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Antibacterial Phytosterols and Alkaloids from *Lycoris radiata*

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Abstract – This research aimed to investigate the antibacterial activity of *Lycoris radiata*. The methanol extract and solvent fractions from *L. radiata* exhibited antibacterial activities against *Escherichia coli*, *Staphylococcus aureus*, and *Helicobactor pylori*. Open-column chromatography was used to isolate phytochemical constituents from *L. radiata*; spectroscopic analysis elucidated their structures as β-sitosterol (**1**), daucosterol (**2**), *O*-methyllycorenine (**3**), lycorenine (**4**), lycoricidinol (**5**), lycorine (**6**), and lycoricidine (**7**). Further testing of compounds **1** - **7** revealed antibacterial effects against *E. coli*, *S. aureus*, and *H. pylori*, which suggested the potential of these substances as antibacterial agents. We determined that compounds **1** and **2**, isolated from the *n*-hexane fraction, were more effective against *S. aureus* and *H. pylori*. Compound **4**, isolated from the methylene chloride fraction, exhibited noticeable antibacterial effects against *E. coli.* This study is the first report on the antibacterial activities of phytochemical constituents from *L. radiata* against *E. coli*, *S. aureus*, and *H. pylori*.
Keywords – *Lycoris radiata*, Alkaloid, Amaryllidaceae, Bacteria

Introduction

Lycoris species are richly represented in the tropics and have pronounced centers of diversity in South Africa and the Andean region. A particular characteristic of Amaryllidaceae is the consistent presence of an exclusive group of unique alkaloids, which have been isolated from the plants of all genera in this family. Amaryllidaceae have been used for thousands of years as herbal remedies.[1] The Amaryllidaceae alkaloids have also been shown to have a variety of biological activities such as anti-tumor, anti-viral, immuno-stimulant, and anti-malarial activities in addition to activity on the central nervous system.[2]

The genus *Lycoris*, a small Amaryllidaceae group that comprises approximately 20 species, is only distributed in moist, warm-temperature woodlands of Eastern Asia from China to Japan and Korea, with a few species extending to northern Indochina and Nepal.[3] The representative *Lycoris* species in Korea are *L. chinensis* var. *sinuolata*, *L. chejuensis*, *L. flavescens*, *L. uydoensis*, *L. squamigera*, *L. sanguinea* var. *koreana*, and *L. radiata*.[4] The major chemical constituents of this genus are alkaloids, which show acetylcholinesterase-inhibitory, anti-tumor, anti-viral, and anti-malarial activities.[2] *L. radiata* contains biologically active chemicals that include lycorine, lycoricidinoal, lycoricidine, galanthamine, lycoramine, galanthamine *N*-oxide, lycoramine *N*-oxide, vittatine, tazettine, haemanthidine, *O*-demethyllycoramine, *O*-methylycorenine, homolycorine *N*-oxide, *O*-demethyhomolycorine, and dipalmitoylphosphatidylcholine.[5-7] Among these chemicals, lycorine is also known to be a powerful plant growth inhibitor[8] and galantamine hydrobromide is used clinically for the treatment of Alzheimer's disease.[9]

The clinical efficacy of many existing anti-biotics is being threatened by the emergence of multidrug-resistant pathogens.[10] Plant products, either as pure compounds or as standardized extracts, provide promising opportunities for new anti-infective drugs. There is an urgent need to discover new anti-microbial compounds with diverse chemical structures and novel mechanisms of action that can be used to treat new and re-emerging infectious diseases.[11] Therefore, researchers are increasingly investigating natural products, seeking to develop better anti-microbial drugs.[12-17]

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This paper discusses the isolation and identification of phytochemical compounds from *L. radiata* and the antibacterial activities of these compounds against *Escherichia coli*, *Staphylococcus aureus*, and *Helicobacter pylori*.

Experimental

Plant materials – The *L. radiata* bulbs were obtained from Yeong-Gwang Agricultural Technology Center, Korea in 2012.

Instruments and reagents – Electron ionization mass spectrometry (EI-MS) was performed with a mass spectrometer (JEOL JMS-600W, Tokyo, Japan). ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectra were recorded with a NMR spectrometer (Bruker AVANCE 500 NMR, Rheinstetten, Germany) in $CDCl_3$ or pyridine-d_6 using TMS as an internal standard. Chemical shifts were reported in parts per million (δ), and coupling constants (*J*) were expressed in Hertz (Hz). TLC analysis was conducted with Kiesel gel 60 F254 (Art. 5715, Merck Co., Germany) plates (silica gel, 0.25 mm layer thickness), and compounds were visualized by spraying with 10% H_2SO_4 followed by charring at 60°C. Silica gel (200 - 400 mesh, Merck, Germany) was used to isolate the constituents. Medium pressure liquid chromatography (MPLC) and cartridges (KP-SIL, 39 × 225 mm, Biotage, Uppsala, Sweden) was used. All other chemicals and reagents were of analytical grade.

