JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

New Quinolinone Alkaloids from Chestnut (*Castanea crenata* Sieb) Honey

Jeong-Yong Cho,[†,⊥] Sun-Hee Bae,[†,⊥] Hye-Kyung Kim,[‡] Myeong-Lyeol Lee,[‡] Yong-Soo Choi,[‡] Byung-Rae Jin,[§] Hyoung Jae Lee,[†] Hang Yeon Jeong,[†] Yu Geon Lee,[†] and Jae-Hak Moon*[,†]

[†]Department of Food Science & Technology, and Functional Food Research Center, Chonnam National University, Gwangju 500-757, Republic of Korea

[‡]Department of Agricultural Biology, National Academy of Agricultural Science, RDA, Suwon 441-100, Republic of Korea

[§]College of Natural Resources and Life Science, Dong-A University, Busan 604-714, Republic of Korea

ABSTRACT: Two new quinolinone alkaloids and 13 known compounds were isolated from chestnut (*Castanea crenata* Sieb) honey. Two new compounds were determined to be 3-dihydro-spiro[2(1*H*),3′(1′*H*)-diquinoline]-3′,4,4′-trione (spirodiquinolinone) and 3-(2′-piperidine)-kynurenic acid. In addition, 2,3-dihydropyrrolo[1,2-*a*]quinazolin-5(1*H*)-one was identified for the first time from nature. In addition, 2,3-dihydropyrrolo[1,2-*a*]quinazolin-5(1*H*)-one was newly identified from chestnut honey, although this compound has been synthesized before. The structures were determined by the NMR and electrospray ionization−mass spectroscopy (ESI−MS). Three compounds were qualified and quantitated in chestnut honey by selective multiple reaction monitoring (MRM) detection of LC−ESI−MS using the isolated compounds as external standards.

KEYWORDS: *chestnut honey, Castanea crenata, alkaloid, quinolinone, kynurenic acid*

INTRODUCTION

Honey is a natural sweet product produced by honey bees from the nectar of flowers and has long been used as food and folk remedy. Honey quality varies depending on the nectar source of the plants such as chestnut, acacia, apple, cranberry, sunflower, and vegetables.[1] Many studies have reported that honeys exert various biological effects including gastroprotective,[2] anti-inflammatory,[3] antidiabetic,[4] antimicrobial,[5] anticancer,[6] and antioxidative[7] activities. Honey is composed mainly of sugars (glucose and fructose) and various bioactive constituents including minerals, vitamins, phenolics (flavonoids and phenolic acids), and numerous volatile compounds.[7,8] Additionally, several studies have reported that quinolinone alkaloids are present in honeys with the highest concentrations in chestnut honey apart from sugars.[1,9−12] During the course of investigations on chemical constituents of honeys, to understand their quality and botanical origins, we isolated and identified 16 compounds including two new and four known quinolinone alkaloids from chestnut (*Castanea crenata* Sieb) honey. In this study, we determined the structures of two new quinolinone alkaloids from chestnut honey. In addition, we identified a quinazolinone alkaloid, previously known as a synthetic compound and now found as a natural product for the first time.

MATERIALS AND METHODS

General Experimental Procedures. NMR spectra were obtained with a ^{unit}INOVA 500 spectrometer (Varian, Walnut Creek, CA). Pyridine-d_5 and methanol-d_4 (CD_3OD) containing tetramethylsilane were used as analytical solvents. All mass spectra were acquired on a SYNAPT G2 hybrid (Waters, Cambridge, UK) equipped with an electrospray ionization source (ESI−MS). Thin-layer chromatography (TLC) was carried out by using silica gel TLC plates (silica gel 60 F254, 0.25 mm thickness) (Merck, Rahway, NJ) and developed using a mixture of *n*-butanol/acetic acid/H_2O (4:1:2, v/v/v). The fractions were visualized by UV and 1% cerium (**IV**) sulfate ethanol solution spray. Amberlite XAD-2 column (5.0 × 84 cm^2, 521 μm) (Supelco, Bellefonte, PA, USA) was used for column chromatography. Fractions were purified by high-performance liquid chromatography (HPLC) equipped with a Shim-pack Prep-ODS (H) Kit (5 μm, 20 × 250 mm^2) (Shimadzu, Kyoto, Japan). Flow rate was 9.9 mL/min, and eluents were monitored at 254 nm. Solvents used for analyses were of HPLC grade. All other chemicals including trifluoroacetic acid (Sigma Chemical Co., St. Louis, MO) used were of reagent grade.

Materials. Chestnut (*C. crenata* Sieb) honey was collected for 3 weeks in June 2012 at a chestnut farm in Chungju, Korea based on information provided by the beekeepers. The chestnut honey was immediately treated after collection.

Extraction and Solvent Fractionation. The honey (18 kg) was homogenized with methanol (MeOH, 18 L). After a 2 h extraction at room temperature, the MeOH extract was filtered under vacuum through No. 2 filter paper (Whatman, Maidstone, UK). The residue was extracted with MeOH (15 L) for 24 h at 4 °C with continuous agitation and then filtered under vacuum through No. 2 filter paper (Whatman). Finally, the residue was extracted with ethanol (EtOH, 4.5 L) for 24 h at 4 °C with shaking to give the EtOH extract. The solutions extracted with MeOH and EtOH were combined and concentrated under vacuum at 38 °C. The MeOH and EtOH extracts (11.76 kg) were suspended in distilled water (9.4 L) and successively partitioned with *n*-hexane (9.4 L, three times), ethyl acetate (EtOAc, 9.4 L, three times), and water-saturated *n*-butanol (9.4 L, three times). The fractions were respectively then concentrated in vacuo.

