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RESEARCH ARTICLE

Design, formulation and sensory evaluation of a polyphenol-rich food placebo: an example of aronia juice for food intervention studies

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ABSTRACT

Products suitable for use as controls in food interventions designed to demonstrate the role of minor components are largely lacking. In the present study, we aimed to develop a formulation to be used as a placebo in a clinical trial designed to assess the effects of aronia juice polyphenols on platelet function. Three formulations with the same nutrient composition as aronia juice were prepared by mixing various nutrients, artificial colours and flavours with water. The similarity of formulations to aronia juice in terms of taste, colour, smell and texture was assessed by six food panellists. The final placebo was tested for its impact on platelet function, biochemical and anthropometric parameters in a 4-week long study. No significant changes in platelet function, or in several cardiovascular and safety markers were recorded. Formulation suitable for use as a placebo for dietary intervention studies using aronia juice has been developed and demonstrated to be well tolerated in humans.

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Aronia melanocarpa; anthocyanins; platelet function; functional food; bioactives

Introduction

Although placebo-controlled trials are essential for the evaluation of successful interventions, products suitable for use as controls in food interventions designed to demonstrate the role of minor components are largely lacking. A placebo can be defined as an intervention applied to mimic some other treatment in most respects so that a comparison between the treatment and placebo effectively identifies the different components in the treatment as causing any observed physiological effect. In drug trials, the most common forms of placebos are capsules filled with an inert substance such as microcrystalline cellulose, but placebos have been used for other kinds of interventions, even physical interventions such as surgery (Vickers & de Craen 2000). Although the main principles of pharmacological intervention trials should also be followed in non-pharmacological interventions such as those with foods, some concepts are difficult to apply in dietary intervention studies (Yao et al. 2013). Ideally, placebo formulations should be designed in a way to appear largely indistinguishable from the tested materials (appearance, smell, taste) but lack the specific component(s) that putatively have biological activity (de Craen et al. 1999). In the case of dietary intervention studies, developing appropriate placebos for the test foods is more challenging, particularly when the compounds that contribute to the taste and smell are at the same time the putatively active components being assessed (Zick et al. 2005; Yoon et al. 2012). Example bioactive compounds include sulphur-containing compounds present in cruciferous and allium vegetables, highly coloured anthocyanins and carotenoid pigments in various fruits and vegetables and astringent proanthocyandins in apples and grape skins. One of the most accurate approaches to achieve a complex plant food placebo is to use plant genetic modification (GM) to introduce or knockout the synthesis of the bioactive components of interest. The use of such an approach has been demonstrated with the introduction of anthocyanin biosynthesis into tomatoes which generated purple tomatoes that were rich in anthocyanins and subsequently shown to extend the lifespan of cancer-prone P57 knockout mice and reduce the formation of atherosclerotic plaque in the ApoE knockout mouse model (Butelli et al. 2008). However, the acceptability of GM foods for human consumption remains controversial, and current regulatory requirements ensure that such an approach is time consuming and extremely expensive. Among various plant foods,

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aronia (Aronia melanocarpa) has been of special interest since its fruits have been indicated as a rich source of polyphenols. Polyphenols from aronia fruits, mainly anthocyanins and procyanidins, represent one of the most potent natural antioxidants, exerting the positive impact on overall health, but also on diseases and states associated with marked level of oxidative stress, such as cardiovascular diseases (CVD) and metabolic disorders (Chrubasik et al. 2010; Sikora et al. 2014; Kardum et al. 2015). Although beneficial effects of aronia polyphenols on CVD risk factors such as hypertension, elevated blood lipids and glucose, platelet hyperactivity and obesity have been demonstrated, controlled clinical trials on aronia juice are still lacking, partly due to the difficulties in delivering suitable control drink. In the present work, we sought to develop and test a polyphenol-free formulation that could be used as a placebo in a clinical trial designed to assess the effects of the polyphenols in an aronia juice on platelet function and other biomarkers and risk factors for CVD.