Extraction, fractionation, and isolation – The air-dried *L. radiata* bulbs (7 kg) were extracted with MeOH (10 L × 3) under reflux. The resulting extracts were combined and concentrated under reduced pressure to yield 1,387 g of residue. Combined MeOH extract was then suspended in H_2O and successively partitioned with equal volumes of *n*-hexane (78.0 g), methylene chloride (MC) (17.0 g), ethyl acetate (EtOAc) (12.0 g), and *n*-butanol (*n*-BuOH) (74.0 g). The *n*-hexane fraction (78.0 g) was subjected to MPLC eluted with an *n*-hexane/EtOAc gradient (100 : 0 → 0 : 100). Fractions were combined according to their TLC behavior in order to obtain 12 fractions (LRH-1 → LRH-12) including compounds **1** and **2** (LRH-2, 42 mg and LRH-3, 42 mg, respectively). The MC fraction (17.0 g) was subjected to MPLC eluted with an *n*-hexane/EtOAc gradient (100 : 0 → 0 : 100). Fractions were combined according to their TLC behavior in order to obtain 11 fractions (LRC-1 → LRC-11) including compounds **3** and **4** (LRC-10, 31 mg and LRC-11, 27 mg, respectively). The EtOAc fraction (12.0 g) was subjected to MPLC eluted with a $CHCl_3$/MeOH gradient (100 : 0 → 0 : 100). Fractions were combined according to their TLC behavior in order to obtain 12 fractions (LCE-1 → LCE-12) including compound **5** (LCE-1, 25 mg). The *n*-BuOH fraction (74.0 g) was subjected to MPLC eluted with a $CHCl_3$/MeOH gradient (100 : 0 → 0 : 100). The fractions were combined according to their TLC behavior in order to obtain 10 fractions (LCB-1 → LCB-10). LCB-2 was separated in a Sephadex LH-20 column (φ 1.0 × 32 cm) eluted with 100% MeOH eluent to obtain 4 fractions (LCB 2.1-2.4) including compound **6** (LCB-2-3, 35 mg). LCB-7 (450 mg) was separated in a Sephadex LH-20 column (φ 1.0 × 32 cm) eluted with 50% MeOH eluent to obtain 4 fractions (LCB-7.1-7.4) including compound **7** (LCB-7-3, 42 mg).

***β*-Sitosterol (1)** – White crystals; EI-MS (rel. int., %): *m/z* 414 $[M]^+$ (100.0), 396 (49.9), 381 (24.3), 329 (28.0), 303 (32.3), 273 (32.7), 255 (69.3), 213 (37.9), 159 (42.9), 145 (45.1); ^{1}H-NMR (500 MHz, $CDCl_3$): δ 3.53 (m, 3-H), 5.35 (br d, *J* = 4.8 Hz, 6-H), 0.70 (s, 18-H), 1.00 (s, 19-H), 0.92 (d, *J* = 6.3 Hz, 21-H), 0.85 (d, *J* = 6.3 Hz, 26-H), 0.88 (d, *J* = 6.3 Hz, 27-H), 0.79 (t, *J* = 6.0 Hz, 29-H); ^{13}C-NMR (125 MHz, $CDCl_3$): δ 37.4 (C-1), 29.8 (C-2), 72.0 (C-3), 39.9 (C-4), 141.1 (C-5), 122.2 (C-6), 32.0 (C-7), 31.8 (C-8), 50.3 (C-9), 36.6 (C-10), 21.2 (C-11), 40.7 (C-12), 42.4 (C-13), 56.9 (C-14), 24.4 (C-15), 28.4 (C-16), 56.2 (C-17), 11.9 (C-18), 19.1 (C-19), 36.3 (C-20), 18.9 (C-21), 34.1 (C-22), 26.2 (C-23), 46.0 (C-24), 29.3 (C-25), 19.9 (C-26), 19.5 (C-27), 23.2 (C-28), 12.1 (C-29).

