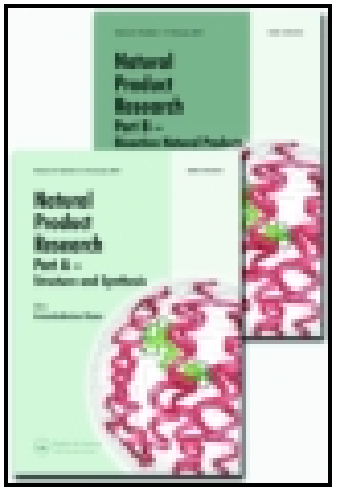

Natural Product Research: Formerly Natural Product Letters

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/gnpl20

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Published online: 27 Jan 2015.

To cite this article: Fei Cong, Khem Raj Joshi, Hari Prasad Devkota, Takashi Watanabe & Shoji Yahara (2015): Dhasingreoside: new flavonoid from the stems and leaves of Gaultheria fragrantissima, Natural Product Research: Formerly Natural Product Letters, DOI: 10.1080/14786419.2014.1003932

To link to this article: http://dx.doi.org/10.1080/14786419.2014.1003932

Natural Product Research, 2015
http://dx.doi.org/10.1080/14786419.2014.1003932

Dhasingreoside: new flavonoid from the stems and leaves of *Gaultheria fragrantissima*

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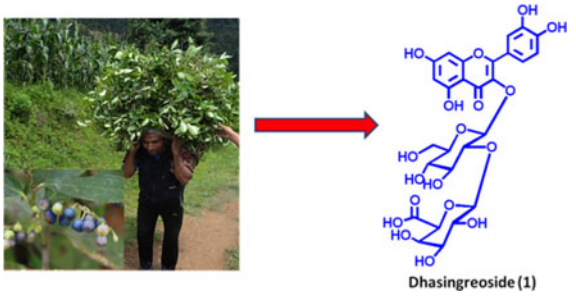
(*Received 9 December 2014; final version received 30 December 2014*)



A new flavonoid, dhasingreoside (**1**) and seven known compounds, quercetin 3-*O*-β-D-galacturonopyranoside (**2**), quercetin 3-*O*-β-D-galactopyranoside (**3**), quercetin 3-*O*-β-D-glucuronopyranoside (**4**), quercetin 3-*O*-α-L-rhamnopyranoside (**5**), (−)-epicatechin (**6**), salicylic acid (**7**) and gaultherin (**8**), have been isolated from the shade-dried stems and leaves of *Gaultheria fragrantissima*, commonly known as 'Dhasingre' in Nepal. The structures were elucidated on the basis of physical, chemical and spectroscopic methods. Among known compounds, five compounds (**3**–**6** and **8**) were isolated for the first time from *G. fragrantissima*. *In vitro* antioxidant activity of all the isolated compounds was evaluated by 1,1-diphenyl-2-picrylhydrazyl free radical-scavenging assay. Dhasingreoside (**1**) and other compounds (**2**–**6**) showed significant free radical-scavenging activity.

Keywords: *Gaultheria fragrantissima*; dhasingreoside; Dhasingre; *in vitro*; DPPH

1. Introduction

Gaultheria fragrantissima Wall. (Family: Ericaceae) is an evergreen aromatic shrub, about 3 m tall, called Wintergreen in English and locally called 'Dhasingre' in Nepal and 'Fangxiangbaizhu' in China. The plant is widely distributed in East Asia from India to Nepal and West China, Sri Lanka and North Vietnam, particularly in shaded woodland between 1200–2600 m (Watanabe et al. 2005). The plant is aromatic, antiseptic, carminative, stimulant and also used in rheumatic, scabies, neuralgia and arthritis treatment (Ranyaphi et al. 2012). Fruits are

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eaten to cure stomachache and crushed leaves are used to treat cough (Watanabe et al. 2005). *G. fragrantissima* is famous for Wintergreen essential oil, obtained by steam distillation of the leaves (Liu et al. 2013). Wintergreen oil is externally applied in liniments to counteract their irritating effects. Wintergreen oil is vermicide towards hookworm and also rubbed on body for rheumatic pain. Very little chemical investigation was performed till the date on title plant. Thus, the detailed chemical analysis and biological activity evaluation on this plant is important from the aspects of natural product chemistry. Therefore, this study was aimed for a detailed chemical analysis of stems and leaves of *G. fragrantissima* and 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenging activity of the isolated compounds.

