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Effects of Infusion and Storage on Antioxidant Activity and Total Phenolic Content of Black Tea

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Abstract: This study determined antioxidant activity in terms of the 1,1-Diphenyl-2-picrylhydrazyl (DPPH) scavenging ability and total phenolic content of black tea under different infusion and storage conditions. High performance liquid chromatography analysis identified caffeine, (−)-epigallocatechin, (−)-epicatechin-3-gallate, (−)-epigallocatechin-3-gallate and (−)-gallocatechin-3-gallate in the tea sample. The water–tea leaves weight ratio did not affect the DPPH scavenging ability. However, infusion temperature affected the DPPH scavenging activity and the total phenolic content. In the present study, the 50% inhibitory concentrations (IC_{50}) for DPPH of black tea infused at 60 to 100 °C ranged from 100.0 ± 13.7 to 28.4 ± 4.8 μg/mL. The total phenolic content of black tea steeped at 60 to 100 °C ranged from 50.4 ± 5.2 to 178.6 ± 16.4 mg gallic acid equivalent/g dry leaf. Black tea exhibited increased antioxidant activity when the infusion temperature was increased. Regarding short-term storage, the DPPH scavenging ability and total phenolic content of black tea did not significantly change within 15 days. This result was consistent for storage temperatures of 4, 9, and 25 °C.

Keywords: antioxidant; black tea; DPPH; storage; time; temperature; total phenolic content

1. Introduction

Tea, a popular beverage, is produced from the leaves of the plant *Camellia sinensis* (L.) O. Kuntze. Tea has been studied for its potential beneficial health effects and has been used for medical purposes in recent decades [1]. Tea may enhance physical health and physiological functions as well as reduce the risk of cardiovascular diseases and cancers [2]. Of the total tea production globally, black tea and green tea account for 78% and 20%, respectively. Black tea is mainly consumed in Western countries and in some Asian countries [3–5]. The biological activity of black tea is related to its chemical profile [3–5]. The typical compounds of black tea include catechins, theaflavins, amino acids and alkaloids [4]. The major polyphenolic components are catechins, theaflavins and thearubigins. Black tea extract products with an ultrahigh content of theaflavins have been marketed as dietary supplements [4].

The antioxidant capacity of black tea is associated with brewing conditions, including the infusion temperature, the amount of stirring and straining, and the particle size of the tea leaves [6,7]. Methods of infusing black tea vary worldwide. For example, infusion of black tea in China involves brewing black tea leaves with hot water at 100 °C for 20–40 s and repeating this infusion several times. In Ireland,

Canada, and the United Kingdom, black tea is mostly prepared using boiling water and consumed with milk and often sugar. A black tea infusion made with a cold steeping temperature (4 or 25 °C) for 2 h has become popular in Taiwan [8,9]. Determining the optimal method of infusing black tea is crucial to achieving high antioxidant activity. A previous paper reported that the total phenolic content of black tea leaves infused at 4 °C for 24 h was lower than those infused at 90 °C for 6 min, and this result correlated with the 1,1-Diphenyl-2-picrylhydrazyl (DPPH) scavenging activity [10]. Black tea produced using a short-duration, hot water extraction method exhibited the highest antioxidant activity. This result concurred with reports by Khokhar and Magnusdottir [6], Venditti et al. [8], and Hajiaghaalipour et al. [9]. However, few reports have discussed the effects of the water–tea leaves ratio on the antioxidant activity of black tea.

A low storage temperature of green tea could considerably extend catechin half-life, and catechins did not undergo significant degradation during a 6-month storage period at 4 °C [11,12]. The result of optimum storage of green tea at 4 °C concurred with another report in which no significant degradation of catechin or caffeine was observed during an 8-week storage period [13]. Moreover, no differences in concentrations were observed for the predominant catechins of green tea over the first 6 weeks of storage at 3 °C in different packages [14]. However, at 25 °C, the (−)-epi-catechin gallate and gallocatechin gallate of green tea decreased rapidly to zero or close to zero after 30 days; (−)-epigallocatechin decreased by 43% after 60 days of storage, and (−)-epigallocatechin gallate decreased by 68% after 90 days [11]. Both the DPPH scavenging ability and total phenolic content of green tea decreased considerably at 25 and 50 °C after a 3-month storage period, and they declined further at 50 °C [15].

