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The Biosynthetic Pathway of Stilbenoids from *Gnetum microcarpum* and SAR Study on Their PGE_2 Inhibitory Activities

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The biosynthesis of stilbenoid type of compounds is derived from the combination of shikimate and acetate malonate pathways. Specifically, resveratrol synthase (STS, EC 2.3.1.95) condenses three malonyl-CoA molecules and one coumaroyl-CoA molecule to form resveratrol. The oligostilbenoid compounds are mostly derived from the oligomerization of resveratrol *via* oxidative coupling reaction followed by several secondary reactions such as cyclization, enolization and oxidation. Stilbenoids isolated from the genus *Gnetum*, however, are rather small, and mainly consist of monomers and dimers. The diversity of the structure of monomers found in *Gnetum* other than resveratrol, also contributed to the oligomerization of oligostilbenoids isolated from the genus. This study focused on the isolation of stilbenoid compounds from *Gnetum* species using various chromatographic and spectroscopic techniques, together with their biosynthesis pathways, their Prostaglandin E_2 inhibitory activities *via* radioimmunoassay method and structure-activity relationship (SAR) study. The phytochemical study on the lianas of *Gnetum microcapum* has led to the isolation of 11 compounds. The proposed biosynthetic pathways of the stilbenoids obtained from the plant are discussed based on literature studies. Gnetol (**6**) (IC_{50} 1.84 µM) showed comparable PGE_2 inhibition to that of the standard indomethacin (IC_{50} 1.29 µM). This study demonstrated the importance of the number of hydroxyl groups and hydrophobicity in stilbenes and oligostilbenes structure for their PGE_2 inhibitory activity.

Keywords: *Gnetum microcarpum*; biosynthetic pathway; oligostilbenoids; SAR, prostaglandin E_2

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A biosynthetic pathway describes the series of chemical reactions that take place when a living organism converts a simpler, smaller molecule into a more complex one. The stilbenoid type of compounds are synthesized by combining shikimate and acetate malonate. The biosynthesis of stilbene phytoalexin starts with phenylalanine through the phenylpropanoid pathway [1]. The final step is carried out by the enzyme stilbene synthase (STS), specifically resveratrol synthase (STS, EC 2.3.1.95), which combines three molecules of malonyl-CoA and one molecule of coumaroyl-CoA to create resveratrol. Furthermore, all oligostilbenoid compounds are formed by the oligomerization of resveratrol through oxidative coupling reactions. The oligomerization of the resveratrol unit occurs through a series of secondary reactions, including cyclization, enolization, and oxidation. In addition, it has been suggested by Sotheeswaran and Pasupathy (1993) [2] that all oligostilbenoids containing a benzofuran ring are derived from dimer ε-viniferin, and those without a benzofuran ring are directly synthesized from resveratrol. However, Takaya et al. (2002) [3] proposed that the biosynthesis route for oligostilbenoid compounds without a benzofuran ring could also be derived from ε-viniferin *via* a rearrangement reaction or through other pathways.

Gnetum which is the sole genus in the family of Gnetaceae and order Gnetales, are distributed pantropically, with the majority of the species being canopy lianas of mostly rainforest or occasionally seasonal forest. Various *Gnetum* species were used for the folk treatment of rheumatism, arthritis, bronchitis, and asthma [4]. The genus *Gnetum* is well known for

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its abundant polyphenolic constituents. Stilbenes and oligostilbenes are the major chemical constituents isolated from the family of Gnetaceae in which their structural formations are unique. The oligostilbenes found in *Gnetum* are composed of heterogenic oligomerization of several stilbene monomers into dimers, trimers and tetramers. They also sometimes occur as glucosides. Stilbenoids isolated from the genus *Gnetum* however are rather small and mainly consist of monomers and dimers. The secondary reactions on resveratrol contribute to the various forms of other monomer structures. The diversity of the structure of monomers found in *Gnetum* other than resveratrol, also contributed to the oligomerization of oligostilbenoids isolated from the *Gnetum*. Oligostilbenes from *Gnetum* have been found to demonstrate a broad range of biological activities, as they are able to lower the level of sugar in the blood [5], induce apoptosis in colon cancer [6], show anti-inflammatory property [7], anti-oxidant [8] and antibacterial activities [9, 10].

EXPERIMENTAL

Isolation of Chemical Compounds

The lianas of *Gnetum microcarpum* Blume were used as plant materials in this study. The sample was collected from Tasik Bera, Pahang with the assistance of botanist Dr. Shamsul Bin Khamis, and the voucher specimen (SK2711/01 for *G. microcarpum*) was deposited at the Herbarium of the Laboratory of Natural Products, Institute of Bioscience, Universiti Putra Malaysia (UPM), Serdang, Selangor.

This study was conducted according to the standard protocols in natural product chemistry. The early step started with plant collection, extraction, fractionation as well as purification. The isolation and purification process were conducted by means of a combination of several chromatography techniques such as column chromatography, radial chromatography and preparative thin-layer chromatography. The analyses of pure compounds were measured by using several spectroscopy techniques such as Nuclear Magnetic Resonance (NMR), Ultraviolet-Visible (UV-Vis), Fourier Transform Infrared (FTIR) and Mass Spectro-meter (MS).

