

Microbiological and physicochemical quality of pasteurized milk supplemented with sappan wood extract (*Caesalpinia sappan L.*)

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Abstract

Caesalpinia sappan L. (Sappanwood) contains antibacterial compounds and antioxidants that inhibit the growth of microbes. This study aimed to investigate the microbiological and physicochemical qualities of pasteurized milk supplemented with 0, 2, 4, 6 and 8% (w/v) sappan wood extract. Data were analyzed using a completely randomized design factorial followed by the Duncan's new multiple range test. Preliminary analysis showed that sappan wood extract contained 44.66 ± 0.09 mg/100g phenols, 0.18 ± 0.01 mg/100mg flavonoids, 46.42 ± 0.23 mg/100g tannins, and antioxidant activity at $85.82 \pm 0.25\%$. The addition of sappan wood extract significantly increased the antioxidant activity ($P < 0.05$) of pasteurized milk during storage. Pasteurized milk supplemented with sappan wood extract had a lower total bacterial count ($P < 0.05$) than that of unsupplemented pasteurized milk, and supplemented milk showed strong antibacterial activities against *Escherichia coli*, *Shigella flexneri*, *Salmonella thypimurium*, *Staphylococcus aureus*, and *Listeria monocytogenes*. The addition of sappan wood slightly increased the protein content but did not affect pH, and viscosity. It is concluded that the addition of sappan wood extract increased the microbiological quality and maintained the physicochemical quality of pasteurized milk, thus extending the product's shelf-life.

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Keywords

Antioxidant

Antibacteria

Sappan wood extracts

Microbiology

Pasteurized milk

Introduction

Fresh milk has a high nutritional content which favours the proliferation of microbes that can trigger milk's spoilage and cause food-borne diseases. Fresh milk spoilage is commonly associated with the presence of *Micrococcus sp.*, *Pseudomonas sp.*, and *Bacillus sp.*, while food-borne diseases are associated with pathogenic microbes, such as *Staphylococcus aureus*, *Escherichia coli*, *Shigella sp* and *Salmonella sp.* Jayarao *et al.* (2006) reported a number of pathogenic bacteria that commonly contaminate fresh milk, included *Listeria monocytogenes*, *Camphlyobacter jejuni*, *Escherichia coli*, and *Salmonella sp.* To eliminate pathogenic bacteria contamination, proper milk processing is required; one of these common processes is pasteurization. According to the Codex Alimentarius (CAC/RCP 57-2004), a pasteurization process using a high temperature short time with a minimum temperature of 72°C for 15 seconds is recommended for continuous flow pasteurization while low temperature long time at 63°C for 30 minutes is required for batch pasteurization. Effective pasteurization kills the pathogenic bacteria but does not eliminate thermo-tolerant spores. As such, pasteurized milk has a limited shelf-life, and consequently also has an

expiry date for human consumption.

One strategy to extend shelf-life and maintain the quality of pasteurized milk is through fortification with naturally recognized antibacterial compounds, such as sappanwood (*Caesalpinia sappan L.*). Sappanwood is an evergreen shrubby plant belonging to the family of Leguminosae (Nirmal *et al.*, 2015). Sappan wood, also known as red wood, is found in tropical climates and its dried heartwood has long been used as a traditional medicine with many health benefits. Previous studies have reported the benefits of sappan wood extracts, including as an anti-inflammatory (Jeong *et al.*, 2008; Washiyama *et al.*, 2009), antibacterial (Xu and Lee, 2004; Srinivasan *et al.*, 2012), antioxidant (Badami *et al.*, 2003; Saenjum *et al.*, 2010), and antiallergic (Yodsaoe *et al.*, 2009). Sappanwood extracts contain bioactive substances such as phenols (Gan *et al.*, 2010), flavonoids (Namikoshi and Saitoh, 1987; Safitri *et al.*, 2003), brazilin (Nirmal *et al.*, 2015), and tannins (Cowan, 1999). The addition of sappan wood extracts has been previously demonstrated to preserve food (Sarayaet *et al.*, 2009); however, their addition to milk to increase its quality and extend its shelf-life has not been reported.

