Lignans and aromatic glycosides from *Piper wallichii* and their antithrombotic activities

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**A R T I C L E   I N F O**

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(-)-Syringaresinol (PubChem CID: 332426)
(+)-Medioresinol (PubChem CID: 181681)
(+)-Epiioresinol (PubChem CID: 637584)
Sinapyl aldehyde (PubChem CID: 5280802)
Isolariciresinol (PubChem CID: 160521)
(-)-Olivil (PubChem CID: 586733)
Prunasin (PubChem CID: 120639)
Conderyl aldehyde (PubChem CID: 5280536)
Aucuparin (PubChem CID: 442508)

Keywords:
*Piper wallichii* (Miq.) Hand.-Mazz. Lignans Aromatic glycosides Antithrombotic effect Zebrafish model

**A B S T R A C T**

Ethnopharmacological relevance: *Piper wallichii* (Miq.) Hand.-Mazz. is a medicinal plant used widely for the treatment of rheumatoid arthritis, inflammatory diseases, cerebral infarction and angina in China. Previous study showed that lignans and neolignans from *Piper* spp. had potential inhibitory activities on platelet aggregation. In the present study, we investigated the chemical constituents of *Piper wallichii* and their antithrombotic activities, to support its traditional uses.

Materials and methods: The methanolic extract of the air-dried stems of *Piper wallichii* was separated and purified using various chromatographic methods, including semi-preparative HPLC. The chemical structures of the isolates were determined by detailed spectroscopic analysis, and acidic hydrolysis in the case of the new glycoside. Determination of absolute configurations of the new compound was facilitated by calculated electronic circular dichroism using time-dependent density-functional theory. All compounds were tested for their inhibitory effects on platelet aggregation induced by platelet activating factor (PAF) in rabbits’ blood model, from which the active ones were further evaluated in vivo antithrombotic activity in zebrafish model.

Results: A new neolignan, piperwalliol A (1), and four new aromatic glycosides, piperwalliosides A–D (2–5) were isolated from the stems of *Piper wallichii*, along with 25 known compounds, including 13 lignans, six aromatic glycosides, two phenylpropyl aldehydes, and four biphenyls. Five known compounds (6–10) showed in vitro antplatelet aggregation activities. Among them, (-)-syringaresinol (6) was the most active compound with an IC50 value of 0.52 mM. It is noted that in zebrafish model, the known lignan 6 showed good in vivo antithrombotic effect with a value of 37% at a concentration of 30 μM, compared with the positive control aspirin with the inhibitory value of 74% at a concentration of 125 μM.

Conclusion: This study demonstrated that lignans, phenylpropanoid and biphenyl found in *Piper wallichii* may be responsible for antithrombotic effect of the titled plant.

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1. Introduction

Platelet plays an important role at sites of vascular injury, while over-aggregation of platelets may lead to pathologic thrombosis (Huang et al., 2014). The majority of cardiovascular diseases, including acute coronary syndrome, ischemic stroke and peripheral vascular diseases, are associated with thrombotic disorders, resulting in serious outcomes such as sudden death or long-term disability (Edelstein et al., 2013; Tsai et al., 2014). The inhibition of platelet aggregation represents an important approach for the treatment of thrombotic diseases (Goto and Tomita, 2013). The quest for safe and efficacious natural leaders with antiplatelet aggregation activity thus remains considerable attention (Tsai et al., 2014).

Lignans, one of the most ubiquitous natural products in terrestrial plants, are commonly derived from oxidative dimerization of two phenylpropanoid units (Saleem et al., 2005). Due to their diversified chemical structures, lignans are functionally diverse, e.g. platelet activating factor (PAF) receptor antagonist, antitumor, anti-AD (Alzheimer’s disease), antiviral, anti-inflammatory, and antifungal activities (Pan et al., 2014; Saleem et al., 2005; Zhu et al., 2013). It is
worth noting that lignans are regarded as one of the vital sources for the discovery of pAF receptor antagonism (Tsai et al., 2014).

The Piper genus is belonging to the family Piperaceae. Alkaloids and lignans are the mainly characteristic metabolites in Piper plants (Gutierrez et al., 2013; Pan et al., 2009). *Piper wallichii* (Miq.) Hand.-Mazz. (Piperaceae) is a perennial herb distributed widely in the Hubei, Hunan, Yunnan, Sichuan, and Gansu provinces of People’s Republic of China, as well as Nepal, India, Bengal and Indonesia. The stems have been used for the treatment of rheumatoid arthritis, inflammatory diseases, cerebral infarction and angina by the local people living in China (Duan et al., 2010; Tamuly et al., 2014). Previously, several lignans, alkaloids, and phenylpropanoids have been isolated from this species (Tamuly et al., 2014; Wei et al., 2011). During our search for bioactive lignans with potential antiplatelet aggregation activities, we investigated on the stems of *Piper wallichii*, and led to the isolation of one new neonigan, piperwaliol A (1) and four new aromatic glycosides, piperwalliosides A–D (2–5), together with 13 known lignans, six aromatic glycosides, two phenylpropyl aldehydes, as well as four biphenyls. Compound 1 features in neonigan skeleton (C_{8–7}C_{8–6}) crosslinked with a tetraoxygenated benzene ring to furnish an extra benzofuran moiety. All compounds were tested for their inhibition against platelet aggregation in vitro and the active compounds were further tested in vivo for their antithrombotic activity in zebrafish model. Herein, we described the isolation, structural elucidation, and bioactivity of all isolates.

