

Article

New Glycosides from the Fruits of *Nicandra physaloides*

Yan Liu, Hai-Bing Jiang, Zhen-Peng Xu, Yan-Gang Cheng, Shao-Wa Lv, Bing-You Yang, Hong-Wei Guo * and Hai-Xue Kuang *

Key Laboratory of Chinese Materia Medica (Ministry of Education), Heilongjiang University of Chinese Medicine, Harbin 150040, China; lifeliuyan@163.com (Y.L.); m15822237055@163.com (H.-B.J.); xu872821578@163.com (Z.-P.X.); chengyg1992@163.com (Y.-G.C.); lswa5599@hotmail.com (S.-W.L.); ybywater@163.com (B.-Y.Y.)

* Correspondence: hongweigu17@163.com (H.-W.G.); hxkuang@hotmail.com (H.-X.K.); Tel.: +86-451-8219-3007 (H.-W.G.); +86-451-8726-7188 (H.-X.K.)

Academic Editor: Isabel C. F. R. Ferreira

Received: 9 April 2017; Accepted: 16 May 2017; Published: 17 May 2017

Abstract: Three new glycosides (1–3) and 15 known ones (4–18) were isolated and identified from the fruits of *Nicandra physaloides*. The structures of these compounds were established by 1D and 2D NMR spectra and HR-ESI-MS. The compounds (4–18) were the first time isolated from the *Nicandra* genus and they (except 8, 10, 14) exhibited inhibitions on the NO release of LPS-induced RAW 264.7 cells with IC₅₀ values from 26.9 to 47.5 μM.

Keywords: *Nicandra physaloides*; phenyl-glycosides; fat glycosides; RAW 264.7; inflammation

1. Introduction

Nicandra physaloides (L.) Gaertn (Solanaceae) was recorded in the Dictionary of Medicinal Plants [1], as being an annual herb of monotypic genus, sweet and acerbity, bitter and natural in flavor, which possessed the various effects of sedation, eliminating phlegm, heat-clearing and detoxifying. Modern researchers have indicated that *Nicandra physaloides* was reported being taken as analgesic, vermifuge, antibacterial agent, antipyretic, diuretic and mydriatic [2–4] and applied to the treatments of hydrophobia, psychosis, epilepsy, rheumatoid arthritis, nasosinusitis, influenza, urinary tract infection, sore and furuncle [5,6]. Meanwhile, it was used in folk medicine for sedation, eliminating phlegm, heat-clearing and detoxifying. Equally important, the Chinese Herbal Medicine Anthology of Yun Nan recorded that the fruits of *Nicandra physaloides* (L.) Gaertn possessed the effects of dispelling wind and anti-inflammation. However, the effective material basic was unclear and indistinct [7,8]. As a part of our ongoing research on structurally diverse and anti-inflammatory ingredients from *Nicandra physaloides*, our study led to the isolation of three new compounds, Niglycoside A–C (1–3), along with fifteen known ones including benzyl α-L-arabinopyranosyl-(1→6)-β-D-glucopyranoside (4) [9], 6-O-L-arabinopyranosyl-β-D-glucopyranoside (5) [10], 2-Phenylethyl-β-D-glucopyranoside (6) [11], (+)-phenylethyl-8-O-α-L-rhamnopyranoside (7) [12,13], Salidroside (4-hydroxy-2-Phenylethyl-O-β-D-glucopyranoside, 8) [14], phenethanol-β-D-gentiobioside (9) [15], Forsythoside E (3,4-dihydroxyl-2-Phenylethyl-O-β-L-arabinopyranosyl-(1→6)-β-D-glucopyranoside, 10) [16], and phenethylalcohol 8-O-β-D-glucopyranosyl-(1→2)-[O-α-L-arabinopyranosyl-(1→6)]-O-β-D-glucopyranoside (11) [17], Helicide (4-β-D-galactopyranosyloxy-benzaldehyde, 12) [18], picein (1-[4-(β-D-glucopyranosyloxy)-phenyl]-ethanone, 13) [19], benzoic acid-β-D-gentiobioside (14) [14], butyl-β-D-glucopyranoside (15) [20], Foliachinenoside I (3-methylbutyl-4-O-α-L-arabinopyranosyl (1→6)-β-D-glucopyranoside, 16) [21], (Z)-3-Hexenyl-1-O-β-D-glucopyranoside (17) [22] and (Z)-3-hexenyl O-α-arabinopyranosyl-(1→6)-O-β-D-glucopyranoside (18) [23], respectively (Figure 1, Table S1). This article is the first time that the phenyl-glycosides and fat glycosides from the fruits of *Nicandra physaloides* have been studied