Jiménez-Zamora et al. [15] also reported a decrease in the DPPH scavenging activity of black tea after 3 months, but the total phenolic content remained almost constant at 25 °C, even after 6 months of storage. Besides this example, few studies have reported the antioxidant properties of black tea following short-term storage. To understand the optimal infusion conditions and storage time of black tea before use, this study investigated the effects of the water–tea leaves weight ratio, infusion temperature, short-term storage, and storage temperature on the antioxidant activity of black tea infusion.

2. Materials and Methods

2.1. Materials

Pali (referring to origin) black tea leaves were obtained from tea trees planted on the campus of the National United University, Miaoli, Taiwan. DPPH, caffeine, epigallocatechin, epicatechin gallate, epigallocatechin gallate, and gallocatechin gallate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Folin–Denis reagent and gallic acid were obtained from Fluka (Neu-Ulm, Germany). Sodium carbonate was obtained from Riedel-de Haën Chemical Co. (Seelze, Germany). Methanol was obtained from Macron Chemical Co. (Washington, DC, USA).

2.2. Sample Preparation

2.2.1. Water–Tea Leaves Weight Ratio

Black tea leaves were added to boiling water (100 °C) with different water–tea leaves weight ratios (25:1, 50:1, 100:1, 125:1, 250:1, and 500:1). After steeping for 1 min, the black tea infusions were filtered through 0.47-μm filter paper and cooled to room temperature rapidly on ice.

2.2.2. Infusion Temperature

A total of 0.4 g of black tea leaves was mixed with 40 mL of water at varying temperatures (60, 70, 80, 90, and 100 °C) for 1 min. Then, the black tea infusions were filtered through 0.47-μm filter paper and cooled to room temperature rapidly on ice. After that, the black tea infusion was diluted to different concentrations as test samples.

2.2.3. Storage Time and Temperature

A total of 1.2 g of black tea leaves was added to 120 mL of boiling water and infused for 1 min. Next, the black tea infusions were filtered through 0.47-μm filter paper and cooled to room temperature rapidly on ice, and then placed in a dark incubator at different temperatures (4, 9, and 25 °C) for 15 days. Each sample's DPPH scavenging activity and total phenolic content was calculated every 3 days.

2.3. Determination of Total Phenolic Content

The total phenolic content of the tea sample was determined using the Folin–Denis method devised in previous reports [16–18]. Every black tea sample was mixed with 0.8 mL of Folin–Denis reagent for 3 min. Then, 0.8 mL of 10% sodium carbonate and 1.6 mL of water were added to induce a reaction. The mixture was shaken and kept in the dark at room temperature for 1 h. The absorbance was measured at 700 nm. Gallic acid was used as a reference standard, and the results were expressed as milligrams of gallic acid equivalent (GAE) per gram of black tea leaf. The measured calibration curve of gallic acid was $y = 0.0171\ x + 0.0999$ ($R^2 = 0.9978$) in the range 0–65 μg/mL, where x and y were concentration and absorbance, respectively.

2.4. High Performance Liquid Chromatography (HPLC) Analysis

The Waters 600 HPLC system (Waters, Milford, MA, USA) with Empower 2 Pro Software (Waters, Milford, MA, USA) had an integrated controller, a quaternary pump, a column temperature controller, an autoinjector, and a photodiode array detector. The mobile phase consisted of a mixture of A (0.085% phosphoric acid), B (acetonitrile), and C (water) [18]. The percentage of the mobile phase was as follows: 0–30 min with the ratio of 98–80% A and 2–20% B; 30–40 min with the ratio of 80–65% A and 20–35% B; 40–55 min with the ratio of 65–0% A, 35–75% B, and 0–25% C; 55–60 min with the ratio of 75–0% B, 25–100% C; and 60–65 min with the ratio of 0–98% A, 0–2% B, and 100–0% C. The flow rate was 1.0 mL/min, the column temperature was controlled at 35 °C, the post-run time of chromatography was 15 min, the analytical column was a Cosmosil 5C18-MS-II column (5 μm, 4.6 × 250 mm, Nacalai tesque), while a Lichrospher RP-18 endcapped column (5 μm, 4.0 × 10 mm, Merck) was used as a guard column, and absorbance was measured at 270 nm.

