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Research article

Antimicrobial effect of Japanese pumpkin (*Cucurbita maxima*) extract on local bovine mastitis pathogen

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Abstract

In Chiang Mai, Thailand, evidence of a high prevalence of bacterial mastitis with antimicrobial resistance has been observed over the past decade. The development of applicable alternative medicine options is needed to address this resistance situation. The crude extraction of natural resources, such as Japanese pumpkin, should be studied for bacterial activity so as to raise the economic value of each substance. The objective of this study was to explore the antimicrobial activity of Japanese pumpkin by-products against bacteria that has caused dairy mastitis to circulate in the Chiang Mai area. Crude polysaccharides and carotenoids were extracted from Japanese pumpkins. Subsequently, 50 isolates of 8 bacterial species were collected from mastitis milk samples obtained from dairy farms in Chiang Mai during August to October, 2018 were screened for antimicrobial activity using the disc diffusion method. Seven bacterial species displaying inhibition zones were then explored to determine the minimum inhibitory concentrations (MICs). Consequently, 300 mg/ml of crude polysaccharide extracts were not found to be able to inhibit most bacteria. However, the MICs of crude carotenoid extracts ranged from 75 to 300 mg/ml. In summary, by-products of the pumpkin extraction displayed effective antimicrobial activity. Thus, these by-products offer an alternative in preventing the bacteria causing mastitis. Additionally, this approach could increase the potential value of a large amount of waste produced during the course of industrial processing.

Keywords: Japanese pumpkin, Polysaccharide, Carotenoids, Antimicrobial activity, Mastitis

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INTRODUCTION

Mastitis is an important economic burden for the dairy industry (Kossaibati and Esslemont, 1997). Damage to the structure of the mammary gland of cattle in most cases can be attributed to bacterial infection (Watts, 1988). *Escherichia coli*, *Streptococcus uberis*, *Staphylococcus aureus*, *Streptococcus dysgalactiae* and *Streptococcus agalactiae* are the major causation agents of this condition worldwide (Bi et al., 2016; Dufour et al., 2019). The impact of the problem has directly affected the cost of treatment, while also resulting in an increase in the culling rate and reductions in milk yield and quality (Bartlett et al., 1990; Bradley, 2002). Several antibiotics have been used for the prevention and treatment of mastitis. Penicillin, cephalosporin, streptomycin, tetracycline are broad spectrum antibiotic acts that have been effectively used against a wide range of disease-causing bacteria (USDA APHIS, 2002). However, antibiotic resistance is a serious and growing problem among both humans and livestock (Molla et al., 2007). Persistent overuse and unreasonable administration of antibiotics have been found in many dairy farms in attempts at the control and treatment of mastitis. This has resulted in an even greater chance in the emergence of an antibiotic resistant pathogen. Consequently, it is imperative that we encourage rational and prudent antibiotic use in the industry (Molla et al., 2007; Nobrega et al., 2018a; Nobrega et al., 2018b; Oliver and Murinda, 2012). In Chiang Mai, Thailand, evidence of a high prevalence of bacterial mastitis with antimicrobial resistance has been identified in several studies that have been conducted over the past decade (Boonyayatra and Chaisri, 2004; Chaisri et al., 2010; Leelahapongsathon et al., 2014; Suriyasathaporn et al., 2012).

Natural extracts have the potential to replace antibiotics and might be employed to address the situation of antimicrobial resistance (Cheng et al., 2014). Interestingly, polysaccharide extracts obtained from the American pumpkin (*Cucurbita moschata*) has displayed antibiotic activity against *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli* (Qian, 2014). Furthermore, it has been found that certain important substances, such as carotenoids that are present in pumpkins, have displayed antimicrobial activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*, *Salmonella* Enteritidis, and *Escherichia coli* (Keceli et al., 2013). In Thailand, a significant amount of by-products is generated by the food industry. An example of this would be the by-products that are produced through the growing and sales of the Japanese pumpkin. However, the antimicrobial activity of the Japanese pumpkin (*Cucurbita maxima*) has not yet been investigated. Hence, it would be of considerable value to examine the antimicrobial activity of the Japanese pumpkin. In addition, the results of this study could be extended to produce a functional extract substance that can be applied in organic animal production or for use in pharmaceutical products at the industrial level. Consequently, the objective of this study was to explore the antimicrobial activity of Japanese pumpkin by-products against bacteria that is suspected of causing dairy mastitis in the Chiang Mai area. These extract substances are likely to possess antimicrobial activity that could raise the value of Japanese pumpkin by-products, and thereby contribute substantially to its economic value.

