Stimulating effect of a new triterpene derived from *Anoectochilus elwesii* on glucose uptake in insulin-resistant human HepG2 cells

Jinyan Cai\textsuperscript{a}, Lin Zhao\textsuperscript{b}, En Zhu\textsuperscript{a} and Jiao Guo\textsuperscript{c,*}

\textsuperscript{a}School of Pharmacy, Guangdong Pharmaceutical University, Guangzhou 510006, P.R. China; \textsuperscript{b}School of Life Science and Bio-pharmaceutical, Guangdong Pharmaceutical University, Guangzhou 510006, P.R. China; \textsuperscript{c}Key Unit of Modulating Liver to Treat Hyperlipemia SATCM (State Administration of Traditional Chinese Medicine), SATCM Level 3 Lab of Lipid Metabolism, Guangdong TCM Key Laboratory for Metabolic Diseases, Institute of Chinese Medicinal Sciences, Guangdong Pharmaceutical University, Guangzhou 510006, P.R. China

(Received 4 April 2014; final version received 1 June 2014)

A new triterpene (1), 3-\(\beta\)-O-olean-11,13 (18)-diene-23,28-dioic acid, together with five known compounds (2–6), was isolated from *Anoectochilus elwesii* and their structures were elucidated by extensive spectroscopic methods and comparison with the literature data. Compound 1 was the first example of highly oxygenated triterpene obtained from *Anoectochilus* genus. The isolated compounds were evaluated on insulin-resistant human HepG2 cells for stimulating glucose uptake activity and the new compound displayed highly potent effect on the stimulation of glucose uptake in human HepG2 cells.

**Keywords:** *Anoectochilus elwesii*; triterpene; insulin-resistant human HepG2 cells

1. **Introduction**

*Anoectochilus elwesii* (Clarke ex Hook. f.) King et Pantl. has been used in Chinese folk medicine in diabetes and nephropathy ailments (Anonymous, 1999). Pharmacological evaluation of the antihyperglycemic activity of the ethanol extract of this plant in rats confirmed the folk information (Cai et al., 2014). A previous phytochemical study on *A. elwesii* has revealed the occurrence of sterols, triterpenes and flavonoids (Zhu et al., 2013). As part of our ongoing investigation for the chemistry of *A. elwesii* growing in Yunnan Province, the south-west of China, here we report on the isolation and characterisation from the herbs of *A. elwesii* of a novel triterpene, 3-\(\beta\)-O-olean-11,13 (18)-diene-23,28-dioic acid (Figure 1).

2. **Results and discussion**

Compound 1 was obtained as a white amorphous powder. It had a molecular formula of \(C_{30}H_{44}O_5\) determined from its pseudo-molecular ion peak at \(m/z\) [M – H] \(^{-}\): 483.3121 (calculated 484.3177) in HR-ESI-MS combined with its \(^{13}\)C NMR data. In the \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) (ppm) spectra, it showed six methyl proton signals at \(\delta\) 1.16 (3H, s), 0.92 (3H, s), 0.98 (3H, s), 1.03 (3H, s) and 0.82 (6H, s). Six \(sp^3\) carbons were assigned to these methyl groups at \(\delta\) 17.54, 18.94, 17.60, 20.46, 32.97 and 24.69. The \(^1\)H NMR spectrum exhibited two olefinic proton signals at \(\delta\) 5.73 (1H, d, \(J = 10.5\) Hz) and 6.69 (1H, dd, \(J = 10.5, 2.5\) Hz), while the \(^{13}\)C NMR spectrum showed four olefinic carbons at \(\delta\) 127.75, 126.63, 137.97 and 133.43, respectively. Two carboxyl carbons and a methine carbon bearing oxygen were found at \(\delta\) 181.26 and 201.45. The \(^1\)H NMR spectrum of 1 showed a signal at \(\delta\) 4.23 (1H, d, \(J = 10.5\) Hz). The \(^{13}\)C NMR spectrum of 1 showed a signal at \(\delta\) 151.54.

