Electronic Journal of Biotechnology 55 (2022) 9–17

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

journal homepage:

Antibacterial activity, stability, and hemolytic activity of heartwood extract from *Caesalpinia sappan* for application on nonwoven fabric ☆

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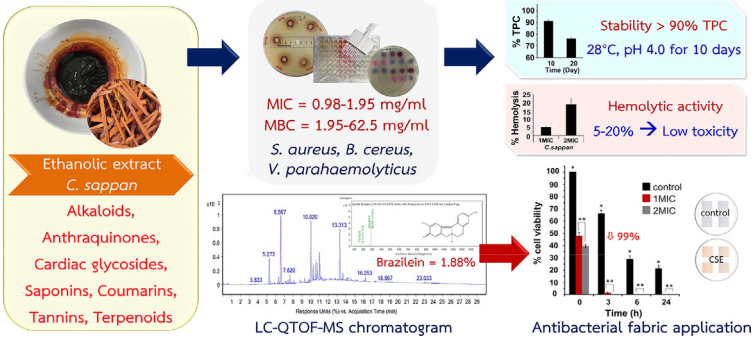
GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 15 February 2021
Revised 27 May 2021
Accepted 5 October 2021
Available online 13 October 2021

Keywords:
Antibacterial activity
Bioactive compounds
Brazilein
Caesalpinia sappan
Heartwood
Hemolytic activity
Inhibitory effect to *Staphylococcus aureus*
Non-woven fabric
Phytochemical
Stability

ABSTRACT

Background: Antimicrobial substances from medicinal plants have been widely applied in many industries, without the concern of side effects. Nonwoven fabric products such as face masks, paper wipes, and wound dressings contain antibacterial substances for reduction of bacterial accumulation. Therefore, the use of bioactive compounds from medicinal plants improves the properties of fabrics. This study used a crude extract from *Caesalpinia sappan* to perform phytochemical screening and investigate its antibacterial activity, stability, hemolytic activity, and preliminary application in nonwoven fabric products.

Results: The alcoholic crude extracts of *C. sappan* heartwood consisted of alkaloids, anthraquinones, coumarins, flavonoids, saponins, tannins, terpenoids, and cardiac glycosides. The *C. sappan* extract (CSE) had the highest inhibitory activity against *Staphylococcus aureus* with inhibition zone of 13.67 ± 1.56 mm in the agar well diffusion assay. The MIC value of CSE was at 1.95, 1.95, and 0.98 mg/ml, while the MBC value was 62.5, 3.91, and 31.25 mg/ml for *S. aureus*, *Bacillus cereus*, and *Vibrio parahaemolyticus,* respectively. The phenolic content was stable at 28 ± 2°C and at pH 4.0 for 12 h (~95%) and 10 d (>90%), and it had low hemolytic activity on human erythrocytes at 5.04–18.95%. The major chemical component was flavonoids, and brazilein was 1.88% area sum. For the application to antibacterial fabrics, the highest inhibitory activity against *S. aureus* was found in 2MIC of CSE-coated fabric with 99% for 3 h.

Peer review under responsibility of Pontificia Universidad Católica de Valparaíso

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https://doi.org/10.1016/j.ejbt.2021.10.002

Conclusions: The CSE had high inhibitory toward 3 bacterial strains and stability in a wide range of pH and temperature. The CSE-coated nonwoven fabric had efficient inhibition to *S. aureus* and a possibility to application in a nonwoven product in the future.

How to cite: 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. https://doi.org/10.1016/j.ejbt.2021.10.002

1. Introduction

Many antimicrobial agents on fabrics provide a special protection such as bacteriostatic, bactericidal, fungistatic and fungicidal properties in textile finishing. The chemical substances are usually coated onto the surface, such as quaternary ammonium, triclosan, and metallic salts (such as copper, zinc, and silver), which possibly affect some consumers and the environment, for example, with allergy, irritation, and chemical residue. Textile consumption worldwide has rapidly increased over the past several decades. The antimicrobial property of a textile product has been highly expected in medical use such as disposable mask, wound dressing, and gown. The microorganisms can contaminate textile products, depending on moisture, nutrients, and temperature. The emergence of antibiotic-resistant pathogenic bacteria is becoming a serious issue in the medical field. Health awareness of contaminating pathogenic microorganisms is increasing, and antimicrobial substances coated onto the surface can diffuse into the fibers and migrate to the external zone to inhibit microorganisms [1,2]. The action of these substances includes damaging the cell wall and inhibiting cell function and protein and nucleic acid synthesis as well as other metabolic processes. Moreover, silver is a known biocide for fabric coatings. The intense use of chemicals leads to a high environmental impact.

Studies on natural products such as medicinal plants are relevant to many fields, and chemical compounds or phytochemical substances in medicinal plants are secondary metabolites such as alkaloids, flavonoids, glycosides, phenolics, and terpenoids, which directly interact with the receptors, cell membranes, and nucleic acids [3]. In Thailand, several diseases have long been treated by folk medicine that involves the use of many Thai herbal plants. Moreover, Thai medicinal plants have been used as important components of many products such as massage oil or cream, cosmetics, skin lotion, and supplementary food. *Caesalpinia sappan* is a herbal medicine and distributed domestically in Thailand. It has been commonly used in Thai folk medicine for treating yaws, cough, diarrhea, hematonic, hemagogue, apthous ulcer, and bleeding in an organ. It has been also used in textile dyeing and histological staining. Srinivasan et al. [4] reported that the crude extract exhibited a wide range of antimicrobial activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella typhi*, *Enterobacter aerogens*, and *Escherichia coli*. The phytochemicals of *C. sappan* have been reported to be different secondary metabolites, and flavonoids (brazilin and brazilein) are the main compounds that affect the DNA and protein synthesis [5]. Moreover, brazilein can suppress the release of nitric oxide (NO), tumor necrosis factor-α (TNF-α), and interleukin (IL)-6 and reduce the expression of inducible nitric oxide (iNOS) synthase, which may protect against cell injury and anti-inflammatory effect [6]. This medicinal plant has possible applications in fabric production.

