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ORIGINAL ARTICLE

Comprehensive Analysis of Agronomic Characters, Chemical Compounds, and Antioxidant Activity in *Chaenomeles* Fruits at Different Developmental Stages

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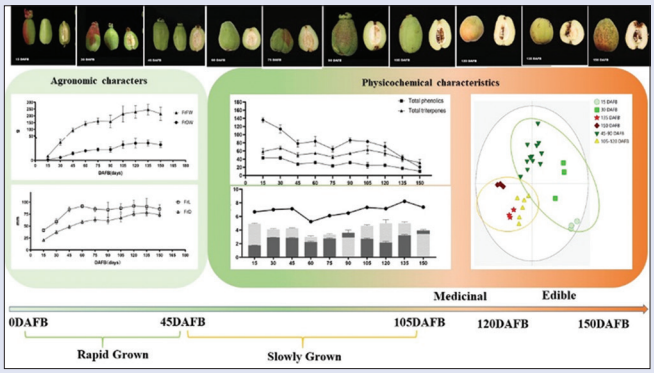
ABSTRACT

Background: *Chaenomeles speciosa*, also known as "*Mugua*" in Chinese, is a medicinal and edible plant with a long history of use. The optimal harvest time for *Chaenomeles* fruits is not clear. **Objectives:** This study aimed to determine the appropriate harvest time for different uses of *Chaenomeles* fruits. **Materials and Methods:** Agronomic characteristics, chemicals, and antioxidant activity of fruits were detected at several developmental stages (15, 30, 45, 60, 75, 90, 105, 120, 135, and 150 days after full bloom [DAFB]). **Results:** Based on agronomic characteristics (length, diameter, fresh weight, and dry weight), the whole development period was divided into rapid growth and slow growth stages. Physicochemical characteristics (flavonoids, triterpenes, and polyphenols) and pentacyclic triterpenoids (oleanolic acid and ursolic acid) were used to distinguish medicinal usage. Organic acid content (shikimic acid, quinic acid, malic acid, protocatechuic acid, and chlorogenic acid) was the major consideration for edible fruits. Principal component analysis and partial least squares-discriminant analysis were performed based on the whole indexes, and all developmental stages were divided into six clusters. Quinic acid, malic acid, shikimic acid, and oleanolic acid showed a positive relationship with antioxidant activity. **Conclusion:** The results showed that the 105–120 DAFB fruits were most suitable for use as Chinese medicine and the 120–150 DAFB fruits might be most suitable for use as edible flavored fruit.

Key words: Antioxidant activity, *Chaenomeles fruits,* chemicals, developmental stage, medicinal and edible plants

SUMMARY

- *Chaenomeles speciosa* remains an important multifunctional plant widely distributed in East Asia. The growth process of fruits can be divided into six steps based on agronomic characters, chemical compounds, and antioxidant activity
- The 105–120 days after full bloom (DAFB) fruits are most suitable for use as Chinese medicine
- The 120–150 DAFB of *C. speciosa* might be harvest as edible flavored fruit
- This study also provides theory foundation for the usage of young fruits.



Abbreviations used: DAFB: Days after full bloom; FrL: Fruit length; FrD: Fruit diameter; FrSI: Fruit shape index; FrFW: Fruit fresh weight; FrDW: Fruit dry weight; FrDR: Fruit desiccation rate; TFs: Total flavonoids; TTs: Total triterpenes; TPs: Total polyphenols; DPPH: Diphenylpicrylhydrazyl; EC_{50}: The concentration for 50% of the maximal effect; HPLC: High-performance liquid chromatography; VIP: Variable importance for projection; PCA: Principal component analysis; PLS-DA: Partial least squares-discriminant analysis.

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INTRODUCTION

Nowadays, with the rapid development of the economy, people from all walks of life face different degrees of tension and stress and the number of people with subhealth is increasing. Due to the increase in the incidence of subhealth in the general population with no obvious organic pathology, there has been a global boom in the concept of returning to nature and the basics of wellness. Owing to their safety, convenience and universality, as well as the effect of curing diseases and maintaining good health, medicinal and edible plants are increasingly favored and recognized in society.[1-3] The term "medicinal and edible plant" refers to a plant that can be used both as food and medicine.[4] There are different names for these plants in different countries. In Western countries, they are called "healthy foods," and in Japan, they are known as "functional foods." In recent

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years, there has been an increasing international research on these plants, such as *Panax ginseng*,[5,6] *Morus alba*,[7] *Lycium chinense*,[8] and others.[9-11]

The fruits of *Chaenomeles speciosa* (Sweet) Nakai (*Mugua* in Chinese), which is an important edible and Traditional Chinese medicine for more than 1000 years, have been of interest to many scholars, especially in Japan,[12] South Korea,[13,14] China,[15] and other East Asian countries. The *Chaenomeles* species belongs to the Rosaceae (subfamily Maloideae) family. Flavonoids, triterpenes,[16] polyphenols, organic acids,[17] and polysaccharides[18] are well-known active compounds in *Chaenomeles* fruits. Modern research has shown that these compounds play a therapeutic role in anti-inflammatory and immunomodulatory antioxidant effects,[19] α-glucosidase inhibitory activity,[20] tumor growth inhibition,[21] anti-influenza[22,23] activity, anti-Parkinson[24] activity, and others.

