doi.org/10.36721/PJPS.2022.35.5.REG.1379-1384.1

Influence of steroidal glycosides from *Cynanchum auriculatum* on antioxidant indicators in H_2O_2-damaged PC12 cells

Mi Zhang*, Dong Wang, Chonglian Chen and Baocai Li
Faculty of Life Science and Technology, Kunming University of Science and Technology, Kunming, China

Abstract: The root of *Cynanchum auriculatum* Royle ex Wight is a traditional Chinese medicine, which is rich in C_{21} steroidal glycosides by phytochemistry research. In this study, the antioxidant effect of 27 C_{21} steroidal glycosides isolated from the root of *C. auriculatum* by our group was evaluated using the H_2O_2-treated PC12 cells. As the result, all tested compounds altered the activities of lactate dehydrogenase, superoxide dismutase, catalase and glutathione peroxidase at concentrations as low as 1 μM in H_2O_2-treated PC12 cells. They also decreased the levels of intracellular reactive oxygen species and Ca^{2+}. Further, the correlation between their structural features described by molecular descriptors and the indicators of bioactivity was analyzed by partial least squares analysis, displaying those six bio-indicators were positive correlated with 13 molecular descriptors and providing some guidance for further study of relationships between steroid structure and antioxidant activity.

Keywords: C_{21} steroidal glycosides; *Cynanchum auriculatum*; antioxidant enzyme; structure–activity relationship; PC12 cell.

INTRODUCTION

Cynanchum is a genus belonging to the Apocynaceae family. This genus includes approximately 200 species of plant that are distributed all around the world. Among these species, 53 plants are grown in China and many of them have been used as Chinese folk medicines (He *et al*., 2015). Previous phytochemical investigations have shown that *Cynanchum* is rich in C_{21} steroids and their glycosides, along with some alkaloids, flavonoids, terpenoids, benzene derivatives and fatty acids. Many of these components, especially the C_{21} steroids and their glycosides, display a variety of effects on biological systems, including immunity enhancement, antitumor, antioxidant and anti-inflammatory effects and analgesia (He *et al*., 2015; Liu *et al*., 2003; Pei *et al*., 1987; Wu and Zhou, 2006).

One particular plant in this genus, the root of *Cynanchum auriculatum* Royle ex Wight, is a traditional Chinese folk medicine that has been used to treat gastric ulcers, dysentery, neurasthenia and other conditions (Jiang and Li, 1977). In order to identify additional bioactive natural products from this plant, we have long carried out systematic chemical research on it and more than forty C_{21} steroidal glycosides have been isolated (Qian *et al*., 2017; Rao *et al*., 2014 and 2015; Zhang *et al*., 2015). Bioassays that we have performed have showed that multiple steroidal glycosides have clear effects on enhancing the viability of H_2O_2-damaged PC12 cells (Qian *et al*., 2017). Based on these early experiments, here, we further investigated the influence of 27 steroidal glycosides on six antioxidant indicators in H_2O_2-treated PC12 cells: lactate dehydrogenase (LDH), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), reactive oxygen species (ROS) and $[Ca^{2+}]_i$. We also explored structure-function relationships of the steroidal glycosides by partial least squares analyses.