The present study investigated phytochemicals from *C. sappan* and its extraction, phytochemical screening, antibacterial activity, stability, hemolytic activity, and preliminary application in active nonwoven fabric products.

2. Materials and methods

2.1. Plant collection

C. sappan L. heartwood was purchased from the Thai medicinal local market (Nakhon Si Thammarat Province, Thailand). Heartwood was then cut and ground into a fine powder using a hammer mill machine.

2.2. Bacterial strains

The bacterial strains used in this study were *S. aureus* TISTR2329, *B. cereus* TISTR747, *E. coli* TISTR527, *P. aeruginosa* TISTR357, and *V. parahaemolyticus* TISTR1596; all these strains were obtained from Thailand Institute of Scientific and Technological Research (TISTR), Thailand. All pathogenic bacteria were cultured in Nutrient Agar (NA) and stored at 4°C until further use.

2.3. Extraction of phytochemicals from C. sappan heartwood

C. sappan heartwood powder (50 g) was extracted with 70% and 95% of both ethanol and methanol (300 mL) at room temperature (28°C) under shaking condition (150 rpm) for 5 days. The extract solution was filtered using a white cloth and a Whatman filter paper No.1 (Sigma Aldrich, USA). The extract was removed from the solvent by a rotary evaporator and dried at 45°C for 3 days. The crude extract of *C. sappan* (CSE) was ground into a powder and stored in amber glass bottle at − 20°C until further use.

2.4. Qualitative phytochemical screening in crude extract

Preliminary phytochemical screening of crude extracts was performed using standard analytical methods described by Evans et al. [7] and Harborne [8].

2.4.1. Test for alkaloids

Crude extract powder (0.2 g) was mixed with 1 mL of 10% sulfuric acid and warmed at 55°C for 5 min before filtration. Five drops of Wagner's and Mayer reagents were added to the filtrate. A reddish brown precipitate (Wagner) and a cream precipitate (Mayer) formation indicated the presence of alkaloids.

2.4.2. Test for flavonoids

Crude extract powder (0.2 g) was dissolved in 50% ethanol (1 mL) and filtered with a filter paper. A small piece of magnesium ribbon and 5 drops of concentrated HCl were added and incubated at 55°C for 5 min. The presence of an intense yellow color indicated the presence of flavones, and a red or pink shade indicated the presence of flavonoids.

2.4.3. Test for anthraquinones

Crude extract powder (0.2 g) was mixed with 10% sulfuric acid (10 mL) and warmed at 55°C for 5 min. After filtering, the anthraquinones in filtrate were extracted with chloroform, and 10% ammonium solution was then added to the upper phase. The

sample solution had a pinkish-red color, which indicated the presence of anthraquinones.

2.4.4. Test for coumarins

Crude extract powder (0.2 g) was dissolved in 50% ethanol (1 mL) and filtered using a filter paper. Then, 6 M sodium hydroxide (1 mL) was added, and the development of an intense yellow color indicated the presence of coumarins.

2.4.5. Test for saponins

Crude extract powder (0.2 g) was dissolved in distilled water (5 mL) and warmed at 55°C for 5 min and filtered using filter paper. The filtrate was vigorously shaken for 3 min, and the stable persistent froth formation indicated the presence of saponins.

2.4.6. Test for tannins

Crude extract powder (0.2 g) was dissolved in distilled water (5 mL) and warmed at 55°C for 5 min. After filtering, 1% $FeCl_3$ (5 drops) was added to the filtered solution, and the greenish-black color after mixing indicated the presence of tannins.

2.4.7. Test for phlobatannins

Crude extract powder (0.2 g) was dissolved in distilled water (5 mL) at 55°C for 5 min and filtered with filter paper. Five drops of 10% HCl were added and mixed well before warming at 55°C for 5 min. The solution was greenish-black or dark blue color, which indicated the presence of phlobatannins.

2.4.8. Test for terpenoids

Crude extract powder (0.2 g) was dissolved in chloroform (1 mL) and warmed at 55°C for 5 min. Concentrated H_2SO_4 (0.5 mL) was slowly added along the side of the test tubes of filtrate. The reddish-brown ring between the extract and H_2SO_4 phase indicated the presence of terpenoids.

2.4.9. Test for steroids

Crude extract powder (0.2 g) was dissolved in chloroform (1 mL) and warmed at 55°C for 5 min. Glacial acetic acid (0.5 mL) was added to the filtrate before adding concentrated H_2SO_4 (3 drops). A blue or blueish-green color indicated the presence of steroids.

2.4.10. Test for cardiac glycosides

Crude extract powder (0.2 g) was dissolved in chloroform (1 mL) and warmed at 55°C for 5 min. Then, 1% $FeCl_3$ (5 drops) and glacial acetic acid (5 drops) were added to the filtrate. Concentrated H_2SO_4 (0.5 mL) was slowly added along the side of the test tubes. A brown ring formation between two layers indicated the presence of cardiac glycosides.

2.5. Determination of antibacterial activity of the crude extract

Antibacterial activity of *C. sappan* crude extract was studied by the agar well diffusion method, and high inhibition efficacy against bacterial strains was then investigated in terms of MIC and MBC. For crude extract preparation, the different alcoholic crude extracts were dissolved in DMSO and diluted with DI water to obtain 125 mg/ml (10% DMSO) and sterilized by filtration before storage at 4°C for further studies.

