Acylphenols and dimeric acylphenols from *Myristica maxima* Warb

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**Abstract**

Giganteone E (1), a new dimeric acylphenol was isolated as a minor constituent from the bark of *Myristica maxima* Warb. The structure of 1 was established on the basis of 1D and 2D NMR techniques and LCMS-IT-TOF analysis. Malabaricones A–C (2–4), giganteones A and C (5 and 6), maingayones A and B (7 and 8), maingayic acid B (9) and β-sitosteryl oleate (10) were also characterized in this plant for the first time. Compound 10 was identified for the first time in the Myristicaceae. Compounds 2 and 5 were active against human prostate cancer cell lines, thus making this the first report on the prostate cancer inhibiting potential of acylphenols and dimeric acylphenols. Compounds 1, 4, 5, 7 and 8 exhibited potent DPPH free radical scavenging activity. This is the first report on their free radical scavenging capacity.

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1. Introduction

Seven dimeric acylphenols have been reported to date in the genus *Myristica*: giganteones A–D and maingayones A–C, all of which were non-symmetrical in nature and derived from either one or two of the following known acylphenols: malabaricones A, B or C which are ubiquitous in this genus [1–4]. The subject of the current paper however is on the isolation and spectroscopic characterization of a new dimeric acylphenol (1) from *Myristica maxima* Warb. with one of its monomers being yet to be isolated from the plant kingdom. *M. maxima* also yielded an additional nine known compounds (2–10) (Fig. 1). Selected compounds were evaluated for their prostate cancer inhibiting and antioxidant potentials. SAR studies were conducted to better understand how the chemical groups in these molecules influenced their activities.

2. Experimental

2.1. General experimental procedures

Analytical and preparative TLC was carried out on Merck 60 F 254 silica gel plates (absorbent thickness: 0.25 and 0.50 mm, respectively). Column chromatography was performed using silica gel (Merck 230–400 mesh, ASTM). Preparative HPLC was carried out using a Gilson HPLC system (GX–271 Preparative HPLC System) equipped with a Binary Gradient Module, a GX Prep Solvent System and an UV detector (scan wavelength range, 200–600 nm) along with a ZORBAX HPLC Eclipse Plus C18 column with the following dimensions; internal diameter 9.6 mm, length 250 mm and particle size 3.5 µm. Recycling HPLC was performed using a Japan Analytical Industry recycling preparative LC System (LC–9130 G NEXT) equipped with a column (JAI–ODS–AP, SP120–15) having the following dimensions; internal diameter 30 mm, length 250 mm and particle size 10.0 µm. IR spectra were recorded using a Perkin-Elmer Spectrum 400 FT–IR Spectrometer. NMR spectra were acquired in CD3OD (Merck) using a JEOL ECA 400 MHz NMR spectrometer. The LCMS–IT–TOF spectra were obtained on a UFLC Shimadzu Liquid Chromatograph with a SPD–M20A diode array detector coupled to an IT–TOF mass spectrometer. UV spectra were recorded using a Shimadzu 1650 PC UV–Vis Spectrophotometer. A Jasco P–1020 polarimeter was used to measure optical rotation. All solvents were of analytical grade and were distilled prior to use.

2.2. Plant material

*M. maxima* was collected from Hutan Simpan Bukit Enggang, Kedah in 2000. The plant was identified by Mr. Teo Leong Eng and its voucher specimen (KL 4974) has been deposited with the University of Malaya herbarium.
2.3. Extraction and isolation

