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Research article

Bioassay-guided fractionation and evaluation of anti-diabetic, anti-oxidant and anti-hyperlipidemic potential of *punica granatum* l. Var nana cultivated in egypt

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ABSTRACT

Punica granatum L. var. nana is an ornamental plant, yet it is underestimated medicinally compared to edible pomegranate. In this study, the ethanolic extract of the leaves(TEL)and its successive fractions were evaluated for anti-diabetic, anti-oxidant and anti-hyperlipidemic potentials in nicotinamide/streptozotocin(NA/STZ)-diabetic rats. Blood was measured for glucose, glycosylated haemoglobin(HbA1c%) and insulin levels.Anti-oxidant activity was assessed *via* measuring malondialdehyde(MDA), nitric oxide(NO), total anti-oxidant activity(TAC) and reduced glutathione(GSH). Anti-hyperlipidemic activity was evaluated *via* monitoring triglycerides(TG), total cholesterol(TC), low density lipoprotein-cholesterol(LDL-c), very low density lipoprotein-cholesterol(VLDL-c), high density lipoprotein-cholesterol(HDL-c) and atherogenic index(AI). Both TEL and ethyl acetate fraction(EAL) reduced glucose, HbA1c%, MDA, NO, TG, TC, LDL-c, VLDL-c and AI but increased plasma insulin, TAC, GSH and HDL-c. Therefore, the potent effect of TEL could be attributed to EAL, from whichseven phenoliccompounds were isolated.Nutritional composition was also investigated. Finally, leaves of the studied plant played a beneficial role in management of diabetes, controlling oxidative stress and improvement of lipid abnormalities.

Keywords: Anti-diabetic, Anti-hyperlipidemic, Anti-oxidant, Bioactive metabolites, Nicotinamide -streptozotocin, Punica granatum L. var. nana

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INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder characterized by chronic hyperglycaemia with disturbances in carbohydrates, fats and proteins metabolism resulting from defects in insulin secretion, insulin action, or both. The long-term specific effects of diabetes include progressive complications of retinopathy, nephropathy, neuropathy, amputation and increased risk of heart diseases, in addition to peripheral and arterial diseases. Type-1 (insulin-dependent) diabetes occurs most often in children and adolescents and is often referred to as juvenile-onset diabetes. Type-2 (non-insulin dependent) diabetes is the most prevalent and accounts for 90% of all patients [1]. Globally, diabetes is among the top 10 causes of deaths in adults [2]. Many of the available hypoglycemic agents do not offer complete control of the disease for their expensiveness or undesirable effects, thus traditional/herbal medicine has been recommended for its effectiveness, less side effects and relatively low cost. Therefore, in a continuation of the search for plant-derived anti-diabetic agents, *Punica granatum* L. var nana was evaluated for its activity.

Punica granatum L. (Pomegranate) family Punicaceae, has long been used in traditional herbal medicine as anti-diarrheal, anti-oxidant, anti-inflammatory, hepatoprotective, neuroprotective and anti-microbial agent [3]. The leaves extracts have been used as anti-diabetic, anti-hyperlipidemic and anti-oxidant due to their valuable metabolites*viz.* tannins (punicalin, punicafolin) and flavone glycosides (luteolin, apigenin) [4].

Punica granatum L. var. nana (Dwarf Pomegranate) is a deciduous ornamental dwarf shrub, native to the region from southern Europe to northern India, but has been naturalized over time around the Mediterranean.Its health benefits are uncertain and only very few reports dealing with the biological and chemical characters of the Egyptian cultivate could be traced reporting the isolation of some phenolic compounds *viz.* 1-galloyl-β-D-glucoside, cyanidin-3,5-O-diglucoside, luteolin-4′-O-β-D-glucoside and luteolin-7-(6″-O-galloyl)-β-D-glucoside, in addition to some triterpenoids and sterols. Moreover, its anti-oxidant nematicidal, fungicidal and anti-inflammatory activities were evaluated [5, 6]. In a previous publication

[7], the authors evaluated the biological efficacy (anti-diabetic, anti-inflammatory and anti-oxidant activities) of the rind of the local plant and isolated four compounds *viz.*rutin, gallic acid, nictoflorin, and tulipanin. In a continuation of our study, the present work was designed to assess the leaves lipid lowering effect by evaluating anti-oxidant and anti-diabetic activities, in addition to investigation of the chemical constituents, in a trial to understand the relevant biological activity of the plant and to justify its potential use in folk medicine

MATERIALS AND METHODS

Plant Material

Leaves of *Punica granatum* L. var. nana were collected during the fruiting stage in September-October from the Experimental Station of Medicinal Plants, Pharmacognosy department, Faculty of Pharmacy, Cairo University, Giza, Egypt. The plant was authenticated by Mrs. Therese Labib, Botanical specialist and consultant at Orman and Qubba botanical gardens and verified by Dr. Reem Samir (Professor of Taxonomy, Botany Department, Faculty of Science, Cairo university). Voucher specimen was kept in the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University (specimen no.: 1852016).

