Original Article

Screening antimicrobial properties against mastitis pathogens of turmeric extract after combination with various antiseptics

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Abstract

The aim of this study was to determine the screening for antimicrobial properties against mastitis pathogens of turmeric extract in combination with various antiseptics. The antibacterial effects of turmeric extract in combination with various antiseptics (5% povidone iodine, 0.5% v/v hydrogen peroxide (H_2O_2), 0.5% v/v chlorine (Cl_2), and 0.5% v/v chlorhexidine) were determined using the agar well diffusion method. Results showed that additional turmeric extract had significantly decreased the antimicrobial activities of either Cl_2 or chlorhexidine against almost all mastitis pathogens, except *S. agalactiae* for Cl_2 and gram negative bacteria for chlorhexidine. In contrast, no negative effect was found between turmeric extract and H_2O_2 against most mastitis pathogens. In addition, a positive antimicrobial effect of turmeric extract and H_2O_2 against other *Streptococcus* spp was found. In conclusion, H_2O_2 was the only antiseptic that can combine with turmeric extract and might support the advantages of the combination to improve wound healing and the antiseptic properties of H_2O_2 for further development for a future antiseptic product for teat dipping, especially for cows with teat end score damage.

Keywords: turmeric extract, antiseptic, mastitis, teat dipping, antibacterial activity

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Introduction

Curcumin, the main active component or rhizome of turmeric (Curcuma longa L.), exhibits an important role in the wound healing process by promoting antioxidant, antimicrobial, anti-inflammatory and skin regeneration activities (Cheppudira *et al.*, 2013; Hewlings and Kalman, 2017; Moghadamtousi *et al.*, 2014; Vanden and Haegeman, 2010). Previous studies on wound healing properties have shown that curcumin can improve the rate of epithelialization, enhance granulation tissue, collagen deposition and wound contact (Cheppudira *et al.*, 2013; Sidhu *et al.*, 1998; Vanden and Haegeman, 2010).

Mastitis in dairy cows has been established as the most costly disease in the dairy industry worldwide. For healthy teats, pre-milking and post-milking teats dipped in appropriate antiseptics for eliminating bacteria on the teat skin has been found to be one of the most effective procedures for reducing the incidence of new intramammary infection (Gleeson *et al.*, 2018; Leslie *et al.*, 2006). Various chemical antiseptics are available in commercial teat disinfectants such as chlorine, iodine, iodophor, hydrogen peroxide, and chlorhexidine (Gleeson *et al.*, 2018; Leslie *et al.*, 2006; Rasmussen and Larsen, 1998). In many cases, however, inappropriate milking procedures and/or the use of milking machines without regularly maintenance causes damage to the teat orifice such as increased callosity, hyperkeratosis, cracks, sores and erosion at the teat ends (Cerqueira *et al.*, 2018; Neijenhuis *et al.*, 2001), and consequently increases risk of new intramammary infections (Mulei, 1999; Neijenhuis *et al.*, 2001).

Correction of milking procedures and milking machines will stop damage to the teat orifice, and the subsequent natural wound healing process will cure the damaged teat end. To accelerate the wound healing process, the use of turmeric extracts and/or in combination with other antiseptics might be an ideal antiseptic teat dipping disinfectant for farms with damaged teat end problems. From our literature reviews, curcumin was found to be synergistic in combination with 8 different antibiotic groups against *S. aureus* (Teow and Ali, 2015), but there were no studies for other incidental mastitis pathogens such as *S. agalactiae*, other streptococci, other staphylococci or those known as coagulase negative staphylococci (CNS), and gram negative bacteria. In screening antimicrobial testing for various plant extracts, the agar diffusion method has been the sufficient accepted method to evaluate the antimicrobial activity (Valgas *et al.*, 2007; Sugathan *et al.*, 2012). Therefore, the objectives of this study were to determine the screening antibacterial effects of turmeric extract and its combination with various antiseptics against microorganisms causing dairy mastitis. In addition, a concentration of curcumin in turmeric extract was determined.

Materials and Methods

Animals: The study was conducted with ethics

Chemicals and reagents Turmeric powders were purchased from a supermarket in Chiang Mai, Thailand. Curcumin standard at 99.8% purification was purchased from Sigma-Aldrich (St. Louis, USA) Analytical grade methanol, HPLC grade of methanol and water were purchased from Labscan (Bangkok, Thailand). Trypticase Soy Broth (TSB), Trypticase Soy Agar (TSA) and Muller-Hinton agar (MHA) were purchased from Merck (Darmstadt, Germany).

