Postprint: Phenolic Content and Antioxidant Activity of Laoying Tea at Different Maturity Stages

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Abstract

To investigate the differences in phenolic compound content and antioxidant activity between hawk teas of two different maturity levels for identification and quality evaluation. This study first employed LC-MS/MS to quantify 15 phenolic compounds in hawk tea, and evaluated the antioxidant capacity of the two tea types using DPPH free radical scavenging assay, ABTS+ free radical scavenging assay, and Fe3+ reducing capacity assay. Statistical data analysis was then conducted to explore the differences in phenolic compound content and antioxidant activity between tender and mature leaf hawk teas, and to further investigate the contribution of different phenolic compounds to antioxidant activity in hawk tea. The results demonstrated: (1) Tender leaf tea exhibited significantly higher contents of catechin, p-coumaric acid, isoquercitrin, hyperoside, nicotiflorin, astragalin, kaempferol, quercetin, and afzelin compared to mature leaf tea, with the average contents of catechin, isoquercitrin, and astragalin being 1,039, 169, and 257 mg · 100 g-1 higher than those in mature leaf tea, respectively. Cluster analysis, principal component analysis, and orthogonal partial least squares discriminant analysis could all distinguish between the two. (2) Significant differences were observed in antioxidant capacity between the two teas in terms of DPPH free radical scavenging rate, ABTS+ free radical scavenging rate, and Fe3+ reducing capacity, with tender leaf tea showing superior performance. (3) Partial least squares regression analysis indicated that isoquercitrin, catechin, astragalin, chlorogenic acid, hyperoside, p-coumaric acid, and kaempferol are the primary chemical components responsible for the antioxidant efficacy of hawk tea. This study provides a reference for the quality control and application promotion of hawk tea.

Full Text

Determination of Phenolic Compounds in Hawk Tea with Different Maturity Levels and Study on Their Antioxidant Activity

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Abstract

This study investigated the differences in phenolic compound content and antioxidant capacity between two maturity levels of Hawk tea to enable their identification and quality evaluation. Fifteen phenolic compounds were quantified using LC-MS/MS, and antioxidant capacity was assessed through DPPH radical scavenging, $ABTS^{+}$ radical scavenging, and Fe^{3+} reducing power (FRAP). Statistical analyses were employed to examine differences in phenolic content and antioxidant activity between tender- and mature-leaf Hawk tea, and to further explore the contribution of individual phenolic compounds to antioxidant efficacy. The results demonstrated: (1) Tender-leaf tea contained significantly higher levels of catechin, p-coumaric acid, isoquercitrin, hyperoside, nicotiflorin, astragalin, kaempferol, quercetin, and afzelin compared to mature-leaf tea, with average contents of catechin, isoquercitrin, and astragalin exceeding those in mature-leaf tea by 1,039, 169, and 257 $mg \cdot 100\ g^{-1}$, respectively. Hierarchical cluster analysis (HCA), principal component analysis (PCA), and orthogonal partial least squares discriminant analysis (OPLS-DA) all successfully distinguished the two tea types. (2) Significant differences in DPPH radical scavenging, $ABTS^{+}$ radical scavenging, and Fe^{3+} reducing capacity were observed between the two teas, with tender-leaf tea exhibiting superior antioxidant activity. (3) Partial least squares regression (PLSR) analysis identified isoquercitrin, catechin, astragalin, chlorogenic acid, hyperoside, p-coumaric acid, and kaempferol as the primary chemical components responsible for Hawk tea' s antioxidant efficacy. These findings provide valuable references for quality control and application development of Hawk tea.

Keywords: Hawk tea; phenolic compounds; LC-MS/MS; antioxidant activity; correlation analysis

Introduction

Hawk tea is produced from the leaves of *Litsea coreana* var. *lanuginosa* (Lauraceae) (Wang et al., 2021). The tender leaves feature grayish-yellow long pubescence on both surfaces, particularly dense on the underside, while the mature leaves have sparse pubescence beneath, making them distinct raw materials for tea production (Ai et al., 2021). Hawk tea possesses multiple health benefits, including thirst-quenching, heat-clearing, digestion-promoting, and cognition-enhancing effects (Tan et al., 2016), along with documented pharmacological activities such as antioxidant, anti-inflammatory, UV-protective, hypoglycemic, hypolipidemic, hepatoprotective, and antimicrobial properties (Feng et al., 2019; Chen et al., 2019; Li et al., 2021; Tao et al., 2022; Xu et al., 2022). Rich in phenolic compounds, these constituents represent the primary bioactive components of Hawk tea (Liu, 2010; Qin et al., 2019).

