Identification of Highly Potent and Selective α-Glucosidase Inhibitors with Antiglycation Potential, Isolated from *Rhododendron arboreum*

Rabia Raza[*1,2], Zaitoon Ilyas[1], Sajid Ali[3], Muhammad Nisar[3], Muhammad Younas Khokhar[2] and Jamshed Iqbal[1]

[1]*Centre for Advanced Drug Research, COMSATS Institute of Information Technology, Abbottabad 22060, Pakistan*
[2]*Institute of Chemical Sciences, Bahauddin Zakariya University, Multan, 60000, Pakistan*
[3]*Institute of Chemical Sciences, University of Peshawar, Peshawar,25120, Pakistan*

Abstract: This study explored antidiabetic potential of eight known pure compounds, isolated from the bark of *Rhododendron arboreum*. *In vitro* studies of these compounds against *α* and *β*-glucosidases revealed them as very potent and selective inhibitors of *α*-glucosidase. Compound 7 (3-*O*-acetylursolic acid) was found to be the most potent inhibitor of *α*-glucosidase with $3.3\pm0.1\mu$M IC_{50} value which was many folds higher than standard inhibitor acarbose. Antiglycation studies of compounds showed that all compounds were also very active antiglycation agents. The studied biological properties of these compounds suggest that they are therapeutically interesting and important tools for treatment of diabetes.

1. Plant Source

Rhododendron arboreum belongs to family Ericaceae. In Pakistan, it is found at Hazara division in Seran valley, Jammu and Kashmir. Plant material was collected from Seran valley Khyber Pakhtoonkhwa, Pakistan, in February 2011, authenticated by Taxonomist at the Department of Botany, University of Peshawar. A specimen was deposited in the herbarium under voucher number 7212/ Bot.

2. Previous Studies

Diabetes mellitus is a group of metabolic disorders characterized by innate or acquired inability to transport glucose from blood stream to cells. During emergence and development of adult onset, diabetes cellular imbalance of lipids and carbohydrates results in eminent post-prandial blood glucose level [1]. Decreasing the post-prandial glucose level by retarding the absorption of glucose by inhibiting the carbohydrate hydrolyzing enzymes (alpha- and beta-glucosidases) in digestive tract is one of the therapeutic approaches to treat diabetes [2].

---

* Corresponding author: Email: rabiakhan_star@yahoo.com (R. Raza), Phone +92-332-7148476

Pathogenesis of diabetic complications is caused by increased protein glycation and the subsequent build-up of tissue advanced glycation end products (AGEs). These Glycation derived end products cause damage to proteins, lipids and nucleic acids and contribute towards oxidative stress in diabetes [3]. Along with *α*-glucosidase inhibitors, use of antiglycation products could also be a useful way to treat diabetes as they may play an efficient role in delay or prevention of diabetic complications. Many of the researchers are focusing on synthesis and discovery of glycation inhibitors that could be used as an efficient drug for diabetes. But still not a single drug based on glycation inhibitors has been able to pass all clinical trials. In such a situation search for antiglycation compounds obtained from natural sources could be a very practical approach towards discovery of biocompatible antiglycation based drugs for treatment of diabetes.

Conventionally, use of plant extracts for the treatment of diabetes was very common. Many studies have shown the effectiveness of crude plant extracts as well as their bioactive compounds in lowering blood glucose levels [4].

