Dereplication-Guided Isolation of New Phenylpropanoid-Substituted Diglycosides from *Cistanche salsa* and Their Inhibitory Activity on NO Production in Macrophage

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**Abstract:** Dereplication allows for a rapid identification of known and unknown compounds in plant extracts. In this study, we performed liquid chromatography-mass spectroscopy (LC-MS)-based dereplication using data from ESI+ QTOF-MS for the analysis of phenylpropanoid-substituted diglycosides, the major active constituents of *Cistanche salsa* (C. A. Mey.) Beck. Using TOF-MS alone, the substructures of these compounds could be unambiguously confirmed based on the characteristic fragmentation patterns of various product ions. HPLC-MS based profiling of *C. salsa* also allowed for the detection of new phenylpropanoid-substituted diglycosides from this plant. Of them, five new phenylpropanoid-substituted diglycosides, named cistansalsides A–E (5, 6, 12, 17 and 18), were isolated. Their structures were elucidated through spectroscopic methods including NMR and MS analysis. All the isolates were tested for their inhibitory activity against NO production in RAW 264.7 cells stimulated by LPS. Of the tested compounds, compounds 5, 11, 13 and 18 showed moderate inhibitory activity on inducible NO synthase. Compounds 11, 13 and 18 also inhibited the phosphorylation of NF-κB in macrophages. None of the compounds displayed significant cytotoxicity.

**Keywords:** dereplication; phenylpropanoid-substituted diglycosides; *Cistanche salsa*; anti-inflammatory

1. Introduction

*Cistanche salsa* (C. A. Mey.) Beck, belonging to the family Orobanchaceae, is a parasitic plant that obtains its nutrition from the root of *Haloxylon ammodendron* (Chenopodiaceae) and other desert plants [1]. This plant has been used in traditional medicine for the treatment of neurasthenia, sexual dysfunction and kidney deficiency [2,3]. In previous phytochemical studies, it has been reported that the whole plant of *C. salsa* contained various types of compounds including phenylethanoid glycosides and iridoid glycosides [4–7]. Phenylethanoid glycosides, such as acteoside and echinacoside, are the major active constituents of the plant [8]. The extracts of *C. salsa* showed beneficial properties, including immunomodulatory, anticancer and antiinflammatory activities [9,10].

Dereplication is a process by which sample mixtures would be tested to differentiate unknown constituents from known compounds. The dereplication strategies are based on the analytical techniques and database searching to identify secondary metabolites early in the phytochemical research process [11]. Of the analytical techniques, ESI-QTOF-MS (electrospray ionization-quadrupole-time of flight-mass spectroscopy) could provide valuable information about chemical structures of secondary metabolites. The LC-MS-based dereplication-guided fractionation
has been demonstrated to enable extraction and purification of target metabolites from crude extracts of plants with high efficiency [12–15].

This study performed the LC-MS-based dereplication using data from ESI+ TOF-MS for analysis of phenylpropanoid-substituted diglycosides, the major active constituents of C. salsa. The TOF-MS data could suggest the substructures of these compounds based on characteristic fragmentation patterns of various product ions. Based on this dereplication, LC-MS profiles of ethyl acetate (EtOAc) fraction and water-soluble fraction were analyzed. The EtOAc fraction was subjected to the dereplication strategy for further separation, resulted in the isolation of five new phenylpropanoid-substituted diglycosides and 13 known compounds (Figure 1). It was confirmed that tentatively predicted structures of phenylpropanoid-substituted diglycosides were correctly matched to their real structures. In addition, the anti-inflammatory activities of the isolates were explored.

2. Results and Discussion

The phenylpropanoid-substituted diglycosides isolated from C. salsa usually have structures based on disaccharide glycosides, which consist of a glucose and a rhamnose with a Rha (1→3) Glc linkage and one cinnamoyl substituent, such as coumaric acid (Cou), caffeic acid (Caf) and feruloyl acid (Fer), at the C-4 or C-6 position of glucose. The aglycone is commonly attached at the C-1 position of glucose. The structures of phenylpropanoid-substituted diglycosides with an acetyl group at the C-2 of glucose have frequently been reported [6,12,16].

To perform the dereplication, MS fragmentation patterns of these compounds were analyzed by positive mode ESI-QTOF-MS. In MS spectra, all the phenylpropanoid-substituted diglycosides produced adduct ion peaks at [M + NH₄]⁺, [M + K]⁺ and [M + Na]⁺, which provided the molecular weight and formula. The pattern of fragment ions could be found by successive losses of aglycone and glycoside residues ([M + H – Aglycone]⁺, [M + H – Aglycone – Rha]⁺ and [M + H – Aglycone – Rha – Glc (or Acetyl-Glc)]⁺), which were useful for predicting the type of cinnamoyl substituent and sugars. The fragment ions at m/z 163 of the caffeoyl group, m/z 147 of the coumaroyl group or m/z 177 of the feruloyl group give the characteristic signal of a cinnamoyl substituent in the

![Structures of compounds 1–18.](image)

**Figure 1.** Structures of compounds 1–18.
phenylpropanoid-substituted diglycosides [4,15] (Figure 2). The analysis of the fragment ions would provide useful information for the identification of the structures of phenylpropanoid-substituted diglycosides. However, their isomers could not be differentiated by MS spectrometry alone. For accurate identification of their complete structures, NMR spectra are required.

Figure 2. The fragmentation pathways of phenylpropanoid-substituted diglycosides. (A) Tubuloside E, C_{31}H_{38}O_{15}, M.W. 650; (B) 2′-Acetylacteoside, C_{31}H_{38}O_{16}, M.W. 666; (C) Cistanoside D, C_{31}H_{40}O_{15}, M.W. 652

C. salsa was analyzed and the fingerprint of the EtOAc fraction was generated using the HPLC-DAD (diode array detector)-ESI-QTOF-MS method (Figure 3). Each peak in the fingerprint of C. salsa was predicted according to MS fragmentation features (Table 1). Many phenylpropanoid-substituted diglycosides were screened out from this fraction, which was subjected to HPLC-QTOF-MS-guided isolation for the discovery of new phenylpropanoid-substituted diglycosides. Eighteen peaks including five new compounds were further identified and their structures were elucidated through extensive spectroscopic analysis.
Figure 3. Base peak chromatogram and extracted ion chromatograms of EtOAc fraction of *C. salsa* analyzed by HPLC-ESI-QTOF-MS in positive mode: (A) Caf at m/z 163; (B) Fer at m/z 177; (C) Cou at m/z 147; (D) base peak chromatogram at 320 nm.

Cistansalside A (5) was obtained as a brown amorphous powder. Its molecular formula was determined to be C$_{30}$H$_{38}$O$_{14}$ by positive mode high resolution (HR) ESI-QTOF-MS based on the adduct ion peak at m/z 645.2146 [M + Na]$^+$ (calcd. for C$_{30}$H$_{38}$O$_{14}$Na, 645.2154). A characteristic ion at m/z 177 suggested that a feruloyl substituent existed in its structure. The fragment ions at m/z 339 and m/z 485 suggested the presence of a rhamnose unit and a glucose unit as well. The $^{13}$C-NMR spectrum showed 30 carbon atoms. Analysis of the $^1$H and HSQC spectra indicated the presence of two anomeric protons at $\delta$H 4.35 (1H, d, $J = 7.9$ Hz, H-1‘) and 5.02 (1H, s, H-1’’), and a methoxy proton at $\delta$H 3.80 (3H, s, 3‘’’-OCH$_3$). The $^1$H-NMR spectrum showed an 1,3,4-trisubstituted benzene ring at $\delta$H 7.29 (1H, d, $J = 1.3$ Hz, H-2’’’), 7.09 (1H, dd, $J = 8.3$, $J = 1.3$ Hz, H-6’’’), and 6.79 (1H, d, $J = 8.3$ Hz, H-5’’’), a trans-olefin group at $\delta$H 7.53 (1H, d, $J = 15.9$ Hz, H-7’’’), and 6.41 (1H, d, $J = 15.9$ Hz, H-8’’’), and a para-substituted benzene ring at $\delta$H 7.05 (2H, d, $J = 8.3$ Hz, H-2, 6) and 6.67 (2H, d, $J = 8.3$ Hz, H-3, 5) (Table 2).
Table 1. Identification of phenylpropanoid-substituted diglycosides in EtOAc fraction of *C. salsa* by HPLC-ESI-QTOF-MS in positive ion mode.