The *Chaenomeles* species is grown widely in many parts of China, such as Anhui, Hubei, Sichuan, Chongqing, and Yunnan provinces. The most famous species is the *Xuan Mugua*, which originates from Xuancheng city in Anhui province. Currently, people are beginning to realize the nutritional value of *Xuan Mugua*, which has been used locally to make many well-received canned foods, preserved fruit, fruit wine, fruit vinegar, etc.[25] According to the pharmacopeia of the People's Republic of China[26] (Chinese Pharmacopoeia Commission, 2020), *Chaenomeles* fruits should be harvested for medicinal purposes. However, the "near maturity" period is not well defined, which makes it impossible for the herbalist to accurately judge the optimal harvest time for *Chaenomeles* fruits. Moreover, *Chaenomeles* fruits have an unpleasant sour taste and cannot be used as edible food directly. Thus, the development process and harvest time of *Chaenomeles* fruits for use as food and medicine are not clear and only a few systematic studies have been conducted.

To our knowledge, the growth period and harvest time are crucial for the different use of plants. Petropoulos[27] focused on the chemicals and antioxidant activity of *Cichorium spinosum* L. leaves during developmental stages, indicating that the leaves can be eaten raw in early stages, while they can be processed into pickled products, water extracts, or decoctions prior to flowering initiation. For the *Chaenomeles* fruits of the Rosaceae family – with similar studies on apples,[28] blackberries, and strawberries[29] – the harvest time is mainly based on traditional experience, which limits the development of the fruits. Therefore, there is an urgent need to study the morphological quality and development characteristics of *Chaenomeles* fruits to identify their appropriate harvest time.

In this study, we examined *Chaenomeles* fruits during different developmental stages in terms of agronomic characteristics to distinguish developmental stages. Chemical components, organic acid content, and antioxidant activities were detected to further understand their characteristics, and multivariate statistical analysis was performed to identify the growth process. The results could help in distinguishing the harvest time of medicinal and edible *Chaenomeles* fruits. This will support the comprehensive utilization of *Chaenomeles* fruits by the food and health industries.

MATERIALS AND METHODS

Plant materials

C. speciosa (sweet) Nakai were collected every 15 DAFB, from March 23 to September 2, 2018. A total of ten batches (15, 30, 45, 60, 75, 90, 105, 120, 135, and 150 days after full bloom [DAFB]) were collected. Under the principle of representativeness, five healthy adult plants of similar age and ecological conditions were selected as samples from the same orchard in Xuancheng, Anhui province (E: 118°72.2470', N: 30°88.3826'). Fruits of similar size were selected for each batch, and one biological repetition was performed for the combined sample fruit, which was repeated three times. All samples were washed, slitted, dried, and kept in plastic bags at 25°C after being shattered and sieved.

Chemicals and reagents

High-performance liquid chromatography (HPLC)-grade methanol and glacial acetic acid were obtained from Concord Technology Co., Ltd. (Tianjin, China). Oleanolic acid, ursolic acid, shikimic acid, malic acid, and quinic acid were purchased from Baoji Herbest Bio-Tech Co., Ltd. (Shannxi, China). Chlorogenic acid and protocatechuic acid were acquired from Chengdu Herbpurify Co., Ltd. (Sichuan, China). All the reference compounds had purities of more than 98% and were used to draw calibration curves by HPLC and UV. Folin–Ciocalteu reagent (purity >99.0%) was acquired from Solebo Biotechnology Co., Ltd. (Beijing, China). Diphenylpicrylhydrazyl (DPPH, purity >98.0%), sodium nitrite, and vanillin were purchased from Shanghai D&B Biotechnology Co., Ltd. (Shanghai, China). Acetonitrile was purchased from Runjie-Corporation (Ocepak, Germany) and deionized water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). All other reagents used in this study were of analytical grade.

Figure 1: *Chaenomeles speciosa* fruits taken at 15, 30, 45, 60, 75, 90, 105, 120, 135, and 150 days after full bloom

Determination of agronomic characters

The typical *Chaenomeles* fruit growth and development dynamics from full bloom to mature stage are shown in Figure 1. Fruit length (FrL) and fruit diameter (FrD) were used to describe the elongation and radial growth of *Chaenomeles* fruits during the growth period. The fruit shape index (FrSI) was defined as the ratio of FrL to FrD to conveniently define fruit shape.

We randomly selected five subsamples of each batch to measure the fruit fresh weight (FrFW). Then, the whole fruit samples were washed and cut and deposited in trays at high temperatures. Subsequently, they were dried in a forced-air oven at 65°C until a constant mass was reached. After weighing the dried subsamples, fruit dry weight (FrDW) was recorded. Fruit desiccation rate (FrDR), the ratio of FrDW to FrFW, were then determined.

Sample preparation

Pretreatment of samples for determination of physicochemical characteristics

Sample powders (25 mg) were extracted with 25 mL of 75% methanol/water mixture solution in reflux extraction for 30 min at 80°C. The filtrate was collected by vacuum suction filtration and then concentrated using a rotary evaporator under vacuum until the solvent was removed. A mixed solvent of methanol and water was added to dissolve the residue and then the solution was transferred to a 10-mL volumetric flask. All sample solutions were stored at 4°C until subsequent testing.

Pretreatment of samples for monomeric compounds

Sample powders (2 g) were mixed with 50% ethanol in a 25 mL erlenmeyer flask and then extracted for 60 min by ultrasound (100 W). Erlenmeyer flask and then extracted for 60 min by ultrasound (100 W). After filtration and concentration, the residue was washed with 20 mL of water and acidified to pH 2.00, with 1 mol/L hydrochloric acid. Then, the residue was enriched and purified twice by *n*-butanol at an equal volume. The total extracts were recovered by solvent decompression and then filled with a mobile phase to a 10-mL volumetric flask. The samples were then filtered through a microfiltration membrane (0.45 μm) before HPLC.

