

Communication

Time-Dependent Modulation of Metabolite Profile and Antioxidant Capacity of Hamamelidis Cortex Extract During Kombucha Fermentation

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Featured Application

This study demonstrates that kombucha fermentation is an effective biotechnological approach for improving the functional properties of Hamamelidis cortex extract. This study demonstrates that kombucha fermentation improves the antioxidant and metal-chelating properties of Hamamelidis cortex extract. The fermented extract represents a promising candidate for further studies on its potential use as a cosmetic ingredient.

Abstract

Fermentation is increasingly used to improve the functional properties of plant-derived cosmetic ingredients. This study evaluated the effect of kombucha fermentation on the phytochemical composition and antioxidant activity of Hamamelidis cortex extract obtained by accelerated solvent extraction (ASE). Changes occurring during 15 days of fermentation with a symbiotic culture of bacteria and yeasts (SCOBY) were monitored by UHPLC–DAD–ESI–MS, while antioxidant activity was assessed using ABTS, DPPH, and metal-chelating assays. The extract was rich in galloylated phenolics, with hamamelitannin as the dominant constituent. Fermentation increased the levels of hamamelitannin (456.4–675.7 µg/mL), gallic acid, galloyl-hexoses, catechin, and protocatechuic acid, whereas high-molecular-weight gallotannins declined. These compositional changes were accompanied by a decrease in pH from 4.4 to approximately 3.0 and by enhanced antioxidant properties. ABTS and DPPH radical scavenging capacity increased, while Fe²⁺ and Cu²⁺ chelating activities reached 69.5% and 85.9%, respectively. Correlation analysis indicated that galloylated phenolics were closely associated with metal-chelating activity. Overall, kombucha fermentation enhanced the antioxidant potential of Hamamelidis cortex extract and improved its functional properties, supporting its application as a multifunctional cosmetic ingredient for protecting the skin against oxidative stress.



Academic Editor: Thomas Lindner

Received: 26 June 2026

Revised: 30 July 2026

Accepted: 31 July 2026

Published: 2 August 2026

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Keywords: gallotannins; microbial biotransformation; metal chelation; *Hamamelis virginiana* bark

1. Introduction

Plant-derived extracts have long been utilized in dermatological and cosmetic formulations due to their rich content of polyphenols, flavonoids, and other secondary metabolites

capable of modulating oxidative stress, exerting anti-inflammatory and skin-protective effects, and generally supporting skin homeostasis [1,2].

Among them, Hamamelidis cortex (*Hamamelis virginiana* bark) is regarded as one of the most valuable botanical raw materials because of its exceptionally high content of hydrolysable tannins, particularly hamamelitannin, together with gallic acid derivatives, flavonoids, and proanthocyanidins [3,4]. These constituents are responsible for the well-documented astringent, anti-inflammatory, antioxidant, wound-healing, antimicrobial, and vasoprotective properties of witch hazel bark, which has been widely incorporated into pharmaceutical and cosmetic formulations for the management of sensitive, irritated, and acne-prone skin [3–5]. Although both leaves and bark are used medicinally, the bark is the official herbal drug recognized by the European Pharmacopoeia and is characterized by a higher content of hydrolysable tannins, particularly hamamelitannin, making it a more suitable substrate for investigating fermentation-induced transformations of tannin-rich extracts. Despite its long-standing therapeutic and cosmetic use, biotechnological approaches aimed at enhancing the biological properties of Hamamelidis cortex remain largely unexplored.

Among the available biotechnological strategies, fermentation has emerged as a promising biotechnological tool for the transformation of plant-derived materials [6,7]. Microbial fermentation enables enzymatic bioconversion of complex phytochemicals into lower-molecular-weight derivatives with improved stability, enhanced skin penetration, and potentially different biological activity [8]. The process may include hydrolysis of glycosidic bonds, depolymerization of tannins, liberation of bound phenolic compounds, and the formation of novel metabolites [9–11]. Consequently, fermentation is increasingly applied in cosmetic biotechnology to improve the efficacy of botanical extracts intended for anti-aging, photoprotective, regenerative, and skin-soothing formulations [12]. Among the different fermentation systems, kombucha fermentation, involving a symbiotic culture of bacteria and yeasts (SCOBY), represents a particularly attractive approach [13]. Recent studies have demonstrated that kombucha-mediated biotransformation can enhance the functional properties of plant extracts, particularly their antioxidant potential, making this process highly relevant for the development of cosmetic ingredients [14]. This approach has been successfully applied to green tea (*Camellia sinensis*), butterfly pea flowers (*Clitoria ternatea*), turmeric (*Curcuma longa*), and various other medicinal plants, where microbial metabolism promoted the conversion of complex phenolic constituents into lower-molecular-weight metabolites, resulting in enhanced antioxidant activity and other beneficial biological properties [15–18]. Interestingly, although spontaneous fermentation of *H. virginiana* leaf extracts has previously been shown to induce extensive biotransformation of hydrolysable tannins and flavonoids, leading to the formation of gallic acid and other low-molecular-weight phenolics [11], comparable studies on Hamamelidis cortex have not yet been reported. Owing to the substantial phytochemical differences between leaves and bark, particularly with respect to the abundance of hydrolysable tannins, the effects of kombucha fermentation on witch hazel bark cannot be directly extrapolated from leaf studies.

Considering the increasing interest in fermented cosmetic actives, the application of kombucha fermentation to Hamamelidis cortex may represent a valuable strategy for modulating its metabolite composition and optimizing its biofunctional properties. Monitoring fermentation-associated changes over time is particularly important, as microbial biotransformation is a dynamic process that may differentially affect the accumulation, degradation, and conversion of phytochemicals at distinct fermentation stages.

Therefore, the aim of this study was to evaluate the temporal effect of kombucha fermentation on the metabolite profile and antioxidant capacity of Hamamelidis cortex.