| No. | t_R  | [M + Na]^+ | M.W. | Molecular Formula | MS Fragment Ions | Fragment ^c | Identification | Abundances ^d (%) |
|-----|------|------------|------|------------------|------------------|-------------|----------------|-------------------|
| X1  | 11.094 | 647.1954   | 624  | C_{29}H_{36}O_{15} | 163, 325, 471, 467, 663 | Caf, Glc, Rha, D | -              | -                |
| X2  | 13.939 | 647.1920   | 624  | C_{29}H_{36}O_{15} | 163, 325, 471, 467, 663 | Caf, Glc, Rha, D | -              | -                |
| X3  | 15.701 | 631.1959   | 608  | C_{29}H_{36}O_{14} | 163, 325, 471, 626, 631, 647 | Caf, Glc, Rha, C | -              | -                |
| 1   | 16.170 | 631.1982   | 608  | C_{29}H_{36}O_{14} | 147, 309, 455, 605, 626, 631, 647 | Caf, Glc, Rha, D | Lipidoside A-I | 1.9              |
| X4  | 16.962 | 661.2103   | 638  | C_{30}H_{38}O_{15} | 163, 325, 471, 656, 661, 679 | Caf, Glc, Rha, E or F | -              | -                |
| 2   | 20.858 | 689.2070   | 666  | C_{31}H_{38}O_{16} | 163, 376, 513, 684, 689, 705 | Caf, Acetyl-Glc, Rha, D | 2′-Acetylated Aseidoside | 50.0 (8.5) |
| 3   | 22.288 | 661.2116   | 638  | C_{30}H_{38}O_{15} | 163, 325, 471, 656, 661, 679 | Caf, Glc, Rha, F | Isocistanoside C | 1.3              |
| 4   | 24.640 | 615.2019   | 592  | C_{29}H_{36}O_{13} | 147, 309, 455, 593, 610, 615, 631 | Caf, Glc, Rha, C | Osmanthuside B | 1.4              |
| 5   | 26.645 | 645.2146   | 622  | C_{29}H_{36}O_{14} | 177, 339, 485, 640, 645, 661 | Caf, Glc, Rha, C | Cistanoside A | <1.0             |
| 7   | 28.901 | 579.2054   | 556  | C_{29}H_{36}O_{13} | 163, 325, 471, 574, 579, 595 | Caf, Glc, Rha, G | Cistanoside B | <1.0             |
| X5  | 27.744 | 689.2027   | 666  | C_{31}H_{38}O_{16} | 163, 376, 513, 684, 689, 705 | Caf, Acetyl-Glc, Rha, D | -              | -                |
| 8   | 28.989 | 673.2247   | 652  | C_{29}H_{36}O_{15} | 177, 339, 485, 653, 670, 675, 691 | Fer, Glc, Rha, F | Epimeridinoside A | <1.0             |
| 6   | 31.963 | 689.2027   | 666  | C_{31}H_{38}O_{16} | 163, 376, 513, 684, 689, 705 | Caf, Acetyl-Glc, Rha, D | -              | -                |
| 9   | 32.824 | 601.1890   | 578  | C_{29}H_{36}O_{15} | 163, 325, 471, 579, 601, 617 | Caf, Glc, Rha, A | Salsaside B | 4.8 (<1.0) |
| 10  | 34.420 | 673.2136   | 650  | C_{31}H_{38}O_{15} | 147, 351, 497, 668, 675, 689 | Cou, Acetyl-Glc, Rha, D | Tubuloside E | 5.3 (1.2) |
| 11  | 40.315 | 645.2168   | 622  | C_{29}H_{36}O_{14} | 147, 309, 455, 640, 645, 661 | Caf, Glc, Rha, F | Cistanoside M | <1.0             |
| 12  | 41.954 | 581.2213   | 558  | C_{29}H_{36}O_{13} | 163, 376, 513, 576, 581, 597 | Caf, Glc, Rha, I | Cistanoside C | <1.0             |
| 13  | 44.636 | 657.2148   | 652  | C_{31}H_{38}O_{15} | 177, 339, 485, 653, 670, 675, 691 | Fer, Glc, Rha, E | Isomartynoside | <1.0             |
| 14  | 50.569 | 585.2004   | 562  | C_{31}H_{38}O_{12} | 147, 309, 455, 563, 580, 585, 601 | Caf, Glc, Rha, A | Salsaside C | <1.0             |
| 15  | 51.199 | 615.2082   | 592  | C_{29}H_{36}O_{13} | 163, 325, 471, 593, 610, 615, 631 | Caf, Glc, Rha, B | Jionoside C | <1.0             |
| 16  | 51.878 | 673.2137   | 650  | C_{31}H_{38}O_{15} | 147, 351, 497, 668, 673, 689 | Cou, Acetyl-Glc, Rha, D | Salsaside F | <1.0             |
| 17  | 53.672 | 657.2147   | 634  | C_{31}H_{38}O_{14} | 147, 351, 497, 652, 657, 673 | Cou, Acetyl-Glc, Rha, C | Cistanoside D | <1.0             |
| 18  | 81.127 | 657.2166   | 634  | C_{31}H_{38}O_{14} | 163, 376, 513, 652, 657, 673 | Caf, Acetyl-Glc, Rha, B | Cistanoside E | <1.0             |

^a new compounds. ^b X1–6 have not been identified yet. ^c Aglycone substituents A-I were shown in Figure 1. ^d Abundances in the EtOAc fraction were measured by LC-PDA (320 nm). Abundances in crude extract were in parenthesis.
Table 2. $^1$H and $^{13}$C-NMR Data of new compounds (DMSO-$d_6$).

