Effects of Fermentation Process on Content and Functions of Total Flavonoids in *Gardenia jasminoides*

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Abstract [**Objectives**] To explore the effects of fermentation process on the content and functions of flavonoids in *Gardenia jasminoides*. [**Methods**] *G. jasminoides* was fermented by microorganisms, and the fermentation process of total flavonoids from *G. jasminoides* was optimized, and the antioxidant activity and hyaluronidase inhibitory activity of the fermentation broth were tested. [**Results**] The best strain for fermentation of total flavonoids in *G. jasminoides* was *Bacillus subtilis*. The optimum fermentation conditions were as follows: the solid-liquid ratio was 1∶30, the inoculation amount was 2%, and the fermentation time was 24 h. Under these fermentation conditions, the content of total flavonoids in *G. jasminoides* reached 36.90 mg/g, which was 45.22% higher than that of the control group without microbial fermentation, and it had good DPPH free radical and hydroxyl free radical scavenging ability, and the inhibition ability of hyaluronidase after fermentation was also improved. [**Conclusions**] This study provides a technical reference for the comprehensive application of *G. jasminoides*.

Key words *Gardenia jasminoides*, Flavonoids, Fermentation, Anti-oxidation

1 Introduction

Gardeniae Fructus is the dry mature fruit of *Gardenia jasminoides* Ellis of Rubiaceae. It is a traditional Chinese medicine. It is recorded in *Compendium of Materia Medica* that it tastes bitter, cold and non-toxic. In addition, it is recorded in *Chinese Pharmacopoeia* that it can be used for the treatment of jaundice, red urine, blood stranguria, metrorrhagia and metrostaxis, febrile disease, vexation, red eyes, swelling and pain, *etc.*[1]. Studies have found that *G. jasminoides* mainly contains flavonoids, iridoids, phenolic acids, triterpenes and organic acid esters and other chemical components[2-3]. Flavonoids have anti-inflammatory, antioxidant, antibacterial, anti-aging and other biological activities[4], and are widely used in the daily chemical industry. At present, the research on *G. jasminoides* mainly focuses on the extraction process and component analysis, and there are few reports on microbial fermentation of *G. jasminoides*.

Microbial fermentation is used as a method to improve product performance, and various biochemical changes in the fermentation process can change plant components and increase the content of active substances[5]. In this study, *G. jasminoides* was fermented by microorganisms, and the effects of strain, solid-liquid ratio, inoculum size and fermentation time on the content of total flavonoids in *G. jasminoides* were explored. Under the optimal fermentation conditions, the *in vitro* antioxidant activity and hyaluronidase inhibitory activity of the fermentation broth were further evaluated in order to provide technical reference for the comprehensive application of *G. jasminoides*.

2 Materials and methods

2.1 Materials

2.1.1 Raw materials and reagents. *G. jasminoides* (produced in Jiangxi, batch No.: 231107), rutin standard (purity 98.5%, Shanghai Nature Standard Biotechnology Co., Ltd.), and other reagents were analytically pure; the water used in the laboratory was ultrapure water. *Bacillus subtilis* was purchased from Shangcheng BNCC Biotechnology Co., Ltd.; yeast was purchased from Angel Yeast Co., Ltd., Lactobacillus plantarum was purchased from Jiangxi Renren Health Micro-ecological Technology Co., Ltd.

2.1.2 Main instruments and equipment. SW-CJ-1G ultra-clean workbench (Suzhou Purification Equipment Co., Ltd.); DNP-9082 electrothermal constant temperature incubator (Shanghai Yiheng Scientific Instrument Co., Ltd.); THZ98C thermostatic oscillator (Shanghai Yiheng Scientific Instrument Co., Ltd.); ZDX-35SBI vertical pressure steam sterilizer (Shanghai Boxun Medical Biological Instrument Corp.); WFZ UV-3802 ultraviolet-visible spectrophotometer (Unico (Shanghai) Instrument Co., Ltd.).

2.2 Methods

2.2.1 Pretreatment of *G. jasminoides*. Weighed a proper amount of traditional Chinese medicine *G. jasminoides*, pulverized in a multifunctional crusher, sieved the pulverized *G. jasminoides* with an 80-mesh sieve, and stored the pulverized *G. jasminoides* at room temperature in a dark and dry environment for later use.

2.2.2 Selection of fermentation strain. Weighed a proper amount of *G. jasminoides* powder, added according to solid-liquid ratio of 1∶20, and sterilized the mixture at 121 ℃ for 20 min. After cooling to room temperature, the mixture was inoculated with 1% of yeast, *Bacillus subtilis* and *Lactobacillus plantarum* seed solution, and fermented at 180 r/min and 37 ℃ for 24 h.

