A New Sucrase Enzyme Inhibitor from Azadirachta indica

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INTRODUCTION

Any defect in insulin secretion or action results in hyperglycemia which leads to a metabolic disease, i.e. diabetic mellitus. Medicinal plants have been used since centuries by various cultures worldwide for the treatment of diabetes. Food and herbal control of postprandial glucose and insulin peaks to reach normoglycemia. Hypoglycemic activity may induce nonenzymatic glycosylation of various proteins, resulting in the improvement of accompanied complications such as macrovascular and cardiovascualr diseases.1-4 Phytochemical constituents isolated from plants exhibit several hypoglycemic mechanisms, through the inhibition of carbohydrate-metabolizing enzymes, manipulation of glucose transporters, regeneration of α-cell, and enhancement of the insulin-releasing activity.5-6 Hyperglycemia could be controlled by decreasing the absorption of glucose through the inhibition or decreasing the effect of certain enzymes responsible for the hydrolysis of carbohydrate such as sucrase enzyme in the digestive tract.7-9

Azadirachta indica A. Juss is a famous plant of the Meliaceae family. It is native to the Indian subcontinent and grows in many countries of the world such as Egypt and the Kingdom of Saudi Arabia (KSA). A massive cultivation of this tree occurred in the plains of Arafat, holy Makkah, the KSA. In 1971, approximately, 50,000 neem trees were cultivated to provide shade for the millions of pilgrims.8 It has enormous therapeutic, biological activity, and ethno-medical significance, so it is considered as a source of many biological active agents, due to its contents of various active constituents with diverse medicinal properties. It was practiced by different types of vaidyas and traditional healers in almost all the countries in the world such as the KSA, China, India, Egypt, Rome, and Greek.9-15 Anti-diabetic activity of medicinal plants has a strong relationship with their antioxidant property and polyphenolic contents.16,17 Our previous study revealed that the leaves of A. indica contain a considerable amount of polyphenolic compounds with significant antioxidant and cytotoxic activity.18 Hence, it is interesting to investigate the in vitro anti-diabetic activity of alcoholic extract of A. indica and the isolated polyphenolic compounds through the performance of sucrase inhibitory activity test.

MATERIALS AND METHODS

Equipment and materials

Pure samples were measured separately as MeOH solutions and various diagnostic shift reagents, Shimadzu ultraviolet (UV) 240 spectrophotometer,19 and with sprayed Naturtstoff reagent.20 Nuclear
magnetic resonance (NMR) analyses were run on Varian Mercury 300 MHz and Bruker 500 MHz spectrometers relative to TMS in different deuterated solvents. Electrospray ionization-mass spectrometry (ESI-MS) spectra were measured according to previously published conditions.[16]

**RESULTS**

The dried residue of 80% EtOH extract, which was extracted with petrol ether for defatting, was chromatographed on a polyamide column followed by successive separation on Sephadex LH-20 CC and cellulose CC for purification. The isolated pure compounds were identified by different chromatographic and spectral techniques such as UV, 1H, 13C NMR, two-dimensional NMR, and negative ESI-MS. As discussed in the published data,[18] the isolated known compounds have been identified as 2, 3-hexahydroxiphenoyl-(α/β)-D-β-D-glucopyranose (2), avicularin (3), castalagin, (4) and quercetin-3-O-glucoside (5). In addition to the new compound isolated for the 1st time from nature identified as 4’-methyl quercetin-7-O-β-D-glucuronopyranoside (1) is identified according to the following:

