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Research article

C30 reversed-phase HPLC analysis and in vitro bioactivity evaluation of decursin and decursinol angelate in Angelicae Gigantis Radix

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ABSTRACT

The roots of *Angelica gigas* (Angelicae Gigantis Radix, AGR) have been used as an herbal medicine for blood stasis and inflammation in Korea. The main ingredients of AGR are decursin (D) and decursinol angelate (DA) belonging to coumarins. However, D and DA present a challenge for separation due to their structural isomerism. The incomplete separation interferes with quantification of D and DA leading to difficulties in quality control of AGR. From these backgrounds, high-performance liquid chromatography (HPLC) method for simultaneous determination of D and DA was established with a C30 column. D and DA were well-separated by a C30 column, resulting in a 2.44 resolution. In addition, the neuroprotective and anti-inflammatory effects of D and DA were compared in HT22 and Raw264.7 cells, respectively. D and DA showed different neuroprotective activities against glutamate-induced HT22 cell death; D showed potent activity, whereas DA was negligible. In contrast, the anti-inflammatory activities of D and DA were similar in lipopolysaccharide-stimulated Raw264.7 cells. This study indicates that separating D and DA is vital for quality control of AGR and pharmaceutical applications based on the differences in the pharmacological properties of each compound.

1. Introduction

The roots of *Angelica gigas* (Angelicae Gigantis Radix, AGR) have a long history of use in Korea as a crude drug for treating anemia, sterility, stomach ailments, and arthritis [1,2]. In the United States, the ethanol extract of AGR is marketed as a dietary supplement for memory enhancement and pain management [2]. Additionally, the main constituents of AGR have been found to have various pharmacological activities, including anti-inflammatory [3], anti-tumor [4], and neuroprotective activities [5].

Decursin (D) and decursinol angelate (DA), dihydropyranocoumarins with a cyclized isoprenyl group at C-6 position of coumarin

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backbone (Fig. 1), are the marker compounds to discriminate Korean angelica (*A. gigas*) from the others such as Chinese angelica (*A. sinensis*) and Japanese angelica (*A. acutiloba*) [6,7]. In addition, Korean Pharmacopoeia defined AGR as a drug containing nodakenin and total decursin (D and DA), and the sum of three compounds should not be less than 6 % on the dried basis [8]. However, D and DA are structural isomers having the same chemical formula, making it difficult to separate and quantify each compound. Therefore, most analysis have been often conducted without distinguishing between D and DA [9–13]. High-performance liquid chromatography (HPLC) methods have been mainly reported for separating these two compounds using a C18 reversed-phase column, but most studies showed insufficient separation with poor resolution due to structural isomerism [9–11,13] and relatively long run time, even taking approximately 60 min [6]. On the other hand, some studies evaluated that D and DA have distinct biological potencies [5,14], although they have similar structures. Therefore, it is necessary to establish a facile and reliable HPLC analytical method for the proper quality control and usage of AGR.

Herein, we employed a C30 column for more efficient separation and rapid determination of D and DA in the methanolic extract of AGR. The C30 column provides sufficient phase thickness to enhance interaction with analytes, and has been used for the separation of hydrophobic molecules [15]. In addition, the neuroprotective and anti-inflammatory activities of D and DA were compared in glutamate-stressed HT22 and lipopolysaccharide (LPS)-stimulated Raw264.7 cell systems, respectively.

2. Materials and methods

2.1. HPLC analysis

HPLC was performed on a Thermo Scientific Ultimate 3000 HPLC system (Sunnyvale, CA, USA). To determine the optimal stationary phase for separating D and DA, three reversed-phase columns were compared; Gemini 5 μm C18 110 Å (C18; Phenomenex, Torrance, CA, USA), Capcell Pak UG120 (C18, Shiseido, Tokyo, Japan), and YMC Carotenoid S-5 μm (C30; YMC, Kyoto, Japan). All columns were 4.6 × 250 mm with a particle size of 5 μm. The mobile phase comprised a mixture of 55 % acetonitrile (ACN) and 45 % water at a flow rate of 1.0 mL/min. The column temperature was 40 °C and eluate was detected at UV 330 nm for 20 min. Peak identity and purity of D and DA were determined by comparing retention times and peak areas with commercial standards D (purity, ≥98 %) and DA (purity, ≥98 %) obtained from Selleckchem (Houston, TX, USA).

