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SCIENTIFIC PAPER

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KOMBUCHA FERMENTATION OF SIX MEDICINAL HERBS: CHEMICAL PROFILE AND BIOLOGICAL ACTIVITY

Article Highlights

- Kombucha on medicinal plants was characterized
- Higher antioxidant activity of herbal kombucha compared to traditional kombucha was established
- Higher phenolic and flavonoids contents in herbal kombucha were determined
- Elderberry kombucha beverage had the highest ACE inhibitory activity

Abstract

Bioactive properties, as well as chemical composition and sensory characteristics of traditional and alternative kombucha broths were examined. Samples were produced by adding 10% of kombucha starter to sweetened (7% sucrose) decocts of black and green teas (traditional substrates) and infusions of winter savory (Satureja montana), peppermint (Menthaׅ×piperita), stinging nettle (Urtica dioica), wild thyme (Thymus serpyllum), elderberry (Sambucus nigra) and quince (Cydonia oblonga), at 25 °C. Fermentation lasted the shortest with elderberry (3 days) and the longest with quince (10 days). The samples with black tea and peppermint had the best sensory quality. In general, alternative products had better antioxidant activity to hydroxyl radicals than the traditional ones, reducing power and angiotensin-converting enzyme inhibitory activity. The most pronounced acetic acid content was obtained with elderberry substrate. Moreover, peppermint substrate showed the highest values both for total phenols and total flavonoids contents. Taken together, the samples produced with the alternative substrates highlighted a higher phenolic and flavonoid contents, compared to the ones obtained with the traditional substrates.

Keywords: ACE, antioxidant, kombucha, medicinal herbs, functional food.

Kombucha is a fermented product that is obtained by metabolic activity of a starter composed of bacteria and yeasts [1-3]. The most common substrates for kombucha fermentation are black or green tea water decoct sweetened with 70 g sucrose per L. The typical fermentation period is 7 days at room temperature (25 °C) [1]. Bacteria and yeasts use substrates in complementary metabolic activity. Yeasts produce ethanol *via* hydrolysis of sucrose followed by glycolysis. Acetic acid bacteria produce acetic acid from ethanol. The obtained beverage contains sugars, organic acids, amino acids, vitamins, carbon dioxide, polyphenols, minerals etc. The product has a pleasant, sour, sweet and refreshing taste. As testified by consumers, this beverage has numerous benefits to the human organism and it is used as herbal tonic [1,3-5].

Black and green teas are used as traditional substrates in kombucha fermentation process. They are very well known for their beneficial properties and are consumed worldwide. These substrates are very good sources of nitrogen compounds needed for kombucha fermentation [6,7]. Winter savory, peppermint, stinging nettle, wild thyme, elderberry and quince are all well-known medicinal herbs that are used in traditional medicine. Medicinal plants are

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known as the source of natural antioxidant compounds [8]. Nitrogen compounds are not characteristically part of medicinal herb composition. Some studies suggested that it is possible to perform kombucha fermentation using them as substrates [9,10]. Application of these alternative substrates can widen the range of kombucha products in the market [11–15].

In order to gain the insight into the potential functional properties of new beverages, *in vitro* biological activity examination was used as the first step. This paper investigated possible antioxidant activity and ACE (angiotensin-converting enzyme) inhibitory activity. Products with antioxidant properties are useful in cases when the oxidant-antioxidant balance of a cell is disturbed due to excess generation of free radicals [16]. The inhibition of ACE activity possesses an overall antihypertensive effect, and ACE inhibitors point to great significance in the therapy of hypertension, in its prevention and in the initial treatment of mildly hypertensive individuals [17].

The initial substrates used for kombucha fermentation, as well as different types of kombucha beverages, possess antioxidant activity [1,6]. Shahbazi *et al.* [18] concluded that the use of medicinal plants, such as Shirazi thyme, cardamom and cinnamon, increased the bioactive potential of kombucha.

The aim of this paper was to investigate the possibility of herbal water extracts application in kombucha beverages production and to gain insight into the chemical composition, sensory characteristics and *in vitro* biological activity of the obtained beverages.

MATERIAL AND METHODS

Initial substrates for kombucha fermentation

Traditional and alternative initial substrates were used.

Traditional substrates were black and green tea, prepared as decocts: in 1 L of boiling tap water 1.5 g of black, *i.e.*, green tea, and 70 g of sucrose were added. After boiling for 5 min, the teas were left steeping for 15 min and then were filtered and left to cool at room temperature (25 °C).

Alternative initial substrates were winter savory herb, peppermint leaves, stinging nettle leaves, wild thyme herb, elderberry flowers and quince leaves. These herbal water extracts were prepared as infusions: in 1 L of boiling tap water, 2.26 g of winter savory, peppermint, stinging nettle, wild thyme, *i.e.*, quince and 3 g of elderberry and 70 g of sucrose were added. The obtained herbal preparation was left steeping for 15 min and then it was filtered and left to cool to room temperature.

Kombucha

The local kombucha contains bacteria of the *Acetobacter* genus and yeast strains that include: *Saccharomycodes ludwigii*, *Saccharomyces cerevisiae*, *Saccharomyces bisporus*, *Torulopsis* sp. and *Zygosaccharomyces* sp. [19].

Fermentation process

Fermentation was performed in a 600 mL glass beaker. Inoculum (kombucha starter) was added to the initial substrates in the amount of 10 vol.%. In order to follow the fermentation progress, the analyzed samples included initial substrates, start of process and broths of the third, seventh and tenth day. All samples were analyzed on the sampling day.

Samples were marked depending on the used substrate and fermentation day, as follows: black tea (BT), green tea (GT), winter savory (WS), peppermint (P), stinging nettle (SN), wild thyme (WT), elderberry (E), quince (Q); 0 – start of fermentation, 3 – third day of fermentation, 7 – seventh day of fermentation, 10 – tenth day of fermentation.

pH determination

pH was measured using a pH-meter (WTW series Inolab pH 720, Weilheim, Bayern, Germany).

Total acidity determination

Total acidity was measured by titration with a standard solution of 0.1 mol/L sodium hydroxide using phenolphthalein as indicator [20]. Results were expressed in grams of acetic acid per liter of sample.

Organic acids analysis

HPLC analysis of ascorbic, acetic, succinic, oxalic, tartaric, formic, lactic, malic, malonic and citric acid was performed with Agilent 1100 Series HPLC, Santa Clara, CA, USA, using reversed phase chromatography, according to Vitas *et al.* [10]. The system had a degasser, binary pump, Zorbax® SB-C18 column (4.6 mm×150 mm, 5 μm) and UV-DAD detector. Liquid chromatography was performed in isocratic mode with 6 mmol/L phosphoric acid (pH 2.1) as mobile phase and parameters were as follows: flow rate 1.0 mL/min, detection wavelength 220 nm and column temperature 28 °C. External standard method calibration was done. Results were expressed in g of organic acid per L of sample.

Total phenolic determination

Total phenolic content was determined spectrophotometrically (Jenway 6300, Dunmow, Essex, UK), using the Folin-Ciocalteu method, with some modifications [21]. The reaction mixture was prepared by

pipetting 0.5 mL of the sample, 0.5 mL of Folin-Ciocalteu reagent and 1.5 mL of sodium carbonate (20 mass%), in a 10 mL volumetric flask, which was then filled to the final volume with distilled water and shaken well. Incubation lasted for 120 min in the dark, at room temperature. Absorbance was measured at 750 nm. The blank was prepared using distilled water instead of the sample. Gallic acid was used as a calibration standard and the results were expressed in gallic acid equivalents per mL of sample (mg GA/mL).

