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POLYPHENOLS AND ANTIOXIDANT ACTIVITIES OF KOMBUCHA BEVERAGE ENRICHED WITH COFFEEBERRY® EXTRACT

Article Highlights

- Polyphenols were identified by HPLC in kombucha beverage enriched with CoffeeBerry®
- Kombucha beverage enriched with CoffeeBerry® exhibited good antioxidant properties
- Kombucha beverage enriched with CoffeeBerry® can be regarded as a potential nutraceutic source

Abstract

Kombucha is a traditional beverage obtained by fermenting sweetened black tea with tea fungus, which represents a consortium of acetic acid bacteria and yeasts. Also, CoffeeBerry® products, derived from the whole fruit of the coffee plant, are valuable ingredients with nutritional and health-enhancing potential. Samples of fermentation broths enriched with CoffeeBerry® extract and traditional Kombucha were analysed. The fermentation was performed in a bioreactor at 28±1 °C for nine days. The results showed that the CoffeeBerry® extract contributed to a faster fermentation of cultivation medium. Some individual polyphenolic compounds and catehins in fermentation broth samples were identified and quantified by high performance liquid chromatography (HPLC). Among the bioactive compounds present in investigated samples obtained during Kombucha fermentation of the sweetened black tea enriched with CoffeeBerry® extract, chlorogenic acid (188.94-458.56 μg/mL) was the predominant. The antioxidant activity of investigated samples was tested by measuring their ability to scavenge DPPH and reactive hydroxyl radicals by electron spin resonance (ESR) spectroscopy. The scavenging activities on DPPH and hydroxyl radicals were increased with the duration of fermentation. IC_{50} values for Kombucha fermentation broth enriched with CoffeeBerry®, based on DPPH and hydroxyl radical scavenging activities, were in the ranges 26.33--170.13 and 11.33-102.22 μL/mL, respectively.

Keywords: Kombucha, CoffeeBerry®, polyphenols, antioxidant activity, HPLC analysis.

Kombucha is a traditional beverage that originated in Manchuria in 220 BC and was then spread to Russia, Germany and the rest of the world. A lot of beneficial effects on human health are attributed to Kombucha. It is claimed that it helps in the treatment of metabolic disorders, in curing cancer and arteriosclerosis, strengthening of cells, regulation of the intestinal flora, etc. These effects have not quite been validated scientifically, but they could be attributed to the presence of gluconic acid, glucuronic acid, vitamins, amino acids, and micronutrients produced during fermentation [1].

Kombucha is typically prepared by fermenting sweetened (usually sucrose) black tea with tea fungus as starter culture. The substrate is inoculated with previous fermented liquid tea broth at a level of 10-20 vol.% or tea fungus pellicle. Cultivation of tea fungus is performed statically under aerobic conditions, usu-

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ally for 10-12 days at 20-28 °C [2,3]. To obtain a pleasantly sour beverage, the fermentation should be terminated when the titratable acidity reaches 4-4.5 g/L, as confirmed by long-time consumers of Kombucha beverage [4].

The tea fungus is a consortium of acetic acid bacteria (*Acetobacter aceti*, *Acetobacter pasteurianus* and *Gluconobacter oxydans*) and yeasts (*Saccharomyces* spp., *Torulopsis* spp., *Pichia* spp., *Brettanomyces* spp., *Zygosaccharomyces kombuchaensis*, etc.) [5]. Sucrose as carbon source in the cultivation medium is hydrolyzed by the enzyme invertase from tea fungus yeasts. The yeasts ferment glucose and fructose to ethanol, which is then oxidized by acetic acid bacteria to acetic acid. This is the main metabolic path of Kombucha fermentation, and acetic acid, ethanol and gluconic acid are the main tea fungus products [3]. Other components present in Kombucha beverage are sugars, ethyl gluconate, oxalic, saccharic, lactic, 5-ketogluconic acid, 2,5-ketogluconic acid, water soluble vitamins (B_1, B_6, B_{12} and C) tea components (catehins, theaflavins, flavonols, etc.) and hydrolytic enzymes (invertase, amylase, etc.) [6].

Sweetened black or green tea (*Camellia sinensis* L.) has been the traditional medium for preparing Kombucha. These teas have high levels of nitrogen sources (like purine derivatives, caffeine and theophylline), which are necessary for growth and reproduction of tea fungus cells. Although Hoffmann [7] noticed that herbal teas cannot be considered very suitable for making Kombucha due to the lack of purine derivatives, Cvetković showed that sweetened echinacea (*Echinacea purpurea* L.) tea or winter savory (*Satureja montana* L.) tea have more stimulating effects on the Kombucha fermentation than black tea, yielding a fermentation product in a shorter time and comparable with traditional beverage [4]. Also, lemon balm (*Melissa officinalis* L.) tea can be successfully used as a nitrogen source for Kombucha fermentation [8]. All of these herbs possess many bioactive compounds, provide the desired antioxidant status and prevent diseases that occur as a result of oxidative stress [9,10]. These functional plants can be successfully used in combination with black or green tea as the medium for Kombucha fermentation, yielding a beverage of significant antioxidant and antimicrobial properties.

CoffeeBerry® products are derived from the whole fruit and include ground whole powder, a water extract, and more recently developed water-ethanol extract [11]. Namely, the fruit of the coffee plant, *Coffea arabica*, has high phenolic antioxidant and phytonutrient contents and could be a beneficial food ingredient. This fruit has long been recognized as having inherent nutritional and health-enhancing potential including antioxidant capacity, immunomodulation, and perhaps tumor suppression [11]. The CoffeeBerry® technology has created a range of non-roasted whole coffee fruit-based food and nutritonal ingredient products that can deliver high levels of coffee phenolic acids, monosaccharides, and other coffee nutrients. The CoffeeBerry®, as a source of caffeine and polyphenols, can contribute to the beneficial effects of Kombucha and justifies its classification as a functional beverage.

The aim of this study was to investigate the contribution of CoffeeBerry® extract as functional supplement on Kombucha fermentation and antioxidant activities of beverage. Changes of pH, titratable acidity (TA), total count of yeasts and acetic acid bacteria, content of polyphenols compounds during fermentation of sweetened black tea enriched with CoffeeBerry® extract were determined. Antioxidant activities of broth samples against hydroxyl and DPPH radicals during Kombucha fermentation were also tested. Kombucha obtained from traditional medium (sweetened black tea) under the same fermentation conditions, served as the control.

