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Full Length Research Paper

Antibacterial activity of water-phase extracts from bamboo shavings against food spoilage microorganisms

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Water-phase extract of bamboo shavings (WEBS), by supercritical carbon dioxide extraction, was evaluated for its antimicrobial action against the range of food borne and food spoilage pathogens using agar disc diffusion assay in nutrient agar and Czapek Dox Agar media. The WEBS exhibited antimicrobial activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Aspergillus niger*, *Penicilliun citrinum* and *Saccharomyces cerevisiae* with a concentration-dependent relationship. The minimum inhibitory concentrations (MICs) of the WEBS against the tested bacterial strains were found in the range of 4.9 - 32 mg/ml using the two-fold dilution method. Different heat treatment conditions have no significant influence on the antibacterial activity. Emodin was taken as the standard sample to test for the content of total anthraquinone compound and preliminarily verify its antibacterial mechanism, so as to lay a theoretical foundation for development of its natural preservatives.

Key words: Water-phase extract of bamboo shavings (WEBS), antimicrobial activity, natural preservative.

INTRODUCTION

Food can be easily affected by microorganisms like bacteria, yeasts and moulds during processing, preservation and consumption, which will further lead to food spoilage and human disease. Food is preserved when the basic causes of its spoilage are controlled (Enzo et al., 2006). Improvements in the shelf life of product can have an important economic impact by reducing losses attributed to spoilage and by allowing the products to reach distant and new markets (Rhodehamel, 1992).

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Abbreviations: MICs, Minimum inhibitory concentrations; **WEBS,** water-phase extract of bamboo shavings; **EBS,** extract from bamboo shavings; **EEBS,** ethanol extract from bamboo shavings.

Food spoilage can be prevented or postponed through preservatives function of restraining or eliminating microorganisms. The preservatives are also capable of improving the color, smell and taste of food as well as maintain good quality of food. In addition to requiring functional foods, there is also an increasing demand for microbiologically safe foods that are not perceived as 'chemical' or 'artificial' (Gary, 2002). To meet the wide array of challenges imposed by these trends, the multiple functional properties (antioxidative, antimutagenic and antimicrobial) of many natural food ingredients, particularly plant extracts, are being investigated and exploited (Cutter, 2000; Edenharder et al., 2001), so the search for more natural antimicrobials have led food scientists to investigate the effectiveness of inhibitory compounds such as organic acids, essential oils, bacteriocins, and dried fermentation-based products (Lemay et al., 2002).

It has been well-known that various extracts made of

different parts of bamboos have multiple biological activities such as antioxidant, scavenging oxygen radicals, protecting human being from cardiovascular disease and cancer, as well as anti-bacteria and anti-virus (Lu et al., 2005, 2006; Zhang et al., 2007). There are many effective components in bamboo leaves, shavings and shoots including flavonoids, phenolic acids, polysaccharides, anthraquinones, coumarins, special amino acids and peptides, etc (Lu et al., 2005, 2006; Zhang et al., 2004, 2007). Among these, phenolic compounds, anthraquinones and coumarins are all of relatively strong anti-bacterial and bactericidal functionalities. Bamboo shavings (*Caulis bamfusae* in taeniam), which are the intermediate layer of the stems of *Bambusa tuldoides* Munro, *Sinocalamus beecheyana* var. *pubescens* P.F. Li or *Phyllostachys nigra* (Lodd.) Munro var. *henonis* (Mitf.) Stapf ex Rendle, are perennial plants of the family Gramineae (Zhang et al., 2004). Our studies in recent years show that there are abundant bio-active components in bamboo shavings such as triterpenoids, saponins and sterols. The safety of EBS, a triterpenoid-rich extract from bamboo shavings and EEBS, a polyphenol-rich ethanol extract from bamboo shavings were evaluated (Zhang et al., 2004; Gong et al., 2010). Our previous study also found that EBS has excellent anti-fatigue, antihyperlipidemic and antihypertensive activities (Zhang et al., 2006; Jiao et al., 2007). WEBS, which contains abundant biological active components such as triterpenoids, flavonoids, anthraquinones and phenolic acids, is prepared by supercritical carbon dioxide extraction from bamboo shavings.