Total flavonoids determination

Total flavonoids content was measured using the spectrophotometric method described by Markham, with minor modifications [22]. Samples in the amount of 3 mL were mixed with 4 mL of distilled water and 0.5 mL of sodium nitrite solution (5%). After 5 min, 0.3 mL of aluminum chloride hexahydrate (10%) was added and allowed to stand for 6 min. Sodium hydroxide (1 mol/L, 1 mL) and 1.4 mL of distilled water were added to the mixture. The blank was prepared by replacing the sample with distilled water. Absorbance was measured at 510 nm. Rutin was used as a calibration standard and results were expressed as rutin equivalents per mL of sample (mg RE/mL).

Catalase activity determination

Catalase activity measurement was based on a method described in literature [23], with some modifications. Sample, in the amount of 25 mL was pipetted in a glass beaker. Peroxide substrate (100 mL) was rapidly added and the solution was mixed for 5 to 10 s. The beaker was covered and incubated at 25 °C until the end of reaction. After 30 min, reaction solution was thoroughly stirred for 5 s and 4 mL were transferred to the Erlenmeyer flask. Sulfuric acid (1 mol/L) was added in the amount of 5 mL, mixed and then 5 mL of 40% potassium iodide and 1 drop of 1% ammonium molybdate was added. The reaction mixture was stirred and titrated with sodium tiosulphate solution. The blank sample was analyzed in the same way, and 4 mL of peroxide substrate were used instead of the sample. Catalase activity was expressed in Baker units per mL of the sample (BU/mL).

Sensory mark determination

Sensory analysis was performed according to Vitas *et al.* [10]. Category scale of 5 points (1 - the lowest and 5 - the highest) and a descriptive test were used. Taste, color, odor and appearance were evaluated.

DPPH radical scavenging ability determination

DPPH radical scavenging ability was measured using the spectrophotometric method described by Morales and Jimenez-Perez [24], with certain modifications. Standard solution of DPPH radical was 80 μmol/L ethanol solution of DPPH radical. Reaction mixture consisted of 4.8 mL standard DPPH solution and 1 mL of sample. Blank sample used 1 mL of distilled water. Reaction mixtures were incubated for 1 hour at the room temperature and absorbance was measured at 515 nm. DPPH radical scavenging ability was expressed in percentages (RSA_{DPPH}) and calculated by Eq. (1):

$$RSA_{DPPH}(\%) = 100\frac{A_{blank} - A_{sample}}{A_{blank}} \quad (1)$$

where A_{blank} is the absorbance of the blank and A_{sample} is the absorbance of the sample.

Hydroxyl radical scavenging ability determination

Hydroxyl radical scavenging ability was measured according to Deeseenthum and Pejovic [25] with minor modifications. The 100 μL of sample was mixed with 450 μL of 0.2 mol/L sodium phosphate buffer (pH 7.00), 150 μL of 10 mmol/L 2-deoxyribose, 150 μL of 10 mmol/L EDTA disodium salt dihydrate, 150 μL of 10 mmol/L $FeSO_4 \cdot 7H_2O$, 150 μL of 10 mmol/L H_2O_2, and 525 μL of distilled water. Reaction mixtures were incubated at 37 °C for 2 h after which 750 μL of 2.8% trichloroacetic acid and 750 μL of 0.1% thiobarbituric acid was added. Afterwards, the test tubes were kept in boiling water for 10 min. Absorbance was measured at 520 nm. Blank sample used 100 μL of distilled water. Hydroxyl radical scavenging ability was given in percentages ($RSA_{\bullet OH}$) and calculated by Eq. (1).

Reducing power ability determination

Reducing power ability was measured according to Yildirim *et al.* [26], using a slightly modified method. The 300 μL of sample was mixed with 2.5 mL of 0.2 mol/L phosphate buffer (pH 6.60) and 2.5 mL of 1% potassium ferricyanide. Reaction mixtures were incubated at 50 °C during 30 min. Afterwards, 2.5 mL of 10% trichloroacetic acid was added. The 2.5 mL of this solution was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% $FeCl_3 \cdot 6H_2O$. Absorbance was measured at 700 nm. Higher absorbance indicates higher reducing power.

ACE inhibitory activity determination

The ACE-inhibitory activity of the samples was determined by Yoshie-Stark *et al.* [27] The method is

based on the reaction of hippuryl-L-histidyl-leucine (HHL) into hippuric acid (HA) by the action of angiotensin converting enzyme (ACE) under defined conditions (80 min at 37 °C). Briefly, 60 μL of sample was mixed with 80 μL of HHL in 0.2 mol/L potassium phosphate buffer containing 300 mmol/L NaCl at pH 8.3 and 10 μL of ACE solution. The mixture was incubated at 37 °C for 80 min; the reaction was stopped with 110 μL of 1 mol/L HCl. The reaction was followed without and in the presence of a potential inhibitor substance (sample). The product of the reaction was quantitated on the HPLC equipment (1100 Agilent Santa Clara, CA, USA, with an Agilent DAD detector). The chromatographic conditions were: flow rate 1 mL/min, temperature 23 °C, injection volume 20 μL and UV detection at 228 nm. Mobile phase was 50% of methanol with 1 vol.% trifluoroacetic acid in double-distilled water. Inhibition of ACE was calculated by Eq. (2):

$$\text{ACE inhibition (\%)} = 100\frac{HA_{blank} - HA_{sample}}{HA_{sample}} \quad (2)$$

where HA_{blank} is the peak area without ACE inhibition and HA_{sample} is the peak area in the sample.

Statistics

All analyses in this investigation were repeated three times and results were given as mean ± standard deviation using Microsoft Office Excel 2010. Also, using the same program, regression and correlation analyses were performed. One-way ANOVA and Tuckey's test ($p < 0.05$) were performed using Statistica 13.2.

RESULTS AND DISCUSSION

pH values and total acidity

pH value is a standard parameter of kombucha beverage production. It is used to indicate the success of the fermentation [1].

pH values of all initial substrates were in the narrow range, from 7.64 (BT) to 8.60 (WT) (Figure 1a-c). These values are in accordance to pH of yarrow infusions which were used in kombucha beverages production [10]. During the fermentation, initial pH values dropped for all samples which indicated kombucha culture activity on all herbal extracts.

Kombucha beverage is usually consumed at the pH value around 3.00 [28]. Based on the pH value it can be concluded that kombucha beverages can be consumed after 3 days for elderberry extract, 10 days for quince extract and 7 days, which is the most usual case, using black tea, green tea, winter savory, peppermint, stinging nettle and wild thyme extract (Figure 1a-c). The change in pH values, at the beginning of fermentation, amounted approximately 3-4 pH units for all initial substrates, except for the peppermint extract (2 pH units). These results were in accordance to literature data obtained for yarrow extracts [10]. Similar changes in pH values were established for kombucha fermentation of African mustard leaves [29]. In this experiment pH decreased from initial value of 7 to final value of 3, after 14 days of the fermentation process [29].

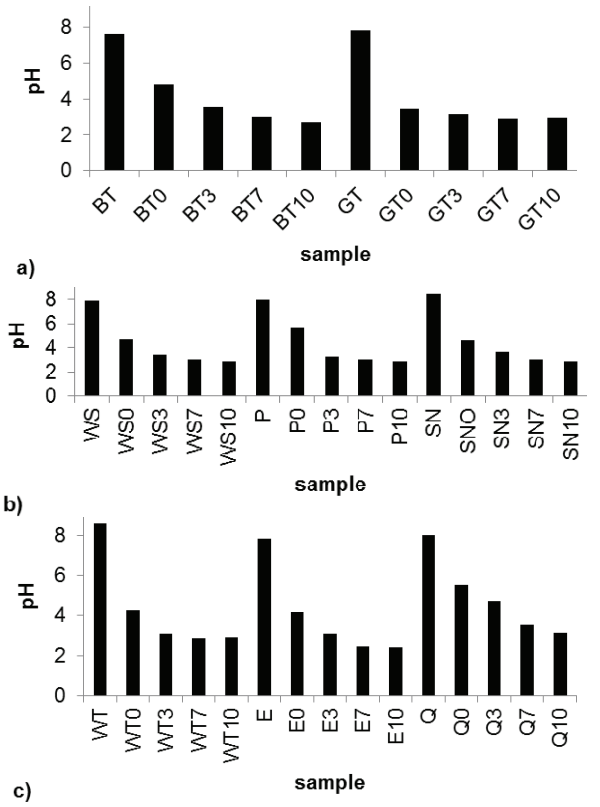

Figure 1. pH values of initial substrates and kombucha products during fermentation: a – black and green tea; b – winter savory, peppermint and stinging nettle; c – wild thyme, elderberry and quince.