Standard solutions of caffeine, (−)-epigallocatechin, (−)-epicatechin-3-gallate, (−)-epigallocatechin-3-gallate and (−)-gallocatechin-3-gallate were prepared at 1.0 mg/mL in 70% methanol, and a series of diluted standards were prepared for HPLC calibration curves (caffeine: 0.05, 0.10, 0.15, 0.20, 0.25, and 0.30 mg/mL; (−)-epigallocatechin: 2.5, 5.0, 10.0, 15.0, 20.0, and 25.0 μg/mL; (−)-epicatechin-3-gallate: 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 μg/mL; (−)-epigallocatechin-3-gallate: 0.5, 1.0, 2.0, 4.0, 5.0, 6.0 μg/mL; (−)-gallocatechin-3-gallate: 0.4, 0.5, 1.0, 1.5, 2.0, and 2.5 μg/mL). Calibration graphs (peak area, y, versus concentration, x) were constructed to obtain the regression equations and their correlation coefficients (r) as follows: caffeine, $y = 55772937x + 64886$ ($r = 0.9999$); (−)-epigallocatechin, $y = 3474x - 3320$ ($r = 0.9961$); (−)-epicatechin-3-gallate, $y = 25471x + 118$ ($r = 0.9987$); (−)-epigallocatechin-3-gallate, $y = 19181x - 6239$ ($r = 0.9995$); and (−)-gallocatechin-3-gallate, $y = 15542x - 2883$ ($r = 0.9963$).

2.5. Determination of DPPH Scavenging Activity

The DPPH scavenging activity of the tea sample on a DPPH radical was estimated according to the procedure developed in previous studies [16,17,19]. The stock solution was prepared by dissolving 80-mg DPPH with 100 mL of methanol. A 2-mL aliquot tea sample was mixed with 2 mL of DPPH solution. The mixture was shaken and incubated for 30 min in the dark at room temperature. The absorbance was measured spectrophotometrically at 517 nm. The DPPH scavenging activity was calculated using the following equation, and the antioxidant activity was evaluated based on IC_{50}

(μg/mL) value, which was determined from the calibration curve inhibition of DPPH free radical (%) vs. infusion concentration (μg/mL).

$$\text{DPPH scavenging ability } (\%) = \left[1 - \left(\frac{A_{Sample}}{A_{Blank}}\right)\right] \times 100\% \quad (1)$$

2.6. Statistical Analysis

To ensure statistical representation, all of the experiments were performed at least in triplicate, and means between the treatment levels were compared using the SAS software (version 9.4, SAS Institute, Cary, NC, USA). Statistical analyses were based on the analysis of variance (ANOVA). If the treatment was significant ($p < 0.05$), the treatment mean was compared with the Tukey's test. The correlation among the variables was measured using the CORR procedure in SAS.

3. Results and Discussion

3.1. HPLC

Black tea contains many functional compounds. Figure 1 shows a HPLC chromatogram of the tea sample with a 100:1 water–tea leaves weight ratio and a 100 °C infusion for 1 min. The identified compounds were caffeine, (−)-epigallocatechin, (−)-epicatechin-3-gallate, (−)-epigallocatechin-3-gallate, and (−)-gallocatechin-3-gallate. By analyzing the peak-area ratios of individual peaks and their corresponding standards, the contents of these compounds were 0.347 mg for caffeine, 0.013 mg for (−)-epigallocatechin, 0.001 mg for (−)-epicatechin-3-gallate, 0.002 mg for (−)-epigallocatechin-3-gallate, and 0.001 mg for (−)-gallocatechin-3-gallate per g of infusion. All the detection limits calculated by the signal-to-noise ratio greater than 3 of compounds were 0.001 mg/g.

