New phenolic glycosides from *Anemone chinensis* Bunge and their antioxidant activity

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**ABSTRACT**

Nine compounds, five phenolic glycosides (1, 2, 4–6), three phenylpropanoids (7–9), and a furanone glycoside (3), were isolated from aqueous soluble extract of the dried roots of *Anemone chinensis* Bunge. The structures of new compounds (1–4) were elucidated by comprehensive spectroscopic data analysis as well as chemical evidence. Pulsatillanin A (1) demonstrated significant antioxidant effects through scavenging free radical in DPPH assay, and relieved the oxidative stress in LPS-induced RAW 264.7 cells by reducing ROS production, enhancing antioxidant enzyme SOD activity, replenishing depleted GSH in a dose-dependent manner. Western blot analysis revealed that 1 showed antioxidant activity via activating Nrf2 signaling pathway.

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**1. Introduction**

*Pulsatillae Radix*, the dried roots of *Anemone chinensis* Bunge (synonym *Pulsatilla chinensis* (Bunge) Regel), is officially listed in the Chinese Pharmacopoeia as a well-known Traditional Chinese Medicine. *A. chinensis* is traditionally used to treat intestinal amebiasis, dysentery, and enteritis (Muluye et al. 2014; Li et al. 2020). Modern
pharmacological studies revealed that the chemical constituents of *A. chinensis* exhibited antitumor (Zhou et al. 2020), antibacterial (Feng et al. 2018), anti-inflammatory, and immunomodulatory effects (Kang et al. 2019). Especially, the clinical efficacy of *A. chinensis* in ulcerative colitis treatment received significant attention (Li et al. 2020; Ma et al. 2020). Phytochemical investigations showed triterpenoid saponins are the main type of chemical component of *A. chinensis* and responsible for the bioactivities of this traditional medicine (Mimaki et al. 2001; Shu et al. 2013; Xu et al. 2013).

With the aim of discovering new bioactive compounds from a natural source, the chemical constituents of *A. chinensis* were investigated. Nine phenolic glycosides and phenylpropanoids were obtained from the aqueous soluble fraction by sequential column chromatographic method (Figure 1). They are pulsatillanin A (1), pulsatillanin B (2), (S)-dihydro-5-(β-D-glucopyranosylxymethyl)furanone (3), tanshinol-3-O-β-D-glucopyranoside (4), cimidahurinine (5) (Zhou et al. 2014), calophymembranside B (6) (Yadikar et al. 2017), β-(4-hydroxyphenyl) lactic acid (7) (Zou et al. 2005), ferulic acid (8) (Salum et al. 2010), and isoferulic acid (9) (Prachayasittikul et al. 2009). The structures of new compounds 1–4 were elucidated by comprehensive spectroscopic data analysis as well as chemical evidence.

Phenolic compounds are well-known antioxidant active ingredients in vegetables, fruits, herb medicine, and other natural sources. They play a key role in the herb medicine to prevent oxidative damage under pathological conditions including cellular injury, aging, cancer, and cardiovascular disorders (Sytar 2015; Aryal et al. 2019). To investigate the potential of isolated phenolic compounds, the antioxidant activity of isolates 1–9 were evaluated by DPPH assay. Meanwhile, the effects of pulsatillanin A (1) scavenging ROS, increasing superoxide gasification enzyme (SOD) and non-enzyme
antioxidant glutathione (GSH) levels by activating Nrf2 signaling pathway were investigated.