Preparation of Leaves Ethanolic Extract and its Successive Fractions for Biological and Phytochemical Studies

The shade-dried powdered (1kg) leaves were exhaustively extracted with 95% ethanol by cold maceration. The combined extracts were concentrated under reduced pressure at 40˚C to give a dark green residue of total ethanolic extract of leaves (TEL) (15%w/w, on dry weight basis (DW)). An aliquot of TEL (130g) was suspended in water and partitioned successively with solvents of increasing polarity to yield 15.24g of petroleum ether (PEL), 5.84g of methylene chloride (MCL), 35.11g of ethyl acetate (EAL), as well as 58.41g of remaining aqueous (RAqL) fractions. The extract and fractions were kept at -20˚C untill use for phytochemical analysis. For biological study, the extract and fractions were separately dissolved in 5% gum acacia, in distilled water, at the appropriate concentration prior to administration.

Chemicals, Drugs and Biochemical Kits

Streptozotocin (STZ), nicotinamide (NA), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), ascorbic acid, apigenin, gallic acid, catechin, rutin and quercetin were purchased from Sigma Co (St. Louis, MO). Gliclazide (Diamicron tablets®) were purchased from Servier Egypt Industries Ltd., Giza, Egypt. All biodiagnostic kits were purchased from Biodiagnostic Inc. (River Falls, WI, US). All solvents, chemicals and reagents used, were of analytical grade (Sigma Co, USA). Folin-ciocalteu reagent, used for determination of phenolic content, was obtained from Sigma Co. (USA). Sodium nitrite (5%), aluminium chloride (10%) and sodium hydroxide (1M), used for determination of total flavonoid content. Vanillin (0.5%) and hydrochloric acid (4%), for total proanthocyanidins content investigation. Potassium chloride buffer (0.025M, pH=1) and sodium acetate buffer (0.4M, pH=4.5), for assessment of total anthocyanin content (Sigma Co., USA).

In-Vitro Evaluation for Anti-oxidant Activity

The *in-vitro* anti-oxidant activity was evaluated adopting the radical scavenging 2,2-Diphenyl-1-picrylhydrazyl (DPPH) method [8]. Results were expressed as inhibition percentages and IC_{50} (µg/ml) value (the concentration needed to cause 50% inhibition) was calculated.

In-Vivo Biological Evaluation

Animals

Male albino Sprague-Dawley rats (150±20g) obtained from National Organization for Drug Control and Research (NODCAR), were housed in standard cages with controlled temperature (20-25˚C), 12/12 hours' light/dark with free access to water and standard pellets diet. The investigation complies with the guidelines for care and use of laboratory animals published by the US National Institutes of Health (1996) [9] and was approved by the Ethics Committee for Animals Experimentation at Faculty of Pharmacy, Cairo University (approval no. MP 442).

Oral acute toxicity study

Oral acute toxicity was performed according to The Organization of Economic Cooperation and Development (OECD) guideline no.[423 (2001)] [10]. A group of 6 adult male rats were kept fasting overnight then received orally a sole dose of 2000 mg/kg body weight of TEL after dissolving in the vehicle (5% gum acacia in distilled water). The incidence of mortality was recorded up to 14 days after administration.

Induction of diabetes mellitus (Type-2)

Non-insulin dependent diabetes mellitus (type-2 diabetes) was induced by a single injection of nicotinamide (NA) (110mg/Kgb.wt, intraperitoneal injection(ip)) followed bystreptozotocin (STZ) (55mg/Kgb.wt,ip) freshly prepared, by dissolving in an ice-cold citrate buffer (0.1 M, pH 4.5).[11]Overnight fasted rats were provided with 10% glucose solution after 6h of STZ injection for the next 24h to prevent fatal hypoglycemia. Animals with fasting glucose level ≥ 250mg/dL after a week served as diabetic rats and were selected for the study [12].