| Position | $^1$H (δ in ppm) | $^1$C (δ in ppm) | $^1$H (δ in ppm) | $^1$C (δ in ppm) | $^1$H (δ in ppm) | $^1$C (δ in ppm) | $^1$H (δ in ppm) | $^1$C (δ in ppm) |
|----------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| 1        | 128.5            | 64.5             | 128.5            | 64.5             | 128.6           | 138.8           | 128.6           | 138.8           |
| 2        | 129.8            | 7.05             | 120.7            | 5.13             | 129.7           | 6.98             | 128.9           | 7.21             |
| 3        | 115.0            | 6.67             | 136.4            | 24.4             | 114.9           | 6.65             | 128.2           | 7.29             |
| 4        | 155.7            | 25.5             | 17.1             | 22.5             | 155.6           | 126.0           | 7.20             | 7.20             |
| 5        | 115.0            | 6.67             | 17.8             | 22.5             | 114.9           | 6.65             | 128.2           | 7.29             |
| 6        | 129.8            | 7.05             | 129.7            | 6.98             | 129.7           | 6.98             | 128.9           | 7.21             |
| 7        | 34.7             | 2.76             | 34.4             | 2.66             | 35.2            | 2.80             | 35.2            | 2.80             |
| 8        | 70.2             | 3.90             | 69.8             | 3.54             | 69.4            | 3.63             | 69.4            | 3.63             |
| 9        | 102.3            | 4.35             | 100.9            | 4.29             | 99.2            | 4.61             | 99.2            | 4.63             |
| 10       | 74.4             | 3.21             | 74.5             | 3.18             | 73.4            | 4.69             | 73.5            | 4.71             |
| 11       | 79.2             | 3.68             | 79.0             | 3.68             | 78.0            | 3.95             | 77.9            | 3.95             |
| 12       | 69.1             | 4.71             | 69.2             | 4.70             | 68.9            | 4.80             | 69.0            | 4.81             |
| 13       | 74.5             | 3.44             | 74.5             | 3.44             | 74.4            | 3.56             | 74.5            | 3.58             |
| 14       | 60.7             | 3.34             | 60.8             | 3.38             | 60.4            | 3.41             | 60.5            | 3.40             |
| 15       | 101.3            | 5.02              | 101.2            | 5.01              | 102.0           | 4.60             | 102.0           | 4.60             |
| 16       | 70.5             | 3.68             | 70.5             | 3.66             | 70.7            | 3.39             | 70.7            | 3.38             |
| 17       | 70.4             | 3.27             | 70.4             | 3.27             | 70.1            | 3.22             | 70.1            | 3.22             |
| 18       | 71.6             | 3.11             | 71.6             | 3.10             | 71.4            | 3.08             | 71.4            | 3.08             |
| 19       | 68.8             | 3.36             | 68.7             | 3.34             | 68.7            | 3.34             | 69.2            | 3.29             |
| 20       | 18.1             | 0.97             | 18.1             | 0.95             | 18.2            | 0.95             | 18.1            | 0.99             |
| 21       | 125.6            | 125.5            | 125.5            | 124.9            | 125.4           | 125.4           | 125.4           | 125.4           |
| 22       | 122.2            | 7.29             | 122.2            | 7.02             | 114.7           | 7.02             | 115.8           | 6.79             |
| 23       | 149.7            | 148.5            | 149.7            | 148.5            | 130.3           | 7.53             | 130.3           | 7.53             |
| 24       | 149.4            | 149.0            | 149.3            | 160.0            | 148.7           | 148.7           | 148.7           | 148.7           |
| 25       | 115.5            | 6.79             | 115.8            | 6.76             | 115.8           | 6.76             | 115.8           | 6.76             |
| 26       | 123.2            | 7.09             | 123.2            | 6.97             | 121.4           | 6.97             | 121.5           | 6.98             |
| 27       | 145.5            | 7.53             | 145.6            | 7.45             | 145.4           | 7.55             | 145.4           | 7.55             |
| 28       | 114.1            | 6.41             | 113.5            | 6.19             | 113.5           | 6.19             | 113.5           | 6.19             |
| 29       | 165.8            | 165.7            | 165.7            | 165.5            | 165.6           | 165.6           | 165.6           | 165.6           |
| 30       | 55.6             | 3.80             | 55.6             | 3.80             | 55.5            | 3.80             | 55.5            | 3.80             |

$^a$ $^1$H and $^{13}$C-NMR spectra for 5, 12 and 18 were obtained with a Bruker Avance 800 HD spectrometer (Bruker, Ettlingen, Germany) coupled with a cryoprobe. $^b$ $^1$H and $^{13}$C-NMR spectra for 6 were obtained with a Bruker Avance-500 (Bruker, Ettlingen, Germany). $^c$ $^1$H and $^{13}$C-NMR spectra for 17 were obtained with a Jeol LA 300 (Jeol, Tokyo, Japan).
A 3,4-dihydroxyphenyl group was suggested by the HMBC correlations between H-2‴‴ and a quaternary aromatic carbon at δC 149.4 (C-4‴‴) and between H-5‴‴ and C-1‴‴ (δC 125.6) and C-3‴‴ (δC 147.9). From the HMBC NMR spectrum, the correlations between a carbonyl carbon at δC 165.8 and H-8‴‴ and between H-6‴‴ and C-7‴‴ (δC 145.5) suggested a 3,4-dihydroxylated cinnamoyl group. The HMBC correlation between the methoxy proton and C-3‴‴ and the NOESY correlation between the methoxy proton and H-2‴‴ confirmed the cinnamoyl substituent to be an (E)-feruloyl group.

The 4-hydroxyphenyl group was suggested by the HMBC correlations between H-3,5 and quaternary aromatic carbons at δC 155.7 (C-4) and 128.5 (C-1). A hydroxyethyl group was confirmed by the COSY correlations among H-7 (2H, δH 2.76, m), H-8a (1H, δH 3.90, m) and H-8b (1H, δH 3.61, m). From the HMBC NMR spectrum, the correlation between C-7 (δC 34.7) and H-2, 6 suggested a 4-hydroxyphenethyl group, as an aglycone substituent.

Two sugar moieties, suggested by the MS fragment pattern, were double-checked by the NMR spectra and HPLC analysis of the acid hydrolysate. The absolute configurations of them were determined to be D-glucose and L-rhamnose using HPLC analysis of the acid hydrolysate [17]. A β-glucose moiety and an α-rhamnose moiety were established by coupling constants of the anomeric protons. The 1H-1H COSY spectrum showed the sequential correlations from H-1′ to H-5′ and from H-1‴ to H-6‴ (Figure 4).

From the 1H-NMR spectrum, the downfield shift of H-4′ (δH 4.71) suggested an acyl-substituent on glucose. The HMBC correlation between H-4′ and C-9‴‴ (δC 165.8) confirmed that a feruloyl substituent was located at the C-4′ position. The aglycone was located at C-1′ according to the HMBC correlation between H-1′ and C-8 (δC 70.2). The HMBC correlation between H-1‴ and C-3′ (δC 79.2) gave us the position of rhamnose in this structure. Thus, the structure of 5 was established to be 4-hydroxyphenethyl-O-α-L-rhamnopyranosyl-(1→3)-4-O-(E)-feruloyl-β-D-glucopyranoside and the compound was named cistansalside A.

![Figure 4. Key 1H-1H COSY (bold line) and HMBC (blue arrow) correlations of new compounds.](image-url)

Cistansalside B (6) was obtained as a brown amorphous powder. Its molecular formula was determined to be C_{26}H_{36}O_{13} based on the 13C-NMR data and a (+)-HR-ESI-QTOF-MS peak at m/z 579.2054 [M + Na]^+ (calcd. for C_{26}H_{36}O_{13}Na, 579.2048). Fragment ions including a [M + H – Aglycone – Rha – Glc]^+ ion at m/z 163, a [M + H – Aglycone – Rha]^+ ion at m/z 325 and a [M + H – Aglycone]^+ ion at m/z 471 were also detected. A characteristic ion at m/z 163 suggested that a caffeoyl group existed in the structure.

The 1H-NMR spectrum showed an 1,3,4-trisubstituted benzene ring at δH 7.02 (1H, s, H-2‴‴), 6.97 (1H, d, J = 8.3 Hz, H-6‴‴) and 6.75 (1H, d, J = 8.3 Hz, H-5‴‴), a trans-olefin at δH 7.47 (1H, d, J = 15.8 Hz, H-7‴‴) and 6.19 (1H, d, J = 15.8 Hz, H-8‴‴). The 1H and HSQC NMR spectra showed two anomic protons at δH 4.29 (1H, d, J = 7.9 Hz, H-1‴) and 5.01 (1H, s, H-1‴), an olefinic proton at δH 5.13 (1H, dd, J = 7.6, 6.5 Hz, H-2), two methylene protons at δH 4.23 (1H, dd, J = 12.2, 6.5 Hz, H-1a) and 4.13 (1H,
dd, J = 12.2, 7.6 Hz, H-1b) and germinal methyl groups at δH 1.71 (3H, s, H-4) and 1.63 (3H, s, H-5) (Table 2).

From the HMBC spectrum, the 3,4-dihydroxyphenyl group was suggested by the correlations between H-2'' and a quaternary aromatic carbon at δC 149.0 (C-4'') and between H-5'' and another quaternary carbons at δC 125.5 (C-1'') and 148.5 (C-3''). From the HMBC NMR spectrum, the correlations between H-6'' and C-7''' (δC 145.6) and between the carbonyl carbon at δC 165.7 (C-9'''') and H-8' suggested an (E)-caffeoyl group.

A 3-methylbutenyl group, an aglycone substructure, was suggested by the COSY correlations of H-2 with H-1a and H-1b and the HMBC correlations between H-4 and H-5 and the olefinic carbons, at δC 120.7 (C-2) and 136.4 (C-3).