Material and methods

Development of placebo formulations

The aim of this study was to synthesise a formulation that would have the same nutrient composition as aronia juice, but that would lack bioactive polyphenols. First we determined the composition of the nutrients in aronia juice. We used a commercial juice, prepared by Nutrika d.o.o. (Belgrade, Serbia), which has been registered at the Serbian Ministry of Health as a dietary supplement. The content of sugars was measured by HPLC by previously described method with slight modifications (Mikulic-Petkovsek et al. 2012), total acidity (expressed as citric acid) was determined by potentiometric titration, and minerals were quantified using atomic absorption spectrometry, as previously described (Vidović et al. 2013). After the chemical characterisation of aronia juice, we prepared potential placebos using three different recipes containing exactly the same content of sugars, citric acid, vitamins and minerals as in the juice. The placebo formulations were prepared by adding these nutrients to water. All of the ingredients were safe and allowed for human consumption and commonly used in the food industry. Glucose and fructose were purchased from LG Hemija (Serbia) and Barentz (the Netherlands), respectively, while sorbitol and acidic acid were purchased from Comcen (Serbia). Potassium, calcium and magnesium were provided from LG Hemija (Serbia), Applichem (USA) and Mitoku (Japan), respectively, in the form of their corresponding salts. Sodium was added from kitchen salt, and all of the vitamins were obtained from Pharmanova (Serbia). Artificial colours ("Bordeaux", "Blue", "Strawberry Red", "Brilliant Black") and flavours ("Aronia" and "Blueberry") were used to mimic the appearance and taste of aronia juice. They were supplied by "Frutarom Etol d.o.o." (Slovenia) and included components in accordance with EC directives, in amounts permitted by the EU legislation for liquid food supplements (Directive 94/36/EC). The only differences between the three placebo formulations were the type and ratio of artificial colours and flavours used for their preparation.

Sensory evaluation of placebo formulations

Sensory evaluation of placebo formulations was performed by six independent food panellists. The panellists were asked to compare the similarity of the three placebo formulations to the aronia juice, which was given first, at the start of the sensory testing. In order to choose the best matching placebo, panellists were served with three different formulations in random order, in a single-blinded manner. An unsalted crackers and water were offered in between.

First, panellists were asked to rate the degree of similarity to aronia juice for the following parameters: taste, colour, smell, texture and overall acceptability. For this purpose the Likert category scale graded from 1 to 5 was used, with following meaning: 1 = not at all, 2 = not really, 3 = undecided, 4 = somewhat, 5 = very much. Afterwards, panellists graded the flavour of tested liquids in terms of astringency, sourness and sweetness. The degree of intensity for each attribute was assessed with following scale: 0 = not present, 1 = just recognisable or threshold, 2 = weak, 3 = moderate, 4 = strong, 5 = very strong. Finally, a third scale was used to assess the overall impression of matching the tested placebo formulations with aronia juice by choosing one of the following values: 1 = low, 2 = medium, 3 = high.

Testing of the final placebo formulation: pilot study

The final placebo formulation was further tested in a study population which included 20 healthy non-diabetic volunteers (10 males, 10 females) aged 30–50 years. Subjects were pre-obese (body mass index: 25–30 kg/m^2) with normal or high normal blood pressure (systolic: 120–139 mmHg) (Mansia et al. 2007) and an increased waist circumference ($\geq$ 80 cm for females and $\geq$94 cm for men). The study had been designed as open label, non-controlled with participants consuming 100 ml of beverage daily for four

weeks (Ethical approval No 2125, Clinical Hospital Zemun EB, 26th September 2013). The purpose of the study was testing the possible impact of placebo consumption on platelet function. Besides that, biomarkers of liver and kidney function as additional markers of placebo inertness were measured. Blood samples were obtained at baseline and at the end of the consumption period, after an overnight fast. Additionally, anthropometric measurements, including blood pressure level, were performed during both study visits.