2. Material and methods

2.1. General

The details for different instruments, column chromatography (CC), thin-layer chromatography (TLC), preparative HPLC and semi-preparative HPLC used in this study were shown in the Supporting information.

2.2. Plant material

The stems of *Piper wallichii* were collected from Henan Province, People’s Republic of China, in June 2012, and were identified by Mr. Xiao-Ming Fang from South China Botanical Garden, Chinese Academy of Sciences. A voucher specimen (HITBC_015077) was deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences.

2.3. Extraction and isolation

The air-dried stems of *Piper wallichii* (19.0 kg) were powdered and extracted with MeOH at 60 °C. After removal of the solvent under reduced pressure, the crude extract (2.1 kg) was partitioned between EtOAc and H_{2}O (1:1, v/v) to furnish an EtOAc-soluble fraction (600 g) and an aqueous phase (1.4 kg). The EtOAc extract was applied to a silica gel CC, eluting with a CHCl_{3}–MeOH (99:1–5:1, v/v), to give eight fractions A–H. Fr. E (75.2 g) was subjected to a RP-18 column using a step gradient of MeOH–H_{2}O (from 40% to 90% MeOH) to afford eight Fr. E1–E8. Fr. E1 (1.8 mg) was chromatographed on silica gel with petroleum ether–acetone (5:1, v/v) to afford 18 (1.8 mg). Fr. E3 (3.0 g) was chromatographed over Sephadex LH-20 column, eluting with MeOH to give eight subfractions Fr. E3A–E3H. Fr. E3D (111 mg) was purified by semi-preparative HPLC (CH_{3}CN–H_{2}O, 22:78, v/v) to yield 6 (1.1 mg), 7 (3.8 mg), and 11 (2.0 mg). Fr. E3E (205 mg) was separated by RP-18 column using a step gradient of MeOH–H_{2}O (from 40% to 80% MeOH), followed by semi-preparative HPLC (CH_{3}CN–H_{2}O, 35:65, v/v) to yield compounds 8 (2.4 mg), 12 (29.2 mg), 27 (5.1 mg), and 28 (12.7 mg).

Fr. F (45.3 g) was subjected to Diaion HP20SS CC, using a step gradient of MeOH–H_{2}O (from 40% to 100%) to afford 13 Fr. F1–F13. Fr. F8 (2.6 g) was purified by Sephadex LH-20, eluting with MeOH to yield 19 (4.8 mg). Fr. F9 (14.3 g) was separated by Sephadex LH-20 eluting with MeOH to afford five fractions Fr. F9A–F9E. Fr. F9B (500 mg) and Fr. F9D (108 mg) was purified with preparative HPLC (CH_{3}CN–H_{2}O, 30:70, v/v) and semi-preparative HPLC (CH_{3}CN–H_{2}O, 20:80, v/v), respectively, to afford 13 (11.0 mg), 14 (5.2 mg), 15 (9.9 mg), and 29 (2.5 mg), 29 (0.8 mg, 30 (57.0 mg).