Research has demonstrated that maturity significantly influences the bioactive components and antioxidant activity of Hawk tea, with tender-leaf tea showing higher contents of total flavonoids and carbohydrates, as well as superior antioxidant efficacy compared to mature-leaf tea (Yuan et al., 2014; Xiao et al., 2017). Consequently, tender-leaf tea is regarded as a premium alternative tea and commands higher prices. However, mature-leaf tea has also been reported to possess considerable bioactivity (Chen et al., 2019) and remains popular due to its lower cost and greater availability (Dai et al., 2022). Current quality control approaches for Hawk tea primarily rely on liquid chromatography to quantify a limited number of components (Liu, 2010), which fails to comprehensively reflect tea quality. Moreover, few studies have investigated the differences in active components between tender and mature leaves or elucidated the material basis underlying their antioxidant activity. Therefore, comprehensive chemical profiling and investigation of the differential material basis of active components between tender- and mature-leaf teas are essential for establishing robust quality standards and promoting application development.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) offers high sensitivity, selectivity, simplicity, and rapid analysis, making it widely applicable for natural product profiling, particularly for simultaneous detection of multiple components in complex matrices. This study employed LC-MS/MS to quantify major phenolic compounds in two maturity levels of Hawk tea leaves and evaluated antioxidant capacity using DPPH radical scavenging, $ABTS^{+}$ radical scavenging, and Fe^{3+} reducing antioxidant power (FRAP). Statistical methods including principal component analysis (PCA), hierarchical cluster analysis (HCA), orthogonal partial least squares discriminant analysis (OPLS-DA), analysis of variance (ANOVA), and partial least squares regression (PLSR) were utilized to address: (1) differences in phenolic composition and in vitro antioxidant activity between the two Hawk tea types, and (2) the contribution of individual phenolic compounds to antioxidant activity.

Materials and Methods

1.1 Materials and Reagents Reference standards of neochlorogenic acid, catechin, chlorogenic acid, epicatechin, p-coumaric acid, rutin, hyperoside, isoquercitrin, nicotiflorin, astragalin, hesperidin, afzelin, quercetin, naringenin, and kaempferol (purity $ $98%) were purchased from Chengdu Glip Biotechnology Co., Ltd. Acetonitrile (LC-MS grade), formic acid (LC-MS grade), DPPH, ABTS, Trolox, potassium persulfate, ferric chloride, sodium acetate, glacial acetic acid, 2,4,6-tripyridyl-s-triazine (TPTZ), sodium carbonate, and Folin-Ciocalteu reagent were obtained from Aladdin Biochemical Technology Co., Ltd. (Shanghai). Ultrapure water was prepared using a pure water system. Eighteen batches of Hawk tea were hand-harvested from farmers during April-May 2022, comprising fresh tender and mature leaves (tender-leaf tea: single bud to one bud with three leaves; mature-leaf tea: remaining lower leaves) from Guizhou, Chongqing, Sichuan, and Anhui provinces. Sample details are presented in Table 1 .

1.2 Solution Preparation 1.2.1 Reference Standard Solutions
Reference standards were accurately weighed and dissolved in methanol to prepare 2 $mg \cdot mL^{-1}$ stock solutions, stored at -20 °C. Before use, stock solutions were diluted with 80% methanol (v/v) to appropriate concentrations for mixed reference solution injection.

1.2.2 Sample Solutions
Freshly harvested Hawk tea leaves were dried at 40 °C, pulverized, and passed through a 60-mesh sieve. Accurately weighed powder (0.75 g) was extracted with 25 mL of 80% methanol (v/v) by ultrasonication for 60 min, allowed to cool, and reweighed to compensate for solvent loss. After centrifugation (10,000 $r \cdot min^{-1}$, 10 min), the supernatant was filtered through a 0.22 m membrane and diluted 4-fold with 80% methanol (v/v) prior to injection.

1.3 LC-MS/MS Conditions 1.3.1 Chromatographic Conditions
Analysis was performed on an I-Class-TQ-S UPLC-triple quadrupole mass spectrometer (Waters, USA) equipped with a Waters Acquity UPLC BEH C_{18} column (100 mm × 2.1 mm, 1.7 m). The mobile phase consisted of acetonitrile (A) and 0.1% formic acid in water (B) with gradient elution (0–15 min, 5%$→30→90^{-1}$, and injection volume at 1 L.

1.3.2 Mass Spectrometry Conditions
Negative ion mode electrospray ionization (ESI^-) was employed with full scan and multiple reaction monitoring (MRM) modes. Parameters: capillary voltage 3.5 kV, desolvation temperature 450 °C, and gas flow 750 $L \cdot h^{-1}$.

