SUPPLEMENTARY MATERIAL

Bioassay-guided fractionation and identification of antidiabetic compounds from the rind of Punica granatum var. nana

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Abstract

This study aimed to evaluate the antidiabetic potential of the rind of Punica granatum var. nana. Acute oral toxicity test revealed the safety profile of its ethanolic extract. The extract was administered at 200 mg/kg b.wt to streptozotocin-induced diabetic rats. Serum diagnostic markers of diabetes (insulin, glucose and glycated hemoglobin), inflammatory mediators (tumor necrosis factor-α, interleukin-6, and nitric oxide), and oxidative stress (total antioxidant capacity and reduced glutathione and malondialdehyde) were assayed. The ethanolic extract was further fractionated and assessed for the aforementioned bioactivities at two different doses (100 and 200 mg/ kg b.wt). The results revealed that the ethyl acetate fraction of rind exhibited the highest activities. Using different chromatographic techniques, four compounds were isolated and identified as rutin, gallic acid, nictoflorin, and tulipanin. In conclusion: The ethyl acetate fraction of the rind of Punica granatum var. nana may provide a potential therapeutic approach for hyperglycemia.

Keywords: Antidiabetic; anti-inflammatory; antioxidant; Punica granatum var. nana; rind
Material

Plant Material

*Punica granatum* var. nana was collected during the fruiting stage (September and October, 2016) from plants cultivated in the Experimental Station of Medicinal Plants, Pharmacognosy department, Faculty of Pharmacy, Cairo University, Giza, Egypt. The plant was authenticated by Mrs. Terez Labib (Consultant in Orman botanical garden, Giza, Egypt) and verified by Prof. Reem Samir (Plant Taxonomy and Egyptian Flora Department, Faculty of Science, Cairo university, Giza, Egypt). Voucher specimens (18-5-2016) were kept at the Herbarium of Pharmacognosy Department, Faculty of Pharmacy, Cairo university, Egypt.

Preparation of Plant Extracts and Fractions

The shade-dried powdered rind (850 g) of *Punica granatum* L. var. nana was exhaustively extracted with 95% ethanol (10 × 1L) by cold maceration. The combined extracts were concentrated under reduced pressure at 40°C to give, 90 g (equivalent to, 10.6% on dry weight basis DW) of ethanolic residues. The dried residue of the rind (90 g) was suspended in water and partitioned successively with solvents of increasing polarity to yield 7.8 g, 1.6 g, 19.5 g, and 35.2 g of petroleum ether, methylene chloride, ethyl acetate and the remaining aqueous fractions (8.67, 1.78, 21.67 and 39.11% DW), respectively. The dried ethanolic extracts and successive fractions were kept in sealed glass vials in a refrigerator until use for biological and phytochemical studies. For biology study, aliquots of the ethanolic extract, as well as, its fractions were separately dissolved in 5% gum acacia in distilled water, respectively at the appropriate concentration.

Biological Study

Chemicals and drugs

Streptozotocin (STZ) and nicotinamide (NA) were purchased from Sigma Co (St. Louis, MO). Gliclazide (Glic.; Diamicron tablets) was obtained from Servicer Egypt Industries Ltd., Giza, Egypt.
**Animals**

Adult male albino Sprague-Dawley rats weighting 180 ± 20 g were obtained from National Organization for Drug Control and Research (NODCAR). Animals were housed in standard cages under controlled temperature (20 - 25°C) and 12/12 hours (light/dark) with free access to water and standard pellets diet. The animals were acclimatized to the experimental conditions for a week before starting the experiment. The investigation complies with the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996). The experiment protocol was approved (approval sheet number MP 442) by the Ethics Committee for Animal Experimentation, Faculty of pharmacy, Cairo university, Giza, Egypt.