G. microcarpum lianas (2 kg) were air-dried, chopped and crushed before being extracted five times with acetone (10 L) over three days at room temperature. To reduce tannins, the dried crude extract was treated in methanol and fractionated with diethyl ether to create ether-soluble and insoluble layers. The ether-soluble material was then concentrated in vacuo at 40 °C. The tannin-less crude extract obtained (52.5 g) was separated using vacuum liquid chromatography (VLC) over silica gel, which was eluted with increasing polarity (from 7:3 to 0:10) mixes of Hex: EtOAc, followed by EtOAc: MeOH (9:1 and 8:2) to yield five main fractions (GM1-GM5). Fraction GM2 (4.68 g) was chosen and subsequently isolated and purified using VLC with Hex: EtOAc (from 9:1 to 0:10) and EtOAc: MeOH (9:1) solvent systems were used to produce six subfractions (GM21-GM26). Further purification of the GM23 (900 mg) subfraction utilizing a repeated radial chromatography approach with a Hex: Acetone (8:2) solvent solution gave compound **3** (2.4 mg) and **11** (2.5 mg). The GM24 (1.50 g) subfraction was also treated to the repeated radial chromatography technique, yielding 5 (6.5 mg) and 9 (3.2 mg). Fraction GM3 is subjected to VLC using Hex: EtOAc (from 7:3 to 2:8) and EtOAc: MeOH (9:1) as solvent systems, yielding 9 subfractions (GM31-GM39). GM31 was found to be compound **7** (12.2 mg). On GM35 (317.4 mg), repetitive radial chromatography ($CHCl_3$: Acetone (8:2)) yielded compound **6** (3.5 mg) and **8** (8 mg). Meanwhile, GM 36 (666.4 mg) was column chromatographed with Sephadex (100% MeOH as the solvent system) to produce six fractions (GM361-GM366). The fraction GM364 weighing 43.3 mg was further purified using radial chromatography with $CHCl_3$: Acetone: MeOH (8.5:1:.5) to obtain compound **4** (10 mg). GM364 (43.3 mg) was purified further using radial chromatography with $CHCl_3$: Acetone: MeOH (8.5:1:.5). Following VLC of fraction GM4 (9.29 g), four subfractions (GM41-GM44) were produced. Radial chromatography was used to purify subfraction GM44 (1.29 g) using $CHCl_3$: MeOH (8.5:1.5) as the solvent system, yielding compound **10** (4.8 mg), **2** (62.5 mg), and **1** (6.2 mg).

Analysis of PGE_2 Inhibitory Activity

The inhibition of PGE_2 production indicated by the concentration of PGE_2 in human whole blood was measured according to the validated radioimmunoassay (RIA) method [11]. The Ethics Committee of Universiti Kebangsaan Malaysia (UKM) permitted human blood application with approval number NF-016-2013.

Blood samples were collected from healthy volunteers who had not taken any medication or supplements for the past two weeks and had fasted for 8 hours. To prevent coagulation, 10% of 2% EDTA was added to the blood in a polypropylene tube. 1 mL aliquots of EDTA-human whole blood samples were transferred into test tubes and incubated with 10 µL of sample or indomethacin for 15 minutes at 37°C before lipopolysaccharide (LPS) addition. The effect of samples or indomethacin on PGE_2 production was studied by incubating each sample with whole blood-EDTA in the presence of LPS (10 µg/mL in 0.9% normal saline) for 24 hours. The concentration of samples was adjusted in five serial dilutions over a concentration range of 0.625 to 10 µg/mL for IC_{50}. After 24 hours of incubation, the plasma was separated by centrifugation at 2600 x g for 15 minutes at 4°C. Triplicates of 100 µL aliquots were transferred into test tubes and added with anti-PGE2 (100 µL; diluted with ratio of 1:50000) and [^{3}H]-PGE_2 (100 µg/mL;

5000 cpm) and incubated for 18-24 hours at 4°C. Dextran-charcoal solution (200 µL) was added to the mixture and incubated once again for 10 minutes at 0°C. The supernatant was then separated by centrifugation at 3000 x g for 15 minutes at 4°C. Triplicates of 300 µL aliquots of the supernatant were pipetted into liquid scintillation cocktail (3 mL). The radioactivity was measured using a liquid scintillation analyzer. The percentage inhibition was calculated by the following formula:

% inhibition = (1 − [PGE_2 in samples or indomethacin] / [PGE_2 in negative control]) × 100

RESULTS AND DISCUSSION

Oligostilbenoids from *G. microcarpum*

The acetone extract of the lianas of *G. microcarpum*, when subjected to various conventional chromatographic techniques, has yielded 11 oligostilbenoid compounds, namely, malaysianol F (**1**)[12], malaysianol D (**2**) [12], malaysianol E (**3**) [13], ε-viniferin (**4**) [13], resveratrol (**5**) [14], gnetol (**6**) [13], gnetucleistol C (**7**) [14], isorhapontigenin [15] (**8**), cuspidan B (**9**) [16], parvifolol D (**10**) [17], and gnetifolin M (**11**) [18]. The structures of these compounds (Figure 1) were elucidated by various spectroscopic analyses as well as comparison of physical and spectroscopic data with previous literature values.

Malaysianol F (**1**). Brown amorphous powder (6.2 mg), HRESITOFMS (positive mode) $[M+H]^+$: m/z 697.2093 (Calc. for $C_{42}H_{32}O_{10}$). ^{1}H NMR (300 MHz, acetone-d6): δ_H7.24 (2H, d, *J*=8.7 Hz, H-2a/6a), 7.08 (1H, d, *J*=8.4 Hz, H-6c), 6.86 (2H, d, *J*=8.7 Hz, H-2b/6b) 6.83 (2H, d, *J*=8.7 Hz, H-3a/5a), 6.60 (2H, d, *J*=8.7 Hz, H-3b/5b), 6.57 (2H, s, H-7b, 8b), 6.48 (1H, s, H-12b), 6.45 (1H, d, J=2.4 Hz, H-3c), 6.33 (1H, dd, J=2.4,7.5 Hz, H-5c), 6.30 (2H, d, *J*=2.4 Hz, H-10c/14c), 6.23 (2H, d, *J*=1.8 Hz, H-10a/14a), 6.22 (1H, t, *J*=1.8 Hz, H-12a) 6.19 (1H, t, *J*=2.4 Hz, H-12c), 4.53/5.77 (1H each, d, *J*=4.2,3.9 Hz, H-7c/8c), 4.51/5.44 (1H each, d, *J*=5.4,5.7 Hz, H-7a/8a). ^{13}C NMR (75 MHz, acetone-d6): 163.8 (C-13b), 163.3 (C-11b), 161.0 (C-11a/13a), 160.6 (C-11c/13c), 160.0 (C-4c), 159.3/159.2 (C4b/4a), 157.3 (C-2c), 148.9 (C-9c), 148.2 (C-9a), 134.8 (C-7b), 134.1 (C-9b), 134.1 (C-1a), 129.5 (C-1b), 129.5 (C-2b/6b), 129.0 (C-2a/6a), 128.9 (C-6c), 123.4 (C-8b), 121.6 (C-1c), 121.5 (C-14b), 120.9 (C-10b), 117.2 (C-3a/5a), 117.1 (C-3b/5b), 108.3 (C-5c), 108.2 (C-10c/14c), 108.0 (C-10a/14a), 104.6 (C-3c), 103.1 (C-12c), 102.9 (C12a), 95.1 (C-7a), 92.3 (C-12b), 90.5 (C-7c), 59.0 (C-8a), 57.5 (C-8c).

