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Original Article

# EFFICACY OF GUAVA LEAF EXTRACT AS ALTERNATIVE PRE-MILKING TEAT DIPPING IN REDUCING TEAT–END BACTERIAL LOAD OF MILKING DAIRY COWS

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**ABSTRACT**

**Objective:** To investigate the efficacy of aqueous methanolic guava leaf extracts used as pre-milking teat disinfectant on teat-end bacterial load reduction in lactating dairy cows.

**Methods:** Two test-skin swabs of before and after teat dipping with 50% aqueous methanolic guava leaf extract were evaluated for the bacterial loads, i.e. total bacterial count (TBC), staphylococcal count (STA), streptococcal count (STR) and coliform count (COL), using 16 lactating cows on a smallholder dairy farm in Khon Kaen, Thailand. Commercially available common chemical disinfectants were also comparatively investigated.

**Results:** Guava leaf extracts and the two others dis infectants showed reduction of the teat-end TBC and STA significantly while there was the decrease in teat-end STR and COL but the result was not significant in statistical analysis. Overall, applying the pre-milking teat dipping showed significant reduction of teat-end bacterial loads when compared to routine udder sanitization without teat dipping.

**Conclusion**: The guava leaf extract can be used as an alternative of pre-milking teat disinfectant for reducing the teat-end bacterial loads. This may lead to lower chemical uses, which may promote more hygienic, safe milk for consumers, and decrease costs of mastitis risk control, especially in the developing and the third world countries where guava is native and easy to obtain.

**Keywords:** Dairy Cow, Disinfectant, Efficacy, Guava, Pre-milking, Teat Bacteria.

## INTRODUCTION

Mastitis is a major economically important disease that affects the health and welfare of cows as well as causes substantial losses to dairy farmers resulting from milk discard, increased number of culled cows, antibiotic treatment costs and reducing in milk quality and price [1]. *Staphylococcus aureus*, *Streptococcus agalactiae* and *Escherichia coli* are well recognized as the major and virulent mastitis-causing bacteria. The bacteria usually inhabit inside and outside the udder as well as the milking equipment surfaces and can pass directly to raw milk and increase health risk to milk consumers [2, 3]. Pre-milking hygiene practice is the most important part not only to reduce udder health problem but also to reduce a risk of bacterial contamination in milk. In addition, teat disinfection is usually recommended to be applied before and after milking to reduce the number of bacteria on teat skin and in milk [4-7]. Proper teat preparation can minimize bacterial contamination from the environment and other infected animals to the milking cows.

Several studies have shown that the use of an effective disinfectant is the most important part of effective pre-milking teat-cleaning regimes in addition to washing and drying teats [4, 8, 9]. Various disinfectant products such as iodophor solution, iodine based gel, sodium hypochlorite, dodecyl benzene sulfonic acid (DDBSA), chlorine, chlorhexidine, phenolic compounds and alcohol have been used as pre-milking teat dipping [4, 5, 9-11]. Pre-milking disinfection can reduce major pathogen intramammary infections from mastitis pathogens including *S. aureus*, *S. agalactiae* and coliforms [12, 13]. However, high concentrations of iodine in dips have raised the concern of potential residues in milk, especially with the advent of pre-milking application of dips.

*Psidium guajava* Linnaeus (guava) is a native plant of tropical America but now grows worldwide throughout tropical and subtropical countries. Guava has been being used as human food and traditional medicine [14-16] due to containing a variety of pharmacologically active ingredients such as flavonoids, guayavolic acid, guavanoic acid, guajadial, guajaverin and other active principles [16-18]. A previous study [18] showed that the methanol extracts of the guava leaves exhibited inhibitory activity against gram-positive bacteria although it was ineffective to the gram-negative bacteria. It was hypothesized that the guava leaf crude extract could be used as an alternative teat disinfectant to decrease the common bacterial species on teat skin and minimize bacterial contamination in raw milk. Currently, no scientific publication regarding the application of guava leaf extracts as teat disinfectant is available. The objective of the present study was to determine the antimicrobial activity of the aqueous methanolic extract of guava leaves against bacterial strains isolated from mastitis milk and to evaluate the efficacy of guava leaf extract as teat disinfectant on reduction of bacterial load on teat-end in lactating dairy cows.

## MATERIALS AND METHODS

### Plant and extraction

Fresh guava leaves were grown natively in Khon Kaen University campus, Khon Kaen, Thailand were collected for the purpose of this study. After washing, cleaned leaves were air-dried at 45-50 °C for 48 h and then pulverized using the electric blender. Phytochemical constituents of guava leaf were crude extracted using modified maceration as described below. Briefly, 2 kg of the ground guava leaf was soaked in 9 l of 50% aqueous methanol at room temperature for 3 days with occasional agitating.