and reported. Here, the isolation and structural elucidation of compounds 1–18, as well as their anti-inflammatory activities, were elaborated and provided.

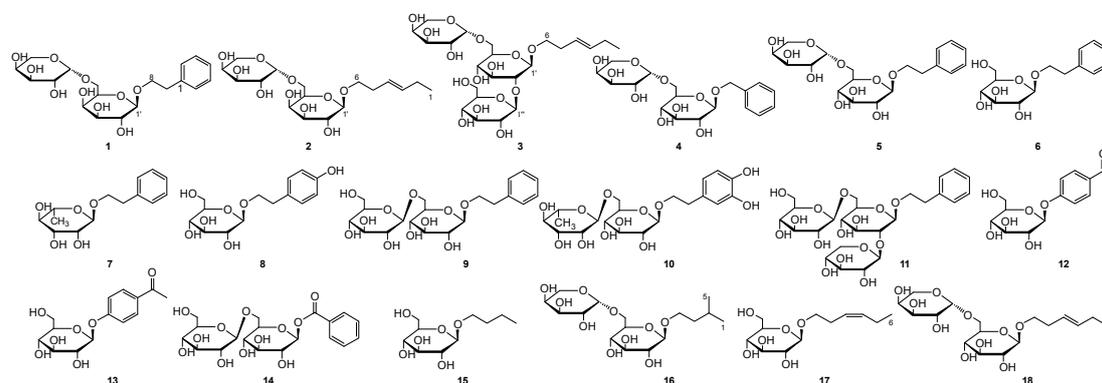


Figure 1. Structures of compounds 1–18 from *Nicandra physaloides*.

2. Results

2.1. Structure Elucidation

Compound 1 possessed the molecular formula of $C_{19}H_{28}O_{10}$ according to the HR-ESI-MS at m/z 417.1755 $[M + H]^+$. The 1H -NMR spectrum (Table 1) of 1 showed the characteristic signals of mono-substituted benzene ring at δ_H 7.24 (4H, overlap, H-2, 3, 5, 6) and 7.16 (m, H-4). In addition, two protons signals of anomeric carbon of β -galactose and α -arabinose were observed at δ_H 4.65 (d, $J = 7.9$ Hz, H-1') and 4.29 (d, $J = 6.8$ Hz, H-1''), respectively. Combined with the DEPT, ^{13}C -NMR spectrum of 1 showed 19 signals, including a group of benzene at δ_C 140.2, 130.0 ($2 \times C$), 129.4 ($2 \times C$) and 127.2, two methylene signals at δ_C 71.8 and 37.3, and two groups of galactopyranose and arabinopyranose glycosyls at δ_C 102.0, 72.3, 72.8, 68.8, 74.5, 69.8 and 105.2, 72.4, 74.2, 69.5, 66.7. The HMBC correlations (Figure 2) between H-1' and C-8, H-1'' and C-6' suggested the attachment position of the galactosyl at C-8 and C-1'' of arabinose at C-6' of galactosyl [24]. Assignments of all groups of 1 were achieved by 1H - 1H COSY, HSQC and HMBC (Figure 2). The absolute configuration of the glycosyls group of compound 1 was determined by GC analysis according to the same t_R at 12.9 and 32.5 min with standard L-arabinopyranose and D-galactopyranoside, respectively. From the above data, the structure of 1 was elucidated as 2-phenylethyl O- α -L-arabinopyranosyl-(1 \rightarrow 6)- β -D-galactopyranoside, named Nicglycoside A.