2.2.3 Selection of inoculation dosage. Weighed appropriate amount of *G. jasminoides* powder, added with water according to solid-liquid ratio of 1∶20, sterilized at 121 ℃ for 20 min, cooled to room temperature, and inoculated with *B. subtilis* seed solution at 1%, 2%, 3%, 4% and 5% of the total inoculation dosage, respectively; fermented at 180 r/min, 37 ℃ for 24 h.

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2.2.4 Selection of solid-liquid ratio. Weighed appropriate amount of *G. jasminoides* powder, added water according to solid-liquid ratio of 1∶5, 1∶10, 1∶15, 1∶20, 1∶25 (g∶mL), sterilized at 121 ℃ for 20 min, cooling to room temperature, inoculated *B. subtilis* seed solution according to 1% inoculation dosage, fermented at 180 r/min, 37 ℃ for 24 h.

2.2.5 Selection of fermentation time. Weighed appropriate amount of *G. jasminoides* powder, added water at solid-liquid ratio of 1∶20, sterilized at 121 ℃ for 20 min, cooled to room temperature, and inoculated with *B. subtilis* seed solution at 2%, and fermented at 180 r/min, 37 ℃. The fermentation broth was sampled every 4 h to determine the content of flavonoids.

2.2.6 Orthogonal experiment design. On the basis of previous single factor experiment, the inoculation dosage of *B. subtilis*, fermentation time and solid-liquid ratio were used as factors, and the content of flavonoids was used as the investigation value, and the experiment was carried out according to the orthogonal design table. Other fermentation parameters were 180 r/min, 37 ℃.

Table 1 Orthogonal experimental design for fermentation process of *Gardenia jasminoides*

Level	Factor		
	Inoculation dosage (A)	Fermentation time (B)	Solid-liquid ratio (C)
	%	h	g/mL
1	1	20	1∶20
2	2	24	1∶25
3	3	28	1∶30

2.2.7 Extraction of total flavonoids. At the end of fermentation, absolute ethanol was added until the ethanol concentration in the solution was 70%, and then the solution was placed in an ultrasonic cleaning machine and extracted for 30 min under the conditions of ultrasonic power of 100 W and ultrasonic frequency of 40 kHz, and filtered and determined the total flavonoid content in the solution[6].

2.2.8 Determination of content of total flavonoids. The content of total flavonoids was determined by ultraviolet spectrophotometry: 10.0 mg of rutin standard substance was accurately weighed, dissolved in 60% ethanol, and the constant volume was 100 mL to prepare 0.1 mg/mL of rutin standard solution. Pipetted 0.0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0 mL of rutin standard solution and 1.0 mL of sample solution separately into a 10 mL test tube with a stopper, added 60% ethanol solution to make up to 5 mL, added 0.3 mL of 5% sodium nitrite solution, shook up and let stand for 6 min. Added 0.3 mL of 10% aluminum nitrate solution, shook well, and then let stand for 6 min. Added 4 mL of 5% sodium hydroxide solution, shook well, and then let stand for 15 min. Measured the absorbance at 510 nm[7-8]. The standard curve was plotted with the concentration of rutin as the abscissa and the absorbance value as the ordinate, and the standard equation was $y = 12.612x - 0.035\ 4$ ($R^2 = 0.997\ 7$). Finally, calculated the content of the total flavonoids in the sample using the Formula (1):

$$\text{Content of the total flavonoids (mg/g)} = (C \times V \times n)/m \quad (1)$$

where C represent the concentration of the flavone in the extracting solution, V denotes the volume of the extracting solution, n represents the dilution multiple, m represents the mass of *G. jasminoides* leaves.

2.2.9 Determination of *in vitro* antioxidant activity. DPPH free radical scavenging activity: determined according to the method of Mollaei *et al.*[9] with minor modifications. First, 2 mL of DPPH solution (0.1 mmol/L) was mixed with 2 mL of the diluted fermentation broth of *G. jasminoides* (10 mg/mL). After shaking, the mixed solution was placed in the dark for reaction for 30 min, and the absorbance was determined at 517 nm after the reaction. Vitamin C was used as positive control instead of *G. jasminoides* fermentation broth in the same way. All experiments were repeated three times. The DPPH radical scavenging ability was determined by the Formula (2):

$$\text{DPPH free radical scavenging rate (\%)} = (A_0 - A_1)/A_0 \times 100 \quad (2)$$

where A_0 represents the absorbance of the control (sample without *G. jasminoides* fermentation broth); A_1 represents the absorbance of the sample.