EXPERIMENTAL

Chemicals

Standards compounds used for HPLC analysys were of analytical grade and purchased from Sigma-Aldrich Chemical Company (Sigma Co., St. Louis, MO, USA). HPLC-grade solvents (methanol, formic acid and acetonitrile) were purchased from J.T. Baker (Deventer, Netherlands). All standard and solutions were prepared with p.a. chemicals and 18 MΩ redistilled and deionised water (Millipore, Bedford, MA, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and 5,5-dimethyl-1-pyroline-*N*-oxide (DMPO), were purchased from Sigma Chemical Co. (St. Louis, MO, USA). These chemicals were reagent of analytical grade. Other chemicals and solvents used in determination of antioxidant activity were of the highest analytical grade and obtained from "Zorka" (Šabac, Serbia).

Tea fungus

Fermentation was performed using the local tea fungus culture, for which previous investigations showed that it contained at least five yeast strains (*Saccharomycodes ludwigii, Saccharomyces cerevisiae, Saccharomyces bisporus, Torulopsis* sp. and *Zygosaccharomyces* sp.) [12] and two strains of ace-

tic acid bacteria: *Gluconobacter oxydans* (also known as *Acetobacter hansenii*) and *Gluconacetobacter hansenii* [13].

CoffeeBerry® extract

CoffeeBerry® extract which was used in this study was produced by VDF FutureCeuticals Inc. (Momence, IL, USA) and supplied by Fructus (Bačka Palanka, Serbia). Characteristics of extract deffined by product specification were: tan/brown powder, total phenolic acids 20% minimum, caffeine 1.0-1.5%, ORAC (oxygen radical absorption capacity) 2500 μmol TE/g average.

Fermentation conditions

The substrate for tea fungus cultivation was prepared by adding 70 g of sucrose in 1 L of boiled tap water. To the boiled water, 3 g/L of black tea (Fructus, Bačka Palanka, Serbia) and 1.5 g/L of CoffeeBerry® extract were added. After cooling to room temperature, the medium was inoculated with 10 vol.% of the fermentation broth from the previous fermentation which was performed on sweetened black tea as cultivation medium. Bioreactors made of glass (volume 0.72 L, diameter 80 mm) were filled in with 0.33 L of inoculated liquid phase. The bioreactors were covered with cheesecloth, and the fermentation at 28±1 °C was monitored for nine days. Control sample was traditional Kombucha prepared as described above without the addition of CoffeeBerry® extract.

Sampling

Sampling of fermentation broth was performed under aseptic conditions at the beginning of fermentation and during nine days of fermentation. Each bioreactor was sampled only once in order to avoid contamination. Two groups of samples were prepared: the first group consisted of samples obtained during traditional Kombucha fermentation (TK) and marked as: T - sweetened black tea; TK0 - sweetened black tea after inoculation (0 day of fermentation); TK2 - fermentation broth after 2 days of fermentation; TK4 - fermentation broth after 4 days of fermentation; TK6 - fermentation broth after 6 days of fermentation; TK8 - fermentation broth after 8 days of fermentation; TK9 - fermentation broth after 9 days of fermentation. The second group consisted of samples obtained during Kombucha fermentation of the sweetened black tea enriched with CoffeeBerry® extract: TCB - sweetened black tea+CoffeeBerry® extract; CBK0 - sweetened black tea+CoffeeBerry® extract after inoculation (0 day of fermentation); CBK2 - fermentation broth after 2 days of fermentation; CBK4 – fermentation broth after 4 days of fermentation; CBK6 – fermentation broth after 6 days of fermentation; CBK8 - fermentation broth after 8 days of fermentation; CBK9 - fermentation broth after 9 day of fermentation.

Samples for the determination of total polyphenols, HPLC analyses and antioxidant tests, were filtered through a sterile microfilter (0.22 μm) to remove the cells.

Determination of pH and titratable acidity (TA)

The pH values were measured using an electronic pH meter (HI 9321, Hanna Instruments, USA) calibrated at pH 4.0 and 7.0. The TA was determined according to Jacobson [14]. After removing CO_2 from the fermentation broth, a 20-mL aliquot was taken and titrated with 0.1 mol/L NaOH. The TA was expressed in grams of acetic acid per liter of the sample.

Microbiological analysis

Total count of yeasts and acetic acid bacteria in the fermentation broth were determined by plate counting method [3,15]. For yeasts, the medium was Sabouraud-4% Maltose Agar (Merck, Darmstadt, Germany) with addition of 50 mg/L of chloramphenicol (Sigma-Aldrich, St. Louis, CA, USA). The plates were incubated for 72 h at 28 °C. The medium for determining total count of acetic acid bacteria was Yeast Peptone Mannitol Agar (Difco, Detroit, MI, USA), containing 500 mg/L cycloheximide (actidione; Sigma-Aldrich, St. Louis, CA, USA) to inhibit yeasts growth. The incubation at 28 °C lasted 5-7 days.

Determination of total polyphenol compounds (Tph)

Content of total polyphenol compounds was determined spectrophotometrically according to Folin-Ciocalteu colorimetric method [16] and expressed as mg/mL of chlorogenic acid equivalent (*CAE*) for all investigated samples.

HPLC analysis of the polyphenol compounds

Quantification of individual phenolic compounds was done by reversed phase HPLC analysis using modified, previously reported method [17]. Samples were injected in a Waters HPLC system consisted of 1525 binary pumps, thermostat and a 717+ autosampler connected to the Waters 2996 diode array and an EMD 1000 single quadropole detector with ESI probe (Waters, Milford, MA, USA). Separation of polyphenols was performed on a Symmetry C-18 RP column whose dimensions are 125 mm×4 mm size with 5 μm particle diameter (Waters, Milford, MA, USA) connected to the appropriate guard column. Two mobile phases, A (0.1% formic acid) and B (acetonitrile) were used at flow rate of 1 mL/min with the

following gradient profile: the first 20 min from 10 to 22% B; next 20 min of linear rise up to 40% B, followed by 5 min reverse to 10% B and additional 5 min of equilibration time. A post column flow splitter (ASI, Richmond, CA, USA) with 5/1 split ratio was used to obtain optimal mobile phase inflow for ESI probe. Chromatograms were gathered in 3D mode with extracted signals at specific wavelengths for different compounds (367, 326, 309 and 240 nm). Resolving of overlapping caffeine/chlorogenic acid peaks was done by LC/MS analysis. Signals for caffeine (*m*/*z* 195) and chlorogenic acid (*m*/*z* 355) were detected in positive ESI single ion recording mode with following parameters: capillary voltage 3.0 kV, cone voltage 25 V, extractor and RF lens voltages were 3.0 and 0.3 V, respectively. Source and desolvation temperatures were 120 and 380 °C, respectively, with N_2 gas flow of 450 L/h. Detected compounds were qualitatively analyzed through comparison of literature data for their retention times as well as characteristic UV and MS spectra with ones recorded from our samples. Individual components were analyzed quantitatively by the external standard method using pure standard compounds as references for concentration, retention time and characteristic UV/MS spectra, respectively. The data acquisition and spectral evaluation for peak confirmation were carried out by the Waters Empower 2 Software (Waters, Milford, USA).