Determination of total flavonoid content

The total flavonoid (TF) content was confirmed by the aluminum chloride colorimetric method[30] at different developmental stages of *Chaenomeles*. The sample solution (2.5 mL) was mixed with 10% sodium nitrite solution (0.75 mL) approximately 6 min after the reaction, and 10% aluminum nitrate solution (0.75 mL) was added to the mixed system. Subsequently, all the mixed solutions were added to the line in a 25-mL volumetric flask with 1 mol/L sodium hydroxide solution. Fifteen minutes later, the color solution was transferred and measured using a spectrophotometer and the absorbance was determined at λ = 506 nm. Rutin was used as the standard to draw the calibration curve, and the TF data were expressed as mg rutin per gram dry weight.

Determination of total triterpene content

The total triterpene (TT) content was measured according to the method described by Moraes Pedrosa *et al.*[31] A 0.2-mL portion of the vanillin-glacial acetic acid solution and 1 mL of sample solution was placed in a centrifuge tube using a pipette and then 1 mL sulfuric acid was added and the solution was incubated in a 60°C water bath for about 15 min. Subsequently, 5 mL of glacial acetic acid was added to the system. Absorbance was measured at λ = 550 nm using a spectrophotometer. Ursolic acid was used as a standard to draw the calibration curve, and the TT data were expressed as mg ursolic acid per gram dry weight.

Determination of total polyphenol content

Total polyphenol (TP) content was tested with Folin–Ciocalteu reagent using a previously reported method.[32] A 0.4-mL portion of Folin–Ciocalteu reagents was mixed with 0.4 mL sample solutions in a test tube for 3 min. Then, 1.2-mL sodium carbonate solution (0.188 mol/L) was added. The whole system was incubated under photophobic conditions for 2 h at 25°C. The solution was diluted with 2-mL ultrapure water, and the absorbance was measured at λ = 765 nm using a spectrophotometer. Gallic acid was used as a standard to draw the calibration curve, and the TP data were expressed as mg gallic acid per gram dry weight.

Quantification analysis of monomeric compounds by high-performance liquid chromatography

Many chemical compounds in *Chaenomeles* have provided beneficial activity. Shikimic acid, quinic acid, malic acid, protocatechuic acid, and chlorogenic acid were qualitatively and quantitatively analyzed by HPLC according to a previously described method[33] with minor modifications. The contents of the five compounds were quantitatively analyzed using an Agilent 1260 with a diode array detector (DAD) and a reversed-phase C_{18} column (250 mm × 4.6 mm, 5 μm, ultimate) at a flow rate of 1.0 mL/min. A binary solvent system was employed, consisting of acetic acid/water (0.1/99.9, v/v) as solvent A and acetonitrile as solvent B, and the gradient program was 0–5 min with 2% solvent B, 5–25 min with 2%–8% B, 25–45 min with 8%–12% B, and 45–60 min with 12%–24% B while the DAD detection wavelength was adjusted to the procedure as follows: 0–20 min with 210 nm, 20–40 min with 280 nm, and 40–60 min with 325 nm.

Oleanolic and ursolic acids were quantitatively tested based on the standard protocols of the People's Republic of China (Chinese Pharmacopoeia Committee, 2020). The mobile phase (0.6 mL/min) consisted of methanol–ultrapure water–acetic acid–triethylamine (v/v/v/v = 265/35/0.1/0.05). The column temperature was 30°C, and the sample injection volume was 10 μL at a 210-nm detection wavelength.

All the compounds in the HPLC chromatograms were identified by retention time using standard curves. Concentrations of oleanolic acid, ursolic acid, shikimic acid, quinic acid, malic acid, protocatechuic acid, and chlorogenic acid are shown in mg per gram dry weight.

Determination of antioxidant activity by diphenylpicrylhydrazyl radical scavenging activity

Sample powders (0.5 g) were extracted with 30-mL ethanol/water (v/v = 75/25) in an ultrasonic bath for 30 min. Then, the mixed liquids were dried under vacuum at room temperature and 75% ethanol was added to dissolve the residue to 10 mL. All sample solutions were stored at 4°C until subsequent testing.

DPPH free radical scavenging capacity was measured using the method described by Brand-Williams[34] with slight modifications. During the assay, 0.1-mL sample extracts with varying concentrations (0.5, 1, 1.5, 2, 4, 6, 8, and 10 mg/mL) were added to the DPPH ethanolic solution (0.1 M, 0.1 mL) in a microplate. The mixture was automatically shaken and then incubated for 20 min in the dark at 25°C using a microplate reader. The absorbance was read at λ = 517 nm, and the DPPH free radical scavenging capacity was calculated according to the following formula:

DPPH radical scavenging ratio (%) = $1-(A_{sample}-A_{control})/(A_{blank}-A_{control})\times100\%$

- A_{sample}: The absorbance of the sample solution and DPPH solution
- $A_{control}$: The absorbance of the sample solution and ethanol solution
- A_{blank}: The absorbance of water and DPPH solution.

The concentration for 50% of the maximal effect (EC_{50}) was used to represent the antioxidant activity using GraphPad Prism software.

Statistical analysis

All assays were performed in triplicate. The data set consisted of a matrix, in which rows represented the *Chaenomeles* fruit samples from ten different stages and columns indicated the contents of the chemical properties. The data matrix was imported into SIMCA-P software (ver. 13, Umetrics, Umea, Sweden) for multivariate statistical analysis, including principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA), to the division for development and maturity stages. The plot of variable importance for projection (VIP) from the PLS-DA was used to discover typical differential compounds for growth and development. Pearson's correlation analysis was performed using R statistics environment.

