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Eugenia punicifolia (Kunth) DC. as an Adjuvant Treatment for Type-2 Diabetes Mellitus: A non-Controlled, Pilot Study

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Type-2 diabetes mellitus (DM) is a highly prevalent disease with significant morbidity and mortality around the world. However, there is no universally effective treatment, because response to different treatment regimens can vary widely among patients. In this study, we aimed to investigate whether the use of the powdered dried leaves of *Eugenia punicifolia* (Kunth) DC. (Myrtaceae) is effective as an adjuvant to the treatment of patients with type-2 DM. Fifteen patients were enrolled in a pilot, non-controlled study, and received *E. punicifolia* for 3 months. After treatment, we observed a significant decrease in glycosylated hemoglobin, basal insulin, thyroid-stimulating hormone, C-reactive protein, and both systolic and diastolic blood pressure. There were no changes in fasting and postprandial glycemia. The compounds myricetin-3-*O*-rhamnoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-xyloside, quercetin-3-*O*-rhamnoside, kaempferol-3-*O*-rhamnoside, phytol, gallic acid, and trans-caryophyllene present in the powdered dried leaves of *E. punicifolia* may be responsible for the therapeutic effect. In conclusion, the powdered leaves of *E. punicifolia* are promising as an adjuvant in the treatment of type-2 DM and deserve further investigation. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: myrtaceae; phytotherapeutics; hypoglycemic agents; herbal medicine.

INTRODUCTION

Type-2 diabetes mellitus (DM) is a highly prevalent disease, affecting more than 20 million people in the USA (Blonde, 2010). It is responsible for significant morbidity and mortality (Yeh *et al*., 2003; Blonde, 2010). However, there is no universally effective treatment, because response to different treatment regimens can vary widely among patients (Blonde, 2009; Blonde, 2010).

The use of complementary and alternative treatments, including the use of herbal medicines, is increasing among diabetic people, because of poor adherence to treatment, poor disease control, side effects of conventional drugs, and cultural beliefs (Yeh *et al*., 2003; Hui *et al*., 2009). Many plant species have been identified as promising sources of new hypoglycemiant drugs (Yeh *et al*., 2003; Negri, 2005; Rao *et al*., 2010). Despite this, only a few herbal medicines have been proven useful in high-quality studies (Hui *et al*., 2009).

Eugenia punicifolia (Kunth) DC. (Myrtaceae, 'pedra-ume-kaá', Brazil) is a 1–4 m high shrub. From about 500 species of *Eugenia*, 400 are located in Brazil. Its leaves have been traditionally used in the treatment of diabetic patients (Brunetti *et al*., 2006) and possess antiinflammatory and nicotinic antagonist activities (Grangeiro *et al*., 2006; Leite *et al*., 2010). The methanolic and aqueous extracts of *E. punicifolia*, studied in an animal model of diabetes, showed anorexic effect and improved carbohydrate and protein metabolism without toxicity (Brunetti *et al*., 2006). However, to date, this has never been investigated in humans.

In this pilot, longitudinal, non-controlled study, we aimed to investigate the effects of *E. punicifolia* on fasting and 2 h postprandial glycemia, glycosylated hemoglobin, and other laboratorial tests in adults with type-2 DM. We hypothesized that *E. punicifolia* can enhance disease control.

MATERIALS AND METHODS

Study design. This is a pilot, longitudinal, non-controlled, open, phase-2 interventional study, comparing patients with themselves before and after the intervention. The study was approved by our local institutional review board (Research Ethics Committee of HCRP and FMRP-USP, process #737/2011). A signed informed consent was obtained from all patients.

Patients. All adult patients with a clinical diagnosis of type-2 DM treated in our institution (Ambulatory of Phytotherapeutics 'Farmácia da Natureza', Jardinópolis, Brazil) were eligible for the study. Inclusion criteria

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were age older than 18 years and a diagnostic of type-2 DM. The diagnosis of type-2 DM was made using the World Health Organization recommendations (WHO, 2006): fasting glycemia ≥ 126 mg/dL or 2 h postprandial glycaemia ≥ 200 mg/dL. The exclusion criterion was known allergy to *E. punicifolia*. Patients were withdrawn from the study in case of serious or severe adverse events. The patients were followed for 3 months. All but one patients were on regular treatment for DM (Table 1).