Fraction H (440.1 g) was subjected to a Diaion HP20SS column using a step gradient of MeOH–H_{2}O (from 20% to 80% MeOH) to afford five Fr. H1–H5. Fr. H1 (70.5 g) was chromatographed on Sephadex LH-20 (from 20% to 50% MeOH) to obtain ten subfractions H1A–H1J. Fr. H1C (5.2 g) was separated by RP-18 with a solvent system of MeOH–H_{2}O (from 25% to 40% MeOH) to afford compound 9 (446.7 mg) and other five fractions Fr. H1C1–H1C5. Fr. H1C2 (135 mg) was purified by preparative TLC (CHCl_{3}–MeOH, 5:1, v/v) to yield compounds 21 (52.7 mg) and 23 (23.0 mg). Fr. H1C3 (75 mg) was subjected to semi-preparative HPLC (CH_{3}CN–H_{2}O, 14:86, v/v) to afford compounds 1 (2.4 mg), 22 (5.1 mg), and 24 (25.7 mg). Fr. H1J (2.3 g) was separated by preparative HPLC (CH_{3}OH–H_{2}O, 15:85, v/v) to yield compounds 4 (27 mg), 25 (565.7 mg), and a mixture which was further purified by semi-preparative HPLC (CH_{3}OH–H_{2}O, 15:85, v/v) to afford compounds 3 (8.3 mg), 5 (2.4 mg), and 26 (10.1 mg). Fr. H3 (13.4 g) was separated by RP-18 using a step gradient of MeOH–H_{2}O (from 20% to 100% MeOH), followed by Sephadex LH-20 eluting with MeOH and preparative TLC (CHCl_{3}–MeOH, 1:1, v/v) and finally purified by semi-preparative HPLC (CH_{3}CN–H_{2}O, 13:87, v/v) to yield 16 (18.4 mg) and 17 (13.1 mg). Fr. H5 (598 mg) was purified by preparative HPLC using a step gradient of CH_{3}CN–H_{2}O (from 5% to 40% for 45 min) to yield compounds 2 (98.3 mg), and 10 (1.3 mg).

2.4. Acid hydrolysis of 2

Compound 2 (6 mg) was hydrolyzed by acid to give D-glucose and L-rhamnose as sugar residues, which were determined by GC analysis of their corresponding trimethylsilylated L-cysteine adducts (Supporting information).

2.5. ECD calculation

The theoretical calculations of compound 1 were performed using Gaussian 09 (Frisch et al., 2010). Conformational analysis was initially carried out using Discovery Studio 3.5 Client. The optimized conformational geometries, thermodynamic parameters, and populations of all conformations were provided in the supporting information (Figs. S53–S54 and Tables S1–S8). The conformers were then optimized at B3LYP/6-31G(d) level. Room-temperature equilibrium populations were calculated according to Boltzmann distribution law. The theoretical calculation of ECD was performed using TDDFT (Bringmann et al., 2009) at B3LYP-SCRF/6-31G+(d,p) level with PCM in MeOH solvent. The ECD spectra of compound 1 were obtained by weighing the Boltzmann distribution rate of each geometric conformation.

The ECD spectra are simulated by overlapping Gaussian functions for each transition according to the following equation:

\[
\Delta \varepsilon(\varepsilon) = \frac{1}{2.297 \times 10^{-30}} \times \frac{1}{\sqrt{2\pi\sigma}} \sum_{i=1}^{A} \Delta \varepsilon_i e^{-\frac{|\varepsilon - \varepsilon_i|}{2\sigma^2}}
\]

where \( \sigma \) represents the width of the band at 1/e height, and \( \Delta \varepsilon_i \) and \( R_i \) are the excitation energies and rotational strengths for
transition \( i \), respectively. \( \sigma = 0.20 \text{ eV} \) and \( K_{\text{velocity}} \) have been used in this work.

2.6. MO analysis

The orbital information (NBO plot files) was generated by NBO program of Gaussian 09 (Glendening et al., 2009). The predominantly populated conformers were selected for MO analysis. NBO plot files were used to generate corresponding Gaussian-type grid file by Multiwfn 2.4 (Lu and Chen, 2012). After that, the isosurface of generated grid date was afforded by VMD software (Humphrey et al., 1996).

2.7. Rabbits’ blood and zebrafish

The New Zealand white rabbits were purchased from Experimental Animal Center of Kunming Medical University of China. The rabbits were anaesthetized by pentobarbital sodium, and the blood was collected by cardiac puncture. The experiments were reviewed and approved by the Animal Ethics Committee of Kunming Institute of Botany, Chinese Academy of Sciences.

A breeding stock of healthy mature zebrafish (4–5 pairs) was used for naturally embryos production. Each pool can yield up to 200–300 embryos and the dead embryos were removed 6 and 24 h post-fertilization (hpf). The rest suitable embryos, according to the embryonic stage of development (Kimmel et al., 1995), were selected and then incubated in the fish water (1 L reverse osmosis water containing soluble salt (200 mg), with conductivity of 7.2, and hardness of 53.7–71.6 mg/L CaCO\(_3\)) under 28 ± 0.2°C. Since the embryos can nutrients obtained from their own yolk sac, it is not necessary to feed them for nine days post-fertilization (dpf). The 3 dpf larval zebrafish were anaesthetized by MESAB (ethyl-m-amino-benzoate methanesulphonate, Sigma-Aldrich) and then dyed with diansisidine (sigma, MKBG4648V) as staining agent. The quantitative analysis of staining power (S) of cardiac red blood cells was carried on a fluorescence microscope (SMZ645, Nikon Company) and analyzed by Nikon NIS-Elements D3.10 software. The inhibition ratio was calculated by the following equation, and before calculations the \( S \) (test group) and \( S \) (model group) should minus the background values. The results were shown as \( X \pm SE \). Statistical evaluation included one-way analysis of variance followed by Dunnett’s t-test for multiple comparisons. \( P < 0.05 \) (*) were taken as significant.

