Identification of the active components inhibiting the expression of matrix metalloproteinase-9 by $\text{TNF}\alpha$ in ethyl acetate extract of *Euphorbia humifusa* Willd

Seunghyun Ahn¹ · Hyeryoung Jung¹ · Yearam Jung¹ · Junho Lee¹ ·
Soon Young Shin² · Yoongho Lim¹ · Young Han Lee²

Received: 27 May 2019 / Accepted: 23 October 2019 / Published Online: 31 December 2019
© The Korean Society for Applied Biological Chemistry 2019

Abstract *Euphorbia humifusa* Willd (EuH), called Ttang-Bin-Dae in Korea, is a traditional medicinal plant widely used for its anti-inflammatory and antiviral activity. Ethyl acetate (EA) extracts of EuH (EA/EuH) inhibit invasion and metastasis by inhibiting tumor necrosis factor $\text{TNF}\alpha$ -induced matrix metalloproteinases (MMP)-9 expression in human breast cancer cells. However, the bioactive components of EA/EuH mediating the inhibition of MMP-9 expression have not been identified. In the present study, three bioactive constituents of EA/EuH were isolated using high-performance liquid chromatography. Nuclear magnetic resonance spectroscopy revealed isoquercetin, avicularin, and astragalin as the bioactive compounds responsible for preventing $\text{TNF}\alpha$ -induced MMP-9 mRNA expression in breast cancer cells. These findings suggest that isoquercetin, avicularin, and astragalin could be used as valuable anti-metastatic agents against metastatic cancers.

Keywords Astragalin · Avicularin · *Euphorbia humifusa* Willd · Isoquercetin · Matrix metalloproteinases-9

Introduction

Euphorbia humifusa Willd (EuH) is an annual plant widely distributed in eastern Asia including Korea. Previously, we demonstrated that the ethyl acetate (EA) extract of EuH (EA/EuH) inhibited invasion and metastasis by inhibiting $\text{TNF}\alpha$ -induced matrix metalloproteinase (MMP)-9 expression in highly metastatic MDA-MB-231 human breast cancer cells [1]. However, the bioactive components mediating inhibition of $\text{TNF}\alpha$ -induced MMP-9 expression have not been elucidated. In this study, we isolated and identified three bioactive constituents, isoquercetin, avicularin, and astragalin, from EA/EuH, which are responsible for preventing $\text{TNF}\alpha$ -induced MMP-9 mRNA expression.

Materials and Methods

The ethyl acetate (EA) extract of the whole plant of *E. humifusa* Willd (EA/EuH) from the first methanol extraction was prepared in a yield of 13.7% as described in our previous report [1]. The EA/EuH fraction was further separated using preparative high-performance liquid chromatography (prep-HPLC) with a Luna C18 column (10×250 mm, $\phi=5\ \mu\text{m}$) using an Agilent 1100 instrument (Santa Clara, CA, USA). The mobile phase, flow rate, and injection volume were 18% aqueous acetonitrile containing 1% formic acid, 3.0 mL/min, and 70 μL , respectively. The chromatogram was monitored at an ultraviolet (UV) wavelength of 254 nm.

MDA-MB-231 human breast cancer cell line was obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), 100 U/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin.

The chemical structures were analyzed using a Bruker Avance 400 nuclear magnetic resonance (NMR) spectrometer (Karlsruhe,

Young Han Lee (✉)
E-mail: yhlee58@konkuk.ac.kr

¹Division of Bioscience and Biotechnology, BMIC, Konkuk University, Seoul 05029, Republic of Korea

²Department of Biological Sciences, Sanghuh College of Life Sciences, Konkuk University, Seoul 05029, Republic of Korea

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0/>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Germany, 9.4 Tesla). The NMR spectroscopic analyses consisting of ^1H -NMR, ^{13}C -NMR, distortionless enhancement by polarization transfer (DEPT), correlated spectroscopy (COSY), total correlated spectroscopy (TOCSY), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bonded connectivities (HMBC) were based on methods we reported previously [2]. To confirm the chemical structures determined using NMR spectroscopy, ultraperformance LC-hybrid quadrupole-time-of-flight mass spectrometry (UPLC-QTOF/MS) was performed using a Waters Acquity UPLC system (Waters Corp., Milford, MA, USA) [3].