2. Results and discussion

Successive extraction of the shade-dried stems and leaves (60 g) of *G. fragrantissima* with 70% MeOH (three times) gave total 12 g of extract. The extract was suspended in water and applied to repeated column chromatography (CC) on MCI gel CHP20P, Sephadex LH-20, ODS and silica gel to isolate compounds **1**–**8**.

Compound **1** was obtained as a pale-yellow amorphous powder, $[\alpha]_D^{21}$ − 41.6° ($c = 0.50$, $CH_3OH–H_2O = 1:1$). Its molecular formula was determined as $C_{27}H_{28}O_{18}$ on the basis of an HR-FAB-MS peak of $[M + Na]^+$ at 663.1218 (calcd for $C_{27}H_{28}O_{18}Na$, 663.1173), indicating the molecular weight to be 640. Compound **1** was positive towards ferric chloride reagent (greenish colour) and gave pink colour with magnesium–hydrochloric acid (Mg–HCl) reagent, suggesting a flavonol derivative. Acid hydrolysis of compound **1** afforded glucose and galacturonic acid confirmed on the basis of co-TLC with authentic samples. The ^{1}H NMR (DMSO-d_6) spectrum of compound **1** showed proton signals due to an ABX system: δ_H 7.64 (1H, d, $J = 1.8$ Hz), 7.54 (1H, dd, $J = 1.8$, 8.2 Hz), 6.85 (1H, d, $J = 8.2$ Hz) and two meta-coupled aromatic protons at δ_H 6.34 (1H, d, $J = 1.5$ Hz) and δ_H 6.19 (1H, d, $J = 1.5$ Hz) which were assignable to 1,3,4-trisubstituted B ring and 5,7-disubstituted A ring of flavonol backbone (Park et al. 2012). The proton signals at δ_H 5.35 (1H, d, $J = 7.3$ Hz) and δ_H 4.59 (1H, d, $J = 7.9$ Hz) were assignable to two anomaric protons of two monosaccharide moieties and coupling constants ($J = 7.3$, 7.9 Hz) suggesting that both are in β-configuration. Moreover, two proton signals at δ_H 3.71 (1H, dd, $J = 2.1$, 12.2 Hz) and δ_H 3.62 (1H, dd, $J = 4.8$, 12.2 Hz) (in $CD_3OD + D_2O$, 1:1) were assignable to the methylene (CH_2) protons of glucopyranosyl moiety (Calis et al. 1999; Andersen & Fossen 2006; Díaz et al. 2008).

The ^{13}C NMR (DMSO-d_6) spectra of compound **1** showed signals equivalent to a total of 27 carbons, in which 15 carbon signals at δ_C (177.3, 164.1, 161.0, 156.1, 155.6, 148.4, 144.7, 133.0, 121.6, 120.9, 116.2, 115.3, 103.7, 98.5, 93.4) were assignable to a 3-*O*-glycosyl substituted quercetin moiety (Markham and Chari 1982). Other characteristic signals of the ^{13}C NMR and distortionless enhancement by polarisation transfer spectra displayed the presence of C-2″-substituted glucopyranosyl [98.3 (CH), 82.3 (CH), 76.6 (CH), 76.4 (CH), 69.4 (CH), 60.5 (CH_2)] (Andersen & Fossen 2006; Wang et al. 2010) and a galacturonopyranosyl unit [104.1 (CH), 71.3 (CH), 74.2 (CH), 69.4 (CH), 76.4 (CH), 171.0 (—O—C═O)]. Substitution by glucopyranosyl moiety at C-3 position of quercetin was supported by the downfield shift of C-2 carbon signal (Markham and Chari 1982). Connectivities of galacturonopyranosyl moiety with C-2″ position of glucopyranose was supported by glycosidation shifts, showing an α-effect on C-2″ and β-effects on C-1″ and C-3″ (Faizi et al. 2011). Thus, C-2″ resonated at a higher frequency (δ_C 82.3) and C-1″ and C-3″ appeared at lower frequencies (δ_C 98.3 and δ_C 76.4, respectively). From the ^{1}H–^{1}H correlation spectroscopy (COSY), the anomaric glucopyranosyl proton signal at δ_H 5.35 (Glc-1H) was correlated with the proton signal at δ_H 3.58 (Glc-2H). From the heteronuclear multiple quantum coherence (HMQC) spectrum, these two protons, δ_H 5.35 and 3.58, were correlated to the carbons at δ_C 98.3 (Glc-1C) and 82.3 (Glc-2C), respectively. This was also

