

Quality markers based on chromatographic fingerprinting and anti-neuroinflammatory screening: A spectrum–effect correlation for *Nardostachys jatamansi* DC. with anti-neuroinflammatory potential

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Abstract

Background

Nardostachys jatamansi DC. (NJ) has long been prescribed to treat neurodegenerative diseases, including Alzheimer's disease and Parkinson's disease, in traditional Chinese medicine and other orient ethnomedicinal systems. However, the anti-neuroinflammatory components and the quality markers (Q-markers) underlying NJ remained unclear.

Objective and design

This study aimed to reveal the Q-markers of NJ in treating neuroinflammation-related diseases by developing 'spectrum–anti-neuroinflammatory effect' correlation for NJ against neuroinflammation.

Methods

First, a Griess method was applied to evaluate the anti-neuroinflammatory potentials of common NJ extracts and components, discovering the dominant anti-neuroinflammatory component of NJ (NJ_1A). The spectrum–effect correlation of NJ_1A was then accomplished by Pearson's correlation, GCA, and PLSR modeling between the UPLC–PDA fingerprints and the inhibitory rates of batches of NJ_1A on NO production in BV-2 cells. Finally, the potentially effective constituents were screened and their anti-neuroinflammatory potentials were further verified.

Results

The fingerprint similarity of NJ_1A as well as the content of nardosinone would gradually decrease along with the prolongation of the NJ storage time. Ten promising anti-neuroinflammatory-correlated peaks were screened accordingly by the spectrum–effect correlation of NJ_1A. And seven of them were identified and validated to exert varying degrees of anti-neuroinflammatory effect. Finally, nardosinone, desoxo-narchinol A, and nardosinonediol stood out to be the major active constituents and key Q-markers for NJ_1A in treatment of neuroinflammation.

Conclusion

The current study demonstrated that spectrum–effect correlation was a powerful approach to investigate the active components dedicated for the anti-neuroinflammation underlying NJ, and provided a solid basis for the Q-markers of NJ against neurodegenerative diseases.

Highlight

1. The anti-neuroinflammatory activities of NJ extracts and fractions were evaluated simultaneously
2. The anti-neuroinflammatory component of NJ was fingerprinted by UPLC analysis

3. Spectrum–effect correlation uncovers the anti-neuroinflammatory Q-markers for NJ

4. Nardosinone-type sesquiterpenoids were the potential Q-markers of NJ

1. Introduction

Neuroinflammation, an inflammatory response triggered by infection or injury, is a prominent pathological hallmark of various neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis [1]. In the brain, neuroinflammation manifests as elevated levels of pro-inflammatory cytokines, microglia activation, peripheral leukocyte infiltration, and damage to nervous tissue [2]. Microglia (innate immune brain macrophage) is regarded as the key element contributing to neuroinflammation [3]. It is prone to be activated by external pathological stimulus to release pro-inflammatory cytokines and their metabolic products, which in turn leads to the cytotoxicity and neurotoxicity [4]. Therefore, suppressing the neuroinflammation mediated by activated microglia cells could potentially facilitate the treatment of neuroinflammatory diseases.

Nardostachys jatamansi DC. (NJ) is an indigenous medicinal herb in China, India, Nepal, and other countries near the Himalayas, and has long been prescribed to treat neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease [5], in traditional Chinese medicine (TCM) and other orient ethnomedicinal systems. Previous studies have revealed that the ethyl acetate extract of the water decoction of NJ [6] and the diverse constituents, including nardosinone, isonardosinone, kanshones B, E, J, and K, desoxo-narchinol A, narchinol B, bullatantriol, and jatamanin A, have been recognized to exhibit varying levels of anti-neuroinflammatory effects by inhibiting NO production of the LPS-injured BV-2 microglial cells [7–9]. Despite all this, the Q-markers of NJ against neuroinflammation still remain uncovered.

In terms of the intricate composition of TCM, spectrum-effect correlation has been developed as a rational approach uncovering the dominantly effective constituents by modeling the relationship between the chromatographic fingerprint and the bioactivity, with multiple chemometric methods, such as gray correlation analysis (GCA) [10], bivariate correlation analysis (BCA) [11], multiple linear regression analysis (MLRA) [12], back propagation-artificial neural network modeling (BP–ANN) [13], and partial least squares regression analysis (PLSR) [14]. A common strategy for the spectrum-effect correlation includes: (i) establishment of fingerprinting method and acquisition of the chromatographic fingerprints, (ii) acquisition of the quantitative bioactivity data, (iii) screening of the candidate constituents by appropriate multivariate statistical methods, and (iv) verification of bioactivities of the candidate constituents [13]. The spectrum-effect correlation enables the development of Q-marker constituents by observing the overall interaction among the phytoconstituents before further goal-oriented phytochemical separation and identification, with the advantages of time and effort saving, low-cost, and high adaptability [15].

In the present work, a Griess method was applied to evaluate the anti-neuroinflammatory potentials of common NJ extracts and components, discovering the dominant anti-neuroinflammatory component of

NJ (NJ_1A). The spectrum–effect correlation of NJ_1A was then accomplished by Pearson’s correlation, GCA, and PLSR modeling between the UPLC–PDA fingerprints and the inhibitory rates of batches of NJ_1A on NO production in BV-2 cells. Finally, the potentially effective constituents were screened and their anti-neuroinflammatory potentials were further verified and proposed as Q-markers for future development and application of NJ and its related products against neuroinflammation.

2. Materials and methods

2.1 Plant materials

The dried roots and rhizomes of NJ for initial analysis were purchased from Beijing Tong Ren Tang Tianjin Nankai Pharmacy Co. Ltd. (origin: Sichuan province, China) and were authenticated by Prof. Miaomiao Jiang of the Institute of Traditional Chinese Medicine at the Tianjin University of Traditional Chinese Medicine. The voucher specimen (No. 20211220) was deposited in the State Key Laboratory of Component-based Chinese Medicine, Tianjin University of Traditional Chinese Medicine, China. Besides, 18 batches of dried NJ with different storage time and origins were collected with detailed information provided in the supplementary Table S1. The moisture and volatile oil of all the samples conformed to the standards of Chinese Pharmacopoeia when purchased.