Two sugar moieties were established by the NMR spectra analysis and HPLC spectra analysis of the acid hydrolysate, with MS fragment pattern. The absolute configuration of them were determined to be D-glucose and L-rhamnose using HPLC analysis of the acid hydrolysate [17]. These sugar moieties were defined as a β-glucose and an α-rhamnose by coupling constants of the anomeric protons. The 1H-1H COSY spectrum showed the sequential correlations from H-1’ to H-6’ and from H-1'' to H-6'' (Figure 4). A downshifted glucose proton at δH 4.70 (H-4') suggested an acyl-substituent on glucose. From the HMBC spectrum, the correlation between H-4’ and C-9'' confirmed the position of caffeoyl substituent. The HMBC correlations between H-1’ and C-1 (δC 64.5) and between H-3’ (δH 3.68) and C-1’'' (δC 101.2) suggested the positions of each substituents. Accordingly, the structure of 6 was determined as 3-methylbutenyl-O-α-L-rhamnopyranosyl-(1→3)-4-O-(E)-caffeoyl-β-D-glucopyranoside and named cistansalside B.

Cistansalside C (12), a brown amorphous powder, was determined to have a molecular formula of C_{26}H_{33}O_{13} by (+)-HR-ESI-QTOF-MS, which showed a peak at m/z 581.2213 [M + Na]^+ (calcd. for C_{26}H_{33}O_{13}Na, 581.2205). A characteristic ion at m/z 163 suggested that a caffeoyl substituent existed in the structure. The fragment ions at m/z 325 and m/z 471 suggested the existence of a rhamnose unit and a glucose unit.

Comparison of the NMR spectra of 12 with those of 6 showed that they were similar except for the aglycone structure. In the NMR spectra of 12, two paraffinic carbons at δC 38.0 (C-2') and 24.4 (C-3') were observed instead of two olefinic carbons at δC 120.7 (C-2) and 136.4 (C-3) in the aglycone of 6. The germinal methyl groups (δH 0.88) in the aglycone of 12 were shifted upfield relative to H-4 and H-5 (δH 1.71 and 1.63) in the aglycone of 6 (Table 2). The aglycone of 12 was suggested to be a 3-methylbutyl group, which was confirmed by the COSY NMR spectra of the acid hydrolysate. Peaks of 3-methylbutyl group were observed at δH 3.81 (1H, m, H-1a), 3.48 (1H, m, H-1b), 1.71 (1H, m, H-3), 1.44 (2H, m, H-2) and 0.88 (H-4, 5).

Two sugar moieties were reaffirmed by the HPLC analysis of the acid hydrolysate and the NMR spectra analysis as well as MS fragment pattern. The absolute configurations of the sugars were identified as D-glucose and L-rhamnose using HPLC analysis of the acid hydrolysate [17]. A β-glucose moiety and α-rhamnose moiety were confirmed by coupling constants of the anomeric protons. The 1H-1H COSY spectrum showed the sequential correlations from H-1’ to H-6’, from H-1’’ to H-3’’ and from H-6’’ to H-4’’ (Figure 4).

The position of substituents were confirmed by means of the HMBC analysis. In the HMBC spectrum, the correlations between H-1’ and C-1 (δC 67.2), between H-3’ (δH 3.68) and C-1’’ (δC 101.2) and between H-4’ (δH 4.70) and C-9’’ (δC 165.7) were detected. Consequently, the structure of 12 was established to be 3-methylbutyl-O-α-L-rhamnopyranosyl-(1→3)-4-O-(E)-caffeoyl-β-D-glucopyranoside and named cistansalside C.

Cistansalside D (17), an amorphous brown powder, was determined to have a molecular formula of C_{31}H_{39}O_{14} by the positive mode high-resolution ESI-QTOF-MS, which showed an adduct ion peak at m/z 657.2147 [M + Na]^+ (calcd. for C_{31}H_{39}O_{14}Na, 657.2154). Fragment ions including a [M + H - Aglycone - Rha - Acetyl Glc]^+ ion at m/z 147, a [M + H - Aglycone - Rha]^+ ion at m/z 351 and a [M...
+ H – Aglycone]⁺ ion at m/z 497 were also detected. A characteristic ion at m/z 147 suggested that a coumaroyl substituent was present in its structure. The fragment ions at m/z 351 and m/z 497 suggested the existence of a rhamnose unit and an acetyl-substituted glucose unit.

The ¹³C-NMR spectrum revealed the presence of 31 carbon atoms. Two anomic protons at δ_H 4.61 (1H, m, H-1') and 4.60 (1H, s, H-1'') were observed in the ¹H and HSQC spectra. The ¹H-NMR spectrum indicated the presence of a methyl group at δ_H 1.97 (3H, s, acetyl-CH₃), a trans-olefin at δ_H 7.55 (1H, d, J = 15.9 Hz, H-7''') and 6.34 (1H, d, J = 15.9 Hz, H-8''') and two para-substituted benzene rings at δ_H 7.53 (2H, d, J = 6.9 Hz, H-3'', 5'') and 6.79 (2H, d, J = 6.9 Hz, H-2'', 6'')/ δ_H 6.98 (2H, d, J = 8.0 Hz, H-2, 6) and 6.65 (2H, d, J = 8.0 Hz, H-3, 5) (Table 2). From the HMBC NMR spectrum, the correlations between a carbonyl carbon at δ_c 165.5 (C-9'') and H-8'' and between H-2'', 6'' and C-7'' (δ_c 145.4) suggested an (E)-coumaroyl group. The HMBC correlation between a methyl proton peak and a carbonyl carbon at δ_c 169.0 confirmed the presence of an acetyl group.

A 4-hydroxyphenyl group was suggested based on the HMBC correlations between H-3, 5 and quaternary aromatic carbons at δ_c 155.6 (C-4) and 128.6 (C-1). A hydroxylated ethyl group was confirmed by the COSY NMR signals at δ_H 2.66 (2H, t, J = 6.1 Hz, H-7), 3.90 (1H, m, H-8a) and 3.54 (1H, m, H-8b). The HMBC correlations between H-7 and C-1 (δ_C 128.6) and C-2, 6 (δ_C 129.7) suggested a 4-hydroxyphenylethyl group, as an aglycone substructure.

Two sugar moieties, suggested from MS fragment pattern, were reconfirmed by the HPLC analysis of the acid hydrolysate and NMR spectra. D-glucose and L-rhamnose were elucidated using HPLC analysis of the acid hydrolysate [17]. A β-glucose moiety and an α-rhamnose moiety were established by coupling constants of the anomeric protons. The ¹H-¹H COSY spectrum showed the sequential correlations from H-1' to H-5' and from H-1'' to H-6'' (Figure 4).

From the ¹H-NMR spectrum, the downfield shift of H-2' (δ_H 4.69) and H-4' (δ_H 4.80) suggested the acyl-substituted position on glucose. The connections between glucose and two acyl groups were confirmed by the HMBC correlations between H-4' (δ_H 4.80) and C-9'' and between H-2' and a carbonyl carbon of an acetyl group (δ_C 169.0). The positions of an aglycone and a rhamnose were given by the HMBC correlations between H-3' (δ_H 3.95) and C-1'' and between H-8 and C-1'. Accordingly, the structure of 17 was assigned as 4-hydroxyphenylethyl-2-O-acetyl-O-α-L-rhamnopyranosyl-(1→3)-4-O-(E)-coumaroyl-β-D-glucopyranoside and named cistansalside D.

Cistansalside E (18) was isolated as an amorphous brown powder. Its molecular formula was determined to be C₃₁H₅₉O₁₄ by ¹³C-NMR data and the positive mode high-resolution ESI-QTOF-MS peak at m/z 657.2166 [M + Na]⁺ (calcld. for C₃₁H₅₈O₁₄Na, 657.2154). Additionally, fragment ions including a caffeoyl ion at m/z 163, an [M + H – Aglycone – Rha]⁺ ion at m/z 367 and an [M + H – Aglycone]⁺ ion at m/z 513 were also detected. The fragment ions suggested the existence of a rhamnose unit and an acetyl-substituted glucose unit.

