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Juices from non-typical edible fruits as health-promoting acidity regulators for food industry

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a b s t r a c t

The study verifies the possibility of application of juices from selected fruits characterized by the high antioxidant potential as natural acidity regulators with improved nutritional properties. The tested non-typical fruits included mirabelle plum, sea buckthorn and blue-berried honeysuckle. Beetroot juice whose pH is about 6.0 served as a model food product. Potentiometric titration was used to compare the efficacy of tested juices as acidity regulators with that of citric acid, a widely applied acidity regulator. The antioxidant activity of tested mixtures of juices was determined by spectrophotometric $ABTS^{\bullet+}$ (2,2-azinobis-(ethyl-2,3-dihydrobenzothiazoline-6-sulphonic acid) diammonium salt) test and their cytotoxic activity was assessed by MTT (thiazolyl blue tetrazolium bromide) test. The potentiometric titration revealed that the efficacy of the juices proposed as acidity regulators matched that of citric acid. Among the mixtures of beetroot juice and titrants studied, the addition of blue-berried honeysuckle juice ensured the highest antioxidant activity, followed by sea buckthorn and mirabelle plum juices. The same order was observed also for biological activity assessed in HT29 cells. The results of all tests confirmed that studied juices can successfully replace currently used acidity regulators, which may be of special interest in the case of functional foods.

Keywords: Beetroot juice, Antioxidant activity, Cytotoxicity, Bioactive phytochemical

Chemical compounds studied in this article: Vulgaxanthin I (PubChem CID: 5281217) Betanin (PubChem CID: 90657360) Isobetanin (PubChem CID: 6325438) Cyanidin-3-O-glucoside (PubChem CID: 12303203)

1. Introduction

The relationship between nutritional, as well as non-nutritional food components and human health is at the core of current research on disease prevention. Such studies concentrate typically on the search for specific edible compounds, especially bioactive phytochemicals exhibiting defined biological effects, with the aim of introducing them into human diet in a form of supplements or fortified foods. However, the possible impact of small yet sustainable changes in food production technology, e.g. salt content reduction, has been recognized as a means ensuring health benefits not less important than those offered by so called novel or functional foods. The ubiquitous ingredients that could be replaced with healthier alternatives are food additives that are present in all kinds of alimentary products. One of such an omnipresent additive is citric acid.

Citric acid is the most frequently applied acidity regulator in food industry, where it serves many different purposes, from acid and acidity regulation, stabilization, preservation to buffering. Until recently, citric acid has been regarded as indifferent to the human organism. This view has been changed by the results of investigations, which demonstrated, that the addition of citric acid and glycine to fried starch foods reduces the formation of acrylamide, a carcinogen arising during baking and frying, also a known cause of damage to neurons. Although the addition of citric acid and glycine to fried foods separately affect adversely taste of such products, the combination of the mentioned two additives reduces the formation of acrylamide without negative change of the taste (Low et al., 2006). Another indicated benefit is associated with nutritional therapy of urinary tract, where citric acid in the form of citrus fruit juice administrated orally is used to prevent kidney stone formation. Owing to its capability of binding to the surface of calcium oxalate crystals, it inhibits their growth and aggregation, as well as reduces adhesion to renal epithelial cells. Citric acid is currently regarded as the most potent and clinically significant

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kidney stone inhibitor (Penniston, Nakada, Holmes, & Assimos, 2008).

Some researchers suggest however, that the consequences of citric acid consumption should be better recognized. Unfortunately, apart from confirming advantages, citric acid was not subjected to any broader controlled investigations to examine its potential disadvantages to human health. Despite this ambiguity in safety assessments, the amount of citric acid used in food industry as an acidity regulator is staggering. Even if its presence does not decrease healthiness of food products, it seems to offer no significant health benefits. Therefore, it appears reasonable to seek alternatives to citric acid, such that could guarantee both desirable technological properties and improved health quality of food products. Since many edible fruits contain high levels of organic acids, including citric acid, being at the same time a rich source of bioactive phytochemicals, their use for pH regulation may fulfill both requirements.

In this study, we tested the suitability of juices from non-typical edible fruits, *i.e*. mirabelle plum, sea buckthorn and blue-berried honeysuckle, as alternatives to citric acid. The main purpose of this paper was to verify their capacity of acidity regulation along with the assessment of probable health benefits. As a medium whose acidity was to be regulated, the beetroot juice with natural pH of about 6.0 was chosen. The ability of the fruit components to regulate pH was compared to that of the most popular industrial acidity regulators, that is citric acid and potassium sodium tartrate. Then, the descriptors frequently associated with health quality were examined for beetroot juice acidified to pH around 3.5 with the studied juices. The following chemical and biological indices were determined: the total antioxidant activity measured by $ABTS^{\bullet+}$ test, HPLC analysis of bioactive phytochemicals, profiling of antioxidants by post-column derivatization and the assessment of biological activity by MTT assay.

