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THE FIRST EVALUATION OF TYROSINASE AND ELASTASE INHIBITORY ACTIVITIES OF GRAYANANE-TYPE DITERPENOIDS ISOLATED FROM *Rhododendron brachycarpum*

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Abstract. From the methanol extract of the aerial parts of *Rhododendron brachycarpum*, five grayanane-type diterpenoids were isolated. These grayanane-type diterpenoids were elucidated by detailed analyses of NMR and MS data and identified as grayanoside B (**1**), rhodomoside A (**2**), piersformoside B (**3**), grayanotoxin III (**4**), and grayanotoxin I (**5**), based on comparison with previously reported spectral data. All compounds were evaluated for inhibitory activity against tyrosinase and elastase. The results revealed that the compounds **1**-**5** exhibited weak tyrosinase inhibitory activity, whereas compounds **1**, **3**, and **4** showed strong elastase inhibition, with inhibition rates of 71.78%, 71.11%, and 85.19%, respectively, at a concentration of 1mM. This study represents the first report of the anti-tyrosinase and anti-elastase activities of grayane-type diterpenoids isolated from *R. brachycarpum*.

Keywords: *Rhododendron brachycarpum*, grayanane-type diterpenoids, tyrosinase, elastase enzymes.

1. Introduction

Rhododendron brachycarpum G. Don (Ericaceae), an evergreen broad-leaved shrub, belongs to the family Ericaceae and is mainly distributed in the northern parts of Korea and the central parts of Japan [1]. In Korea, this plant is commonly known as “man byoung cho”. Traditionally, the leaves of *R. brachycarpum* have been used in the treatment of diabetes, hypertension, and rheumatoid arthritis [2]. Phytochemical investigations have identified several bioactive constituents, including triterpenoids such as ursolic acid, which exhibit notable anti-inflammatory and anticancer activities [3]. Flavonoids such as hyperoside and quercetin, characteristic compounds of this species, have been reported to protect cardiomyocytes and alleviate pain [4]-[6], hyperoside also shows potential for treating severe vascular inflammatory conditions such as septic shock [7].

Moreover, phenolic glycosides from this plant have exhibited antiseptic effects by inhibiting HMGB1 release and HMGB1-mediated inflammatory responses [8]. Additionally, triterpenoids such as rhododendric acid A, corosolic acid, and ursolic acid isolated from *R. brachycarpum* have exhibited antidiabetic properties [9].

Grayanane-type diterpenoids are characterized by a distinctive 5/7/6/5 tetracyclic skeleton and are recognized as characteristic secondary metabolites in plants in the Ericaceae family [10], [11]. To date, more than 400 grayanane diterpenoids with a 25-carbon framework have been isolated and structurally characterized from species within this family [11]. These metabolites have attracted considerable interest among phytochemists and synthetic organic chemists owing to their structural complexity, chemical diversity, and notable biological activities [10]. Numerous studies have demonstrated that grayanane-type diterpenoids exhibit a broad range of biological activities, including insecticidal, analgesic, antinociceptive, anticancer, antiviral, antioxidant, and anti-inflammatory effects [10], [12].

In our ongoing efforts to discover potential drug leads and bioactive natural products, five grayanane-type diterpenoids were isolated from *R. brachycarpum* using a combination of chromatographic and spectroscopic methods. These compounds were subsequently evaluated for anti-tyrosinase and anti-elastase activities to further investigate their bioactive diversity and potential applications.

2. Content

2.1. Materials and methods

2.1.1. Plant material

The leaves of *R. brachycarpum* were collected from a farm in Gongju, Korea, in 2011. The plant material was authenticated by Prof. Min-Kyun Na (College of Pharmacy, Chungnam National University). A voucher specimen (CNU00195) has been deposited in the Pharmacognosy Laboratory, the College of Pharmacy, Chungnam National University, Daejeon, Korea.