The mechanisms of the antimicrobial activity of WEBS are poorly understood, but have been shown to be dependent on both the bamboo and the bacterial species or strain against which it is tested. The present study was undertaken to further assess the potential of this extract by determining the occurrence of any antimicrobial action that the extract has on a range of food-related bacteria to expand its uses in foods. Meanwhile, the effect of different heat treatment on the antibacterial activity and anthraquinone compounds were determined to preliminarily analysis of the active components of its anti-bacterial function.

MATERIALS AND METHODS

Experimental materials

The materials used for this study are: Nutrient agar medium, Czapek's medium, sodium benzoate, methanol (analytical pure), 1.5% magnesium acetate-methanol solution and standard sample (emodin).

Instrument and equipment

Some of the Instrument and equipment used are: Constant temperature incubator, sterile operating room, high pressure sterilizer, UV-visible spectrophotometer, etc.

Sample preparation

WEBS was prepared from bamboo shavings by supercritical carbon dioxide extraction. WEBS were diluted to 32, 16, 11, 6.4, 5.3 and 4.9 mg/ml with distilled water, respectively. The insoluble fraction was separated by filtration. Distilled water and sodium benzoate was selected as negative control and positive control to evaluate the antibacterial activity.

Microorganisms and sources

Six bacterial strains, mostly food borne including *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia.coli*, *Penicilliun citrinum* and *Saccharomyces cerevisiae* were obtained from the College of Biological and Environmental Engineering, Zhejiang University of Technology, Hangzhou, China. *Aspergillus niger* was collected from College of Biosystems Engineering and Food Science, Zhejiang University. The stock cultures were kept in a refrigerator (4℃) on nutrient agar and Czapek Dox agar media, respectively.

Test methods

The following methods were applied to study the antimicrobial effect of bamboo shavings extract:

Components analysis of WEBS

Total phenolic content of WEBS was determined by the Folin–Ciocalteu method (Meda et al., 2005). The total flavonoid content was determined according to the aluminum chloride colorimetric method described by (Chang et al., 2002). The triterpenoid content of WEBS was determined by Colorimetry using ginsenoside as a standard and phenol-sulfuric acid method was chosen to determine its total sugar.

Preparation of culture medium

Thirty four (34 g) nutrient agar and 50 g Czapek's medium powder was put in 1000 ml distilled water and then heat with mild fire till the powders dissolved, sterilize at the temperature of 121℃ for 20 min and then can be used, pH was 7.3 ± 0.2 and 5.8 ± 0.1, respectively.

Preparation of bacterial suspension

Respective suitable slant medium was used to activate the bacterial species to be tested by means of sterile operation and then select four bacterial lawns on each slant of fresh bacteria species and inoculate them into the corresponding liquid medium. It was taken as stock solution after culturing in the constant temperature incubator at the most suitable temperature for 16 – 18 h. Bacterial suspensions containing bacteria of about 10^6 CFU/ml was prepared with sterile physiological saline for further use.

Antimicrobial activity determined by the agar diffusion method

Antimicrobial activity was measured using the agar diffusion method (Kirby–Bauer method). Inocula were prepared from every pure cultures, incubated in nutritive agar and czapek dox agar for their optimum time. Bacterial suspension was obtained diluting with sterilized physiological saline solution. One milliliter (1 ml) of the obtained cellular suspension was added to 10 ml cultures previously melted, mixed, poured in Petri dishes and left 1 h to

Table 1. Antibacterial activity of extract from bamboo shavings (WEBS).