2. Results and discussion

2.1. Chemical

Compound 1 was isolated as a white solid. Its molecular formula was established as C_{19}H_{26}O_{9} by HRESIMS and ^{13}C NMR data with six degrees of unsaturation. The IR spectrum of 1 showed absorption bands for hydroxyls at 3301 cm\(^{-1}\) and a carboxyl at 1706 cm\(^{-1}\). The \(^{1}H\) NMR (Supplementary material Table S1) spectrum of 1 displayed two aromatic hydrogens at \(\delta_H 6.84 \text{ (d, } J=8.8 \text{ Hz)}\) and \(\delta_H 6.63 \text{ (d, } J=8.8 \text{ Hz)}\), an olefinic hydrogen at \(\delta_H 4.98 \text{ (t, } J=6.0 \text{ Hz)}\), two methylenes at \(\delta_H 3.68 \text{ (d, } J=16.1 \text{ Hz)}\), 3.50 (d, \(J=16.1 \text{ Hz)}\) and \(\delta_H 3.27 \text{ (dd, } J=15.0, 6.0 \text{ Hz)}\), 3.17 (m), two singlet methyls at \(\delta_H 1.71, 1.62\). Moreover, seven oxygenated hydrogens belonging to a sugar moiety were observed at \(\delta_H 3.13-4.49\). With the aid of HSQC experiment, the 19 carbon signals observed in \(^{13}C\) NMR spectrum (Supplementary material Table S1) of 1 were attributed to a carboxyl (\(\delta_C 174.3\)), a phenyl (\(\delta_C 150.6, 149.7, 127.7, 125.9, 115.2, 113.8\)), an olefinic double bond (\(\delta_C 130.6, 123.4\)), a hexose (\(\delta_C 103.8, 77.5, 77.1, 74.0, 70.3, 61.4\)), two methylenes (\(\delta_C 32.8, 25.8\)), and two methyls (\(\delta_C 26.0, 18.2\)). The \(^{1}H\) and \(^{13}C\) NMR data of 1 showed it is a phenolic glycoside and almost identical to the known 3-(\(\beta\)-D-glucopyranosyloxy)-6-hydroxy-2-(3-methyl-2-buten-1-yl)benzeneacetic acid except for the position of glycosyl substituent (Ren et al. 2011). Acid hydrolysis of 1 and further GC-MS analysis of the trimethylsilyl ether derivative of sugar moiety revealed the presence of D-glucose. HMBC correlations (Supplementary material Figure S1) observed from H-7 (\(\delta_H 3.68, 3.50\)) to C-1 (\(\delta_C 125.9\)), C-2 (\(\delta_C 127.7\)), C-6 (\(\delta_C 149.7\)) and C-8 (\(\delta_C 174.3\)), from H-9 (\(\delta_H 3.27, 3.17\)) to C-2 (\(\delta_C 127.7\)), C-3 (\(\delta_C 150.6\)) and C-11 (\(\delta_C 130.6\)), from H-10 (\(\delta_H 4.98\)) to C-2 (\(\delta_C 127.7\)), C-12 (\(\delta_C 26.0\)), C-13 (\(\delta_C 18.2\)) and from H-1’ (\(\delta_H 4.49\)) to C-6 (\(\delta_C 149.7\)) confirmed the location of carboxyethyl, isopentenyl, and glycosyl moieties. The \(\beta\)-configuration of the glucose was determined based on the coupling constant of anomeric hydrogen (\(\delta J_{H-1/H-2} = 7.2 \text{ Hz}\)). Therefore, the structure of 1 was determined to be 6-(\(\beta\)-D-glucopyranosyloxy)-3-hydroxy-2-(3-methyl-2-buten-1-yl)benzeneacetic acid and named pulsatillanin A.