The purpose of this study was to investigate the addition of sappan wood extracts to pasteurized milk

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and to assess the qualities and shelf-life of the milk during storage.

Materials and Methods

Fresh milk, sappan wood and bacterial strains

Fresh milk was obtained from a local dairy farm owned by the Faculty of Animal Science Universitas Gadjah Mada. Sappanwood (*Caesalpinia sappan* L.) was collected from the local district of Imogiri in Yogyakarta, Indonesia. The following bacterial strains were used for microbiological and antibacterial studies: *Shigella flexneri* ATCC 12022, *Salmonella thypimurium* FNCC 0157, *Staphylococcus aureus* FNCC 0047 (obtained from Research Centre for Food and Nutrition Universitas Gadjah Mada), *Escherichia coli* and *Listeria monocytogenes* (provided by Health Laboratory of Yogyakarta Local Government).

Extraction of sappan wood

Extraction of sappan wood was conducted according to the method developed by Xu and Lee (2004). The main part of sappan wood – the heartwood – was dried under the sun for \pm 4.5 hours, chopped and blended into small pieces, and powdered using a grinder. 100 g of sappanwood powder was mixed with 1.5 L sterile water and then heated at 100°C for 3 hours. The boiling water solution obtained was filtered using a cotton cloth to separate the filtrate which was subsequently used for further experiments.

Fresh milk pasteurization

Sappan wood extract was added to fresh cow's milk at concentrations of 0, 2, 4, 6, and 8% (w/v), and then pasteurized at 63°C for 30 minutes. The pasteurized milk was cooled at room temperature and then stored at 8°C for 0, 3, 7 and 14 days. The milk was then analyzed for total phenol, flavonoid and tannin content, antioxidant and antibacterial activity, total plate count (TPC), and physicochemical qualities.

Total phenol, flavonoid, and tannin analysis

Total phenolic compound were determined by using the Folin-Ciocalteu method (Senter *et al.*, 1989). Total flavonoid content was determined by using aluminium chloride colorimetric method ($AlCl_3$) according to Stankovic (2011) with slight modifications using quercetin as standard. The tannin content was determined using spectrophotometry method according to Ranganna (1977).

Antioxidant analysis

Antioxidant activity was analyzed based on

the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method developed by Brand-Williams *et al.* (1995) as cited by von Gadov *et al.* (1997).

Antibacteria and TPC analysis of the pasteurized products

Antibacterial activity was measured by a commonly used agar diffusion bioassay technique according to Wolf and Gibbon (1996) with modifications. A 40 μ L culture of 24 hour old *Escherichia coli*, *Shigella flexneri*, *Salmonella typhimurium*, *Staphylococcus aureus*, and *Listeria monocytogenes* with concentrations of 10^8 CFU/mL was separately inoculated into 20 mL nutrient agar (NA), mixed and poured onto a sterile petri dish. After solidification, two holes were drilled in the agar and 200 μ L pasteurized milk supplemented with sappan wood extract was poured into each hole. The agar was then incubated at 37°C for 24 hours, and the inhibitory zones were measured. Total viable bacteria in pasteurized milk with or without the addition of sappan wood extract was measured on days 0, 3, 7 and 14 using TPC. To perform TPC, pasteurized milk was diluted up to $1/10^3$ with sterile 0.9% NaCl, plated on plate count agar and incubated at 37°C for 24 hours. The colonies were then counted and total bacterial numbers per sample were estimated.

Physicochemical analysis of pasteurized milk

Three physicochemical parameters of pasteurized milk were measured: protein content, pH, and viscosity. Viscosity measurements were performed using Viscotester VT-03F (Rhion, Japan). All viscosity measurements were expressed in centipoise (cP), performed in triplicate and averaged. Protein content was determined using the Lowry method for protein quantification, as cited by Plummer (1987). Potential hydrogen (pH) was measured at room temperature using a potentiometric pH meter (Eutech 510).

Data analysis

Data were analyzed using a completely randomized factorial design and followed by Duncan's new Multiple Range Test, with statistical significance accepted at $P < 0.05$.