supported by the upfield of anomaric carbon Glc-1C (δ_C 98.3) and downfield shift of Glc-2C (δ_C 82.3). The anomaric proton of galacturonopyranosyl moiety at δ_H 4.59 (1H, d, $J = 7.9$ Hz) was correlated to the anomaric carbon at δ_C 104.1 by the HMQC spectrum. Heteronuclear multiple bond correlation (HMBC) correlated to the anomaric proton of GalA-1H (δ_H 4.59) with Glc-2C (δ_C 82.3). Thus, the complete assignment of the proton and carbon atoms, their positions, and the linkage between the glucopyranosyl and galacturonopyranosyl moiety in the compound **1** were determined on the basis of chemical shifts, $^1H-^1H$ COSY, HMQC and HMBC spectra. Thus, from the physical and spectral data, the structure of compound **1** was elucidated as quercetin 3-*O*-(2″-*O*-β-D-galacturonopyranosyl)-β-D-glucopyranoside and named as dhasingreoside (Figure 1).

The structures of known compounds were elucidated on the basis of their physical and spectral data and comparison with literature values as quercetin 3-*O*-β-D-galacturonopyranoside (**2**) (Hansen et al. 1999), quercetin 3-*O*-β-D-galactopyranoside (**3**) (Bouktaib et al. 2002), quercetin 3-O-β-D-glucuronopyranoside (**4**) (Markham and Chari 1982), quercetin 3-*O*-α-L-rhamnopyranoside (**5**) (Lu & Foo 1997), (−)-epicatechin (**6**) (Zhang et al. 2012), salicylic acid (**7**) and gaultherin (**8**) (Moon et al. 1996).

All of the isolated compounds were tested for their *in vitro* antioxidant activity towards DPPH free radical-scavenging assay. The 50% inhibition concentration (IC_{50}) for DPPH radical-scavenging activity was calculated and provided in Table 1. The results were compared with Trolox as a positive control (IC_{50}, 96.1 μM). Dhasingreoside (**1**), quercetin 3-*O*-β-D-galacturonopyranoside (**2**), quercetin 3-*O*-β-D-galactopyranoside (**3**), quercetin 3-*O*-β-D-glucuronopyranoside (**4**), quercetin 3-*O*-α-L-rhamnopyranoside (**5**) and (−)-epicatechin (**6**) showed potent free radical-scavenging activity. These findings were similar to the previous reports regarding the free radical-scavenging activity of flavonoids (Okawa et al. 2001; Cho et al. 2003). Thus, the presence of such strong antioxidants in the stems and leaves of *G. fragrantissima* might be able to protect against oxidative damage.