The analysis focused on changes occurring at defined fermentation intervals in order to identify time-dependent biotransformation patterns and assess their relevance for the development of enhanced plant-based cosmetic ingredients.

2. Materials and Methods

2.1. Plant Material, Extract Preparation and Fermentation Procedure

The plant material was purchased from a Polish supplier—Plantago (Poland)—in three separate batches. The individual batches were combined to ensure sample homogeneity and then ground into a fine powder using a laboratory mill (IKA M20, IKA-Werke GmbH & Co. KG, Staufen, Germany). Extraction was performed using accelerated solvent extraction (ASE) with a Dionex ASE 350 extractor (Thermo Fisher Scientific Inc., Sunnyvale, CA, USA). A methanol/water (8:2, *v/v*) solvent system was used for extraction, with the extraction temperature set to 65 °C and a static extraction time of 5 min. The resulting extracts were freeze-dried using a Christ Alpha 2–4 LDplus freeze-dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) and stored at +4 °C for further analysis. The kombucha starter culture (SCOBY) was purchased from a commercial supplier in Poland and stored at 4 °C until fermentation according to the supplier's recommendations. The commercial starter consisted of a cellulose SCOBY pellicle and the accompanying fermented kombucha liquid, both of which were used for inoculation. According to the supplier, the culture comprised a symbiotic consortium of acetic acid bacteria (*Acetobacter* spp. and *Gluconobacter* spp.) and yeasts (*Saccharomyces*, *Saccharomyces*, *Schizosaccharomyces*, *Zygosaccharomyces*, *Dekkera*, *Candida*, *Torulospora*, *Kloeckera*, *Pichia*, *Mycotorula*, and *Mycoderma*). The freeze-dried extract (yield 21.7%) was weighed into sterile Falcon tubes in amounts ranging from 50 mg to 300 mg. The extracts were then dissolved in 20 mL of deionized water, followed by the addition of 2 g of sucrose (10.0% *w/v*), 100 mg of SCOBY pellicle and 2 mL of fermented kombucha starter liquid. The samples prepared in this manner were covered with sterile gauze, and fermentation was carried out for 15 days at room temperature (approximately 25 °C) in the dark. Every other day (on days 1, 3, 5, 7, 9, 11, 13, and 15), the pH of the fermented extracts was measured using a Mettler Toledo FiveGo F2 pH meter (Mettler Toledo, Greifensee, Switzerland). Samples for analysis were collected every 3 days (on days 3, 6, 9, 12, and 15) in a volume of 1.5 mL, and 1.5 mL of methanol was added to them to stop the fermentation process. After each sampling, the liquid level was marked, and before the subsequent sampling, evaporative losses were replenished with distilled water to restore the original volume. The fermented extracts were filtered through 0.22 µm membrane filters. All analytical standards were purchased from Sigma-Aldrich (St. Louis, MO, USA), except for 1,6-di-O-galloyl-β-D-glucose, which was obtained from MedChemExpress (Monmouth Junction, NJ, USA).

2.2. Determination of Biologically Active Compounds

An ultra-high performance liquid chromatography (UHPLC) Infinity Series II with a DAD detector and high resolution mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) was used to identify the metabolites. The conditions were as follows: an RP18 reversed-phase a Kinetex C18 column (1.7 µm, 150 mm × 2.1 mm; Phenomenex, Torrance, CA, USA); the column temperature was set to 30 °C, and the flow rate was 0.2 mL/min. The mobile phase consisted of a mixture of water containing 0.1% formic acid (solvent A) and acetonitrile (solvent B). The compounds were separated using gradient elution according to the following program: 0–8 min from 98% to 90% A, (from 2% to 10% B), 8–15 min from 90% to 88% A, (from 10% to 12% B), 15–29 min from 88% to 85% A, (from 12% to 15% B), 29–40 min from 85% to 80% A, (from 15% to 20% B), and 40–50 min from 80% to 75% A, (from 20% to 25% B). Chromatograms were recorded in the wavelength range from 220 to

500 nm. The mass spectrometry conditions were as follows: electrospray ionization (ESI) was performed in the negative ion mode, the drying gas temperature was set to 325 °C with a flow rate of 8 L/min, the nebulizer pressure was 30 psi, the capillary voltage was 3500 V, the skimmer voltage was 65 V, and the fragmentor voltages were set to 200 and 280 V. Mass spectra were acquired over an m/z range of 100–1200.

2.3. Antioxidant Assays

To evaluate the free radical scavenging capacity of the fermented extracts, a test was conducted using the DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical [19]. For this purpose, samples of the fermented extracts and extract before fermentation were prepared at appropriate dilutions of 50 for ratios of 50 mg, 160 for a ratio of 100, 150 mg and 500 for ratios of 200, 250 and 300 mg. Next, 50 µL of the diluted extracts and extract before fermentation were dispensed into 96-well plates, after which 200 µL of 0.2 mM DPPH in methanol (Merck KGaA, Darmstadt, Germany) was added to each well and mixed on an orbital shaker. The control sample consisted of DPPH solution and methanol. The Trolox (Merck KGaA, Darmstadt, Germany) standard curve was prepared in a range of concentrations (0–300 µM). After incubation in the dark for 30 min at room temperature, absorbance was measured in a microplate reader (BioTek Epoch, Agilent Technologies, Santa Clara, CA, USA) at a wavelength of $\lambda = 517$ nm. The results are presented as the equivalent of mg of Trolox per gram of dry weight (DW) and a percentage of DPPH radical scavenging activity relative to the control sample (Equation (1)) [20]. DW refers to the dry weight of the initial freeze-dried Hamamelidis cortex extract used for fermentation.

$$\% \text{ DPPH scavenging} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100 \quad (1)$$