Malaysianol D (**2**). Dark brown amorphous powder (62.5 mg), HRESITOFMS (positive mode) $[M+H]^+$: m/z 681.2173 (Calc. for $C_{42}H_{32}O_9$). ^{1}H NMR (300 MHz, acetone-d6): δH7.23 (4H, d, *J*=8.7 Hz, H-2a/6a, 2c/6c), 6.85 (2H, d, *J*=8.4 Hz, H-2b/6b), 6.83 (4H, d, *J*=8.7 Hz, H-3a/5a, 3c/5c), 6.59 (2H, d, *J*=8.7 Hz, H-3b/5b), 6.56 (1H, d, *J*=16.5 Hz, H-7b), 6.50 (1H, d, *J*=16.8 Hz, H8b), 6.45 (1H, s, H-12b), 6.23 (4H, d, *J*=2.1 Hz, H-10a/14a, 10c,14c), 6.21 (2H, t, *J*=2.1 Hz, H-12a/12c), 5.45 (2H, d, *J*=4.8 Hz, H-7a/7c), 4.53 (2H, d, *J*=5.1 Hz, H8a/8c). ^{13}C NMR (75 MHz, acetone-d_6): 163.5 (C-11b/13b), 161.0 (C-11a/13a, 11c/13c)), 159.3 (C-4a/4c), 159.2 (C-4b), 148.3 (C-9a/9c), 135.0 (C-7b), 134.7 (C1a/1c), 134.1 (C-9b), 131.0 (C-1b), 129.6 (C-2b/6b), 128.9 (C-2a/6a, 3c/6c), 123.2 (C-8b), 121.0 (C-10b/14b), 117.2 (C-3a/5a, 3c/5c), 117.1 (C-3b/5b), 108.0 (C10a/14a, 10c/14c), 103.1 (C-10b/14b), 95.1 (C-7a, 7c), 92.3 (C-12b), 59.0 (C-8a, 8c).

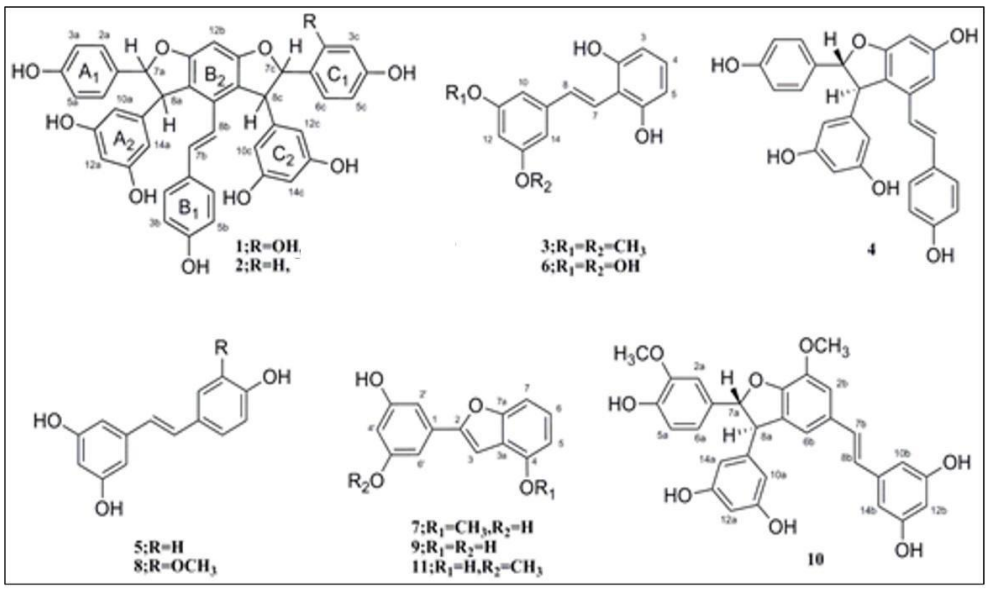

Figure 1: Chemical structures of compounds isolated from *G. microcarpum*.

Malaysianol E (**3**). Brown amorphous powder (2.4 mg), HRESITOFMS (positive mode) m/z: $[M+H]^+$ 273.1119 (Calc. for $C_{16}H_{17}O_4$). ^{1}H NMR (300 MHz, acetone-d_6): δ_H8.74 (2H, s, OH), 7.61 (1H, d, *J*=16.7 Hz, H-7), 7.49 (1H, d, *J*=16.7 Hz, H-8), 6.88 (1H, t, *J*=8.1 Hz, H-12), 6.67 (2H, d, *J*=2.1 Hz, H-2, 6), 6.44 (2H, d, *J*=8.1 Hz, H-11, 13), 6.36 (1H, t, *J*=2.1 Hz, H4) and 3.81 (6H, s, OCH_3) . ^{13}C NMR (75 MHz, acetone-d_6): 161.2 (C-3, 5), 156.9 (C10, 14), 141.7 (C-1), 130.9 (C-7), 128.0 (C-12), 121.2 (C-8), 111.9 (C-9), 107.1 (C11, 13), 103.9 (C-2, 6), 99.0 (C-4), 54.7 (OCH_3).