Isolation. A portion (34.7 g) of the EtOAc fraction (48.16 g) was fractionated by chromatography on an Amberlite XAD-2 column (5.0 × 84 cm^2) eluted with a stepwise system of H_2O/MeOH (10:0, v/v, 5.2 L) and H_2O/MeOH (8:2, 6:4, 8:2, 0:10, v/v, each 2.6 L). The nine

Received: February 25, 2015
Revised: March 20, 2015
Accepted: March 21, 2015
Published: March 21, 2015

DOI: 10.1021/acs.jafc.5b01027
J. Agric. Food Chem. 2015, 63, 3587−3592

Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data of 13 and 14[a]

13			14		
position	δ_H (int., mult., J in Hz)	δ_c	position	δ_H (int., mult., J in Hz)	δ_c
1	8.52 (1H, s)		1	ND[b]	
2		68.0	2		143.0
3	3.76 (1H, d, 13.5)	40.9	3		125.5
	3.59 (1H, d, 13.5)				
4		189.5	4		176.3
4a		117.7	4a		128.1
5	8.15 (1H, dd, 7.0, 1.5)	126.8	5	8.32 (1H, d, 8.0)	126.5
6	6.75 (1H, br. t, 7.0)	116.1	6	7.46 (1H, m)	125.7
7	7.30 (1H, td, 7.0, 1.5)	134.7	7	7.76 (1H, br. d, 3.5)[c]	134.1
8	7.06 (1H, br. d, 7.0)	114.7	8	7.76 (1H, br. d, 3.5)[c]	120.5
8a		149.6	8a		141.9
1′	12.26 (1H, s)		2′		162.6
2′		169.8	2″	4.46 (1H, dd, 11.8, 3.8)	58.9
4′		191.3	3″	2.78 (1H, m)	32.6
				1.09 (1H, m)	
4′a		117.9	4″	1.98 (1H, m)	24.2
				1.75 (1H, m)	
5′	8.02 (1H, dd, 7.0, 1.0)	125.4	5″	1.90 (1H, m)	27.0
				1.44 (1H, m)	
6′	7.01 (1H, br. t, 7.0)	121.5	6″	4.37 (1H, dd, 13.0, 5.0)	41.5
				3.14 (1H, dd, 13.0, 3.5)	
7′	7.40 (1H, td, 7.0, 1.5)	133.7			
8′	7.04 (1H, br. d, 7.0)	115.1			
8′a		141.0			

[a]Frequency was 500 MHz for ^{1}H NMR and 150 MHz for ^{13}C NMR in pyridine-d_5 for **13** and CD_3OD for **14**. [b]ND, not detected. [c]The chemical shifts of H-7 and H-8 overlapped.

fractions (G1−G9) were grouped according to the TLC profile. Fraction G3 (H_2O/MeOH, 6:4, v/v, 248.4 mg) was subjected to a semipreparative ODS-HPLC, using a linear gradient system of 5% MeCN (pH 2.65 by TFA, eluent A) and 60% MeCN (pH 2.65 by TFA, eluent B), from 100% A to 17% B for 30 min and increased to 100% B for 45 min, to yield fraction G3a (t_R 22.8 min, 5.2 mg), **2** (t_R 28.2 min, 9.1 mg), a mixture of **2** and **3** (t_R 28.8 min, 13.6 mg), a mixture of **4** and **5** (t_R 30.0 min, 10.1 mg), and **6** (t_R 33.5 min, 7.6 mg). Compound **1** (t_R 31.5 min, 2.4 mg) was isolated from fraction G3a (5.2 mg) by semipreparative ODS-HPLC, using a linear gradient system of 2% MeCN (pH 2.65 by TFA, eluent C) and 40% MeCN (pH 2.65 by TFA, eluent D), from 100% C to 17% D for 30 min.

Fraction G8 (H_2O/MeOH, 2:8, v/v, 1216.2 mg) was suspended in distilled water (100 mL) and partitioned with $CHCl_3$ (3 L, three times) to obtain G8-C ($CHCl_3$ layer, 779.4 mg). Fraction G8-C (779.4 mg) was subjected to semipreparative ODS-HPLC, using a linear gradient system of 30% MeOH (eluent E) and 70% MeOH (eluent F), from 100% E to 100% F for 30 min, to yield **7** (t_R 17.2 min, 4.0 mg), **8** (t_R 19.7 min, 7.6 mg), **9** (t_R 21.0 min, 75.0 mg), **10** (t_R 23.0 min, 67.5 mg), G8-C7 (t_R 24.7 min, 16.7 mg), G8-C8 (t_R 27.1 min, 20.2 mg), and G8-C9 (t_R 29.4 min, 32.1 mg). Through further purification by semipreparative ODS-HPLC, using a linear gradient system of 20% MeCN (pH 2.65 by TFA, eluent G) and 40% MeCN (eluent H), from 100% G to 100% H for 30 min and held at 100% H for 60 min, **11** (t_R 24.2 min, 1.1 mg), **12** (t_R 27.7 min, 3.0 mg), and **13** (t_R 29.5 min, 1.0 mg) from G8-C7 (16.7 mg), **14** (t_R 19.4 min, 1.1 mg) and **15** (t_R 26.8 min, 1.6 mg) from G8-C8 (20.2 mg), and **16** (t_R 31.4 min, 1.6 mg) from G8-C9 (32.1 mg) were isolated, respectively.