2.1.2. General experimental procedures

Vacuum liquid chromatography (VLC) was performed on silica gel (Merck, 70−230 mesh). Medium-pressure liquid chromatography (MPLC) was conducted with a Biotage Isolera™ system equipped with reversed-phase C_{18} SNAP Cartridges KP-C18-HS (340 g, Biotage). Open-column chromatography was conducted with a glass column (1.5 × 60.0 cm). Preparative reversed-phase HPLC (prep. HPLC) separation was carried out on a YMC C_{18} column (250.0 × 20.0 mm, 5 μm) at a flow rate of 5 mL/min. ^{1}H NMR (600 MHz, 300 MHz) and ^{13}C NMR (150 MHz, 75 MHz) spectra were recorded on a Bruker Avance Ultrashield spectrometer. High-resolution mass spectrometry (HRMS) data were acquired by using an electrospray ionization (ESI) quadrupole time-of-flight (Q-TOF) tandem mass spectrometry (MS/MS) system (AB SCRIEX Triple). HPLC-grade solvents (Merck, Ltd.) were used for prep-HPLC, while the solvents for VLC and MPLC separations were distilled prior to use. Kojic acid, *L*-tyrosine, oleanolic acid, and *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide (AAAPVN) were purchased from Sigma-Aldrich.

2.1.3. Extraction and isolation

The isolation of compounds was described in a previous publication [13]; however, a brief overview is provided here for reference. Dried leaves of *R. brachycarpum* (25 kg) were extracted twice with methanol (250 L × 2), and the combined extracts were filtered to yield a crude MeOH extract (6 kg). Half of this extract (3 kg) was conducted to chromatographic separation, yielding five grayanane-type diterpenoids.

2.1.4. Tyrosinase inhibitory assay

The tyrosinase inhibition assay was conducted as previously described [14]. Briefly, 130 µL of tyrosinase (approximately 46 units/mL), prepared in 0.05 mM phosphate buffer (pH = 6.8), was added to each well of a 96-well microplate. Then, 20 µL of each test compound (1 mM) was introduced. Afterward, the enzymatic reaction was initiated by adding 50 µL of 1.5 mM *L*-tyrosine substrate (also prepared in phosphate buffer). The reaction mixtures were incubated at room temperature, and absorbance was recorded at 475 nm using a UV-Vis spectrophotometer for 20 min.

The tyrosinase inhibitory activity was calculated using the following formula:

$$\text{Inhibition activity rate (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \text{ x } 100$$

where, $A_{control}$ is the absorbance value of the control (without inhibitor); $A_{control}$ is the absorbance value of the sample (with inhibitor).

2.1.5. Elastase inhibitory assay

The elastase inhibition assay was performed as previously described [15], [16], with slight modifications. Briefly, 140 µL of 0.1M Tris-HCl buffer (pH = 8.0) was added to each well of a 96-well microplate. Then, 20 µL of each test compound (1 mM) and 20 µL of *N*-Succinyl-Ala-Ala-Ala-*p*-nitroanilide (1mM prepared in Tris-HCl buffer) were introduced. Afterward, the enzymatic reaction was initiated by adding 20 µL of Elastase (0.34 unit/mL). The reaction mixtures were incubated at 37 ^{0}C temperature in 20 minutes, and absorbance was recorded at 410 nm using a UV-Vis spectrophotometer for 30 minutes at 60s intervals.

The elastase inhibitory activity was calculated using the following formula:

$$\text{Inhibition activity rate (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \text{ x } 100$$

where, $A_{control}$ is the absorbance value of the control (without inhibitor); $A_{control}$ is the absorbance value of the sample (with inhibitor).

2.2. Results and discussions

The dried leaves of *R. brachycarpum* were extracted with methanol. Using various chromatographic to afford five compounds (**1-5**) from the MeOH extract of the dried leaves of *R. brachycarpum.* All compounds were elucidated as grayanoside B [17], rhodomomoside A [18], piersformoside B [19], grayanotoxin III [20], and grayanotoxin I [21]. Their structures are shown in Figure 1.