Microorganism	Inhibition zone (mm)	Blank control (distilled water)	Positive control (sodium benzoate)
Staphylococcus aureus	16.8 ± 3.6	-	21.6 ±1.2
Bacillus subtilis	18.1 ± 1.0	-	19.2 ± 1.8
Escherichia.coli	14.7 ± 3.1	-	16.4 ± 1.1
Aspergillus niger	17.0 ± 0.4	-	18.0 ± 0.8
Penicilliun citrinum	9.1 ± 0.5	-	10.4 ± 0.5
Saccharomyces cerevisiae	8.5 ± 0.5	-	9.2 ± 0.3

solidify. A sterile coating bar was used for even coating and a sterile tweezer was used to steadily place 3 sterilized cups on the solid culture medium with equal distance. One hundred microlitres (100 µl) of sample was added in cup holes with distilled water and sodium benzoate as negative and positive control. Each treatment was repeated for three times, and culture was carried out at the temperature that was most suitable for each bacteria. After incubation, the inhibition zones were measured to 1 mm accuracy and the effect was calculated as the mean of the duplicate experiments. Three replicates were used for each bacterial strain. After incubation, the zones of growth inhibition around every cup were measured (including cup) in mm with a vernier caliper.

Minimum inhibitory concentrations (MICs) determined by plate culture

The MICs of WEBS were determined for all bacteria by the two-fold dilution method (Murray et al., 1995). A loopful of the each bacterial culture was inoculated in the nutrient agar and Czapek Dox agar media for their optimum time. For sample preparation, briefly, the potential antimicrobial agents were subject to a twofold dilution series with microbial growth media in microtitre plate wells.

The wells of media containing the diluted potential antimicrobial agents were subsequently inoculated with an appropriate level of the organism of interest and incubated for 24 - 48 h under the conditions appropriate for individual bacteria. The minimum concentrations of WEBS at which no visible growth was observed were defined as MICs which were expressed in mg/ml. All the tests for MICs determinations were performed in triplicate.

Effect of different treatment on the antibacterial activity

The sample was treated with different heating method to testify the application in the food processing. A (90°C, 60 min treatment), B (100°C, 45 min treatment) and C (121°C, 30 min treatment) were used to handle the sample with untreated one as comparison.

Determination of emodin standard curve

The content of anthraquinones in WEBS was determined by colorimetric method with emodin as the standard. 3.2 mg standard sample of emodin was dried to constant weight at the temperature of 105°C and then methanol added with ultrasonic treatment to promote dissolution. Standard solutions of 0.1, 0.2, 0.4, 0.8, 1.2 and 1.6ml were respectively put in evaporating dishes and dried up by evaporation in a water bath; 2 ml of magnesium acetate-methanol solution (1.5%) was added and then shaken evenly, the absorbance at 510 nm was measured with 1.5% magnesium acetate-methanol solution as blank.

Preparation and determination of total anthraquinone of WEBS

Five milliliter (5 ml) of the sample solution to be tested was evaporated and 50 ml of sulfuric acid (20%) was added to dissolve it in water for 2 h, ethyl acetate was extracted for 3 times with 10ml per time after cooling.

1.5% magnesium acetate-methanol solution was used to show color after reducing pressure and evaporating to dry, total content of anthraquinone compounds was calculated with the value of absorbance (A) was measured at 510 nm.

RESULTS AND DISCUSSION

Components of WEBS

The PH, total solids, refractive index, specific gravity, triterpenoid, total sugar, flavonoids, protein and phenolic was 2.97, 3.2%, 0.6 Brix0, 0.98 mg/ml; 3.65, 0.8, 0.69, 0.625 and 0.465 mg/ml, respectively.

