Methyl syringate and isovanilic acid from the bark of *Alphonsea elliptica*

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Abstract
A phytochemical study of dichloromethane extract of Alphonsea elliptica has led to the isolation of two compounds previously unreported from the species methyl syringate and isovanilic acid. Stigmasterol which has been previously reported was also isolated. The structures were determined by spectroscopic techniques and by comparison with data reported in the literature. The findings of this study will contribute to the chemotaxonomic and phylogenetic aspects of the Alphonsea sp.

Keywords: *Alphonsea*, Phytochemical, Annonaceae, Spectroscopic, Isolation.

Introduction
Alphonsea sp is a genus belonging to the family Annonaceae. Annonaceae family are flowering plants which consist of 130 genus and 2300-2500 species; encompassing trees, shrubs and climbers. This plant family is commonly called custard apple family and locally known as Mempisang in Malaysia.

In Asia, *Alphonsea sp*. are distributed throughout India, Sri Lanka, Myanmar, Thailand, Laos, Vietnam, Cambodia, Malaysia, Indonesia and Papua New Guinea[18,21]. Malaysia has reported *A. borneensis, A. curtisii, A. cylindrica, A. elliptica, A. johorensis, A. maingayi* and *A. rugosa* as available species of *Alphonsea*[14,23-25]. In Malaysia, *A. elliptica* is reported from Terengganu, Pahang, Selangor, Negeri Sembilan, Melaka and Johor with a tree height reaching 25 metres[13].

Previous phytochemical studies on *Alphonsea* species revealed isolation of mostly alkaloids, steroids and flavonoids[1,3,4,8,16,17,20,22,27]. Pharmacological studies of *Alphonsea* species revealed biological activities such as anti-oxidant[12,15], anticancer[11,16], antibacterial[21] and antifungal[9]. In addition, there was limited number of phytochemical study on *A. elliptica*.

Material and Methods
Plant materials: Barks of *A. elliptica* Hk. f. et Th. (KL 5285) were collected from Hutan Simpan Sembarong, Kluang, Johor, Malaysia. The species was taxonomically identified by the phytochemical group of Chemistry Department, University of Malaya, Kuala Lumpur where the voucher specimen was deposited.

Extraction and isolation: Sample was extracted with five litres of hexane, dichloromethane and methanol each for three days. Upon each extractions, samples were dried using rotary evaporator and were concentrated under reduced pressure. This yielded 10 g of hexane, 20 g of dichloromethane and 25 g of methanol extracts of *A. elliptica.*

For purification, dichloromethane crude extract of *A. elliptica* (20 g) by gravity column chromatography was eluted with gradient solvents hexane/DCM (100:0, 80:20, 50:50, 20:80, 0:100), mixture DCM/MeOH (100:0, 99:1,98:2, 97:3, 96:4, 95:5, 94:6, 92:8, 90:10, 80:20, 70:30, 50:50) and 100% MeOH. From the first column chromatography, one fraction was identified as stigmasterol (3) (5 mg) and sixty two fractions were yielded labelled as FR_1 to FR_62.

After that, it was combined based on the color and the result of TLC which yielded into fourteen fractions. Fraction FR_7 (0.15 g) was subjected to column chromatography and afforded methyl syringate or methyl 4-hydroxy-3,5-dimethoxybenzoate (1) (3.7 mg). Mixture fraction FR_5 and FR_6 (0.11 g) were subjected to column chromatography and afforded isovanilic acid or 3-hydroxy-4-methoxy-benzoic acid (2) (2.7 mg).

Methyl syringate or Methyl 4-hydroxy-3,5-dimethoxybenzoate (1): ^{1}H-NMR (500 MHz, $CDCl_3$), δ ppm: 3.94 (6H, *s*, OCH_3-3 and OCH_3-5); 3.91 (3H, *s*, α-OCH_3); 5.90 (1H, *s*, OH); and 7.32 (2H, s, Ar-H); ^{13}C-NMR (125 MHz, $CDCl_3$), δ ppm: 56.5 (OCH_3 C-3 and C-5), 52.2 (α-OCH_3), 106.6 (C-2 and C-6), 139.2 (C-4), 121.2 (C-1), 146.8 (C-3 and C-5) and 168.2 (C=O); ESI-MS: *m/z* 212.1 $[M+H]^+$, $C_{10}H_{12}O_5$.