A molecular formula of C_{25}H_{36}O_{14} was determined for compound 2 deduced from HRESIMS as well as \(^{13}C\) NMR data. Analysis of \(^{1}H\) and \(^{13}C\) NMR data revealed that 2 was also a phenolic glycoside and very similar to compound 1. An additional sugar moiety signals including six carbons at \(\delta_C 102.7, 77.5, 77.3, 73.9, 70.2, 61.3\) and seven hydrogens corresponding to carbons of sugar residue at \(\delta_H 3.14-4.67\) were observed, which indicated 2 was a phenolic diglycoside. To further identify the sugar moieties of compound 2, the similar protocol of hydrolysis and GC-MS assay were carried out. The results indicated both of hexose moieties were glucosyl groups. Main HMBC correlations (Supplementary material Figure S1) observed between H-7 (\(\delta_H 3.83, 3.43\)) and C-1 (\(\delta_C 125.4\)), C-2 (\(\delta_C 130.9\)), C-6 (\(\delta_C 151.7\)), C-8 (\(\delta_C 173.9\)), between H-9 (\(\delta_H 3.57, 3.12\)) and C-2 (\(\delta_C 130.9\)), C-3 (\(\delta_C 151.2\)), C-10 (\(\delta_C 123.3\)), between H-10 (\(\delta_H 4.99\)) and C-2 (\(\delta_C 130.9\)), C-12 (\(\delta_C 26.0\)), C-13 (\(\delta_C 18.2\)), between H-1’ (\(\delta_H 4.57\)) to C-6 (\(\delta_C 151.7\)), between H-1’’ (\(\delta_H 4.67\)) and C-3 (\(\delta_C 151.2\)) determined carboxyethyl, isopentenyl were located at C-1, C-2, and two glycosyls were located at C-3, C-6, respectively. The anomer
β-configurations of two glucose residues were identified through analysis of the coupling constants of two anomeric hydrogens ($^{3}J_{H-1/H-2} = 6.6$ Hz). Thus, the structure of compound 2 was identified as 3,6-di-(β-D-glucopyranosyloxy)-2-(3-methyl-2-buten-1-yl)-benzeneacetic acid, and named pulsatillanin B.

Compound 3 was obtained as a colorless solid and assigned a molecular formula of $\text{C}_{11}\text{H}_{18}\text{O}_{8}$ according to HRESIMS and $^{13}$C NMR data. Its IR spectrum revealed the presence of hydroxy groups (3375 cm$^{-1}$) and a carbonyl (1758 cm$^{-1}$). The $^{1}$H NMR spectrum presented signals for six oxygenated methines at $\delta_{H}$ 4.68–2.96, four methylenes at $\delta_{H}$ 3.96–2.02. The $^{13}$C NMR and HSQC spectra demonstrated signals for all eleven carbons and assigned as one lactone carbonyl at $\delta_{C}$ 177.7, one oxygenated methine at $\delta_{C}$ 79.1, one oxygenated methylene at $\delta_{C}$ 70.6, two methylenes at $\delta_{C}$ 28.2, 23.4), and a group of carbon signals for a hexose moiety $\delta_{C}$ 103.4, 77.0, 76.8, 73.6, 70.1, 61.2. All the hydrogen signals were assigned to the corresponding carbons through carefully HSQC spectral analysis. The $^{1}$H NMR, $^{13}$C NMR, COSY, and HMBC spectra indicated 3 was a γ-lactone alcohol glycoside. The aglycone was identified as in figure 1 based on acid hydrolysis product 3a, which showed nearly identical optical rotation value and NMR data with those of (S)-dihydro-5-((hydroxymethyl)furanone (Wrona et al. 2010). The configuration of C-4 was further confirmed to be S based on the experimental ECD spectrum of 3a similar to the calculated ECD curve of S (Supplementary material Figure S2). The β-D-glucose was determined by the same chemical method as 1 and analysis of coupling constant of its anomeric proton. The connectivity of aglycone and D-glucopyranose was established from HMBC correlation observed between H-1' ($\delta_{H}$ 4.18) and C-5 ($\delta_{C}$ 70.6), between H-5 ($\delta_{H}$ 3.96, 3.57) and C-1' ($\delta_{C}$ 103.4). Based on the above evidence, the structure of 3 was determined as (S)-dihydro-5-(β-D-glucopyranosyloxymethyl)furanone.