2.5.1. Primary screening of antibacterial activity

CSE was preliminarily screened for antibacterial activity against 5 bacterial strains by using the agar well diffusion method. The bacterial inoculum was prepared to obtain 10^8 cfu/mL by direct suspension (0.5 McFarland), and was then swabbed onto Muller–Hinton agar (MHA) (4 mm thickness). Five-millimeter wells were prepared, and the sample solution was added to each well (125 mg/mL, 50 μL) in duplicate and repeated in thrice. Kanamycin (750 μg/mL) and 10% DMSO were used as the control. All plates were incubated at 35°C for 24 h, and the diameter of the inhibition was measured in millimeters.

2.5.2. Determination of the minimum inhibitory concentration and minimum bactericidal concentration

The minimum inhibitory concentration (MIC) of crude extracts was determined using the broth microdilution method according to the Clinical and Laboratory Standards Institute [9]. Eight dilutions of CSE from 0.49 to 62.50 mg/mL (100 μL each) were prepared using serial two-fold dilutions with Muller–Hinton broth (MHB) (MHB + 3% NaCl for *V. parahaemolyticus*) in 96-well microplates. Ten microliters of bacterial inoculum (1×10^6 CFU/mL) was added to each well and incubated at 35°C for 20 h, and 0.18% of resazurin was then added to each well before continuing incubation for 4 h. Kanamycin, 10% DMSO, and distilled water were used as positive, negative, and sterile controls, respectively. The minimum concentration inhibiting the bacterial growth or the last blue well was the MIC.

The minimum bactericidal concentration (MBC) was determined using an agar spot on agar medium. All blue wells and several pink wells (10 μL) were dropped onto ager medium. The minimum concentration of CSE with no bacterial growth was MBC value. All experiments were performed in duplicate and repeated thrice.

2.6. Determination of total phenolic content

The total phenolic content (TPC) in CSE was determined using the Folin–Ciocalteu method by a modified method of Maurya and Singh [10]. CSE solution (aqueous DMSO solution) (50 μL, triplicate) was mixed with 10% Folin–Ciocalteu reagent (2.5 mL) and incubated at room temperature for 15 min. Next, 7.5% (w/v) Na_2CO_3 (2 mL) was added and incubated at room temperature for 15 min. The absorption was measured at 765 nm using a spectrophotometer. Total phenolic content was expressed in terms of gallic acid equivalents (GAEs) and calculated as GAEs per gram of the dry sample.

2.7. Stability of the total phenolic content in crude extract

2.7.1. Effect of temperature

CSE solutions (1.95 mg/ml, aqueous DMSO solution) were incubated at room temperature (28°C), 37°C, 50°C, and 75°C for 12 h and autoclaving at 121°C for 15 min. The remaining TPC was calculated from the TPC at 0 h. All samples were analyzed in triplicate.

2.7.2. Effect of pH

CSE powder was dissolved in DMSO and diluted in 50 mM of each buffer solution (acetate buffer (pH 4.0 and 5.5), phosphate buffer (pH 7.4), and glycine buffer (pH 10.0)) to obtain 1.95 mg/ml and incubated at room temperature (28°C) for 12 h. The remaining TPC was calculated from the TPC at 0 h. All samples were analyzed in triplicate.

2.8. Hemolytic activity of crude extract

The preliminary toxicity of phytochemical substances from plant extract was determined using a hemolysis assay on human erythrocytes [11]. Moreover, this assay was indicated for the possible application in medical material product. The blood (type O blood from Thasala hospital, Thailand) was used for erythrocyte suspension preparation. For an *in vitro* hemolysis test, 50 μL of the erythrocyte suspension (pH 7.4, 10% erythrocytes) was mixed

with 100 μL of the crude extract and incubated at 37°C for 1 h. Then, 850 μL of phosphate buffered saline (pH 7.4) was added and centrifuged at 3,000 rpm for 3 min. The supernatant was collected, and the hemolysis was measured the absorbance at 540 nm. Water and phosphate buffered saline (pH 7.4) were used as positive (maximum hemolysis) and negative control (minimum hemolysis). All tests were performed in triplicate.

$$\%\,\text{hemolysis} = A_{\text{crude extract}} - -A_{\text{negative control}} \times 100$$

$$A_{\text{positive control}} - -A_{\text{negative control}}$$

2.9. Analysis of major chemical constituents by LC-QTOF-MS

Liquid chromatograph-quadrupole time-of-flight mass spectrometry technique (LC-QTOF-MS) was used for chemical component analysis in ethanolic CSE. The sample was analyzed by LC-QTOF-MS system consisting of Agilent 1290 Infinity II LC with 6545 Quadrupole-TOF (Agilent Technologies, USA) equipped with an Agilent Dual Jet Stream electrospray ionization (Dual AJS ESI) interface in the negative ionization mode. The chemical component was separated using Agilent Zorbax Eclipse Plus C18 Rapid Resolution HD column (150 mm length × 2.1 mm inner-diameter, particle size 1.8 μm) at 25°C. Chromatographic conditions were as follows: 1% acetic acid + water (solvent A) and acetonitrile (solvent B). The gradient elution was as follows: 0 min, 80:20, (A: B v/v) for 5 min, 70:30 for 5 min, 65:35 for 5 min, 55:45 for 5 min, 25:75 for 5 min and 5:95 for 5 min. The flow rate was set at 200 μL/min and the sample volume injected was 2 μL. The MS operating parameters were as follows: gas temperature 325°C, gas flow 13 L/min, nebulizer 35 psig, capillary 4000 V, fragmentor 175 V, skimmer 65 V, Octopole RF Peak 750. All acquisition and analysis of data were performed using MassHunter Workstation software (Agilent Technologies, USA). The METLIN Personal Compound Database and Personal Compound Database and Library (version 8) included all compounds and additionally accurate mass Q-TOF MS/MS library reference spectra.