Preparation of the medicine. Leaves of *E. punicifolia* were collected at 9 AM and dried in an oven with circulating air at 45°C for 36 h. They were then ground in a knife mill and sieved to standardize the particle size (40 mesh). The powdered dried leaves were administered in capsules containing 200 mg each.

The plant was identified according to an authentic sample by Dr Marcos Eduardo Guerra Sobral (taxonomist, Federal University of São João Del-Rei, expert on Myrtaceae). A voucher of *E. punicifolia* species was deposited at the herbarium of the Biotechnology Department of the University of Ribeirão Preto (UNAERP), with the reference number HPMU-1539.

Analytical procedures. *Solvents and reagents.* All solvents and reagents were of the highest purity needed for each application. Silylation reagents, *N*,*O*-bis (trimethylsilyl) trifluoroacetamide (BSTFA) and trimethyl-cholorosilane (TMCS), were purchased from Supelco and Sigma-Aldrich (St. Louis, MO, USA), respectively.

Extraction and isolation. Dried leaves (0.2 kg) were exhaustively extracted with MeOH at room temperature. The obtained extract was concentrated under reduced pressure to remove organic solvents, affording 17.20 g of crude extract, which was suspended in MeOH/H_2O (8:2) and partitioned with solvents of increasing polarities, yielding hexane and ethyl acetate fractions.

Table 1. Demographic data

Variable	Result
Age (years)	60 ± 10
Time since diagnostic (years)	10 ± 8
Time between visits (days)	109 ± 18
Female gender	9 (60%)
Complications	4 (27%)
Alcohol consumption (currently)	2 (13%)
Smoking (currently)	0
Family history of diabetes mellitus	7 (47%)
Sedentary habits (currently)	8 (53%)
Stress (currently)	9 (60%)
Medications	
Metformin	5 (33%)
Metformin + glibenclamide	4 (26%)
Metformin + glimepiride + vildagliptine	1 (6%)
Metformin + glimepiride	1 (6%)
Metformin + glicazide	1 (6%)
Glicazide	1 (6%)
Repaglinide	1 (6%)
None	1 (6%)

Results are expressed as mean ± standard deviation or number (proportion).

Sample derivatization. Five milligrams of fraction was dissolved in 100 μL of pyridine. The residue was derivatized by adding 200 μL of BSTFA:TMCS/98:2 and heating for 90 min at 80°C. After cooling down to room temperature, an aliquot of 200 μL was transferred into the vials and diluted to 1 mL with EtOAc for gas chromatography–mass spectrometry (GC-MS) analysis.

HPLC-DAD/ESI-MS analysis. Phenolic profile of methanolic extract of *E. punicifolia* was investigated according Ferreres (Ferreres *et al.*, 2012). For flavonoids identification, 1 mg of ethyl acetate fraction was re-dissolved in 1 mL of MeOH:H_2O (1:1), and sample was sonicated and filtered (0.22 μ). High-performance liquid chromatography–diode array detection/electrospray ionization-mass spectrometry (HPLC-DAD/ESI-MS) analysis was developed on a Shimadzu LC-6 AD apparatus with a diode array detector (SPD-M10Avp; Shimadzu), coupled with an auto-injector (SIL-10AF; Shimadzu) and Class-VO 6.14 controller. Chromatographic analysis was carried out on a Luna C18 column (250 × 4.6 mm × 5 μm particle size; Phenomenex, Macclesfield, UK). The mobile phase consisted of water–acetic acid (1%) (A) and methanol (B), starting with 20% B and using a gradient to obtain 50% B at 30 min and 90% B at 35 min. The injection volume was 20 μL, and the flow rate was 1 mL/min. Spectral data from all peaks were accumulated in the range 240–400 nm, and chromatograms were recorded at 340 nm.

GC-MS analyses. For volatile compounds identification, 5 mg of hexane and ethyl acetate fractions were dissolved, separately, in 100 μL of pyridine. The residues were derivatized by adding 200 μL of BSTFA:TMCS/98:2 and heating for 90 min at 80°C. After cooling down to room temperature, an aliquot of 200 μL of each silanized fraction was transferred into the vials and diluted to 1 mL with EtOAc for GC-MS analyses. Chromatographic analyses were carried out using a gas chromatograph–mass spectrometer (model QP-2010, Shimadzu Corp., Kyoto, Japan); column DB-5 ms (30 × 0.25 mm × 0.25 μm) Agilent J&W (Santa Clara, CA, USA); carrier gas helium; split 1:40; flow rate of 1.1 mL/min; oven program: total run time: 73 min; initial temperature at 100°C; ramp 3°C/min to 290°C; hold 10 min; injection volume 1 μL; injector temperature was set at 260°C. MS was operated in the positive ion mode, with an ionization voltage of 70 eV. The MS data were obtained in full scan mode (50–600 amu). A comparison of the mass spectra of silylated compounds with those of the MS fragmentation patterns of the National Institute of Standards and Technology (Nist 08 lib.) spectral database was carried out.

