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Effects of betalains on atherogenic risk factors in patients with atherosclerotic cardiovascular disease

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This study evaluated the potential impacts of supplementation with betalain-rich extracts of foods on some atherosclerotic risk factors in coronary artery disease patients. During an acute phase, 48 male patients received about 50 mg betalain/betacyanin, and their blood and urine samples were collected at 3, 8, and 24 hours after supplementations. Also, in a pilot randomized crossover trial, these participants were allocated to two-week interventions (a betacyanin-rich supplement of *Opuntia stricta*, a betalain-rich supplement of red beetroot and a placebo) with two-week washout periods. Then, their plasma samples were collected at the baseline after a two-week period. The concentrations of betanin in plasma and urine samples were determined using HPLC. Also, homocysteine and glucose levels, lipid profile, and blood pressure were analyzed. Additionally, quality of life and dietary intake were assessed. After these interventions, minimal amounts of betanin were found in plasma and about 0.13–0.93% in urine. Also, both supplements significantly decreased the concentration of homocysteine, glucose, total cholesterol, triglyceride, and LDL. Also, betalain-rich supplements lowered both systolic and diastolic blood pressures. Nevertheless, the clinically meaningful changes were only found in the case of Hcy, LDL, and non-HDL-c concentrations. It seems that food sources of betalains can be considered as functional foods because they improve the lipid profile and levels of homocysteine, glucose, blood pressure, and quality of life to some extent.

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Introduction

Cardiovascular diseases (CVDs) and mainly coronary artery disease (CAD) are the first causes of mortality all over the world. Hypertension, diabetes, dyslipidemia, and obesity have been considered as modifiable risk factors of CAD and have close relationships with the quality of our diet and lifestyle. CAD as a macrovascular complication is one of the injurious effects of hyperglycemia in diabetes patients. CAD patients have elevated levels of homocysteine (Hcy) compared to healthy humans. Elevated levels of Hcy can induce the formation of reactive oxygen species (ROS), inflammation, and thrombogenicity.[1] Also, low-density lipoprotein (LDL) is a causal factor of atherosclerosis. Besides, high triglyceride (TG) and low high-density lipoprotein cholesterol (HDL-c) levels make individuals more prone to CVD. The European Atherosclerosis Society Consensus Panel recommends these high-risk people to implement lifestyle modifications together with attention to compliance with pharmacotherapy and secondary causes of dyslipidemia. If inadequately corrected, intensifying LDL-c lowering treatment may be considered.[2]

Lifestyle changes and healthful dietary choices could play active roles in the prevention/management of diabetes, hypertension, dyslipidemia, hyperhomocysteinemia, and CAD. It seems that red beetroot and *Opuntia stricta* (*O. stricta*) can be introduced as functional foods due to their health-promoting betalain content.[3,4] Betalains,[5] generally used as color additives, are composed of betalamic acid. Condensation of betalamic acid with amino acids/derivatives or imino compounds forms a variety of yellow-orange betaxanthins or red-violet betacyanins, respectively. Betalains are cationized antioxidants and have free radical scavenging and anti-inflammatory effects.[4] They improve the lipid profile and endothelial function, too.[4] The stabilized profile and content of betalains have

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been found in human physiological fluids, after the regular and long-term red beetroot intake.[6] In *O. stricta* fruits, only betacyanins (betanin and isobetanin) are detectable, and these fruits have a high betacyanin content (80 mg per 100 g fresh fruit) among the *Opuntia* cacti.[7] But, different amounts of betaxanthins (2.71–4.25 mg g^{-1} dm) and betacyanins (7.18–13.50 mg g^{-1} dm) are found in red beetroot varieties.[8] Thus, in this study, for the first time, we investigate the effects of two edible sources of betalains: *O. stricta* fruits (as a source of betacyanins) and red beetroot (as a source of betalains: betacyanin + betaxanthin) on hypertension, Hcy and glucose levels, lipid profile, quality of life, and dietary intake in CAD patients.

Materials and methods

Sample preparation

Fresh red beetroot and deep red-purple *O. stricta* were purchased from a local market, washed and sanitized using a chlorine solution (0.5%) and then passed through a juice extractor (Tefal, French). After that, −20 °C ethanol (99%) was added to the homogenized mixture and gently blended. Samples were filtered with Whatman paper, centrifuged (Eppendorf, Germany) at 4000*g* for 10 min and the resulting clear supernatants that contain about 0.7–1% of betalains (in red beetroot juice) or of betacyanins (in *O. stricta* juice) were sterilized by membrane filtration (0.22 μm). Then the obtained juices were passed through a rotary evaporator for removing ethanol using a vacuum of 72 mbar, a maximum temperature of 30 °C, and time below one hour. Finally, these concentrates were dried using a freeze dryer (Christ, Germany). By this procedure, about 1.1 ± 0.1 g betalain-rich powder and 1.4 ± 0.2 g betacyanin-rich powder were obtained from 100 g of fresh red beetroot and *Opuntia stricta*, respectively.

A DPPH (2,2-diphenyl-1-picrylhydrazyl) test was performed at 517 nm for analyzing the antioxidant power of 25 μg of prepared powders per 100 μl methanol. The inhibition percent of $DPPH^{\bullet}$ free radicals, which was measured against a control and compared with the standard curve of ascorbic acid (0–30 μM), was about 79 and 72.5% for betacyanin- and betalain-rich powder, respectively.

Finally, the lyophilized powder of *O. stricta* (483 mg) and red beetroot (491.5 mg) was used for the preparation of 500 mg capsule bodies. Each capsule of *O. stricta* and red beetroot contained approximately 25 mg of betacyanins and betalain, respectively. Also, placebo capsules contained only corn starch.

Quantification of betalains

The betalain concentration of the prepared powders was evaluated using UV-visible spectrophotometry (Spectrum, USA), the standard curve of betanin (Sigma Aldrich), and also, reversed-phase chromatography using HPLC instrument[9] (Agilent HPLC1200).

Briefly, the absorbance was recorded at the wavelengths of 536 and 474 nm for betacyanin and betaxanthins, respectively. Then, their concentrations were calculated using the Beer–Lambert equation based on the molar extinction coefficient of each pigment and standard of betanin (betacyanins: ε_{535} = 65 000 M^{-1} cm^{-1}, molecular weight = 551.48 g mol^{-1}, and betaxanthins: ε_{480} = 48 000 M^{-1} cm^{-1}, molecular weight = 339.304 g mol^{-1}).