2.2. Validation of HPLC method

The optimized HPLC method using C30 column (YMC Carotenoid S-5 μm, 4.6 × 250 mm, 5 μm, YMC, Kyoto, Japan) was validated in accordance with the International Conference on Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) validation protocols (Q2: Validation of analytical procedures). Isocratic elution was carried out with 55 % ACN in water as a mobile phase at a flow rate of 1.0 mL/min. The column temperature was 40 °C and eluate was detected at UV 330 nm for 20 min. Each D and DA was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 1 mg/mL as stock solutions. The solutions were further diluted serially to concentrations ranging from 10 to 200 μg/mL in DMSO and stored at 4 °C until use. Calibration curves were generated from triplicate experiments, correlating peak areas and the corresponding concentrations (μg/mL) of D or DA as y and x functions, respectively. Linearity was assessed using a simple linear regression method (GraphPad Prism 9.4.1; La Jolla, CA, USA). The limit of detection (LOD) was determined by taking 3 times the response's standard deviation (SD) divided by the slope of each regression equation. The limit of quantitation (LOQ) was determined by multiplication the ratio of response's standard deviation to the slope of regression equation by 10. The method precision was analyzed for intra- and inter-day variations. The intra-day precision analysis was conducted in triplicate on samples prepared on a single day. In contrast, the inter-day precision analysis was conducted in triplicate on samples prepared on three different days. Variations are expressed as relative standard deviations (RSD).

2.3. Preparation of AGR extract

AGR (voucher specimen #NPM2021-AGR-01 deposited at College of Pharmacy, Kyungpook National University), cultivated in Jinbu (Gangwon, Republic of Korea), was identified and supplied by Rural Development Administration (Eumseong, Republic of

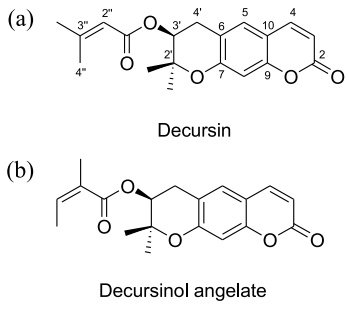

Fig. 1. The chemical structures of (a) decursin (D) and (b) decursinol angelate (DA).

Korea). The dried AGR (5 g) was refluxed with 100 mL MeOH for 3 h thrice to extract as much of the constituents as possible, and the solutions were combined to be evaporated. Then, the MeOH extract (AGR extract) was dissolved in DMSO to the concentration of 1 mg/mL. For the quantification of D and DA, AGR extract was diluted to 0.5 mg/mL in DMSO.

2.4. Cell culture

HT22 cells (RRID: CVCL 0321), a mouse-derived hippocampal neuronal cell line, were kindly donated from Prof. Dong-Seok Lee (College of Natural Sciences, Kyungpook National University, Daegu, Republic of Korea). Raw264.7 cells (RRID: CVCL 0493), a macrophage cell line, were provided by the Korean Cell Line Bank (Seoul, Republic of Korea). Both cells were tested for mycoplasma before being received and authenticated one year before the start of this study. To authenticate the cells, HT22 and Raw264.7 cells were observed morphology by microscope to check whether cells have healthy morphology or not, with comparing to other reports (HT22, neural shape [16]; Raw264.7, rounded or cuboidal shape [17]). In addition, the morphological changes and cell responses were also observed. Since HT22 cells are sensitive to glutamate, the shrunk morphology and cell damage mediated by glutamate were analyzed by western blotting, flow cytometry, and confocal microscopy [18,19]. On the other hand, the spindle-shaped morphology and increased production of nitric oxide were confirmed in LPS-stimulated Raw264.7 cells [20]. The culture medium was Dulbecco's Modified Eagle's Medium (DMEM; Welgene #LM001-05, Gyeongsan, Republic of Korea) supplemented with 10 % fetal bovine serum (FBS; Gibco #16000, Grand Island, NY, USA) and 1 % antibiotics (penicillin/streptomycin; Welgene #LS202-02). Cells were maintained at 37 °C in 5 % CO_2 with humidified air, and subculturing was performed every other day.