Inhibitory effect

Agar plate diffusion method was used for qualitative measurement of the inhibitory effect of the sample *in vitro*, which makes use of diffusion of the sample in agar to inhibit growth of the surrounding bacteria, thus to form an inhibition zone; whether the bacteria are sensitive to the sample or not may be judged according to the size of the inhibition zone. This experiment adopted oxford cup instead of disk diffusion method, which means that the oxford cup containing quantitative antibacterial sample is placed on the agar plate which has been inoculated with experimental bacteria, and the sample dissolves and continuously diffuses to the surrounding which forms a degressive concentration gradient. If the growth of the experimental bacteria is inhibited surrounding the oxford cup, a transparent inhibition zone will be formed. The size of inhibition zone and the sensitivity of experimental bacteria to the sample are in positive correlation. The antibacterial activity of different concentrations of WEBS on the growth of various strains of bacteria tested by cup method is represented in Table 1, and Figures 1 and 2.

As shown in Table 1, Figures 1 and 2, the WEBS was found to be effective against all the strains of bacteria tested. The inhibitory effect of undiluted sample on *S.*

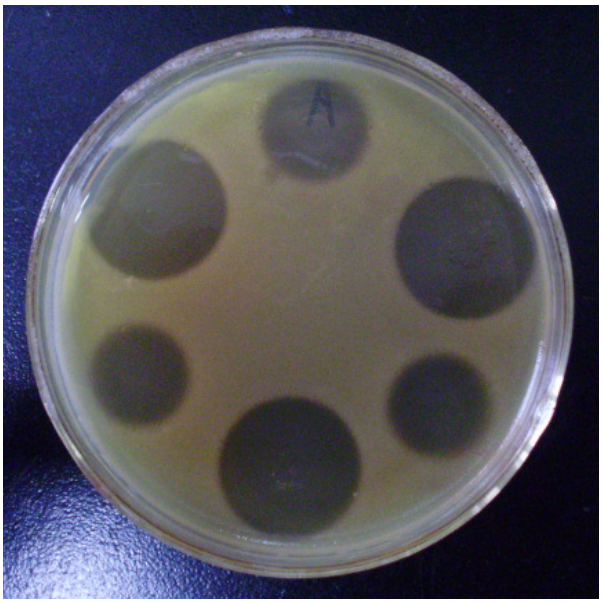

Figure 1. Test of inhibition of the sample on *S. aureus*.

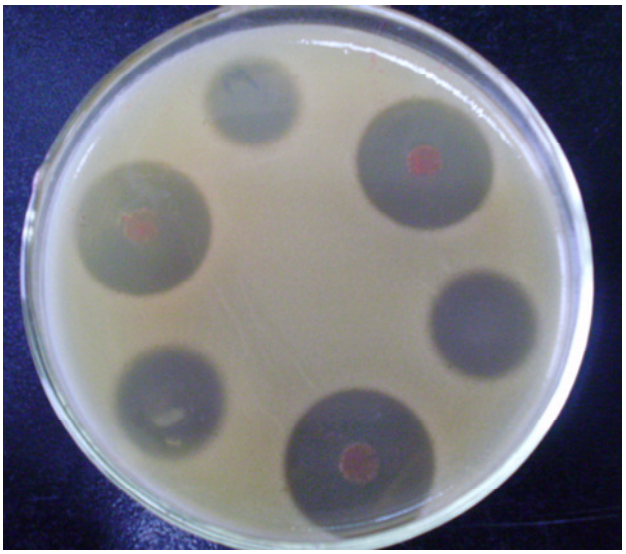

Figure 2. Test of inhibition of the sample on *E. coli.*

aureus, *B. subtilis*, *E. coli* and *A. niger* was relatively strong, and diameters of the inhibition zones are 16.8 ± 3.6, 18.1 ± 1.0, 14.7 ± 3.1 and 17.0 ± 0.4 mm, respectively. The inhibitory effect of undiluted sample on *P. citrinum* and *S. cerevisiae* is relatively weak, and diameters of the inhibition zones are 9.1 ± 0.5 and 8.5 ± 0.5 mm, respectively. The inhibitory effect of WEBS is a little lower than the positive control sodium benzoate which is very effective in the inhibition of bacterial spoilage in the food processing. So the results revealed that it maybe used in the food products to extend the shelf-life.