For the HPLC analysis, all of the solvents and chemicals used in the HPLC method were of HPLC-grade (Fluka Chemie, Buchs, Switzerland). The betanin standard (Cat. No. 901266) and other chemicals were purchased from Sigma Aldrich and Merck, Germany, respectively. Before HPLC analysis, the standards and samples were filtered through a 0.2 μm filter to avoid the contamination of the HPLC system. Furthermore, the brown vials of HPLC were used to avoid light degradation. The samples were analyzed *via* reversed-phase chromatography using HPLC instrument. The retention times of the HPLC chromatogram were applied for qualitative analysis of betalains, and the area under a peak was employed to calculate their relative concentrations. HPLC analysis was performed using a Kinetex XB C18 column with a particle size of 2.6 μm and a pore size of 100 Å. Also, mobile phase A (0.1% formic acid in water), mobile phase B (0.1% formic acid in acetonitrile), and washing buffer (80% acetonitrile in water) were used as the elution and washing solvents. The gradient moves from 1.8% mobile phase B at the start to 80% of it at the end. Other details are mentioned in Table 1. Since both betaxanthins and betacyanins have had a considerable absorbance at the wavelength of 474 nm, the HPLC analysis was performed at the wavelength of 474 nm instead of 536 nm (Fig. 1).

HPLC analysis of plasma and urine samples was also conducted and checked in the same way.

Table 1 HPLC instrument setting

Instrument part	Item	Setting
Injector	Draw and eject speed	100 μL min^{-1}
	Draw position	2.2 mm
	Wait time after drawing	2.0 s
	Sample flush out factor	5.0
	Injection mode	Standard
	Injection volume	1 μL
	Stop time	As a pump
Binary pump	Flow	0.2 mL min^{-1}
	Stroke mode	Synchronized
	Low pressure limit	0 bar
	High pressure limit	500 bar
	Max flow ramp up	100 mL min^{-2}
	Max flow ramp down	100 mL min^{-2}
	Stop time	32 min
	Post time	4 min
	Column temp.	25 °C
Mobile phase	A	0.1 formic acid in H_2O
	B	0.1 formic acid in ACN
Gradient program	Time (min)	Component A/B
	Start	98.2% A : 1.8% B
	3 min	98.2% : 1.8% B
	20 min	88% A : 12% B
	25 min	70% A : 30% B
	28 min	20% A : 80% B
	30 min	20% A : 80% B
	32 min	98% A : 2% B

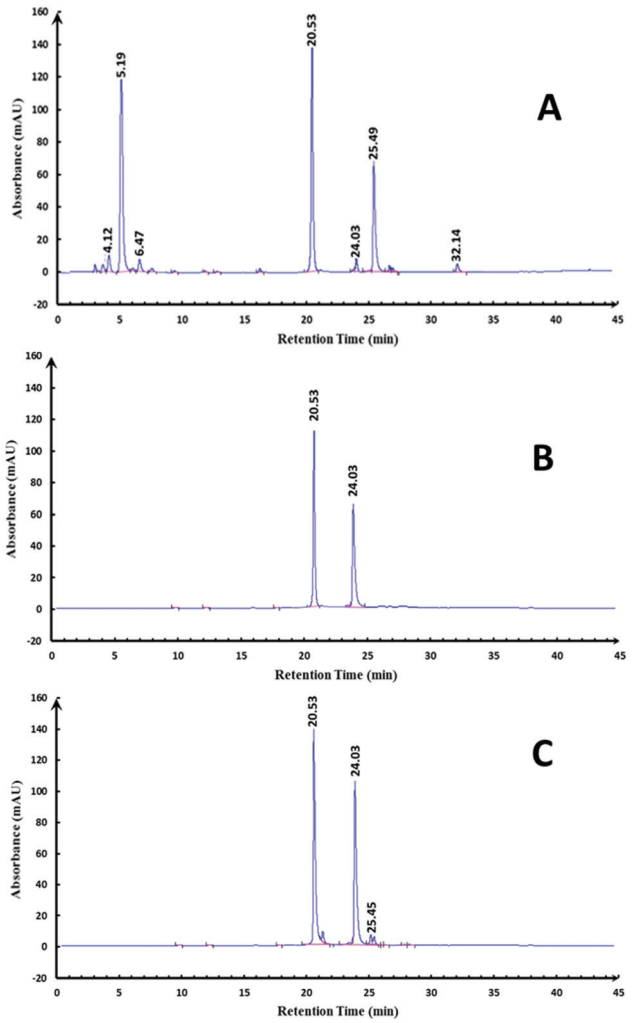

Fig. 1 HPLC chromatogram of betalain- and betacyanin-rich extracts and betanin standard. HPLC analysis was performed at the wavelength of 474 nm. A: Betalain-rich extract of red beetroot; the 4–6 min peaks present betaxanthins with the maximum absorption at 474 nm, and 20–25 min peaks present betacyanins with the maximum absorption at 536 nm. B: Betacyanin-rich extract of *Opuntia stricta*; the 20 min peak presents betacyanins with the maximum absorption at 536 nm. C: Betanin standard (Sigma Aldrich). The 20-, 24-, and 25 min peaks, with the maximum absorption at 536 nm, present the betanin, isobetanin, and betanidin, respectively, while the peak eluting with a retention time of 5 min, with the maximum absorption at 474 nm, shows vulgaxanthin I.

Subjects

Non-smoker men (45–55 years, BMI: 25–35 kg m^{-2}) with CAD for at least six months (with no myocardial infarction experience) were recruited for this study. They had no current or previous history of any other acute or chronic illness requiring prescription medication and never consumed alcohol, tobacco, and steroid/anticoagulant drugs. Subjects were excluded if they displayed any of the following: allergy, inflammatory conditions, any changes in drug administration, supplementation with vitamins or antioxidants, fasting, and strenuous exercise. Since menstrual changes in female patients might affect their mood and especially their responses to the emotional questions of quality of life questionnaire, only males have been recruited for the study.

Participant recruitment

Participants were recruited from cardiologist referrals at the Shahid Madani Medical Research Training Center of Iran. The study lasted from November 2017 to March 2019. These patients were treated according to the currently accepted guidelines for the secondary prevention of CAD. After conducting eligibility screening over telephone, additional information about the study were provided to volunteers. Fig. 2 demonstrates the flow diagram of the study. Among 57 volunteers, 52 of them were eligible and fulfilled the inclusion criteria. Informed consent was obtained from human participants of this study. Anthropometric measurements, blood pressure measurement, fasting blood glucose test, and routine laboratory analyses of fasting blood samples were performed after obtaining the answers of a standard medical history questionnaire. Out of 52 individuals, 4 participants were withdrawn during the trial. One subject was excluded due to gastrointestinal discomfort, another was excluded due to taking an antibiotic drug for treating his tooth abscess, and two subjects declined to participate without any explanation. Therefore, 48 subjects completed this trial.

