Antibacterial activity of anthraquinone from cassia seed on spiced pig head

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Abstract. [Objective] To optimize the extraction of anthraquinone from cassia seed by ultrasonic extraction and its antibacterial activity. [Method] The influences of different extraction time and ethanol concentration, on anthraquinone content were evaluated by a single factor experiment. And anthraquinone content was determined by ultraviolet spectrophotometry. The bacteriostasis of anthraquinone on spiced pig head’s common putrefying bacteria: Staphylococcus, Serratiaeae, Bacillus, Proteus and the minimal inhibitory concentration (MIC) were studied by oxford plate assay system. [Result] The best extraction time was 30 minutes and the best ethanol concentration was 80\%. The antibacterial activity of the cassia seed anthraquinone on Staphylococcus Aureus, Bacillus Proteus is obviously, the minimum inhibitory concentrations were 0.125 g/mL, 0.125 g/mL, 1 g/mL respectively and no inhibitory effect on Serratiaeae.[ Conclusions] The anthraquinones from Cassia seed can inhibit a part of spoilage bacteria in spiced pig heads

1. Introduction
Low-temperature meat products are popular in public, because of its rich nutrition, strong flavor and rich sense of mastication. However, in order to maintain its flavor, the processing of sterilization condition needs to be controlled at 68-70 degrees for 30 minutes. This leads to some spores and some heat-resistant bacteria are not all killed, and their higher water activity was also beneficial for putrefying bacteria to grow and reproduce, which causes that the low-temperature meat products are more perishable than other meat products and affects their shelf life [1-5].

To prolong the shelf life of low-temperature meat products, people generally choose to add some synthetic antibacterial agents, but with the improvement of living standards, people's health awareness gradually increased, more and more pollution-free natural products are demanded by public [6-7].

Cassia seed, also known as cassia obtusifolia, is the dried ripe seeds of legume of cassia seed. It is cultivated in most parts of the country, such as Anhui, Guangxi, Zhejiang and Sichuan. The main active ingredient in cassia seed is anthraquinone. It has many physiological functions such as antioxidation, bacteriostasis and anti mutation [8-10].
In the food industry, the traditional method of anthraquinone from Cassia seed is used ethanol or alkaline aqueous solution to extract, but it has many disadvantages, such as many processes, large losses, long cycle, and the low extraction rate. Ultrasonic extraction has many advantages, such as high extraction rate, easy operation, fast extraction speed and less loss of active components. It has been widely used in the extraction of natural ingredients [11-12]. This experiment was used ethanol as extraction agent, and extracted the anthraquinones form Cassia seed by ultrasonic extraction method. The extraction of anthraquinones in Cassia seed was determined by using 1,8-dihydroxyanthraquinone as the reference substance. The extraction process was optimized. It can provide scientific basis for further research and production. The oxford plate assay system was used to investigate the inhibition of anthraquinone compounds form Cassia seed to the main spoilage bacteria, Staphylococcus, Serratia, Bacillus and Proteus in spiced pig head, for the future, added the Cassia seed as a bacteriostatic agent into the low-temperature meat products to extend its shelf life.

2. Materials and methods

2.1. Materials
Cassia seed blocks are purchased at Mengzheng Pharmacy. All organic reagents came from Beijing land bridge Company and all Inorganic reagents came from Beijing Chemical Works. The indicator bacteria were isolated and purified from spiced pig head by Beijing University of Agriculture.

2.2. Alcohol extract
Took out the dried Cassia seed block and grinded into powder by pulverizer, after 40 mesh sieve, it was placed in a sealed place at normal temperature.

2.2.1 Ethanol concentration. The 2.5g samples were divided into five groups on average. Each group according to the solid-liquid ratio of 1:25, were added for 50%, 60%, 70%, 80%, 90% ethanol, soaking at room temperature, overnight. The samples were extracted by ultrasonic power 480W at 30 °C for 40 min. The extraction with ethanol was repeated thrice. The ethanol extracts were filtered and evaporated by using a rotary evaporator at 50°C, kept at 4 °C.

