Antibacterial effect of flavonoids in *castanea mollissima blume* on common spoilage bacteria in low-temperature meat products

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Abstract. The oxford cup method and filter paper method were used to determine the antibacterial effect of flavonoids extracted from *castanea mollissima blume*, three kinds of common spoilage bacteria in low-temperature meat products were subjects to be tested, named proteus, macrococcus and bacillus. The results showed that flavonoids in *castanea mollissima blume* had an antibacterial effect on proteus and macrococcus. For flavonoid glycosides, the diameters of inhibition zone were 16.32 mm and 17.76 mm, and the minimum inhibitory concentration were 0.00625 g/mL and 0.0125 g/mL, respectively. The effective inhibitory time was 14 h. For free flavonoids, the maximum diameter of the inhibition zone was 7 mm, the minimum inhibitory concentration was 0.0125 g/mL. They had no antibacterial effect on bacillus. As natural bacteriostatic agents, the flavonoids in *castanea mollissima blume* are likely to have many important applications in meat products in the future.

1. Introduction

Chestnut (*Castanea mollissima BL.*) is native to China, mainly in Hebei, Shandong, Henan, etc.

It is sweet and delicious, rich in nutrition. It has good prevention and treatment effects on hypertension, coronary heart disease, atherosclerosis, etc.

*Castanea mollissima blume* is the prickly shell of chestnut. It has rich resources and low cost. Researchers have proved that the bacteria could be inhibited by the water extract of *castanea mollissima blume*[1]. It has been proved that its water decoction, alcohol soluble substance and ethyl acetate extract had strong anti-inflammatory and anti-bacterial activity[2].

With the continuous improvement of people's living standards, more attention is paid to food safety, consumers' demand for natural bacteriostatic agents is increasing. In the mean time, the antibacterial effect of flavonoids has been affirmed by the pharmaceutical industry, there are many studies on Toona sinensis leaves, Moringa oleifera leaves, Dandelion, etc[3,4,5]. However, up to now, there is no systematic study on the bacteriostasis of flavonoids in *castanea mollissima blume*. Thus, in this
experiment, the bacteriostatic properties of flavonoids in castanea mollissima blume were determined, which provided a theoretical basis for the application of chestnut in food natural bacteriostatic agents.

2. Materials and methods

2.1. Treatments of plant
The castanea mollissima blume was purchased from Shandong Guoruun Food Co., Ltd. After sieving, cleaning and drying, pulverized them with a Chinese medicine crusher and passed through a 50-mesh sieve. Placed it in a drying oven for later use.

2.2. Extraction of the flavonoids from castanea mollissima blume
Since there are two types of flavonoids in castanea mollissima blume, one is flavonoid glycosides, soluble in water and diluted alcohol; the other is free flavonoid, soluble easily in alcohol but insoluble in water, the sample was divided into two groups for extraction. Weighed 20 g dry samples. The extracting condition of the flavonoid glycosides (10 g) group was as follows: extracted by water, the ratio of material to liquid was 1:10 and extraction temperature was 60 ℃. The extraction of free flavonoids group (10 g) was in the conditions of 92 % ethanol solution with 1:10 solid-liquid ratio at 62 ℃ for 273 min.

2.3. Treatments of bacteria
Bacillus, proteus and macrococcus, preserved in glycerol at -80 ℃. Microorganisms isolated from low-temperature spoilage meat, which provided by food chemistry laboratory of Beijing University of Agriculture. Returned them to normal temperature, then added to 100 mL sterilized nutrient broth medium, shaked up into suspension, placed in a 37 ℃ incubator (HWS-2000, Ningbo Haishusaifu Experimental Instrument Factory) for 12 hours, waited for the medium to become turbid from transparent. Another 100 mL nutrient broth medium was inoculated with 2 % of the inoculum, and then activated again[6]to reserve.

2.4. Determination of antibacterial effect
The activated bacteria liquid was taken 2 mL into 100 mL of nutrient agar medium about 50 ℃, shaken well, and poured into aseptic plates. Each plate was about 15-20 mL. Placed the sterilized oxford cups on it gently, the 0.1 g/mL water extract and distilled water were added to the oxford cup by a pipette gun with 100 μL. The experiment must be repeated three times. Then observed and measured the diameters of antibacterial circle after incubated for 15 hours at 37 ℃[7].

The antibacterial effect of the free flavonoids group was measured by filter method. Dissolved free flavonoids in methanol to make 0.1 g/mL flavonoid methanol solution, and the solvent was used as a blank control group. The 6 mm filter papers after sterilization at 150 ℃ were placed in the sample solution and methanol, respectively, and soaked for 2 h, then took out and put them in a bacterial plate after drying. The rest of the operation was the same as above[8].

2.5. Determination of minimum inhibitory concentration
Flavonoid glycosides: the antimicrobial agents of 0.1 g/mL were diluted into 2, 4, 8, 16, 32-fold solution in turn in test tubes, then poured 100 μL each into oxford cup slowly by a pipette gun and marked[9]. The sterile distilled water was used as a blank control group, repeated three times.

Free flavonoids: Diluted in the same way in turn to make antibacterial filter papers, placed on the same bacterial plate, made three groups of parallel tests. The plate was cultured at 37 ℃ for 12 h, and the MIC was the minimum sample concentration when there was no bacterial growth in the circle completely[10].

