RESEARCH ARTICLE

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Chemical constituents from the leaves of *Styrax argentifolius* H.L. Li and their antioxidative activity

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ABSTRACT

Searching for bioactive agents from medicinal plants, the phytochemical investigation on the EtOAc extract of the Vietnamese *Styrax argentifolius* leaves has resulted in the isolation and structural determination of five compounds, including one *nor*-neolignan egonoic acid (1), one lignan (+)-pinoresinol (2), one sterol (20*R*)-3*β*-hydroxysitgmasta-5,22-dien-7-one (3), and two triterpenoids lupeol (4), and 2α,3α,24-trihydroxy-urs-12-en-28-oic acid (5). The chemical structures of these secondary metabolites were elucidated by NMR and MS spectral data. All isolated compounds were first observed in *S. argentifolius* species. Sterol 3 and triterpenoid 5 were detected in genus *Styrax* for the first time. With the IC_{50} value of 19.10 µg/mL, compound 2 possessed the strong activity in DPPH radical scavenging assay.

Keywords: *Styrax argentifolius*, leaves, phytochemistry, DPPH antioxidant

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INTRODUCTION

In the family Styracaeae, the genus *Styrax* contains about 130 species of small trees or large shrubs.[1] The plants of this genus have a widespread distribution from tropical to subtropical regions, and are mostly found in Asia.[2] The dried resinous materials from the pierced barks of *S. benzoin*, *S. benzoides*, and *S. tonkinensis* have been regarded as commercial products for perfumes, incense, and folk medicines since ancient time.[3] Searching for bioactive components from *Styrax* species has been performed in many previous publications. As a representative example, Liu and partners (2011) suggested that benzofuran derivatives were the main class of isolated compounds from *S. agrestis*. They also severed as potential agents in acetylcholinesterase inhibitory assays.[4] In other report, triterpenoids were found available in the resin of *S. tokinensis*, especially oleanolic acid can be seen as a promising anti-cancer compound with the significant IC_{50} value of 8.9 µM against HL-60 cell growth.[5]

Of the 13 *Styrax* species were recorded in Vietnam, most of them were found in both South and North.[6] Among these species, the Vietnamese *S. tonkinensis* seems to be the best objective for various types of studies.[7-9] *Styrax argentifolius* H.L. Li, which locally named Bo de la trang, was native to Hagiang-Vietnam.[6] To the best of our knowledge, the phytochemical and pharmacological investigations of *S. argentifolius* has not yet been published till now. In the current paper, we first describe the isolation, NMR structural elucidation of five compounds from the EtOAc extract of the Vietnamese *S. argentifolius* leaves, as well as their DPPH radical scavenging capacity in anti-oxidative assay.

EXPERIMENTAL

General experimental procedures

ESI-MS spectroscopies were measured on a Thermo Scientific LTQ Orbitrap XL spectrometer (USA). NMR data have been acquired at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR on a Bruker 500 MHz spectral tool. Silica gel (200-300 mesh, Germany), Sephadex LH-20 (Bio-Science, Sweden), RP-C18 (40-63 µm, Japan) were used for column chromatography (CC). Preparative TLC analysis was carried out on silica gel 60 F_{254} plates (Merck).

Plant material

The leaves of *S. argentifolius* were collected in Hagiang province in January 2018. The plant material was identified by Prof. Tran The Bach, Institute of Ecology and Biological Resources. A voucher specimen VN-1066 was deposited in Department of Applied Biochemistry, Institute of Chemistry.

Extraction and isolation

The dried powder of *S. argentifolius* leaves (1.5 kg) was immersed by re-fluxing with *n*-hexane [12 L x 3 times, 50°C, 2 h]. In the same manner, this

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powder was then extracted with EtOAc [12 L x 3 times, 55°C, 3 h]. This soluble extract was evaporated under reduced pressure to afford the EtOAc crude residue (62.0 g), which was subjected to silica gel CC (12 x 60 cm, 370.0 g), eluted with a stepwise gradient of CH_2Cl_2-EtOAC (100:0 to 0:100, v/v), to give ten fractions (fr.s) SAE1-SAE10.

