Chinese Chemical Letters 31 (2020) 1254–1258

Contents lists available at ScienceDirect

Chinese Chemical Letters

journal homepage: www.elsevier.com/locate/cclet

Communication

New norlignan enantiomers from the fruit of *Crataegus pinnatifida* with neuroprotective activities

Rui Guo[a], Tianming Lv[a], Fengying Han[a], Zilin Hou[a], Guodong Yao[a], Bin Lin[b], Xiaobo Wang[c], Xiaoxiao Huang[a,c,*], Shaojiang Song[a,*]

[a] *Key Laboratory of Computational Chemistry-Based Natural Antitumor Drug Research & Development, Liaoning Province, School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University, Shenyang 110016, China*
[b] *School of Pharmaceutical Engineering, Shenyang Pharmaceutical University, Shenyang 110016, China*
[c] *Chinese People's Liberation Army Logistics Support Force No. 967 Hospital, Dalian 116021, China*

ARTICLE INFO

Article history:
Received 10 August 2019
Received in revised form 18 September 2019
Accepted 20 September 2019
Available online 21 September 2019

Keywords:
Crataegus pinnatifida
Norlignan enantiomer
Neuroprotective activity
SH-SY5Y cells
Apoptosis

ABSTRACT

(±)-Crataegusnorin A (**1a**/**1b**) and B (**2a**/**2b**), two pairs of rare 8,9′-epoxy-type norlignan enantiomers featuring a γ-butyrolactone ring, were isolated from the fruit of *Crataegus pinnatifida*. Their structures were determined *via* extensive spectroscopic analyses. Gauge-independent atomic orbital (GIAO) NMR chemical shift calculations, combined with the advanced statistical method DP4+ were employed to establish the relative configurations of four compounds. Next, chiral separation was accomplished by chiral chromatographic column and the absolute configurations of the four compounds were unambiguously assigned by comparison between their experimental electronic circular dichroism curves with the quantum-mechanically calculated curves based on time-dependent density functional theory (TDDFT). All the isolates were evaluated for their neuroprotective activities against H_2O_2-induced cell injury in human neuroblastoma SH-SY5Y cells. The results showed that two pairs of enantiomers **1a**/**1b** and **2a**/**2b** displayed diff ;erent effect on neuroprotective activity. Among them, compound **2a** displayed the most potent neuroprotective effect. Further flow cytometry analysis indicated that **2a** could protect SH-SY5Y cells from oxidative damage through inhibiting cell apoptosis.

Crataegus pinnatifida, a traditional Chinese medicinal herb, belongs to the Rosaceae family. It is widely distributed in Asia, Europe and North America [1]. People use it as a medicinal plant to improve digestion, promote blood circulation and resolve blood stasis both in traditional and folk medicine [2]. Modern investigations have demonstrated that *C. pinnatifida* has various pharmacological effects on the cardiovascular, digestive, and endocrine systems as well as killing some pathogenic microorganisms [3–5]. In the research on the chemical constituents of *C. pinnatifida*, a variety of secondary metabolites, such as flavonoids, triterpenoids, steroids, lignans and organic acids, have been isolated and identified [6–9].

Herein, we carried out the continued search for biologically active and structurally unique compounds, which resulted in the isolation of two rare norlignans (**1** and **2**) from the fruit of *Crataegus pinnatifida*. For compounds **1** and **2**, they were assigned as new 8,9′-epoxy-type norlignans featuring a γ-butyrolactone ring. Structurally, they possessed the same C_6C_3-C_2C_6 carbon skeleton and only differed in the substituents present on phenyl moiety (Fig. 1).

Due to their negligible optical activities, compounds **1** and **2** were suggested as racemic mixtures, which was further supported by the absence of Cotton effects in their ECD spectra. The following chiral separation resulted in two pairs of enantiomers (**1a**/**1b** and **2a**/**2b**) using chiral chromatographic column. Their relative configurations were established by employing NMR chemical shifts calculations using Gauge-independent atomic orbital (GIAO) method [10], combined with the advanced statistical method DP4+ [11]. Additionally, their absolute configurations were established by comparison of experimental and calculated ECD spectra. Their neuroprotective activities against H_2O_2-induced oxidative injury in human neuroblastoma SH-SY5Y cells were investigated. This is the first report of the configurational