Results and Discussion

Bioactive compounds in the sappan wood extracts

Sappan wood was extracted using sterile water at 100°C for 3 hours. The obtained extract was analyzed, and the data is presented in Table 1.

Table 1 shows that sappan wood extracts

Table 1. Bioactive compounds present in sappan wood extracts

Bioactive compound	Concentration
Phenol	44.66 ± 0.09 (mg/100g)
Flavonoid	1.84 ± 0.03 (mg/100mg)
Tannin	46.42 ± 0.23 (mg/100g)
Antioxidant	85.82 ± 0.25 (%)

contained phenols, flavonoids, tannins, and antioxidants. The concentration of total phenols in the sappan wood extracts was 44.66 ± 0.09 mg/100g. The total phenol concentrations presented in this study are in agreement with a previous study by Gan *et al.* (2010), who reported a total phenol concentration of 40.97±0.12 mg/100g in sappan wood. Flavonoid, a derivative of phenol, was present at 1.84 ± 0.03 mg/100mg in our sappan wood extracts. Antioxidant activity was present at 85.82 ± 0.25% of DPPH scavenging activity. Widowati (2011) reported that sappanwood had a strong antioxidant activity due to the presence of flavonoids and phenols. The concentration of tannin in the sappan wood extracts was 46.42 mg/100g. Tannins inhibit the growth of bacteria by disrupting the bacterial cell membranes (Akiyama *et al.*, 2001).

Antioxidant activities

Antioxidants are substances that delay or prevent the damage of foodstuffs due to oxidation. These substances are produced naturally or can be added to the product during processing (Gordon, 2001). The addition of sappan wood extracts to pasteurized milk was intended to increase the antioxidant activity. The antioxidant activity of pasteurized milk with the addition of 0, 2, 4, 6, and 8% sappan wood extract after storage for 0, 3, 7, and 14 days is presented in Figure 1.

Figure 1 shows that the addition of sappan wood extracts significantly increased ($P < 0.05$) the antioxidant activity of pasteurized milk. Pasteurized milk without the addition of sappan wood extracts had an antioxidant activity of 2.55%. Albera and Kankofer (2009) reported a number of components contributed for antioxidant activities in milk, including antioxidative enzymes such as glutathione peroxidase and superoxide dismutase, and non-enzymatic antioxidants lactoperoxidase, lactoferrin and ceruloplasmin. Of these antioxidants in milk, a decrease in the activity of antioxidative enzymes—superoxide dismutase and glutathione peroxidase due to pasteurization had previously been reported by

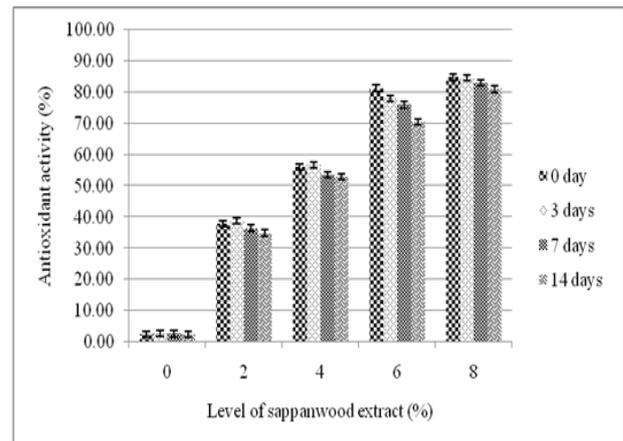


Figure 1. Antioxidant activity (%) of pasteurized milk supplemented with sappan wood extract after various storage times