*Compound **9** (White Amorphous Powder).* $[\alpha]_D^{25}$ −0.03 (c 0.025, $CHCl_3$); UV $CHCl_3$ λ_{max} (nm) (log ε) 336 (3.82); ^{1}H NMR spectrum (500 MHz, pyridine-d_5, TMS) δ 8.48 (1H, d, J = 7.8 Hz, H-5), 7.42 (1H, t, J = 7.8 Hz, H-6), 7.71 (1H, t, J = 7.8 Hz, H-7), 7.83 (1H, d, J = 7.8 Hz, H-8), 4.02 (2H, t, J = 8.0 Hz, H-1′), 1.89 (2H, m, H-2′), 2.96 (2H, t, J = 7.0 Hz, H-3′); ^{13}C NMR (125 MHz, pyridine-d_5, TMS) δ 160.6 (C-2), 161.3 (C-4), 121.8 (C-4a), 127.0 (C-5), 126.6 (C-6), 134.6 (C-7), 127.9 (C-8), 150.8 (C-8a), 47.1 (C-1′), 19.8 (C-2′), 32.9 (C-3′); HRESI−MS (positive) m/z 187.0881 $[M + H]^+$ (calculated for $C_{11}H_{11}N_2O$, m/z 187.0871, +1.0 mDa).

*Compound **13** (White Amorphous Powder).* $[\alpha]_D^{25}$ −0.02 (c 0.013, $CHCl_3$); UV $CHCl_3$ λ_{max} (nm) (log ε) 348 (4.14); ^{1}H and ^{13}C NMR data shown in Table 1; HRESI−MS (positive) m/z 293.0916 $[M + H]^+$ (calculated for $C_{17}H_{13}N_2O_3$, m/z 293.0926, −1.0 mDa).

*Compound **14** (White Amorphous Powder).* $[\alpha]_D^{25}$ +0.08 (c 0.013, $CHCl_3$); UV $CHCl_3$ λ_{max} (nm) (log ε) 349 (4.04), 331 (4.01); ^{1}H and ^{13}C NMR data shown in Table 1; HRESI−MS (positive) m/z 277.0952 $[M + Na]^+$ (calculated for $C_{15}H_{14}N_2O_2Na$, m/z 277.0953, −0.1 mDa)

HPLC−ESI−MS/MS Analysis of 9, 13, and 14 in Chestnut Honey. The chestnut honey (20 g) was homogenized using a homogenizer (BM-2 Nissei biomixer) with MeOH (40 mL). After extraction for 24 h at room temperature, the mixture was filtered under vacuum through No. 2 filter paper (Whatman). The residue was extracted with EtOH (40 mL) for 24 h at 4 °C and then filtered under vacuum through No. 2 filter paper (Whatman). The MeOH and EtOH solutions were combined and concentrated under vacuum at 38 °C. The extracts were suspended in distilled water (60 mL) and partitioned with *n*-hexane (60 mL, three times) and EtOAc (60 mL, three times), successively. The EtOAc fraction was evaporated under vacuum at 38 °C. The EtOAc fraction (60 mg) was suspended in distilled water (50 mL) and partitioned with $CHCl_3$ (50 mL, three times). After evaporation under vacuum at 38 °C, the $CHCl_3$ fraction (11.5 mg) was dissolved in MeOH (4.6 mL). The fraction was analyzed using a high-performance liquid chromatography−electrospray ionization tandem mass spectrometer (HPLC−ESI−MS) (Shimadzu, Kyoto, Japan). Compounds **9**, **13**, and **14** were separated under the following HPLC conditions: column, MG III (C18, 3 μm, 3.0 × 100 mm^2) (Shiseido, Tokyo, Japan); flow rate, 0.2 mL/min (LC10AD, Shimadzu). The sample was eluted using a gradient system of 100% H_2O (containing 1% formic acid, eluent I) to 100% MeCN (containing 1% formic acid, eluent J), starting with 100% I, increasing