RESULTS AND DISCUSSION

Characteristics of *Chaenomeles* fruits at different developmental stages

The *Chaenomeles* fruits at ten stages of fruit development (15, 30, 45, 60, 75, 90, 105, 120, 135, and 150 DAFB) are shown in Figure 1. In terms of FrL, FrD, FrFW, and FrDW, the development of *Chaenomeles* fruits could be classified into two phases [Figure 2]. From 15 to 45 DAFB, *Chaenomeles* fruits grew rapidly. After 45 DAFB, *Chaenomeles* fruit growth slowed down and finally stabilized. Moreover, the fruit peel changed from green to yellow. After 120 DAFB, the *Chaenomeles* fruits entered the mature stage. This growth pattern also exists in other plants of the Maloideae subfamily, such as apples[35] and peaches.[36]

From 15 to 45 DAFB, the detection indexes showed a significant increase at the beginning of the observation period. The length changed from 41.20 ± 3.49 cm to 85.56 ± 3.89 cm, while the transverse diameter changed from 19.87 ± 1.45 cm to 47.31 ± 2.69 cm. This means that the former displayed about 1.44 times more growth than the latter. The fruit development was mainly longitudinal from 15 to 45 DAFB. In a previous study,[37] from 15 to 45 DAFB, *Chaenomeles* fruit pulp cells continually underwent cell division and an increase in the number of cells, which increased the volume of *Chaenomeles* fruits. Gradually, the cells of *Chaenomeles* fruit pulp stopped dividing and began to enlarge continuously. With the increase in cell volume and intercellular space,[38] the fruit mainly developed laterally and finally grew into an oval fruit. According to our results, from 45 to 150 DAFB, the FrL gradually stabilized and FrD increased slowly. According to the above results, the FrSI and FrDR could be deduced [Figure 2c]. The FrSI decreased from 1.99 to 1.16, demonstrating that the fruit changed from an elongated ovate to an oval shape. Interestingly, the FrFW and FrDW increased, meaning that the content of the fruits also increased. As the index of soluble solid content and water content, FrDR indicated that dry matter quality was one of the main indices used to evaluate the quality of fruit varieties. It decreased initially and then gradually increased[39] presenting the highest value of 0.1605 at 105 DAFB. Water has an indispensable influence on fruit development and maturation, and this has been proven in studies on muskmelon[40] and citrus.[41] The results showed that the absolute water content increased significantly with the development time, but the water percentage (83.6%–85.5%) did not increase significantly. From a numerical point of view, the fresh weight of *Chaenomeles* fruits increased 22.6 times, while the dry weight increased 21.7 times from 15 to 150 DAFB.

In summary, the present study shows that the fruits grew rapidly in the early stages of fruit development from 15 to 45 DAFB. The fruits grew steadily and matured gradually from 45 to 150 DAFB. However, it is difficult to determine the optimal harvest time for medicinal or edible *Chaenomeles* fruits based on agronomic traits. Therefore, further experiments are required.

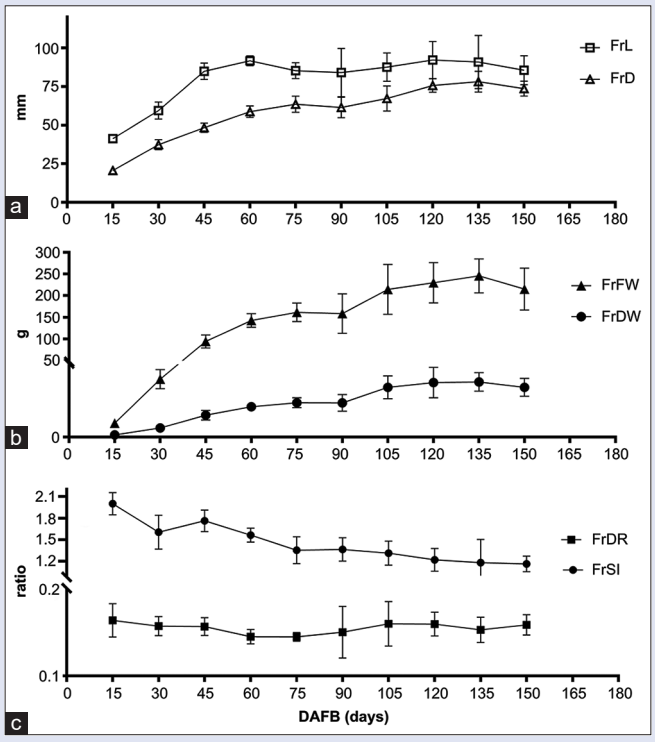

Figure 2: Dynamics of *Chaenomeles speciosa* fruit development at different stages. (a) Fruit elongation (FrL) and radial growth (FrD). (b) Fresh weight (FrFW) and dry weight (FrDW) during fruit development stages. (c) Fruit desiccation rate (FrDR) and fruit shape index (FrSI)

Harvesting time of medicinal *Chaenomeles* fruits revealed by chemical analysis

Previous reports have shown that flavonoids, polyphenols, and triterpenoids are active substances in *Chaenomeles* fruits.[20] The