Total acidity is also an usual parameter of kombucha fermentation that indicates the metabolic activity of used starter [1,6]. Total acidity values of initial substrates ranged from 0.02 (for green tea) to 0.11 g acetic acid/L (for peppermint) (Figure 2a-c), and the obtained results were in accordance with literature data [10]. Kombucha culture caused the gradual increase in total acidity during fermentation, for all beverages.

The biggest difference in total acids content was between initial substrates and the start of fermentation for samples with black tea, green tea, stinging

nettle, wild thyme and elderberry. The significant increase of total acidity was noticed between the start of fermentation and the third day for products with peppermint. The same trend was established between the third and seventh day for samples with stinging nettle. When taking into account the consummation day, the most pronounced fermentation was achieved using stinging nettle (4.05 g acetic acid/L, 7th day of fermentation) and quince extract (4.06 g acetic acid/L, 10th day of fermentation, Figure 2a-c). The obtained results correspond to kombucha products with yarrow obtained using yarrow infusions [10].

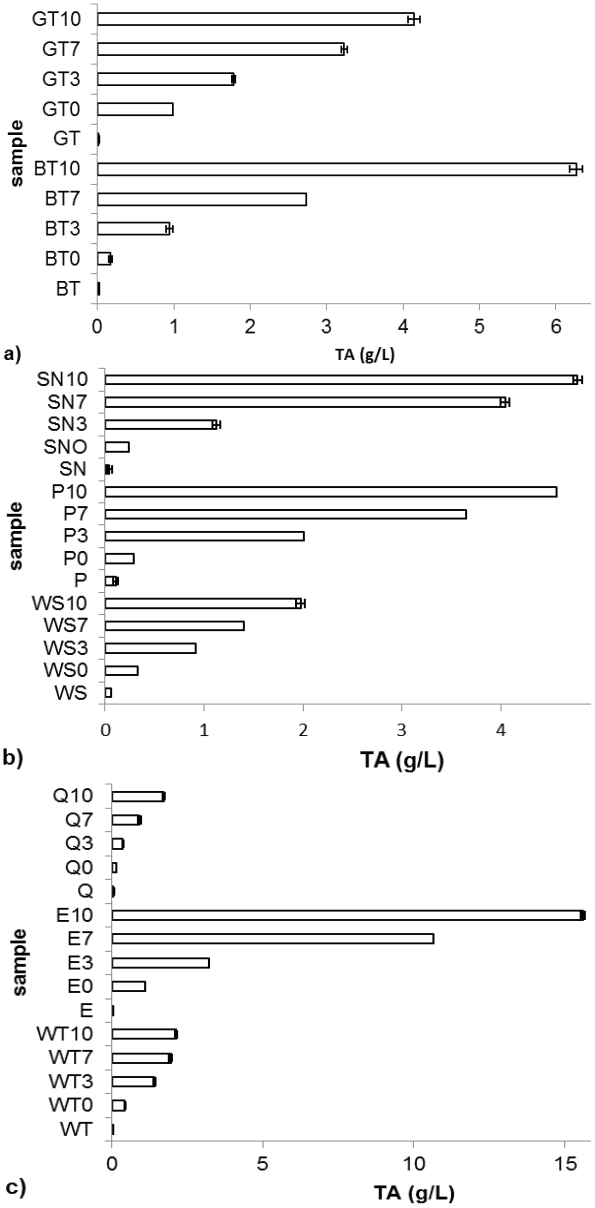

Figure 2. Total acidity initial substrates and kombucha products during fermentation: a - black and green tea; b - winter savory, peppermint and stinging nettle; c - wild thyme, elderberry and quince.

Organic acids

In order to further elucidate the chemical composition of the kombucha beverages, organic acids profiles were determined. Organic acids are important part of kombucha beverage composition because they influence sensory characteristics and have numerous beneficial effects to human organism [10]. The organic acids composition determination included the examination of ascorbic, acetic, succinic, oxalic, tartaric, formic, lactic, malic, malonic and citric acid. Ascorbic, succinic, tartaric and citric acid were not established in any of the samples, using the applied method. Results of organic acids content are given in Tables 1 and 2.

Acetic acid

In kombucha fermentation, ethanol was used by the acetic acid bacteria to produce acetic acid, which is considered the most characteristic metabolite [30]. All herbal extracts, used as initial substrates, do not contain acetic acid. During the fermentation, acetic acid content increased over time. Only samples with wild thyme between the start of fermentation and the third day showed similar content. On the basis of the acetic acid content, the most pronounced fermentation was with the elderberry extract (Table 1). The lowest value was 0.05 g/L for sample BT0 and the highest value had sample E10 (6.90 g/L). Using African mustard leaves in kombucha fermentation, the acetic acid content was increased to reach 15 g/L on the 10th day of fermentation which was around two-fold higher than the highest measured value in this paper. This difference can be attributed to the substrates used in the fermentation process.

Oxalic acid

Oxalic acid was determined in all samples, which suggested bacteria activity of kombucha culture, since they cause oxidation of carbohydrates. Herbal extracts with black tea, green tea, winter savory, wild thyme and quince had considerably lower content in comparison to the appropriate kombucha beverages (Table 1). These results indicate that kombucha stimulates the production of oxalic acid which is in accordance with literature [10]. Extract with stinging nettle showed higher content than the beverages, until the tenth day of process. Extracts with peppermint and elderberry showed a slightly lower content than the fermentation products. During the fermentation process, the content increased in the production of all beverages. The lowest measured value was 0.02 g/L for sample GT0 and the highest was 2.48 g/L (E10).

Table 1. Acetic, oxalic and formic acid content of initial substrates and kombucha products during fermentation; results expressed as average ± SD. Different letters indicate significant difference among the samples ($p < 0.05$)