Turmeric extraction Turmeric powders (1000 g) were extracted with 15 L of methanol using maceration technique for 20 h at room temperature. Subsequently, the extracts were filtered using 0.45 micron filter papers and concentrated using a vacuum rotary evaporator with a controlling temperature of 45°C (Büchi Labortechnik AC, Flawil, Switzerland). The extract were dried in a conventional oven until reaching a constant weight and stored at 4°C until use. The extract was re-dissolved in methanol to obtain the final concentration of 5 µg/ml before being subjected to HPLC analysis.

Determination of curcumin in crude extract The percentage of curcumin in the turmeric crude extract was analyzed using High Performance Liquid Chromatography (HPLC). The separation was implemented on Schimadzu CBM-20A controller, LC-20AD pump, SIL-20AHT auto injector, the fluorescence detector SPD-20A (Shimadzu, Tokyo, Japan) and operating software of LC-solution. The chromatographic method was carried out isocratically with a flow rate of 1 ml/min. The mobile phase consisted of methanol and water (85:15 v/v). Separation was achieved using a Phenomenex® C18 (5 µm; 4.6 mm x 250 mm, 5 µm) analytical column connected to an Inersil® C18 (50 mm x 4.6 mm, 5 µm) guard column. The column temperature was set at 35°C. The detection wavelengths were set at 426 nm and 539 nm for excitation and emission, respectively. The injection volume was 20 µL and the running time was 8 mins. Calibration curves were prepared by dissolving an accurate weight of curcumin standard and further diluted in methanol to obtained concentration ranges of 1-10 µg/ml.

Bacterial preparation For antimicrobial activities of the tested substances, 2 reference strains including *Staphylococcoccus aureus* ATCC25923 and *Escherichia coli* ATCC12228 were purchased and used. Field strain of mastitis bacteria were obtained from a stock of stored bacteria, Faculty of Veterinary Medicine, Chiang Mai University. Prior to study, all selected bacterial isolations had been confirmed for their species by MALDI-TOF Mass Spectrometry (Bruker Daltonics) according to the manufacturer's recommendation. Spectra were analyszed using Bruker Biotyper softwareReal Time Classification 3.0 software as describe by (Barreiro *et al.*, 2010). The isolates with species level identification scores ≥ 2.00 using the manufacture's criteria, log scores ≥ 2.00 were used. In total, fifty field strains comprising five strains for each pathogen of 10 mastitis pathogens including *Streptococcus agalactiae*, *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus chromogenes*, *Staphylococcus hyicus*, *Staphylococcus xylosus*, *Staphylococcus haemalyticus*, *Staphylococcus simulant*,

Escherichia coli and *Klebseilla pneumoniae* were used. These microorganisms were maintained in 15-20% glycerol and kept at -80 °C. All the bacterial strains were recovered by sub-culturing in Trypticase Soy Broth (TSB), incubation at 37 °C for 8-12 h and/or growing on Trypticase Soy Agar (TSA) at 37 °C for 24 h when required.

Antibacterial trial design study The turmeric extract, other antiseptics, turmeric extracts in combination with other antiseptics and a commercial 0.5% iodine teat dipping antiseptic (Iodine teat dip) available in the local market were used to compare their antibacterial activities. The turmeric extracts were dissolved in DMSO to obtain the concentration of 5%w/v and used as a control group (Klawitter *et al.,* 2012). Other antiseptics used in this study included 5% povidone iodine (PVP), 0.5% v/v hydrogen peroxide (H_2O_2) (Rios-Castillo *et al.*, 2017), 0.5% v/v chlorine (Cl_2), and 0.5% v/v chlorhexidine (Gleeson *et al.*, 2009; Hicks *et al.*, 1981), and were prepared using distilled water as dissolved solutions. The combinations of turmeric extract with other antiseptic compounds were prepared using turmeric extract, dissolved with DMSO and Tween, and then mixed with each prepared antiseptic compound to obtain the final concentration of turmeric 5% and antiseptic 0.5% v/v (Gleeson *et al.*, 2009; Zaikin *et al.*, 2013).