Isovanilic acid or 3-Hydroxy-4-methoxy-benzoic acid (2): White amorphous,^{1}H-NMR (500 MHz, $CDCl_3$), δ ppm: 7.59 (1H, *d*, J=8.5 Hz, H-2), 6.96 (1H, *d*, J=2.0 Hz, H-5), 7.72 (1H, *dd*, J=2.0, 8.5 Hz, H-6) and δ 3.96 (OCH_3-4); (^{13}C-NMR (125 MHz, $CDCl_3$), δ ppm: 56.2 (OCH_3-4), 146.2 (C-4), 112.3 (C-2), 125.2 (C-6),127.9 (C-1), 150.7 (C-3), 114.2 (C-5) and 172.5 (C=O); ESI-MS: *m/z* 168 $[M+H]^+$, $C_8H_8O_4$.

Stigmasterol (7):[19] White amorphous. ^{1}H-NMR (500 MHz, $CDCl_3$), δ ppm: 3.53 (1H, *m*, H-3), 5.35 (1H, *m*, H-6), 0.67 (3H, *s*, H-18), 0.92 (3H, *s*, H-19), 1.02 (3H, *d*, H-21), 5.03 (1H, *m*, H-22), 5.13 (1H, *m*, H-23), 0.83 (3H, *d*, H-26), 0.81

(3H, *d*, H-27), 0.78 (3H, *d*, H-29); ^{13}C-NMR (125 MHz, $CDCl_3$), δ ppm: 37.3(C-1), 32.0 (C-2), 71.9 (C-3), 42.3(C-4), 140.8 (C-5), 121.8 (C-6), 31.7(C-7), 31.7(C-8), 50.2(C-9), 36.2(C-10), 21.1(C-11), 39.7(C-12), 42.2(C-13), 56.8(C-14), 29.0(C-15), 25.5(C-16), 56.0(C-17), 21.2 (C-18), 19.4 (C-19), 40.6(C-20), 18.8(C-21), 129.3 (C-22), 138.4 (C-23), 45.9(C-24), 29.2(C-25), 12.0 (C-26), 21.2 (C-27), 24.4(C-28), 12.3 (C-29); ESI-MS *m/z* 413 $[M]^+$.

Results and Discussion

Methyl syringate (3.7 mg) was isolated as pale brown powder. The GC-MS spectrum showed the molecular ion peak at *m/z* 212.1, thus suggesting the molecular formula of $C_{10}H_{12}O_5$. The ^{1}H-NMR spectrum ($CDCl_3$, 500 MHz) of MNS-55-1 revealed the presence of three proton signals. The first signal was appeared as a singlet (2H) of aromatic protons which were assigned to H-2 and H-6 at δ 6.09. The second signal observed at δ 5.90 (1H) which was identified as the hydroxyl group (OH). Meanwhile, the third signal exhibited two strong singlets (9H) of aromatic methoxyl groups at δ 3.82 which belonged to 3-OCH_3 and 5-OCH_3 and an upfield methoxyl signal at δ 3.78 was probably attached to the side chain structure.

The ^{13}C-NMR spectrum ($CDCl_3$, 125 MHz) of MNS-55-1 exhibited the presence of ten carbon signals in the range of δ 52.2 to 168.2. Two methine carbon signals overlapping at δ 106.6 belonged to C-2 and C-6 and four quartenary carbons at δ 121.2, 139.2 and 146.8 were attributable to C-1, C-4 and C-3/C-5 respectively. The downfield carbon signal at δ 168.2 belonged to the carbonyl carbon atom. In addition, three methoxyl carbons corresponded to OCH_3-3, 5 and OCH_3–α side chain appeared at δ 56.5 and 52.2 respectively. The COSY spectrum indicated no correlations in the aromatic region, thus suggesting that there were no neighboring protons in this compound.