The ¹H-NMR spectrum suggested the presence of two anomic protons at δ_H 4.63 (1H, d, J = 8.2 Hz, H-1') and 4.60 (1H, s, H-1'') and two acyl-substituted glucose protons at δ_H 4.71 (1H, dd, J = 8.9, 8.2 Hz, H-2') and 4.81 (1H, dd, J = 9.7, 9.5 Hz, H-4'). The ¹H and HMBC spectra suggested the existence of an acetyl group at δ_H 1.94 (3H, s, acetyl-CH₃), an (E)-caffeoyl moiety at δ_H 7.48 (1H, d, J = 15.9 Hz, H-7'''), 7.02 (1H, d, J = 1.6 Hz, H-2'''), 6.98 (1H, dd, J = 8.1, 1.6 Hz, H-6'''), 6.76 (1H, d, J = 8.1 Hz, H-5''') and 6.21 (1H, d, J = 15.9 Hz, H-8''') and a mono-substituted benzene ring at δ_H 7.29 (2H, m, H-3, 5), 7.21 (2H, m, H-2, 6) and 7.20 (1H, m, H-4) (Table 2). A phenylethyl group, an aglycone substructure, was suggested by the HMBC correlations between H-7 (2H, δ_H 2.80, m) and C-1 (δ_C 138.8) and between H-7 and C-2, 6 (δ_C 128.9) and the COSY NMR signals of H-7 with H-8a (1H, δ_H 3.99, m) and H-8b (1H, δ_H 3.63, m).

Two sugar moieties were reaffirmed by the HPLC analysis of the acid hydrolysate and NMR spectra analysis. The absolute configurations of the sugars were elucidated using HPLC analysis of the acid hydrolysate, which were confirmed to be D-glucose and L-rhamnose [17]. A β-glucose moiety and an α-rhamnose moiety were established by coupling constants of the anomeric protons. The ¹H-¹H
trans peaks at approximately 7.55 ppm (d, J = 15.8 Hz, H-7′′′) and C-9′′′ (δC 165.6) confirmed the position of substituents in the structure. Therefore, the structure of 18 was determined to be phenethyl-2-O-acetyl-O-α-L-rhamnopyranosyl-(1→3)-4-O-(E)-caffeoyl-β-D-glucopyranoside and named cistansalside E.

Most of the trans-cinnamoyl substituents were isomerized to the cis-isooform in vitro. Light has been reported to convert trans-cinnamic acid derivatives into cis-isooforms [18,19]. The equilibrium of the trans-cis conversion of the cinnamoyl substituents was observed to maintain approximately 70% of the isolates in the trans-isooform. For the olefin protons of the cis form, peaks at approximately 6.90 ppm (d, J = 12–13 Hz, H-7′′′) and 5.80 ppm (d, J = 12–13 Hz, H-8′′′) were assignable in the 1H-NMR spectra, whereas peaks at approximately 7.55 ppm (d, J = 15.8 Hz, H-7′′′) and 6.40 ppm (d, J = 15.8 Hz, H-8′′′) were observed for trans form [20]. In the 1H-NMR spectrum of trans-cis mixtures, peaks for two olefinic protons (H-7′′′ and H-8′′′) were observed in the ratio of 7:3 (trans:cis). 13C-NMR peaks of the cis form were similar to those of the trans form.

All the isolates were tested for their inhibitory effects on LPS-induced NO production in RAW 264.7 cells. Dexamethasone was used as a positive control and its IC_{50} was 7.0 M. Of the tested compounds, compounds 5 (IC_{50} 42.7 ± 6.6 M), 11 (IC_{50} 37.3 ± 2.2 M), 13 (IC_{50} 40.0 ± 4.0 M) and 18 (IC_{50} 27.9 ± 0.8 M) showed moderate inhibitory activities on inducible NO synthase, while the other compounds were inactive in this assay (IC_{50} values > 100 M). To verify whether these compounds had cytotoxicity, cell viability was measured employing MTT assay. As a result, none of them displayed significant cytotoxicity (Supplemental Figure S6-1). These four compounds were selected to evaluate for their inhibitory activity against NF-κB pathway in LPS-stimulated RAW 264.7 cells. Stimulation of RAW 264.7 cells with LPS induced the phosphorylation of IkBα and NF-κB (p65) after 0.5 h of incubation. The phosphorylation of NF-κB (p65) was significantly reduced by pretreatment with compounds 11, 13 and 18 as shown by western blot analysis (Figure 5). Therefore, compounds 11, 13 and 18 might exert anti-inflammatory effects via the inhibition of NF-κB in macrophages.

Figure 5. Effect of four compounds on phosphorylation of IkBα and NF-κB (p65) in LPS-stimulated RAW 264.7 cells. (A) The western blots were conducted in LPS and sample-treated RAW 264.7 cells; (B, C) The immunoblot signals were quantified using Molecular Analyst/PC densitometry software (Bio-Rad, Richmond, CA, USA). Densitometric analysis of phosphorylated isoforms is reported. NF-κB in RAW 264.7 cell was normalized to the content of β-actin.

3. Materials and Methods

3.1. General Experiment Procedure

Optical rotations were measured with a Jasco P-2000 digital polarimeter (Jasco, Tokyo, Japan). UV spectra were recorded on a Chirascan plus Circular Dichroism spectrometer (Chirascan, APL, UK). IR spectra were recorded using Jasco FT/IR-4200 spectrophotometer. High-resolution electrospray
ionization quadrupole-time-of-flight mass spectrometry (HR-ESI-qTOF-MS) was performed on an Agilent 6530 Accurate-Mass Q-TOF LC/MS equipped with Agilent 1260 Infinity series (Agilent Technologies, Inc., Palo Alto, CA, USA), and the column used was a Jasco SFCpak Crest C18T-5 column (i.d. 150 × 4.6 mm, 5 µm). MassHunter Workstation Software was used for data acquisition.

1D (1H and 13C) and 2D (1H-1H COSY, HMQC, HMBC, NOESY) NMR spectra were obtained with a Jeol LA 300 (Jeol, Tokyo, Japan), Bruker AVANCE-400, Bruker AVANCE-500, Bruker AVANCE-600 and Bruker AVANCE 800 HD spectrometers coupled with cryoprobe (Bruker, Ettlingen, Germany). DMSO-d6 (Cambridge Isotope Laboratories, Inc. Andover, MA, USA) was used as NMR solvent and reference peaks (δH 2.50 and δC 39.5). Column chromatography (CC) was performed using Sephadex LH-20 (25–100 µm; Pharmacia, Uppsala, Sweden) or Kieselgel 60 silica gel (40–63 µm, 230–400 mesh, Art. 9385; Merck, Darmstadt, Germany). Thin-layer chromatography (TLC) was conducted on pre-coated Kieselgel 60 silica gel F254 plates (Art. 5715; Merck). Spots on TLC were detected using UV lamp at 254 nm and 365 nm (VL-4.LC, 365/254; Vilber Lourmat, Torcy, France). Medium pressure liquid chromatography (MPLC) was performed on a RediSep 120 g silica flash column (Isco, Lincoln, NE, USA) and Kieselgel 60 silica gel (40–63 µm, 230–400 mesh, Art. 9385; Merck) using a Combiflash companion (Isco). The high pressure liquid chromatography (HPLC) system was a Gilson HPLC equipped with a Gilson 321 pump and UV VIS 151 detector (Gilson, Middleton, WI, USA), using semi-preparative ODS columns (Luna 5 µm C18 (2) 100 Å, i.d. 250 × 10 mm, 5 µm, Phenomenex Inc., Torrance, CA, USA; Hypersil GOLD™ aQ 175 Å, i.d. 250 × 10 mm, 5 µm, Thermo Scientific™, Henningsdorf, Germany; Inno C18 column 120 Å, i.d. 250 × 10 mm, 5 µm, Young Jin Biochrom Co., Ltd., Seongnam, Korea). The analytical RP-HPLC system was a Waters 2695 alliance system with a 996 Photodiode Array (PDA) detector (Waters Corp, Milford, MA, USA), and the column used was a Hypersil™ BDS C18 column (130 Å, i.d. 150 × 4.6 mm, 5 µm, Thermo Scientific™). Formic acid was purchased from Daejung Chemicals & Metals Co., Ltd. (Seoul, Korea). H2SO4, Na2CO3 and first grade solvents for extraction, fractionation and isolation were purchased from Daejung Chemical & Metals Co. Ltd. (Seoul, Korea). L- and D-cysteine methyl ester hydrochloride and o-tolylisothiocyanate were purchased from Tokyo Chemical Industry (Tokyo, Japan).

