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Sappan Heartwood (*Caesalpinia sappan* L.) Extract as a Natural Antimicrobial used in Beetroot Juice by Accelerated Solvent Extraction

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Abstract

After accelerated solvent extraction using ethanol-water solvent (at a weight ratio of 3 to 1) at extraction temperatures of 60°C, 120°C, and 180°C for 5 min under 1500 psi extraction pressure, the brazilin content in the extracts was determined and also the obtained sappan heartwood extracts (SHE) were used to inhibit some pathogenic bacteria in food, including *B. cereus, E. coli, S. aureus*, and *S*. Typhimurium, using agar disc diffusion method. According to the findings of this study, the average yield of SHE using ethanol-water solvent at different extraction temperatures of 60°C, 120°C, and 180°C was 9.16, 13.64 and 16.81%, respectively, providing that the brazilin compound was found in the extracts to be approximately 3.36, 2.69 and 2.68%, respectively. SHE samples were found to be antibacterial against all bacteria tested. These extracts' minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) at 37°C for *E*. coli were 3.91 and 5.64-6.51 mg/ml, respectively; *S. aureus*, 3.69-3.91 and 3.69-3.91 mg/ml, respectively; *B. cereus,* 0.15-0.16 and 0.20 mg/ml, respectively, and *S*. Typhimurium, 0.96 and 1.31-1.96 mg/ml, respectively. SHE obtained at 120°C extraction temperature were suitable and selected for addition into beetroot juice stored at 4°C for 7 days and 37°C for 24 hr, with the lowest SHE concentrations found to completely and simultaneously kill *B. cereus, E. coli, S. aureus*, and *S*. Typhimurium in beetroot juice being 11.73 and 3.91 mg/ml, respectively.

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Introduction

Sappan heartwood is used in folk medicine in China and alternative medicine in India. Sappan heartwood is used in Thailand as a food coloring, dye, and cosmetic ingredient.[1] It is also used in the treatment of tuberculosis, diarrhea, dysentery skin diseases, and anemia, among other things, in folk medicine.[2] Many important flavonoids and phenolic compounds are found in sappan heartwood, including brazilin, caesalpin J, protosappanins A, B, and E, sappanone B, and sappanchalcone.[3,4,5,6,7] Brazilin is an active compound found in sappan heartwood that has been used in traditional medicine in China to improve blood circulation, provide pain relief, and for its anti-inflammatory properties. Brazilin has been linked to a variety of biological benefits, including antibacterial, anti-inflammatory, anti-aging, anti-allergy, anti-acne, and antioxidant properties.[6]

Many groups of researchers are interested in the use of sappan heartwood extract (SHE) as an antimicrobial agent. Antimicrobial capacity of SHE has been investigated, and it was discovered that SHE can be used to inhibit certain microorganisms including *Pseudomonas aeruginosa*, *Salmonella* Typhi, *Staphylococcus aureus*, *Enterobacter aerogenes*, *Candida albicans*, *Aspergillus niger,*[8] *Klebsiella pneumoniae*, *Bacillus subtilis*, *Escherichia coli*, and *Salmonella* Ebony.[9] Sireeratawong[2] found that when 5,000 mg/kg of SHE was given to rats, it did not cause toxicity or changes to the rats' visceral organs. A sappan heartwood herbal beverage study and development was also carried out, and it was discovered that the formula that was most accepted by consumers and suitable for beverage production contained a 1.7% stevia sweetener, 9.83% *Caesalpinia sappan* extract, 29.49% water, and 58.97% tamarillo extract.[10]

Traditional solvent extraction methods, which are commonly used to extract various bioactive compounds, have disadvantages; for example, low selectivity, long analysis times, low extract yields, and the use of toxic solvents in large amounts. Recently, the emphasis has shifted to the use of GRAS (generally recognized as safe) solvents, as well as subcritical and supercritical fluids. Accelerated solvent extraction is one of the most promising extraction methods.[11,12] Among the benefits of accelerated solvent extraction are ease of automation, improved repeatability, faster sample analysis, the low solvent volume required, low risk of solvent exposure, and preservation of bioactive compounds that are sensitive to oxygen, light, and heat.[11,12,13,14] The operator of an accelerated extraction system can control the extraction temperature, extraction pressure, extraction time, and several extractions, potentially increasing the content of extracted bioactive compounds from plants.[11,12,13,14] This technique also has improved solvent diffusion into the sample due to the high-pressure of breaking a cell wall, and lower solvent viscosity at higher pressures and temperatures, which leads to improved solubility, advanced mass transfer, and shorter extraction times. However, because of the solvent's limited volume, accelerated solvent extraction may be incomplete, and higher temperatures may result in lower extracted thermolabile component yields.[13]

Aside from food safety, consumers are aware of nutritional value, which is another concern. Consumers today prefer to consume more healthy foods and beverages. Fruit and vegetable juice consumption, on the other hand, is another option that consumers prefer for health maintenance. Beetroot juice is one of the healthiest vegetable juices due to its high concentration of vitamins and antioxidants. However, to produce beetroot juice, the root portion of the beetroot is juiced and extracted. As a result, there is a risk of pathogenic bacteria from the soil contaminating the juice. Furthermore, heat sterilization, such as pasteurization, may result in the loss of specific vitamins or nutrients. Thus, this study aimed to extract brazilin from sappan heartwood using an accelerated solvent extraction method. The minimum inhibitory concentrations (MIC) of SHE required to inhibit Bacillus cereus (*B. cereus*), *Escherichia coli (E. coli), Staphylococcus aureus (S. aureus), and Salmonella Typhimurium (S. Typhimurium)*, as well as the minimum bactericidal concentrations (MBC) required to kill *B. cereus, E. coli, S. aureus,* and *S. Typhimurium,* were also determined. In addition, in this study investigated the addition of the obtained SHE as a natural antimicrobial in beetroot juice product during storage.

Materials and Methods

Microorganisms and Media

Thailand Institute of Scientific and Technological Research (TISTR) provided *Bacillus cereus* (TISTR 008), *Escherichia coli* (TISTR 780), *Staphylococcus*

aureus (TISTR 118), and *Salmonella* Typhimurium (TISTR 1469). McFarland standard No. 0.5 was purchased from bioMérieux, France. Mueller–Hinton agar (MHA), Mueller Hinton Broth (MHB), and Tryptic Soy Broth (TSB) were supplied from Merck, Germany. Plate Count Agar (PCA) was purchased from Difco, France. Agar was purchased from Unionsciences, Thailand.