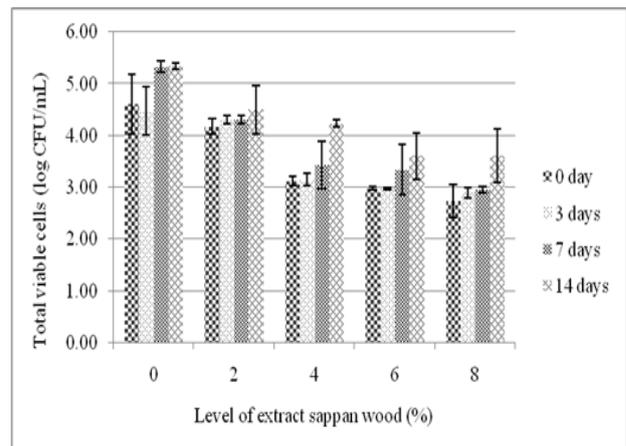


Figure 2. Total viable microbes (measured by total plate count; log CFU/mL) in pasteurized milk supplemented with sappan wood extract during various lengths of storage

Marinkovic *et al.* (2016). The antioxidant activities of pasteurized milk supplemented with 0, 2, 4, 6, and 8% of sappan wood extracts were 2.55±0.69, 37.0±2.99, 54.7±4.13, 76.4±7.81, and 83.5 ± 3.35%, respectively. The antioxidant activity in pasteurized milk increased with increasing concentrations of sappan wood extract. This is likely because the sappan wood extracts contain phenolic and flavonoid compounds which are very strong natural antioxidants. Badami *et al.* (2003) has previously demonstrated that flavonoids and phenolic compounds have a strong antioxidant activity.

Total plate count

Total plate count was conducted to evaluate the number of total viable microbes in pasteurized milk supplemented with sappan wood extracts at concentrations of 0, 2, 4, 6, and 8% (w/v) after storage for 0, 3, 7, and 14 days. Data on total viable microbes is presented in Figure 2. Figure 2 shows that the addition of sappan wood extracts significantly decreased ($P < 0.05$) the number of total

Table 2. Inhibitory zones of sappan wood extracts against five tested bacteria

Sappanwood extract (%)	Inhibitory zones (mm)				
	<i>Escherichia coli</i>	<i>Shigella flexneri</i>	<i>Salmonella typhimurium</i>	<i>Staphylococcus aureus</i>	<i>Listeria monocytogenes</i>
0	0.00±0.00 ^a	0.00±0.00 ^a	0.00±0.00 ^a	0.00±0.00 ^a	0.00±0.00 ^a
2	20.3±3.38 ^b	15.3±1.49 ^b	18.1±1.82 ^b	17.9±1.03 ^b	20.4±1.08 ^b
4	22.7±3.34 ^c	19.6±0.73 ^c	21.2±1.59 ^c	21.3±1.30 ^c	23.3±1.32 ^{bc}
6	25.0±3.59 ^d	23.3±0.68 ^d	24.7±1.54 ^d	23.5±1.21 ^d	25.4±0.82 ^d
8	26.8±3.54 ^d	24.6±0.77 ^d	27.7±0.71 ^d	25.0±1.79 ^d	25.8±2.15 ^d

a,b,c,d different superscript in the same raw/column indicates $P < 0.05$

viable microbes in pasteurized milk. The average number of total viable microbes without the addition of sappan wood was 4.60 ± 0.58 log CFU/mL, while the total viable microbes in pasteurized milk after the addition of 2, 4, 6 and 8% (w/v) sappan wood extracts was 4.32 ± 0.24 , 3.48 ± 0.51 , 3.22 ± 0.39 , and 3.04 ± 0.43 log CFU/mL, respectively. This data shows that the microbiological quality of pasteurized milk supplemented with sappan wood extracts remains well within the Indonesian national standard for pasteurized milk (SNI, 1995), which requires a maximum total plate count of 3×10^4 or 4.48 log CFU/mL.

The decrease in total microbes in pasteurized milk supplemented with a high concentration of sappan wood extracts indicates high antibacterial activity. The high antibacterial activity of sappan wood extracts is in accordance with the high content of phenols and tannins. A previous study by Miksusanti *et al.* (2011) reported the ability of phenol-containing sappan wood to inhibit the activity of *Bacillus cereus*. Another study by Xu and Lee (2004) demonstrated that the active antibacterial compound in sappan wood – brazilin – actively inhibited the growth of bacteria without affecting the viability of mammalian cells.

