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Organic beet leaves and stalk juice attenuates HDL-C reduction induced by high-fat meal in dyslipidemic patients: A pilot randomized controlled trial

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ABSTRACT

Objectives: Beet leaves and stalks are rich in polyphenols; however, their effect on risk factors for cardiovascular disease in humans, to our knowledge, has not yet been investigated. The aim of this study was to analyze the acute effect of beet leaves and stalk juice, containing different concentrations of polyphenols, on lipemia, glycemic control, nitric oxide concentration, and blood pressure in patients with dyslipidemia after a high-fat meal.
Methods: In a randomized double-blind, placebo-controlled, crossover pilot study, patients 20 to 59 y of age with dyslipidemia were fed a single high-fat meal supplemented with either a placebo or one of two organic beet leaves and stalk juices rich in polyphenols (32 or 77.5 mg EAG/100 mL) with a 1-wk washout. Thus, each group was composed of 13 patients. Blood samples were obtained at fasting and 30, 60, 120, and 180 min after intervention. Total cholesterol, high-density lipoprotein, triacylglycerols, glucose, insulin, nitrite and nitrate, and blood pressure were assessed at each time period. The high-fat meal increased triacylglycerol levels after 120 ($P < 0.001$) and 180 min ($P < 0.001$) and reduced high-density lipoprotein cholesterol after 60 min ($P < 0.05$). This reduction was attenuated in both groups that received BLS juices after 120 min ($P = 0.005$). A reduction in diastolic blood pressure within groups that received BLS juice was also observed.
Results: There was no significant difference between groups for other biomarkers.
Conclusion: The beet leaves and stalk juice attenuated the reduction of high-density lipoprotein cholesterol induced by a high-fat meal.

Introduction

Cardiovascular disease (CVD) is the leading cause of death worldwide [1]. In addition to *well-known* CVD risk factors [1], other relevant risk factors include changes in postprandial lipemia [2], endothelial dysfunction, and subclinical inflammation [3].

The term *postprandial lipemia* is attributed to metabolic events occurring after digestion and absorption of lipids from the diet. Individuals might spend up to 18 h of their day in a postprandial state owing to the average number of daily meals [4]. Different studies have demonstrated that a high-fat meal (10–15.5 g of saturated fatty acids [SFAs]) may change lipid profile after 2 h [5,6] and increase blood pressure (BP) after 1 to 4 h [6].

Once these acute changes happen daily, there could be long-term implications on vascular health, and overall CVD risk can be affected

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[4]. Some dietary components, such as polyphenols (1.77 mg/g) [7] and dietary nitrate (250 mg/100 g) [8] present in red beet, for example, may regulate these changes in postprandial homeostasis, thereby reducing CVD risk [6,8].

Ninfali et al. [7] showed that beet leaves and stalks (BLS) have higher concentrations of polyphenols and total antioxidant capacity than beetroot. However, to our knowledge, studies investigating the effect of BLS on metabolism in humans have not yet been developed [9]. In addition, increasing consumption of this plant is an efficient way to encourage the use of all parts of food, minimizing the waste.

Therefore, the aim of this study was to evaluate the acute effect of organic BLS juice on lipemia, glycemic control, BP, and nitric oxide (NO) concentration in patients with dyslipidemia after consuming a high-fat meal.

Methods

BLS juice

Organic BLS were harvested weekly in a farm located in Goiania, Brazil. They were sanitized and the total phenolic content was analyzed by the Folin-Ciocalteu method [10]. The BLS were stored in plastic bags in a refrigerator at 4°C to be used during the week. Every week, the BLS quantities were readjusted according to the amount of polyphenols in that batch. Moreover, to ensure that all participants received the same amount of polyphenols, previous tests were performed to evaluate the maintenance of the amount of polyphenols in the BLS stored in a refrigerator during the week.

During the clinical trial, juices were prepared daily at two concentrations composed of 100 mL of mineral water and ~45.6 g of BLS and 100 mL of mineral water and ~101.1 g of BLS. The juices were filtered and stored in a refrigerator at 4°C until the moment of consumption. Because there are no human studies with BLS, the concentrations were chosen based on the amount of leaves that was considered viable for daily human consumption.