Acetic acid				Oxalic acid				Formic acid			
Sample	g/L	Sample	g/L	Sample	g/L	Sample	g/L	Sample	g/L	Sample	g/L
BT^{a}	-	SN^{a}	-	BT^{a}	0.02±0.00	SN^{f,g,h}	0.55±0.03	BT^{a,b}	0.02±0.00	SN^{a,b}	0.01±0.00
BT0^{a,b}	0.05±0.00	SN0^{b,c,d}	0.18±0.01	BT0^{a-f}	0.22±0.02	SN0^{b-h}	0.44±0.05	BT0^{h,i}	0.21±0.03	SN0^{a,b,c}	0.04±0.02
BT3^{c,d,e}	0.27±0.02	SN3^{n}	1.12±0.05	BT3^{a-e}	0.19±0.01	SN3^{b-h}	0.46±0.03	BT3	0.16±0.00	SN3^{a,b,c,d}	0.06±0.00
BT7^{k}	0.66±0.00	SN7^{q}	1.98±0.10	BT7^{g,h}	0.60±0.03	SN7^{g,h}	0.57±0.03	BT7^{k,l}	0.32±0.01	SN7^{d,e}	0.12±0.00
BT10^{l}	0.84±0.01	SN10^{s}	3.03±0.16	BT10^{i}	1.01±0.06	SN10^{i}	1.15±0.07	BT10^{m}	0.52±0.03	SN10^{k,l}	0.32±0.03
GT^{a}	-	WT^{a}	-	GT^{a}	0.02±0.00	WT^{a}	0.02±0.00	GT^{a,b}	0.02±0.00	WT^{a,b}	0.02±0.00
GT0^{d,e,f}	0.30±0.01	WT0^{e,f,g,h}	0.41±0.02	GT0^{a,b}	0.12±0.01	WT0^{a-f}	0.22±0.01	GT0^{c,d,e}	0.10±0.01	WT0^{f,g,h,i}	0.19±0.00
GT3^{i,j,k}	0.56±0.05	WT3^{e,f,g,h}	0.37±0.02	GT3^{a-d}	0.15±0.00	WT3^{a-f}	0.21±0.02	GT3^{d,e}	0.12±0.01	WT3^{i,j}	0.23±0.01
GT7^{o}	1.31±0.02	WT7^{h,i,j}	0.50±0.02	GT7^{a-f}	0.22±0.01	WT7^{a-h}	0.32±0.02	GT7^{e,f}	0.13±0.02	WT7^{i,j}	0.25±0.01
GT10^{p}	1.66±0.07	WT10^{j,k}	0.60±0.02	GT10^{a-h}	0.31±0.02	WT10^{c-h}	0.47±0.02	GT10^{g,h,i}	0.20±0.05	WT10^{l}	0.36±0.01
WS^{a}	-	E^{a}	-	WS^{a}	0.03±0.01	E^{a-h}	0.34±0.01	WS^{a,b}	0.02±0.00	E^{a,b}	0.03±0.00
WS0^{b,c,d}	0.18±0.04	E0^{l,m}	0.91±0.02	WS0^{a-d}	0.14±0.00	E0^{e-h}	0.50±0.02	WS0^{c,d,e}	0.10±0.00	E0^{e,f,g}	0.14±0.00
WS3^{e,f,g}	0.35±0.03	E3^{r}	2.43±0.03	WS3^{a-h}	0.31±0.02	E3^{i}	1.31±0.06	WS3^{e,f,g}	0.14±0.02	E3^{k,l}	0.33±0.02
WS7^{f,g,h,i}	0.42±0.02	E7^{t}	4.80±0.02	WS7^{d-h}	0.48±0.02	E7^{j}	2.34±0.10	WS7^{h,i}	0.21±0.00	E7^{n}	1.67±0.09
WS10^{g,h,i,j}	0.48±0.00	E10^{u}	6.90±0.04	WS10^{h}	0.61±0.04	E10^{j}	2.48±0.10	WS10^{j,k}	0.28±0.02	E10^{a}	-
P^{a}	-	Q^{a}	-	P^{a,b}	0.12±0.00	Q^{a}	0.03±0.00	P^{a,b}	0.01±0.00	Q^{a,b}	0.02±0.01
P0^{a,b}	0.12±0.01	Q0^{a,b}	0.14±0.02	P0^{a-e}	0.20±0.01	Q0^{a}	0.07±0.00	P0^{b,c,d}	0.11±0.04	Q0^{a,b,c,d}	0.06±0.01
P3^{m,n}	1.01±0.04	Q3^{d,e,f}	0.32±0.05	P3^{a-g}	0.24±0.02	Q3^{a}	0.08±0.01	P3^{e,f,g,h}	0.15±0.00	Q3^{a,b,c}	0.05±0.00
P7^{p}	1.56±0.09	Q7^{p}	1.57±0.01	P7^{b-h}	0.43±0.03	Q7^{a,b,c}	0.13±0.00	P7^{e,f,g,h}	0.16±0.01	Q7^{a}	-
P10^{q}	1.94±0.11	Q10^{r}	2.39±0.03	P10^{g,h}	0.58±0.03	Q10^{a-e}	0.20±0.01	P10	0.16±0.00	Q10^{d,e}	0.12±0.01

Table 2. Lactic, malic and malonic acid content of initial substrates and kombucha products during fermentation; results expressed as average ± SD. Different letters indicate significant difference among the samples ($p < 0.05$)

Lactic acid				Malic acid				Malonic acid			
Sample	g/L	Sample	g/L	Sample	g/L	Sample	g/L	Sample	g/L	Sample	g/L
BT^{d,e,f}	0.08±0.00	SN^{a}	-	BT^{a}	-	SN^{a}	0.01±0.00	BT^{a}	-	SN^{a}	-
BT0^{e,f,g}	0.10±0.01	SN0^{a}	-	BT0^{a}	-	SN0^{a}	0.02±0.01	BT0^{a}	-	SN0^{a}	-
BT3^{e,f,g}	0.10±0.01	SN3^{a,b}	0.01±0.00	BT3^{a}	0.03±0.00	SN3^{a}	0.09±0.05	BT3^{a}	-	SN3^{a}	-
BT7^{a}	-	SN7^{f,g}	0.11±0.00	BT7^{a}	0.03±0.00	SN7^{a}	0.03±0.01	BT7^{e}	0.10±0.01	SN7^{a}	-
BT10^{a}	-	SN10^{i}	0.22±0.05	BT10^{a}	0.04±0.00	SN10^{a}	0.08±0.05	BT10^{f}	0.12±0.00	SN10^{c}	0.04±0.00
GT^{a,b,c,d,e}	0.05±0.00	WT^{a,b}	0.01±0.00	GT^{a}	-	WT^{a}	0.03±0.00	GT^{a}	-	WT^{a}	-
GT0^{b,c,d,e,f}	0.06±0.01	WT0^{a,b}	0.01±0.01	GT0^{a}	-	WT0^{a}	0.03±0.00	GT0^{a}	-	WT0^{a}	-
GT3^{c,d,e,f}	0.07±0.01	WT3^{a}	-	GT3^{a}	-	WT3^{a}	0.02±0.00	GT3^{a}	-	WT3^{b}	0.02±0.00
GT7^{a}	-	WT7^{a}	-	GT7^{a}	0.01±0.01	WT7^{a}	0.03±0.00	GT7^{d}	0.06±0.01	WT7^{b}	0.02±0.00
GT10^{a}	-	WT10^{a}	-	GT10^{a}	0.02±0.01	WT10^{a}	0.03±0.00	GT10^{e}	0.09±0.02	WT10^{b}	0.02±0.00
WS^{j}	0.29±0.01	E^{g,h}	0.14±0.01	WS^{b}	0.65±0.89	E^{a}	0.07±0.00	WS^{a}	-	E^{a}	-
WS0^{j}	0.30±0.07	E0^{h,i}	0.18±0.01	WS0^{a}	0.03±0.01	E0^{a}	0.08±0.01	WS0^{a}	-	E0^{a}	-
WS3^{j}	0.33±0.03	E3^{a}	-	WS3^{a}	0.03±0.01	E3^{a}	0.07±0.00	WS3^{a}	-	E3^{g}	0.18±0.01
WS7^{a}	-	E7^{a}	-	WS7^{a}	0.01±0.00	E7^{a}	0.11±0.00	WS7^{h}	0.28±0.01	E7^{g}	0.17±0.01
WS10^{a,b,c}	0.02±0.02	E10^{a}	-	WS10^{a}	0.02±0.00	E10^{a}	0.12±0.01	WS10^{i}	0.31±0.00	E10^{h}	0.27±0.01
P^{a}	0.01±0.01	Q^{a,b}	0.01±0.00	P^{b}	0.66±0.90	Q^{a}	0.01±0.00	P^{a}	-	Q^{a}	-
P0^{a,b}	0.01±0.00	Q0^{a,b,c}	0.02±0.01	P0^{a}	0.03±0.00	Q0^{a,b}	0.05±0.05	P0^{a}	-	Q0^{a}	-
P3^{a,b,c}	0.02±0.00	Q3^{a}	-	P3^{a}	0.02±0.01	Q3^{a}	0.02±0.00	P3^{a}	-	Q3^{b}	0.02±0.00
P7^{a,b,c,d}	0.03±0.00	Q7^{a}	-	P7^{a}	0.03±0.00	Q7^{a}	0.02±0.00	P7^{b}	0.02±0.00	Q7^{b,c}	0.03±0.00
P10^{a,b,c,d,e}	0.05±0.00	Q10^{a}	-	P10^{a}	0.04±0.00	Q10^{a}	0.02±0.00	P10^{b}	0.02±0.00	Q10^{b,c}	0.03±0.00

Formic acid

Formic acid was determined in all samples, except in samples E10 and Q7. The presence of formic acid indicates that reduction of carbon dioxide occurred. All initial substrates had lower content of formic acid in comparison to the produced beverages. Content increased during the fermentation process in all kombucha beverages (Table 1). Sample SN0 had the lowest (0.01 g/L) and sample BT10 the highest content of formic acid (0.52 g/L). The obtained values were higher in comparison to kombucha beverages produced using yarrow infusions [10].

