# Comparative Study on Characteristics of Mandarin Peel Extracts by Biological Processing

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**Research Article**

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# Comparative study on characteristics of mandarin peel extracts by biological processing

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**Abstract**

[Background] Mandarin peel is a by-product from mandarin canning industry containing multiple functional substances with useful properties such as antibacterial and antioxidant activities. [Objective] To evaluate the effect of bioprocessing, fresh mandarin peels were fermented by *Rhizopus stolonifer* JP13 for 4 days and then the peels' antioxidant and antimicrobial activities were tested. The flavonoiuds, hesperidin and VC contents in dry peels were also determined. [Results]The data showed that the fermented mandarin peel had promoted antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, *Aspergillus flavus*, and *Candida albicans*. An increased scavenging effect on free radicals, with 73.0% of ·OH scavenging activities were obtained when compared with fresh mandarin peels. We also observed a significant increase on content of flavonoid (334% )and hesperindin (253.7% ), a reduced scavenging effect on $O^{2-}$ free radicals(13.94%) and decrease content of VC (13.7%). [Conclusion] The presaging of mandarin peel by *Rhizopus stolonifer* JP13 strain will promote the functional activities of mandarin peel and accelerate the process of manufacture *Citri Reticulatae Pericarpium*.

**Keywords:** Mandarin peel, Fermentation, Antimicrobial, Hesperidin, Antioxidant activity

Mandarin(*Citrus reticulated*) peel, as a by-product of mandarin canning industry, is widely used in a variety of fields, like food industry, traditional Chinese medicine, condiment, etc[1]. Rich in various nutrients and multiple functional factors, mandarin peel has been used to make *Citri Reticulatae Pericarpium*(a traditional Chinese medicine and also a common flavoring) since ancient times. According to Compendium of Materia Medica, *Citri Reticulatae Pericarpium* has the effects of regulating breathe-flowing for strengthening spleen, drying dampness and resolving phlegm, treating abdominal distention, vomiting, diarrhea and cough[2]. Nowadays the effective components of *Citri Reticulatae Pericarpium* have been identified as flavonoids, terpenes and alkaloids. As a matter of fact, flavonoids in mandarin peel is quite abundant which rendering it significant anti-oxidant, anti-microbial, anti-inflammatory and anti-tumor activities as protection medicine against certain diseases[3−4]. The major flavonoids present in Citrus species are narirutin, hesperidin, naringin and eriocitrin[5]. Separation and purification those flavonoids from mandarin peel will help exert its huge potential value as clinical drugs and make full use of the mandarin peel residue[6]. However, studies have shown that both processing and extracting methods of flavonoids from mandarin peel will affect their bioactivities[8]. Varies ways to process mandarin peel and to extract its bioactive compounds, such as supercritical $CO_2$ [9], ultrasound-assisted[10], microwave[11], heating[12] and different solvents extraction were applied[13]. All of these methods either cost energy or rely on specific equipment, which are not fit for large scale processing of mandarin peel as a seasonal product. In this study, the fresh raw peel was pretreated by a selected bacteria strain and then flavonoids were extracted after microbial processing. The efficiency of extraction process was analyzed, the anti-microbial and anti-oxidant activities of extracts were tested and compared.

# 1 Materials and methods

## 1.1 Material

### 1.1.1 Mandarin

Fresh mandarin were collected from Nanbei Lake, Jiaxing, China.

### 1.1.2 Strains

Fermentation strain *Rhizopus stolonifer* JP13 was separated from soil in Jiaxing university campus. Strains *Escherichia coli* ATCC25922,*Staphyloccocus aureus* ATCC25923, *Canidia Albicans* ATCC14053 were bought from China Center for Type Culture Collection, Wuhan. Strains *Lactobacillus paracasei* 54,*Lactobacillus plantarum*55,*Lactobacillus rhamnosus* 216,*Lactobacillus sakei* 217,and *Aspergillusflavus* ATCC 14895 were preserved in our laboratory.

### 1.1.3 Culture media

PDA medium[14],Beef extract peptone medium[15], MRS medium[16].