\[
\text{Antithrombotic ratio (\%) = } \frac{S\text{(test group)} - S\text{(model group)}}{S\text{(solvent group)} - S\text{(model group)}} \times 100\%
\]

3. Results

3.1. Compounds isolated from Piper wallichii

The methanolic extract of air-dried stems of Piper wallichii was suspended into H\(_2\)O and partitioned with EtOAc. The EtOAc layers were collected by column chromatography over silica gel, Sephadex LH-20, and reversed-phase C18 to afford five new compounds (1–5). In addition, 25 known compounds were identified as (--) syringaresinol (6) (He et al., 2012), (++)-medioresinol (7) (Deyama et al., 1985), (++)-epi-pinoresinol (8) (Rahman et al., 1990), sinapyl aldehyde (9) (Zhou et al., 2013), 3,5'-dimethoxybiphenyl-3,4'-diol (10) (Bao et al., 2010), (++)-lirioresinol A (11) (Zhou et al., 2007), (++)-pinoresinol (12) (Roy et al., 2002), prinsepil (13) (Piccinnelli et al., 2004), (++)-4-epi-pinoresinol (14) (Ohashi et al., 1994), isolirioresinol (15) (Abe and Yamauchi, 1989), (++)-lyoniside (16), (--)-nudiposide (17) (Šnite et al., 1995), (--)-pregestane B (18) (Meragelman et al., 2001), (--)-olivil (19) (Yeo et al., 2004), neolivil (20) (Schöttner et al., 1997), 3,4,5-trimethoxyphenyl-\( \beta \)-o-glucopyranoside (21) (Jin et al., 2015), kemapaposide A (22) (Miyamura et al., 1983), benzyl \( \beta \)-o-glucopyranoside (23) (Goncalves et al., 2004), prunasinamide (24) (Sendker and Nahrstedt, 2009), prunasin (25) (Nahrstedt and Rockenbach, 1993), garcinosigane D (26) (Huang et al., 2001), coniferyl aldehyde (27) (El-Hassan et al., 2003), 4'-methoxyaucaparinc (28) (Kokubun et al., 1995), aucaparin (29) (Borejsza-Wysocki et al., 1999), and garcibiphyllin C (30) (Chen et al., 2006), respectively, by comparing their spectroscopic data with those reported previously in literatures.

3.2. Structure elucidation of new compound

Compound 1 was obtained as a yellow oil. The molecular formula of 1 was determined as \( C_{32}H_{52}O_{8} \) by a quasimolecular ion peak at \( m/z \) 467.1337 [M+H]+ (calcld. for \( C_{32}H_{52}O_{8} \), 467.1342) in the negative HRESIMS, indicating 14 degrees of unsaturation. The IR spectrum of 1 indicated the presence of hydroxy (3452 cm\(^{-1}\)) and aromatic (1631 and 1453 cm\(^{-1}\)) groups. The \(^{13}\)C NMR spectrum displayed the occurrence of 25 carbon resonances ascribable to four methoxyls (\( \delta c \): 56.7, 56.7, 61.3, 57.2), one aliphatic methylene (\( \delta c \): 63.4), two tested compounds were added separately as the test group with a final concentration of 30 \( \mu \)M. The negative control group was the solvent group, which not only represented the background value of the test group, but also made sure that the cardiac red blood cells of larval zebrafish were normal. Blank control group was used to prove that the solvent did not have harmful effects on the larval zebrafish. Aspirin was used as positive control. The experiment was carried on 96-well cell culture plates, on which it included a blank control, a solvent control, a model, a positive control and the test samples’ groups. Each group processed 8 wells, and the larval zebrafish were seeded 4 fish per well. After a treatment period of 48 h until 5 dpf. The larval zebrafish were anesthetized by MESAB (ethyl-\( \alpha \)-amino-benzoate methanesulphonate, Sigma-Aldrich) and then dyed with diansisidine (sigma, MKBG4648V) as staining agent. The quantitative analysis of staining power (S) of cardiac red blood cells was carried on a fluorescence microscope (SMZ645, Nikon Company) and analyzed by Nikon NIS-Elements D3.10 software. The inhibition ratio was calculated by the following equation, and before calculations the \( S \) (test group) and \( S \) (model group) should minus the background values. The results were shown as \( X \pm SE \). Statistical evaluation included one-way analysis of variance followed by Dunnett’s t-test for multiple comparisons. \( P < 0.05 \) (*) were taken as significant.