Essential oil analysis. The essential oil was extracted by hydro-distillation in a Clevenger device during 2 h, using 100 g of fresh leaves into 1 L of distilled water. The GC-MS analysis of the essential oil of *E. punicifolia* was performed on a Varian Saturn 3900 GC/2100MS/CP8410 injector (Agilent Technologies, Santa Clara, CA, USA), using a VF-5 ms (WCOT fused silica) capillary column (30 m × 0.25 mm i.d.) under the following conditions: helium as carrier gas at 1.0 mL/min, injector split

at 240°C (split ratio of 1/10), electron impact ionization at 70 eV (trap temperature 220°C), and oven temperature programmed from 60 C up to 240 C at 3 C/min. Injection volume was 1 μL of solution (1 μL essential oil/1 mL hexane). Compounds of the oil were identified by comparison of their mass spectra with those of Nist 62 library mass spectra and confirmed by comparison of their retention indices relative to C8-C20 *n*-alkanes (Adams, 1995).

Treatment protocol. All patients took one capsule (200 mg) of the powdered plant material (leaves) of *E. punicifolia*, orally, three times a day (after breakfast, lunch, and dinner), for 3 months, in addition to their current treatment. This regimen was arbitrarily chosen to correspond to the main daily meals. Adherence was assessed by questioning the subjects during the medical interview. Blood pressure was recorded only in the visits.

Laboratory evaluation and toxicity. The following laboratory tests were performed in all patients before and after 3 months of treatment: fasting and 2 h postprandial glycemia, glycosylated hemoglobin, basal insulin, 24 h microalbuminuria, ultra-sensible C-reactive protein (usCRP), thyroid-stimulating hormone (TSH), cholesterol (total and fractions), and triglycerides. Toxicity was assessed by measuring plasma aspartate transaminase, alanine transaminase, gamma-glutamyl transferase, bilirubins, urea, creatinine, and creatine-phospho-kinase.

Data collection. The following data were collected from medical charts and patient interviews: age, gender, body weight and height, body mass index, time since diagnostic, complications, current alcohol use, current smoking, level of physical activity, family history of DM, and current treatment.

Statistical analysis. Analysis was per-protocol. The primary outcome was a reduction of at least 1% in glycosylated hemoglobin. In order to detect such a difference (paired analysis) after the intervention, with a standard deviation of 1, significance of 5% and power of 80%, ten subjects needed to be studied. Secondary outcomes included fasting and 2 h postprandial glycemia. Results are expressed as median (range), mean (±SD), or counts (percentages). Continuous variables were compared (before versus after treatment) using paired Student's *t*-tests or Wilcoxon's test, according to data distribution. Categorical variables were compared using Fisher's exact tests. Results were considered significant when $p < 0.05$.

RESULTS

Fifteen patients were screened and enrolled in the study between May 2010 and August 2011. Their demographic data are presented in Table 1. No patient was withdrawn from the study.

After treatment, there was a significant reduction from baseline on both systolic and diastolic blood pressure, glycosylated hemoglobin, basal insulin, TSH, and usCRP, whereas there was no significant change on the other studied parameters (Table 2 and Figs 1 and 2).