Chemicals and Reagents

Standard brazilin was supplied by Anatech Ltd., Michigan, USA. Chloramphenicol and Tetracycline were purchased from Oxoid, England. Acetonitrile HPLC grade, dimethyl sulfoxide (DMSO), phosphoric acid, and ethanol were purchased from RCI Labscan Limited, Bangkok, Thailand. Sodium chloride was supplied by Merck, Germany. All of the chemicals and reagents used in this study were analytical grade.

Accelerated Solvent Extraction

Sappan heartwood samples were collected (Warorot Market, Chang Moi Road, Muang District, Chiang Mai Province, Thailand), with a moisture content of about 10.03% by weight, and ground into a powder of 40 mesh size. Each ground sappan heartwood sample (23 g) was combined with diatomaceous earth (5.75 g) and put in an extraction vessel (66 mL) before being loaded into an accelerated extractor with ethanol-water solvent (Dionex, ASE350, USA) (modification of the previous study[15]). Under high extraction pressure, the diatomaceous earth will aid in dispersing powder samples in the extraction vessel and preventing sample compression. After that, the extraction vessel was closed and warmed for 5 min before the extraction step began. The ethanol-water solvent (at a weight ratio of 3 to 1) was used as the solvent for extraction at temperatures ranging from 60°C to 180°C. The solvent was fed into the extractor to maintain a constant intracellular pressure of 1500 psi throughout the 5 min extraction. After the extraction was completed, a rotary vacuum evaporator (Model RKTS-23050-SNW, Genevac Rocket Evaporation, Pennsylvania, USA) set to 40°C was used to remove the solvent from the extract samples. The percentage extract yield was calculated using Equation 1 after the solvent had been completely evaporated.

$$\text{Percentage extract yield (\%)} = m_0/m_s \times 100 \quad \ldots(1)$$

where m_o is the extract weight (g), and m_s is the dried sappan heartwood weight (g) before the accelerated solvent extraction.

Before moving on to the next phase, all extract samples were stored in amber glass vials at -18 °C.

Determination of Brazilin Content by HPLC Technique

The high-performance liquid chromatography (HPLC) method, as explained by Kitdamrongtham,[16] was used to determine the brazilin compound in SHE samples obtained from the "Accelerated Solvent Extraction" section. The Phenomenex Luna® 5 µm C18(2) 100 Å column with a flow rate of 1 ml/min was used for the HPLC analysis. The volume of injection was set at 20 µl, and the deuterium lamp wavelength was set at 254 nm. The percentage yield of brazilin in the extract was compared to the standard curve for brazilin. The extract samples were dissolved in the mobile phase to 10 mg/ml concentration before being filtered and placed in vials for HPLC injection. Standard brazilin (4 mg) dissolved in the mobile phase (1 ml) was diluted to concentrations of 0.25, 0.5, 1.0, 2.0, and 4.0 mg/ml. To create a standard curve, the HPLC injection vial was inserted. Brazilin was determined using HPLC with acetonitrile in water (a ratio of 10:90, v/v) and phosphoric acid (0.1% v/v) as a mobile phase.

Antibacterial Activity of SHE

Agar Diffusion Assay

The antibacterial capacity of SHE samples was determined using the agar disc diffusion technique (modification of the Clinical and Laboratory Standards Institute[17]). To obtain the extract solution (500 mg/ml), DMSO (1 ml) was used to dissolve the obtained extract (0.5 g). Tryptic soy broth (TSB) medium was used to culture *B. cereus*, *E. coli*, *S. aureus*, and *S*. Typhimurium for 24 hours at 37°C. The bacterial solution's turbidity was adjusted in sterile 0.85% NaCl solution to a turbidity comparable to 0.5 McFarland Standard of 1.5×10^8 CFU/ml. After swabbing the inoculum suspension, excess fluid was removed, and the entire Muller-Hinton Agar (MHA) surface was swabbed with a sterile cotton swab in three directions, and allowed to dry for 3-5 minutes on the plate. Sterilized and qualitative filter paper discs (6 mm diameter) were contaminated

on the surface of the MHA with 10 µl of the extracts at 500 mg/ml concentration using sterile forceps. Test samples and bacteria were incubated on plates for 24 hr at 37°C. Finally, measuring the inhibition zone (or clear zone) around the disc revealed the sensitivity and resistance of the microorganisms to antibacterial activity testing. The inhibition zone's diameter was measured. The positive controls were 30 µg chloramphenicol and 30 µg tetracycline, and the negative controls were DMSO solution which were compared to SHE samples.

Minimum Inhibition Concentration of SHE

MIC value of SHE samples was measured using the broth dilution method (modification of the Clinical and Laboratory Standards Institute[17]), and the four types of bacteria (*B. cereus*, *E. coli*, *S. aureus*, and *S.* Typhimurium) were cultured in Tryptic soy broth (TSB) for 24 hr at 37°C. The turbidity of each culture was then adjusted to McFarland Standard No. 0.5. The SHE concentration of 500 mg/ml was prepared at in DMSO. In MHB, serial dilutions of SHE were combined with the standardized bacterial suspensions (500 µl) at a ratio of 1:1 (v/v). In the negative controls, MHB and microbial suspension were found. The findings were determined after a 24-hour incubation period at 37°C. The MIC is the concentration at which the microorganism does not develop turbidity. The MIC of an extract is the lowest concentration that totally inhibits the growth of a bacterial sample. The lower the MIC value, the greater the extract's activity. In independent experiments, all MIC analyses were done in triplicate.

Minimum Bactericidal Concentration of SHE

For the MBC determination (modification of the Clinical and Laboratory Standards Institute[17]), using the streak plate approach and the tube with no turbidity from the section "Minimum Inhibition Concentration of SHE", the bacterial samples were cultured on tryptic soy agar. To observe the bacterial sample growth, the bacterial samples were streaked over. The MBC was calculated from the broth with no bacteria growth on tryptic soy agar plates after incubation for 24 hr at 37°C. The MBC is the concentration at which the microorganism stops growing. In independent experiments, all MBC analyses were done in triplicate.

Application of SHE to Inhibit and Kill Pathogenic Bacteria in Beetroot Juice

Fresh and undamaged beetroot samples were thoroughly washed, peeled, cut into pieces, and beetroot juice was squeezed out. Beetroot juice was sterilized at 121°C for 15 min. Then, in sterile test tubes, beetroot juice (2 ml) was added and mixed with bacteria solution (2 ml) turbidity-adjusted to McFarland Standard No. 0.5. After that, the bacteria solution was then treated with SHE at various concentrations, including those equal to the MIC values, as well as those decreased and increased to 2, 3, and 4 times the MIC values. At 37°C, the prepared mixture solution was incubated for 24 hr. The bacterial samples were counted using the pour plate technique. Subsequently, the concentration of SHE that kills all four types of bacteria, *B. cereus*, *E. coli*, *S. aureus*, and *S. Typhimurium,* was determined at 37°C for 24 hr. This concentration, as well as two and three times this concentration, was mixed into beetroot juice and refrigerated at 4°C for 7 days. The pour plate technique was used to count the bacterial samples.