MS Parameters for 15 Target Compounds
Detailed MS parameters are presented in Table 2 .

1.4 Antioxidant Activity Assays 1.4.1 DPPH Radical Scavenging Assay

Following published methods (Pan et al., 2021; Mary & Merina, 2021) with minor modifications, 1 mL DPPH working solution (100 mg·L^{-1} in 80% methanol) was mixed with 0.5 mL diluted sample solution. After 30 min reaction at room temperature in darkness, absorbance (A_1) was measured at 519 nm. Control absorbance (A_0) was obtained using 0.5 mL 80% methanol (v/v) instead of sample. DPPH radical scavenging rate (%) was calculated as: (A_0 - A_1)/A_0 × 100.

1.4.2 $ABTS^+$ Radical Scavenging Assay

Based on reported methods (Wolosiak et al., 2021; Xiao et al., 2022) with adjustments, ABTS working solution was prepared by mixing ABTS aqueous solution with potassium persulfate, reacting for 12–16 h in darkness at room temperature, and diluting to an absorbance of 0.8 ± 0.05 at 734 nm. One mL ABTS working solution was combined with 0.5 mL diluted sample solution, reacted for 30 min in darkness, and absorbance (A_1) measured at 734 nm. Control absorbance (A_0) was determined using 80% methanol (v/v). Calculation followed the same formula as for DPPH.

1.4.3 Ferric Reducing Antioxidant Power (FRAP) Assay

Following Chen et al. (2020), 100 L test sample was mixed with 300 L FRAP working solution (prepared by mixing 300 mmol·L^{-1} sodium acetate buffer pH 3.6, 10 mmol·L^{-1} TPTZ solution, and 20 mmol·L^{-1} ferric chloride solution at 10:1:1 v/v/v). After 10 min incubation at 37 °C, absorbance was measured at 593 nm. A standard curve was constructed using Trolox: y = 0.0141x + 0.151, r^2 = 0.996. FRAP values were expressed as mg Trolox equivalents per gram dry weight (mg TE·g^{-1} DW).

Results

2.1 Identification of Major Phenolic Compounds in Hawk Tea Using the chromatographic conditions described in Section 1.3, total ion current chromatograms were obtained in negative ion mode (scan range m/z 100–800 amu) as shown in Figure 1 [Figure 1: see original paper]. By comparing MRM fragmentation patterns with reference standards, 15 compounds were identified: neochlorogenic acid, catechin, chlorogenic acid, epicatechin, p-coumaric acid, rutin, hyperoside, isoquercitrin, nicotiflorin, astragalin, hesperidin, afzelin, quercetin, naringenin, and kaempferol. These compounds exhibited relatively large chromatographic peak areas in negative ion mode, indicating they are the predominant phenolic constituents in Hawk tea.

2.2 Method Validation Linearity, Precision, and Accuracy

Mixed standard solutions prepared from the stock solution described in Section 1.2.1 were analyzed using the method in Section 1.3. Linear regression

was performed between peak area (y) and mass concentration (x) for each compound. Method precision was evaluated by six consecutive injections of the mixed standard solution, calculating relative standard deviation (RSD) of peak areas. Accuracy was assessed through recovery tests by spiking mixed standards into known Hawk tea samples, processing according to Section 1.2.2, and analyzing with Section 1.3 conditions (n = 6). Results are summarized in Table 3 . All 15 compounds showed good linearity ($r^2 > 0.99$) within their respective linear ranges, with detection limits (S/N = 3) of 0.1–1.3 $g \cdot L^{-1}$. Precision RSD values ranged from 0.7% to 2.38%, indicating excellent instrumental precision. Mean recovery rates for the 15 compounds were 93.2%–109.7% with RSD < 6.6%, confirming satisfactory method accuracy.

2.3 Quantification of Samples The validated LC-MS/MS method in MRM mode was applied to quantify 15 phenolic compounds in 8 batches of tender-leaf and 10 batches of mature-leaf samples. Normalized chromatograms of reference standards and samples are shown in Figure 2 [Figure 2: see original paper]. Detailed quantitative results are presented in Table 4 .