2.2.2 Ultrasonic time. The 2.5g samples were divided into five groups on average. Each group according to the solid-liquid ratio of 1:25, were added for 60% ethanol soaking at room temperature, overnight. Each group was extracted by ultrasonic power 480W at 30 °C for 20min, 30min, 40min, 50min, 60min. The extraction with ethanol was repeated thrice. The ethanol extracts were filtered and evaporated by using a rotary evaporator at 50°C, kept at 4 °C.

2.3. Ultraviolet spectrophotometry
1, 8-dihydroxyanthraquinone standard was accurately weighed 10mg and dissolved in 100mL methanol, which was the standard solution of 1,8-dihydroxyanthraquinone. Added 0.5 magnesium acetate-methanol solution as chromogenic agent, with 0.5 Magnesium Acetate-methanol solution as blank sample, the standard solution was spectrally scanned in 300-700nm [13], to determine the optimum absorption wavelength.

The standard solution was removed 0.5mL, 1.0mL, 1.5mL, 2.0mL, 2.5mL, 3.0mL, 3.5mL, 4.0mL and added 0.5 magnesium acetate-methanol solution as chromogenic agent, then constant-volumed in 10mL volumetric flask. With 0.5 Magnesium Acetate-methanol solution as a blank solution, it was measured the absorbance. Absorbance (A) and concentration (C) was used as coordinates and fitted equation of linear regression.
2.4. Actication of culture
Strains (Staphylococcus, Serratiaeae, Bacillus, Proteus) were recovered to normal temperature and poured into 50mL sterilized nutrient broth, cultured 12 h at 30 °C. And then the strain added to new nutrient broth medium with 2% inoculation amount, activated again.

2.5. Bacteriostat of anthraquinone by Oxford cup method
Sterile nutrient agar was inoculated with microbial cells (1 mL of microbial cell suspension in 100 mL agar medium) at 46 °C and then poured into sterile petri dishes each about 15-20mL.

The sterilized Oxford cup was placed on the carrier plate gently. 100 μL of 1 g/mL of Cassia seed active ingredients and distilled water were added to the Oxford cups (three times). After incubating at 37℃ for 12h, the antibacterial activity was evaluated by the diameter of inhibition zones.

2.6. Determination of minimum inhibitory concentration
1g/mL of Cassia seed active ingredients were diluted 2 times into different mass concentration gradient. 100mL Cassia seed extraction of different concentrations were added in different Oxford cups on the same plate, and the purified distilled water was used as blank control (three times). After culturing at 30℃ for 12h, the antibacterial activity was evaluated by the diameter of inhibition zones [14].

3. Results and discussion
As seen in figure 1, with the increase of ethanol concentration, the concentration of anthraquinone in the extract is also slowly increasing, and when the increase to 60%, the concentration of anthraquinone drops rapidly. When the concentration of ethanol was 60%, the concentration of the extract was the highest. The reason is that the form of anthraquinone in Cassia seed is conjugated anthraquinone glycoside and free anthraquinone, and the content of conjugated anthraquinone glycoside is higher. The conjugated anthraquinone glycosides are dissolved in water easily, while the free anthraquinone is soluble in organic solvent easily [15]. So when the ethanol concentration is less than 80%, with the increase of the concentration of ethanol, the free anthraquinone is soluble more. Total concentration of anthraquinone is gradually increased. But when the ethanol concentration is higher than 60%, anthraquinone glycosides which dissolved in water easily are dissolved decreased, making the concentration of the anthraquinone decreased.

![Figure 1](image1.jpg) **Figure 1.** The effect of ethanol concentration on extraction of Anthraquinone.

![Figure 2](image2.jpg) **Figure 2.** The effect of extracted time time on extraction of Anthraquinone.