2. Materials and methods

2.1. Chemicals and reagents

The following chemicals and biochemicals were used: citric acid, hydrochloric acid (HCl), thiazolyl blue tetrazolium bromide (MTT), dimethyl sulphoxide (DMSO), 2,2-azinobis-(ethyl-2,3-dihydrobenzothiazoline-6-sulphonic acid) diammonium salt ($ABTS^{\bullet+}$), sodium potassium tartrate, phosphate buffered saline (PBS), fetal bovine serum (FBS), penicillin–streptomycin solution and McCoy's media from Sigma–Aldrich (USA). Standard compounds: 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and betanine were purchased from Sigma–Aldrich (USA). HPLC grade methanol and pure p.a. methanol were purchased from Chempur (Poland), ethyl acetate pure p.a. was from POCH (Poland). Formic acid was from Merck (Germany). Water was purified with a QPLUS185 system from Millipore (USA). Stock solution of Trolox was prepared in HPLC grade methanol at a concentration of 1 mg/mL and diluted with this solvent as required.

2.2. Plant material

Juices prepared from five plants: blue-berried honeysuckle (*Lonicera caerulea* L. var. *edulis*), lemon (*Citrus limon* (L.) Burm.), mirabelle plum (*Prunus domestica* subsp. *syriaca* (Borkh.) Janch. ex Mansf.), sea buckthorn (*Hippophaë rhamnoides* L.) and beetroot (*Beta vulgaris* L. ssp. *vulgaris* var. *rubra* L.), were used throughout this study. Most of the plant material (except for lemons, that were bought in the nearby grocery store) came from local private plantations. To obtain juices from fruits and beetroot, a typical home juice extractor was used. The plant material was washed, lemons and beetroots were also peeled and roughly chopped, then placed in the juice extractor (DeLonghi) operated according to the producer's manual. To remove particulates, the juices were centrifuged at 2800 × *g* for 20 min at 15 °C (Heraeus Megafuge 16R Centrifuge). The clear juices were kept at −20 °C until studied to diminish degradation of phytochemicals. Beetroot juice was additionally pasteurized by heating up to the boiling point for 10 min in order to increase its microbial and enzymatic stability during storage at 4 °C over the experimentation period (about 2 weeks).

2.3. Sample preparations

For the assessment of potential health benefits of fruit juices used as acidity regulators, two kinds of mixtures were prepared. The "pH 3.5 MIX-es", in which the tested fruit based acidity regulators and beetroot juice were combined in such proportions, so as to obtain the required pH 3.5 level (Table 1). The other kind of mixtures – "9:1 MIX-es" - were chosen to determine how the fruit based acidity regulators may affect the beetroot juice composition, and therefore health promoting properties of the final mixtures, when added in the proportion expected for typical acidity regulators. In the case of pH regulation of commercial fruit juices, the volume of solution of such additives does not exceed 100 mL/L.

In order to learn in what way the addition of natural acidity regulators may affect the stability of beetroot juice components and its bioactivity, both kinds of prepared mixtures were stored at 4 °C for 1 and 2 weeks.

2.4. Cell culture

HT29 (human colon adenocarcinoma) cells from ATCC collection were grown in McCoy's medium supplemented with L-glutamine (2 mol/L), sodium pyruvate (200 g/L), fetal bovine serum (100 mL/L) and antibiotics (100 U/mL penicillin and 100 g/L streptomycin). The cells were grown at 37 °C in humidified atmosphere containing 5% CO_2 in the Smart cell incubator (Heal Force).

2.5. Potentiometric titration

To compare the ability of fruit juices studied to regulate pH level with that of typical acidity regulators, 10 mL of beetroot juice was titrated with either sea-buckthorn juice, lemon juice, blue-berried honeysuckle juice, mirabelle plum juice or citric acid (0.16 mol/L) and 0.01 mol/L sodium potassium tartrate. HCl (1 mol/L) was used to examine the effect of acid with lower pKa value. Beetroot juice

Table 1
The composition of "pH 3.5 MIX-es" that is mixtures of beetroot juice with the tested fruit juices or 0.16 mol/L citric acid combined to obtain the final pH 3.5.

Tested acidifier	Acidity regulator [mL]	Beetroot juice [mL]	Water [mL]
Citric acid	2.75	10.0	–
Citric acid control I	–	10.0	2.75
Citric acid control II	2.75	–	10.0
Lemon	1.75	10.0	–
Lemon control I	–	10.0	1.75
Lemon control II	1.75	–	10.0
Mirabelle plum	3.0	10.0	–
Mirabelle plum control I	–	10.0	3.0
Mirabelle plum control II	3.0	–	10.0
Sea buckthorn	2.25	10.0	–
Sea buckthorn control I	–	10.0	2.25
Sea buckthorn control II	2.25	–	10.0
Blue-berried honeysuckle	2.5	10.0	–
Blue-berried honeysuckle control I	–	10.0	2.5
Blue-berried honeysuckle control II	2.5	–	10.0

was selected as food matrix with neutral pH requiring acidification. After adding each portion (1 mL) of titrant, pH of the mixture was measured using a Mettler Toledo Seveneasy pH-meter and the titration curves were plotted for each mixture.

2.6. Spectrophotometric determination of antioxidant activity

The popular method employing $ABTS^{\bullet+}$ radical was employed for the colorimetric determination of antioxidant activity as described earlier (Kusznierewicz, Piasek, Bartoszek, & Namieśnik, 2011). The solution of $ABTS^{\bullet+}$ reagent (7 mmol/L in 2.45 mmol/L Na_2SO_4) was prepared a day before measurements. Just before use, $ABTS^{\bullet+}$ solution was diluted with methanol to adjust the level of the final solution absorbance to 0.7 at 734 nm. Trolox stock solution in methanol (4 mmol/L) was used to generate the standard line (concentration range 0—4.0 mmol/L). The results of antioxidant activity determinations are expressed as Trolox Equivalents (TE). All determinations were carried out in disposable cuvettes at room temperature, and the absorbance was measured with Thermo Scientific NanoDrop 2000c spectrophotometer.