2.2 Chemicals, reagents, cells, and apparatuses

Water was provided by Guangzhou Watson’s Food & Beverage Co., Ltd., Guangzhou, China. Chromatographic-grade acetonitrile and formic acid were obtained from Thermo Fisher Scientific, Waltham, MA, USA. Other solvents used for this work were of analytical grade, and were purchased from Concord Technology Co., Ltd., Tianjin, China. Chlorogenic acid, caffeic acid, desoxo-narchinol A, 2-deoxokanshone L, nardosinonediol, nardonoxide, isonardosinone, nardoaristolone B, 1-hydroxylaristolone, debilone, nardosinone, kanshone H and (-)-aristolone were prepared in our lab with UPLC purities of above 98% [16–19]. Essential oil was extracted using Clevenger-type apparatus (Shanghai Leigu Instrument Co., Ltd., China); DE-100g/650W herbal medicine shredder (Ruian Baixin Pharmaceutical Machinery Co., Ltd., China) was used to pulverize NJ materials. EYELA CCA-1111 (EYELA Co., Ltd., Japan) and BUCHI R-125 (BUCHI, Flawil, Switzerland) rotary evaporator was employed to evaporate the solvent from extraction. SB-4200D Ultrasonic apparatus (Xinzhi Bio-tech Co., Ltd., Ningbo, China) was applied to yield NJ methanol extract.

For the anti-neuroinflammatory assay, the BV-2 microglia cell line was obtained from the Cell Resource Center, IBMS, CAMS/PUMC, Beijing. FORMA 3111 CO₂ incubator was supplied by Thermo Scientific Co., Ltd., Waltham, MA, USA. Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), trypsin, penicillin, streptomycin, and LPS were purchased from Gibco BRL (Grand Island, NY, USA). Minocycline was obtained from Beijing Solarbio Science & Technology Co., Ltd., Beijing, China. Cell Counting Kit-8 (CCK-8) was supplied by Dojindo, Beijing, China. And the Nitric Oxide (NO) Content Assay Kit was obtained from Beyotime Biotechnology Co., Ltd., Shanghai, China.

2.3 Extraction and fractionation

Two equivalent amount of 30 g NJ homogenized powder filtered through a 50-mesh sieve were accurately weighed. Then they were extracted with 600 mL methanol ultrasonically and equal volume of distilled water under refluxing, respectively, two times and each 2 h, before the resultant solution being filtered through 15–20 μm medium-speed filter paper, combined the extractions and concentrated to dryness at 40 °C by rotary evaporator to obtain the NJ methanol extract (NJ_1) and water-refluxing extract (NJ_2). NJ_1 was then resuspended in 10-times distilled water (w/v) and extracted three times with equal volumes of ethyl acetate to afford the ethyl acetate fraction (NJ_1A) and water-soluble fraction (NJ_1B), before the similar filtration and concentration procedures. Eighteen batches of NJ_1A were named as GS1–GS18. The light yellowish green essential oil of NJ (NJ_3) was extracted from 30 g NJ powder by hydro-distillation for 4 h in 600 mL distilled water, using the Clevenger-type apparatus following the standard procedure described in the Chinese Pharmacopoeia [20].

2.4 Sample preparation

Samples NJ_1 and NJ_1A were accurately weighted and dissolved in methanol, and samples NJ_1B and NJ_2 were accurately weighted and dissolved in water, to obtain the test solutions at a concentration of 10 mg/mL, which were then centrifuged at 14, 000 rpm for 10 min, filtered through 0.22 μm microporous membrane, and stored in sealed vials at 4°C before the injection for UPLC analysis. 10 μL NJ_3 was dissolved in ethyl acetate (1:500, v/v), filtered through a 0.22 microporous membrane, and kept in an amber sealed vial at 4°C before GC–MS analysis. The thirteen standard compounds, including chlorogenic acid, caffeic acid, desoxo-narchinol A, 2-deoxokanshone L, nardosinonediol, nardonoxide, isonardosinone, nardoaristolone B, 1-hydroxylaristolone, debilone, nardosinone, kanshone H, and (-)-aristolone, were dissolved in methanol to prepare the single and mixed standards solution with suitable concentration for qualitative analysis.

2.5 UPLC analysis

A Waters Acquity UPLC® H class plus system (Waters Corporation, Milford, MA, USA) was employed to undertake the chromatographic separation using an Acquity UPLC BEH C18 column (2.1 mm $\times$ 100 mm, 1.7 μm) at 40 °C. The mobile phase composed of acetonitrile (A) and 0.1% formic acid in water (B) ran at a flow rate of 0.3 mL/min. The samples were detected at a wavelength of 254 nm with an injection volume of 3 μL and pre-equilibrated time of 10 min. The optimized gradient program was set as follows: 0–2 min, 5–10% A; 2–5 min, 10% A; 5–5.5 min, 10–14% A; 5.5–10 min, 14% A; 10–15 min, 14–20% A; 15–22 min, 20–35% A; 22–27 min, 35% A; 27–30 min, 35–40% A; 30–32 min, 40% A; 32–40 min, 40–70% A; 40–45 min, 70–95% A.

Method validation and similarity evaluation of UPLC chromatographic fingerprint were performed with the detailed procedure presented in the supplementary S1–S2.

2.6 GC–MS analysis

NJ_3 dissolved in ethyl acetate (1:500, v/v), was subjected to GC–MS analysis following the reported methods using the same chromatographic condition in one of our previous studies [17].

