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Bioactive and Anti-carcinogenic Properties of Kombucha Prepared with Aronia Melanocarpa Juice

Aronia Melanocarpa Suyu Kullanılarak Hazırlanan Kombu Çayının Biyoaktif ve Anti-karsinojenik Özellikleri

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Abstract

Aim: Polyphenolics derived from Aronia Melanocarpa may have beneficial effects in reducing the risk of several diseases, such as diabetes, cardiovascular diseases, and cancer. Kombucha is a symbiotic system comprising cultures of bacteria and yeasts known as SCOBY. It is prepared by fermenting sugar and tea leaves. In this study, we aimed to enhance the beneficial aspects of Kombucha by preparing it using aronia juice as a fermentation substrate.

Methods: The total phenolic content was measured quantitatively through the Folin-Ciocalteu method, with Gallic acid as the standard. The antioxidant capacity was determined using the ABTS and DPPH methods. Cell viability in colon cancer and normal fibroblasts was determined using the MTT assay.

Results: The mean values of pH and total acidity of aronia juice used in Kombucha production were determined as 5.24±0.00 and 0.45±0.05, respectively. The total phenolic content was highest in the sample containing green tea and aronia juice. The antioxidant capacity of the sample containing green tea and aronia juice had the highest values in extractable (TEACABTS: 13.39 µmol Trolox/mL), hydrolyzable (TEACDPPH: 15.15 µmol Trolox/mL) and bioaccessible phenolics (TEACABTS: 4.77 µmol Trolox/mL). However, we found a decline by about 56% in the viability of colon cancer cells (HT-29) after treatment with the Kombucha sample containing green tea and aronia juice.

Conclusion: Enrichment of Kombucha with aronia juice is a good alternative for producing new fermented drinks with high antioxidant capacity. Kombucha enriched with aronia juice has high total phenolic content and antioxi-

Öz

Amaç: Aronia Melanocarpa'dan elde edilen polifenolikler diyabet, kardiyovasküler hastalıklar ve kanser gibi çeşitli hastalıkların riskini azaltmak için faydalı etkilere sahip olabilmektedir. Kombu çayı, SCOBY olarak bilinen bakteri ve maya kültürlerinden oluşan simbiyotik bir sistemdir. Şeker ve çay yapraklarının fermente edilmesiyle hazırlanır. Bu çalışmada, Kombu çayını fermantasyon substratı olarak aronia suyu ile hazırlayarak yararlı yönlerini geliştirmeyi amaçladık.

Yöntem: Toplam fenolik madde miktarları gallik asit standardına göre Folin-Ciocalteu yöntemi ile kantitatif olarak ölçülürken, antioksidan kapasite ABTS ve DPPH yöntemleri kullanılarak belirlenmiştir. Kolon kanseri ve normal fibroblastların hücre canlılıkları MTT testi kullanılarak belirlenmiştir.

Bulgular: Kombu çayı üretiminde kullanılan aronya suyunun ortalama pH ve toplam asitlik değerleri sırasıyla 5.24±0.00 ve 0.45±0.05 olarak belirlenmiştir. Yeşil çay ve aronya suyu içeren örnekte toplam fenolik madde içeriği en yüksek bulunmuştur. Yeşil çay ve aronya suyu içeren örneğin antioksidan kapasitesi değerlendirildiğinde, ekstrakte edilebilir (TEACABTS: 13.39 µmol Trolox/mL), hidrolize edilebilir (TEACDPPH: 15.15 µmol Trolox/mL) ve biyoerişilebilir fenolikler (TEACABTS: 4.77 µmol Trolox/mL) açısından en yüksek değerleri göstermiştir. Bununla birlikte, yeşil çay ve aronia suyu içeren Kombucha örneği ile muameleden sonra kolon kanseri hücrelerinin (HT-29) canlılığında yaklaşık %56 oranında bir azalma tespit ettik.

Sonuç: Kombu çayının aronia suyu ile zenginleştirilmesi, yüksek antioksidan kapasiteye sahip yeni fermente içecek-

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dant capacity. Besides, it may be considered in the context of colon cancer prevention.