The antibacterial activities of all prepared compounds were determined using the agar diffusion method according to The National Committee for Clinical Laboratory Standard (NCCLS, 1997). Briefly, prepared bacterial strains were separately inoculated in 0.9% normal saline, incubated at 37°C for 24 h and the turbidity adjusted with 0.5 McFarland to obtain a final concentration of 10^8 colony-forming units (CFU)/ml. To confirm the viability of bacteria, counting the number of bacteria was performed before experiment. The bacterial suspension was inoculated at the surface of Muller-Hinton agar using a sterile cotton swab. Wells with a 6.0 mm diameter were made using a croxi broiler. The wells were filled with fifty microliters of each compound and incubated for 24 h. The diameters of inhibition zones were measured in millimeters after incubation

Statistical analysis: The concentration of curcumin from turmeric extract was described in percentage by weight. All pathogens were separated into 5 groups including 1) *S. agalactiae*, 2) other streptococci from *Streptococcus uberis, Streptococcus dysgalactiae*, 3) *S. aureus*, 4) coagulase negative staphylococci (CNS) from *Staphylococcus epidermidis*, *Staphylococcus chromogenes*, *Staphylococcus hyicus*, *Staphylococcus xylosus*, *Staphylococcus haemalyticus*, *Staphylococcus simulant*, and 5) Gram negative bacteria from *Escherichia coli* and *Klebseilla pneumoniae.* Antibacterial activities separated for bacterial group of PVP teat dip, turmeric extract, H_2O_2, Cl_2, and chlorhexidine were determined by their diameters of inhibition zones and were analyzed using analysis of variance (ANOVA). Pairwise comparisons among groups were performed using Duncan's test and the significant difference was defined at $P < 0.05$.

Results

Curcumin extraction: The extraction yield of turmeric powder in this study was 27.2%. The HPLC analysis of curcumin showed single peaks at retention times of 4.11 mins (Figure 1). The value of curcumin yield was 1.73%.

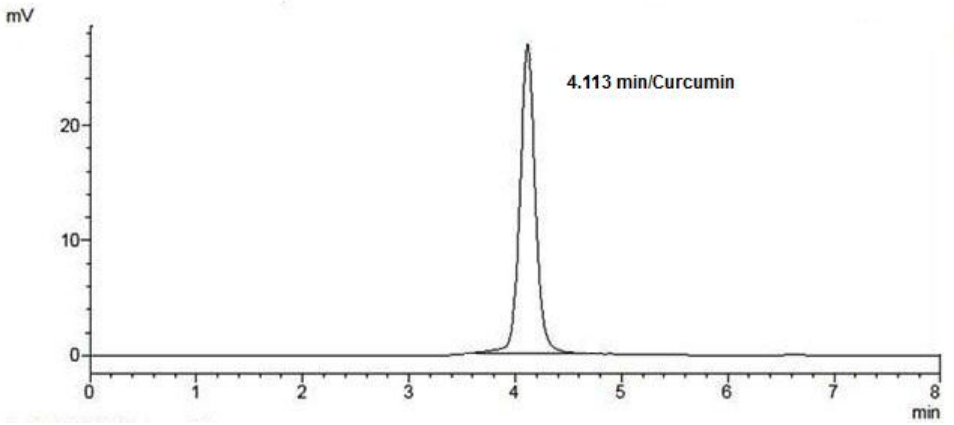

Figure 1 Chromatogram of curcumin in turmeric extract

Antibacterial activities of turmeric extract and its combination with other antiseptic compounds: Antibacterial activities indicated by the inhibition zone of turmeric extracts and all antiseptic compounds with and without combinations with turmeric extracts, separated for bacterial groups, are shown in Figure 2. Averages and standard error of means (SEM) of the inhibition zones of commercial iodine teat dips were 15 ± 0.55, 16 ± 0.70, 18.4 ± 0.81, 20.8 ± 0.95 and 20 ± 3.42 mm for *S. agalactiae*, other streptococci, *S. aureus*, CNS and Gram negative bacteria, respectively. The inhibition zones for all bacterial groups of turmeric extracts ranged from 6.46 mm for CNS to 11.7 mm for other streptococci. The most effective antibacterial compounds against mastitis pathogens were chlorhexidine for *S. agalactiae* (25.2 ± 0.97mm) and other streptococci (23.5 ± 0.37 mm), H_2O_2 for *S. aureus* (40.2 ± 0.2 mm) and CNS (35.9 ± 1.06 mm), and Cl_2 for gram negative bacteria (28.6 ± 2.28 mm). On all bacterial groups, turmeric extracts had significantly lower inhibition zones than those of the iodine teat dip. In comparison to the iodine teat dip, inhibition zones

of PVP were not significantly lower in other streptococci and gram negative bacteria, but were significantly lower in *S. agalactiae*, *S. aureus* and CNS.