2.7. HPLC analysis of bioactive components

The composition of phenolic compounds and betalains was determined according to the analytical procedure described earlier (Kusznierewicz et al., 2011). Analysis was performed using the Agilent 1200 Series HPLC-DAD-MS system (Agilent Technologies, USA). After the photodiode array detector, the eluent flow was guided to mass spectrometer with electrospray ionization interface (ESI), operated in both positive and negative ionization mode. Drying gas temperature was 350 °C and nitrogen was the sheath gas. The mass detector was used in a positive and negative scanning mode from 100 to 800 atomic mass units (amu). Data from the mass detector were collected, compiled and analyzed using Chemstation Rev.B.04.02. Phytochemicals were separated on an Agilent Eclipse XDB-C8 column, size 150 × 4.6 mm, particle size 3.5 μm. The mobile phase consisted of two components: Solvent A (48 mL/L formic acid in water), and Solvent B (HPLC grade methanol). The mobile phase flow was 1 mL/min and a gradient mode was used for all analyses. The initial conditions of the gradient were 98% A and 2% B; the proportion reached 50% A and 50% B over 30 min. The total run time was set to 30 min. The injection volume of samples studied was 3 μL. If the standard compounds were not available, the comparison of retention time, mass signal ($[M+H]^+$, $[M+H]^-$) and (m/z) fragment with available literature data were the basis for identification of individual components.

2.8. Profiling of antioxidants by post-column derivatization

The chromatographic profiles of antioxidants in plant juices studied were obtained by post-column online derivatization with $ABTS^{\bullet+}$ reagent injected to HPLC eluate using Pinnacle PCX Derivatization Instrument (Pickering Laboratories, Inc., USA) according to Kusznierewicz et al. (2011). The $ABTS^{\bullet+}$ derivatization reagent was prepared by dissolving $ABTS^{\bullet+}$ in aqueous sodium persulphate (2.45 mmol/L) to obtain concentration of 7 mmol/L and diluting it with methanol after 12 h to the stock concentration of 2 mmol/L. The flow rate of the derivatization reagent was set at 0.2 mL/min. In all experiments, the 0.5 mL coil (PTFE, 0.25 mm, 10 m) was used and derivatization reaction was carried out at 130 °C. The profiles showing bleaching of $ABTS^{\bullet+}$ following reaction with antioxidant compounds present in the eluate from HPLC column were monitored at 734 nm using a multiple wavelength detector (Agilent 1200 Series MWD, USA).

2.9. MTT cytotoxicity assay

The cytotoxicity of beetroot juice, alone or combined with fruit juices (pH 3.5 MIX-es), towards HT29 cells was determined by MTT assay. The exponentially growing cells were seeded in 96-well tissue culture plates (about 25,000 cells/well in 0.15 mL). The cells were allowed to settle for 24 h at 37 °C, then they were treated for 24 or 72 h with 0.05 mL of different concentrations ranging from 10 to 250 mL/L of tested plant samples diluted with PBS. In the case of the shorter exposure, the medium was aspirated after 24 h from the wells, replaced with fresh medium and the cells were incubated at 37 °C for further 48 h. After 72 h of the total incubation time, 0.05 mL of MTT solution (4 g/L) was added and cells were left for another 4 h at 37 °C. Finally, medium was carefully removed from wells, and formazan crystals formed dissolved in 0.05 mL of DMSO. The absorption of the resultant solutions was determined at 540 nm with TECAN Infinite M200 plate reader (Tecan Group Ltd., Switzerland). The cytotoxicity was expressed as growth inhibition of cells exposed on tested plant samples compared to control non-treated cells whose growth was regarded as 100%.

2.10. Statistical analysis

Results of determinations of total antioxidant activity obtained by spectrophotometric $ABTS^{\bullet+}$ assay were examined by a two-tailed Student's t-test (pH 3.5 MIX-es) or One-Way ANOVA with Dunnett's test (9:1 MIX-es). All statistical analyses were performed using the Prism 4.0 software package (GraphPad Software, Inc.). The level of statistical significance was set at $p \leq 0.05$.

