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Inhibition of foodborne bacterial pathogens in meat products using bioplastic with *Ceasalpinia sappan* heartwood crude extract

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Abstract The bioplastic packaging is found to be inhibited the growth of bacteria causing spoilage of meat products. The result showed antibacterial activity of the crude extract of *C. sappan* heartwood. The results showed that the MIC values for *E. coli*, *B. cereus* and *S. aureus* were 1,875 mg/ml. In addition, the MBC values for *E. coli* and *S. aureus* were found to be 7,500 mg/ml but the MBC values for *B. cereus* were found to be more than 60,000 mg/ml. The result indicated that the inhibition zone diameter for bioplastics from corn starch and *C. sappan* heartwood crude extract inhibited *E. coli*, *B. cereus* and *S. aureus* were 5.4, 10.4 and 13.4, respectively. Therefore, bioplastic and crude extract of *C. sappen* heartwood could develop into packaging to prolong the meat product spoilage.

Keywords: Bioplastic, Foodborne spoilage, Disc diffusion method

Introduction

Nowadays, the meat products are highly demanded due to their high nutrient value, reasonable price, good taste and easily eating. The meat products are important bioactive sources as protein, fat, essential amino acids, minerals and vitamins. The quality, quantity, safety and shelf-life of meat products are developed through processing, marketing, storage and preparation for consumption. (Eldin Shaltout *et al*., 2022). Meat products are highly perishable food and contaminated food is one of factor the shelf-life, quality and safety. Thereby, products of meat must store, process, and pack and distribute in order to prevent microbial growth. Microbes present in meat products have several species. The most frequently identified microorganisms associated meat products are bacterial pathogens such as *Escherichia coli*, *Bacillus cereus* and *Staphylococcus aureus* (Pal *et al*., 2018). The plant products exhibiting strong antimicrobial could be considered as sustainable health. Researchers conclude

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the potential of bioactive compounds in crude extract as food preservatives. *Caesalpinia sappan* heartwood properties have been found to prolong the shelf life of some food products for their antimicrobial activities and inhibitors of micro-pathogen. *Caesalpinia sappan* heartwood containing brazillin exhibits antibacterial properties against several gram-positive bacteria, such as *S. aureus*, *B. subtilis* and gram-negative bacteria, such as *Klebsiella pneumoniae*, *E. coli*, and *Proteus vulgaris* (Seephonkai *et al*., 2021).

Furthermore, the significance retarding spoilage, prolong shelf-life, and preserving the quality of packaged food. The physical, chemical, or biological damage are essential facter to protect of food packaging. Treatment quality and safety of food packaging are the time of manufacture to the time it is used by the consumer. Food packaging use single use and discarded after a short time. These environmental problems caused by food packaging impede the sustainability of packed food. Sustainability concern in the food packaging, drives the more use of bioplastics. Thus, the biodegradability of bioplastics is a result to decrease pollution and support eco-friendly (Emadian *et al*., 2017).

Therefore, the development of bioplastics with *C. sappan* heartwood crude extract has promote environmental sustainability and antimicrobial activity in meat products. The aim of this work was to investigate the addition *C. sappan* heartwood crude extract of the bioplastic packaging for inhibiting of foodborne bacterial pathogens in meat products.

Materials and methods

Bacterial pathogens

Bacterial pathogens in meat products consisted of *B. cereus, S. aureus and E. coli.* were obtained from Department of Biochemistry, Faculty of Science Mahidol University, Thailand. The pathogenic bacteria were cultured on nutrient agar (NA) at 37°C for 5 days.

Extraction from C. sappan heartwood powder

A powder of *C. sappan* heartwood were collected from Kabinburi district, Prachin Buri province. The *C. sappan* heartwood powder sample of 1.2 kg was weighed and soaked in 6,000 ml of 95% ethanol in a separating funnel for 5 days, with intermittent shaking. The resulting 95% ethanol extract was completed, the rotary evaporator at 50°C was used to remove 95% ethanol from the extract sample. The crude extract was cooled at room temperature.