2.7. Anti-neuroinflammatory assay

BV-2 murine microglial cell line was cultured in DMEM supplemented with penicillin (100 U/mL)/streptomycin (100 µg/mL) and 10% heat-inactivated FBS at 37°C in 5% CO₂ incubator. Cells were passaged with trypsin digestion every other day (1:6). The cells were seeded on 48-well plates at a density of 2×10^5 cells/well, and pretreated with 10 µM minocycline [21] as a positive group, or with extracts, fractions and 10 µM major compounds of *N. jatamansi* as drug group (NJ_1, NJ_1A, NJ_3 and tested compounds were dissolved in DMSO; NJ_1B and NJ_2 were dissolved in water), and then stimulated with 0.1 µg/mL LPS (lipopolysaccharide, an immune-stimulating factor) in serum-free DMEM. The untreated culture medium was determined as the blank control group, while the culture medium only stimulated with LPS was regarded as the model group. After incubating for 24 h, the culture supernatant was collected and the NO Content Assay Kit was utilized to determine the anti-neuroinflammatory capacity by the Griess reaction [22] according to the manufacturer's protocol. The CCK-8 assay kit [23] was employed to clarify cell viability of BV-2 microglia for 30 minutes of incubation, and the absorbance of 450 nm was read. The relative production of NO compared with the model group and the inhibition ratio on NO were calculated according to the following formula:

The production of NO (% of LPS) = $(C_{\text{treatment}} - C_{\text{control}}) * 100 / (C_{\text{LPS}} - C_{\text{control}})$

The inhibition ratio on NO (%) = $1 - (C_{\text{treatment}} - C_{\text{control}}) * 100 / (C_{\text{LPS}} - C_{\text{control}})$

Where the C_{control} , C_{LPS} , and $C_{\text{treatment}}$ are the contents of NO in the control, the model (LPS-stimulated) and the positive/drug-treatment groups, respectively.

2.8 'Spectrum–anti-neuroinflammatory effect' correlation

Two chemometrics approaches (GCA and PLSR) were employed in the modeling of the fingerprint-effect relationship between the relative contents (percentage, %) of twenty-one common peaks in 18 batches of NJ_1A and the anti-neuroinflammatory activity data using Excel 2016 and RStudio software, respectively. Pearson's correlation analysis was carried out using Origin 2021 software. The average NO inhibition rate of each NJ_1A at the concentration of 50 µg/mL was taken into account due to the considerable and significant effects under this concentration. The combination of several methods enabled a mutual verification of the spectrum-effect correlation analysis between pharmacodynamic indices and chromatographic peaks, enhancing the credibility of these efficacy-associated compounds selected out of the constituents in the NJ_1A. The detailed principles and procedures of GCA and PLSR can be checked in our previous research [17].

2.9 Statistic analysis

The experiment data were analyzed statistically using Graph Pad Prism 8.0 software (Graph Pad Software, CA), and the results were expressed as the mean ± SEM. One-way ANOVA was carried out for

multi-group comparisons of the data with statistical significance set at $p < 0.05$.

3. Results and discussion

3.1 Evaluation of the anti-neuroinflammatory activities of the NJ extracts and fractions

The representative UPLC-PDA chromatograms of samples NJ_1, NJ_1A, NJ_1B, and NJ_2, and the GC-MS total ion current chromatogram of sample NJ_3, were shown in Fig. 1A, which demonstrated that there were different profiles of constituents in different extracts and fractions: NJ_1A contains the most liposoluble constituents with long retention behavior; NJ_1B and NJ_2 have predominantly water-soluble constituents with quite short retention behavior; And NJ_3 includes mainly the volatile constituents. The effects of samples NJ_1, NJ_1A, NJ_1B, NJ_2, and NJ_3 on the viability and the LPS-stimulated secretion of NO in BV-2 microglia cells were evaluated (Fig. 1B). The results showed that samples NJ_1, NJ_1A, NJ_1B, and NJ_3 dose-dependently suppressed the LPS-stimulated NO production in BV-2 microglia cells, while sample NJ_2 didn't show any significant activities until its concentration reached to the extremely high concentration of 100 $\mu\text{g/mL}$. Specifically, samples NJ_1 and NJ_1A promisingly suppressed the LPS-stimulated NO production in BV-2 microglia cells at the concentration of 10–1 $\mu\text{g/mL}$ and 50–5 $\mu\text{g/mL}$, respectively. Sample NJ_1B showed an inferior significant activity at the concentration of 100–10 $\mu\text{g/mL}$. Notably, cytotoxicity on BV-2 cells had not been found in samples NJ_1, NJ_1A, NJ_1B, and NJ_2. However, sample NJ-3 exhibited significant cytotoxicity on the cells when being administrated at a concentration above 100 $\mu\text{g/mL}$. By comprehensive consideration, sample NJ_1A showed the most potent anti-neuroinflammatory activity with no significant cytotoxicity on BV-2 cells, and it was selected as the key active component in NJ and subjected to subsequent studies.

Similar as our discovery of the anti-neuroinflammatory capacities of NJ_1 and NJ_2, previous studies have revealed that the pretreatment with the ethyl acetate fraction from the hot water extract [6], 20% aqueous ethanol extract [24], as well as several major constituents from the methanol extract [7–9, 25] of NJ could inhibit the LPS-induced excessive production of NO without cytotoxicity on BV-2 cells.

3.2 Fingerprinting of samples of NJ_1A and the spectrum-effect correlation

3.2.1 UPLC–PDA fingerprinting and the multivariate statistical analysis

As shown in the supplementary Table S2, the yields of 18 batches of NJ_1, NJ_1A, and NJ_1B were 15.73–32.39%, 7.86–16.17%, and 4.52–10.91%, respectively, which indicated that NJ-1A was the major component of NJ_1. Subsequently, a UPLC–PDA fingerprinting method of NJ_1A was established with methodological properties including the precision, repeatability, and stability validated as demonstrated in the supplementary Tables S3–S5, with all relative standard deviation (RSD) values of relative peak

area less than 0.9%. As shown in Fig. 2A, there were 21 common chromatographic peaks in the UPLC–PDA fingerprints of 18 batches of NJ_1A. By compared to the reference chromatogram of NJ_1A (supplementary Fig. S1), the similarity coefficients of the 18 batches of NJ_1A were calculated as presented in the supplementary Table S6. Eighteen batches of NJ_1A possessed poor quality consistency with similarity coefficients ranging from 0.339 to 0.993, and samples GS9 (0.464), GS11 (0.664), GS13 (0.339), and GS15 (0.540) possessed exceedingly low similarities. The greater the difference in peak intensities, the lower the similarity. For example, the contents of peak **18** (nardosinone), the most prominent constituent in NJ_1A (t_R = 28.80 min), varied dramatically among different batches of NJ_1A, with extremely lowest relative-contents in samples GS9 (2.19%), GS11 (3.89%), GS13 (1.81%), and GS15 (3.08%), and conversely the highest relative-contents in samples GS1 (39.45%), GS2 (49.70%), GS3 (50.34%), GS6 (34.11%), and GS7 (48.46%).