To evaluate the free radical scavenging capacity of the fermented extracts, a test was conducted using the ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) free radical [21]. First, a solution was prepared consisting of 7 mM ABTS (Merck KGaA, Darmstadt, Germany) with 2.4 mM potassium persulfate (Merck KGaA, Darmstadt, Germany) in a 1:1 volume ratio. The mixture was left at room temperature for 14 h. After incubation, the solution was diluted with methanol. Samples of the fermented extracts and extract before fermentation were prepared in the same dilutions as those used for the DPPH radical assay. Next, 20 µL of the diluted extracts and extract before fermentation were dispensed into 96-well plates, after which 180 µL of the prepared ABTS solution were added to each well and mixed on an orbital shaker. The control sample consisted of ABTS solution and methanol. The Trolox (Merck KGaA, Darmstadt, Germany) standard curve was prepared in a range of concentrations (0–300 µM). After incubation in the dark for 6 min at room temperature, absorbance was measured in a microplate reader (BioTek Epoch, Agilent Technologies, Santa Clara, CA, USA) at a wavelength of $\lambda = 734$ nm. The results are presented as the equivalent of mg of Trolox per gram of dry weight (DW) and a percentage of ABTS radical scavenging activity relative to the control sample (Equation (2))

$$\% \text{ ABTS scavenging} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100 \quad (2)$$

2.4. Ion Chelating Activity

Fe^{2+} chelating activity was measured according to the ferrozine-based method with minor modifications. Briefly, 0.5 mL of sample solution at different concentrations was mixed with 0.05 mL of FeCl_2 solution (2 mM). The reaction mixture was incubated for 5 min at room temperature, after which 0.1 mL of ferrozine solution (5 mM) was added. The final volume was adjusted with distilled water, and the mixture was incubated for

an additional 10 min at room temperature. The absorbance was recorded at 562 nm. The ability to chelate Fe^{2+} ions was calculated as the percentage inhibition of the Fe^{2+} –ferrozine complex formation.

Cu^{2+} chelating activity was determined using the pyrocatechol violet (PCV) assay with slight modifications. In brief, 1.0 mL of sample solution was mixed with 0.1 mL of CuSO_4 solution (2 mM) and incubated for 5 min at room temperature. Subsequently, 0.1 mL of pyrocatechol violet solution (0.1 mM) was added, and the reaction mixture was adjusted with an ammonium acetate buffer (pH 6.0) to the final volume. After incubation for 10 min at room temperature, the absorbance was measured at 632 nm. Metal-chelating activity was expressed as the percentage inhibition of the Cu^{2+} –PCV complex formation.

2.5. Statistical Analysis

All experiments were performed in triplicate ($n = 3$), and the results are presented as mean $\pm$ standard deviation (SD). Statistical differences between groups were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons. Pearson correlation coefficients were calculated to assess the relationships between the contents of selected phytochemicals and the antioxidant and metal-chelating activities. Differences were considered statistically significant at $p < 0.05$.

3. Results and Discussion

3.1. UHPLC-DAD-MS Characterization of Phenolic Constituents

Consistent with our previous study and published literature [4], the methanolic extract of Hamamelidis cortex is rich in phenolic compounds, predominantly hydrolysable tannins, with hamamelitannin being the major constituent [22]. However, the composition of the fermentation medium may substantially influence the solubility, stability, and availability of plant metabolites. Therefore, to evaluate the effect of the kombucha-containing medium on the phenolic profile of the extract, a qualitative UHPLC–ESI–MS analysis was performed. The analysis compared solutions prepared by dissolving the dried methanol/water extract in water (sample A) and in water supplemented with sugar and a kombucha culture (sample B). Detailed MS data are presented in Table 1, while representative BPC chromatograms are shown in Figure 1. Representative UV and HRMS spectra are provided in the Supplementary Materials (Figure S1).

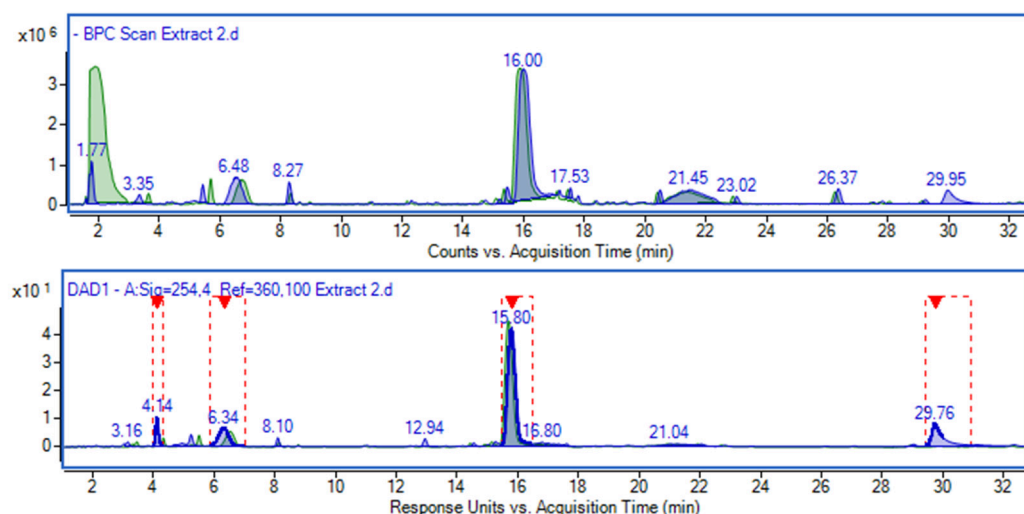


Figure 1. Overlaid base peak chromatograms (BPC, (**upper panel**)) of the extract dissolved in water (blue) and in kombucha fermentation medium (green), together with the DAD chromatogram recorded at 254 nm (**lower panel**). Red dashed boxes indicate gallic acid (RT $\approx$ 4.1 min), galloyl hexose (RT $\approx$ 6.3 min), hamamelitannin (RT $\approx$ 15.8 min), and ellagic acid (RT $\approx$ 29.8 min).