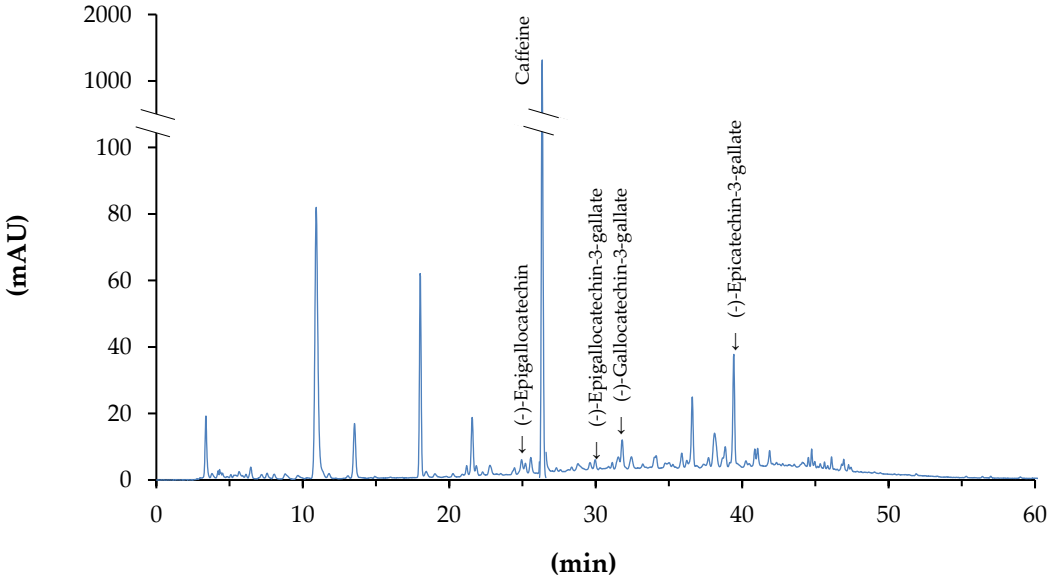

Figure 1. High performance liquid chromatography (HPLC) chromatogram of the tea sample.

3.2. Effect of the Water–Tea Leaves Weight Ratio

Figure 2 shows the effects of the water–tea leaves weight ratio on DPPH scavenging activity. IC_{50} is a value revealing the 50% inhibitory concentrations for DPPH, and a low IC_{50} indicates that strongly antioxidant compounds are present in black tea. Results showed that IC_{50} for the varying weight ratios 25, 50, 100, 125, 250, and 500 were 30.4 ± 3.3, 29.6 ± 5.3, 28.4 ± 4.8, 28.0 ± 3.4, 26.6 ± 3.9, and 28.6 ± 3.9 μg/mL. The results indicate that no difference is observable among these IC_{50} values with weight ratios from 25 to 500, as shown in Figure 2. The IC_{50} for DPPH scavenging ability

ranged from 30.4 ± 3.3 to 26.6 ± 3.9 μg/mL. Therefore, the black tea infusion was fixed with a 100:1 weight ratio for determining the antioxidant capacity in this study to allow comparison with previous reports [6,8,20–22].