Principal component analysis (PCA) clustered the 18 batches of NJ_1A into two separate groups (Fig. 2D) by using the SIMCA (Version 14.1): Samples GS1–GS8 in group I, and GS9–GS18 in group II. Among them, GS1–GS7 were obtained from different origins in year 2021–2022, while others were purchased in year 2017–2019 with the relatively longer storage time. It can be speculated that the contents of certain constituents in NJ_1A changed considerably along with the prolongation of the storage time.

As reported, nardosinone (peak **18**) is extremely unstable under conditions of high temperature, high humidity, and highly light radiation [26, 27]. The content of nardosinone would gradually decrease along with the increase of the storage time, resulting in the low similarity coefficients of the 18 batches of NJ_1A, and the clustering into two groups by the PCA analysis.

By comparing with chromatographic behaviors and UV spectra of reference compounds (Fig. 2B), thirteen of the twenty-one common characteristic peaks have been assigned and identified as chlorogenic acid (**P1**, t_R = 4.24 min), caffeic acid (**P2**, t_R = 4.84 min), desoxo-narchinol A (**P5**, t_R = 17.29 min), 2-deoxokanshone L (**P9**, t_R = 19.84 min), nardosinonediol (**P10**, t_R = 20.87 min), nardonoxide (**P11**, t_R = 21.81 min), isonardosinone (**P12**, t_R = 22.30 min), nardoaristolone B (**P14**, t_R = 23.60 min), 1-hydroxylaristolone (**P15**, t_R = 23.92 min), debilone (**P16**, t_R = 24.90 min), nardosinone (**P18**, t_R = 28.80 min), kanshone H (**P19**, t_R = 31.59 min), and (-)-aristolone (**P20**, t_R = 34.84 min), with chemical structures as presented in Fig. 2C.

3.2.2 Correlation between the UPLC-PDA fingerprints and the anti-neuroinflammatory effects of 18 batches of NJ_1A.

As shown in Fig.s 2E and 2F, the anti-neuroinflammatory effects of 18 batches of NJ_1A, at a concentration of 50 $\mu\text{g/mL}$, was evaluated and the result indicated that all batches of NJ_1A could promisingly inhibit LPS-stimulated NO secretion in BV-2 microglia cells at a low concentration without any effects on the cell viability. Specifically, almost all batches of NJ_1A exhibited considerable NO inhibitory effects (inhibition ratio: 85.25–111.54%) equivalent to that of the 10 μM minocycline (67.35 $\pm$

9.28%), except for sample GS5 and GS13 with the inhibitory rates of $71.53 \pm 28.24\%$ and $65.37 \pm 3.34\%$, respectively.

The UPLC spectra (*viz.* relative contents of the normalized 21 common peaks) of 18 batches of NJ_1A and their anti-neuroinflammatory effects at a concentration of $5 \mu\text{g/mL}$ were correlated by three chemometric methods of Pearson's correlation analysis (Fig. 3A), GCA and PLSR (Fig. 3B).

A heat-map of Pearson's correlation coefficients was plotted as shown in Fig. 3A, the dark brown and green points indicate the positive and negative correlations to effects, respectively. As a result, fourteen peaks (**P2**, **P3**, **P4**, **P5**, **P7**, **P10**, **P11**, **P12**, **P14**, **P15**, **P17**, **P18**, **P19**, and **P20**) with correlation coefficient more than zero showed positive correlations to the anti-neuroinflammatory effect, which can be speculated that these constituents exert beneficial perturbations on the anti-neuroinflammation activity of NJ_1A. As illustrated in the supplementary Table S7 and Fig. 3B, GCA correlation degrees of the 21 common characteristic peaks relative to the effect ranged from 0.7598 to 0.9441, indicating that almost all the observed common constituents possessed high relevance to the anti-neuroinflammatory activity with correlation degrees above 0.6. When a correlation degree of 0.85 was taken as the cut-off, fourteen peaks with relatively high contributions to the anti-neuroinflammatory effect were accordingly screened out, including **P1**, **P2**, **P5**, **P9**, **P10**, **P11**, **P12**, **P14**, **P15**, **P16**, **P18**, **P19**, **P20**, and **P21**. Further, PLSR correlation was performed, and the results was shown in supplementary Table S8 and Fig. 3B. The correlation coefficients of the twenty-one characteristic peaks to the anti-neuroinflammation efficacy ranged from -0.4871 to 0.3371 . The result was consistent with the findings of the Pearson correlation analysis (Fig. 3A).

By a comprehensive consideration of the aforementioned GCA and PLSR results, ten common constituents, including **P2** (caffeic acid), **P5** (desoxo-narchinol A), **P10** (nardosinonediol), **P11** (nardonoxide), **P12** (isonardosinone), **P14** (nardoaristolone B), **P15** (1-hydroxylaristolone), **P18** (nardosinone), **P19** (kanshone H), and **P20** (aristolone), finally stood out as the key facilitators for anti-neuroinflammatory activity of NJ.

In addition, it can be observed that the relative content of **P18** (nardosinone) was negatively correlated to those of most common peaks except for **P19** (kanshone H) and **P20** (aristolone). Our previous work has demonstrated that nardosinone is unstable and is prone to a ring-opening reaction, occurring with the major degradation products, including **P9** (2-deoxokanshone L), **P5** (desoxo-narchinol A), **P10** (nardosinonediol), and **P12** (isonardosinone) [17]. This intrinsic degradation in nardosinone can explain reasonably the significant negative correlations from **P18** to **P5** (-0.51), **P9** (-0.64), **P10** (-0.34), and **P12** (-0.48). Further to say, more intrinsic degradation may exist in NJ samples along with the process of storage. And there might be a synergistic and antagonistic effects among the NJ constituents to exert the anti-neuroinflammatory activity.