Lactic acid

Lactic acid is a result of fermentation by lactic acid bacteria. Lactic acid determined in traditional substrates and traditional kombucha beverages showed increase in content, until the third day of fermentation. It was not measured in the samples from the seventh and tenth day. Samples with wild thyme, elderberry and quince contained lactic acid only in the initial substrates and at the beginning of the production process and the content was lower in the initial substrates. Products with stinging nettle showed lactic acid from the third day of fermentation, with gradual increase. Lactic acid was measured in all samples with peppermint and content increased during the fermentation process. In the samples with winter savory, lactic acid content increased until the third day of fermentation and after that it decreased. The lowest content was determined in sample WS7 (0.005 g/L) and the highest in sample WS3 (0.33 g/L) (Table 2). Literature data show that black tea kombucha contained higher amounts of lactic acid in comparison to green tea kombucha, during fermentation from 6^{th} to 18^{th} day [30].

Malic acid

Malic acid was determined in all samples obtained using alternative substrates. Samples WS and P had malic acid in more pronounced amounts in comparison to the appropriate kombucha beverages. Products with SN showed increase until third day and then decreased in content. Beverages with P had an increase in malic acid content during the whole fermentation. Samples SN, WT, E and Q had lower malic acid content in initial substrates and an increase in content during the fermentation (Table 2). Samples with black and green tea had malic acid after third and seventh day of fermentation, respectively. Sample Q showed the lowest malic acid content (0.01 g/L) and sample P the highest (0.66 g/L). Kombucha beverages with yarrow infusions showed higher malic acid content [6]. Since malic acid can be produced by yeasts [31], the obtained results suggest that the herbal water extracts used are more suitable for kombucha fermentation than black and green tea, in terms of kombucha yeasts activity. Malic acid is one of the typical organic acids in black tea kombucha [2].

Malonic acid

Malonic acid is aliphatic dicarboxylic acid whose importance for food products relates to the maintaining of acidity levels [32]. Malonic acid was determined in kombucha beverages with black and green tea, winter savory and peppermint on the seventh and tenth day of fermentation. The content was higher on the tenth day for all products, except for beverages with peppermint which showed the same malonic acid content on both days. In samples with stinging nettle, it was determined only on the tenth day of fermentation. Products with wild thyme, elderberry and quince contained malonic acid from the third to the tenth day of fermentation and the amount increased during this time period (Table 2). The lowest content of malonic acid had sample WT7 (0.02 g/L) and the highest content showed product WS10 (0.31 g/L). Kombucha beverages with peppermint showed higher total phenolic content than traditional kombucha products.

Total phenolic content

Phenolic compounds are secondary metabolites of plant origin that possess biological activity [8]. In kombucha, they are believed to be a product of hydrolysis of a polyphenolic compounds originating from the herbal raw material [30]. Traditional kombucha products had higher total phenols content than the initial substrates (Table 3). This pattern was established for alternative kombucha beverages as well, except for samples SN3 and E0 which showed slightly lower values in comparison to the appropriate initial substrates. The most pronounced influence of the fermentation process was observed for products with winter savory. Total phenols value was eight times higher than those measured in the initial substrate. During the fermentation, differences in total phenols content were more pronounced for alternative kombucha beverages, when compared to the traditional ones (Table 3). The lowest value was determined in the sample WS (0.01 mg GAE/mL), and the highest in P10 (0.15 mg GAE/mL). The highest value measured in this investigation approximately corresponded to the lowest values given in literature [10]. Previous data suggested that the highest total phenolic content was found in the ethyl acetate fraction of the kombucha fermented African mustard leaves [29] in com-

Table 3. Total phenols, total flavonoids content and catalase activity of initial substrates and kombucha products during fermentation; results expressed as average ± SD. Different letters indicate significant difference among the samples ($p < 0.05$)