Experimental design

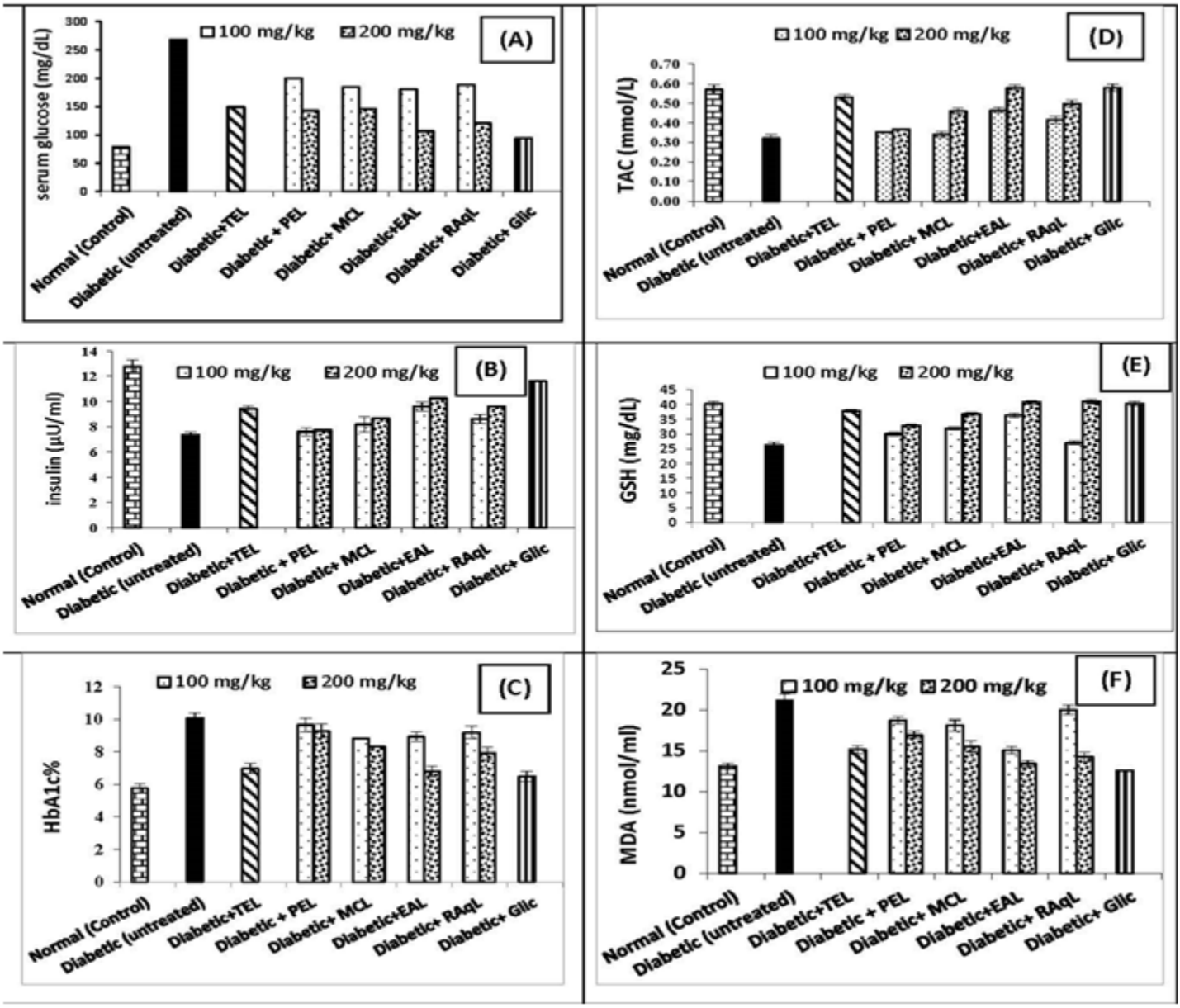
A total of 96 rats were randomly divided into twelve groups (8 rats/ group). Group 1; normal control administered 1 ml/day of vehicle (5% gum acacia in distilled water), Group 2; untreated diabetic rats, Groups 3; diabetic rats + TEL (200mg/kgb.wt/day), Groups 4-11; diabetic rats + PEL/MCL/EAL/RAqL (100 and 200mg/kgb.wt/day), respectively, Group 12; diabetic rats + Gliclazide (7.2 mg/kgb.wt/day). The extract/fractions/standard drug

were administered by oral gavage for 4 weeks. At the end of the experiment, blood samples were withdrawn from retro-orbital plexus veins under light anesthesia and serum samples were separated by centrifugation at 3000 r.p.m for 10 minutes and stored at –20°C, untill used for the biochemical estimations. In case of the determination of blood glutathione, 0.1ml of the whole blood was taken and lysed directly in 0.9ml of ice- cold distilled water [13].

Biochemical analysis

The separated sera were used to assess the anti-diabetic activity through the estimation of fasting serum glucose,glycated haemoglobin(HbA1c%) and insulin levels [14,15,16]. Results are illustrated in **Figure 1**.

Figure 1: (A-C): Anti-diabetic and (D-F) anti-oxidant activities of total ethanolic extract of leaves (TEL) and its fractions of Punica granatum L. var. nana in STZ-induced diabetic rats, (n=8). Results are expressed as mean values ± Standard error (SEM). Results were statistically analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT), by using the Statistical Package for the Social Sciences (SPSS, version 21). Different letters are significantly different at $P < 0.05$ by DMRT. HbA1c%: glycated haemoglobin, TAC: total antioxidant capacity, GSH: reduced glutathione, MDA: malondialdehyde, TEL: Total ethanolic extract of leaves, PEL: Petroleum ether fraction, MCL: Methylene chloride fraction, EAL: Ethyl acetate fraction, RAqL: remaining aqueous fraction, Glic.: Reference drug; Gliclazide



The lipid profile was estimated by evaluation of the serum levels of triglycerides (TG),[17]total cholesterol (TC),[18]high density lipoprotein cholesterol (HDL-c), very low densitylipoprotein cholesterol(VLDL-c),[19]low density lipoprotein cholesterol (LDL-c) and atherogenic index (AI),[20]spectrophotometrically. Furthermore, the anti-oxidant activity markers*viz.* serum total antioxidant capacity (TAC),[21]reduced glutathione (GSH)[22]and malondialdehyde (MDA)[23]levels were also determined.

Results are presented in Table 1.

Table 1 : Anti-hyperlipidemic activity of total ethanolic extract of leaves of *Punica granatum* L. var. nana and its fractions in STZ-induced diabetic rats, (n=8).