MATERIALS and METHODS

Isolates

In this study, fifty bacterial isolates were obtained from mastitis milk samples collected from dairy farms in Chiang Mai during the period of August to October, 2018. The local isolates were comprised of *Acinetobacter calcoaceticus* (n=2), *Acinetobacter lwoffi*i (n=1), *Bacillus cereus* (n=1), *Bacillus* spp. (n=6), *Corynebacterium* spp. (n=4), *Escherichia coli* (n=11), *Klebsiella pnuemoniae* (n=4) and cougulase-negative *Staphylococci* (n=21).

Crude polysaccharide extractions obtained from Japanese pumpkin by-products

The method used in this research study was modified from that which was employed in the study of Gu and Pan (2017). The white and green skin of fresh fully grown (105-110 days) Japanese pumpkin by-products were macerated with 95% ethanol in ration 1:10 (w/v) for 24 hours. Then, the residue was boiled in distilled water at 60 oC for 1 hr. The residue was then precipitated by ethanol 95% (1:10 ratio) for 24 hrs and centrifuged 2 times at 3000 rpm 10 min. The supernatant was filtrated through Whatman's filter paper No. 1 (Whatman®, USA) and then dried in a vacuum evaporator. Finally, both the dried supernatant and the pellets were freeze dried to yield the crude polysaccharide.

Crude carotenoid extractions obtained from Japanese pumpkin by-products

Here, the method was modified from that which was employed in the study of Yolmeh et al. (2016). The white and green skin of fresh Japanese pumpkin by-products were macerated with acetone in ration 1:10 (w/v) for 24 hours. The filtrated residue was dried with a vacuum evaporator. Acetone extracts were collected and carotenoids were then extracted with petroleum ether (1:10 ratio) followed by being centrifuged 2 times at 3000 rpm 10 min. Finally, the supernatant was evaporated to remove petroleum ether. Carotenoid extracts were then collected.

Testing of antimicrobial susceptibility of Japanese pumpkin by-products using disc diffusion method

All isolates were screened for antimicrobial activity using the disc diffusion method. Subsequently, 10^5 McFarland of bacteria were inoculated on a Mueller-Hinton agar plate (MHA, MERCK, Germany). Papers comprised of 100 mg crude polysaccharide of the green skin of Japanese pumpkin by-products (GPJP) and 300 mg of crude carotenoid extracts obtained from the green and white skin of Japanese pumpkin by-products (GCJP and WCJP) and crude polysaccharide extracts obtained from the white skin of Japanese pumpkin by-products (WPJP) discs were placed on the inoculated agar surface. 100% dimethyl sulfoxide (DMSO) (Pipatphatsakorn et al., 2018) inoculated disc was used as a control for confirming that the solvent had no effect on antimicrobial activity. Plates were incubated for 24 hrs at 37°C prior to obtaining the desired results. Bacterial isolates with the inhibition zone were chosen to further the process of agar microdilution testing.

Testing of antimicrobial susceptibility of Japanese pumpkin by-products using agar microdilution

The agar microdilution method was modified from the study of Golus et al. (2016) according to the Clinical and Laboratory Standards Institute (2015). Previously prepared 2X molten Mueller-Hinton agar were added 96-well plate (50 µl per well). The crude polysaccharide extracts obtained from WPJP, GCJP and WCJP were diluted by sterile distilled water from stock solution with 2-fold serial dilution broth at concentrations of 300, 150, 75, 37.5, 18.75, 9.38 and 4.69 mg/ml in 96-well microtiter plates. Additionally, crude polysaccharide extract obtained from the GPJP was diluted sterile distilled water from the stock solution with 2-fold serial dilution broth at concentrations of 100, 50, 25, 12.5, 6.25, 3.13 and 1.56 mg/ml in 96-well microtiter plates. Each well contained the same volume of 200 µl including the control. After that, 2 µl of 105 McFarland of bacteria was inoculated to each well and the lid was closed. Subsequently, the 96-well microtiter plates were incubated for 24 hrs at 37°C prior to determining the results. Minimum inhibitory concentrations were observed with regard to the turbidity of the wells indicating the growth of bacteria.