Compound 4 was confirmed to have the molecular formula $\text{C}_{15}\text{H}_{20}\text{O}_{10}$ by its HRESIMS and $^{13}$C NMR data. The $^{1}$H and $^{13}$C NMR data (Supplementary material Table S1) of 4 showed it was a phenolic glycoside. Acid hydrolysis of 4 afforded two products, aglycone (4a) and a sugar residue. 4a was elucidated as in Figure 1 by comparing its optical rotation value and NMR data with those of (R)-3-(3,4-dihydroxyphenyl)-2-hydroxypropanoic acid (tanshinol) (Kelley et al. 1976; Dai et al. 2010). Comparing the experimental and calculated ECD spectra (Supplementary material Figure S2) of 4a further determined the absolute configuration of C-8 was R. The sugar was identified as a D-glucose through using the same GC-MS analysis of the sugar derivative as those of compounds 1–3. HMBC cross-peaks found from H-1' ($\delta_{H}$ 4.59, d, $J = 7.2$ Hz) to C-3 ($\delta_{C}$ 144.9) confirmed the sugar residue linkage with C-3. The β-configuration of glucose was determined according to the coupling constant of anomeric hydrogen ($^{3}J_{H-1/H-2} = 7.2$ Hz). The structure of 4 was further confirmed by $^{1}$H-$^{1}$H COSY and HMBC data analysis (Supplementary material Figure S1). As shown in Figure S1 (Supplementary material), the structure of 4 was elucidated and named as tanshinol-3-O-β-D-glucopyranoside.

### 2.2. Antioxidant activity determined by DPPH assay

To explore the antioxidant capacity of other isolates, the DPPH assay was carried out. As the result (Supplementary material Table S2), besides FA (8) and IFA (9),
pulsatillanin A (PA, 1) showed effective antioxidant activity with IC$_{50}$ value at 177.17 ± 8.18 μM. Whereas, no significant antioxidant effect was observed among compounds 2–7 (IC$_{50}$ > 200 μM). Subsequently, new phenolic glycoside PA (1) was selected to evaluate the antioxidant effects in RAW 264.7 cells. FA (8) was used as the reference standard, which was reported to exhibit antioxidant ability by activating Nrf2 signaling pathway (Mahmoud et al. 2020).

2.3. Effects of PA on cell viability and oxidative stress in RAW 264.7 cells

Prior to investigate the antioxidant activity of PA (1), the cytotoxicity of PA on RAW 264.7 cells was evaluated by MTT assay. The result showed that there was no significant effects on cell viability after exposure to PA (1) at the concentration of 0~100 μM for 24 h (Supplementary material Figure S3A).

The ROS level in LPS-stimulated RAW 264.7 cells increased significantly comparing to control group, which was in turn attenuated in a dose-dependent manner after treated with PA for 24 h (Supplementary material Figure S3B and C). The data further verified the protective effect of PA against oxidative stress in LPS-induced cells. Superoxide dismutase (SOD) and non-enzyme antioxidant glutathione (GSH) play the key role in the protection of cells against damage induced by oxidative stress (Li et al. 2020). SOD is one of the key antioxidant involved in scavenging ROS and promoting the decomposition of hydrogen peroxide (Gür et al. 2020). The ratio of reduced glutathione and oxidized glutathione (GSH/GSSG) is widely used to determine the oxidative stress status. To investigate the effect of PA on the enzymatic and non-enzymatic antioxidant ability, the expression levels of SOD and GSH/GSSG were determined in LPS-stimulated RAW 264.7 cells with PA treatment. According to the experimental result (Supplementary material Figure S3D), an obvious elevation in SOD level was observed in PA-treated group. Besides, PA also significantly reversed GSH level and GSH/GSSG ratio compared with LPS group (Supplementary material Figure S3E and F).

2.4. Effects of PA on Nrf2 signaling pathway in LPS-induced RAW264.7 cells

Under normal condition, Nrf2 is inactive in the cytoplasm and interacted with Kelch-like ECH-associated protein 1 (Keap1). Once exposed to oxidative stress, Nrf2 dissociated from Keap1 and translocated into the nucleus, subsequently combined with antioxidant response element (ARE) resulting in the expression of antioxidant/detoxification enzymes, including NAD(P)H, quinone oxidoreductase 1 (NQO1), superoxide dismutase (SOD), glutamate cysteine ligase catalytic subunit (GCLC) (Choi et al. 2020; Sun et al. 2020).