3. Experimental

3.1. *Instruments and chemicals*

Optical rotations were measured with a JASCO DIP-1000KUY polarimeter (JASCO Corporation, Tokyo, Japan). NMR spectra were measured on a JEOL JNM α-500 spectrometer

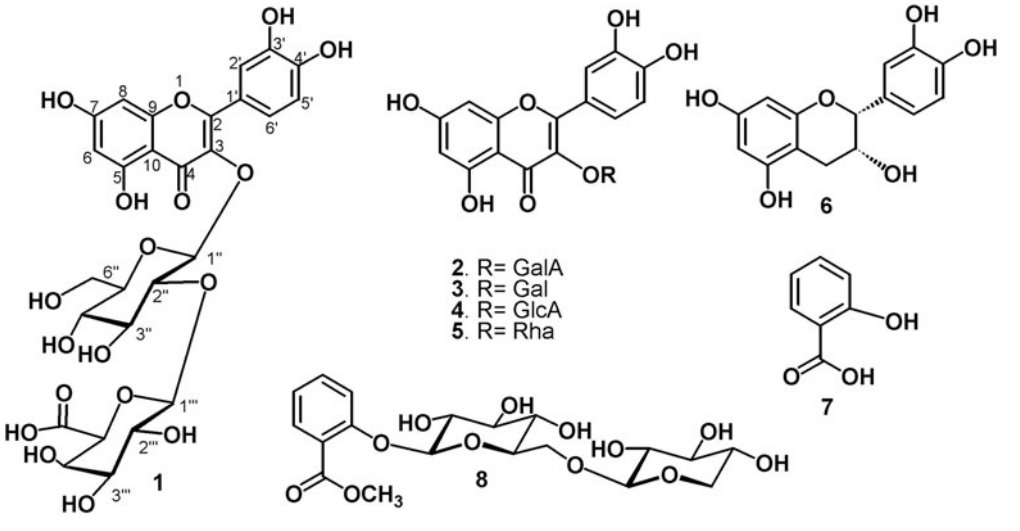

Figure 1. Structures of compounds **1**–**8**.

Table 1. IC_{50} (μM) value of compounds **1**–**6** and Trolox.

Compounds	IC_{50} (μM)
1	41.6
2	48.6
3	51.4
4	54.6
5	48.6
6	138.3
Trolox	96.1

(JEOL Ltd, Tokyo, Japan) (^{1}H: 500 MHz and ^{13}C: 125 MHz). Chemical shifts are given in ppm with reference to TMS. Mass spectra were recorded on a JEOL JMS 700 MStation mass spectrometer (JEOL Ltd., Tokyo, Japan). CC was carried out with silica gel 60 (0.040–0.063 mm, Merck, Tokyo, Japan), MCI gel CHP20P (75–150 μm, Mitsubishi Chemical Industries Co., Ltd, Tokyo, Japan), Sephadex LH-20 (Amersham Pharmacia Biotech, Tokyo, Japan) and Chromatorex ODS (30–50 μm, Fuji Silysia Chemical Co., Ltd, Aichi, Japan). TLC was performed on a precoated silica gel 60 F_{254} (0.2 mm, aluminium sheet, Merck). Authentic sample of sugars were obtained from Sigma-Aldrich, Tokyo, Japan. DPPH was used from Wako Pure Chemicals, Osaka, Japan. Trolox was purchased Calbiochem, Tokyo, Japan, and 2-(*N*-morpholino)ethanesulphonic acid (MES) buffer was obtained from Dojindo Chemical Research, Kumamoto, Japan.

3.2. *Plant material*

The fresh stems and leaves of *G. fragrantissima* were collected from Nagarkot, Bhaktapur, Nepal in July 2014. The voucher specimen (Voucher No.: KUNP20140709-002) was deposited on Graduate School of Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan.