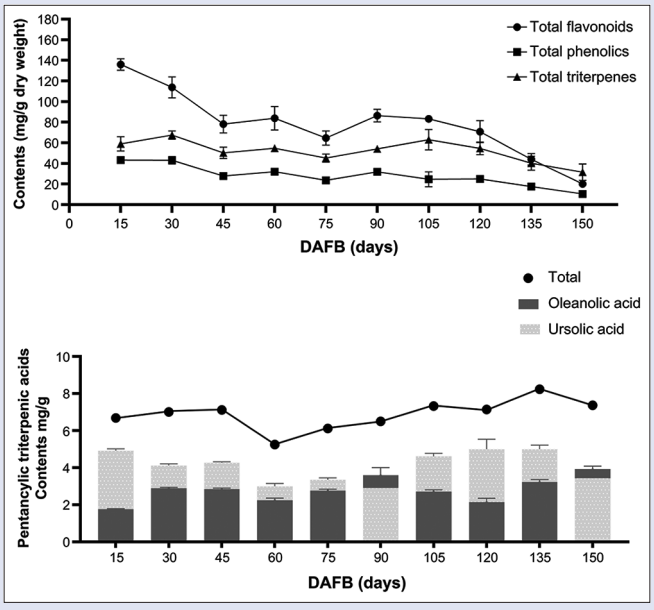

Figure 3: The contents of physicochemical characteristics and (b) pentacyclic triterpenic acid in different developmental stages of *Chaenomeles speciosa* fruits

measured total flavonoids, phenolics, and triterpenes are shown in Figure 3. Each data group had an average of three replicates and the precision was expressed as the standard error, as displayed. The X-axis shows the DAFB, while the Y-axis shows the equivalent of reference materials.

According to Figure 3, total flavonoids (TFs) ranged from 135.95 ± 5.56 to 20.05 ± 3.45 mg rutin per g dry weight. The change in TF content could be divided into three stages during the whole fruit growth process. The highest content of TFs was during the developmental stage at 15 DAFB. They decreased by 50% after 30 days. From 45 to 105 DAFB, the content fluctuated in the range of 78 mg and reached the highest value at 90 DAFB. From 105 to 120 DAFB, the level of TFs decreased sharply with the ripening of *Chaenomeles* fruits and remained at only 20.05 ± 3.45 mg/g, which was about 14% of the initial results. The contents of total phenolics (TPs) changed from 43.12 ± 1.54 to 10.26 ± 0.49 mg/g during the ten stages of the *Chaenomeles* fruit growth process. During the rapid growth stage, the amount declined by 40%. From 45 to 105 DAFB, the average value was approximately 27 mg/g. At the end of the developmental curve, the TP content was the lowest, which was one-fourth of the initial value at 150 DAFB. In addition, the contents of TTs, which had the smallest range of variation, also fluctuated around 55.05 mg/g. Moreover, from 105 DAFB, the contents of TTs decreased continuously till it reached the lowest value at 31.42 ± 7.97 mg/g, which was only about 61% of the average value during at 150 DAFB.

The results showed that the contents of these three substances decreased during the developmental process. Despite the differences in the levels of the three physicochemical substances in the stages of fruit development, some similarities were seen as follows: all the values began to decrease gradually at 90 or 105 DAFB. Both TFs and TPs had the highest value in the initial stage (15 DAFB), and all three physicochemical types had the lowest value at 150 DAFB. The TF content was 25% higher than the sum of contents of TPs and TTs at 15 DAFB, while the content of TFs reached only two-third of the content of TTs and twice the content of TPs at 150 DAFB. This indicates that flavonoids were lost rapidly during fruit development. The results showed that the contents of these three kinds of substances decreased with the development process, among which flavonoids, polyphenols, and triterpenoids decreased by 85.2%, 76.19%, and 46.67%, respectively. Studies have shown that flavonoids within plants play important roles, such as nutritional quality, stress response,[42] and disease resistance.[43,44] Therefore, we speculated that the sharp decrease in flavonoids during fruit development is related to various physiological changes that can be studied further. In the analysis of the results, we thought that the transformation of these three substances was regular. At 15–45 DAFB, the average decline rate of the three substances was the largest, followed by 45–105 DAFB, where the growth curve was relatively gentle and the fluctuation of substances was the minimal and maintained an average value. At 105–150 DAFB, the contents of all the substances dropped sharply. In fact, different extraction methods also affect the results.

Ursolic acid and oleanolic acid are the index components of *Chaenomeles* fruits (Chinese Pharmacopoeia Commission, 2020). They are pentacyclic triterpenoids and a pair of isomers. In order to accurately determine the appropriate harvest time for medicinal *Chaenomeles* fruits, we further analyzed ursolic acid and oleanolic acid at different developmental stages. Oleanolic acid content fluctuated from 1.765 ± 0.03 to 3.93 ± 0.157 mg/g, with an average of 2.8 mg/g. However, the content of ursolic acid changed from 4.922 ± 0.099 to 3.439 ± 0.125 mg/g. After 75 DAFB, the contents of ursolic acid and oleanolic acid continued to increase and gradually stabilized after DAFB 105. These results were consistent with those of previous studies.[45]

Overall, the results revealed that 105 DAFB is a critical period for fruit quality during the developmental stage of *Chaenomeles* fruits. In this period, there was a higher content of TFs, TTs, TPs, and pentacyclic triterpenoids compared with other stages. Consequently, 105 DAFB is the optimal time to harvest medicinal *Chaenomeles* fruits.

Harvesting time of edible *Chaenomeles* fruits in terms of organic acids

Chaenomeles fruits have a sour taste because of the high concentration of organic acids. Therefore, the optimal harvest time of edible *Chaenomeles* fruits was determined by analyzing the dynamic changes in organic acids during the development stages. Based on previous studies, we tested the main organic acids with a high content of dried fruit using HPLC with DAD. The accumulation trends of various components are shown in Figure 4.

