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Analysis of the Flavor and Active Compounds in *Lonicera japonica* Wine Produced by a New Extraction and Fermentation Method

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Abstract *Lonicera japonica* species have been used as an adjuvant therapy for physical fitness, due to their extensive biological activity and pharmaceutical properties. For example, this species demonstrates antimicrobial, anti-inflammatory and detoxifying effects. Therefore, the changes in the chlorogenic acid, amino acid, and total polyphenol contents and in the antioxidant activity were analyzed. The results showed that the content of chlorogenic acid was 2378.63 mg/L without residue fermentation, 2039.05 mg/L with residue fermentation and 1476.74 mg/L with liquor extraction, which were increases of 1.86, 1.53 and 0.87 times more, respectively, than the chlorogenic content in unfermented *L. japonica* broth. The total content of polyphenols during the fermentation time had a positive linear correlation and the following values were obtained; fermentation without residue was 0.73 mg/mL, fermentation with residue was 0.62 mg/mL, and liquor extraction was 0.45 mg/mL. The clearance rate of ABTS and DPPH reached approximately 95%. Moreover, flowers could be used to avoid the production of methanol and fusel oil. Therefore, the nutrients and active functional components in L. japonica and the flower flavor of L. japonica could be preserved. This suggested fermentation process is better than the liquor extraction process and is a safe and controllable metabolic process.

Keywords: *Lonicera japonica, chlorogenic acid, polyphenol, antioxidant activity, liquid fermentation*

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1. Introduction

L. japonica is a species of the *Lonicera* genera of dry buds and primary flowers, which are also known as honeysuckle in China, and commonly used in traditional Chinese herbal medicine [1] to treat pneumonia, swelling, infection, and breast cancer [2,3]. This species has important potential uses in pharmaceutical preparations, cosmetics and health care products, such as toothpastes, herbal teas and food additives in beverages [4,5]. This species produces various natural bioactive compounds with effects on human health, such as antiviral and anti-inflammatory reactions [6,7,8] Additionally, these bioactive compounds can significantly enhance the immunological function of human cells and body fluids, exhibiting a high effect in aqueous solution. Previous studies have shown that *L. japonica* has a significant inhibitory effect on *Staphylococcus aureus* and *Streptococcus pneumonia* [9]. Additionally, *L. japonica* extracts have anticoagulation, hypolipidemic and hypoglycemic effects [10]. These pharmacological effects are derived from the various functional active substances, such as chlorogenic acid, volatile oil, flavonoids, pectin [11], saponins, inositol, luteolin, polysaccharides, phenolic acids and iridoids [12,13].

In particular, the most important active ingredient is chlorogenic acid, which is an ester compound formed by the condensation of quinic acid (QA) with trans-cinnamic acid (t-CA) [14]. A previous study revealed for the first time the structural formula of chlorogenic acid. Common trans-cinnamic acids are caffeic acid, p-co-phthalic acid (p-CoA) and ferulic acid [15]. The type and amount of hydride affects the esterification of trans-cinnamic acid with quinic acid. Thus, chlorogenic acid has a variety of isomeric compounds, the most common of which is 3-O-caffeoylquinic acid (3-CQA), which is commonly known as chlorogenic acid [16].

At present, the biological function of chlorogenic acid has been widely used in the food, beauty, medicine and

chemical industries [17] because chlorogenic acid has the following wide range of benefits for the treatment of clinical diseases: antioxidant, analgesic, and antibacterial behavior [18]; liver and nerve protection [19]; heat removal; detoxification; antiradiation, anti-obesity, and antihypertensive behavior [20,21]; immune system regulation and nerve center activation [22,23]. In addition, studies have shown that chlorogenic acid can regulate lipid metabolism and the physiological metabolism of glucose as well as diseases related to a healthy metabolism, thereby assisting in the treatment of many diseases, such as liver steatosis, cardiovascular disease, diabetes and obesity [24].

L. japonica wine is a type of deeply processed product with flowers as raw materials. People commonly drink *L. japonica* tea or other *L. japonica* products to gain certain effects. The idea of brewing *L. japonica* wine is based on an ancient Chinese pharmacopeia that included some flower-soaked wines that could cure some diseases according to the characteristics of the active ingredients in different flowers [25]. However, the active ingredients in these wines are only obtained through a simple extraction method, and the pharmacological effects have been poorly studied. The development of a new type of *L. japonica* wine and its effects constitute a new focus on the production of low-grade wine with health care functions. In this study, we aimed to compare two methods of fermentation and extraction and their effect on the bioactive components in *L. japonica* wine, thus providing a basis for the development of wine with health benefits.

2. Materials and Methods

2.1. Chemicals and Reagents

Chlorogenic acid and amino acid standards (purity 98.0%, HPLC grade) were obtained from Shanghai Yuanye Bio-Technology Co., Ltd. (China) and Alta Technology Co., Ltd (USA). Formic acid was obtained from Merck (Germany). Other analytically pure reagents used in this study were from Sinopharm Group Chemical Reagent Co., Ltd (Shanghai, China).

2.2. Microorganisms and Culture Conditions

*Saccharomyces cerevisiae*EC1118 was obtained from Presque Isle Wine Cellars (Pennsylvania, USA). The use of this yeast is advantageous due to its good comprehensive fermentation ability, while having the best tolerance for high alcohol concentrations that are between 18 and 20%. Furthermore, *EC1118* yeast can ferment a variety of vegetables and flowers but not fruits.

Aroma-producing yeast was obtained from Angel Yeast Co., Ltd., Hubei, China. Additionally, yeast can enhance wine flavor.