Daucosterol (2) – White powder; FAB-MS: *m/z* 577 $[M + H]^+$; ^{1}H-NMR (500 MHz, $CDCl_3$): δ 3.59 (m, 3-H), 5.26 (br d, *J* = 4.8 Hz, 6-H), 0.66 (s, 18-H), 0.99 (s, 19-H), 1.00 (d, *J* = 5.6 Hz, 21-H), 0.86 (d, *J* = 7.1 Hz, 26-H), 0.84 (d, *J* = 7.1 Hz, 27-H), 0.91 (t, *J* = 8.0 Hz, 29-H), 4.22 (d, *J* = 7.8 Hz, H-1'); ^{13}C-NMR (125 MHz, $CDCl_3$): δ 36.8 (C-1), 29.3 (C-2), 78.7 (C-3), 38.3 (C-4), 140.4 (C-5), 121.2 (C-6), 31.4 (C-7), 31.3 (C-8), 49.6 (C-9), 36.2 (C-10), 20.6 (C-11), 40.1 (C-12), 41.8 (C-13), 56.2 (C-14), 23.9 (C-15), 27.8 (C-16), 55.1 (C-17), 11.7 (C-18), 19.1 (C-19), 35.5 (C-20), 18.6 (C-21), 33.3 (C-22), 25.4 (C-23), 45.1 (C-24), 28.7 (C-25), 18.9 (C-26), 19.7 (C-27), 22.6 (C-28), 11.8 (C-29), 100.8 (C-1'), 73.5 (C-2'), 76.9 (C-3'), 70.1 (C-4'), 76.7 (C-5'), 61.1 (C-6').

***O*-Methyllycorenine (3)** – White powder; EI-MS (rel. int., %): *m/z* 331 $[M]^+$ (0.5), 299 (11.8), 280 (1.4), 191 (10.4), 145 (10.1), 109 (100.0); ^{1}H-NMR (500 MHz, $CDCl_3$): δ 2.12 (3H, s, NMe), 3.16 (1H, m), 3.55 (3H, s, OMe), 3.87, 3.89 (each, 3H, s, OMe), 4.31 (1H, m, 1-H), 5.50 (1H, m, 3-H), 5.52 (1H, s, 6β-H), 6.79, 6.88 (ea. 1H, s, 7-H, 10-H); ^{13}C-NMR (125 MHz, $CDCl_3$): δ 66.7 (C-1), 32.1 (C-2), 115.5 (C-3), 140.8 (C-4), 67.5 (C-4a), 98.7

(C-6), 130.1 (C-6a), 109.7 (C-7), 148.1 (C-8), 148.8 (C-9), 112.8 (C-10), 125.0 (C-10a), 44.9 (C-10b), 28.6 (C-11), 56.0 (C-12), 55.1 (OMe), 55.3 (OMe), 55.5 (OMe), 43.6 (NMe).

Lycorenine (4) – White powder; EI-MS (rel. int., %): *m/z* 317 $[M]^+$ (1.2), 299 (23.5), 281 (2.4), 266 (4.3), 256 (1.8), 191 (1.6), 109 (100.0); ^{1}H-NMR (500 MHz, $CDCl_3$): δ 2.12 (3H, s, NMe), 3.17 (1H, m, 12α-H), 3.55 (3H, s, OMe), 3.88, 3.89 (each, 3H, s, OMe), 4.31 (1H, m, 1-H), 5.49 (1H, m, 3-H), 5.53 (1H, s, 6β-H), 6.79, 6.88 (each 1H, s, 7-H, 10-H); ^{13}C-NMR (125 MHz, $CDCl_3$): δ 67.7 (C-1), 32.0 (C-2), 115.3 (C-3), 141.1 (C-4), 67.4 (C-4a), 91.7 (C-6), 130.0 (C-6a), 108.9 (C-7), 148.0 (C-8), 148.7 (C-9), 112.5 (C-10), 124.9 (C-10a), 45.0 (C-10b), 28.7 (C-11), 55.9 (C-12), 55.0 (OMe), 55.2 (OMe), 55.5 (OMe), 43.6 (NMe).

Lycoricidinol (5) – White powder; EI-MS (rel. int., %): *m/z* 307 $[M]^+$ (6.2), 289 (12.6), 271(100.0); ^{1}H-NMR (500 MHz, DMSO): δ 12.23 (1H, s, phenolic-OH), 4.40 - 5.50 (3H, 3 × OH), 7.85 (1H, s, NH), 3.70 - 4.20 (4H, complex, 2,3,4-H), 6.87 (1H, s, H-aromatic), 6.18 (1H, m, 1-H), 6.12 (2H, br s, O-CH_2-O); ^{13}C-NMR (125 MHz, DMSO): δ 168.4 (C-6), 151.8 (C-9), 144.3 (C-7), 132.8 (C-8), 131.6 (C-10a), 128.7 (C-10b), 124.2 (C-1), 105.0 (C-6a), 95.3 (C-10), 71.8 (C-3), 68.3 (C-4), 68.2 (C-2), 52.4 (C-4a).

