Investigation of anti-inflammatory lignans from the leaves of Symplocos sumuntia Buch-Ham ex D Don (Symplocaceae)

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Abstract

**Purpose:** To investigate the anti-inflammatory activity of Symplocos sumuntia Buch.-Ham. ex D. Don and identify the main secondary metabolites responsible for this effect.

**Methods:** The in vitro anti-inflammatory activity of the plant extract and isolated compounds was determined in terms of the ability to inhibit the production of nitric oxide (NO), and expressions of iNOS and COX-2 proteins in RAW264.7 cells stimulated by lipopolysaccharide (LPS). Compounds were isolated and identified by spectroscopic methods.

**Results:** The methanol extract of S. sumuntia leaves showed strong inhibitory effects on nitric oxide (NO) production and expression of iNOS and COX-2 proteins in LPS-induced RAW264.7 cells. A phytochemical assay-guided fractionation of the methanol extract of S. sumuntia leaves led to the isolation of four lignans which are arctigenin (1), matairesinol (2), monomethylpinoresinol (3) and pinoresinol (4). These compounds were identified for the first time from S. sumuntia. All four compounds inhibited the production of nitric oxide (NO), with arctigenin showing the most potent activity with half-maximal inhibitory concentration (IC₅₀) value of 4.08 µM.

**Conclusion:** S. sumuntia is a promising source of anti-inflammatory agents, which may clarify to the therapeutic use of this plant in Vietnamese traditional medicine.

**Keywords:** Symplocos sumuntia, Symplocos caudata, Lignan, Arctigenin, Anti-inflammatory

INTRODUCTION

Symplocos is a genus of flowering plants with about 300 species distributed in Asia, Australia and America [1]. The genus Symplocos is well known for their traditional uses in the treatment of various diseases such as leprosy, gynecological disorders, ulcers, leucorrhea, menorrhagia, malaria, and tumefaction. Previous investigations indicated that those plants contain terpenoids, flavonoids, lignans, phenols, steroids, alkaloids, and iridoids [2]. These plants exhibited antipyretic, anti-inflammatory, antibacterial, antioxidant, and anticancer activities [2,3]. Symplocos sumuntia Buch.-Ham. ex D. Don (synonym Symplocos caudata Wall. ex G. Don, Symplocos tonkinensis Brand) is a Vietnamese traditional medicinal herb that grows in the mountainous regions at 700-1500 m altitude [4]. Roots, leaves and flowers of this plant have been used in traditional medicine to treat cough, tonsillitis, stomachache, inflammation,
hyperlipidemia and hypertension [5]. This plant is exploited from natural source for medicinal purpose. There are only few reports on the chemical composition of *S. sumuntia*. Nortriterpenoid saponins, neolignans, phenolic glycosides, daucosterol, glucose, sucrose and inositol were isolated [6,7]. Two other studies revealed the presence of neolignan glycosides, preneolignan glycoside, phenylpropanoid glycosides and cerebrosides [8,9]. However, literature searches have shown no report on biological activities of this plant.

Inflammation is a normal protective process that responds to tissue injury. In this process, cytokines, such as interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, and inflammatory mediator, such as NO and PEG2, are increased by activated inflammation cells. It is known that inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), two major inflammatory mediators, are expressed in a variety of cells including macrophages in response to pro-inflammatory stimuli such as lipopolysaccharide (LPS), ILs, and TNF-α [10]. They are responsible for the production of NO and prostaglandins, which have been implicated in the tissue destruction and pathogenesis of a number of immunological and inflammatory diseases [10]. Overproduction of NO causes numerous human diseases, such as arthritis, asthma, inflammation, diabetes and cancer [11]. Therefore, an assay for inhibition of NO production is one of the possible ways to screen anti-inflammatory agents [12,13].

In our continuing search for anti-inflammatory agents of natural origin, we found that a methanol extract of *Symplocos sumuntia* strongly suppressed the production of NO in LPS-induced RAW264.7 cells (with 60.1% at a concentration of 30 μg/mL). The present study aims to investigate the NO inhibition of this extract and identify the main secondary metabolites responsible for the anti-inflammatory activity of this plant.

**EXPERIMENTAL**

**Plant material**

The leaves of *Symplocos sumuntia* were collected at VinhPhuc Province, Vietnam in March 2013, and authenticated by Prof Huy Thai Tran, Institute of Ecology and Biological Resources, VAST. A voucher specimen (no. BK-B02) was deposited at the herbarium of the School of Chemical Engineering, Hanoi University of Science and Technology.