MICs of WEBS against bacteria

Concentrations of antimicrobial required for inhibiting growth of all bacteria tested in the experiment is referred to as MICs. MIC is general evaluation on the micro-organisms inhibiting performance or bacteria inhibiting effect of the antimicrobial, which represents the capacity of inhibiting and preventing microbial reproduction of the antimicrobial. The minimal inhibitory concentrations (MICs) of the sample to be tested to the bacteria for testing were shown in Table 2.

As shown in Table 2, the MICs of WEBS against *S. aureus*, *B. subtilis*, *E. coli*, *P. citrinum*, *S. cerevisiae* and *A. niger* was 4.9, 5.3, 6.4, 16, 16, and 4.9 mg/ml, respectively. It means, the sample still had certain inhibitory effect on *S. aureus*, *B. subtilis*, *E. coli* and *A. niger* after being diluted 5 times, but no inhibitory effect was shown on *P. citrinum* and *S. cerevisiae*.

Influence of sample concentration on inhibitory effect

The curves of inhibitory effects of samples of different concentrations on bacteria, moulds and yeasts were shown in Figures 3, 4 and 5, respectively. As can be seen, the samples all had relatively strong inhibitory effect on *S. aureus*, *B. subtilis* and *E. coli*. The change trend of the three inhibition curves were relatively consistent, which was to say, with the increase of dilution times and decrease of concentration, the inhibitory effect reduced. However, the increase of dilution times and decrease of concentration had no significant influence on the inhibitory effect of *E. coli*, the diameter of the inhibition zone after 5 times dilution was still 10.9 ± 1.4 mm. It indicated that it had much better inhibiting effect on *E. coli*, which had special meaning to prevent food pollution and food borne diseases. With the decrease of concentration, the inhibitory effect on *S. aureus* and *B. subtilis* reduced rapidly. As the dilution was further increased, the diameter of the inhibition zone became smaller rapidly, and the inhibitory effect strikingly reduced.

As shown in Figure 4, the inhibitory effect of WEBS on *A. niger* was more than that on *Penicilliun citrinum*. Diameters of inhibition zones when diluted by 1 time and 2 times were 13.7 ± 1.2, 8.3 ± 0.6, 11.3 ± 1.0 and 7.3 ± 0.8 mm, respectively, and the inhibitory effect and the concentration were in positive correlation (the inhibitory effect strikingly reduced with decrease of the concentration). The sample had no inhibitory effect when diluted by 4 times.

As shown in Figure 5, the inhibitory effect of WEBS on *S. cerevisiae* was relatively poor. The diameter of the inhibition zone of undiluted sample was only 8.5 ± 0.5 mm, and the inhibitory effect strikingly reduced with decrease of the sample concentration. There was no obvious inhibition zone that could be measured when the sample was diluted by 3 times.

Table 2. Minimal inhibitory concentrations (MIC) of WEBS against bacteria.

Microorganism	Minimal inhibitory concentrations (MIC, mg/ml)	Cultures (pH)
Staphylococcus aureus	4.9 ± 0.2	7.0
Bacillus subtilis	5.3 ± 0.3	7.0
Escherichia.coli	6.4 ± 0.1	7.0
Aspergillus niger	4.9 ± 0.2	6.5
Penicilliun citrinum	16.0 ± 0.5	6.5
Saccharomyces cerevisiae	16.0 ± 0.8	6.5