Study design and intervention

The study followed a randomized, double-blind, crossover design and has been registered at the Iranian Registry of Clinical Trials website as IRCT2017092836469N1. All experiments were performed in accordance with the guidelines of Helsinki, and experiments were approved by the ethics committee at Tarbiat Modares University (approval ID: IR.TMU.REC.1396.609). This study was carried out in two steps. In step I, after a one-week washout period (the diet of patients was free of any product containing betalains), patients received a single dose of the betalain-rich supplement of red beetroot (containing 50 mg betalain) and the betacyanin-rich supplement of *Opuntia stricta* (containing 50 mg betacyanin). Blood and urine samples were collected after 3, 8, and 24 hours of supplement intake. Step II of the study lasted for ten weeks and was divided into five periods. Subjects were allocated *via* random block assignment using STATA 14 software-generated random codes devised by a statistician to study arms where each arm consisted of a sequence of two treatments given consecutively. Participants received three treatments (one capsule of the betalain-rich supplement of red beetroot, the betacyanin-rich supplement of *Opuntia stricta*, and the placebo; twice daily for two weeks) on three occasions which were separated by at least two-week washout periods (Fig. 2). Since each capsule contained 25 mg of betalains/betacyanins, participants received a safe dose of about 50 mg day^{-1} of betalains/betacyanins in line with the previous clinical trials.[8,10,11] Subjects were randomly assigned (by the chief investigator) to consume capsules, preferably with lunch and dinner. Patients who consumed capsules of red beetroot in the

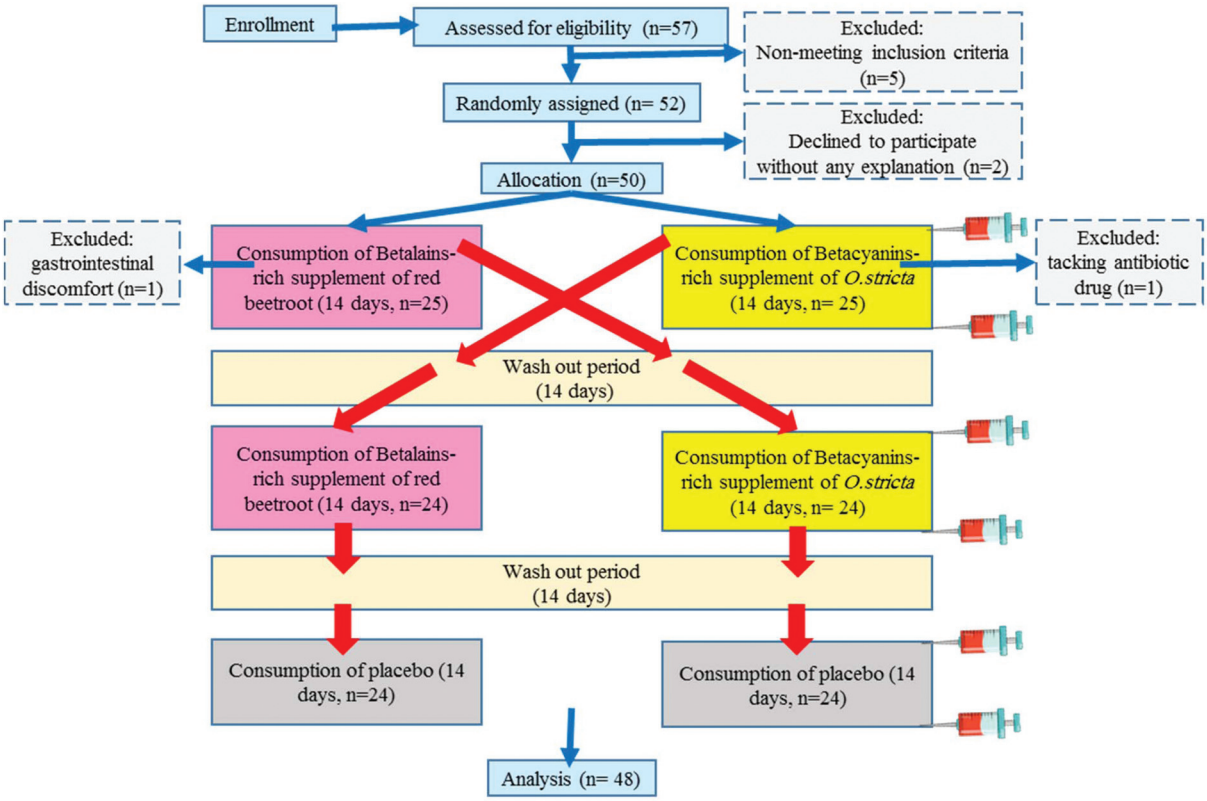

Fig. 2 Consort diagram of the study (phase II).

first two weeks received the capsules of *O. stricta* in the third two weeks and *vice versa*. Finally, both groups consumed placebo capsules which contained only corn starch, after at least a two-week washout period. One week before and during the study, subjects were on their daily diet and asked to abstain from changes in their diet, lifestyle factors, physical activity, and medications. Also, they were instructed to avoid consuming products containing betalain pigments, high-antioxidant supplements, tobacco, alcohol, high caffeine, and processed meats. All individuals, except one investigator, were blinded to the treatment allocation. Moreover, all capsules and their bottles were identical in appearance to blind individuals. Each patient completed a total of 8 visits to the Shahid Madani hospital. All of the visits were scheduled in the morning, at the beginning and the end of each intervention. Adherence to the study protocol was verified at all the visits *via* a standard questionnaire. The subjects were also contacted by telephone during the period of the study.

Blood and urine collection

All samples were collected after a 12 hour fasting period, on days 0, 14, 28, 42, 56 and 70, in EDTA containing tubes appropriate for plasma isolation. Then, plasma was separated using a refrigerated centrifuge (10 minutes at 1000–2000*g*). Aliquots of plasma samples were frozen at −80 °C in sterile tubes until the analyses were performed. Urine samples were collected at 3, 8, and 12 hours after a single dose consumption of supplements and also after a 12 hour fasting period at the baseline and the end of two-week interventions.

Biochemical analysis

Plasma TG, total cholesterol (TC), and HDL-c were measured photometrically by enzymatic methods using commercial kits (Pars Azmoon Co, Tehran, Iran). The Friedewald formula was used to determine LDL-c levels. Besides, plasma fasting blood glucose (FBG) and Hcy were measured using a spectrophotometric-based kit (Pars Azmoon Co, Tehran, Iran) and a sandwich ELISA kit (LS-F10591, LifeSpan BioSciences, Seattle, WA, USA), respectively.