2.4 Differential Analysis Quantitative data were imported into SPSS 23.0 for one-way ANOVA to examine differences in the 15 phenolic components between the two tea types. As shown in Table 5 , significant differences ($P < 0.05$) were observed for catechin, p-coumaric acid, hyperoside, isoquercitrin, nicotiflorin, astragalin, afzelin, quercetin, and kaempferol, with tender-leaf tea containing markedly higher levels of these nine components. Notably, tender-leaf tea showed elevated average contents of catechin [(1,258.46 ± 280.64) $mg \cdot 100\ g^{-1}$], astragalin [(309.65 ± 52.54) $mg \cdot 100\ g^{-1}$], isoquercitrin [(246.75 ± 42.18) $mg \cdot 100\ g^{-1}$], hyperoside [(134.08 ± 70.73) $mg \cdot 100\ g^{-1}$], and quercetin [(89.50 ± 45.42) $mg \cdot 100\ g^{-1}$]. The most pronounced differences were observed for catechin, isoquercitrin, and astragalin, which were higher in tender-leaf tea by 1,039.43, 169.12, and 257.35 $mg \cdot 100\ g^{-1}$, respectively, compared to mature-leaf tea. No significant differences ($P > 0.05$) were found for neochlorogenic acid, chlorogenic acid, epicatechin, rutin, hesperidin, or naringenin between the two tea types.

2.5 Hierarchical Cluster Analysis (HCA) HCA is commonly employed to analyze multiple chemical components, bioactivities, and functional properties to discriminate relationships among samples (Wang et al., 2022). Using the quantitative data for 15 phenolic compounds from 18 batches of Hawk tea as variables, a cluster heatmap was generated in Origin software using between-group linkage and squared Euclidean distance. As shown in Figure 3 [Figure 3: see original paper], tender- and mature-leaf teas were clearly distinguished based on their phenolic profiles. HCA results suggest that the two tea types can be discriminated using the 15 phenolic compounds, though geographic origin showed limited discriminatory power, indicating that maturity differences outweigh regional variations.

2.6 Principal Component Analysis (PCA) PCA was performed using the contents of 15 phenolic compounds as variables to calculate eigenvalues, cumulative contribution rates, initial factor loading matrices, and comprehensive scores. As presented in Table 6 , components with eigenvalues > 1 accounted for 80.210% of total variance, indicating adequate representation of the overall dataset. The first principal component (PC1) had an eigenvalue of 7.418, explaining 49.450% of variance, with absolute loading values > 0.5 for catechin, p-coumaric acid, hyperoside, isoquercitrin, nicotiflorin, astragalin, afzelin, quercetin, naringenin, and kaempferol, suggesting PC1 primarily reflects these 10 components. The second principal component (PC2) had an eigenvalue of 3.299, accounting for 21.996% of variance, with epicatechin, quercetin, chlorogenic acid, and rutin showing absolute loading values > 0.5, indicating PC2 mainly represents these four compounds. The third principal component (PC3) had an eigenvalue of 1.315 (8.764% variance), primarily reflecting neochlorogenic acid and naringenin. Detailed factor loadings are provided in Table 7 .

Using five principal components with eigenvalues > 0.5 (cumulative contribution rate = 90.942%), comprehensive scores (F) were calculated as: $F = 0.49451F_1 + 0.21996F_2 + 0.08764F_3 + 0.06556F_4 + 0.04117F_5$. Results in Table 8 show that most tender-leaf samples scored higher than mature-leaf samples, with the highest scores for S2 and S1 (both from Xuancheng, Anhui). Notably, mature-leaf samples S16 and S13 scored higher than tender-leaf sample S8, demonstrating that some mature-leaf teas also exhibit good quality.

A two-dimensional scatter plot using PC1 and PC2 (Figure 4 [Figure 4: see original paper]) showed relatively concentrated and independent distributions of tender- and mature-leaf teas, consistent with HCA results.

2.7 OPLS-DA Analysis OPLS-DA combines orthogonal signal correction with partial least squares to remove irrelevant variation and achieve classification (Kang et al., 2022). Based on the 15 phenolic compounds, the OPLS-DA model showed $R^2X(cum) = 0.759$, $R^2Y(cum) = 0.948$, and $Q^2(cum) = 0.837$, indicating a stable and reliable model (Yan et al., 2021). Correlation coefficients indicate positive/negative contributions to discrimination, while variable importance in projection (VIP) values represent variable weights (VIP > 1 indicates significant influence) (Li et al., 2021). As shown in Figure 5 [Figure 5: see original paper], the OPLS-DA score plot achieved complete separation of the two tea types. Components positively contributing to tender-leaf discrimination with VIP > 1 were catechin, p-coumaric acid, hyperoside, isoquercitrin, nicotiflorin, astragalin, and afzelin, consistent with ANOVA and PCA results. These compounds can thus differentiate the two tea types. Conversely, neochlorogenic acid, chlorogenic acid, epicatechin, rutin, and naringenin showed negative correlation coefficients and VIP < 1, aligning with compounds showing no significant differences in ANOVA and PC2 composition, suggesting these components may contribute to the antioxidant activity of mature-leaf tea.