Total RNA was isolated using Isol-RNA lysis reagent (NucleoZOL; Clontech, Mountain View, CA, USA), and cDNA synthesis was carried out using an iScript cDNA synthesis kit according to the manufacturer's instruction (Bio-Rad, Hercules, CA, USA). Quantitative real-time polymerase chain reaction (qPCR) was performed using the TaqMan-iQ supermix kit with the Bio-Rad iCycler iQ thermal cycler according to the manufacturer's instruction (Bio-Rad). TaqMan fluorogenic probes and RT-PCR primers for MMP-9 and GAPDH were synthesized as described previously [4].

The statistical significance was analyzed using GraphPad Prism version 7.04 software (La Jolla, CA, USA). A p -value <0.05 reflects statistical significance.

Results and Discussion

Three peaks at 20.73, 30.85, and 33.11 min (named F1, F2, and F3, respectively) in the chromatogram obtained from prep-HPLC (Fig. 1) could be separated and collected, freeze-dried, and their dried amounts were approximately 15, 20, and 30 mg, respectively. We identified the chemical structure of each compound using NMR spectroscopy and confirmed them using UPLC-QTOF/MS. Because the F3 fraction was the most abundant among three fractions collected here, we first analyzed its chemical structure using NMR spectroscopy. The ^1H -NMR spectrum (Fig. 2A) shows that F3 was a single compound, and it consisted of a saccharide and aromatic ring based on peaks at 3–4 ppm and 5–8 ppm, respectively. Twenty peaks were observed in the ^{13}C NMR spectrum (Fig. 2B), including two at 115.3 and 131.0 ppm, which showed double intensities in the comparison of their neighboring peaks, indicating that F3 contained 22 carbons. The DEPT (Fig. 2C) revealed that F3 consisted of one quartet, one triplet, nine doublet, and nine singlet carbons. The interpretation of the ^1H -NMR, ^{13}C -NMR, and DEPT spectra, assigned F3 as a glycosylated flavonoid. To clarify the structure, two-dimensional (2D) NMR experiments, COSY (Fig. 2D), TOCSY (Fig. 2E), HMQC (Fig. 2F), and HMBC (Fig. 2G) were performed. The spectral data of F3 are summarized as follows: ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 12.61 (s, 1H, 4-OH), 8.04 (d, 2H, H-2/H-6, $J=8.8$ Hz), 6.89 (d, 2H, H-3/H-5, $J=8.8$ Hz), 6.44 (s, 1H, H-8), 6.21 (s, 1H, H-6), 5.46 (d, 1H, H-1, $J=7.4$ Hz), 3.57 (d, 1H, H-6 α , $J=11.4$ Hz), 3.34 (d,

Fig. 1 Chromatogram of EuH ethyl acetate extract (EA/EuH). Three compounds showing main peaks at 20.73, 30.85, and 33.11 min (F1, F2, and F3, respectively) were collected. y-axis, milli-Absorbance Unit (mAU); x-axis, retention time (min)