The fr. SAE1 (7.60 g) was chromatographed on silica gel CC [*n*-hexane-CH_3COCH_3 (9:1 to 1:1, v/v)], to give five fr.s SAE1.1-SAE1.5. White amorphous solids were precipitated out of the fr. SAE1.3 (110 mg), then washed with MeOH (100%), to yield compound 5 (10.3 mg). Compound 1 (4.2 mg) was purified from the fr. SAE1.6 (1.1 g) by silica gel CC [CH_2Cl_2-CH_3COCH_3, 8:1 (v/v)].

Similarly, the fr. SAE4 (2.5 g) was then separated on silica gel CC, eluting with CH_2Cl_2-CH_3COCH_3 (10:1 to 1:1, v/v), to give five fr.s SAE4.1-SAE4.5. Compound 2 (5.1 mg) was purified from the fr. SAE4.2 (80.5 mg) by RP-C_{18} CC [MeOH-H_2O (2:1, v/v)]. The fr. SAE4.4 (0.7 g) was chromatographed on Sephadex LH-20 [MeOH (100%)], to give compound 3 (6.0 mg). Lastly, the fr. SAE8 (3.1 g) was separated on silica gel CC [EtOAc-CH_3COCH_3 (30:1 to 1:1, v/v)], to produce compound 4 (white amorphous solids, 8.0 mg).

Egonoic acid (1): White amorphous powders; ESI-MS (+): 341 $[M+H]^+$; ^{1}H-NMR (500 MHz, CD_3OD, δ_H ppm): 7.39 (1H, dd, 2.0, 8.5 Hz, H-6'), 7.31 (1H, d, 2.0 Hz, H-2'), 6.99 (1H, d, 1.5 Hz, H-4), 6.91 (1H, s, H-3), 6.89 (1H, d, 8.5 Hz, H-5'), 6.75 (1H, d, 1.5 Hz, H-6), 5.99 (2H, s, -OCH_2O-), 4.00 (3H, s, 7-OMe), 2.96 (2H, t, 7.5 Hz, H-1"), 2.55 (2H, t, 7.5 Hz, H-2"); ^{13}C-NMR (125 MHz, CD_3OD, δ_C ppm): 180.0 (C-3"), 157.1 (C-2), 149.6 (C-3'), 149.4 (C-4'), 146.1 (C-7), 143.8 (C-7a), 139.0 (C-5), 132.4 (C-3a), 126.1 (C-1'), 120.0 (C-6'), 113.3 (C-4), 109.5 (C-5'), 108.7 (C-6), 106.1 (C-2'), 102.7 (-OCH_2O-), 101.5 (C-3), 56.6 (7-OMe), 39.7 (C-2"), 33.4 (C-1").

(+)-Pinoresinol (2): Yellow amorphous powders; ESI-MS (+): *m/z* 359 $[M+H]^+$; ^{1}H-NMR (500 MHz, CD_3OD, δ_H ppm): 6.96 (1H, d, 1.5 Hz, H-2, H-2'), 6.82 (1H, dd, 1.5, 8.0 Hz, H-6, H-6'), 6.80 (1H, d, 8.0 Hz, H-5, H-5'), 4.71 (1H, d, 4.5 Hz, H-7, H-7'), 4.23 (1H, dd, 6.0, 8.5 Hz, H_a-9, H_a-9'), 3.86 (3H, s, 3-OMe, 3'-OMe), 3.83 (1H, dd, 3.5, 8.5 Hz, H_b-9, H_b-9'), 3.13 (1H, m, H-8, H-8'); ^{13}C-NMR (125 MHz, CD_3OD, δ_C ppm): 148.4 (C-3, C-3'), 145.9 (C-4, C-4'), 134.5 (C-1, C-1'), 120.0 (C-6, C-6'), 115.7 (C-5, C-5'), 110.6 (C-2, C-2'), 87.1 (C-7, C-7'), 72.4 (C-9, C-9'), 56.3 (3-OMe, 3'-OMe), 55.1 (C-8, C-8').