Keywords: *Aronia melanocarpa*, Kombucha, anti-carcinogenic effect, antioxidant capacity, bioaccessibility

ler üretmek için iyi bir alternatiftir. Aronia suyu ile zenginleştirilmiş Kombu çayı yüksek toplam fenolik içeriğe ve antioksidan kapasiteye sahiptir. Ayrıca, kolon kanserinin önlenmesi bağlamında da değerlendirilebilir.

Anahtar Kelimeler: *Aronia melanocarpa*, kombu çayı, anti-karsinojenik etki, antioksidan kapasite, biyoerişilebilirlik

1. Introduction

With a growing global population, the increase in people with health problems has led to a greater interest in nutritious food with health-promoting functions. Moreover, due to the devastating consequences of the COVID-19 pandemic, immunity is one of the most significant concerns of the time. To meet the rising demand, food manufacturers are developing new functional foods containing bioactive compounds such as probiotics and fibers for intestinal health, antioxidants and polyphenols for oxidative stress reduction, and flavonoids for anti-carcinogenic properties.

Kombucha is a symbiotic culture of bacteria (namely *Acetobacter* and *Gluconobacter*), and yeast (SCOBY). It is a mildly sweet, acidic, carbonated beverage made from fermenting tea leaves (black, green, white, or oolong) (1). The yeast present in the symbiotic culture facilitate the conversion of sucrose into ethyl alcohol and carbon dioxide by the action of the enzyme invertase. On the other hand, the acetobacteria in the culture use aldehyde dehydrogenase enzymes to convert the ethyl alcohol produced by the yeast into acetic acid. In contrast, Gluconobacteria are capable of gluconate production but lack the necessary succinate and α-ketoglutarate enzymes for acetic acid oxidation. *Gluconobacter* and *Acetobacter* are known to enzymatically convert glucose into gluconic acid, whereas the conversion of fructose and ethanol leads to the production of acetic acid (1, 2). Kombucha is composed of several constituents including sugars, organic acids, ethanol, carbon dioxide, fiber, amino acids, essential elements including copper, iron, manganese, nickel, and zinc, as well as vitamin B derivatives, vitamin C, hydrolytic enzymes, and polyphenols produced from green tea leaves (3, 4). Antioxidant compounds are accountable for Kombucha's health benefits. Regarding phenolic compounds, flavonoids, and phenolic acids were the most prevalent substances in all Kombucha varieties (5). Catechins are a subgroup of polyphenols, the primary group of flavonoids, and they are found in green tea leaves. They are responsible for increased antioxidant properties, including free radical scavenging activity (6, 7). Organic acids such as glucuronic and acetic acids are also essential components of Kombucha (5). In addition to the fermentation of tea leaves, the enrichment of Kombucha by bioactive ingredients increases its health benefits.

Aronia Melanocarpa has many beneficial health effects including reducing blood sugar and lipid levels, and lowering blood pressure. Also, it has antioxidant, anti-carcinogenic, antibacterial, and anti-inflammatory properties (8-10). Phenolic and non-phenolic components of aronia may suppress cancer-related processes such as cell proliferation, cell cycle arrest, and apoptosis. Yu et al. (2021) reported that aronia anthocyanins effectively inhibited the proliferation of Caco-2 colon cancer cells by regulating glutamine metabolism (11). Another study showed that aronia leaf extract has strong cytotoxic activity against Caco-2 cells (12). In addition, aronia fruit and leaf extracts have been reported to inhibit the growth of human colorectal adenocarcinoma LS-174T cells more strongly than anticancer drug cisplatin (13). Also, it exhibits anticancer properties on different cancers, including breast cancer, intestinal cancer, and leukemia (14).

Mixing traditional Kombucha with fruits and herbs with bioactive substances has yet to be extensively studied. Therefore, in this study, we aimed to investigate the potential health benefits and anti-carcinogenic properties of Kombucha enriched with aronia juice (10%). Kombucha samples made from green tea and/or aronia juice were compared regarding pH and total acidity, total phenolic content, antioxidant capacity ($TEAC_{ABTS}$ and $TEAC_{DPPH}$), and cytotoxicity (cell viability).