Saccharomyces cerevisiae EC1118 strain activation: Dry yeast (1 g) was dissolved in 9 mL of a sterile saline solution 100 times, and then 100 μL of the yeast solution was absorbed and coated on a solid plate of yeast extract/peptone/dextrose (YPD) medium and placed in an incubator at 28 ℃ for 48 h. *Saccharomyces cerevisiae* with a full colony shape was selected and inoculated in wort medium (wort 70 mL, natural pH of 6.4, the plate was inverted and sterilized at a high temperature for 20 min) for activation and cultured in a shaking table at 28 ℃ and 150 r/min for 16 h to obtain a seed liquid. The seed liquid inoculation scale was 5% (v/v), which was then joined with a new wort medium to continue culturing according to the culture method, thereby obtaining the yeast activation solution.

Aroma-producing yeast activation: A 2.5% saccharose solution (mass fraction) was mixed with 10 times the amount of dry yeast, and the temperature was adjusted to 35 ℃. The yeast was dissolved in the saccharose solution and activated at 35 ℃ for approximately 1 h. In the process of fermentation, the inoculation scale was 0.1% (v/v).

2.3. Preliminary Treatment of Raw Materials and Fermentation Pathways of *L. japonica*

The optimum time to harvest *L. japonica* is early June in the morning, when the buds are not completely opened, remain full of nutrients, and have a strong smell and good color. Harvested buds were vacuum dried at 50 ℃ and 0.09 MPa for 16 h.

2.4. Dry Processing

First, *L. japonica* was stored at -80 ℃ and dried at 50 ℃ and 0.09 MPa for 16 h in a vacuum drying oven according to the vacuum drying method [26]. This process was used to obtain dried *L. japonica*. After treatment, the water content of *L. japonica* was less than 5%, so its fragrance was completely released.

2.5. Preparation of Fermentation Broth

L. japonica broth was divided into three batches.

The first was fermented without residue. *L. japonica* (50 g) and pure water (200 mL) were mixed and crushed in a beater for 2 min at 60000 r/min. The samples were filtered through gauze to obtain a clarified broth.

The second was fermented with residue. *L. japonica* (50 g) and pure water (200 mL) were mixed and crushed in a beater for 2 min at 60000 r/min. It was not filtered in the process and contained residue.

Liquor extraction was kept as the control group. *L. japonica* (50 g) and 200 mL anhydrous liquor were mixed and crushed in a beater for 2 min at 60000 r/min.

These three batches of *L. japonica* broth were poured into consecutive fermentation tanks, and 800 g of pure water was added. Each treatment had three replicates.

2.6. Fermentation Process

The addition of 150 g glucose was dissolved fully, and the food grade citric acid was adjusted to pH=3.5-4. To dissolve the *L. japonica* plant and release the fermentable sugars, 1 g pectinase and 1.5 g cellulase were added to hydrolyze *L. japonica* at 40 ℃ for 30 min. After that, the broth was sterilized at 115 ℃ for 20 min, inoculated with *Saccharomyces cerevisiae* EC1118 (0.3% v/v) and aroma-producing yeast (0.1% v/v), and then shaken well at a rotation speed of 150 r/min for 12 h. Alcohol fermentation was carried out under aseptic conditions for

30 days at 25 ℃. The fermentation process was performed according to referenced methods [27,28,29].

Finally, *L. japonica* wine was obtained by fermentation and extraction (Figure 1).

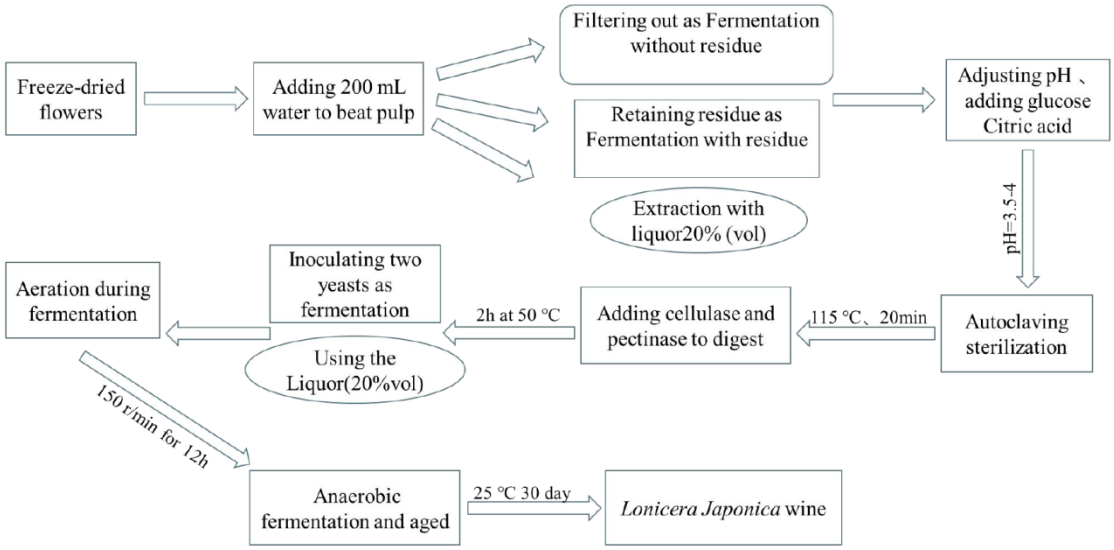

Figure 1. Brewing process of *Lonicera japonica* wine

2.7. Component Detection

Qualitative analysis of flavor compounds by GC-MS in *L. japonica* wine

Chromatographic conditions: Inlet temperature of 220 ℃, column temperature of 35 ℃ for 5 min, ramp rate of 5 ℃/min to 100 ℃ for 2 min, ramp rate of 15 ℃/min to 230 ℃ for 10 min, carrier gas flow rate of 1 mL/min, and column db-wax (60 m×250 μm×0.25 μm).

Mass spectrometry conditions: The 70 eV electron ionization (EI) mode of the EI ion source at 230 ℃ and a scanning mass range of 30-500 amu.

After centrifugation, the sample passed through a 0.22 μm filter membrane, and then 5 mL was injected into an analysis bottle for machine testing.