Scanning Electron Microscopy of Characterization of *B. cereus*, *E. coli*, *S. aureus*, and S. Typhimurium Cells in Beetroot Juice

In sterile test tubes, a 1:1 ratio of prepared beetroot juice sample and bacteria solution turbidity adjusted to McFarland Standard No.4 was used. The obtained solution samples were divided into two examples: (a) unpasteurized beetroot juice (b) beetroot juice with SHE added

All beetroot juice samples were carbon dioxide-dried using the critical point drying (CPD) method (modification of Wang[18]). The procedures are as follows. Before washing with 0.1 M phosphate buffer, each beetroot juice sample was pre-treated in 2.5% glutaraldehyde for 2 hr. A second treatment was carried out by immersing the sample in 1% osmium tetroxide for 2 hr. Each sample was then extracted from water once every 30 min by immersing it in ethanol concentrations of 30%, 50%, 70%, 80%, 90%, 95%, and 100%. Using a critical point dryer, all obtained samples were dried with carbon dioxide at the critical point. Each dried sample was attached to the stub with carbon tape and gold-coated.

Finally, a scanning electron microscope (SEM) at 20,000 times magnification was used to image *B. cereus*, *E. coli*, *S. aureus*, and *S*. Typhimurium cells from dried samples.

Statistical Analysis

Three iterations were performed for each experiment. Duncan's Multiple Range Test was employed for analysis of variance (ANOVA) and comparing average values. The findings were presented as average value ± standard deviation. The Statistical Package for the Social Sciences (SPSS) version 16.0 with a 95% confidence level was utilized for statistical data analysis.

Results and Discussion

Accelerated Solvent Extraction of Sappan Heartwood

The sappan heartwood was extracted for 5 min under constant extraction pressure of 1500 psi using an ethanol/water solvent mixture (ratio by weight 3 to 1) at extraction temperatures of 60°C, 120°C, and 180°C. The percentage yield of SHE obtained from all three extraction temperatures differed statistically ($p \leq 0.05$). Sappan heartwood extracted at 180°C yielded the highest percentage extract yield of 16.18, followed by sappan heartwood extracted at 120°C and 60°C, which yielded 13.64 and 9.16, respectively.

As shown in Table 1, when the content of brazilin in SHE samples was investigated, it was discovered that sappan heartwood extracted at 60°C yielded the highest statistically significant percentage of brazilin yield in the extracts of 3.36, followed by sappan heartwood extracted at 120°C and 180°C, which yielded brazilin yields of 2.69 and 2.68, respectively. The percentage SHE yields increased when the extraction temperature was raised. More extracts will be released as temperatures rise. However, extremely high extraction temperatures may cause the brazilin compound and other antimicrobial compounds to degrade. According to Xia,[19] as the extraction temperature increased above 50°C and 60°C when the ultrasonic extraction method was used, the percentage yield of brazilin in the heartwood extract decreased. In addition, the color of dried SHE darkened at higher extraction temperatures.

Table 1: Appearance of SHE, percentage yield of SHE, and percentage yield of brazilin in SHE

Temperature (°C)	The appearance of SHE	The yield of SHE (%)	Brazilin yield of SHE (%)
60	dried dark red powder	9.16 ± 1.01^{c}	3.36 ± 0.17^{a}
120	dried dark red powder	13.64 ± 0.25^{b}	2.69 ± 0.03^{b}
180	dried dark brown powder	16.18 ± 0.58^{a}	2.68 ± 0.36^{b}

Values with different superscript lowercase letters in the same column differ significantly ($p \leq 0.05$).

Furthermore, as shown in Table 1, the accelerated solvent extraction method has the potential to degrade plant cell walls and release soluble phytochemical compounds. One of the most important factors influencing phytochemical extraction was solvent. As a result, functional extracts with high yields are produced. The ethanol/water solvent mixture used in this experiment may also aid in the extraction of brazilin and antimicrobial chemical compounds. Many previous studies found that extracting with 95% ethanol yielded a high content of important phytochemical compounds with potent antibacterial activity.[8,20,21] Thus, the accelerated solvent extraction technique used in this study, which used an ethanol/water solvent mixture for a shorter extraction time of 5 min under high pressure, is an intriguing method for extracting brazilin and antibacterial compounds. Furthermore, the variation in extract yield and brazilin content is caused not only by the type of solvent and extraction temperature but also by the age and geographic location of the heartwood tree.

Inhibiting Activity of Pathogenic Bacteria in Certain Foods Containing SHE by Agar Disc Diffusion Method

Several studies have found brazilin to be an important active compound in sappan heartwood, with the ability to inhibit pathogenic bacteria; for example, *Enterobacter aerogens*, *E. coli*, *Staphylococcus aureus*, *Salmonella Typhi*,[8] and

Salmonella Typhimurium in certain foods.[22] It would be interesting to investigate the use of SHE in suppressing pathogenic bacteria contaminating food products and reducing consumer harm caused by pathogenic bacteria in food.

The inhibitory effect of SHE samples obtained at extraction temperatures of 60°C, 120°C, and 180°C on the growth of pathogenic bacterial samples, namely *B. cereus*, *E. coli*, *S. aureus*, and *S*. Typhimurium, was investigated. It was discovered that these extracts at 500 mg/ml concentrations containing the content of brazilin of 16.80, 13.45, and 13.40 mg/ml, respectively, were capable of inhibiting the growth of four types of pathogenic bacteria. As shown in Table 2, the mean inhibition zone's diameter of SHE samples obtained at extraction temperatures of 60°C, 120°C, and 180°C for testing *B. cereus*, *E. coli*, *S. aureus*, and *S*. Typhimurium were approximately 12.33, 12.89, and 12.33 mm, 29.67, 30.00, and 29.89 mm, 25.56, 26.11, and 25.67 mm, and 30.44, 30.89, and 30.67 mm, respectively.