To explore the mechanism responsible for the antioxidant activity of PA, the protein expression levels of Nrf2 and its target genes GCLC, NQO-1 were detected by western blot. As shown in Figure S4 (Supplementary material), the protein expression level of Nrf2 was slightly elevated, but GCLC and NQO1 was no significant changes in RAW 264.7 cells after LPS treatment. The observation indicate that oxidative stress occurred after LPS stimulation, and compensatory increase of Nrf2 was insufficient to induce antioxidant defense in cells. Nevertheless, compared with LPS group, the protein
expression levels of Nrf2, GCLC and NQO1 significantly increased in a dose-dependent manner in LPS-induced cells with PA treatment. Hence, the antioxidant effect of PA might be attributed to activating Nrf2 signal pathway, which resulted in an enhancement of detoxification and antioxidant capacity in cells.

3. Conclusion

In summary, four new compounds, pulsatillanin A (1), pulsatillanin B (2), (5)-dihydro-5-((β-D-glucopyranosyloxymethyl)furanone (3) and tanshinol-3-O-β-D-glucopyranoside (4), as well as five known compounds, cimidahurinine (5), calophymembranside B (6), β-(4-hydroxyphenyl) lactic acid (7), ferulic acid (8) and isoferulic acid (9) were obtained from the dried roots of Anemone chinensis Bunge. New phenolic glycoside, pulsatillanin A (1), showed potently antioxidant activity in LPS-stimulated RAW 264.7 cells and relieved the oxidative stress in cells by reducing ROS level, enhancing antioxidant enzyme SOD activity, replenishing the depleted GSH by activating Nrf2 signal pathway.

Disclosure statement

The authors declare no competing financial interest.

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References

Aryal S, Baniya MK, Danekhu K, Kunwar P, Gurung R, Koirala N. 2019. Total phenolic content, flavonoid content and antioxidant potential of wild vegetables from Western Nepal. Plants. 8(4): 96.

Choi JW, Kim GJ, Kim HJ, Nam JW, Kim J, Chin J, Park JH, Choi H, Park KD. 2020. Identification and evaluation of a napyradiomycin as a potent Nrf2 activator: anti-oxidative and anti-inflammatory activities. Bioorganic Chem. 105:104434.

Dai JQ, Sorribas A, Yoshida WY, Williams PG. 2010. Sebestenoids A–D, BACE1 inhibitors from Cordia sebestena. Phytochemistry. 71(17-18):2168–2173.

Feng QM, Yu Y, Tang MX, Zhang TY, Zhang MY, Wang HF, Han YQ, Zhang YX, Chen G, Pei YH. 2018. Four new hybrid polyketide-terpenoid metabolites from the Penicillium sp. SYPF7381 in the rhizosphere soil of Pulsatilla chinensis. Fitoterapia. 125:249–257.

Gür F, Gür B, Erkayman B, Halıcı Z, Karakoç A. 2020. Investigation of serum and brain superoxide dismutase levels depending on atomoxetine used in attention-deficit/hyperactivity disorder treatment: a combination of in vivo and molecular docking studies. Bioorganic Chem. 105:104435.

Kang NX, Shen WH, Zhang Y, Su ZT, Yang SL, Liu YL, Xu QM. 2019. Anti-inflammatory and immune-modulatory properties of anemoside B4 isolated from Pulsatilla chinensis in vivo. Phytomedicine. 64:152934.
Kelley CJ, Harruff RC, Carmack M. 1976. Polyphenolic acids of Lithospermum ruderale. II. Carbon-13 nuclear magnetic resonance of lithospermic and rosmarinic acids. J Org Chem. 41(3): 449–455.

Li WB, Qiao XP, Wang ZX, Wang S, Chen SW. 2020. Synthesis and antioxidant activity of conjugates of hydroxytyrosol and coumarin. Bioorganic Chem. 105:104427.

Li YH, Zou M, Han Q, Deng LR, Weinshilboum RM. 2020. Therapeutic potential of triterpenoid saponin anemoside B4 from Pulsatilla chinensis. Pharmacol Res. 160:105079.