2.2.1. Structural determination of isolated compounds

Compound **1** was obtained as a white amorphous powder. Its molecular formula was established as $C_{26}H_{42}O_9$ based on HRESIMS analysis, which showed a $[M+Na]^+$ ion at

m/z 521.2234 (calcd. for $[C_{26}H_{42}O_9Na]^+$, $[M+Na]^+$, *m/z* 521.2727). The ^{1}H NMR spectrum displayed characteristic signals including three methyl resonances at δ_H 1.32 (3H, s, H-17); 1.56 (3H, s, H-18); and 1.79 (3H, s, H-19), two olefinic methylene protons at δ_H 5.14 (1H, s, H-20a) and 5.08 (1H, s, H-20b); and one anomeric proton at δ_H 5.01 (1H, d, J = 7.8 Hz, H-1'). Consistently, the ^{13}C-NMR and DEPT spectra exhibited 26 carbon signals, comprising three methyls, nine methylenes, nine methines (including seven oxygenated), and five quaternary carbons. Structural elucidation was accomplished through detailed analysis of the 1D and 2D NMR data (HSQC, HMBC experiments). The proton signals were assigned to the corresponding carbons through direct ^{1}H NMR and ^{13}C-NMR correlations in the HSQC spectrum. The HMBC correlations from H_3-17 (δ_H 1.32) to C-16 (δ_C 79.7), C-15 (δ_C 63.4), and C-13 (δ_C 48.0) were observed. A pair of *germinal* tertiary methyl groups connected to C-4, was indicated by the HMBC correlations between H_3-18,19/C-3 (δ_C 89.1), C-4(δ_C 51.0), and C-5(δ_C 82.7). The sugar moiety was assigned to C-3 (δ_C 89.1) based on HMBC correlations from the anomeric proton H-1' (δ_H 5.01) to C-3 (δ_C 89.1), as well as between the proton H-3 (δ_H 4.20) to C-1' (δ_C 105.9). In addition, the comparison of the NMR data of **1** with those reported in the literature [17], confirmed the identity of **1** as grayanoside B.

Compounds **2** and **3** were also isolated as white amorphous powders. Their structures were elucidated through detailed NMR spectroscopic analyses. A comparison of the spectral data of compounds **1** and **2** revealed a slight difference at the C-14 position. In the ^{1}H-NMR and ^{13}C-NMR spectra of **2**, the chemical shift for C-14 was observed to shift downfield from δ_C 36.1 (in compound **1**) to 80.8 (C-14), suggesting the presence of a hydroxyl group at this position. Further comparison of the NMR data of **2** with previously reported literature values [18] confirmed its identity as rhodomoside A.

Similarly, comparison of the NMR data of compounds **1** and **3** indicated the absence of a methine signal, replaced by a methylene signal. This change suggests that compound **3** has a structure closely related to that of **1**, but with a hydroxyl substitution at the C-6 position. The structure of **3** was also determined to be piersformoside B based on a comparison of its NMR data with previously reported literature values [19].

Compound **4** was yielded as a white amorphous powder. The ^{1}H-NMR spectrum of **4** exhibited signals for four methyl groups at δ_H 1.13 (3H, s, H-19); 1.50 (3H, s, H-18); 1.67 (3H, s, H-19); and 1.85 (3H, s, H-20). Comparison of the ^{1}H NMR data of **4** with that of **2** revealed the disappearance of two olefinic methylene protons, which were replaced by a methyl and a hydroxy group at the C-20 position. In addition, the absence of the sugar moiety was observed. This is also confirmed with the ^{13}C NMR spectrum, which displayed 20 carbon signals. Moreover, the comparison of the NMR data of **4** with previously reported literature values [20] confirmed that the structure of **4** was identified as grayanotoxin III**.**

Compound **5** was identified as grayanotoxin I by comparison of its NMR data with those of **4** and literature values [21]. The NMR spectrum of **5** showed the presence of an acetyl group, evidenced by characteristic resonances at δ_H 1.94 (3H, s, H-22)/ δ_C 21.6 (C-22) and δ_C 170.7 (C-21). These slight differences were attributed to the substitution at the C-14 position, as supported by downfield shifts in the NMR data.

2.2.2. Tyrosinase and elastase inhibitory effects of isolated compounds

Tyrosinase is a key regulatory enzyme in melanin biosynthesis within melanocytes [22]. However, excessive melanin production can lead to hyperpigmentation disorders, including melasma, lentigines, freckles, and even melanoma, and may have genotoxic implications [23]. Therefore, tyrosinase inhibitors have attracted considerable attention in the cosmetic and pharmaceutical industries, particularly as skin-whitening agents and for the treatment of pigmentary disorders [24]. Meanwhile, elastase, a serine protease, plays a critical role in the degradation of elastin, a structural protein essential for maintaining the elasticity of the skin, lungs, and blood vessels. Proper regulation of elastase activity is crucial for tissue homeostasis, as its dysregulation has been implicated in the pathogenesis of various disorders, including emphysema, skin aging (wrinkles), and atherosclerosis [25].