As seen in figure 2, before 20min, the concentration of anthraquinone increases with the increase of extracted time. After 30min, the concentration of anthraquinone decreases slowly with the increase of extraction time. The reason may be that some anthraquinones are unstable in heat. With the increase of extraction time, it will lead to decomposition [16].
Table 1. Inhibitory zone diameter of anthraquinone in Cassia seed.

| strain        | Inhibitory zone diameter /mm |
|---------------|-----------------------------|
| Proteus       | 12 ± 0.1                    |
| Staphylococcus| 12 ± 0.2                    |
| Bacillus      | 18 ± 0.5                    |
| Serratieae    | 0                           |

*Note: 0 means no inhibition zone.

As can be seen from table 1, 1g/mL of anthraquinone in Cassia seed has obvious inhibitory effect on Proteus, Staphylococcus, and Bacillus, but it has no bacteriostasis effect on Serratieae.

Table 2. MIC of anthraquinone in Cassia seed.

| anthraquinone in Cassia seed (g/mL) | 1  | 0.05 | 0.25 | 0.125 | 0.0625 | 0.03125 |
|-------------------------------------|----|------|------|-------|--------|---------|
| Inhibitor                           |    |      |      |       |        |         |
| Proteus                             | 12 ± 0.1 | 0  | 0   | 0 | 0 | 0 |
| Bacillus                            | 12 ± 0.2 | 10 ± 0.5 | 8 ± 0.3 | 6 ± 0.1 | 0 | 0 |
| Serratieae                          | 18 ± 0.5 | 11 ± 0.2 | 10 ± 0.2 | 6 ± 0.1 | 0 | 0 |

*Note: 0 means no inhibition zone.

As can be seen from table 2, the anthraquinones in Cassia seed has well bacteriostatic efficacy on Staphylococcus which minimum inhibitory concentration is 0.125g/mL and the maximum inhibition zone is 18mm; the effect of Bacillus is second which minimum inhibitory concentration is 0.125g/mL and the maximum inhibition zone is 12mm; the bacteriostatic efficacy on Proteus is ordinary which minimum inhibitory concentration is 1g/mL and the maximum inhibition zone is 12mm. The reason may be that Staphylococcus and Bacillus are gram positive bacteria, while Proteus and Serratieae belong to gram negative bacteria and the anthraquinone in Cassia seed is better for gram positive bacteria.

The components of anthraquinones in Cassia seed is complex. There are more than 20 kinds of substances [17] at least, such as aloin, Cassia seed sin, emodin and so on. The antibacterial mechanism of anthraquinone in Cassia seed might be two. One is to change the permeability of cell membrane leading to the cell damage: bacteria are through the selective permeability of cell to absorb nutrition from the outside world for its growth and reproduction. When the permeability of the cell membrane changing, the leakage of nucleic acids causes the cell deformation and nutrient flowing will result irreversible damage to bacteria themselves, and make bacterial die ultimately [18-19]. The other one is to inhibit the respiratory metabolism of bacteria: the growth and reproduction of bacteria cannot be separated from respiratory metabolism. Respiratory metabolism is the carbohydrates being oxidized and discomposed to release energy. Anthraquinone in Cassia seed can inhibit the Krebs cycle of oxidation of sugar and inhibit the synthesis of ATP and NADH in cell, which obstructs the oxidation of sugar. The respiratory metabolism of bacteria is affected, and then the bacteria can even lead to death [20].
4. Conclusion
In the single factor analysis of ultrasonic extraction, it can be concluded that 60% ethanol concentration is the best concentration of extraction and the extraction time of 20min is the best time. The main component of the extraction liquid is anthraquinone.

The anthraquinone in Cassia seed has better bacteriostatic efficacy for gram positive bacteria. And with the decrease of the concentration of active ingredients, the inhibitory effect gradually decreased, which indicated that high concentration extracts have certain bacteriostatic effect.

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