**Corresponding author:* e-mail: mizhangkmust@126.com

MATERIALS AND METHODS

Compounds

The 27 steroidal glycosides were obtained from the root of *C. auriculatum* as described in our previous investigation (Qian *et al*., 2017). These compounds were identified as saccatol D (1), saccatol E (2), saccatol F (3), saccatol G (4), saccatol H (5), saccatol I (6), 20-*O*-acetyl-12-*O*-cinnamoyl-3-*O*-*β*-D-digitoxopyranosyl-8,14-secosarcostin-8,14-dione (7), saccatol J (8), cynsaccatol I (9), cynsaccatol J (10), cynsaccatol K (11), cynsaccatol L (12), cynsaccatol M (13), wilfoside A (14), cynsaccatol N (15), cynsaccatol O (16), gagaminin (17), cynsaccatol P (18), cynsaccatol R (19), sarcostin 3-O-*β*-D-oleandropyranosyl-(1→4)-*β*-D-oleandropyranosyl-(1→4)-*β*-D-cymaropyranoside (20), 12-O-benzoylsarcostin3-O-*β*-D-oleandropyranosyl-(1→4)-*β*-D-oleandropyranosyl-(1→4)-*β*-D-digitoxopyranosyl (21), cynsaccatol S (22), cynsaccatol T (23), cynsaccatol U (24), cynsaccatol V (25), cynsaccatol W (26) and caudatin 3-*O*-*β*-D-digitoxopyranoside (27). Each compound was dissolved in dimethyl sulfoxide (DMSO) to prepare a mother liquor at a concentration of 10 mM and was further diluted in cell culture medium to obtain test samples at the dosages of 1, 5 and 10 μM (fig. 1).

Cell Culture and Reagents

PC12 cells were obtained from Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, China. They were cultured in high glucose Dulbecco's modified Eagle medium (DMEM; Gibco and Inc.).