Dried powdered bark (1.5 kg) of *M. maxima* was extracted with dichloromethane (15.0 L, 2×) followed by ethyl acetate (15.0 L, 2×) at room temperature, affording 22.15 g and 25.08 g of extracts, respectively. 20 g of the dichloromethane extract was chromatographed on a silica gel column (600 g, 5 × 60 cm). Elution was carried out using mixtures of dichloromethane: acetone in proportions of 95:5 (v/v, 2800 mL), 90:10 (v/v, 5000 mL), 85:15 (v/v, 2000 mL), 80:20 (v/v, 2300 mL), 70:30 (v/v, 4000 mL), 50:50 (v/v, 4500 mL) and 0:100 (v/v, 2000 mL) sequentially to afford fractions FA (0.47 g), FB (1.88 g), FC (2.71 g), FD (4.14 g), FE (1.42 g), FF (1.20 g), FG (2.44 g), FH (1.69 g) and FI (1.91 g), respectively. FA was further chromatographed on a silica gel column (25.0 g, 5 × 60 cm) with successive elutions using mixtures of hexane: ethyl acetate in ratios of 100:0 (v/v, 250 mL) and 97:3 (v/v, 250 mL), to afford sub-fractions FA-1 (0.15 g), FA-2 (0.08 g) and FA-3 (0.17 g). Prep-TLC of FA-1 using hexane led to the isolation of 10 (10.0 mg). FB was subjected to column chromatography over a 60 g silica gel column (3.5 × 51 cm) eluting successively with mixtures of dichloromethane: acetone in ratios of 85:15 (v/v, 400 mL), 80:20 (v/v, 300 mL) and 70:30 (v/v, 300 mL) to afford sub-fractions FE-1 (0.10 g), FE-2 (0.15 g) and FE-3 (1.13 g). FE-3 was repetitively separated over a 30 g silica gel column (3.5 × 51 cm) with dichloromethane: acetone (85:15) (v/v, 400 mL) to yield 4 (863.9 mg). FG was separated over a 70 g silica gel column (3.5 × 51 cm) eluting successively with mixtures of dichloromethane: acetone in ratios of 80:20 (v/v, 800 mL) and 75:25 (v/v, 300 mL), providing sub-fractions FG-1 (0.30 g), FG-2 (0.50 g), FG-3 (0.40 g) and FG-4 (0.30 g). FG-1 (0.30 g) was passed through a Sephadex LH-20 column (20 g) using 120 mL of methanol to give FG-1.1 (0.20 g) which was further subjected to repetitive prep-TLC using dichloromethane: acetone (80:20 v/v) to furnish FG-1.2 (6.0 mg). Column chromatography of FG-2 to give FG-2.1 (0.07 g) and FG-2.2 (0.10 g) was achieved through Sephadex LH-20 (10 g) using 150 mL of methanol. Sub-fraction FG-2.1 was later purified via prep-HPLC (8 injections of 70 mg in 7 mL) on a semi preparative C-18 column using mobile phases A: water + 0.2% formic acid and B: acetonitrile. An isocratic system of 30% A and 70% B for 21 mins with a flow rate of 3 mL/min was employed to yield 5 (9.0 mg) (tR 14.3 min) and 7 (8.0 mg) (tR 16.3 min). FG-2.2 was passed through a Sephadex LH-20 column (20 g) using 120 mL of methanol to give FG-2.2.1 (0.04 g) which was further subjected to repetitive prep-TLC using dichloromethane: acetone (70:30 v/v) to furnish 6 (6.0 mg). G-4 was fractionated over a Sephadex LH-20 column (20 g) with 120 mL of methanol to yield G-4.1 (0.08 g), G-4.2 (0.07 g) and G-4.3 (0.05 g). G-4.3 was purified via recycling preparative LC (1 injection of
49.3 mg in 4 mL) using an isocratic mobile phase of 100% methanol for 200 mins with a flow rate of 6 mL/min to obtain 1 (4.9 mg) (tR 185 mins).

2.3.1. Giganteone E (1)
Brown viscous oil, UV \( \lambda_{\text{max}} \) (MeOH) nm (log e): 206 (4.16), 265 (3.05); IR \( \nu_{\text{max}} \) (NaCl) cm\(^{-1}\): 3399, 2922, 2852, 1722, 1625, 1452; LCMS-IT-TOF m/z: 737.3276 [M + Na]+ (calcd. for C\(_{42}\)H\(_{50}\)O\(_{10}\)Na\(_{x}\)). For \(^1H\) and \(^{13}C\) NMR spectroscopic data; see Table 1.