3.2. Plant Material

The whole plants of C. salsa, which were collected from the Shinjang Uyghur, were imported through Daerim Pharmaceutical Wholesale Company (Cheongju, Korea). They were identified by Prof. Dr. Jehyun Lee (Dongguk University, Seoul, Korea). The voucher specimen (SNUPH2016-03) was deposited in the Herbarium of Medicinal Plant Garden, College of Pharmacy, Seoul National University.

3.3. Extraction and Isolation

The dried whole plants of C. salsa (5.7 kg) were chopped and extracted three times with MeOH (20 L) at room temperature for 99 min. After removal of the solvent in vacuo, the crude extract (1.35 kg) was suspended in H2O (5 L), then partitioned with EtOAc (5 L). The EtOAc residue (55.3 g) was separated into 16 fractions (E01-16) on silica gel chromatography eluting with CHCl3/MeOH (50:1–0:1, step-gradient system).

E08 (611.7 mg) was subjected to silica gel medium-pressure liquid chromatography (25 g) and eluted with CHCl3/MeOH (18:1–0:1, step-gradient system) and gave 11 fractions (E08a-k). From E08g, compounds 13 (0.7 mg) and 18 (0.6 mg) were purified using a Luna 5 µm HPLC column and isocratic elution with 28% aq. MeCN. HPLC purification (Hypersil GOLD, 25% aq. MeCN) of E08h (138.5 mg) furnished compounds 7 (10.6 mg), 8 (17.2 mg), 14 (13.4 mg) and 17 (4.9 mg).

E09 (1.15 g) was further purified on a silica-MPLC column (20 g) eluting with CHCl3/MeOH (10:1–0:1, step-gradient system) to give 11 subfractions (E09a-k). E09e (398.7 mg) was subjected to Sephadex LH-20 (MeOH) and yielded eight subfractions (E09e1-8). E09e6 was subsequently purified using a Hypersil GOLD HPLC column (22% aq. MeCN) to afford compound 11 (0.4 mg). From E09e7,
compound 5 (0.6 mg) was purified by isocratic elution from a Luna 5 µm HPLC column with 40% aq. MeOH.

E10 (1.20 g) was separated into nine fractions (E10a-i) on Sephadex LH-20 column eluting with MeOH. From E10g (147.5 mg), compounds 4 (13.4 mg), 9 (8.0 mg) and 15 (5.0 mg) were isolated by HPLC separation (Inno, 23% aq. MeCN). Fraction E10h (470.0 mg) was purified on a Hypersil GOLD column by isocratic elution (40% aq. MeOH) to yield compounds 1 (3.8 mg), 10 (42.1 mg) and 16 (3.0 mg).

E11 (2.96 g) was subjected to Sephadex LH-20 eluting with MeOH to give nine fractions (E11a-i). E11e was separated by Sep-Pak C18 cartridge eluting stepwise with 10%, 20%, 30%, 50% and 100% aq. MeOH to yield seven fractions (E11e1-7), followed by Luna 5 µm HPLC (28% aq. MeCN) to give compounds 3 (3.4 mg), 6 (1.4 mg) and 12 (1.2 mg).

E12 (19.0 g) was subjected to silica MPLC (120 g) using a CHCl3/MeOH step-gradient system to give six fractions (18:1–0:1, E12a-f). E12f was chromatographed on a Sephadex LH-20 column (i.d. 150 × 4.6 mm, 5 µm, Jasco, Japan). The mobile phase consisted of 0.1% (v/v) formic acid in MeCN (A) and water (B) using a gradient elution of 0–35 min (23% A), 35–45 min (23–28% A), 45–75 min (28% A) and 75–80 min (90% A). The flow rate was kept at 0.3 mL/min. The absorbance was measured at 320 nm. The conditions of the ESI source were as follows: drying gas (N2) flow rate, 10 L/min; drying gas temperature, 350 °C; nebulizer, 30 psig; sheath gas flow rate, 12.0 L/min; sheath gas temperature, 350 °C; capillary, 4000 V; skimmer, 60 V; octopole RF, 750 V; fragmentor voltage, 180 V; positive mode. The system was operated under Masshunter workstation software. The mass range was set at m/z 50–1000.

### 3.4. Characterization

**Cistansalside A (5):** brown amorphous powder; [α]D20 −33.7 (c 0.1, MeOH); UV(MeOH) λmax nm (log ε) 322 (3.18); IR (neat) νmax 3359, 1748, 1705, 1602, 1516 cm−1; 1H-NMR (800 MHz) and 13C-NMR (200 MHz) data, see Table 2; HRMS (ESI-TOF) m/z [M + Na]+ 654.2146 (calcd. for C30H38O14Na, 645.2154).

**Cistansalside B (6):** brown amorphous powder; [α]D20 −72.9 (c 0.1, MeOH); UV(MeOH) λmax nm (log ε) 333 (3.32); IR (neat) νmax 3400, 1705, 1603, 1516 cm−1; 1H-NMR (800 MHz) and 13C-NMR (125 MHz) data, see Table 2; HRMS (ESI-TOF) m/z [M + Na]+ 579.2054 (calcd. for C26H36O13Na, 579.2048).

**Cistansalside C (12):** brown amorphous powder; [α]D20 −61.6 (c 0.1, MeOH); UV(MeOH) λmax nm (log ε) 337 (3.25); IR (neat) νmax 3359, 1704, 1602, 1508 cm−1; 1H-NMR (800 MHz) and 13C-NMR (200 MHz) data, see Table 2; HRMS (ESI-TOF) m/z [M + Na]+ 581.2213 (calcd. for C26H36O13Na, 581.2205).

**Cistansalside D (17):** brown amorphous powder; [α]D20 −51.1 (c 0.1, MeOH); UV(MeOH) λmax nm (log ε) 221 (3.53), 315 (3.52); IR (neat) νmax 3588, 1746, 1722, 1603, 1516, 1232, 1157, 1039 cm−1; 1H-NMR (400 MHz) and 13C-NMR (75 MHz) data, see Table 2; HRMS (ESI-TOF) m/z [M + Na]+ 657.2147 (calcd. for C31H38O14Na, 657.2154).

**Cistansalside E (18):** brown amorphous powder; [α]D20 −59.2 (c 0.1, MeOH); UV(MeOH) λmax nm (log ε) 336 (3.22); IR (neat) νmax 3370, 1741, 1712, 1602, 1231, 1157 cm−1; 1H-NMR (800 MHz) and 13C-NMR (200 MHz) data, see Table 2; HRMS (ESI-TOF) m/z [M + Na]+ 657.2166 (calcd. for C31H38O14Na, 657.2154).