Determination of antioxidant activity

DPPH radical assay. A blank probe was obtained by mixing 0.2 mL of distilled water and 0.4 mL of 0.4 mM methanol solution of DPPH•. The effect of fermentation broth or tea on DPPH• was studied in the concentration range 20-300 μL/mL. After that, the mixture was stirred for 2 min and transferred to an ER-160FT quartz flat cell. The ESR spectra were recorded on an ESR spectrometer (model 300E, Bruker, Rheinstetten, Germany) under the following conditions: field modulation 100 kHz, modulation amplitude 0.256 G, time constant 40.96 ms, conversion time 335.544 ms, center field 3440.00 G, sweep width 100.00 G, x-band frequency 9.45 GHz, power 7.96 mW, temperature 23 °C. The scavenging activity (*SA*) value of investigated fermentation broth samples for DPPH• was defined as:

$$SA_{DPPH\bullet} = 100(h_0 - h_x)/h_0 \quad (1)$$

where h_o and h_x are the heights of the second peak in the ESR spectrum of DPPH radicals of the blank and the probe, respectively [13].

Hydroxyl radical scavenging activity. Hydroxyl radicals were obtained by the Fenton reaction and detected by spin trapping in a system consisting of: 0.2 mL H_2O_2 (2 mM), 0.2 mL $FeCl_2$ (0.3 mM), 0.2 mL of *N*,*N*-dimethyl formamide (DMF) and 0.2 mL of 5,5--dimethyl-1-pyrroline-*N*-oxide (DMPO) (112 mM) as spin trap (probe). The influence of investigated two groups of samples on the amount of hydroxyl radicals trapped by DMPO was studied by adding them to the reaction system in the concentration range of 20-250 μg/mL (probe). The ESR spectra were recorded 2.5 min after mixing on the above mentioned ESR spectrometer, with the following settings: modulation amplitude 0.512 G, receiver gain 1×10^4, time constant 81.92 ms, conversion time 163.84 ms, center field 3440.00 G, sweep width 100.00 G, x-band frequency 9.64 GHz, power 20 mW, temperature 23 ×C. The $SA\bullet_{OH}$ value of the all fermentation broth samples was calculated according to Eq. (1), where h_0 and h_x are the hights of the second peak in the ESR spectrum of DMPO/•OH spin adduct of the blank and the probe, respectively [13].

Antioxidant activities of investigated samples against both, DPPH and OH radicals are expressed as IC_{50} values. The IC_{50} value, defined as the concentration of extract required for 50% scavenging of DPPH and hydroxyl radicals under experimental condition employed, is a parameter widely used to measure the free radical scavenging activity. The sample concentration providing 50% of *SA* (IC_{50}) was calculated from the graphic of *SA* (%) against extract concentration (mg/mL).

Statistical analyses

All experiments were performed in duplicate (two independent series of Kombucha fermentation were performed), under the same conditions, while each quantity was measured three times and the results were expressed as means±*SD*. Statistical analyses were done by using Origin 7.0 SRO software package (OriginLab Corporation, Northampton, MA, USA, 1991-2002) and Microsoft Office Excel 2003 software. The significance of differences was calculated by ANOVA test and then by least significant difference (*LSD*) test ($p < 0.05$), unless noted otherwise.

RESULTS AND DISCUSSION

Values of pH, TA and total count of yeasts and acetic acid bacteria

The changes of the basic parameters of fermentation broth during Kombucha fermentations are presented in Figure 1.

The decrease of pH values (Figure 1) follows the increase in acidity due to the Kombucha ferment-

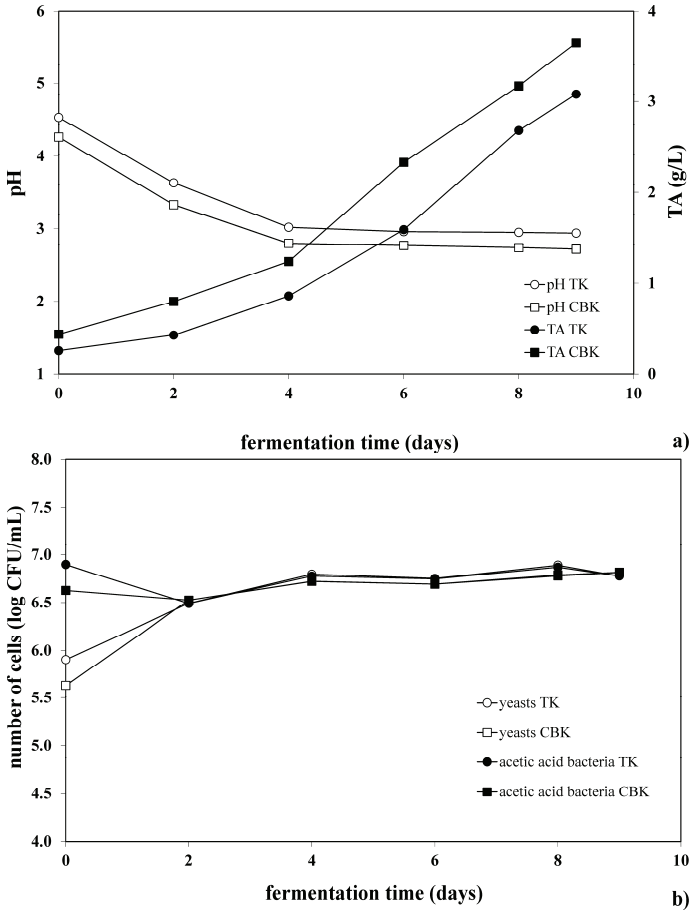

Figure 1. Rate of change in pH and TA (a) and total count of yeasts and acetic acid bacteria (b) during traditional Kombucha fermentation (TK) and Kombucha fermentation of sweetened black tea enriched with CoffeeBerry® extract (CBK).