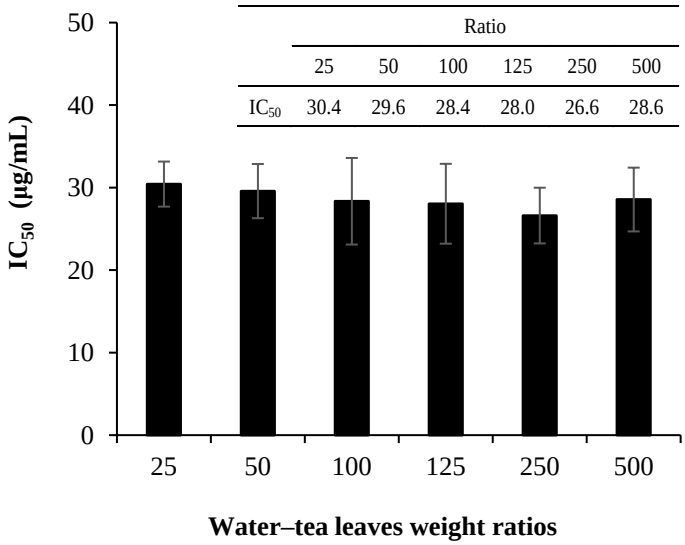

Figure 2. Relationship between 1,1-Diphenyl-2-picrylhydrazyl (DPPH) IC_{50} and the water–tea leaves weight ratio.

3.3. Effect of Infusion Temperature

The antioxidant capacity for DPPH scavenging activity and the total phenols of black tea are dependent on infusion temperature. Figure 3 confirms that the DPPH scavenging ability of black tea was affected by infusion temperature. A higher DPPH scavenging ability was noted at higher temperatures, revealing that the scavenging activity for the DPPH radical of black tea prepared with boiling water was better than the others at the same tea concentration. However, Figure 3 indicates that the DPPH scavenging activity increased with tea concentration regardless of infusion temperature. Furthermore, the effects of tea concentration on the DPPH scavenging activity were more prominent at a higher infusion temperature.

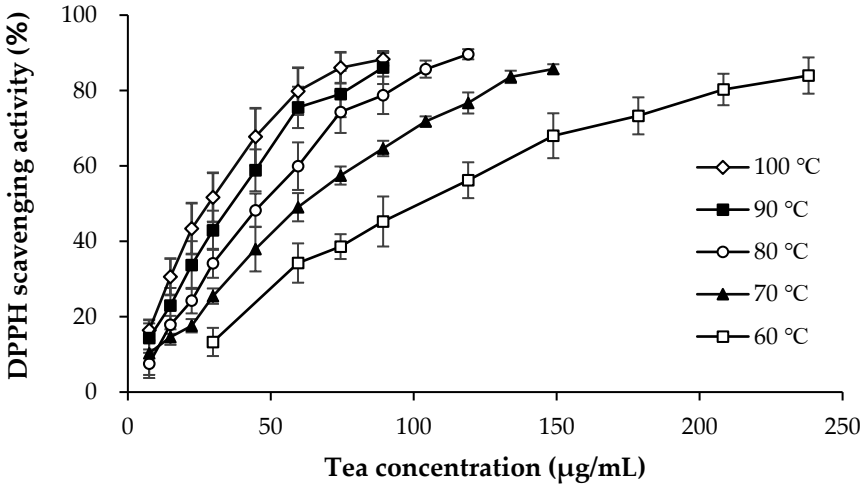

Figure 3. Effects of temperature on the DPPH scavenging activity of black tea.

According to Figure 3, the IC_{50} values were 100.0 ± 13.7, 63.0 ± 4.2, 46.2 ± 5.5, 35.3 ± 5.2, and 28.4 ± 4.8 μg/mL for temperatures from 60 to 100 °C (Figure 4). The total phenolic contents for 60 to 100 °C

were 50.4 ± 5.2, 72.6 ± 7.9, 104.1 ± 7.0, 135.2 ± 8.5, and 178.6 ± 16.4 mg GAE/g dry leaf, respectively (Table 1). An increase in the DPPH scavenging ability and the total phenolic content associated with an increase in the infusion temperature concurred with the findings in previous reports [6,8,10,23–25]. Thus, the infusion temperature of black tea is critical in the preparation process when aiming for high antioxidant capacity. Black tea with lower DPPH IC_{50} had higher total phenols, and the IC_{50} of DPPH scavenging ability showed a negative correlation with the value of the total phenolic content, as seen in Figure 5. This result concurred with previous reports [23,26–28]. Theaflavins, a type of phenolic compound in black tea, may contribute to strong antioxidant properties that affect the DPPH scavenging activity and total phenols [23,29–32]. However, the therapeutic activities of black tea are attributed not only to the antioxidant activity of its components but also to the ability of bioactive molecules to interact with several biological targets [5,33]. At a high temperature, chemical degradation can occur, changing the structure of temperature-sensitive molecules such as (−)-epigallocatechin-3-gallate.