The level of quinic acid decreased from 11.984 ± 1.016 to 3.487 ± 0.613 mg/g with each development stage. Chlorogenic acid decreased from 4.109 ± 0.33 to 0.253 ± 0.033 mg/g with each developmental stage. The opposite trend was observed for malic acid. The value of malic acid increased gradually during 15–60 DAFB, and following a short period of fluctuation, it reached its highest value of 16.152 ± 3.182 mg/g at 120 DAFB and then decreased gradually. The trend of protocatechuic acid was similar to that of malic acid. In the early development stage, protocatechuic acid gradually increased, reaching a peak value of 1.338 ± 0.075 mg/g at 45 DAFB and decreased rapidly to the lowest level of 0.343 ± 0.011 mg/g with fruit ripening. Shikimic acid showed a high level in the middle and early stages of fruit development (15–90 DAFB), with an average value of 2.54 mg/g. Later, it dropped rapidly to 1.708 mg/g at 105 DAFB.

In the initial stage of fruit development, quinic acid and protocatechuic acid represented the highest and lowest proportions, respectively. In the end stage of sampling (150 DAFB), malic acid was present in the highest proportion and chlorogenic acid was present in the lowest proportion. Over the development process, the largest difference in content was seen for chlorogenic acid, with a difference of 4.45 between the highest and the lowest levels, followed by quinic acid, protocatechuic acid, shikimic acid, and malic acid. It must be noted that there was a high quinic acid data error in 30, 45, 90, and 120 DAFB. This may be attributed to the strong polarity and sole terminal absorption of quinic acid, which causes unstable results.

In our study, it was determined that after 105 DAFB, the fruit entered the mature stage according to the agronomic characteristics, as well as the chemical properties of *Chaenomeles* fruits. These results are consistent with those in the literature.[39] It should be noted that the change in the levels of the total organic acids was extremely significant between 105 and 150 DAFB. The average content of organic acids at 105–120 DAFB was 31.29 mg/g while the content dropped to 25.52 mg/g at 135–150 DAFB. It could be concluded that the organic acids of *Chaenomeles* fruits participate in important physiological and biochemical activities during fruit development and ripening and the decrease in total organic acids may lead to the inhibition of organic acid synthesis, which were used for respiratory consumption and sugar conversion.[46,47] In addition, malic acid was the main contributor of organic acid in mature *Chaenomeles* fruits. This is similar to other Rosaceae fruits including apples,[48] pears,[49] and nectarines.[50] The sharp decline of total acids in the last growth period indicated that *Chaenomeles* fruits are more suitable for food development and utilization after 120 DAFB.

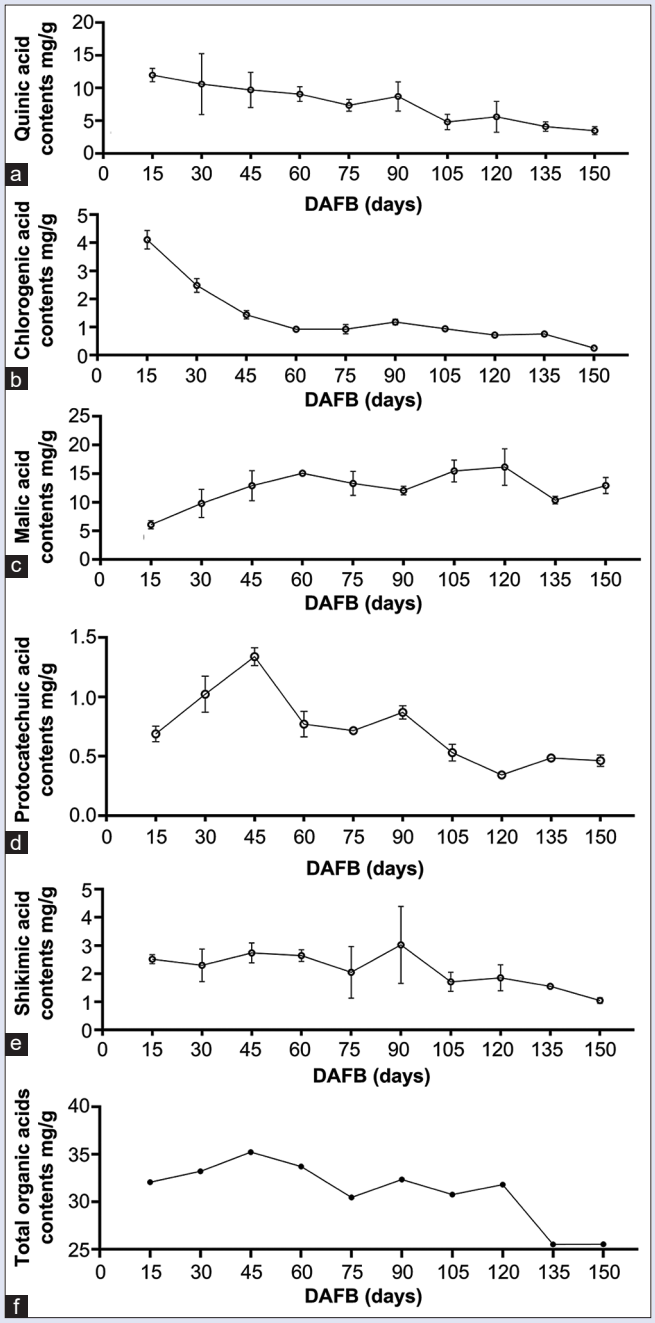

Figure 4: Accumulation law of organic acids in different samples of *Chaenomeles speciosa* fruits (a-f) represent quinic acid, chlorogenic acid, malic acid, protocatechuic acid, shikimic acid, and total organic acid contents