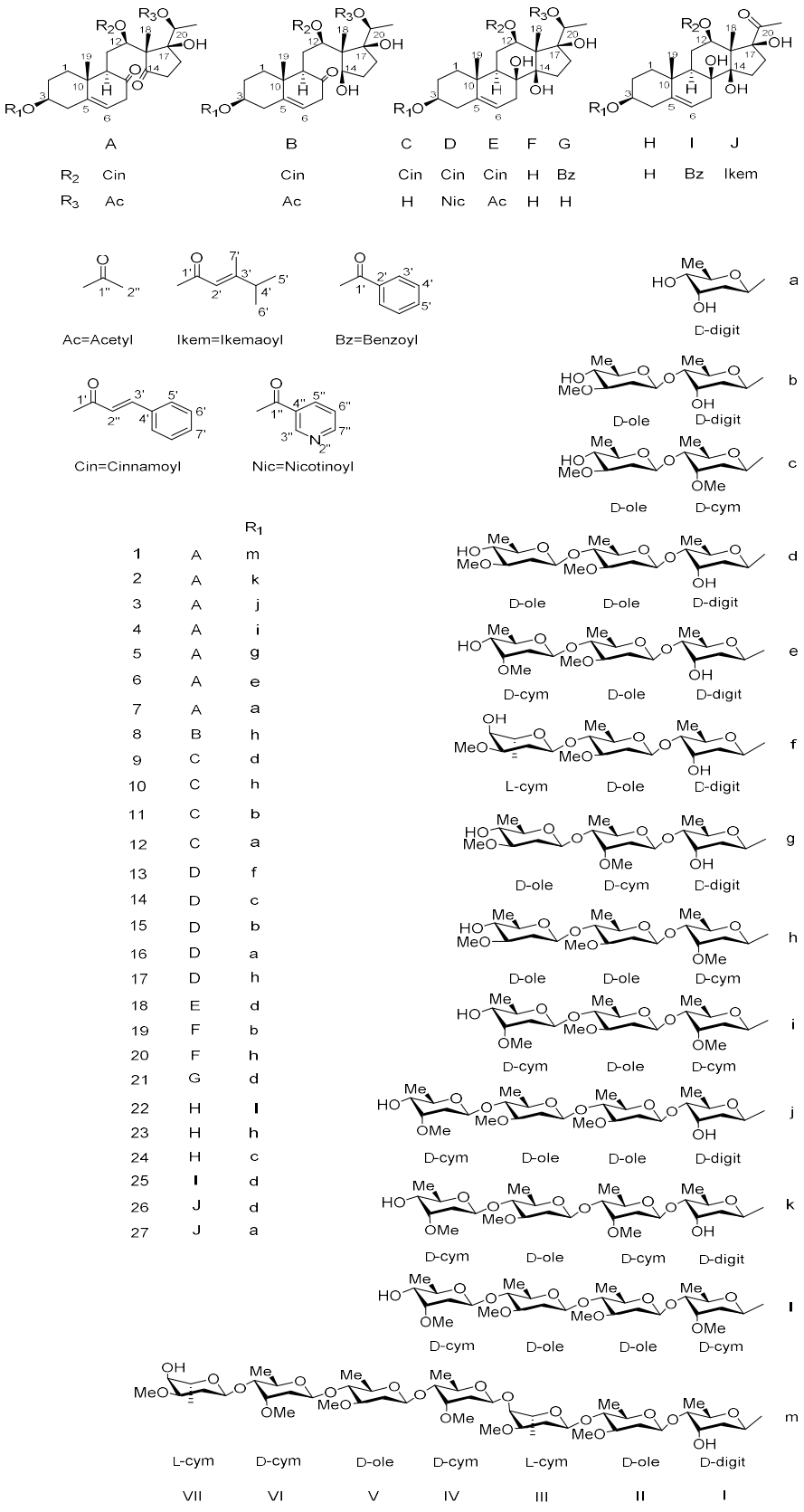

Fig. 1: The structures of C_{21} steroidal glycosides (1-27).

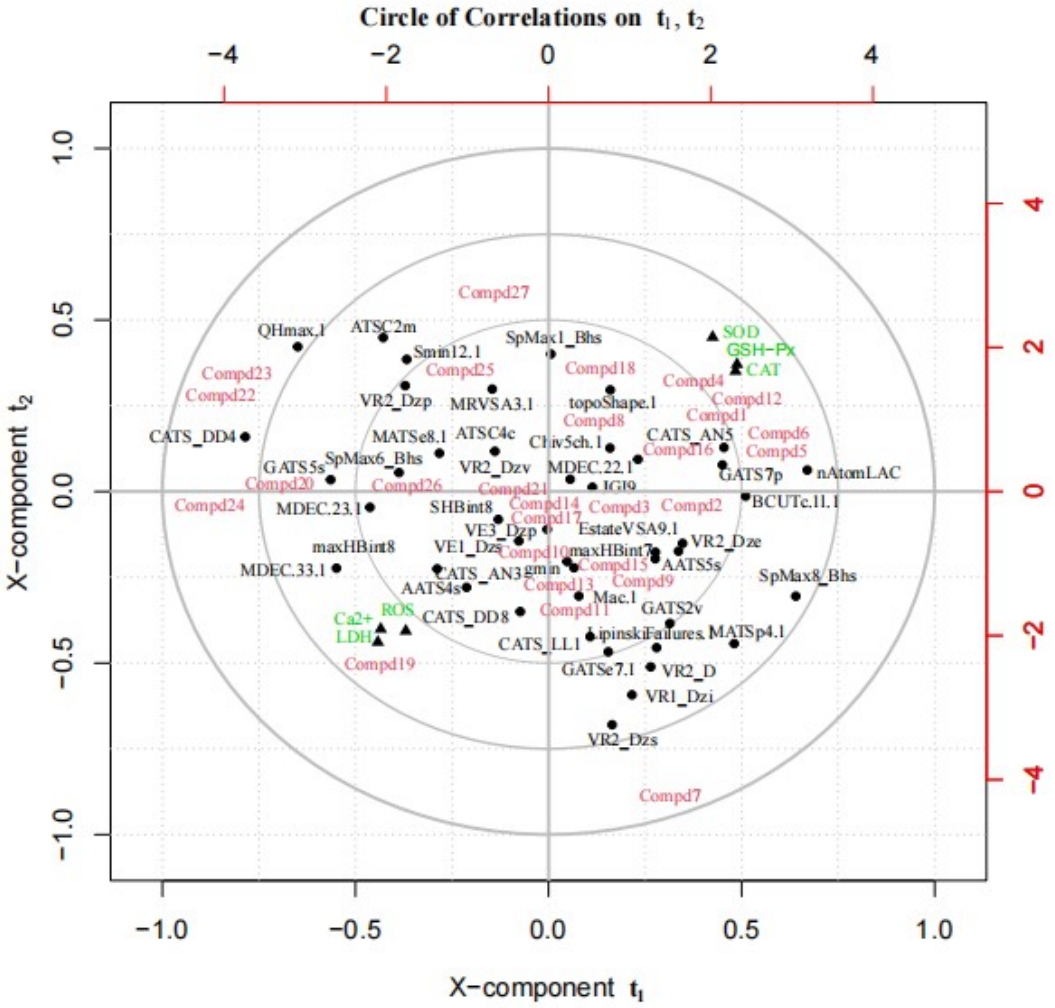

Fig. 2: Relationship between bio-indexes and 44 selected molecular descriptors of C_{21} steroidal glycosides (1-27).