2.5. HT22 cell treatment

2.5.1. Cell viability assay

To examine the neuroprotective effect of D and DA against glutamate toxicity, HT22 cells were seeded at a density of 0.75×10^4 cells/well in a 96-well plate. After 15 h of incubation, cells were treated with D and DA diluted in culture medium for 9 h. Cell death was induced by exposure to 10 mM glutamate (Sigma-Aldrich #49621, St. Louis, MO, USA) for 15 h. After removal of the medium, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Biosesang #MC1029, Seongnam, Republic of Korea) solution was added at a concentration of 0.5 mg/mL in phenol red- and serum-free DMEM (Welgene #LM001-10) and incubated for 1 h. The residual MTT solution was removed, and the purple formazan produced was dissolved in DMSO to measure the absorbance (Abs) at 575 nm using a microplate reader (SpectraMax ABS Plus; Molecular Devices; Radnor, PA, USA). Cell viability was expressed as a percentage relative to that of the vehicle-only treated group (control). Quercetin (QC; Sigma-Aldrich #Q4951) at 50 μM was used as a positive control [21].

2.5.2. Lactate dehydrogenase assay

The CytoTox 96® non-radioactive cytotoxicity assay kit (Promega #G1780, Madison, WI, USA) was used to assess the leakage of cytosolic lactate dehydrogenase (LDH) according to the manufacturer's instruction. Phenol red-free DMEM containing 10 % FBS served as the culture medium for this assay. At the end of the treatment, 50 μL of medium was collected to evaluate the released LDH. After removing the residual culture medium, cells were treated with 1 × lysis solution for 45 min at 37 °C. The cell lysate was transferred into a 1.5 mL tube and centrifuged at 250×*g* for 4 min at 4 °C for the detection of cytosolic LDH. Each aliquot (50 μL) was mixed with 50 μL CytoTox 96® reagent, then incubated for 20 min at room temperature in the dark. The reaction was finished by treatment of 50 μL stop solution, followed by the measurement of Abs at 490 nm. The experimental controls were the culture medium for the detection of released LDH and 1 × lysis solution for the detection of intracellular LDH, respectively. LDH leakage was calculated as the ratio of Abs value in the supernatant to that in the cytosol.

2.5.3. Observation of cell morphology

After treatment, cell morphology was observed under an optical microscope (magnification × 100). The scale bar in the capture indicates 100 μm.

2.6. Raw264.7 cell treatment

2.6.1. Cell viability assay

To evaluate the anti-inflammatory activity of D and DA under LPS (from *Escherichia coli* O55:B5; Sigma-Aldrich #L6529)-stimulated conditions, Raw264.7 cells were seeded at a density of 1×10^5 cells/well in a 96-well plate and incubated for 15 h. D and DA were prepared by dilution in serum- and phenol red-free DMEM. After compound treatment for 2 h, cells were stimulated with 500 ng/mL LPS for 22 h. The medium was collected for the quantification of nitric oxide (NO) followed by MTT treatment for 45 min. QC at 25 μM was used as a positive control [20].

2.6.2. Quantification of nitric oxide

To quantify the NO produced by the cells, a commercial kit (iNtRON Biotechnology #21023, Seongnam, Republic of Korea) was used according to the manufacturer's instructions. In brief, 100 μL of collected medium was added to a 96-well plate and treated with 50 μL N1 buffer (sulfanilamide in the reaction buffer). After incubation for 5 min at room temperature, 50 μL N2 buffer (naphthylethylenediamine in the stabilizer) was added, and the Abs was measured at 540 nm. The quantity of NO was calculated based on

the nitrite standard curve. The nitrite standard solution was prepared by successively diluting a 2 mM nitrite standard in ultrapure water (Welgene #ML019-02) to a concentration range of 3.13–200 μM. The standard curve was generated by plotting each concentration of nitrite standard as a function x and the Abs value as a function y. The formula for the standard curve was $y = 0.0100x + 0.0517$ ($R^2 = 0.9995$).

2.7. Statistical analysis

Cell experiment data were presented as mean ± standard deviation (SD) of three-independent experiments. Statistical significance ($p < 0.05$) was determined by one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test, conducted with GraphPad Prism 9.4.1 (La Jolla, CA, USA).

3. Results & discussion

3.1. HPLC method development

In HPLC analysis using a C30 reversed-phase column, D and DA were well separated, yielding a resolution of 2.44 and a peak asymmetry of 0.97 (Fig. 2a). Conversely, when C18 columns were employed, the resolution between D and DA fell below 1.5, indicating incomplete separation (resolutions of 1.21 and 1.35 for Gemini C18 and Capcell Pak C18, respectively). Additionally, C30 column showed faster analyte retention time (D, 11.033 min; DA, 11.845 min) compared to the C18 columns. When the AGR extract was analyzed with a C30 column, the complete separation of D and DA was also observed (Fig. 2b). In addition, the retention times and peak shapes of D and DA in the AGR extract were identical to those of the standards, indicating that there were no other interfering components (Fig. S1). Finally, a C30 column was selected as an optimal stationary phase to separate D and DA in AGR extract.