Among all combination compounds, combination between turmeric extracts and H_2O_2 was the only compound without shorter inhibition zones than the commercial iodine teat dip against all bacterial groups. After combination with turmeric extracts, most antibacterial activities of both Cl_2 and chlorhexidine were significantly decreased, excepting *S. agalactiae* for Cl_2 and gram negative bacteria for chlorhexidine. In contrast, the inhibition zones of H_2O_2 against all pathogens were not decreased after combination with turmeric extracts ($p > 0.05$). In addition, the inhibition zone of H_2O_2 for other streptococci (11.8 ± 0.2 mm) was significantly increased after combination with turmeric extract (16 ± 0.63 mm).

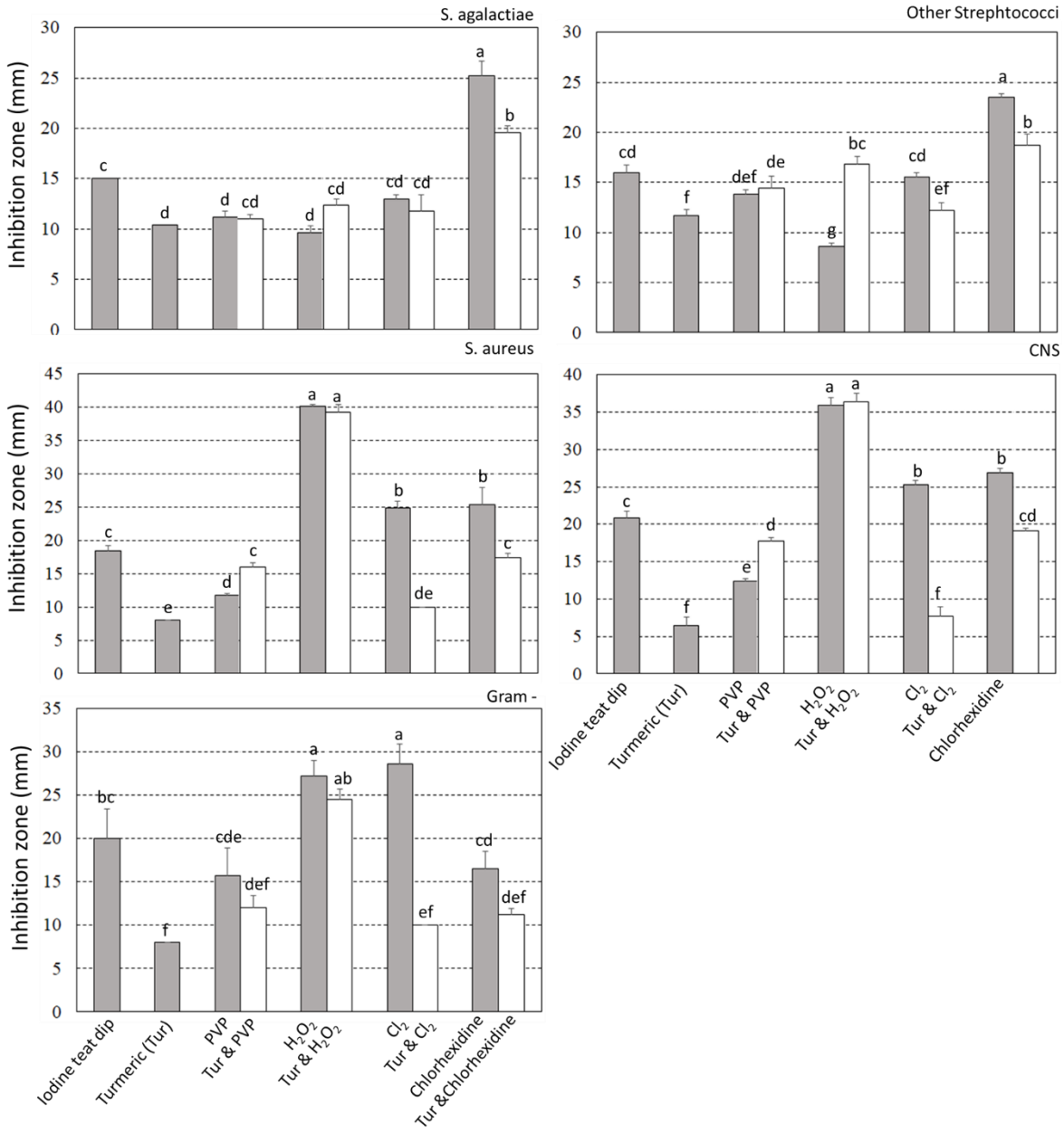

Figure 2 Antibacterial activities indicating by inhibition zone of turmeric extracts and all antiseptic compounds with and without combinations with turmeric extracts separated for bacterial groups. [a,b,c,d,e,f] different letters indicated significant difference at $P < 0.05$.