3.3 Evaluation of the anti-neuroinflammatory potentials of the major constituents in NJ_1A

As shown in the Fig. 4, at the concentration of 10 μ M, all of the 13 major constituents in NJ_1A didn't affect the cell viability of BV-2 microglia cells. And among them, nardosinone (**P18**) and desoxo-narchinol A (**P5**) exhibited extremely significant anti-neuroinflammatory activities, similar to the positive drug of minocycline, with the NO inhibitory rates at $96.61 \pm 2.75\%$ and $92.47 \pm 12.50\%$, respectively; Nardosinonediol (**P10**) and kanshone H (**P19**) showed moderate anti-neuroinflammatory activity with the NO inhibitory rates of $47.84 \pm 10.25\%$ and $47.24 \pm 13.13\%$, respectively; Caffeic acid (**P2**), nardonoxide (**P11**), and isonardosinone (**P12**) possessed mild anti- neuroinflammatory effects with the inhibitory rates of $26.85 \pm 7.23\%$, $35.10 \pm 1.40\%$, and $35.53 \pm 1.79\%$, respectively; While other compounds, such as chlorogenic acid (**P1**), 2-deoxokanshone L (**P9**), nardoaristolone B (**P14**), 1-hydroxylaristolone (**P15**), debilone (**P16**), and aristolone (**P20**) showed no significant anti-neuroinflammatory activities. Specifically, seven out of the ten above-mentioned key facilitators for anti-neuroinflammation activity of NJ were clarified and validated, further convincing the feasibility of the spectrum–effect correlation. Our study suggested that NJ_1A exerts the anti-neuroinflammatory activity through the joint contributions of multiple constituents.

In this work, the anti-neuroinflammatory activities of the NJ extracts and fractions were evaluated firstly. Sample NJ_1A was selected as the dominant active component in NJ and subjected to subsequent studies. Then the fingerprinting of the 18 batches of NJ_1A, the anti-neuroinflammatory evaluation, the spectrum–effect correlation, and the activity verification were accomplished successively in our study, enabling the screen of anti-neuroinflammatory Q-markers for NJ. Finally, as demonstrated in the Fig. 5A, three nardosinane-type sesquiterpenoids (**P5**, **P10**, **P18**) stood out as the promisingly key facilitators with Q-marker potential for the anti-neuroinflammation activity of NJ along with their high relative contents and significant effects. Subsequently, the content level of these three constituents in different batches of NJ_1A was analyzed (Fig. 5B). We found that the relative-contents of nardosinone (**P18**) presented in samples from GS1–GS7 (purchase time: 2021–2022) were relatively higher than those in samples from GS8–GS18 (purchase time: 2021–2022), indicating that nardosinone is unstable along with the process of storage. Another interesting phenomenon was observed that, in the samples with the lower relative-content of nardosinone (**P18**), the relative contents of desoxo-narchinol A (**P5**) and nardosinonediol (**P10**) were more likely higher than those in other samples. As reported in our recent study [17], nardosinone (**P18**) is prone to degrading into desoxo-narchinol A (**P5**), nardosinonediol (**P10**), 2-deoxokanshone L (**P9**), and other products. As shown in the Fig. 5B, samples from GS5 and GS13 exerted more weaker anti-neuroinflammation activities than others due to the lower contents of the above three chosen makers (**P18**, **P5**, and **P10**). We speculated that in the process of the anti-neuroinflammatory effect exerted by NJ, the transformation and/or degradation, synergistic and/or antagonistic interaction among the constituents exists, and the exertion of the activity is the result of the joint action of multiple components.

4. Conclusions

In this study, sample NJ_1A was selected as the key anti-neuroinflammatory component in NJ. As a result of the correlation analysis on the relationship between the UPLC spectrum and the anti-neuroinflammatory activity of NJ, ten promising activity-related peaks were screened, and seven of them

were identified and evidenced to exert varying degrees of anti-neuroinflammatory effect. Nardosinone, desoxo-narchinol A, and nardosinonediol hold a considerable contribution weight and occupy an exceedingly vital position in the process of resisting neuroinflammation by NJ. This work could lay a valuable technical and methodological foundation for the future quality assessment, exploitation and application of NJ for the neurological and related disorders.

Declarations

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Author contributions

BXX: Performed research, data analysis, results visualization, writing the manuscript; XJL: writing the manuscript; CYD: performed research, data analysis; LHZ: reviewed and edited the manuscript; SXW: provided the technical support of cell experiment, reviewed and edited the manuscript; HHW: conception and design of the research, wrote the manuscript, reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Data availability

The authors will supply the relevant data in response to reasonable requests.

No conflicts of interest are declared by the authors.