**Reagents and cell culture**

All culture media and reagents were supplied by Invitrogen (Grand Island, NY). Primary and secondary antibodies were bought from Santa Cruz Technology (USA). The macrophage cell line, RAW264.7, was purchased from ATCC (USA).

**Assay for inhibition of NO production**

The inhibitory effect of tested samples against NO production was evaluated in RAW264.7 cells stimulated by lipopolysaccharide (LPS) as previously described [14]. The Griess reagent was used for quantification of NO levels and cell viability was evaluated by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) uptake method.

**Western blot analysis**

RAW264.7 cells were plated in 6 cm dishes at 70 % confluence and incubated at 37 °C overnight. After pre-treatment with tested samples for 30 min, the cells were incubated with 1 μg/mL LPS for another 24 h. The cells were harvested, washed twice with ice-cold phosphate-buffered saline by centrifugation, and then lysed in lysis buffer containing 50 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, 5 mM sodium orthovanadate, 1 % NP-40 and protease inhibitor cocktail. The cell lysates were subjected to electrophoresis on 10 % sodium dodecyl sulfate-polyacrylamide gel and then the proteins were transferred to a polyvinylidenedifluoride membrane. The membrane was blocked with 5 % skim milk, and incubated overnight with primary antibodies of iNOS, COX-2 and α-tubulin at 4 °C. The corresponding HRP-conjugated secondary antibody was then added for 1 h incubation at room temperature. The signals were detected by enhanced chemiluminescent system (Intron, Seongnam, Korea) [15].

**Extraction and bioassay-guided isolation**

Air-dried and powdered leaves of *Symplocos sumuntia* (4 kg) were extracted three times with methanol at 40 °C under ultrasonic condition. The combined extracts were concentrated to obtain 300 g methanol residue, which was suspended in water (2 L), and then successively partitioned with n-hexane (1.5 L x 3 times) and ethyl acetate (1.5 L x 3 times). The organic layers were concentrated to give 36.6 and 66.7 g of n-hexane and ethyl acetate residues, respectively. The ethyl acetate residue (71 % inhibition of NO production at 30 μg/mL) was subjected to chromatography on a silica gel
column eluted with a mixture of n-hexane and acetone (from 100:0 to 0:100 v/v) to give ten fractions, F1-F10. Fraction F5 (87 % inhibition of NO production at 30 μg/mL) was separated on a silica gel column eluting with n-hexane-acetone (4:1, v/v) to give four fractions, F5.1-F5.4. Fraction F5.1 (98 % inhibition of NO production at 30 μg/mL) was further separated by using a silica gel column with n-hexane-dichloromethane-ethyl acetate (2:6:1, v/v/v) as eluent, followed by a silica gel column with n-hexane-dichloromethane-ethyl acetate (8:32:1, v/v/v) to obtain compound 1 (885.8 mg) and compound 3 (198.3 mg). Fraction F5.3 (92 % inhibition of NO production at 30 μg/mL) was isolated on a silica gel column eluting with n-hexane-dichloromethane-ethyl acetate (5:40:4, v/v/v), followed by a RP-C18 column with methanol-water (1:1.2, v/v) as eluent to obtain compound 2 (374.3 mg) and compound 4 (7.6 mg).

Statistical analysis

Each assay was repeated at least three times. Means were checked for statistical differences by Student’s t-test with error probability set at p < 0.05

RESULTS

In a screening test for the inhibition of NO production in LPS-stimulated RAW 264.7 cells, the methanol extract of S. sumuntia leaves (SS) showed remarkable inhibitory effect in a dose-dependent manner. SS did not inhibit NO production at low concentration (3 μg/mL). When cells were treated with higher concentrations of SS (10, 30 and 100 μg/mL), the inhibition increased to 23, 44 and 73%, respectively (Figure 1). Since COX-2 and iNOS are two major inflammatory mediators, and the induced expression of iNOS is primarily responsible for the NO production, we examined SS for subsequent study on the expression of COX-2 and iNOS. Consistently, in Western blot analysis, SS also prevented the LPS-induced expressions of COX-2 and iNOS at 10 and 30 μg/mL (Figure 2). These results suggested that S. sumuntia might contain strong anti-inflammatory compounds.