2.4. Cell culture
The human prostate adenocarcinoma cell line, PC3 (ATCC, CRL-14352, Manassas, VA) was purchased from American Type Culture Collection (ATCC). PC3 cells were maintained in Roswell Park Memorial Institute medium (RPMI-1640, Gibco, Life Technologies, Thermo Fisher Scientific, Inc., Rockville, MD) enriched by 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were incubated overnight at 37 °C in a humidified cell culture 5% CO\(_2\) incubator. Cells were split 1:2 once every three days to reach 70–80% confluence for experimental purposes.

2.5. Cytotoxicity assay
MTT assay was performed to determine the cytotoxicity of compounds 1–5 and 7–10 on PC3 cells. Summarily, 1 × 10\(^4\) cells/well were seeded in a 96-well plate and incubated at 37 °C in 5% CO\(_2\). The day after, the cells were treated with various dosages of compounds 1–5 and 7–10 (1 to 1600 μM). Since DMSO was used as the solvent, 0.2% of DMSO was used as vehicle control. Doxorubicin was selected as the positive control. Cells were incubated for 24 and 48 h in a CO\(_2\) incubator at 37 °C. Subsequently, 50 μL/well of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 2 mg/mL) was prepared, added and incubated for 2 h in an incubator. Furthermore, media and MTT were discarded and 100 μL of DMSO was replaced into each well to dissolve the formazan crystals. Later, the plates were read in a Chameleon™ multitechnology microplate reader (Hidex, Turku, Finland) at 570 nm. The experiments were performed in triplicates. Mean values ± SD for each concentration was determined.

2.6. DPPH free radical scavenging activity
The free radical scavenging activity of compounds 1–5 and 7–8 was measured in terms of hydrogen donating ability using DPPH radical as described [5]. Briefly, 40 μL of each compound at different concentrations (47.85–1400.56 μM) were mixed with 200 μL of 50 μM DPPH solution in methanol. The mixture was immediately shaken and incubated for 15 min in the dark at room temperature. The decrease in the absorbance was measured at 517 nm with a microplate reader (Tecan Sunrise, Austria). Ascorbic acid (35.51–568.18 μM) was used as the standard and the control was methanol. The percentage inhibition activity of compounds 1–5 and 7–8 was calculated according to the following equation:

\[
\% \text{ of DPPH radical scavenging activity} = \left( \frac{A_0 - A_1}{A_0} \right) \times 100.
\]

where, \(A_0\) was the absorbance of the control, and \(A_1\) was the absorbance of the compound or standard.

The concentration of the compound required to scavenge 50% of the DPPH radical (IC\(_{50}\)) was estimated from the graph plotted against the percentage inhibition and compared with the standard. All tests were carried out in triplicates, and the results were expressed in μM.

3. Results and discussion
Giganteone E, 1, was isolated by recycling preparative LC as an optically inactive viscous oil. The positive LCMS-IT-TOF which exhibited a pseudo-molecular ion [M + Na]+ at m/z 737.3276 (calcd. for C\(_{42}\)H\(_{50}\)O\(_{10}\)Na\(_{x}\)) with 18 degrees of unsaturation to be proposed (Supplementary material S1). On this basis, it was logical to hypothesize that compounds 1 and 5 were isomers owing to the fact that they shared the same molecular formula. The IR, \(^1H\) NMR (Supplementary material S2) and \(^{13}C\) NMR (Supplementary material S3) spectroscopic data (Table 1) of compound 1 were almost superimposable with those of compound 5 (Table 1), thus implying that compound 1 was also a non-symmetrical dimeric acylenol which was structurally related to compound 5. There was however a significant difference between ring b of compound 1 upon comparison with that of compound 5. Unlike the latter whose ring b bore ortho-dihydroxyl groups, the corresponding substructure in compound 1 bore meta-dihydroxyl groups instead. This was evident from the presence of a pair of meta-coupled doublets at δ\(_{\text{H}}\) 6.54 (d, J = 2.0 Hz, H-13; \(\delta_{\text{C}}\) 119.0, C-13) and δ\(_{\text{H}}\) 6.68 (d, J = 2.0 Hz, H-11; \(\delta_{\text{C}}\) 117.2, C-11) in the \(^1H\) NMR spectrum of compound 1 and the absence of the singlets at δ\(_{\text{H}}\) 6.53 (H-14) and δ\(_{\text{H}}\) 6.68 (H-11) which indicated them to be para to one another in the spectrum of compound 5 (Table 1). The 25 ppm downfield shift of the C-14 and the 25 ppm upfield shift of the C-13 resonances of compound 1 upon comparison
with the corresponding atoms in compound 5 suggested that the hydroxyl group in compound 1 was at C-14 rather than at C-13. The H-13/C-18 and H-19/C-15 correlations as observed in the HMBC experiment unambiguously established that the inter-acylphenol linkage in 1 was between C-15 and C-18 (Fig. 2) (Supplementary material S4–S6). Thus, the structure of 1 was established as 1-(2,6-dihydroxyphenyl)-9-(2-[3-[9-(3,4-dihydroxyphenyl)-nonanoyl]-2,6-dihydroxyphenyl]-3,5-dihydroxyphenyl)-nonanone or trivially named as giganteone E, a new dimeric acylphenol.  