Group		Dose (mg/kg b.wt/day)	TG (mg/dL)	TC (mg/dL)	HDL-c (mg/dL)	VLDL-c (mg/dL)	LDL-c (mg/dL)	AI
1	**Normal control**	**1 ml vehicle**	57.79 ± 2.49^{a}	86.6 ± 1.29^{a}	41.89 ±1.91^{bc}	11.56 ± 0.50^{a}	33.16 ± 2.72^{a}	1.10 ± 0.10^{a}
2	**Diabetic (untreated)$^{\#}$**	**45***	131.88±2.02^{f} (+128.21)$^{\bullet}$	210.40±5.11^{h} (+143.01)$^{\bullet}$	33.31 ± 1.43^{a} (-20.50)$^{\bullet}$	26.38 ± 0.40^{f} (+128.22)$^{\bullet}$	150.71 ± 5.28^{h} (+354.61)$^{\bullet}$	5.41 ± 0.34^{i} (+391.81)$^{\bullet}$
3	**Diabetic+ TEL$^{\#*}$**	**200**	81.88 ± 1.22^{b} (-37.91)$^{\bullet}$	130.65±2.22^{cd} (-37.90)$^{\bullet}$	43.55 ± 1.67^{c} (+30.74)$^{\bullet}$	16.38±0.24^{b} (-37.91)$^{\bullet}$	70.73 ± 2.34^{cd} (-53.07)$^{\bullet}$	2.03 ± 0.11^{bc} (-62.47)$^{\bullet}$
4	**Diabetic+ PEL$^{\#*}$**	**100**	123.14±5.32^{ef} (-6.62)$^{\bullet}$	187.23±2.80^{g} (-11.01)$^{\bullet}$	33.55 ± 1.53^{a} (+0.72)$^{\bullet}$	24.63±1.06^{ef} (-6.62)$^{\bullet}$	129.05 ± 3.62^{fg} (-14.37)$^{\bullet}$	4.67 ± 0.28^{gh} (-13.68)$^{\bullet}$
5		**200**	114.04±4.92^{de} (-13.52)$^{\bullet}$	166.1 ± 2.48^{e} (-21.06)$^{\bullet}$	33.83 ±1.57^{a} (+1.56)$^{\bullet}$	22.81 ± 0.98^{de} (-13.52)$^{\bullet}$	109.46 ± 3.36^{e} (-27.37)$^{\bullet}$	3.99 ± 0.25^{fg} (-26.25)$^{\bullet}$
6	**Diabetic+ MCL$^{\#*}$**	**100**	124.87±1.91^{ef} (-5.31)$^{\bullet}$	190.35±4.62^{g} (-9.53)$^{\bullet}$	33.63 ± 1.51^{a} (+0.94)$^{\bullet}$	24.98±0.38^{ef} (-5.31)$^{\bullet}$	131.75 ± 5.55g (-12.58)$^{\bullet}$	4.75 ± 0.32h (-12.20)$^{\bullet}$
7		**200**	109.16±4.71^{d} (-17.22)$^{\bullet}$	154.67±2.31^{d} (-26.49)$^{\bullet}$	35.31 ±1.61^{a} (+6.00)$^{\bullet}$	21.83 ± 0.94^{d} (-17.22)$^{\bullet}$	97.52 ± 3.27^{d} (-35.29)$^{\bullet}$	3.45 ± 0.32^{ef} (-36.23)$^{\bullet}$
8	**Diabetic+ EAL$^{\#*}$**	**100**	95.38 ± 4.12^{c} (-27.67)$^{\bullet}$	130.03 ± 1.93^{c} (-38.20)$^{\bullet}$	36.92 ± 1.66^{a} (+10.82)$^{\bullet}$	19.08 ± 0.82^{c} (-27.67)$^{\bullet}$	74.04 ± 2.99^{c} (-50.88)$^{\bullet}$	2.58 ± 0.17^{cd} (-52.31)$^{\bullet}$
9		**200**	**80.10 ±3.46^{b} (-39.27)$^{\bullet}$**	**128.77 ± 1.92^{bc} (-38.80)$^{\bullet}$**	**43.84 ±2.00^{c} (+31.60)$^{\bullet}$**	**16.02 ± 0.69^{b} (-39.27)$^{\bullet}$**	**68.92 ± 3.29^{bc} (-54.27)$^{\bullet}$**	**1.98 ± 0.15^{bc} (-63.40)$^{\bullet}$**
10	**Diabetic + RAqL$^{\#*}$**	**100**	118.62±1.82^{de} (-10.05)$^{\bullet}$	178.34 ± 4.33^{f} (-15.24)$^{\bullet}$	35.5 ±1.59^{a} (+6.56)$^{\bullet}$	23.72±0.36^{de} (-10.05)$^{\bullet}$	119.11 ± 5.32^{ef} (-20.97)$^{\bullet}$	4.11 ± 0.28^{fgh} (-24.03)$^{\bullet}$
11		**200**	90.84 ± 3.92^{c} (-31.12)$^{\bullet}$	146.79 ± 2.19^{d} (-30.23)$^{\bullet}$	38.29±1.74^{ab} (+14.93)$^{\bullet}$	18.17 ± 0.78^{c} (-31.12)$^{\bullet}$	90.34 ± 3.29^{d} (-40.06)$^{\bullet}$	2.89 ± 0.19^{de} (-46.58)$^{\bullet}$
12	**Diabetic+Glic$^{\#*}$**	**7.2**	77.96 ± 1.84^{b} (-40.88)$^{\bullet}$	120.39±2.13^{b} (-42.78)$^{\bullet}$	44.09 ± 1.40^{c} (+32.35)$^{\bullet}$	15.59 ± 0.37^{b} (-40.88)$^{\bullet}$	60.71 ± 1.59^{b} (-59.72)$^{\bullet}$	1.74 ± 0.06^{ab} (-67.84)$^{\bullet}$