3.3. *Extraction and isolation*

The shade-dried stems and leaves (60 g) were extracted successively with 70% MeOH (3 L three times), at 55°C (5 h) and room temperature (20°C, for 22 h) each time. The combined extract was evaporated under reduced pressure to give 12 g extract. The extract (21 g) was suspended in water and was subjected to MCI gel CHP20P CC and eluted successively with water, 40%, 60%, 80% and 100% MeOH and $CHCl_3$ to give 13 fractions (1–13). Fraction 3 (3.15 g, 40% MeOH eluate) was subjected to Sephadex LH-20 CC (50% MeOH) to give five subfractions (3-1–3-5). Fraction 3-2 (399 mg) was subjected to ODS CC (H_2O, 5–8% MeOH) to give 11 subfractions (3-2-1–3-2-11). Fraction 3-2-3 was obtained as compound **7** (12 mg). Fraction 3-2-10 (33 mg, 8% MeOH eluate) was applied on Sephadex LH-20 CC (H_2O) followed by MCI gel CHP20P CC to afford compound **1** (10 mg). Fraction 4 (3.01 g, 40% MeOH eluate) was subjected to Sephadex LH-20 CC (50% MeOH) to give six subfractions (4-1–4-6). Fraction 4-2 (1.31 g) was subjected to ODS CC (10–15% MeOH) to give six subfractions (4-2-1–4-2-6). Fraction 4-2-6 (1.13 g, 15% MeOH eluate) was applied on silica gel CC ($CHCl_3$–MeOH–H_2O = 8:2:0.1) to afford compound **8** (165 mg). Fraction 4-5 (123 mg) was applied on ODS CC (15% MeOH) to afford compound **4** (17 mg). Fraction 4-6 (208 mg) was applied on ODS CC (25% MeOH) to afford compounds **2** (3 mg) and **6** (36 mg). Fraction 7 (424 mg, 60% MeOH eluate) was subjected to Sephadex LH-20 CC (MeOH) to afford compound **3** (57 mg). Similarly, fraction 9 (243 mg, 60% MeOH eluate) was subjected to Sephadex LH-20 CC (MeOH) to afford compound **5** (29 mg).

3.4. *Dhasingreoside (1)*

Pale-yellow amorphous powder; $[\alpha]_D^{21}$ − 41.6° (c = 0.50, $CH_3OH–H_2O$ = 1:1); HR-FAB-MS (positive mode) $[M + Na]^+$ at 663.1218 (calcd for $C_{27}H_{28}O_{18}Na$, 663.1173); ^1H NMR (500 MHz, DMSO-d_6): δ 7.64 (1H, d, J = 1.8, H-2′), 7.54 (1H, dd, J = 1.8, 8.2, H-6′), 6.85 (1H, d, J = 8.2, H-5′), 6.34 (1H, d, J = 1.5, H-8), 6.13 (1H, d, J = 1.5, H-6), 5.65 (1H, d, J = 7.3, Glc H-1), 4.59 (1H, d, J = 7.9, GalA H-1), 3.58 (1H, t, J = 7.3, Glc H-2), 3.46 (1H, dd, J = 4.5, 11.6, Glc H-6a), 3.12–3.55 (7H, m, Glc H-3, Glc H-4, Glc H-5, Glc H-6b, GalA H-3, GalA H-4, GalA H-5); ^{13}C NMR (125 MHz, DMSO-d_6): 177.3 (C-4), 171.0 (C-GalA-6), 164.1 (C-7), 161.0 (C-5), 156.1 (C-2), 155.6 (C-9), 148.4 (C-4′), 144.7 (C-3′), 133.0 (C-3), 121.6 (C-6′), 120.9 (C-1′), 116.2 (C-5′), 115.3 (C-2′), 104.1 (C-GalA-1), 103.7 (C-10), 98.5 (C-6), 98.3 (C-Glc-1), 93.4 (C-8), 82.3 (C-Glc-2), 76.6 (C-Glc-5[a]), 76.4 (C-Glc-3[a]), 76.2 (C-GalA-5), 74.2 (C-GalA-3), 71.3 (C-GalA-2), 69.4 (C-Glc-4), 69.4 (C-GalA-4), 60.5 (C-Glc-6); [a]assignments may be reversed.

