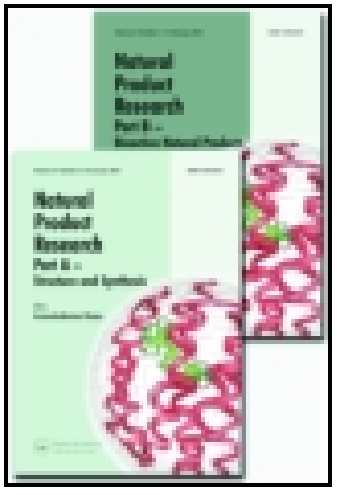

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A new flavonol triglycoside derived from Anoectochilus elwesii on stimulating glucose uptake in insulin-induced human HepG2 cells

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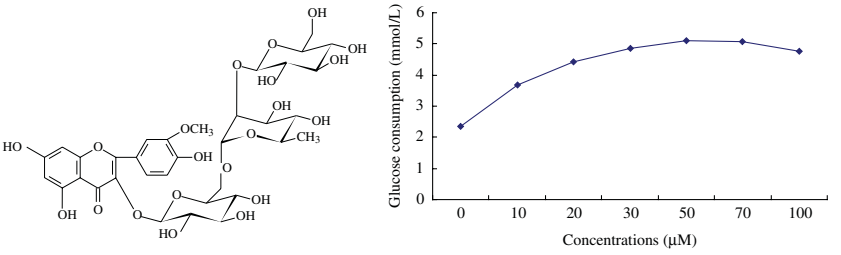

A new compound, elwesoside A, the first example of flavonol triglycosides isolated from *Anoectochilus* genus.

A novel flavonol triglycoside (**4**), isorhamnetin-3-*O*-β-D-glucopyranosyl (1→2)-α-L-rhamnopyranosyl (1→6)-β-D-glucopyranoside, named elwesoside A, together with six known flavonols (**1**–**3**, **5**–**7**) was isolated from *Anoectochilus elwesii* (Clarke ex Hook. f.) King et Pantl. and its structure was elucidated by extensive spectroscopic methods and comparison with the literature data. All compounds were first reported in this plant and two of them (**4** and **5**) were the first examples of flavonol triglycosides isolated from *Anoectochilus* genus. The effects of **1**–**7** were evaluated on insulin-treated human HepG2 cells under high glucose conditions for stimulating glucose uptake activities. The novel compound (**4**) displayed highly potent dose-dependent effect on the stimulation of glucose uptake in insulin-resistant human HepG2 cells.

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

1. Introduction

Type-2 diabetes mellitus (T2DM) or non-insulin-dependent diabetes is one of the fastest growing public health problems worldwide. It is marked by abnormal glucose and lipid metabolism due in part to insulin resistance in skeletal muscle, liver and fat, leading to elevated hepatic glucoproduction, hyperglycemia and hyperlipidemia (Bukhari et al. 2014). Insulin resistance is characterised as the failure of tissues to respond to insulin, resulting in reduced glucose intake in the peripheral tissues and increased hepatic glucose output (Zia-Ul-Haq, Ahmad, et al. 2014; Zia-Ul-Haq, Riaz, et al. 2014). It was found that the metabolic effects of insulin on glucose uptake and glycogen synthesis were inhibited by high glucose conditions. Currently, many therapeutic agents exist for the treatment of T2DM. However, in addition to

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insufficient effectiveness and durability, some of the current agents have side effects, including hypoglycemia, weight gain, edema, fractures, lactic acidosis, gastrointestinal intolerance and so on (Zhang et al. 2009). Thus, there is an emerging necessity to explore novel agents for the treatment of T2DM to prevent the spread of this global epidemic.

Insulin resistance in liver cells principally causes impaired glycogen synthesis and fails to suppress glucose production, which is the major contribution to hyperglycemia (Kola et al. 2008). HepG2 cells are hepatocellular carcinoma cells and have been proven to be valuable in investigating liver-derived functions. They maintain most functions of liver and are steady through many passages (Gupta et al. 2007). HepG2 cells have been used to investigate T2DM via an insulin-resistant model (Luo et al. 2009). In this study, we have also utilised insulin-resistant HepG2 cells to investigate effects of the isolated compounds from *Anoectochilus elwesii* on stimulating glucose uptake under high glucose condition.