2. Method

100% aronia juice was obtained from Cherry&Berry Co. (Plovdiv, Bulgaria), organic tea leaves were supplied from Caykur Co., (Rize, Türkiye), and the sucrose (beet sugar) was obtained from Torku Co., (Konya, Türkiye). SCOBY was supplied by Kombucha 2200 (Istanbul, Türkiye).

2.1. Kombucha Production

Kombucha production was conducted according to Yildiz et al. (15). For Kombucha production, one liter of sterile water was boiled at 95°C for 15 min, and then organic green tea (14 g/L) was added and brewed for 15 min. After removing the tea leaves, the tea mixture was filled in sterile glass jars, and allowed to cool down to room temperature, and sucrose (30 g/L) was added. Afterwards, SCOBY and Kombucha (10%) were added as inoculums. Aronia Kombucha added 10% aronia juice after brewing and removing tea leaves. All fermentation jars were covered with cheesecloth and kept at 30±2°C in dark condition for 12 days.

2.2. pH and Total acidity

The pH of the samples was determined by FiveEasy™ model Mettler Toledo (Ohio, USA) according to AOAC Method No: 981.12 (16), and the total acidity of samples was determined according to AOAC Method No 942.15 (16). Aronia juice expresses citric acid equivalent, while Kombucha samples in acetic acid equivalent.

2.3. Evaluation of Antioxidant Capacity and Total Phenolic Content

The antioxidant capacity (AC) and total phenolic content (TPC) of the samples were analyzed regarding phenolics' extractable, hydrolysable, and bioaccessible fractions. 2 mL of sample was extracted with HCl/methanol/water (1:80:10, v/v/v) at 20 °C in a stirring water-bath (Thermo Fisher Scientific Inc., Waltham, MA, USA; 250 rpm, 2 h) and centrifuged (Sigma centrifuge 3 K 30, Germany; 3500 rpm, 4 °C, 10 min). The supernatant was taken as an extractable fraction, and the residue was treated with methanol/sulfuric acid (10:1), heat treated in stirring water bath (250 rpm) at 85 °C for 20 h, and then centrifuged at 3500 rpm for 10 min. The hydrolysable fraction was obtained by collecting the supernatant. In order to determine the bioaccessible fraction, a mimic *in-vitro* digestion process was used, which included enzymatic extraction of the materials (17). Pepsin enzyme (40 mg/mL in 0.1 M HCl; Merck, Germany) was used to treat 2 mL of sample in stirring water-bath (250 rpm) at 37 °C for 2 h. The extraction underwent the intestinal digestion procedure using porcine pancreatic enzyme (2 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) and porcine bile mixture (12 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) at 37 °C (250 rpm) for 2 h, followed by centrifugation at 15 °C, 3500 rpm, for 10 minutes. The extracts were kept at -18 °C and utilized in AC and TPC assays.

AC analysis is determined according to ABTS and DPPH assays (18). The UV–Vis spectrophotometer was used to quantitatively measure the absorbance of the extracts (Jenway, 6405 UV/Vis, UK), Trolox equivalent calibration curve was derived for analyses in between the range of 0.02-0.08 μmol Trolox (Sigma-Aldrich, St. Louis, MO, USA). The results were reported as mol of Trolox equivalent (TE) per millilitre of sample. Apak et al. (19) utilized the Folin-Ciocalteu method to determine the concentration of TPC. The absorbance of the extracts was measured using gallic acid (Sigma-Aldrich, St. Louis, MO, USA) as standard, and the results were expressed as mg gallic acid equivalents (GAE) per 100 mL sample. The bioaccessibilities % of AC and TPC were calculated from

Table 1. Formulations of Kombucha samples

Samples	Abbreviation	Aronia Juice	Green Tea Leaves
Green Tea Kombucha	GK	-	14 g/L
Aronia Kombucha	AK	10 %	-
Aronia-Green Tea Kombucha	GAK	10 %	14 g/L

the results of extractable, hydrolysable, and bioaccessible phenolic fractions (20).