RESULTS

Testing of antimicrobial susceptibility of Japanese pumpkin by-products using disc diffusion method

All isolates were screened for antimicrobial activity using the disc diffusion method. Some bacteria species, consisting of *A. calcoaceticus*, *A. lwoffii*, *B. cereus*, *Corynebacterium* spp., *E. coli* and coagulase-negative *Staphylococci*, were inhibited using polysaccharide and carotenoid extracts obtained from Japanese pumpkin by-product. Inhibition zone diameters were measured to determine the antimicrobial activity of the extracted substances. coagulase-negative *Staphylococci* (n=1; 4.8%) was inhibited by 100 mg/ml GPJP with 10 mm of an inhibition zone. Additionally, coagulase-negative *Staphylococci* (n=1; 14.8%) and *K. pnuemoniae* (n=1; 25%) were inhibited by 300 mg/ml WPJP with 10 mm and 8 mm of an inhibition zone, respectively. Furthermore, many isolates were inhibited by 300 mg/ml of GCJP and WCJP with inhibition zones of varying inhibition zone diameters. Conversely, *Bacillus* spp. was not inhibited by both crude polysaccharide and carotenoid extracts (Table1).

Table 1 Antimicrobial activity of Japanese pumpkin by-product extract on 50 bacterial isolates from bovine mastitis using disc diffusion method

Species	N[5]	GPJP 100 mg/ml[1]		WPJP 300 mg/ml[2]		GCJP 300 mg/ml[3]		WCJP 300 mg/ml[4]	
		n (%)[6]	Mean±SD[7]	n (%)	Mean±SD	n (%)	Mean±SD	n (%)	Mean±SD
Acinetobacter calcoaceticus	2	-	-	-	-	1 (50%)	10±NA	2(100%)	10.5±2.12
Acinetobacter lwoffii	1	-	-	-	-	1 (100%)	12±NA	1 (100%)	10±NA
Bacillus cereus	1	-	-	-	-	1 (100%)	9±NA	1 (100%)	7±NA
Bacillus spp.	6	-	-	-	-	-	-	-	-
Coagulase-negative *Staphylococci*	21	1 (4.8%)	10±NA	1 (4.8%)	10±NA	6 (28.6%)	9.17±4.36	5 (23.8%)	8.6±0.55
Corynebacterium spp.	4	-	-	-	-	2 (50%)	11±1.41	3 (75%)	9±1.73
Escherichia coli	11	-	-	-	-	1 (12.5%)	8±NA	-	-
Klebsiella pnuemoniae	4	-	-	1 (25%)	8±NA	-	-		

[1] 100 mg/ml of polysaccharide extract obtained from the green skin of Japanese pumpkin by-products
[2] 100 mg/ml of polysaccharide extract obtained from the white skin of Japanese pumpkin by-products
[3] 300 mg/ml of carotenoid extract obtained from the green skin of Japanese pumpkin by-products
[4] 300 mg/ml of carotenoid extract obtained from the white skin of Japanese pumpkin by-products
[5] Total number of isolates tested
[6] Number and percentage of isolates that showed inhibition zone when tested with Japanese pumpkin by-product extract
[7] Mean and standard deviation of inhibition zone diameter (mm), isolates with no inhibition zone were excluded

Testing of antimicrobial susceptibility of Japanese pumpkin by-products using agar microdilution