The aim of developing placebo beverage was its application in a clinical trial assessing the effects of the polyphenols in an aronia juice on platelet function and other risk factors of CVD. In accordance with the duration of intervention phases in this clinical trial, we have chosen 4-week intervention period for our pilot study. In addition, our previous research indicated that 4-week interventions with Aronia juice could express beneficial effects on blood pressure, blood lipids, including fatty acid status, and pro-oxidant/antioxidant status (Kardum et al. 2014a, 2015). The beverage size in our pilot study was chosen in accordance with the amount of aronia juice (100 ml) to be consumed in mentioned clinical trial testing platelet function. This was rationalised by the amount of total polyphenols, as active compounds of interest and in accordance with amounts that have been shown as effective in our previous research (Kardum et al. 2014a, 2014b, 2015).

Sample collection and analyses

Venous blood was obtained after an overnight fast and an initial 20-min rest. For the platelet analyses, whole blood samples were collected into tubes with sodium citrate as an anticoagulant, according to the appropriate guidelines (Krueger et al. 2002). Additional blood samples were collected into sample tubes for serum in order to evaluate lipid status and glucose level. The activity of liver enzymes and levels of urea, creatinine and uric acid were determined from the same samples. These analyses were performed with a clinical chemistry analyser (Cobas c111, Roche Diagnostics, Basel, Switzerland) and Roche's diagnostic kits according to the manufacturer's instructions

Flow cytometry/determination of platelet activation and aggregation

The platelet activation markers (P-selectin and GPIIb-IIIa) and platelet-leukocyte aggregates were measured by whole-blood flow cytometry according to a previously published protocol (Michelson et al. 2000; Krueger et al. 2002; Barnard et al. 2003). For the determination of platelet activation, aliquots of whole blood dissolved (1:10) in Hepes-Tyrode Buffer (HTB) were incubated with peridinin chlorophyll protein (PerCP)-conjugated anti-CD61, phycoerythrine (PE)-conjugated CD62P (anti-P-selectin) and fluorescein isothiocyanate (FITC)-conjugated PAC1 (anti-GPIIb–IIIa) monoclonal antibodies with or without a suboptimal concentration of platelet agonists (0.5 μM adenosine diphosphate, ADP) for 20 min in the dark, at room temperature. After the incubation with antibodies, the samples were fixed in fixing buffer for 15 min and analysed. Platelet-monocyte (PMA) and platelet-neutrophil aggregates (PNA) were analysed form whole blood samples, incubated with monoclonal antibodies FITC-conjugated anti-CD61, PE-conjugated anti-CD11b and PerCP-conjugated anti-CD14 with or without suboptimal concentration of platelet agonist (0.5 μM ADP) for 15 min in the dark, at room temperature. Afterwards, samples were treated with erythrocyte-lysing buffer for 10 min, washed twice in HTB, fixed for 15 min in fixing buffer and analysed. Sample analysis was performed using a FACS Calibur flow cytometer with CellQuest software (Becton Dickinson). The monoclonal antibodies, FACS Lysing Solution and Cell Fix Solution were purchased from Becton Dickinson (Heidelberg, Germany).

Statistical analysis

Prior to comparisons, the normality of variables distribution was tested using the Shapiro–Wilk test. Variables with normal distribution were compared by paired samples *t*-test and data are shown as mean values ± standard deviation (SD). Non-parametric Wilcoxon test was used for the comparisons of variables that were not normally distributed and these data are presented as median and 25th–75th percentile. In the case of platelet activation and aggregation markers, data are shown graphically with Tukey "box and whiskers" plots. On these plots, boxes present interquartile range (IQR; the difference between the 25th and 75th percentiles), horizontal lines are plotted at the median, and vertical lines (whiskers) extend to the largest and the smallest values. When there are outliers detected, defined as values differing more than 1.5 × IQR from the 75th and the 25th percentiles, they are presented as individual dots on graphs. In this case, the upper whisker is drawn to the largest value less than the sum of the 75th percentile and 1.5 IQR and the lower whisker to the lowest value greater than

the 25th percentile minus 1.5 IQR. Analyses were performed using the GraphPad Prism Software and SPSS software (SPSS, Chicago, IL). p values <0.05 were used to indicate statistical significance.