Table 2. Biochemical tests (mean ± SD) before and after treatment

Variable	Before treatment	After treatment	*p*-value
Body weight (kg)	72 ± 17	71 ± 16	0.121
BMI (kg/m^2)	26.9 ± 5.2	26.5 ± 4.8	0.118
Systolic blood pressure (mmHg)	130 ± 11	119 ± 9	0.004[a]
Diastolic blood pressure (mmHg)	75 ± 7	69 ± 8	0.029[a]
Fasting glycemia (mg/dL)	142 ± 30	134 ± 28	0.120
2 h postprandial glycemia (mg/dL)	150 ± 49	148 ± 47	0.911
Glycosylated hemoglobin (%)	7.6 ± 1.0	6.7 ± 0.9	0.013[a]
Basal insulin (μU/mL)	11.2 ± 10.2	8.5 ± 8.3	0.028[a]
TSH (μIU/mL)	3.2 ± 2.6	2.5 ± 1.6	0.044[a]
usCRP (mg/L)	5.5 ± 6.4	2.5 ± 3.8	0.035[a]
AST (IU/L)	20 ± 8	22 ± 6	0.256
ALT (IU/L)	21 ± 13	23 ± 15	0.373
GGT (IU/L)	24 ± 14	27 ± 18	0.527
Bilirubins (total, mg/dL)	0.61 ± 0.27	0.68 ± 0.29	0.362
CPK (IU/L)	87 ± 42	91 ± 52	0.801
Microalbuminuria (mg/24 h)	12.3 ± 14.0	14.6 ± 11.6	0.454
Urea (mg/dL)	35 ± 12	36 ± 9	0.832
Creatinine (mg/dL)	0.92 ± 0.21	0.87 ± 0.24	0.354
Cholesterol (mg/dL) total	170 ± 31	165 ± 23	0.396
HDL	45 ± 11	46 ± 7	0.599
LDL	97 ± 26	92 ± 25	0.970
Triglycerides (mg/dL)	144 ± 105	138 ± 85	0.798

BMI, body mass index; TSH, thyroid-stimulating hormone; usCRP, ultra-sensible C-reactive protein; AST, aspartate transaminase; ALT, alanine transaminase; GGT; gamma-glutamyl transferase; CPK, creatine phospho-kinase; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

[a]Significant difference.

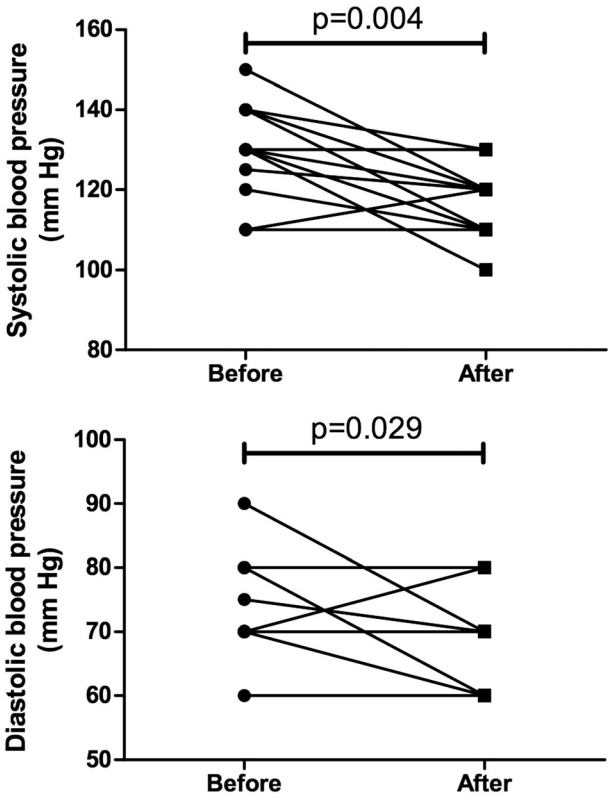

Figure 1. Systolic and diastolic blood pressure before and after 3 months of treatment ($N = 15$). Notice that almost all patients experienced a reduction on systolic blood pressure after 3 months. On diastolic blood pressure, the effect was less intense. Some symbols and lines are superimposed, given the impression that there are fewer than 15 observations.

Interestingly, the effects were stronger in patients with more abnormal tests. The mean difference on glycosylated hemoglobin was of −0.96 (±1.32, with 95% confidence interval of −0.30 to −1.63) %. There was no evidence of toxicity. Only three patients had minor adverse events: diarrhea ($n = 2$) and flatulence ($n = 1$).

Online Supplementary Table S1 shows the proportion of patients fulfilling the 2010 American Diabetes Association (ADA) goals for the management of type-2 DM (American Diabetes Association, 2009).