Lycorine (6) – White powder; EI-MS (rel. int., %): *m/z* 287 $[M]^+$ (55.0), 268 (29.8), 256 (6.8), 240 (5.2), 226 (100.0); ^{1}H-NMR (500 MHz, DMSO): δ 6.80 (s, 11-H), 6.67 (s, 8-H), 5.95 (s, 12-H), 5.36 (s, 3-H), 4.88 (br s, 1-H), 4.78, 4.27 (1H each, 7-H), 4.01 (m, 2-H), 3.97 (*d*, *J* = 11.8 Hz, 11c-H), 3.32, 3.18 (1H each, m, 5-H), 2.60 (d, J = 11.8 Hz, 11b-H), 2.51 (m, 4-H); ^{13}C-NMR (125 MHz, DMSO): δ 145.6 (C-9), 145.1 (C-10), 141.6 (C-3a), 127.7 (C-7a), 129.6 (C-11a), 118.4 (C-3), 106.9 (C-8), 105.0 (C-11), 100.5 (C-12), 71.6 (C-2), 70.1 (C-1), 60.7 (C-11c), 56.8 (C-5), 53.2 (C-7), 48.2 (C-11b), 28.0 (C-4).

Lycoricidine (7) – White powder; EI-MS (rel. int., %): *m/z* 291 $[M]^+$ (21.4), 271 (25.2), 255 (94.4), 231 (100.0); ^{1}H-NMR (500 MHz, DMSO): δ 8.40 (1H, br s, NH), 7.94 (1H, s, 7-H), 7.22 (1H, s, 10-H), 6.60 (1H, m, C=CH-), 6.50 - 6.00 (3H, OH), 5.98 (2H, dd, OCH_2O), 5.20-4.60 (4H, m, 2,3,4,4a-H); ^{13}C-NMR (125 MHz, DMSO): δ 163.4 (C-6), 150.1 (C-9), 147.7 (C-8), 131.7 (C-6a), 130.0 (C-10a), 123.6 (C-10b), 121.9 (C-1), 106.1 (C-7), 103.3 (C-10), 72.0 (C-3), 69.2 (C-4), 69.2 (C-2), 52.4 (C-4a).

Microorganisms and media preparation – *E. coli* and *S. aureus* were provided by Korean Culture Center of Microorganisms (KCCM, Seoul, Korea). Trypticase Soy Agar (TSA) was purchased from BD Difco (NJ, USA), and disc paper was obtained from Adabantec (Tokyo, Japan). The TSA culture medium contained 15 g casein pancreatic digest, 5 g papaic soybean digest, 5 g NaCl, 15 g sodium chloride, and 15 g agar in 1 L of distilled water. Microaerophilic conditions were maintained at 37 ºC. *H. pylori* was provided by Korean Type Culture Collection (KTCC, Daejeon, Korea), and was cultured in Brucella broth (Difco, NJ, USA) containing 10% horse serum (Welgene, Daegu, Korea) and, for testing, was grown on a medium prepared with (per liter) BD Bactodextrose (1 g), BD Bactoyeast extract (2 g) (Becton, Dickinson and Company [BD], Franklin Lakes, NJ, USA), sodium chloride (5 g), and sodium bisulfate (0.1 g).

Antibacterial activity – Antibacterial activity against *S. aureus*, *E. coli*, and *H. pylori* was tested by the disc agar method.[18] Plates of medium were spread with 0.1 mL of culture broth, and 50 and 100 μg/30 μL of the fractions and 15 and 30 μg/30 μL of compounds were pipetted onto sterile filter paper discs (8 mm). Inhibition zones were determined after 24 hr at 37 ºC.

Minimum inhibitory concentration (MIC) – The assessment of MIC test was based on the measurement of diameter of inhibition zone formed around the disc. The discs (diameter, 8 mm) were each impregnated with 7 kinds of compounds at a concentration 1, 5, 10, 15, and 30 μg/30 μL placed on the inoculated agar, and incubated at 37 ºC for 24 hr.