Anoectochilus elwesii (Clarke ex Hook. f.) King et Pantl. has been used in Chinese folk medicine in diabetes and nephropathy ailments (Flora Republicae Popularis… 1999). Pharmacological evaluation of the antihyperglycemic activity of the ethonal extract of this plant in rats confirmed the folk information. A preliminary phytochemical study on *A. elwesii* has revealed the occurrence of flavonoids, triterpenoids and sterols. As part of our ongoing investigation for the chemistry of *A. elwesii*, here we report on the isolation and characterisation from the herbs of *A. elwesii* of some flavonoids, including a new flavonol triglycoside, isorhamnetin-3-*O*-β-D-glucopyranosyl (1→2)-α-L-rhamnopyranosyl (1→6)-β-D-glucopyranoside (**4**).

2. Results and discussion

Compound **4**, yellowish powder from MeOH, R_f 0.53 ($CHCl_3$–MeOH–H_2O 65:35:10, lower phase). IR_{max} (KBr): 3327, 2934, 2890, 1660, 1610, 1567, 1511, 1452, 1364, 1283, 1210, 1183, 1080, 1016, 993, 836, 811 cm^{-1}. ^1H and ^{13}C NMR, see Table S1. The ^1H NMR spectrum displayed three aromatic protons at δ 7.84 (1H, d, $J = 2$ Hz), 7.52 (1H, dd, $J = 2$ and 8 Hz) and 6.91 (1H, d, $J = 8$ Hz) for H-2′, H-6′ and H-5′, respectively. Two doublets at δ 6.20 (1H, d, $J = 2$ Hz) and 6.42 (1H, d, $J = 2$ Hz) integrating single protons were assigned to H-6 and H-8, respectively. A signal at δ 3.83 (3H, s) indicated the presence of a methoxyl substituent on the flavonol skeleton. The number and characteristic shifts of the ^{13}C glycosidic signals indicated the presence of two hexoses and a methyl hexose system in the pyranose form. The signals at δ 4.17 (1H, d, $J = 7.5$ Hz), 5.45 (1H, d, $J = 7.5$ Hz) and 4.73 (1H, s) were attributed to H-1 of two β-glucosyl units and an α-rhamnosyl unit, respectively. The existing two glucose units had β-glycosidic linkages, as concluded from the magnitude of the vicinal proton couplings (7.5 Hz) of the anomeric protons in the ^1H spectrum. The ^{13}C NMR spectrum showed a singlet which resonated at δ 56.32, and its protons (δ 3.83) showed a correlation with C-3′ in HMBC spectrum, which was assigned to the methoxyl substituent at C-3′. The signal at δ 178.02 was attributed to the carbonyl carbon (C-4). The signals of the aglycone and saccharides were assigned by HMBC (as Table S1). In the ^{13}C NMR spectrum, signals of the two β-glucopyranosyl and an α-rhamnopyranosyl moieties could be detected at 106.24, 101.77 and 100.22. Furthermore, the glucose linked to the hydroxyl group at C-3 of the aglycone was unambiguously confirmed by a long-range correlation between the proton of H-1″ (δ 5.45) and C-3 (δ 133.76) in the HMBC spectrum. The downfield shift of the C-6″ and the upfield shift of C-1‴, and furthermore, the correlation between H-6″ and C-1‴ (δ 100.22) suggested the position of attachment of the rhamnosyl moiety at C-6″ of glucose. The anomeric proton H-1‴ (δ 4.73) showed a correlation with C-6″ (δ 67.71), and similarly for the third sugar moiety, a long-range correlation between H-1⁗ (δ 4.18) and C-2‴ (δ 81.48) was observed. Hence, the glycosidic linkages were proven to be the two-linked glucopyranose and rhamnopyranose with a terminal glucopyranose. Acid hydrolysis

of **4** with 2M HCl afforded glucose, rhamnose and isorhamnetin. In addition, the sugar moiety was unambiguously determined to include two β-D-glucose and one α-L-rhamnose according to GC analysis. Based on the above interpretation, compound **4** was elucidated as isorhamnetin-3-*O*-β-D-glucopyranosyl (1→2)-α-L-rhamnopyranosyl (1→6)-β-D-glucopyranoside (As Figure 1) and was named elwesoside A.

