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Full Length Research Paper

Antimicrobial action of purified raspberry flavonoid

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In this paper, the bacteriostatic action of purified raspberry flavonoid was explored. A study was conducted on the effect of raspberry flavonoid on *Escherichia coli, Bacillus subtilis*, *Staphylococcus aureus, Saccharomyces cerevisiae, Rhizopus, Mucor* and *Penicillium*. Raspberry flavonoid had the best inhibition effect on *S. aureus*, followed by *B. subtilis*, *E. coli* and *Penicillium*. However, it cannot inhibit the growth of *S. cerevisiae*, *Rhizopus* and *Mucor*. The minimal inhibitory concentration was 0.16 mg/ml for *E. coli*, 0.08 mg/ml for *B. subtilis*, 0.04 mg/ml for *S. aureus* and 0.64 mg/ml for *Penicillium*. At 100°C, temperature does not have an obvious effect on the bacteriostatic action of raspberry flavonoid. The rate of inhibition increases with time and the increase of concentration of raspberry flavonoid. Raspberry flavonoid has a stable bacteriostatic action pH from 4 to 8.

Key words: Raspberry, flavonoid, bacteriostasis, *Escherichia coli*, *Staphylococcus aureus*.

INTRODUCTION

Raspberry, also called palm leaf raspberry fruit, is a kind of rubus berry plant of rosaceae. Raspberries are abundant in nutrients essential to the human body, such as amino acids, mineral elements, organic acids and V_E, which is one of the components of raspberry (Ancos et al., 2000). Moreover, raspberries contain functional components such as flavonoids, polysaccharides and triterpenoids (Kazuhiro et al., 1991; Carmen et al., 2000).

Raspberry flavonoid is a type of important organic compound generated during the metabolic process of the raspberry and is one of its major active ingredients. Generally, the flavonoid compound of plants is weak (Gao et al., 2005). However, raspberry flavonoid can coagulate and denaturalize protein, and has bacteriostatic and bactericidal action. Presently, some studies have been conducted on the bacteriostatic and bactericidal action of flavonoid compound (Hazra et al., 2010; Wang et al., 2005). Several high-quality investigations have examined the relationship between flavonoid structure and antibacterial activity and these are in close agreement. In addition, numerous research groups have sought to elucidate the antibacterial mechanisms of action of selected flavonoids (Cushnie et al., 2005), but there have been no reports on the bacteriostatic and bactericidal action of raspberry flavonoid. In this paper, the purified raspberry flavonoid is selected as the object of study and its bacteriostatic action is explored with the aim of setting a solid foundation for future R&D and application of raspberry flavonoid in food.

MATERIALS AND METHODS

Dried raspberry (first grade, bought from the drugstore of Hengshui Municipal Hospital of Traditional Chinese Medicine) was milled to 60-mesh granularity and dried at 50°C for later use. All chemical reagents adopted during the analysis of the sample ingredients were analytically pure or biochemical reagents.

Strains

Bacteria used were *Escherichia coli* (TTHR 17033), *Bacillus* (Ti-2) and *Saccharomyces* (TCCC 32005). Yeast used was *Staphylococcus* (21600) and moulds used were *Rhizopus* (TCCC 41044), *Moucor* (TCCC 41023) and *Penicillium cyclopium.*

Culture medium

PDA culture medium: Potatoes 200 g, glucose 20 g, agar 15 g;

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Czapek's medium: sucrose 3 g, $NaNO_3$ 0.3 g, K_2HPO_4 0.1 g, KCl 0.05 g, $MgSO_4.7H_2O$ 0.05 g, 0.001 g $Fe_2(SO_4)_3$, and 2 g agar filled with 1 L of distilled water; Wort culture medium: 12º fresh wort and 2% agar.

Preparation of bacterial suspension

100 g milled raspberry powder was weighed and placed in a conical flask. Next, 1 L of 95% ethanol was added to extract of the raspberry flavonoid at 50°C for 36 h. During the process, ultrasonic extraction was carried out at the 12^{th} and 24^{th} h at the ultrasonic power of 300 W for 25 min (He et al., 2005; Herrera et al., 2005). Shaking was required when the extraction occurred at 50°C. After extraction, the paste was obtained through vacuum filtration and distillation. Purified water was added for ultrasonic dissolving and petroleum ether was used three times for extraction. The upper water layer was taken and then extracted with ethyl acetate three times. Yellow powder was obtained after vacuum distillation. For ultrasonic dissolution, 70% ethanol was used and passed through the AB-8 resin column for purification. A yellow powder was obtained after another vacuum distillation.