2.10. Application of active fabric coated with crude extract

The antibacterial property of ethanolic CSE was used as a coating substance on fabric, and an effective inhibition against *S. aureus* was determined according to Taki et al. [12]. The nonwoven textile product was cut into 2-cm^2 patches, which were then autoclaved at 121°C for 15 min and dried in the oven. The crude extract solutions at 1MIC and 2MIC were absorbed onto each set of patches (which was performed in triplicate) and distilled water was used as a control. All samples were allowed to dry in laminar flow. The bacterial inoculation was prepared by direct suspension and the turbidity was adjusted using 0.5 McFarland Standard (~1.0 × 10^8 CFU/mL). The bacterial suspension (50 μL) was inoculated onto each piece of fabric in sterile petri dish which was then incubated at 35°C for 12 h. Each fabric set was collected and introduced into and mixed with sterile phosphate buffered saline (pH 7.4, 10 mL). The sample was diluted to 10^{-1} to 10^{-3}, and the viable bacterial number were determined using the drop plate technique. The results were calculated based on the remaining bacteria compared with the control at the given time.

3. Results and discussion

3.1. Phytochemical extraction from C. sappan heartwood

The phytochemical substances in *C. sappan* heartwood were extracted using a maceration technique to break the plant's cell wall and release the soluble phytochemicals. The solvent was one of the main factors that influenced phytochemical extraction, resulting in high-yield extracts with functional properties. Additionally, both 95% and 70% ethanol or methanol were used in this experiment because the main chemical constituents having an antimicrobial activity contained polar parts in the structure such as phenol, flavonoids, saponins, and tannins [13]. *C. sappan* heartwood extracts were obtained with 6.00–9.48% yield and a red-brown viscous characteristic (Table 1). For this extraction, the higher solvent concentration resulted in the higher yield of crude extract. Methanol could extract a higher yield of CSE, and the highest yield was 9.48% from extraction by 95% methanol, while the lowest yield was 6.00% from extraction by 70% ethanol. However, the high amount of TPC was detected in 95% ethanolic crude extract. Many previous studies widely reported that the use of 95% ethanol for extraction led to a high yield of important phytochemical substances with an effective antibacterial activity [4,14,15].

Phytochemical screening showed that the CSE was composed of alkaloids, anthraquinones, coumarins, flavonoids, saponins, tannins, terpenoids, and cardiac glycosides (Table 2). The detection of alkaloid in this extraction was compared to the literature data of phytochemical substances from other sources such as alcoholic CSE from India with undetected alkaloid [16,17,18]. However, alkaloid could be found in an aqueous or alcohol extraction in some reports [19,20]. The difference in the antibacterial efficacy of the phytochemical contents in CSE was interesting in the next step.

3.2. Antibacterial screening of CSE

3.2.1. Primary screening using the agar well diffusion assay

Both alcoholic crude extracts of *C. sappan* heartwood were measured the primary antibacterial activity against 5 pathogenic bacteria by the agar well diffusion method. These bacteria were both gastrointestinal pathogens and wound infectious bacteria and could easily contaminate the fabric from environment and hospi-

Table 1
Yield and total phenolic content of crude extract of *C. sappan* with 95% and 70% of ethanol and methanol.

Solvent	Conc. (%)	Weight (g)	Yield (%)	TPC (mg/100 mg)
Methanol	95	50	9.48	1.06 ± 0.02
	70	50	9.03	0.98 ± 0.01
Ethanol	95	50	6.31	1.61 ± 0.03
	70	50	6.00	1.54 ± 0.00

TPC: Total Phenol Content as GAE mg/100 mg (Gallic Acid Equivalent).
Each value was an average of three determinations.

Table 2
Preliminary phytochemical screening of crude extract from *C.sappan* heartwood.

Phytochemicals	CSE			
	Methanol		Ethanol	
	95%	70%	95%	70%
Alkaloid	+	++	+	+
Flavonoid	+	+	+	+
Anthraquinone	+	+	+	+
Coumarin	+	+	+	+
Saponin	+++	++	++	++
Tannin	+++	+++	+++	+++
Phlobatannin	–	–	–	–
Terpenoid	–	+	–	+
Steroid	–	–	–	–
Cardiac glycoside	+	+	++	++

Table 3
Preliminary determination of the antibacterial activity of crude extracts of *C. sappan* by the agar well diffusion assay.

Bacterial test strain	Zone of inhibition (mm)				
	Methanol		Ethanol		Kanamycin
	95%	70%	95%	70%	
S. aureus TISTR2329	11.58 ± 1.62	9.92 ± 1.88	13.67 ± 1.56	7.00 ± 0.63	23.33 ± 0.87
B. cereus TISTR747	11.42 ± 0.90	10.42 ± 0.79	11.67 ± 0.24	7.08 ± 0.89	23.00 ± 1.52
E. coli TISTR527	–	–	–	–	20.22 ± 1.09
P. aeruginosa TISTR357	–	–	–	–	8.67 ± 1.67
V. parahaemolyticus TISTR1596	8.89 ± 0.93	6.89 ± 1.69	8.58 ± 2.71	9.56 ± 0.53	14.22 ± 1.39