### 3.5. HPLC-QTOF-MS Analysis

Chromatographic-mass-spectrometry analysis was performed on an Agilent 1260 Infinity series LC system (Agilent Technologies, Inc., USA). The analytical column was a SFCpak Crest C18T-5 column (i.d. 150 × 4.6 mm, 5 µm, Jasco, Japan). The mobile phase consisted of 0.1% (v/v) formic acid in MeCN (A) and water (B) using a gradient elution of 0–35 min (23% A), 35–45 min (23–28% A), 45–75 min (28% A) and 75–80 min (90% A). The flow rate was kept at 0.3 mL/min. The absorbance was measured at 320 nm. The conditions of the ESI source were as follows: drying gas (N2) flow rate, 10 L/min; drying gas temperature, 350 °C; nebulizer, 30 psig; sheath gas flow rate, 12.0 L/min; sheath gas temperature, 350 °C; capillary, 4000 V; skimmer, 60 V; octopole RF, 750 V; fragmentor voltage, 180 V; positive mode. The system was operated under Masshunter workstation software. The mass range was set at m/z 50–1000.
3.6. Acid Hydrolysis

Compounds were hydrolyzed using 1 N H$_2$SO$_4$ (100 µL) heated with a water bath at 90 °C for 2 h, then neutralized with saturated aqueous Na$_2$CO$_3$ solution. After the solutions were dried under a stream of N$_2$, the products and standard sugars (D-Glc, L-Glc, L-Rha) were dissolved in pyridine (100 µL) containing L-cysteine methyl ester hydrochloride (0.5 mg). An L-rhamnose sample was dissolved in pyridine (100 µL) containing D-cysteine methyl ester hydrochloride (0.5 mg). After that, they were heated at 60 °C for 1 h. The solutions were treated with 1 µL (1.11 mg) of o-tolylisothiocyanate, which were heated again at 60 °C for 1 h. Each final mixture was directly analyzed by analytical RP-HPLC (Hypersil™ BDS C18 column, 17% aq. MeCN, 0.8 mL/min, 40 min, 35 °C). The $t_R$ of the peak at 21.9 and 40.4 min coincided with that of the thiocarbamoyl thiazolidine derivative of D-glucose and L-rhamnose, respectively.

3.7. Cell Culture

Murine macrophages, RAW 264.7, were obtained from the Korean Research Institute of Bioscience and Biotechnology (Daejeon, Korea), and grown in RPMI medium containing 10% fetal bovine serum and 100 U/mL penicillin/streptomycin sulfate. Cells were incubated in a humidified 5% CO$_2$ atmosphere at 37 °C.

3.8. Drugs and Chemicals

RPMI, penicillin and streptomycin were purchased from HyClone (Logan, UT, USA). Bovine serum albumin and LPS were purchased from Sigma (St. Louis, MO, USA).

3.9. Measurement of NO Production

The nitrite concentration in the culture medium was measured as an indicator of NO production according to the Griess reaction. The cells were seeded at $2 \times 10^5$ cells/well in 96-well culture plates. After pre-incubation of the RAW 264.7 cells for 18 h, the cells were pretreated with compounds (50 µM, 10 µM, 5 µM or 1 µM) and stimulated with LPS (500 ng/mL) for 24 h. Test compounds dissolved in DMSO. Cells were also treated with 0.05% DMSO as a vehicle control. RAW 264.7 cells ($2 \times 10^5$ cells/well) were cultured in 96-well plates using RPMI without phenol red, and pretreated with samples for 0.5 h. Cellular NO production was induced by the addition of 500 ng/mL final concentration LPS and a 24 h incubation. Following incubation, 100 µL of conditioned media was mixed with the same volume of Griess reagent and incubated for 15 min. The absorbance of the mixture at 540 nm was measured with an ELISA microplate reader (Benchmark, Bio-Rad Laboratories, Richmond, CA, USA). The values obtained were compared with those of standard concentrations of sodium nitrite dissolved in RPMI, and the concentrations of nitrite in the conditioned media of sample-treated cells were calculated.

3.10. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay for Cell Viability

Cells were seeded into 96-well plates at a density of $5 \times 10^4$ cells/well and incubated with serum-free media in the presence of samples. Test compounds dissolved in DMSO. Cells were also treated with 0.05% DMSO as a vehicle control. Following incubation for 24 h, 10 µL MTT (5 mg/mL in saline) was added and incubation was continued for further 4 h. Mitochondrial succinate dehydrogenase in live cells converts MTT into visible formazan crystals during incubation. The formazan crystals were then solubilized in dimethyl sulfoxide and the absorbance was measured at 540 nm using an enzyme-linked immunosorbent assay (ELISA) microplate reader (Benchmark, Bio-Rad Laboratories). Relative cell viability was calculated compared with the absorbance of the untreated control group. All experiments were performed in triplicate.
3.11. Immunoblot Analysis

Protein expression was assessed by western blotting according to standard procedures. Briefly, RAW264.7 cells were cultured in 60 mm culture dishes (2 × 10^6/mL), following by pretreatment 50 μM of compounds. Cells were washed twice in ice cold PBS (pH 7.4), the cell pellets were resuspended in lysis buffer on ice for 15 min, and the cell debris was then removed by centrifugation. Protein concentration was determined using Bio-Rad protein assay reagent according to the manufacturer’s instructions. Protein (20–30 μg) was mixed 1:1 with 2× sample buffer (20% glycerol, 4% SDS, 10% 2-ME, 0.05% bromophenol blue, and 1.25 M Tris [pH 6.8]), loaded onto 8 or 15% SDS-PAGE gels, and run at 150 V for 90 min. Cellular proteins were transferred onto ImmunoBlot polyvinylidene difluoride membranes (Bio-Rad) using a Bio-Rad semi-dry transfer system according to the manufacturer’s instructions. The membranes were then incubated overnight with the respective p-NF-κB, NF-κB, p-IκBα and β-actin primary antibodies (Abcam, Cambridge, UK) in Tris-buffered saline containing 5% skimmed milk and 0.1% Tween 20. The following day, the blots were washed three times with Tris-buffered saline (0.1% Tween 20) and incubated for 1 h with an HRP-conjugated secondary anti-IgG antibody (diluted 1:2000–1:20,000). The blots were washed again three times with Tris-buffered saline (0.1% Tween 20), and immunoreactive bands were developed using the chemiluminescent substrate ECL Plus (Amersham Biosciences, Piscataway, NJ, USA).

3.12. Statistical Analysis

Experimental data are presented as the mean ± SEM. The level of statistical significance was determined by analysis of variance (ANOVA) followed by Dunnett’s t-test for multiple comparisons. p Values less than 0.05 were considered significant.

4. Conclusions

In this study, we isolated and elucidated the structures of five new phenylpropanoid-substituted diglycosides, named cistansalside A-E (5, 6, 12, 17 and 18), in addition to isolating and identifying 13 known compounds, using a dereplication strategy. The known compounds were determined to be lipedoside A-I (1) [21], 2′-acetylacteoside (2) [22], isocistanoside C (3) [15], osmanthuside B (4) [21], epimeridinoside A (7) [15], cistanoside D (8) [23], salsaside B (9) [6], tubuloside E (10) [16], cistanoside M (11) [15], isomartynoside (13) [24], salsaside C (14) [6], jionoside C (15) [25] and salsaside F (16) [6]. Their structures were established through the analysis of extensive spectroscopic data and by comparison to reported data in the literature. It was confirmed that tentatively predicted structures of phenylpropanoid-substituted diglycosides were correctly matched to their real structures.

In NO inhibitory assay, compounds 5 (IC_50 42.7 ± 6.6 μM), 11 (IC_50 37.3 ± 2.2 μM), 13 (IC_50 40.0 ± 4.0 μM) and 18 (IC_50 27.9 ± 0.8 μM) showed moderate inhibitory activities. Of these compounds, compounds 11, 13 and 18 were found to inhibit the phosphorylation of NF-κB in macrophages and might thus exert an anti-inflammatory activity which will have to be proven in further experiments.

Supplementary Materials: Supplementary data associated with this article can be found in the PDF file (supplementary material).