Table 1: Effects of compounds on LDH induced by H2O2 in PC12 cells

No.		1 μM	5 μM	10 μM	No.	1 μM	5 μM	10 μM
Control	35.10±1.50				13	53.55±2.36***	57.23±1.26**	52.70±2.05***
H_2O_2	70.60±1.29$^{###}$				14	58.44±1.52**	53.69±0.90***	50.23±1.78***
Vit E		43.81±1.23***			15	47.07±1.76***	50.06±1.20***	44.87±1.88***
1		37.34±1.11***	35.76±1.15***	36.15±1.39***	16	50.09±2.15***	37.46±0.90***	33.25±1.56***
2		47.48±0.90***	38.69±1.04***	40.09±1.34***	17	65.08±2.02*	63.04±1.59*	61.88±1.75*
3		58.08±0.97**	53.54±0.93***	49.41±1.66***	18	39.27±1.90***	37.15±1.68***	36.89±2.33***
4		36.22±1.41***	39.89±1.41***	38.02±1.33***	19	61.84±2.08*	57.28±1.00**	54.06±2.16**
5		45.26±1.03***	48.17±1.25***	55.84±2.01**	20	41.30±2.01***	37.96±0.99***	37.08±1.83***
6		39.86±2.18***	46.97±1.67***	50.73±1.57***	21	56.78±1.34**	58.32±0.74**	46.08±1.60***
7		57.86±1.80**	59.72±1.22**	61.82±1.41*	22	49.97±1.51***	55.55±2.91**	57.02±1.64**
8		47.36±1.35***	42.08±1.03***	42.22±1.49***	23	63.44±0.66*	54.94±1.61***	60.17±1.67*
9		42.58±1.31***	39.03±1.46***	41.36±0.94***	24	70.53±1.15	57.76±1.68**	40.83±1.21***
10		58.78±1.23**	53.98±1.52***	48.27±0.97***	25	32.12±1.86***	30.68±1.33***	47.25±1.39***
11		45.26±1.61***	41.72±1.11***	37.95±1.55***	26	59.77±1.49**	65.39±1.49	68.12±2.08
12		38.25±1.37**	41.86±1.46***	44.54±1.93***	27	57.92±0.98**	45.87±1.36***	40.79±1.57***

Data were presented as mean ± SD (n = 3). *p<0.05, **p<0.01 ***p<0.001 vs. H_2O_2 group or $^{###}$p<0.001 vs. control. Cells were incubated with 400 μM H_2O_2, and 1, 5, 10 μM of compounds for 24 h. 1 μM Vit E as the positive control.