Table 2: Inhibition zone of SHE obtained by accelerated solvent extraction at temperatures of 60°C, 120°C, and 180°C for inhibiting *B. cereus, E. coli*, *S. aureus*, and *S.* Typhimurium at 500 mg/ml extract concentration compared to tetracycline, chloramphenicol, and DMSO solution

Samples	Inhibition zone's diameter (mm)			
	B. cereus	***E. coli***	***S. aureus***	***S*. Typhimurium**
SHE from an extraction temperature of 60°C	12.33 ± 0.47^{dD}	25.56 ± 0.50^{bC}	29.67 ± 0.47^{bB}	30.44 ± 0.68^{bA}
SHE from an extraction temperature of 120°C	26.11 ± 0.74^{bC}	12.89 ± 0.31^{cD}	30.00 ± 0.47^{bB}	30.89 ± 0.87^{bA}
SHE from an extraction temperature of 180°C	25.67 ± 0.47^{bC}	12.33 ± 0.47^{dD}	29.89 ± 0.57^{bB}	30.67 ± 0.94^{bA}
Tetracycline antibiotics	31.89 ± 0.31^{aA}	25.44 ± 0.50^{aC}	30.56 ± 0.68^{aB}	30.22 ± 0.63^{bB}
Chloramphenicol antibiotics	25.67 ± 0.47^{bB}	24.89 ± 0.74^{bC}	24.78 ± 0.42^{cC}	34.67 ± 0.67^{aA}
DMSO solution	0.00 ± 0.00^{cA}	0.00 ± 0.00^{eA}	0.00 ± 0.00^{dA}	0.00 ± 0.00^{cA}

Values with different superscript lowercase letters in the same column and values with different superscript uppercase letters in the same row differ significantly ($p \leq 0.05$).

The bacteria inhibition test of all SHE samples, as shown in Table 2, revealed that SHE samples at 500 mg/ml concentration of inhibited bacteria. It inhibited *E. coli* less effectively than tetracycline and chloramphenicol antibiotics and had a higher inhibitory effect on *S. aureus* than chloramphenicol antibiotics. It inhibited *B. cereus* less effectively than tetracycline antibiotics but not as effectively as chloramphenicol antibiotics. It was similar to tetracycline antibiotics in terms of inhibition of *S.* Typhimurium but less effective than chloramphenicol antibiotics.

SHE samples suppressed *S*. Typhimurium the most compared to other strains with the largest diameter of inhibition zone, followed by *S. aureus*, *B. cereus*, and *E. coli*, respectively, at each extraction temperature. Furthermore, when each inhibitory strain was considered, it was discovered that the SHE samples obtained at extraction temperatures of 60°C, 120°C, and 180°C inhibited *S. aureus*, *B. cereus*, and *S*. Typhimurium which were insignificantly different ($p > 0.05$). However, SHE samples obtained at extraction temperatures of 60°C, 120°C, and 180°C had statistically different inhibitory effects on *E. coli* ($p > 0.05$). SHE at 120°C inhibited *E. coli* more effectively than extracts at 60°C and 180°C. This study discovered that SHE samples, as well as brazilin in the extracts, were found to inhibit pathogenic bacteria in certain foods (as shown in Table 2).

Romruen[23] found that SHE added to gelatin film inhibited *S. aureus* as gram-positive bacteria more than *E. coli* as gram-negative bacteria, with a diameter of 10.33-20.33 mm for the inhibition zone against *S. aureus*. According to Srinivasan,[8] the antibacterial activity of SHE samples obtained using different solvents (ethanol, water, and petroleum ether) was determined using the Soxhlet extraction method and the agar disc diffusion method. SHE samples derived from ethanol were found to inhibit *S. aureus* and *E. coli* more effectively than water and petroleum ether extracts. The ethanolic SHE inhibits *S. aureus* with a 31.0 mm inhibition zone, as well as a 15.0 mm inhibition zone against *E. coli*. It is conceivable that the SHE had different antimicrobial activity, which could be attributed to the different cell wall structures of gram-positive and gram-negative bacteria.

Table 3: Minimum inhibition concentration of SHE and standard brazilin for inhibiting *B. cereus*, *E. coli*, *S. aureus*, and *S.* Typhimurium

Samples	Minimum inhibition concentration (mg/ml)			
	B. cereus	***E. coli***	***S. aureus***	***S.* Typhimurium**
SHE from an extraction temperature of 60°C	0.15 ± 0.05^{bC}	3.91 ± 0.00^{bA}	3.91 ± 0.00^{bA}	0.96 ± 0.00^{bB}
SHE from an extraction temperature of 12°C	0.15 ± 0.05^{bC}	3.91 ± 0.00^{bA}	3.69 ± 0.61^{bA}	0.96 ± 0.00^{bB}
SHE from an extraction temperature of 180°C	0.16 ± 0.06^{bC}	3.91 ± 0.00^{bA}	3.91 ± 0.00^{bA}	0.96 ± 0.00^{bB}
Standard brazilin	0.49 ± 0.00^{aD}	26.04 ± 7.3^{aA}	5.21 ± 1.84^{aB}	1.96 ± 0.00^{aC}

Values with different superscript lowercase letters in the same column and values with different superscript uppercase letters in the same row differ significantly ($p\leq0.05$).

Table 4: Minimum bactericidal concentration of SHE and standard brazilin for killing *B. cereus,* E. coli, S. aureus, and *S.* Typhimurium

Samples	Minimum bactericidal concentration (mg/ml)			
	B. cereus	***E. coli***	***S. aureus***	***S.* Typhimurium**
SHE from an extraction temperature of 60°C	0.20 ± 0.06^{bD}	6.08 ± 1.94^{bA}	3.91 ± 0.00^{bB}	1.31 ± 0.46^{bC}
SHE from an extraction temperature of 120°C	0.20 ± 0.06^{bD}	5.64 ± 1.94^{bA}	3.69 ± 0.61^{bB}	1.31 ± 0.46^{bC}
SHE from an extraction temperature of 180°C	0.20 ± 0.06^{bD}	6.51 ± 1.84^{bA}	3.91 ± 0.00^{bB}	1.96 ± 0.00^{bC}
Standard brazilin	0.65 ± 0.23^{aD}	26.04 ± 7.37^{aA}	7.81 ± 0.00^{aB}	3.26 ± 0.92^{aC}

Values with different superscript lowercase letters in the same column and values with different superscript uppercase letters in the same row differ significantly ($p\leq0.05$).