A previous study [11] observed that the main compound present in BLS extract was Vitexin-2-O-Rhamnoside (VR; MW 578) and vitexin derivatives. The quantification of VR was performed by high-performance liquid chromatography using the EXTRACT-US analysis system (FAPESP 2013/04304-4, patent pending), as described by Lorizola et al. [11]. Separation was performed on a Kinetex C18 column (150 × 4.6 mm, 2.6 um, Phenomenex, Torrance, CA, USA). The gradient of water with 0.1% of phosphoric acid (solvent A) to acetonitrile with 0.1% of phosphoric acid was the following: 2 min, 88% A; 4 min, 80% A; 6 min, 70% A; 8 min 40% A; 10 min, 20% A; 13 min, 20% A; and 14 min, 95% A. The equilibration time between runs was 3 min. Flow rate was 1.2 mL/min and injection volume was 5 L. Peaks were recorded and integrated at 320 nm. The software for the control of the system was developed by Kalatec (Campinas, Brazil). The ChromNav software from Jasco (Tokyo, Japan) was used for data acquisition and processing. The VR was identified by comparing the retention times of the peak obtained in the analysis of juices to the peak obtained in the analysis of the authentic standard. The calibration curve (6 points: 100, 50, 25, 2.5, 1, 0.5 ppm) of the compound was prepared by plotting concentration versus area. The concentration of the other compounds detected in the samples was expressed as vitexin 2-rhamnoside equivalents (VRE). The analysis was performed in duplicate.

The antioxidant capacity of juices was evaluated by oxygen radical absorbance capacity method according to Ou et al [12]. We added 30 μL of Trolox (12.5–400 μM) or the same amount of sample to 60 μL of fluorescein (508.25 μM) using a microplate. The plate was incubated at the temperature of 37°C during 30 min. After this period, 110 μL of 2,2′-Azobis(2-amidinopropane; 76 mM) were added and shaken for 10 s. The fluorescence signal was monitored every minute for 2 h at the emission wavelength at 528 ± 20 nm with excitation at 485 ± 20 nm in a plate reader system (BioTek, model Synergy). The net protection provided by the juices or standard was calculated using the difference between the area under the fluorescence decay curve in the presence of the sample (AUCantioxidant) and in its absence (AUCblank). Regression equations between net AUC and the concentration of the sample were calculated. Final results were expressed in mM Trolox equivalents/mg extract.

Participants

Initially, 47 men and women were recruited by announcements in social networks and in the Faculty of Nutrition at the Federal University of Goiás, Brazil, and 25 attended eligibility criteria. The inclusion criteria were a diagnosis of dyslipidemia and an age of 20 to 59 y. The exclusion criteria were use of insulin, antihypertensive, or lipid-lowering agents; hormone replacement; treatment for weight control; presence of diabetes, kidney disease, liver disease, or thyroid disorders; menopause; a cardiovascular event in the previous 6 mo; pregnancy; or breastfeeding. In addition, anthropometric measurements were evaluated, and blood samples were collected to analyze serum triacylglycerols (TGs), total cholesterol (TC), very low-density lipoprotein, low-density lipoprotein, and high-density lipoprotein (HDL) to confirm the reported dyslipidemia.

The current pilot study was performed in accordance with the principles in the Declaration of Helsinki. Ethical approval was obtained from the Ethics Committee

Table 1
Characterization of organic beet leaves and stalks juice phenolic profile

Analysis	Low dose (319.7 mg GAE/L)*	High dose (775.2 mg GAE/L)*
Phenolic total (mg VRE/L)†	105 ± 3.91	144.36 ± 5.18
Vitexin-2-O-rhamnoside (mg/L)	17.42 ± 0.33	22.32 ± 1.08
ORAC (mM Eq.trolox/g)	5.93 ± 0.78	12.15 ± 1.79

GAE, Gallic acid equivalent; ORAC, oxygen radical absorbance capacity; VRE, Vitexin-2-O-rhamnoside equivalent.
*Analyzed by Folin- Ciocalteu method.
†Analyzed by high-perfomance liquid chromatography.