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Conflicts of Interest: The authors declare no conflict of interest.
References

1. Shimamura, H.; Miyazawa, M.; Enomoto, K.; Nakamura, S.-I.; Kameoka, H. Suppression of SOS-Inducing Activity of Trp-P-1 by Fatty Acids from Cistanche salsa in the Salmonella Typhimurium TA1535/pSK1002 umu Test. Nat. Prod. Lett. 1997, 10, 261–265. [CrossRef]

2. Jeon, E.; Chung, K.-S.; An, H.-J. Anti-Proliferation Effects of Cistanches salsa on the Progression of Benign Prostatic Hyperplasia. Can. J. Physiol. Pharmacol. 2016, 94, 104–111. [CrossRef] [PubMed]

3. Xu, C.; Jia, X.; Xu, R.; Wang, Y.; Zhou, Q.; Sun, S. Rapid Discrimination of Herba Cistanches by multi-step infrared macro-fingerprinting combined with soft independent modeling of class analogy (SIMCA). Spectrochim. Acta Part A Mol. Biomol. Spectrosc. 2013, 114, 421–431. [CrossRef] [PubMed]

4. Jiang, Y.; Li, S.P.; Wang, Y.T.; Chen, X.J.; Tu, P.F. Differentiation of Herba Cistanches by fingerprint with high-performance liquid chromatography-diode array detection-mass spectrometry. J. Chromatogr. A 2009, 1216, 2156–2162. [CrossRef] [PubMed]

5. Kobayashi, H.; Karasawa, H.; Miyase, T.; Fukushima, S. Studies on the Constituents of Cistanchis Herba. V. Isolation and Structures of Two New Phenylpropanoid Glycosides, Cistanosides E and F. Chem. Pharm. Bull. 1985, 33, 1452–1457. [CrossRef]

6. Lei, L.; Jiang, Y.; Liu, X.-M.; Tu, P.-F.; Wu, L.-J.; Chen, F.-K. New Glycosides from Cistanche salsa. Helv. Chim. Acta 2007, 90, 79–85. [CrossRef]

7. Karbaceaeva, E.B.; Sakipova, Z.B.; Ibragimova, L.N.; Kapsalyamova, E.N.; Ternynko, I.I. Compositional study of phenolic compounds of Cistanche salsa (C.A. Mey) G. Beck, growing in the Republic of Kazakhstan. J. Chem. Pharm. Res. 2015, 7, 120–122.

8. Liu, J.-Y.; Guo, Z.-G.; Zeng, Z.-L. Improved accumulation of phenylethanoid glycosides by precursor feeding to suspension culture of Cistanche salsa. Biochem. Eng. J. 2007, 33, 88–93. [CrossRef]

9. Maruyama, S.; Akasaka, T.; Yamada, K.; Tachibana, H. Cistanche salsa extract acts similarly to protein-bound polysaccharide-K (PSK) on various types of cell lines. J. Tradit. Med. 2008, 25, 166–169.

10. Tian, X.-F.; Pu, X.-P. Phenylethanoid glycosides from Cistanches salsa inhibit apoptosis induced by 1-methyl-4-phenylpyridinium ion in neurons. J. Ethnopharmacol. 2005, 97, 59–63. [CrossRef] [PubMed]

11. Michel, T.; Halabalaki, M.; Skaltsounis, A.L. New Concepts, Experimental Approaches, and Dereplication Strategies for the Discovery of Novel Phytoestrogens from Natural Sources. Planta Med. 2013, 79, 514–532. [CrossRef] [PubMed]

12. De Medeiros, L.S.; Abreu, L.M.; Nielsen, A.; Ingmer, H.; Larsen, T.O.; Nielsen, K.F.; Rodrigues-Filho, E. Dereplication-guided isolation of depsides thielavins S-T and lecanorins D-F from the endophytic fungus Setophoma sp. Phytochemistry 2015, 111, 154–162. [CrossRef] [PubMed]

13. Rakotondraibe, L.H.; Rasolomampianina, R.; Park, H.-Y.; Li, J.; Slebodnik, C.; Brodie, P.J.; Blasiak, L.C.; Hill, R.; TenDyke, K.; Shen, Y.; et al. Antiproliferative and antiplasmodial compounds from selected Streptomyces species. Bioorg. Med. Chem. Lett. 2015, 25, 5646–5649. [CrossRef] [PubMed]

14. Jiang, Y.; Liu, F.-J.; Wang, Y.-M.; Li, H.-J. Dereplication-guided isolation of novel hepatoprotective triterpenoid saponins from Celosiae Semen by high-performance liquid chromatography coupled with electrospray ionization tandem quadrupole–time-of-flight mass spectrometry. J. Pharm. Biomed. Anal. 2017, 132, 148–155. [CrossRef] [PubMed]

15. Zhang, J.; Li, C.; Che, Y.; Wu, J.; Wang, Z.; Cai, W.; Li, Y.; Ma, Z.; Tu, P. LTQ-Orbitrap-based strategy for traditional Chinese medicine targeted class discovery, identification and herbomics research: A case study on phenylethanoid glycosides in three different species of Herba Cistanches. RSC Adv. 2015, 5, 80816–80828. [CrossRef]

16. Yoshizawa, F.; Deyama, T.; Takizawa, N.; Usmanghani, K.; Ahmad, M. The Constituents of Cistanche tubulosa (Schrenk) Hook. f. II. Isolation and Structures of a New Phenylethanoid Glycoside and a New Neolignan Glycoside. Chem. Pharm. Bull. 1990, 38, 1927–1930. [CrossRef]

17. Tanaka, T.; Nakashima, T.; Ueda, T.; Tomii, K.; Kouno, I. Facile Discrimination of Aldose Enantiomers by Reversed-Phase HPLC. Chem. Pharm. Bull. 2007, 55, 899–901. [CrossRef] [PubMed]

18. Wong, W.S.; Guo, D.; Wang, X.L.; Yin, Z.Q.; Xia, B.; Li, N. Study of cis-cinnamic acid in Arabidopsis thaliana. Plant Physiol. Biochem. 2005, 43, 929–937. [CrossRef] [PubMed]

19. Kahnt, G. Trans-Cis-Equilibrium of Hydroxycinnamic Acids during Irradiation of Aqueous Solutions at Different pH. Phytochemistry 1967, 6, 755–758. [CrossRef]
20. Hanai, K.; Kuwae, A.; Takai, T.; Senda, H.; Kunimoto, K.-K. A comparative vibrational and NMR study of cis-cinnamic acid polymorphs and trans-cinnamic acid. *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* 2001, 57, 513–519. [CrossRef]

21. He, Z.-D.; Ueda, S.; Akaji, M.; Fujita, T.; Inoue, K.; Yang, C.-R. Monoterpenoid and Phenylethanoid Glycosides from *Ligustrum pedunculare*. *Phytochemistry* 1994, 36, 709–716. [CrossRef]

22. Han, L.; Boakye-Yiadom, M.; Liu, E.; Zhang, Y.; Li, W.; Song, X.; Fu, F.; Gao, X. Structural Characterisation and Identification of Phenylethanoid Glycosides from *Cistanthes deserticola* Y.C. Ma by UHPLC/ESI-QTOF-MS/MS. *Phytochem. Anal.* 2012, 23, 668–676. [CrossRef] [PubMed]

23. Zhongjian, J.; Zimin, L.; Changzeng, W. Phenylpropanoid and Iridoid Glycosides from *Pedicularis lasiophrys*. *Phytochemistry* 1992, 31, 263–266. [CrossRef]

24. Bai, H.; Li, S.; Yin, F.; Hu, L. Isoprenylated Naphthoquinone Dimers Firmianones A, B, and C from *Firmiana platanifolia*. *J. Nat. Prod.* 2005, 68, 1159–1163. [CrossRef] [PubMed]

25. Sasaki, H.; Nishimura, H.; Chin, M.; Mitsuhashi, H. Hydroxycinnamic acid esters of phenethylalcohol glycosides from *Rehmannia glutinosa* var. *purpurea*. *Phytochemistry* 1989, 28, 875–879. [CrossRef]

**Sample Availability:** Not available.

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