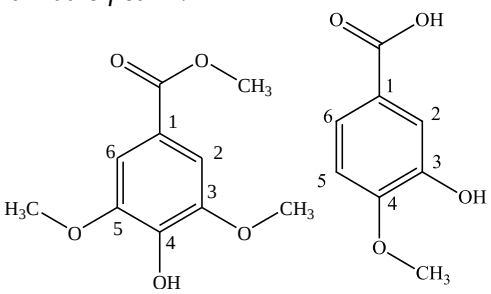
The HMQC spectrum showed very strong correlation between H-2 with C-2, H-6 with C-6, OCH_3 with C-3, OCH_3 with C-5 and OCH_3/C=O. The HMBC spectrum revealed the long range coupling between H-2 (δ 6.09) to C-2 (δ 106.6), C-4 (δ 139.2) and C=O (δ 168.2). Meanwhile, H-6 (6.09) showed J_2 coupling with C-2 (δ 106.6.) and C-5 (δ 146.8) and J_3 coupling with C=O (δ 168.2).

Based on the ^{1}H-NMR, complete assignment of the 2D NMR and by comparison with literature, the compound was identified as a known compound namely methyl 4-hydroxy-3,5-dimethoxybenzoate or methyl syringate which was isolated earlier from *Betula alba*[10] and *Buxus Sempervirens*[2].

Isovanilic acid (2.7 mg) was obtained as pale brown and showed a molecular ion peak at *m/z* 168.0 in the GC-MS and support the molecular formula of $C_8H_8O_4$. The ^{1}H-NMR spectrum ($CDCl_3$, 500 MHz) of MNS-55-1 showed an ABX-type aromatic ring at δ 7.72 (1H, *dd*, J=8.5, 2.0 Hz, H-6), 7.59 (1H, *d*, J=2.0 Hz, H-2) and 6.96 (1H, *d*, J=8.5 Hz, H-5). In addition, it revealed a singlet signal due to a methoxy proton (OCH_3-4) at δ 3.96. The ^{13}C-NMR spectrum indicated that compound MNS-58-18 contains eight carbon atoms. Signal at δ 172.5 attributed to a carbonyl group while signals at δ 112.3, 114.2, 125.2, were assigned to C-2, C-5 and C-6 respectively. Three quartenary carbon atoms located at δ 127.9, 146.2 and 150.7 were attributable to C-1, C-4 and C-3. On top of that, the presence of methoxy carbon was confirmed with the signal at δ 56.2.

Furthermore, the evidence from 2D NMR including COSY, HMQC and long range coupling HMBC supported the assignments of protons and carbons in the molecule. The COSY spectrum of MNS-58-18 showed that, there was a correlation between H-5 and H-6 meaning that these two protons are next to each other. The HMQC spectrum of MNS-58-18 showed the direct correlation between H-2 (δ 7.59) with C-2 (δ 112.3), H-5 (δ 6.96) with C-5 (δ 114.2), H-6 (δ 7.72) with C-6 (δ 125.2) and OCH_3-4 (δ 3.96) with 4-OCH_3- (δ 56.2). ^{1}H-^{13}C long range correlation signals in HMBC spectrum *of* MNS-58-18 displayed correlation between OCH_3-4 (δ 3.96) with C-4 (δ 146.2). At the same time, H-5 (δ 6.97) coupled with C-4 (δ 146.2).

In addition, the spectrum showed cross peak of H-2 with C-6 (δ 125.2), C-3 (δ 150.7) and C=O (δ 172.2). The positions of H-6 were further confirmed due to the cross peak with C-3 (δ 150.7), C-2 (δ 112.3) and C=O (δ 172.2). All the observations gathered suggested that compound is 3-hydroxy-4-methoxybenzoic acid or known as isovanilic acid and comparison with literature confirmed the hypothesis. This compound was previously isolated from the *Selaginella stautoniana*[26], *Diathus superbus*[28], the stems of *Annona cherimola*[7], the aril of *Cassia fistula* L[6] and the roots of *Synsepalum dulcificum*[5].



(1) (2)

Conclusion

Phytochemical study of *A. elliptica* revealed two compounds which are reported from *A. elliptica*: methyl syringate and isovanilic acid.

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