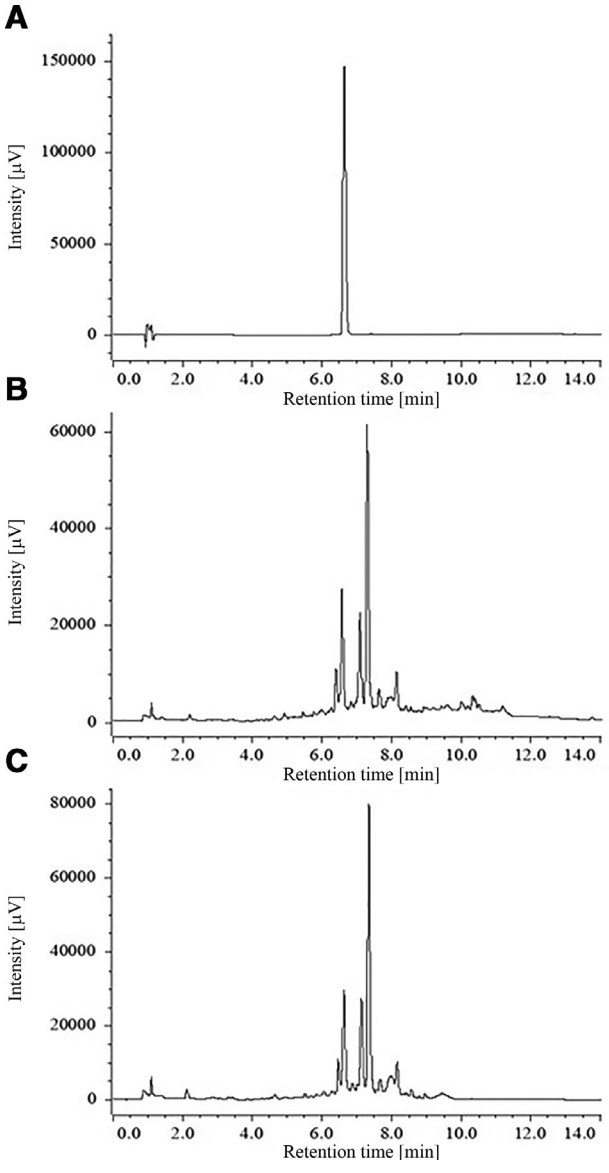

Fig. 1. Representative chromatogram of polyphenols present in BLS juice. (**A**) Vitexin-2-O-rhamnoside standard; (**B**) low-dose group; (**C**) dose 2: high-dose group.

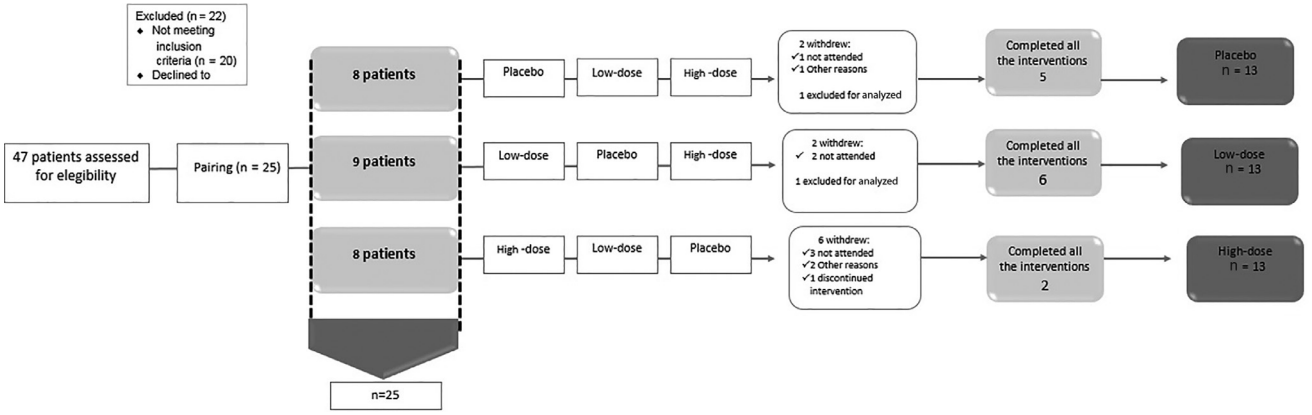

Fig. 2. Flowchart of the study participants.

of the Federal University of Goiás. The pilot study was registered at the Brazilian Registry of Clinical Trials. All participants provided written informed consent.