Table 2: Effects of compounds on SOD induced by H_2O_2 in PC12 cells

No.		1 μM	5 μM	10 μM	No.	1 μM	5 μM	10 μM
control	100				13	71.85±1.03***	68.05±1.30***	70.65±0.99***
H_2O_2	40.61±1.33###				14	65.25±1.83***	67.22±1.35***	70.78±1.52***
Vit E		77.84±0.38***			15	78.99±1.37***	76.83±1.74***	83.56±1.12***
1		80.78±1.43***	80.15±1.93***	83.15±0.90***	16	83.02±2.08***	86.34±2.15***	88.78±1.22***
2		75.22±1.52***	82.98±1.52***	84.01±0.78***	17	67.62±1.33***	65.36±1.88***	65.42±1.39***
3		68.82±2.28***	72.54±1.65***	81.04±1.75***	18	83.34±1.49***	87.04±1.86***	83.71±1.21***
4		83.69±1.83***	84.98±1.73***	84.60±2.22***	19	60.76±1.45***	63.96±1.26***	66.77±1.46***
5		85.10±1.10***	83.37±1.57***	74.20±2.36***	20	82.91±1.14***	86.80±1.41***	87.68±1.61***
6		84.85±2.58***	80.19±1.63***	77.24±1.80***	21	76.02±1.70***	72.24±1.85***	71.71±1.29***
7		68.65±1.58***	68.80±2.68***	64.79±2.28***	22	81.05±1.91***	75.82±1.06***	71.79±1.42***
8		76.18±0.93***	83.73±1.44***	81.66±0.81***	23	50.17±2.25*	63.65±1.92***	56.63±1.77***
9		74.79±2.34***	85.09±1.70***	80.72±0.47***	24	55.79±2.77**	76.42±2.41***	81.56±1.30***
10		61.62±1.76***	58.05±1.49***	68.20±1.76***	25	82.08±1.73***	82.06±2.05***	74.22±0.87***
11		75.45±1.93***	77.93±1.52***	81.68±1.59***	26	72.64±1.60***	64.02±2.88***	61.18±2.24***
12		84.12±1.92***	81.44±2.39***	78.38±1.11***	27	70.83±1.96***	81.61±1.37***	84.95±1.68***

Table 3: Effects of compounds on CAT induced by H_2O_2 in PC12 cells

No.		1 μM	5 μM	10 μM	No.	1 μM	5 μM	10 μM
control	100				13	58.14±1.20***	53.11±1.66**	60.08±1.66***
H_2O_2	42.60±2.00###				14	58.01±1.61***	57.66±2.65***	61.81±1.50***
Vit E		65.11±1.49***			15	63.64±1.78***	60.76±1.66***	67.11±1.51***
1		74.23±1.16***	77.93±1.68***	78.33±2.17***	16	63.00±1.74***	78.44±1.59***	84.77±1.00***
2		61.26±1.26***	77.84±1.99***	77.04±1.29***	17	49.16±1.69*	48.70±2.46*	47.05±1.57
3		54.14±1.91**	56.75±1.40***	57.38±1.50***	18	76.88±1.48***	80.63±0.89***	82.33±1.08***
4		74.48±1.47***	72.58±1.85***	73.81±1.18***	19	50.09±1.34**	52.02±1.73***	53.30±2.91**
5		74.96±1.30***	76.66±1.12***	62.02±3.22***	20	77.91±1.09***	80.49±1.35***	80.32±2.60***
6		80.43±1.43***	76.29±1.80***	65.43±2.07***	21	57.76±1.45***	56.92±1.78***	53.56±0.86**
7		60.85±1.17***	61.72±1.53***	56.48±1.02***	22	51.90±1.55**	50.73±1.52**	48.67±0.59*
8		62.63±0.75***	75.37±2.90***	76.80±1.71***	23	44.03±1.72	50.56±0.69**	47.85±0.97
9		63.90±1.35***	77.46±1.47***	74.37±1.98***	24	40.71±2.00	55.11±1.53***	74.86±1.65***
10		51.58±0.86**	52.22±0.82**	56.40±2.23***	25	81.70±1.16***	82.56±1.78***	74.77±1.22***
11		60.23±1.69***	60.30±2.29***	75.22±1.09***	26	51.85±0.59**	49.95±1.24**	45.17±2.00
12		63.24±2.59***	57.61±2.27***	56.60±1.22***	27	56.63±1.60***	66.02±1.90***	73.70±1.45***