3. Results

3.1. The selection of fruit juices

Two properties were taken into account while choosing fruit juices as health promoting acidity regulators: the content of organic acids, as well as the content and composition of bioactive phytochemicals. Three fruits were selected that satisfactorily fulfilled both requirements. These were sea buckthorn (*Hippophaë rhamnoides* L.) with a total content of organic acids amounting to 27.5 g/kg dw (dry weight) (Beveridge, Harrison, & Drover, 2002; Beveridge, Li, Oomah, & Smith, 1999; Zeb, 2004), blue-berried honeysuckle (*L. caerulea* L. var. *edulis*) with a total content of organic acids reaching 17.9 g/kg dw (Wojdyło, Jáuregui, Carbonell-Barrachina, Oszmiański, & Golis, 2013) and mirabelle plum (*P. domestica* subsp. *syriaca* (Borkh.) Janch. ex Mansf.), whose total organic acid content (22.3 g/kg dw) was assumed based on published data for *P. domestica* (Gorsel, Kerbel, Smits, & Kader, 1992) (Table 2). These three fruits have been also shown to be a rich source of bioactive phytochemicals and their health promoting properties are widely appreciated (Celli, Ghanem, & Brooks, 2014; Fujii, Ikami, Xu, & Ikeda, 2006; Guthri, Morley, Shin Hasegawa, Manner & Venderberg, 2000; Klewicka, 2012; Manners, 2007; Shuckla et al., 2006; Stacewicz-Sapuntzakis, Bowen, Hussain, Damayanti-Wood, & Farnsworth, 2001; Suleyman et al., 2002). Lemon fruits were selected because of their well known high content of organic acids (37.4 g/kg dw) (Nisperos-Carriedo, Buslig, & Shaw, 1992; Nour, Trandafair, & Ionica, 2010; Penniston et al., 2008). The average contents of the most common organic acids in tested fruits is shown in Table 2.

3.2. Potentiometric titration of beetroot juice with tested acidity regulators

The titration curves obtained for beetroot juice titrated with

Table 2
The average content of organic acids in tested fruits.

Acid component	Composition [g/kg d.w]			
	[b]*Citrus limon* L.	[c]*Hippophaë rhamnoides* L.	[d]*Lonicera caerulea* L. var. *edulis*	[e]*Prunus domestica* subsp. *syriaca* (Borkh.) Janch. ex Mansf.)
Citric acid	35	0.42–23.4	9.4–1.3	ND
Malic acid	2	0.01–0.06	1.9–4.1	4.90–21.80
Ascorbic acid	0.42	3.6–25	0.03–0.32	0.53
Oxalic acid	ND	ND	0.72–0.97	ND
Tartaric acid	ND	0.13–0.14	ND	ND
Water [g/kg m.w][f]	–	830	850	900
[a]pH	2.26	2.61	3.06	2.68

[a] pH values refer to the actual juices used in the study.
[b] (Nisperos-Carriedo et al., 1992; Nour et al., 2010; Penniston et al., 2008).
[c] (Beveridge et al., 1999, 2002; Zeb, 2004).
[d] (Wojdyło et al., 2013).
[e] (Gorsel et al., 1992).
[f] m.w – moisture weight.

either typical acidity regulators, 1 mol/L HCl or juices of lemon, mirabelle plum, blue-berried honeysuckle and sea buckthorn are shown in Fig. 1. The addition of all titrants, with the exception of sodium–potassium tartrate, caused a dose dependent drop in the pH value of beetroot juice. In the case of HCl, the drop was the most pronounced, because of the lower pKa value of this acid and no buffering properties. The ability of tested juice titrants to control pH level turned out to be very similar to that of 0.16 mol/L citric acid (typical concentration in food industry). The titration curves for potential fruit based acidity regulators and the curve for citric acid are almost overlapping. This probably results from the high content of citric acid in the used fruits, and the fact that the initial pH value of all organic titrants tested is very similar, as shown in Table 2. Thus, all tested juices seem to fulfill the requirements expected from the efficient acidity regulators. Sodium–potassium tartrate did not lower pH value; its role in food processing is not to decrease, but to stabilize the acidity of food products. Since all the tested juices seemed to represent the appropriate replacements of citric acid, the next step was aimed at the assessment of health benefits associated with their use.

3.3. Total antioxidant activity of "pH 3.5 MIX-es"

The determination of the total antioxidant activity of beetroot juice mixed with fruit based acidity regulators to obtain final pH of about 3.5 was carried out by spectrophotometric batch test employing $ABTS^{\bullet+}$ radical. Reference samples were also prepared by mixing beetroot juice with water or water with the fruit juices in corresponding proportions (Table 1). These solutions served as controls of the input of individual diluted components into antioxidant potential of the respective mixtures. This allowed to recognize whether the tested fruit based regulators contained antioxidants that act synergistically with beetroot components – increasing, or antagonistically - lowering the antioxidant activity of the mixture, compared to the additive effect. The latter occurs when antioxidant activity is equal to the sum of determined activities for the individual components.

The total antioxidant activities of the "pH 3.5 MIX-es" are shown in Table 3. The most commonly used acidity regulator, *i.e.* citric acid, did not display a positive influence on antioxidative potential of the respective "pH 3.5 MIX". Only after two weeks' storage, it seemed to