Antioxidant activity of *Chaenomeles* fruits in different growth stages

In this study, DPPH was used to evaluate the antioxidant activity at different developmental stages of *Chaenomeles* fruits. We prepared a series of samples with DPPH solutions reacting in the dark. The EC_{50} was calculated using GraphPad Prism software to deduce antioxidant capacity. The higher the EC_{50} value, the larger the sample concentration that would be used to consume the DPPH solution, which indicated a lower antioxidant capacity. The results are shown in Figure 5 where the abscissa represents development time and the ordinate shows the antioxidant capacity, which was recorded

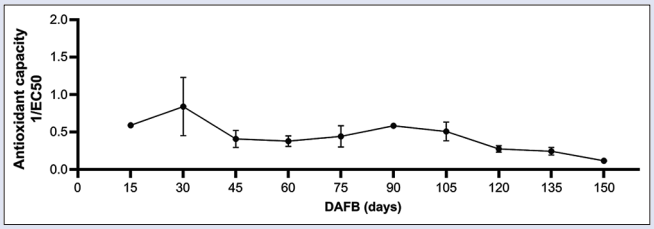

Figure 5: Antioxidant activity of *Chaenomeles speciosa* fruits in different developmental stages

as $1/EC_{50}$. According to the results, in the early development stages, the EC_{50} value was approximately 1.8 mg/mL. At 45–75 DAFB, the EC_{50} value increased by 1.7 times to 2.6 mg/mL, which showed that the antioxidant capacity decreased slightly. After that, the EC_{50} value showed a downward pattern and the lowest point of the curve occurred at 90–105 DAFB. From 105 DAFB, it increased sharply to 8.77 mg/mL. The results showed that the antioxidant capacity of fruits decreased with the maturation process.

The results were divided into two stages. Antioxidant activity was maintained at 2.0 mg/mL during 15–105 DAFB but decreased sharply with time after 105 DAFB, and the antioxidant capacity changed with the total organic acid content.[51] In addition, we know that *Chaenomeles* fruits are bioactive[15,16] and have a high content of ursolic acid, oleanolic acid, chlorogenic acid, protocatechuic acid, and quinic acid, which have excellent antioxidant effects.[20,52] Our research also showed that the young fruit of the *Chaenomeles* has a very good antioxidant effect. Cui *et al.*[53] reported that falling fruits are not only typical agronomic traits in crop cultivation and breeding but also one of the major forms of plant shedding. Shedding often permits young fruit to fall, leading to large amount of wastage. This experiment can also provide a theoretical basis and reference for the reuse of *Chaenomeles* fruit resources in early developmental stages.

Discovery of differential metabolites and developmental processes for *Chaenomeles* fruit samples

To elucidate the intrinsic similarities and differences between the *Chaenomeles* fruit samples at ten different developmental stages, a PCA and PLS-DA were performed based on the agronomic traits, chemical contents, and $1/EC_{50}$ value. As shown in the PCA, the samples can be classified into six groups and the PCA results show a gradual transition from 15 to 150 DAFB. To further identify differential metabolites for the six different developmental stages of the fruit, a supervised PLS-DA model was established using the same data as in the PCA. As illustrated in Figure 6, the samples were divided into six clusters in terms of their developmental stages, which also corresponded to the PCA results. The six developmental processes of *Chaenomeles* fruits could be divided as follows: 15, 30, 45–90, 105–120, 135, and 150 DAFB. Based on the VIP plot shown in Figure 6, ursolic acid, malic acid, protocatechuic acid, oleanolic acid, TT value, shikimic acid, and FrL with VI*P* values greater than 1 contributed to the separation of the ten different developmental stages. Combined with the agronomic traits of *Chaenomeles* fruits, the compounds, especially those with a VIP >1, were involved in important physiological and biochemical reactions, which led to significant differences between 15 and 30 DAFB. The fruit expansion stage, from 45 to 90 DAFB, was characterized by relatively stable properties. The fruits from 105 to 120 DAFB could be harvested as a Chinese medicine because of the presence of high secondary metabolites and active compounds in them. From 135 to 150 DAFB, the fruits had the lowest organic acid content and were suitable for use as a flavored fruit or ingredient in processed products in theory.

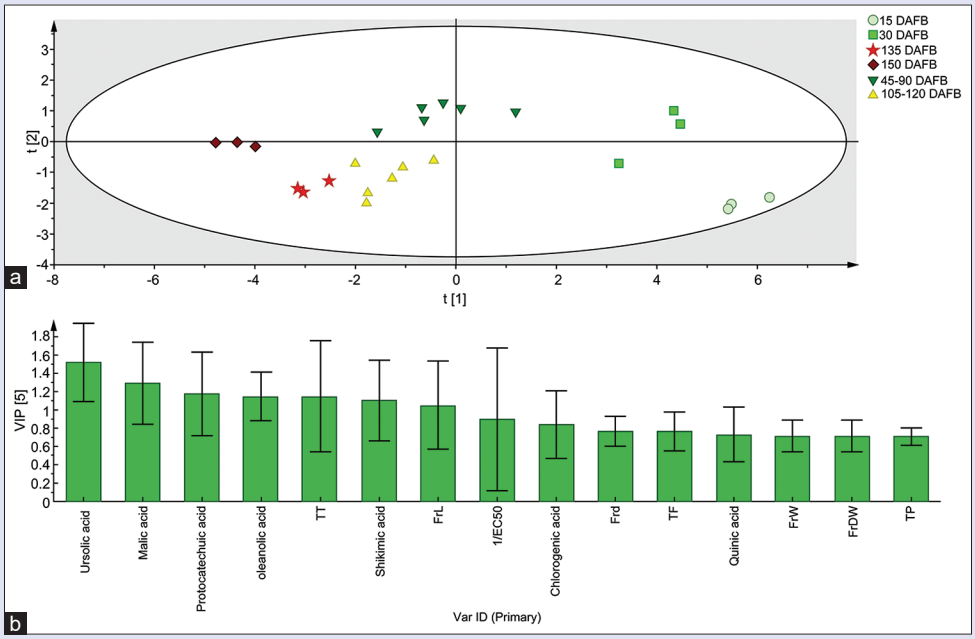

Figure 6: (a) The partial least squares-discriminant analysis scores of *Chaenomeles speciosa* fruits divided into six different growth processes and (b) the variable importance for projection plot of all indices in different developmental stages