ations. These changes are result of metabolic activity of tea fungus yeasts and acetic acid bacteria that produce mainly acetic acid. The pH values of sweetened T and TCB were 6.60 and 6.80, respectively. It dropped after the inoculation with the fermentation broth from previously Kombucha fermentation. In first four days of fermentation the pH decreased by about 1.5 unit and in next five days decreasing was less than 0.2 units. Changes in pH during tea fungus fermentation were similar for both medium (Figure 1a). The TA of sweetened T and TCB was 0.09 g/L TK and 0.05 g/L CBK, respectively, and increased constantly from the beginning till the end of the fermentation process for both medium (Figure 1a). The TA in broth with CoffeeBerry® extract during fermentation, on average, was in range from 1.18-1.69 g/L higher compared to that in traditional Kombucha. Values of TA of the investigated cultivation medium were significantly different ($p < 0.05$). The incompatible changes in the pH and TA could be explained by the buffer capacity of the fermentation broth. Namely, during the fermentation, carbon dioxide is released, and the obtained water solution of CO_2 dissociates and produces the amphiprotic hydrocarbonate anion (HCO_3^-). This anion easily reacts with hydrogen ions from the present organic acids in the fermentation broth, preventing further changes in the pH, thus contributing to the buffer character of the system [15]. This trend for the changes of pH and TA is typical for Kombucha fermentation and it was also observed by some other authors who used similar cultivation conditions [18,19]. In view of the above, TA should be used as a critical parameter which determines the end of Kombucha fermentation instead of the pH.

The total number of yeasts and acetic acid bacteria as a function of fermentation time is presented in Figure 1b. The starting number of yeasts in both medium was at a level of 5log CFU/mL. During first 48 h of process count of yeasts was increased for 0.9 (CBK) and 0.59 (TK) unit. At the same time, total count of acetic acid bacteria was decreased for 0.1 (CBK) and 0.41log CFU/mL (TK) from initial values of 6.63 (CBK) and 6.9log CFU/mL (TK). By the end of the process, the total number of yeasts and acetic acid bacteria in fermentation mediums were uniform until the end of process reached the values of about

6.8log CFU/mL. The changes in the total count of yeasts and acetic acid bacteria during fermentations are comparable with previous investigations. During Kombucha fermentation the viable population of yeasts followed a standard growth curve pattern, in which yeasts grew exponentially for up to 8-10 days, dying off as nutrients became limiting and the pH decreased [20]. Count of individual yeast species in fermentation broth was 4log units at the beginning of process and reached maximum value of 7log units [20]. On the other hand, the number of acetic acid bacteria during Kombucha fermentation increased rapidly through 4 days of fermentation (from 4log units at start of fermentation to more than 7log units after 4 days), declined drastically by 6 days of fermentation, and thereafter continued to decrease [18]. However, the differences in the total number of tea fungus cells in fermentation liquid can be explained by the differences in the applied Kombucha cultures, inoculums (fermentation broth or pellicle wherein the cells are immobilized), as well as in the sampling procedure.

Content of polyphenol compounds and HPLC analysis

During the fermentation, fermentation broth samples were taken for the determination of total polyphenol compounds and individual polyphenols. The Folin-Cioacalteu method is a rapid and widely--used assay to investigate the total polyphenols. This method is based on reducing power of hydroxyl groups, but it is known that different polyphenol compounds have different responses to the Folin-Cioacalteu reagent [16]. The contents of total polyphenol compounds are expressed as mg of chlorogenic acid equivalent (CAE) per mL of investigated samples and varied depending on the duration of the fermentation and type of the fermentation medium (Figure 2). The content of total polyphenol compounds varied from 0.309-0.519 mg CAE/mL in all TK samples and 1.037-1.139 mg CAE/mL in all CBK samples, depending on the duration of fermentation. Comparing the obtained results, in all samples enriched with CoffeeBerry® extract the content of total polyphenols was higher. Only minor differences in content values were found between the individual samples in the groups.

Major polyphenols present in investigated samples were identified and quantified by HPLC analysis. Catehins (Table 1) were identified in all TK and all CBK samples. The high content of catehin and epicatehin were obtained in all investigated samples. Based on the obtained results, it can be concluded that the contents of catehins, especially epicatehin, in CBK samples is higher (with the exception of epigallocatehin gallate).

The catechin profile in tea has been found to differ depending on the method of tea preparation. The concentration of thermal isomers reaches significant levels, and, as such, the physiological function of these isomers is also of interest. Therefore, catechins and their active metabolites may act at different sites and so contain diverse actions, which will subsequently increase the versatility of catechins as potential therapeutic interventions.

Also, TK and CBK samples are very rich with other polyphenol bioactive compounds (Table 2).

Chu and Chen showed that the content of total polyphenol compounds increased linearly with the fermentation time [21]. Additionally, four catechins –

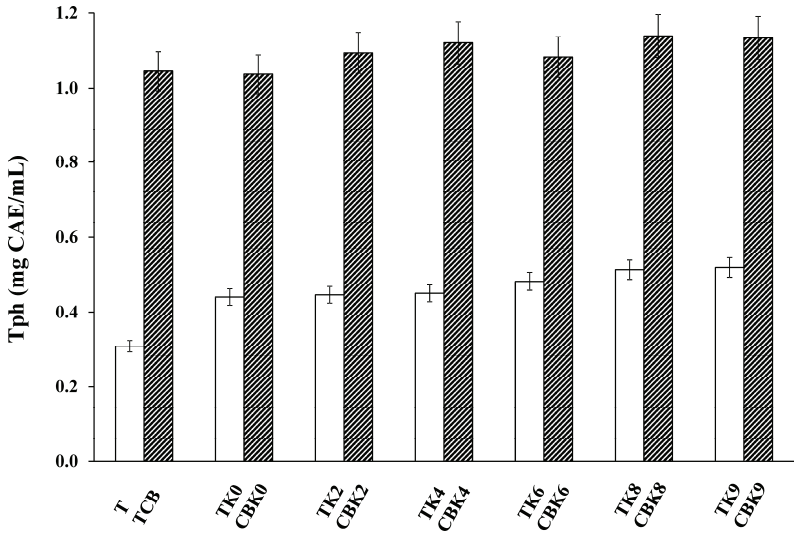

Figure 2. Content of total polyphenol compounds (Tph) in fermentation broth samples obtained during traditional Kombucha fermentation (TK) and Kombucha fermentation of sweetened black tea enriched with CoffeeBerry® extract (CBK).