Blood pressure (BP) measurements

Systolic and diastolic blood pressures (SBP and DBP) were measured three times in subjects who sat quietly for at least 5 minutes in a chair, with feet on the floor, and arms above the heart level. They avoided caffeine, exercise, and smoking for at least 30 minutes before measurement in order to ensure accuracy. The average of two measurements[12] was recorded using a large Omron's monitor (Omron M2 Basic, UK).

Anthropometric measurements

Anthropometric measurements included height, weight, and BMI. Height was measured to the nearest 0.1 cm using a stadiometer fixed to a wall. Weight was measured using Omron scales (Omron HBF-508 weighing scale, Japan) and recorded to

the nearest 0.1 kg. Participants were asked to remove all extra clothes and take off their shoes. Body Mass Index (BMI) defined as the weight in kilograms, which is divided by the square of the height in meters, was also recorded. According to the WHO reports, overweight or class I of obese adults have a BMI of 25–34.99 kg m^{-2}.

Assessment of quality of life (QoL), physical activity and dietary intakes

Measurement of changes in health-related quality of life is an essential aspect in assessing secondary prevention programs in heart patients. In this study, it was evaluated using a validated MacNew instrument,[13] which is the most appropriate measure to investigate changes in the quality of life following various interventions of ischemic heart disease. Also, the validated International Physical Activity Questionnaire was used for the assessment of physical activity (low: <600, moderate: 600–3000 and high: >3000 (metabolic equivalents × minutes per week)) at the baseline. Furthermore, dietary intakes were assessed using 24 h dietary recalls completed for three days (two weekdays and one weekend day) per week. Energy and macronutrient compositions were analyzed using Nutritionist IV software (the Hearst Corporation, CA).

Statistical analysis

Power analysis for the paired *t*-test on the primary outcome of this trial (SIRT1 expression levels) showed that a total sample size of 24 subjects (α error = 0.05 and power = 0.90) is required.[14] Changes (%) of this value after supplementation with betalain- and betacyanin-rich supplements compared to the baselines were −23 and −25%, respectively. Results of SIRT1 analysis are not reported here. All of the tests were replicated three times under similar conditions. Pre- and post-intervention characters were reported through both descriptive ((means ± SDs) or (number or percent when appropriate)) and inferential statistics (means ± SEMs). The normality of the data was tested using the Kolmogorov–Smirnov test and also *via* Skewness and Kurtosis measures. Intra-group comparisons were made using the 2-tailed paired Student's *t*-test and Wilcoxon test for normal and non-normal data, respectively. Inter-group comparisons were assessed using the one-way analysis of variance (ANOVA) for normal baseline data, the Kruskal–Wallis for non-normal pre-treatment variables, and the analysis of covariance (ANCOVA) tests for post-intervention data. Within- and between-group differences were expressed as means and 95% CIs. The two-way ANOVA for repeated measures was performed for comparison between the measures obtained by changes in the sequence of treatments (carryover effect or interaction effect), between before and after treatments with the betacyanin- and betalain-rich supplements (time effects), as well as between the effects of the betacyanin- and betalain-rich supplements (treatment effect) on the studied variables. The two-tailed *P* values <0.05, and the variable changes >−10 to 10 percent were considered as statistically and clinically significant (Fig. 3), respectively. All analyses were performed using Graph Pad Prism software (San Diego, CA, USA), version 7, and SPSS software (Chicago, IL, USA), version 25 for windows.

Results

Quantification of betalains

The results indicate that there are two main groups of peaks with retention times of around 5 and 20 min. The UV-visible spectrum of these peaks suggested that the 20- and 25 min peaks comprise betacyanins with the maximum absorption at 536 nm, while the 4-, 5-, and 6 min peaks are composed of betaxanthins with the maximum absorption at 474 nm. Furthermore, the percentages of betaxanthin and betacyanin contents of the provided extracts have been determined using the area under the curve analysis. Accordingly, the red beetroot extract was recognized as a mixture of betaxanthins and betacyanins (40.86 ± 1.05% and 59.13 ± 1.21% of the total pigments, respectively). However, the extract of *O. stricta* had only betacyanin components. The UV-visible spectrum of the 20 min peak (Fig. 1) confirms that it might be betanin because of its maximum absorption at 536 nm. Also, the comparison of this peak with the betanin standard's main peak proves that the 20 min peak in both curves belonged to betanin. Furthermore, the 4 min peak which emerged only in red beet was visible at 474 nm, and it must be belonging to betaxanthins. Interestingly, the UV-visible spectrum of this emerged peak strongly confirms this idea, as this peak has a maximum absorbance at the wavelength of 474 nm and a minimum absorbance at 530 nm.

Finally, the lyophilized powder of *O. stricta* contained 5.18% (w/w) betacyanins and that of red beetroot contained 5.09% (w/w) betalains (3.01% w/w of betacyanins + 2.08% w/w of betaxanthins). Also, the main compound found in both supplements was betanin which covered almost 32.4 (about 16.2 mg) and 70 (about 35 mg) % of total betalains (50 mg) identified in the betalain-rich supplement of red beetroot and the betacyanin-rich supplement of *O. stricta*, respectively.

Furthermore, before a single dose consumption of supplements, betalains were not found in the urine and plasma samples demonstrating that the one-week washout period with a betalain-free diet was sufficient and adequate. Also, after acute and short-term consumption of betalain-rich supplements of red beetroot and betacyanin-rich supplements of *Opuntia stricta*, a tiny amount/nothing of betanin was present in blood plasma. Also, 24 hours after supplementations, betanin was almost undetectable in plasma and urine samples. However, urine contained betanin after eight hours and two weeks. Table 3 shows the results.

Biochemical and clinical analysis

The screening characteristics of participants who completed the study are presented in Table 2. Baseline characteristics were similar in both groups. The interventions were well-tolerated and without adverse effects. Also, no significant change was found after placebo consumption. Table 4 and Fig. 3 show the results.