tal. The bacteria attachment on fabric can cause change in fabric and unpleasant odors as well as health problems. Therefore, fabric should be protected to bacterial attachment. The results are shown in Table 3. The crude extracts moderately inhibited three bacterial test stains (*S. aureus*, *B. cereus*, and *V. parahaemolyticus*), but they could not inhibit *E. coli* or *P. aeruginosa*. The CSE extracted with 95% alcohol (8.58–13.67 mm) showed a higher efficient inhibition than extraction by 70% alcohol (6.89–10.42 mm), except for inhibition to *V. parahaemolyticus*. The antibacterial activity of plant extracts depended on the chemical composition apart from the TPC. Many studies have reported that the ethanolic and aqueous extracts of *C. sappan* heartwood had inhibitory effects against *S. aureus*, *B. cereus*, *B. subtilis*, *E. coli*, *P. aeruginosa*, and *Listeria monocytogenes* [13,17,21,22]. The results were similar to those reported by Kadchumsang et al. [23] who showed no inhibition of gram-negative bacterial strains using the agar well diffusion method. In addition, an effective antibacterial activity was observed against gram-positive bacteria, which was due to more susceptibility and permeability of cell wall structure than that of gram-negative bacteria [24]. *P. aeruginosa* is a gram-negative and multidrug resistant bacterium with several resistant mechanisms such as low outer membrane permeability, expression of efflux pumps for removing the antibiotics, and antibiotic-inactivating enzyme production [25]. *Vibrio* sp. is normally considered to be highly susceptible to all antibiotics [26].

3.2.2. Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC of crude extracts against *S. aureus*, *B. cereus*, and *V. parahaemolyticus* was investigated by broth microdilution method. The results are shown in Table 4. The crude extract concentrations from 0.49–62.50 mg/mL were prepared using serial two-fold dilutions, and the inhibitory pattern increased according to the sample concentration. The 95% alcoholic extract had a lower MIC value than 70% alcoholic extract, and CSE showed more inhibition. For CSE, 95% ethanolic crude extract had a more effective inhibitory against bacterial strains than methanolic crude extract, and both samples showed the lowest MIC to *V. parahaemolyticus* (0.98 mg/mL) followed by *B. cereus* and *S. aureus* (1.95 mg/mL). The lowest MBC was found for *B. cereus* (3.91 mg/mL) followed by *V. parahaemolyticus* (31.25 mg/mL), and *S. aureus* (62.5 mg/mL). From the literature, the antibacterial activity of CSE affected both gram-positive and gram-negative bacteria. The MIC value of ethanolic extract of *C. sappan* heartwood was reported in the range of 0.62 to 125 mg/mL for *S. aureus* [27,28], 125 μg/mL for *B. subtilis*, and 1000 μg/mL for *E. coli* [29]. Moreover, gram-negative bacteria had lower susceptibility and permeability than gram-positive bacteria [30]. It was concluded that the CSE showed high effective inhibition, which might be caused by the difference in the chemical components as the result of the above phytochemical screening. Moreover, the type of organic solvent and extraction method were also important factors affecting the type, amount, and purity of extracted bioactive compound.

3.2.3. Thermal and pH stabilities of total phenolic content of crude extract

The physical parameters including temperature, pH, and time considerably affected the stability of phenolic compounds, [31,32] and the results are used for practical applications. The stability of crude extracts was observed in term of the remaining of TPC for 12 h. The results are shown in Fig. 1. For thermal stability, the phenolic content in ethanolic CSE decreased with an increasing temperature and incubation time, and the lowest remaining TPC was achieved at 75°C for 12 h. The TPC was slightly decreased at 29–50°C for 12 h of incubation as well as autoclave conditions (121°C for 15 min) (Fig. 1A and C), which remained >90%. The highest stability at 12 h was found at 29°C with 96.5% of the TPC. During long-term incubation, the remaining TPC was 76.4% and 63.9%for 10 and 20 d of incubation, respectively (Fig. 1B). The rings of phenolic structure can degrade into other components through oxidation and hydrolysis under heating and storage condition, thus indicating a higher instability of crude extract [33].

For pH stability, the different pH conditions critically affected the structure of phenolic compounds, and the active structure at a suitable pH has a high stability for application. The results are shown in Fig. 2. The pH value had less influence than temperature.

Table 4
Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of crude extract from *C. sappan* against pathogenic bacteria.

Sample		*S. aureus TISTR2329*		*B. cereus TISTR747*		*V. parahaemolyticus TISTR1596*	
		MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
CSE	Methanol 95%	1.95	62.5	1.95	15.63	1.95	1.95
	Methanol 70%	1.95	62.5	1.95	7.81	1.95	1.95
	Ethanol 95%	1.95	62.5	1.95	3.91	0.98	31.25
	Ethanol 70%	3.91	62.5	3.91	3.91	3.91	62.5

The lowest MIC value in each bacterial strain is indicated by the grey shade area.

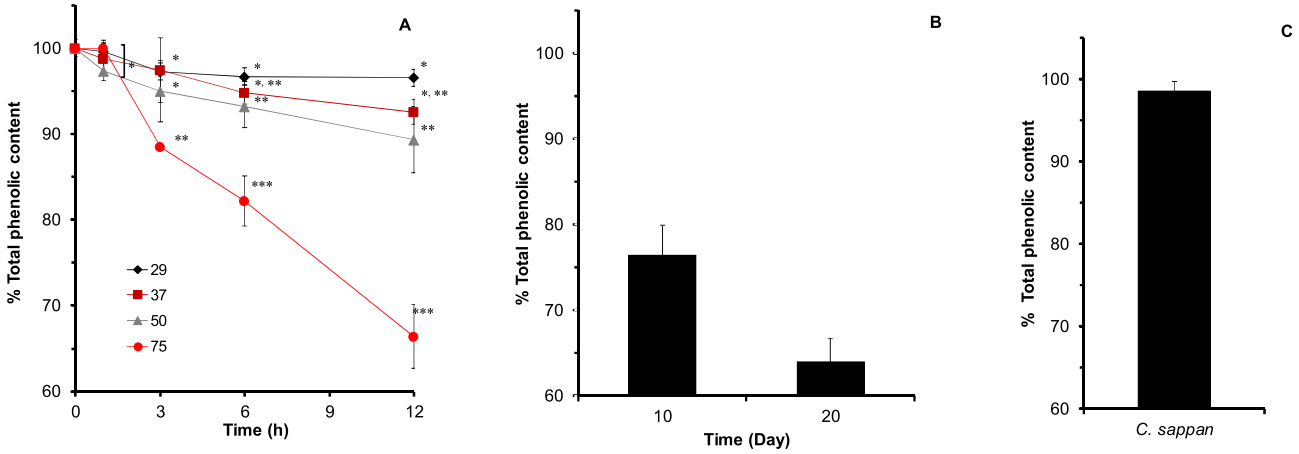

Fig. 1. Thermal stability of total phenolic content for 12 h of CSE (A) at 29°C for 10 and 20 days (B), and in autoclave (121°C for 15 min) (C).

