STILBENOIDS FROM *PINUS KESIYA* AND THEIR POTENTIAL TO INHIBIT NO PRODUCTION IN LPS-ACTIVATED RAW264.7 CELLS

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TÓM TẮT

CÁC STILBENOID TỪ GỖ THÔNG BA LÁ (*PINUS KESIYA*) VÀ KHẢ NĂNG ỨC CHẾ SẢN XUẤT NO TRONG TẾ BÀO RAW264.7 ĐƯỢC KÍCH HOẠT BỞI LPS CỦA CHÚNG

*Hai hợp chất stilbenoid đã được phân lập từ chiết xuất ethyl acetate của gỗ thông ba lá (Pinus kesiya) sau khi tiến hành phân tách nhiều lần bằng phương pháp sắc ký cột. Hai hợp chất này được xác định là (E)-piceid (**1**) và isorhapontin (**2**). Cấu trúc của các hợp chất này đã được làm sáng tỏ sau khi phân tích các dữ liệu phổ như NMR và MS. Đáng chú ý, hợp chất isorhapontin (**2**) lần đầu tiên được phân lập từ loài thực vật này. Cả hai chất đều có khả năng ức chế ở mức vừa phải khả năng sản sinh NO trong các tế bào RAW264.7 được kích hoạt bằng LPS (lipopolysaccharide).*

Keywords. *Pinus kesiya, stilbenoid, (E) piceid, isorhapontin, anti-inflammatory activity*

1. INTRODUCTION

The genus *Pinus*, the largest within the pine family (Pinaceae), encompasses 114 species worldwide [1]. In Vietnam, there are eight known species of *Pinus*, thriving across diverse habitats ranging from lowland forests to mountainous areas. Each of these species possesses distinct attributes concerning size, morphology, and needle morphology, enriching the nation's botanical diversity. Conservation initiatives are actively addressing the protection of these invaluable pine species and their associated ecosystems against pressing challenges like deforestation and climate variability.

Pinus kesiya Royle ex Gordon, a member of the Pinaceae family, is a coniferous species with widespread distribution across regions including India, Myanmar, Thailand, Laos, Vietnam, and the Philippines. Its discovery in Vietnam traces back to the Langbiang plateau, where it thrives extensively, particularly

in regions like Son La, Ha Giang, and Central Highlands (Kon Tum, Gia Lai, Daklak). Notably, the Langbiang plateau harbors the largest concentration, covering approximately 90% of the area. Characterized by needle-shaped leaves typically arranged in clusters of three on short branches, each needle measures around 20-25 cm in length and exhibits a distinctive turquoise hue. The tree's reproductive structures, manifested as flower heads attached to short leaves, are approximately 1.5 cm long and clustered in whorls on larger branches. Primarily cultivated for its wood, *Pinus kesiya* serves various industrial purposes, including paper manufacturing, construction, and artistic endeavors [2]. Additionally, it holds significance in traditional medicine, where diverse plant parts are utilized to address conditions such as meridian blockages, blood stasis, inflammation, detoxification, and relaxation. Despite its medicinal significance, there have been limited studies exploring the chemical composition and therapeutic properties of the essential oils derived from this species [3]. In China, only one publication has reported the chemical composition of *Pinus kesiya* pinecone, revealing three diterpenoids: 15-hydroxylabd- 8(17)-en-19-oic acid, 15-hydroxydehydroabietic acid, junicedric acid, daucosterol, and *β*-sitosterol [4]. In recent studies, our researchers have isolated and structurally elucidated abietane diterpenoids, other diterpenoids, and phenolic compounds from the roots of *P. kesiya* [5, 6]. Continuing this line of research, two stilbenoids were identified from the woods of this plant, with findings indicating their mild effects on inhibiting nitric oxide (NO) production. This research opens up further possibilities for understanding the therapeutic potential and chemical diversity of *P. kesiya*.

2. EXPERIMENTAL

2.1. Instruments

NMR spectra were obtained using a Bruker Avance 500 Ultrashield NMR Spectrometer. ESI-MS analysis was performed using an Agilent LC-MSD-Trap SL instrument. TLC was conducted using Silica gel 60 F254 (0.25mm, Merck). Column chromatography involved the use of Silica gel 60 (230-400 mesh, Merck) for the first column, and silica gel 60, 40-63 μm (Merck) and Sephadex LH_{20} for the subsequent columns.