After the maceration period, the liquid extract was obtained and clarified by gross filtration and centrifugation. Subsequently, methanolic extractant was evaporated out at 45-50 °C and subjected for freeze-drying to gain dry extract powder using a freeze dryer (Telstar® LyoAlfa 6-50, Terrassa, Spain). The dry extract powder was weighed and stored at-20 °C until used. Prior to utilizing, stock guava crude extract solution (256 000 µg/ml) was prepared by reconstituting the crude extract powder with 50% aqueous ethanol and then sterilized by filtering through 0.45 µm cellulose acetate membrane filter. Ethanol was purposefully used in place of methanol for preparation because of its lesser biotoxicity.

### *In vitro* antibacterial activity of guava extract

Three isolates of each mastitis-causing pathogen *i.e. E. coli*, *S. aureus* and *S. agalactiae*, isolated from mastitis milk samples, were used. The bacteria were propagated and kept in aliquots at-20 °C. Prior to antibacterial activity testing, frozen bacteria were thawed and grown in brain heart infusion (BHI) broth at 37 °C for 2-6 h. The growing bacteria were suspended with Mueller-Hinton Broth (MHB) to obtain desired density using spectro photometrical adjustment. The optical density (OD) of 0.1 at 625 nm corresponded to $10^8$ CFU/ml for *E. coli*, OD of 0.3 at 625 nm corresponded to $10^8$ CFU/ml for *S. aureus* [19], while OD of 0.1 at 550 nm equated with $10^6$ CFU/ml for *S. agalactiae* [20].

Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the extract were determined using broth micro-dilution methods [21, 22]. Briefly, triplicate of 2-fold serial dilution of the extract with MHB were performed at concentrations ranging from 64 000 to 125 µg/ml in 96-wells microplate. An equal volume of the bacterial suspension containing $10^6$ CFU/ml was added into each well. After overnight incubation at 37 °C, bacterial growth was examined by adding *p*-iodo nitro tetrazolium violet (INT) (Sigma-Aldrich®, Sigma-Aldrich Co., St. Louis, USA) to reveal viable bacteria. Before INT adding, all wells revealing no turbidly visible bacterial growth were subjected to sub-culture on Mueller-Hinton agar (MHA) plate to determine MBC exhibiting by no bacterial colony growth on the overnight subculture at 37 °C.

### Efficacy of pre-milking disinfectants on reduction of teat-end bacterial load

### Preparation of pre-milking disinfectants

Guava leaf crude extract at the concentration of 4000 µg/ml used as teat dipping solution was prepared by diluting the stock solution of the extract prepared aseptically as described above with sterile distilled water. In addition, two commercially available teat disinfectants including 0.5% iodine (Chokchai™, Chokchai Farm, Thailand) and combined solution of 1.5% w/v chlorhexidine gluconate and 15% w/v cetrimide (Septichlor® New Life Pharma Co., Ltd.) were used as comparing teat dipping disinfectants. The former preparation was ready to use while the latter was diluted to a concentration of 0.5% w/v chlorhexidine gluconate and 5% w/v cetrimide before teat dipping.

### Animals and experimental design

Sixteen lactating Holstein-Friesian cows which were free from clinical mastitis raised at a smallholder dairy farm were allocated into 4 groups using a 4x4 latin square experimental design as follows; group 1) control with no teat dipping; group 2) dipping with 0.5% iodine; group 3) dipping with 0.5% chlorhexidine combined with 5% cetrimide and; group 4) dipping with 4000 µg/ml guava leaf crude extract. Animal care was performed based on the guidance of the Ethic of Animal Experimentation of the National Research Council of Thailand and the procedure was ethically reviewed and approved by the Animal Ethics Committee of Khon Kaen University (Record No. AEKKU 99/2555; Reference No. 0514.1.12.2/105).

Before applying teat dipping, the cow's entire udder was sanitized using standard recommended procedure by the same milker for all cows. The udders were washed with chlorinated water, equivalent to 200 µg/ml available chlorine as stated by US Government regulations; 21 CFR 178.1010 followed by thoroughly wipe and dry using a dry and single used sterile towel.

Both hind quarters were used for comparison of the bacterial load on the teat-end as before and after applying teat dips. The preliminary trial indicated that there were no differences in the bacterial load on teat-end of both hind quarters of each cow. Teat-end swabs subjected for bacterial counts were taken and collected at one week apart before the afternoon milking.