## 1.2 Methods

### 1.2.1 Mandarin peel processing

Fresh mandarin peels were cut into pieces (15mm*15mm) and collected. All fermentation box with 20g peel pieces each were sterilized and then inoculated with strain JP13 at a dose of 5%(v/w). The fermentation was carried under 30℃ for 96h. All peel pieces in each fermentation box were pressed and filtered afterwards to prepare fermented peel juice as test sample. For contrast samples, 20g fresh peels were directly pressed and filtered into juice. Both test and contrast samples were prepared in triplet.

### 1.2.2 The antimicrobial testing

The microorganisms for antimicrobial test were activated and coated on the corresponding medium agar plates. Then the susceptibility papers pretreated with fermented peel juice samples were put on the surface, with 3 parallels in each group. The susceptibility papers infiltrated with the fresh peel juice and 1.0$\mu$g/mL amoxicillin were set as contrast and control samples, respectively. Afterwards, pathogenic bacteria plates were cultured under 37℃ for 24h; fungi plates were cultured under 30℃ for 72h; lactic acid bacteria plates were cultured under 30℃ for 24h. And antimicrobial zones of those samples were measured immediately by caliper after cultivation.

### 1.2.3 Reducing power measurement

Modified potassium ferricyanide reduction method[6] was applied to determine the reducing power of the samples. Briefly, to build the calibration curve, the 1.0 mL of solutions with 0.0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0 mg/mL VC were put into test tubes, then 2.5 mL of pH 6.6 phosphate buffer solution and 2.5 mL of 1% potassium ferricyanide solution were added and mixed respectively. The tubes were heated at 50℃ water bath for 20 min, then 2.5 mL 10% TCA solution were added to terminate the reaction. The tubes were centrifuged and 2.5 mL of supernatant from each tube was moved into a new tube, then 2.5 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$ solution were added and mixed evenly. The absorbances of 700 nm ($A_{700}$) of the mixed solutions were measured. The reducing power of samples were measured for 3 times as described above. The standard curve was drawn with the concentration of VC solutions as the abscissa and the absorbance as the ordinate. The higher the absorbance got, the greater the reducing power was.

### 1.2.4 $O_2^-$ scavenging capacity measurement

Pyrogallol autoxidation method[8] with some modifications was used as follows: 0.3 mL of 3 mmol/L pyrogallol solution was added into a 10 mL volumetric

flask, then 2.0 mL of sample solution was added, followed with pH 8.3 tris-HCl buffer added to scale mark. $A_{400}$ was detected for every 30 s till 5 min. The linear regression was carried out with time as abscissa and $A_{440}$ value as ordinate, and the slope of the line was the auto-oxidation rate. The antioxidant capacity of fresh and fermented peels were detected as above. 3 groups were set as parallels. Clearance rate is calculated as below:

$$Clearancerate(\%) = [(\delta A_0 - \delta A)/\delta A_0] * 100\% \quad (1)$$

$\delta A_o$ is the slope of pyrogallol auto-oxidation curve; $\delta A$ is the slope of pyrogallol auto-oxidation curve added test sample[8].

### 1.2.5 -OH scavenging capacity measurement

Fenton colorimetric method[11] was applied to detect the •OH scavenging capacity. Briefly,in a 25.0 mL colorimetric tube, 2.0 mL of 2 mmol/L ferrous sulfate solution and 2.0 mL of 6.0 mmol/L hydrogen peroxide solution were successively transferred and mixed evenly. Then 6.0 mmol/L salicylic acid ethanol solution was added to scale mark. The mixed solution was set in 37℃ water bath for 15min, then $A_{700}$ was detected and marked as $A_O$.In the above process, 5.0ml sample was added after adding hydrogen peroxide solution, and other conditions remained unchanged, that is $A_X$.Replace the hydrogen peroxide solution added in the preparation of $A_X$ with the pure water, other conditions remained unchanged, that is $A_{X0}$.
The scavenging capability can be calculated according to the following formula:

$$Scavengingrate(\%) = (A_0 - (A_X - A_{X0}))/A_0 * 100\% \quad (2)$$

## 1.3 Total flavonoids and VC content measurement

The tested peels were dried under 60℃ for 24h by drying oven and shattered by pulverizer. The content of total flavonoids was measured by aluminum trichloride method, and the content of total VC was measured by 2, 6-dichlorophenol indigophenol method[7].