DOI: 10.1021/acs.jafc.5b01027
J. Agric. Food Chem. 2015, 63, 3587−3592

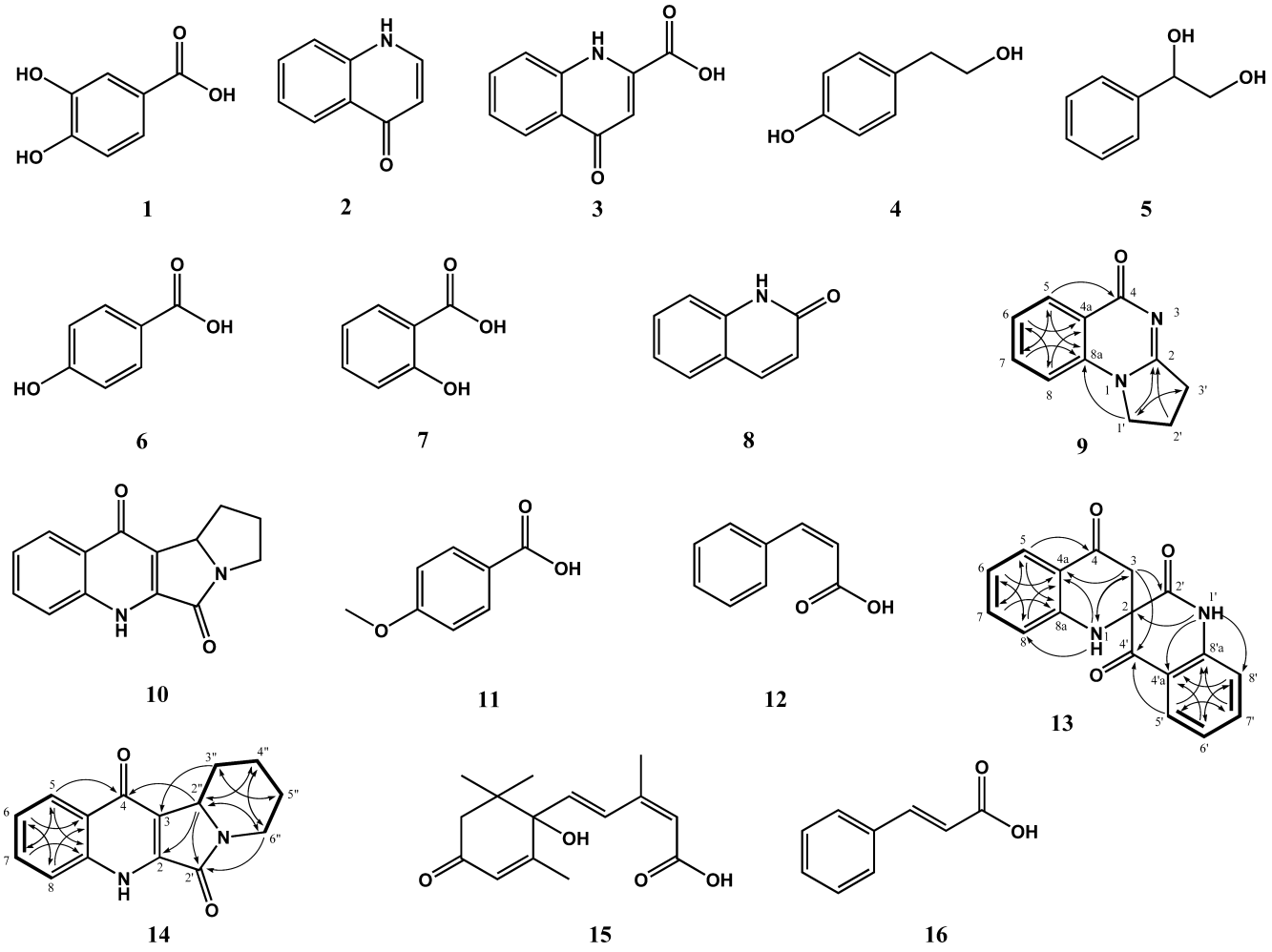

Figure 1. Structures of the isolated compounds from chestnut honey and the important $^{1}H-^{1}H$ correlation spectroscopy (COSY, bold lines) and heteronuclear multiple bond correlation (HMBC, arrows) correlations for **9**, **13**, and **14**.

to 20% J for 10 min, increasing to 50% J for 20 min, increasing to 100% J for 22 min, and holding at 100% J for 25 min. The mass spectrometer (positive ion mode) was set up for multiple reaction monitoring (MRM) to monitor m/z 187.0 → 92.1 for **9**, m/z 293.2 → 247.2 for **13**, and m/z 255.4 → 89.1 for **14** with the a dwell time of 0.1 s per transition. The optimal MS conditions for **9**, **13**, and **14** were employed; ESI source voltage 3.5 kV, detector voltage 45 V, heat block temperature 400 °C, and desolvation line temperature 250 °C. Nebulizing gas and drying gas flows were 3.0 L/min and 15.0 L/min, respectively. Argon was used as the collision gas at a pressure of 230 kPa. The optimized collision energies for **9**, **13**, and **14** were −36 eV, −26 eV, and −50 eV, respectively.

The content of **9**, **13**, and **14** in chestnut honey was analyzed quantitatively by HPLC−ESI−MS/MS analysis at the analytical scale. Sample and standard solutions were prepared just prior to analysis. The calibration curves (n = 6) were constructed using **9**, **13**, and **14** (0.1−50 ng) that were isolated from chestnut honey in this study. Accuracy and reproducibility were evaluated using the standard spike method. External standards of **9**, **13**, and **14** were added to aliquots of chestnut honey at three concentrations (n = 3) to determine their precision. The contents of **9**, **13**, and **14** in chestnut honey were determined in triplicate experiments.