ε-viniferin (**4**). Dark brown amorphous powder (10 mg), m.p 191°C, HRESITOFMS (positive mode) m/z: $[M+H]^+$ 453.1341 (Calc. for $C_{28}H_{22}O_6$). ^{1}H NMR (300 MHz, acetone-d_6): δ_H7.21 (2H, d, *J*=8.4 Hz, H-2a/6a), 7.18 (2H, d, *J*=9 Hz, H-2b/6b), 6.93 (1H, d, *J*=16.2 Hz, H-7b), 6.84 (2H, d, *J*=8.7 Hz, H-3a/5a), δH6.74 (2H, d, *J*=8.7 Hz, H-3b/5b), 6.73 (1H, d, *J*=2.7 Hz, H-14b), 6.67 (1H, d, *J*=16.2 Hz, H-8b), 6.32 (1H, d, *J*=2.1 Hz, H-12b), 6.24 (3H, brs, H-10a/12a/14a), 5.42 (1H, d, *J*=5.4 Hz, H-7a and 4.47 (1H, d, *J*=5.4 Hz, H-8a) . ^{13}C NMR (75 MHz, acetone-d6): 163.4 (C-11b), 160.9 (C-11a/13a), 160.6 (C-13b), 159.3 (C-4a/4b), 146.4 (C-9a), 137.4 (C-9b), 134.8 (C-1a), 131.0 (C-7b), 130.8 (C-1b), 129.7 (C-2b/6b), 128.9 (C-2a/6a), 124.4 (C-8b), 120.8 (C-10b), 117.3 (C-3b/5b), 117.1 (C-3a/5a), 111.9 (C-9), 107.9 (C-10a/14a), 105.2 (C-14b), 103.1 (C-12a), 97.8 (C-12b), 94.9 (C-7a), 58.1 (C-8a).

Resveratrol (**5**). Off-white amorphous powder (6.5 mg), HRESITOFMS (positive mode) m/z: $[M+H]^+$ 229.0880 (Calc. for $C_{14}H_{12}O_3$). ^{1}H NMR (300 MHz, acetone-d_6): δ_H8.71 (1H, s, OH), 8.40 (2H, s, OH), 7.39 (1H, d, *J*=8.6 Hz, H-2, 6), 6.98 (1H, d, *J*=16.5 Hz, H-7), 6.83 (1H, d, *J*=16.5 Hz, H-8), 6.81 (2H, d, *J*=8.6 Hz, H-3, 5), 6.52 (2H, d, *J*=2.4 Hz, H-10, 14), and 6.25 (1H, t, *J*=2.4 Hz, H-12). ^{13}C NMR (75 MHz, acetone-d_6): 158.8 (C-11, 13), 157.4 (C-4), 139.9 (C-1), 129.0 (C-9), 128.2 (C-7), 127.8 (C-2, 6), 125.9 (C-8), 115.5 (C-3, 5), 104.7 (C-10, 14), 101.8 (C-12).

Gnetol (**6**). Brown amorphous powder (10.5 mg), HRESITOFMS (positive mode) m/z: $[M+H]^+$ 245.0817 (Calc. for $C_{14}H_{12}O_4$). ^{1}H NMR (300 MHz, acetone-d_6): δH8.92 (2H, s, OH), 8.44 (2H, s, OH), 7.51 (1H, d, *J*=16.7 Hz, H-7), 7.41 (1H, d, *J*=16.7 Hz, H-8), 6.83 (1H, t, *J*=8.1 Hz, H-4), 6.51 (2H, d, *J*=2.1 Hz, H-10, 14), 6.41 (2H, d, *J*=8.1 Hz, H-3, 5) and 6.22 (1H, t, *J*=2.1, Hz, H-12). ^{13}C NMR (75 MHz, acetone-d_6): 160.5 (C-3, 5), 158.7 (C-10, 14), 143.5 (C-1), 132.9 (C-7), 129.5 (C-4), 122.4 (C-8), 113.8 (C-9), 108.9 (C-3, 5), 106.4 (C-10, 14), 103.3 (C-12).

Gnetucleistol C (**7**). Brown amorphous powder (12.2 mg), HRESITOFMS (positive mode) m/z: $[M+H]^+$ 257.0825 (Calc. for $C_{15}H_{12}O_4$). ^{1}H NMR (300 MHz, acetone-d_6): δ_H7.23 (1H, t, *J*=8.4, 8.1 Hz, H-6), 7.14 (1H, d, *J*=0.9 Hz, H-3), 7.12 (1H, dd, *J*=8.4, 0.9 Hz, H-7), 6.91 (2H, t, *J*=2.1 Hz, H-2', 6'), 6.76 (1H, d,*J*=8.1 Hz, H-5), 6.39 (1H, t, *J*=2.1 Hz, H-4') and 3.95 (3H, s, OCH3) . ^{13}C NMR (75 MHz, acetone-d_6): 161.0 (C-3,5), 157.6 (C-7a), 156.6 (C-2), 155.4 (C4), 133.9 (C-1), 127.1 (C-6), 121.1 (C-3a), 105.9 (C-7), 105.5 (C-5), 105.0 (C2',4',6'), 100.5 (C-3), 57.0 (OCH3).