HPLC for identification in C. sappan heartwood crude extract

Analysis of main constituents of *C. sappan* heartwood extract using high performance liquid chromatography (HPLC) were measured for brazilin with the modified method (Warinhomhaun *et al*., 2018). The standard solution of brazilin®SIGMA was diluted with methanol at concentration of 60 µg/ml. Sample solution of *C. sappan* heartwood extract was dissolved in methanol at concentration of 0.1 mg/ml and filtered through a 0.45 µm membrane filter before before injection in HPLC analysis. The HPLC system using a Shimadzu LC-20A HPLC system (Shimadzu Corporation, Kyoto, Japan) consisting of SIL20 AC auto-sampler, SPD-M20A detector and CTO-20A column oven. The HPLC conditions followed durind the experiment, a Halo C18 column (4.6 × 150 mm, 5 µm) controlled at 35°C with gradient elution at a flow rate of 1.0 ml/min. The mobile phases were performed by varying the proportion of methanol (A) and 2.5% v/v acetic acid (B). The gradient was as follows: initial 10% A and 90% B, 12 min, then automatically ramped to 100% A, 13 min, and automatically decreased to initial mobile phase ratio, 12 min. The compounds were detected wavelength at 280 nm. The injection volume was 20 µl. Finally, *C. sappan* heartwood crude extract had the same peak with the standard brazilin and was used for further experiment, and therefore it was confirmed as brazilin.

Antibacterial activities

The sensitivity of different bacterial pathogens to *C. sappan* heartwood crude extract was measured in terms of zone of inhibition using a paper-disc agar diffusion method (Salayová *et al.*, 2021). All bacterial pathogens mentioned above were incubated on NA at 37°C for 5 days. Each bacterial pathogen was adjusted to 10^8CFU/ml with sterile saline solution compared to the McFarland 0.5 turbidity standard and 100 µl of bacterial solution was spread on nutrient agar plates. Experiments were performed in duplicate and repeated three times for each combination of plant crude extract and bacterial pathogens. 1% dimethyl sulfoxide (DMSO) was used as the negative control, while 200 mg/ml streptomycin was used as the positive control. Sterile paper discs shape with a diameter of 6 mm were added 30 µl of *C. sappan* heartwood crude extract (final concentrations 600, 6,000, and 60,000 mg/ml). The dried discs were placed onto into the agar media and incubated for 24 hours at 37°C. Data Analysis of variance was performed by SPSS software. Means values were compared using one-way analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT). Statistical significance accepted at a level of $P < 0.01$.

Microbiological parameters were the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of antimicrobial efficacy of plant crude extract. Determination of MIC was examined using colorimetric broth microdilution. (Coelho *et al*., 2021). A two-fold serial dilution of each plant crude extract in 1% dimethyl sulfoxide (DMSO) was prepared and obtained the final concentrations ranging from 117.19 to 60,000 mg/ml with adjusted bacterial concentration (10^8CFU/ml) in microbial broth culture. Another set of wells containing only the growth medium and each of the test bacteria with no crude extract was set up separately as a control. The plate incubated at 37 °C for 24 hours. After each incubation for 37 °C for 24 hours, 50 µl of resazurin solution was added per well. A change in color from blue (oxidized state) to pink (reduced state) indicated growth of the bacteria. The MIC was regarded as the lowest concentration of the plant crude extract in the series of dilutions which did not change in color from blue to pink. The MBC of *C. sappan* heartwood crude extract was determined subcultures made from 10 µl samples, obtained from wells which did not change in color from blue to pink in the MIC determination of plant crude extract, was inoculated onto freshly NA agar plates after further 37 °C for 24 hours. The MBC was regarded as the lowest concentration of an antimicrobial agent that killed the original inoculum and did not grow onto the medium used. The criteria were selected even the plant crude extract was sterilized a bacterial population.

Preparation of bioplastics

The corn starch bioplastic formulations were prepared by mixing corn starch, glycerin, gelatin, distilled water, vinegar, and coconut oil. The formulation mixture was stirred for 8 minutes at 80 °C and added *C. sappan* heartwood crude extract. For the negative and control bioplastic production, 1% dimethyl sulfoxide only and 24% acetic acid were used, respectively. The resulting solution was poured and spread onto oiled aluminum foil and incubated in a hot air oven at 35 °C for 2 days on a flat surface, to ensure a consistent film thickness. The drying the spread of bioplastic was enabled the process of spread removal from aluminum foil and further involved into next tests.

Antibacterial activity test of bioplastics

Determination of antibacterial property bioplastics containing 7,500 mg/ml of plant crude extract. 0.1% DMSO bioplastic were used as negative control, while 24% acetic acid bioplastic was used as positive control. The antibacterial in *vitro* activity of the bioplastics was tested using growth inhibition

studies against *B. cereus*, *S. aureus* and *E. coli* at a concentration of $1X10^8$ CFU/ml by modified a paper-disc agar diffusion method (Salayová *et al*., 2021). Data were presented as mean values for zone of inhibition and multiple comparisons were performed using one-way analysis of variance (ANOVA) followed by DMRT at $P<0.01$.