The results are expressed as mean values ± standard error (SEM). Results were statistically analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT), by using the Statistical Package for the Social Sciences (SPSS, version 21). Different letters are significantly different at $P < 0.05$ by DMRT;; (*): single dose; PEL Petroleum ether fraction; MCL: Methylene chloride fraction; EAL: Ethyl acetate fraction; RAqL: remaining aqueous fraction; Glic: Gliclazide.(Reference drug);vehicle**:** 5% gum acacia in distilled water; TG : triglycerides; TC: total cholesterol; HDL-c: high density lipoprotein; VLDL-c: very low density lipoprotein; LDL-c: low density lipoprotein; AI.: atherogenic index; $()^{\bullet}$: % of change of untreated group regards normal group ($^{\#}$**)** and of extract/ fractions /Gliclazide groups regards untreated group ($^{\#*}$), (+) represents percentage of increase and (-): represents percentage of decrease in each value when compared to STZ-induced diabetic rats group.

Phytochemical Evaluation

General

Column chromatography (CC) was performed over Silica gel 60 and sephadex LH-20 (Sigma Co, USA). Thin layer chromatography (TLC) and preparative TLC (p-TLC) were performed on silica gel F254 (Fluka) using solvent systems; S1 (CH_2Cl_2: Ace: HCOOH, 50:33:17), S2 (ETOAc: HCOOH: H_2O, 12:2:3), S3 (n-BuOH: AcOH: H2O, 4:1:5, upper phase), S4 (petroleum ether: CH_2Cl_2, 70:30) and S5 (CH_2Cl_2: ETOAc, 50:50). Compounds were visualized under UV light (254 and 365 nm) and by spraying with aluminium chloride. Melting points were determined on Electrothermal 9100 equipment. UV spectra were measured using Unicam spectrophotometer (UK), EI-MS spectra were measured on Agilent Triple Quadrupole mass spectrometer (UK), 1H-NMR and 13C-NMR spectra were measured in CD3OD/ DMSO-d6 with a Mercury plus (400 MHz, USA) and Bruker (400 MHz, Switzerland), respectively.

Chromatographic fractionation

The bioactive EAL (20g) fraction was chromatographed on Sephadex LH-20, eluted with CH_3OH and fractions (100ml/each) were collected into 7 main fractions (Fr. I-VII). Fr. III (3.35g) was re-chromatographed on silica gel 60 column, eluted with gradients of CH_2Cl_2, ETOAc, CH_3OH and H_2O to yield 8 subfractions (S.Fr. 1-8). S.Fr. 7 (401.5mg) was washed with $CH_2Cl_2$to yield compound L1 from the supernatant layer, while the residue and S.Fr. 8 (230mg) were rechromatographed on Sephadex LH-20 eluting with CH_3OH, to afford compounds L2 and L3, respectively. Fr. IV (6.52g) was subjected to p-TLC using S_1 as solvent system, five bands were scraped off, dissolved in CH_3OH, filtered and dried under reduced pressure. Compounds L4 and L5 afforded from bands 3 and 4, respectively, revealed one single spot/each, while band 5 was purified on Sephadex LH-20 using CH_3OH for elution to give compound L6. Fraction V (1.85g) was rechromatogrphed on Sephadex LH-20 column using gradients of CH_3OH-H_2O for elution to give compound L7. The structures of the isolated compounds were elucidated using their physical properties and/or co-chromatography in addition to spectral analyses.

Quantitative estimation of total phenolic, flavonoid, proanthocyanidin and anthocyanin contents

TEL extract was subjected to determination of the total phenolic content using Folin-Ciocalteu reagent [24] and expressed as mg gallic acid equivalentsper gram of dried weight (mg/g DW). Total flavonoid content was measured by aluminum chloride colorimetric

assay [24] and was expressed as mg quercetin equivalents (mg/gDW). Total proanthocyanidin content was measured by vanillin assay method,[8] expressed as mg catechin equivalents (mg/g DW).Total anthocyanin content was measured by pH differential method, [24] calculated as cyanidin-3-glucoside equivalents on fresh weight basis (µg/g FW).

Quantitative estimation of nutritive profile

The dried leaves of *Punica granatum* L. var. nana were analyzed for crude proteins, fats, fibres, total carbohydrates, [25] minerals,[26]as well as, the contents of vitamins (A and E) [27]and amino acids [28] .The analyses were carried out in the Regional Center for Food & Feed, Cairo University, Giza, ARE.