The structures of the known compounds were identified as malabaricones A–C (2–4), giganteones A and C (5 and 6), maingayones A and B (7 and 8), maingayic acid (9) and β-sitosteryl oleate (10) by comparison of their spectroscopic data with those reported in the literature [1–36]. Compounds 2–4 were the major metabolites in the bark while the remaining compounds were obtained in smaller amounts.  

The genus Myristica (Myristicaceae) consists of 120 species [7]. However, only 10 species have been phytochemically investigated to date which were inclusive of Myristica fragrans Houtt., Myristica malabarica Lam., Myristica gigantea King, Myristica maingayi Hk. F., Myristica cinnamomea King, Myristica dactyloides Gaertn., Myristica ceylanica A. DC., Myristica cagayanensis Merr., Myristica argentea Warb and Myristica crassa King [1–4,8–17]. This is the first phytochemical report on M. maxima, leading to the isolation of three acylphenols (2–4), five dimeric acylphenols (1, 5–8), a carboxylic acid (9) and a sterol ester (10). It is noteworthy to mention that this is the second report in the isolation and characterization of dimeric acylphenols from the bark of a Myristica species. The first report was from the bark of M. cinnamomea [4]. Furthermore, β-sitosteryl oleate (10) was isolated for the first time from the Myristicaceae.  

Acylphenols have been characterized in certain species such as M. cagayanensis from Taiwan, M. ceylanica and M. dactyloides from Sri Lanka, M. crassa, M. cinnamomea, M. gigantea and M. maingayi from Malaysia, M. malabarica from India and M. fragrans from Vietnam [1–4,8–15]. Until present, dimeric acylphenols were only reported to be present in Myristicaceae species collected from Malaysia such as M. crassa, M. cinnamomea, M. gigantea and M. maingayi [1–4]. The five dimeric acylphenols isolated in the current investigation (1, 5–8), were characterized and categorized into two types. Type I was inclusive of giganteones (1, 5 and 6) while type II was inclusive of maingayones (7 and 8). Types I and II were both present in the leaves and fruits of M. crassa and the fruits of M. gigantea while type I and type II were respectively obtained from the bark of M. cinnamomea and the fruits of M. maingayi [1–4].  

Apart from acylphenols and dimeric acylphenols, the genus Myristica is also a source of other classes of compounds such as lignans, neolignans and flavonoids [9,11–13,16–20]. These compounds also represent the main components of several other species in this genus. For example, M. argentea, M. dactyloides and M. fragrans were documented to yield lignans and neolignans whereas lignans and flavanoids were characterized in M. ceylanica from Sri Lanka and M. cinnamomea from Thailand [9,11–13,16–20]. In addition to lignans and neolignans, acylphenols and dimeric acylphenols should also be used as chemotaxonomic markers for Myristica. More chemotaxonomic studies that involve a range of compounds could help define the relationship of more species in this genus.  