Study design and blood sampling

A randomized double-blind, placebo-controlled crossover pilot study was carried out with 25 individuals with dyslipidemia. Using the sample function of the R software, participants were randomized into three groups: the placebo group received flavored water; the low-dose group received ~32 mg polyphenols (319.7 mg/L); and the high-dose group received ~77.5 mg polyphenols (775.2 mg/L), with 1-wk washout period between each step. During the intervention, 10 patients withdrew and 2 were excluded. Thus, at the end of study, each group was composed of 13 participants.

A black cup with a lid and straw containing the juice had the same appearance for all groups. An independent research group not involved in the study was responsible for administering the juices according to the randomization protocol. Thus, the investigators and participants were blinded.

At each visit, BP was measured and then blood was collected from the peripheral vein after a 12-h fasting period. Individuals received a high-fat breakfast, which consisted of a pastel (fried dough) stuffed with pepperoni sausage, bacon, and cheddar cheese, and a coconut candy. This breakfast contained 720.6 kcal, 38.5 g (21%) of carbohydrates, 19.7 g (11%) of protein, and 54.2 g (68%) of total fat, including 17.7 g of saturated fat.

Within 5 min of the end of the meal, placebo or organic BLS juice was provided. New blood collections were performed 30, 60, 120, and 180 min after the first blood collection and BP was measured 60, 120, and 180 min after the first reading. The plasma and serum was separated for biochemical analysis and the other part was aliquoted into Eppendorf tubes and stored at −80°C for measurement of NO. The participants were instructed not to perform vigorous physical exercise 24 h before collection, nor drink alcohol 72 h before collection. They were also instructed to maintain their lifestyle during the study and to avoid antioxidant-rich food for 48 h before testing. To ensure that these instructions were followed, a list containing antioxidant-rich food was provided and possible alterations in dietary intake were monitored throughout the study.

Anthropometric and body composition

Body mass and height were measured (according to Heyward and Stolarczyk) [13] using a digital anthropometric scale (Filizola, Brazil), and body mass index was calculated. Waist circumference was measured according to Heyward and Stolarczyk [13]. Dual-energy x-ray absorptiometry (DXA) assessments of fat mass (FM), lean mass (LM), body fat percentage, and android and gynoid fat were conducted using a General Electric Lunar Prodigy scanner (DPX NT, GE) with Encore 2011 software (version 13.60, GE Healthcare, Chicago, IL, USA). The coefficient of variation for the dual-energy x-ray absorptiometry tests of LM and FM were 0.75% and 1.03%, respectively.

Blood pressure measurements

BP was measured as recommended by the American Heart Association [14] with an Omron pressure monitor (HEM-7200 model Kyoto, Japan).

Biochemical analyses

TC, HDL cholesterol (HDL-C), TG, glucose, and insulin were determined by the enzymatic colorimetric method, with an automatic System Vitros Chemistry 950 XRL (Johnson & Johnson, New Brunswick, NJ, USA) analyzer on the same day as the blood samples were collected.

Dosage of nitrite and nitrate in plasma

The nitrite and nitrate (NOx) concentrations were determined using the Griess reaction method and expressed as μmol/L. Samples were deproteinized with acetonitrile (1:1, v/v) and 50 μL of the supernatants were distributed in a 96-well plate and then equal volumes of Griess reaction solutions (1% sulfanilamide, 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride in 2.5% phosphoric acid) were added. The reaction was performed for 15 min at room temperature. The absorbance was read at 540 nm using an automatic microplate reader (BioTek Instruments, Inc, Winooski, VT, USA) [15].

Statistical analysis

Posteriori power calculation was performed based on change in HDL concentration, considering a Δ of 6, variance of 50, and absolute error (α) of 5%. The test power ($1-\beta$) was 99.8%.

For data distribution verification, the Lilliefors test was performed. The significance of carryover was evaluated, as recommended by Rosner [16]. Net change was obtained from the difference between each time and baseline. Thus, data were presented as mean ± SE and differences between groups in each point were determined by an analysis of variance (ANOVA) test, followed by Tukey post hoc test.