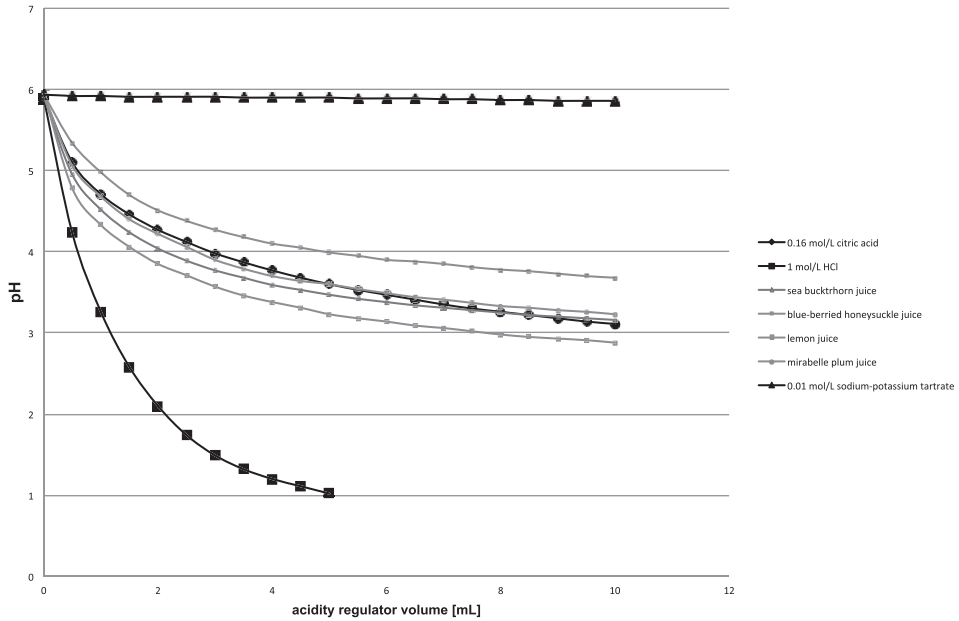

Fig. 1. Titration curves for beetroot juice titrated with 1 mol/L HCl, 0.16 mol/L citric acid, 0.01 mol/L sodium–potassium tartrate or potential fruit based acidity regulators: sea buckthorn juice, blue-berried honeysuckle juice, lemon juice, mirabelle plum juice. The points are means of three independent measurements. SD did not exceed 5%.

Table 3
The changes of the total antioxidant activity determined by ABTS test for mixtures of beetroot juice and potential fruit based acidity regulators with final pH of about 3.5 (pH 3.5 MIX-es) and for mixtures of beetroot juice and selected juices combined at a final concentration of 100 mL/L (9:1 MIX-es) for three storage periods: on the day 1 (D1) and after 1 week (D8) or 2 weeks (D15) at 4 °C. The results are means ± SD of three independent determinations. Significantly different values determined by two-tailed Student's t-test were marked as: *p < 0.05,**p < 0.01, and by ONE-WAY ANOVA Dunnett's test were marked as:[a] p < 0.05,[b] p < 0.01.

Juice mixture studied	Trolox equivalents [mmol/L]		
	D1	D8	D15
pH 3.5 MIX-es			
Beetroot + citric acid	3.50 ± 0.09*	2.17 ± 0.19	1.99 ± 0.09**
Control I: beetroot + water	3.74 ± 0.07	2.22 ± 0.11	1.18 ± 0.12
Control II: water + citric acid	0.012 ± 0.009	0.011 ± 0.008	0.012 ± 0.009
Beetroot + lemon	5.22 ± 0.27*	2.75 ± 0.13	2.71 ± 0.16**
Control I: beetroot + water	4.15 ± 0.32	2.56 ± 0.03	2.01 ± 0.11
Control II: water + lemon	0.097 ± 0.003	0.095 ± 0.004	0.091 ± 0.002
Beetroot + mirabelle plum	3.79 ± 0.05**	2.47 ± 0.20**	2.85 ± 0.21**
Control I: beetroot + water	3.23 ± 0.18	1.66 ± 0.34	1.98 ± 0.13
Control II: water + mirabelle plum	0.34 ± 0.10	0.34 ± 0.10	0.31 ± 0.10
Beetroot + sea buckthorn	5.00 ± 0.17	2.04 ± 0.03**	3.17 ± 0.14*
Control I: beetroot + water	4.81 ± 0.26	1.09 ± 0.25	2.78 ± 0.05
Control II: water + sea buckthorn	2.72 ± 0.26	1.25 ± 0.11	1.83 ± 0.06
Beetroot + blue-berried honeysuckle	6.07 ± 0.24**	3.10 ± 0.23**	4.83 ± 0.09**
Control I: beetroot + water	4.20 ± 0.24	2.55 ± 0.51	2.04 ± 0.02
Control II: water + blue-berried honeysuckle	3.00 ± 0.14	1.52 ± 0.14	2.09 ± 0.11
9:1 MIX-es			
Control I: beetroot + water	4.14 ± 0.05	3.12 ± 0.23	3.14 ± 0.14
Beetroot + citric acid	4.11 ± 0.31	2.77 ± 0.09	2.84 ± 0.08
Control II: water + citric acid	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
Beetroot + lemon	4.74 ± 0.38[a]	2.34 ± 0.14[b]	3.15 ± 0.26
Control II: water + lemon	0.075 ± 0.003	0.073 ± 0.002	0.069 ± 0.003
Beetroot + mirabelle plum	4.36 ± 0.11	2.47 ± 0.14[b]	3.56 ± 0.03[b]
Control II: water + mirabelle plum	0.254 ± 0.007	0.244 ± 0.005	0.230 ± 0.006
Beetroot + sea buckthorn	4.82 ± 0.12[b]	2.96 ± 0.17	3.78 ± 0.02[b]
Control II: water + sea buckthorn	2.35 ± 0.09	0.77 ± 0.04	1.17 ± 0.05
Beetroot + blue-berred honeysuckle	5.53 ± 0.13[b]	3.91 ± 0.09[b]	4.39 ± 0.09[b]
Control II: water + blue-berried honeysuckle	1.65 ± 0.03	0.30 ± 0.08	0.70 ± 0.18

decrease the degradation of beetroot antioxidants; TE value was 69% higher than in pure beetroot juice. Among the samples studied, the highest values of TE parameter were measured for beetroot juice mixed with blue-berried honeysuckle juice, next in order was the "pH 3.5 MIX" of beetroot juice with sea buckthorn juice.