3. Present Study

In the current study, we have evaluated antidiabetic potential of eight natural compounds isolated from the bark of *Rhododendron arboreu*m (Figure 1)
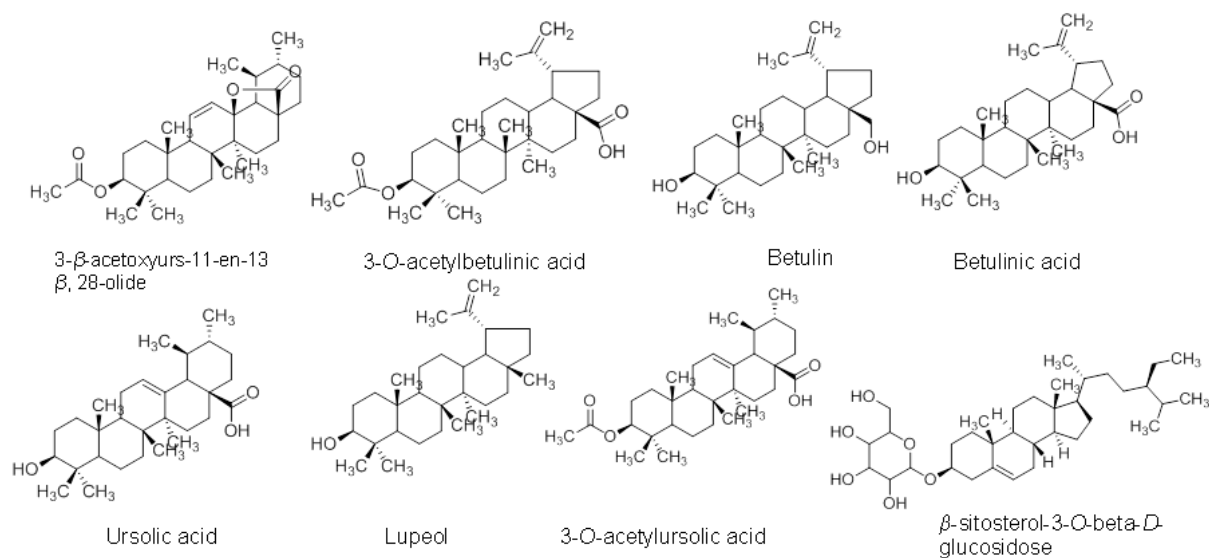
Figure 1. Structure of compounds isolated from the bark of *Rhododendron arboreum*

The dried plant material (bark; 5 Kg) was pulverized by a mechanical grinder, sieved through 40 mesh and extracted by maceration with methanol at room temperature for 15 days with occasional shaking. The methanolic extract was filtered and concentrated by rotary evaporator at low temperature, resulting in crude methanolic extract (650 g, 13.0% w/w). The crude methanolic bark extract (650 g) was redissolved in distilled water and successively extracted with hexane (9.5 % w/w), chloroform (17.46 % w/w), ethyl acetate (36.5 % w/w), butanol (17.46 % w/w) and finally with water (22.22 % w/w) to afford the respective fractions. The ethyl acetate fraction was subjected to column chromatography. The column was eluted with n- hexane: ethyl acetate with increasing polarity and finally with methanol: chloroform, which yielded a total of twenty sub-fractions EA-EU. The sub-fraction E-R (473mg) was subjected to flash column chromatography (silica gel, 6 g) and eluted with n- hexane: ethyl acetate (60:40) and it yielded two crystalline compounds **1** (10 mg) and compound **2** (20 mg). Sub-fraction E-T (503 mg) of ethyl acetate fraction was subjected to flash chromatography and eluted with n-hexane: ethyl acetate (30:70) to yield a white amorphous compound **3** (25 mg). Similarly, the fraction E-N (403 mg) on further purification through flash column chromatography and repeated preparative TLC yielded compound **4** (50 mg), compound **5** (15 mg) and compound **6** (10

mg), respectively. The sub fraction E-R (3.7g) on purification through flash column using n-hexane: chloroform (30:70) yielded colorless amorphous powder compound **7** (2.7 g), while fraction SFM (800 mg) on elution though pencil column using methanol: chloroform (5:95) resulted in compound **8** (25mg) as colorless powder. Spectroscopic data of compounds is given in supplementary information.