Table 4: Effects of compounds on GSH induced by H_2O_2 in PC12 cells

No.		1 μM	5 μM	10 μM	No.	1 μM	5 μM	10 μM
control	100				13	71.86±1.38***	70.75±1.82***	71.70±1.09***
H_2O_2	55.85±1.57###				14	71.41±0.93***	72.56±0.72***	78.74±1.45***
Vit E		76.40±1.49***			15	81.92±1.48***	79.73±0.43***	83.54±1.51***
1		81.43±0.86***	82.93±2.19***	84.05±2.35***	16	79.40±1.06***	84.73±0.87***	88.98±1.74***
2		76.85±0.90***	84.80±1.34***	84.27±1.32***	17	64.06±1.25*	67.87±1.67**	66.58±1.54*
3		68.35±1.70**	67.57±1.87**	69.42±1.16**	18	83.40±2.27***	87.19±1.57***	85.32±2.14***
4		84.64±1.00***	83.98±1.44***	82.71±1.49***	19	64.10±1.64**	67.78±1.64**	67.34±1.83**
5		82.48±2.10***	82.53±1.13***	78.74±0.76***	20	81.86±1.86***	87.19±1.43***	85.69±2.15**
6		87.44±1.38***	78.77±1.73***	75.16±1.30***	21	78.20±2.03***	77.27±1.89***	76.08±1.14***
7		70.63±1.00**	70.35±1.84***	69.36±0.86**	22	77.04±1.69***	70.73±1.34***	69.31±0.99**
8		77.98±1.41***	85.08±2.34***	83.65±1.14***	23	60.39±1.64*	66.86±1.59**	62.66±0.87*
9		80.87±1.62***	84.30±1.61***	82.74±1.54***	24	53.29±1.96	71.99±2.04***	80.98±1.46***
10		70.09±1.34**	71.90±1.18***	77.68±2.08***	25	85.64±2.33***	84.53±2.22***	77.20±1.57***
11		80.09±1.45***	81.69±0.77***	80.04±5.76***	26	71.18±1.44***	60.82±2.16*	60.45±0.69
12		82.26±1.84***	81.18±1.50***	78.69±1.44***	27	66.61±1.33**	79.97±1.64***	83.05±1.69***

Data were presented as mean ± SD (n = 3). *p< 0.05, **p< 0.01 ***p< 0.001 vs. H_2O_2 group or ###p<0.001 vs. control. Cells were incubated with 400 μM H_2O_2, and 1, 5, 10 μM of compounds for 24 h. 1 μM Vit E as the positive control.

Supplemented with 10% fetal bovine serum (FBS; Solar Bioscience & Technology Co., Ltd., Beijing, China) and 1% penicillin and streptomycin at 37°C and 5% CO_2.

Analysis of LDH, SOD, CAT and GSH

PC12 cells were inoculated into 96-well plates at densities of 10000 cells/well and were incubated for 24h. The cells

glycosides resulted in enhancement of the activities of SOD, CAT and GSH (tables 2-4), consistent with a model in which these compounds have protective effects against oxidative damage by regulating the activities of antioxidant enzymes *in vitro*.

Table 5: Effects of compounds on ROS induced by H_2O_2 in PC12 cells

No.		1 μM	5 μM	10 μM	No.	1 μM	5 μM	10 μM
control	100				13	143.15±2.17***	146.16±1.77***	145.64±2.22***
H_2O_2	196.75±2.04$^{###}$				14	153.95±1.84***	152.19±2.24***	146.60±2.14***
Vit E		135.08±1.66***			15	136.67±1.10***	140.05±1.39***	135.50±0.97***
1		133.37±1.69***	129.28±1.14***	128.61±1.37***	16	143.86±1.62***	128.84±1.54***	122.45±0.82***
2		145.17±1.30***	129.78±2.68***	133.94±1.80***	17	158.72±1.35***	156.77±1.69***	152.86±1.82***
3		156.71±0.94***	152.94±1.61***	146.94±1.79***	18	133.99±1.08***	126.85±1.74***	123.77±1.91***
4		130.29±1.53***	135.02±1.79***	133.21±2.31***	19	161.89±1.70**	150.40±2.21***	150.12±1.84***
5		136.60±1.30***	138.67±1.47***	143.80±1.98***	20	135.94±1.38***	128.94±1.83***	128.03±1.62***
6		127.47±0.94***	137.15±1.22***	144.12±1.81***	21	150.34±2.38***	154.93±1.29***	159.48±1.39***
7		154.09±1.68***	151.31±1.41***	157.15±3.16***	22	142.94±1.15***	146.98±1.63***	150.72±0.70***
8		146.27±2.15***	130.60±2.13***	132.17±1.92***	23	178.03±2.09	152.41±2.20***	157.07±1.93***
9		137.43±0.77***	129.02±1.33***	133.89±1.64***	24	178.61±2.21	148.67±1.99***	134.22±1.48***
10		161.58±1.13***	157.05±1.71***	147.60±0.73***	25	126.73±1.78***	131.68±0.71***	137.40±0.71***
11		136.76±1.35***	133.87±1.46***	131.32±0.98***	26	150.18±1.85***	157.88±1.81***	164.73±1.50**
12		135.32±1.96***	141.98±1.29***	146.55±0.81***	27	150.06±2.20***	135.97±1.43***	132.97±2.37***