\[
\text{Antithrombotic ratio (\%) = } \frac{S\text{(test group)} - S\text{(model group)}}{S\text{(solvent group)} - S\text{(model group)}} \times 100\%
\]
aliphatic methines ($\delta_C$ 89.3, 54.1), and 18 aromatic carbons ($\delta_C$ 97.6–154.3) due to three benzene rings. The $^1$H NMR spectroscopic data (Table 1) revealed the presence of a benzofuran-type lignan skeleton ($\delta_H$ 5.71 (1H, d, $J=6.0$ Hz, H-7), 3.88 (1H, m, H-8), 4.17 (1H, dd, $J=11.2, 4.0$ Hz, H-9a), 4.00 (1H, dd, $J=11.2, 7.4$ Hz, H-9b), which was supported by the three aliphatic resonances in the $^{13}$C NMR spectrum ($\delta_C$ 89.3 (CH, C-7), 54.1 (CH, C-8), 63.4 (CH$_2$, C-9)) [Yang et al., 2007]. Further analysis of the $^1$H NMR spectrum assigned the three aromatic

### Table 1
The $^1$H (600 MHz) and $^{13}$C (150 MHz) NMR spectroscopic data for compounds 1 and 2 (methanol-$d_4$).

<table>
<thead>
<tr>
<th>position</th>
<th>$\delta_C$</th>
<th>$\delta_H$ (mult., $J$ in Hz)</th>
<th>$\delta_C$</th>
<th>$\delta_H$ (mult., $J$ in Hz)</th>
</tr>
</thead>
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</tr>
<tr>
<td>2</td>
<td>103.9, CH</td>
<td></td>
<td>159.5, C</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>149.4, C</td>
<td></td>
<td>95.6, CH</td>
<td>6.04 (br s)</td>
</tr>
<tr>
<td>4</td>
<td>136.3, C</td>
<td></td>
<td>164.9, C</td>
<td></td>
</tr>
<tr>
<td>5</td>
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<td></td>
<td>98.3, CH</td>
<td>5.97 (br s)</td>
</tr>
<tr>
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<td>6.71 (s)</td>
<td>161.2, C</td>
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<td>7.71 (d, 8.3)</td>
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<td>4.17 (11.2, 4.0) 4.00 (dd, 11.2, 7.4)</td>
<td>7.60 (br d, 7.3)</td>
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<td></td>
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<td>7.40 (m)</td>
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<td>7.30 (t, 7.3)</td>
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<tr>
<td>1’a</td>
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<td>130.6, CH</td>
<td>7.60 (br d, 7.3)</td>
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<td>3.81 (s)</td>
<td>69.9, CH</td>
<td>3.39 (dq, 9.2, 6.1)</td>
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<td>3.43 (s)</td>
<td>179.9, CH$_3$</td>
<td>0.88 (d, 6.1)</td>
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</tbody>
</table>

* Data were measured at 400 MHz for $^1$H NMR and 100 MHz for $^{13}$C NMR spectra, respectively.

Fig. 1. Structures of compounds 1–10 from Piper wallichii.
1g calculated ECD curve for (7R,8R)-1. The aforementioned data revealed that 1 was a neolignan possessing one more aromatic ring. Apart from the furan ring and the three benzene rings accounting for 13 degrees of unsaturation, 1 should have an additional ring in the molecule. Comparison 1D NMR data of 1 with those of 4,6-dimethoxy-5-hydroxy-3-hydroxymethyl-2-(3,4,5-trimethoxyphenyl)-2,3-dihydrobenzofuran isolated from Tarenna attenuata (Yang et al., 2007) suggested that it shared the same A and B rings. This was further confirmed by the $^1$H–$^1$H COSY correlations of H-7/H-8/H-9, and the HMBC correlations from H-2 to C-3 (δC 161.2)/C-2 (δC 139.0), from H-7 to C-1 (δC 134.1)/C-26 (δC 103.9)/C-9 (Fig. 2). However, the $^{13}$C NMR resonances due to the C ring were different with those of the known neolignan because 1 had an additional aromatic ring. The structure of C ring and the linkage of the additional aromatic ring was further determined by the $^1$H–$^1$H COSY correlation of H-5'/H-6', the HMBC correlations from H-7 to C-1' (δC 161.2)/C-2' (δC 110.5), from H-5' to C-1'/C-3' (δC 154.3)/C-6' (δC 117.3), from H-6' to C-2'/C-' (δC 120.4), and from H-5' to C-4'/C-1' (δC 144.5)/C-3' (δC 139.0), and the ROESY correlations of H-5' with H-5 (Fig. 2). The HMBC correlations from OMe-3 (δC 38.1) to C-3, OMe-5 (δC 38.1) to C-5, and OMe-4' (δC 39.4) to C-4' (δC 147.4), together with the ROESY correlations of OMe-3,5 with H-2/6 and OMe-4' with H-5' clearly revealed the locations of these three methoxyl groups (Fig. 2). Although there were no available ROESY correlations for the downfield-shifted methoxyl group at δC 41.1, the HMBC correlations of H-2/6 to δC 136.3 (C-4'), H-5' to δC 139.0 (C-3') and OMe-2' (δC 41.1) to δC 134.6 (C-2') favoredly supported this methoxyl group was linked to C-2' and not C-4. Therefore, the planar structure of 1 was established as shown in Fig. 1.