Sample processing: The fermentation and extraction broth was directly taken and centrifuged for 5 min at 5000 r/min. Then, approximately 1 mL of supernatant was decanted, passed through a 0.45 μm filter membrane, and injected into the sample bottle with a syringe. Approximately 2 mL of the wine sample was placed into a 15 mL headspace bottle, and 5 mL of deionized water was added, saturated with NaCl, and extracted with ultrasonication for 30 min.

Injection sample method: Headspace solid phase microextraction.

2.8. Quantitative Analysis

Determination of methanol, ethanol, total acid, pH value, and ethyl acetate in *Lonicera japonica* wine [30].

The glucose content was measured by using the SBA-90 biosensor.

2.9. Determination of the Total Polyphenol Content

The total polyphenol content of *L. japonica* wine was determined using the Folin-Ciocalteu method [31].

Preparation of the standard curve: The standard sample was diluted 0, 10, 50, and 100 times. Additionally, 5.0 mL of 10% Folin reagent was added, and the reaction was shaken for 5 min. Then, 4.0 mL of sodium carbonate solution (7.5%) was added and shaken to blend the constituents together. After reacting for 60 min at room temperature, the absorbance was measured at 765 nm. Finally, according to the experimental OD value to confirm the optimal dilution, a standard curve of polyphenols was established. Additionally, the fermentation broth was determined using the method described above.

2.10. Detection of Chlorogenic Acid

The content of chlorogenic acid was determined according to the national standard detection method [32]. A high-performance liquid chromatography (HPLC-20A, SHIMADZU) instrument was used for detection.

Liquid chromatography conditions: ODS C18 column (250 mm×4.6 mm, 5 μm), flow rate of 1.0 ml/min, column temperature of 35°C, sample volume of 10 μL, detection wavelength of 325 nm, and a 9:1 ratio of 0.5% acetic acid solution:acetonitrile. The standard curve of chlorogenic acid was established based on the peak area, and according to the standard curve, the content of chlorogenic acid in the *L. japonica* wine samples was calculated.

2.11. Analysis of the Antioxidant Activities of *Lonicera japonica* Wine

ABTS (2,2'-diazo-bis-3-ethylbenzothiazolin-6-sulfonic acid): The ABTS radical scavenging activity was determined using a modified method [33]. The prepared working fluid (ABTS+$K_2S_2O_8$) was diluted 40 times with 95% ethanol (analytical grade) so that the absorbance was 0.7±0.02 at 734 nm.

ABTS reserve solution (7.4 mmol/L): A mixture of 96 mg of ABTS and 25 mL of distilled water.

$K_2S_2O_8$ reserve solution (2.6 mmol/L): Briefly, 378.4 mg of K2S2O8 and 10 mL of distilled water were mixed

together. Then, 5 ml of 7.4 mmol/L ABTS reserve fluid and 88 μL of 2.6 mmol/L $K_2S_2O_8$ were mixed and allowed to stand for 12-16 h to produce the ABTS working fluid.

Sample experiment: Briefly, 3.9 mL of the diluted solution was mixed with 0.1 mL of sample, and the absorbance was measured at 734 nm after standing at room temperature for 6 min in the dark. The result was recorded as the absorbance of sample A. Then, 3.9 mL of the dilution was mixed with 0.1 mL of 95% ethanol solution for 6 min, and the absorbance was measured at 734 nm. As a result, A_0 was recorded as a blank control group and A was recorded as a sample result.

$$Clearance = (A_0 - A) / A_0 \times 100\%$$

DPPH (1,1-diphenyl-2-trinitrophenylhydrazine): The DPPH radical scavenging activity was determined using a modified method [34]. In brief, 7.8 mg of DPPH was weighed in a volumetric flask, dissolved in anhydrous ethanol and diluted to the desired volume in the dark. Then, 3 mL of DPPH-ethanol solution was added to 3 mL of sample solution A. It was well mixed and left in the dark at 37 ℃. The absorbance value was measured at 517 nm after 30 min. Absolute ethanol instead of DPPH was taken as the control A_1, and absolute ethanol instead of sample solution was taken as the blank A_0.

The clearance rate of DPPH was calculated according to the following formula: DPPH clearance rate (%) = $[1-(A-A_0)/A_1] \times 100\%$.

2.12. Determination of Amino Acids

Preparation of the standard solution: Each standard sample of a single amino acid was weighed in the same beaker, dissolved in 8.3 ml of 6 mol/L hydrochloric acid solution, and accurately transferred to a 250 mL volumetric flask. The standard reserve solution of amino acids was prepared by diluting the volume to the desired scale with water. A total of 1.0 mL of standard amino acid reserve solution was accurately transferred to a 10 ml volumetric flask, a pH 2.2 sodium citrate buffer solution was added, and then the solution was mixed; the resulting solution was the standard analysis liquid.

Samples were measured with ninhydrin, and the OD values of the samples were controlled between 0.1-1.5. Samples were filtered through a 0.45 μm membrane and transferred into liquid phase sample vials for measurement. An automatic amino acid analyzer (L-8900 basic amino acid analyzer: Hitachi High-tech Co., LTD., Tokyo, Japan) was used to detect amino acids based on the reference method [35].

2.13. Observation of the *Lonicera japonica* Internal Structure by Scanning Electron Microscopy

Sample preparation: The samples of *L. japonica* wine were absorbed as drops and placed on a quartz plate. The samples were dried completely at room temperature and sprayed with platinum to improve the conductivity of the samples.

2.14. Statistical Analysis

Each experiment was repeated three times. All the data are expressed as the mean ± SD (standard deviation). IBM SPSS Statistics was applied for statistical and variance (ANOVA) analysis with a significance level. Additionally, Graph Pad Prism 6.01 was used.