**Oral acute toxicity study**

Oral acute toxicity was determined according to the Organization for Economic Co-operation and Development guideline-423 (OECD 2001) for the acute toxicity class method. A total number of 6 rats each were kept overnight fasting then separately received a fixed dose of 2000 mg/kg body weight of rind total ethanolic extract. All animals were observed individually for any sign of toxicity or mortality once during the first 30 min after dosing, then monitored for a period of 24 and 72 hours with a daily observation up to 14 days after administration.

**Induction of type-2 diabetes in experimental rats**

Non-insulin dependent diabetes mellitus; type 2 diabetes (T2D) was induced by injection of NA (110 mg/Kg b.wt, i.p) 15 min before injection of STZ (45 mg/kg b.wt., i.p) in overnight fasted rats. STZ was freshly prepared in an ice-cold citrate buffer (0.1 M, pH 4.5). Animals were provided with 10% glucose solution after 6 hours of STZ injection for the next 24 h to prevent fatal hypoglycemia. Animals with fasting glucose level ≥ 250 mg/dl after a week was served as diabetic rats (Masiello 2006).

**Experimental design**

To achieve the goal of this study two separated biological experiments were conducted as follows:

*Assessment of the ethanolic extract of Punica granatum var. nana:* Thirty-two rats were randomly divided into 4 groups (8 rats/each): Group 1(Normal) received vehicle (5% gum acacia in distilled water) and served as control for 4 weeks. Group 2 (STZ) served as
untreated diabetic rats. Groups 3 (STZ+Rind) diabetic rats were treated with the ethanolic extracts of the rind of *P. granatum* var. nana at a sole concentration (200 mg/ kg b.wt/day, p.o) for 4 weeks (Paget and Barnes 1964). Group 4 (STZ+ Glic) diabetic rats were treated with Gliclazide (as a reference antidiabetic drug) at a dose of 7.2 mg/kg bw/day, p.o. (equivalent to human therapeutic dose) for the same period.

**Assessment of the successive fractions of the ethanolic extract:** Eighty-eight rats were randomly divided equally into 11 groups (8 rats/each). Group 1 and 2 were treated as previously mentioned. Groups 3-10: NA/STZ- induced diabetic rats were separately treated once daily with two different doses (100 and 200 mg/kg b.wt, p.o) of petroleum ether, methylene chloride, ethyl acetate and the remaining aqueous fractions of the rind, respectively for 4 weeks. Group 11: diabetic rats were treated with gliclazide (7.2 mg/kg b.wt/day, p.o) for the same period.

**Handling of Blood Samples:** After 4 weeks, blood samples were collected from the retroorbital sinus of overnight fasted animals under light ether anesthesia. Sera were separated by centrifugation at 800 ×g for 10 min, and kept at -20°C for the different biochemical analyses.

**Assessment of antidiabetic effect:** Antidiabetic potential was measured by colorimetric method using Unicum spectrophotometer according to instruction of commercial kits measuring fasting serum levels of glucose (Spinreact S.A., Sant Esteve de Bas, Spain), and HbA1C% (Biosystems Inc. Barcelona, Spain) while insulin was assayed by ELISA (ALPCO Diagnostics, Salem, NH).

**Assessment of anti-inflammatory effect:** The anti-inflammatory potential was measured by ELISA according to manufacturers instruction, the serum levels of tumor necrosis factor-alfa (TNF-α), Interleukin (IL)-6 (Quantikine R&D, USA) while nitric oxide (NO) was assayed by colorimetric method (Biodiagnostic Inc., Egypt).

**Assessment of antioxidant effect:** Antioxidant potential was measured calorimetrically by determination of serum total antioxidant capacity (TAC), reduced glutathione (GSH) and a marker of lipid peroxidation as malondialdehyde (MDA) (Biodiagnostic Inc., Egypt).