RESULT AND DISCUSSION

***In-Vitro* Evaluation for Anti-oxidant Activity**

TEL exhibited strong anti-oxidant activity (IC_{50}=6µg/ml), whichsurpassedthe reference drug; ascorbic acid (IC_{50}=11.04µg/ml). Consequently, for this promising potent activity of the extract, the authors were encouraged to further investigate its *in-vivo* biological activities.

***In-Vivo* Biological Evaluation**

Oral Acute Toxicity Study

This studyrevealednotoxicity signs after administration of 2000mg/kg of TEL, for 14 days. Hence, the pharmacological evaluation of TEL wascarried out at a fixed daily dose of 1/10 of the tolerated dose i.e. 200mg/kg/b.wt.

Anti-diabetic activity

A marked increase in serum glucose, HbA1c% and low insulin levels**(Figure 1A-C)**were observed in diabetic rats compared to normal rats. Gliclazide-treated rats showed significantly decreased glucose and HbA1c%, as well as,significant increase in insulin compared to diabetic rats. Similarly, TEL significantly prevented the elevation of serum glucose and HbA1c%,andshowed moderate increase in insulin compared to diabetic rats.

Allstudied successive fractions (PEL,MCL, EAL, RaqL),at 200mg/kg/day, showed significant potent effect than the lower dose (100mg/kg/day) indicating their dose-dependent activity. All fractions (200mg/kg/day) significantly decreased glucose, HbA1c% and increased insulin, compared to diabetic rats, except for PEL. EAL showed lowest glucose and HbA1c%, besides,highest insulin level.

Anti-oxidant activity

Significant reduction of TAC and GSH, in addition to increased MDA, compared to control group, was exhibited after administration of STZ indicating generation of oxidative stress (**Figure 1D-F**). All fractions, specifically EAL, displayed remarkable increase in TAC and GSH, but significantly decreased MDA, but PEL showed non-significant effect compared to diabetic group. Furthermore, the fractions showed dose-dependant anti-oxidant activity and EAL (200mg/kgb.wt/day) was the most potent.

Anti-hyperlipidemic activity

From **Table 1**, diabetic rats displayed marked elevation in TG, TC, VLDL-c, LDL-c and AI, while HDL-c was reduced compared to control group. Meanwhile, groups treated with gliclazide and TEL, showed dropping in TG, TC, VLDL-c, LDL-c and AI, however, HDL-c was raised up.

Likewise, administration of EAL (200mg/kgb.wt/day) showed similar actions to the reference drug and was the most potent fraction.

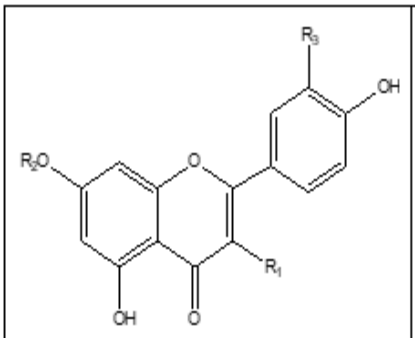
From the previous data, it could be concluded that the anti-diabetic, anti-oxidant and anti-hyperlipidemic activities of the plant were mostly localized in its EAL fraction. Therefore, EAL was subjected to further fractionation for isolation of its active constituents.

Phytochemical Investigation

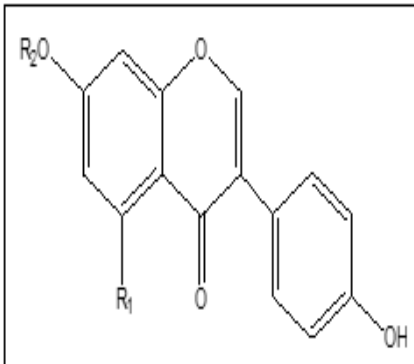
Investigation of EAL

Seven phenolic compounds were isolated from the bioactive fraction(EAL);apigenin**(L1)**, rutin**(L2)**, genistein**(L3)**, daidzin**(L4)**, astragalin**(L5)**, quercetin**(L6)** and antirrhinin**(L7)**. The identification was based on their physical and spectral data, in addition to comparison of these data with the previous reports. Compounds**L1-L7(Figure 2)** were isolated for the first time.

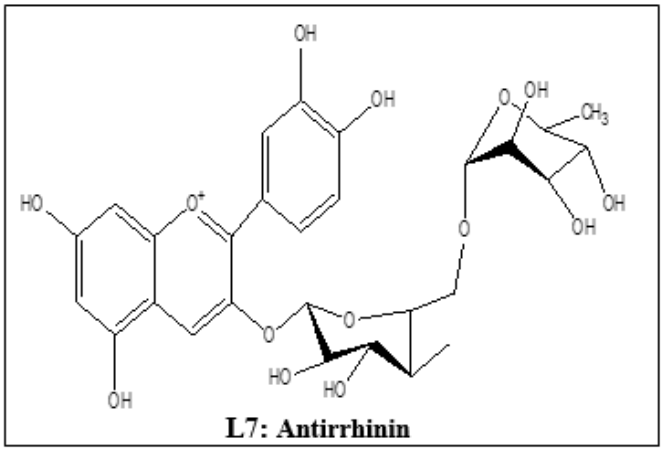
Figure 2. Isolated compounds from *Punica granatum* L. var. nana