3. Results

3.1. GC-MS Analysis Results for *Lonicera japonica* Wine

GC-MS was used to identify the volatile flavor compounds (Table 1). These compounds included alcohols, esters, acids, aldehydes, ketones, and volatile compounds. The differences in flavor compounds between fermentation with residue and fermentation without residue were not obvious. The main metabolites consisted of eighteen types of esters, five alcohols, two aldehydes, six terpene classes, and four acids, while the composition of flavor substances after liquor extraction was lower, with eight esters, four alcohols, two aldehydes, two terpenes, and three acids. Table 1 analyzes the differences in the flavor substances of untreated *L. japonica* wine, including the following common substances: isoamyl caprylate, hexadecanoic acid ethyl ester, ethanol, 2-methylpropanol, 2-methylbutanol, benzene ethanol, acetal, benzaldehyde, transeugenene, imidodicarbonic acid, 2,4,5-trimethyl-1,3-dioxalane, acetic acid, 2,3-O-benzyl-d-mannosan, and thiosemicarbazone.

In terms of qualitative flavor compounds, *L. japonica* wine had the largest number of ester compounds, and these esters mostly came from the fermentation process that consisted of the esterification of alcohol with acid. Due to the high content of ethanol in liquor, more ethyl ester was formed through yeast fermentation, and this ethyl ester accounted for a large proportion of the ester.

The results of the physicochemical characterization of *L. japonica* wine after full brewing are shown in Table 2. As *L. japonica* itself contains a low sugar content, some exogenous food - grade sugar was added in the brewing process, which raised the final alcohol content to approximately 17 vol% after fermentation.

There was no significant difference in the alcohol content of the experimental groups. The extracted alcohol level of *L. japonica* wine remained the same because liquor has no microorganisms; thus, the *L. japonica* broth did not undergo secondary fermentation, and the total acid content was higher with a low pH.

3.2. Polyphenol Comparison

The content of polyphenols in the samples was calculated through the use of ultraviolet spectrophotometry, which was based on the calibration curve of gallic acid and with the OD value of the sample measured at a wavelength of 765 nm. The change trend of the polyphenol content with increasing fermentation time was found to be linear (Figure 2, left Y).

Table 1. Main volatile flavor compounds identified in *Lonicera japonica* wine using GC-MS

Compound	With residue	Without residue	Chinese liquor extraction	Flavor description
Ethyl acetate	++	++	+	Pineapple aroma, fruity
Ethyl octanoate	+	+	-	Fruity and sweet
Ethyl valerate	++	++	+	The fruit is sweet
Ethyl hexanoate	++	+	-	Fruity
Ethyl caprate	++	+	-	Fruity
Ethyl 3-methyl butyrate	++	+	-	Ester and fruity
Ethyl laurate	+	+	-	Floral, fruity
Dodecyl isoamyl ester	+	+	-	Fat flavor
Ethyl tetradecanoate	+	+	-	Iris incense
Ethyl hexapecate	++	++	+	Fruity
Ethyl hexadecanoate	+	+	-	Cream sweet
Linoleic acid ethyl ester	+	+	-	Flowers fragrant
Ethyl oleate	+	+	-	Flowers fragrant
Pelargonic acid ethyl ester	+	+	-	
Linalyl acetate	++	++	+	Osmanthus fragrance
Ethyl benzoate	+	+	-	Honey, flowers
3-methyl- 1-Butanol	+	+	-	Grape wine
4-heptyl phenol	+	+	-	Chinese medicine
Ocimene	+	+	-	Lilac flowers
Linalool	+++	++	+	
Terpineol	+	+	-	
Terpine oil	+	+	-	Lemon zest
Beta - Malaysia	+	+	-	Sweet candy day
Geranyl ethyl ether	+	+	-	Rose fragrance
1-[3-Aminopropyl]-pyridone	+	+	-	
5-Aminohexanoic acid	+	+	+	
Succinic acid,	+	+	-	Sour fragrance
Sebacicdihydrazide	++	++	+	
1-Ethyl-3-thiourea	+	+	-	
Phenylethyl Alcohol	-	-	+	
4-Methyl-2-hexanol	-	-	+	
Citronellol	++	++	+	
D-Glucitol	-	-	+	

[+] indicates that the compound was present; [-] indicates that the compound was not present; The more +, the higher the content.

Table 2. Detection of physiochemical indicators in *Lonicera japonica* wine

Indicators	Without residue	With residue	Liquor extraction
Methanol(mg/L)	-	-	234±0.86
Ethanol(%voL)	17±1.26	16±1.15	18±0.79
Total acidity (Acetic acid, g/L)	6.65±0.04	5.15±0.28	6.36±0.15
pH value	3.86±0.07	3.65±0.13	3.91±0.12
Glucose (g/L)	12.1±0.06	11.6±0.04	85.56±4.11
Ethyl acetate(mg/L)	30±0.09	28±0.17	17±0.15

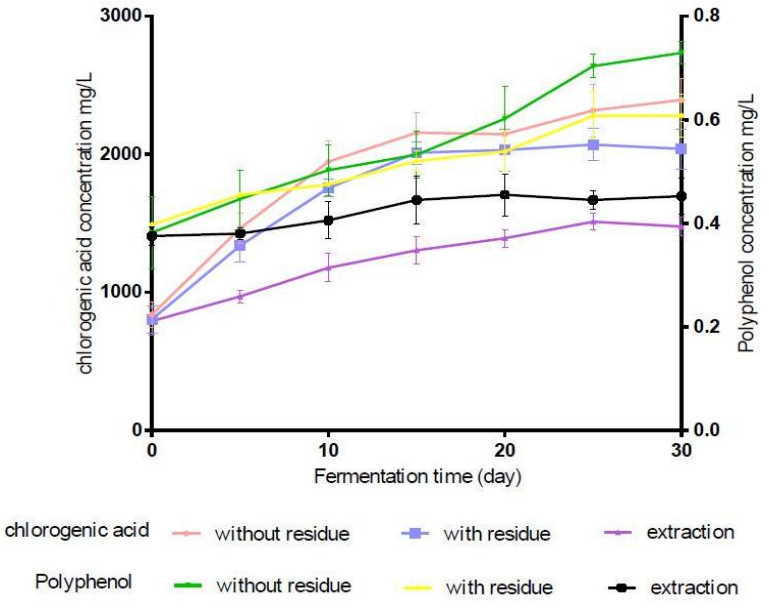

Figure 2. Relationship of polyphenols changes in *Lonicera japonica* wine at different fermentation time