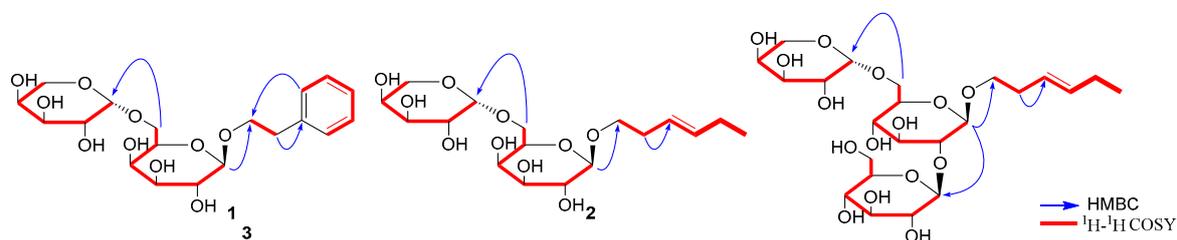


Figure 2. Key HMBC and 1H - 1H COSY correlations of compound 1–3.

Compound 2 was obtained as an amorphous powder with the molecular formula assigned as $C_{17}H_{30}O_{10}$ by positive HR-ESI-MS from the $[M + H]^+$ signal at m/z 395.1901, indicating 3 degrees of unsaturation. The 1H -NMR spectrum (Table 1) of 2 showed two groups characteristic signals of galactose and arabinose glycosyls at δ_H 4.61 (1H, d, $J = 8.0$ Hz, H-1') and 4.30 (1H, d, $J = 6.7$, H-1''), which were in accordance with the glycosyl of compound 1; a pair signals of double bonds δ_H 5.42 (2H, m), three methylene at δ_H 2.07 (2H, m), 2.37 (2H, m), 3.52 (1H, o), 3.84 (1H, o) and one methyl at

δ_{H} 0.96 (3H, t, 7.6). In the ^{13}C -NMR, the glycosyl signals of galactopyranose and arabinopyranose existed at δ_{C} 102.0, 72.3, 72.8, 68.8, 74.4, 69.8 and 105.2, 72.4, 74.2, 69.5, 66.7. The HMBC correlations (Figure 2) between H-1' and C-6, H-1'' and C-6' suggested the attachment position of the galactosyl was at C-6 and C-6' of galactosyl was substituted connecting with the C-1''' of arabinose. Its absolute configurations of the glycosyls group were determined by GC analysis as with the compound 1. From the above data and combined with the literatures [24,25], the structure of 2 was elucidated as (Z)-hex-3-en-1-ol- α -L-arabinopyranosyl-(1 \rightarrow 6)- β -D-galactopyranoside, named Nicglycoside B.

Table 1. ^1H - and ^{13}C -NMR Data of 1–3 (CD_3OD).