Hydroxyl radical scavenging activity was determined by salicylic acid method[10], 0.5 mL of diluted *Gardenia jasminoides* fermentation broth (10 mg/mL), 0.5 mL of 2 mmol/L salicylic acid-absolute ethanol solution, 0.5 mL of 1 mmol/L Fe_2SO_4 solution, 1 mL of 2 mmol/L H_2O_2 solution was added into the test tube in turn, fully shaken and mixed, and then stood for 30 min, and the absorbance was measured at the wavelength of 510 nm. The hydroxyl radical scavenging capacity was calculated using the Formula (3).

$$\text{Hydroxyl free radical scavenging rate (\%)} = [1 - (A_1 - A_2)/A_0] \times 100 \quad (3)$$

where A_0 represents the absorbance of the control (sample without *G. jasminoides* fermentation broth); A_1 represents the absorbance of the sample; A_2 represents the absorbance without ferrous sulfate solution.

2.2.10 Determination of hyaluronidase inhibitory activity. With reference to Zhang Xingqi's method for hyaluronidase inhibition[11], and the specific steps are as follows: take 0.1 mL $CaCl_2$ solution (0.25 mmol/L) and 0.5 mL hyaluronidase solution (1 000 U/mL) in water bath at 37 ℃ for 20 min; add 0.5 mL of sample solution, keep the temperature for 20 min, then add 0.5 mL of sodium hyaluronate solution (0.5 g/L), react for 30 min, take out and cool for 5 min; add 0.1 mL of sodium hydroxide solution (4 mol/L) and 0.5 mL of acetylacetone solution (3.5 mL of acetylacetone dissolved in 50 mL of 1.0 mol/L sodium carbonate solution), and immediately transfer to an ice-water bath for 5 min after a boiling water bath for 15 min; add dropwise 1 mL of Ehrli-

ch reagent (0.8 g of p-dimethylaminobenzaldehyde dissolved in 15 mL of concentrated hydrochloric acid and 15 mL of absolute ethanol), dilute with 1 mL of absolute ethanol, place at room temperature for 20 min to develop color, and measure the absorbance at 540 nm, then calculate that inhibition rate of the extract to hyaluronidase according to the Formula (4):

$$\text{Inhibition rate of hyaluronidase} = [(A-B)-(C-D)]/(A-B)\times 100 \quad (4)$$

where A is the absorbance of the reference solution (acetic acid buffer solution is used to replace the sample solution); B is the absorbance of the reference blank solution (acetic acid buffer solution is used to replace the sample solution and enzyme solution); C is the absorbance of the sample solution; D is the absorbance of the sample blank solution (acetic acid buffer solution is used to replace the enzyme solution).

3 Results and analysis

3.1 Effects of different strains fermentation on total flavonoids in *G. jasminoides* It can be seen from Fig. 1 that the fermentation of *Lactobacillus plantarum* has no effect on the content of total flavonoids in *G. jasminoides*, but reduces it. The fermentation of yeast and *B. subtilis* is helpful to improve the content of total flavonoids in *G. jasminoides*, of which *B. subtilis* has the best effect, and the content of total flavonoids in *G. jasminoides* is 29.95 mg/g after fermentation. Therefore, it is more appropriate to select *B. subtilis* as the fermentation strain.

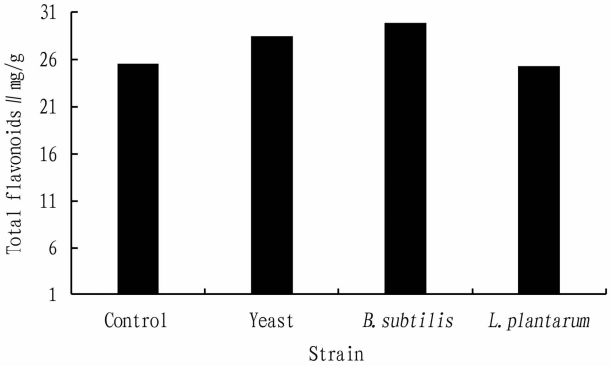

Fig. 1 Effect of different strains on the content of total flavonoids in *Gardenia jasminoides*

3.2 Effects of different inoculation dosages on total flavonoids in *G. jasminoides* The inoculation dosage is related to the growth and metabolism of the strain, and the appropriate inoculation dosage will affect the number of viable bacteria in the fermentation substrate, thereby affecting the fermentation effect. When the inoculation dosage is low, the amount of bacteria in the fermentation broth increases slowly, and when the inoculation dosage is high, there will be competitive inhibition among the bacteria, so it is necessary to find a suitable inoculation dosage. The experimental results are shown in Fig. 2. When the inoculation dosage of *B. subtilis* is 2%, the content of total flavonoids in *G. jasminoides* is the highest.