(20***R***)-3β-Hydroxysitgmasta-5,22-dien-7-one (3): White amorphous solids; ESI-MS (+): $C_{29}H_{46}O_2Na$ *m/z* 449 $[M+Na]^+$; ^{1}H-NMR (500 MHz, CD_3OD, δ_H ppm) and ^{13}C-NMR (125 MHz, CD_3OD, δ_C ppm): See Table 1.

Lupeol (4): White amorphous solids; ESI-MS (+): *m/z* 427 $[M+H]^+$; ^{1}H-NMR (500 MHz, CD_3OD, δ_H ppm) and ^{13}C-NMR (125 MHz, CD_3OD, δ_C ppm): See Table 1.

2α,3α,24-Trihydroxy-urs-12-en-28-oic acid (5): White amorphous solids; ESI-MS (+): *m/z* 475 $[M+H]^+$; ^{1}H-NMR (500 MHz, CD_3OD, δ_H ppm) and ^{13}C-NMR (125 MHz, CD_3OD, δ_C ppm): See Table 1.

DPPH antioxidative assays

All isolated compounds 1-5 were subjected to the DPPH radical scavenging assay, when the protocol has been carefully described in our previous reports.[10-12]

RESULTS AND DISCUSSION

Compound 1 was obtained as white amorphous powders. Its molecular formula was elucidated as $C_{19}H_{16}O_6$ by the pseudo-molecular ion peak at *m/z* 341 $[M+H]^+$ in the ESI-MS (+) spectrum. The ^{1}H-NMR spectral data of 1 showed a pattern of *nor*-neolignan type benzofuran derivative, in which benzofuran nucleus included a singlet signal at δ_H 6.91 (H-3), and two doublet signals (J = 1.5 Hz) at δ_H 6.99 (H-4) and δ_H 6.75 (H-6). The ^{1}H-NMR data were also characteristic of an ABX spin system at [δ_H 6.89 (d, 8.5 Hz, H-5'), δ_H 7.39 (dd, 2.0, 8.5 Hz, H-6'), and δ_H 7.31 (d, 2.0 Hz, H-2')], a dioxygenated methylene at δ_H 5.99 (-OCH_2O-), two aliphatic methylenes at δ_H 2.55 (H-1") and δ_H 2.96 (H-2"), and a methoxy group at 4.00 (7-OMe). The ^{13}C-NMR spectral data has composed of six aromatic methine groups, three methylene groups, eight quaternary carbon groups, one methoxy group and one carbonyl group (Figure 1). The chemical structure of 1 was further confirmed by 2D-NMR evidence (HSQC, HMBC, and COSY). The key HMBC correlations H-3/C-2 and C-3a, H-4/C-3a and C-5, H-6/C-5 and C-7 were remarkable features of benzofuran skeleton, while the characteristic HMBC correlations -OCH_2O-/C-3' and C-4', H-2'/C-3', H-6'/C-2' and C-4', together with the COSY cross-peak H-5'/H-6' affirmed the presence of a 3',4'-methylenedioxyphenyl unit. Furthermore, H-2' and H-6' have the HMBC correlations to C-2, which determined the position of this phenyl ring at carbon C-2. The HMBC correlation 7-OCH_3/C-7 showed that methoxy group substituted at carbon C-7. The COSY cross-peak H-1"/H-2" and the HMBC correlations H-2"/C-3", and H-1"/C-5 suggested that carboxyethyl group located at carbon C-5. Based on these findings and comparison with literature data, compound 1 was elucidated as a benzofuran derivative, which named egonoic acid. Secondary metabolite 1 presented in several *Styrax*