Compound 1 was obtained as pale yellow amorphous powder (17 mg) with the following chromatographic properties: Rp values; 0.32 (S), 0.51 (S); purple color under UV light turned to green color with FeCl₃ and orange color with Naturstoff spray reagents. UV-spectral data λmax (nm) (MeOH): 281, 352; (+NaOMe): 287, 382; (+NaOAc): 280, 351; (+AlCl₃): 280, 302 (sh), 352, 391; (+AlCl₃/HCl): 281, 301 (sh), 348, 390. 1H NMR (500 MHz, DMSO-d6): δ ppm 12.49 (1H, s, H-β), 8.17 (1H, d, J = 7.4 Hz, H-α), 7.68 (1H, d, J = 8.4 Hz, H-β), 6.74 (1H, d, J = 1.9 Hz, H-α), 6.67 (1H, d, J = 1.9 Hz, H-α), 5.68 (1H, d, J = 6.1 Hz, H-β), 3.76 (3H, s, O-Me), 4.29-2.85 (remaining sugar protons). 13C NMR (125 MHz, DMSO-d6): δ ppm 177.24 (C-4), 172.02 (C-6), 164.2 (C-2), 163.21 (C-5), 156.22 (C-7), 156.06 (C-9), 148.65 (C-4'), 148.60 (C-3'), 131.71 (C-3), 131.21 (C-6), 120.07 (C-1'), 116.24 (C-5'), 113.63 (C-2'), 104.06 (C-10), 100.85 (C-15), 97.65 (C-6), 96.48 (C-8), 78.63 (C-3'), 76.45 (C-5'), 71.06 (C-2'), 70.26 (C-4'), 54.78 (OCH₃-4)-ve ESI-MS: m/z 491.18 (M-H)+, 477.39 (M-CH₃)+, 315.44 (M-deoxyglucuronide)+, 300.43 (quercetin-H)+.

**Assay of sucrase inhibitory activity**

A sucrase enzyme solution of rat intestine has been prepared according to Dahlqvist's method. It occurred as a complex of sucrase and isomaltase, this hydrolyzes sucrose into glucose and fructose.[21] Honda and Hara created a method to assay the effect of samples on sucrase activity.[22] Enzyme solutions (10 µL) were incubated together with buffered solubilized sample (25–200 µg/ml in maleate buffer with pH 6.0) for 10 min at 37°C, while the volume was completed to 200 µL with maleate buffer (pH 6.0) in case of control, then the reaction was initiated by the addition of 100 µL of sucrose solution (60 mM). About 30 min later, the reaction was stopped by the addition of 200 µL of 3, 5-dinitrosalicylic acid reagent. The mixture was incubated in a boiling water bath for 5 min. The absorbance of each reaction was read at 540 nm. The percentages of inhibitory activities were calculated using the following formula:

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\text{% Inhibition} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100
\]

Abs control represents the absorbance of the control reaction (containing all reagents except the tested sample), whereas the Abs sample is the absorbance of the tested sample. An untreated enzyme solution was used as control. All experiments were carried out 3 times.

**Table 1:** Percentage sucrase inhibitory activity using Honda and Hara assay

| Conc. 25 µg/ml | Conc. 50 µg/ml | Conc. 100 µg/ml | Conc. 200 µg/ml |
|---------------|---------------|----------------|----------------|
| Total extract | 31.72±1.63    | 34.64±1.54     | 41.34±2.37     | 55.63±2.88     |
| Compound 1    | 37.52±0.92    | 42.56±2.03     | 62.72±1.83     | 71.35±3.21     |
| Compound 2    | 32.27±2.02    | 36.43±1.87     | 43.67±1.47     | 50.29±2.65     |
| Compound 3    | 28.62±1.07    | 31.47±0.86     | 52.38±2.46     | 58.34±1.64     |
| Compound 4    | 25.73±0.77    | 28.43±2.13     | 48.28±2.37     | 52.77±2.04     |
| Compound 5    | 21.39±2.14    | 30.82±1.06     | 54.64±1.34     | 61.36±3.02     |