## 1.4 Hesperidin extraction, purification and content measurement

### 1.4.1 Extraction of hesperidin

Soxhlet extraction method[25] was applied in hesperidin extraction process: dried fresh and fermented peel powder as tested samples. 2.0g of each sample was weighed and transferred in extractor and from which hesperidin was extracted by 75% ethanol solution with the material-to-liquid ratio of 1:35 (w/v) under 85°C for 2.5h. The extraction solution was filtered under reduced pressure, then the filtrate was filtered through a microporous membrane

(organic, 0.45$\mu$m) twice before hesperidin determination by HPLC (Dionex ultimate 3000 with welch Xtimate $C_{18}$,USA).

### 1.4.2 Hesperidin measurement[17]

2.0g/L hesperidin stock solution was prepared by acetic acid and dimethyl methylamide solvent (acetic acid : dimethyl methylamide=4:1, v/v ). Hesperidin calibration curve was established by using 10 fold series gradient dilutions from 1.2 g/L to 0.012mg/L.
HPLC analysis was performed using a welch $C_{18}$ column (5 $\mu$m, 4.6 mm $\times$250 mm) for separation.The mobile phase was composed by 5% acetic acid and methanol at a 65:35 (v/v) ratio. The column temperature was set at 35 and the flow rate was 0.80 mL/min. The injection volume for all samples was 10 $\mu$L. The detection wavelength was 283 nm. The content of hesperidin in the sample was calculated from peak area by calibration curve established above.

### 1.4.3 Statistical analysis

All the experiments were carried out in triplicate. The relative standard deviation and the mean values were calculated using Microsoft Excel 2016 software. Tests of significant differences were determined by one way ANOVA of Tukey method (SPSS 16.0). Significant differences are labeled accordingly($p<0.05^{*}$, $p<0.01^{**}$, $p<0.001^{***}$)

# 2 Results and analysis

## 2.1 Antimicrobial activity

### 2.1.1 Antimicrobial activity

Both fermented and fresh mandarin peel were found inhibitory effect on *Escherichia coli* and *Staphylococcus aureus*(Fig1).The antibacterial experiment showed that the fermented mandarin peel had significantly enhanced inhibitory effect on*Escherichia coli*,*Staphylococcus aureus* and *Lactobacillus rhamnosus*than the fresh peel, but poorer antibacterial activity than that of amoxicillin. The antibacterial activity of fermented peel was enhanced comparing to fresh peel with an inhibition zone diameter of 8.17$\pm$0.24 mm ($P<0.05$) vs. 6.66$\pm$0.23 nm ($P<0.05$)for *Escherichia coli* and 9.50$\pm$0.41 mm ($P<0.05$) vs. 6.5$\pm$0.40 nm ($P<0.05$) for *Staphylococcus aureus*. It was speculated that the bacteriostasis promotion after fermentation was due to: (1) the relative concentration of bacteriostatic substances increased; (2) modification of antibacterial substances by *Rhizopus Stemphylium* JP13 in mandarin peel fermentation. On the other hand, study on a variety of *Lactobacillus* showed that the inhibitory effect of peel juice on *Lactobacillus rhamnosus* was significantly improved after fermentation. The inhibition zone diameter was expanded from 8.67$\pm$0.24mm to 12.17$\pm$0.24mm($P<0.05$). This fact indicates there might be new anti-*lactobacillus rhamnosus* metabolite generated during fermentation.