Results and Discussion

This study evaluated the antibacterial properties of *L. radiata* against *E. coli*, *S. aureus*, and *H. pylori*. Inhibitory activities of the MeOH extract and solvent fractions from *L. radiata* on microbial growth are summarized in Tables 1 and 2. The MeOH extract and fractions inhibited the growth of *E. coli* and *S. aureus*, forming inhibition zones 11 - 13 mm at 100 μg/30 μL concentration. The *n*-hexane fraction showed the greatest zone of inhibition against *S. aureus* (13 mm) and *H. pylori* (11 mm). In addition, the MC fraction produced the highest inhibition zone against *E. coli* at 13 mm. A chromatographic separation of the MeOH extract from *L. radiata* led to the isolation of compounds **1** - **7** (Fig. 1). Their structures were elucidated

Table 1. Antibacterial activity of *L. radiata* MeOH extract against *E. coli*, *S. aureus*, and *H. pylori*

Extract	Concentration (μg/30 μL)	Clear zone (mm)		
		E. coli	*S. aureus*	*H. pylori*
MeOH	50	10	12	9
	100	11	13	9

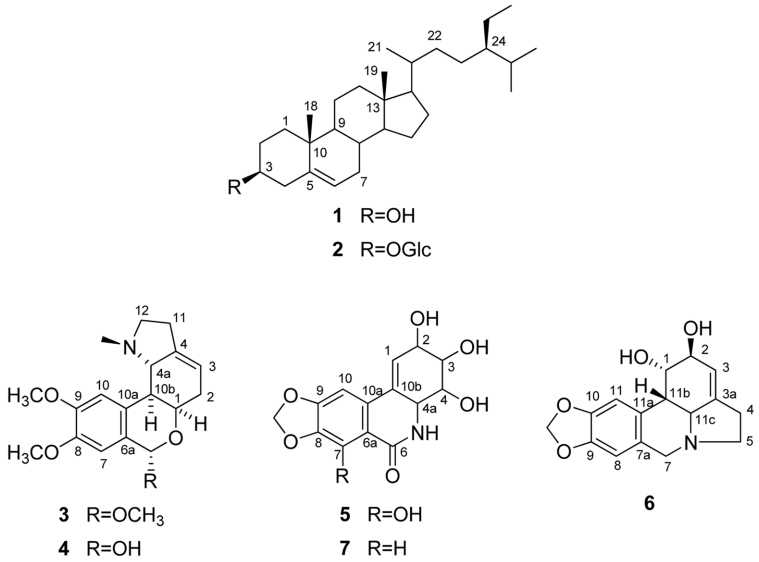

Fig. 1. Structures of compounds **1** - **7**.

Table 2. Antibacterial activity of *L. radiata* fractions against *E. coli*, *S. aureus*, and *H. pylori*

Fractions (100 μg/30 μL)	Clear zone (mm)		
	E. coli	*S. aureus*	*H. pylori*
n-Hexane	11	13	11
MC	13	12	10
EtOAc	12	12	–
n-BuOH	11	10	–

–: no growth

as β-sitosterol (**1**), daucosterol (**2**), *O*-methyllycorenine (**3**), lycorenine (**4**), lycoricidinol (**5**), lycorine (**6**), and lycoricidine (**7**) by comparing the spectral data as described in the literature.[19-24]

The anti-microbial activities of compounds **1** - **7** from *L. radiata* against *E. coli*, *S. aureus*, and *H. pylori* are shown in Table 3. The compounds were treated with concentrations of 15 and 30 μg/ 30 μL for 24 hr. The antibacterial activities of all compounds against *E. coli*, a Gram-negative bacterium, are shown with inhibition zones of 9 - 14 mm and 12 - 15 mm at concentrations of 15 and 30 μg/30 μL, respectively. In particular, compound **4** exerted the greatest clear zone, which showed 15 mm at a concentration of 30 μg/30 μL. In comparison, the anti-bacterial effects against *S. aureus*, a Gram-positive bacterium, were observed with 3 units (compounds **1**, **3**, and **4**) that inhibited zones larger than 14 mm at a concentration of 30 μg/30 μL. Specifically, compound **1** showed the greatest inhibition zone (15 mm) against *S. aureus* at both concentrations. The results of the activity

Table 3. Antibacterial activity of compounds **1** - **7** from *L. radiata* against *E. coli*, *S. aureus*, and *H. pylori*