The area under the curve (AUC) was calculated by the trapezoidal method. Differences between groups were also determined by an ANOVA test, followed by Tukey post hoc test. Statistical analyses were performed using the R software, version 3.1.1.

Results

Characterization of organic BLS juice phenolic profile

VR was one of the major compounds found in the BLS juice, corresponding to ~16% of the total compounds detected. Moreover, a

Table 2
Baseline characteristics of participants (n = 13)

Variables	Value
Age, y	40.6 ± 2.3
Body mass index, kg/m^2	32.7 ± 1.5
Waist circumference, cm	96.8 ± 3.1
Body fat, %	46.2 ± 2.2
Total cholesterol, mg/dL	211 ± 9.8
HDL cholesterol, mg/dL	47.1 ± 3.3
LDL cholesterol, mg/dL	126.5 ± 8.8
VLDL cholesterol, mg/dL	37.4 ± 2.9
Triacylglycerol, mg/dL	187 ± 14.6
Glucose, mg/dL	89.8 ± 3.5
Insulin, μU/mL	11.5 ± 1.8
Diastolic blood pressure, mm Hg	73.4 ± 2
Systolic blood pressure, mm Hg	114.7 ± 3.6

HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL,very low-density lipoprotein.
Values are expressed as means ± SEMs.

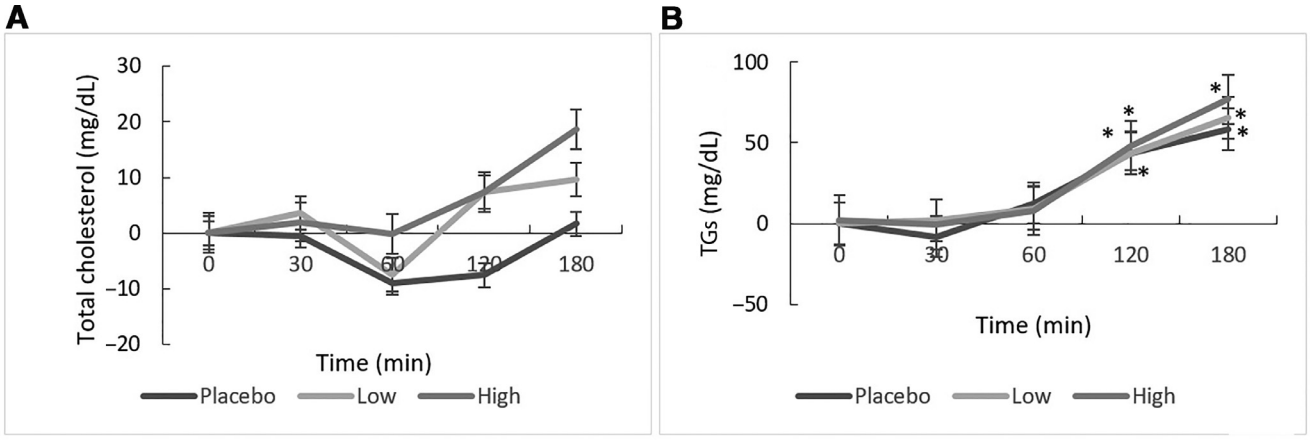

Fig. 3. Monitoring of changes in (**A**) total cholesterol and (**B**) TGs for participants receiving low (319.7 mg/L) and high (775.2 mg/L) levels of polyphenols from beet leaf and stalks juice compared with placebo group, after 30, 60, 120, and 180 min of diet intake with high lipid content. *Levels of significance ($P < 0.001$) comparing the value at 0 h to the values of the intervals within each group. Data are expressed as mean ± SEMs (n = 13 per group). TG, triacylglycerol.

higher antioxidant capacity was observed in the high-dose juice (Table 1 and Fig. 1).

Characteristics of volunteers

In the screening stage, 91 volunteers were recruited, from which 47 agreed to participate and 25 were selected according to the eligibility criteria. During the intervention, 10 patients withdrew: 1 for a surgical procedure, 6 because of difficulties with blood collection, and 3 for other reasons. Moreover, 2 patients were excluded from statistical analyses. As a result, 13 participants completed the study (Fig. 2). The baseline characteristics of the volunteers enrolled in this study are presented in Table 2. No carryover effect was found among the three periods for any variables.