A large number of traditional medicinal plants and plant derived constituents for example terpenes, alkaloids, curcuminooids, anthocyanins, flavonoids and phenols have been known to inhibit the growth of prostate cancer [21]. However, to date, acylphenols have never been investigated for their inhibiting potential. Therefore, with regard to this, the prostate cancer inhibiting potential of the dichloromethane and ethyl acetate extracts of M. maxima and compounds 1–5 and 7–10 against PC3 cell lines were evaluated. Preliminary screening results revealed that the inhibition of the growth of the PC3 cell lines by the dichloromethane extract (20.16 ± 2.2 μg/mL and 9.12 ± 4.2 μg/mL, respectively) was more effective compared to the ethyl acetate extract (91.00 ± 3.1 μg/mL and 76.00 ± 2.1 μg/mL, respectively) after 24 and 48 h of treatment, respectively. Subsequently, the dichloromethane extract was selected for further investigation.  

The MTT assay confirmed the cytotoxic activity of compounds 2 and 5 against the human prostate cancer cells, PC3. In this study we discovered that compound 2 potentially inhibited the PC3 cell growth at a half maximal inhibitory concentration (IC50) of 26.0 ± 3.3 μM and 9.2 ± 2.4 μM after 24 and 48 h of treatment, respectively. As for compound 5, it inhibited the PC3 cell proliferation at an IC50 value of 17.5 ± 1.7 μM and 6.3 ± 1.2 μM after 24 and 48 h of treatment, respectively (Table 2). In contrary, except for compound 7 (IC50 = 13.4 ± 4.6 μM), the remaining compounds did not show specific cytotoxicity against the PC3 cells (IC50 > 30 μM) even after 48 h of treatment (Table 2). The DMSO (solvent)-treated samples did not show any inhibitory effect. Doxorubicin was chosen as the positive control. The IC50 values of

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC50 (μM) in 24 h</th>
<th>IC50 (μM) in 48 h</th>
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<tbody>
<tr>
<td>1</td>
<td>&gt;200</td>
<td>151.1 ± 4.5</td>
</tr>
<tr>
<td>2</td>
<td>26.0 ± 3.3</td>
<td>9.2 ± 2.4</td>
</tr>
<tr>
<td>3</td>
<td>73.4 ± 3.9</td>
<td>31.8 ± 3.2</td>
</tr>
<tr>
<td>4</td>
<td>143.1 ± 2.8</td>
<td>50.5 ± 2.1</td>
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<tr>
<td>5</td>
<td>17.5 ± 1.7</td>
<td>6.3 ± 1.2</td>
</tr>
<tr>
<td>7</td>
<td>31.6 ± 5.3</td>
<td>13.4 ± 4.6</td>
</tr>
<tr>
<td>8</td>
<td>124.7 ± 5.2</td>
<td>80.6 ± 8.0</td>
</tr>
<tr>
<td>9</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>10</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>9.7 ± 2.2</td>
<td>2.3 ± 1.2</td>
</tr>
</tbody>
</table>

Fig. 2. Key COSY and HMBC correlations of 1.
doxorubicin were 9.7 ± 2.2 μM and 2.3 ± 1.2 μM after 24 and 48 h of treatment, respectively.

A closer look at the structures of compounds 1–5 and 7–9 provided further insight as to how the activities of these acylphenols (2–4) and dimeric acylphenols (1, 5, 7 and 8) were influenced by the chemical groups in their respective structures (Supplementary material S7). The inhibiting potential of compounds 2–4 decreased with the increase in the number of hydroxyl groups in their ring b (Supplementary material S7). When the activity of compounds 2–4 were compared to that of compound 9, the inactivity of compound 9 led to the assumption that the presence of two aromatic rings was a prerequisite for the activity. The dimeric acylphenols on the other hand were classified into two groups; group 1 (1 and 5) and group 2 (7 and 8) in order to simplify the discussion on their SAR studies. For group 1, compound 5 was significantly more effective in inhibiting the growth of the PC3 cells as compared to compound 1. Based on this observation, it can be deduced that the potency of compound 5 could have resulted from the ortho-dihydroxyl groups in its ring b in contrast to compound 1 whose ring b bore meta-dihydroxy groups instead (Supplementary material S7). As for group 2, the cytotoxicity of compound 7 which was stronger compared to compound 8 may have been attributed to the presence of the two hydroxyl groups in its ring b unlike the latter which only bore a single hydroxyl group in its ring b (Supplementary material S7). The 2-acylresorcinol moieties (rings a and a’) which were present in the acylphenols (2–4) and dimeric acylphenols (1, 5, 7 and 8) did not contribute to their varying degrees of inhibiting potentials.