The C30 column-based HPLC method was validated with commercial standards of D and DA through linearity, limit of detection (LOD), limit of quantitation (LOQ), precision, and accuracy. Regression equations for D and DA are presented in Table S1. Both D and DA showed good linearity ($R^2 = 0.9998$) with linear ranges spanning 10–200 μg/mL. The LOD for D and DA were determined as 1.22 and 0.16 μg/mL, respectively. The LOQ for D and DA were determined as 4.05 and 0.53 μg/mL, respectively. Intra-day relative standard deviations (RSDs) of D and DA ranged from 0.15 to 0.96 % and 0.29–0.55 %, with accuracies spanning 96.5–100.4 % and 100.3–101.3 %, respectively. Inter-day RSDs of D and DA ranged from 0.4 to 1.4 % and 0.4–0.8 %, with accuracies spanning 99.5–103.0 % and 101.6–102.5 %, respectively (Table S2). These results affirm the precision and accuracy of the C30 column-based HPLC method for determining D and DA in the samples.

3.2. Quantification of D and DA in AGR

Using the validated HPLC method, the contents of D and DA in the AGR extract cultivated at Jinbu site were analyzed. The amounts of D and DA were found to be 44.9 ± 0.2 mg/g dry weight (4.5 %) and 38.8 ± 0.2 mg/g dry weight (3.9 %), respectively. These contents exceed those in the previous report that the contents of D and DA were 3.92 and 2.95 %, respectively, in the methanolic extract of AGR cultivated at Jinbu. In that study, a silica column and a mixture of hexane:$CHCl_3$:EtOAc = 20:1:1 were used for HPLC analysis resulting in insufficient resolution [22]. In addition, the contents of D and DA in the methanolic extract of AGR cultivated in Duil-ri and Topdong-ri, Pyeongchang, Gangwon, Republic of Korea, were ranged from 2.39 to 3.09 % and 1.63–1.93 %, respectively,

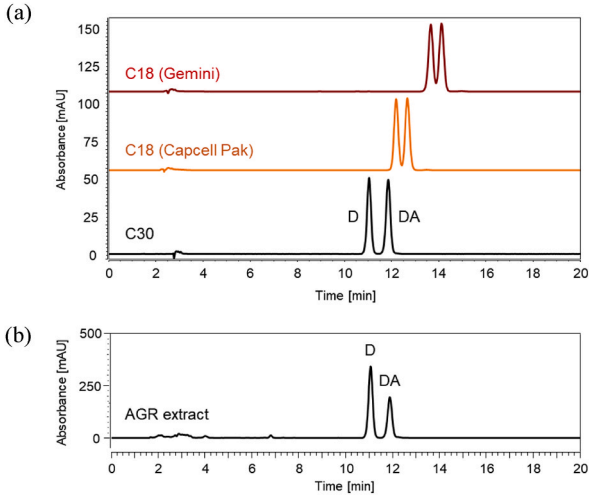

Fig. 2. HPLC analysis of D and DA. (a) HPLC chromatograms of a standard mixture of D and DA, obtained from different stationary phases. The resolution between D and DA was 2.44 by a C30 column. (b) HPLC chromatogram of the methanolic extract of AGR resulted by a C30 column.

resulted by a C18 column and gradient elution with ACN and water each containing 1 % formic acid [23].

Jeong et al. (2015) reported HPLC method using a C18 column and a mixture of ACN and water containing 0.1 % formic acid under gradient mode for 60 min. According to their result, D and DA contents in 70 % ethanolic extract of AGR were 0.9–2.2 % and 0.5–1.6 %, respectively [6], which were relatively lower than those of current and other studies [22,23]. This is presumably due to the lack of clear baseline separation in HPLC chromatogram because of the lower resolution of two compounds. In addition, the water-containing extraction solvent, 70 % ethanol (EtOH), might be disadvantageous to extract the hydrophobic D and DA, relative to MeOH. Lee et al. (2004) also reported the quantification method using C18 columns and 50 % ACN solution containing 0.01 M sodium dodecyl sulfate and sodium dihydrogen phosphate (pH 5.0). However, the resolution between D and DA was 1.66 in the methanolic extract of AGR with 10 min longer run time compared to current study [24]. Moreover, Kang et al. (2003) described the use of 50 % ACN containing sodium phosphate and sodium lauryl sulfate with a C18 column. The run time was over 40 min [25]. Generally, buffer solutions are known to be bothersome tasks to prepare and often cause issues such as line blocking.