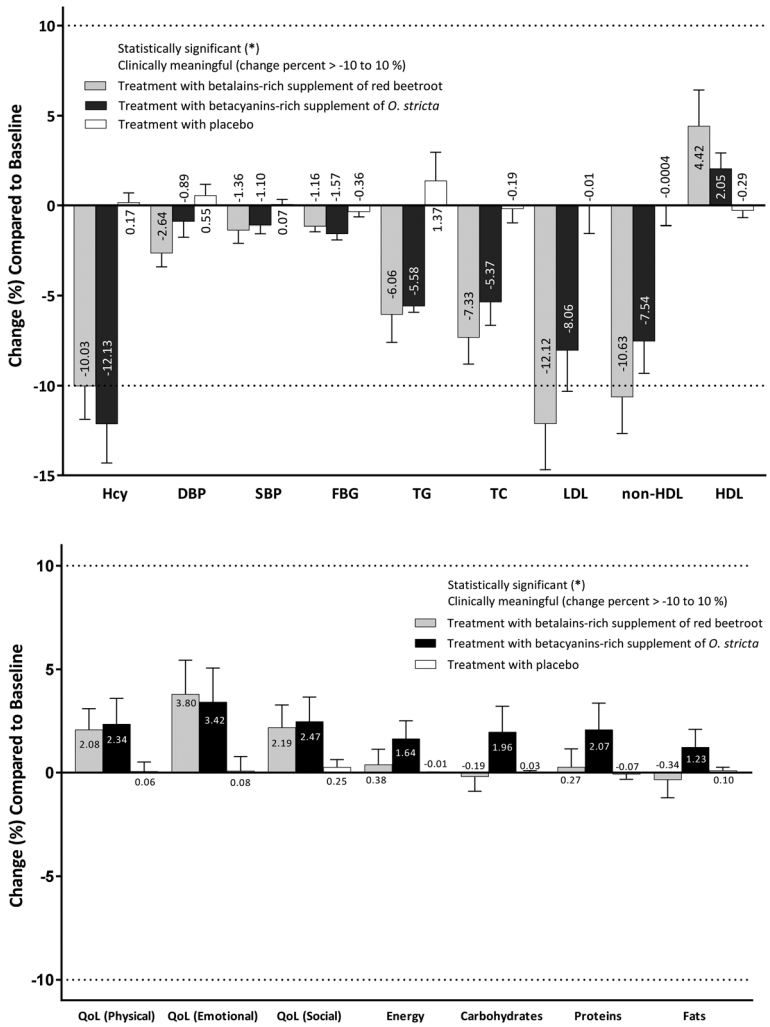

Fig. 3 The effect of two weeks' treatments on the study variables. Percentage change = $((value_{after} - value_{before})/value_{before}) \times 100$.

Table 2 Baseline characteristics of patients

Variables	Group 1 (n = 24) (Red Beet first)	Group 2 (n = 24) (*O. stricta* first)	P
Age (year)	48.91 ± 2.02	50.03 ± 2.08	0.189[a]
Weight (kg)	82.08 ± 14.37	83.70 ± 12.96	0.773[a]
Height (m)	1.63 ± 0.11	1.65 ± 0.21	0.654[a]
BMI (kg m^{-2})	30.64 ± 3.47	30.51 ± 2.73	0.858[a]
First diagnosis of CAD (year)	1.22 ± 0.81	1.11 ± 0.26	0.518[a]
Physical activity			
Low	5 (20.83)	6 (25.00)	
Moderate	16 (66.66)	17 (70.83)	>0.999[b]
High	3 (12.50)	1 (4.16)	
Secondary prevention drug treatment			
Anti-hypertensive medication use	15 (62.50)	18 (75.00)	>0.999[b]
Lipid-lowering medication use	20 (83.33)	17 (70.83)	>0.887[b]

Abbreviations: BMI: body mass index; CAD: coronary artery disease; *O. stricta*: *Opuntia stricta*; Red Beet: red beetroot. All data were presented as means ± standard deviation or N (%) when appropriate. [a] Between group comparison (unpaired t-test/Mann–Whitney test). [b] Comparison of frequencies between two groups (Fisher's exact test).

Biochemical analysis. The plasma concentrations of Hcy (P < 0.0001), FBG (P < 0.0001), non-HDL-c (P < 0.0001), TG (P < 0.0001), total cholesterol (TC) (P < 0.0001), and LDL-c (P = 0.0004 for *O. stricta*, and P = 0.0002 for red beetroot) and the ratios of TC/HDL-c (P < 0.0001) and LDL-c/HDL-c (P < 0.0001) significantly decreased after both supplementations. Besides, HDL-c concentration increased after the consumption of red beetroot and *O. stricta*; however, this elevation was not statistically significant (P = 0.063 for *O. stricta* and P = 0.062 for red beetroot). Nevertheless, the clinically significant changes (Fig. 3) were only found for Hcy (both treatments), non-HDL-c (betalain-rich supplement), and LDL (betalain-rich supplement) concentrations. There were no carryover effects between treatments (sequence of the betalain-rich supplement and betacyanin-rich supplement; Table 4, the P-value for interaction) and no significant difference between the effects of two supplementations (Table 4, the P-value for the treatment effect) for any of the variables. Furthermore, no significant change was found after placebo consumption. Table 4 and Fig. 3 show the results.

Blood pressure measurement. Table 4 and Fig. 3 demonstrate the results of blood pressure measurements. On the one side, SBP considerably declined after both treatments ($P < 0.0001$), without any significant difference between the effects of two supplementations (Table 4, the *P*-value for treatments effect), on the other side, DBP significantly decreased after red beetroot intake ($P = 0.0004$). No significant changes in blood pressure were observed after the consumption of *O. stricta* ($P = 0.188$). Nevertheless, these changes were not clinically meaningful (Fig. 3). Also, no carryover effect was found between interventions (Table 4, the *P*-value for the interaction of treatments = 0.572 for SBP, and 0.179 for DBP).

Anthropometric parameters. Table 4 shows the results of the anthropometric measurements. There were no significant changes in the BMI of the participants.

Quality of life and dietary intakes. No statistically and clinically meaningful changes were observed in the dietary intakes as well as the quality of life of the patients. Significant differences were observed between the effects of two supplementations (Table 4, the *P* value for treatments effect) only for energy, carbohydrates, and protein scores (dietary intake). Furthermore, no carryover effect (Table 4, the *P* value for interaction) was found between both treatments. Table 4 and Fig. 3 present the results.

Discussion

This is the first clinical trial that is in line with the previous *in vitro* and pre-clinical studies conducted to explore the consumption effects of red beetroot and prickly pears, especially their betalain content in atherosclerosis. Herein, we found that two-week consumption of the betalain- and betacyanin-rich supplements have the potential to improve the lipid profile, blood pressure, and homocysteine and glucose levels in CAD patients.