α-glucosidase inhibition study: Assay for α-glucosidase inhibition was performed by slight modification of a previously published method [5]. Briefly, solutions of α-glucosidase (from *Saccharomyces cerevisiae*) and its substrate *p*-nitrophenyl α-D-glucopyranoside (pNPG) were prepared in phosphate buffer (70 mM, pH 6.8). Methanol was used as a preferred solvent for preparation of inhibitor solutions. The inhibition assays were conducted by adding inhibitor solution (10 µL) to 70 µL buffer and 10 µL of enzyme solution (2.5 unit/mL) in 70 mM phosphate buffer (pH 6.8) followed by preincubation at 37 °C for 5 min. After preincubation, 10 µL of 10 mM substrate (pNPG) prepared in phosphate buffer was added to the mixture to initiate enzymatic reaction. The reaction mixture was incubated at 37 °C for 30 min, and the reaction was stopped by addition of 80 µL of 0.2M Na_2CO_3. Acarbose was used as a positive control. The α-glucosidase activity was determined by measuring the *p*-nitrophenol released from pNPG at 405 nm using an Elx 800 Micro plate reader. The % inhibition was calculated using the following equation:

$$\text{Inhibition (\%)} = [1 - (A_{sample} / A_{control})] * 100$$

IC$_{50}$ values of potent inhibitors were determined by testing 10-12 serial dilutions of inhibitors and were calculated by using the program PRISM 5.0 (GraphPad, San Diego, California, USA).

β-glucosidase inhibition study: To determine the inhibitory activity against β-glucosidase activity, the assay was performed with slight modification of the previously published method [6]. β-glucosidase (from sweet almonds) and *p*-nitrophenyl β-D-glucopyranoside (pNPG) as substrate were prepared in 0.07 M phosphate buffer (pH 6.8). The inhibition assays were conducted by adding inhibitor solution (10 µL) to 70 µL buffer and 10 µL of enzyme solution (2.0 unit/mL) in 0.07 M phosphate buffer (pH 6.8) followed by preincubation at 37 °C for 5 min. Following preincubation, 10 µL of 10 mM *p*-nitrophenyl glucopyranoside (pNPG) as a substrate in phosphate buffer was added to the mixture to start the reaction. The reaction mixture was then incubated at 37 °C for 30 min and stopped by adding 80 µL of 0.2 M Na_2CO_3. Negative control contained 10 µL of distilled water instead of inhibitor. Acarbose was used as a positive control.

Antiglycation study: Antiglycation activity was determined using a previously described method with appropriate modifications [7]. Briefly, to 250 µL of bovine serum albumin (1mg/mL) equal amount of 500 mM glucose was added. Test compounds dissolved in DMSO (50 µL) were added to this mixture and the contents were subjected to incubation at 60°C for 24 h. 100% TCA (10 µL) was added to each sample to stop the reaction. Samples were centrifuged at 10000 rpm 4 °C for 15 min. Supernatants were removed and pellets were collected. Phosphate buffer saline (PBS) at pH 10 (50 µL) was added to dissolve the pellets. Finally the fluorescence intensity was measured at 360 nm excitation and 460 nm emissions by using a Biotek Flx 800 spectrofluorometer.

All the tested compounds were highly potent inhibitors of α-glucosidase with IC$_{50}$ values in low micromolar ranges (Table 1). Compound **7** (3-*O*-acetyl ursolic acid), which is a triterpenoid, was found to be the most potent inhibitor of α-glucosidase with IC$_{50}$ value as low as 3.32 ± 0.10 µM. To the best of our knowledge this is the first report of α-glucosidase inhibition potency of this compound and interestingly it was much higher than the standard inhibitor acarbose, which is 445 ±7.9µM. Compound **1**(3-β-acetoxyurs-11-en-13 β, 28-olide), compound **2** (3-*O*-Acetyl-betulinic acid), compound **4** (Betulinic acid) and compound **5** (Ursolic acid) also showed good and comparable inhibition potencies against α-glucosidase.

In order to check the selectivity of compounds, all of these compounds were also tested against β-glucosidase obtained from sweet almonds. All compounds showed less than 13% inhibition when tested at 0.1 mM end concentration, indicating that they do not inhibit β-glucosidase (Table 1). Hence these compounds can be studied further as potential antidiabetic agents with selective inhibition against α-glucosidase.