Discussion

The yield of curcumin found in this study at 1.73% was considered as a low yield level as obtained from Paulucci and colleagues (2013) at 0.1 to 1.8 %. In contrast, Sogi *et al.* (2010) obtained a curcumin yield of 4.5-12.9% that was 3 to 7 times the yield from the present study. The low curcumin yields in this study might be due to several factors such as different sources of turmeric powder, extraction conditions such as solvent types, solvent to turmeric ratio, times, and temperature of storages (Paulucci *et al.*, 2013; Sogi *et al.*, 2010). In this study, 50 field strains used comprising 5 strains for each pathogen from 10 mastitis pathogens represented the majority of mastitis pathogens worldwide including this area (Leelahapongsathon *et al.*, 2016; Suriyasathaporn, 2011; Suriyasathaporn *et al.*, 2012). The results from this study might be used for the

further development of antiseptic teat dipping for mastitis.

Efficacies of antiseptics and their combination with turmeric extracts determined by inhibition zones are shown in Figure 2. The control iodine compound, as the commercial iodine teat dip, had high inhibition zones for all pathogens. Iodine has been commonly used as a commercially effective disinfectant for teat dipping around the world (Foret *et al.*, 2005; Galton, 2004). The effectiveness of our prepared PVP against bacteria was lower than the control, and this might have been caused by the different molecular weight polymer of iodine (Zaikin *et al.*, 2013). Excepting gram negative bacteria, chlorhexidine had significantly better antibacterial efficiency than the control in all pathogen groups (Figure 2). For H_2O_2 and Cl_2, both compounds had significantly higher inhibition zones than the control in *S. aureus*, CNS and gram negative bacteria, but significantly lower in both *S. agalactiae* and other streptococci for H_2O_2.

Curcumin has been known for a long time for its antibacterial properties (Teow and Ali, 2015). In this study, turmeric extracts expressed low effective antibacterial activities, which might be caused by the low concentration of curcumin in our extract. After combination with turmeric extracts, significant decreases of antibacterial activity were shown in combinations of turmeric and either Cl_2 or chlorhexidine against almost all of the mastitis pathogens. With limitation on knowledge of antibacterial efficacy of combinations of turmeric and other antiseptics, a report showed that curcumin reduced the antimicrobial activity of ciprofloxacin, an antibiotic, against *Salmonella thyphi* and *S.thyphimurium* (Marathe *et al.*, 2013). In contrast to Cl_2 or chlorhexidine, H_2O_2 did not reduce its antimicrobial efficacy after combination with turmeric extracts (Figure 2). Unlike any other antiseptics in this study, H_2O_2 acts as an oxidant by producing hydroxyl free radicals (•OH) which attack essential cell components of bacteria, causing disturbances in the structure and permeability of the cell wall and the cytoplasmic membrane. These different antibacterial qualities of H_2O_2 might be related to the penetration of curcumin enhancing their antibacterial activities (McDonnell and Russell, 2001).

In conclusion, H_2O_2 was the only antiseptic that can combine with turmeric extract, for use in promoting the wound healing process, without any loss of antibacterial efficacy. Although chlorhexidine had the highest antibacterial efficacy, especially for streptococci, it was previously reported that chlorhexidine could induce biofilm development of *E. coli*, *S. aureus* and *S.agalactiae* (Ebrahimi *et al.*, 2014). In contrast, H_2O_2 was able to destroy both microorganisms and their biofilm matrix (Lineback *et al.*, 2018). This might support the advantages of combining turmeric extract and H_2O_2 for further development of future antiseptic teat dipping products. Due to the screening antimicrobial testing in this study, the establishment of synergistic and/or antagonistic properties of turmeric extract in various antiseptics need further studies for example the determination of minimal inhibitory concentration (MIC) to be confirmed.

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