Lipid profile

The high-fat meal induced an increase in TG levels after 120 ($P = 0.000$) and 180 min ($P = 0.000$) but none of the juices were able to attenuate these increases (Fig. 3B). Furthermore, a reduction of HDL-C after 60 min ($P = 0.003$), 120 ($P = 0.003$), and 180 min ($P = 0.027$) was observed in the placebo group and this reduction was attenuated after 120 min ($P = 0.005$) in the participants who received the organic BLS juice (Fig. 4A). Postprandial changes in TC did not differ among the groups at any time point (Fig. 3A), and no differences in AUC–HDL-C values ($P = 0.07$) were observed (Fig. 4B).

Glycemic profile and blood pressure

Postprandial glucose and insulin levels are shown in Figure 5A, B. There was an increase in glycemia concomitantly with an increase in insulin in all groups after 30 min.

There was a significant reduction of diastolic BP (DBP) within the low-dose group after 60 ($P = 0.004$), 120 ($P = 0.007$), and 180 min ($P = 0.004$). In the high-dose group, DBP was significantly reduced after 60 ($P = 0.011$) and 120 min ($P = 0.020$). However, postprandial changes in systolic BP (SBP), DBP, and NOx concentrations did not differ among groups (Fig. 5C, D).

Discussion

To our knowledge, this was the first clinical trial to evaluate the effect of BLS on postprandial lipemia, glycemic control, BP, and NO concentration in humans. The most important finding in this study was that the BLS juice was able to attenuate the reduction of HDL-C and the increase of BP induced by a high-fat meal.

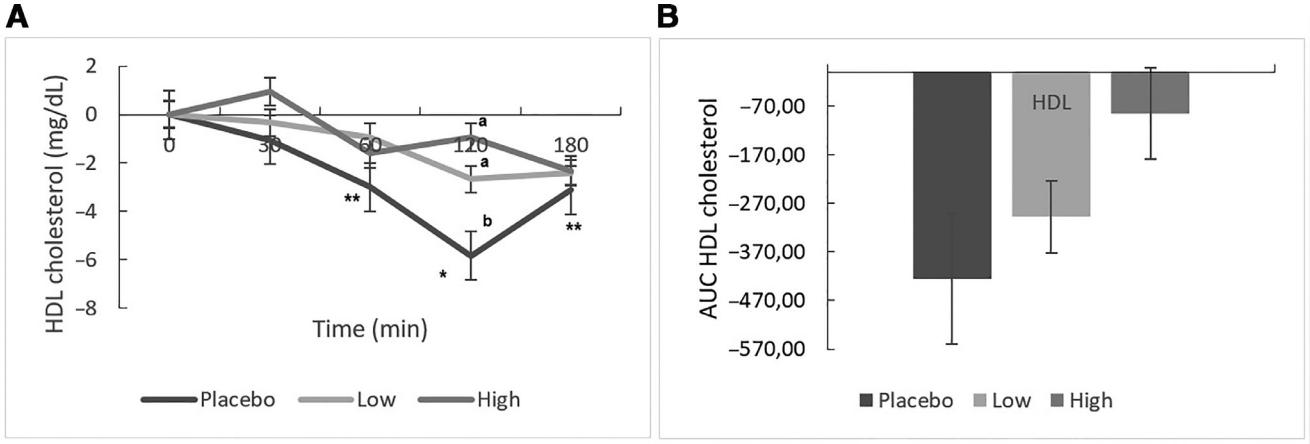

Fig. 4. Changes in HDL cholesterol for subjects receiving low (319.7 mg/L) and high (775.2 mg/L) levels of polyphenols from beet leaf and stems juice compared with placebo group after 30, 60, 120, and 180 min of dietary intake with high lipid content. (a, b) The mean value was significantly different among groups ($P < 0.01$). *Levels of significance ($P < 0.05$) and †Levels of significance ($P < 0.001$) comparing the value at 0 h to the values of the intervals within each group. Data are means ± SEMs (n = 13 per group). AUC, area under the curve; HDL, high-density lipoprotein.