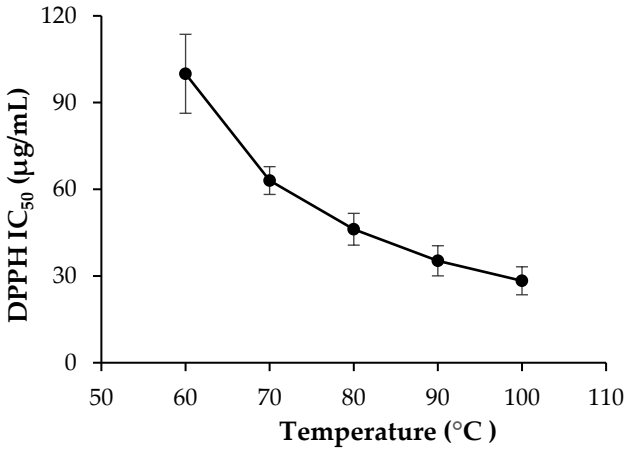

Figure 4. Effects of temperature on the DPPH IC_{50} of black tea.

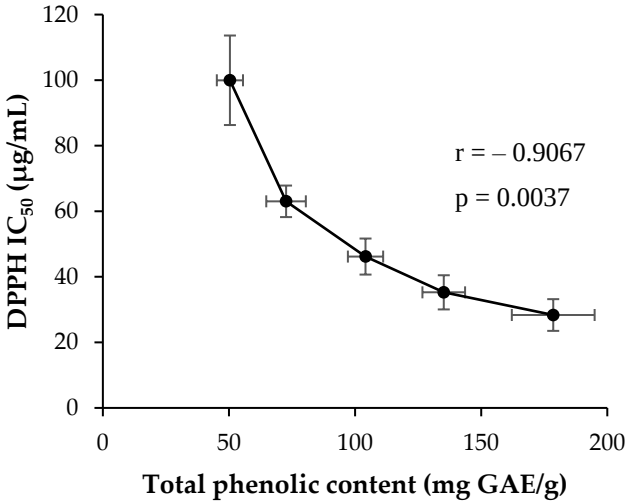

Figure 5. Correlation between DPPH IC_{50} and total phenolic content.

Table 1. Effects of temperature on the total phenolic content of black tea.

	Temperature (°C)				
	60	**70**	**80**	**90**	**100**
Total phenolic content (mg GAE/g dry leaf)	50.4 ± 5.2	72.6 ± 7.9	104.1 ± 7.0	135.2 ± 8.5	178.6 ± 16.4

Wide variations are noted among different kinds of black tea in terms of DPPH scavenging ability [9,22,34–38] and total phenolic content [6,20,32,36,39]; these are highly dependent on the genetic background and growing conditions (such as temperature, nitrogen availability, and light conditions) of the plant material. The IC_{50} of black tea in this study was 28.4 ± 4.8 μg/mL, and it falls within the range of 12.7–118.7 μg/mL found by other authors [34,38]. The total phenolic content in this study was 178.6 ± 16.4 mg GAE/g dry leaf, and it was in the range from 50.9 ± 4.1 mg GAE/g dry leaf [32] to 274.3 ± 0.0 mg GAE/g dry leaf [20]. Different brewing conditions, including infusion temperature and time, may affect the DPPH IC_{50} and total phenolic content. Pastoriza et al. [7] reported that an increase in the infusion temperature increased antioxidant activity, and Ramalho et al. [20] demonstrated that the total phenolic content increased with infusion time.