Results

Placebo characterisation and sensory evaluation

The final polyphenol-free placebo beverage had the same nutrient composition (i.e. content of sugars, citric acid, minerals and vitamins) as aronia juice. Along with the aforementioned nutrients, artificial colours (30 mg of "Strawberry red" – SR and 10 mg of "Brilliant black" –BB/100 ml) and artificial flavour (100 mg of "Blueberry" flavour/100 ml) were added to the water solution (Table 1). Since all the colouring and flavouring agents were synthetic, no phenols were present in the placebo formulation. The colours consisted of dextrose and food additives. More precisely SR was made of dextrose, sodium sulphate an food additives: Ponceau 4R (E124), Sunset Yellow FCF (E110), silicon dioxide (E551); while BB contained dextrose, Brilliant Black BN (E151) and silicon dioxide (E551).The flavour present in final placebo formulation consisted of water, ethanol, Azorubine (E122) and Brilliant Black BN (E151).

Characteristics of the subjects

General characteristics of the subjects who participated in the pilot study are presented in Table 2. None of the measured anthropometric parameters -including blood pressure- and biochemical parameters was significantly changed during the course of the study.

Table 1. Composition of the final placebo formulation.

Parameter	Content (/L)
Sugars	
Glucose	45 g
Fructose	28 g
Sorbitol	50 g
Citric acid	1.21 g
Minerals	
Potassium	2.2 g
Calcium	152 mg
Magnesium	139 mg
Natrium	30 mg
Vitamins	
Vitamin C	29.2 mg
Thiamine(B1)	381 µg
Riboflavin(B2)	708 µg
Niacin(B3)	394 µg
Pyridoxine(B6)	442 µg
Colours	
"Strawberry red"	300 mg
"Brilliant black"	100 mg
Flavours	
"Blueberry"	1.0 ml

Effects on platelet activation and aggregation

In the case of platelet activation, there were no significant changes observed in the expression of either of the markers: P-selectin and GPIIbIIIa, both in basal state or after responding to an agonist. Percentages of antigen-positive platelets (from a platelet pool of 20,000 analysed cells) at baseline and after the 4 weeks long consumption period are presented with box and whisker plots in Figure 1. The formation of platelet-leukocyte aggregates was evaluated by measuring the expression of platelet-specific antigen CD61 in the population of monocytes (assessed from a monocyte pool of 1000 analysed cells) and neutrophils (assessed from a neutrophil pool of 10,000 analysed cells). No significant changes were found in the proportion of platelet-monocyte and platelet-neutrophil aggregates, either in basal state or after responding to an agonist. The percentages of platelet-leukocyte aggregates at baseline and at the end of the consumption period in the basal state and after activation with ADP are presented at Figure 2.

Discussion

The present study indicates that, besides some limitations in mimicking the astringent taste, each of the sensory characteristics of aronia juice can be successfully matched to create a placebo beverage potentially suitable for use in clinical trials to investigate the physiological effects of consuming polyphenols in aronia juice. All three placebo formulations were graded with a weak presence of astringent taste, while overall similarity to the aronia juice taste was evaluated with a grade 2 (not really). However, the panellists evaluated other parameters with moderate and strong similarity to aronia juice. Additionally, overall impression of matching the placebo formulations with aronia juice was evaluated as high for each of the tested placebos. The colour of the final placebo was graded at level 5- very strong similarity to aronia juice. The presence of "Blueberry" flavour partly contributed to this, since it contained artificial colour (3% of its content), unlike the "Aronia" flavour used for preparation of the other two placebo formulations.

Furthermore, we showed that this placebo was well tolerated in 20 apparently healthy human volunteers without any reported side effects. Besides the evaluation of blood lipids and glucose, activities of liver enzymes: alanine aminotransferase (ALT), aspartate

aminotrasnferase (AST) and gamma-glutamyltransferase (GGT), as indicators of liver function were also monitored. Additionally, the effects on kidney function were examined by measuring concentrations of creatinine, urea and uric acid in blood samples obtained at the baseline and at the end of the consumption period. Finally, we demonstrated that the placebo beverage is inert with respect to possible effects on platelet reactivity.

Taking into account obtained results and scarce literature data, we consider this study of great importance for the future research of potential beneficial effects of aronia juice polyphenols in humans, aiming to support their use as complementary therapies.