Compounds	Concentration (μg/30 μL)	Clear zone (mm)		
		E. coli	*S. aureus*	*H. pylori*
1	15	13	15	–
	30	13	15	–
2	15	10	8	–
	30	14	9	9
3	15	9	12	–
	30	14	14	–
4	15	9	10	–
	30	15	14	–
5	15	14	12	–
	30	13	11	–
6	15	11	11	–
	30	13	11	–
7	15	11	11	–
	30	12	11	–
Penicillin*	15	25	23	17
	30	25	28	17

*Penicillin was used as a positive control.
–: no growth

against *H. pylori*, which cause gastritis and gastric cancer, indicated that there was relatively low activity (8 mm). Compound **2** showed an inhibition zone of 9 mm, which demonstrated an anti-microbial effect.[25] The MIC of compounds **1** - **7** against *E. coli*, *S. aureus*, and *H. pylori* are shown in Table 4. The result revealed that compounds **1**-**7** showed MIC values from 5 to 30 μg/30 μL and

Table 4. MIC of compounds **1**-**7** from *L. radiata* against *E. coli*, *S. aureus*, and *H. pylori*

Compounds	Concentration (μg/30 μL)	Clear zone (mm)		
		E. coli	*S. aureus*	*H. pylori*
1	30	++	++	–
	15	++	++	–
	10	+	–	–
	5	+	–	–
	1	+	–	–
	MIC	N/D	15	N/A
2	30	++	+	+
	15	+	–	–
	10	+	–	–
	5	+	–	–
	1	+	–	–
	MIC	N/D	30	30
3	30	++	++	–
	15	+	+	–
	10	+	+	–
	5	+	+	–
	1	-	+	–
	MIC	5	N/D	N/A
4	30	++	++	–
	15	+	+	–
	10	–	–	–
	5	–	–	–
	1	–	–	–
	MIC	15	15	N/A
5	30	++	+	–
	15	++	+	–
	10	+	–	–
	5	+	–	–
	1	–	–	–
	MIC	5	15	N/A
6	30	++	+	–
	15	+	+	–
	10	–	+	–
	5	–	–	–
	1	–	–	–
	MIC	15	10	N/A
7	30	+	+	–
	15	+	+	–
	10	+	+	–
	5	+	+	–
	1	+	-	–
	MIC	N/D	5	N/A
Penicillin*	10	+++	++	++

*Penicillin was used as a positive control.
Inhibition zone: –, none; +, 9~12 mm; ++, 13~15 mm, +++, > 15 mm.
N/D: Not detected within the concentration limit > 1 μg / 30 μL
N/A: Not active within the concentration limit < 30 μg / 30 μL

classified as "+", "++", and "+++". Among them, compounds **3** and **5** showed "+" at the concentration of 5 μg/30 μL against *E. coli*. However, compounds **1**, **2**, and **7** were not detected within the concentration limit > 1 μg/ 30 μL. For compound **7** showed "+" results against *S. aureus*. Furthermore, there were not active against *H. pylori* except for compound **2** (30 μg/30 μL).

In conclusion, compounds **1** and **2** isolated from the *n*-hexane fraction are effective against *S. aureus* and *H. pylori*. Moreover, compound **4** isolated from the MC fraction has strong antibacterial activity against *E. coli*. *β*-Sitosterol (**1**), a well-known plant sterol, reduces serum cholesterol levels and prevents cardiovascular events by inhibiting cholesterol absorption in the intestines.[21] Daucosterol (**2**) has an immunoregulatory effect on disseminated candidiasis caused by *Candida albicans*.[26] Lycorenine (**4**) is a compound that produced a decrease in blood pressure in dogs, cats, and rabbits, a transient increase of contractile force in the isolated toad heart, and an increase in motility of isolated rabbit ileum.[27]

The antibacterial activity of *L. radiata* compounds was previously untested and this study is the first to report the anti-microbial properties of *L. radiata* compounds. There is enormous potential for developing anti-microbials from plant compounds, and these compounds may not produce the same toxicity associated with synthetic antimicrobials. In conclusion, *β*-sitosterol (**1**), daucosterol (**2**), *O*-methyllycorenine (**3**), lycorenine (**4**), lycoricidinol (**5**), lycorine (**6**), and lycoricidine (**7**) were isolated from the *L. radiata* bulbs, and the antibacterial activities of these compounds were confirmed. These biologically active constituents may potentially provide inhibitory agents against *E. coli*, *S. aureus*, and *H. pylori*. Therefore, *L. radiata* can be used as an easily-accessible and natural antibacterial source. Additional studies should be conducted, such as radical scavenging, to further characterize these extracts as biological antioxidants.

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