**Statistical Analysis**

The biochemical results are expressed as means ± standard error (S.E.M). Results were statistically analysed by one-way ANOVA (analysis of variance) followed by Duncan’s multiple range test (DMRT) using the Statistical Package for the Social Sciences (SPSS), version 21. The level of statistical significance was taken at P < 0.05.
Phytochemical Study

Materials for chromatographic analysis

Column chromatography was performed over silica gel 60 (Merck) and Sephadex LH20 (Fluka). TLC was carried out using silica gel plates 60 F254 (Reide-de Haen, Germany). The following solvent systems were prepared from analytical grade chemicals purchased from El–Gomhoreya Co., Egypt; S1 (chloroform: acetone: formic acid; 50: 33: 17 v/v/v), S2 (ethyl acetate: formic acid: water; 12: 2: 3 v/v/v) S3 (butanol: acetic acid: water; 4:1:5 v/v/v, upper phase), S4 (petroleum ether: chloroform; 70:30 v/v) and S5 (chloroform: ethyl acetate; 50:50 v/v). The spraying reagents used were p-anisaldehyde-sulphuric acid, ferric chloride, aluminium chloride and ammonia vapour. Gallic acid and rutin (Sigma Co, St. Louis, MO) were used as authentics.

Apparatus

Unicam UV spectrophotometer was used for determining the UV spectra. NMR spectra were recorded in DMSO and CD3OD, using Mercury plus (400 MHz) and BRUKER (400 MHz) using TMS as internal standard and chemical shifts were given in δ value. The mass spectra were accomplished by using Agilent Triple Quadrupole mass spectrometer (UK-EI) and electron energy 150 eve. Electrothermal 9100 apparatus (Electrothermal Engineering Ltd, Essex, England) was utilized for determination of the melting point of the isolated compounds.

Isolation and identification of the compounds

The ethyl acetate fraction obtained from partitioning of the ethanolic extract of the rind (19.5 g) was concentrated under reduced pressure at 40°C to yield a yellow precipitate (Fr. I 1.56 g) and mother liquor (Fr. II). Fr. I was purified on a column of Sephadex LH20 using methanol for elution and afforded compound R1 (55.4 mg). Fr. II was evaporated to dryness to yield a residue (5.65g) which was then subjected to column chromatography on silica gel 60 using petroleum ether, dichloromethane, ethyl acetate and methanol. Similar fractions were pooled together then concentrated under reduced pressure yielding subfractions (S. Fr). S. Fr (33-45) (1.21g) which was eluted with ethyl acetate (100%) to ethyl acetate: methanol (80:20%) was further purified on column chromatography of Sephadex LH20 using methanol for elution to
give compound R2 (40.2 mg). S. Fr (46-59) (1.72 g) eluted with ethyl acetate: methanol (70:30%) was purified on column of silica gel 60 using dichloromethane and methanol in gradient elution manner. S.fr. (20-23) 0.46 g, eluted from dichloromethane-methanol (70:30) was further purified on Sephadex LH20 column, eluted by methanol-water (80:20%) to yield compound R3 (40 mg). S.fr. (60-65) (1.15 g) eluted from ethyl acetate: methanol (70:30%) to (100 %) methanol, was rechromatographed on a Sephadex LH20 column using methanol (100%) for elution to afford compound R4 (white powder, 30.20 mg).

Compound R1: is a yellow powder, soluble in methanol with melting point 242°C and Rf =0.26 in S1, 0.33 in S2 and 0.47 in S3. UV λ max nm (MeOH): 259, 266(sh), 299(sh), 360; NaOMe: 273, 327, 410; NaOAc: 272, 325, 390; AlCl3: 275, 303(sh), 430; AlCl3/HCl: 273, 300(sh), 405. From the previous physical, spectral data, co chromatography with rutin standard and by comparing the obtained data with those reported for rutin (Mabry et al. 1996), it could be concluded that compound R1 may be identified as Quercetin 3- O-rutinoside (Rutin)

Compound R2: white powder, soluble in methanol with Rf = 0.78 in S1 and 0.97 in S3. UV λ max nm (MeOH): 273. 1H NMR (400 MHz, CD3OD) δ ppm 7.54 (2H, s, H-2 & H-6). From the above spectral data, and its comparison with published data with those reported for gallic isolated from Punica granatum L. (Al-Mosawe and AL-Saadi 2012), and by co-chromatography with authentic sample it could be concluded that compound R2 was identified as Gallic acid.