2.4. Cell Culture

The Department of Biochemistry at Istanbul University supplied the human colon cancer cell line (HT-29) as well as the non-tumorigenic human umbilical vein endothelial cell line (HUVEC). In this study, cells that had been stored in liquid nitrogen were thawed gradually on ice and subsequently cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin-streptomycin, and 2 mM L-glutamine. The cell culture was maintained in a fully humidified environment at a temperature of 37°C with 5% CO_2. The medium underwent daily changes. The cells underwent sub-culturing twice each week, specifically when they achieved 80% confluency. A single cell suspension was acquired by using 0.5% trypsin.

2.5. MTT Assay

Cytotoxic activities of Kombucha samples were tested against HUVEC and HT-29 cells using MTT assay. The MTT test is based on the mitochondrial dehydrogenases of living cells converting the yellow colour formazan crystals in MTT solution [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] to a purple colour. The reduction activity is dependent on the concentration of intracellular NADH and NADPH.

HT-29 and HUVEC cells were seeded into each well of a 96-well plate at a density of approximately $1x10^4$ cells/mL per 100 μL of medium. Then, cells were incubated for 24 h at 37 °C in a 5% CO_2 incubator, and the medium was changed. Before treatment, Kombucha samples were diluted with DMEM. Then, cells were incubated with 0.1 and 0.2% (v/v) Kombucha samples for 48 h. After the incubation, the medium was changed to remove non-adherent cells. The cells underwent a washing procedure using phosphate-buffered saline (PBS), followed by the addition of 100 μL of new medium containing 10 μL of MTT (0.25 mg/mL). Following a 4-hour incubation period, the MTT solution was subsequently aspirated, and 100 μL of dimethyl sulfoxide (DMSO) was added to facilitate the dissolution of formazan crystals. The measurement of absorbance for the coloured solution was conducted using an ELISA microplate reader, with a wavelength of 570 nm. The findings were presented in the form of a viability percentage relative to the control group that did not receive any treatment. The experiments were conducted a minimum of three times, and the mean results were calculated. The formula (Eq.1) was used to determine the proportion of viable cells.

$$\text{The percentage of the viable cells (\%)} = \frac{Average\ absorbance\ of\ treated\ cells}{Average\ absorbance\ of\ untreated\ cells}\ x\ 100$$

2.6. Statistical Analysis

The data is presented as mean (M) ± standard deviation (SD) from one representative of three independent experiments. Results were compared using the one-way analysis of variance (ANOVA). Determination of the significance level among the means ($p \leq 0.05$) was determined using the least significant difference (LSD) test. The statistical analyses were conducted using GraphPad Prism software, version 8.0.1 (San Diego, CA, USA).

3. Results

The pH and the total acidity values of aronia juice (10%) were 5.24±0.00 and 0.45±0.05, respectively (data not shown in tables). The total acidity value of aronia juice is indicated as citric acid equivalent. Table 2 shows the pH

Table 2. pH and total acidity values of Kombucha samples

Sample	pH	Total acidity
Green Tea Kombucha	5.23±0.00^{c}	0.12±0.00^{c*}
Aronia Kombucha	5.52±0.05^{b}	0.36±0.00^{a*}
Green Tea-Aronia Kombucha	6.33±0.00^{a}	0.17±0.00^{b*}

*Values are given as mean±SD. Different letters (a-c) for each column indicate the statistically significant differences between samples. *Total acidity values of Kombucha samples indicated as acetic acid equivalent with statistical evaluation.*

Table 3. Antioxidant capacity and total phenolic content of aronia juice

	Extractable Phenolics	Hydrolizable Phenolics	Bioaccessible Phenolics	Bioaccessibility %
Total phenolic content (mg GAE/100mL)	9.96±0.05^{b*}	13.39±0.05^{a}	10.75±0.00^{a}	46.04±0.05^{b}
ABTS (μmol TE/mL)	0.78±0.04^{c}	3.16±0.02^{c}	1.41±0.39^{c}	80.20±0.62^{a}
DPPH (μmol TE/mL)	13.30±0.04^{a}	11.36±0.04^{b}	3.33±0.12^{b}	13.51±0.50^{c}

**Values are given as mean±SD. Different letters (a-c) indicate the statistically significant differences between samples in terms of phenolic fractions and bioaccessibility % values.*

and total acidity values of Kombucha samples.