Results

Identification in C. sappan heartwood crude extract using HPLC

The HPLC method was developed and optimized to obtain a suitable chromatographic condition and Brazilin in plant crude extract samples were determined. Chromatographic analysis by HPLC of the crude extract allowed the identification of brazilin, in comparison with brazilin standard. Chromatographic analysis showed that the *C. sappan* heartwood crude extract had many peaks at different retention times. The peak for brazilin in the sample allowed the identification to a retention time of 5.1 min, which matched the retention of brazilin in the standard chromatogram (Figure 1. A and 1. B).

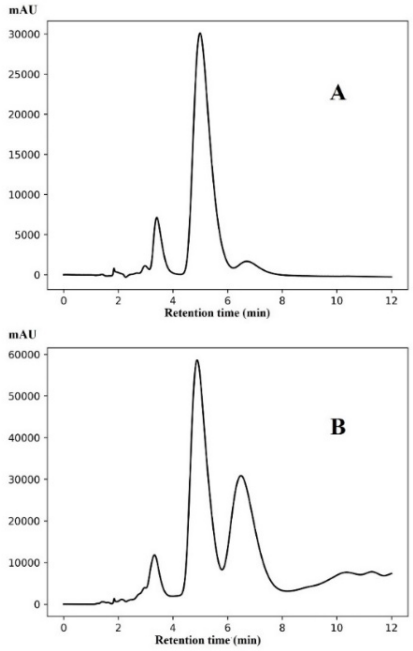
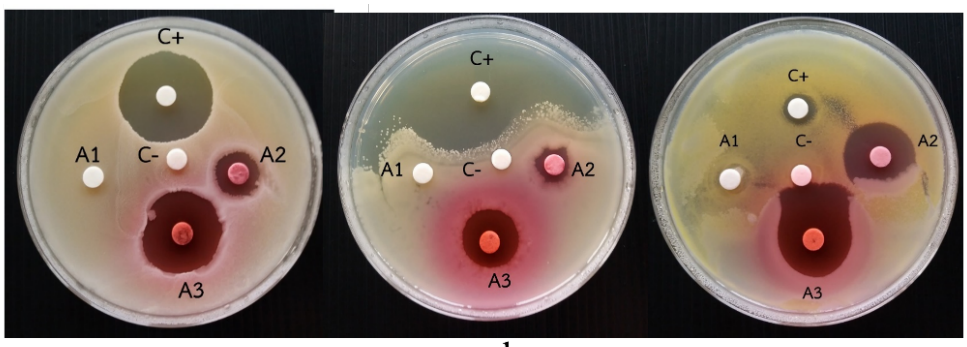


Figure 1. HPLC chromatograms obtained from (A) 60 µg/ml brazilin standard and (B) sample solutions (0.1 mg/ml) of *C. sappan* heartwood extract, presenting the brazilin peaks at retention time of 5.1min

Antibacterial activity of extract

Results showed the antibacterial activity of plant extract tested against *B. cereus*, *S. aureus* and *E. coli* were presented in Figure 2. The plant crude extract showed antibacterial activity against the growth of bacterial pathogens at concentrations of 6,000 mg/ml and 60,000 mg/ml. Antibacterial activity against *B. cereus*, *S. aureus* and *E. coli* with diameter zone of inhibition between the crude extract at a concentration of 6,000 and 60,000 mg/ml was significantly differed (Table 1).



a b c

Figure 2. Effects of crude extract of *C. sappan* heartwood by different concentration; A1= 600 mg/ml, A2= 6,000 mg/ml, A3= 60,000 mg/ml, C^+ = 200 mg/ml streptomycin, C^- = 1% dimethyl sulfoxide (DMSO) on the growth of *B. cereus* (a), *E. coli* (b) and *S. aureus* (c)

Table 1. The diameter of the inhibition zone for bacterial pathogens by plant crude extract

Substances	The diameter of inhibition zone (Mean ± standard deviation) (mm)		
	B. cereus	*E. coli*	*S. aureus*
600 mg/ml crude extract	0.0±0.0^{c*}	0.0±0.0^{d*}	0.0±0.0^{d*}
6,000 mg/ml crude extract	7.2±0.2^{b}	10.0±0.2^{c}	12.0±0.4^{b}
60,000 mg/ml crude extract	16.2±0.2^{a}	20.6±0.2^{b}	22.4±0.4^{a}
200 mg/ml streptomycin	18.6±0.4^{a}	30.4±0.3^{a}	4.8±0.1^{c}
1% DMSO	0.0±0.0^{c}	0.0±0.0^{d}	0.0±0.0^{d}