Figure 2 also shows that total viable microbes increased after 14 days of storage at refrigerator temperatures (8°C). The average number of total bacteria in pasteurized milk at 0, 3, 7, and 14 days was 3.51 ± 0.79 , 3.55 ± 0.73 , 3.87 ± 0.92 , and 4.25 ± 0.73 log CFU/mL, respectively. According to Harding (1999), pasteurized milk maintains its quality for 6 – 8 days when stored at 8°C, with spoilage occurring afterward. This data suggests that low storage temperatures did not kill microbes; rather they only slowed down microbial metabolisms, thus retarding microbial growth.

Antibacterial activity

Antibacterial activity was measured based on the

inhibitory zones against the growth of pathogenic microbes. Table 2 shows that pasteurized milk supplemented with sappan wood extract inhibited the growth of Gram-positive *Listeria monocytogenes* and *Staphylococcus aureus*, as well as Gram-negative *Escherichia coli*, *Shigella flexneri*, and *Salmonella typhimurium*.

Table 2 also shows that pasteurized milk without the addition of sappan wood extracts did not inhibit the growth of any of the bacteria tested. Treating pasteurized milk with sappan wood extracts at 2, 4, 6, 8%, and 100% (a positive control) resulted in inhibitory zones against all tested Gram-positive and Gram-negative bacteria (data not shown). The highest inhibitory zones against *Escherichia coli*, *Shigella flexneri*, *Salmonella typhimurium*, *Staphylococcus aureus* and *Listeria monocytogenes* were 26.8 ± 3.54 ; 24.6 ± 0.77 ; 27.7 ± 0.71 ; 25.0 ± 1.79 ; 25.8 ± 2.15 mm, respectively. Based on the National Committee for Clinical Laboratory Standards (NCCLS), Coyle (2005) classified all pathogenic bacteria tested are susceptible to pasteurized milk supplemented with sappan wood extracts at and 4, 6 and 8%.

Testing the antibacterial activity of ethanolic extract of sappan wood, Srinivasan *et al.* (2012) previously reported inhibitory zones of 20.0 ± 1.3 mm against *Salmonella typhi*, 28.0 ± 2.3 mm against *Staphylococcus aureus*, and 9.0 ± 0.7 mm *Escherichia coli*. Except for *Escherichia coli*, *Salmonella typhi* and *Staphylococcus aureus* tested by Srinivasan *et al.* (2012) were susceptible to sappan wood extract; and this was in agreement with our findings. The antibacterial activity of sappan wood extract reported in this study was associated with the active phenolic compounds present in the sappan wood extracts. Kim and Fung (2004) showed that the mechanism of bacterial growth inhibition by sappanwood extract was due to cell membrane disruption causing the cell to die.

Table 3. Physicochemical quality of pasteurized milk supplemented with sappan wood extracts