Table 1. The content of catehins (μg/mL) in fermentation broth samples obtained during traditional Kombucha fermentation and Kombucha fermentation of sweetened black tea enriched with CoffeeBerry® extract; nd – not detected

Sample	Gallocatechin	Epigallocatechin	Catehin	Epicatehin	Gallocatechin gallate	Epigallocatechin gallate	Catehin gallate	Epicatehin gallate
T	0.01±0.001	2.04±0.10	3.76±0.18	0.52±0.03	2.01±0.10	0.25±0.01	0.28±0.01	1.00±0.05
TK0	0.02±0.001	0.75±0.04	3.45±0.17	0.84±0.04	1.78±0.09	0.31±0.02	1.79±0.08	4.61±0.23
TK2	0.01±0.005	1.68±0.08	3.64±0.18	0.49±0.02	1.54±0.08	0.27±0.01	0.27±0.01	0.82±0.04
TK4	0.20±0.001	0.71±0.03	3.86±0.19	0.53±0.03	1.72±0.08	0.26±0.01	0.24±0.01	0.79±0.04
TK6	0.10±0,005	1.76±0.08	4.10±0.21	0.67±0.03	2.03±0.10	0.27±0.01	0.23±0.01	0.63±0.03
TK8	0.59±0.02	1.77±0.08	4.43±0.22	1.09±0.05	2.47±0.12	0.29±0.01	1.93±0.09	4.53±0.22
TK9	0.60±0.03	1.40±0.07	4.55±0.23	0.96±0.04	2.61±0.13	0.34±0.02	0.94±0.04	2.40±0.12
TCB	0.29±0.01	2.01±0.10	3.37±0.17	2.29±0.11	2.56±0.12	nd	0.49±0.02	0.07±0.003
CBK0	0.63±0.03	0.49±0.24	3.89±0.19	5.72±0.28	2.70±0.13	nd	1.28±0.06	0.18±0.01
CBK2	0.40±0.02	0.71±0.03	4.15±0.20	4.49±0.22	2.34±0.11	0.17±0.01	1.52±0.08	0.22±0.01
CBK4	0.26±0.01	2.45±0.12	3.28±0.16	2.73±0.14	2.22±0.11	nd	1.59±0.08	0.23±0.01
CBK6	0.71±0.03	1.42±0.07	4.14±0.20	5.42±0.26	3.24±0.16	nd	1.74±0.08	0.25±0.01
CBK8	0.58±0.03	1.31±0.06	4.16±0.20	4.99±0.24	3.36±0.16	nd	1.60±0.08	0.23±0.01
CBK9	0.63±0.03	1.82±0.09	4.28±0.21	7.47±0.37	3.59±0.18	0.28±0.01	1.28±0.06	0.18±0.01

Table 2. The content of individual polyphenolic compounds (μg/mL) in fermentation broth samples obtained during traditional Kombucha fermentation and Kombucha fermentation of sweetened black tea enriched with CoffeeBerry® extract; nd – not detected

Sample	Gallic acid	Caffeine	Rutin	*p*-Coumaric acid	Caffeic acid	Ferulic acid	Isoferulic acid	Neohloro-genic acid	Cryptochloro-genic acid	Chlorogenic acid
T	1.40±0.04	69.87±1.54	1.23±0.02	0.53±0.01	nd	nd	0.30±0.01	nd	nd	nd
TK0	20.00±0.36	61.97±1.98	4.67±0.11	0.47±0.02	nd	nd	0.53±0.02	nd	nd	nd
TK2	13.33±0.24	67.17±2.08	1.47±0.04	0.33±0.01	nd	nd	0.30±0.01	nd	nd	nd
TK4	13.43±0.40	70.97±1.56	1.70±0.04	0.37±0.01	nd	nd	0.27±0.01	nd	nd	nd
TK6	10.90±0.27	75.70±3.33	1.40±0.06	0.33±0.01	nd	nd	0.30±0.01	nd	nd	nd
TK8	19.60±0.59	73.93±1.63	5.27±0.19	0.37±0.02	nd	nd	0.60±0.01	nd	nd	nd
TK9	19.46±0.58	82.33±3.05	4.67±0.17	0.40±0.01	nd	nd	0.40±0.01	nd	nd	nd
TCB	9.23±0.092	68.20±2.80	5.03±0.21	1.25±0.04	48.40±2.36	96.80±3.64	3.12±0.14	20.00±0.75	34.90±1.72	188.94±8.65
CBK0	18.93±0.76	63.40±1.24	5.67±0.14	0.57±0.01	191.1±8.92	77.63±3.54	1.90±0.09	12.00±0.31	25.00±0.19	423.36±14.90
CBK2	19.36±0.64	64.30±1.70	6.43±0.29	0.43±0.01	166.6±7.05	7.20±0.17	1.90±0.08	12.10±0.28	26.50±1.20	430.61±14.00
CBK4	12.53±0.15	66.90±2.44	1.03±0.02	0.37±0.09	24.63±1.11	1.73±0.06	2.15±0.05	11.10±0.17	20.20±0.71	342.66±14.56
CBK6	20.10±0.40	67.10±2.90	6.87±0.16	0.43±0.02	88.93±3.76	2.01±0.05	2.15±0.03	13.10±0.48	26.10±1.20	409.85±10.49
CBK8	20.36±0.41	67.50±1.73	6.72±2.38	0.43±0.02	63.22±2.16	2.03±0.05	2.03±0.05	13.20±0.59	26.90±0.98	435.55±14.18
CBK9	19.83±0.60	69.00±2.84	6.67±0.28	0.33±0.01	43.12±1.95	2.05±0.05	1.80±0.08	14.00±0.48	28.00±0.91	458.56±16.74

epicatechin, epicatechin gallate, epigallocatechin and epigallocatechin gallate (as well as other complex polyphenols) – can be biotransformed by the enzymes extracted from tea fungus cells [1]. Also, catechins are released from acid-sensitive cells during Kombucha fermentation on black tea, which could be the reason for the increase in tea polyphenols content during the fermentation. On the other hand, catechins may polymerize to molecules of higher molecular weights, and thus lower the content of polyphenols [21]. This can be explained by the nonuniform changes in the polyphenols concentration during Kombucha fermentation.

Presence of polyphenols in inoculum can be the reason for the increase of some polyphenols in samples TK0 and CBK0 (compared with T and TCB). On the other hand, decrease in polyphenols content in samples TK0 and CBK0 can be explained by the influence by the enzymes originated from inoculum.

Caffeine, epigallocatechin gallate, and epigallocatechin are mentioned as the main compounds in black tea methanolic extracts [22]. Caffeine is the main polyphenol component in black tea infusion, followed by gallic acid, 5-galloylquinic acid, 4-*p*-coumaroylquinic acid, some flavonols, etc. [23]. Certain differences in tea and extract composition are exp-

ected and influenced by a number of factors such as species, season, age of the leaves, climate, and horticultural conditions [22].