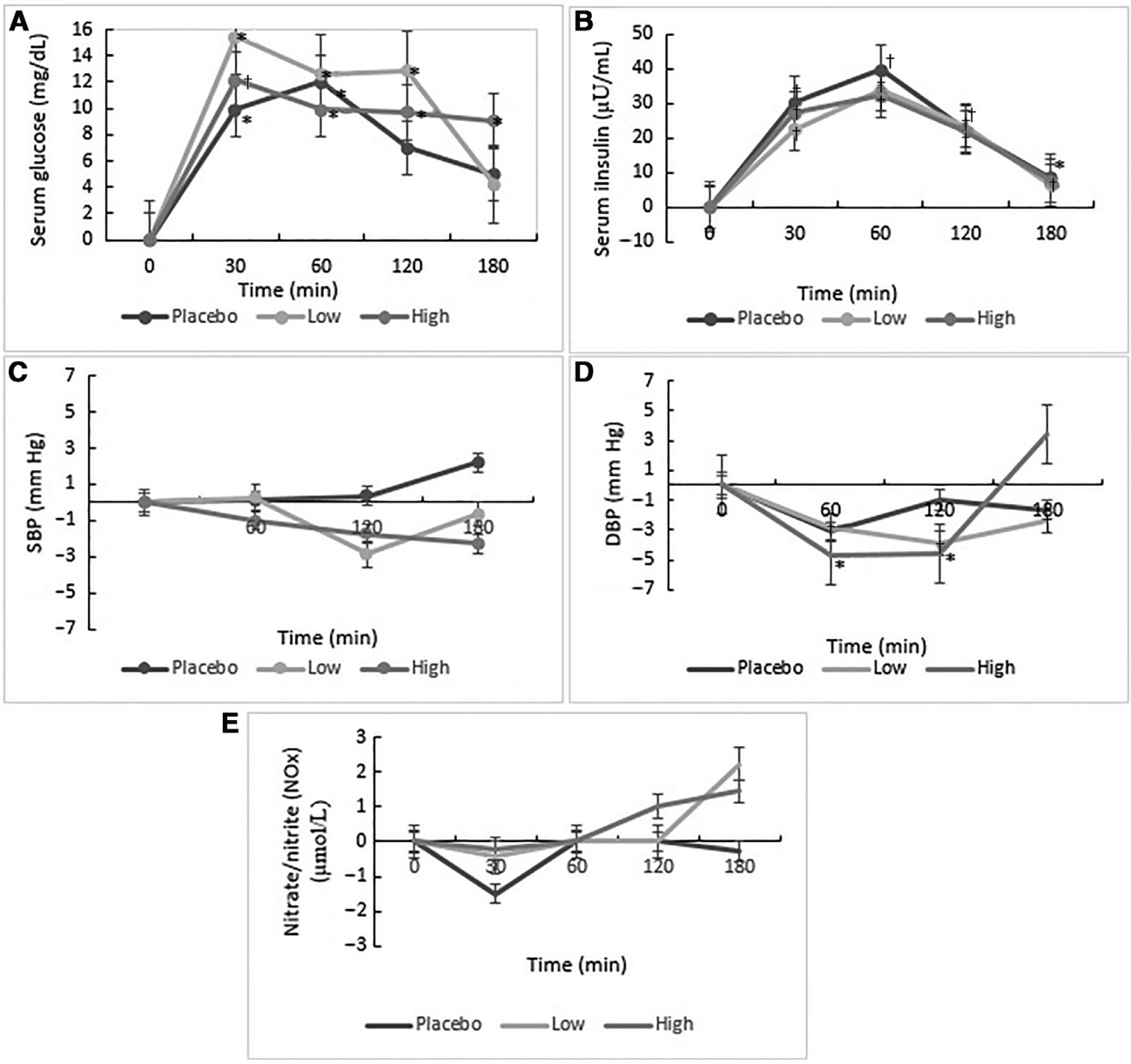

Fig. 5. (**A**) Changes in serum glucose, (**B**) insulin, (**C**) SBP, (**D**) DBP, and (**E**) NOx for participants receiving low (319.7 mg/L) and high (775.2 mg/L) levels of polyphenols from beet leaf and stems juice compared with placebo group after 30, 60, 120, and 180 min of dietary intake with high lipid content. *Levels of significance ($P < 0.001$) comparing the value at 0 h to the values of the intervals min within each group. †Levels of significance ($P < 0.05$). Data are expressed as means ± SEMs (n = 13 per group). DBP, diastolic blood pressure; NOx, nitrate/nitrite; SBP, systolic blood pressure.