The TPC in ethanolic CSE showed the highest stability in acid (~95–96%, pH 4.4 and 5.5) and slightly reduced at neutral conditions (~93%, pH 7.4), while it drastically reduced to 83% at pH 10.0 for 12 h. According to Friedman and Jürgens [31], the pH value affected the spectrum absorption of phenolic compounds because of the change in their structure, and the different susceptibility of plant phenolic compounds to pH strongly depended on the phenolic structure. Moreover, the different phenolic groups underwent degradation in different manners, which could be attributed to their structural differences such as the position of the functional groups [34]. The degradation likely resulted from oxidation, complex formation, and various reactions of other phenols, amino acids, proteins, and metal ions [35]. The result of TPC reduction lower than 30% for 10 d might be an issue for the application. Moreover, it had to be further studied for stability improvement such as the combination with other substance or preparation technique as emulsion or coating method.

3.2.4. Hemolytic activity of the crude extract

The toxicity study of plant extract was conducted by *in vitro* red blood cell lysis analysis. The 1MIC and 2MIC of ethanolic CSE were examined for the hemolytic or antihemolytic activity on human erythrocytes. The results are shown in Fig. 3. The hemolytic activity of CSE showed a dose-dependent increase in its activity, and the 2MIC exhibited the highest activity to 18% of hemolysis followed by 1MIC with a low amount of hemolysis (5%). Many plants have been studied for their hemolytic property such as the crude extract of *Croton bonplandianum* (25 μg/mL, 18% of hemolysis) [36], *Daphne gnidium* (500 μg/mL, 7.06% of hemolysis), and *Acacia nilotica* (IC_{50} > 500 μg/mL) [37], indicated a little hemolytic activity. The strong hemolytic effect of plant extract could induce lytic properties or membrane instability, leading to hemolytic anemia. For fabric application, the active coatings for smart textile in the finishing process was also evaluated for the toxicological properties because the human skin is in prolonged contact with the textile fabric during various activities in everyday life when touching or wearing fabric as well as for medical application [38]. In this study, the 1MIC of CSE showed potential for the next study.

3.3. Analysis of major chemical constituents by LC-QTOF-MS

The phytochemical profile of the crude extract of *C. sappan* was analyzed using LC-QTOF-MS. The chromatogram profile highlighted the presence of a large group of compounds corresponding to the protonated molecular ions of different flavonoids (Fig. 4). Individual components were identified by their m/z values in the total ion count (TIC) profile by matching their spectra with library reference spectra (Table 5). The chromatogram showed 5 main peaks corresponding to hematoxylin, heliannone C, (2S,2″S,3S,3″R,4S)-3,4′,5,7-tetrahydroxyflavan(2->7,4->8)-3,4′,5,7-tetrahydroxyflavan (Geranin A), silymonin, and pyllodulcin. All these compounds are members of the flavonoid group as the main compound in the *C. sappan* extract. Moreover, the brazilein was found at 10.433 min with 1.88% of area sum. Liang et al. [39]

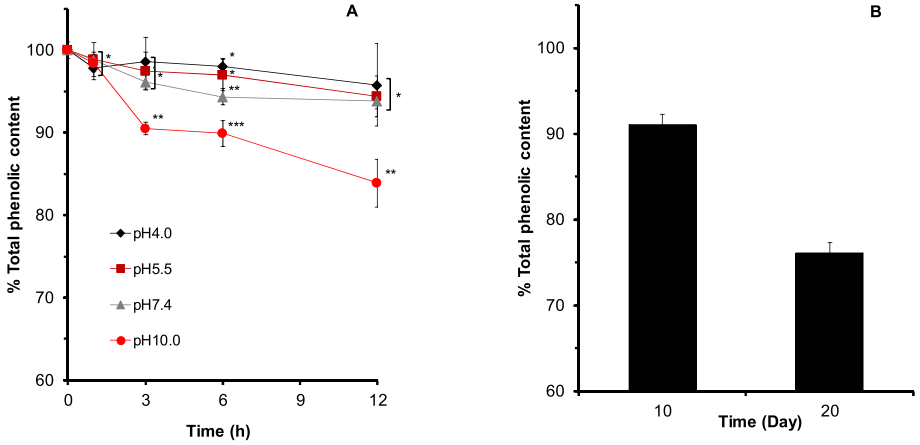

Fig. 2. pH stability of total phenolic content for 12 h of CSE (A) and at 29°C for 10 and 20 days (B).