Table 1. Qualitative profile of phenolic compounds detected in solutions prepared by dissolving the dry extract in water (A) and in fermentation medium containing kombucha culture and sugar (B). The symbols (+) and (−) indicate the presence and absence of a given compound.

RT (min.)	Mass Data (<i>m/z</i> [M−H] [−])	Formula	Mass Error (ppm)	Component	A	B
3.65	331.06744	C ₁₃ H ₁₆ O ₁₀	1.11	Galloyl hexose	+	+
4.52	169.01504	C ₇ H ₆ O ₅	4.66	Gallic acid *	+	+
5.70	343.06622	C ₁₄ H ₁₆ O ₁₀	−2.47	Galloylquinic acid	+	+
6.48	331.06731	C ₁₃ H ₁₆ O ₁₀	0.72	Galloyl hexose	+	+
8.31	483.07851	C ₂₀ H ₂₀ O ₁₄	0.99	Digalloyl hexose	+	+
8.77	153.01928	C ₇ H ₆ O ₄	−0.34	Protocatechuic acid *	+	+
14.63	343.06642	C ₁₄ H ₁₆ O ₁₀	−1.89	Galloylquinic acid	+	+
14.65	495.07655	C ₂₁ H ₂₀ O ₁₄	−2.98	Di-O-galloylquinic acid	+	+
15.10	289.07238	C ₁₅ H ₁₄ O ₆	2.13	Catechin *	+	+
15.35	635.08890	C ₂₇ H ₂₄ O ₁₈	−0.14	Tri-O-galloylhexose	+	+
15.45	343.06657	C ₁₄ H ₁₆ O ₁₀	−1.45	Galloylquinic acid	+	+
16.00	483.07909	C ₂₀ H ₂₀ O ₁₄	2.19	Hamamelitannin *	+	+
21.26	635.08887	C ₂₇ H ₂₄ O ₁₈	−0.18	Tri-O-galloylhexose	+	+
22.90	467.08263	C ₂₀ H ₂₀ O ₁₃	−1.03	Ginnalin A	+	+
29.76	300.99853	C ₁₄ H ₆ O ₈	−1.53	Ellagic acid *	+	−
31.30	787.09798	C ₃₄ H ₂₈ O ₂₂	−2.49	Tetra-O-galloylhexose	+	+
39.17	939.109459	C ₄₁ H ₃₂ O ₂₆	−1.40	Penta-O-galloylhexose	+	+

Authentic analytical standards were used for compounds marked with an asterisk (*).

The chromatographic profiles of both solutions were generally comparable, indicating that the majority of phytochemicals remained detectable regardless of the medium composition. Hamamelitannin, galloyl hexose, and gallic acid were identified as the predominant constituents in both samples. Nevertheless, notable differences were observed for selected metabolites. In particular, solution B exhibited a lower abundance of gallic acid, whereas ellagic acid was not detected. These differences are likely associated with matrix-dependent effects resulting from the presence of sugars, organic acids, and microbial metabolites in the kombucha-containing medium, as such components may influence the solubility and stability of phenolic compounds. Ellagic acid is known to exhibit limited solubility in aqueous systems and a tendency to form intermolecular aggregates, which may decrease its availability in solution and consequently its detectability. In addition, interactions with medium constituents may further reduce the concentration of freely dissolved ellagic acid [23]. The reduced abundance of gallic acid may similarly result from interactions with carbohydrates and other matrix constituents present in the kombucha-containing medium, which are known to affect the availability of phenolic compounds in complex food systems [24].

3.2. Effect of Extract Concentration on Physicochemical and Compositional Changes During Fermentation

In the next stage of the study, we evaluated whether increasing the amount of extract added to the fermentation medium influenced the physicochemical and compositional changes occurring during fermentation. A control fermentation without the plant extract was also conducted to evaluate the contribution of the Kombucha starter itself to the observed metabolite profile. Since the Kombucha starter fluid originated from a previously fermented tea infusion, it contained trace amounts of tea-derived phenolic compounds, including phenolic acids (e.g., gallic, chlorogenic, and galloylquinic acids), catechins, and flavonoid glycosides of quercetin and kaempferol [25]. During fermentation, only minor changes in the metabolite profile of the control were observed. Therefore, the starter

itself was considered to make only a minor contribution to the overall phytochemical profile. Representative chromatograms of the control fermentation are provided in the Supplementary Materials (Figures S2 and S3).

To evaluate the effect of the extract-to-medium ratio, 50–300 mg of extract was added to the fermentation medium. This range was selected to ensure complete dissolution of the extract at the upper limit while avoiding excessive dilution at the lower limit, which could have resulted in insufficient signal intensity of low-abundance metabolites for reliable monitoring during fermentation. Hamamelitannin, galloyl hexoses, and gallic acid were selected as representative markers to monitor fermentation-induced changes in the phytochemical profile. The results are presented in Figure 2 as the percentage increase in analyte content relative to the initial extract (day 0), which was normalized to 100%.