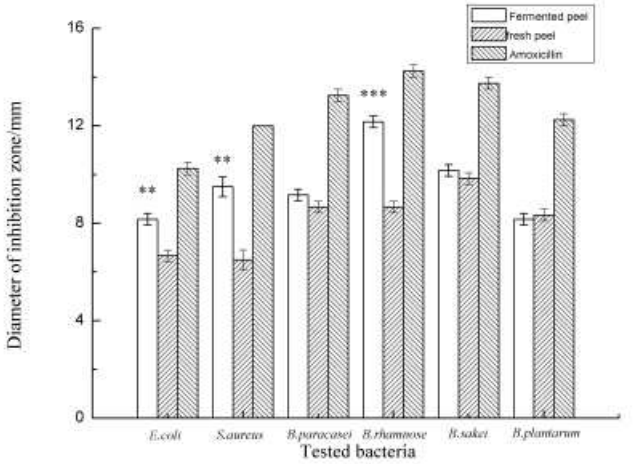


**Fig. 1** Antimicrobial activities of fermented and fresh peel

### 2.1.2 Antifungal activity

According to Fig.2,little antibacterial zone was observed in the fresh peel juice sample, as obvious inhibition zone was observed in fermented peel juice sample group. The diameters of the inhibition zone against *Aspergillus flavus* and *Candida albicans* were 9.17±0.289mm and 9.83±0.289mm, respectively. The anti-fungal effect of the mandarin peel was significantly improved after fermentation, which indicating there were new antifungal substances generated by *Rhizopus Stemphylium* JP13.

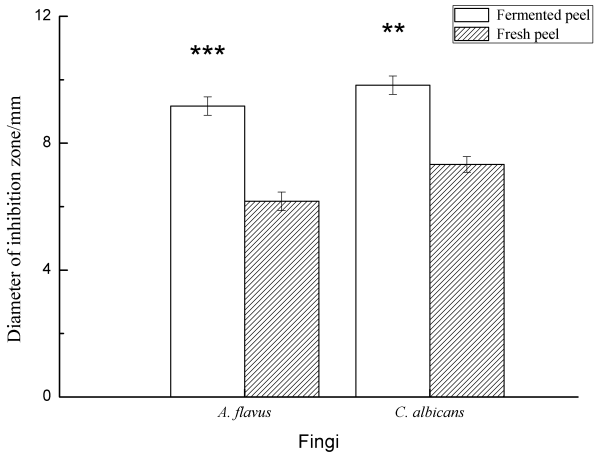


**Fig. 2** Antifungal effects of fermented mandarin peel and fresh mandarin peel on fungi

## 2.2 Antioxidant activity of fermentation peel

### 2.2.1 Determination of reducing power

(1) **VC standard curve**

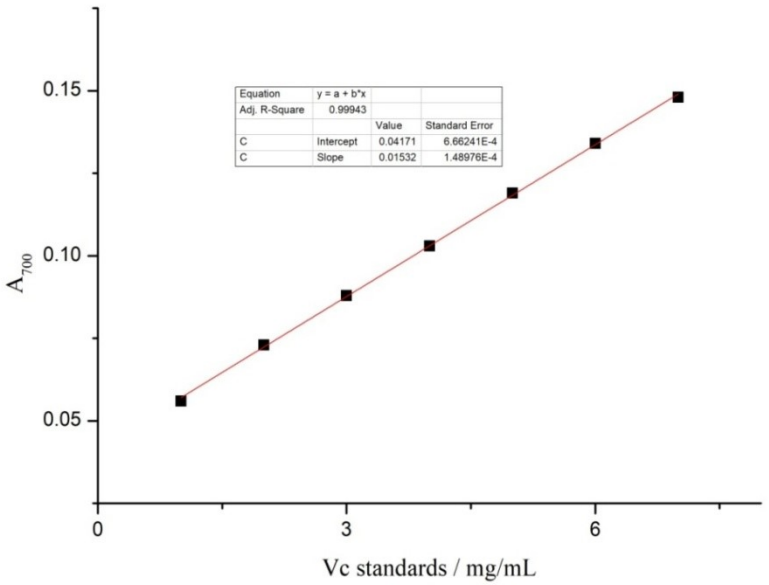


**Fig. 3** Standard curve of reducing power by VC solution

The VC solution reducing power between concentration 1.0-7.0 mg/mL fits the equation Y=0.0153X + 0.0417, $R_2$=0. 9994(Fig.3).Samples reducing power were measured by the VC standard curve.