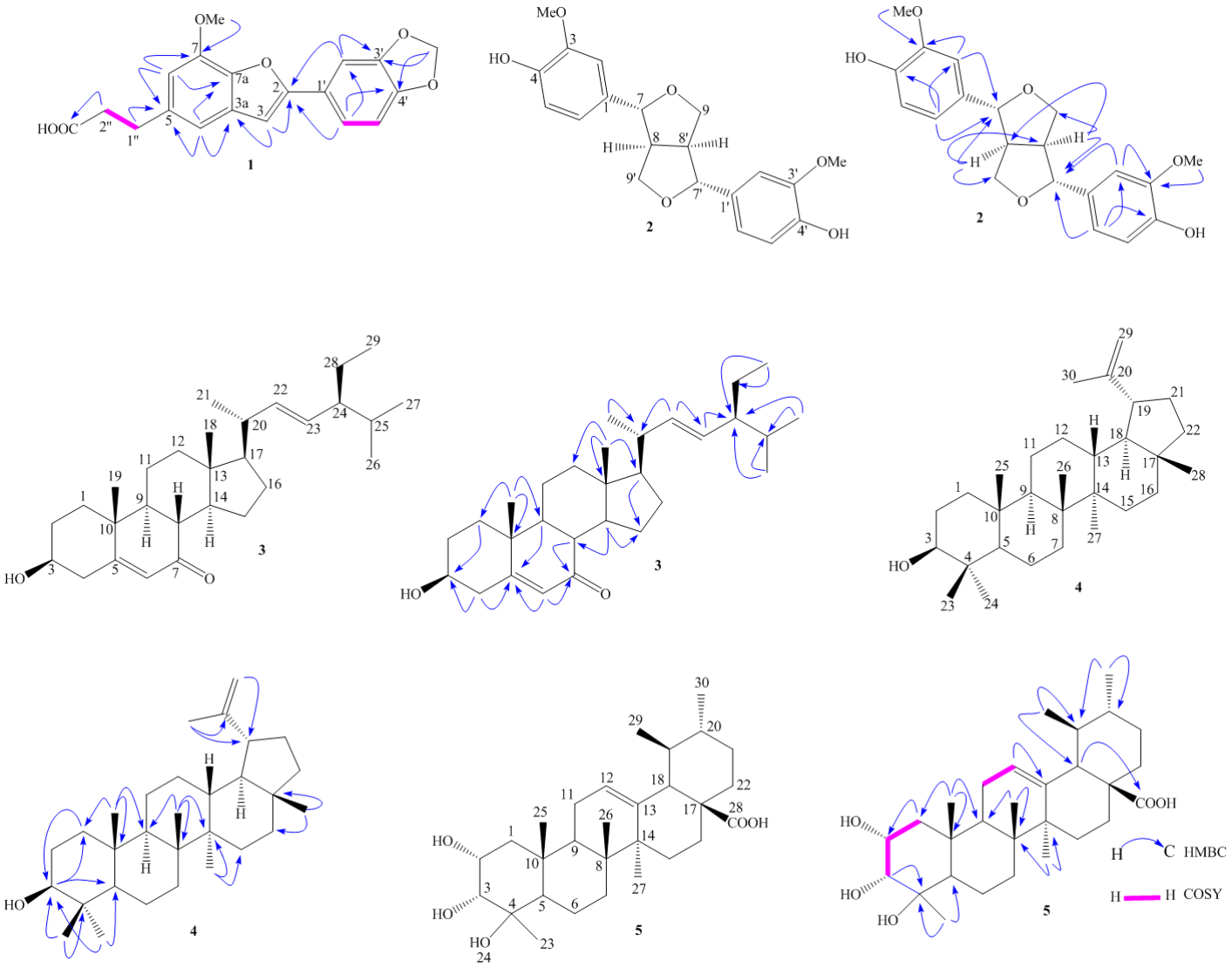

Figure 1 The isolated compounds from *S. argentifolius* leaves and their key HMBC and COSY correlations

plants, such as *S. annamensis* and *S. agrestis*,[3,4] but this is the first time to isolate from *S. argentifolius*.