* Corresponding authors at: Key Laboratory of Computational Chemistry-Based Natural Antitumor Drug Research & Development, Liaoning Province, School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University, Shenyang 110016, China.
E-mail addresses: xiaoxiao270@163.com (X. Huang), songsj99@163.com (S. Song).

https://doi.org/10.1016/j.cclet.2019.09.042
1001-8417/

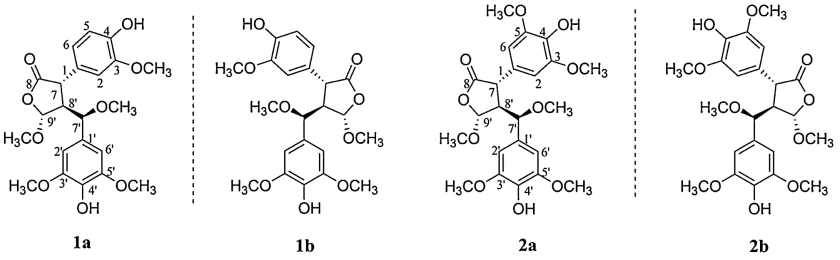

Fig. 1. The structures of compounds **1a**/**1b** and **2a**/**2b**.

Table 1
^{1}H NMR data (400 MHz) and ^{13}C NMR data (100 MHz) for compounds **1a**/**1b**-**2a**/**2b** in $CDCl_3$.

Position	**1a**/**1b**		**2a**/**2b**	
	δ_H (multi, *J* in Hz)	δ_C	δ_H (multi, *J* in Hz)	δ_C
1		129.3		128.6
2	6.31, d (2.0)	110.7	6.07, br s	104.9
3		146.6		147.1
4		145.1		134.2
5	6.78, d (8.2)	114.7		147.1
6	6.48, dd (8.2, 2.0)	121.1	6.07, br s	104.9
7	3.41, d (5.7)	49.2	3.36, d (5.4)	49.6
8		176.5		176.4
1′		129.0		129.0
2′/6′	6.44, br s	104.2	6.45, br s	104.3
3′/5′		147.4		147.4
4′		134.9		135.0
7′	4.02, d (8.4)	83.6	4.00, d (8.7)	83.8
8′	2.69, ddd (8.4, 5.7, 2.6)	57.4	2.69, ddd (8.7, 5.4, 2.4)	57.4
9′	5.52, d (2.6)	106.3	5.52, d (2.4)	106.5
3-OCH_3	3.74, s	55.8	3.75, s	56.2
5-OCH_3			3.75, s	56.2
3′/5′-OCH_3	3.81, s	56.5	3.81, s	56.5
7′-OCH_3	3.26, s	57.0	3.27, s	57.0
9′-OCH_3	3.55, s	57.4	3.57, s	57.4

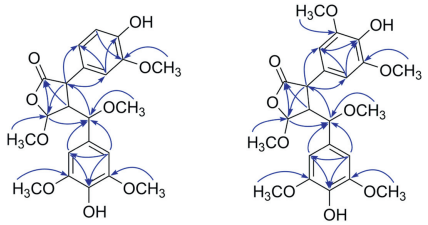

Fig. 2. Key HMBC correlations of compounds **1** and **2**.

assignment of 8,9′-epoxy-type norlignans with a flexible skeleton by quantum chemical calculations.