The content of polyphenols reached the maximum value, and the content of polyphenols in *L. japonica* wine reached a satisfactory level. After the 30-day fermentation period, the polyphenol content increased significantly and exhibited the following values: fermentation without residue was 0.73±0.02 mg/mL, fermentation with residue was 0.61±0.03mg/mL, and liquor extraction was 0.45±0.03mg/mL. Figure 3 illustrates the statistical significance between the fermentation and extraction data. The content of polyphenols was clearly higher than that of unleavened broth, which might reflect the impact of the yeast strain on the phenolic extraction and bioconversion during fermentation by regulating their metabolism and interaction processes.

3.3. Comparison Results of Chlorogenic Acid

In the process of fermentation, the chlorogenic acid content was positively correlated with increasing fermentation time (Figure 3, left X). After fermentation was terminated, the concentration of chlorogenic acid in the fermented samples was significantly higher than the chlorogenic acid concentrations after liquor extraction and in the unfermented broth; the yield of chlorogenic acid increased by 1.85, 1.53, and 0.87 times (for fermentation with residue, fermentation without reside, and liquor extraction, respectively) compared with that of unfermented broth. Compared with direct extraction, yeast fermentation was more effective in improving the content of chlorogenic acid, and the rate of formation was also more rapid than that with liquor extraction. SPASS software was used to analyze the data of each group, and the results showed that the experimental data had statistical significance (Figure 4). Therefore, fermentation and extraction had significant effects on the composition and content of ***L. japonica*** wine.

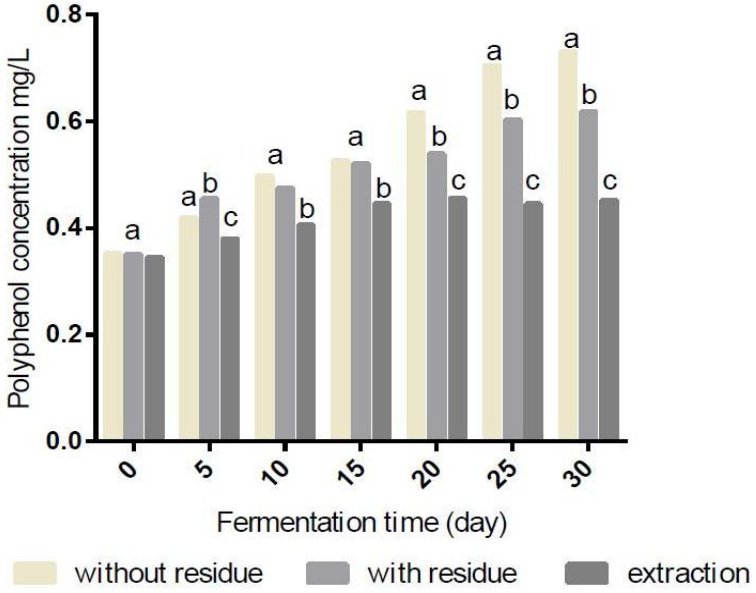

Figure 3. Effects of different treatment methods on polyphenol content in *Lonicera japonica* wine. Different letters above the bars indicate significant difference according to least significant difference test at $p<0.05$

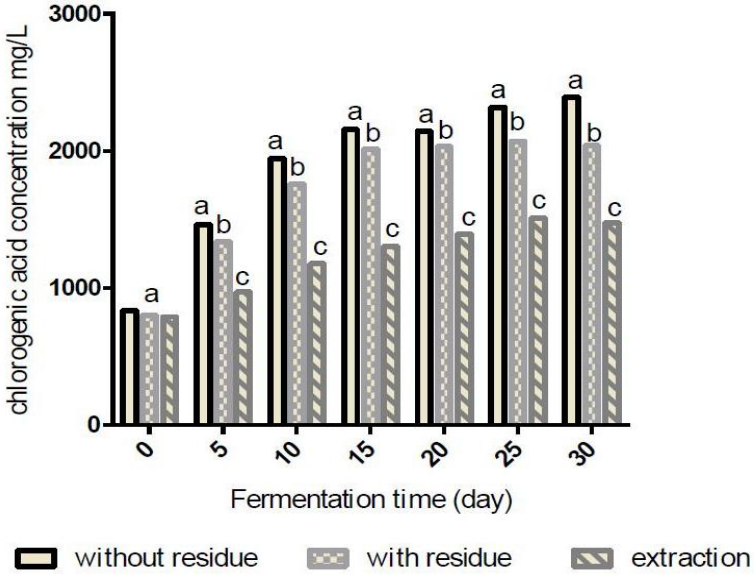

Figure 4. Effects of different treatment methods on chlorogenic acid content in *Lonicera japonica* wine. Different letters above the bars indicate significant difference according to least significant difference test at $p<0.05$