	R_1	R_2	R_3
L1: Apigenin	H	H	H
L2: Rutin	O-rutinose	H	OH
L5: Astragalin	O-glucose	H	H
L6: Quercetin	OH	H	OH



	R_1	R_2
L3: Genistein	OH	OH
L4: Daidzin	H	O-glucose



L7: Antirrhinin

Quantitative estimation of total phenolic, flavonoid, proanthocyanidin and anthocyanin contents

TEL displayed high phenolic (144.53mg/gDW**)** and flavonoid contents (88.22mg/gDW), but considerable amounts of proanthocyanidin (3.057mg/gDW) and anthocyanin contents (42.58µg/100gFW).

Quantitative estimation of nutritive profile

One hundred grams serving of powdered leaves **(Table 2)**, contained 9.4g total proteins and 5.8g fats, 13.21g fibers and 52.89g carbohydrates..

Table 2: Nutritive profile of leaves of *Punica granatum* L. var. nana

Item		Content (mg/100g D.W)	Daily value (DV=mg/day)	Percentage of daily value (%DV)
Crude fibres		13210	28000	**47.20**
Fats		5800	78000	7.44
Proteins		9400	50000	18.80
Total carbohydrates		52890	275000	17.63
Minerals	Calcium	300	1300	23.08
	Iron	12.90	18	**71.67**
	Potassium	2600	4700	**55.32**
	Phosphorus	66.57	1250	5.33
	Sodium	320	2300	13.91
Vitamins	Vitamin A	0.970	0.900	**107.78**
	Vitamin E	0.600	15	4.0
Essential amino acids	Histidine	260	700	37.14
	Isoleucine	480	1400	34.29
	Leucine	1010	2730	37.0
	Lysine	650	2100	30.95
	Methionine	310	728	42.58
	Phenylalanine	530	875	60.57
	Threonine	580	1050	55.24
	Tyrosine	570	875	65.14
	Valine	980	1820	53.85
Non- essential amino acids	Aspartic	1080	-	-
	Alanine	660	-	-
	Arginine	490	-	-
	Cysteine	180	-	-
	Glutamic	1350	-	-
	Glycine	700	-	-
	Proline	510	-	-
	Serine	550	-	-

They were found to be rich in potassium, sodium and calcium, while iron and phosphorus were present in lower concentrations. Also, they contained 0.6 and 0.097mg/100g of vitamins E and A, respectively. The identified amino acids were histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tyrosine, valine, aspartic acid, alanine, arginine, cysteine, glutamic acid, glycine, proline and serine that mounted to 10.89%. Non-essential amino acids content (5.52%) was found to be slightly higher than the essential amino acids (5.37%). Leucine and valine were the predominant essential amino acids, whereas glutamic and aspartic acids were the major non-essential amino acids.

Daily values are listed in The Complete Guide to Recommended Daily Intakes and Daily Values (2021), %DV=(content/DV)*100, (-): not applicable

Phenolic compounds constitute the most numerous and ubiquitously distributed bioactive secondary metabolites. The present study, revealed the presence of high contents of phenolic and flavonoid compounds which elucidate the anti-oxidant and free radical scavenging abilities [8]. Considering anti-diabetic, anti-oxidant and anti-hyperlipidemic protective principles from herbal medicinal plants, the present study focused on *Punica granatum* L. var nana.

In this study, the effect of TEL extract on serum levels of NA/STZ-induced diabetic rats, was investigated.NA/STZ-diabetic rats' model is appropriate to assess the mechanism of diabetes as it simulates the biochemical abnormalities of the human disease [29]. Type-2diabetes was induced by intraperitoneal injection of NA followed by STZ in rats. In this model, the pancreatic β-cells are selectively destructed by the highly cytotoxic agent; STZ, leading to reduction in the release of insulin, but partially protected by NA, thus a case of stable hyperglycaemia is produced [30].

HbA1c% affords a time-average integral of the blood glucose concentration through the 120-day life span of red blood cells. Its increase is directly proportional to blood glucose level, thus, it provides an objective measurement for monitoring glycemic control in patients with diabetes. Consequently, HbA1c% can be used as a diagnostic test for diabetes and a value of 6.5% is recommended as the cut point for diagnosing diabetes [31].

In the current study,NA/STZ-diabetic rats showed significant increase in blood glucose and HbA1c%, in addition to marked decrease in serum insulin. Oral administration of TEL extract and its EAL fraction (200mg/kgb.wt.), significantly increased serum insulin and decreased HbA1c%, as well as reduced blood glucose compared to diabetic rats.The present findings support that EAL fraction exerted a significantanti-diabetic effect. This activity may be correlated to the phenolic compounds, namely flavonoids. In

fact, antirrhinin was reported to alleviate hyperglycemia by inhibition of intestinal α-glucosidase[32]. Ahmed et al. [33, 34] demonstrated that rutin and quercetin could ameliorate hyperglycemia *via*free radical scavenging ability, protecting the integrity of pancreatic β-cells manifested by increasing insulin secretion and decreasing blood glucose. Moreover, genistein showed significant increase in insulin release from islets of Langerhans and decrease elevated serum glucose and HbA1c%levels [35].