2.2. Plant material

The *P. kesiya* woods were collected in September 2019 in Daklak province of Vietnam and identified by Prof. Do Huu Thu from the Institute of Ecology and Biological Resources (IEBR), VAST. Voucher specimen (No. Zo.02) is stored in the Laboratory of Drug Research and Discovery, Institute of Chemistry, VAST, Hanoi, Vietnam. The woods sample was dried, minced, and pulverized for analysis.

2.3. Extraction and isolation

The powdered wood sample was then subjected to extraction with a solvent to isolate the bioactive compounds present. The extract obtained will undergo further analysis to determine its potential pharmacological properties and potential applications in drug discovery.

The sample of *Pinus kesiya* wood powder (2.1 kg) underwent triplicate extraction with 90% methanol (MeOH) over three-day intervals. The pooled methanolic extracts were concentrated under reduced pressure, yielding a crude extract weighing 250 g. This crude extract was subsequently partitioned between *n*-hexane, ethyl acetate (EtOAc), and *n*-butanol following the addition of water

(H_2O). The resulting fractions were concentrated at 45°C under reduced pressure to yield their respective residues.

The EtOAc fraction (21.0 g) was subjected to silica gel column chromatography (CC) employing a gradient of solvents with increasing polarity (*n*-hexane:EtOAc:MeOH from 100:1:0 to 0:50:50) [7], which resulted in 18 fractions (F1-F18). Fraction 4 (2.3 g) was further purified using silica gel chromatography with solvent systems comprising dichloromethane (CH_2Cl_2) and methanol (MeOH) in ratios of 100:1, 100:5, and 100:20, followed by MeOH alone, and subsequently by Sephadex LH_{20} column chromatography using an acetone mixture MeOH (1:10), ultimately isolating compound **1** (74 mg).

Fraction 5 was subjected to silica gel CC using a CH_2Cl_2:MeOH gradient (100:1, 100:2, 100:5, 100:10, up to 50:50, and 100% MeOH), yielding three subfractions (F5.1-F5.3). Subfraction 5.2 (2.0 g) underwent additional purification via silica gel CC (CH_2Cl_2) and Sephadex LH_{20} column chromatography (acetone : MeOH = 1:10), resulting in the isolation of compound **2** (152 mg).

2.4. Spectral data of isolated compounds

Compound 1: (*E*) piceid, $C_{20}H_{22}O_8$, colorless crystal ($MeOH/CH_2Cl_2$); mp. 136 ^oC, ESI-MS *m/z* 391.1 $[M+H]^+$, NMR data (detailed in table 1)

Compound 2: isorhapontin, $C_{21}H_{24}O_9$, colorless crystal ($MeOH/CH_2Cl_2$); mp 193 ^oC, ESI-MS *m/z* 443.1 $[M+Na]^+$, NMR data (detailed in table 1).

3. Results and discussion

Compound **1** was isolated in the form of colorless crystals. Its molecular formula, $C_{20}H_{22}O_8$, was elucidated through comprehensive analysis, including positive electrospray ionization mass spectrometry ESI-MS at *m/z* 391.1 $[M+H]^+$ ^{1}H-NMR, ^{13}C-NMR spectra. The sugar moiety was readily discerned from its characteristic signals in the ^{1}H-NMR and ^{13}C-NMR spectra, indicating the presence of *β*-D-glucopyranose and suggesting $C_{14}H_{12}O_3$ as the aglycone. The *β*-configuration of the sugar moiety was determined by the coupling constant of the anomeric proton (δ_H 4.92, *d*, *J* = 7.0 Hz, H-1″). Analysis of the ^{13}C-NMR and HSQC spectra, excluding the glucose moiety, revealed 14 carbons, comprising 5 quaternary carbons and 9 methine carbons. The presence of two distinct aromatic rings was established by the observation of an AA'BB' type system at δ_H 7.38 (2H, *d*, *J* = 8.5 Hz, H-2′/H-6′) and 6.79 (2H, *d*, *J* = 8.5 Hz, H3′/H5′), indicative of a 1,4-disubstituted benzene ring. Additionally, three protons at δ_H 6.80 (1H, *brs*, H-2), 6.63 (1H, *br s*, H-6), and 6.48 (1H, *br s*, H-4) suggested a 1,3,5-trisubstituted benzene ring. Two *trans* olefinic protons appeared as an AB spin system with a large coupling constant at 7.04 and 6.88 (each 1H, *d*, 16.5 Hz, H-8 and H-7, respectively). Cross-peaks observed in the HSQC spectrum facilitated the assignment of protons to their corresponding carbons in compound **1**. Furthermore, the NMR data of compound **1** closely resemble those of (*E*)-piceid, implying their structural similarity [8]. It's noteworthy that this compound has been identified not only in red wine but also in the roots of *Polygonum cuspidatum*. Both this compound and its aglycone, resveratrol, are prevalent in grape juice and are recognized for their potent antioxidant and free radical scavenging activities. Additionally, these compounds have been noted for their potential therapeutic benefits in neurological disorders, including Parkinson's disease,