Compound 2 was obtained as yellow amorphous powders. Its molecular formula was to be $C_{20}H_{22}O_6$, deducing from the pseudo-molecular ion peak at *m/z* 359 $[M+H]^+$ in the ESI-MS (+) spectrum. The ^{1}H-NMR spectrum of 2 displayed a model of a symmetrical furofuran lignan, in which two oxygenated methylenes resonated at δ_H 4.23 (dd, 6.0, 8.5 Hz, H_a-9, H_a-9') and δ_H 3.83 (dd, 3.5, 8.5 Hz, H_b-9, H_b-9'); a multiplet signal at δ_H 3.13 was assigned to two methines H-8 and H-8'; a doublet signal at δ_H 4.71 belongs to two methines H-7 and H-7'. The ^{1}H-NMR data of 2 have further composed of a superimpose ABX spin system [δ_H 6.80 (d, 8.0 Hz, H-5, H-5'), δ_H 6.82 (dd, 1.5, 8.0 Hz, H-6, H-6'), and δ_H 6.96 (d, 1.5 Hz, H-2, H-2')], and a superimpose methoxy group [δ_H 3.86 (s, 3-OMe, 3'-OMe)]. The ^{13}C-NMR spectrum of 2 is in agreement with this, the chemical shifts of carbons of furofuran nucleus ranged from δ_C 55.1 to δ_C 87.1 ppm, while those of two symmetric phenyl units were found in δ_C 110.6-148.4 ppm. Methoxy carbons 3-OMe and 3'-OMe resonated at δ_C 56.3. The chemical structure of this compound was also confirmed by 2D-NMR evidence (HSQC and HMBC). Proton methine H-8 had the key correlations with C-7, C-8' and C-9', whereas H-8' correlated to C-8, C-9, and C-7'. Methoxy group located at carbons C-3 and C-3' with the crucial HMBC cross-peaks 3-OMe/C-3, and 3'-OMe/C-3'. Because of the HMBC correlations H-2 and H-6/C-7, and H-2' and H-6'/C-7', two phenyl moieties connected to bridge carbons C-7 and C-7', respectively. Compared to literature, compound 2 was elucidated as (+)-pinoresinol.[13] Compound 2 is now available in several *Styrax* plants,[9,14] but this is the first time to isolate from *S. argentifolius*.

Compound 3 was purified as white amorphous solids. The pseudo-molecular ion peak at