No.	1		2		3	
	δ_{C}	δ_{H} mult (J, Hz)	δ_{C}	δ_{H} mult (J, Hz)	δ_{C}	δ_{H} mult (J, Hz)
1	140.2		14.6	0.96 (3H, t, 7.6)	14.7	0.97 (3H, t, 7.6)
2	129.4	7.24 overlap	21.6	2.07 (2H, m)	21.6	2.08 (2H, m)
3	130.0	7.24 overlap	134.5	5.42 (m)	134.5	5.42 (m)
4	127.2	7.16 (m)	125.9	5.42 (m)	126.0	5.42 (m)
5	130.0	7.24 overlap	28.8	2.37 (2H, m)	28.8	2.38 (2H, m)
6	129.4	7.24 overlap	70.6	3.52 overlap 3.84 overlap	70.6	3.54 overlap 3.85 overlap
7	37.3	2.92 (t, 7.4)				
8	71.8	3.74 overlap 4.07 overlap				
1'	102.0	4.65 (d, 7.9)	102.0	4.61 (d, 8.0)	105.0	4.59 (d, 7.8)
2'	72.3	3.34 overlap	72.3	3.51 (m)	83.0	3.20–3.80 (m)
3'	72.8	4.04 (m)	72.8	4.05 (m)	78.2	3.20–3.80 (m)
4'	68.8	3.55 (m)	68.8	3.55 (m)	71.4	3.20–3.80 (m)
5'	74.5	3.83 (m)	74.4	3.82 (m)	77.7	3.20–3.80 (m)
6'	69.8	4.09 overlap 3.70 overlap	69.8	4.06 (dd, 11.2, 2.0) 3.72 (dd, 11.2, 5.0)	69.5	4.09 (dd, 11.2, 1.9) 3.70 (dd, 11.4, 5.1)
1''	105.2	4.29 (d, 6.7)	105.2	4.30 (d, 6.7)	105.1	4.30 (d, 6.7)
2''	72.4	3.57 (m)	72.4	3.59 (m)	72.4	3.20–3.80 (m)
3''	74.2	3.47 (m)	74.2	3.50 (m)	74.2	3.20–3.80 (m)
4''	69.5	3.78 (m)	69.5	3.80 (m)	69.5	3.20–3.80 (m)
5''	66.7	3.50 (dd, 12.5, 3.2) 3.85 (dd, 12.5, 2.0)	66.7	3.52 overlap 3.87 overlap	66.7	3.51 overlap 3.87 overlap
1'''					103.0	4.43 (d, 7.6)
2'''					76.0	3.20–3.80 (m)
3'''					77.7	3.20–3.80 (m)
4'''					71.4	3.20–3.80 (m)
5'''					76.7	3.20–3.80 (m)
6'''					62.7	3.52 overlap 3.87 overlap

Compound 3 possessed the molecular formula of $\text{C}_{23}\text{H}_{40}\text{O}_{15}$ according to the HR-ESI-MS at m/z 557.2419 $[\text{M} + \text{H}]^+$. The ^1H -NMR spectrum (Table 1) of 3 showed three characteristic signals of glycosyls at δ_{H} 4.59 (1H, d, $J = 7.8$ Hz, H-1'), and 4.30 (1H, d, $J = 6.7$, H-1''), and 4.43 (1H, d, $J = 7.6$, H-1'''), assigned to glucose, arabinose and glucose, respectively. Moreover, a group of parent nucleus signals existed in accordance with 2 at δ_{H} 5.42 (2H, m), 2.08 (2H, m), 2.36 (2H, m), 3.52 (1H, o), 3.84 (1H, o) and 0.97 (3H, t, 7.6). Combined with the DEPT, ^{13}C -NMR spectrum of 3 showed 23 signals, including a fatty chain group, which is the same as compound 2, at δ_{C} 14.7, 21.6, 134.5, 126.0, 28.8, 70.6, and two groups of glucose and a group of arabinose glycosyls at δ_{C} 105.0, 83.0, 78.2, 71.4, 77.7, 62.7, 103.0, 76.0, 77.7, 71.4, 76.7, 69.5 and 105.1, 72.4, 74.2, 69.5, 66.7. The HMBC correlations between H-1' and C-6, H-1'' and C-6', and H-1''' and C-2' suggested the attachment position of the arabinosyl at C-8 and C-1''' of glucose at C-6' of glucose. Assignments of all groups of 3 were achieved by ^1H - ^1H COSY, HSQC and HMBC (Figure 2). The absolute configuration of the glycosyls group of compound 3 was determined by GC analysis according to the same t_{R} at 16.8 min and 12.9 min with standard D-glucose and L-arabinose, respectively. From the above data and combined with the

literature [26,27], the structure of **3** was elucidated as (Z)-3-hexenyl O-β-D-glucopyranosyl-(1→2)-O-α-L-arabinopyranosyl-(1→6)-O-β-D-glucopyranoside, named Nicglycoside C.

2.2. Anti-Inflammatory Activity

All compounds **1–18** were evaluated for their anti-inflammatory activities (Table 2) inhibiting NO production of LPS-induced RAW 264.7 cells in vitro [27]. NO, as a key pro-inflammatory mediator, could suppress inducible enzyme expression via inhibition of the mitogen-activated protein kinase pathway and nuclear translocation of critical transcription factors [28,29]. The results suggested that the isolated compounds (**1–18**) possessed different degrees of activities in inhibiting NO production. For the compounds with the same mother nucleuses (**1, 5, 6, 7, 9, 11**), compounds **7** and **11** showed weaker activities than others, which might be due to the moieties of rhamnose or trisaccharide. Compounds **8** and **10** showed weaker activities than other phenylethanoid glycosides, which might be ascribed to the hydroxy-substituted benzene rings.