It should be noted that caffeic, ferulic, neochlorogenic and cryptochlorogenic acid were detected at relatively high levels only in fermentation broth samples enriched with CoffeeBerry® extract. Also, in these samples during 9-days of Kombucha fermentation, chlorogenic acid was detected at relatively high levels (188.94-458.56 μg/mL). Green coffee beans contain important amounts of chlorogenic acid (CAE), accounting for up to 10% of the weight of green coffee [24]. Chlorogenic acid, which is an ester of caffeic acid and quinic acid (5-*O*-caffeoylquinic acid), is one of the main hydroxycinnamates found in CoffeeBerry® extract. Coffee brew, a complex mixture of more than a thousand bioactive compounds, is known to exhibit distinct antioxidant activity [25]. The original coffee constituents chlorogenic acids, *e.g.*, the quantitatively most relevant caffeoylquinic acids (CQAs), and their polyphenol degradation products, e.g. caffeic acid (CA), have been found to exhibit distinct radical scavenging activity *in vitro* [26]. Chlorogenic acid and caffeic acid, originated from CoffeeBerry® extract, are components of samples CBK0-CBK9.

The content of total polyphenol compounds were higher than the sum of all the individual polyphenols identified using HPLC (Tables 1 and 2). This difference can be explained by the fact that the Folin-Ciocalteau method is not an absolute measurement of the amount of polyphenols because some other substances such as organic acids, residual sugars, amino acids, proteins and other hydrophilic compounds interfere with this assay [16].

Antioxidant activity

The free radical scavenging activity of samples obtained during traditional Kombucha fermentation and Kombucha fermentation of sweetened black tea with CoffeeBerry® extract was evaluated on two different free radical species: 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) and hydroxyl radical using ESR spectroscopy.

Stable DPPH radicals have been used to evaluate the radical scavenging ability of the investigated samples. In all cases a typical ESR spectrum of DPPH radicals, with five lines of relative intensities 1:2:3:2:1 and hyperfine splitting constant $a_N = 9.03$, was observed [27]. The H-transfer reactions from antioxidants, present in fermentation broth samples, to DPPH• were monitored by recording the decay of the DPPH• ESR signal. The $SA_{DPPH•}$ of different concentrations of all fermentation broth samples is presented in Figure 3. IC_{50} values for the fermentation broth samples enriched with CoffeeBerry®, based on DPPH radical scavenging activities, were in the range 26.33-170.13 μL/mL. The scavenging activities on DPPH radical were increased with the duration of fermentation.

As hydroxyl radicals are highly reactive, with relatively short half-lives, the concentrations found in natural systems are usually inadequate for direct detection by ESR spectroscopy. Spin-trapping is a chemical reaction that provides an approach to help overcome this problem. These reactive radicals are identified because of their ability to form nitroxide adducts (stable free radicals form) from the commonly used DMPO as the spin trap.

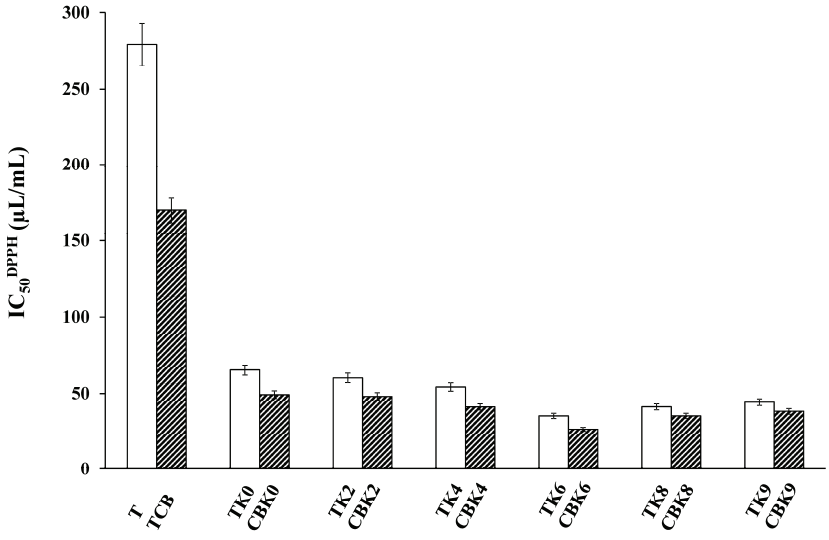

Figure 3. IC_{50}^{DPPH} values of fermentation broth samples obtained during nine days of traditional Kombucha fermentation (TK) and Kombucha fermentation of sweetened black tea enriched with CoffeeBerry® extract (CBK) in DPPH radical-scavenging assay.

Production of hydroxyl radical was reliable and stable using the classic Fenton Fe^{2+}/H_2O_2 radical generating system, and the amounts of DMPO-OH spin adduct formed in this system were proportional with ESR signal intensity, which was measured at the second peak of the ESR curve in the spectra. In all cases a typical ESR spectrum of DMPO-OH spin adduct, with four lines of relative intensities 1:2:2:1 and hyperfine splitting constant $a_N = a_H = 14.9$ G was observed [28].

The scavenging activities on hydroxyl radicals (Figure 4) were increased with duration of time of fermentation. IC_{50} values for Kombucha beverage enriched with CoffeeBerry® extract, based on hydroxyl radical scavenging activities were in the range 11.33--102.22 μL/mL.

However, the changes in the IC_{50} values during the fermentation are not equal to the changes in the content of polyphenol compounds. It is proposed that some tea fungus metabolites such as vitamins, organic acids, etc. contributed to the antioxidant activities of Kombucha beverages [21]. Also, the scavenging ability is determined by the key extracellular enzymes that are involved in the structural modification of the medium components during Kombucha fermentation [21].

Reactive oxygen species, primarily highly reactive and short-lived hydroxyl radicals, have been implicated as substances leading to cell damage. On the other hand, DPPH• is a stable free radical that has been used to test the ability of various natural extracts to transfer hydrogen atom, to reduce the Mo^{6+} to Mo^{5+} and to subsequently form a green phosphate [29]. All TK and CBK samples demonstrated good antioxidant capacities in •OH and DPPH• assays, whereas the activity towards •OH was expressed in lower concentration range (11.33-102.22 μL/mL) than towards DPPH• (26.33-170.13 μL/mL). This could be explained by the fact that naturally occuring compounds remove different free radical species employing different mechanisms. In this study, •OH are generated in Fenton reaction system, which includes the presence of Fe^{2+}, while DPPH radicals are synthetic radicals. Different interactions between reactants and compounds present in extracts are possible, *e.g.*, chelation of Fe^{2+}, hydrogen donation, scavenging, reduction, etc.