Alzheimer's disease, and Huntington's disease. Moreover, studies have highlighted the anti-inflammatory and anti-cancer properties of this compound, indicating its potential as a candidate for the prevention and treatment of various diseases [9].

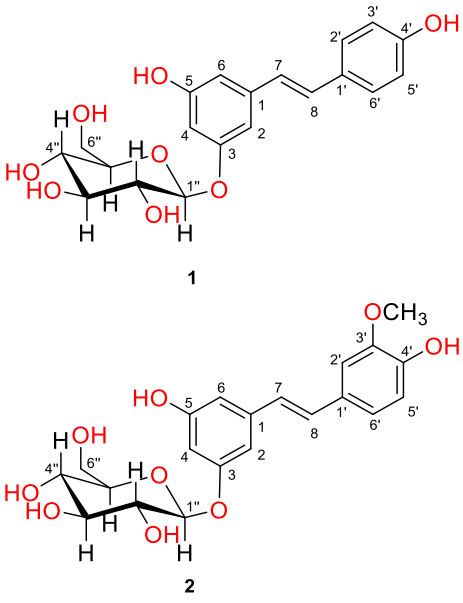

*Figure 1. Structures of compounds **1** and **2** isolated from the wood of Pinus kesiya.*

Compound **2** was isolated as colorless crystals. The molecular formula, $C_{21}H_{24}O_9$, was established through electrospray ionization mass spectrometry (ESI-MS), which exhibited a molecular ion peak at *m/z* 443.1 $[M+Na]^+$. Detailed analysis of the nuclear magnetic resonance (NMR) data of **2** revealed a structure similar to that of compound **1.** On standard ^{1}H-NMR and HSQC spectra, substance **2** exhibits signals for six aromatic hydrogens from two cyclic benzenes, signals for two *trans*-olefinlic hydrogens, one signal for a methoxy group, and six signals for a hexose sugar. In the first benzene ring (ring A), a 1,3,5-trisubstituted pattern was inferred from the meta-positioned proton signals observed at δ_H 6.65 (1H, *br s*, H-2), 6.82 (1H, *br s*, H-6), and 6.48 (1H, *br s*, H-4). The second benzene ring (ring B) displayed a 1,3,4-trisubstituted pattern, as indicated by proton signals at δ_H 6.99 (1H, *dd*, 8.4, 1.8 Hz, H-6'), 6.79 (1H, *d*, 8.4 Hz, H-5'), and 7.13 (1H, *d*, 1.8 Hz, H-2'). The two *trans*-olefinic protons were distinctly observed at δ_H 7.03 and 6.89, with a significant coupling constant of 16.5 Hz. The sugar moiety is identified as glucopyranose, with the anomeric hydrogen H-1″ oriented along the bond axis, displaying a doublet peak at δ_H 4.92 and a coupling constant of 7.5 Hz. A comparison with the NMR data of common compounds listed in the document reveals substantial similarities [10]. Comprehensive assignment of proton and carbon signals was achieved using two-dimensional NMR techniques such as HSQC and HMBC spectra. The HMBC spectrum provided key insights into the connectivity of the methoxy group, revealing interactions with C-3', thereby confirming the methoxy group's attachment at the C-3' position on the aromatic ring.