3.4. *Effect of Storage Time and Storage Temperature*

The storage time and storage temperature have a great influence on determining the antioxidant activity and total phenolic content of black tea. Figures 6 and 7 show the DPPH scavenging activity and total phenolic content of black tea at different temperatures for different storage times, respectively. As shown in Figure 6, the difference between the two means of the DPPH scavenging ability is not statistically significant at 4 °C and 25 °C. In addition, at 9 °C, the trend was similar for 0-day to 15-day storage periods, except for the 9-day storage period, which was lower than the other storage times. These results indicated that the DPPH scavenging activity of black tea did not decline within 15 days even though it was kept at 25 °C. No notable difference was observed in the scavenging activity against the DPPH radical during 15-day storage. Jiménez-Zamora et al. [15] reported that the DPPH scavenging activity of black tea exhibited considerable decline at 25 °C after 3 months; however, no perceptible difference was observed in the DPPH scavenging activity among the three storage temperatures in this study because of the relatively short 15-day storage period.

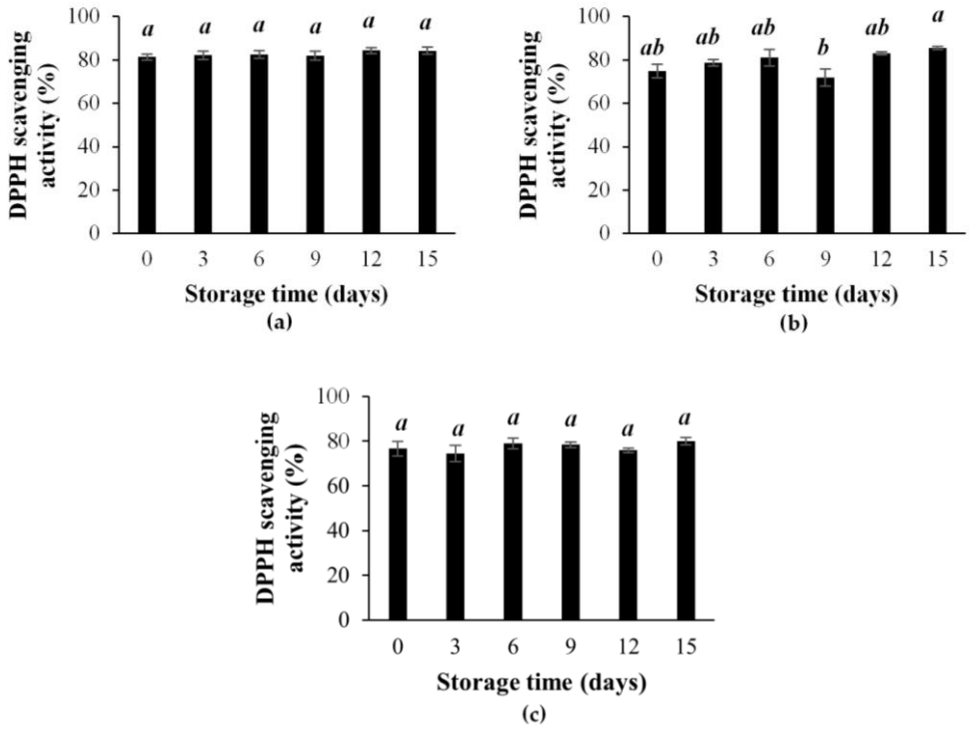

Figure 6. Effects of storage time on the DPPH scavenging activity of black tea: (**a**) 4 °C, (**b**) 9 °C and (**c**) 25 °C. Means with the same capital alphabetical letters are not significantly different according to the results of the Tukey's test.