Also, our study showed that after a single dose intake of the betalain-rich supplement of red beetroot and the betacyanin-rich supplement of *O. stricta*, the plasma concentration of betanin is almost unmeasurable. In the case of urine samples, almost similar to the study of Frank *et al.* (2005), in volunteers who consumed a single dose of red beet juice,[15] about 0.13% (after 3 h) and 0.21% (after 8 h) of total betanin was detected (Table 3). Also, after two weeks' consumption of supplements, about <1–3 μg and 120–325 μg of betanin were present in the plasma and urine samples, respectively. These outcomes are in line with Sawicki *et al.* whose study investigated the content and profile of betalains in physiological fluids of participants who consumed a fermented red beet juice (0.7 mg of betalains per kg body weight) for six weeks.[6] Most importantly, Sawicki *et al.* found that after regular and long-term consumption of betalains, native betalains and their dehydrogenated/decarboxylated metabolites, mainly glycoside derivatives, are present in physiological fluids.

Although there has been no study about the effects of betalains on the circulating levels of homocysteine, the present trial indicated that consumption of betalain-/betacyanin-rich extracts significantly reduced Hcy concentration in CAD patients. There is an independent and positive association between Hcy concentrations and CVD risk. Hcy likely affects CAD through several mechanisms including oxidative stress,[16] activation of pro-inflammatory factors,[17] oxidative modification of LDL-c, inhibition of ApoA-I protein expression,[18] reduction of HDL-c concentration,[18,19] and formation of procoagulant compounds and thrombotic events.[20] However, HDL-c independently counteracts the adverse effects of Hcy in CAD patients.[18] A study of 8335 US adults shows that higher uptake of antioxidants lowers the concentrations of Hcy and CRP in blood and has beneficial effects on CVD.[16] Betalains are potential antioxidant[21,22] and anti-inflammatory agents.[4,23–25] It appears that treatments with betalains can reduce Hcy and alleviate the Hcy-related mentioned mechanisms. For example, in several human studies, consumption of *Opuntia ficus indica* fruit juice or betalain-rich red beetroot extract significantly reduced inflammatory factors[26] and had positive effects on the body's redox balance and reduced lipid oxidation, and these effects may be due to their betalain content.[27–29] Similarly, an *in vivo* study showed that the presence of both natural and semi-synthetic betalains in the medium of *Caenorhabditis elegans*, with an ED_{50} value up to 10 μM for betaxanthins and around 25 μM for betacyanins, reversed the oxidative stress caused in the fluorescent strain TJ375.[30] Likewise, betalain-enriched LDL are more resistant than homologous native LDL to oxidative stress.[31]

In the present study (Table 4 and Fig. 3), betalain supplementations significantly reduced TC, TG, non-HDL-c, and LDL concentrations, and also, the ratios of TC/HDL-c (Castelli I) and LDL-c/HDL-c (Castelli II). Two inventors patented a group

Table 3 The concentration of betanin in the plasma and urine samples after the consumption of supplements

Betanin (%)		After a single dose consumption of supplements			After 2 weeks' consumption of supplements
		3 h	8 h	24 h	
Betalain-rich supplement of red beetroot, contained 50 mg betalain (about 16.2 mg betanin)	**Plasma**	nd	<1 μg	nd	<1 μg
	Urine	21 μg	34 μg	nd	120 μg
Betacyanin-rich supplement of *O. stricta*, contained 50 mg betacyanins (about 35 mg betanin)	**Plasma**	nd	<3 μg	nd	<3 μg
	Urine	47 μg	74 μg	nd	325 μg

nd: not detectable.

Table 4 The effects of two weeks' interventions on the study variables

Variables	Pre-intervention (n = 48)	Post-intervention (n = 48)	P^a	Differences[b]	P for treatment[c]	P for time[d]	P for interaction[e]
HDL-c (mg dl^{-1})							
O. stricta	45.17 ± 6.26 [f]	45.92 ± 0.79 [g]	0.063	0.75 (−0.04, 1.54)	0.357	0.013	0.406
Red Beet	44.17 ± 8.66	45.65 ± 1.17	0.062	1.48 (−0.08, 3.04)			
Placebo	46.00 ± 7.51	45.90 ± 1.12	0.520	−0.10 (−0.42, 0.22)			
P^h	0.493	0.169					
Non-HDL-c (mg/dl)							
O. stricta	142.90 ± 41.45	131.50 ± 5.95	<0.0001	−11.33 (−16.43, −6.23)	0.734	<0.0001	0.279
Red Beet	144.10 ± 43.30	127.80 ± 5.72	<0.0001	−16.38 (−23.61, −9.14)			
Placebo	140.10 ± 41.85	138.90 ± 5.62	0.406	−1.20 (−4.11, 1.69)			
P^h	0.854	<0.001					
LDL-c (mg dl^{-1})							
O. stricta	112.20 ± 40.41	102.60 ± 5.93	0.0004	−9.64 (−14.68, −4.60)	0.926	<0.0001	0.268
Red Beet	114.50 ± 41.88	99.64 ± 5.69	0.0002	−14.85 (−22.18, −7.52)			
Placebo	110.50 ± 39.79	109 ± 5.44	0.324	−1.44 (−4.36, 1.47)			
P^h	0.889	0.002					
LDL-c/HDL-c							
O. stricta	2.55 ± 1.00	2.28 ± 0.14	<0.0001	−0.26 (−0.39, −0.13)	0.500	<0.0001	0.223
Red Beet	2.69 ± 1.10	2.27 ± 0.15	<0.0001	−0.42 (−0.64, −0.20)			
Placebo	2.46 ± 0.92	2.45 ± 0.13	0.736	−0.01 (−0.07, 0.05)			
P^h	0.529	<0.001					
TC (mg dl^{-1})							
O. stricta	188.00 ± 40.53	177.40 ± 5.83	<0.0001	−10.58 (−15.58, −5.58)	0.612	<0.0001	0.334
Red Beet	188.30 ± 43.43	173.40 ± 5.69	<0.0001	−14.90 (−21.76, −8.03)			
Placebo	186.10 ± 41.81	184.80 ± 5.55	0.368	−1.31 (−4.21, 1.59)			
P^h	0.963	0.001					
TC/HDL-c							
O. stricta	4.26 ± 1.13	3.94 ± 0.15	<0.0001	−0.490 (−0.72, −0.25)	0.501	<0.0001	0.233
Red Beet	4.41 ± 1.24	3.92 ± 0.16	<0.0001	−0.322 (−0.46, −0.18)			
Placebo	4.15 ± 1.07	4.14 ± 0.15	0.963	−0.002 (−0.07, 0.07)			
P^h	0.533	<0.001					
TG (mg dl^{-1})							
O. stricta	153.10 ± 52.09	144.70 ± 7.21	<0.0001	−8.45 (−9.87, −7.03)	0.206	<0.0001	0.642
Red Beet	148.30 ± 47.39	140.60 ± 7.13	<0.0001	−7.60 (−11.10, −4.10)			
Placebo	148.30 ± 46.81	149.50 ± 6.84	0.600	1.18 (−3.33, 5.71)			
P^h	0.853	<0.001					
FBG (mg dl^{-1})							
O. stricta	87.58 ± 8.37	86.17 ± 1.16	<0.0001	−1.41 (−1.97, −0.86)	0.334	<0.0001	0.123
Red Beet	87.65 ± 8.76	86.58 ± 1.20	<0.0001	−1.06 (−1.55, −0.56)			
Placebo	87.9 ± 8.50	87.52 ± 1.15	0.140	−0.37 (−0.87, 0.12)			
P^h	0.982	0.009					
Hcy (μmol l^{-1})							
O. stricta	22.74 ± 2.89	19.91 ± 0.53	<0.0001	−2.82 (−3.72, −1.92)	0.070	<0.0001	<0.204
Red Beet	21.99 ± 2.62	19.76 ± 0.48	<0.0001	−2.23 (−2.98, −1.48)			
Placebo	21.94 ± 2.53	21.97 ± 0.37	0.137	0.03 (−0.19, 0.26)			
P^h	0.307	<0.001					
SBP (mmHg)							
O. stricta	126.9 ± 11.87	123.8 ± 1.50	<0.0001	−3.16 (−4.32, −2.01)	0.770	0.0028	<0.572
Red Beet	127.6 ± 13.13	121.3 ± 1.52	<0.0001	−6.29 (−8.09, −4.48)			
Placebo	127.90 ± 11.53	128.00 ± 1.66	0.960	0.06 (−0.56, 0.69)			
P^h	0.739	0.037					
DBP (mmHg)							
O. stricta	83.99 ± 8.00	83.02 ± 1.02	0.188	−0.97 (−2.44, 0.49)	0.919	<0.0001	<0.179
Red Beet	84.81 ± 7.19	82.29 ± 0.67	0.0004	−2.51 (−3.85, −1.18)			
Placebo	84.42 ± 7.07	84.75 ± 0.92	0.506	0.33 (−0.66, 1.33)			
P^h	0.653	0.008					
QoL (emotional)[i]							
O. stricta	4.21 ± 0.91	4.32 ± 0.12	0.105	0.11 (−0.01, 0.24)	0.232	0.047	0.681
Red Beet	4.10 ± 1.08	4.19 ± 0.13	0.061	0.08 (−0.04, 0.20)			