Strains activation

The strains were inoculated into the slant medium on the clean bench and the molds cultured were at 28°C for 48 h, bacteria at 36°C for 24 h, and yeasts at 30°C for 24 h. After culturing, the strains were placed into the refrigerator at 4°C for later use.

Plate preparation

The wet heat sterilization method was adopted for the prepared culture medium and then cooled to 60°C. The culture medium was then poured into the sterile culture dishes, reaching up to one third of each dish under aseptic conditions. They were stored for later use after setting.

Preparation of bacterial suspension

Under aseptic conditions, the inoculating loop was used to pick a small amount of mold spores, bacteria and yeast mycelium out of the activated strains and then inoculated into the sterile water separately. The bacterial suspension was shaken and completed for later use (Zhai et al., 2005).

Filter paper method for determination of total raspberry flavonoid

The filter paper was changed into a round one with a diameter of 6 mm using a puncher and then placed in the conical flask. After high-pressure steam disinfection, the filter paper was submerged in each liquid under aseptic conditions and then taken out with aseptic tweezers 12 h later. After the redundant liquid was drained away, it was pasted clockwise onto the plate of the culture medium. After being cultured at 37°C for 24 h, the medium was checked for zones of inhibition. The diameters of existing zones were measured with a slide caliper.

Determination of minimal inhibitory concentration

About 2 ml liquid medium was placed in eight test tubes. Next, 2 ml (0.64 mg/ml) raspberry flavonoid extract was placed in the first test tube, shaken, and then 2 ml of the mixture was taken out of the first test tube and placed in the second test tube. This procedure was repeated successively for the five other test tubes. For the 8th test tube, 2 ml was taken out and abandoned. Then 200 ul bacteria (the optical density were respectively 10^6 cfu/ml) was inoculated into each test tube and one test tube without bacteria was chosen as the blank control. They were cultured at 37°C for 24 h. The diluted liquid was observed and recorded for the minimal concentration necessary to inhibit bacterial growth, that is, the minimal inhibitory concentration (Yan et al., 2004).

Study of the factors affecting bacteriostatic action of raspberry flavonoid

Effect of temperature on bacteriostatic action of raspberry flavonoid

Five sterile conical flasks were taken under aseptic condition and 5 ml raspberry flavonoid diluent of the minimal inhibitory concentration was placed in the five flasks. The flasks were then heated in a water bath of 20, 40, 60, 80, and 100°C, respectively, for 20 min. Next, 200 ul bacterial suspension was inoculated into each flask to carry out the bacteriostatic activity test for the minimal inhibitory concentration of each kind of bacteria (the optical density were respectively 10^6 cfu/ml). Comparison of the effect of different temperatures on the bacteriostatic activity of raspberry flavonoid was then done.

Relationship between action length and inhibition rate of raspberry flavonoid

The raspberry flavonoid extract was diluted into different concentrations with sterile water. The effect of the extract of different concentrations on the relationship between action length and inhibition rate was assayed based on *Escherichia coli* and *Bacillus subtilis*. About 2 ml diluent of different concentrations was mixed with 200 ul bacterial suspension. The mixture was then shaken and allowed to act for 2, 4, 6, 8, 10 and 12 h, and then poured into flat dishes with nutrient agar medium. Next, 2 ml sterile water and 200 ul bacterial suspensions were mixed and poured into the flat dish with nutrient agar. This was taken as the control group. All dishes were cultured at 37°C for 24 h. The inhibition rate was calculated according to the colony count.

Inhibition rate (%) = (colony count in control group - colony count in test group) / colony count in control group × 100

Effect of pH value on bacteriostatic action of raspberry flavonoid

The pH value of nutrient agar medium was adjusted with diluted HCl or NaOH to 5, 6, 7, 8 and 9. The bacteriostatic activity test was carried out based on the minimal inhibitory concentration of the raspberry flavonoid for each kind of bacteria. The effects of different pH values on bacteriostatic activity of raspberry flavonoid were then compared.

RESULTS AND DISCUSSION

Study of bacteriostatic action of raspberry flavonoid

The raspberry flavonoid exhibited bacteriostatic action on *E. coli, B. subtilis* and other bacteria, as shown in Table 1.

Table 1. Bacteriostatic action of raspberry flavonoid.