Table 2. Inhibitory on NO production in LPS-induced RAW 264.7 cells of compounds **1–18**.

Compounds	IC ₅₀ (μM)	Compounds	IC ₅₀ (μM)
NMMA ^b	19.6 ± 2.4	Compound 10	>50
Compound 1	31.1 ± 3.5	Compound 11	41.7 ± 7.6
Compound 2	32.9 ± 5.6	Compound 12	29.8 ± 5.7
Compound 3	41.2 ± 4.1	Compound 13	36.6 ± 3.9
Compound 4	30.2 ± 4.7	Compound 14	>50
Compound 5	26.9 ± 5.1	Compound 15	25.1 ± 4.4
Compound 6	37.5 ± 4.7	Compound 16	38.9 ± 5.9
Compound 7	41.2 ± 6.6	Compound 17	33.4 ± 2.7
Compound 8	>50	Compound 18	31.4 ± 4.2
Compound 9	34.8 ± 6.3		

IC₅₀ was defined as the concentration that resulted in a 50% inhibition on NO production. The IC₅₀ greater than 50 μM was deemed inactive. ^b Positive control.

3. Experimental Section

3.1. General Experimental Procedures

UV spectra were recorded on a Shimadzu UV-1601 instrument. HR-ESI-MS was performed on a Waters Xevo-TOF-MSTM.1D and 2D NMR spectra using a Bruker DPX 400 instrument with TMS as an internal standard. Preparative HPLC (Waters, Milford MA, USA, 515-2414) was performed on Sunfire (10 μm, 19 × 250 mm, Waters). ODS was obtained from YMC Company Ltd., Japan. Silica gel was used Qingdao Marine Chemical Ltd., Qingdao, China. All the solvents were of analytical grade and were purchased from Tianjinfuyu Company Ltd., Tianjin, China. ELISA reader was used from PerkinElmer, Waltham, MA, USA. The RAW 264.7 cells were from China Center for Type Culture Collection in Wuhan University, Hubei, China. DMEM was purchased from Corning, New York, NY, USA. The integrant biological agents were prepared, such as, MTT (Biotopped, Beijing, China), LPS (Sigma-Aldrich, St. Louis, MO, USA), DMSO (Sigma-Aldrich), Penicillin Streptomycin Solution (Corning, New York, NY, USA), Fetal bovine serum (Sijiqing, Hangzhou, China), NMMA (Sigma-Aldrich), Sulfanilic acid anhydrous (Tianli, Tianjin, China), N-(1-naphthyl) enylenediamine dihydrochloride (Damao, Tianjin, China), PBS (Biotopped).

3.2. Plant Material

The fruits of *Nicandra physaloides* (L.) Gaertn were harvested from Harbin, Heilongjiang Province of China, in September 2014, which was identified by Prof. Ruifeng Fan of Heilongjiang University of Chinese Medicine. The voucher specimen (20140911) had been deposited at Heilongjiang University of Chinese Medicine.