Data were analyzed using two-factorial ANOVA, including first-order interactions (two-way ANOVA), followed by the Tukey’s post hoc test for multiple comparisons. P<0.05 indicated statistical significance. Conc.: Concentration of the tested sample; ANOVA: Analysis of variance.
According to the chromatographic properties and UV-spectral data, compound 1 was expected to be 4',7-quercetin derivative. The UV spectrum in MeOH exhibited the two characteristic absorption bands at $\lambda_{\text{max}}$ (nm) 281 nm (band II) and 352 nm (band I) of quercetin nucleus. Upon addition of NaOAc, no bathochromic shift of band II was observed which is diagnostic of a substituted 7-OH group. The remaining diagnostic shift reagents were in complete agreement with the 3', 5 dihydroxy-4'-7 disubstituted flavonol structure.$^{190}$ Negative ESI-MS spectrum exhibited the molecular ion peak at m/z 491 [M-H] - which corresponds to a molecular weight of 492 and a molecular formula of C$_{15}$H$_{10}$O$_{7}$. In addition, a fragment ion peak at m/z 477 after a loss of a methyl moiety indicates a methyl quercetin glucuronide structure. Mild acid hydrolysis and CoPC showed the presence of glucuronic acid in the aqueous phase and quercetin in the organic phase. $^1$H NMR spectrum showed the AM coupling system of the two meta-coupled doublets at $\delta$ ppm 6.74 and 6.67 assignable for H-8 and H-6, respectively, characteristic for ring-A of flavonol, in addition to the signals at $\delta$ ppm 7.58 (dd), 7.47 (d), 7.12 (d), assignable to H-6', H-2', and H-5', respectively, of ring-B with the absence of H-3' resonance signal suggesting the presence of quercetin moiety. The resonance singlet signal which integrated for three protons at $\delta$ ppm 3.76 was indicative for the presence of methoxy group, the location of the methoxy group on C-4' (ring-B) was deduced from the downfield shift of H-5' at $\delta$ ppm 7.12 (+0.3), in comparison with previously published data describing related structures.$^{24,25}$ This was approved by the heteronuclear multiple bond correlation (HMBC) that showed $^{23}$J$_{\text{C} \text{H}}$ coupling between O-Me protons at $\delta$ ppm 3.76 with C-4' at $\delta$ ppm 148.65. Further, structure confirmation was proved by the two-dimensional spectrums of $^1$H-$^1$H COSY, HSQC, NOESY and HMBC, and by comparison with related compounds reported in published data.$^{24-26}$ This complete assignment confirmed the structure of compound 1 to be 4'-methyl Quercetin-7-O-β-D-glucuronopyranoside (1), 2,3,hexahydroxyphenyl-(α/β)-D-C$_1$-glucopyranose (2), avicularin (3), castalagin (4), and quercetin-3-O-glucoside (5) significantly decreased the sucrase enzyme activity $^{[1]}$. These findings are in accordance with the previous study as the sucrase-inhibitory activity of tested plants supposed to be due to the presence of flavonoid glycosides and/or hydrolysable tannins.$^{[2]}$ Due to the rise in the incidence of diabetic patients around the world, it appears that more anti-diabetic drugs with complementary actions should be discovered and improved to reduce the blood glucose level by inhibiting the hydrolysis of carbohydrates in a reversible way. The World Health Organization suggested the evaluation of traditional plant treatments for diabetes as they are effective, less toxic, with fewer side effects, and are considered to be excellent candidates for oral therapy.$^{[29]}$ The antihyperglycemic activity of the plants is mainly due to their ability to restore the function of pancreatic tissues by causing an increase in insulin output or inhibiting the intestinal absorption of glucose or to the facilitation of metabolites in insulin-dependent processes. Glycosides, flavonoids, and carotenoids, from the plants, are frequently implicated in having anti-diabetic effect.$^{[30]}$ In the present study, the hydroalcoholic extract of A. indica and its isolated polyphenolic compounds 1-5 may have anti-diabetic activity due to their potent inhibitory activity against sucrase enzyme as shown in Figure 1.

**CONCLUSION**

The hydroalcoholic extract of the leaves of A. indica (family Meliaceae) and the tested compounds; 4'-methyl quercetin-7-O-β-D-glucuronopyranoside (1), 2,3,hexahydroxyphenyl-(α/β)-D-C$_1$-glucopyranose (2), avicularin (3), castalagin (4), and quercetin-3-O-glucoside (5) exhibited a significant in vitro anti-diabetic activity using sucrase enzyme inhibitory activity test. The hydroalcoholic extract of the leaves of A. indica contains a considerable amount of polyphenolic compounds that have significant antioxidant, cytotoxic,$^{[31]}$ and sucrase-inhibitory activities, thus have a great potential as a source for natural health products. Hence, the authors recommend in vitro and in vivo toxic tests to be done to evaluate the safety of A. indica to be used as a complementary medicine.

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Conflicts of interest
There are no conflicts of interest.

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