RESULTS AND DISCUSSION

Thirteen known compounds isolated from chestnut honey were additionally identified as protocatechuic acid (**1**), 4(1*H*)-quinolinone (**2**), kynurenic acid (**3**), 4-(2-hydroxyethyl)phenol (**4**), 1-phenyl-1,2-ethanediol (**5**), *p*-hydroxybenzoic acid (**6**), salicylic acid (**7**), 2(1*H*)-quinolinone (**8**), 3-pyrrolidinyl kynurenic acid (**10**), 4-methoxybenzoic acid (**11**), *cis*-cinnamic acid (**12**), *trans*-abscisic acid (**15**), and *trans*-cinnamic acid (**16**) (Figure 2) based on NMR and MS spectroscopic data (Figure 1). All of these compounds have been found in chestnut honey or other honeys.[1,11−17]

The molecular formula of **9** was $C_{11}H_{10}N_2O$ (MW, 186), as established by a pseudomolecular ion peak in the HRESI−MS (positive). The ^{1}H NMR spectrum exhibited four sp^2 carbon proton signals of *ortho*-substituted benzene ring at δ 8.48 (1H, d, J = 7.8 Hz, H-5), 7.42 (1H, t, J = 7.8 Hz, H-6), 7.71 (1H, t, J = 7.8 Hz, H-7), and 7.83 (1H, d, J = 7.8 Hz, H-8). A propane moiety was also assigned by coupling patterns of three sp^3 methylene carbon protons at δ 4.02 (2H, t, J = 8.0 Hz, H-1′), 1.89 (2H, m, H-2′), and 2.96 (2H, t, J = 7.0 Hz, H-3′) in the ^{1}H NMR spectrum and their proton−proton correlations (bold lines) in the $^{1}H-^{1}H$ correlation spectroscopy (COSY) spectrum (Figure 1). This result was also supported by the ^{13}C NMR spectrum, which contained 11 carbon signals including eight sp^2 carbons with four quaternary carbons at δ 175.9−121.8 and three sp^3 carbons at δ 47.1 (C-1′), 32.9 (C-2′), and 19.8 (C-3′). Considering the molecular formula of **9**, the chemical shifts of four sp^2 quaternary carbons indicated that three of them were nitrogen-bearing carbons at δ 160.6 (C-2) and 150.8 (C-8a) with evidence of a carbonyl carbon at δ 161.3

DOI: 10.1021/acs.jafc.5b01027
J. Agric. Food Chem. 2015, 63, 3587−3592

(C-4). From the HRESI−MS and 1D NMR spectra, **9** was suggested to be quinazolinone connected with a propane moiety. The ^{1}H and ^{13}C NMR spectra of **9** were similar to those of 2,3-dihydropyrrolo[1,2-b]quinazolin-5(1*H*)-one, which has been synthesized as reported previously.[18] Complete NMR assignment and connectivity of **9** were further determined by ^{1}H−^{1}H COSY, heteronuclear single quantum correlation (HSQC), and heteronuclear multiple bond correlation (HMBC) experiments. Quinazolinone and propane moieties were also confirmed from the spectra. In particular, correlations (arrows) from H-1′ to C-8a and C-2 and from H-2′ to C-2 observed in the HMBC spectrum indicated that the propane moiety was connected with the 1 and 2 positions of the quinazolinone (Figure 1). Consequently, the structure of **9** was unambiguously determined to be 2,3-dihydropyrrolo[1,2-a]quinazolin-5(1*H*)-one (Figure 1). This compound has been synthesized, as reported previously.[19,20] However, this compound was identified for the first time in nature.

The molecular weight of **13** was determined to be 292 by the pseudomolecular ion peaks detected at *m*/*z* 293.1 $[M + H]^+$ and 315.1 $[M + Na]^+$ in the ESI−MS (positive) spectrum. The molecular formula ($C_{17}H_{12}N_2O_3$) was established by a pseudomolecular ion peak in the HRESI−MS (positive). The ^{1}H NMR spectrum of **13** showed the presence of eight proton signals occurring from two *ortho*-substituted benzene rings at δ 8.15−6.75. In addition, one methylene proton signal at δ 3.76 (1H, d, *J* = 13.5 Hz, H-3a) and 3.59 (1H, d, *J* = 13.5 Hz, H-3b) and two nitrogen-bearing proton signals at δ 8.52 (1H, s, H-1) and 12.26 (1H, s, H-1′) were observed. Considering the molecular formula and the chemical shifts of 17 carbon signals observed in the ^{13}C NMR spectrum of **13**, two *ortho*-substituted benzene ring moieties were assigned from 12 carbons at δ 149.6−114.7. Three carbonyl carbons at δ 189.5 (C-4), 169.8 (C-2′), and 191.3 (C-3′), one quaternary sp^3 carbon at δ 68.0 (C-2), and one sp^3 methylene carbon at δ 40.9 (C-3) were also observed, and four of them were nitrogen-bearing carbons at δ 169.8 (C-2′), 149.6 (C-8a), 141.0 (C-8′a), and δ 68.0 (C-2). From the MS and 1D-NMR data, the skeleton structures of **13** were suggested to be dihydroquinolinone and quinolinedione. In particular, the presence of one quaternary sp^3 carbon at δ 68.0 (C-2) indicated that **13** was a spiro compound consisting of dihydroquinolinone and quinolinedione. The complete NMR assignment and connectivity of **13** were further determined by the ^{1}H−^{1}H COSY, HSQC, and HMBC. The important correlations observed in ^{1}H−^{1}H COSY (bold lines) and the HMBC (arrows) spectra are shown in Figure 1, and the two partial structures of **13** were determined to be 2,3-dihydroquinolin-4(1*H*)-one and quinoline-2,4(1*H*,3*H*)-dione (Figure 1). In particular, correlations between H-1/C-8 and C-4a and H-3/C-4a were observed in the HMBC spectrum. Therefore, the planar structure of **13** was determined to be 3-dihydro-spiro[2(1*H*),3′(1′*H*)-diquinoline]-3′,4,4′-trione (spirodiquinolinone) (Figure 1), which is a new compound. However, structural determination for the absolute stereochemistry of **13** was impossible in the nuclear Overhauser effect spectroscopy experiment. Therefore, unambiguous determination of the absolute stereochemistry for **13** isolated in this study should be studied further with X-ray crystallography.