Recently, the simultaneous analysis of D and DA using an ultra-HPLC (UHPLC)-MS/MS [26] and ultra-performance convergence chromatography (UPC^2) [27] have been reported. These methods require expensive instruments and were optimized for the trace amount of sample with less reliable validation indexes, such as correlation coefficients, accuracies, and precision. Therefore, the HPLC method established in this study could be more suitable for the quantitative determination of ordinary herbal drug analysis, even the sensitivity of UHPLC-MS/MS and UPC^2 was much better than UV detectors.

It has been important to develop the analytical method for discrimination of Korean angelica from Chinese angelica and Japanese angelica, since it is not easy to distinguish each origin of dried and cut roots through the external view. This challenge has partially

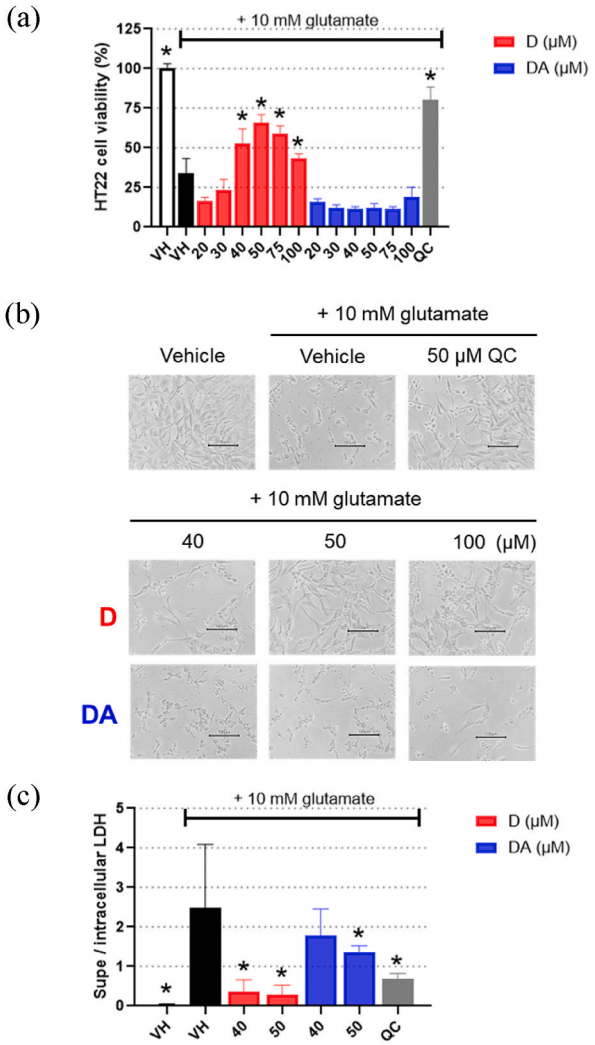

Fig. 3. Comparison of neuroprotective effects between D and DA against glutamate-induced HT22 cell death. (a) Cell viability assay ($n \geq 9$). (b) Cell morphology. Each scale bar indicates 100 μm. (c) LDH assay ($n \geq 6$). *, $p < 0.05$ compared to vehicle (VH) plus glutamate-treated group by one-way ANOVA with Dunnett's multiple comparison test. Quercetin (QC; 50 μM) was used as a positive control.

been resolved by establishment of analytical methods [6,12]. Basically, the developed methods to discriminate Korean angelica focused on detection of specific components for Korean angelica, D and DA, while the others contain (*Z*)-ligustilide and ferulic acid. However, the methods could not separate completely D and DA in AGR extract. The other challenge is that the distinguishment of geographical origin of Korean angelica cultivated in Korean and China. It was confirmed that both Korean angelica from Korea and China contain D and DA [7,28]. However, Korean angelica cultivated in Korea revealed higher content of D (4.6 %) compared to that collected from Jilin Province, China (3.3 %) in UHPLC analysis. The principal component analysis (PCA) indicated that the ratio of D and DA is the main effector to classify Korean angelica according to the geographical origin [28]. Thus, the proper quantification of D and DA in AGR extract via simple and reliable analytical method is important for quality control and origin determination of AGR.