Minimum Inhibition Concentration and Minimum Bactericidal Concentration of SHE

The MIC values for *B. cereus*, *E. coli*, *S. aureus*, and S. Typhimurium inhibition obtained from SHE samples using extraction temperatures of 60°C, 120°C and 180°C were approximately 0.15, 3.91, 3.91, and 0.96 mg/ml, 0.15, 3.91, 3.69, and 0.96 mg/ml, and 0.16, 3.91, 3.91, and 0.96 mg/ml,

respectively, as shown in Table 3. Meanwhile, the MBC values for *B. cereus*, *E. coli*, *S. aureus*, and *S.* Typhimurium inhibition obtained from SHE samples extracted at 60°C, 120°C and 180°C were approximately 0.20, 6.08, 3.91, and 1.31 mg/ml, 0.20, 5.64, 3.69, and 1.31 mg/ml, 0.20, 6.51, 3.91, and 1.96 mg/ml, respective, as illustrated in Table 4

SHE obtained using extraction temperatures of 60°C, 120°C, and 180°C showed no significant differences in MIC and MBC when inhibiting and killing *B. cereus*, *E. coli*, *S. aureus*, and *S.* Typhimurium. SHE samples obtained using extraction temperatures of 60°C and 120°C had the lowest MIC and MBC in inhibiting *B. cereus* growth (0.15 mg/ml) and killing *B. cereus* (0.20 mg/ml). When the MIC and MBC values were considered, it was found that SHE samples were the most effective in inhibiting and killing *B. cereus*, followed by *S*. Typhimurium, *S. aureus*, and *E. coli*.

SHE samples had lower MIC and MBC values than standard brazilin, as shown in Table 3 and Table 4. As a result, SHE samples were more effective than standard brazilin at inhibiting and killing *B. cereus*, *E. coli*, *S. aureus*, and S. Typhimurium. The comparison of the antibacterial activity of some foodborne pathogenic bacteria of the SHE samples and the standard brazilin confirms that the SHE samples may contain other active compounds besides brazilin that could inhibit *B. cereus*, *E. coli*, *S. aureus*, and S. Typhimurium. The obtained results were consistent with those of Nirmal,[6] Srinivasan,[8] and Xu and Lee[22], who found that SHE containing brazilin compound could inhibit *S. aureus*, *E. coli*, and S. Typhimurium. Moreover, Hemthanon and Ungcharoenwiwat[24] reported that Caesalpinia sappan heartwood was extracted for 5 days with 70% and 95% ethanol and 70% and 95% methanol, at a temperature of 28°C and 150 rpm shaking. It was found that 95% ethanolic crude extract inhibited bacterial strains more effectively than methanolic crude extract. The 95% alcoholic crude extract of sappan heartwood had the highest inhibitory capacity against *S. aureus* in the agar well diffusion assay, with a 13.67 mm inhibition zone. This obtained SHE had MIC values of 1.95 and 1.95 mg/ml, and MBC values of 62.5 and 3.91mg/ml for *S. aureus* and *B. cereus*, respectively. According to previous research,[25] sappan heartwood samples were extracted using the subcritical solvent extraction technique. The optimal subcritical solvent extraction, at 100°C for 50 min using a solvent as water: ethanol mixture (1:3 w/w) and a sappan heartwood: solvent ratio of 1:9 (w/w), yielded the highest percentage of dried crude extract yield and the highest brazilin content, with values of approximately 9.95% and 84.65 mg/g dry heartwood, respectively. The growth of *S. aureus*, *B. cereus*, *B. subtilis*, and *E. coli*. were all inhibited by the SHE. Additionally, the optimal subcritical solvent extraction yielded SHE with MIC values of 0.98, 1.96, 3.91, and 3.91 mg/ml to inhibit *S. aureus*, *B. subtilis*, *B. cereus*, and *E. coli*., respectively, and MBC values of 1.45, 2.94, 3.91, and 3.91 mg/ml to kill *S. aureus*, *B. subtilis*, *B. cereus*, and *E. coli*., respectively. Under this optimal extraction condition, the SHE had the lowest IC_{50} DPPH value of 11.42 µg/ml, the lowest IC_{50} ABTS value of 8.62 µg/ml, and the greatest FRAP value of 12.11 µM FeSO4 equivalents/100 µl.

The results above demonstrated that the SHE could effectively inhibit *B. cereus*, *E. coli*, *S. aureus*, and *S.* Typhimurium. However, it was not investigated in this study which important substances in the SHE contributed to the inhibition of each bacterial. As a result, further research into the screening of various antibacterial substances in the SHE should be conducted.

Additionally, the type of organic solvent and extraction methods had an impact on the amount of SHE as well as the type, quantity, and purity of extracted bioactive compounds. However, while this study benefited from a shorter extraction time of 5 min, it did not identify any other important substances that should be investigated further in the future.

Application of SHE in Beetroot Juice

SHE obtained at a temperature of 120°C were found to be more effective than others in inhibiting *B. cereus*, *E. coli*, *S. aureus*, and *S.* Typhimurium, and thus were chosen to be added to beetroot juice. Furthermore, before the accelerated solvent extraction, total plate count testing for microbial contamination in sappan heartwood samples

revealed a total microbial content of 4.13×10^3 CFU/g. Thus, the accelerated solvent extraction at 120°C could be one method of killing microorganisms found in sappan heartwood, which is likely safe if used as an ingredient in food or beverages.

Table 5: Concentration of SHE to kill *B. cereus, E. coli, S. aureus,* and *S.* Typhimurium in beetroot juice stored at 37°C for 24 hr

Bacterial	The concen tration of SHE (mg/ml)	Bacterial content (log CFU/ml)	Bacterial	The concen tration of SHE (mg/ml)	Bacterial content (log CFU/ml)
B. cereus	0.04	7.08 ± 0.02	*S. aureus*	0.92	4.95 ± 0.09
	0.05	6.91 ± 0.03		1.23	3.78 ± 0.07
	0.08	6.64 ± 0.04		1.85	0.00 ± 0.00
	0.15	1.54 ± 0.06		3.69	0.00 ± 0.00
	0.30	0.00 ± 0.00		7.38	0.00 ± 0.00
	0.45	0.00 ± 0.00		11.07	0.00 ± 0.00
	0.60	0.00 ± 0.00		14.76	0.00 ± 0.00
E. coli	0.98	7.38 ± 0.14	*S*. Typhimurium	0.24	7.85 ± 0.03
	1.30	6.66 ± 0.09		0.32	5.85 ± 0.14
	1.96	4.81 ± 0.09		0.48	5.27 ± 0.02
	3.91	0.00 ± 0.00		0.96	0.00 ± 0.00
	7.82	0.00 ± 0.00		1.92	0.00 ± 0.00
	11.73	0.00 ± 0.00		2.88	0.00 ± 0.00
	15.64	0.00 ± 0.00		3.84	0.00 ± 0.00