The molecular weight of **14** was determined to be 254 by the pseudomolecular ion peaks detected at *m*/*z* 255.1 $[M + H]^+$ and 277.1 $[M + Na]^+$ in the ESI−MS (positive) spectrum. The molecular formula ($C_{15}H_{14}N_2O_2$) was established by a pseudomolecular ion peak in the HRESI−MS (positive). The ^{1}H- and ^{13}C NMR spectra of **14** except for one methylene group were closely related to those of 3-(2′-pyrrolidinyl)-kynurenic acid isolated from chestnut reported previously[12] and in this study. That is, the ^{1}H NMR spectrum of **14** showed the presence of four *ortho*-substituted benzene ring proton signals at δ 8.32−7.46. In addition, one methine proton signal δ 4.46 (1H, dd, *J* = 11.8, 3.8 Hz, H-2″) and four methylene proton signals at δ 4.37−1.09 were observed. The ^{1}H NMR spectrum of **14** was supported by the ^{13}C NMR spectrum, which showed the presence of 15 carbon signals including 10 sp^2 carbons with six quaternary carbons at δ 176.3−120.5 and five sp^3 carbons at δ 58.9−24.2. Considering the molecular formula of **14**, the chemical shifts of six sp^2 quaternary carbons indicated that three of them were nitrogen-bearing carbons at δ 162.6 (C-2′), 143.0 (C-2), and 141.9 (C-8a) with two carbonyl carbons at δ 176.3 (C-4) and 162.6 (C-2′). The MS and 1D-NMR data suggested that the skeleton of **14** was kynurenic acid. In addition, a piperidine moiety was assigned as the partial structure from the split patterns of five sp^3 protons at δ 4.46−1.09 in the ^{1}H NMR spectrum and the correlations (bold lines) of H-2″/H-3″, H-3″/H-4″, H-4″/H-5″, and H-5″/H-6″ detected in the ^{1}H−^{1}H COSY spectrum (Figure 1). Therefore, the MS and 1D NMR data suggested that **14** was a pyrrolidinylkynurenic acid. The complete NMR assignment and connectivity of **14** were further determined by the HSQC and HMBC experiments. On the basis of the proton−carbon correlations (arrows) observed in the HMBC spectrum (Figure 1), **14** was determined to be 3-(2′-piperidine)-kynurenic acid (Figure 1), which is a new compound.

Qualitative and Quantitative Analyses of 9, 13, and 14 in Chestnut Honey. Several studies have already reported the presence of quinolinone alkaloids **2**, **3**, **8**, **10** in honey,[1,9−12] and these compounds were also confirmed in this study. We additionally isolated and identified three quinolinone derivatives **9**, **13**, and **14** from chestnut honey in this study. Compound **9** and its similar derivatives have been synthesized, as reported previously.[19] However, this is the first study where **9** was identified in nature. Furthermore, of the quinolone alkaloids isolated in the present study, **13** and **14** were determined to be new compounds. Thus, a further investigation on the occurrence and content of three compounds **9**, **13**, and **14** in chestnut honey was required. The three compounds **9**, **13**, and **14** were analyzed qualitatively and quantitatively by HPLC−ESI−MS. We used HPLC system and selective MRM detection of tandem MS using the isolated compounds as external standards to confirm the occurrence of three compounds in chestnut honey. The $CHCl_3$ fraction obtained after solvent fractionation of the chestnut honey MeOH and EtOH extracts was analyzed by MRM via HPLC−ESI systems. The three peaks of **9**, **14**, and **13** were detected at t_R 11.4, 17.0, and 14.3 min, respectively, on the MRM chromatogram of chestnut honey (Figure 2A−C). These data were in agreement with the retention times of authentic compounds (**9**, **14**, and **13**) purified from chestnut honey in the present study (Figure 2D). These results indicate that the occurrence of **9**, **13**, and **14** in chestnut honey was unambiguously revalidated.