As described above, D and DA contents of AGR vary depending on the cultivation sites, extraction method, and analytical methods. However, fast and reliable HPLC quantification method with sufficient resolution has not been established. In this study, a rapid and facile HPLC-UV method to determine D and DA in AGR was successfully validated concerning specificity, LOD, LOQ, precision, and accuracy. This HPLC method can be used to reliably evaluate the quality of AGR and AGR-containing medicinal preparations within 20 min. In addition, it could be applied to a preparative scale for the isolation of D and DA, as well.

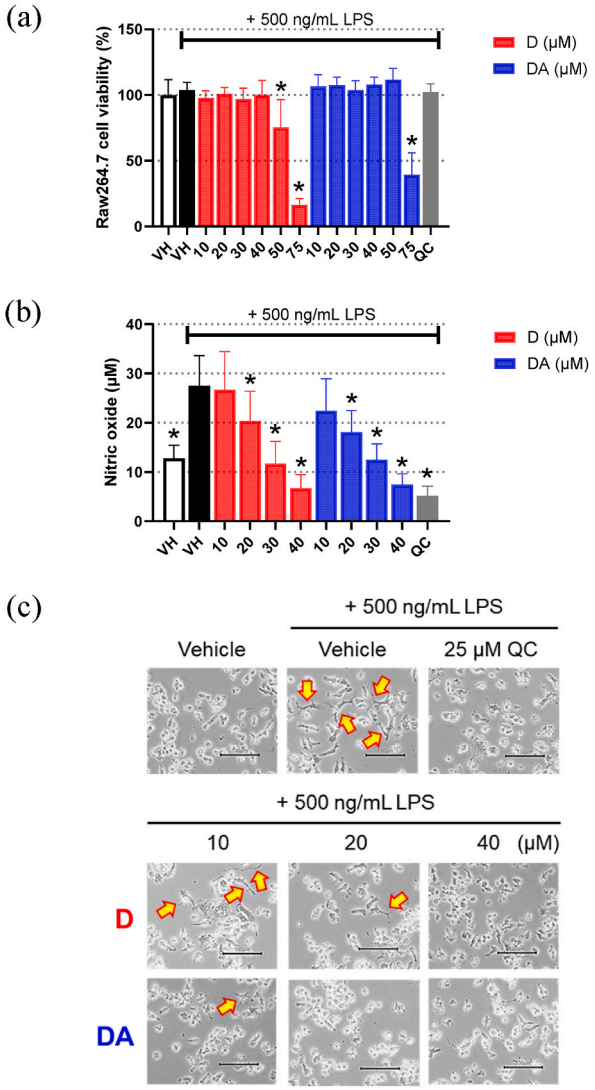

Fig. 4. Comparison of anti-inflammatory effects between D and DA on Raw264.7 stimulated by LPS. (a) MTT cell viability assay ($n \geq 6$). (b) Quantification of nitric oxide ($n \geq 6$). (c) Cell morphology. Arrows represent the activated cells. Each scale bar indicates 100 μm *, $p < 0.05$ compared to vehicle (VH) plus LPS-treated group by one-way ANOVA with Dunnett's multiple comparison test. Quercetin (QC; 25 μM) was used as a positive control.

3.3. Comparison of neuroprotective effect between D and DA

As shown in Fig. 3a, D effectively restored HT22 cell viability against the glutamate-induced cell death (control, 100.0 ± 2.9 %; glutamate only, 33.2 ± 9.4 %), while DA was negligible. The highest recovery of D was observed at 50 μM (66.1 ± 4.9 %). Cell morphology revealed that cells treated with 50 μM D exhibited relatively healthy morphology compared to those treated with glutamate only or DA (Fig. 3b). In addition, 50 μM D significantly attenuated intracellular LDH leakage to 0.3 ± 0.2, while 50 μM DA exhibited relatively lower potency as 1.4 ± 0.1 (control, 0.04 ± 0.01; glutamate only, 2.5 ± 1.6) (Fig. 3c).