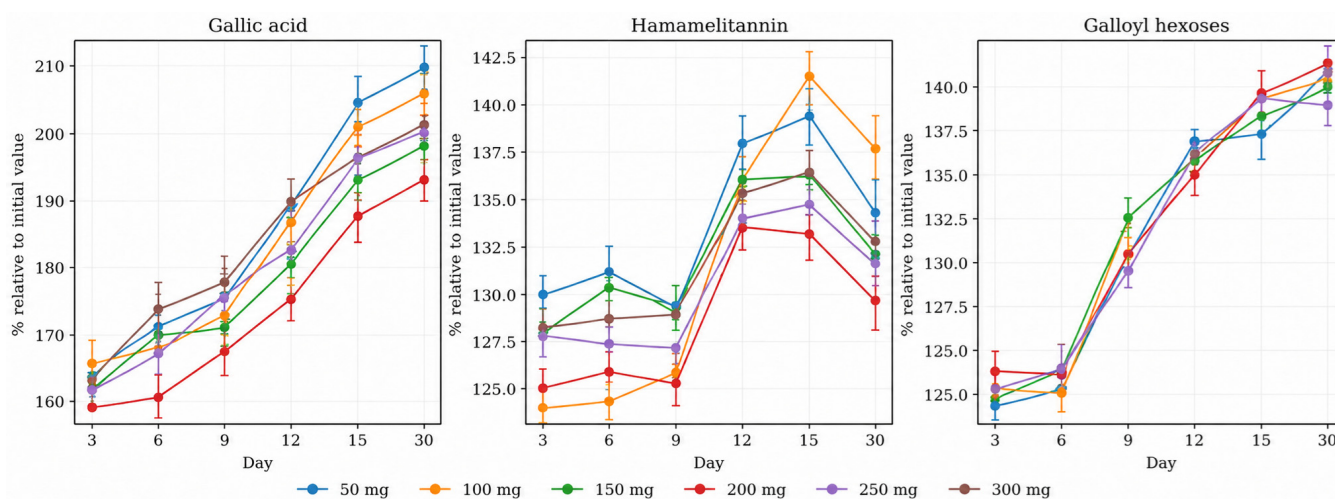


Figure 2. Changes in the content of gallic acid, hamamelitannin, and galloyl hexoses during kombucha fermentation at different extract concentrations. Results are expressed as the percentage relative to the initial value (day 0 = 100%).

Regardless of the extract-to-medium ratio, kombucha fermentation increased the content of all analyzed compounds compared with the initial extract. The most pronounced changes were observed for hamamelitannin, whose levels increased by approximately 24–30% relative to baseline values. Although the upward trend was maintained throughout fermentation, the rate of increase gradually slowed over time. In samples containing lower extract concentrations (50 and 100 mg), hamamelitannin levels continued to increase until day 15, whereas in samples with higher extract concentrations, a plateau was reached around day 12. After day 15, a decrease in hamamelitannin content was observed in all samples. Gallic acid content also increased markedly during fermentation. The increase was particularly evident after day 9, and the highest values were generally observed between days 15 and 30. Depending on the extract concentration, gallic acid levels reached approximately 198–211% of the initial value by day 30. A similar trend was observed for galloyl glucoside, although the changes were less pronounced. Its content gradually increased throughout fermentation, reaching approximately 138–142% of the initial value by day 30. The most substantial increase occurred between days 6 and 12, after which the levels stabilized, suggesting that the biotransformation of galloylated compounds occurred mainly during the early and intermediate stages of fermentation.

Changes in pH throughout the process were also monitored. A significant decrease in pH was observed already on the first day of fermentation compared with the initial value of 4.4. Thereafter, pH gradually declined until approximately day 11, reaching a value of

around 3.0, and remained relatively stable for the remainder of the fermentation period. Extract concentration had no noticeable effect on the observed pH changes.

The observed decrease in pH during fermentation is consistent with the typical metabolic activity of kombucha cultures, where acetic acid bacteria and yeasts convert sugars into organic acids, primarily acetic, gluconic, and glucuronic acids [13]. The rapid drop in pH within the first days of fermentation is associated with intense metabolic activity, followed by the establishment of a dynamic equilibrium between acid formation and microbial adaptation. Similar pH trends have been widely reported in kombucha fermentation systems, where an initial sharp acidification phase is followed by a slower decline and eventual stabilization of the system [26,27].

To precisely monitor fermentation-induced changes in the phytochemical profile, the process conducted with 100 mg of extract was selected for detailed analysis, as this concentration exhibited the most favorable hamamelitannin accumulation. Quantitative LC–MS analysis revealed that fermentation significantly affected the content of most identified phenolic constituents (Table 2).

Table 2. Quantitative analysis of the compounds identified in the samples ($\mu\text{g/mL}$). Values are expressed as mean $\pm$ SD ($n = 3$).

tR (min)	m/z [M–H] [–]	Component	0 Day	3 Day	6 Day	9 Day	12 Day	15 Day
3.65; 6.71	331.06739	Galloylhexoses *	71.0 $\pm$ 4.3 ^a	87.9 $\pm$ 5.1 ^b	86.5 $\pm$ 5.4 ^b	94.1 $\pm$ 5.2 ^c	96.5 $\pm$ 5.6 ^c	99.0 $\pm$ 6.0 ^c
4.52	169.01498	Gallic acid	14.0 $\pm$ 0.8 ^a	22.8 $\pm$ 1.3 ^b	23.4 $\pm$ 1.5 ^b	24.0 $\pm$ 1.4 ^b	25.6 $\pm$ 1.5 ^c	27.5 $\pm$ 1.7 ^c
5.70; 14.63; 15.45	343.06634	Galloylquinic acids *	20.5 $\pm$ 1.2 ^a	25.9 $\pm$ 1.5 ^b	24.9 $\pm$ 1.6 ^b	26.4 $\pm$ 1.5 ^b	28.1 $\pm$ 1.7 ^c	28.8 $\pm$ 1.8 ^c
8.77	153.01921	Protocatechuic acid	5.4 $\pm$ 0.3 ^a	7.4 $\pm$ 0.4 ^b	8.0 $\pm$ 0.5 ^b	8.0 $\pm$ 0.5 ^b	9.4 $\pm$ 0.6 ^c	9.7 $\pm$ 0.6 ^c
8.31	483.07857	Digalloyl hexoses *	5.5 $\pm$ 0.3 ^a	6.5 $\pm$ 0.4 ^b	6.7 $\pm$ 0.4 ^b	7.4 $\pm$ 0.5 ^c	7.4 $\pm$ 0.5 ^c	8.2 $\pm$ 0.5 ^c
14.65	495.07649	di-O- galloylquinic acid *	5.5 $\pm$ 0.3 ^a	7.0 $\pm$ 0.4 ^b	7.9 $\pm$ 0.5 ^c	8.2 $\pm$ 0.5 ^c	8.2 $\pm$ 0.5 ^c	7.9 $\pm$ 0.5 ^c
15.10	289.07221	Catechin	18.6 $\pm$ 1.1 ^a	26.6 $\pm$ 1.6 ^b	28.0 $\pm$ 1.7 ^b	28.7 $\pm$ 1.8 ^b	30.2 $\pm$ 1.9 ^c	31.6 $\pm$ 1.9 ^c
15.35; 21.26	635.08891	Tri-O- galloylhexoses *	52.8 $\pm$ 3.0 ^a	58.8 $\pm$ 3.4 ^b	63.9 $\pm$ 3.8 ^b	69.9 $\pm$ 4.2 ^c	73.0 $\pm$ 4.3 ^c	82.1 $\pm$ 4.9 ^d
15.85	483.07905	Hamamelitannin	456.4 $\pm$ 25.1 ^a	617.3 $\pm$ 35.2 ^b	590.1 $\pm$ 34.4 ^b	597.6 $\pm$ 33.8 ^b	646.5 $\pm$ 37.1 ^c	675.7 $\pm$ 39.5 ^c
22.90	467.08251	Ginnalin A *	2.6 $\pm$ 0.1 ^a	2.7 $\pm$ 0.2 ^a	2.8 $\pm$ 0.2 ^a	3.5 $\pm$ 0.3 ^b	3.6 $\pm$ 0.2 ^b	3.9 $\pm$ 0.3 ^b