In this study, the inhibitory activities of five grayanane-type diterpenoids isolated from *R. brachycarpum* were evaluated against tyrosinase and elastase enzymes. The tyrosinase inhibition by the compounds ranged from 4.28 to 11.03% at a concentration of 1 mM (Table 1), indicating relatively weak activity. In contrast, compounds **1**, **3**, and **4** exhibited notable elastase inhibitory effects, with inhibition rates of 71.78%, 71.11%, and 85.19%, respectively. This suggests that compounds **1**–**3** share a similar core structure, with primary differences at the C-14 position. The presence of a hydroxy group at C-14 in compound 2 may account for its weaker activity. In contrast, compounds **4** and **5** also differ at C-14, bearing hydroxy and methyl acetate groups, respectively, which may explain their differences in inhibitory activity. Further, evaluation of the elastase inhibitory activity of compounds **1**, **3**, and **4** at lower concentrations revealed IC_{50} values of 245.35 µM, 250.01 µM, and 160.35 µM, respectively. In comparison, oleanolic acid, used as a positive control, exhibited an IC_{50} value of 105.76 µM (Table 2). These results suggest the potential of these compounds as elastase inhibitors for dermatological applications.

Table 1. Inhibition rate of compounds 1-5 on tyrosinase and elastase at 1mM

Compounds	Inhibition rate (%)[a]	
	Tyrosinase	***Elastase***
1	4.28 ± 1.12	71.78 ± 3.16
2	5.08 ± 1.41	-
3	7.25 ± 2.01	71.11± 2.09
4	10.16 ± 1.42	85.19 ± 1.70
5	11.03 ± 1.48	-
Kojic acid[b]	99.55 ± 0.54	
Oleanic acid[b]		91.56 ± 0.73

[a]All experiments were conducted in triplicate; [b]Positive control

Table 2. IC_{50} value of compounds 1-5 on tyrosinase and elastase

Compounds	IC_{50} (μM)[a]	
	Tyrosinase	***Elastase***
1	> 1000	245.35 ± 2.06
2	> 1000	-
3	> 1000	250.01± 1.91
4	> 1000	160.35 ± 1.93
5	> 1000	-
***Kojic acid*[b]**	25.40 ± 1.21	
***Oleanic acid*[b]**		105.76 ± 1.46

[a]All experiments were conducted in triplicate; [b]Positive control

2.2.3. Key of spectroscopic data of isolated compounds

Grayanoside B (1): ^{1}H-NMR (600 MHz, pyridine-d_5) δ_H 5.14 (1H, s, H-20a), 5.08 (1H, s, H-20b), 5.01 (1H, d, J = 7.8 Hz, H-1'), 4.59 (1H, dd, J = 11.7, 2.3 Hz, H-6'a), 4.42 (1H, dd, J = 11.7, 5,3 Hz, H-6'b H-14), 4.26 (2H, m, H-3', H-6), 4.20 (2H, m, H-4', H-3), 4.02 (1H, m, H-2', H-5'), 2.95 (1H, dd, J = 10.7, 8.3 Hz, H-1), 2.63 (1H, m, H-9), 2.23 (1H, m, H-13), 2.12 (1H, d, J = 7.8 Hz, H-15a), 1.97 (1H, d, J = 13.9 Hz, H-15b), 1.79 (1H, s, H-19), 1.56 (3H, s, H-18), 1.32 (3H, s, H-17). ^{13}C-NMR (150 MHz, pyridine-d_5) δ_C 43.5 (C-1), 38.0 (C-2), 89.1 (C-3), 51.0 (C-4), 82.7 (C-5), 72.1 (C-6), 47.0 (C-7), 44.8 (C-8), 54.7 (C-9), 152.1 (C-10), 24.2 (C-11), 26.7 (C-12), 48.0 (C-13), 36.1 (C-14), 63.4 (C-15), 79.7 (C-16), 27.3 (C-17), 26.0 (C-18), 20.6 (C-19), 113.2 (C-20), 105.9 (C-1'), 75.9 (C-2'), 78.9 (C-3'), 72.0 (C-4'), 78.8 (C-5'), 63.2 (C-6'). ESI-MS: $[M+Na]^+$, $[C_{26}H_{42}O_9Na]^+$ calcd.: *m/z* 521.2727, found: *m/z* 521.2234.