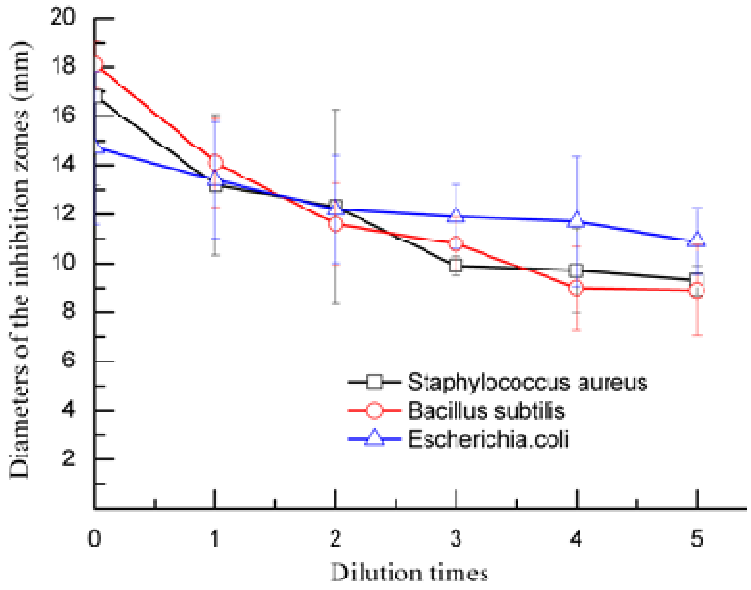

Figure 3. Influence of sample concentration on bacteria inhibitory effect.

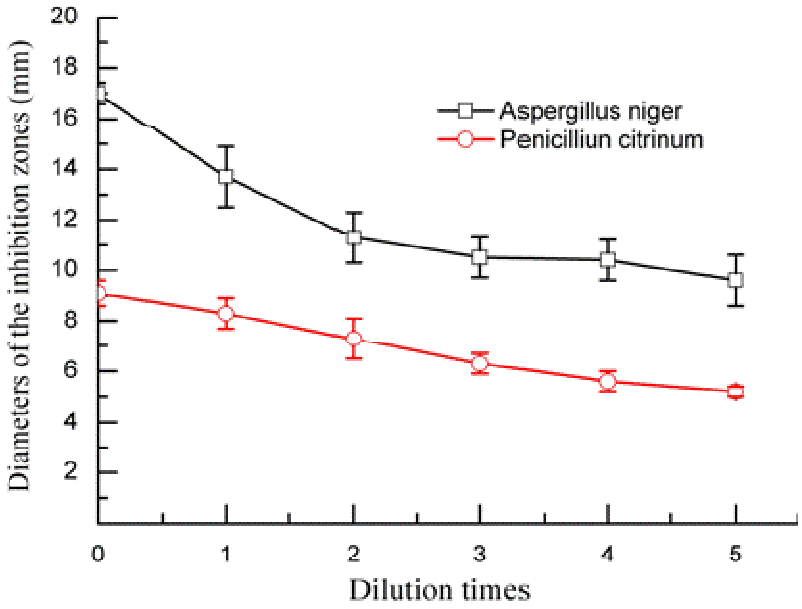

Figure 4. Influence of sample concentration on moulds inhibitory effect.