However, on the sixth day, both groups of fermentation broth samples had the highest antioxidant activity. It can be suggested that some other non-polyphenols, formed on the sixth day of fermentation contribute to the higher antioxidant activity of TK6 and CBK6.

The structure of polyphenol compounds is a key determinant of their radical scavenging and metal chelating activity, and this is referred to as structure-activity relationships. There is a wealth of literature that suggests that the potency of the catechins' free radical scavenging abilities relates directly to the chemical structure of each compound, namely, the gallate moiety esterified at the 3 position of the C ring, the catechol group (3,4-dihydroxyl groups) on the B ring and the hydroxyl groups at the 5 and 7 positions on the A ring. The galloylated catechins were more active antioxidants due to their higher phospholipid/ /water partition coefficients and so affected the properties of the phospholipid bilayers of membranes and hence increased solubilization [30]. Based on

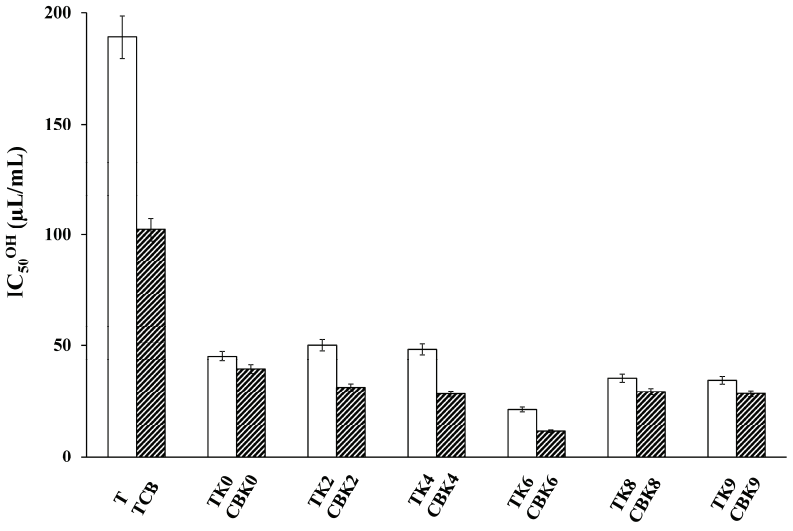

Figure 4. IC_{50}^{OH} values of fermentation broth samples obtained during nine days of traditional Kombucha fermentation (TK) and Kombucha fermentation of sweetened black tea enriched with CoffeeBerry® extract (CBK) in OH radical scavenging assay.

literature data, catechins which are present in the samples affect their beneficial effect on the human health and investigated samples CBK0-CBK9 might be classified as highly valuable products. The antioxidant activity of all present polyphenol compounds is due to their ability to scavenge free radicals, donate hydrogen atoms or electron, or chelate metal cations [31]. Previous structure-activity relationship studies on cinnamic acids and derivatives have pointed out the importance of *o*-3c,4c-dihydroxyl groups to the antiradical efficiency. In addition, it is of interest to note that caffeic acid contains the *o*-3c,4c-dihydroxyl groups, In addition, it is of interest to note that caffeic acid containing the *o*-3c,4c-dihydroxyl groups [32]. The enhanced antioxidant potential of the CoffeeBerry® polyphenols was probably due to increased hydrophilicity of the compounds by esterification [33]. Additionally, it is known that the total antioxidant potential of fruits, vegetables and tea and herbal infusions is more important than the level of any individual specific antioxidant constituent. Because of that, the antioxidant properties of a single compound within a group can vary remarkably, so that the same levels of polyphenols do not necessarily correspond to the same antioxidant responses [34]. The observed antioxidant activity is also due to a synergistic action of the different compounds present [35].

The antioxidant potential of samples obtained during Kombucha fermentation of sweetened black tea enriched with CoffeeBerry® extract are attributed to chlorogenic acid and their derivatives (Table 2), which has strong capacity for scavenging free radicals [36,37]. Chlorogenic and caffeic acid are antioxidants *in vitro*, and they inhibit the formation of mutagenic and carcinogenic *N*-nitroso compounds [38]. Further, chlorogenic acid can inhibit DNA damage *in vitro* [39]. Therefore, chlorogenic acid, the major polyphenol compound in coffee, might be involved in the inverse association between coffee consumption and colon cancer that was found by some epidemiological studies [39].

CONCLUSION

In this study, polyphenols profile and antioxidant activities of kombucha enriched with CoffeeBerry® extract during fermentation were analysed. The HPLC results proved the presence of polyphenols and catechins in fermentation broth samples. All samples enriched with CoffeeBerry® possess chlorogenic acid, one of the most abundant dietary polyphenols, and showed remarkable antioxidant activities on stable DPPH and toxic OH radicals. The classification of Kombucha beverage enriched with CoffeeBerry® extract, a source of bioactive polyphenols, as a functional beverage is justified. These findings were noteworthy because such compounds could be well adapted to the pathogenesis of diseases that are characterised by an overproduction of free radicals.

REFERENCES

[1] R. Jayabalan, S. Marimuthu, K. Swaminathan, Food Chem. **102** (2007) 392-398

[2] J. Reiss, Z. Lebensm. Unters. Forsch.**198** (1994) 258-261

[3] M. Sievers, C. Lanini, A. Weber, U. Schuler-Schmid, M. Teuber, Syst. Appl. Microbiol. **18** (1995) 590-594

[4] D. Cvetković, PhD Thesis, University of Novi Sad, Novi Sad, 2008 (in Serbian)

[5] C.J. Greenwalt, K.H. Steinkraus, R.A. Ledford, J. Food Prot. **63** (2000) 976-981

[6] B. Bauer-Petrovska, L. Petrushevska-Tozi, Int. J. Food Sci. Tech. **35** (2000) 201-205

[7] N. Hoffmann, Basic Building Blocks, Nutrients and Growth Factors, What the Kombucha culture needs to survive, http://www.kombu.de/nutrient.htm (accessed 12 February 2008)

[8] D.D. Četojević-Simin, S.A. Velićanski, D.D. Cvetković, L.S. Markov, Ž.J. Mrđanović, V.V. Bogdanović, V.S. Šolajić, Food Bioprocess Technol. **5** (2012) 1756-1765

[9] G. Cetkovic, J. Canadanovic-Brunet, S. Djilas, V. Tumbas, S. Markov, D. Cvetkovic, Int. J. Mol. Sci. (2007) 1013-1027

[10] A.S. Velićanski, D.D. Cvetković, S.L. Markov, V.T. Tumbas Šaponjac, J. J. Vulić, Food Technol. Biotechnol. **52**(4) (2014) FTB-3611