The relative configuration of 1 was elucidated on the basis of the coupling constants observed in the $^1$H NMR spectrum and ROESY experiment. The value as 6.0 Hz of JH-7, H-8 indicated a 7R,8R-7,8-trans configuration (Yang et al., 2007), which was confirmed by the ROESY correlation of H-7 with H-8. A theoretical calculation of electronic circular dichroism (ECD) spectrum of 1 was performed to determine the absolute configurations, using time-dependent density-functional theory (TDDFT) method. A pair of enantiomers, (7S,8R)-1 and (7R,8S)-1, were calculated at the B3LYP-SCRF/6-31 G(d,p)/B3LYP/6-31 G(d) level with polarizable continuum model (PCM) in MeOH. As illustrated in Fig. 3, the calculated ECD curve for (7S,8R)-1 was in good agreement with the experimental one. In order to comprehend the generation of the experimental ECD spectrum of 1, molecular orbital (MO) analysis using conformer 1 as an example was carried out at the B3LYP/6-31+G(d,p) level with PCM in MeOH (Fig. 4). The positive Cotton effect at 205 nm could be ascribed to the positive rotatory strengths at 210.76 and 211.94 nm, which were caused by the electronic transitions from MO120 to MO125 and from MO122 to MO130 involving two π→π* transitions in the aromatic rings. The negative rotatory strength at 225.86 nm contributed to the negative Cotton effect at 223 nm, which was associated with the electronic transitions from MO118 to MO124 involving a π→π* transition. Accordingly, the absolute configurations of 1 were established as 7S,8R and the compound was named piperwalliol A. To the best of our knowledge, piperwalliol A (1), represents the first example possessing a 5/6/5/6 ring system in the lignan family.

Compound 2, obtained as a yellow oil, had a molecular formula of C25H30O13, as determined by the HREIMS (m/z 538.1683 [M]+; calcd for C25H30O13, 538.1686). The $^{13}$C NMR (DEPT) spectra of 2 (Table 1) showed the presence of 12 aromatic carbon signals (δC 78.3–166.1), a conjugated carbonyl group at δC 201.1 (C-7), and 12 carbon signals at δC 77.9 (C-4), 80.6 (C-2'), 78.3 (C-3'), 77.9 (C-4'), 71.1 (C-5'), 62.6 (C-6'), 102.3 (C-1'), 72.1 (C-2'), 72.2 (C-3'), 74.2 (C-4'), 69.9 (C-5'), and 17.9 (C-6'), arising from two hexosyl groups. The $^1$H NMR spectrum (Table 1) exhibited a typical monosubstituted benzene ring [δH 7.60 (2H, br d, $J$ = 7.3 Hz, H-9, 13), 7.30 (2H, t, $J$ = 7.3 Hz, H-10, 12), and 7.40 (1H, m, H-11)], one two-proton aromatic broad singlets at δH 6.04 (1H, br s, H-3) and 5.97 (1H, br s, H-5) due to an asymmetric 1,2,4,6-tetrasubstituted phenyl, two anomeric protons at δH 4.85 (1H, d, $J$ = 7.8 Hz, H-1') and 4.63 (1H, br s, H-5'), and the characteristic rhamnosyl signals at δH 3.39 (1H, dq, $J$ = 9.2, 6.1 Hz, H-5') and 0.88 (3H, d, $J$ = 6.1 Hz, H-6') (Pathak et al., 2004). Acid hydrolysis of 2 gave o-glucose and r-rhamnose as sugar residues that were determined by GC analysis of their corresponding trimethylsilylated L-cysteine adducts. The aforementioned data revealed that compound 2 was a benzophenone glycoside similar to garcinamangoside D from the fruit hulls of...
Garcinia mangostana (Huang et al., 2001). The only difference was the presence of an additional rhamnosyl unit in 2. The benzophenone moiety in 2 was further established on the basis of the HMBC correlations from H-3 to C-1 (δC 108.8)/C-4 (δC 164.9)/C-5 (δC 98.3)/C-7 (δC 200.1) and from H-9 and H-13 to C-7/C-11 (δC 132.9) (Fig. 5). The location and sequence of the sugar moieties were readily confirmed by the HMBC correlations from H-1α to C-2 (δC 159.5) and from H-1″ to C-20 (δC 80.6) (Fig. 5), which was supported by the ROESY correlation of H-3 with H-1α. Thus, the structure of 2 was established as 4,6-dihydroxy-2-O-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl]benzophenone and named piperwallioside A.