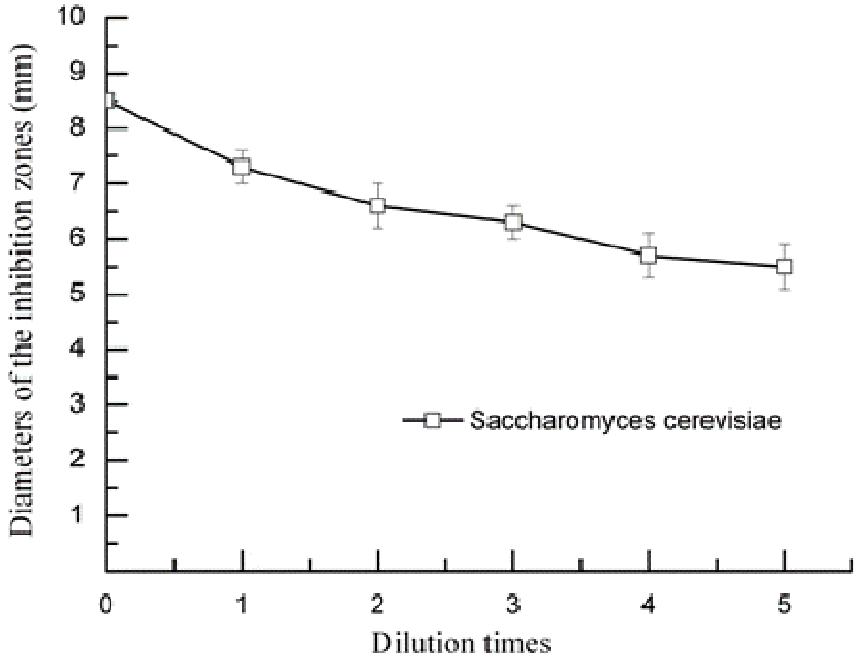

Figure 5. Influence of sample concentration on yeast inhibitory effect.

Table 3. Effect of different heating conditions on **antibacterial activity.**

Microorganism	Stock solution (mm)	A (mm)	B (mm)	C (mm)
Staphylococcus aureus	20.6 ± 0.5	21.5 ± 0.6	17.5 ± 0.4	21.9 ± 0.6
Bacillus subtilis	17.0 ± 0.4	19.0 ± 0.3	15.0 ± 0.2	18.2 ± 0.2
Escherichia.coli	15.2 ± 0.2	14.8 ± 0.6	14.6 ± 0.3	13.6 ± 0.4
Aspergillus niger	14.0 ± 0.1	14.2 ± 0.2	13.5 ± 0.4	13.8 ± 0.3
Penicilliun citrinum	8.5 ± 0.3	8.0 ± 0.1	8.3 ± 0.2	8.4 ± 0.2

A represents 90°C, 60 min treatment; B represents 100°C, 45 min treatment and C represents 121°C, 30 min treatment.

Effect of different heating treatment on the antibacterial activity

Heating is a commonly used method for food processing and the study on the influence of heat treatment conditions on the antibacterial activity was of great significance. Whether the antibacterial activity was declined will be known by measuring the zones of growth inhibition around every cup, thus to study the effect of heat treatment has on the antibacterial activity. The results of influence of different heat treatment conditions on the antibacterial activity were shown in Table 3.

As can be seen from the Table 3, the three treatment methods, namely; A (90°C, 60 min treatment), B (100°C, 45 min treatment) and C (121°C, 30 min treatment), had no significant influence on the antibacterial activity of antibacterial components in the extract solution of WEBS. Particularly, the treatment method C (121°C, 30min) had no influence on the antibacterial activity of the extract. After high-temperature treatment, the inhibitory effect of part of the test groups was obviously strengthened, so it was suitable to add WEBS to food requiring high-temperature treatment.

Determination of the total anthraquinones

An ethanol/water extract of bamboo leaf mainly contains flavone glycosides, phenolic acids, coumarin lactones, anthraquinones and amino acids (Zhou, 1992; Zhang and Ding, 1996a, b; Chen et al., 2002; Meng et al., 2002; Li et al., 2003; Luo and Chen, 2003; Lu and Liao, 2003). Various anthraquinone substances have antibacterial functions of different degrees, of which, emodin, rhein and aloe-emodin have relatively strong antibacterial functions. Because of the complexity of existing form of