Correlation analysis

A Pearson's correlation analysis was performed for all characteristics using the "complot" R package. The correlation was color-coded according to the value of the coefficient of warm and cool colors with positive and negative correlations, and the size of the circle was scaled according to its absolute value. The darker and larger the circle, the higher the value and the stronger the correlation.

As Figure 7, strong positive correlations were observed among the three physicochemical characteristics (TFs, TPs, and TTs) and the Pearson's correlation coefficients were all >0.71. In addition, chlorogenic acid was positively correlated with TFs, TPs, TTs, and quinic acid. TPs were positively correlated with protocatechuic acid and shikimic acid. In addition, malic acid had positive correlation coefficients of more than 0.51 with FrD, FrL, FrFW, and FrDW. FrSI showed positive correlations with TFs, TPs, TTs, quinic acid, protocatechuic acid, shikimic acid, chlorogenic acid, and oleanolic acid. These results indicate that quinic acid, protocatechuic acid, shikimic acid, and chlorogenic acid are related to the weight and volume of fruit development. Furthermore, obvious negative correlations were observed between malic acid and TFs and TPs and between oleanolic acid and TFs, TPs, TTs, quinic acid, and chlorogenic acid. Quinic acid, protocatechuic acid, shikimic acid, chlorogenic acid, TFs, TPs, and TTs were all negatively correlated with FrD, FrL, FrFW, and FrDW. In terms of activity, TF, TP, TT, quinic acid, chlorogenic acid, and protocatechuic acid were all related to the antioxidant capacity of DPPH, with Pearson's correlation coefficients >0.4. These results showed that flavonoids, polyphenols, and triterpenoids were the important active ingredients in *Chaenomeles* fruits for their key role in promoting antioxidants.[28,54] Quinic acid and shikimic acid also significantly promoted the DPPH scavenging ability of fruits.

Perhaps, the most surprising result in this study was the correlation between ursolic acid and DPPH scavenging ability. Pentacyclic triterpenoids are the index components of *C. speciosa* medicinal materials, and they were not significantly related to antioxidant activities during development.

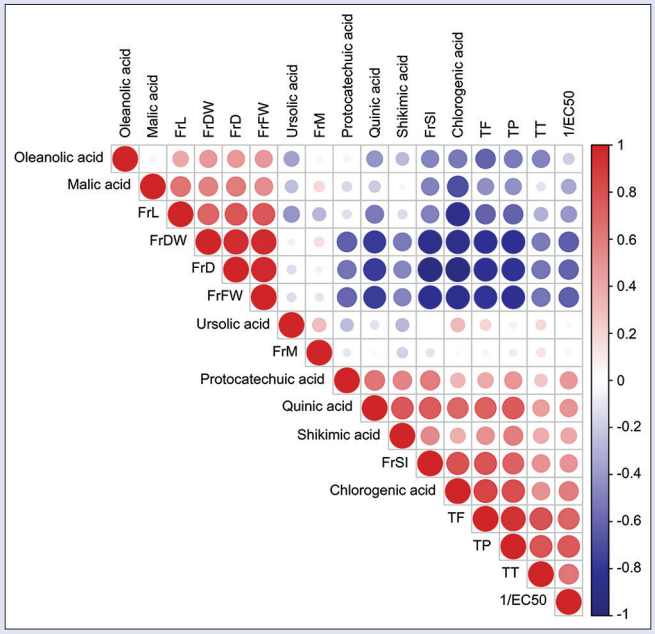

Figure 7: Pearson's correlation analysis of the determination indices in different developmental stages of *Chaenomeles speciosa* fruits

CONCLUSION

This study proposed the agronomic characteristics, chemical quality, and bioactivity of *Chaenomeles* fruits at different developmental stages. This is the first study to analyze the growth periods and harvesting times of medicinal and edible *Chaenomeles* fruits. The results showed that the developmental processes of *Chaenomeles* fruit can be divided into six clusters: 15 DAFB and 30 DAFB, which represent the fast growth period, 45–90 DAFB, which represent the slow growth period, 105 DAFB, which represent the harvesting time and most critical period, 105–120 DAFB,

which represent the more suitable for medicinal use, and 120 DAFB, post which the organic acid content sharply decreases making the fruit conducive to taste and use as an edible fruit. This is consistent with the information provided in pharmacopeia for *Chaenomeles* fruit. Our study contributes to identifying the appropriate harvest time of *Chaenomeles* fruits for medicinal and edible purposes. We recommend that more research is done on the potential utilization of young *Chaenomeles* fruits.

Although organic acids can affect the flavor, they are not the only determinants of the best time for eating *Chaenomeles* fruits. In this paper, the growth stages were segregated based on the components at different developmental stages of *Chaenomeles* fruits, as they can provide evidence for the optimal harvesting time of medicinal and edible *Chaenomeles* fruits. For practical application, this result still needs to be verified.

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Conflicts of interest

There are no conflicts of interest.

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