### Teat-end swabs collection and analysis of bacterial count

Two teat-end swabs from each cow were collected individually from both hind quarters by rotating a moistened cotton swab, covering an area of 2 cm² outside the teat orifice. The first swab was taken immediately after udder sanitization whereas the second swab was performed on another quarter after applying with teat dip solution and removing the surplus teat dip solution. Teats were dipped in a cup of dipping solution and allowed contacting cow's teat skin for 30 s and subsequently dried using a dry and single use sterile towel to remove the surplus teat dip solution. The teat-end swabs were placed in a separated test tube containing 0.1% peptone water and stored in ice container until analyzed.

All teat-end swabs were analyzed for bacterial counts, *i.e.* total bacterial count (TBC), staphylococcal count (STA), streptococcal count (STR) and coliform count (COL). The swab tubes were vigorously shaken using vortex mixer for 30 s to extract the bacteria from the cotton swab and the cotton tips were then removed.

The retaining samples were 10-fold serial diluted and 1 ml of each were plated to bacterial count. TBC, STA, and STR were carried out using spread plate technique plating on Plate Count agar (Difco™), Baird-Parker agar (Difco™), and modified Edwards agar (Oxoid®), respectively [4] while COL was placed on Violet Red Bile agar (Difco™) with a cover layer using pour plate technique according to manufacturer direction referring to American Public Health Association. After incubation at 37 °C for 48 h, viable bacterial counts for each bacterium were manually enumerated and were expressed as CFU/ml.

### Statistical analysis

Teat-end bacterial loads (CFU/ml) were transformed to natural logarithm (ln) of CFU/ml and statistical differences in TBC, STA, STR and COL between, before and after applying teat dip were analyzed using paired t-test, Analysis of Variance (ANOVA). In addition, reduction rates (%) of bacterial loads after teat-dipping were also calculated. All statistical measures were performed using SPSS for Windows, version 17.0 (SPSS Inc.).

## RESULTS

### *In vitro* antibacterial activity of guava leaf extract

The aqueous methanol (50%) guava leaf extracts yielded the components at 17.25% of the dry leaf mass (345 g/2000 g). The guava leaf crude extracts exhibited both growths inhibitory and killing effects on the 3 selected mastitis-causing bacteria although MICs and MBCs varied considerably among the bacteria tested.

**Table 1: MICs and MBCs of guava leaf extract and tannic acid on mastitis-causing bacteria**

| Bacteria | Strain | Guava leaf extract (µg/ml) | | Tannic acid (µg/ml) | |
|---|---|---|---|---|---|
| | | MIC | MBC | MIC | MBC |
| *E. coli* | 1 | 1000 | 4000 | 1000 | 4000 |
| | 2 | 1000 | 4000 | 500 | 4000 |
| | 3 | 1000 | 8000 | 500 | 4000 |
| *S. aureus* | 1 | <125 | 250 | <125 | <125 |
| | 2 | <125 | 125 | <125 | <125 |
| | 3 | <125 | 250 | <125 | <125 |
| *S. agalactiae* | 1 | 125 | 500 | 125 | 500 |
| | 2 | 125 | 500 | 125 | 500 |
| | 3 | 125 | 500 | 125 | 500 |

The guava leaf extract possessed higher antibacterial potency against gram-positive cocci (MIC≤125; MBC=125 -250 µg/ml for *S. aureus*, MIC=125; MBC=500 µg/ml for *S. agalactiae*) than gram-negative bacilli, *i.e. E. coli* (MIC=1000; MBC 4000–8000 µg/ml). In addition, at the effective concentrations of guava leaf extract it was proved that incorporated ethanol (as solvent) had no effect on bacterial growth. Also, tannic acid was *in vitro* tested in parallel, showing the similar antibacterial effect on all bacteria as that of the guava leaf extract.

**Efficacy of pre-milking disinfectants on reduction of teat-end bacterial loads**

The effect of various possibly confounding factors was statistically tested and shown in table 2. Before testing, teat-end bacterial loads, i.e. TBC, STA, STR, and COL, of all cows between applying no dip (control) and dip (treatments) after udder sanitizing preparation were analyzed and had no significant difference. Teat-end score (TES), staging of the visible health status of teat-end and cow's cleanliness confounders was also tested and it revealed that there was no effect on teat-end bacterial loads.

Furthermore, all types of teat-end bacterial loads of quarter position between the 2 teats of the control (designed corresponding to before and after dipping in test groups) were also not different (table 2).

The efficacy on teat-end bacterial loads reduction of the 3 pre-milking dipping disinfectants was shown in table 3. Overall, there was the significant reduction (P<0.05) for TBC and STA after teat dipping, whereas no significant differences for STR and COL were detected. The reduction of teat-end bacterial loads, i.e. TBC, STA, STR and COL, after teat dipping were 84, 78, 32 and 18%, respectively.