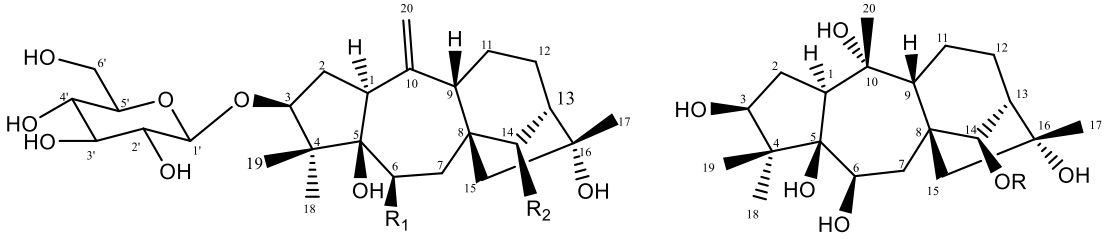
Rhodomoside A (2): ^{1}H-NMR (600 MHz, pyridine-d_5) δ_H 5.18 (1H, s, H-20a), 5.08 (1H, s, H-20b), 5.01 (1H, d, J = 7.8 Hz, H-1'), 4.59 (2H, m, H-6'a, H-5), 4.46 (1H, m, H-14), 4.42 (1H, dd, J = 11.7, 5,4 Hz, H-6'b), 4.26 (2H, m, H-4', H-5'), 4.17 (1H, m, H-3), 4.03 (1H, m, H-2'), 4.00 (1H, m, H3'), 3.06 (1H, t, J = 9.3 Hz, H-1), 2.96 (1H, s, H-9), 2.61 (1H, m, H-2a), 2.51 (1H, m, H-6), 2.48 (1H, m, H-2b), 2.43 (1H, d, J = 14.3 Hz, H-15a), 2.38 (1H, s, H-13), 2.16 (1H, d, J = 14.3 Hz, H-15b), 1.76 (3H, s, H-19), 1.54 (3H, s, H-17), 1.20 (3H, s, H-18). ^{13}C-NMR (150 MHz, pyridine-d_5) δ_C 45.2 (C-1), 37.9 (C-2), 89.1 (C-3), 51.3 (C-4), 83.3 (C-5), 71.1 (C-6), 42.5 (C-7), 50.7 (C-8), 53.3 (C-9), 152.3 (C-10), 25.7 (C-11), 24.9 (C-12), 54.4 (C-13), 80.8 (C-14), 61.6 (C-15), 81.3 (C-16), 25.2 (C-17), 26.6 (C-18), 20.2 (C-19), 113.0 (C-20), 105.9 (C-1'), 76.0 (C-2'), 79.0 (C-3'), 72.2 (C-4'), 78.8 (C-5'), 63.3 (C-6'). ESI-MS: *m/z* at 515.2861 $[M+H]^+$. ESI-MS: $[M+H]^+$, $[C_{26}H_{43}O_{10}]^+$ calcd.: *m/z* 515.2856, found: *m/z* 515.2861.

Piersformoside B (3): ^{1}H-NMR (600 MHz, pyridine-d_5) δ_H 5.27 (1H, s, H-20a), 5.22 (1H, s, H-20b), 5.01 (1H, d, J = 7.7 Hz, H-1'), 4.14 – 3.96 (6H, m, H-2' – H-6'), 4.81 (1H, dd, J = 9.6, 4.8 Hz, H-3), 3.12 (1H, t, J = 10.0 Hz, H-1), 2.01 (1H, m, H-9), 1.55 (3H, s, H-17), 1.44 (3H, s, H-18), 1.25 (3H, s, H-19). ^{13}C-NMR (150 MHz, pyridine-d_5) δ_C 50.9 (C-1), 35.5 (C-2), 88.8 (C-3), 51.5 (C-4), 82.2 (C-5), 33.2 (C-6), 40.6 (C-7), 47.4 (C-8),