In the case of all fruit based acidity regulators, a similar pattern of changes in antioxidant activity of tested "pH 3.5 MIX-es" after different storage periods was observed. After one week at 4 °C, the antioxidant potential of all samples decreased, which may indicate degradation of some reducing components. After subsequent week of storage, for samples containing juices of mirabelle plum, sea buckthorn and blue-berried honeysuckle, the antioxidant activity increased by 15–55 % in comparison to the previous storage period. This may result from the fact that betalains in acidic environment degrade and after some storage period they are being resynthesized. In the controls containing citric acid or lemon juice, the decrease of antioxidant activity continued also after the second storage period. However, no clear synergistic/antagonistic effects between fruit and beetroot juices was seen. Most of the mixtures showed lower antioxidant potential than the sum of their individual components (Table 3), though the exceptions were observed, especially in the case of "pH 3.5 MIX-es" containing lemon (TE value 23% above the additive effect) or mirabelle plum (TE value 6% above the additive effect) juices.

3.4. Total antioxidant activity of "9:1 MIX-es"

The antioxidative potential among tested "9:1 MIX-es" (Table 3), was the highest for beetroot juice combined with blue-berried honeysuckle juice. Next in line were the mixtures of beetroot juice with sea buckthorn juice and mirabelle plum juice. As in the case of "pH 3.5 MIX-es", the lowest antioxidant potential was determined for the mixture of beetroot juice with citric acid solution. Moreover, when combined in 1:9 proportion, 0.16 mol/L citric acid solution reduced the own antioxidant activity of beetroot juice. The storage of "9:1 MIX-es" for one week significantly decreased the content of antioxidants, which in the case of samples containing fruit juices was restored to some extent after subsequent week.

3.5. HPLC analysis of bioactive phytochemicals

Fig. 2 shows chromatograms obtained as a result of HPLC-DAD analysis followed by post-column derivatization with $ABTS^{\bullet+}$ reagent for "9:1 MIX-es" of beetroot juice with water (panel A), beetroot juice with sea buckthorn juice (panel B) and beetroot juice with blue-berried honeysuckle juice (panel C). These particular chromatograms were chosen, because they showed clear differences in antioxidant profiles resulting from the presence of fruit phytochemicals.

The addition of sea buckthorn juice to beetroot juice resulted in the enrichment of the final mixture in ascorbic acid (Fig. 2B, peak 1). Sea buckthorn is known to be a very good source of vitamin C, whose amount in this fruit ranges between 3.6 and 25 g/kg dw (Beveridge et al., 1999, 2002; Zeb, 2004). In the case of combination with blue-berried honeysuckle juice, the presence of cyanidin-3-*O*-glucoside can be observed (Fig. 2C, peak 6). This compound is a major anthocyanin typical for this berry, where its concentration may reach up to 42 g/kg dw (Kusznierewicz et al., 2012). Other chromatographic profiles, *i.e.* for citric acid, lemon or mirabelle plum containing mixtures, were identical with that determined for beetroot juice combined with water.

The relative changes in the quantity of beetroot betalains in mixtures studied upon storage are given in Table 4. Because of the lack of all betalain standards, the identification of major peaks if not

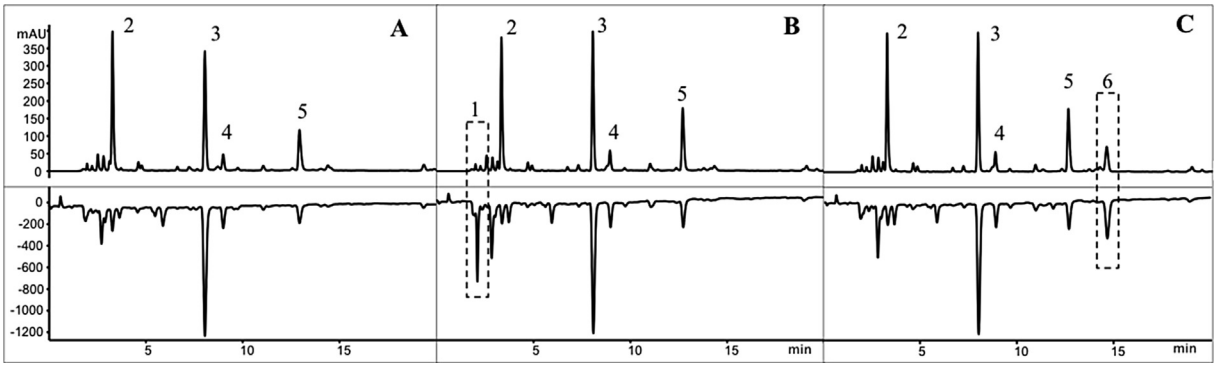

Fig. 2. Combined plots of profiles before - (top chromatograms traced at 270 nm) and after derivatization with ABTS reagent - (bottom chromatograms traced at 734 nm) obtained for "9:1 MIX-es" of beetroot juice with water (panel A), beetroot juice with sea buckthorn juice (panel B) and beetroot juice with blue-berried honeysuckle juice (panel C). Numbered peaks represent: ascorbic acid (1), vulgaxanthin I (2), betanin (3), isobetanin (4), neobetanin (5), and cyanidin-3-O-glucoside (6).