Table 6: Effects of compounds on $[Ca^{2+}]_i$ induced by H_2O_2 in PC12 cells

No.		1 μM	5 μM	10 μM	No.	1 μM	5 μM	10 μM
control	100				13	157.33±0.64***	160.11±1.20***	158.12±1.63***
H_2O_2	178.62±0.89$^{###}$				14	153.41±1.90***	149.53±1.23***	152.27±2.11***
Vit E		144.03±1.68***			15	141.41±0.97***	145.26±1.57***	138.91±1.51***
1		139.45±1.40***	133.47±0.95***	131.86±1.36***	16	142.84±1.09***	130.89±1.20***	126.35±0.95***
2		152.72±1.78***	134.35±1.24***	136.99±1.38***	17	162.12±1.63***	165.44±1.85***	166.03±1.59**
3		158.66±1.46***	157.92±3.19***	150.78±1.87***	18	138.16±1.39***	132.64±2.54***	132.09±1.74***
4		136.07±1.74***	139.97±2.53***	136.88±0.96***	19	166.83±1.33***	163.47±2.73***	164.94±1.50***
5		139.87±1.64***	142.17±1.14***	153.03±2.64***	20	141.29±1.87***	135.88±2.37***	136.08±2.53***
6		131.04±2.24***	142.71±1.79***	150.82±1.45***	21	155.54±0.98***	159.68±0.88***	162.26±1.71***
7		157.52±0.59***	156.34±1.10***	160.76±1.39***	22	149.34±1.79***	156.98±1.58***	160.41±1.59***
8		154.15±1.23***	138.55±1.29***	141.41±0.77***	23	172.01±1.62*	157.76±2.33***	163.19±1.47***
9		146.49±1.52***	136.38±1.72***	138.23±1.48***	24	175.40±1.47	153.74±1.29***	136.95±1.57***
10		165.11±1.84***	168.52±2.31*	151.95±2.35***	25	129.00±1.65***	130.49±2.12***	156.24±2.63***
11		150.00±1.71***	145.73±1.45***	140.14±1.79***	26	156.85±1.78***	165.87±1.29***	168.82±1.09*
12		141.99±1.45***	148.53±2.23***	151.81±1.41***	27	155.15±1.53***	148.38±2.35***	142.25±1.82***

Data were presented as mean ± SD (n = 3). *p< 0.05, **p< 0.01 ***p< 0.001 vs. H_2O_2 group or $^{###}$p < 0.001 vs. control. Cells were incubated with 400 μM H_2O_2 and 1, 5, 10 μM of compounds for 24 h. 1 μM Vit E as the positive control.

RESULTS

The influence of compounds on the levels of ROS and $[Ca^{2+}]_i$

The influence of compounds on the levels of ROS and intracellular Ca^{2+} was determined by fluorescence labeling assays. In H_2O_2-treated cells that were not exposed to steroidal glycosides, the levels of ROS and Ca^{2+} were significantly increased as compared to cells not treated with H_2O_2 (tables 5 and 6). However, treatment with each of the 27 steroidal glycoside compounds was found to decrease levels of intracellular ROS and Ca^{2+}, further supporting the anti-oxidant effect of these compounds.