The external calibration curve of each compound at a concentration range of 0.1−50 ng produced a good linear correlation (R^2 > 0.99). Therefore, the optical dilution of chestnut honey in MeOH was 1:920. The precision of **9** (3.5%), **14** (5.6%), and **13** (4.1%) ranged from 3.5−5.6%. The recovery percentage was calculated by comparing the slope of

DOI: 10.1021/acs.jafc.5b01027
J. Agric. Food Chem. 2015, 63, 3587−3592

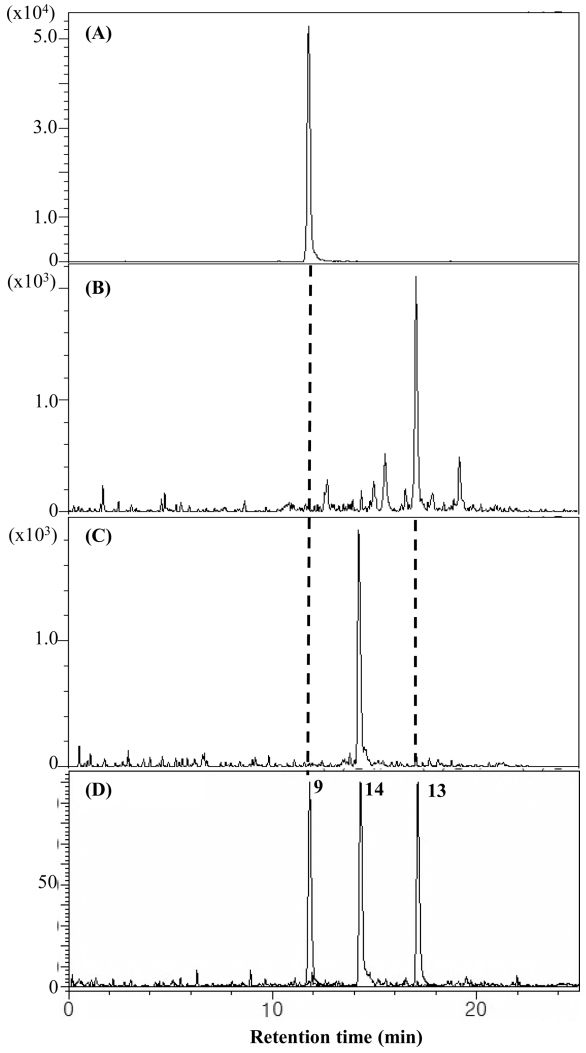

Figure 2. Multiple reaction monitoring (MRM) chromatograms of: (A) **9**, (B) **13**, and (C) **14** in chestnut honey and (D) standards.

the standard addition and the calibration curve for each compound. As a result of this analysis, the recovery of **9** (102.2 ± 3.9%), **13** (101.0 ± 3.44%), and **14** (98.8 ± 2.1%) ranged from 98.8−102.2%. Three compounds **9**, **13**, and **14** in chestnut honey were quantitatively analyzed by HPLC system and selective MRM detection of tandem MS. In the present study, the contents (μg/100 g) of **9**, **13**, and **14** in chestnut honey were 630.5 ± 62.5, 10.8 ± 0.7, and 734.8 ± 28.5, respectively. Compounds **9**, **13**, and **14** were present in very small amounts in chestnut honey when compared to quinolinone alkaloids **2** (2.3−211.5 mg/100 g) and **3** (8.1−64.4 mg/100 g), as reported previously.[11]

Quinoline alkaloids are widely distributed in nature and possess antimalarial, antimicrobial, anti-inflammatory, and analgesic activities.[21−23] In addition, kynurenic acid derivatives, which included **3**, **10**, and **14** identified in this study, are endogenous nonselective antagonists of α-7-nicotinic acetylcholine and *N*-methyl-D-aspartate receptors.[24] Furthermore, quinazolinone derivatives including **9** have anti-inflammatory and analgesic activities.[18] Therefore, quinolinone alkaloids including the two new compounds **13** and **14** identified in this study may be biologically active substances. These molecular-level investigations may offer very useful information for establishing the chemical profiles of honeys.

AUTHOR INFORMATION

Corresponding Author

*Phone: +82-62-530-2141. Fax: +82-62-530-2149. E-mail: nutrmoon@jnu.ac.kr.

Author Contributions

⊥J.Y.C. and S.H.B. contributed equally to this work.

Funding

This research was supported by the Rural Development Administration (PJ00826601).

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Schievano, E.; Moelato, E.; Fachin, C.; Mammi, S. Characterization of markers of botanical origin and other compounds extracted from unifloral honeys. *J. Agric. Food Chem.* **2013**, *61*, 1747−1755.

(2) Gharzouli, K.; Amira, S.; Gharzouli, A.; Khennouf, S. Gastroprotective effects of honey and glucose−fructose−sucrose−maltose mixture against ethanol-, indomethacin-, and acidified aspirin-induced lesions in the rat. *Exp. Toxicol. Pathol.* **2002**, *54*, 217−221.

(3) Kassim, M.; Achoui, M.; Mustafa, M. R.; Mohd, M. A.; Yusoff, K. M. Ellagic acid, phenolic acids, and flavonoids in Malaysian honey extracts demonstrate in vitro anti-inflammatory activity. *Nutr. Res. (N.Y.)* **2010**, *30*, 650−659.

(4) Al-Wailli, N. S. Natural honey lowers plasma glucose, C-reactive protein, homocysteine, and blood lipids in healthy, diabetic, and hyperlipidemic subjects: Comparison with dextrose and sucrose. *J. Med. Food* **2004**, *7*, 100−107.

(5) Koc, A. N.; Silici, S.; Kasap, F.; Hormet-Oz, H. T.; Mavus-Buldu, H.; Ercal, B. D. Antifungal activity of the honey bee products against *Candida* spp. and *Trichosporon* spp. *J. Med. Food* **2011**, *14*, 128−134.