On the basis of the results of HPLC-DAD-ESI-MS analysis (Online Supplementary Table S2), five flavonoids were identified in the leaves of *E. punicifolia* methanolic extract: myricetin-3-*O*-rhamnoside (myricetin-3-*O*-R), quercetin-3-*O*-galactoside (quercetin-3-*O*-Ga), quercetin-3-*O*-xyloside (quercetin-3-*O*-X), quercetin-3-*O*-rhamnoside (quercetin-3-*O*-R), and kaempferol-3-*O*-rhamnoside (kaempferol-3-*O*-R). The GC-MS analyses of hexane and ethyl acetate fractions showed the presence of two major compounds: gallic acid and phytol (Online Supplementary Figs S1 and S2). The essential oil from the leaves of *E. punicifolia* contains, as major component, (-) trans-caryophyllene (Table S3).

DISCUSSION

This study showed that, in patients with type-2 DM, a 3-month-long treatment with the powdered dried leaves of *E. punicifolia* resulted in a significant reduction on glycosylated hemoglobin, basal insulin, TSH, usCRP, and both systolic and diastolic blood pressure.

It is interesting that we did not observe any effect on fasting or postprandial glycemia in our patients. However, according to recent studies, besides controlling glycemia, it is equally important to control other cardiovascular risk factors such as overweight, hypertension, and dyslipidemia (Kuritzky, 2010). The 2010 ADA guidelines for the management of type-2 DM recommend achieving a glycosylated hemoglobin <7.0%, fasting glycemia of 70–130 mg/dL, postprandial glycemia <180 mg/dL, LDL cholesterol <100 mg/dL, HDL cholesterol >40 (women) or >50 (men) mg/dL, and triglycerides <150 mg/dL (American Diabetes Association, 2009). According to

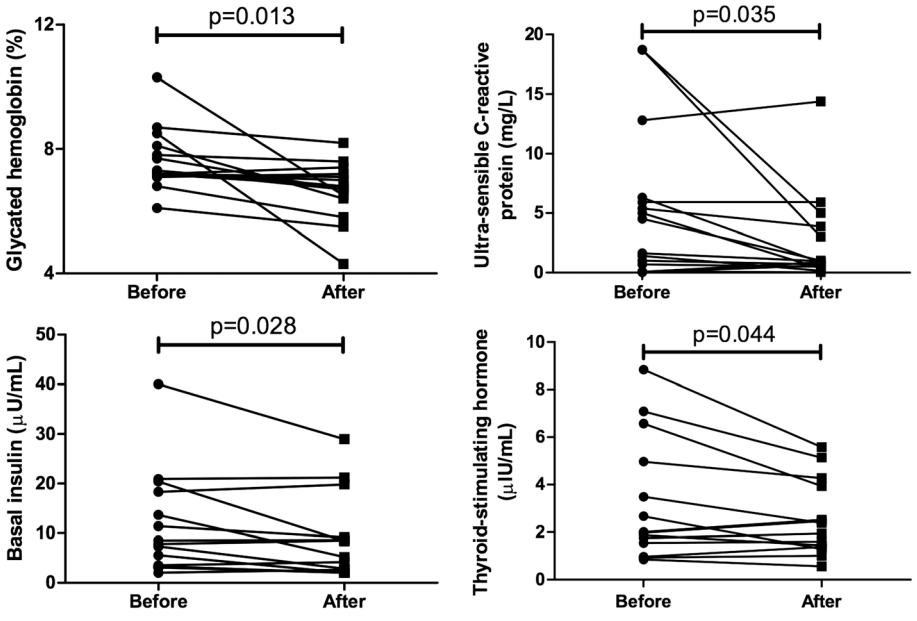

Figure 2. Glycosylated hemoglobin, basal insulin, thyroid-stimulating hormone (TSH), and ultra-sensible C-reactive protein (usCRP) before and after 3 months of treatment. Notice that glycosylated hemoglobin levels fell dramatically in two patients, whereas only moderately in the others. Basal insulin fell in a few patients but was mostly unchanged. usCRP and TSH also fell dramatically in few patients whereas they were slightly lower or unchanged from baseline in the remaining.

the data from 1999–2006 in the National Health and Nutrition Examination Survey, only 12.2% of patients with DM achieved the recommended combined targets for glycosylated hemoglobin, blood pressure, and LDL cholesterol during this period (Kuritzky, 2010). In our study, there was a significant increase in the proportion of patients achieving the 2010 ADA therapeutic goals (Table S3).