Table 2. Characteristics of subjects at baseline and after 4 weeks of regular placebo consumption.

	Baseline	4 weeks	P value
Gender (M/F)	10/10		
Age (years)[a]	38.6 ± 6.7		
Body mass index (kg/m^2)[a]	27.7 ± 2.7	27.6 ± 2.6	0.379
Waist circumference (cm)[a]	94.3 ± 9.1	94.5 ± 9.1	0.812
SBP (mmHg)[a]	128.4 ± 13.6	125.6 ± 15.3	0.122
DBP (mmHg)[a]	79.9 ± 11.5	77.2 ± 12.9	0.107
Glucose (mmol/L)[b]	5.07 (4.77–5.36)	5.00 (4.70–5.44)	0.212
Triglycerides (mmol/L)[b]	1.36 (0.94–1.67)	1.24 (0.76–2.06)	0.332
Total cholesterol (mmol/L)[a]	5.61 ± 1.09	5.43 ± 0.78	0.200
LDL cholesterol (mmol/L)[a]	3.92 ± 1.11	3.70 ± 0.77	0.132
HDL cholesterol (mmol/L)[a]	1.51 ± 0.47	1.56 ± 0.52	0.608
ALT (U/L)[a]	31.3 ± 10.9	31.0 ± 12.9	0.867
AST (U/L)[b]	21.7 (18.5–25.8)	21.4 (18.2–25.2)	0.478
GGT (U/L)[b]	18.7 (11.2–31.5)	15.4 (10.0–28.6)	0.247
Creatinine (μmol/L)	71.10 (58.88–76.05)	63.30 (53.48–74.80)	0.211
Urea (mmol/L)	3.44 ± 1.11	3.23 ± 0.93	0.365
Uric acid (μmol/L)	279.24 ± 71.58	261.36 ± 84.64	0.323

[a]Data are presented as mean ± SD.
[b]Data are presented as median and 25th–75th percentile.