![Figure 1](image1.png)

**Figure 1:** Effect of S. sumuntia extract (μg/mL) on the NO production in RAW264.7 cells. Cells were pretreated with tested samples for 30 min, then incubated with 1 μg/mL LPS for 24 h. NO levels were quantified by Griess assay. Bars are standard deviations obtained from triplicated experiments; *p < 0.05 compared to LPS-treated control

![Figure 2](image2.png)

**Figure 2:** Effect of S. sumuntia extract (μg/mL) on iNOS and COX-2 expression. RAW264.7 cells were pretreated with tested samples for 30 min, then incubated with 1 μg/mL LPS for indicated times. The levels of iNOS and COX-2 proteins were analyzed by Western blot. α-Tubulin was used as protein loading control. Bars are standard deviations obtained from triplicated experiments; *p < 0.05 compared to LPS-treated control

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Bioassay-guided fractionation of the methanol extract of *S. sumuntia* led to the isolation of four lignans (Figure 3). By means of spectroscopic methods including mass (MS) and nuclear magnetic resonance (NMR) spectroscopy, the isolated compounds were identified as arctigenin (1), matairesinol (2) [16], monomethylpinoresinol (3) and pinoresinol (4) [17]. These four compounds were isolated for the first time from *Symlocos sumuntia*.

The isolated compounds were tested for their inhibition of NO production in LPS-stimulated RAW264.7 cells. As shown in Table 1, arctigenin (1) was the most active compound with IC$_{50}$ value of 4.08 ± 0.26 μM. This effect was comparable with the reference control, cardamonin. Monomethylpinoresinol (3) exhibited moderate inhibitory effect while matairesinol (2) and pinoresinol (4) showed weaker inhibition. In the MTT assay, these compounds did not affect cell viability of RAW264.7 cells at the tested concentrations (data not shown). The inhibitory effect of compounds 1 and 3 were stronger than that of 2 and 4, respectively, suggesting that the replacement of a hydroxyl group by a methoxy group increased the inhibition of NO production.

<table>
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<tr>
<th>Compound</th>
<th>IC$_{50}$ (μM$^*$)</th>
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<tr>
<td>Arctigenin (1)</td>
<td>4.08 ± 0.26</td>
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<tr>
<td>Matairesinol (2)</td>
<td>36.9 ± 1.85</td>
</tr>
<tr>
<td>Monomethyl pinoresinol (3)</td>
<td>13.3 ± 0.94</td>
</tr>
<tr>
<td>Pinoresinol (4)</td>
<td>25.2 ± 1.17</td>
</tr>
<tr>
<td>Cardamonin (control)</td>
<td>2.80 ± 0.18</td>
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*Values are mean ± SD (n = 3)*

**DISCUSSION**

It has been reported that the plants of *Symlocos* genus exhibited strong anti-inflammatory activity. Jung *et al.* reported that several flavonoids isolated from the aerial parts of *S. racemosa* moderately inhibited NO production with IC$_{50}$ values in the range of 42.1 - 88.2 μM [18]. A triterpene and a sterol isolated from the stem bark of *S. paniculata* exhibited potent anti-inflammatory activity in carrageenan induced acute paw edema in rats [19]. In a similar study, α-spinnerosterol isolated from *S. spicata* stem bark prevented the development of paw carrageenan edema [20]. An ethanol extract of *S. racemosa* barks exhibited anti-inflammatory activity at 100-500 mg/kg in formalin and carrageenan induced mice models [21]. Consistently, our study showed that *S. sumuntia* strongly inhibited NO production by decreasing the expression of iNOS.

Another major inflammatory mediator, COX-2, was also inhibited by *S. sumuntia*. Dibenzybutyrolactone lignans have been known to possess a wide range of biological activities including anti-inflammation [22]. Arctigenin, one of the most common dibenzybutyrolactone lignan, is commonly found in natural sources such as plants, animals or microorganisms. It has been shown to possess anti-inflammatory, anticancer, anti-obesity, neuroprotection, and free radical scavenging properties [23-27]. In the present study, arctigenin exhibited potent inhibitory effect against NO production. Among four compounds isolated from *S. sumuntia* leaves, this compound was found in high content, and therefore might
be an important biomarker for quality control of this plant.

CONCLUSION

The findings of the present study show that the anti-inflammatory activity of *S. sumuntia* occurs via the inhibition of NO production and the expression of iNOS and COX-2 proteins. This study is the first report on the biological activity of *Symlocos sumuntia*. The results also explained the effective use of this plant for treatment of inflammation in traditional medicine. Thus, *S. sumuntia* is a promising source of anti-inflammatory agents.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

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REFERENCES