Table 4 *(Contd.)*

Variables	Pre-intervention (n = 48)	Post-intervention (n = 48)	P^a	Differences[b]	P for treatment[c]	P for time[d]	P for interaction[e]
Placebo	4.10 ± 1.09	4.09 ± 0.15	0.943	−0.009 (−0.05, 0.03)			
P^h	0.608	0.165					
QoL (physical)[i]							
O. stricta	4.12 ± 0.89	4.19 ± 0.12	0.078	0.06 (−0.02, 0.16)	0.872	0.045	0.964
Red Beet	4.15 ± 0.93	4.21 ± 0.13	0.069	0.06 (−0.01, 0.14)			
Placebo	4.22 ± 0.89	4.22 ± 0.13	0.220	0.001 (−0.03, 0.03)			
P^h	0.644	0.124					
QoL (social)[i]							
O. stricta	4.34 ± 0.99	4.40 ± 0.12	0.188	0.06 (−0.02, 0.15)	0.638	0.123	0.883
Red Beet	4.32 ± 1.11	4.37 ± 0.14	0.329	0.05 (−0.03, 0.14)			
Placebo	4.25 ± 0.97	4.26 ± 0.14	0.475	0.01 (−0.02, 0.04)			
P^h	0.833	0.430					
Energy[j] **(kcal d^{-1})**							
O. stricta	2418 ± 381.80	2448 ± 50.37	0.128	30.57 (−9.18 70.32)	0.006	0.226	0.345
Red Beet	2516 ± 310	2521 ± 42.78	0.809	4.78 (−34.93, 44.50)			
Placebo	2463 ± 322.7	2463 ± 46.63	0.589	−0.23 (−1.09, 0.62)			
P^h	0.998	0.356					
Carbohydrate[j] **(g d^{-1})**							
O. stricta	359.6 ± 58.06	364.20 ± 7.36	0.186	4.61 (−2.30, 11.52)	0.008	0.512	0.150
Red Beet	375 ± 48.11	373.40 ± 6.48	0.577	−1.56 (−7.16, 4.04)			
Placebo	375.50 ± 49.68	375.70 ± 7.14	0.637	0.11 (−0.38, 0.61)			
P^h	0.238	0.206					
Protein[j] **(g d^{-1})**							
O. stricta	66.61 ± 9.95	67.97 ± 1.62	0.111	1.35 (−0.32, 3.04)	<0.0001	0.210	0.197
Red Beet	72.50 ± 11.04	72.54 ± 1.57	0.944	0.04 (−1.25, 1.34)			
Placebo	68.10 ± 9.05	68.06 ± 1.32	0.797	−0.04 (−0.37, 0.29)			
P^h	0.013	0.898					
Total fat[j] **(g d^{-1})**							
O. stricta	79.60 ± 13.50	80.31 ± 1.85	0.318	0.71 (−0.70, 2.12)	0.484	0.745	0.275
Red Beet	81.04 ± 8.63	80.66 ± 1.31	0.599	−0.37 (−1.81, 1.06)			
Placebo	76.66 ± 10.15	76.72 ± 1.45	0.618	0.05 (−0.17, 0.29)			
P^h	0.140	0.311					
BMI (kg m^{-2})							
O. stricta	30.49 ± 3.02	30.43 ± 0.42	0.095	−0.06 (−0.13, 0.01)	0.443	0.136	0.969
Red Beet	30.57 ± 2.89	30.50 ± 0.42	0.150	−0.07 (−0.17, 0.02)			
Placebo	30.46 ± 2.90	30.46 ± 0.41	0.905	0.004 (−0.06, 0.07)			
P^h	0.981	0.359					