Total phenols				Total flavonoids				Catalase activity			
Sample	mg GA/mL	Sample	mg GA/mL	Sample	mg RE/mL	Sample	mg RE/mL	Sample	BU/mL	Sample	BU/mL
BT^{a,b}	0.03±0.00	SN^{a,b,c,d}	0.08±0.00	BT^{a,b}	0.02±0.00	SN^{c,d,e,f,}	0.06±0.00	BT^{h-n}	0.04±0.00	SN^{e-m}	0.04±0.00
BT0^{a,b,c}	0.04±0.00	SN0^{a,b,c,d}	0.09±0.00	BT0^{a,b}	0.02±0.00	SN0^{e,f,g}	0.08±0.00	BT0^{e-m}	0.04±0.00	SN0^{d-l}	0.04±0.00
BT3^{a,b,c}	0.04±0.00	SN3^{a,b,c,d}	0.08±0.00	BT3^{a,b}	0.02±0.00	SN3^{d,e,f,g}	0.07±0.00	BT3^{o,p,q}	0.05±0.00	SN3^{j-o}	0.04±0.00
BT7^{a,b,c}	0.04±0.00	SN7^{a,b,c,d}	0.10±0.00	BT7^{a,b,c}	0.03±0.00	SN7^{e,f,g}	0.08±0.00	BT7^{i-o}	0.04±0.00	SN7^{m-p}	0.05±0.00
BT10^{a,b,c}	0.04±0.00	SN10^{b,c,d}	0.11±0.06	BT10^{a,b,c}	0.03±0.00	SN10^{c,d,e,f}	0.06±0.00	BT10^{p,q}	0.05±0.00	SN10^{c}	0.03±0.01
GT$^{a ,b}$	0.03±0.00	WT^{a,b,c}	0.05±0.00	GT^{a,b,c}	0.03±0.00	WT^{b,c,d}	0.04±0.00	GT^{d-j}	0.04±0.00	WT^{d-k}	0.04±0.00
GT0^{a,b,c}	0.04±0.00	WT0^{a,b,c,d}	0.09±0.00	GT0^{a,b,c}	0.03±0.00	WT0^{b,c,d}	0.04±0.00	GT0^{c,d,e}	0.04±0.00	WT0^{d-i}	0.04±0.00
GT3^{a,b,c}	0.05±0.00	WT3^{b,c,d}	0.11±0.00	GT3^{b,c,d}	0.04±0.00	WT3^{a,b,c}	0.03±0.00	GT3^{d-l}	0.04±0.00	WT3^{d-h}	0.04±0.00
GT7^{a,b,c,d}	0.06±0.00	WT7^{a,b,c,d}	0.10±0.01	GT7^{a,b,c}	0.03±0.00	WT7^{d,e,f,g}	0.07±0.00	GT7^{d-h}	0.04±0.00	WT7^{d,e,f}	0.04±0.00
GT10^{a,b,c,d}	0.07±0.00	WT10^{b,c,d}	0.11±0.13	GT10^{c,d,e,f}	0.06±0.00	WT10^{f,g,h}	0.09±0.00	GT10^{e-l}	0.04±0.00	WT10^{d-i}	0.04±0.00
WS^{a}	0.01±0.00	E^{a,b,c,d}	0.09±0.00	WS^{a}	0.00±0.00	E^{b,c,d,e}	0.05±0.00	WS^{k-o}	0.05±0.00	E^{d-g}	0.04±0.00
WS0^{a,b,c,d}	0.08±0.00	E0^{a,b,c,d}	0.08±0.00	WS0^{b,c,d,e}	0.05±0.00	E0^{c,d,e}	0.06±0.00	WS0^{c,d}	0.04±0.00	E0^{d-h}	0.04±0.00
WS3^{a,b,c,d}	0.07±0.00	E3^{b,c,d}	0.10±0.05	WS3^{c,d,e,f}	0.06±0.00	E3^{d,e,f,g}	0.07±0.00	WS3^{o,p,q}	0.05±0.00	E3^{f-m}	0.04±0.00
WS7^{a,b,c,d}	0.10±0.00	E7^{b,c,d}	0.12±0.03	WS7^{c,d,e,f}	0.06±0.00	E7^{f,g,h}	0.09±0.00	WS7^{m-p}	0.05±0.00	E7^{f-m}	0.04±0.00
WS10^{e}	0.06±0.00	E10^{b,c,d}	0.12±0.01	WS10	0.05±0.00	E10^{b,c,d}	0.04±0.00	WS10^{m-p}	0.05±0.00	E10^{q}	0.05±0.00
P^{a,b,c,d}	0.08±0.00	Q^{a,b,c,d}	0.10±0.00	P^{b,c,d}	0.04±0.00	Q^{f,g,h}	0.09±0.00	P^{d-l}	0.04±0.00	Q^{g-n}	0.04±0.00
P0^{a,b,c,d}	0.10±0.00	Q0^{b,c,d}	0.12±0.01	P0^{c,d,e,f}	0.06±0.00	Q0	0.05±0.00	P0^{l-p}	0.05±0.00	Q0^{d-i}	0.04±0.00
P3^{b,c,d}	0.12±0.07	Q3^{b,c,d}	0.11±0.00	P3^{f,g,h}	0.09±0.00	Q3^{b,c,d,e}	0.05±0.00	P3^{n,o,p}	0.05±0.00	Q3^{f-n}	0.04±0.00
P7^{c,d}	0.13±0.02	Q7^{b,c,d}	0.11±0.00	P7^{g,h}	0.10±0.02	Q7^{b,c,d,e}	0.05±0.00	P7^{a}	0.00±0.00	Q7^{d-h}	0.04±0.00
P10^{d}	0.15±0.03	Q10^{b,c,d}	0.12±0.00	P10^{h}	0.12±0.01	Q10^{b,c,d,e}	0.05±0.00	P10^{b}	0.02±0.00	Q10^{d-l}	0.04±0.00

parison to *n*-butanol and aqueous extract of the same sample.

Total flavonoids content

Flavonoids are a group of phenolic compounds whose beneficial effects to human organism is related to synergistic activity with other compounds [8]. Kombucha products with black tea, winter savory, peppermint and stinging nettle had higher total flavonoids content than the appropriate substrates (Table 3). Samples GT7, WT0, WT3 and E10 showed slightly lower flavonoids content than the initial substrates, while all kombucha products with quince had lower total flavonoids values in comparison to the appropriate initial substrates. Results obtained for products with quince were in accordance with literature data [10]. The lowest value was measured for sample WS (0.005 mg RE/mL) and highest in sample P10 (0.12 mg RE/mL). Kombucha products with winter savory showed approximately the same pattern in content that was established for total phenols and measured values were up to around ten times higher than in the initial substrate. In average, alternative kombucha beverages had higher total flavonoids than traditional products (Table 3).

Catalase activity

Catalase is widely distributed and a well-known antioxidant enzyme. To our knowledge, catalase was identified in kombucha products only in the paper by Danielova [33].

All of the examined samples in this investigation showed catalase activity. The highest value (0.0456 BU) of all the initial substrates showed winter savory extract. Kombucha beverages with highest catalase activity were samples with winter savory and stinging nettle. During the fermentation these values varied depending on the used initial substrate but the differences were not pronounced. On the average, products with black tea had the highest catalase activity (Table 3).

In order to examine the potential bioactive properties of kombucha beverages produced using water herbal extracts, two *in vitro* methods were applied. Antioxidant activity and ACE inhibitory activity was determined for consumption day samples. It is reasonable to test biological activity for samples that are going to be consumed, but it is also valuable to gain the insight into the biological activity development during fermentation, because of the production process parameters selection. Antioxidant activity tests were chosen because more information was gathered.

Development of radical scavenging ability during fermentation

Literature data suggest that traditional kombucha products showed increased free-radical scavenging ability during the fermentation process [34]. Results of antioxidant tests are given in Figures 3a-d and 4a and b.

Radical scavenging ability towards DPPH and hydroxyl radical

DPPH radical is very well known as a stable synthetic product. It is widely used in preliminary investigations of antioxidant potential of different products of natural origin [35]. Average values measured during the fermentation process suggested that kombucha products with quince had the highest radical