Isorhapontigenin (**8**). White amorphous powder (8 mg), HRESITOFMS (positive mode) m/z: $[M+H]^+$ 259.0980 (Calc. for $C_{15}H_{14}O_4$). ^{1}H NMR (300 MHz, acetone-d_6): δ_H8.43 (2H, s,OH), 7.90 (1H, s, OH), 7.21 (1H, d, *J*=2.1 Hz, H-3), 7.05 (1H, d, *J*=16.4 Hz, H-7), 6.99 (1H, dd, *J*=2.1, 8.4 Hz, H-5), 6.89 (1H, d, *J*=16.4 Hz, H-8), 6.79 (1H, d, *J*=8.1 Hz, H-6), 6.53 (2H, d, *J*=2.3 Hz, H-10, 14), 6.27 (1H, d, *J*=2.3 Hz, H-12), and 3.89 (3H, s, OCH3). ^{13}C NMR (75 MHz, acetone-d_6): 158.8 (C-11,13), 147.8 (C-2), 146.7 (C-4), 139.9 (C1), 129.5 (C-9), 128.5 (C-7), 126.2 (C-8), 120.3 (C-5), 115.1 (C-6), 109.3 (C-3), 104.7 (C-10,14), 101.9 (C-12), 55.4 (OCH3).

Cuspidan B (**9**). Brown amorphous powder (3.2 mg), HRESITOFMS (positive mode) m/z: $[M+H]^+$ 243.0656 (Calc. for $C_{14}H_{10}O_4$). ^{1}H NMR (300 MHz, acetone-d_6): δ_H8.84 (1H, s, OH), 8.45 (2H, s, OH), 7.19 (1H,s, H-3), 7.12 (1H, t, *J*=7.8, 8.1 Hz, H6), 7.02 (1H, d, *J*=8.4 Hz, H-7), 6.90 (1H, d, *J*=2.1 Hz, H-2',6'), 6.66 (1H, d, *J*=7.8 Hz, H-5), 6.39 (1H, t, *J*=2.1 Hz, H-4). ^{13}C NMR (75 MHz, acetone-d_6): 160.9 (C-105 3',5'), 156.2 (C-7a), 154.8 (C-2), 153.0 (C-4), 134.2 (C-1'), 127.1 (C-6), 120.8 (C-3a), 109.8 (C-5), 105.1 (C-2',6'), 104.9 (C-4'), 104.5 (C-7), 100.6 (C-3).

Parvifolol D (**10**). Brown amorphous powder (4.8 mg), $C_{30}H_{26}O_8$. 1H NMR (300 MHz, acetone-d_6): δ_H8.32 (2H, s, OH), 8.30 (2H, s, OH), 7.77 (1H, s, OH), 7.17 (1H, d, *J*=1.2 Hz, H-2b), 7.04 (1H, d, *J*=1.5 Hz, H-2a), 7.01 (1H, d, *J*=16.2 Hz, H-7b), 6.89 (1H, d, *J*=16.5 Hz, H-8b), 6.83 (1H, d, *J*=1.8 Hz, H-6b), 6.82 (2H, m, H-5a, 6a), 6.52 (2H, d, *J*=2.1 Hz, H-10b, 14b), 6.27 (1H, t, *J*=2.1 Hz, H-12a), 6.25 (1H, t, *J*=2.1 Hz, H-12b), 6.20 (2H, d, *J*=2.1 Hz, H-10a, 14a), 5.44 (1H, d, *J*=8.7 Hz, H-7a), 4.50 (1H, d, *J*=8.4 Hz, H-8a), 3.93 (3H, s, OCH_3) and 3.81 (6H, s, OCH_3) . ^{13}C NMR (75 MHz, acetone-d_6): 160.8 (C-11a/13a), 160.6 (C-11b/13b), 150.1 (C-9a), 149.5 (C-3a), 148.7 (C-4a), 146.4 (C-3b), 145.9 (C-4b), 141.6 (C-9b), 134.0 (C-1b), 133.7 (C-5b), 133.6 (C-1a), 130.3 (C-7b), 126.5 (C-8b), 121.2 (C-6a), 117.7 (C-6b), 116.7 (C-5a), 112.6 (C-2b), 111.8 (C-2a), 108.5 (C-10a/14a), 106.7 (C-10b/14b), 103.8 (C-12b), 103.4 (C12a), 95.5 (C-7a), 59.1 (C-8a), 57.4 (OCH_3-3a), 57.3 (OCH_3-3b).

Gnetifolin M (**11**). Brown amorphous powder (2.5 mg), HRESITOFMS (positive mode) m/z: $[M+H]^+$ 273.1119 (Calc. for $C_{15}H_{12}O_4$). ^{1}H NMR (300 MHz, acetone-d_6): δ_H9.01 (1H, s, OH), 8.74 (1H, s, OH), 7.15 (1H, d, *J*=0.6 Hz, H-3), 7.01 (1H, t, *J*=7.8, 8.1 Hz, H-6), 6.91 (1H, d, *J*=8.1 Hz, H-7), 6.88 (1H, t, *J*=2.1 Hz, H-2'), 6.85 (1H, t, *J*=2.1 Hz, H-6'), 6.56

(1H, d, *J*=8.4 Hz, H-5), 6.32 (1H, t, *J*=2.1 Hz, H-4') and 3.73 (3H, s, OCH_3).

Biosynthesis Pathway

As previously mentioned in introduction section, resveratrol (**5**) was synthesized from the phenylalanine pathway. The formation of isohapontigenin (**8**) can be suggested from a series of hydroxylation, followed by O-methylation on ring A of **5** for the addition of a methoxy group. The hydroxylation of natural compounds is commonly performed by O_2 in the presence of NADPH as sources of hydroxyl group *via* epoxidation reaction [19]. The O-methylation of various secondary metabolites is mainly catalyzed by S-adenosyl-l-methionine (SAM)-dependent O-methyl- transferase (OMT) proteins that are encoded by the O-methyltransferase gene family. In this route, once **5** has been hydroxylated at C-3, O-methylation took place *via* nucleophilic substitution SN_2 reaction of an electrophilic methyl group under basic condition. On the other hand, a dehydroxylation on C-4 of resveratrol (**5**), followed by hydroxylation on C-2 and C-6, forming gnetol (**6**) structure. So far, 4-hydroxybenzoyl-CoA reductase (dehydroxylating, 4-HBCR) from the denitrifying bacterium *Thauera aromatica* represents the best characterised enzyme catalysing the reductive removal of a functionality from the benzene ring [20]. Then, malaysianol E (**3**) was produced by O-methylation process at C-11 and C-13 of compound **6**.