Table 1 ^{1}H and ^{13}C-NMR data of compounds 3-5

	3		4		5	
No	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C
1	1,21 (1H, m) 2.05 (1H, m)	37.6	1.70 (1H, m) 0.97 (1H, dd, 5.0, 9.0 Hz)	40.7	1.29 (1H, m) 1.69 (1H, m)	42.8
2	1.68 (1H, m) 1.91 (1H, m)	31.9	1.64 (1H, m) 1.60 (1H, m)	28.0	4.10 (1H, dt, 3.5, 10.5)	66.9
3	3.58 (1H, m)	71.2	3.12 (1H, dd, 5.0, 10.0)	79.8	3.45 (1H, d, 2.5)	78.8
4	2.43 (1H, dt, 2.0, 7.0) 2.49 (1H, dd, 2.5, 7.5)	42.8	-	40.0	-	76.5
5	-	169.1	0.71 (1H, t, 9.0)	56.8	1.21 (1H, t, 8.0)	48.5
6	5.67 (1H, s)	126.3	1.57 (1H, m) 1.45 (1H, m)	19.4	1.52 (1H, m) 1.61 (1H, m)	18.5
7	-	204.6	1.48 (1H, m) 1.46 (1H, m)	35.2	1.38 (1H, m) 1.62 (1H, m)	34.1
8	2.33 (1H, m)	46.6	-	42.2	-	40.9
9	1.34 (1H, m)	51.6	1.32 (1H, t, 1.5)	51.8	1.72 (1H, m)	47.8
10	-	39.7	-	38.1	-	39.0
11	1.68 (2H, m)	22.3	1.42 (1H, m) 1.26 (1H, m)	22.2	2.01 (2H, dd, 5.5, 9.0 Hz)	24.3
12	1.18 (1H, m) 2.07 (1H, m)	39.9	1.71 (1H, m) 1.15 (1H, dd, 4.5, 12.5)	26.6	5.27 (1H, t, 3.5)	127.0
13	-	44.2	1.73 (1H, m)	39.4	-	139.8
14	1.52 (1H, m)	51.5	-	44.1	-	43.6
15	1.29 (1H, m) 2.34 (1H, m)	30.2	1.73 (1H, m) 1.05 (1H, m)	28.7	0.98 (1H, m) 1.98 (1H, m)	29.2
16	1.36 (1H, m) 1.79 (1H, m)	27.5	1.60 (1H, m) 1.42 (1H, m)	36.8	1.70 (1H, m) 2.05 (1H, dd, 6.0, 13.0)	25.3
17	1.17 (1H, m)	56.1	-	44.2	-	48.8
18	0.75 (3H, s)	12.6	1.41 (1H, m)	49.6	2.30 (1H, d, 11.5)	54.4
19	1.26 (3H, s)	17.7	2.41 (1H, m)	48.6	1.39 (1H, m)	40.4
20	2.11 (1H, m)	41.7	-	151.9	1.03 (1H, m)	40.3
21	1.07 (3H, d, 6.5)	21.9	1.97 (1H, m) 1.38 (1H, m)	31.1	1.37 (1H, m) 1.54 (1H, m)	31.8
22	5.22 (1H, dd, 9.0, 15.0)	139.6	1.41 (1H, m) 1.24 (1H, m)	41.2	1.71 (2H, m)	38.1
23	5.08 (1H, dd, 9.0, 15.0 Hz)	130.8	0.79 (3H, s)	16.3	1.26 (3H, s)	27.5
24	1.59 (1H, m)	52.8	0.98 (3H, s)	28.8	-	-
25	1.58 (1H, m)	33.2	0.90 (3H, s)	17.0	1.13 (3H, s)	16.7
26	0.90 (3H, d, 6.5)	21.5	1.10 (3H, s)	16.7	0.87 (3H, s)	18.1
27	0.85 (3H, d, 7.0)	19.5	1.01 (3H, s)	15.1	1.15 (3H, s)	24.2
28	1.17 (1H, m) 1.49 (1H, m)	26.5	0.85 (3H, s)	18.3	-	181.8
29	0.86 (3H, d, 7.0)	12.6	4.70 (1H, d, 3.0) 4.60 (1H, dd, 1.5, 3.0)	110.0	0.91 (3H, s)	17.6
30			1.70 (3H, s)	19.7	0.99 (3H, s)	21.6

m/z 449 $[M+Na]^+$ in the ESI-MS (+) spectral data revealed that the molecular formula of 3 was to be $C_{29}H_{46}O_2$. The ^{1}H and ^{13}C-NMR spectral data aided by the HSQC and HMBC spectroscopic data indicated that compound 3 is a sterol, which named (20*R*)-3***β***-hydroxysitgmasta-5,22-dien-7-one (Table 1).[15] Methine carbinol group (δ_H 3.58) was substituted at carbon C-3 (δ_C 71.2) with the

characteristic HMBC correlations H-1 and H-4/C-3. The fragment C5=C6(H6)-CO was associated with the key HMBC correlations H-6 (δ_H 5.67)/C-5 (δ_C 169.1) and C-7 (δ_C 204.6). The ^{1}H-NMR data was also remarkable with the presence of the other *E*-double bond [H-22 (δ_H 5.22), H-23 (δ_H 5.08), and J = 15.0 Hz]. This double bond was accompanied by the HMBC evidence H-22/C-20 and C-23, and H-23/C-24. Sterol 3 also contained six methyl groups [H-18 (δ_H 0.75), C-18 (δ_C 12.6); H-19 (δ_H 1.26), C-19 (δ_C 17.7); H-21 (δ_H 1.07), C-21 (δ_C 21.9); H-26 (δ_H 0.90), C-26 (δ_C 21.5); H-27 (δ_H 0.85), C-27 (δ_C 19.5); and H-29 (δ_H 0.86), C-29 (δ_C 12.6)]. These methyl groups induced the important HMBC cross-peaks H-18/C-12, C-13, and C-17, H-19/C-1, C-9, and C-10, H-21/C-20, H-29/C-24 and C-28, and H-26 and H-27/C-24 and C-25. (20*R*)-3***β***-hydroxysitgmasta-5,22-dien-7-one (3) was first isolated from genus *Styrax*, to date.