*Corresponding author. Email: gyguoyz@163.com*
(assigned to C-28), 180.49 (C-23) and 73.95 (C-3), respectively. The signal at \( \delta_H \) 3.75 (t) was assigned to the methine protons at C-3. Detailed analysis of the spectral data indicated that heteroannular conjugated diene carbons were at C-11 and C-13 (18), which was further confirmed to be olean-11,13 (18)-diene-23,28-dioic acid by comparison with the aglycone moiety of the literature data. So far, there are two saponins which are reported to be its bisdesmosidic glycosides, glycosylated at C-3 and C-28: 3-\( \beta \)-D-glucopyranosyl-olean-11,13 (18)-diene-23,28-dioic acid and 3-\( \beta \)-D-xylopyranosyl-olean-11,13 (18)-diene-23,28-dioic acid-28-\( \beta \)-D-glucopyranosyl-(1 \( \rightarrow \) 3)-\( \beta \)-D-glucopyranosyl-(1 \( \rightarrow \) 6)-\( \beta \)-D-glucopyranoside (Koike et al. 1999). After extensive NMR analysis and literature survey, this structure was established to be 3-\( \beta \)-O-olean-11,13 (18)-diene-23,28-dioic acid, a new triterpenoid sapogenin. The differences of the chemical shifts of C-3 and C-28 between compound 1 and the literature (Koike et al. 1999; Chen et al. 2010) revealed the glycosidation shifts.

Five other known compounds were isolated from the leaves of A. elwesii and their structures were established on the basis of comparison with the published data as sorghumol (2) (Han et al. 2008), epifriedelanol (3) (Yuan & Sun 1999), oleanoic acid (4) (Yuan & Yi 2005), (6R,9S)-9-hydroxy-megastigma-4, 7-dien-3-one-9-\( \beta \)-D-glucoside (5) Zhao et al. 2012) and dibutylphthalate (6) (Liu et al. 2007). All compounds were first obtained from this species of A. elwesii.

Metabolic syndrome is a complex and chronic disease associated with adverse functioning of many organs. Among the metabolic syndrome components, hyperglycemia caused by insulin resistance is the main contributor to the associated disorders (Zaid et al. 2008). Thus, regulating glucose uptake is important for the treatment of diabetes and metabolic syndrome. A. elwesii is one of the commonly used Chinese folk medicinal herbs and has been reported to have several pharmacological effects (Zhang & Li 2010; Cai et al. 2012). However, the pharmacological effects of individual components are largely unknown. Hence, in this study, we obtained six compounds from A. elwesii and examined their effects on glucose uptake in insulin-resistant HepG2 cells under high glucose conditions. We found that, of the six compounds, the novel compound showed the most potent stimulatory effects on glucose uptake. It significantly increased glucose uptake in a good dose-dependent manner, which increased more than 50% over basal level during 20–100 \( \mu \)M. Metformin brought about an increase in glucose uptake, and was utilised as a positive control, which the glucose uptake increased about 50% over the basal level in response to metformin (Table 1). Compounds 2–6 also showed moderate effects on the stimulation of glucose uptake. Meanwhile, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) tests revealed the tested six compounds did not show any cellular toxicity up to 100 \( \mu \)M concentration.

3. Experimental

3.1. Plant material

The herbs of A. elwesii were collected in September of 2010 from Yunnan province, south-west China. The plant was authenticated by Hongyan Ma, School of Traditional Chinese Medicine,
Table 1. Glucose consumption (mmol L\(^{-1}\)) of the tested samples in different concentrations (μM).