1H, H-6 β , $J=11.4$ Hz), 3.21 (s, 1H, H-4), 3.20 (d, 1H, H-2, $J=7.4$ Hz), 3.11 (s, 1H, H-3), 3.09 (s, 1H, H-5); ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) δ 177.6 (C-4), 164.3 (C-7), 161.4 (C-5), 160.1 (C-4), 156.5 (C-9), 156.4 (C-2), 133.3 (C-3), 131.0 (C-2/C-6), 121.0 (C-1), 115.3 (C-3/C-5), 104.2 (C-10), 101.0 (C-1), 98.9 (C-6), 93.8 (C-8), 77.6 (C-3), 76.5 (C-4), 74.4 (C-2), 70.0 (C-5), 61.0 (C-6). The saccharide included in F3 was determined to be glucopyranoside by comparing the ^{13}C NMR peaks with the reference [5]. The flavonoid part in F3 was determined to be kaempferol based on the comparison of the NMR assignments obtained here with those of our previous NMR data [6–8]. The connection between kaempferol and glucopyranoside was determined based on the long-range coupling between C-3 and H-1 in the HMBC spectrum. Based on the interpretation of the 2D spectra, proton-proton and proton-carbon connectivities were determined, as shown in Fig. 3A. Therefore, F3 was identified as a kaempferol-3-O- β -D-glucopyranoside with a molecular formula and calculated molecular mass of $\text{C}_{21}\text{H}_{20}\text{O}_{11}$ and 448.1006, respectively. The molecular formula was determined by UPLC-QTOF/MS. The molecular mass in the negative mode obtained from UPLC-QTOF/MS was found at 447.0961 (Fig. 4A). Because the calculated molecular mass of $\text{C}_{21}\text{H}_{19}\text{O}_{11}$ in the negative mode is 447.0927, the structure of F3 determined based on the interpretation of the NMR data was correct. Collectively, these results determined F3 to be kaempferol-3-O- β -D-glucopyranoside, which is a known compound, astragalin (Fig. 5A). A comparison of the ^{13}C -NMR data of F3 with the data published previously [9] was listed in Table 1.

The NMR and HR/MS analyses of F1 and F2 fractions were similarly carried out. The spectral data of F1 are summarized as follows: ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ ppm: 7.59 (s, 1H, H-2), 7.58 (d, 1H, H-6, $J=9.0$ Hz), 6.85 (d, 1H, H-5, $J=8.6$ Hz), 6.41

Fig. 2 The one-dimensional and two-dimensional NMR spectra of the fraction F3. (A) ^1H -NMR spectrum, (B) ^{13}C -NMR spectrum, (C) DEPT spectrum, (D) COSY spectrum, (E) TOCSY spectrum, (F) HMQC spectrum, (G) HMBC spectrum

Fig. 3 The important proton-proton and proton-carbon connectivities of (A) F3, (B) F1, and (C) F2, based on the interpretation of the COSY, TOCSY, and HMBC experiments

(s, 1H, H-8), 6.21 (s, 1H, H-6), 5.46 (d, 1H, H-1, $J=7.2$ Hz), 3.59 (d, 1H, H-6 α , $J=11.4$ Hz), 3.34 (dd, 1H, H-6 β , $J=3.4$, 11.5 Hz), 3.25 (d, 1H, H-3, $J=4.6$ Hz), 3.24 (d, 1H, H-2, $J=2.2$ Hz), 3.11 (d, 1H, H-5, $J=4.5$ Hz), 3.10 (d, 1H, H-4, $J=2.3$ Hz); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 177.6 (C-4), 164.3 (C-7), 161.4 (C-5), 156.5 (C-9), 156.4 (C-2), 148.6 (C-4), 144.9 (C-3), 133.5 (C-3), 121.7 (C-6), 121.3 (C-1), 116.4 (C-2), 115.4 (C-5), 104.1 (C-10), 101.0 (C-1), 98.8 (C-6), 93.7 (C-8), 77.7 (C-5), 76.6 (C-3), 74.2 (C-2), 70.1 (C-4), 61.1 (C-6); HR/MS (m/z): calculated for $\text{C}_{21}\text{H}_{19}\text{O}_{12}$ (M-H) $^+$: 463.0877; found 463.0862 (Fig. 4B). Because the mass data were collected in the negative mode, the molecular formulae of F1 should be $\text{C}_{21}\text{H}_{20}\text{O}_{12}$. The molecular formula was determined by UPLC-QTOF/MS. As mentioned above, the saccharide contained in F1 was determined to be glucopyranoside by comparing the ^{13}C NMR peaks with the reference values [5]. Furthermore, the flavonoid part was confirmed based on the comparison of the NMR assignments obtained here with our previous NMR data, and it was determined to be quercetin [6–8].