Compound R3: yellow powder soluble in methanol with Rf = 0.54 in S1, and 0.61 S2 and 0.81 S3. UV λ max nm (MeOH): 255, 364; NaOMe: 264, 324, 410; NaOAc: 263, 310, 402; AlCl3:263, 303(sh), 360; AlCl3/HCl:263, 303(sh), 358; NaOAc/H3BO3: 255, 360. 1H NMR (400 MHz, CD3OD) δ ppm sugar moiety 5.36(1H, d, J=7, H-1''), 4.24 (1H, d, J=1.2, H-1'') 3.07-3.39 (sugar protons, m), 0.97(3H, J=7.4, protons of rhamnosyl CH3). 13C-NMR (400 MHZ, CD3OD) δ ppm, sugar moiety 101.24(c-1''), 78.02(C-3''), 76.28(C-5''), 75(C-2''), 70.78(C-4''), 68.29(C-6''), 100.26(C1''), 70.45(C-3''), 70.38(C-2''), 67.33(C-5''), 18.6(C-6''). On comparing the spectral data with the published data (Mabry et al. 1996), compound R3 was identified as Kaempferol 3-O-rutinoside (nictoflorin).

Compound R4: dark purple crystals, soluble in water with Rf = 0.17 in S1, 0.36 S2 and 0.45 S3. UV λ max nm (MeOH): 276, 540. 1H NMR (400 MHz, DMSO) δ ppm 8.96 (s, H-4), 7.71 (2H, s, H-2' & H-6'), 7.02(1H, br s, H-8), 6.68(1H, m, H-6), 4.91(1H, d, J=7.6, H-1''), 4.28
(1H, d, J=3, H-1'') 3.49-3.18 (sugar protons, m), 1.04 (3H, J=6.8, protons of rhamnosyl CH3). 13C-NMR (400 MHz, DMSO) δ ppm 168.46 (C-7), 164.40(C-2), 160.65(C-5), 157.57(C-9), 147.47(C-3' & C-5'), 145.41(C-4'), 144.89(C-3), 135.59(C-4), 120.43(C-1'), 113.04(C-2' & C-6'), 112.75(C-10), 103.36(C-6), 95.70(C-8), 101.49(c-1''), 83.77 (C-2''), 78.29(C-5''), 76.45(C-3''), 72.44(C-4''), 68.96(C-6''), 100.06(C1''), 76.17(C-3''''), 73.17(C-2'''), 70.22 (C-4''''), 67.95(C-5'''), 17.63(C-6''''). The spectral data were matched with the published data (Li-Li et al. 2014), compound R4 was identified as Delphinidin 3-O-rutinoside (tulipanin).

Table S1, Fig S1 & S2 here

References
Al-Mosawe EHA, Al-Saadi IIA. 2012. The extraction and purification of gallic acid from pomegranate rind. MJS. 23(6):53-60.
Table S1. Effect of the ethanolic extract of the rind of *Punica granatum* L. var. nana on serum levels of the different biochemical parameters