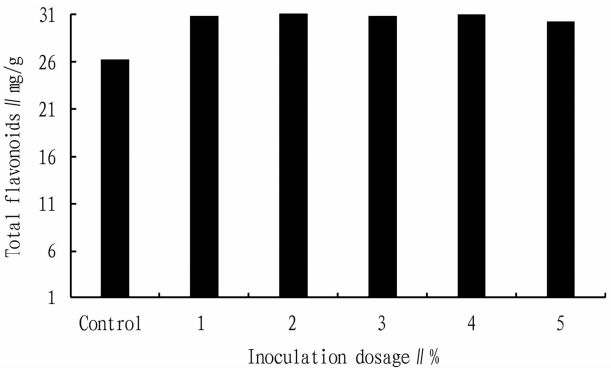

Fig. 2 Effects of different inoculation dosages on total flavonoids in *Gardenia jasminoides*

3.3 Effects of different solid-liquid ratios on total flavonoids in *G. jasminoides* The solid-liquid ratio also has a great influence on the extraction of the active components and a proper solid-liquid ratio is beneficial to the dissolution of the active components. The effect of different solid-liquid ratios on the content of total flavonoids in *G. jasminoides* is shown in Fig. 3. When the solid-liquid ratio was less than 1∶25 (g∶mL), the content of total flavonoids increased with the increase of solid-liquid ratio; When the solid-liquid ratio was 1∶25, the content of total flavonoids reached the maximum of 30.63 mg/g. When the solid-liquid ratio continued to increase, the content of total flavonoids did not continue to increase. Water film was formed due to excessive water content, which hinders the oxygen circulation inside the culture medium and weakens the fermentation capacity[12].

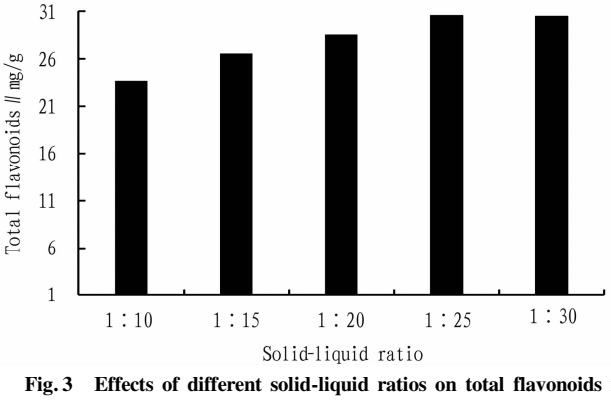

Fig. 3 Effects of different solid-liquid ratios on total flavonoids in *Gardenia jasminoides*

3.4 Effects of fermentation time on total flavonoids in *G. jasminoides* The fermentation time is very important for the proliferation and metabolism of the strain. After a certain period of fermentation, the cell wall of *G. jasminoides* is destroyed, and the small molecular substances in the cells are separated out, which increases the content of active components[14]. The results are shown in Fig. 4. With the increase of fermentation time, the content of total flavonoids increased gradually, and reached the maxi-

mum of 35.77 mg/g at 28 h of fermentation. With the increase of fermentation time, the content of total flavonoids began to decrease.

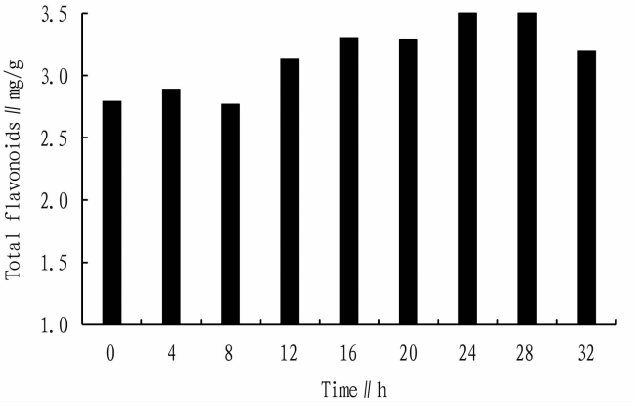

Fig. 4 Effect of fermentation time on total flavonoids in *Gardenia jasminoides*

3.5 Orthogonal experiment optimization Three factors of inoculation dosage, fermentation time and solid-liquid ratio were selected, and the content of total flavonoids in *G. jasminoides* was used as the indicators. On the basis of single factor experiment, orthogonal experiment design was carried out to determine the optimal process conditions for *G. jasminoides* fermentation. The results of orthogonal test are shown in Table 2. It can be seen from the results of the range data R in the table that the factors affecting the content of total flavonoids in *G. jasminoides* are: solid-liquid ratio > inoculation dosage > fermentation time, and the optimal fermentation condition is $A_2B_2C_3$, that is, the inoculation dosage is 2%. Fermentation time 24 h, solid-liquid ratio 1∶30.