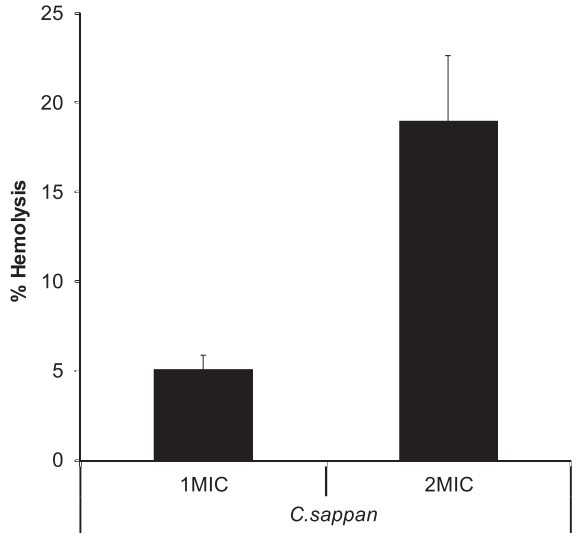

Fig. 3. Hemolytic activity of CSE on human erythrocytes.

studied the extraction and purification of brazilein from 33 kg of *C. sappan* heartwood and obtained 127.5 g of brazilein with 99.3% purity. Although, brazilin had easily oxidized leading to stuctural tranformation to brazilein, there were fewer report of brazilein. .

3.4. Application for active fabric coated with crude extract

The plant crude extracts were preliminarily applied on nonwoven fabric products. The antibacterial property from a natural source is an alternative product for customers, and this may motivate farmers and increase the value of the product. The coating technique was completed using an absorption method, and the percentage of viable *S. aureus* was measured for 24 h. The results are shown in Fig. 5. The bacterial inhibition had strongly increased depending on the crude extract concentration that was most effective at 2MIC of CSE. The 1MIC of CSE-coated nonwoven fabric had strongly inhibited the cell viability to 53% at the beginning and increased more than 95% at 3 h, whereas the 2MIC of CSE coated fabric had immediately inhibited to 60% at 0 h and no growth at 3 h.

However, the percentage of bacterial viability in control treatment had continuously reduced during incubation period because the patch of fabric had without culture medium or another substance supporting the bacterial viability. However, the results indicated that the crude extract-coated fabric treatment had negative effect on bacterial viability. The effective inhibition of CSE-coated fabric might be the large amounts of various phenolic groups and other components acting as an active component. The modes of action of plant crude extract were reported including the interference of the phospholipoidal cell membrane to increase the permeability profile, damage cellular metabolism and energy production enzymes, destroy genetic material, and affect the cytoplasmic

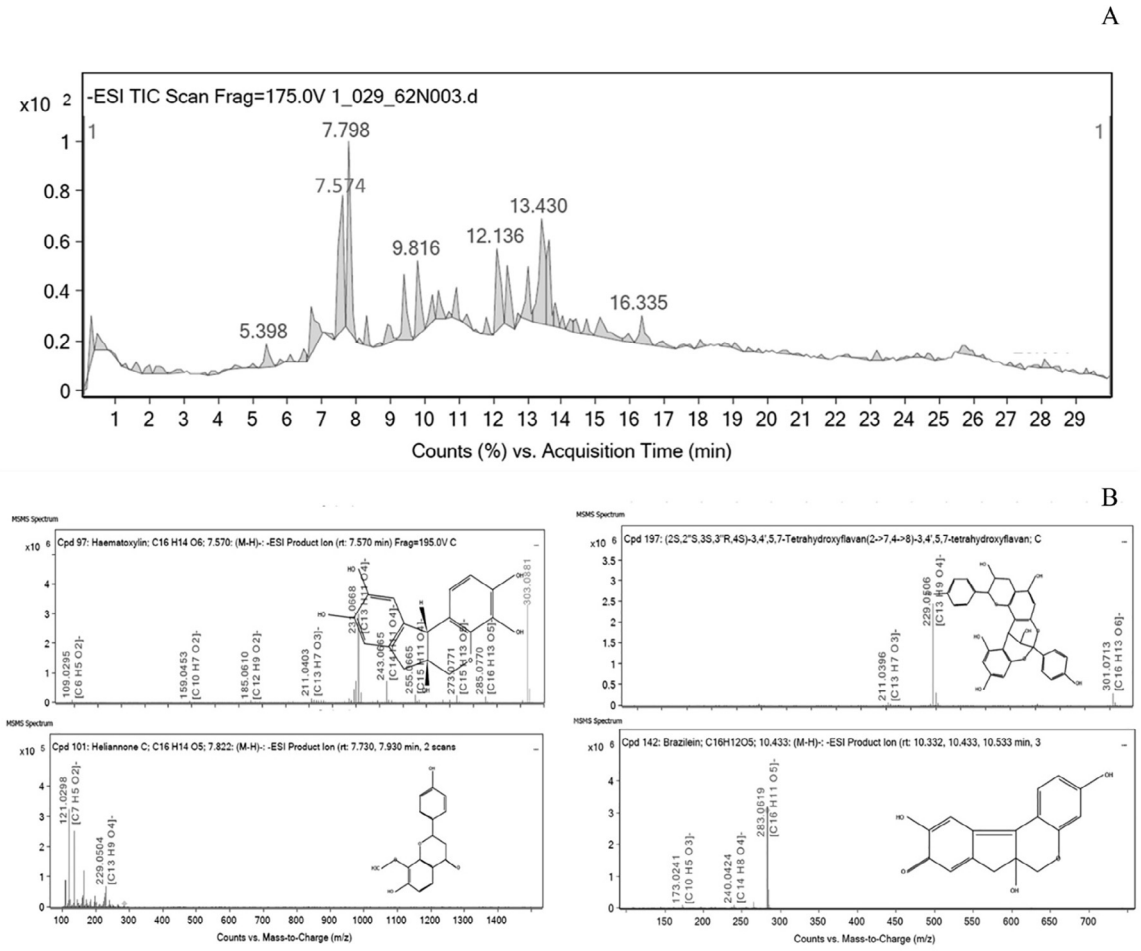

Fig. 4. LC-QTOF-MS chromatogram of ethanolic CSE monitored for 30 min (A) and the MS/MS fragmentation of the 3 main compounds and brazilein (B).