Test organism	Diameter of inhibition zone (mm)	Diameter of inhibition zone of blank control group (mm)
Escherichia coli	9.22	0
Bacillus subtilis	11.64	0
Staphylococcus aureus	13.50	0
Saccharomyces cerevisiae	+	0
Rhizopus SP	+	0
Mucor	+	0
Penicillium	5.26	0

+ Means the bacteriostatic action was not obvious.

Table 2. Minimal inhibitory concentration of raspberry flavonoid for microorganism.

Test organism	Number							
	1	2	3	4	5	6	7	8
Escherichia coli	—	—	—	—	—	+	++	+++
Bacillus subtilis	—	—	—	—	—	—	+	++
Staphylococcus aureus	—	—	—	—	—	—	—	+
Saccharomyces cerevisiae	++	+++	+++	+++	++++	++++	++++	++++
Rhizopus SP	+	++	++	++	++	++	++	++
Mucor	++	++	++	++	+++	+++	+++	+++
Penicillium	—	—	—	+	+	++	+++	+++

"—" Means no bacteria were grown in the culture dish; "+" means a small amount of bacteria was grown in the culture dish; "++" means bacteria less than 1/5 of the area were grown in the culture dish; "+++" means bacteria less than 1/3 of the area were grown in the culture dish; "++++" means bacteria less than 1/2 of the area were grown in the culture dish.

Table 1 shows that raspberry flavonoid has different inhibition effects on different bacteria. It can inhibit the growth of *E. coli, B. subtilis, S. aureus* and *Penicillium*. It has the best inhibition effect on *S. aureus* and the diameter of the inhibition zone reaches up to 13.5 mm. However, its inhibition effect on *Saccharomyces cerevisiae*, *Rhizopus* and *Mucor* were not obvious. Raspberry flavonoid has the best inhibition effect on *S. aureus*, followed by *B. subtilis, E. coli* and *Penicillium*.

Determination of minimal inhibitory concentration of raspberry flavonoid

The results of the minimal inhibitory concentration of raspberry flavonoid are shown in Table 2. Table 2 shows that raspberry flavonoid had a strong inhibition effect on bacteria. The minimal inhibitory concentrations were 0.16 mg/ml for *E. coli*, 0.08 mg/ml for *B. subtilis* and 0.04 mg/ml for *S. aureus*. Results from the bacteriostatic activity test for minimal inhibitory concentration indicated that raspberry flavonoid had the best inhabitation effect on *S. aureus*, followed by *B. subtilis*, *E. coli* and *Penicillium*. The result was consistent with that of the cup-plate method. *B. subtilis*, *S. aureus* and *E. coli;* all contain both gram - positive and negative bacteria. The results obtained in this study indicated that raspberry flavonoid had an inhibition effect on both gram - positive and negative bacteria, and the results implied that raspberry flavonoid had good inhibition effect on bacteria.

Study of the factors affecting the bacteriostatic action of raspberry flavonoid

Effect of temperature on bacteriostatic action of raspberry flavonoid

Temperature greatly affects the stability of substances. Many substances may decompose or deteriorate easily under high temperatures and their actions may be decreased accordingly. The bacteriostatic action of raspberry flavonoid under different temperatures is shown in Table 3.

Table 3 shows that the bacteriostatic action of raspberry flavonoid did not decline with the rise of temperature to 100°C. However, its bacteriostatic action decreased at temperatures above 100°C. Overall, the bacteriostatic action of raspberry flavonoid had thermal

Table 3. Effect of different temperature on bacteriostatic activity of raspberry flavonoid.

Test organism	Temperature (°C)				
	20	40	60	80	100
Escherichia coli	—	—	—	—	+
Bacillus subtilis	—	—	—	—	+
Staphylococcus aureus	—	—	—	—	+
Penicillium	—	—	—	+	+