The antioxidant capacity and total phenolic content of aronia juice were assessed concerning extractable, hydrolysable, and bioaccessible phenolics. The findings are shown in Table 3, with statistical significance set at $p<0.05$. This method is effective in extracting a higher quantity of bioaccessible phenolics.

The antioxidant capacity, total phenolic content, and bioaccessibility % values of Kombucha samples were assessed concerning extractable, hydrolysable, and bioaccessible phenolics. The findings are shown in Table 4, with statistical significance set at $p<0.05$. The highest total phenolic content was detected in GAK regarding extractable, hydrolysable, and bioaccessible phenolics. Regarding total phenolic content, extractable, hydrolysable, and bioaccessible phenolics of GAK sample increased by 39%, 49% and, 75%, respectively, comparing to AK sample. The results of the antioxidant capacity analysis of Kombucha samples indicate that GK and GAK exhibited similar values with significantly higher levels of extractable and hydrolysable phenolic compounds. Regarding $TEAC_{ABTS}$ and $TEAC_{DPPH}$ antioxidant capacity, bioaccessible phenolics of GAK sample increased by 133% and 72% compared to AK sample. Regarding total phenolic

Table 4. Antioxidant capacity and total phenolic content of Kombucha samples

		GK*	AK	GAK
Total Phenolic Content (mg GAE/100mL)	Extractable phenolics	21.58±0.07^{b**}	10.46±0.05^{c}	27.08±0.52^{a}
	Hydrolysable phenolics	29.39±0.04^{b}	15.98±0.05^{c}	32.71±0.05^{a}
	Bioaccessible phenolics	10.75±0.00^{c}	12.81±0.03^{b}	17.09±0.02^{a}
	Bioaccessibility (%)	31.35±0.04^{b}	48.45±0.01^{a}	28.58±0.08^{c}
ABTS (μmol TE/mL)	Extractable phenolics	14.39±0.14^{a}	0.84±0.00^{c}	13.39±0.22^{a}
	Hydrolysable phenolics	7.24±0.05^{a}	3.21±0.00^{c}	6.72±0.02^{b}
	Bioaccessible phenolics	2.81±0.11^{c}	3.58±0.43^{b}	4.77±0.66^{a}
	Bioaccessibility (%)	50.14±0.24^{b}	79.18±0.00^{a}	50.09±0.49^{b}
DPPH (μmol TE/mL)	Extractable phenolics	13.40±0.04^{a}	12.87±0.05^{b}	13.18±0.20^{a}
	Hydrolysable phenolics	15.29±0.37^{a}	11.24±0.04^{b}	15.15±0.42^{a}
	Bioaccessible phenolics	5.57±0.81^{a}	2.86±0.16^{c}	3.99±0.11^{b}
	Bioaccessibility (%)	19.44±2.93^{a}	11.85±0.69^{c}	14.09±0.16^{b}

**GK: Green tea Kombucha; AK: Aronia Kombucha; GAK: Green tea-aronia Kombucha.*

***Values are given as mean±SD. Different letters (a-c) indicate the statistically significant differences between samples in terms of phenolic fractions for relevant assay.*