| Concentrations (μM) | Compound 1 | Compound 2 | Compound 3 | Compound 4 | Compound 5 | Compound 6 | Metformin |
|---------------------|------------|------------|------------|------------|------------|------------|-----------|
| 0                   | 2.381 ± 0.267 |           |            |            |            |            |           |
| 10                  | 4.401 ± 0.420 | 3.421 ± 0.356 | 3.488 ± 0.300 | 3.631 ± 0.386 | 3.267 ± 0.272 | 2.126 ± 0.643 | 4.193 ± 0.167 |
| 20                  | 4.464 ± 0.448 | 3.453 ± 0.722 | 3.519 ± 0.451 | 2.830 ± 0.734 | 3.833 ± 0.273 | 3.856 ± 0.209 | 3.991 ± 0.560 |
| 30                  | 4.505 ± 0.593 | 3.512 ± 0.726 | 4.251 ± 0.220 | 3.739 ± 0.491 | 3.702 ± 0.647 | 2.242 ± 0.294 | 4.550 ± 0.511 |
| 50                  | 4.401 ± 0.699 | 3.948 ± 0.560 | 3.926 ± 0.617 | 3.061 ± 0.195 | 4.108 ± 0.348 | 2.350 ± 0.748 | 4.217 ± 0.270 |
| 70                  | 4.965 ± 0.578 | 3.602 ± 0.249 | 3.886 ± 0.549 | 4.424 ± 0.701 | 3.984 ± 0.136 | 2.515 ± 0.342 | 4.816 ± 0.545 |
| 100                 | 4.606 ± 0.529 | 3.821 ± 0.447 | 3.495 ± 0.539 | 3.705 ± 0.547 | 3.939 ± 0.563 | 2.435 ± 0.363 | 4.500 ± 0.290 |
| Normal control      | 3.215 ± 0.433 |           |            |            |            |            |           |
Guangdong Pharmaceutical University. A voucher specimen (2010-XN1002) has been deposited in the herbarium of the School of Pharmacy, Guangdong Pharmaceutical University.

### 3.2. General methods

NMR spectra were recorded on a Bruker DRX-400 NMR spectrometer (Bruker Biospin Gmbh, Rheistetten, Germany) with the solvent residual peaks of dimethyl sulphoxide (DMSO)-d$_6$ at $\delta$H 2.50 and $\delta$C 39.52 as references. High-resolution electrospray ionisation mass spectrometry (HR-ESI-MS) was obtained on a Bruker Bio TOF IIQ mass spectrometer (Bruker Daltonics, Inc., Billerica, MA, USA). Column chromatography was performed over silica gel (100–200 mesh, Qingdao Haiyang Chemical Co., Ltd, Qingdao, China), Develosil ODS (S-75 μm, Nomura Chemical Co., Ltd, Seto, Japan), macroporous resin Diaion HP-20 (Mitsubishi Chemical Corporation, Tokyo, Japan) and Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden). High-performance liquid chromatography was carried out on a Shimadzu LC-6AD liquid chromatograph equipped with a Shimadzu RID-10A refractive index detector (Shimadzu Corp., Kyoto, Japan). YMC-Pack ODS-A columns (S-5 μm, 250 mm × 4.6 mm and 250 mm × 20 mm inner diameter, YMC Co., Ltd, Tokyo, Japan), were used for analytical and preparative purposes, respectively. Thin-layer chromatography was conducted on precoated silica gel GF254 plates (Yantai Jiangyou Silica Gel Development Co., Ltd, Yantai, China) and reversed-phase-18 F254 plates (Merck Japan Ltd, Tokyo, Japan), and spot detection was performed under fluorescent light ($\lambda$ = 254 and 365 nm) and then spraying 10% H$_2$SO$_4$ in EtOH, followed by heating. MTT was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Roswell Park Memorial Institute -1640 medium was purchased from Gibco BRL (Gaithersburg, MD, USA). Foetal calf serum was obtained from Guangzhou Jinan Biomedicine Research and Development Center (Guangzhou, China).