<table>
<thead>
<tr>
<th>Activity</th>
<th>Groups</th>
<th>Normal (control) (1 ml vehicle)</th>
<th>STZ (untreated) (110/45*)</th>
<th>STZ + Rind (200 mg/kg b.wt.)</th>
<th>STZ + Glic. (7.2 mg/kg b.wt.)</th>
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<tbody>
<tr>
<td><strong>Antidiabetic</strong></td>
<td>Insulin (µU/ml)</td>
<td>14.1 ± 0.36&lt;sup&gt;d&lt;/sup&gt;</td>
<td>7.03 ± 0.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.79 ± 0.29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.4 ± 0.73&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>Glucose (mg/dL)</td>
<td>83.5 ± 2.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>274.3 ± 3.98&lt;sup&gt;e&lt;/sup&gt;</td>
<td>134.1 ± 7.92&lt;sup&gt;b&lt;/sup&gt;</td>
<td>94.5 ± 2.94&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>HbA1c %</td>
<td>5.58 ± 0.31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.82 ± 0.26&lt;sup&gt;d&lt;/sup&gt;</td>
<td>6.92 ± 0.31&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>6.13 ± 0.19&lt;sup&gt;ab&lt;/sup&gt;</td>
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<tr>
<td><strong>Anti-inflammatory</strong></td>
<td>TNF-α (pg./ml)</td>
<td>39.8 ± 0.70&lt;sup&gt;a&lt;/sup&gt;</td>
<td>73.0 ± 0.87&lt;sup&gt;c&lt;/sup&gt;</td>
<td>43.2 ± 1.51&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>42.6 ± 0.75&lt;sup&gt;ab&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>IL-6 (pg/ml)</td>
<td>37.4 ± 0.52&lt;sup&gt;a&lt;/sup&gt;</td>
<td>66.9 ± 0.99&lt;sup&gt;d&lt;/sup&gt;</td>
<td>46.1 ± 1.31&lt;sup&gt;b&lt;/sup&gt;</td>
<td>39.9 ± 0.94&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>NO (µmol/L)</td>
<td>29.9 ± 0.79&lt;sup&gt;a&lt;/sup&gt;</td>
<td>56.3 ± 1.30&lt;sup&gt;c&lt;/sup&gt;</td>
<td>34.4 ± 0.60&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>32.4 ± 1.13&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Antioxidant</strong></td>
<td>TAC (mmol/L)</td>
<td>0.58 ± 0.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.29 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.57 ± 0.02&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.57 ± 0.03&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>GSH (mg/dL)</td>
<td>40.9 ± 0.98&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>26.0 ± 0.79&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38.7 ± 0.96&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>40.5 ± 1.21&lt;sup&gt;d&lt;/sup&gt;</td>
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<td></td>
<td>MDA (nmol/ml)</td>
<td>12.8 ± 0.36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.7 ± 0.97&lt;sup&gt;c&lt;/sup&gt;</td>
<td>14.9 ± 0.43&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.8 ± 0.24&lt;sup&gt;a&lt;/sup&gt;</td>
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</table>

Each value represents the mean ± standard error (S.E.M), values within the same row followed by different superscripted letters are significantly different at $P < 0.05$, tested by one-way ANOVA (analysis of variance) followed by DMRT (Duncan’s multiple range test); ); vehicle: 5% gum acacia in distilled water; (110/45*): dose of nicotinamide / STZ; Glic: Gliclazide, GSH: reduced glutathione; MDA: malondialdehyde; STZ: Streptozotocin-induced diabetic rats; TAC: total antioxidant capacity.
Figure S1. Effect of successive fractions of the rind of *Punica granatum* var. nana on: serum insulin (A), serum glucose (B), glycated hemoglobin (HbA₁C %) (C) TNF-α tumor necrosis factor (D); IL-6: interleukine-6 (E); NO: nitric oxide (F), TAC: total antioxidant capacity (G), GSH: reduced glutathione (H) and MDA: malondialdehyde (I) levels, (n=8).

Aq.: remaining aqueous fraction; E.Ac.: ethyl acetate fraction, Glic: Gliclazide; Meth.chl.: methylene chloride fraction; Pet.ether: petroleum ether fraction; STZ (untreated): Streptozotocin-induced diabetic group. Different letters are significantly different at *P* < 0.05 by DMRT (Duncan’s multiple range test).
Figure S2. Chemical structure of the isolated compounds

(R1): Quercetin 3- O-rutinoside (Rutin)  
(R2): Gallic acid

(R3): Kaempferol 3-O-rutinoside (nictoflorin)  
(R4): Delphinidin 3-O- rutinoside (tulipanin)