*Means with different superscripts in a column indicate significant difference at $P<0.01$

The MIC and MBC values of plant crude extract against the three tested bacteria were shown in Table 2. *Ceasalpinia sappan* heartwood crude extract was recorded that the MIC value against *B. cereus*, *E. coli* and *S. aureus* was 1,875 mg/ml and the MBC of plant crude extract against *E. coli* and *S. aureus* was 7,500 mg/ml. On the other hand, the MBC of plant crude extract against *B. cereus* was >60,000 mg/ml.

Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of crude extract against *B. cereus*, *E. coli* and *S. aureus*

Microorganism	MIC (mg/ml)	MB (mg/ml)
B. cereus	1,875	> 60,000
E. coli	1,875	7,500
S. aureus	1,875	7,500

Antibacterial activity test of bioplastics

Each bioplastic was selected to determine its antimicrobial activity against foodborne bacterial species. The inhibition to microbial species was seen in Table 3. Bacterial pathogens were inhibited by the bioplastic production by employing *C. sappan* heartwood extract-based at a concentration of 7,500 mg/ml. Moreover, the diameter of the zone of inhibition of the bioplastic with plant extract showed significantly higher values than acetic acid at a concentration of 24% and the bioplastic with 1% DMSO.

Table 3. The average diameter of inhibition zone for bacterial pathogens by bioplastics

Bioplastics	The diameter of the zone of inhibition (Mean ± Standard deviation) (mm)		
	B. cereus	*E. coli*	*S. aureus*
1% DMSO	0.0± 0.0^{b*}	0.0± 0.0^{b*}	0.0± 0.0^{b*}
7,500 mg/ml plant crude extract	5.4± 0.6^{a}	10.4±0.1^{a}	13.4±0.2^{a}
24% Acetic acid	0.0 ± 0.0^{b*}	0.0 ± 0.0^{b*}	0.0 ± 0.0^{b*}

*Data are mean of different superscripts in a column indicate significant difference at $P<0.01$

Discussion

The HPLC chromatogram monitored for 12 min could completely resolve brazilin at 5.1 min from any other impurities presented in the *C. sappan*

heartwood crude extract. The result showed that brazilin is an active compound found in *C. sappan* heartwood heartwood (Muangrat and Thipsuwan, 2023). Methods for HPLC analysis: HPLC methods using different mobile phases (methanol and 2.5% v/v acetic acid) selected on the basis of varying gradations of solvent systems in specific retention times and elute detections were used (Nirmal *et al*., 2015). The brazilin in crude extract separated *via* HPLC was not the possible interference of the organic solvent. The HPLC methodology was investigated as validation, suitability and applicability.

Brazilin in *C. sappan* heartwood extract by 95% ethanol could inhibit *B. cereus*, *E. coli* and *S. aureus*. 95% ethanol is the most effective solvent for extracting *C. sappan* heartwood. The ethanol used in the extraction of brazilin and antimicrobial chemical compounds. This supports the investigations showed that extracting with 95% ethanol yielded a high content of important phytochemical compounds with potent antibacterial activity (Warinhomhaun *et al*., 2018). *Ceasalpinia sappan* heartwood extracts against foodborne bacterial species including *B. cereus*, *E. coli* and *S. aureus*. Plant heartwood showed strong inhibitory activity against bacteria because brazilin showed inhibiting DNA and protein synthesis (Nirmal *et al*., 2015). Brazilin had antibacterial activity against both Gram-negative bacteria and Gram-positive bacteria. Previous research conducted that *C. sappan* heartwood extract had antibacterial activity against both Gram-positive and Gram-negative bacteria, such as *S. aureus* and *E. coli* (Vij *et al*., 2023) and the brazilin extract from *C. sappan* heartwood extract was evaluated for efficacy in inhibiting growth of Gram-positive and Gram-negative bacteria, such as *S. aureus* and *Pseudomonas aeruginosa* (Nirmal *et al*., 2014).

Ceasalpinia sappan heartwood extract showed antibacterial activity against *B. cereus*, *E. coli* and *S. aureus* due to its had brazilin. Bioplastic with the addition of *C. sappan* heartwood crude extract was showed to be effective against bacterial pathogen in meat products. The development of bioplastic with *C. sappan* heartwood crude extract promoted the environmental sustainability and could be useful for food packaging in meat products.

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Conflicts of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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