54.5 (C-9), 151.3 (C-10), 26.3 (C-11), 25.0 (C-12), 49.6 (C-13), 36.6 (C-14), 59.2 (C-15), 79.8 (C-16), 25.0 (C-17)21.3 (C-18), 19.5 (C-19), 109.1 (C-20), 106.7 (C-1'), 76.0 (C-2'), 79.1 (C-3'), 72.0 (C-4'), 78.6 (C-5'), 63.1 (C-6'). ESI-MS: $[M+Na]^+$, $[C_{26}H_{42}O_8Na]^+$ calcd.: *m/z* 505.2777, found: *m/z* 505.2782.

Gyayanotoxin III (4): ^{1}H-NMR (600 MHz, pyridine-d_5) δ_H 4.53 (1H, dd, J = 10.7, 3.4 Hz, H-7), 3.16 (1H, dd, J = 11.0, 4.9 Hz, H-3), 2.86(1H, dd, J = 13.4, 3.6 Hz, H-14), 1.85 (3H, s, H-20), 1.67 (3H, s, H-17), 1.50 (3H, s, H-18), 1.13 (3H, s, H-19). ^{13}C-NMR (150 MHz, pyridine-d_5) δ_C 52.0 (C-1), 36.0 (C-2), 82.9 (C-3), 52.8 (C-4), 84.9 (C-5), 74.4 (C-6), 44.5 (C-7), 51.9 (C-8), 55.4 (C-9), 79.6 (C-10), 23.5 (C-11), 28.5 (C-12), 56.6 (C-13),78.4 (C-14), 60.6 (C-15), 80.0 (C-16), 24.1 (C-17), 22.7 (C-18), 20.0 (C-19), 27.3 (C-20). ESI-MS: $[M+Na]^+$, $[C_{20}H_{34}O_6Na]^+$ calcd.: *m/z* 393.2253, found: *m/z* 393.2265.

Grayanotoxin I (5)*:* ^{1}H-NMR (300 MHz, pyridine-d_5) δ_H 4.21 (1H, dd, J = 10.4, 4.6 Hz, H-7), 3.93 (1H, m, H-14), 3.29 (1H, t, J = 8.2 Hz, H-3), 1.94 (3H, s, H-22), 1.87 (3H, s, H-20), 1.69 (3H, s, H-17), 1.50 (3H, s, H-18), 1.29 (3H, s, H-19). ^{13}C-NMR (75 MHz, pyridine-d_5) δ_C 51.7 (C-1), 36.2 (C-2), 83.0 (C-3), 52.1 (C-4), 84.8 (C-5), 74.0 (C-6), 44.5 (C-7), 51.4 (C-8), 56.0 (C-9), 78.3 (C-10), 23.7 (C-11), 28.6 (C-12), 55.4 (C-13), 83.2 (C-14), 61.6 (C-15),78.9 (C-16), 24.3 (C-17), 22.8 (C-18), 20.1 (C-19), 27.7 (C-20), 170.7 (C-21), 21.6 (C-22). ESI-MS: $[M+Na]^+$, $[C_{22}H_{36}O_7Na]^+$ calcd.: *m/z* 435.2359 found: *m/z* 435.2370.



1. R_1 = OH; R_2 = H
2. R_1 = OH; R_2 = OH
3. R_1 = H; R_2 = H

4. R = H
5. R = $COCH_3$

Figure 1. Structures of isolated compounds

3. Conclusions

In this study, five grayanane-type diterpenoids were isolated from *R. brachycarpum.* Their structures were elucidated by detailed spectroscopic analyses and confirmed by comparison with previously reported data. The compounds were identified as grayanoside B (**1**), rhodomoside A (**2**), piersformoside (**3**), grayanotoxin III (**4**), and grayanotoxin I (**5**). The evaluation of the anti-tyrosinase and elastase activities of these compounds is reported for the first time. The results provided valuable preliminary data that may serve as a foundation for future biological screenings and pharmacological investigations.

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