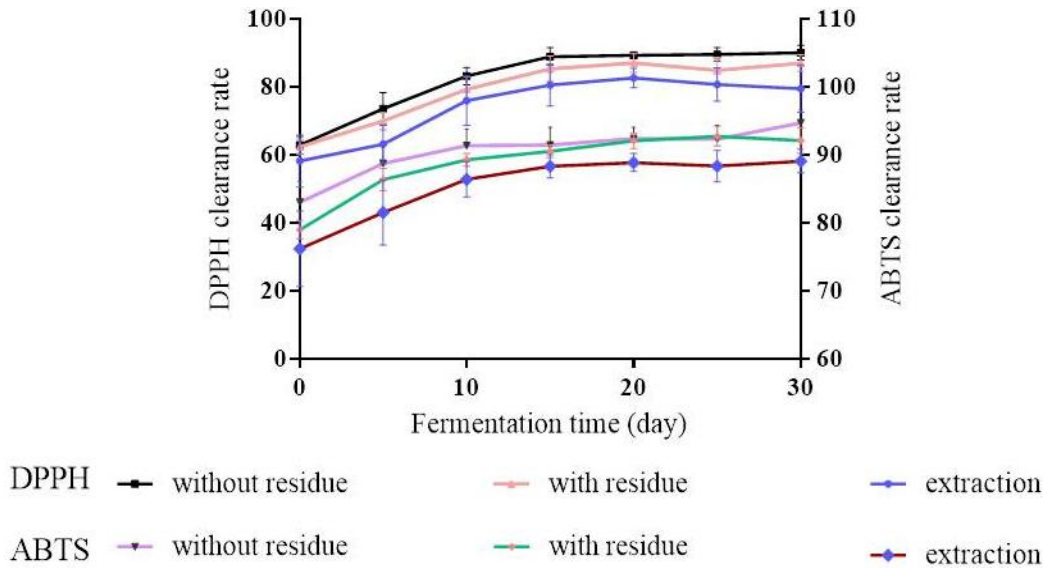

Figure 5. Comparison of ABTS and DPPH clearance rate among three wine samples in Lonicera japonica wine

3.4. Comparison of Antioxidant Activities

The linear correlation between the total polyphenols and antioxidant activity (ABTS and DPPH values) of the *L. japonica* wine was analyzed. The ability of polyphenols to scavenge free radicals of ABTS and DPPH in three types of *L. japonica* wines was observed (Figure 5). This indicated that the *L. japonica* broth could improve the rate that ABTS scavenges free radicals after yeast fermentation. The initial clearance rate of the raw broth was approximately 87%, and the three types of wines after fermentation reached 94%.

According to this characteristic, many cosmetic industries could use fermented *L. japonica* in certain amounts as a raw material in various products that may benefit from an increased antioxidant capacity to delay aging.

Our results suggested that the variations in the total polyphenol content and antioxidant activity among the *L. japonica* wines with yeast fermentation were distinct from those in the control group that underwent liquor extraction.

3.5. Results of the Amino Acid Analysis

The changes in amino acids in *L. japonica* wine during the brewing process were detected by an automatic amino acid analyzer (Table 3). Either the number of amino acid species sharply decreased or most of the amino acid content decreased, thereby showing that the amino acid type, content and production wine yeast microbes, were closely related. In the brewing process, in addition to Asp, Thr, Glu, Gly, Cys, Val, Tyr, and Lys, which are not used by yeast, the rest of the present amino acids would be consumed in a large amount by microbial activities, and the decarboxylation reduction reaction would occur; this reaction would result into corresponding advances in the wine and the production of some volatile esters. The amount of amino acids also clearly decreased in the liquor extraction control group because most of the free amino acids were soluble in ethanol, thus effectively solving the damage of ethanol to the human body. The decrease in free amino acids is beneficial for increasing the flavor of *L. japonica* wine. The next step was to further explore the specific metabolic process of amino acids under the action of yeast.

Table 3. Determination of free amino acid components (unit mg/L)

name	original broth	Without residue	With residue	Liquor extract
Asp	2.97±0.01	0.06±0.00	0.05±0.00	-
Thr	7.28±0.07	0.17±0.01	0.09±0.01	0.15±0.01
Glu	7.82±0.10	1.91±0.05	1.48±0.02	1.71±0.07
Gly	1.27±0.04	0.02±0.00	0.02±0.01	0.04±0.01
Ala	12.97±1.07	-	-	-
Cys	0.30±0.11	0.48±0.03	0.42±0.06	0.47±0.07
Val	3.22±0.14	0.46±0.01	0.43±0.02	-
Ile	1.34±0.05	-	-	-
Leu	1.02±0.01	-	-	-
Tyr	0.93±0.03	0.66±0.02	0.82±0.04	-
Phe	1.81±0.12	-	-	-
Lys	2.04±0.17	0.60±0.01	0.64±0.03	1.70±0.06
His	1.05±0.02	-	-	0.10±0.01
Arg	2.23±0.40	-	-	-
Pro	29.27±3.56	-	-	-

not detection.

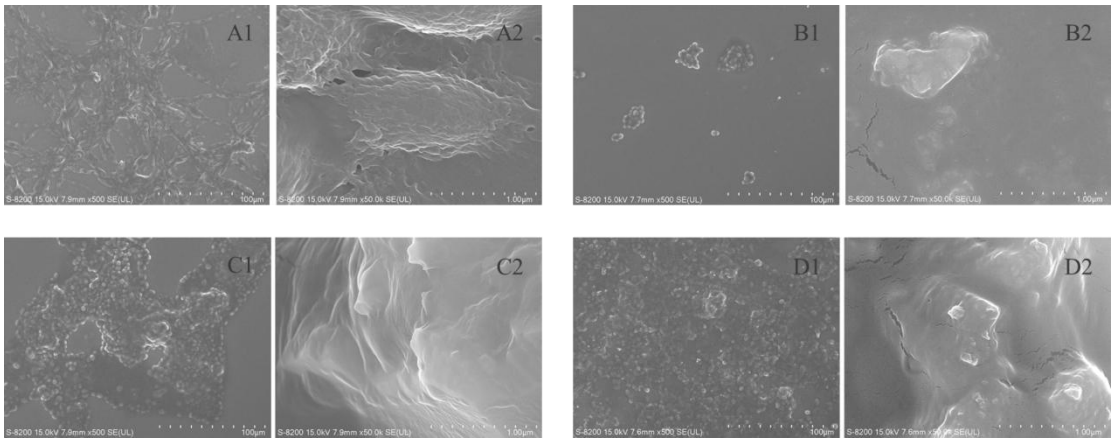

Figure 6. Morphological changes of *Lonicera japonica* wine by treatment with fermentation and extraction during the experiment, photos were taken at the same location. A represents *Lonicera japonica* solution without any treatment; B represents *Lonicera japonica* wine of fermentation without slag; C stands for *Lonicera japonica* wine of fermentation with slag; D represents *Lonicera japonica* wine of extraction by liquor. 1, 2 stands for observation using scanning electron microscope at 500, 50000x respectively