Table 4
The comparison of contents of major beetroot betalains expressed as peak areas determined by HPLC-DAD-MS for beetroot juice combined with the fruit based acidity regulators following different storage periods: freshly prepared mixtures, stored for 1 week or 2 weeks.

Relative changes in the content[a] of betalains[b]												
	Fresh MIX-es				After 1 week				After 2 weeks			
	Vulg	Bet	Iso	Neo	Vulg	Bet	Iso	Neo	Vulg	Bet	Iso	Neo
Beetroot juice combined with: pH 3.5 MIX												
Citric acid	95	107	107	248	72	104	105	247	193	162	171	309
Citric acid control I	100	100	100	100	100	100	100	100	100	100	100	100
Lemon	79	116	117	212	51	132	148	249	106	185	244	5303
Lemon control I	100	100	100	100	100	100	100	100	100	100	100	100
Mirabelle plum	80	123	125	338	54	135	149	255	119	196	267	3439
Mirabelle plum control I	100	100	100	100	100	100	100	100	100	100	100	100
Sea buckthorn	78	119	129	241	64	150	195	543	140	118	175	251
Sea buckthorn control I	100	100	100	100	100	100	100	100	100	100	100	100
Blue-berried honeysuckle	82	115	112	387	112	141	128	5261	137	178	155	8749
Blue-berried honeysuckle control I	100	100	100	100	100	100	100	100	100	100	100	100
9:1 MIX												
Control (water)	100	100	100	100	100	100	100	100	100	100	100	100
Citric acid	98	100	99	131	94	115	113	180	168	129	135	2022
Lemon	92	102	101	135	70	128	131	251	130	166	187	3097
Mirabelle plum	92	101	100	138	78	123	124	237	145	161	171	3145
Sea buckthorn	92	101	102	131	76	126	133	222	133	165	192	2467
Blue-berried honeysuckle	93	99	98	131	81	121	115	244	140	150	143	3007

[a] The content of betalains is expressed in Arbitrary Units estimated based on the areas under chromatographic peaks corresponding to indicated analytes; the values are results of a single determination.
[b] The abbreviated betalains names are: Vulg – vulgaxanthin, Bet – betanin, Iso – isobetanin, Neo - neobetanin.

confirmed with available standard compounds was achieved by comparison with published data. The structures of unknown compounds were deduced according to UV and MS spectra and based on literature data (Georgiev et al., 2010; Herbach, Stintzing, & Carle, 2004; Kujala, Vienola, Klika, Loponen, & Pihlaja, 2002). The mass spectra collected during analyses revealed fragmentation patterns characterized by a major molecular ion peak, that in the case of positive ionization occurs as $[M+H]^+$ and amounts respectively for vulgaxanthin I - 338 m/z, betanin – 551 m/z, isobetanin – 551 m/z and neobetanin – 549 m/z. The changes in the content of the major beetroot analytes during time of storage, in the presence or absence of fruit juices, are presented as the differences in peak areas only for four betalains. The decrease of content of betalains with time of storage was observed in both "pH 3,5 MIX-es" and "9:1 MIX-es". In the case of "9:1 MIX-es", after one-week of storage, this decrease is not very pronounced (no more than 30% of initial value). However, after two-weeks of storage period, in samples of beetroot juice and beetroot juice with citric acid, the decrease of vulgaxanthin I, betanin and isobetanin became clearly noticeable. Other "9:1 MIX-es" did not show much decrease in betalain content. It can thus be presumed that the addition of fruit juices prevents degradation of betalains. In the case of "pH 3,5 MIX-es", the decrease of betalain content after both storage periods was also observed. The protective effect of added fruit based acidity regulators on betalains in these samples was assessed by comparison of MIX-es with control samples in which the potential acidity regulators were replaced with deionized water. It has been shown in the case of "pH 3,5 MIX-es" (Table 4), that the decrease of betalain content depended not only on the kind of added acidity regulator, but also on its amount which determined pH of the final mixture. As mentioned before, it is well known that betalains are more stable in low pH environment, so the observed protective effect of fruit based acidity regulators may be explained in terms of the optimal pH level.