Different superscript letters within a row indicate statistically significant differences between groups ($p < 0.05$), as determined by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. * Ginnalin A was quantified using the calibration curve of hamamelitannin. Galloylquinic acids and galloyl hexoses were quantified using the calibration curve of gallic acid. Tri-O-galloylhexoses were quantified using the calibration curve of 1,3,6-tri-O-galloyl- β -D-glucose, whereas di-O-galloylquinic acid and digalloyl hexose were quantified using the calibration curve of 1,6-di-O-galloyl- β -D-glucose.

Among the analyzed metabolites, hamamelitannin was the dominant compound throughout the experiment. Its concentration increased significantly from 456.4 $\mu\text{g/mL}$ in the non-fermented extract to 617.3 $\mu\text{g/mL}$ after 3 days of fermentation and reached the highest level of 675.7 $\mu\text{g/mL}$ after 15 days, corresponding to an overall increase of approximately 48%. Similarly, galloyl hexoses increased from 71.0 to 99.0 $\mu\text{g/mL}$, while tri-O-galloylhexoses showed a pronounced increase from 52.8 to 82.1 $\mu\text{g/mL}$. Gallic acid and galloylquinic acid also accumulated during fermentation, increasing by approximately 96% and 40%, respectively. Significant increases were additionally observed for protocatechuic acid, catechin, digalloyl hexoses, and Ginnalin A. Moreover, a decrease in the abundance of high-molecular-weight tannins, including tetra-O-galloylhexose and penta-O-galloylhexose, was observed during fermentation. Due to the lack of authentic reference standards, quantitative analysis of these compounds could not be performed. Nevertheless,

changes in their abundance were monitored using extracted ion chromatograms (EICs) (Figure 3), which indicated a gradual decline in their signal intensity over the course of fermentation. This decrease may suggest the progressive biotransformation or hydrolysis of highly galloylated tannins into lower-molecular-weight phenolics during fermentation. Representative extracted ion chromatograms obtained after 0, 3, and 12 days of fermentation are presented in Figure 3.

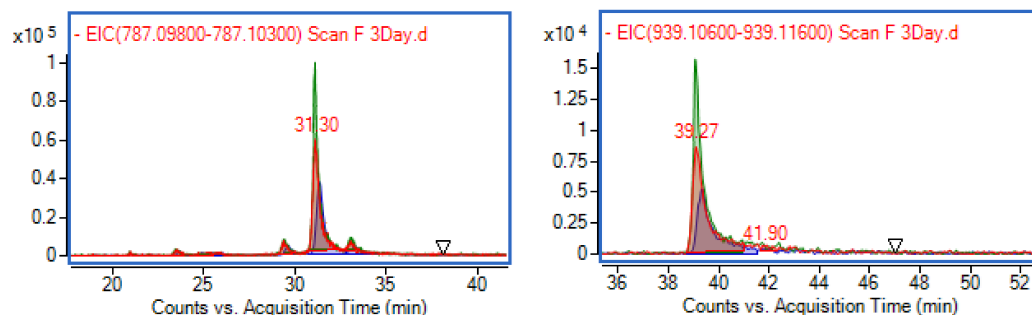


Figure 3. Extracted ion chromatograms (EICs) of Hamamelidis cortex extract in the mass range for tetra-O-galloylhexose (787.09800–787.10300), and penta-O-galloylhexose (939.10600–939.11600); green line—the extract before fermentation (day 0), red line—the extract after 3 days of fermentation, blue line—the extract after 12 days of fermentation.

A similar phenomenon has been observed in studies on hydrolysable tannins, where enzymatic and chemical hydrolysis leads to the cleavage of ester bonds and the stepwise release of gallic acid and other small phenolic units [28]. In fermentation systems, tannase and related microbial enzymes are known to catalyze the degradation of gallotannins and ellagitannins, resulting in a progressive reduction in polymerization degree and an increase in simpler phenolic acids in the medium. Such transformations have been widely reported in plant fermentation models and tannin-rich matrices, where complex tannins are converted into low-molecular-weight phenolics over time [29]. For example, during the fermentation of tea (*Camellia sinensis*), galloylated phenolics are progressively hydrolysed, leading to increased levels of free gallic acid [30]. Comparable transformations have also been observed during the fermentation of tannin-rich persimmon (*Diospyros kaki*) products, where complex hydrolysable tannins are converted into simpler phenolic compounds [31].