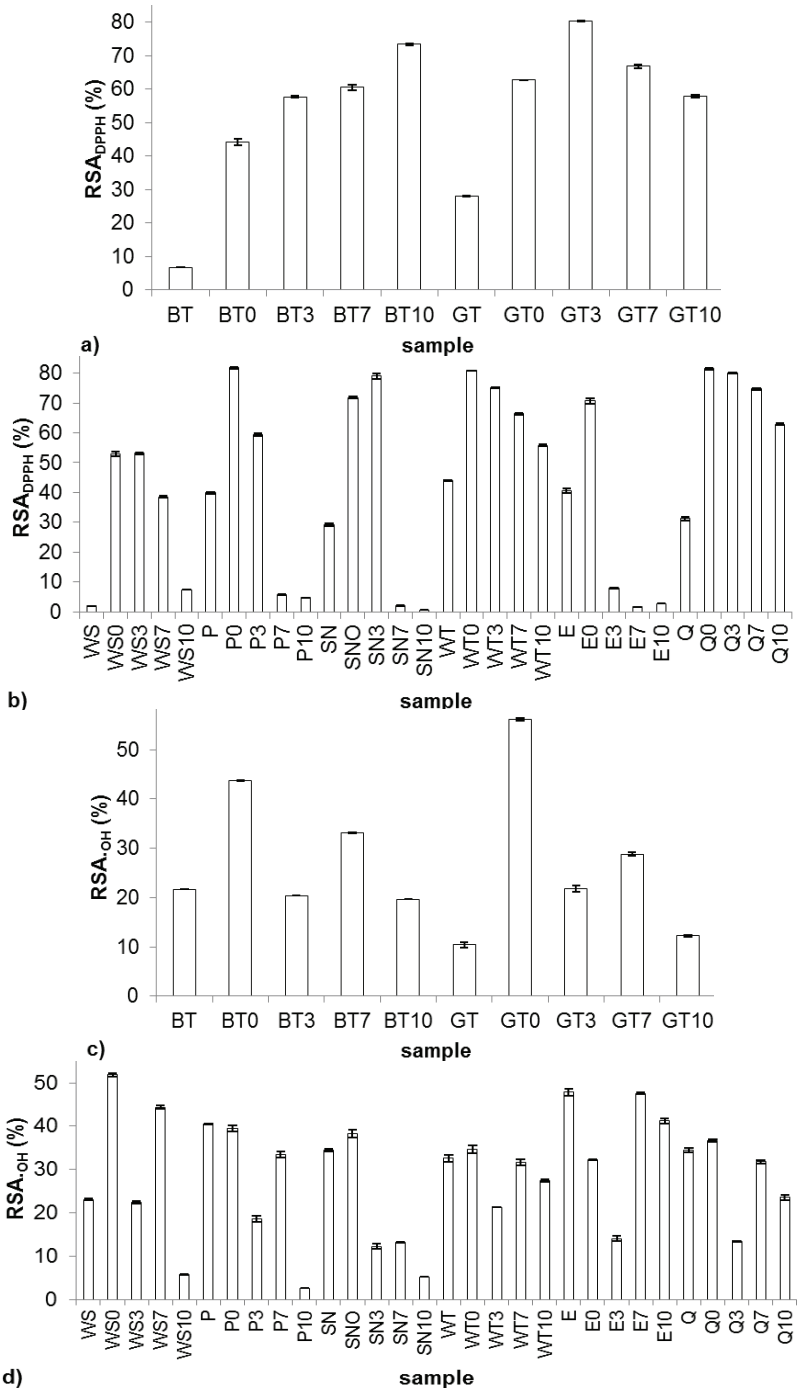

Figure 3. Radical scavenging ability towards DPPH and hydrohyl radical of initial substrates and kombucha products during fermentation: a - DPPH traditional; b - DPPH alternative; c - OH traditional; d - OH alternative.

scavenging ability towards DPPH radical (Figure 3b). The fermentation process showed the most pronounced effect in the production of samples with black tea and winter savory.

Only beverages with black tea showed a linear increase in values during the fermentation time period (Figure 3a). Samples with green tea, winter savory, and stinging nettle showed increase in values up to the third day, and then a decrease in activity. Products with peppermint, wild thyme, elderberry and quince had an increase in radical scavenging ability at the start of fermentation, and after that a decrease in values was established. The lowest value had sample SN10 (0.55%) and the highest sample P0 (81.81%). Some authors found that antioxidant capacity of ethyl-acetate, *n*-butanol and aqueous extracts of initial substrate and kombucha products with African mustard leaves had low ability of DPPH radical neutralization [29].

Hydroxyl radical is unstable, extremely reactive and oxidizing and is produced by Fenton reaction [36]. Average values determined during fermentation indicated that there is no difference between kombucha products with black and green tea (Figure 3c). Also, based on the average values, samples with elderberry showed the highest antioxidant potential to the hydroxyl radical (Figure 3d).

The activity increased on day zero and the 7th day of fermentation and decreased on the 3rd and 10th day in traditional products and products with winter savory, stinging nettle, wild thyme and quince. Samples with peppermint and elderberry showed a decrease in values until seventh day, followed by an increase on the seventh day and then again decreased by the tenth day of fermentation. Only kombucha products with green tea had higher $RSA\bullet_{OH}$ than the initial substrate during the entire fermentation process. Other samples showed higher, as well as lower values, in comparison to the initial substrates. The lowest value of $RSA\bullet_{OH}$ had sample P10 (2.58%) and the highest showed the product GT0 (56.11%). Compared on day 7 (typical consumption day) BT, GT, PP and WT had similar values (around 30%), while WS had highest and SN had the lowest value [37].

Reducing power

Antioxidant characteristics can be evaluated by the reducing power method since the possibility of electron transfer is established [34]. Kombucha beverages with green tea, wild thyme, elderberry and quince showed higher reducing power values than the initial substrates (Figure 4a and b). Products with black tea and winter savory had lower reducing power than the appropriate initial substrates. Beverages with

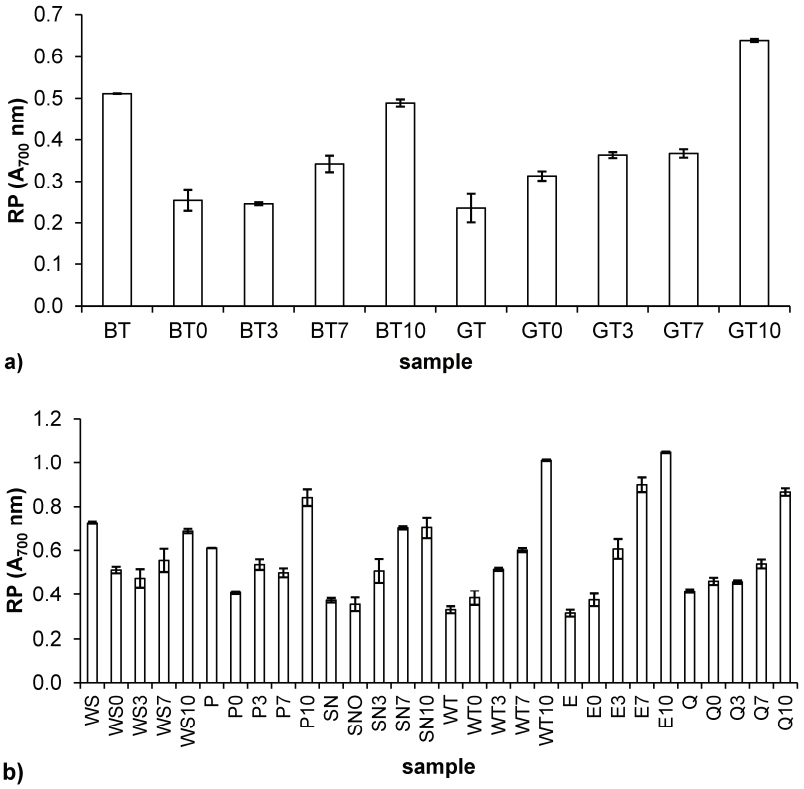

Figure 4. Reducing power of initial substrates and kombucha products during fermentation: a - traditional; b - alternative.

peppermint had higher values than substrates only on the tenth day of fermentation. On day 7, the highest reducing power had SN with the value of 0.701, while WT, WS and PP had values between 0.5 and 0.6. BT and GT had significantly lower values, 0.342 and 0.367, respectively [37].

Products with stinging nettle showed higher values than initial substrates from the third day of fermentation. Samples with green tea, wild thyme and elderberry showed linear increase in reducing power values. The lowest reducing power showed the sample GT (0.235) and highest E10 (1.050). Products with stinging nettle showed linear increase from the third day of fermentation, whilst beverages containing quince had approximately same values at the beginning and on the third day, after which linear increase was established. When average values are taken into account, it can be seen that alternative kombucha products showed higher reducing power than traditional kombucha products.

ACE inhibitory activity

The antihypertensive potential of obtained beverages was evaluated through the inhibition of the angiotensin-converting enzyme (ACE) activity. ACE is a carboxypeptidase, and it works by participating in regulating blood pressure by converting an inactive form of the decapeptide angiotensin I to a potent vasopressor octapeptide, angiotensin II. ACE inhibitory activity was established for some types of kombucha products [38]. Figure 5 shows the ACE inhibitory activity of obtained beverages.

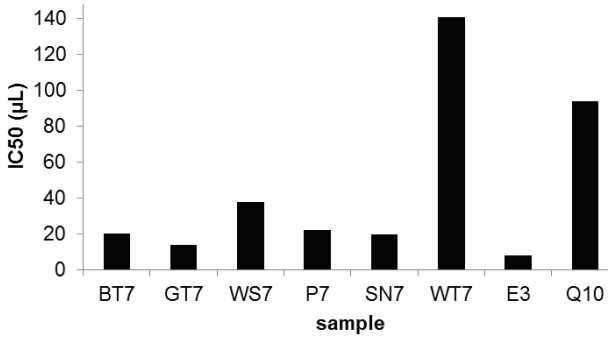

Figure 5. ACE inhibitory activity of kombucha products on consumption day.