3.6. *Lonicera japonica* Degradation

L. japonica was degraded by yeast fermentation. The morphological structure of the four samples was observed by using scanning electron microscopy (Figure 6), which could directly show the difference of the samples before and after fermentation. Figure A shows the *Lonicera japonica* broth without fermentation. The *L. japonica* fibrous tissue was very tight, with no decomposition by external forces, and the whole surface structure was complete. Figures B and C show the *L. japonica* broth after fermentation. It could clearly be seen from the graph that the structure of the plant tissue had been destroyed and the internal organization of *L. japonica* was broken down to form a new structure. Therefore, we provided evidence of a new transformation produced by fermentation. Figure D shows that the plant tissue of *L. japonica* is not completely destroyed and becomes loose after liquor extraction.

These results demonstrate that the *L. japonica* broth would undergo changes in its internal structure after yeast fermentation because of the transformation of some enzymes. Thus, the more valuable substances in *L. japonica* were decomposed and incorporated into the wine.

4. Discussion

L. japonica was subjected to a fermentation treatment, which significantly increased the amount of active substances in the *Lonicera japonica* wine without affecting its original compounds. The production of *L. japonica* wine through yeast metabolism could produce many beneficial flavor substances (Figure 7).

On the other hand, the chlorogenic acid and total polyphenol contents in all the fermentation-treated *L. japonica* samples were significantly higher than those from the liquor extraction control group.

Polyphenols are very important molecules in many wines and are responsible for their quality and sensorial characteristics, such as taste and color. Compared with other wines with health benefits, the polyphenol concentration in Tower brand yellow wine was 0.544 mg/mL; Huizelong yellow rice wine was 0.303 mg/mL; and Kuaijishan was 0.430 mg/mL [36]. The total content of polyphenols in wine were closely related to the antioxidant capacity. Thus, the content of polyphenols affected the antioxidant capacity. Many studies have reported positive correlations between the total content of polyphenols and the antioxidant activity of different wines, including white, red, fruit and glutinous rice wines [37,38]. The results showed that the antioxidant activity of the *Lonicera japonica* wine reached a relatively high level.

Chlorogenic acid is the main active ingredient in *L. japonica*. Its leaching rate was very low in the initial test. After fermentation, this active ingredient of *L. japonica* was extracted, and its nutritional value was higher than that of simply drinking *L. japonica* tea. Throughout the fermentation process, the content of chlorogenic acid reached a maximum value and then started to decline until reaching a steady state. This result was satisfactory and demonstrated a certain theoretical basis for the medicinal value and extraction of chlorogenic acid. It could be further speculated that the increase in chlorogenic acid content was attributed to three major factors. One was that chlorogenic acid in plant cells was extracted by organic solvents such as ethanol produced during fermentation. The second was that chlorogenic acid was converted into shikimic acid by the addition of glucose under the catalysis of enzymes in plant cells. In this work, we identified a third factor in that a chlorogenic acid precursor compound was formed during fermentation. The new chlorogenic acid formed by the esterification, substitution, and condensation reactions of quinic acid and ferulic acid, caffeic acid, and shikimic acid was then converted to phenylalanine before a new chlorogenic acid was finally synthesized [39]. Furthermore, 3-O-feruloylquinic acid (3-FQA), 3,4-di-O-caffeoylquinic acid (3,4-CQA), and 3,5-di-O-caffeoylquinic acid (3,5-CQA) were metabolically produced [40,41]. These main secondary chlorogenic acid metabolites were one of the reasons for the increase in chlorogenic acid content. This result was verified by the literature in which 3,5-di-O-caffeoylquinic acid, 3-O-caffeoylquinic acid, 4,5-di-O-caffeoylquinic acid and 3,4-di-O-caffeoylquinic acid were reported to be the main secondary metabolites in suspension culture cells [42].

In addition, the *L. japonica* broth was treated at high temperature and pressure, and some active substances

might be released during sterilization. It has been reported that when *L. japonica* is treated under high pressure, the contents of major phenolic acids and flavonoids, such as chlorogenic acid, caffeic acid and luteolin, significantly increase. This observation is probably due to the release of cell wall-bound phenolic compounds. The antioxidant activities of *L. japonica* were significantly increased after the high-pressure treatment [43]. Figure 8 shows the metabolic condensation reactions of quinic acid and caffeyl-CoA. It was first discovered that quinic acid, coenzyme A and ATP were essential for the chlorogenic acid synthesis pathway, and it was believed that chlorogenic acid would not be produced without quinic acid. Moreover, Stckigt *et al*. found that caffeyl-CoA was an inevitable intermediate in the chlorogenic acid synthesis pathway. Later, Ulbrich *et al*. isolated hydroxylated cinnamic acid-CoA:hydroxycinnamoyl-CoA (quinate hydroxycinnamoyl transferase, HQT) from two plant cells, and this enzyme could catalyze caffeyl-CoA and quinine to produce chlorogenic acid; thus, they believed that HQT was a key enzyme that directly produced chlorogenic acid.

The solubility of chlorogenic acid would increase in aqueous solution. Chlorogenic acid is soluble in ethanol, acetone, and methanol, slightly soluble in ethyl acetate, but hardly soluble in chloroform, ether, benzene and other lipophilic organic solvents. Chlorogenic acid is a polar organic acid that is not very stable, so it is easy to isomerize in the process of extraction or fermentation. Therefore, this study greatly improved the content of chlorogenic acid by fermenting *L. japonica* broth with yeast, and the medicinal value of *L. japonica* was correspondingly improved.