3.3. Extration and Isolation

The dry fruits (15 kg) of *Nicandra physaloides* were extracted with 70% ethanol 3 times and the condensed crude (1167.2 g) was fractionated by AB-8 macroporous resin column chromatography and eluted with 10% EtOH (439.5 g), 30% EtOH (209.4 g) and 95% EtOH (176.1 g). The 10% EtOH elution (100.0 g) was concentrated and separated. Thirteen obtained fractions (Fr. I–XIII) were combined based on the TLCs. Fr. V was separated by ODS chromatography, eluted with H₂O/MeOH (0:1 to 0:1), to afford Fr. V-1 to V-17. Fr. V-9 was subjected to ODS chromatography (H₂O/MeOH, 1:0 to 0:1) to afford **3** (12 mg), **10** (8 mg) **12** (9 mg) and **15** (10 mg). Fr. V-16 was separated by ODS chromatography (H₂O/MeOH, 1:0 to 0:1) to afford Fr. V-16-1 to V-16-10. Fr. V-16-2 was purified by to afford **2** (10 mg), **4** (8 mg), **5** (9 mg) and **17** (9 mg). Fr. VI was separated by ODS chromatography, eluted with H₂O/MeOH (0:1 to 0:1), to afford Fr. VI-1 to VI-10. Fr. VI-5 were purified by preparative HPLC to afford **9** (11 mg), **13** (8 mg), **16** (12 mg) and **18** (10 mg). Fr. X was purified by ODS chromatography (H₂O/MeOH, 1:0 to 0:1), to afford Fr. X-1 to X-15. Fr. X-11 and X-13 were repeatedly subjected to ODS chromatography (H₂O/MeOH, 1:0 to 0:1) and then purified by preparative HPLC to afford **1** (12 mg), **6** (9 mg), **7** (10 mg), **8** (11 mg), **11** (9 mg) and **14** (9 mg), respectively.

Nicglycoside A (**1**). White amorphous powder. $[\alpha]_{\text{D}}^{25} -8.9$ ($c = 1.30$, MeOH); IR (KBr) 3435, 2921, 1750, 1260 cm⁻¹; UV (MeOH) λ_{max} 212, 258, 271 nm; ¹H- and ¹³C-NMR (MeOH, 400, 100 MHz) data, see Table 1; HR-ESI-MS m/z 417.1755 [M + H]⁺ (calcd. for C₁₉H₂₉O₁₀, 417.1761) (Figures S1 and S2).

Nicglycoside B (**2**). White amorphous powder. $[\alpha]_{\text{D}}^{25} -46.4$ ($c = 1.57$, MeOH); IR (KBr) 1732, 1464, 1263, 710 cm⁻¹; ¹H- and ¹³C-NMR (MeOH, 400, 100 MHz) data, see Table 1; HR-ESI-MS m/z 395.1901 [M + H]⁺ (calcd. for C₁₇H₃₁O₁₀, 395.1917) (Figures S3 and S4).

Nicglycoside C (**3**). White amorphous powder $[\alpha]_{\text{D}}^{25} -51.8$ ($c = 1.60$, MeOH); IR(KBr) 1730, 1458, 1260, 704 cm⁻¹; ¹H- and ¹³C-NMR (MeOH, 400, 100MHz) data, see Table 1; HR-ESI-MS m/z 557.2419 [M + H]⁺ (calcd. for C₂₃H₄₁O₁₅, 557.2445) (Figures S5 and S6).

3.4. Acid Hydrolysis and GC Analysis

The isolated glycosides (**1–18**) (2.0 mg) were refluxed with 2 mL H₂O and 2 N aqueous 1 mL HCl for water bath (3 h). Then, the reaction mixtures were extracted with ethyl acetate for 3 times (5 mL). The aqueous layer was neutralized and evaporated with MeOH and then dissolved in anhydrous pyridine (5 mL) and treated with L-cysteine methyl ester hydrochloride (1.5 mg). After being stirred for 1 h at 60 °C, the mixture was added into 150 µL of HMDS–TMCS (3:1) and then stirred for another 30 min at 60 °C. The supernatant was concentrated under N₂ stream after being centrifuged off. The residue was partitioned between *n*-hexane and H₂O (0.1 mL each), and the hexane layer (1 µL) was analyzed by GC [30,31], respectively. The configurations of D-glucose for compounds **3–18** were determined by the same t_{R} of standard D-glucose ($t_{\text{R}} = 16.8$ min), L-arabinose for compounds **1–5**, **16**, **18** ($t_{\text{R}} = 12.9$ min), D-galactose for compound **1–2** ($t_{\text{R}} = 32.5$ min), and L-rhamnose for compound **7**, **10** ($t_{\text{R}} = 14.8$ min).