### 3.3. Extraction and isolation

Dry powdered herbs (1.6 kg) of *A. elwesii* were refluxed with 95% (v/v) ethanol for 2 h, and each filtrate was concentrated to dryness in vacuo to render the total EtOH extract, which was suspended in distilled water and partitioned in sequence with petroleum ether (PE), ethyl acetate (EtOAc) and n-BuOH, thus yielding four fractions. The respective yields (%) of the PE fraction, EtOAc fraction, n-BuOH fraction and H$_2$O fraction were 18.05%, 15.90%, 25.38% and 41.67%. The EtOAc fraction (10.90 g) was chromatographed over silica gel (200–300 mesh) using petroleum–EtOAc mixtures of increasing polarity. Repeated chromatography with the same eluent over silica gel (400 mesh) afforded compounds 1–3: 1 (8 mg), 2 (103 mg) and 3 (15 mg). The 50% petroleum–EtOAc part (6.0 g) was chromatographed over silica gel (200–300 mesh) using petroleum–EtOAc mixtures of increasing polarity. Repeated chromatography with the same eluent over silica gel (400 mesh) afforded compounds 1–3: 1 (8 mg), 2 (103 mg) and 3 (15 mg). The 50% petroleum–EtOAc part (6.0 g) was chromatographed over silica gel (200–300 mesh) using petroleum–EtOAc mixtures of increasing polarity. Repeated chromatography with the same eluent over silica gel (400 mesh) afforded compounds 1–3: 1 (8 mg), 2 (103 mg) and 3 (15 mg). The 50% petroleum–EtOAc part (6.0 g) was chromatographed over silica gel (200–300 mesh) using petroleum–EtOAc mixtures of increasing polarity. Repeated chromatography with the same eluent over silica gel (400 mesh) afforded compounds 1–3: 1 (8 mg), 2 (103 mg) and 3 (15 mg).

![](image)

**Olean-11,13 (18)-diene-23, 28-dioic acid** (1) white amorphous powder, m/z [M – H]$^-$: 483.3121 (calculated 484.3177). $^1$H NMR (500 MHz, DMSO-d$_6$, ppm) δ: 1.16 (s, 3H, H-24), 0.92 (s, 3H, H-25), 0.98 (s, 3H, H-26), 1.03 (s, 3H, H-27), 0.82 (s, 6H, H-29, 30), 5.68 (1H, d, J = 10.5 Hz, H-11), 6.69 (1H, dd, J = 10.5, 2.5 Hz, H-12), 3.75 (t, 2H, H-3), 2.21 (s, 2H, H-19). $^{13}$C NMR (125 MHz, DMSO-d$_6$, ppm): 38.22, 26.27, 73.95, 52.38, 51.50, 20.46, 32.93, 41.55, 55.83, 36.85, 127.75, 126.63, 137.97, 42.62, 22.33, 26.09, 45.44, 133.43, 43.53, 33.53, 37.36, 34.06, 180.49, 12.75. 17.54, 18.94, 17.60, 20.46, 181.26, 32.97, 24.69.
3.4. Biological assays

3.4.1. Cell culture
Human HepG2 cells were maintained in DMEM supplemented with 10% foetal bovine serum (FBS), 100 U mL\(^{-1}\) penicillin, 100 \(\mu\)g mL\(^{-1}\) streptomycin, 2 mM L-glutamine (Invitrogen, CA, USA), and kept at 37\(^\circ\)C; in a humidified atmosphere of 5% CO\(_2\) in air. Cells were grown to 70% confluence and then preincubated in serum-free medium for 24 h before treatment.

3.4.2. Insulin-resistant HepG2 cell model
The HepG2 cells were seeded into 96 multi-well plates in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 \(\mu\)g/mL streptomycin. The cells were cultured in a humidified incubator (5% CO\(_2\)) at 37\(^\circ\)C, and were allowed to attach for 24 h. Insulin-resistant cell model was induced according to the previous method (Chen et al. 2006) with a slight modification. In brief, HepG2 cells were incubated with fresh medium containing 1% FBS and 5 \(\times\) 10\(^{-7}\) mol/L bovine insulin for 24 h. Subsequently, the medium was exchanged with medium containing 10\(^{-9}\) mol/L insulin and test compounds, or metformin, and then incubation was conducted for 12 h.