The connection between quercetin and glucopyranoside was determined based on the long-range coupling between C-3 and H-1 in the HMBC spectrum. Based on the interpretation of the 2D spectra, including COSY, TOCSY, and HMBC, proton-proton and proton-carbon connectivities were determined, as shown in Fig. 3B. Therefore, F1 was identified as quercetin-3- O - β -D-glucopyranoside (Fig. 5B), which is a known isoquercetin. A comparison of the ^{13}C -NMR data of F2 with the data published previously [10] was listed in Table 2.

The spectral data of F2 are summarized as follows: ^1H NMR (400 MHz, DMSO-d_6) δ ppm: 7.56 (d, 1H, H-6, $J=8.3$ Hz), 7.50 (s, 1H, H-2), 6.87 (d, 1H, H-5, $J=8.4$ Hz), 6.42 (s, 1H, H-8), 6.21 (s, 1H, H-6), 5.59 (s, 1H, H-1), 4.17 (d, 1H, H-4, $J=2.7$ Hz), 3.74 (dd, 1H, H-3, $J=3.8$, 6.5 Hz), 3.58 (dd, 1H, H-2, $J=4.9$, 9.1 Hz), 3.35 (dd, 1H, H-5 α , $J=3.1$, 11.8 Hz), 3.29 (dd, 1H, H-5 β , $J=5.0$, 11.9 Hz); ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 177.9 (C-4), 164.4 (C-7), 161.4 (C-5), 157.1 (C-9), 156.5 (C-2), 148.6 (C-4), 145.2 (C-3), 133.5 (C-3), 121.9 (C-6), 121.1 (C-1), 115.7 (C-2),

Fig. 4 The UPLC-QTOF/MS spectra of (A) F3, (B) F1, and (C) F2

Table 1 A comparison of the ^{13}C -NMR data of F3 with the data published previously [9]. The ^{13}C -NMR data obtained from the current study were collected in dimethylsulfoxide-d6, and those published previously were in methanol-d4

position	^{13}C -NMR data obtained from the current study collected in dimethylsulfoxide-d6/ppm	^{13}C -NMR data published previously in methanol-d4/ppm	error/ppm
2	156.4	158.0	1.6
3	133.3	135.3	2.0
4	177.6	179.2	1.6
5	161.4	162.7	1.3
6	98.9	99.8	0.9
7	164.3	165.7	1.4
8	93.8	94.8	1.0
9	156.5	158.8	2.3
10	104.2	105.6	1.4
1'	121.0	122.5	1.5
2'/6'	131.0	132.1	1.1
3'/5'	115.3	115.9	0.6
4'	160.1	161.2	1.1
1''	101.0	104.0	3.0
2''	74.4	75.6	1.2
3''	77.6	78.0	0.4
4''	76.5	78.2	1.7
5''	70.0	71.2	1.2
6''	61.0	62.4	1.4

Table 2 A comparison of the ^{13}C -NMR data of F1 with the data published previously [10]. The ^{13}C -NMR data obtained from the current study were collected in dimethylsulfoxide-d6, and those published previously were in dimethylsulfoxide-d6

position	^{13}C -NMR data obtained from the current study collected in dimethylsulfoxide-d6/ppm	^{13}C -NMR data published previously in DMSO-d6/ppm	error/ppm
2	156.4	156.5	0.1
3	133.5	133.9	0.4
4	177.6	177.5	-0.1
5	161.4	161.2	-0.2
6	98.8	99.1	0.3
7	164.3	165.1	0.8
8	93.7	94.0	0.3
9	156.5	156.8	0.3
10	104.1	103.9	-0.2
1'	121.3	122.9	1.6
2'	116.4	116.4	0
3'	144.9	144.9	0
4'	148.6	148.7	0.1
5'	115.4	115.5	0.1
6'	121.7	121.8	0.1
1''	101.0	101.2	0.2
2''	74.2	74.3	0.1
3''	76.6	76.7	0.1
4''	70.1	70.2	0.1
5''	77.7	77.8	0.1
6''	61.1	61.0	-0.1