Table 6: Concentration of SHE to kill *B. cereus, E. coli, S. aureus*, and *S.* Typhimurium in beetroot juice stored at 4°C for 7 days

Bacterial	The concentration of SHE (mg/ml)	Bacterial content (log CFU/ml)			
		Day 1	Day 3	Day 5	Day 7
B. cereus	3.91	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	7.82	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	11.73	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
E. coli	3.91	5.31 ± 0.10	4.56 ± 0.25	4.46 ± 0.07	2.37 ± 0.18
	7.82	3.87 ± 0.13	2.25 ± 0.17	0.00 ± 0.00	0.00 ± 0.00
	11.73	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
S. aureus	3.91	2.54 ± 0.06	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	7.82	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	11.73	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
S. Typhimurium	3.91	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	7.82	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	11.73	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

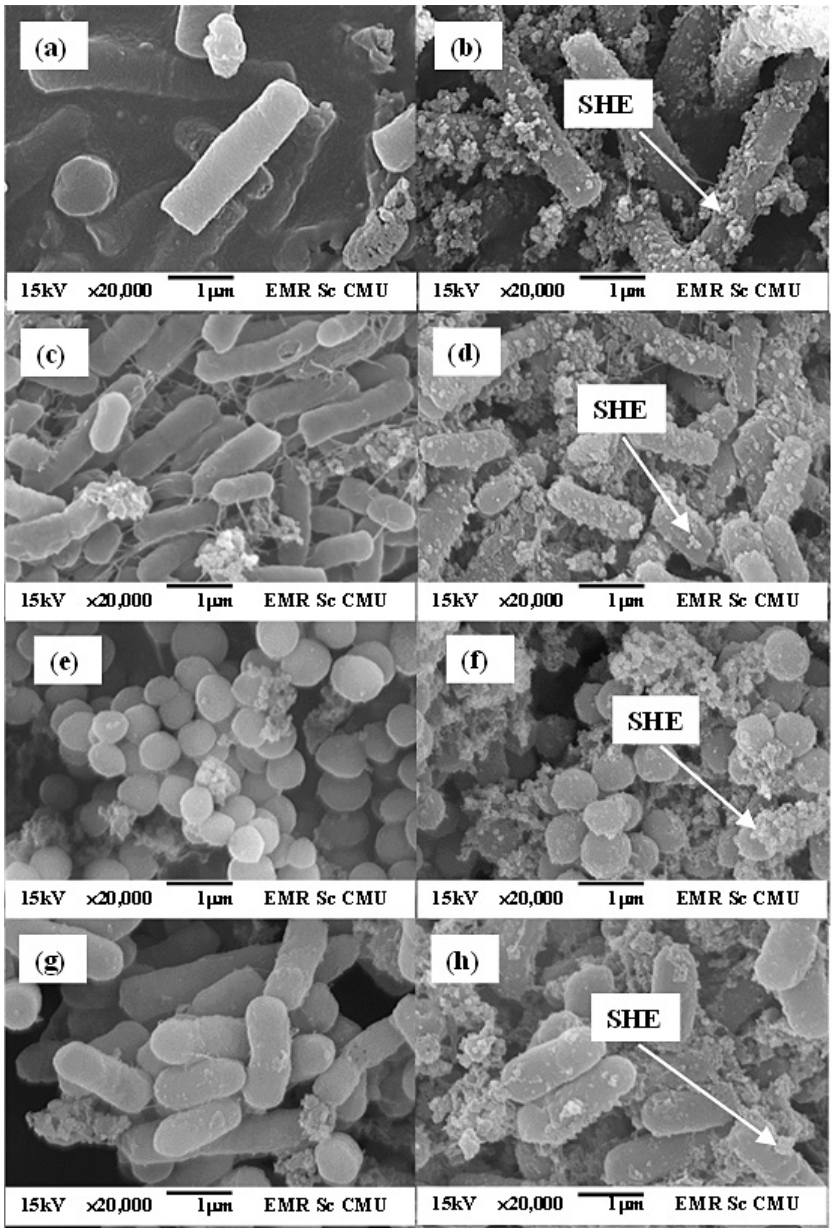

Fig. 1: SEM micrographs (magnification 20,000×) of); *B. cereus* cell wall in beetroot juice (a) without the addition of SHE (b) with the addition of SHE (11.73 mg/ml); *E.coli* cell wall in beetroot juice (c) without the addition of SHE (d) with the addition of SHE (11.73 mg/ml); *S. aureus* cell wall in beetroot juice (e) without the addition of SHE (f) with the addition of SHE (11.73 mg/ml), and *S.* Typhimurium cell wall in beetroot juice (g) without the addition of SHE (h) with the addition of SHE (11.73 mg/ml)

SHE samples were prepared in a variety of concentrations, including those that were equal to the MIC values for *B. cereus*, *E. coli*, *S. aureus*, and *S.* Typhimurium, and those that were reduced and increased to 2, 3, and 4 times their MIC values. The inhibitory MIC values of *B. cereus*, *E. coli*, *S. aureus*, and S. Typhimurium for SHE samples extracted at 120°C were 0.15, 3.91, 3.69, and

0.96 mg/ml, respectively (as shown in Table 3). After being added and mixed with bacteria solution and SHE samples, the prepared beetroot juice samples were incubated for 24 hr at 37°C. The concentrations of SHE samples used to kill *B. cereus*, *E. coli*, *S. aureus*, and *S.* Typhimurium in beetroot juice were shown in Table 5. According to Table 5, it was found that the lowest concentrations of SHE capable of killing *B. cereus*, *E. coli*, *S. aureus*, and *S.* Typhimurium in beetroot juice stored at 37°C for 24 hr were 0.30, 3.91, 1.85, and 0.96 mg/ml, respectively.

The SHE concentration capable of killing *B. cereus*, *E. coli*, *S. aureus*, and *S.* Typhimurium all at once was 3.91 mg/ml at 37°C for 24 hr (as shown in Table 5). Subsequently, beetroot juice samples were stored at 4°C for 7 days after being mixed with four types of bacterial samples (*B. cereus*, *E. coli*, *S. aureus*, and *S.* Typhimurium) and SHE samples at 3.91 mg/ml concentration and two and three times that concentrations. As shown in Table 6, the concentration of SHE samples capable of simultaneously killing all four types of bacterial samples (*B. cereus*, *E. coli*, *S. aureus*, and *S.* Typhimurium) in beetroot juice stored at 4°C for 7 days was 11.73 mg/ml.