A series of six other known flavonoids was isolated from *A. elwesii* and its structure was established by comparison with reported data as follows: isorhamnetin (**1**) (Gu et al. 2004), isorhamnetin-3-*O*-β-D-glucoside (**2**) (Lee et al. 2008), isorhamnetin-3-*O*-β-D-rutinoside (**3**) (Gong et al. 2009), kaempferol 3-*O*-β-D-glucopyranosyl (1→2)-α-L-rhamnopyranosyl (1→6)-β-D-glucopyranoside (**5**) (Murai et al. 2013), quercetin (**6**) (Yang & Teng 2007) and rutin (**7**) (Liu et al. 2007). Compounds **4** and **5** were the first examples of flavonol triglycosides isolated from the genus of *Anoectochilus*, and both have the same sugar chains with each other.

In order to evaluate the activities of compounds **1**–**7** in glucose metabolism, we first examined their effects on glucose uptake in HepG2 cells under high glucose conditions. Metformin was utilised as a positive control, and brought about a maximum increase of 102.5% ± 23.1% over the basal level of model control in glucose uptake at 70 μM. The maximum increase in glucose consumption of compounds **1**–**7** achieved 76.5% ± 24.8%, 62.6% ± 18.9%, 73.5% ± 9.2%, 113.9% ± 14.7%, 72.7% ± 14.7%, 89.9% ± 14.3% and 114.7% ± 22.7%, respectively. Rutin (**7**) caused a highest value at a low concentration of 10 μM, but the new flavonol (**4**) caused a significant and dose-dependent elevation in the glucose uptake in HepG2 cells. It led to continuous increases of more than 50% over the basal level of model control during 20–100 μM, and especially increased the maximum of 113.9% ± 14.7% at 50 μM, which were almost comparable with or more potent than metformin. Whereas the glucose uptake increased about 102.5% ± 23.1% at its peak when the dosage was 70 μM over the basal level of model control in response to metformin (Table S2). Compounds **1**–**3** and **5**–**6** also showed moderate effects on the stimulation of glucose uptake.

Meanwhile, MTT tests revealed that the experimented seven compounds did not show any cellular toxicity up to 100 μM concentration.

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

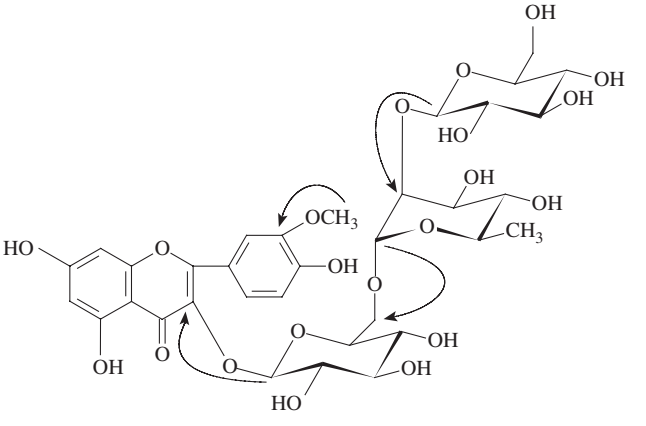

Figure 1. Structure of compound **4** and its key correlations observed from HMBC spectrum.

effects of individual components are largely unknown. Most recent studies of flavonols such as isorhamnetin, quercetin, kaempferol and their glycosides have focused on their antioxidant or anti-inflammatory activities and so on (Boesch-Saadatmandi et al. 2011; Sharma et al. 2014). In the present study, we isolated seven flavonols and their glycosides from *A. elwesii* and examined their effects on glucose uptake in insulin-induced HepG2 cells. Two compounds (**4** and **5**) were the first examples of flavonol triglycosides isolated from *Anoectochilus* genus. The novel compound elwesoside A showed a good stimulatory profile on glucose uptake. It increased the maximum of 113.9% ± 14.7% at 50 μM, which were more potent than metformin, increasing a peak of 102.5% ± 23.1% at 70 μM. Meanwhile, elwesoside A significantly increased glucose uptake in a good dose-dependent manner, better than the other tested flavonols including compound **5**, which was merely absent of a methoxyl on C-3′ of the aglycone. This methoxyl group may contribute to its stimulating effect.

3. Conclusion

The pharmacological profile on stimulating glucose uptake provides a scientific support for the claimed ethnomedical use of *A. elwesii* in diabetes, which might be indicative of the potential of some flavonol glycosides in improving the cells' resistant state. Further studies are warranted to bring insight into the accuracy mechanisms of action.

Supplementary material

Experimental details relating to this article are available online, alongside Tables S1–S2.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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