Crataegusnorin A (**1**) was obtained as pale yellow oil. Its molecular formula was established as $C_{22}H_{26}O_9$ from the ^{13}C NMR data and an HRESIMS ion at *m/z* 457.1472 $[M + Na]^+$ (calcd. for $C_{22}H_{26}NaO_9$: 457.1469), indicating 10 indices of hydrogen deficiency. The ^{1}H NMR data of **1** (Table 1 and Fig. S4 in Supporting information) showed two sets of aromatic protons [δ_H 6.31 (d, 1H, *J* = 2.0 Hz, H-2), 6.78 (d, 1H, *J* = 8.2 Hz, H-5), 6.48 (dd, 1H, *J* = 8.2, 2.0 Hz, H-6)] and [δ_H 6.44 (br s, 2H, H-2′/H-6′)] arising from a 1,3,4-trisubstituted and a symmetrical 1,3,4,5-tetrasubstituted aromatic ring system, respectively. In addition, four aliphatic proton signals were evident at δ_H 3.41 (d, 1H, *J* = 5.7 Hz, H-7), 4.02 (d, 1H, *J* = 8.4 Hz, H-7′), 2.69 (ddd, 1H, *J* = 8.4, 5.7, 2.6 Hz, H-8′), and 5.52 (d, 1H, *J* = 2.6 Hz, H-9′), and the protons of five methoxy groups resonated at δ_H 3.26 (s, 3H), 3.55 (s, 3H), 3.74 (s, 3H), and 3.81 (s, 6H). The ^{13}C NMR data of **1** (Table 1 and Fig. S7 in Supporting information) showed 22 carbon signals comprising two benzene rings, five methoxy groups, an ester carbonyl group, and four methines (two of which were oxygenated). The HMBC correlations of H-7, H-8′ and H-9′ with C-8 revealed the presence of the γ-butyrolactone ring in **1** (Fig. 2 and Fig. S8 in Supporting information). The multiplet centered at δ_H 2.69 (ddd, 1H, *J* = 8.4, 5.7, 2.6 Hz, H-8′) displayed correlations with C-1/C-7/C-1′/C-8, indicating the γ-butyrolactone moiety of cyclo[C_7-C_8-*O*-$C_{9'}$-$C_{8'}$], with C-8′ connected to the C-7′ methine and C-7 to the phenyl ring at C-1. According to the above observations, this structure was determined as a norlignan compound with a C_6C_3-C_2C_6 skeleton. The HMBC correlations for CH_3O-3/C-3, CH_3O-3′/C-3′, CH_3O-5′/C-5′, CH_3O-7′/C-7′, and CH_3O-9′/C-9′ revealed that these methoxy groups were located at C-3, C-3′, C-5′, C-7′, C-9′, respectively. Thus, the planar structure of **1** was established and named crataegusnorin A.

In the NOESY experiment of **1** (Fig. 3 and Fig. S10 in Supporting information), the correlations of H-8′ with H-2/6, and CH_3O-9′ indicated anti-configuration between H-7 and H-8′, H-8′ and H-9′. In addition, the lack of NOE between H-7′ and CH_3O-9′ also confirmed an opposite spatial orientation of H-8′ with H-9′. However, due to the free rotation of the flexible chain ($C_{1'}$-$C_{7'}$), the

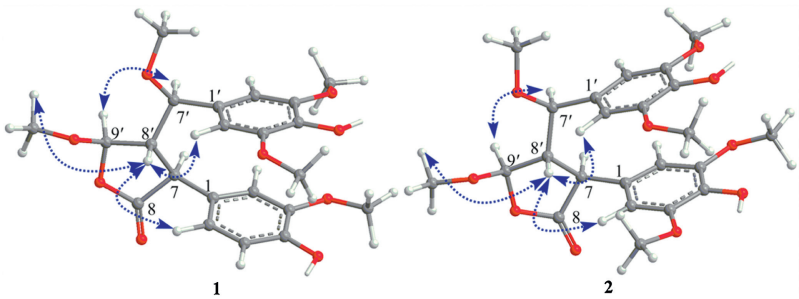

Fig. 3. Key NOESY correlations of compounds **1** and **2**.

relative configuration assignment of C-7′ could not be determined when solely based upon interproton distances deduced from NOESY or *J*-based NMR spectroscopic analysis. The configurational establishment of these molecules has always been a challenge in the structure elucidation of natural products. We therefore turned to quantum mechanical calculations for the determination of the relative configuration.

In recent years, quantum chemical calculations coupled with several correlative approaches, such as CP3, DP4, and DP4+, have been successfully employed in natural products elucidation. We performed such calculations here. The relative configuration at C-7′ was assigned by employing calculations of shielding tensor values with support from DP4+ probability analysis (Fig. 4 and Tables S1 and S2 in Supporting information). The theoretical calculations of ^{1}H and ^{13}C NMR chemical shifts of the two possible isomers (7*S**,7′*S**,8′*S**,9′*R**)-**1** and (7*S**,7′*R**,8′*S**,9′*R**)-**1** were predicted using the GIAO method with the Gaussian 09 software at the B3LYP/6-311+G(d,p) level utilizing the polarizable continuum model (PCM) in chloroform [12]. This assignment was confirmed by comparing the experimental and calculated NMR chemical shifts. With a DP4+ probability of approximately 100%, the relative configuration of **1** was defined as 7*S**,7′*R**,8′*S**,9′*R**.