The meal used in the present study was effective in promoting postprandial hypertriacylglycerolmia after 30 min, similar to other studies [5,17]. SFAs can stimulate sterol regulatory element-binding protein 1 c, a transcription factor that increases the expression of enzymes involved in fatty acid synthesis, promoting an increase in serum TG and very low-density lipoprotein [18]. However, BLS did not attenuate the increase in postprandial TG. Our finding is consistent with previous studies, which showed that polyphenols from a beverage of Asiatic plantain extracts and beetroot juice also did not prevent this increase of TG after a high-fat meal [6,17].

In contrast, BLS juice was able to attenuate the reduction of HDL, probably because of the presence of VR, a flavonoid glycoside derived from apigenin. Several studies have established a relationship between decreased postprandial HDL-C levels after a high-fat meal [19,20] and an increase in this lipoprotein concentration after treatment with dietary polyphenols [21–23]. An increase of TG-rich lipoproteins (TRLs) and a delay in their clearance resulting from a high-fat meal can activate cholesteryl ester transfer protein (CETP) and induce TG transfer from TRLs to HDL-C, and cholesteryl esters from HDL-C to TRL. This reshuffle in the lipid levels of HDL-C make its appropriate substrates for hepatic lipase, favoring the formation of lower HDL-C particles that are rapidly removed from circulation [24].

The increase in HDL-C levels as an effect of polyphenol intake can be explained by mechanisms such as an increased hepatic expression and secretion of apolipoprotein A1 [25] and inhibition of CETP activity [22,24]. Polyphenols from apple significantly inhibited CETP activity in vivo and were associated with increased HDL-C levels [22]. As apples [26], BLS juices are a source of flavonoid glycosides (dose 1: 17.42 ± 0.33 mg/L and dose 2: 22.32 ± 1.08

mg/L). Thus, based on this evidence, VR present in BLS may have reduced the increased CETP activity induced by a high-fat meal, thereby attenuating the decrease in HDL-C levels.

However, it is important to highlight that measuring HDL-C levels provides information about HDL pool, but does not predict HDL composition or function [27]. Some authors also suggest that HDL-C levels are affected by many different variables that also influence CVD risk [28]. Therefore, the assessment of HDL function is required to complement the measurement of HDL-C levels in the further studies.

No significant effects in the glycemic parameters were found. Similar results were reported by Hobbs et al. [8], who treated 24 healthy men in a crossover study with white bread or bread with 100 g of beet and did not observe significant effects on postprandial glycemia. However, overweight subjects were treated with a polyphenol-rich Asian plant tea (Mesona chinensis) and a significant reduction in postprandial blood glucose was verified [29]. However, the dose of polyphenols offered was superior 212.37 ± 5.64 mg EAG/g and type of polyphenol is different. Thus, it is feasible to suggest that the food matrix, the concentration and type of polyphenol and genetic characteristics of the population may explain these divergent results.

We observed a reduction in DBP within both groups that received the juice, but contrary to our expectations, no changes in serum NOx and DBP were found among groups. NO is an important vasodilator whose bioavailability may become compromised in the presence of lipids [6]. Therefore, the high-fat meal used in the present study may have reduced the NO bioavailability, contributing to the lack of significance among groups. However, we can observe a clinical relevance in the response of this biomarker because the NO concentration peak (T120) coincides with the reduction in SBP and DBP (T120). Moreover, this reduction might occur through the inhibition of the renin–angiotensin pathway by phenolic acids [30] or flavonoid glycosides derived from apigenin as vitexin (i.e., VR and Vitexin-2-O-xyloside) [31,32], the latter being present in BLS juice.

It is important to highlight that an increase in postprandial BP reflects a cardiovascular risk [33] and, therefore, strategies should be developed for the control, not only of BP, but also of the postprandial variability of this pressure.

This study's main limitation was the high dropout rate during the intervention period and the absence of analysis of NO in urine and feces. Strengths included thorough control in terms of behavioral confounders and the study design.

Therefore, beet leaves and stalks presented therapeutic potential once their juice was able to attenuate the reduction of HDL-C induced by a high-fat meal.

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