roots. These previous studies revealed that the *S. cathayensis* contained three major categories of secondary metabolites involving triterpenoid (Zhou et al. 2002), flavonoid and polyphenolic compound (Qiu et al. 2020). Being consistent with these observations, we recently isolated resveratrol derivatives, triterpenoids, flavonoids and phenolic glycosides from *S. chingii*, another species of *Semiliquidambar* (Zhang et al. 2022). Therefore, the total contents of flavonoid, phenolic compound, and triterpenoid saponin in WESCL were investigated, respectively in the present study. Our results indicated that the WESCL contained high amounts of these kinds of compounds, with TFC, TPC, and TTS being 38.18 mg QE/g, 30.6 mg GAE/g and 100.5 mg OAE/g, respectively. The triterpenoid saponins have been reported to exhibit anti-inflammatory effects by inhibiting the production of ROS, TNF-\(\alpha\), IL-8 and iNOS in LPS-stimulated cells (Jeong et al. 2013; Fu et al. 2015). Similarly, the anti-inflammatory effects of flavonoids and phenolic compounds already had well-studied (Kırmızıbekmez et al. 2019; Jucá et al. 2020). Taken together, these results allowed us to suppose that the anti-inflammatory effect of WESCL might be attributable to its high content of flavonoid, phenolic compound, and triterpenoid saponin. However, a more comprehensive phytochemical investigation of WESCL should be carried out to justify this assumption in future work.

**3. Conclusion**

The results presented here revealed that the WESCL demonstrated an anti-inflammatory effect by significantly inhibiting the production of ROS and NO, alleviating the disease development of OA and slightly decreasing the level of IL-6 in mice, which supported the folk medicinal application of *S. cathayensis* for the treatment of inflammation-engaged disease. Furthermore, the leaf of *S. cathayensis* was identified as having anti-inflammatory activity in the present study, suggesting that it might be used as an alternative resource to replace the bark or root as folk medications, which might help the conservation of this rare plant.

**Disclosure statement**

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