Table 2 Orthogonal experimental results of optimum fermentation conditions of *Gardenia jasminoides*

Experiment No.	Factors and levels A (inoculation dosage)	B (fermentation time)	C (solid-liquid ratio)	D (Blank)	Extraction of total flavonoids mg/g
1	1	1	1	1	28.56
2	1	2	2	2	31.76
3	1	3	3	3	34.81
4	2	1	2	3	34.95
5	2	2	3	1	35.92
6	2	3	1	2	32.39
7	3	1	3	2	34.38
8	3	2	1	3	29.25
9	3	3	2	1	34.36
T_1	31.710	32.630	30.067	32.947	
T_2	34.420	32.310	33.690	32.843	
T_3	32.663	33.853	35.037	33.003	
R	2.710	1.543	4.970	0.160	

3.6 *In vitro* antioxidant activity of *G. jasminoides* fermentation broth The *in vitro* antioxidant activity of the optimal group of *G. jasminoides* fermentation broth was investigated, and the results are shown in Table 3. The results showed that *G. jasminoides* flavonoids had a certain ability to scavenge DPPH free radicals and hydroxyl free radicals. After fermentation according to the optimal process, the total flavonoid content of *G. jasminoides* increased by 45.22% compared with the unfermented, the antioxidant capacity of the fermentation broth was improved, and the DPPH free radical scavenging rate increased by 9.46% compared with the unfermented. The hydroxyl radical scavenging rate was increased by 29.24%.

Table 3 *In vitro* antioxidant activity of *Gardenia jasminoides* fermentation broth

Group	Total flavonoids // mg/g	DPPH free radical scavenging rate // %	Hydroxyl free radical scavenging rate // %
Unfermented	25.41 ± 0.65	59.06 ± 1.34	30.78 ± 1.20
G. jasminoides fermentation broth	36.90 ± 0.45	64.65 ± 2.57	39.78 ± 2.0

3.7 Inhibition of hyaluronidase activity *in vitro* by *G. jasminoides* fermentation broth Studies have shown that the inhibition of hyaluronidase activity can reflect the anti-inflammatory activity of substances to a certain extent. The higher the inhibition rate of hyaluronidase, the stronger the anti-inflammatory activity[14]. Table 4 lists the inhibition of hyaluronidase activity of *G. jasminoides* fermentation broth *in vitro*. *G. jasminoides* has a certain inhibition of hyaluronidase activity. Compared with unfermented group, the hyaluronidase inhibition rate of the *G. jasminoides* fermentation broth was improved by 90.30%.

Table 4 Inhibition of hyaluronidase activity *in vitro* by *Gardenia jasminoides* fermentation broth

Group	Total flavonoids mg/g	Hyaluronidase inhibition rate // %
Unfermented	25.41 ± 0.65	46.39 ± 4.79
G. jasminoides fermentation broth	36.90 ± 0.45	88.28 ± 8.61

4 Discussion

In this study, the traditional Chinese medicine *G. jasminoides* was fermented by microorganisms, and the factors such as strain selection, solid-liquid ratio, inoculation dosage and fermentation time were optimized. On the basis of single factor experiment, the fermentation conditions were optimized by orthogonal experiment, and the optimum fermentation conditions were as follows: *B. subtilis*, inoculation dosage 2%, solid-liquid ratio 1∶30, and fermentation time 24 h. Under these fermentation conditions, the content of total flavonoids was 36.91 mg/g, which was 45.22% higher than that of the control group. The antioxidant activity of the fermentation broth of *G. jasminoides* was evaluated by DPPH radical scavenging rate and hydroxyl radical scavenging rate, and the results showed that the fermentation broth of *G. jasminoides* had better antioxidant activity, which was increased by 9.46% and 29.24% compared with the unfermented group, and the inhibition rate of

(To page 27)

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(From page 22)

G. jasminoides on hyaluronidase was also increased by 90.30% after fermentation. The results showed that the fermentation could increase the content of total flavonoids in *G. jasminoides* and improve the efficacy of its active components, which provided a reference for the comprehensive development and utilization of active components in *G. jasminoides*.

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