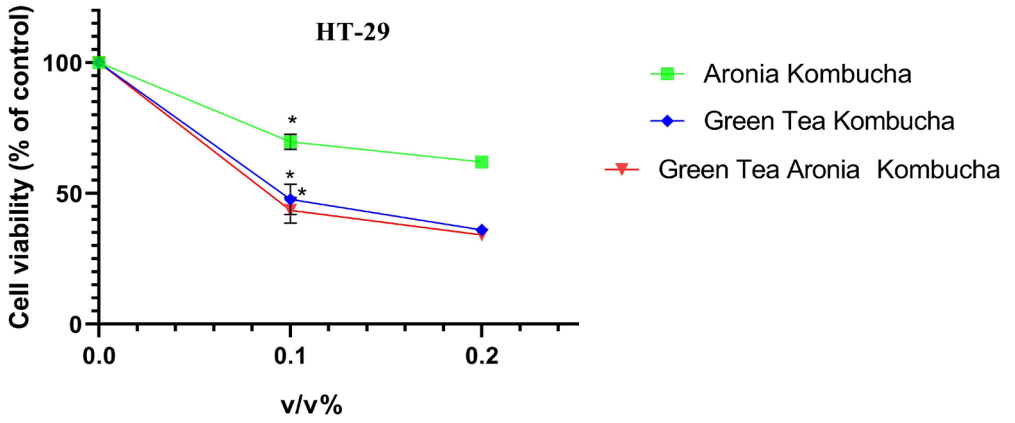

Figure 1. Cell viability was determined by MTT assay. HT-29 cells were treated with indicated concentrations of Kombucha samples along with control after 48 h of incubation. Results are expressed as a percentage of control (untreated cells). Data points represent mean ± SD from three independent experiments. *p<0.05

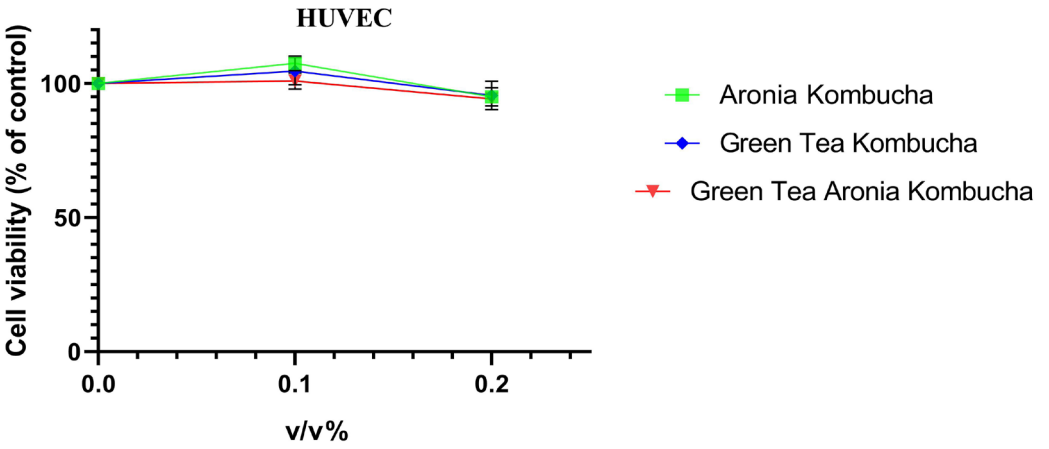

Figure 2. Cell viability was determined by MTT assay. HT-29 cells were treated with indicated concentrations of Kombucha samples along with control after 48 h of incubation. Results are expressed as a percentage of control (untreated cells). Data points represent mean ± SD from three independent experiments.

content, the same sample increased by 75% compared to the AK sample.

As depicted in Figure 1, treatment with AK, GK and, GAK samples (0.1% v/v) decreased cell viability of HT-29 cells by about 30%, 52% and, 56%, respectively. The effect of Kombucha samples on the cell viability of HUVEC cells was insignificant (Figure 2). These results indicate that Kombucha samples decrease the cell viability of HT-29 cells while leaving HUVEC cells unaffected.

4. Discussion

In this paper, Kombucha enriched with aronia juice was evaluated for the first time regarding antioxidant activity,

total phenolic content, and cytotoxicity. This means that we cannot compare our results directly to the previous work. The results suggested that enrichment of Kombucha with aronia juice could increase the $TEAC_{ABTS}$, $TEAC_{DPPH}$, and TPC values of Kombucha samples. In the literature, it is common to conduct experiments of the antioxidant potential of Kombucha by single extraction methods, focusing on the quantification of extractable phenolics. Also, there needs to be more investigations on *in-vitro* bioaccessibility assays.