3.6. Cytotoxicity by MTT assay for "pH 3.5 MIX-es"

Another approach of testing health promoting properties of fruit based acidity regulators relied on the assessment of biological potential determined as capability of growth inhibition of HT29 cells by "pH 3.5 MIX-es". Similarly to common practice, it was assumed that higher cytotoxicity marks greater concentration of biologically

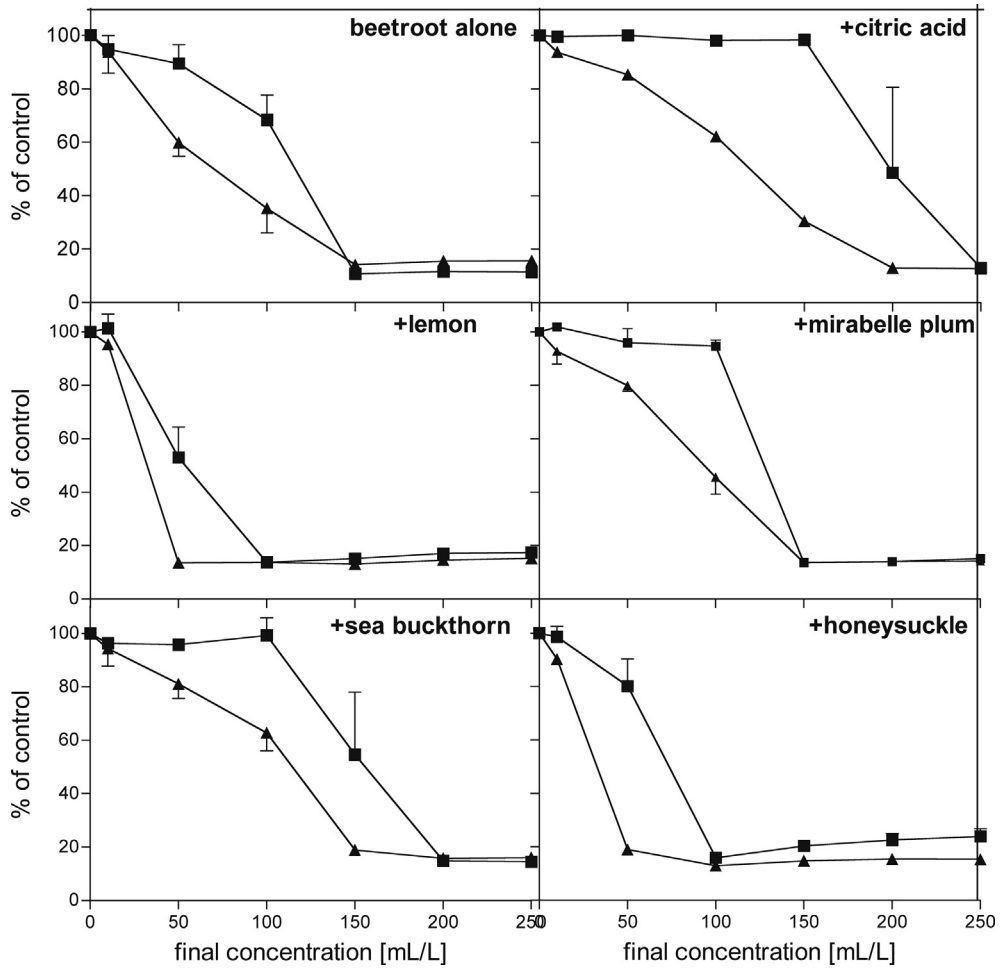

Fig. 3. Inhibition of growth of HT29 cells determined by MTT assay for "pH 3.5 MIX-es" after two treatment periods: 24 h (squares) and 72 h (triangles). Results represent means ± SD of three independent experiments carried out in triplicates.

active components.

Fig. 3 presents the dose response curves for the "pH 3.5 MIX-es" in HT29 cell line. In all cases, the prolonged exposure on plant juices resulted in the stronger biological effect. The lowest cytotoxicity exhibited the mixture containing citric acid, whose addition reversed significantly growth inhibition induced by beetroot juice alone *i.e.*, the sample used as a reference in these experiments. This may suggest that citric acid decreased biological potential of the tested juice. Among "pH 3.5 MIX-es" acidified with fruit juices, those containing lemon and blue-berried honeysuckle juices exhibited the strongest ability to inhibit cell growth. This is not suprising, since these fruit juices contain bioactive compounds that are known to impose cytotoxic effect on cancer cells: lemon is the rich source of flavonol glycosides, while blue-berried honeysuckle flavan-3-ols and anthocyanins. Most probably betalains were not phytochemicals responsible for cell growth inhibition because these two mixtures did not contain the highest amounts of betalains (Table 4). Cytotoxicity assessments again confirmed superiority of fruit based acidity regulators over citric acid in terms of bioactivity.

4. Conclusions

The study showed that fruit juices may successfully replace currently used acidity regulators, which would be of special value in the case of functional food production. The best fruit based acidity regulator turned out to be the blue-berried honeysuckle juice. Not only it displayed comparable ability to control pH as citric acid, but also ensured the highest antioxidant activity of the final mixtures. In the case of beetroot juice as a model food item, such a combination exhibited the strongest cytotoxic effect against cancer cells, but also gave the final food product an interesting flavor. Moreover, blue-berried honeysuckle juice enriched the mixture in a number of bioactive phytochemicals characterized by a range of reported chemopreventive properties that may have a very positive impact on consumers' health. The only drawback is the high cost of blue-berried honeysuckle due to rather limited harvest and low availability of this plant. Thus, the economic issues can restrict the potential production of acidity regulators based on extracts from this fruit. However, also very promising seemed sea buckthorn juice and mirabelle plum juice. Both of these plants are known for their positive impact on human health and in the presented experiments exhibited very close capacity of controlling pH of food products to citric acid. For economic reasons and because of the widespread occurrence, a minor degree of current utilization, and apparent ease of collection, mirabelle plum appears to be a replacement of current acidity regulators particularly worthy of interest.

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