Parameter	Storage (days)	Level extract addition sappanwood					Mean
		0%	2%	4%	6%	8%	
Protein (%)	0	2.52±0.07	2.75±0.09	2.69±0.28	2.78±0.06	2.75±0.08	2.69±0.15 ^P
	3	2.83±0.59	2.83±0.45	2.72±0.21	3.12±0.30	3.85±0.74	3.03±0.61 ^P
	7	2.98±1.10	3.05±0.67	3.65±0.63	4.48±0.40	4.33±0.71	3.70±0.90 ^Q
	14	3.20±0.15	3.03±0.56	3.32±0.52	3.86±0.20	4.76±0.12	3.63±0.72 ^Q
	Mean	2.87±0.59 ^a	2.87±0.45 ^a	3.09±0.70 ^a	3.56±0.72 ^b	3.91±0.90 ^b	
pH	0	6.60±0.02 ^c	6.53±0.07 ^{bc}	6.51±0.09 ^{bc}	6.48±0.08 ^{bc}	6.44±0.09 ^b	6.51±0.09 ^b
	3	6.57±0.07 ^{bc}	6.53±0.06 ^{bc}	6.52±0.08	6.48±0.01 ^{bc}	6.45±0.11 ^b	6.51±0.08 ^b
	7	6.55±0.05 ^{bc}	6.53±0.06 ^{bc}	6.51±0.05 ^{bc}	6.47±0.02 ^{bc}	6.44±0.09 ^b	6.50±0.06 ^b
	14	6.24±0.03 ^a	6.48±0.07 ^{bc}	6.52±0.08 ^{bc}	6.47±0.06 ^{bc}	6.43±0.06 ^b	6.42±0.11 ^b
	Mean	6.48±0.15 ^{ns}	6.51±0.06 ^{ns}	6.52±0.07 ^{ns}	6.47±0.05 ^{ns}	6.44±0.07 ^{ns}	
Viscosity (cP)	0	2.82±0.16	2.76±0.31	3.00±0.44	2.93±0.13	2.88±0.24	2.87±0.25 ^P
	3	5.56±2.36	4.04±0.34	3.68±0.18	4.00±0.49	4.04±0.60	4.26±1.17 ^Q
	7	4.59±1.04	4.00±0.60	4.04±0.56	4.36±1.94	3.52±0.14	4.10±0.96 ^Q
	14	4.23±0.76	3.92±0.27	3.98±0.21	3.98±0.30	4.88±1.39	4.19±0.72 ^Q
	Mean	4.30±1.54 ^{ns}	3.68±0.65 ^{ns}	3.68±0.54 ^{ns}	3.81±1.03 ^{ns}	3.83±1.00 ^{ns}	

^{a, b, c} different superscript on the same line indicated P<0.05

^{P, Q} different superscript in the same column indicates P<0.05

Physicochemical quality

Table 3 shows that there was a slight increase in protein concentration in pasteurized products supplemented with sappan wood extracts at 6 and 8%. The protein concentration without sappan wood addition was 2.87±0.59% (w/v), while the concentration was 2.87±0.45; 3.09±0.70; 3.56±0.72 and 3.91±0.90% after 2, 4, 6 and 8% sappan wood addition, respectively. The protein concentration in pasteurized milk supplemented with sappan wood extracts reported in this study is within the standards of Indonesian national standards (SNI, 1995) (i.e. < 2.5%). A little increase in protein concentration was also observed during storage at 8°C over 7 and 14 days. The protein concentrations of pasteurized milk supplemented with sappan wood extracts at 0, 3, 7, and 14 days of storage were 2.69±0.15, 3.03±0.61, 3.70±0.90, and 3.63±0.72%, respectively.

The average pH of pasteurized milk supplemented with sappan wood extracts was between 6.44 and 6.52 (Table 3). There was no difference in pH between pasteurized products without (0%) and with the addition of 2, 4, 6 and 8% sappan wood extracts. The pH reported in this study was within the standard limits of pH (6.3 to 6.8) of the Indonesian national standards (SNI, 1995). Table 2 also shows that prolonged storage at 8°C for 14 slightly decrease the pH of pasteurized milk. The pH values during storage on day 0, 3, 7, and 14 were 6.51±0.09, 6.51±0.08,

6.50±0.06, and 6.42±0.11, respectively. A slight decrease in pH was coincidence with the increase of total viable microbes during storage (Figure 2).

The supplementation of sappan wood extract also did not affecton the viscosity (Table 3). The apparent viscosity of pasteurized products without (0%) and with the addition of 2, 4, 6 and 8% sappan wood extracts were 4.30 ±1.54; 3.68 ± 0.65; 3.68 ± 0.54; 3.81 ± 1.03; 3.83 ± 1.00 cP; respectively. The apparent viscosity reported in this study was in agreement with the viscosity of pasteurized milk reported by Bateman and Sharp (1928) at around 2.0. A slight increase of apparent viscosity was observed during storage. The apparent viscosity during storage on day 0, 3, 7, and 14 were 2.87±0.25, 4.26±1.17, 4.10±0.96, and 4.19±0.72, respectively.

Conclusion

The addition of sappan wood extracts to pasteurized milk increased the microbiological quality, maintained the physicochemical quality and improved the shelf-life of this product.

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