In the biosynthetic route for the formation of arylbenzofuran type of monomer, compound **6** seems to be the key molecule. Initially, **6** undergone epoxidations forming the *trans*-stilbene oxide which then in an acid-base catalysis condition, a nucleophilic attack on the epoxide carbon activated by base occurred, followed by stabilization of the negative charge on the epoxide oxygen catalyzed by acid, causing an epoxide opening cyclization to form a 5-membered ring cyclic ether. As dehydration reaction took place to remove a molecule of water, the structure of cuspidan B (**9**) was formed. Hydroxylation followed by O-methylation of **9** produced gnetucleistol C (**7**) structure.

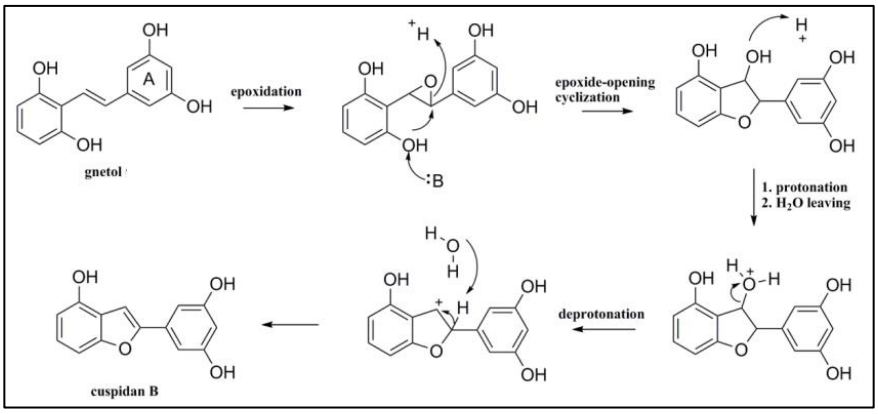

Figure 2: Proposed mechanism on formation of arylbenzofuran monomer.

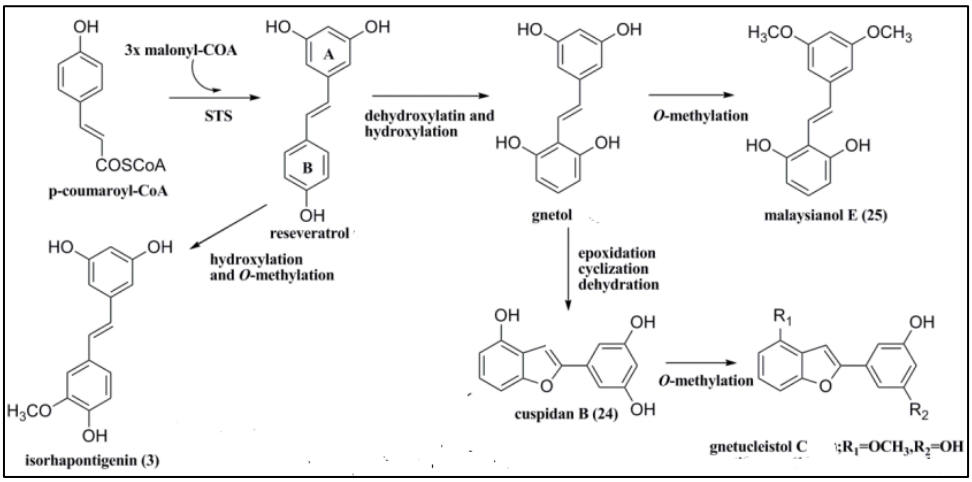

Figure 3: Plausible biosynthesis of stilbene monomer of *G. microcapum*.

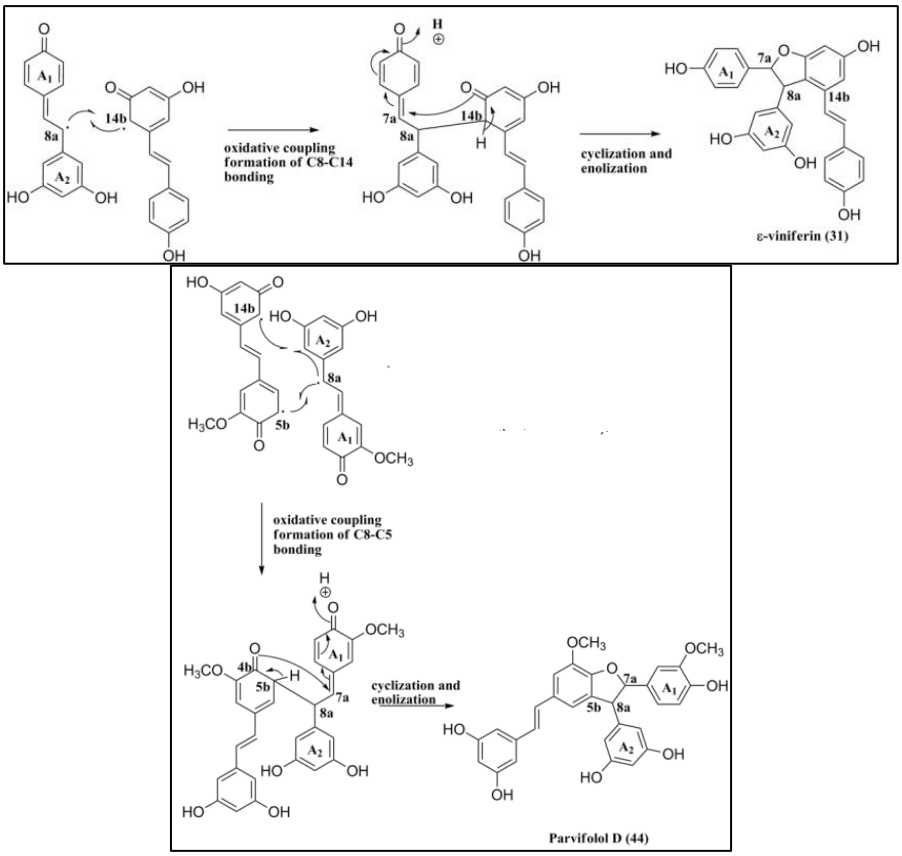

Figure 4: Plausible biosynthesis of stilbene dimer of *G. microcarpum*.