Correlations of bio-indicators and molecular descriptors

In order to identify structure-function relationships of the steroidal glycosides, 44 molecular descriptors were selected and used to characterize the physical, chemical and structural information of compounds 1 through 27. PLS analysis was used to determine correlations of the six tested bio-indicators with these 44 selected molecular descriptors. According to this analysis (fig. 2), positive correlations were observed between biological impacts on SOD, CAT and GSH and the molecular descriptors topoShape 1, Chiv5ch.1, MDEC.22.1, JGI 9, GATS7p, nAtomLAC and CATS_AN5. Similarly, positive correlations were observed between LDH, ROS and $[Ca^{2+}]_i$ and CATS_DD8, AATS4s, CATS_AN3, VE1_Dzs, VE3_Dzp, SHBint8.

DISCUSSION

The model of H_2O_2 induced PC12 cells has been widely used in screening neuroprotective materials or antioxidants in vitro associating oxidative stress. Based on the traditional effects and previous investigation of *C. auriculatum*, it indicated this plant contains potentially active ingredients on the aspects of neuroprotection or antioxidation. Thus, C_{21} steroidal glycosides, as the major

constituents in the roots of *C. auriculatum*, were evaluated their antioxidative effects using H_2O_2-treated PC12 cell model. On the other hand, LDH, SOD, CAT and GSH are major indexes reflecting the level of oxidative stress in the cell. The results in this study showed the 27 C_{21} steroidal glycosides from the roots of *C. auriculatum* could decease LDH activity, increase SOD, CAT and GSH activities and reduce the levels of intracellular ROS and Ca^{2+}, which supported that C_{21} steroidal glycosides are the ingredients with antioxidation relating to the traditional effects of *C. auriculatum*. In addition, the correlation between 44 molecular descriptors characterizing 27 compounds and six tested bio-indicators was analyzed by PLS analysis, which provide information to guide further investigation into the relationships between molecular features of steroidal glycosides and several biological indicators related to oxidative stress.

CONCLUSION

C_{21} steroidal glycosides 1 through 27 from the roots of *C. auriculatum* were associated with protective effects on H_2O_2-treated PC12 cells by increasing SOD, CAT and GSH activities and deceasing LDH activity and levels of intracellular ROS and Ca^{2+}. PLS analyses showed that the SOD, CAT and GSH indicators were positively correlated with 7 molecular descriptors, while the LDH, ROS and Ca^{2+} indicators are positively correlated with 6 molecular descriptors.

REFERENCES

He D, Yang S, Luo H, Qin X, Ma Y and Zhang X (2015). Progress in steroid constituents of *Cynanchum* and pharmacological effects of the steroids. *J. Southwest Univ. Nat. (Natural Science Edition)*, **41**(4): 423.

Jiang Y and Li BT (1977). Chinese Flora (Zhongguo Zhiwu Zhi), *Beijing: Science Press*, **63**: 322.

Liu WW, Zhang ZH, Wu LY, Dai YJ and Wu QZ (2003). Advance on chemical constituents and pharmacological activities of *Cynanchum* plants. *J. Chin. Med. Mat.*, **26**(3): 216.

Pei YQ, Dai J, Cheng WX and Lu ZQ (1987). Study on the pharmacological effect of three kinds in *Cynanchum* L. (Asclepiadaceae). *J. Beijing Med. Univ.*, **19**(1): 29.

Qian X, Li B, Li P, Wang D, Dai W and Zhang M (2017). C_{21} steroidal glycosides from *Cynanchum auriculatum* and their neuroprotective effects against H_2O_2-induced damage in PC12 cells. *Phytochemistry*, **14**: 1.

Rao L, Zhang M, Li P and Li B (2014). Studies on chemical constituents of *Cynanchum saccatum*. *J. Kunming Univ. Sci. Technol. (Natural Science Edition)*, **39**(5): 82.

Rao L, Zhang M, Xiang C, Li B and Li P (2015) Steroid glycosides and phenols from the roots of *Cynanchum saccatum*. *Phytochemistry Lett.*, **11**: 49.

Wu Y and Zhou HL (2006). Advance on chemical constituents of *Cynanchum* plants. *Central South Pharm.*, **4**(5): 371.

Zhang M, Rao L, Xiang C, Li B and Li P (2015). C_{21} steroidal glycosides from the roots of *Cynanchum saccatum*. *Steroids*, **101**: 28.