Most bacterial isolates were found to be inhibited by crude carotenoid extracts. The minimum inhibitory concentrations (MICs) of the crude carotenoid extract obtained from Japanese pumpkin by-products varied from 75 to more than 300 mg/ml. However, the crude polysaccharide extract obtained from Japanese pumpkin by-products could not inhibit most bacteria at a concentration of 300 mg/ml. Additionally, only one of the 21 isolates of coagulase-negative *Staphylococci* was inhibited by 100 mg/ml of GPJP, while the others were not inhibited at the same concentration value. The MICs of WPJP for some *K. pnuemoniae* (n=1; 25%) and coagulase-negative *Staphylococci* (n=1; 4.76%) isolates were 300 mg/ml. Furthermore, the MICs of WPJP for most of the strains were found to be more than 300 mg/ml, and these were comprised of *A. calcoaceticus* (n=2; 100%), *A. lwoffii* (n=1; 100%), *B. cereus* (n=1; 100%), *Corynebacterium* spp. (n=4; 100%), *E. coli* (n=11; 100%), *K. pnuemoniae* (n=3; 75%) and coagulase-negative *Staphylococci* (n=20; 95.24%). The MICs of GCJP were observed at 150 mg/ml in some of the isolates, namely *A. calcoaceticus* (n=1; 50%), *A. lwoffii* (n=1; 100%), *B. cereus* (n=1; 100%), *Corynebacterium* spp. (n=2; 50%), *E. coli* (n=1; 9.09%) and coagulase-negative *Staphylococci* (n=1; 4.76%). The MICs of WCJP were measured at 150 mg/ml for some strains of *Corynebacterium* spp. (n=1; 25%) and coagulase-negative *Staphylococci* (n=4; 19.05%). The MICs of WCJP were measured at 75 mg/ml for some strains of *A. calcoaceticus* (n=2; 100%), *A. lwoffii* (n=1; 100%), *B. cereus* (n=1; 100%) and *Corynebacterium* spp. (n=1; 100%) (Table 2).

Table 2 Frequency distribution of minimum inhibitory concentrations (MICs) of Japanese pumpkin by-product extract for 44 bacterial isolates from bovine mastitis

Species	N[1]	Extraction substances	Number and percentage of isolates			
			>300[2]	300[3]	150[4]	75[5]
Acinetobacter calcoaceticus	2	GCJP[6]	1 (50%)	-	1 (50%)	-
		WCJP[7]	-	-	-	2 (100%)
		WPJP[8]	2 (100%)	-	-	-
Acinetobacter lwoffii	1	GCJP	-	-	1 (100%)	-
		WCJP	-	-	-	1 (100%)
		WPJP	1 (100%)	-	-	-
Bacillus cereus	1	GCJP	-	-	1 (100%)	-
		WCJP	-	-	-	1 (100%)
		WPJP	1 (100%)	-	-	-
Corynebacterium spp.	4	GCJP	2 (50%)	-	2 (50%)	-
		WCJP	2 (50%)	-	1 (25%)	1 (25%)
		WPJP	4 (100%)	-	-	-
Escherichia coli	11	GCJP	10 (90.91%)	-	1 (9.09%)	-
		WCJP	11 (100%)	-	-	-
		WPJP	11 (100%)	-	-	-
Klebsiella pnuemoniae	4	GCJP	4 (100%)	-	-	-
		WCJP	4 (100%)	-	-	-
		WPJP	3 (75%)	1 (25%)	-	-
Coagulase-negative *Staphylococci*	21	GCJP	20 (95.24%)	-	1 (4.76%)	-
		WCJP	17 (80.95%)	-	4 (19.05%)	-
		WPJP	20 (95.24%)	1 (4.76%)	-	-

[1] Total number of isolates tested in each extraction substances
[2] At 300 mg/ml of extraction substances, bacterial isolates were not inhibited
[3] 300 mg/ml of extraction substances
[4] 150 mg/ml of extraction substances
[5] 75 mg/ml of extraction substances
[6] Carotenoid extract obtained from the green skin of Japanese pumpkin by-products
[7] Carotenoid extract obtained from the white skin of Japanese pumpkin by-products
[8] Polysaccharide extract obtained from the white skin of Japanese pumpkin by-products

DISCUSSION

To our knowledge, this is the first study on the crude polysaccharides and carotenoids extracted from the white and green skin of Japanese pumpkin by-products with regard to their antimicrobial activity. Notably, 300 mg/ml represented the beginning of GCJP, WCJP and WPJP concentration values for the disc diffusion test. Nonetheless, 100 mg/ml represented the beginning of the GPJP concentration value. This was because a higher concentration value could not be prepared due to the insolubility of the substance in DMSO. Differences in the degree of crude polysaccharide solubility between the green and white skins of Japanese pumpkin by-products is still unknown. However, 100 % DMSO used as control in this study showed no effect on antimicrobial activity (Pipatphatsakorn et al., 2018).

Interestingly, many strains of bacteria, including *A. calcoaceticus*, *A. lwoffii*, *B. cereus*, *Corynebacterium* spp., *E. coli* and coagulase-negative *Staphylococci*, were found to be inhibited by GCJP and WCJP extractions within a range of 75-300 mg/ml. However, other bacterial strains included in this study may be inhibited by GCJP and WCJP extractions at concentrations higher than 300 mg/ml. This may have been caused by several factors related to the species and its ability to resist the antibiotic that is specific to the infection. This inhibition may have also been influenced by differences in the cell wall structure, the lipid composition of the cytoplasmic membrane and the composition of different types of bacteria (Devine and Hancock, 2002; Karpiński and Adamczak, 2019). Furthermore, purified extraction methods should be employed in further studies.