In diabetes, oxidative stress develops through overproduction of reactive oxygen species (ROS), which leads to reduction of the anti-oxidant mechanism and thus impair glucose metabolism. Therefore, oxidative stress is involved in the progression of diabetes and its complications [36]. It is evidenced that, ROS induces lipid peroxidation, membrane damage, degradation of polyunsaturated lipids and formation of MDA;a reliable biomarker of oxidative stress [37]. In addition, previous studies established that the development of diabetic complications is closely related to the increased generation of NO,[38] reduction of TAC and depletion of GSH [39]. In our study, lipid peroxidation markers; MDA and NO, were significantly increased with marked reduction in TAC, as well as, depletion of GSH in diabetic rats. Supplementation of TEL and EAL (200mg/kg b.wt.), markedly ameliorated all the aforementioned biomarkers resulting in reduction of oxidative stress. The prominent anti-oxidant activity can be related to the presence ofthe isolated phenolic metabolites. They may offer protection to cells against oxidative stress by scavenging free radicals and initiation of the anti-oxidant defense mechanism through significant enhancement in GSH and TAC, and thusincreasing the anti-diabetic activity *via* the anti-oxidant potential [24].

In type-2 diabetes, deficiency in insulin or insulin resistance might be responsible for hyperlipidaemia [40]. Therefore,hyperglycemia is associated with lipid and lipoprotein abnormalities including elevated levels of TG, TC, LDL-c, VLDL-c, AI and reduced HDL-c[41, 42]. Oral administration of TEL extract and EAL fraction (200mg/kgb.wt.) significantly restored TC, TG, LDL-c and HDL-c levels in diabetic rats towards normal compared with gliclazide-treated rats. Moreover, the marked decrease in AIdisplayed byEAL revealed its activity as anti-hyperlipidemic agent.The improvement in lipid profile may be due to thepresence of phenolic compounds such as rutin, which enhance insulin secretion and increase peripheral glucose uptake, as well as,significantly reducing intestinal glucose and cholesterol absorptionas describedby Ahmed et al[33] Moreover, antirrhinin has been reported to possess lipid-lowering activity through inhibition of intestinal lipid digestion and absorption.[43]Furthermore,apigenin[44], quercetin [34] and genistien[35]are also known for their anti-hyperlipidemic activities through free radical scavenging effect and regulation of anti-oxidant defenses of pancreatic β-cells.Our findings suggest that the bioactive EAL fraction exerted lipid-lowering action as well as anti-atherogenic potential and may be beneficial in reducing the incidence of atherosclerosis and decreasing the risk of cardiovascular diseases complications [40].

The present findings are in accordance with Salweet al,[45]who reported the anti-diabetic, anti-oxidantand hypolipedimic activities of hydroalcoholic extracts of leaves for edible pomegranate in STZ-induced diabetic rats through decreasing blood glucose.Phytochemical investigation of EAL fraction led to isolation of phenolic compounds which may contribute to its anti-hyperglycemic, anti-oxidant and anti-hyperlipidmic activities.

In this work, the evaluation of the nutritive profile of the leaves revealed it to be a functional ingredient and a good source of crude fibers**,** which provide numerous health benefits as their ability to decrease serum LDL-c, improve glucose tolerance and the insulin response, reduce hyperlipidemia and hypertension, contribute to gastrointestinal health and the prevention of certain cancers such as colon cancer. The concentration of potassium is relatively high, exceeding half the amount needed daily by humans. Such mineral plays an important role in muscle contraction, maintain the body's proper electrolyte and prevent incident cardiovascular disease or coronary heart disease. Furthermore, the leaves could participate by a valuablepercentage of the recommended daily need of iron and thus could combat iron defficiency. Also, they contain adequate amount of calcium that is required for normal muscle function, transmitting messages through the nerves and the release of hormones. Long-term calcium deficiency can lead to ricketes and poor blood clotting. Vitamin A content could cover its daily need, while the leaves could be considered as a rich dietary source of essential amino acids needed to maintain good health and normal body functioning [46].

CONCLUSION

According to the aforementioned findings, it is obvious that the anti-oxidant effects of the bioactive EAL fraction, resulted in decreased oxidative stress, which subsequently revealed significant anti-diabetic and anti-hyperlipidemic activities. Hence, *Punica granatum* L. var. nana leaves augmented with such biological activities and good nutritional values could be considered as a promising source of natural therapeutic agent or safe dietary supplementrich in health promoting polyphenols that canmanage diabetes, protect against oxidative stress and control lipid abnormalities, as well as, prevent cardiovascular diseases.However, further application in medical preparationshould be confirmed through additional laboratory investigation, preclinical and controlled clinical studies.

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Ethics statement

The *in-vivo* biological investigation was approved by the Ethics Committee for AnimalExperimentation at Faculty of Pharmacy, Cairo University, Cairo, Egypt. (approval no. MP 442).

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