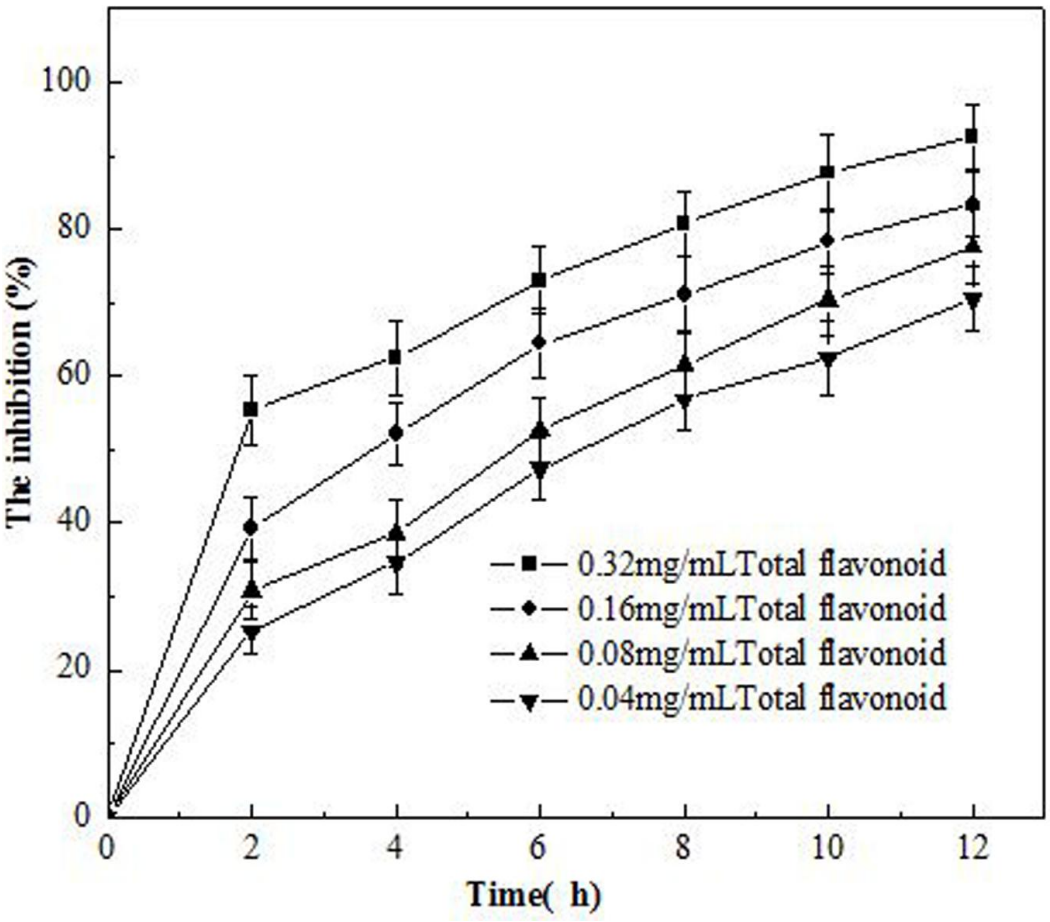

Figure 1. The inhibition of raspberry flavonoid on *E. coli*.

stability within 100°C.

Relationship between action length and inhibition rate of raspberry flavonoid

The relationship between action length and inhibition rate of raspberry flavonoid are shown in Figures 1 to 4. As shown in the figures, the inhibition rate of raspberry flavonoid on several organisms increases with time. The inhibition rate also rises with the increase in raspberry flavonoid concentration for the same kind of bacteria during the same action length. Raspberry flavonoid has the best bacteriostatic action on *S. aureus*. During the same action length, the inhabitation rate of raspberry flavonoid on *S. aureus* is higher than in other test organisms when the concentration is kept unchanged.

Effect of pH values on bacteriostatic action of raspberry flavonoid

The effect of pH values on bacteriostatic action of raspberry flavonoid is shown in Table 4. The table shows that