(6) Othman, N. H. Honey and cancer: Substainable inverse relationship particularly for developing nations—A review. *J. Evidence-Based Complementary Altern. Med.* **2012**, *2012*, No. 410406.

(7) Erejuwa, O. O.; Sulaiman, S. A.; Ab Wahab, M. S. Honey: A novel antioxidant. *Molecules* **2012**, *17*, 4400−4423.

(8) Ball, D. W. The chemical composition of honey. *J. Chem. Educ.* **2007**, *84*, 1643−1646.

(9) Beretta, G.; Caneva, E.; Facino, R. M. Kynurenic acid in honey from arboreal plants: MS and NMR evidence. *Planta Med.* **2007**, *73*, 1592−1595.

(10) Beretta, G.; Caneva, E.; Regazzoni, L.; Bakhtyari, N. G.; Facino, R. M. A solid-phase extraction procedure coupled to 1H NMR, with chemometric analysis, to seek reliable markers of the botanical origin of honey. *Anal. Chim. Acta* **2008**, *620*, 176−182.

(11) Beretta, G.; Artali, R.; Caneva, E.; Orlandini, S.; Centini, M.; Facino, R. M. Quinoline alkaloids in honey: Further analytical (HPLC−DAD−ESI−MS, multidimensional diffusion-ordered NMR spectroscopy), theoretical, and chemometric studies. *J. Pharm. Biomed. Anal.* **2009**, *50*, 432−439.

(12) Beretta, G.; Vistoli, G.; Caneva, E.; Anselmi, C.; Facino, R. M. Structure elucidation and NMR assignments of two new pyrrolidinyl quinolone alkaloids from chestnut. *Magn. Reson. Chem.* **2009**, *47*, 456−459.

(13) Gasic, U.; Keckes, S.; Dabic, D.; Trifkovic, J.; Milojkovic-Opsenica, D.; Natic, M.; Tesic, Z. Phenolic profile and antioxidant activity of Serbian polyfloral honeys. *Food Chem.* **2014**, *145*, 599−607.

(14) Alissandrakis, E.; Kibaris, A. C.; Tarantilis, P. A.; Harizanis, P. C.; Polissiou, M. Flavour compounds of Greek cotton honey. *J. Sci. Food Agric.* **2005**, *85*, 1444−1452.

(15) Jerković, I.; Marijanović, Z.; Kezić, J.; Gugić, M. Headspace, volatile, and semi-volatile organic compounds diversity and radical scavenging activity of ultrasonic solvent extracts from *Amorpha fruticosa* honey samples. *Molecules* **2009**, *14*, 2717−2728.

(16) Stephens, J. M.; Schlothauer, R. C.; Morris, B. D.; Yang, D.; Fearnley, L.; Greenwood, D. R.; Loomes, K. M. Phenolic compounds

DOI: 10.1021/acs.jafc.5b01027
J. Agric. Food Chem. 2015, 63, 3587−3592

and methylglyoxal in some New Zealand manuka and kanuka honeys. *Food Chem.* **2010**, *120*, 78−86.
(17) Tuberoso, C. I. G.; Bifulco, E.; Caboni, P.; Cottiglia, F.; Cabras, P.; Floris, I. Floral markers of strawberry tree (*Arbutus unedo* L.) honey. *J. Agric. Food Chem.* **2010**, *58*, 384−389.
(18) Azimvand, J. Synthesis of novel 1,2,3,5-tetrahydropyrrole[2,1-b]quinazoline-5-one analogs by using lithiation-intramolecular electrophilic reaction. *J. Chem. Pharm. Res.* **2012**, *4*, 3924−3928.
(19) Arya, V. P.; Dave, K. G.; Khadse, V. G.; Shenoy, S. J. Synthesis of new heterocycles: Part XXI. Synthesis of some quinazolones. *Indian J. Chem., Sect. B: Org. Chem. Incl. Med. Chem.* **1976**, *14B*, 879−882.
(20) Eun, S. M. Synthesis of quinazolin-4-one derivatives from 2-aminobenzamide. II. Reaction with γ-lactone and diketones. *Yakhak Hoechi* **1986**, *30*, 203−207.
(21) Michael, J. P. Quinoline, quinazoline, and acridone alkaloids. *Nat. Prod. Rep.* **2008**, *25*, 166−187.
(22) Marella, A.; Tanwar, O. P.; Saha, R.; Ali, M. R.; Srivastava, S.; Akhter, M.; Shaquiquzzaman, M.; Alam, M. M. Quinoline: A versatile heterocyclic. *Saudi Pharm. J.* **2013**, *21*, 1−12.
(23) Souto, A. L.; Tavares, J. F.; da Silva, M. S.; Diniz, M. F. F. M.; de Athayde-Filho, P. F.; Filho, J. M. B. Anti-inflammatory activity of alkaloids: An update from 2000 to 2010. *Molecules* **2011**, *16*, 8515−8534.
(24) Stone, T. W. Development and therapeutic potential of kyrurenic acid and kynurenine derivatives from neuroprotection. *Trends Pharmacol. Sci.* **2000**, *21*, 149−154.

DOI: 10.1021/acs.jafc.5b01027
J. Agric. Food Chem. 2015, 63, 3587−3592