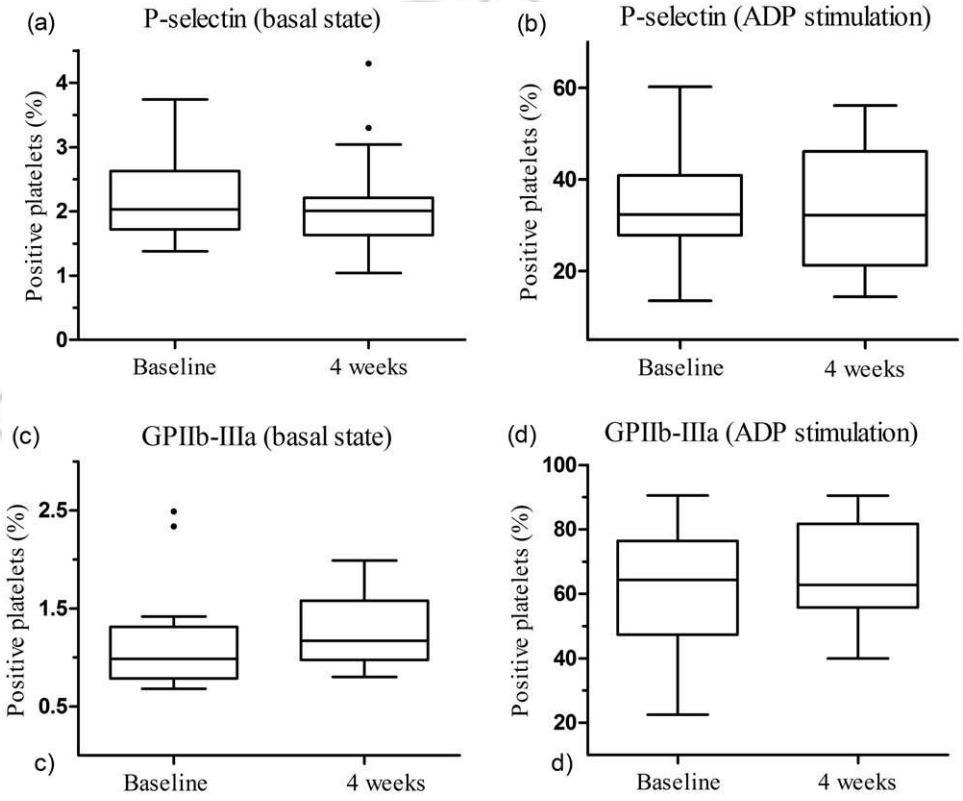

Figure 1. Platelet activation at baseline and after 4 weeks of regular placebo consumption; the percentage of P-selectin-positive plateletes at basal state (a) and after stimulation with adenosine diphosphate – ADP (b); The percentage of GPIIb-IIIa-positive platelets at basal state (c) and after stimulation with adenosine diphosphate – ADP (d).

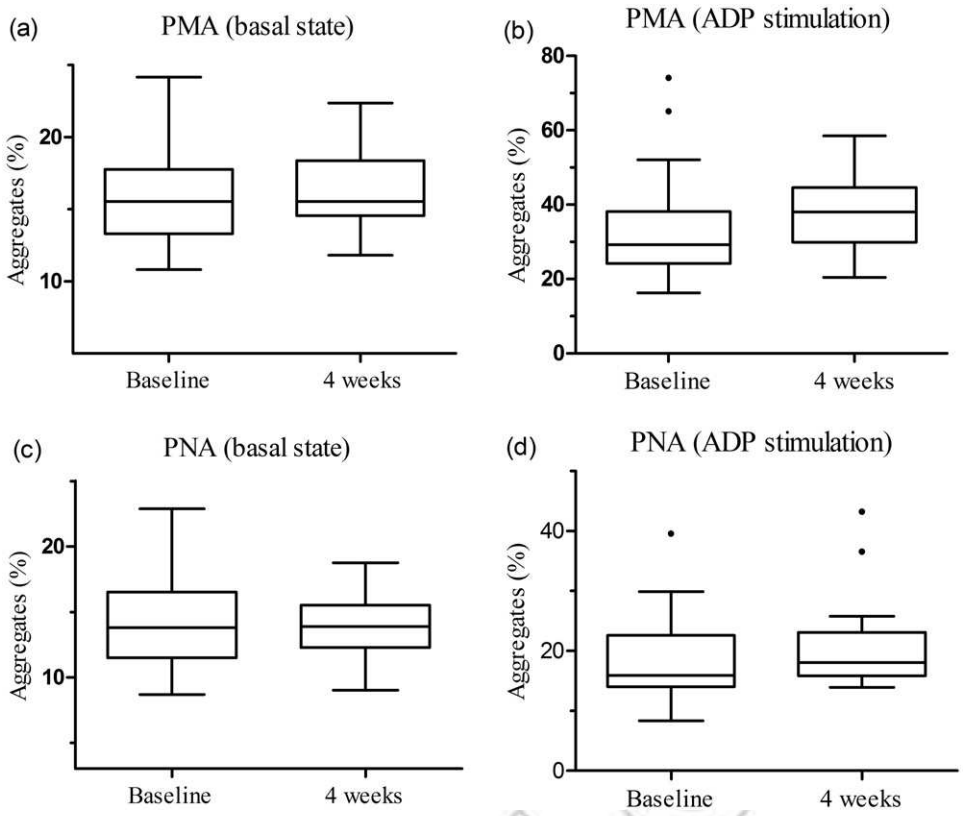

Figure 2. Platelet aggregation at baseline and after 4 weeks of regular placebo consumption; The percentage of platelet-monocyte (PMA) aggregats at basal state (a) and after stimulation with adenosine diphosphate – ADP (b); The percentage of platelet-neutrophil aggregates at basal state (c) and after stimulation with adenosine diphosphate – ADP (d).