[11] J.T. Heimbach, P.A. Marone, J.M. Hunter, B.V. Nemzer, S.M. Stanley, E. Kennepohl, Food Chem. Toxicol. **48** (2010) 2517-2525

[12] S.L. Markov, R.V. Malbaša, M.J. Hauk, D.D. Cvetković, Acta Periodica Technol. **32** (2001) 133-138

[13] A. Velićanski, PhD Thesis, University of Novi Sad, Novi Sad, 2012 (in Serbian)

[14] J.L. Jacobson, Introduction to Wine Laboratory Practices and Procedures, Springer Science Business Media, Inc., New York, 2006, pp. 257-277

[15] D. Cvetković, S. Markov, M. Djurić, D. Savić, A. Velićanski, J. Food Eng. **85** (2008) 387-392

[16] V.L. Singleton, R. Orthofer, R.M. Lamuela-Raventos, in Methods in Enzymology, L. Packer (Ed.), Academic Press, San Diego, CA, 1999, pp. 152-178

[17] M. Vuletić, V. Hadži-Tašković Šukalović, K. Marković, N. Kravić, Ž Vučinić, V. Maksimović, Plant Biol. **16** (2014) 88-96

[18] G. Sreeramulu, Y. Zhu, W. Knol, J. Agr. Food Chem. **48** (2000) 2589-2594

[19] G. Belloso-Morales, H. Hernández-Sánchez, Rev. Latinoam. Microbiol. **45** (2003) 5-11

[20] A.L. Teoh, G. Heard, J. Cox, Int. J. Food Microbiol. **95** (2004) 119-126

[21] S.-C. Chu, C. Chen, Food Chem. **98** (2006) 502-507

[22] C. Cabrera, R. Giménez, M.C. López, J. Agr. Food Chem. **51** (2003) 4427-4435

[23] D. Del Rio, A.J. Stewart, W. Mullen, J. Burns, M.E.J. Lean, F. Brighenti, A. Crozier, J. Agr. Food Chem. **52** (2004) 2807-2815

[24] L. Ji, P. Jiang, B. Lu, Y. Sheng, X. Wang, Z. Wang, J. Nutr. Biochem. **24** (2013) 1911-1919

[25] J.G. Dórea, T.H.M. da Costa, Brit. J. Nutr. **93** (2005) 773-782

[26] S.K. Bøhn, R. Blomhoff, I. Paur, Mol. Nutr. Food Res. **58** (2014), 915-930

[27] G. Ćetković, S. Djilas, J. Čanadanović-Brunet, V. Tumbas, Food Res. Int. **37** (2004) 643-650

[28] J.M. Čanadanović-Brunet, G.S. Ćetković, S.M. Djilas, V.T. Tumbas, S.S. Savatović, A.I. Mandić, S.L. Markov, D.D. Cvetković, Int. J. Food Sci. Technol. **44** (2009) 269--278

[29] Y.-C. Yang, J. Li, Y.-G. Zu, Y.-J. Fu, M. Luo, N. Wu, X.-L. Liu, Food Chem. **122** (2010) 373-380

[30] B.A. Sutherland, R.M.A. Rahman, I. Appleton, J. Nutr. Biochem. **17** (2006) 291-306

[31] K. Apel, H. Hirt, Annu. Rev. Plant Biol. **55** (2004) 373--399

[32] R.M. Olthof, P.C.H. Hollma, M.B. Katan, J. Nutr. **131** (2001) 66-71

[33] M.-H. Kweon, H.-J. Hwang, H.-C. Sung, Food Chem. **49** (2001) 4646-4655

[34] A. Farah, C.M. Donangelo, Braz. J. Plant Physiol. **18** (2006) 23-36

[35] R.H. Liu, Am. J. Clin. Nutr. **78** (2003) 517S–520S

[36] A. Yashin, Y. Yashin, J.Y. Wang, B. Nemzer, Antioxidants **2** (2013) 230-245

[37] J.A. Gomez-Ruiz, J.M. Ames, D.S. Leake, Eur. Food Res. Technol. **227** (2008) 1017-1024

[38] M. Daglia, M. Racchi, A. Papetti, C. Lanni, S. Govoni, G. Gazzani, J. Agric. Food Chem. **52** (2004) 1700-1704

[39] T. Bakuradze, R. Lang, T. Hofmann, H. Stiebitz, G. Bytof, I. Lantz, M. Baum, G. Eisenbrand, C. Janzowski, Mol. Nutr. Food Res. **54** (2010) 1734-1743.

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NAUČNI RAD

POLIFENOLI I ANTIOKSIDATIVNA AKTIVNOST KOMBUHA NAPITKA OBOGAĆENOG COFFEEBERRY® EKSTRAKTOM

Kombuha je tradicionalni napitak koji se dobija fermentacijom zaslađenog crnog ili zelenog čaja aktivnošću čajne gljive, koja predstavlja konzorcijum bakterija sirćetnog vrenja i autohtonih vrsta kvasaca. CoffeeBerry® proizvod se dobija od celog ploda kafe i sadrži komponente koje imaju nutritivnu vrednost i zdravstveni značaj. U radu su ispitani uzorci fermentativne tečnosti tokom pripremanja Kombuha napitka na tradicionalan način, kao i oni dobijeni obogaćenjem te fermentativne tečnosti CoffeeBerry® ekstraktom. Fermentacija se odvijala u bioreaktoru na 28±1 °C u trajanju od 9 dana do postizanja optimalne konzumne kiselosti. Rezultati su pokazali da CoffeeBerry® ekstrakt doprinosi bržoj fermentaciji medijuma za kultivaciju. HPLC analizom uzoraka fermentativnih tečnosti identifikovana su i kvantifikovana pojedina polifenolna jedinjenja i katehini, a takođe i kofein. U fermentativnoj tečnosti obogaćenoj CoffeeBerry® ekstraktom detektovana je hlorogenska kiselina u visokom sadržaju (188,94-458,56 μg/ml). Ispitana je antioksidativna aktivnost fermentativnih tečnosti na DPPH i hidroksil radikale elektron-spin rezonantnom (ESR) spektroskopijom. Antioksidativna aktivnost na DPPH i hidroksil radikale povećava se tokom trajanja fermentacije. U fermentativnoj tečnosti obogaćenoj CoffeeBerry® ekstraktom, izračunate IC_{50} vrednosti za DPPH i hidroksil radikale iznosile su 26,33-170,13, odnosno 11,33-102,22 μL/mL.

Ključne reči: Kombuha, CoffeeBerry®, polifenoli, antioksidativna aktivnost, HPLC analiza.