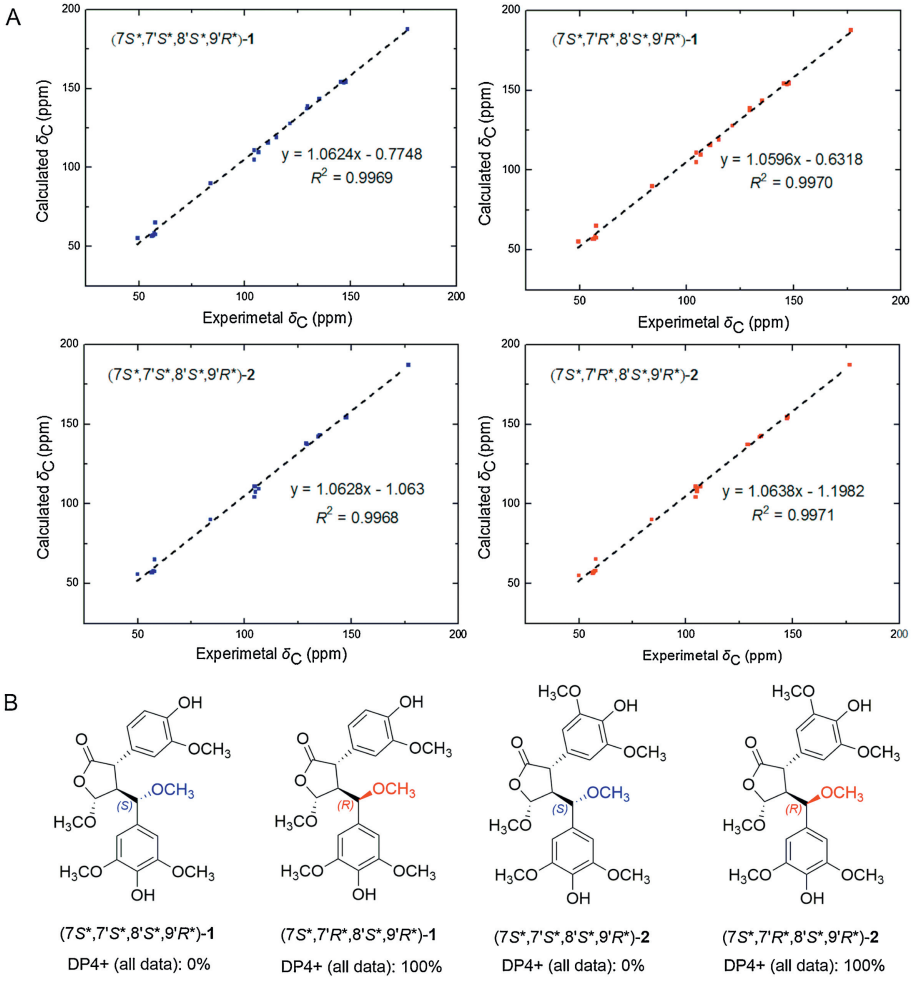

Fig. 4. (A) Calculated ^{13}C NMR spectroscopic data of two pairs of C-7′ epimers of (7*S**,7′*S**,8′*S**,9′*R**)-**1** and (7*S**,7′*R**,8′*S**,9′*R**)-**1**, (7*S**,7′*S**,8′*S**,9′*R**)-**2** and (7*S**,7′*R**,8′*S**,9′*R**)-**2** at B3LYP/6-311+G(d,p) level in $CDCl_3$. (B) DP4+ probability of ^{1}H and ^{13}C NMR chemical shifts of **1** and **2**.

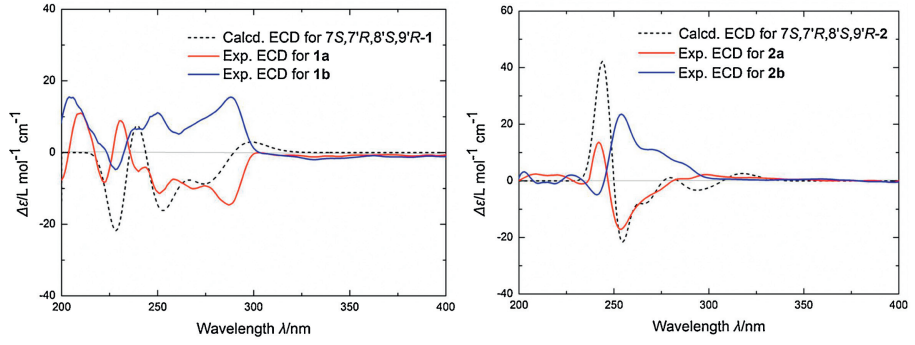

Fig. 5. Experimental and calculated ECD spectra for **1a**/**1b** and **2a**/**2b** in MeOH.