In the study conducted by Karpiński and Adamczak (2019), fucoxanthin, a marine carotenoid extracted from brown algae and diatoms, was found to be effective against some Gram-positive pathogens including *S. agalactiae*, *S. epidermidis* and *S. aureus*, but was found to be weaker against Gram-negative ones (e.g., *E. coli*, *K. oxytoca*, *K. pneumoniae*). Additionally, the carotenoid extract obtained from Rhodotorula glutinis yeast inhibited the growth of *S. aureus*, *B. subtilis*, *B. cereus*, *S.* Enteritidis and *E. coli* (Keceli et al., 2013). Carotenoid extracts could be effective against many species of bacteria that are similar to those included in our study. However, the MICs of fucoxanthin for gram-positive bacteria reached values of between 62.5 and 250 µg/mL, while the gram-negative values were from 125 to 500 µg/ml. In addition, the MICs of carotenoid extracts obtained from *Rhodotorula glutinis* for bacteria values varied from 0.56 to 0.95 ug/ml. These findings were different from those of our study possibly because of the degree of purification in the extraction process. The mechanism of carotenoids is unknown; however, carotenoids are also known to be antioxidants. Importantly, the possible mechanisms of the antimicrobial activity of antioxidants include three basic attributes: outer membrane permeability, cytoplasm leakage, and inhibition of nucleic acid formation (Karpiński and Adamczak, 2019).

The MIC of WPJP was found to be as high as 300 mg/ml, which may be able to inhibit the growth of some bacteria. This outcome was different from that of the study conducted by Qian (2014), wherein 100 mg/ml of polysaccharide extracted from *C. moschata* was found to be protective against *B. subtilis*, *S. aureus* and *E. coli*. This may have been due to the extraction technique used for polysaccharides and the species of pumpkin being investigated. Interestingly, polysaccharides can also be extracted from certain animal constituents such the cuttlebone, wherein the degree of antimicrobial activity has been recorded in *B. subtilis*, *E. coli*, *K. pneumoniae*, *V. cholerae*, *V. parahaemolyticus*, *S. aureus*, *P. aeruginosa*, *S. typhi* and *Shigella* spp. (Shanmugam et al., 2008). The antimicrobial mechanism of polysaccharides can damage the bacterial cell wall and the cell membranes, thereby increasing cell permeability which can be the cause of structural lesions and the release of cell components, both of which can lead to cell death (Zhang et al., 2017). The antimicrobial activity of polysaccharides depends on certain factors related to their distribution, molecular weight, charge density and sulphate content (in sulphated polysaccharides) (Pérez et al., 2016).

Nevertheless, the antimicrobial activity of carotenoids and polysaccharides that was investigated in this study may significantly depend upon the source of isolation and the composition of the carotenoids and polysaccharides present in Japanese pumpkin by-products. Notably, pure extraction methods are quite costly. This study demonstrated that crude extracts also display antimicrobial activity but require a high degree of concentration. However, this finding can increase the value of the relevant by-products.

CONCLUSION

The crude carotenoid extraction substances investigated in this study exhibited antimicrobial activity against both gram positive and negative bacteria. The development of these substances has potential to influence the efficiency of dairy farming in Thailand with regard to the quality of milk production. This can be of great importance to the agricultural industry, as well as being beneficial to the health of both producers and consumers. It is imperative that we develop standardized products that directly contribute to the sustainability of dairy farming. The by-products obtained during pumpkin extraction display antimicrobial activity and could be a beneficial alternative in the prevention of the bacterial infection that is known to cause mastitis. Additionally, this approach could increase the potential value of a large amount of waste that is produced from pumpkin processing factories.

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CONFLICT of INTEREST

All authors declare no conflict of interest

AUTHOR CONTRIBUTION

Conceived and designed the experiments: PT, KC, DA. Performed the experiments: PT,KC,AP,NK. Analyzed the data: PT. Contributed reagents/materials/analysis tools: PT,KC,DA. Wrote the paper: PT.

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