Compound 3, a colorless oil, [α]D21 –87.9, possessed a molecular formula of C15H20O8, as deduced by the HREIMS at m/z 328.1159 [M]+ (calcd for C15H20O8, 328.1158). The 13C NMR and DEPT spectra of 3 showed a set of signals attributed to a glucopyranosyl moiety (δC 100.9, 74.8, 78.2, 71.6, 77.6 and 62.8), and nine carbon signals comprising of six aromatic (δC 129.1–136.4), one carboxyl (δC 173.3), one methoxy (δC 53.0) and one oxymethine (δC 78.3) carbons. The 1H NMR spectrum of 3 (Table 2) exhibited the presence of one mono-substituted aromatic ring [δH 7.48 (2 H, m, H-2,6) and 7.37 (3 H, m, H-3,4,5)], one anomic proton [δH 4.09 (d, J = 7.7 Hz, H-1′)] and a methoxy group (δH 3.69). In the HMBC spectrum of 3, correlations from the oxymethine proton (δH 5.51, s, H-7) to the carboxyl carbon at δC 173.3 and aromatic carbons at C-1 (δC 136.4)/C-2 (δC 129.1)/C-6 (δC 129.1)/C-8 (δC 173.3) and the methoxy proton (δH 3.69) to the carboxyl carbon (δC 173.3) established the aglycone moiety as methyl 2-hydroxy-2-phenylacetate (Fig. 5). The glucosyl moiety was determined to be attached to the C-7 hydroxyl group by the HMBC correlation from H-1′ to C-7 (δC 78.3) (Fig. 5). Further comparison of the NMR and optical rotation data with those of d-mandelic acid β-D-glucopyranoside ([α]D21 –126) (Kitajima and Tanaka, 1993) concluded that compound 3 was a methyl ester of d-mandelic acid β-D-glucopyranoside, which was given a trivial name of piperwallioside B.

Compound 4 was isolated as colorless oil. The molecular formula of 4 was determined as C15H20O8, on the basis of its HREIMS (m/z 328.1150 [M]+, calcd for C15H20O8, 328.1158). The 13C NMR and DEPT spectra of 4 resembled those of 3 (Table 2), except for the chemical shift of the anomeric carbon, which was downfield shifted to δC 103.0 in 4. The anomeric carbon of the glucosyl residue was deduced to be β by the coupling constant of anomeric proton (J = 7.6 Hz) and it should have α-form as that of 2 from a biogenic standpoint. The aforementioned data suggested that 4 should be a C-7 configurational epimer of 3. Since the experimental ECD spectrum of 4 showed an obvious positive Cotton effect at 222 nm being opposite to that of 3 (Fig. 6), compound 4 should possess an S absolute configuration at C-7. Therefore, the structure of piperwallioside C (4) was determined as l-mandelic acid methyl ester β-D-glucopyranoside.

Compound 5, a colorless oil, had a molecular formula of C14H19NO7 as deduced from HREIMS at m/z 313.1160 [M]+ (calcd for C14H19NO7, 313.1162). The 13C and 1H NMR data of 5 (Table 2) were similar to those of prunasinamide [(2R)-2-β-D-glucopyranosylomandelamide] from Cyanogenic glucosides, except for the downfield-shifted anomic carbon of 5.
3.3. Physical and spectroscopic data of new compound

Piperwallioside A (1): yellow oil; [α]D25 +18.7 (c 0.2, MeOH); UV (MeOH) λmax (log ε): 316 (3.65), 209 (4.27) nm; ECD (c 0.06, MeOH) λmax nm (Δε): 223 (~5.32), 205 (+9.00); IR (KBr) νmax: 3452, 1641, 1631, 1134 cm⁻¹; ¹H and ¹³C NMR data, see Table 1; negative ESIMS: m/z 467 [M + H]⁺; HRESIMS: m/z 467.1337 [M⁺] (calcd for C25H30O13, 467.1342).

Piperwallioside A (2): yellow oil; [α]D25 +128.9 (c 0.3, MeOH); UV (MeOH) λmax (log ε): 303.5 (3.28), 248.5 (3.32), 204.5 (3.94) nm; IR (KBr) νmax: 3440, 1624, 1600, 1451, 1277 cm⁻¹; ¹H and ¹³C NMR data, see Table 1; negative ESIMS: m/z 537 [M⁺] (calcd for C25H29O13, 538.1683); HREIMS: m/z 538.1683 [M⁺] (calcd for C25H29O13, 538.1686).