115.7 (C-5), 108.0 (C-1), 104.1 (C-10), 98.9 (C-6), 93.8 (C-8), 86.0 (C-2), 82.3 (C-4), 77.1 (C-3), 60.82 (C-5); HR/MS (m/z): calculated for $\text{C}_{20}\text{H}_{17}\text{O}_{11}$ ($\text{M}-\text{H}$) $^+$: 433.0771; found 433.0772 (Fig. 4C). Because the mass data were collected in the negative mode, the molecular formula of F2 should be $\text{C}_{20}\text{H}_{18}\text{O}_{11}$. The molecular formula was determined by UPLC-QTOF/MS. The saccharide contained in F2 was determined to be arabinofuranoside by comparing the ^{13}C NMR peaks with reference values [5]. The flavonoid part was confirmed to be quercetin based on comparison

of NMR assignments obtained here with previous NMR data [6–8]. The connection between quercetin and arabinofuranoside was determined based on the long-range coupling between C-3 and H-1 in the HMBC spectrum. Based on the interpretation of the 2D spectra, proton-proton and proton-carbon connectivities were determined, as shown in Fig. 3C. Collectively, F2 was quercetin-3- O - α -L-arabinofuranoside (Fig. 5C), which is known as avicularin.

Fig. 5 Structures of (A) F3 (kaempferol-3- O - β -D-glucopyranoside, astragalin), (B) F1 (quercetin-3- O - β -D-glucopyranoside, isoquercetin), and (C) F2 (quercetin-3- O - α -L-arabinofuranoside, avicularin)

Table 3 A comparison of the ^{13}C -NMR data of F2 with the data published previously [11]. The ^{13}C -NMR data obtained from the current study were collected in dimethylsulfoxide- d_6 , and those published previously were in methanol- d_4

position	^{13}C -NMR data obtained from the current study collected in dimethylsulfoxide- d_6 /ppm	^{13}C -NMR data obtained from the current study collected in methanol- d_4 /ppm	error/ppm
2	156.5	159.3	2.8
3	133.5	134.9	1.4
4	177.9	179.9	2.0
5	161.4	163.0	1.6
6	98.9	99.8	0.9
7	164.4	165.9	1.5
8	93.8	94.7	0.9
9	157.1	158.5	1.4
10	104.1	105.6	1.5
1'	121.1	123.0	1.9
2'	115.7	116.4	0.7
3'	145.2	146.3	1.1
4'	148.6	149.8	1.2
5'	115.7	116.8	1.1
6'	121.9	122.9	1.0
1''	108.0	109.5	1.5
2''	82.3	83.2	0.9
3''	77.1	78.6	1.5
4''	86.0	88.0	2.0
5''	60.8	62.5	1.7

A comparison of the ^{13}C -NMR data of F1 with the data published previously [11] was listed in Table 3.

Previously, we have demonstrated that the EA/EuH fraction exhibited anti-metastatic activity by suppressing MMP-9 mRNA expression in MDA-MB-231 breast cancer cells [1]. To determine the biological activity of the fractions, F1, F2, and F3 identified in this study, MDA-MB-231 cells were treated with 5 $\mu\text{g/mL}$ F1, F2, or F3 before adding 10 ng/mL TNF- α . The EA/EuH fraction (5 $\mu\text{g/mL}$) was a positive control. The reverse transcription (RT)-PCR analysis shows that the TNF- α -induced MMP-9 mRNA expression was substantially decreased by the F1, F2, or F3 fraction (Fig. 6A). To further quantitate the effect of each fraction on MMP-9 mRNA expression, the relative expression level of MMP-9 mRNA was measured using qPCR. Treatment with TNF- α alone resulted in a 336.7 $\pm$ 37.86-fold increase in the MMP-9 mRNA level; however, this effect was significantly reduced to 136.7 15.28-, 231.3 27.79-, and 35.33 5.508-fold (all $p < 0.001$, $n=3$) by pre-exposure to 5 $\mu\text{g/mL}$ F1, F2, and F3, respectively (Fig. 6B). These data suggest that the three fractions contained bioactive components that mediate the inhibition by EA/EuH.