(2) **Determination of relative reducing power of fermented and fresh mandarin peel**

The absorbance of fresh and fermented mandarin peel were 0.326 and 0.130, which was equivalent to the reducing power of VC at concentration of 92.8 mg/mL and 13.8 mg/mL. The relative reducing power of fermented peel was distinctly reduced, which remained about 15% of the fresh peel only. Result suggested that the reducing power consumed during the fermentation. There were two main reasons deduced: (1) VC consumption by the microoganism *Rhizopus Stolonifer* JP13 during the bioprocessing, (2) the self-oxidation of VC occurred during fermentation.

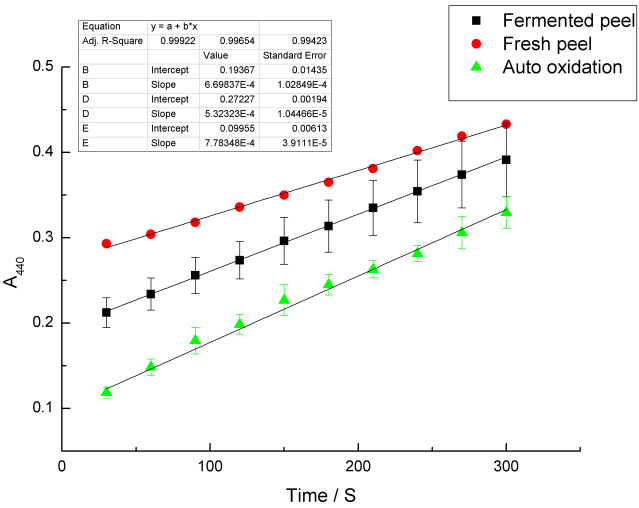


**Fig. 4** Pyrogallol oxidation rate of auto, with fermented peel and fresh peel

### 2.2.2 Determination of $O_2^-$ scavenging activity

The $O_2^-$ scavenging activities were compared for fresh and fermented peel juice. Similar to autooxidation of pyrogallol, the absorbance values of both fermented and fresh peel juice systems gradually increased at 440nm and kept a linear growth to 300s ( Fig. 4). The slopes of the lines described by the fermented, fresh and auto-oxidation samples activities were different, showed the varied $O_2^-$ scavenging activities of these systems.

The scavenging rates of fermented mandarin peel and fresh mandarin peel for $O_2^-$ can be calculated by formula ① using data from the Fig. 4 The values are 13.94% and 31.61%,respectively. Mandarin peel before and after fermentation has certain ability to scavenge $O_2^-$,but the effect decreased after fermentation. This might be due to the consumption of VC in the fermentation process. And the loss of polyphenols and flavonoids in mandarin peel cells during fermentation might also have certain negative effects to clear $O_2^-$. However, our results on $O_2^-$ scavenging rate of fermented mandarin peel was promoted when compared with Li's [22] research, where $O_2^-$scavenging capacity was measured after flavonoids were extracted from mandarin peel. On the other hand, the antioxidant and free radical scavenging abilities of flavonoids might be related to their structures, especially the structure of phenolic hydroxyl group, which can effectively capture and donate hydrogen ion to free radicals, thus inhibiting the further oxidation[20].

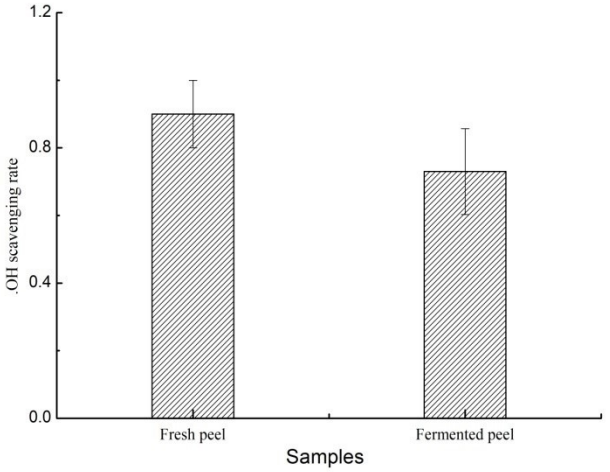


**Fig. 5** -OH scavenging ability of fermented and fresh mandarin peel

### 2.2.3 -OH scavenging activity determination

In Fig.5, ·OH scavenging activity results showed that the scavenging rates of ·OH in fresh and fermented mandarin peel were 90.5±10.0% and 73.0±12.7%, respectively. After fermentation, the ·OH scavenging capacity of mandarin peel decreased slightly, remained as 81% of that in fresh one. Compared data of those with the flavonoids extracted from mandarin peel by Jin et al[21] and the essential oil extracted from mandarin peel by Hao et al[1], the scavenging rate of ·OH in our study was higher, indicating that the bioprocess extraction method we conducted giving a promoted •OH scavenging ability.