Piperwallioside B (3): colorless oil; [α]D25 -87.9 (c 0.3, MeOH); UV (MeOH) λmax (log ε): 257.6 (1.45), 203.6 (3.11) nm; ECD (c 0.3, MeOH) λmax nm (Δε): 218 (~6.76); IR (KBr) νmax: 3421, 1741, 1631, 1384, 1078 cm⁻¹; ¹H and ¹³C NMR data, see Table 2; positive ESIMS: m/z 351 [M + Na⁺]; HREIMS: m/z 328.1159 [M⁺] (calcd for C15H20O8, 328.1158).

Piperwallioside C (4): colorless oil; [α]D25 +4.94 (c 0.2 MeOH); UV (MeOH) λmax (log ε): 257.4 (1.69), 204.6 (3.2) nm; ECD (c 0.2, MeOH) λmax nm (Δε): 222 (+10.49); IR (KBr) νmax: 3428, 1741, 1630, 1076, 1038 cm⁻¹; ¹H and ¹³C NMR data, see Table 2; positive ESIMS: m/z 351 [M + Na⁺]; HREIMS: m/z 328.1159 [M⁺] (calcd for C15H20O8, 328.1158).

Piperwallioside D (5): colorless oil; [α]D25 -0.88 (c 0.3, MeOH); UV (MeOH) λmax (log ε): 257.4 (1.83), 204.2 (3.3) nm; ECD (c 0.2, MeOH)
3.4. Inhibition activities on platelet aggregation induced by PAF

All the isolates were evaluated for their inhibition activities on platelet aggregation induced by PAF, with ginkgolide B (GB) used as a positive control (IC$_{50}$ = 0.016 mM). It showed that five known compounds, (–)-syringaresinol (6), (+)-medioresinol (7), (+)-epi-pinoresinol (8), sinapyl aldehyde (9), and 3,5-di-methoxy-biphenyl-3,4-diol (10) exhibited antiplatelet aggregation activities in a concentration-dependent manner (see SI, Table S9). Among which, compound 6 was the most active one with an IC$_{50}$ value of 0.52 mM. (–)-Lirioresinol A (SI, Fig. S52) with different configurations from 6 presented no activity. Compound 7 showed the maximum platelet aggregation and inhibition of aggregation with 52.7 ± 4.0% and 16.8 ± 6.1%, respectively, at a concentration of 0.50 mM, while in the case of 8 at a concentration of 0.40 mM, it was 46.3 ± 4.0% and 24.1 ± 1.0%, respectively. Moreover, (+)-pinoresinol showed no activity, which might be correlated with the absence of a phenolic methoxy group. Compounds 9 and 10 displayed moderate antiplatelet aggregation activities with IC$_{50}$ values of 2.4 and 2.0 mM, respectively. The aforementioned results implied that configurations and methoxy group of lignans should be crucial for the antiplatelet aggregation activities of these compounds.

3.5. In vivo antithrombotic effect of compounds 6, 9 and 10

The active compounds in platelet aggregation induced by PAF model were further tested for their in vivo antithrombotic effects at a concentration of 30 μM on zebrafish model. Due to limited amounts, only compounds 6, 9 and 10 were further tested. The results were shown in Fig. 7 and Table S10 of SI. (–)-Syringaresinol (6) and sinapyl aldehyde (9) showed good in vivo antithrombotic effect with inhibitory values of 37% and 11%, at a concentration of 30 μM, respectively. Although 3,5-di-methoxy-biphenyl-3,4-diol (10) displayed better activity than compound 9 in PAF model, compound 10 showed no antithrombotic effect on zebrafish model, at a concentration of 30 μM. Among them, compound 6 was also the most potential active compound on zebrafish model, compared with the positive control aspirin with an inhibitory value of 74% at a concentration of 125 μM. Moreover, all of them showed no cytotoxicity on zebrafish at a concentration of 30 μM and 1.1% DMSO did not affect the thrombus in zebrafish.

4. Discussion and conclusion

This study on *Piper wallichii* led to the isolation and identification of a new neolignan, piperwalliol A (1), and four new aromatic...
glycosides, piperwalliosides A–D (2–5), along with 25 known compounds. Being the first neolignan with 5/6/5/6 ring system, piperwalliol A (1) features an unique architecture, which represents one of an interesting work during decades of the studies on Piper family. Three known lignans, (−)-syringaresinol (6), (+)-meioresinol (7), and (+)-epipinoresinol (8), and two phenolic compounds, sinapyl aldehyde (9) and 3',5'-dimethoxybiphenyl-3,4'-diol (10) showed in vitro antplatelet aggregation activities. It is noted that (−)-syringaresinol (6) also displayed in vivo antithrombotic effect on zebrafish model, which represents one new scaffold with antplatelet aggregation activity. The activities of compounds from Piper wallichii supported the traditional use of this herbal medicine.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2014.12.038.

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