On the other hand, the content of amino acids in the *L. japonica* wine were mainly from the enzymatic hydrolysis of the raw material proteins during the fermentation of metabolites and the autolysis of yeast cells after fermentation [44,45,46]. During the fermentation of *L. japonica*, the transformation and formation of various compounds were related to the presence of certain amino acids. For the flavor analysis of the *L. japonica* wine, aromatic ester substances mainly presented a fruity aroma, which was most of the aromatic substance variety in flower wine. Most of the aromatic compounds were floral, which could enhance the aroma and coordination of the wine body. Additionally, these compounds mainly came from the catabolism of aromatic amino acids in the raw material. Due to the sugar and high carbon content in the raw materials used for producing the *Lonicera japonica* wine, the carbon atoms formed saturated hydrocarbon chains after yeast fermentation. There was little alcohol variety, which mainly stemmed from the conversion of sugars and amino acids in the fermentation stage

In addition, the *L. japonica* wine also contained some synthesized drug substances, namely, linalool, terpineol, and terpene oil compounds. This study showed that linalool was one of the major pharmacological substances in *L. japonica*, and as a functional active substance, linalool has been proven to reduce pulmonary inflammation [47]. Most terpenoids show inhibitory activity against HBsAg and HBeAg secretion and HBV DNA replication. These results indicated that *L. japonica* flower buds could serve as a functional food for antihepatoma and anti-HBV activities [48]. Thus, *Lonicera japonica* wine has potential for being applied in a health care role.

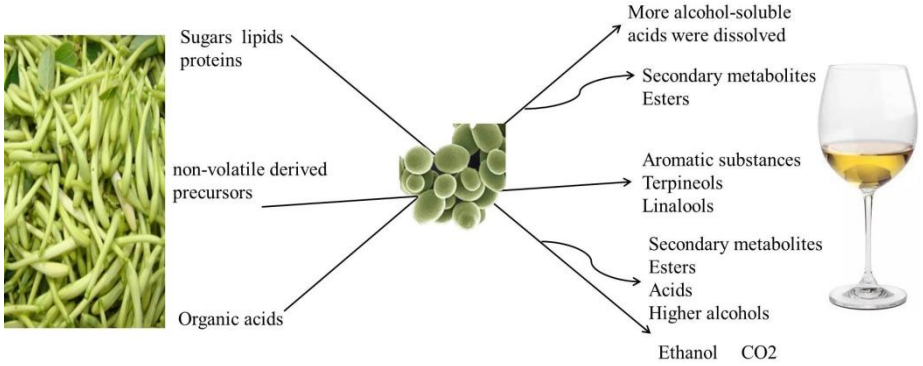

Figure 7. Main metabolites of the *Lonicera japonica* fermentation process and produces some flavor through yeast metabolism

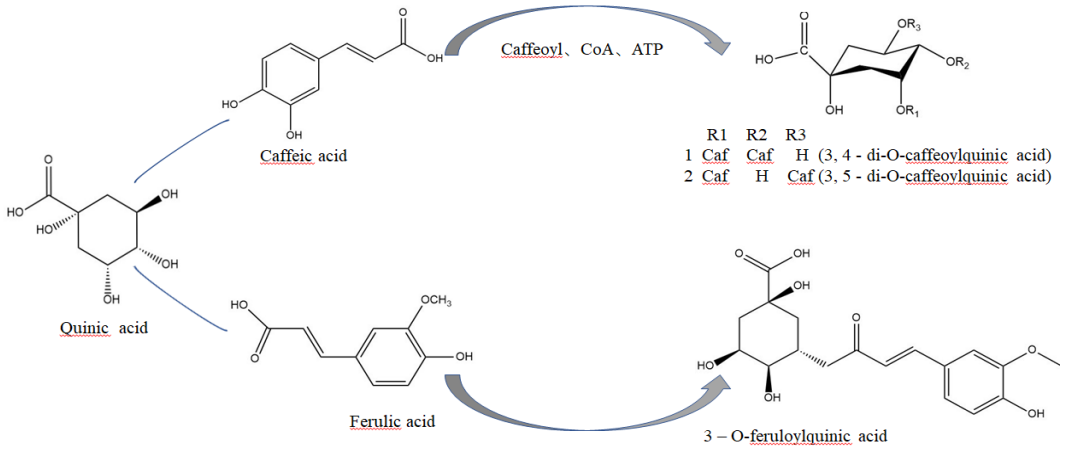

Figure 8. Biochemestry formation pathway of chlorogenic acid

5. Conclusion

In this work, we developed a healthy functional wine through a new liquid fermentation method with a low alcohol content; furthermore, the flavor and active components in the *L. japonica* wine were analyzed.

The pharmacological composition and function of *L. japonica* showed that yeast fermentation increased the main nutritional components, namely, the contents of chlorogenic acid and polyphenols, the oxidation resistance activity, and the number of metabolites and flavor compounds. The *L. japonica* wine contained various esters, alcohols, aldehydes, terpene classes and acids. Another feature was that flowers were used as the raw material to avoid the production of methanol and fusel oil. Methanol and fusel oil are produced when grain is added as an auxiliary material, which is common in the production of conventional flower wine. Thus, the production of methanol was eliminated from the raw material, and the flavor compounds and active functional components were synthesized by yeast metabolism during fermentation. Therefore, the nutrients and active functional components in *L. japonica* and the flower flavor of *L. japonica* could be preserved. This suggested fermentation process is better than the liquor extraction process and is a safe and controllable metabolic process.

Author Contribution

Thanks to all authors for their contributions to this manuscript, Xinli Liu designed the research; Xiaolong Zhou, Wenting Ruan, Guoxiang Lin performed the experiments; Xiaolong Zhou analyzed the data and wrote this manuscript. In the end, Borras Orlando and Jing Zhao provided guidance and revision of the manuscript.

Disclosure Statement

The authors declare no conflicts of interest.

This study does not involve any human or animal testing.

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