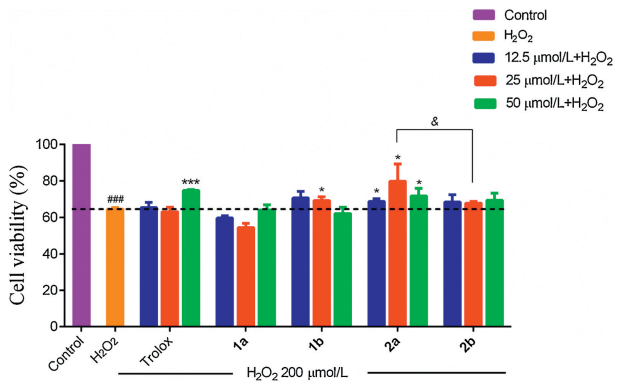

Fig. 6. Neuroprotective effects of compounds **1a/1b** and **2a/2b** against H_2O_2-induced cell growth inhibition of SH-SY5Y cells. In the presence or absence of the tested compounds at different concentrations (12.5, 25, and 50 μmol/L), the MTT assay was used to examine the cell viability after H_2O_2 (200 μmol/L) treatment for 4 h. $^{###}P < 0.001$ *vs.* control group. $^{*}P < 0.05$, $^{***}P < 0.001$ *vs.* the H_2O_2 treated group. $^{\&}P < 0.05$ was considered statistically significant when compared to its enantiomer.

With an optical rotation value close to zero and no Cotton effect in its ECD spectrum, compound **1** was considered as a racemic mixture. Subsequent chiral resolution of **1** afforded the anticipated enantiomers **1a** and **1b**, which showed mirror image-like ECD curves and specific rotations (**1a**: $[\alpha]_D^{20}$ −25.0, *c* 0.10, MeOH; **1b**: $[\alpha]_D^{20}$ +27.0, *c* 0.10, MeOH). The absolute configuration of the enantiomers were determined by comparison of its experimental and quantum chemical ECD spectra calculated at the B3LYP/6-311++G(2d,p) level in MeOH. The calculated ECD of 7*S*,7′*R*,8′*S*,9′*R*-**1** is consistent with the measured ECD of **1a** but opposite to that of **1b** (Fig. 5). Therefore, the structure of (−)-**1**, namely, (−)-crataegusnorin A, was determined to be (7*S*,7′*R*,8′*S*,9′*R*), and (+)-**1**, namely, (+)-crataegusnorin A, was determined to be (7*R*,7′*S*,8′*R*,9′*S*), respectively.