Compound 4 was separated as white amorphous solids. This compound was not visible with UV lamp (256 nm), and gave the pink color in vanillin indicator [R_f = 0.7 in *n*-hexane-acetone (5:1, v/v)]. Its molecular formula $C_{30}H_{50}O$ was based on the pseudo-molecular peak at *m/z* 427 $[M+H]^+$ in the ESI-MS (+) spectrum. In comparison with authentic sample, compound 4 was determined to be a well-known triterpenoid, which named lupeol.[16] The ^{1}H and ^{13}C-NMR data of 4 were fully assigned and provided in Table 1. Lupeol established a great role in many biological activities, since it was used for anti-inflammation, anti-bacteria, cholesterol-lowering, especially in terms of anti-cancer treatment.[17]

Compound 5 was isolated as white amorphous solids. On the basis of the pseudo-molecular ion peak at *m/z* 475 $[M+H]^+$ in the ESI-MS (+) spectral data, its molecular formula was determined as $C_{29}H_{46}O_5$. The 1D-NMR spectral data (^{1}H, and ^{13}C-NMR) assisted by the 2D-NMR (HSQC, HMBC, and COSY) identified that secondary metabolite 5 was categorised as a triterpenoid, namely 2α,3α,24-trihydroxy-urs-12-en-28-oic acid.[18] In details, three hydroxy methines induced NMR chemical shifts at [δ_H 4.10 (H-2), δ_C 66.9 (C-2); δ_H 3.45 (H-3), δ_C 78.8 (C-3); and δ_C 76.5 (C-4)], and have the key COSY cross-peak H-2/H-3 and the key HMBC correlation H-3/C-4 (Figure 1). The positions of six methyl groups at six carbons C-4, C-8, C-10, C-14, C-19, and C-20 can be possibly observed by the important HMBC correlations H-24 (δ_H 1.26)/C-4 and C-5, H-26 (δ_H 0.87)/C-8 and C-9, H-25 (δ_H 1.13)/C-1, C-9, and C-10, H-27 (δ_H 1.15)/C-8 and C-14, H-29 (δ_H 0.91)/C-19, and H-30 (δ_H 0.99)/C-20, respectively. The chemical structure of 5 was also structurally formulated by an olefinic double bond [δ_H 5.27 (H-12), δ_C 127.0 (C-12); and C-13 (δ_C 139.8)] and a carboxy group [C-28 (δ_C 181.8)]. The positions of these groups were confirmed by the key 2D-NMR correlations, in which H-12 has the COSY cross-peak to H-11 and the HMBC correlation to C-13, H-18 has the HMBC correlation to C-28. Previously, compound 5 has been only isolated from *Isodon macrophyllus* leaves.[18] However, its ^{1}H-NMR data is not fully available. The current paper has resulted in detail NMR data in MeOD.

All isolated compounds 1-5 have been subjected to antioxidative assay for capturing DPPH radical agent (Figure 2). (+)-Pinoresinol (2) has the strong activity with the IC_{50} value of 19.10 μg/mL, as compared with that of the positive control quercetin (IC_{50} 9.34 μg/mL). The remaining tested compounds fail to do so (inactive, (IC_{50} > 128.0 μg/mL). It may possibly conclude that *Styrax* lignans are likely to be better than *nor*-neolignans, sterols, and triterpenoids in anti-oxidative purposes.

CONCLUSION

From the EtOAc extract of the Vietnamses *S. argentifolius* leaves, five compounds, comprising of one *nor*-neolignan egonoic acid (1), one lignan (+)-pinoresinol (2), one sterol (20*R*)-3***β***-hydroxysitgmasta-5,22-dien-7-one (3), and two triterpenoids lupeol (4), and 2α,3α,24-trihydroxy-urs-12-en-28-oic acid (5), were isolated. This is the first time that compounds 1-5 were detected in *S. argentifolius*. Compounds 3 and 5 have never been isolated from genus *Styrax* before. *Styrax* lignans would be superior to *nor*-neolignans, sterols, triterpenoids in DPPH radical scavenging assay.

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