3.4. Comparison of anti-inflammatory effect between D and DA

Prior to quantifying nitric oxide, the appropriate concentration ranges for quantifying nitric oxide were selected as 10–40 μM for D and DA via cell viability assay (Fig. 4a). The LPS-induced elevation of nitric oxide (27.5 ± 6.2 μM; control, 12.8 ± 2.6 μM) was concentration-dependently mitigated by D and DA. Particularly, 40 μM of D and DA significantly decreased the nitric oxide to 6.8 ± 2.8 and 7.4 ± 2.2 μM, respectively. The positive control, 25 μM QC, also remarkably reduced nitric oxide to 5.2 ± 1.9 μM (Fig. 4b). The cell morphology altered by LPS treatment was effectively recovered upon treatment with D and DA at 20–40 μM (Fig. 4c).

It has been investigated to the structure-activity relationships (SAR) in neuroprotection of coumarins [29,30]. Skalicka-Woźniak et al. (2016) concluded that the cyclized isoprenyl structure containing dihydropyran and dihydrofuran plays an important role in promoting the neuroprotective effect of coumarins via its lipophilicity. In addition, dihydropyranocoumarins have higher efficacy relative to dihydrofuranocoumarins in neuroprotection [30]. Kang and Kim (2007) also emphasized that coumarins substituted with lipophilic moiety help to increase the neuroprotective activity as well as to penetrate the cellular membranes such as blood-brain barrier [31]. Therefore, D and DA belonging to dihydropyranocoumarins have structural advantages in exhibiting neuroprotective effect.

In this study, we confirmed that D effectively protected HT22 cells from glutamate toxicity, whereas DA had less of an effect. This tendency is similar with the results of the other group, Tran et al. (2023), HT22 protective efficacy of D against glutamate-induced cell death is more potent than that of DA, but decursinol (DOH) is negligible [5]. In addition, the permeability properties of D were better than those of DA in both Caco-2 and MDR-MDCK cell monolayers [14]. On the contrary, the primary rat cortical cells were significantly protected by D and DOH from glutamate toxicity, however, the efficacy of DOH is better than D [32].

In contrast, most studies have reported similar potencies of D and DA in human glutamate dehydrogenase activity [33], amyloid $\beta_{25\text{-}35}$-induced PC12 cell death [34], carbon tetrachloride (CCl_4)-induced hepatic damage in rats [35], apoptotic effects on human breast cancer cell lines (MCF-7 and MDA MB-231) [36], and LPS-stimulated Raw264.7 cells [31]. We also confirmed that D has similar potency to DA in LPS-induced nitric oxide production in Raw264.7 cells. This tendency was also observed in the report of Ma et al. (2019), which explained that the inhibitory effects of D and DA on nitric oxide production in LPS-challenged Raw264.7 are comparable to each other, but the efficacy of D and DA are more potent than DOH [31]. Structurally, D and DA are substituted with senecioylic acid and angeloylic acid, respectively, instead of hydroxyl group at C-3' in DOH [37]. Therefore, it can be inferred that the potent structure, whether the C-3' position is prenylated in DOH backbone, varies according to the assay system applied, including cell type and stimulated system. These varying efficacies of D and DA also support the necessity of complete separation analysis for D and DA, along with proper quality control of AGR.

Therefore, the establishment of optimal condition to separate two isomers D and DA is important to quality control of AGR and bioactivity assessment for each compound. It is concluded that the rapid and simple HPLC-UV condition using a C30 column is useful and valuable.

4. Conclusion

This study presents a straightforward HPLC method using a C30 column for the complete separation-based quantification of D and DA in AGR. Using this validated HPLC method, we determined AGR cultivated in Jinbu (Gangwon, Republic of Korea) contains 4.5 % D and 3.9 % DA. These established HPLC conditions facilitate rapid quality control of medicinal preparations containing AGR. Additionally, our investigation revealed distinct neuroprotective activities between D and DA against glutamate-induced HT22 cell death, with D exhibiting higher efficacy compared to DA. However, D and DA showed similar inhibitory potency in LPS-induced nitric oxide production in Raw264.7 cells. These findings underscore the importance of elucidating the pharmacological properties of D and DA, as well as the necessity of separately analyzing the contents of D and DA for quality control of AGR.

CRediT authorship contribution statement

Eun-Ju Yang: Writing – original draft, Investigation. **Sang-Eun Shin:** Validation, Formal analysis. **Jiwon Park:** Formal analysis. **Haewon Park:** Software. **Min Kim:** Formal analysis. **Kyung-Sik Song:** Supervision, Funding acquisition. **Dong Hee Na:** Writing – review & editing, Supervision, Conceptualization.

Data availability statement

Data will be made available on request.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2025.e43093.

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