2.9 Antioxidant Activity Assessment and Differential Analysis DPPH radical scavenging, $ABTS^{+}$ radical scavenging, and FRAP values for different Hawk tea batches are presented in Table 9 . Statistically significant differences ($P < 0.05$) were observed between the two tea types for all three antioxidant assays. Further ANOVA analysis (Table 10) confirmed that tender-leaf tea demonstrated stronger antioxidant activity, though mature-leaf tea also exhibited considerable antioxidant capacity based on mean values and standard deviations.

2.10 PLSR Analysis of Antioxidant Substances in Hawk Tea PLSR is a common correlation analysis method for determining compound contributions to bioactivity. To identify key antioxidant compounds, PLSR was performed with standardized regression coefficients indicating positive/negative relationships with antioxidant activity, and VIP values representing contribution weights (higher values = greater contribution) (Burnett et al., 2021). As shown in Figure 6 [Figure 6: see original paper], isoquercitrin, catechin, astragalin, chlorogenic acid, hyperoside, p-coumaric acid, and kaempferol showed positive regression coefficients with VIP > 1, indicating these seven compounds are critical for Hawk tea' s antioxidant activity. Notably, chlorogenic acid content showed no significant difference between tea types, with mature-leaf tea containing substantial levels [(201.69 ± 161.24) mg $\cdot$ 100 g^{-1}], which may explain the appreciable antioxidant activity of mature-leaf tea.

Discussion and Conclusion

The results demonstrate that due to different harvest positions, tender-leaf tea exhibits superior total phenolic content and in vitro antioxidant activity compared to mature-leaf tea. Integrating ANOVA, PCA, and OPLS-DA findings, catechin, p-coumaric acid, isoquercitrin, hyperoside, nicotiflorin, astragalin, kaempferol, afzelin, and quercetin showed significant differences between the two tea types, with markedly higher levels in tender-leaf tea. The most prominent difference was observed for catechin, whose content in tender-leaf tea far exceeded that in mature-leaf tea. Tender-leaf tea production follows traditional tea harvesting practices using buds and young leaves. Since catechin is highly water-soluble (Cuevas-Valenzuela et al., 2014) and abundant in tender-leaf tea, brewing with boiling water yields desirable taste and efficacy. In contrast, mature-leaf tea contains lower catechin levels, prompting consumers to employ decoction methods—boiling the leaves in cold water for several minutes —to extract less water-soluble flavonoids (Dai et al., 2022). Flavonoids and phenolic acids are primary antioxidant components in natural plants (Pérez-Torres et al., 2021; Arzola-Rodriguez et al., 2022). The identified and quantified compounds in Hawk tea were predominantly flavonoids, while caffeic acid derivatives (neochlorogenic acid and chlorogenic acid) showed no significant maturity-related differences. Combined with the observed antioxidant activity differences,

flavonoids appear to be the main contributors to activity variation between tea types.

PLSR analysis identified isoquercitrin, catechin, astragalin, chlorogenic acid, hyperoside, p-coumaric acid, and kaempferol as compounds closely associated with antioxidant activity. Among these, chlorogenic acid was present at high levels in both tea types without significant differences, suggesting that non-differential components like chlorogenic acid may contribute to the antioxidant activity of mature-leaf tea. These compounds have been individually reported to correlate with antioxidant activity: Morais et al. (2022) found coca leaf antioxidant activity proportional to hyperoside and isoquercitrin contents; catechin antioxidant effects represent a major research focus (Thammarat et al., 2021; Liang et al., 2021; Xia et al., 2022); Du et al. (2022) reported astragalin' s ability to inhibit insulin resistance and oxidative stress; Kluska et al. (2022) demonstrated kaempferol' s activation of antioxidant genes and proteins; Taha et al. (2020) confirmed p-coumaric acid' s hepatoprotective effects via antioxidant mechanisms; and chlorogenic acid compounds are recognized for diverse bioactivities including antioxidant, hepatoprotective, anti-inflammatory, and antimicrobial effects (Rojas-Gonzalez et al., 2022). Currently, limited attention has been paid to phenolic differences between Hawk tea maturity levels, and specific quality standards remain unestablished. Therefore, these bioactive compounds hold significant importance for discriminating tea types and controlling production quality standards.

In summary, this study quantified 15 phenolic compounds in Hawk tea using LC-MS/MS and established HCA, PCA, and OPLS-DA models to differentiate tender- and mature-leaf teas. PLSR analysis identified key antioxidant components, providing valuable references for quality control and development of Hawk tea products.

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Note: Figure translations are in progress. See original paper for figures.