The molecular formula of compound **2** was determined to be $C_{23}H_{28}O_{10}$ in agreement with its HRESIMS data (*m/z* 487.1572 $[M+Na]^+$, calcd. for $C_{23}H_{28}NaO_{10}$: 487.1575). The ^1H and ^{13}C NMR spectroscopy data (Table 1, Figs. S15 and S18 in Supporting information), along with the HSQC experiment, showed resonances for two benzene rings, six methoxy groups, an ester carbonyl group, and four methines. The NMR spectra of **2** are similar to those of **1**, except for the presence of the C-5 methoxy group in **2**, which were confirmed by HMBC correlations from the methoxy protons (δ_H 3.75) to C-5. A comparative analysis of the remaining data in the two-dimensional NMR spectra (HSQC and HMBC) allowed us to assign all the resonances of **2** (Fig. 2, Figs. S19 and S20 in Supporting information). Similar to **1**, the 7,8′-*anti* and 8′,9′-*anti* configuration were determined by NOESY experiment in which correlations of H-8′/H-2 and H-8′/CH_3O-9′ were observed (Fig. 3 and Fig. S21 in Supporting information). The unobserved correlation of H-7′/CH_3O-9′ also confirmed the orientation of H-8′ and H-9′. In addition, the DP4+ protocol was again applied to the simulated ^1H and ^{13}C NMR chemical shifts of the two possible epimers (Fig. 4 and Tables S3 and S4 in Supporting information). The statistical results indicated that the epimer possessing the *R* configuration at C-7′, was the correct structure for **2**, with 100% probability. The optical rotation value of **2** was very close to zero and there was no Cotton effect in its ECD spectrum, suggesting that it was a racemic mixture. Subsequently, the chiral HPLC purification of **2** afforded the enantiomers **2a** and **2b** with opposite specific rotation (**2a**: $[\alpha]_D^{20}$ −27.0, *c* 0.10, MeOH); **2b**: $[\alpha]_D^{20}$ +28.0, *c* 0.10, MeOH) and mirror imaged Cotton effects in their ECD spectrum. To determine the absolute configurations of **2a** and **2b**, the ECD spectra of **2a** and **2b** were measured in MeOH and compared with the calculated ECD spectra of the enantiomers (Fig. 5). On the basis of the matching of the experimental and computed ECD spectra, the absolute configurations of (−)-**2** and (+)-**2** were determined as (7*S*,7′*R*,8′*S*,9′*R*)-**2** and (7*R*,7′*S*,8′*R*,9′*S*)-**2**, and were assigned the names (−)-crataegusnorin B and (+)-crataegusnorin B, respectively.

The neuroprotective activities of norlignan enantiomers **1a**/**1b** and **2a**/**2b** against H_2O_2-induced oxidative injury in SH-SY5Y cells were evaluated by MTT assay, and the cell viabilities of all compounds were shown in Fig. 6. The results showed that 200 μmol/L H_2O_2 could significantly reduce the cell viability compared with the control group. Among them, enantiomers **2a** and **2b** exhibited varying degrees of neuroprotective effects, while enantiomers **1a**/**1b** exhibited no obvious protective effects in the bioassay. Comparison of compounds **1a**/**1b** with **2a**/**2b** indicated that the presence of methoxy group at C-5 could increase neuroprotective effects. As shown in Fig. 6, compound **2a** showed

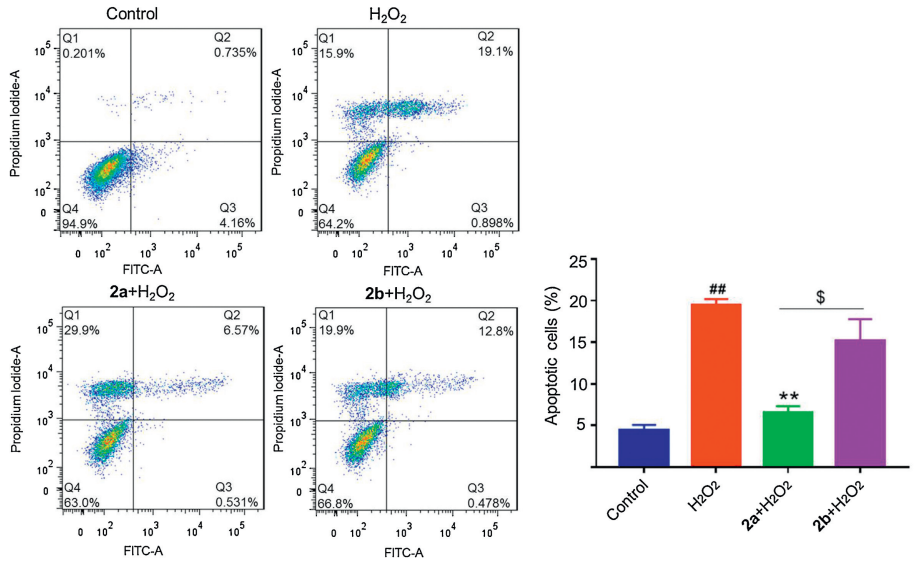

Fig. 7. Effects of enantiomers **2a** and **2b** on the apoptosis ratio in H_2O_2-treated SH-SY5Y cells. The cells were pretreated with enantiomers **2a** and **2b** and then incubated with H_2O_2 for 4 h. Flow cytometry was used to examine the apoptotic ratio after Annexin V-FITC/PI staining. The percentage of apoptotic cells was calculated in the right. $^{##}P < 0.01$ *vs.* the control group. $^{**}P < 0.01$ *vs.* the H_2O_2-treated group. $^{\$}P < 0.05$ was considered statistically significant when compared to its enantiomer.

the most potent neuroprotective activity, the cell viability of which at 25 μmol/L was 79.79%, much more potent than the positive control Trolox (63.1% at 25 μmol/L) and its enantiomer **2b** had a weaker protective effect at a certain concentration. These results highlighted the fact that the absolute configurations of enantiomers possess remarkable influences on their neuroprotective activities.