In conclusion, isoquercetin, avicularin, and astragalin as the bioactive components of the EA/EuH mediate the inhibition of TNF- α -induced MMP-9 mRNA expression in MDA-MB-231 breast cancer cells. These results suggest that EuH can be used as a functional adjuvant to prevent or attenuate invasion and metastasis in the early stage of breast cancer.

Acknowledgment This article was written as part of Konkuk University's research support program for its faculty on sabbatical leave in 2018 (YH Lee).

Fig. 6 Effect of EuH ethyl acetate extract (EA/EuH) and three fractions, including F1, F2, and F3. (A) MDA-MB-231 cells were untreated or treated with TNF α (10 ng/mL) in the absence or presence of EA/EuH, F1, F2, or F3 (5 $\mu\text{g/mL}$ each) for 24 h. Total RNA was isolated, and then RT-PCR was performed. GAPDH mRNA was determined as an internal control. (B) MDA-MB-231 cells were treated as in (A). MMP-9 mRNA levels were measured using qPCR. Relative fold changes were normalized to GAPDH mRNA in the same sample. Data are means $\pm$ standard deviation (SD). ** $p < 0.001$ ($n=3$)

References

1. Shin SY, Kim CG, Jung YJ, Jung Y, Jung H, Im J, Lim Y, Lee YH (2016) *Euphorbia humifusa* Willd exerts inhibition of breast cancer cell invasion and metastasis through inhibition of TNF α -induced MMP-9 expression. *BMC Complement Altern Med* 16: 413
2. Jung H, Ahn S, Jung Y, Noh HJ, Kim SY, Koh D, Lim Y (2015) ^1H and ^{13}C NMR spectral assignments of 18 novel polymethoxylated hydroxy-naphthopyrazolylchalconoids. *Magn Reson Chem* 53: 391–397
3. Jung H, Ahn S, Jung Y, Koh D, Lim Y (2016) ^1H and ^{13}C NMR spectral assignments of 19 novel polymethoxylated diphenylnaphthyl-pyrazolylcarbothioamides. *Magn Reson Chem* 54: 246–251
4. Shin SY, Lee JM, Lim Y, Lee YH (2013) Transcriptional regulation of the growth-regulated oncogene α gene by early growth response protein-1 in response to tumor necrosis factor α stimulation. *Biochim Biophys Acta* 1829: 1066–1074
5. Breitmaier E, Voelter W, Breitmaier E (1987) Carbon-13 NMR spectroscopy: high-resolution methods and applications in organic chemistry and biochemistry. 3rd completely rev. edn. VCH Publishers, New York
6. Moon BH, Lee Y, Ahn JH, Lim Y (2005) Complete assignment of ^1H and ^{13}C NMR data of some flavonol derivatives. *Magn Reson Chem* 43: 858–860
7. Kim H, Moon BH, Ahn JH, Lim Y (2006) Complete NMR signal assignments of flavonol derivatives. *Magn Reson Chem* 44: 188–190
8. Park Y, Moon BH, Lee E, Lee Y, Yoon Y, Ahn JH, Lim Y (2007) ^1H and ^{13}C -NMR data of hydroxyflavone derivatives. *Magn Reson Chem* 45: 674–679
9. Seo K, Jung J, Thi NN, Lee Y, Baek N (2016) Flavonoid glycosides from the glowers of *Pulsatilla koreana* Nakai. *Nat Prod Sci* 22(1): 41–45
10. Dawidar A, Abdel-Mogib M, El-Naggar M, Mostafa ME (2014) Isolation and characterization of *Polygonum equisetiforme* flavonoids and their acaricidal activity against *Tetranychus urticae* Koch. *Res J Pharm Biol Chem Sci* 5(4): 140–148
11. Pereira C, Kuster C, Simas KS, Sakuragui CM, Wessjohann A (2012) Flavonoids and a neolignan glucoside from *Guarea macrophylla* (meliaceae). *Quim Nova* 35(6): 1123–1126