The formation of dimer ε-viniferin (**4**) was discussed by Wibowo (2014) [21], where two radical molecules of resveratrol (**5**) with active site at C-8a and C-14a undergone an oxidative coupling reaction to form C-8-C-14 bonding which then continued with intramolecular cyclization reaction of nucleophilic oxygen at aromatic carbon C-13b and olefinic carbon C-7a to form benzofuran ring followed by enolization of p-hydroxybenzene ring which finally gave out the structure of **4.** The formation of dimer parvifolol D (**10**) formed from the oxidative coupling reaction of two molecules of isorhapontigenin (**8**) radicals. In the formation of **10**, two radicals of **8** containing active site at C-8a and C-5b oxidized into C-8-C-5 bond and formed benzofuran ring *via* intramolecular cyclization of nucleophilic oxygen at C-4b and olefinic carbon C-7a and enolization which completed the structure.

The biosynthesis of trimer malaysianol D (**2**) can be suggested from the oxidative coupling reaction of resveratrol (**5**) radical with active site at C-8c and ε-viniferin (**4**) radical with active site at C-14b [21]. As the bond formed, the formation of benzofuran ring takes place via intramolecular cyclization reaction in acidic condition which involved the nucleophilic oxygen of O-13b and olefinic carbon C-7c, to give the final structure of **2**. However, the difference between stereochemistry at C-7a/C-8a of 4 and 2 resulted in another pathway which is more reliable. Compound **2** can also be formed from the three steps oxidative coupling reaction of three **5** radicals, one of which with active site at C-14a and two with active site at C-8b including the formation of two hypothetical intermediates. This pathway will yield a more varied stereochemistry of the benzofuran ring formed. The same process suggested for the formation of malaysianol F (**1**) except that, the radical resveratrol (**5**) with active site C-8 undergone hydroxylation process which added one hydroxyl group at C-2 of ring A to form oxyresveratrol. Then oxidative coupling reaction occurred between ε-viniferin (**4**) radical with active site at C-14b and oxyresveratrol radical with active site at C-8c. Malaysianol F (**1**) can also be formed from direct hydroxylation of 9 at C-2c. Since the stereochemistry of benzofuran ring A3 and C3 of **1** is the same as **2**, direct hydroxylation on **2** is preferred.

PGE_2 Inhibitory Activity and SAR Study

Five compounds were found to inhibit the production of PGE_2 induced by LPS as shown in **Figure 6**. Isorhapontigenin (**8**) showed the highest percentage of PGE_2 inhibition (74.86%), while malaysianol D (**2**) displayed the lowest (43.05%). All the monomeric stilbenoid compounds that were subjected to the PGE_2 inhibitory test in this study displayed anti-inflammatory properties. Resveratrol (**5**) is known for its anti-inflammatory property and has been shown to be a non-selective inhibitor of COX-1 and COX-2 [22].