Scanning Electron Microscopy of *B. cereus*, *E. coli*, *S. aureus*, and *S.* Typhimurium Cell Wall in Beetroot Juice

The bacterial cell characteristics of *E. coli*, *B. cereus*, and *S.* Typhimurium are rod-shaped, whereas *S. aureus* is spherical as presented in Fig. 1. All four bacteria had smooth cell wall surfaces. Fig. 1 depicts the characteristics of *B. cereus*, *E. coli*, *S. aureus*, and *S.* Typhimurium in beetroot juice supplemented with SHE at a concentration of 11.73 mg/ml, revealing that SHE presented on the surface of their cell wall may help to inhibit bacterial growth.

Brazilin, protosappanin, and hematoxylin are water-soluble flavonoids found in sappan heartwood, with brazilin being the major homoisoflavonoid.[26] Brazilin has antibacterial activity because of its ability to bind intracellular protein, soluble protein, as well as bacterial cell walls, and then cause leakage.[27] The SHE may then enter bacteria cells, where key extract compounds, such as brazilin, may affect the intracellular structure of the bacteria. Brazilin could inhibit bacterial DNA and protein synthesis, according to the report of Xu and Lee.[22] A radiolabel incorporation assay was used to quantify DNA and protein formation in methicillin-resistant *S. aureus* (MRSA) after brazilin was extracted from sappan heartwood. This assay looked at DNA synthesis using radioactively labeled thymidine (3H-thymidine) and protein synthesis using radioactively labeled serine (3H-serine) in agar with MRSA and a brazilin concentration of 32 µg/ml. Brazilin was discovered to inhibit 3H-thymidine or 3H-serine transport into cells for use in DNA and protein synthesis. Bacterial growth should include cell division as well as the cellular synthesis of various substances such as carbohydrates, lipids, proteins, RNA, and DNA. Before cell division, DNA replication occurs, and thymidine is one of the essential substances that cells use in DNA synthesis. As a result, measuring 3H-thymidine intracellular activity could be used to calculate the DNA synthesis rate, a bacterial growth indicator.[28] As a result, when brazilin inhibits the processes of DNA and protein synthesis, bacteria cannot grow or live.

Conclusions

This study explains the development of a method for extracting brazilin as the main compound in sappan heartwood using accelerated solvent extraction at extraction temperatures of 60°C, 120°C, and 180°C for 5 min at a constant pressure of 1500 psi and an ethanol and water solvent mixture (weight ratio of 3 to 1). Sappan heartwood extracted at 180°C yielded the highest average percentage of extract yield (16.18), followed by sappan heartwood extracted at 120°C and 60°C, which yielded 13.64 and 9.16, respectively. When the content of brazilin in SHE samples was investigated, it was discovered that extracts produced at 60°C had the greatest mean percentage yield of brazilin in the extracts, equal to 3.36. The MIC and MBC values for *B. cereus* at 37°C were 0.15-0.16 mg/ml and 0.20 mg/ml, respectively; *E. coli*, 3.91 mg/ml and 5.64-6.51 mg/ml, respectively; *S. aureus*, 3.69-3.91 mg/ml and 3.69-3.91 mg/ml, respectively; and *S.* Typhimurium, 0.96 mg/ml and 1.31-1.96 mg/ml, respectively.

SHE obtained from using accelerated solvent extraction at 60°C, 120°C, and 180°C could inhibit pathogenic bacterial growth in all four foodborne pathogens, namely *B. cereus*, *E. coli*, *S. aureus*, and *S.* Typhimurium. SHE obtained through accelerated

solvent extraction at 120°C was used to kill *B. cereus*, *E. coli*, *S. aureus*, and *S.* Typhimurium in beetroot juice stored at 4°C for 7 days and 37°C for 24 hr. The concentration of SHE required to kill all four types of bacteria simultaneously at storage temperatures of 4°C for 7 days and 37°C for 24 hr was 11.73 mg/ml and 3.91 mg/ml, respectively. The lower the storage temperature, the higher the concentration of SHE that can kill bacteria in beetroot juice products. Therefore, the accelerated solvent extraction method can be applied for extracting brazilin compound from sappan heartwood as well as bioactive compounds from other plants. SHE has the potential to be used as a natural antimicrobial in beverages such as beetroot juice. In the following phase, this research will then create a prototype beetroot juice mixed with SHE and evaluate its marketability.

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Conflicts of Interest
The author(s) declares no conflict of interest.

References

1. Wetwitayaklunga P., Phaechamud T., Keokitichaic S. The antioxidant activity of Caesalpinia sappan L. heartwood in various ages. *NUJST.* 2005; 13(2): 43-52.
2. Sireeratawong S., Piyabhan P., Singhalak T., Wongkrajang Y., Temsiririrkkul R., Punsrirat J., Ruangwises N., Saraya S., Lerdvuthisopon N., Jaijoy K. Toxicity evaluation of sappan wood extract in rats. *J Med Assoc Thai*. 2010; 93: S50-S57.
3. Miyahara K., Kawasaki T., Kinjo J. E., Shimoka T., Yamahara J., Yamasaki M., Harano K., Nohara T. The X-ray analysis of cesalpin J from Sappan Lignum. *Chem Pharm Bull.* 1986; 34(10): 4166-4169. https://doi.org/10.1248/cpb.34.4166
4. Batubara I, Mitsunaga T, Ohashi H. Brazilin from Caesalpinia sappan wood as an antiacne agent. *J Wood Sci.* 2010; 56: 77–81. https://doi.org/10.1007/s10086-009-1046-0
5. Wang Z, Sun J. B., Qu W, Guan F. Q., Li L. Z., Liang J. Y. *Caesappin* A and B, two novelprotosappanins from *Caesalpinia sappan* L. *Fitoterapia.* 2014; 92: 280-284. https://doi.org/10.1016/j.fitote.2013.12.004
6. Nirmal N. P., Rajput M. S., Prasad R. G. S. V, Ahmad M. Brazilin from Caesalpinia sappan heartwood and its pharmacological activities: A review. *Asian Pac J Trop Med.* 2015; 8(6): 421-430. https://doi.org/10.1016/j.apjtm.2015.05.014
7. Zeng K. W., Liao L. X., Zhao M. B., Song F. J., Yu Q., Jiang Y., Tu, P.F. Protosappanin B protects PC12 cells against oxygen–glucose deprivation-induced neuronal death by maintaining mitochondrial homeostasis via induction of ubiquitin-dependent p53 protein degradation. *Eur J Pharmacol.* 2015; 751: 13–23. https://doi.org/10.1016/j.ejphar.2015.01.039
8. Srinivasan R., Ganapathy selvam G., Karthik S., Mathivanan K., Baskaran R., Karthikeyan M., Gopi M., Govindasamy C. *In vitro* antimicrobial activity of *Caesalpinia sappan* L. *Asian Pac J Trop Biomed.* 2012; 2(1): S136-S139. https://doi.org/10.1016/S2221-1691(12)60144-0
9. Bukke A. N., Hadi F. N., Produtur C. S. Comparative study of in vitro antibacterial activity of leaves, bark, heart wood and seed extracts of Caesalpinia sappan L. *Asian Pac J Trop Med.* 2015; 5(11): 903-907. https://doi.org/10.1016/S2222-1808(15)60954-9
10. Angelica C., Pangestu O. O., Kurniawan J., Meindrawan B., Hutabarat D. J. C. Design formula and product prototype of beverage made from tamarillo (*Solanum betaceum*) fruit and sappan wood (*Caesalpinia sappan*) using Kano method. The 2nd International Conference on Agriculture and Rural Development. IOP *Conf Ser*: *Earth Environ Sci.* 2021; 715: 012070. https://doi.