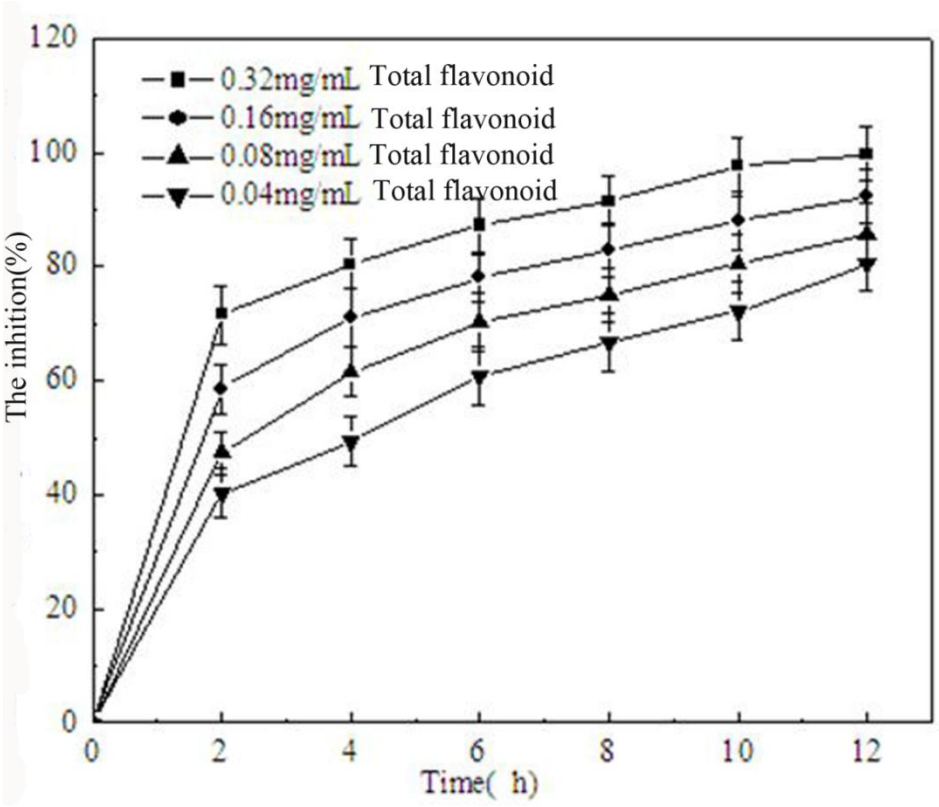

Figure 2. The inhibition of raspberry flavonoid on *B. subtilis*.

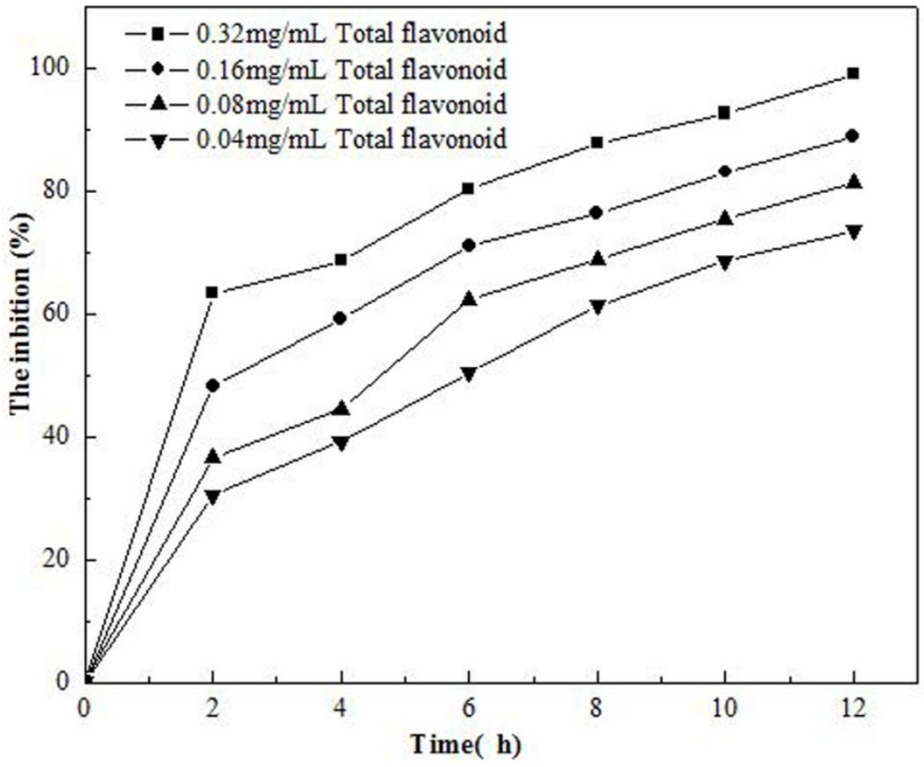

Figure 3. The inhibition of raspberry flavonoid on *S. aureus*.