Moreover, in order to further investigate the apoptosis-inhibiting effects of enantiomers **2a**/**2b** in SH-SY5Y cells, Annexin V-FITC/PI staining using flow cytometry was applied to quantify the number of the apoptotic cells (Fig. 7). It can be seen that significant apoptosis occurred in model group and the apoptosis ratio reached to 20.00% compared to the control group (4.90%). Then, pretreatment of compound **2a** (25 μmol/L) could decrease the apoptosis ratio to 7.10%, while **2b** had no obvious effect on the apoptotic ratio in H_2O_2-treated cells. Consistent with the MTT results, these results indicated that **2a** showed stronger neuroprotective effect compared with its enantiomer **2b**. Taken together, compound **2a** exerted neuroprotective effect against H_2O_2-induced SH-SY5Y cellular damage by inhibiting apoptosis.

In summary, two pairs of rare 8,9′-epoxy-type norlignan enantiomers (**1a**/**1b** and **2a**/**2b**) featuring a γ-butyrolactone ring, were isolated from the fruit of *Crataegus pinnatifida*. Their structures as well as the absolute configurations of four compounds were established *via* extensive spectroscopic analyses and quantum chemical calculations. This is the first report of the configurational assignment of 8,9′-epoxy-type norlignans with the flexible skeleton by quantum chemical calculations. In addition, all the isolates were evaluated for their neuroprotective effects against H_2O_2-induced oxidative injury in SH-SY5Y cells. The results showed that compound **2a** displayed the most potent neuroprotective effect and the two pairs of enantiomers **1a**/**1b** and **2a**/**2b** displayed diff ;erent effects on neuroprotective activity. Further flow cytometry analysis indicated that **2a** could protect SH-SY5Y cells from oxidative damage through inhibiting cell apoptosis. Overall, this Chinese medicine contains promising candidates for the treatment of neurodegenerative diseases.

Declaration of competing interest

The authors declare that they have no conflicts of interest to this work.

Acknowledgments

This work was supported by Career Development Support Plan for Young and Middle-aged Teachers in Shenyang Pharmaceutical University (No. ZQN2018006) and the Project of Innovation Team Foundation (No. LT2015027).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.cclet.2019.09.042.

References

[1] P. Liu, B. Yang, H. Kallio, Food Chem. 121 (2010) 1188–1197.
[2] P.C. Zhang, S.X. Xu, Chin. Chem. Lett. 13 (2002) 337–340.
[3] Q. Chang, Z. Zhong, M.S.S. Chow, et al., Food Chem. 98 (2006) 426–430.
[4] Q. Chang, Z. Zhong, F. Harrison, et al., J. Clin. Pharmacol. 42 (2002) 605–612.
[5] J. Wu, W. Peng, R. Qin, et al., Molecules 19 (2014) 1685–1712.
[6] N. Nikolov, O. Seligmann, H. Wagner, et al., Planta Med. 44 (1982) 50–53.
[7] P.C. Zhang, S.X. Xu, Phytochemistry 57 (2001) 1249–1253.
[8] S.J. Song, L.Z. Li, P.Y. Gao, et al., Food Chem. 129 (2011) 933–939.
[9] X.X. Huang, M. Bai, L. Zhou, et al., J. Agric. Food Chem. 63 (2015) 7252–7260.
[10] K. Wolinski, J.F. Hinton, P. Pulay, J. Am. Chem. Soc. 112 (1990) 8251–8260.
[11] N. Grimblat, M.M. Zanardi, A.M. Sarotti, J. Org. Chem. 80 (2015) 12526–12534.
[12] M.J. Frisch, G.W. Trucks, H.B. Schlegel, et al., Gaussian 09, Revision D.01, Gaussian, Inc., Wallingford CT, 2013.