The compounds were also tested for their antiglycation activity. The data obtained (Table 1) revealed a considerable antiglycation potential of these compounds. Highest antiglycation activity was exhibited by compound **1** (3-*β*-acetoxyurs-11-en-13 *β*, 28-olide) with 76.3±6% glycation inhibition.

Table 1. Inhibition potency of isolated compounds against *α*-glucosidase, *β*-glucosidase and glycation.

	Compound	*α*-glucosidase (IC$_{50}$ μM ± SEM)	*β*-glucosidase (%inhibition ± SEM)	Glycation (%inhibition ± SEM)
1	3-*β*-acetoxyurs-11-en-13 *β*, 28-olide	14.7±1.3	13.8%	76.3±6
2	3-*O*-acetylbetulinic acid	12.3±2.6	10.08%	35.4±2.1
3	Betulin	26.9±1.2	6.91%	25.9±4.6
4	Betulinic acid	16.5±0.6	5.38%	8.15±2.4
5	Ursolic acid	12.4±2.1	7.2%	40.6±7.2
6	Lupeol	27.4±3.6	4.84%	16.8± 4.6
7	3-*O*-acetylursolic acid	3.3±0.1	4.54%	69.6±9.2
8	*β*-sitosterol-3-*O*-beta-*D*-glucosidose	NT	2.84%	29.9±4.9
9	Acarbose (*α*-glucosidase Standard)	545 ±7.9	---	---
10	Castanospermine (*β*-glucosidase Standard)	---	59.98% [8]	---
11	Rutin (Antiglycation standard)	---	---	80.5±6.3

SEM= Standard error mean of three experiments.
NT= Not tested.

The study reports of natural compounds that inhibit *α*-glucosidase selectively as well as glycation. The data suggest that they are potential antidiabetic compounds that can lead to the discovery of novel potential drugs for diabetes with dual action.

Acknowledgments

Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

References

[1]   U. D. Palanisamy, L. T. Ling, T. Manaharan and D. Appleton (2011). Rapid isolation of geraniin from Nephelium lappaceum rind waste and its anti-hyperglycemic activity, *Food Chem.* **127**, 21-27.
[2]   S. Cheplick, Y. I. Kwon, P. Bhowmik and K. Shetty (2010). Phenolic-linked variation in strawberry cultivars for potential dietary management of hyperglycemia and related complications of hypertension, *Bioresour. Technol.* **101**, 404-413.
[3]   A. Elosta, T. Ghous and N. Ahmed (2012). Natural products as anti-glycation agents: possible therapeutic potential for diabetic complications, *Curr. Diabetes. Rev.* **8**, 92-108.
[4]   G. Kaushik, S. Satya, R. K. Khandelwal and S. N. Naik (2010). Commonly consumed Indian plant food materials in the management of diabetes mellitus, *Diabetes & Metabolic Syndrome: Clinical Research & Reviews.* **4**, 21-40.

[5]   H. Y. Ma, H. Y. Gao, L. Sun, J. Huang, X. M. Xu and L. J. Wu (2011). Constituents with alpha-glucosidase and advanced glycation end-product formation inhibitory activities from *Salvia miltiorrhiza* Bge, *J. Nat. Med.* **65**, 37-42.

[6]   M. Pérez, F. J. Muñoz, E. Muñoz, M. Fernández, J. V. Sinisterra and M. J. Hernáiz (2008). Synthesis of novel glycoconjugates and evaluation as inhibitors against β-glucosidase from almond, *J. Mol. Catal. B: Enzym.* **52–53**, 153-157.

[7]   M. Xi, C. Hai, H. Tang, M. Chen, K. Fang and X. Liang (2008). Antioxidant and antiglycation properties of total saponins extracted from traditional Chinese medicine used to treat diabetes mellitus, *Phytother. Res.* **22**, 228-237.

[8]   N. Verma, B. C. Behera and B. O. Sharma (2012). Glucosidase inhibitory and radical scavenging properties of lichen metabolites salazinic acid, sekikaic acid and usnic acid, *Hacett. J. Biol. Chem.* **40**, 7–21.