3.4.3. Extracellular glucose in HepG2 cells
After treatment, glucose was assayed in 10 \(\mu\)L of medium by enzymatic methods with diagnostic kits (Nanjing Jiancheng Bioengineering Inst., Nanjing, China). Data were expressed as consumption of extracellular glucose content (mmol L\(^{-1}\)) = [extracellular glucose content (mmol L\(^{-1}\))\(_{0h}\) – extracellular glucose content (mmol L\(^{-1}\))\(_{24h}\)] (Chen et al. 2006).

3.4.4. Cell viability assay
Cells were seeded into 96-well plates at a density of 2 \(\times\) 10\(^3\) cells/well and cultured for 24 h. After incubation of the cells with the isolated compounds 1–6 at different concentrations for 48 h, the cytotoxicity of them was determined by the MTT assay as previously described (Mosmann 1983). Percentage cell viability was calculated based on the absorbance measured relative to the absorbance of control cells exposed to the vehicle alone.

3.4.5. Statistical analysis
Data are presented as the means \(\pm\) SD.

4. Conclusions
In conclusion, our study on isolation of triterpenes and their evaluation for stimulating glucose uptake activities are indicative of the potential of the oxygenated triterpene for improving the cells’ resistant state, which may be further developed to be good proponents for the treatment of diabetes.

**Supplementary material**
Supplementary material relating to this article is available online.

**Acknowledgements**
The authors thank Guangdong Natural Science Foundation (grant number S2013010014771) and National Natural Science Foundation of China (grant number 81001628) for financial support.
Conflict of interest
We declare that we have no conflict of interest.

References
Anonymous in Editorial Committee of the Flore of China of Chinese Academy of Science. 1999. Peking: Science Press; Vol. 17, p. 224.
Cai JY, Zhu E, Wang YN. 2012. Effect of extracts from three different species of Anoectochilus on blood lipid and renal function in diabetic rats induced by high lipid diet plus STZ. Tradit Chin Drug Res Clin Pharm. 23:271–274.
Cai JY, Zhu E, Zhao L, Zhang T. 2014. Hypoglycemic effect of three different extracts from Anoectochilus genus. Herald Med. 33:116–120.
Chen Q, Xia YP, Qiu ZY. 2006. Establishment of insulin resistant HepG2 cell model and pharmacological evaluation of pioglitazone. Chin Pharmacol Bull. 22:248–251.
Chen X, Luo JG, Kong LY. 2010. Two new triterpenoids aponins from Dianthus superbus L. J Asian Nat Prod Res. 12:458–463.
Han MH, Yang XW, Jin YP. 2008. Novel triterpenoid acyl esters and alkaloids from Anoectochilus roxburghii. J Phytochem Anal. 19:438–443.
Koike K, Jia ZH, Nikaido T. 1999. New triterpenoid saponins and sapogenins from Saponaria officinalis. J Nat Prod. 62:1655–1659.
Liu YF, Yang XW, Wu B. 2007. Chemical constituents of the flower buds of Tussilago farfara. J Chin Pharm Sci. 16:288–290.
Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 65:55–63.
Yuan GJ, Yi YK. 2005. Studies on chemical constituents of the roots of Salacia hainanensis. J Chin Med Mater. 28:27–28.
Yuan JZ, Sun QS. 1999. Chemical constituents from leaves of Quercus acutissima Carruth. J Shenyang Pharm Univ. 16:60–62.
Zaid H, Antonescu CN, Randhawa VK, Klip A. 2008. Insulin action on glucose transporters through molecular switches, tracks and tethers. Biochem J. 413:201–215.
Zhang T, Li HC. 2010. Tissue culture and rapid propagation of Anoectochilus elwesii. J Wenshan Univ. 23:143–144.
Zhao M, Chen CC, Yang SQ, Zhang SJ. 2012. Chemical constituents from leaves of Morus alba Linn. Chin Trad Patent Med. 34:1126–1131.
Zhu E, Cai JY, Wang YN, Zhang DZ. 2013. Study on chemical constituents from Anoectochilus elwesii. Nat Prod Res Dev. 25:1–3.