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Figures

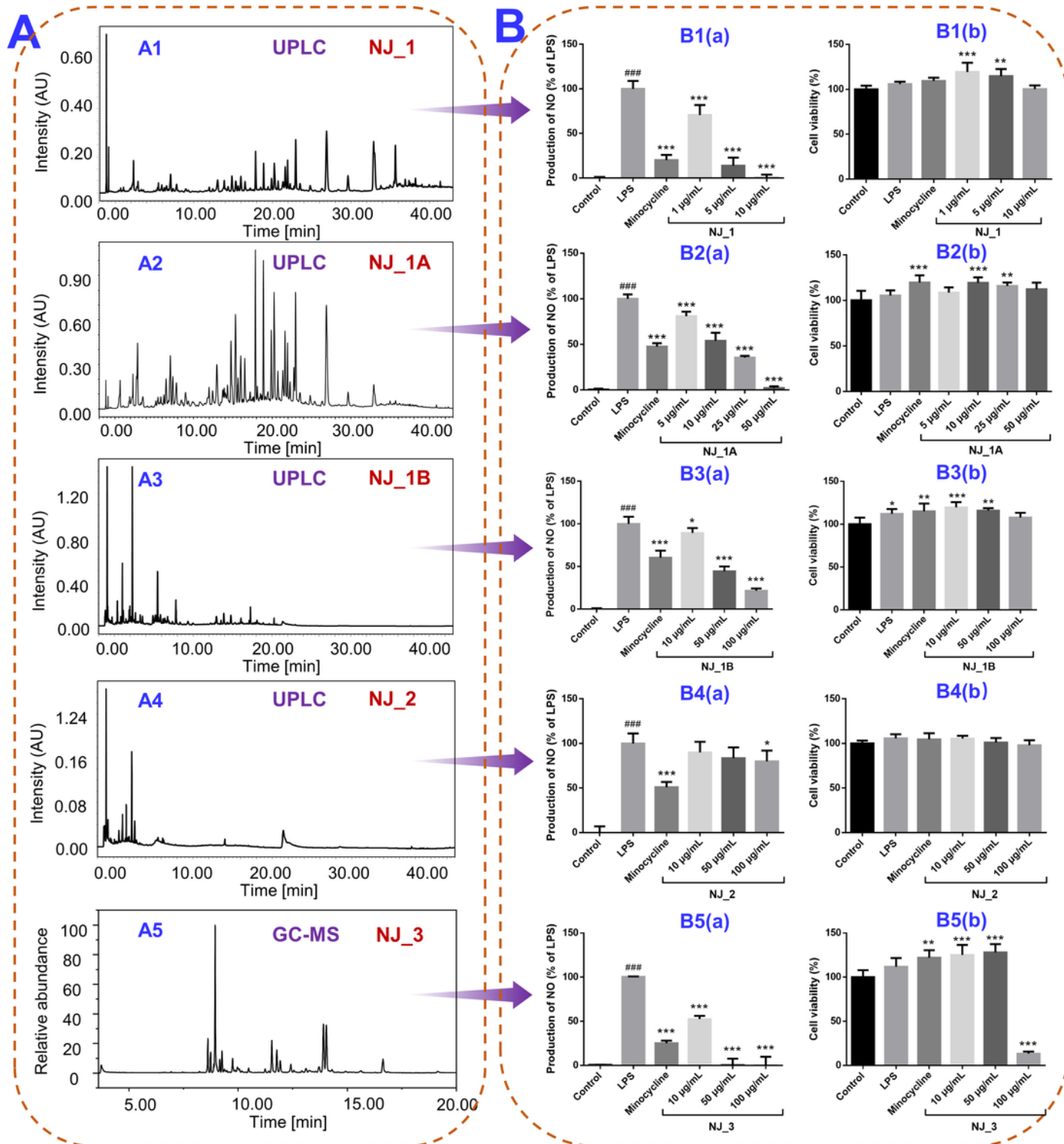


Figure 1

Chromatographic analysis (**A**) and anti-neuroinflammatory activity evaluation (**B**) of the NJ extracts and fractions. [UPLC chromatogram of NJ_1 (**A1**), NJ_1A (**A2**), NJ_1B (**A3**) and NJ_2 (**A4**); GC-MS total ion current chromatogram of NJ_3 (**A5**); The production of NO (% of LPS) and cell viability of NJ_1 (**B1**), NJ_1A (**B2**), NJ_1B (**B3**), NJ_2 (**B4**), NJ_3 (**B5**) on BV-2 cells. $n = 6$, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. control group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. LPS group]