Although some *in vitro* studies have demonstrated a positive impact of aronia products on platelet aggregation and their adhesion to collagen and fibrinogen (Ryszawa et al. 2006; Olas et al. 2008; Malinowska et al. 2013; Sikora et al. 2014), these effects have not been confirmed in placebo-controlled trials so far. Further, there is a limited number of human studies investigating the effects of Aronia products on cardiovascular health using the pure aronia juice (Simeonov et al. 2002; Skoczyñska et al. 2007; Poreba et al. 2009; Kardum et al. 2015). Most often these studies are uncontrolled, or even if they do report use of appropriate placebo, they lack the detail description of the control drink' composition (Handeland et al. 2014). What distinguishes our work is the inclusion of the recipe for placebo – given in details – along with the description of its sensory evaluation in terms of taste, small and colour testing. The main challenge of our study was to develop a placebo which could sufficiently match the astringent taste of aronia juice. This is not surprising, considering that aronia juice, as a complex mixture of flavour and pigmenting compounds, is characteristic for its astringency, which is hard to mimic since it is caused by the bioactive compounds (Kulling & Rawel 2008). Like ours, placebo drink applied in a study evaluating effects of aronia juice on urinary tract, contained blueberry aroma, in addition to the grape one. However, since sensory evaluation of this drink is lacking we could not conclude whether it matched the astringency of aronia juice better or at the same level as the placebo described in our study (Handeland et al. 2014). Similar difficulties in developing liquid placebo have been reported for other natural and herbal products. Clinical study, aimed to develop and evaluate a placebo for a complex liquid herbal supplement, failed to achieve the specific mint flavour and smell of the product (Yoon et al. 2012). Comparing with the pharmaceuticals, herbal materials are a much serious challenge for developing credible placebos. However, even for pharmaceuticals placebos can differ from active forms – especially when active forms affect smell or taste (e.g. antibiotics, antidepressants and diuretics) (Doty & Bromley 2004). The challenges of demonstrating the activity of a single compound in the context of complex foods, researchers often revert to the reductionist approach and use pure compounds or crude extracts rather than whole foods. When using extracts, placebos are much more easily formulated and most commonly applied as capsules prepared

from a simple blend of inorganic salts or other inert substances (Dube et al. 2007; Chang et al. 2010). This approach can lead to false conclusions; it has been demonstrated that some components express their effects only when they are delivered along with and are able to interact with other food components (Jacobs & Tapsell 2007). The results of a pilot study showed no significant effect on liver and kidney function, indicating that placebo was well tolerated and caused no observed side effects. This was in accordance with our expectations, since the placebo was prepared from nutrients allowed for human consumption and commonly used in the food industry. Additionally, artificial colours and flavours, present in placebo formulation, were applied in amounts permitted by the EU legislation for liquid food supplements.

The main purpose of developing the placebo was its use in a study that aimed to assess the impact of aronia juice polyphenols on platelet function; thus, we also tested for possible effects of the placebo on platelets. Cardiovascular disease (CVD), as one of the most frequent chronic diseases, is, among other factors, influenced by activated platelets. Altered platelet function, manifested by an increase in circulating platelets, which express more adhesion molecules on cell surface, has been found in subjects with hypercholesterolaemia, hypertension, diabetes mellitus and metabolic syndrome (Lip 2003; Angiolillo et al. 2007; Santilli et al. 2012; Bröijersén et al. 1998a). Furthermore, enhanced platelet activity was also detected after consumption of meals containing fat, as well as after treatment with high glucose levels in vitro (Bröijersén et al. 1998b; Sudic et al. 2006). Here, we showed that placebo consumption caused no changes in platelet activity, including platelet activation and aggregation with other blood cells. Our study also showed no effects of placebo consumption on the levels of blood glucose and lipids, which is noteworthy because glucose and lipid status affect not only CVD development, but platelet function as well.

Conclusions

Despite obvious challenges, we developed a polyphenol-free placebo formulation that was well matched to Aronia juice in terms of taste, smell and appearance. The placebo was well tolerated and its consumption had no side effects in humans. Furthermore, we demonstrated that consumption of the placebo had no effects on platelet activation, platelet aggregation with other blood cells, and biochemical parameters related to the platelet functionality. We conclude that this well characterised beverage can be used as a placebo to facilitate controlled human intervention studies assessing the effects of aronia juice polyphenols on platelet function and on other biomarkers of health and risk factors for diseases.

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Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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