3.5. *Known compounds*

Quercetin 3-*O*-β-D-galacturonopyranoside (**2**): pale-yellow amorphous powder, $[\alpha]_D^{20}$ − 42.6° (c = 0.13, CH_3OH); quercetin 3-*O*-β-D-galactopyranoside (**3**): pale-yellow amorphous powder, $[\alpha]_D^{20}$ − 43.5° (c = 0.44, pyridine); quercetin 3-*O*-β-D-glucuronopyranoside (**4**): pale-yellow amorphous powder, $[\alpha]_D^{20}$ − 18.6° (c = 0.44, CH_3OH), quercetin 3-*O*-α-L-rhamnopyranoside (**5**): pale-yellow amorphous powder, $[\alpha]_D^{20}$ − 17.5° (c = 0.98, CH_3OH); (−)-epicatechin (**6**): $[\alpha]_D^{20}$ − 50.3° (c = 0.97, CH_3OH); salicylic acid (**7**): white needle crystals; and gaultherin (**8**): white amorphous powder, $[\alpha]_D^{20}$ − 52.3° (c = 1.14, CH_3OH).

3.6. *Antioxidative activity*

The DPPH radical-scavenging activity was measured by the method described by Li and Seeram (2011), with slight modifications. Briefly, 80 μL of each compound at various concentrations (in DMSO–EtOH = 1:1) was mixed with 40 μL of MES buffer (200 mM, pH 6.0) and 40 μL of DPPH solution (800 μM in EtOH) in a 96-well plate. The reaction mixture was shaken vigorously and left for 30 min at room temperature in the dark. The antioxidative activity corresponding to the scavenging of DPPH radicals was measured at 510 nm with a UV spectrophotometer using the following formula:

$$\text{Radical scavenging activity (\%)} = 100 \times \frac{A - B}{A},$$

where A is the control absorbance of DPPH radicals without samples and B is the absorbance after reacting with samples. Trolox was used as the positive control. The result is expressed as the mean of four experiments. From these data, a curve was plotted and the concentration (μM) of the sample required for 50% reduction of the DPPH radical absorbance (IC_{50}) was calculated.

3.7. *Acid hydrolysis of compounds 1*

Compound **1** (1.0 mg) in 2 M HCl (0.5 mL) was heated at 70°C for 3 h in a sealed tube. Co-TLC in silica gel together with the authentic samples was performed using $CHCl_3–MeOH–H_2O$ (6:4:1, v/v/v) and *n*-BuOH–AcOEt–H_2O (5:1:4, v/v/v, upper phase) as the developing solvents. Aqueous H_2SO_4 (10%) was used as the detection reagent. Glucose, galacturonic acid and quercetin were detected from compound **1**.

4. Conclusion

Eight phenolic compounds (**1–8**) were isolated as major constituents of leaves and stems of *G. fragrantissima*: a new compound, dhasingreoside (**1**) and seven known compounds; quercetin 3-*O*-β-D-galacturonopyranoside (**2**), quercetin 3-*O*-β-D-galactopyranoside (**3**), quercetin 3-*O*-β-D-glucuronopyranoside (**4**), quercetin 3-*O*-α-L-rhamnopyranoside (**5**), (−)-epicatechin (**6**), salicylic acid (**7**) and gaultherin (**8**). Among them, five compounds (**3–6** and **8**) were isolated for the first time from *G. fragrantissima*. *In vitro* antioxidant activity of all the isolated compounds from *G. fragrantissima* was evaluated by DPPH free radical-scavenging assay. New compound, dhasingreoside (**1**) and other phenolic compounds (**3–6**) showed significant free radical scavenging activity. Thus, these phenolic compounds may contribute to the antioxidant activities of the leaves and stems of *G. fragrantissima* and might be used as natural antioxidants. This study provides some evidences for the use of *G. fragrantissima* plant as traditional medicine.

Supplementary material

Supplementary material relating to this paper is available online, alongside Figures S1–S7 and Tables S1–S8.

Acknowledgements

The authors thank KOHNAN Asia Scholarship Foundation for the scholarship to Ms Fei Cong and the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan for the scholarship to Mr Khem Raj Joshi. This work was supported in part by the Program for Leading Graduate Schools 'HIGO' (Health life science: Interdisciplinary and Glocal Oriented), MEXT, Japan.

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