To date, no studies have described the fermentation of Hamamelidis cortex. Nevertheless, Duckstein et al. reported comparable changes during the spontaneous fermentation of aqueous *H. virginiana* leaf extracts, where higher gallotannins were progressively hydrolysed into lower galloylated derivatives and gallic acid, which became the predominant phenolic constituent after one month of fermentation. These transformations were attributed to the activity of the natural microbial flora and tannase-mediated hydrolysis of gallotannins [11].

3.3. Free Radical Scavenging Activity

Two complementary assays were used to investigate changes in the antioxidant capacity of the extracts during fermentation. The ABTS assay, which measures the ability of compounds to quench the ABTS radical cation, and the DPPH assay, which evaluates the capacity to scavenge DPPH free radicals, were applied. The results are presented in Figures 4a and 4b, respectively.

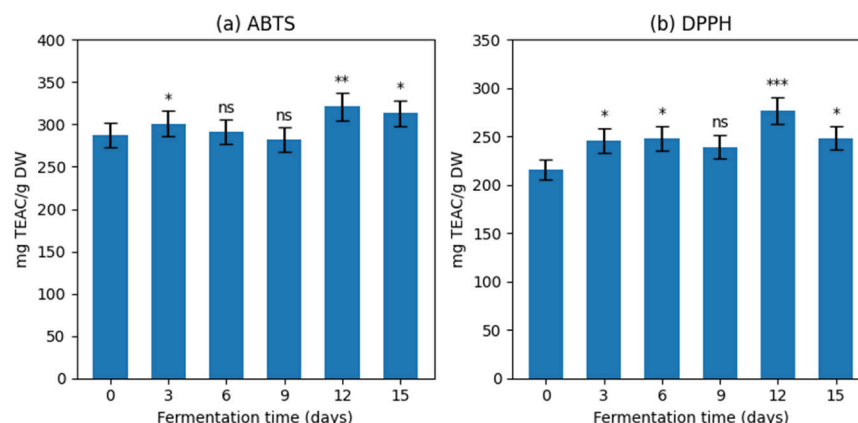


Figure 4. Changes in antioxidant activity determined using the ABTS and DPPH radical scavenging assays during fermentation. Results are expressed as mg TEAC/g DW. Values represent mean $\pm$ SD ($n = 3$). Statistical significance was determined relative to day 0 (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns—not significant).

Antioxidant activity determined by the ABTS assay increased from 287.4 mg TEAC/g DW in the non-fermented extract to a maximum value of 321.2 mg TEAC/g DW after 12 days of fermentation (Figure 4a) and then remained stable. A similar trend was observed in the DPPH assay, where antioxidant activity increased from 215.9 to 276.4 mg TEAC/g DW after 12 days, representing an improvement of approximately 28%. However, a slight decrease was observed after 12 days. Overall, the most significant differences relative to the non-fermented extract were observed after 12 days of the fermentation process.

3.4. Effect of Fermentation on Fe^{2+} and Cu^{2+} Chelating Activity

In the next step, the ability of the extracts to bind Cu^{2+} and Fe^{2+} ions was evaluated as it reflects the ability of compounds to inhibit metal-catalyzed oxidative processes. The results are shown in Figures 5a and 5b, respectively.

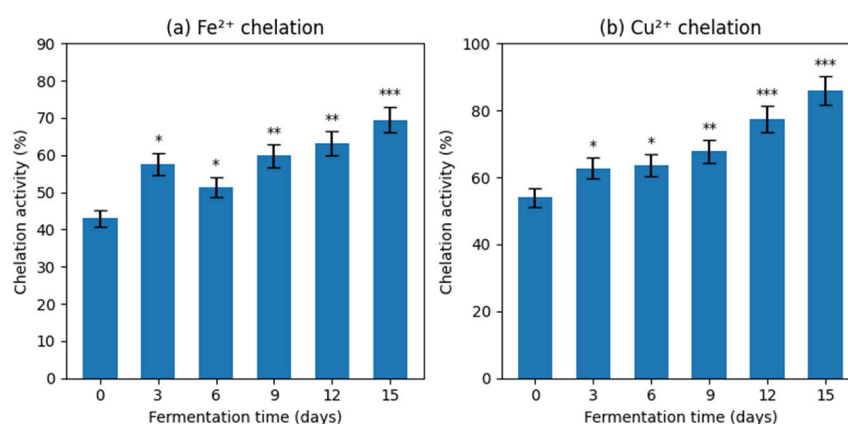


Figure 5. Changes in chelating activity toward (a) Fe^{2+} and (b) Cu^{2+} ions during fermentation. Results are expressed as mean $\pm$ SD ($n = 3$). Statistical significance was determined relative to the non-fermented extract (day 0) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Fermentation enhanced the metal-chelating properties of the extracts (Figure 5). Fe^{2+} chelating activity increased from 43.0% in the non-fermented sample to 69.5% after 15 days of fermentation. Likewise, Cu^{2+} chelation increased from 54.0% to 85.9%, indicating a stronger affinity toward copper ions than ferrous ions. The highest chelating activity for both metals was observed after 15 days of fermentation.

3.5. Correlation Between Metabolite Content and Biological Assay

The observed changes in metabolite composition were accompanied by enhanced biological activity, as demonstrated by correlation analysis (Figure 6). The correlation analysis revealed that metal-chelating activity showed the strongest positive associations with galloylated phenolic compounds. In particular, Fe^{2+} chelating activity was most closely associated with galloyl-hexoses, galloylquinic acid, digalloyl hexoses, and hamamelitannin, whereas Cu^{2+} chelation was primarily related to tri-O-galloylhexoses, digalloyl hexoses, protocatechuic acid, and ginnalin A. In contrast, the ABTS and DPPH antioxidant assays also exhibited positive correlations with these phytochemicals, although these relationships were generally weaker than those observed for metal-chelating activity.