org/10.1088/1755-1315/715/1/012070

11. Saha S., Walia S., Kundu A., Sharma K., Paul R. K. Optimal extraction and fingerprinting of carotenoids by accelerated solvent extraction and liquid chromatography with tandem mass spectrometry. *Food Chem.* 2015; 177: 369-375. https://doi.org/10.1016/j.foodchem.2015.01.039
12. Gomes S. V. F., Portugal L. A., dos Anjos J. P., de Jesus O. N., de Oliveira E. J., David J. P., David J. M. Accelerated solvent extraction of phenolic compounds exploiting a Box-Behnken design and quantification of five flavonoids by HPLC-DAD in Passiflora species. *Microchem J.* 2017; 132: 28–35. https://doi.org/10.1016/j.microc.2016.12.021
13. Repajić M., Cegledi E., Kruk V., Pedisić S., Çınar F., Kovačević D. B., Žutić I., Dragović-Uzelac V. Accelerated solvent extraction as a green tool for the recovery of polyphenols and pigments from wild nettle leaves. *Processes.* 2020; 8(7): 803. https://doi.org/10.3390/pr8070803
14. Fan Y., Li X., Ding L., Zhou W., Xu G., Wang Y., Zhang Y., Ni Q. Accelerated solvent extraction of antioxidant compounds from gardeniae fructus and its acetylcholinesterase inhibitory and PC12 cell protective activities. *Foods*. 2021; 10(11): 2805. https://doi.org/10.3390/foods10112805
15. Uoonlue N., Muangrat R. Effect of different solvents on subcritical solvent extraction of oil from Assam tea seeds (Camellia sinensis var. assamica): Optimization of oil extraction and physicochemical analysis. *J Food Process Eng.* 2019; 42: e12960. https://doi.org/10.1111/jfpe.12960
16. Kitdamrongtham W., Manosroi A., Akazawa H., Gidado A., Stienrut P., Manosroi W., Lohcharoenkal W., Akihisa T., Manosroi J. Potent anti-cervical cancer activity: Synergistic effects of Thai medicinal plants in recipe N040 selected from the MANOSROI III database. *J Ethnopharmacol.* 2013; 149(1): 288-296. https://doi.org/10.1016/j.jep.2013.06.037
17. Clinical and Laboratory Standard Institute (CLSI). Methods for dilution antimicrobial susceptibility test for bacteria that grow aerobically; Approved Standard-Eight edition. CLSI documents M07-A8. Wayne, PA. Clinical and Laboratory Standard Institute; 2009.
18. Wang Z., de la Fuente-Núñez C., Shen Y., Haapasalo M., Hancock R.E.W. Treatment of Oral Multispecies Biofilms by an Anti-Biofilm Peptide. *PLoS ONE*. 2015; 10(7): e0132512. https://doi.org/10.1371/journal.pone.0132512
19. Xia Z., Li D., Li Q., Zhang Y., Kang W. Simultaneous determination of brazilin and protosappanin B in Caesalpinia sappan by ionic-liquid dispersive liquid-phase microextraction method combined with HPLC. *Chem Cent J.* 2017; 11(114): 1–11. https://doi.org/10.1186/s13065-017-0342-9
20. Sukprasert J., Subtaeng S. The effect of solvent on antimicrobial activity of medicinal plant extraction. *Bull Appl Sci*. 2012; 1: 99–109.
21. Warinhomhaun S., Sritularak B., Charnvanich D. A simple high-performance liquid chromatographic method for quantitative analysis of brazilin in *Caesalpinia sappan* L. extracts. Thai *J Pharm Sci.* 2018;.42(4): 208–213.
22. Xu H. X., Lee S. F. The antibacterial principle of *Caesalpina sappan. Phytother Res.* 2004; 18(8): 647-651. https://doi.org/10.1002/ptr.1524
23. Romruen O., Kaewprachu P., Karbowiak T., Rawdkuen S. Development of intelligent gelatin films incorporated with sappan (*Caesalpinia sappan* L.) heartwood extract. *Polymers.* 2022; 14(12): 2487. https://doi.org/10.3390/polym14122487
24. Hemthanon T., Ungcharoenwiwat P. Antibacterial activity, stability, and hemolytic activity of heartwood extract from *Caesalpinia sappan* for application on nonwoven fabric. *Electron J Biotechnol.* 2022; 55: 9–17. https://doi.org/10.1016/j.ejbt.2021.10.002
25. Muangrat R., Jirarattanarangsri W., Simapisan P. Response surface methodology applied to subcritical solvent extraction of brazilin compound from *Caesalpinia sappan* L. heartwood. *J Appl Res Med Aromat Plants.* (2022); 31: 100408. https://doi.org/10.1016/j.jarmap.2022.100408
26. Yun J., Lee H. M., Kim S. K., Lee S. Y., Lee C. S., Cho T. S. Formation of Cu (II)–brazilin

complex in the presence of DNA and its activities as chemical nuclease. *J Inorg Biochem.* 2006; 100(9): 1501–1505. https://doi.org/10.1016/j.jinorgbio.2006.04.013

27. Cushnie T. P. T, Lamb A. J. Antimicrobial activity of flavonoids. *Int J Antimicrob Agents.* 2005; 26(5): 343–356. https://doi.org/10.1016/j.ijantimicag.2005.09.002

28. Unanue M., Iriberri J. Limitations of 3H-thymidine incorporation in measuring bacterial production in marine systems. *Sci Mar.* 1996; 61(2): 111-122.