## 2.3 Total flavonoids and VC content determination

### 2.3.1 Total flavonoids content determination

The amount of total flavonoids in fermented mandarin peels was 84.8±5.6mg/g,and the corresponding amount of total flavonoids in fresh mandarin peels was 25.4±2.5mg/g(Fig.6). The amount flavonoids of fermented mandarin peel was as 334% of that of the fresh mandarin peel.

### 2.3.2 VC content determination

VC content in fresh and fermented mandarin peels were shown in Fig.7. The results were contrary to the content of total flavonoids. The total amount of VC in fermented mandarin peel was 12.0±2.5mg/100g,, and in fresh mandarin peels was 87.5±10.5mg/100g.. The amount VC of fermented mandarin peel was only 13.7% of that of the fresh one.

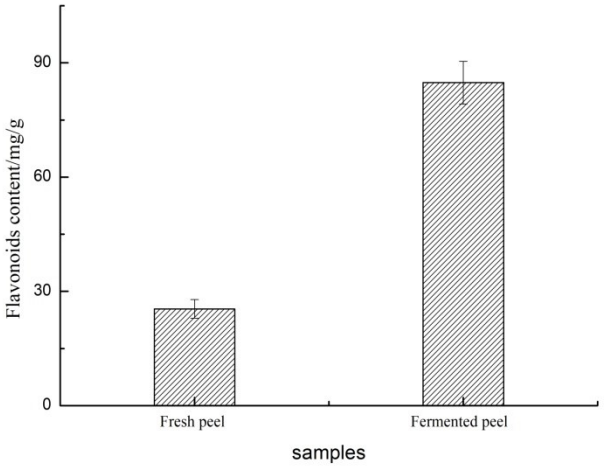


**Fig. 6** Total flavonoids content of fermented and fresh mandarin peel

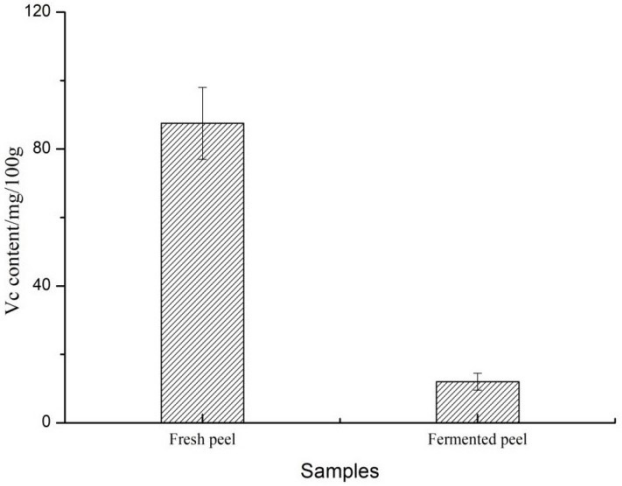


**Fig. 7** VC content of fermented and fresh mandarin peel

## 2.4 Hesperidin content determination

Hesperidin content in fresh and fermented mandarin peel were showed in Fig. 9. The total amount of hesperidin in fresh peel was 3.91±0.10mg/g, and in fermented peel was 9.91±0.21mg/100g. Same as the total flavonoids, the amount of hesperidin from fermented mandarin peel was as 253.7% of that of the fresh one.