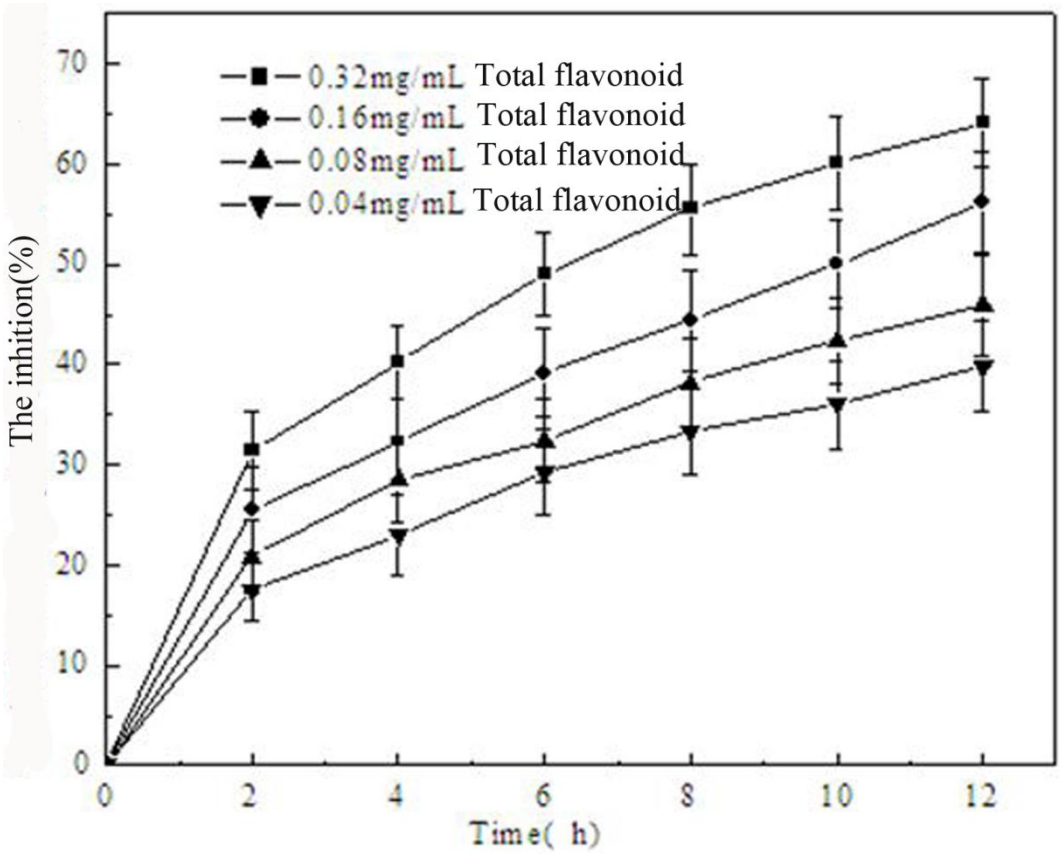

Figure 4. The inhibition of raspberry flavonoid on *Penicillium.*

Table 4. Effect of different pH values on bacteriostatic activity of raspberry flavonoid.

Type	pH value									
	5		6		7		8		9	
	a	b	a	b	a	b	a	b	a	b
Escherichia coli	+	—	++	—	++	—	+++	—	++++	+
Bacillus subtilis	+	—	++	—	+++	—	+++	+	++++	++
Staphylococcus aureus	+	—	++	—	+++	—	++++	—	++++	—
Penicillium	+	—	++	—	+++	—	+++	—	++++	+

“a” Means no raspberry flavonoid was added; “b” means raspberry flavonoid was added; “-” means no bacteria were grown in the culture dish; “+” means a small amount of bacteria was grown in the culture dish; “++” means bacteria less than 1/5 of the area were grown in the culture dish; “+++” means bacteria less than 1/3 of the area were grown in the culture dish; “++++” means bacteria less than 1/2 of the area were grown in the culture dish.

raspberry lavonoid could inhibit the growth of bacteria within a wide scope of pH. It had a strong bacteriostatic action in lower pH; it could also inhibit the growth of bacteria in high pH. With pH from 4 to 8, raspberry flavonoid could inhibit the growth of bacteria very well. Its bacteriostatic activity declines and bacteria begin to grow at pH of 9. However, the speed of growth was still lower than in the control group.

Conclusion

Flavonoid compounds could cause damage to microbial cell, cause the cellular content to release, prevent the synthesis of macromolecules in the cell (Wang et al., 2008) and inhibit the growth of organisms. The bacteriostatic action of raspberry flavonoid had been studied. The test results showed that raspberry flavonoid had a

strong inhibition effect on *E. coli*, *B. subtilis* and *S. aureus*, indicating that the application of raspberry flavonoid could be expanded. The results indicated that raspberry flavonoid could be used as an antiseptic and antistaling agent and applied in medicine and health care products. In this way, its dual efficacy as an antiseptic and a health care product could be studied further.

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