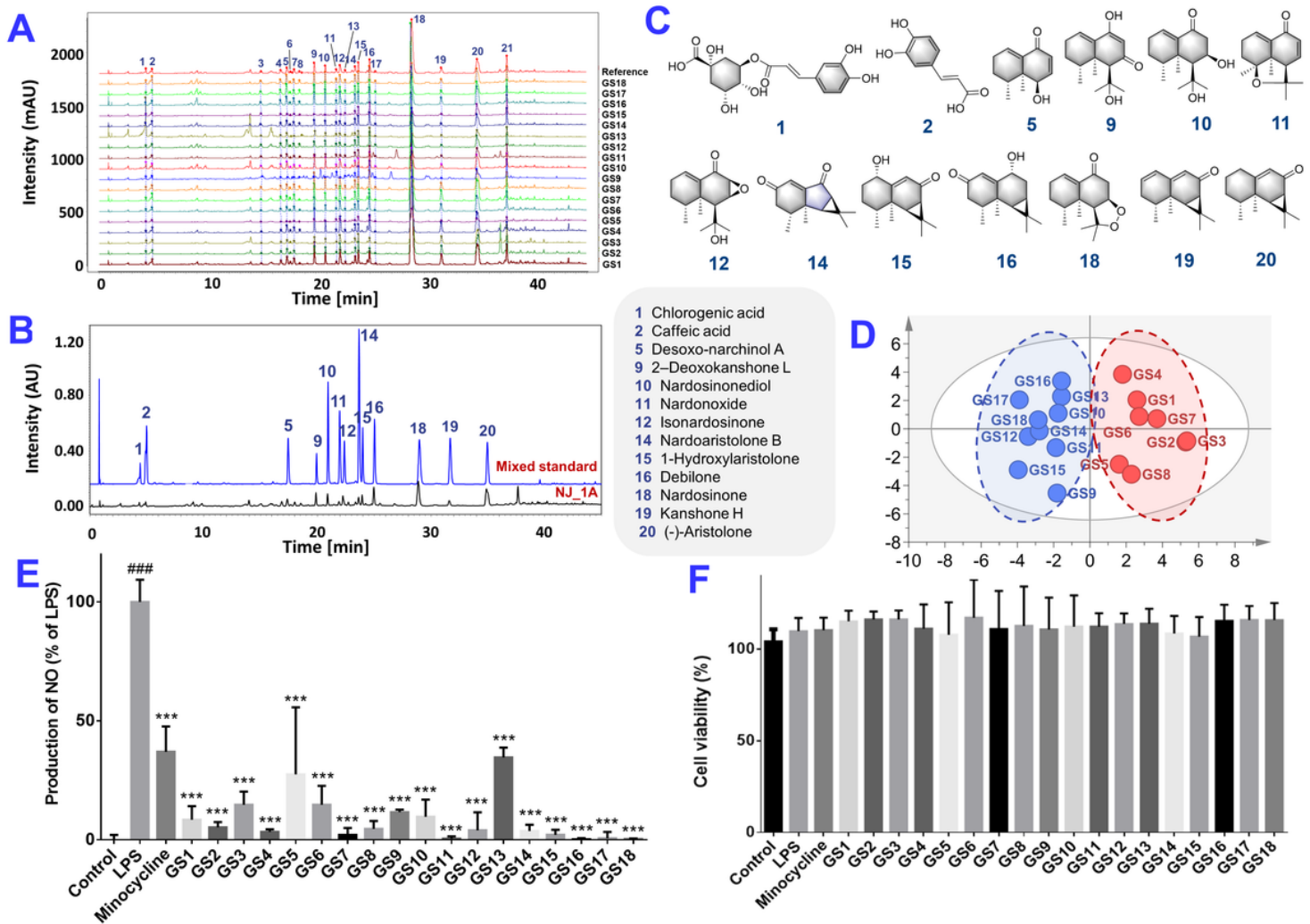


Figure 2

UPLC–PDA analysis, characterization, and the evaluation of anti-neuroinflammatory activity of 18 batches of NJ_1A. [A, UPLC fingerprint; B, identification of the characteristic peaks; C, chemical structures of the 13 identified constituents; D, PCA analysis of the UPLC fingerprints; And (E) the production of NO (% of LPS) and (F) cell viability of BV-2 microglial cells treated with LPS and NJ_1A. $n = 3$, ### $p < 0.001$ vs. control group; *** $p < 0.001$ vs. LPS group]

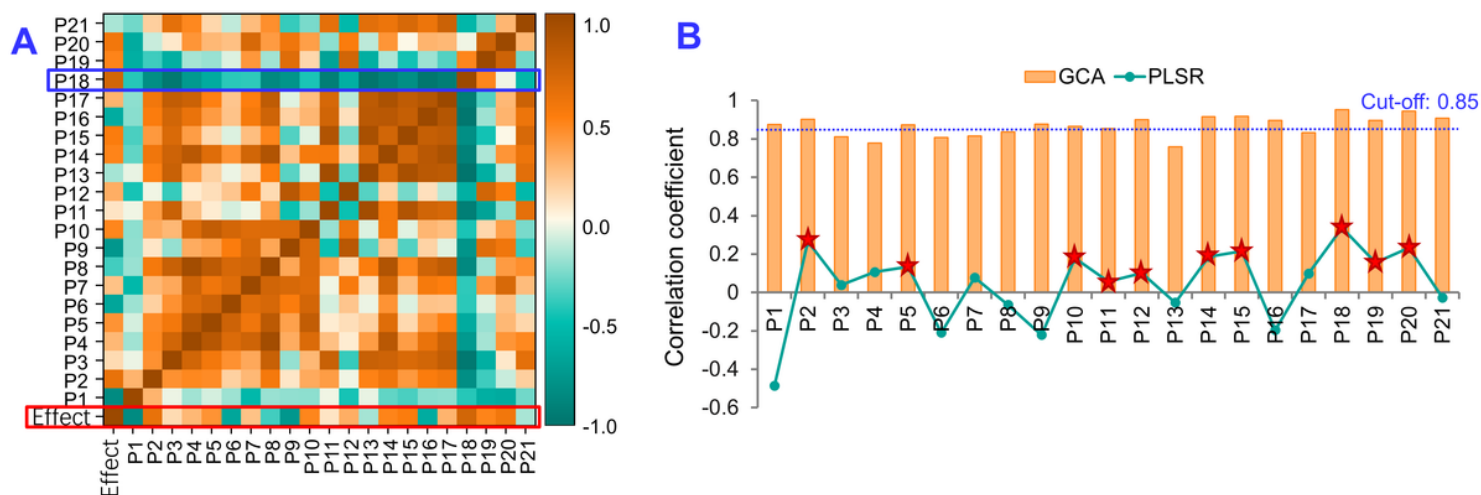


Figure 3

The Pearson's correlation (**A**, heatmap), GCA (**B**, orange bar chart), and PLSR (**B**, cyan line diagram) modeling results of the relationship between the UPLC fingerprints and the anti-neuroinflammatory activity of 18 batches of NJ_1A. Red stars represent characteristic peaks with correlation coefficients greater than 0.85 in GCA as well as larger than zero in PLSR. Blue and red blocks represent the correlation from P18 to other peaks, 21 peaks to effect, respectively.