The bioactive potential of Kombucha is generally associated with the phenolic compounds. In the study by Cardoso et al. (5), the samples of Kombucha made from green tea and black tea were analyzed for their compound phenolic acids. The results revealed that phenolic acids and flavonoids were the predominant bioactive compounds in the Kombucha samples. Specifically, flavonoids accounted for 70.2% of the total content. Furthermore, it was observed that the quantity and activities of these compounds varied depending on the fermentation conditions, as demonstrated in the same study. In the abovementioned investigation, the green tea samples had a $TEAC_{ABTS}$ value of 8.22 μmol TE/mL and a total phenolic compound content of 0.70 mg, as evaluated by GAE/mL (5).

In a study conducted by Pereira et al. (21), the researchers determined the total phenolic content of Kombucha made using green tea and black tea, which were found to be 1080 mg GAE/L and 1120 mg GAE/L, respectively. Similarly, Khokhar and Magnusdottir (22) calculated the total phenol content of Kombucha (with green tea) to be 86.3 mg GAE/g. The findings from our comprehensive analysis of phenolic compounds align with the existing body of research.

In addition to the phenolic content and concentration, the antioxidant activity of Kombucha may be influenced by metabolites, including ascorbic acid and other organic acids, which are generated during fermentation (23). The fermentation temperature (24) and length (25) also impact the antioxidant content of Kombucha. Furthermore, the concentration of total phenolic compounds exhibits a progressive rise during the fermentation period. The enzymatic degradation of complex phenolic compounds found in green and black tea occurs inside the microbiota of SCOBY in the acidic environment of Kombu tea. Degradation of complex polyphenols into small molecules also causes an increase in total phenolic compounds (26) and an increase in the bioaccessibility of phenolic compounds. Aronia is known for its bioactive potential and health-promoting effects. Kombucha fermentation increases the bioaccessibility of aronia juice (AJ), and the green tea content enriches its bioactive potential (GAK). Based on the findings derived from the research, it is hypothesized that the levels of phenolic components and the bioaccessibility and bioavailability of Kombucha samples were acquired via the co-fermentation of aronia juice and green tea.

Colon cancer is one of the most frequent malignancies in both men and women, and it is the world's fourth leading cause of cancer-related death. Chemotherapy is commonly used to treat colon cancer but has many side effects (27). Therefore, it is critical to investigate new anticancer agents with minimal toxicity to prevent and treat colon cancer. Kombucha can be used as a traditional treatment for various types of cancer due to its antiproliferative properties. Active components responsible for the antiproliferative activity could be a variety of organic acids (acetic acid, gluconic acid, lactic acid, and glucuronic acid) and vitamins (B,C) (28, 29). Aronia juice infusion can be successfully used with green tea for Kombucha fermentation, yielding a beverage of strong antioxidant activity. Several studies reported the cytotoxic effects of Kombucha on cancer cells, such as the prostate cancer cell line (29) and bladder cancer cells (30). It was reported that Kombucha prepared with green, oolong, and black tea has effective toxicity against Caco-2 colorectal cancer cells (31). Our MTT results clearly showed that AK, GK, and GAK can potentially induce cytotoxicity towards HT-29 cells without affecting HUVEC cells.

5. Conclusion

Based on the literature review that we have performed, this is the first study investigating the total phenolic content, antioxidant capacity and cytotoxic activity of Kombucha produced with aronia juice enrichment. These results represent preliminary findings that will aid the extraction of the bioactive components in Kombucha beverages to improve the efficacy. Further research needs to be done to understand the molecular mechanism regulated by the Kombucha and aronia regarding cancer prevention. Finally, enrichment of kombucha samples with aronia juice by fermentation with tea residues is

very promising due to their high antioxidant activities, which could be used as functional foods to prevent various diseases caused by oxidative stress, including cancer.

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