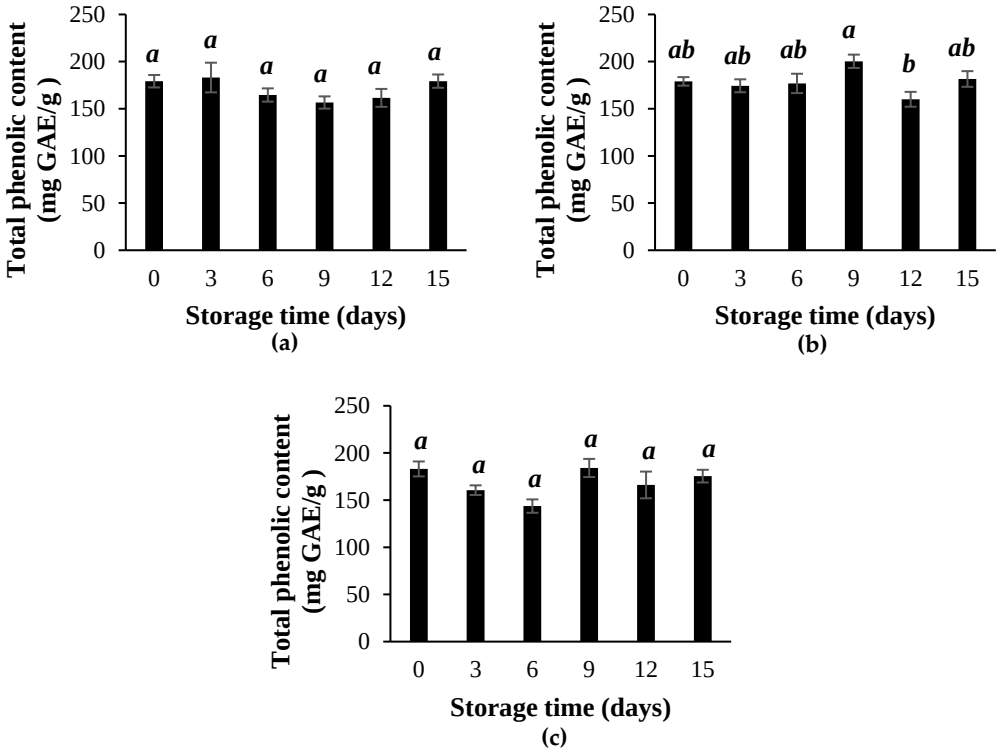

Figure 7. Effects of storage time on the total phenolic content of black tea (**a**) 4 °C, (**b**) 9 °C and (**c**) 25 °C. Means with the same capital alphabetical letters are not significantly different according to the results of the Tukey's test.

In terms of the total phenolic content, no significant difference was observed from 0-day to 15-day storage periods at 4 °C and 25 °C, as shown in Figure 7. As with the result of the DPPH scavenging ability, there was a similar trend for the total phenolic content from 0-day to 15-day storage at 9 °C, except 12-day storage, which was lower than the other storage times.

In summary, the black tea exhibited similar scavenging ability and total phenolic content at 4, 9, and 25 °C regardless of storage time in this study. Although at 9 °C, the performance of DPPH scavenging ability and total phenolic content was lower than the other storage times at 9-day and 12-day, respectively. The situation might have resulted from sampling and the significant level of $p < 0.05$. Moreover, the performance of the total phenolic content in this study concurred with a previous report indicating that the total phenolic content of black tea remained almost constant after storage at 25 °C or 50 °C for 6 months [15].

4. Conclusions

This study investigated the effects of infusion conditions and short-term storage on the antioxidant activity and total phenolic content of black tea. The results indicated that the water–tea leaves weight ratio from 10 to 500 did not have a significant impact on DPPH scavenging ability. However, the DPPH scavenging ability and total phenolic content increased with infusion temperature. The IC_{50} of DPPH scavenging activity and the total phenolic content had a negative correlation. Moreover, the results indicated that the antioxidant capacity and total phenolic content of black tea did not exhibit perceptible changes during 15-day storage. Both the DPPH scavenging ability and the total phenolic content of black tea had negligible variation at 4, 9, and 25 °C storage temperatures.

Author Contributions: Conceptualization, Y.-S.L.; methodology, M.-Y.C., W.-Y.H. and S.-L.H.; formal analysis, M.-Y.C., W.-Y.H. and W.-S.L.; investigation, Y.-S.L.; data curation, Y.-Y.L., Y.-C.C. and W.-S.L.; writing—original draft preparation, Y.-Y.L. and C.-Y.C.; writing—review and editing, Y.-S.L.; supervision, Y.-S.L.; All authors have read and agreed to the published version of the manuscript.

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

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