*Table 1. NMR data of isolated compounds (**1**, and **2**)*

C	HSQC	**1** (measured in methanol d_4)		**2** (measured in methanol d_4)	
		δ_C	**δ_H**	**δ_C**	**δ_H**
1	C_q	141.4	-	141.3	-
2	CH	107.1	6.80 (1H, *br s*)	108.4	6.65 (*t*, *br s*)
3	C_q	160.5	-	159.6	-
4	CH	104.0	6.47 (1H, *br s*)	104.2	6.48 (*br s*)
5	C_q	159.6	-	160.4	-
6	CH	108.4	6.63 (1H, *br s*)	107.1	6.82 (*br s*)
7 (α)	CH	126.7	6.88 (1H, *d*, 16.5 Hz)	126.9	6.89 (*d*, 16.5 Hz)

8 (*β*)	CH	130.1	7.04 (1H, *d*, 16.5 Hz)	130.2	7.03 (*d*, 16.5 Hz)
1′	C_q	130.3	-	130.9	-
2′	CH	128.9	7.38 (2H, d, 8.5 Hz)	110.6	7.13 (*d*, 1.8 Hz)
3′	CH (C_q)	116.4	6.79 (2H, *d*, 8.5 Hz)	149.1	-
4′	C_q	158.5	-	147.7	-
5′	CH	116.4	6.79 (2H, *d*, 8.5 Hz)	116.3	6.79 (d, 8.4, Hz)
6′	CH	128.9	7.38 (2H, d, 8.5 Hz)	121.4	6.99 (*dd*, 8.4 Hz, 1.8 Hz)
-OCH_3		-	-	56.5	3.92 (s)
		3-O-D-glucopiranose		**3-O-D-glucopiranose**	
1″	CH	102.3	4.93 (1H, *d*, 7.0 Hz)	102.4	4.92 (*d*, 7.5 Hz)
2″	CH	75.0	3.40 (m)	75.0	3.41 (m)
3″	CH	78.1	3.43 (m)	78.0	3.44 (m)
4″	CH	71.5	3.46 (m)	71.5	3.47 (m)
5″	CH	78.2	3.52 (m)	78.2	3.54 (m)
6″	CH_2	62.6	3.94 (1H, *dd*, *J* = 12.0 Hz, 2.0 Hz) 3.73 (1H, *dd*, *J* = 12.0 Hz, 5.5 Hz)	62.6	3.95 (*d*, 12.0 Hz, 2.5 Hz) 3.74 (*dd*, 12.0 Hz, 5.4 Hz)

In this investigation, the anti-inflammatory properties of two isolated compounds were examined through Griess assays to quantify the inhibition of nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated RAW264.7 cells. NG-methyl-L-arginine acetate (L-NMMA) from Sigma was employed as the positive control. Initially, cytotoxicity evaluations on RAW264.7 cells were treated with various concentrations (2.0, 5.0, 20.0, and 100.0 μM) of the selected compounds for 24 hours, utilizing the MTT assay to assess cell viability [11]. None of the tested compounds exhibited significant cytotoxic effects, as evidenced by cell viability exceeding 90% at all concentrations. Consequently, these concentrations were utilized in subsequent NO assays for both the isolated compounds and the positive control. Following a 3-hour pre-treatment of RAW264.7 cells with the selected concentrations of the compounds, the cells were stimulated with LPS (1.0 μg/mL) for 24 hours. The majority of the compounds showed modest inhibition of LPS-induced NO production. Notably, at a concentration of 100 μM, compound **2** exhibited a slight decrease in NO production (12.29 ± 0.56%), while compound **1** displayed a slightly higher inhibition effect (25.03 ± 0.89%) compared to compound **2**. This disparity may be attributed to the presence of the methoxy group at the C-3' position of compound **2**.

Table 2. Inhibition effect of compounds 1 and 2 on LPS-activated RAW264.7 cells

Compounds	**NO inhibition at 100 μM (%)**	**Cell viability at 100 μM (%)**
1	25.03 ± 0.89	100.01 ± 2.03
2	12.19 ±0.56	98.88 ± 1.07
Dexamethasone (the positive control)	87.45± 1.75	86.92 ± 2.05

Statistical analysis.

Data were analyzed using GraphPad Prism 5.0 software and presented as mean ± SD. Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparison test, with $P < 0.05$ considered significant.

4. Conclusion

The present study represents the initial documentation of the isolation, structural elucidation, and anti-inflammatory attributes of stilbenoids from *Pinus kesiya*. The identification of these compounds signifies a notable advancement in unlocking the pharmacological capabilities of *Pinus kesiya*, providing insights into its untapped pharmacological characteristics. These stilbenoids exhibited a mild inhibitory impact on NO production, necessitating additional investigations to uncover their mechanism of action and potential therapeutic uses.

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