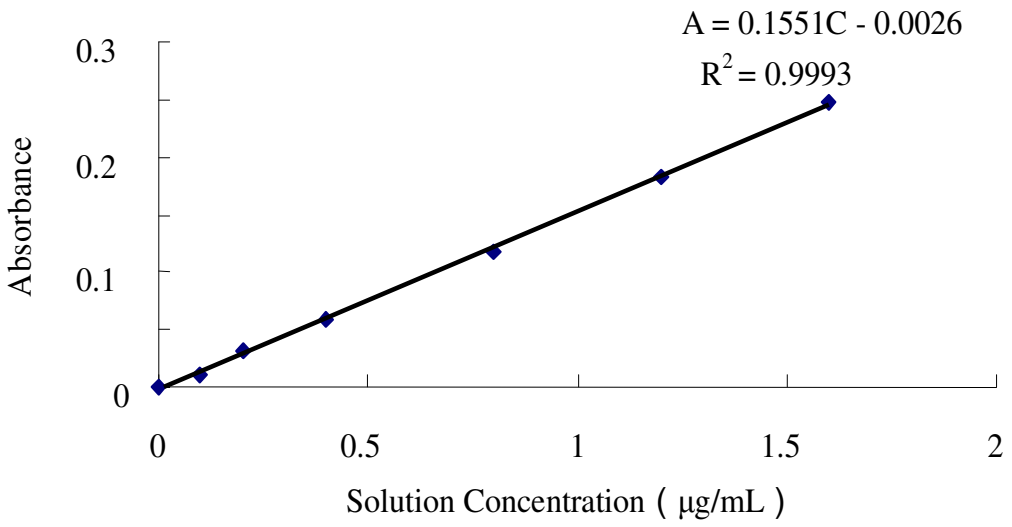

Figure 6. The standard curve of $Mg(AC)_2$ colorimetric method.

anthraquinone compounds in plants and the difference of various types in polarity and solubility, the extraction methods are diversified, which mainly include solvent extraction, ultrasound-assisted extraction, supercritical extraction and microwave exaction. Methods for content determination mainly include colorimetry, paper chromatography, thin layer chromatography, high performance liquid chromatography, etc. Determination of emodin standard curve was shown in Figure 6.

There are many anthraquinone compounds contained in bamboo extracts, which have certain antibacterial effect and other functions beneficial to the health of human bodies. Colorimetry was adopted to measure the total content of anthraquinone compounds and prelim-narily discussed their antibacterial mechanism. The average optical density (OD) value of WEBS by 2 times was 0.123, the total content of anthraquinone compounds in the solution by 2 times was 2.59 μg/ml. With combination of the antibacterial activity tests of WEBS, we know that the antibacterial activity of WEBS, to some extent, was related to the total concentration of anthra-quinones.

Safety evaluation of WEBS

Safety evaluation experiment must be done to expand its uses in the food processing and technology. The acute toxicological, subchronic and developmental toxicity tests will be discussed in the subsequent articles.

Conclusion

In conclusion, we reported here, the novel function of WEBS as an antibacterial agent showing antibacterial activity against food spoilage microorganisms. Our results suggested that use of WEBS can be considered as antibacterial availability for trials in controlling food safety standards. Therefore, WEBS may be useful in controlling number of food borne and food spoilage pathogens as a traditional concern in food systems.

Considering the results, it may be concluded that, WEBS tested in the performed experimental conditions may successfully inhibit the bacterial *in vitro* as safe levels for human consumption and, consequently, it can be useful as natural preserver or unspecific antibacterial food preserver.

Prospects

Supercritical extracts from bamboo shavings contain a large number of effective factors. The antibacterial effect and function vary with the extract concentration and type of bacteria, so they should be applied reasonably in practice. Under different pH values, there is an obvious difference in the antibacterial effect. It is a subject requiring further study as to how to give full attention to the antibacterial effect of effective factors in bamboo shavings so as to achieve the best antibacterial effect. Inhibition on different microorganisms by different effective factors is also a new study field.

At present, the study on the antibacterial mechanism of extracts from bamboo shavings is still not sufficient, and the specific antibacterial factors still require further studies. This paper has just studied the relationship between anthraquinone compounds and antibacterial effect. In future, the contents and specific applications of other effective factors in extracts from bamboo shavings shall be given more attention, and the study on the application in the food antiseptic field will be carried out to enlarge the application scope of WEBS.

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