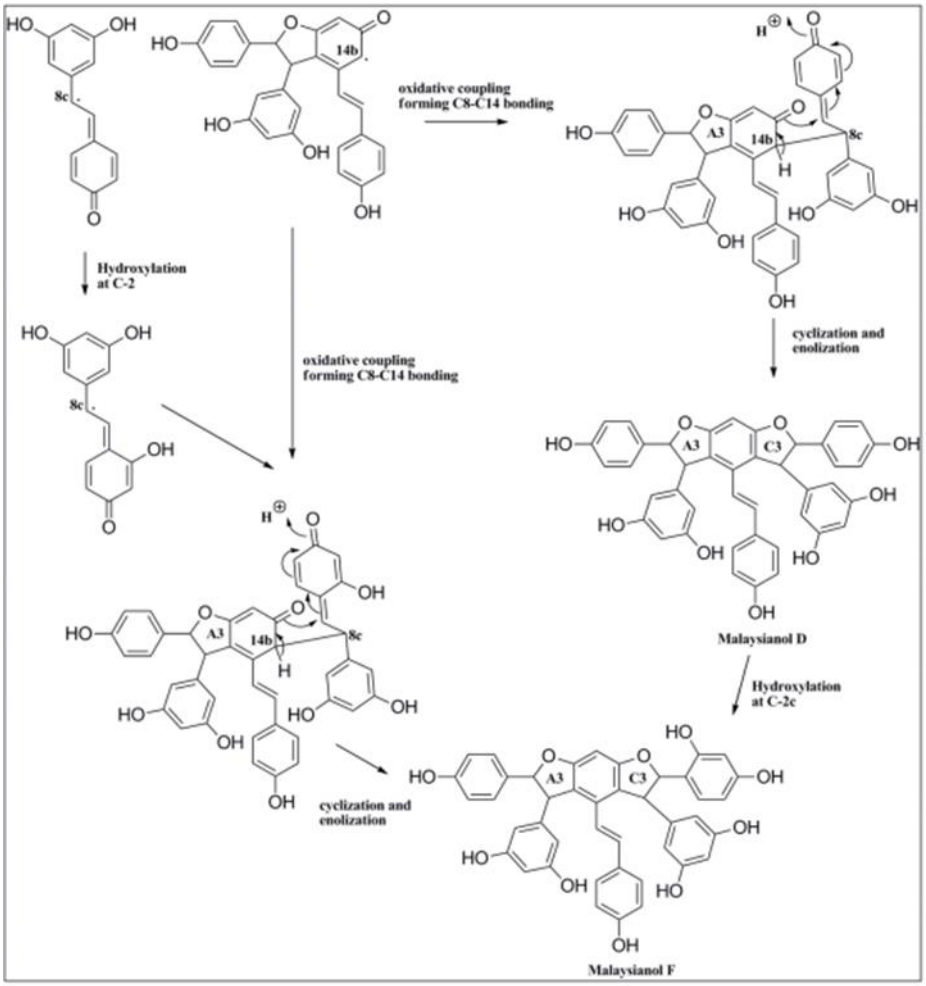

Figure 5: Plausible biosynthesis of stilbene trimer of *G. microcarpum*

Similarly, in this study, resveratrol (**5**) demonstrated good PGE_2 inhibitory activity with an IC_{50} of 6.57 µM. Gnetol (**6**) exhibited higher activity compared to resveratrol (IC_{50} 1.84 µM), followed by isorhapontigenin (**8**) (IC_{50} 3.68 µM). All these three stilbenoids have the structure of the basic stilbene skeleton. The results indicate that an increase in the number of hydroxyl groups in the compound structure results in better activity, a finding that has been proven previously [23]. Additionally, the presence of a methoxy group at the ortho-position in the structure of isorhapontigenin (**8**) also appears to give a positive effect on the reactivity. Similarly, a study has shown that a dimethoxystilbene compound is a potent PGE_2 inhibitor [24]. The methoxy group confers higher lipophilicity to the compounds, which may favor their entry into cells and confer more resistance to degradation, thus improving pharmacokinetics [25]. The hydroxyl group confers more solubility, which allows better interaction with proteins [26], whereas methoxylated groups confer resistance to degradation. However, the number of methoxy and hydroxyl groups must be in equilibrium, as an excessive number of methoxylated groups may impair the interaction with the target protein [27].

The compound ε-viniferin (**4**) demonstrated only moderate activity with a 52.70% inhibition of PGE_2 at a concentration of 10 µg/mL. Similarly, malaysianol D (**2**), a stilbene trimer, also showed moderate PGE_2 inhibitory activity (43.05%) at the same concentration. The results suggest that the presence of a benzofuran ring does not significantly affect the activity of the compounds, as both compound **4** and **2** contain this ring in their structure. On the other hand, the number and position of hydroxyl groups in stilbenes and oligostilbenes had a significant impact on their PGE_2 inhibitory activity. For instance, compound **6**, which has an additional OH in its structure, exhibited higher activity compared to compound **5**. Therefore, the methoxy group in the ortho-position and the number of hydroxyl groups in stilbenes and oligostilbenes are crucial for their PGE_2 inhibitory activity. Furthermore, the size of the stilbenoid compounds was found to have an effect on their activity, with larger molecules exhibiting lower activity.

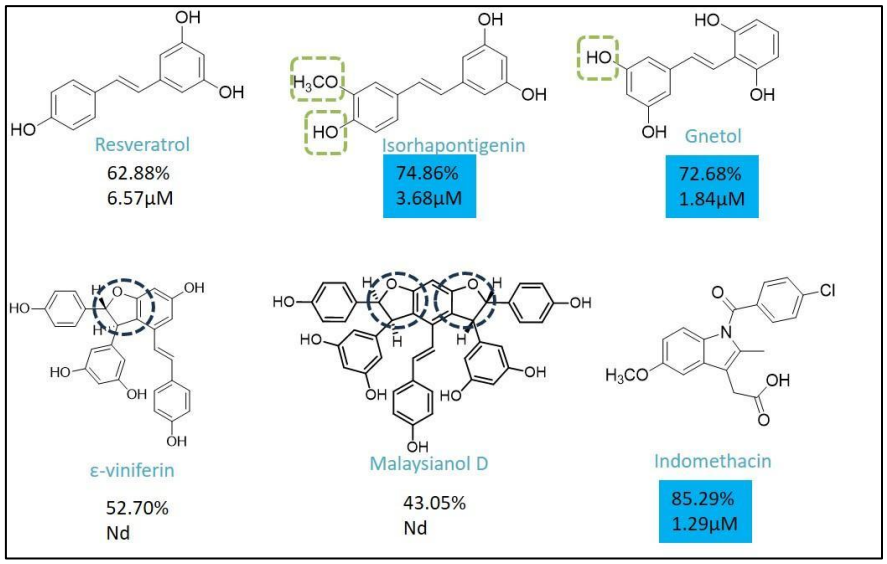

Figure 6: PGE_2 Inhibitory Activity of Tested Stilbenoids

CONCLUSION

During the investigation on the lianas extract of *G. microcarpum*, 11 stilbenoid compounds were isolated, including malaysianol F (**1**), malaysianol D (**2**), malaysianol E (**3**), ε-viniferin (**4**), resveratrol (**5**), gnetol (**6**), gnetucleistol C (**7**), isorhapontigenin (**8**), cuspidan B (**9**), parvifolol D (**10**), and gnetifolin M (**11**). These compounds are produced from a cinnamoyl- CoA starter unit with chain extension from three molecules of malonyl-CoA that gives a polyketide. This polyketide, with the presence of different enzymes, can be folded in two different ways. With stilbene synthase, an aldol reaction takes place, coupling the cinnamoyl-CoA with three malonyl-CoA units, forming the stilbene monomer structure. The oligomerization of this monomer unit *via* oxidative coupling, followed by a series of secondary reactions gives rise to the complex structure of oligostilbenes. Based on their PGE_2 inhibitory activity, gnetol (**6**) (IC_{50} 1.84 µM) showed comparable activity to that of the standard indomethacin (IC_{50} 1.29 µM). It is worth noting that the activity on PGE_2 inhibition depends on the number of hydroxyl groups present in stilbenes. Furthermore, the presence of a fair number of hydroxyl and substituents, which give hydrophobic properties to their structure, are required in order to exert better activity.

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