To evaluate the antioxidant potential of M. maxima, the dichloromethane and ethyl acetate extracts were screened for their in vitro DPPH free radical scavenging activity. Preliminary screening results revealed that the dichloromethane (3.61 ± 0.33 μg/mL) extract possessed a slightly stronger DPPH free radical scavenging activity compared to the ethyl acetate extract (3.83 ± 0.14 μg/mL). Subsequently, the DPPH free radical scavenging activity of seven (1–5 and 7–8) compounds isolated from the dichloromethane extract were tested with the intention of identifying the compound(s) which were responsible in giving rise to its strong antioxidant activity. Compounds 7 (2.90 ± 0.01 μM), 1 (2.92 ± 0.10 μM), 5 (3.17 ± 0.07 μM), 4 (5.28 ± 0.05 μM) and 8 (6.08 ± 0.20 μM) exhibited notably high radical scavenging activity, all being 4 or 9 folds more potent than the positive control, ascorbic acid (26.25 ± 0.34 μM) (Table 3). Overall, the order of the DPPH free radical scavenging ability of the isolated compounds were 7 > 1 > 5 > 4 > 8 > 3 > 2.

DPPH is a stable free radical which is often used to determine the antioxidant activity of natural compounds in an easy, rapid and sensitive way. Basically, the radical scavenging activity of DPPH is attributed to its ability to accept an electron or hydrogen radical and hence, become a more stable molecule. Phenolic compounds which are capable of donating a hydrogen atom are known to be effective in scavenging radicals. It has been reported that high molecular weight phenolic compounds possess a better ability in quenching the radicals and the effectiveness depends on the molecular weight, the number of aromatic rings and the nature of the hydroxyl group substitution [22–23].

In the current study, all of the tested compounds possessed either one or two 2-acylresorcinol moieties (rings a and a’) in their respective skeletons. Evidently, the resorcinol moiety did not influence the free radical scavenging activity which was obvious from the poor activity of compounds 2 and 3 in contrast to compound 4 (Supplementary material S8) [24–25]. The free radical scavenging potential of compounds 2–4 increased with the increase in the number of hydroxyl groups in their ring b. It can also be postulated that dimerization increases the activity. Dimeric acylphenols possess double the number of phenolic hydroxyl groups in their molecules as compared to their respective monomers (acylphenols) and thus a larger conjugated system, whose electron withdrawing effects leads to easy oxidation of the phenolic hydroxyl groups [26]. The free radical scavenging capacity of the dimeric acylphenols isolated from M. maxima increased with the increase in the number of hydroxyl groups present in rings b and b’ (Supplementary material S8). The greater activity of compounds 7, 1 and 5 in comparison to compound 8 could have been due to the fact that the former three compounds bore two hydroxyl groups in each of their rings b and b’ unlike the latter whose ring b only bore a single hydroxyl group.

4. Conclusion

The genus Myristica has been utilized in folk medicine in Asia [2–3, 9]. However, there is no record on the usage of M. maxima in the traditional treatment of a variety of ailments. In conclusion, the potent cytotoxicity of the dichloromethane extract, malabaricone A (2) and giganteone A (5) towards prostate cancer cell lines in addition to the effective DPPH free radical scavenging activity of the dichloromethane extract, malabaricone C (4), giganteone A (5), giganteone E (1) and maingayone A (7) in the present study provided scientific evidence for the possible usage of the bark of M. maxima as traditional medicine.

Conflict of interest

Authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.fitote.2016.04.004.

References