3.5. Anti-Inflammatory Assays

RAW 264.7 cells were cultivated at densities of 5×10^5 cell/wells in 96-well for 24 h, then discarded the supernatants and stimulated by LPS (100 µL, 1 µg/mL) to generate NO for cultivating 24 h. Following incubation of the demonstrated time, the amount of sable nitrite, the end product of NO generation by activated cells, were determined by a modification of the Griess reaction [32,33]. The cells were treated with 100 µL of the compounds by the final concentration of 5, 25, 50, 100 and 200 µg/mL. Briefly, 100 µL of culture supernatants from control or stimulated macrophages were transferred to 96-well plates. Supernatants were mixed with 50 µL of 1% sulfanilic acid anhydrous in 85% phosphoric acid, incubated for 10 min at room temperature, shielded from light, followed by 50 µL of 1 mg/mL *N*-(1-naphthyl) ethylenediamine dihydrochloride for 10 min incubation in light

proof. The absorbance was measured at 540 nm using an ELISA reader, and nitrite concentration was determined by comparison with a sodium nitrite standard curve. NMMA was used as a positive control. No isolates did showed an effect on the assay systems with the final concentration 0.2 (v/v) in DMSO and the MTT assay revealed no significant cytotoxic effects (over 90% cells survival) on cells treated with above compounds at concentrations up to 200 µg/mL.

4. Conclusions

As described in the introduction, *Nicandra physaloides* possessed many kinds of bioactivities such as heat-clearing, detoxifying, antipyresis, and anti-inflammation, and was applied to the treatments of rheumatoid arthritis, and so on. This study obtained the 18 glycosides compounds from the *Nicandra physaloides* fruits, including phenyl-glycosides and fat glycosides. Meanwhile, many researchers have reported that the aromatic glycosides and phenyl-glycosides show significant anti-inflammatory activities [34–37]. Thus, the anti-inflammatory activities of the compounds were evaluated, some of which showed significant activities. These results indicated that these glycosides compounds could be the pharmacodynamic material basis for anti-inflammation from the *Nicandra physaloides* fruits, and played important roles in the treatments of inflammatory diseases such as rheumatoid arthritis, nasosinusitis, influenza, urinary tract infection, sore and furuncle. The systematic studies on the composition in this manuscript will be the foundations and references of further research. We have made contributions to discovering active ingredients and leading compounds and provided experimental and scientific basis of drug design and drug discovery of the *Nicandra physaloides*.

Supplementary Materials: The following are available online: Figures S1–S6 and Table S1: The ¹³C-NMR data of Compounds 4–18.

Acknowledgments: This work was financed by the Changjiang Scholars Back-Up Program of Heilongjiang Universities Colleges (2013CJHB006) and Research Program of Chinese Medicine of Heilongjiang (ZHY16-091).

Author Contributions: H.-W.G. and H.-X.K. designed the experiments; Y.L., H.-B.J. and Y.-G.C. performed the experiments; Y.L. and S.-W.L. wrote the paper. Z.-P.X. and B.-Y.Y modified the paper; All authors read and approved the manuscript.

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

Abbreviations

The following abbreviations are used in this manuscript:

HR-ESI-MS	High-resolution electrospray ionization mass spectrometry
NMR	Nuclear magnetic resonance
DEPT	Distortionless Enhancement by Polarization Transfer
HMBC	Heteronuclear multiple bond correlation
¹ H- ¹ H COSY	Correlation spectroscopy
HSQC	Heteronuclear multiple quantum coherence
GC	Gas chromatography
<i>t</i> _R	Retention time
NMMA	NG-monomethyl Arginine
UV	Ultraviolet
TMS	Tetramethylsilane
HPLC	High performance liquid chromatography
ODS	Octadecylsilyl
ELISA	Enzyme-linked immunosorbent assay
DMEM	Dulbecco's modified eagle medium
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
LPS	Lipopolysaccharides
DMSO	Dimethyl sulfoxide
PBS	Phosphate buffer saline

EtOH	Ethyl alcohol
TLC	Thin Layer Chromatography
MeOH	Methanol
HMDS-TMCS	Hexamethyldisilazane-trimethylchlorosilane

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Sample Availability: Samples of the compounds are available from the authors.



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