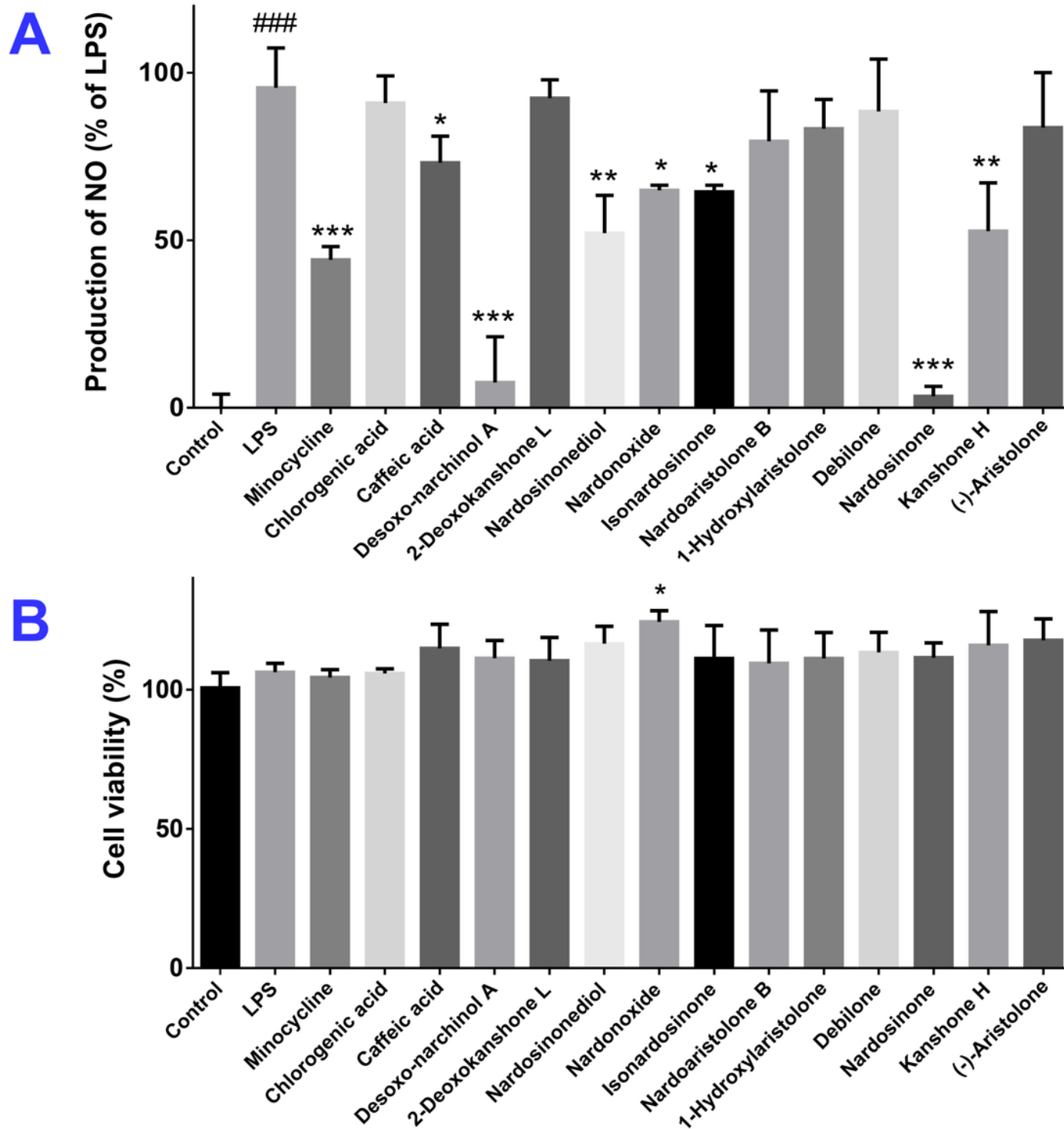


Figure 4

Evaluation of the anti-neuroinflammatory activities for 13 major constituents from NJ_1A. [(A) The production of NO (% of LPS) and (B) cell viability of BV-2 microglial cells treated with LPS and compounds. $n = 6$, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. control group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. LPS group]

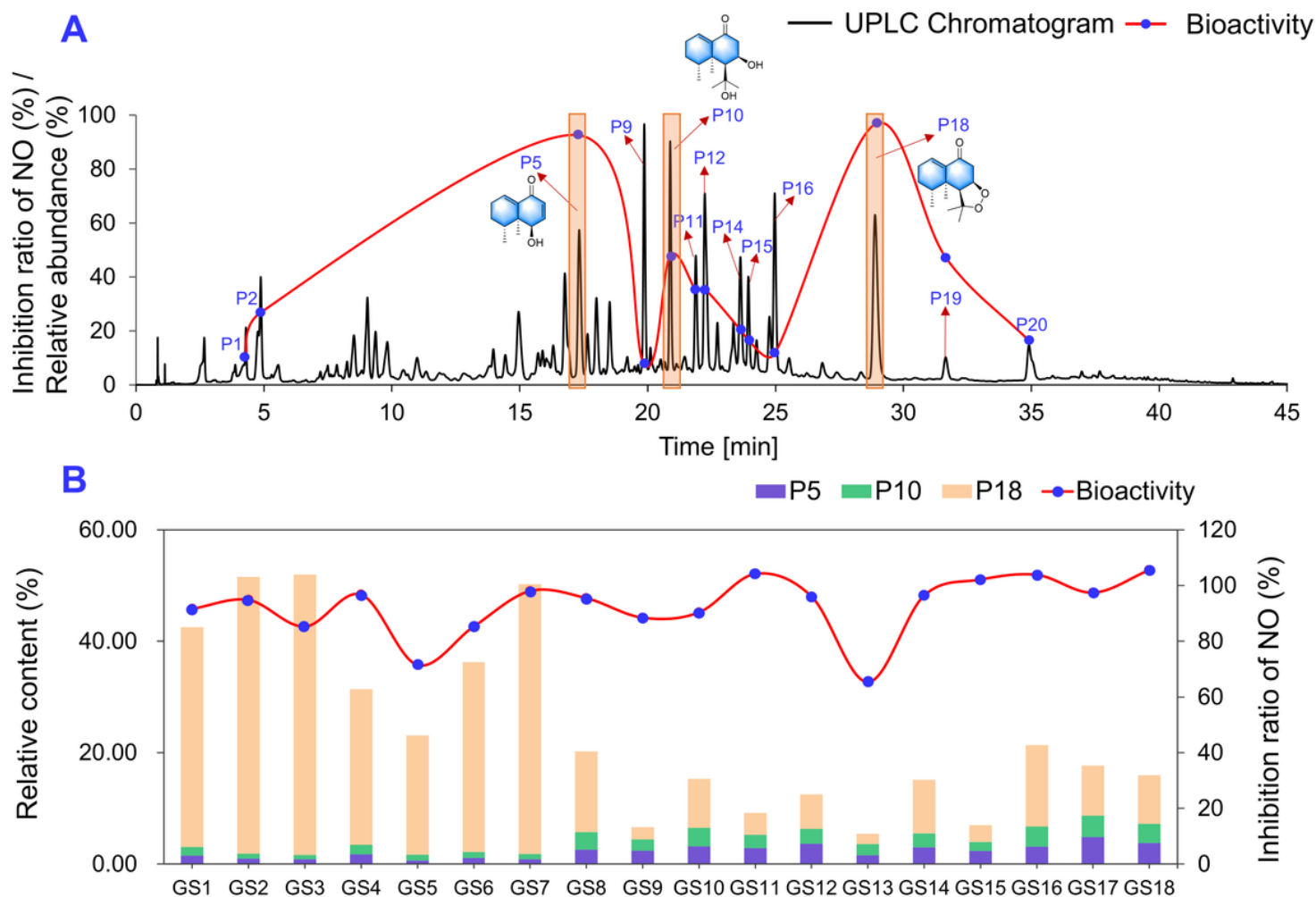


Figure 5

Fitting curves of 'UPLC spectrum of NJ_1A—anti-neuroinflammatory activity of the 13 main constituents' (A) and 'relative contents of P5, P10 and P18—anti-neuroinflammatory activity of 18 batches of NJ_1A' (B)

Supplementary Files

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