The large increased of hesperidin content in mandarin peel after fermentation

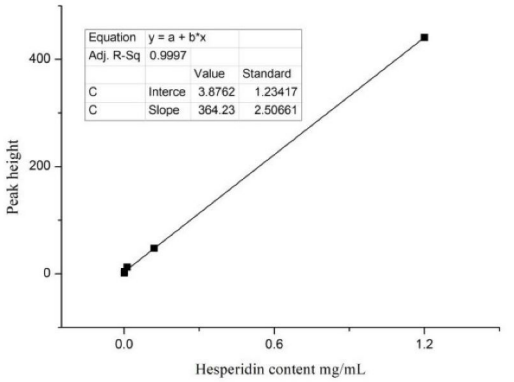


**Fig. 8** Hesperidin standard solution curve (HPLC)

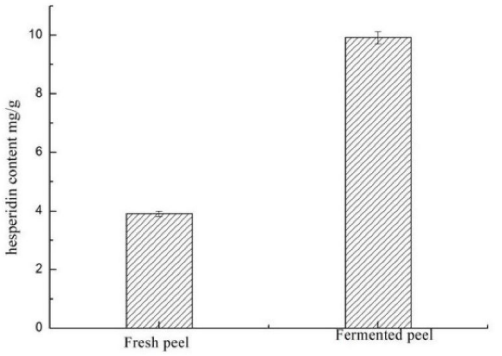


**Fig. 9** Hesperidin content in fresh and fermented peel

indicated some main component like lignocelluloses in mandarin peel had been hydrolyzed and thus the cell wall of the plant was destroyed. The relative content of remained substances increased and more bioactive compounds including hesperidin were released, illustrating that the fermentation of mandarin peel like plant material can effectively improve their biological value.

# 3 Discussion

As "affinal drug and diet" food, mandarin peel has been widely used in East Asia, but the low utilization efficiency of its active ingredients limits its function in the organism[23] [24].Therefore, Chinese medicine adopts the processing method to prepare mandarin peel to give full play to its effect. On market, the price of mandarin peel is often related to the aging time, the longer it ages,

the more expensive it is. Some peels may be stored for decades, which limits the output of aged mandarin peels, and further hinders the development of the mandarin peel industry. Our research shows that fermented mandarin peel by microbial enzymes in four days can effectively decompose the cell wall, increase the relative content of bioactive substances and thus significantly shorten the mandarin peel aging process. Our data showed that compared with fresh mandarin peel, the fermented mandarin peel had higher total flavonoids content and antimicrobial and anti-mildew dual functions. It has great application value and may be used as antibacterial, mouldproof additives to replace antibiotics in feed, food and other industries[19].So far, there were few studies on mandarin peel fermentation technology development and application in antibacterial and antioxidant activities. The biological processing method with single strain, the process optimization as well as the mechanism of action of specific strains or mixed bacteria need to be further studied in the future. On the other hand, traditional Chinese medicines like*Ganoderma lucidum*(“Lingzhi” in Chinese) etc. With high polymer lignocelluloses composed the main part of the plant cell wall, hampered the decomposition and absorption of the effective constituent of the valuable herbal medicine, which are the key common problems of the Chinese pharmaceutical industry. Bioprocess pretreatment with certain microorganisms especially probiotics may accelerate the mature of the herbal medicine and endowed the products with more beneficial substances. Bioprocessing would also enhance the activity of bioactive substances and shorten the production cycle. And thus be beneficial to the further development of traditional Chinese medicine industry.

# 4 Conclusion

After biological processing only 4 days, mandarin peel had significant promotion of antibacterial effects on *Escherichia coli*, *Staphylococcus aureus* and *Lactobacillus rhamnosus on other three bacteria speicies*when compared with the fresh peel. More interestingly, compared with the fresh mandarin peel, the processed mandarin peel has enhanced inhibition effect on fungi, with obvious inhibitory effect to *Aspergillus flavus* and *Candida albicans*.By comparison, the relative reducing power, scavenging superoxide free radical and hydroxyl free radical of biological processed mandarin peel decreased, illustrated that its antioxidant activity decreased due to a decrease of VC content. Large-scale release of total flavonoids did not bring intuitive improvement in relative reducing power and antioxidant activity. Compared with the similar studies, fermented mandarin peel still shows higher antioxidant activity and relative reducing power.

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