Abbreviations: BMI: body mass index; DBP: diastolic blood pressure; FBG: fasting blood glucose; Hcy: homocysteine; HDL-c: high-density lipoprotein-cholesterol; LDL-c: low-density lipoprotein-cholesterol; non-HDL-c: non-high-density lipoprotein-cholesterol; *O. stricta*: betacyanin-rich supplement of *Opuntia stricta*; QoL: quality of life; Red Beet: betalain-rich supplement of red beetroot; SBP: systolic blood pressure; TC: total cholesterol; TG: triglycerides. [a] Within group comparison (2-tailed paired Student's t test/Wilcoxon test). [b] Within group difference; $\bar{x}$ (lower 95% CI, upper 95% CI). [c] Comparison between beetroot and *O. stricta*; treatment effect (repeated-measures ANOVA). [d] Comparison between before and after treatments with red beet and *O. stricta*; time effects (repeated-measures ANOVA). [e] Comparison between measures obtained before and after intervention with beetroot and *O. stricta*; treatment sequence or interaction effect or carryover effect (repeated-measures ANOVA). [f] $\bar{x}$ ± SD (all pre-intervention values). [g] $\bar{x}$ ± SEM (all post-intervention values). [h] Between group comparison (pre-intervention: one-way ANOVA/Kruskal–Wallis test, post-intervention: ANCOVA). [i] Quality of life (QoL) scores according to the MacNew quality of life questionnaire. [j] Dietary intakes were assessed using a 24 h dietary recall questionnaire.

of beetroot-enriched betalain composition (containing about 30% of total betalains), and administration of 100 mg of those supplements, 3 times a day (about 90 mg betalains per day), for 3 consecutive days, significantly increased the ratio of HDL-c/LDL-c and reduced the serum concentrations of TG in healthy humans.[32] In hypertensive subjects, a two-week supplementation with 250 ml red beet juice considerably reduced the concentrations of LDL and TC.[33] Two weeks' consumption of 150 ml day^{-1} *Opuntia ficus indica* juice by healthy athletes significantly decreased their TC, TG, and LDL levels before and after the yo–yo intermittent recovery test.[34] Besides, subcutaneous treatment of AMI rats with 25 or 100 mg kg^{-1} betanin for three days, dose-dependently reduced their LDL level.[35]

A cross-sectional study showed an independent positive association between Hcy and hypertension.[36] Hyperhomocysteinemia causes hypertension through endothelial cell damage, dysfunction of diastolic function of

vessels, reduction of their flexibility, and adverse effects on the biosynthesis and function of vasodilator factors.[20] The present study showed that two weeks' consumption of both supplements decreased SBP; however, betalain-rich extract and betacyanin-rich extract had statistically significant and non-significant reducing effects on DBP, respectively (Table 4 and Fig. 3). In several studies, following a single ingestion of red beet juice, SBP[37,38] and DBP[37] were decreased in healthy subjects. In the same vein, after three weeks of concentrated red beet juice consumption by overweight and obese subjects, SBP was significantly reduced.[39] In healthy men, consumption of beetroot bread, as a part of the dietary regimen, increased endothelium-independent vasodilation and decreased DBP.[40] However, no differences in the 24 h ambulatory blood pressure and home blood pressure were observed after one-week consumption of nitrate-rich beetroot juice by hypertensive subjects.[41] Similarly, two-week supplementation with *Opuntia ficus indica* juice had no effects on the blood pressure in healthy male volunteers.[34] Thus, the improvement of blood pressure by betalain-rich sources may be in part due to the reduction of oxidative damage to vascular cells, alleviation of Hcy-related adverse effects, the improvement of endothelial functions[33] and their nitrate content (red beetroot).

In our study, the consumption of both supplements significantly decreased the concentration of fasting blood glucose (Table 4, Fig. 3). In healthy humans who consumed 100 mg beetroot-enriched betalain supplements containing about 30 mg total betalains (about 90 mg betalains per day) 3 times a day, for three consecutive days, the concentration of blood glucose (fasting and under glucose challenge) reduced/normalized.[32] In healthy individuals, a single dose consumption of red beet juice beverage (225 ml), a rich source of betalain degradation compounds, significantly lowered glucose response in the 0–30 min phase.[42] Besides, four-week administration of red beetroot crisps lowered the serum concentration of glucose in dyslipidemic diet-administered rats.[43] This effect may be in part due to the improvement of insulin sensitivity following the consumption red beet juice,[44] and also the antioxidative[45] and anti-inflammatory[46] effects of betalain sources, and their fiber[47] content. However, in another study, a two-week supplementation with 150 ml day^{-1} *Opuntia ficus indica* juice had no effects on the glucose concentration of healthy athletes.[34]

Surprisingly, first, in rats which were treated with fructose solution to induce heart fibrosis, for 60 days, betanin treatment (25–100 mg kg^{-1}) had anti-fibrotic and antiglycative effects on their hearts.[48] Second, subcutaneous administration of 25–100 mg betanin per kg (for three days) ameliorates AMI and improves infarct size and cardiac function in rats with acute myocardial infarction (AMI).[35] Third, treatment with indicaxanthin can prevent vascular tissue injury and consequent thrombotic complications. Incubated RBCs with oxysterols (to induce eryptosis) plus five μM-indicaxanthin lose the ability to adhere to human umbilical vein endothelial cell monolayers.[49] Fourth, supplementation with red beetroot significantly increases flow-mediated dilation,[33,33] an indicator of improved endothelial function.

In summary, the present study showed that after two weeks' consumption of betalain-/betacyanin-rich supplements, betanin is found to be present in physiological fluids. Thus, treatment with betalain can alleviate and modify some of the atherogenic risk factors such as hyperhomocysteinemia, dyslipidemia, hypertension, and hyperglycemia. As we mentioned above, it seems that all of these benefits are in line with the anti-oxidative and anti-inflammatory properties of betalains. Thus, this study provides new insight into the application of betalains as a promising natural health-promoting candidate. Nevertheless, several limitations of this study should be acknowledged. It was a single-center pilot study with a short period, a small sample size, and few checked factors. Therefore, further investigations using larger sample sizes, more extended periods, yellow/orange prickly pear and beetroot (as the other sources of betalains), and pure betacyanins/betaxanthins are required for evaluating the responsible mechanisms of the mentioned effects. Also, it is necessary to explore the other possible effects of betalain-rich diet in atherosclerosis and other related diseases.

Conclusion

This study showed that the acute and short-term supplementation with betalains causes the presence of betanin in plasma and urine samples. Also, consumption of betalain-/betacyanin-rich food extracts could statistically reduce Hcy levels, SBP, and FBG and improve the lipid profile to some extent in atherosclerotic patients. However, among these factors, only Hcy (after consuming both supplements), non-HDL-c and LDL (after consuming the betalain-rich supplement) levels were clinically decreased. Although the dietary sources of betalains appear to be potential health-promoting functional foods, still, further trials using high purity betalains, larger sample sizes, and more extended periods are required to gain a deeper understanding of their precise biological functions.

Conflicts of interest

None.

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