Table 5
Major phytochemical compounds identified in ethanolic crude extract from *C. sappan* by LC-QTOF-MS.

RT	Height	score	Base peak	Name	Formula	m/z	Mass	Score DB	Difference ppm
5.437	5177109	94.68	191.0563	Chlorogenic Acid	$C_{16}H_{18}O_9$	353.0875	354.0948	98.72	0.7
7.570	59057622	42.39	303.0913	Haematoxylin	$C_{16}H_{14}O_6$	301.0719	302.0792	84.79	-0.65
7.822	21215822	48.94	285.0770	Heliannone C	$C_{16}H_{14}O_5$	285.0770	286.0843	97.88	-0.68
9.755	9632356	92.88	229.0507	Hesperetin	$C_{16}H_{14}O_6$	301.0722	302.0796	96.61	-1.87
9.931	8564772	47.88	341.0661	Epigallocatechin 3-O-p-coumarate	$C_{24}H_{20}O_9$	451.1032	452.1106	95.76	0.23
10.181	3811899	49.06	569.1450	Aurasperone A	$C_{32}H_{26}O_{10}$	569.1454	570.1528	98.13	-0.34
10.433	42102054	46.46	283.0625	Brazilein	$C_{16}H_{12}O_5$	283.0630	284.0703	98.10	-6.51
12.089	411184	48.20	465.1183	Silymonin	$C_{25}H_{22}O_9$	465.1187	466.1259	96.40	1.13
12.792	9718129	48.60	341.0664	Epigallocatechin 3-O-p-coumarate	$C_{24}H_{20}O_9$	451.1033	452.1107	97.21	0.05
13.006	8431869	49.03	297.0407	2-Hydroxypseudo-baptigenin	$C_{16}H_{10}O_6$	297.0408	298.0482	98.05	-1.51
13.307	10363680	95.51	283.0610	Biochanin A	$C_{16}H_{12}O_5$	283.0615	284.0688	98.01	-0.34
13.445	17047574	49.34	301.0717	(2S,2"S,3S,3"R,4S)-3,4',5,7-Tetrahydroxy flavan (2->7,4->8)-3,4',5,7-tetrahydroxy flavan	$C_{30}H_{24}O_{10}$	603.1512	544.1369	98.68	0.03
13.508	25548724	47.25	285.0775	Phyllodulcin	$C_{16}H_{14}O_5$	285.0780	286.0852	94.5	-3.6

The main identified compounds corresponding with LC-QTOF-MS chromatogram are indicated by the grey shade area.

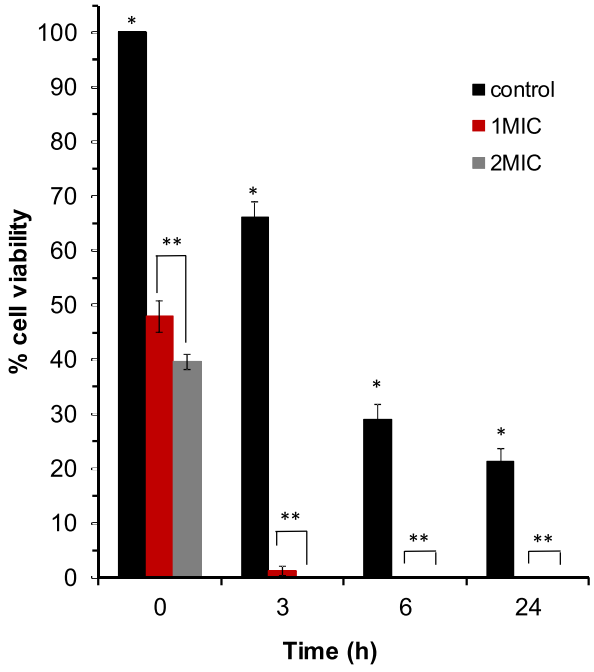

Fig. 5. Cell viability of *S. aureus* on non-woven fabric coated with ethanolic CSE for 24 h.

membrane and electron transport mechanisms [40]. In this study, the CSE had possibility to be applied as antibacterial substance in fabric product, but the stability improvement had to be further studied according to the time of fabric processing and distribution for practical application.

4. Conclusions

The bioactive compounds from *C. sappan* heartwoods were extracted by 95% ethanol and 95% methanol. The main phytochemical components were alkaloids, saponins, tannins, terpenoids, cardiac glycosides, anthraquinones, coumarins and flavonoids. CSE inhibited and killed three bacterial strains: *S. aureus, B. cereus*, and *V. parahaemolyticus*, with 0.98–62.5 mg/ml of crude extract. In terms of stability, the total phenolic content was stable at various temperatures and pH. At 1MIC of CSE had low effect on human erythrocytes. Three major chemical components detected by LC-QTOF-MS were hematoxylin, heliannone C, and (2S,2″S,3S,3″R,4S)-3,4′,5,7-tetrahydroxyflavan(2->7,4->8)-3,4′,5,7-tetrahydroxyflavan (Geranin A), while brazilein was found at 10.433 min with 1.88% of area sum. In addition, the 2MIC of CSE-coated nonwoven fabric inhibited *S. aureus* at 3 and 24 h. In the present study, the crude extract had possibility to be applied as antibacterial substance, but the stability of TPC had to be improved for appropriate fabric processing and prolonged distribution in the future.

Ethical approval

The research in hemolytic activity was approved by the Ethics Committee on Human Rights Related to Research Involving Human Subjects, Walailak University (WUEC-20-171-01). It was conducted under the framework of best ethical practice.

Financial support

The authors would like to thank the Institute of Research and Innovation, Walailak University for financial supports (Grant no. WU63248) and Development and Promotion of Science and Technology Talents Project (DPST) for supporting this research.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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