2.6. Determination of effective inhibiting time
The experimental strains (macrococcus and proteus) were inoculated into nutrient broth medium,
respectively, shaked at 37 °C (150 r/min) to logarithmic growth phase, then they were inoculated in nutritional broth medium containing 10 mL antimicrobial agent. The concentration was 1*MIC tested in 2.5, and inoculation amount was 1 %. Mixed and cultured in a constant temperature shaking table (150 r/min) at 37 °C, measured the OD value every one or two hours at the wavelength of 600 nm (TU-1901, Beijing Purkinje General Instrument,Co., Ltd.). The liquid medium with no bacteriostasis was used as the control group. Made the bacterial growth curve with the time as abscissa and the absorption value as ordinate to determine the effective inhibiting time[11].

3. Results and discussion

3.1. Antibacterial effect

The result indicated the antibacterial effect of flavonoid glycosides in castanea mollissima blume on macrococcus was the greatest, the diameter of antibacterial circle was 17.76 ± 0.20mm, and the second one was on proteus, 16.32 ± 0.12mm. It couldn't against bacillus, as described in Table 1.

The free flavonoids of castanea mollissima blume had the same antibacterial effect on proteus and macrococcus, the diameters of antibacterial circle were all about 7 mm, and there was no antibacterial effect on bacillus, as described in Table 2. Probably because proteus and macrococcus belong to gram-negative bacteria, whereas bacillus is gram-positive bacteria, flavonoids in castanea mollissima blume have better inhibitory activity against gram-negative bacteria.

| Bacterial species | Diameter of antibacterial circle (mm) |
|-------------------|--------------------------------------|
| Proteus           | 16.32 ± 0.12                         |
| Macrococcus       | 17.76 ± 0.20                         |
| Bacillus          | -                                    |

Table 1. Antibacterial effect of flavonoid glycosides from castanea mollissima blume.

Table 2. Antibacterial effect of free flavonoids from castanea mollissima blume.

| Bacterial species | Diameter of antibacterial circle (mm) |
|-------------------|--------------------------------------|
| Proteus           | 7.32 ± 0.18                          |
| Macrococcus       | 7.29 ± 0.23                          |
| Bacillus          | -                                    |

3.2. Minimum inhibitory concentration

It could be seen from Table 3, and Table 4 that different concentrations of bacteriostatic agents had different effects. The lower the concentration of bacteriostatic agents were, the lower the bacteriostatic property was. The MIC of flavonoid glycosides of castanea mollissima blume was 0.00625 g/mL for proteus and 0.0125 g/mL for macrococcus, it showed strong bacteriostasis to both bacteria. The MICs of free flavonoids on proteus and macrococcus were both 0.0125 mm. The antibacterial circles were small, perhaps because free flavonoids were insoluble in water, the diffusion area in the plate with bacteria was limited. It couldn't affect the bacteria in the area where it hadn't spread.

| Extract concentration (g/mL) | 0.1   | 0.05  | 0.025 | 0.0125 | 0.00625 | 0.003125 |
|------------------------------|-------|-------|-------|--------|---------|-----------|
| Diameter of the antibacterial circle (mm) |       |       |       |        |         |           |
| Proteus                      | 16.28±0.12 | 14.32±0.33 | 14.21±0.30 | 10.57±0.23 | 6.47±0.16 | -         |
| Macrococcus                  | 17.89±0.33 | 11.57±0.26 | 6.36±0.25  | 6.71±0.12  | -        | -         |

Table 3. MIC of flavonoid glycosides in castanea mollissima blume.
Table 4. MIC of free flavonoids in castanea mollissima blume.

| Extract concentration (g/mL) | 0.1     | 0.05    | 0.025   | 0.0125  | 0.00625 |
|-----------------------------|---------|---------|---------|---------|---------|
| Diameter of the antibacterial circle (mm) |          |         |         |         |         |
| Proteus                     | 7.28±0.21 | 7.15±0.17 | 7.09±0.23 | 6.49±0.16 | -       |
| Macrococcus                 | 7.16±0.23 | 7.04±0.20 | 6.53±0.16 | 6.23±0.14 | -       |

3.3. Effective inhibiting time

As could be seen from Figure 1., the blank group had typical growth characteristics. However, the OD value of macrococcus with 0.0125 g/mL (MIC) flavonoid glycosides measured in 3.2., was always lower than those of the blank group. The smaller the number of bacteria was, the smaller the corresponding OD value was. It could be inferred that the normal growth state of the bacteria at the beginning was significantly inhibited by flavonoid glycosides, but it could not kill all the bacteria. After a period of effective inhibition, its inhibition of bacteria began to weaken or even completely disappeared. There were two possible reasons for this, one was that the antimicrobial substances may changed the structure of the content, resulted in a decrease in bacteriostasis, the second was that the bacteria slowly adapted to the environment with the bacteriostatic substance added[12].

As the Figure 2., proteus without flavonoid glycosides was typically S-shaped, while the number of proteus with flavonoid glycosides did not increase between 1h and 14h. It showed that flavonoid glycosides could inhibit the growth of proteus and inhibit the growth of proteus for 14 hours.

Figure 1. Effects of flavonoid glycosides in castanea mollissima blume on growth curve of macrococcus.

Figure 2. Effects of flavonoid glycosides in castanea mollissima blume on growth curve of proteus.

4. Conclusions

Flavonoids in castanea mollissima blume had antibacterial effect on proteus and macrococcus, no antibacterial effect on bacillus. For flavonoid glycosides, the maximum antibacterial circle was 16.32 mm and 17.76 mm, the minimal inhibitory concentration was 0.00625 g/mL and 0.0125 g/mL, respectively, and the effective inhibiting time was 14 h. For free flavonoids, the maximum antibacterial circle was 7 mm, the minimum inhibitory concentration was 0.0125 g/mL. Hence, as natural antimicrobial agent, it will have a wider application prospect in the preservation of meat products.

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