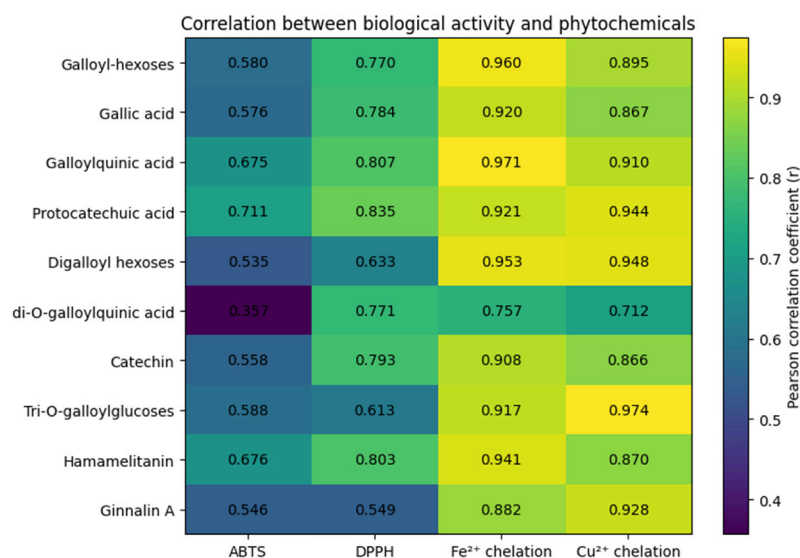


Figure 6. Heatmap presenting Pearson correlation coefficients between antioxidant activity (ABTS and DPPH assays), metal-chelating activity (Fe^{2+} and Cu^{2+} chelation), and the content of selected phytochemicals identified in fermented extracts.

As shown by chromatographic analysis, fermentation induced a marked shift in the gallotannin profile of Hamamelidis cortex extract, characterized by a decrease in highly galloylated derivatives accompanied by a concomitant increase in hamamelitannin, galloylhexose, and gallic acid. The observed increase in metal-chelating activity despite a decrease in highly galloylated tannins suggests that partial enzymatic depolymerization enhances the accessibility and mobility of phenolic ligands. The release of gallic acid, hamamelitannin, and mono-galloyl derivatives increases the effective number of freely accessible catechol- and galloyl-type binding sites, which likely outweighs the loss of multidentate but sterically hindered high-molecular-weight tannins [32,33].

The association between gallotannin transformation and enhanced antioxidant capacity has also been reported during the fermentation of other tannin-rich plant materials. For example, fermentation of *C. sinensis* with *E. cristatum* resulted in the hydrolysis of galloylated catechins, a marked increase in gallic acid content, and a concomitant enhancement of antioxidant activity [34]. Similar observations have been reported for kombucha fermentation of tea, where the release of gallic acid from complex phenolics was accompanied by increased radical-scavenging activity [35]. Likewise, fermentation of persimmon promoted the formation of low-molecular-weight phenolics together with a significant increase in antioxidant capacity [31].

The compositional changes observed during fermentation may also have other functional implications. High-molecular-weight hydrolysable tannins are considered the principal contributors to the astringency of *H. virginiana* bark owing to their ability to form

stable complexes with proteins. Therefore, the observed decrease in highly galloylated tannins may reduce the protein-binding capacity of the extract and consequently attenuate its astringent properties [36]. Furthermore, tannin-induced protein precipitation promotes the formation of a protective layer on the skin, reduces vascular permeability, and thereby contributes to the local anti-inflammatory effects [37]. Consequently, fermentation may modify this particular mechanism of action. However, this does not necessarily imply an overall reduction in anti-inflammatory potential, as lower-molecular-weight phenolics have also been reported to exert anti-inflammatory effects through distinct molecular mechanisms, including modulation of inflammatory signaling pathways such as NF- κ B, MAPK, and Nrf2 [5,38,39]. Therefore, further studies comparing the protein-binding capacity and anti-inflammatory activity of fermented and non-fermented extracts are needed to fully elucidate the biological consequences of these compositional changes.

4. Conclusions

Kombucha fermentation induced time-dependent changes in the phytochemical composition of *Hamamelidis cortex* extract. The process increased the abundance of low-molecular-weight galloylated compounds, including hamamelitannin, gallic acid, galloylhexoses, and tri-O-galloylhexoses, while reducing highly galloylated tannins, consistent with the enzymatic transformation of complex gallotannins during fermentation.

These compositional changes were accompanied by improved antioxidant properties, reflected by higher ABTS and DPPH radical scavenging activity and enhanced Fe²⁺ and Cu²⁺ chelation. The results indicate that kombucha fermentation is an effective approach for tailoring the phenolic profile of *Hamamelidis cortex* extract and increasing its functional potential. The fermented extract represents a promising candidate for further studies on its potential cosmetic application. However, additional studies using relevant biological models and cosmetic formulations are required to confirm its efficacy.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/app16157657/s1>: Figure S1: Representative HRMS and UV-DAD spectra of compounds identified in *Hamamelidis cortex* extract: (a) galloylquinic acid, (b) galloylhexose, (c) tri-O-galloylhexose, and (d) hamamelitannin. Figure S2. Comparison of the kombucha starter solution at the beginning of fermentation (day 0, red) and after 15 days of fermentation (green): (a) base peak chromatograms (BPC) and (b) DAD chromatograms. Figure S3. Overlaid BPC chromatograms of the extract without kombucha at the beginning of the experiment (blue line) and after 15 days of incubation (red line).

Author Contributions: Conceptualization, W.P. and I.S.; methodology, W.P. and I.S.; validation, W.P., J.S. and I.S.; formal analysis, W.P. and J.S.; investigation, W.P., J.S., M.W. and I.S.; data curation, W.P. and I.S.; writing—original draft preparation, W.P., M.W. and I.S.; writing—review and editing, W.P., I.S. and M.W.; visualization, W.P. and J.S.; supervision, W.P., M.W. and I.S. All authors have read and agreed to the published version of the manuscript.

Funding: This study was funded by the Medical University of Lublin (DS 51 and DS 52).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding authors.

Conflicts of Interest: The authors declare no conflicts of interest.

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