In a previously published study, a reduction of 1% on glycosylated hemoglobin was associated with a 37% reduction in the risk of microvascular complications (Stratton *et al*., 2000). Similarly, intensive glycemic control (glycosylated hemoglobin <7.0%) was associated with lower rates of new onset or worsened nephropathy or retinopathy, compared with patients randomized to non-intensive therapy (conventional insulin) during 8 years of follow-up (Shichiri *et al*., 2000). In our study, glycosylated hemoglobin mean decrease was of 0.96%, and after treatment, 60% of patients had their glycosylated hemoglobin <7.0%. Another interesting finding of our study was that usCRP decreased by more than a half from baseline. If we speculate that the treatment caused this reduction, then that might be an important finding because chronic inflammation plays a role on long-term complications of diabetes (Forbes and Cooper, 2013; Tabas and Glass, 2013). Therefore, *E. punicifolia* may be a good candidate for the prevention of complications of type-2 DM. We also observed a reduction on TSH levels, unexplained.

To explain the biological activities of *E. punicifolia*, we conducted a chemical analysis of its components. Flavonoids, gallic acid, and phytol identified in *E. punicifolia* leaf extracts have significant antioxidant activity (Galeno *et al*., 2013; Salgueiro *et al*., 2013; Santos *et al*., 2013). It is well known that cells exposed to natural antioxidants exhibit decreased insulin resistance (Ruhe and McDonald, 2001). Hypoglycemic effects of flavonoids have been demonstrated through glucose uptake in peripheral tissues, regulatory activity and expression of enzymes involved in carbohydrate metabolism pathway (Choi *et al*., 1991; Matsuda *et al*., 2002; Kamalakkannan and Prince, 2006). Histopathological studies of pancreas of diabetic rats treated with gallic acid showed that this compound preserves the pancreatic tissue, enhance insulin secretion, promotes β-cell regeneration, and normalizes biochemical parameters related to the patho-biochemistry of DM (Latha and Daisy, 2011; Punithavathi *et al*., 2011). Phytol and its analogs also exhibit direct effects on diabetes, because they reduce insulin resistance, promote glucose uptake in primary hepatocytes, and are agonists of peroxisome proliferator-activated receptors (Elmazar *et al*., 2013).

The essential oil from the leaves of *E. punicifolia* is rich in oxygenated sesquiterpenes and its major component, the (-) trans-caryophyllene, which has significant antiinflammatory activity (Fernandes *et al*., 2007). It is also effective in reducing TNF-α-induced prostaglandin E_2 release, inducible nitric oxide synthase, and cyclooxygenase-2, all inflammatory mediators that negatively impact diabetes control. Undoubtedly, the substances identified in leaf extracts and essential oil of *E. punicifolia* can explain the clinical results observed here.

Many authors advocate the use of individualized therapies for patients with type-2 DM, taking into consideration adverse effects of medications, medication administration complexity, cost, 'needle phobia', and comorbidities (Kuritzky, 2010). From this standpoint, our results suggest that adding *E. punicifolia* to current treatment regimens may improve control of type-2 DM of at least selected patients. Moreover, there was no evidence of toxicity or significant side effects. The adverse effects reported were diarrhea and flatulence, which are also common in patients using metformin and other hypoglycemiant drugs, including herbal medicines (Dans *et al*., 2007; Hui *et al*., 2009).

Many other herbal interventions have been reported as useful for type-2 DM treatment, including *Panax ginseng*, *Momordica charantia*, and *Coptis chinensis* (Hui *et al*., 2009). However, to our knowledge, this is the first to exhibit an effect of such magnitude on disease control.

Our results have to be considered with caution. Because this study was not placebo-controlled, we cannot exclude a Hawthorne effect, that is, the patients in the study may have been more prone to comply with the treatment and diet. This effect might have been further increased if patients consenting in participate in the study were sympathetic to herbal treatments. In addition, the use of the powdered leaves brings difficulties in reproducibility by other researchers. On the other hand, it was our intention to show that the plant has biological effects 'in natura', without need of any extraction method. Other limitations are the small sample size, which limits power and external validity, and lack of monitoring at multiple time points during treatment. We plan to address these issues in a larger phase-3, randomized, placebo-controlled clinical trial, which is under consideration.

In conclusion, diabetic patients treated with *E. punicifolia* as an adjuvant to standard treatment experienced a marked reduction on their glycosylated hemoglobin, basal insulin, TSH, usCRP, and both systolic and diastolic blood pressure, without changes in fasting and postprandial glycemia. Therefore, *E. punicifolia* deserves to be further investigated in larger, randomized, placebo-controlled clinical trials.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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