Ma HM, Zhou MJ, Duan WB, Chen LY, Wang LL, Liu P. 2020. Anemoside B4 prevents acute ulcerative colitis through inhibiting of TLR4/NF-κB/MAPK signaling pathway. Int Immunopharmacol. 87:106794.

Mahmoud AM, Hussein OE, Hozayen WG, Bin-Jumah M, Abd El-Twab SM. 2020. Ferulic acid prevents oxidative stress, inflammation, and liver injury via upregulation of Nrf2/HO-1 signaling in methotrexate-induced rats. Environ Sci Pollut Res. 27(8):7910–7921.

Mimaki Y, Yokosuka A, Kuroda M, Hamanaka M, Sakuma C, Sashida Y. 2001. New bisdesmosidic triterpene saponins from the roots of Pulatatilla chinensis. J Nat Prod. 64(9):1226–1229.

Muluye RA, Bian YH, Alemu PN. 2014. Anti-inflammatory and antimicrobial effects of heat-clearing Chinese cerbs: a current review. J Tradit Complement Med. 4(2):93–98.

Prachayasittikul S, Suphapong S, Worachartcheewan A, Lawung R, Ruchirawat S, Prachayasittikul V. 2009. Bioactive metabolites from Spilanthes acmella Murr. Molecules. 14(2):850–867.

Ren FX, Zhao YM, Zhang AJ, Yang Y, Zhang Y, inventors; Institute of Pharmacology and Toxicology, Academy of Military Medical Sciences, PLA, Peop. Rep. China, assignee. 2011. Extraction method of effective components from Chinese medicine Pyrola and its application for treating hemorrhage and/or pain-related disease. China CN102204937A.

Salum ML, Robles CJ, Erra-Balsells R. 2010. Photoisomerization of ionic liquid ammonium cinnamates: one-pot synthesis – isolation of Z-cinnamic acids. Org Lett. 12(21):4808–4811.

Shu Z, Chen Z, Liu YL, Zhu WF, Feng YL, Xu QM, Li XR, Yang SL. 2013. A new oleanane-type triterpenoidal saponin from Pulatatilla chinensis. Nat Prod Res. 27(23):2196–2201.

Sun Y, Huang JX, Chen YF, Shang H, Zhang WN, Yu JQ, He L, Xing CG, Zhuang CL. 2020. Direct inhibition of Keap1-Nrf2 protein-protein interaction as a potential therapeutic strategy for Alzheimer’s disease. Bioorganic Chem. 103:104172.

Sytar O. 2015. Phenolic acids in the inflorescences of different varieties of buckwheat and their antioxidant activity. J King Saud Univ Sci. 27(2):136–142.

Wrona IE, Gozman A, Taldone T, Chiosis G, Panek JS. 2010. Synthesis of reblastatin, autolytimycin, and non-benzoquinone analogues: potent inhibitors of heat shock protein 90. J Org Chem. 75(9):2820–2835.

Xu K, Shu Z, Xu QM, Liu YL, Li XR, Wang YL, Yang SL. 2013. Cytotoxic activity of Pulapatilla chinensis saponins and their structure–activity relationship. J Asian Nat Prod Res. 15(6):680–686.

Yadikar N, Bobakulov KM, Eshbakova KA, Aisa HA. 2017. Phenolic compounds from Lavandula angustifolia. Chem Nat Compd. 53(3):562–564.

Zhou XJ, Yan LL, Yin PP, Shi LL, Zhang JH, Liu YJ, Ma C. 2014. Structural characterisation and antioxidant activity evaluation of phenolic compounds from cold-pressed Perilla frutescens var. arguta seed flour. Food Chem. 164:150–157.

Zhou Y, Wen JH, Wang GJ. 2020. Identification of cytochrome P450 isoenzymes involved in the metabolism of 23-hydroxybetulinic acid in human liver microsomes. Pharm Biol. 58(1):60–63.

Zou J, Jin DZ, Chen WL, Wang J, Liu QF, Zhu XZ, Zhao WM. 2005. Selective cyclooxygenase-2 inhibitors from Calophyllum membranaceum. J Nat Prod. 68(10):1514–1518.