It can be seen that all the samples inhibit the activity of ACE with the IC_{50} values in the range from 8.03 to 140.81 µL of beverage. Traditional products such as green and black tea have high activity; green tea kombucha was more potent than black tea kombucha. Beverages with winter savory, peppermint, wild thyme and quince had lower ACE inhibitory activity when compared to traditional products. Samples with elderberry and stinging nettle had significantly higher activity, more than 10 times higher, in comparison with other beverages.

Results of correlation and regression analysis (data not shown) suggested that there is no statistically significant connection between examined *in vitro* antioxidant activity tests and very well-known compounds that possess antioxidative properties: total phenols, total flavonoids and catalase. ACE inhibitory activity was correlated with total phenols and total flavonoids on the basis of available literature data [39–41], and no statistically significant correlation was recognized. Therefore, the established bioactive potential could be a result of synergistic effect of the determined compounds.

Sensory mark

Sensory analysis is important for the commercialization process of the obtained beverages. Sensory mark varied during the fermentation process for all beverages (Figure 6a and b). Initial substrates were marked with 4, except of the beverages with stinging nettle and elderberry. The highest mark, on the consumption day of fermentation, was given to kombucha beverages with black tea and peppermint (5) and the lowest to beverages with elderberry and quince (3) (Figure 6a and b).

Lower marks on days after consumption day can be attributed to the production of organic acids that can cause unpleasant taste. This is in accordance with the results of total acidity. During the fermentation, until the consumption day, sensory marks remained the same for products with wild thyme, elderberry and quince. Products with black tea, winter savory, peppermint and stinging nettle had less pleasingly sensory characteristics in the first days of fermentation, while green tea kombucha beverages showed better sensory characteristics during the first days of fermentation.

CONCLUSION

Kombucha culture was successfully fermented on traditional, as well as alternative substrates and the range of new, potentially functional, products was widened.

Fermentation lasted the shortest with elderberry infusion (3 days), and longest with quince water extract (10 days).

Organic acids profile provided the insight into the more pronounced activity of bacteria on winter savory, peppermint and stinging nettle infusions and

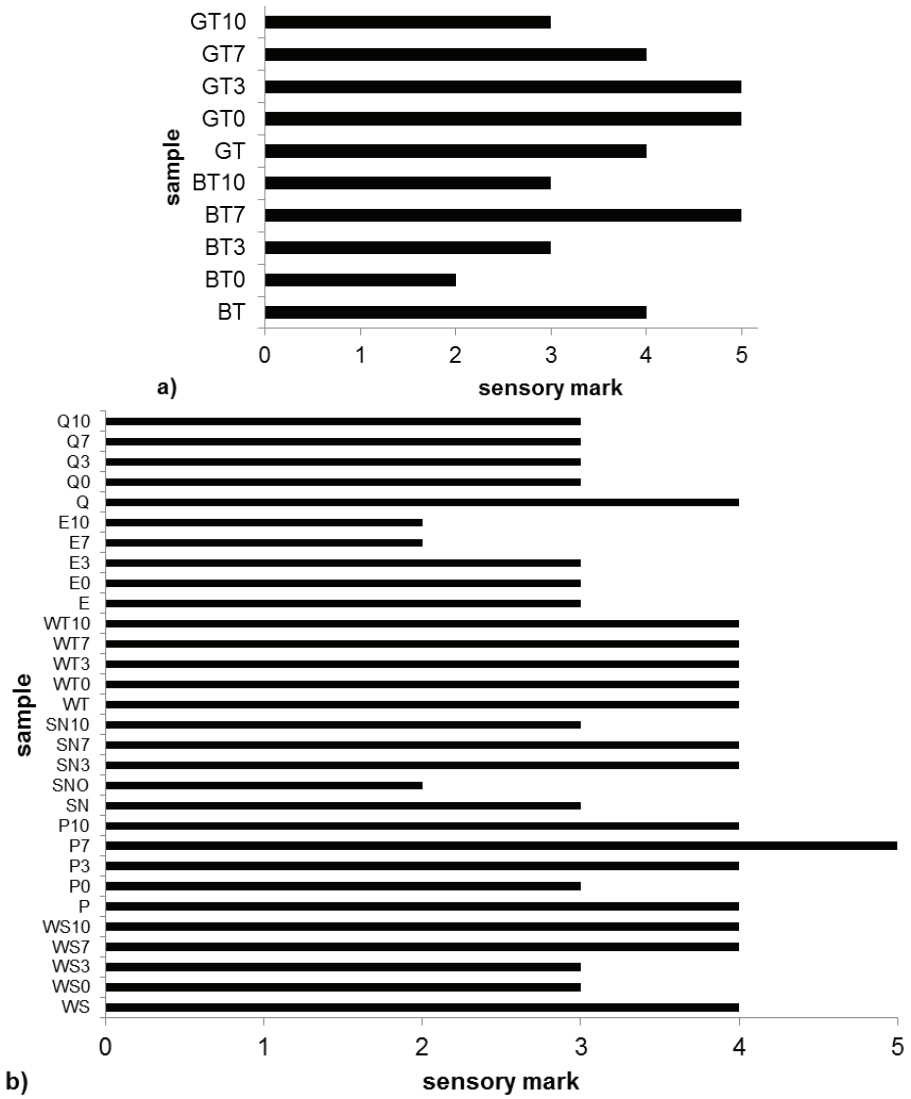

Figure 6. Sensory mark of initial substrates and kombucha products during fermentation: a - traditional; b - alternative.

higher metabolism of yeasts on all alternative substrates.

Total phenols and flavonoids content indicated superiority of water herbal extracts over the traditional substrates for kombucha fermentation.

This is the first measurement of antioxidant enzyme catalase activity determination in these types of kombucha products.

Kombucha beverages with peppermint and black tea had the best sensory characteristics.

When taking into account beverages that can be consumed, it can be concluded that: products with green tea and wild thyme had the highest radical scavenging activity to DPPH radical; the sample with winter savory showed the highest antioxidant activity to hydroxyl radical; the sample with quince had the most pronounced reducing ability.

All samples exhibited a potential antihypertension activity, while samples with elderberry and stinging nettle had significantly higher activity, in comparison to the other beverages.

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NAUČNI RAD

KOMBUHA FERMENTACIJA ŠEST LEKOVITIH BILJAKA: HEMIJSKI PROFIL I BIOLOŠKA AKTIVNOST

U ovom radu ispitivana su bioaktivna svojstva, hemijski sastav i senzorne karakteristike tradicionalnih i alternativnih fermentativnih tečnosti kombuhe. Uzorci su proizvedeni dodavanjem 10% kombuha startera u zaslađene (7% saharoze) dekokte crnog i zelenog čaja (tradicionalne podloge) i infuze rtanjskog čaja (Satureja montana), nane (Mentha x piperita), koprive (Urtica dioica), majčine dušice (Thymus serpyllum), zove (Sambucus nigra) i dunje (Cydonia oblonga), na 25 °C. Fermentacija je najkraće trajala na infuzu zove (tri dana), a najduže na infuzu dunje (10 dana). Najbolje senzorne osobine imali su uzorci dobijeni na crnom čaju i na nani. Ukupno posmatrano, alternativni proizvodi, u odnosu na tradicionalne, imali su izraženiju antioksidativnu aktivnost prema hidroksi radikalu, redukcionu moć i inhibitornu aktivnost prema angiotenzin-konvertujućem enzimu. Najveći sadržaj sirćetne kiseline dobijen je u proizvodima sa zovom, dok su uzorci sa nanom imali najveće vrednosti sadržaja ukupnih fenola i ukupnih flavonoida. Uzeto zajedno, uzorci proizvedeni na alternativnim podlogama imali su viši sadržaj fenola i flavonoida u poređenju s tradicionalnim.

Ključne reči: ACE, antioksidant, kombuha, lekovito bilje, funkcionalna hrana.