**Table 2: Teat-end bacterial counts before teat dipping and associated confounders**

| Confounders | Levels | Teat-end bacterial count; mean (SEM) of ln CF | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | TBC[1] | p-value | STA[2] | p-value | STR[3] | p-value | COL[4] | p-value |
| Treatment | No dip | 6.11 (0.47) | 0.074 | 2.58(0.45) | 0.551 | 0.52 (0.25) | 0.992 | 0.13 (0.07) | 0.013 |
| | Dip | 6.97 (0.23) | | 2.28(0.25) | | 0.52 (0.18) | | 0.44 (0.10) | |
| Teat end score | Normal | 6.32 (0.35) | 0.349 | 2.55(0.36) | 0.479 | 0.79 (0.30) | 0.143 | 0.03 (0.02) | 0.056 |
| | Abnormal | 6.71 (0.25) | | 2.23(0.27) | | 0.31 (0.11) | | 0.28 (0.12) | |
| Cow cleanliness | Clean | 6.28 (0.24) | 0.267 | 2.17(0.37) | 0.432 | 0.29 (0.14) | 0.170 | 0.16 (0.13) | 0.873 |
| | Dirty | 6.74 (0.31) | | 2.52(0.27) | | 0.70 (0.23) | | 0.19 (0.08) | |
| Quarter position | Near | 6.51 (0.28) | 0.829 | 2.19(0.25) | 0.260 | 0.58 (0.20) | 0.548 | 0.10 (0.06) | 0.270 |
| | Far | 6.60 (0.26) | | 2.72(0.43) | | 0.39 (0.19) | | 0.32 (0.19) | |

[1]Total bacterial count; [2]Staphylococcal count; [3]Streptococcal count; and [4]Coliform count, *significant difference when P<0.05

The ln of CFU/ml of teat-end TBC, STA, STR and COL of before and after teat dipping with 4000 µg/ml of guava leaf extract, 0.5% iodine and combined solution of 0.5% w/v chlorhexidine and 5% w/v cetrimide were shown in table 3. The reduction of all bacterial counts after teat dipping were detected for all teat disinfectants, but statistical significances was revealed only for TBC and STA. Dipping with 4000 µg/ml of guava leaf extract as pre-milking teat disinfectant was able to significantly reduce the TBC and STA (P=0.0001 for TBC and P=0.0026 for STA) with bacterial load reductions at 79% and 73%, respectively. Similarly, the significant reductions of TBC and STA were also detected after teat dipping with the other 2 disinfectants. Interestingly, guava leaf extract showed the efficacy in reducing teat-end STR and COL at 39% and 27%, respectively, while the other 2 chemicals exhibited extremely high reduction rate up to 100% but all had no statistical difference.

**Table 3: Efficacy of different teat disinfectants on reduction of teat-end bacterial counts**

| Bacterial count | Treatment | N | Mean (SEM) | | % reduction | P value* |
|---|---|---|---|---|---|---|
| | | | Before (0 sec) | After (30 sec) | | |
| TBC[1] | Control | 16 | 6.11 (0.47) | 6.15 (0.47) | - | 0.9458 |
| | Guava Leaf Extract | 16 | 7.65 (0.29) | 6.09 (0.36) | 79 | 0.0001 |
| | Iodine | 16 | 6.79 (0.46) | 4.20 (0.59) | 92 | <0.0001 |
| | Chlorhexidine & cetrimide | 16 | 6.47 (0.37) | 2.65 (0.72) | 98 | <0.0001 |
| | All dipping | 48 | 6.15 (0.47) | 4.31 (0.39) | 84 | 0.0140 |
| STA[2] | Control | 16 | 2.58 (0.45) | 2.17 (0.41) | - | 0.3073 |
| | Guava Leaf Extract | 13 | 2.58 (0.51) | 1.28 (0.40) | 73 | 0.0026 |
| | Iodine | 15 | 2.38 (0.47) | 0.45 (0.13) | 85 | 0.0003 |
| | Chlorhexidine & cetrimide | 14 | 1.91 (0.33) | 0.32 (0.11) | 80 | 0.0005 |
| | All dipping | 42 | 2.17 (0.41) | 0.66 (0.15) | 78 | 0.0030 |
| STR[3] | Control | 16 | 0.52 (0.25) | 0.40 (0.21) | - | 0.5636 |
| | Guava Leaf Extract | 13 | 0.55 (0.38) | 0.05 (0.05) | 39 | 0.1540 |
| | Iodine | 15 | 0.61 (0.35) | 0.00 (0.00) | 100 | 0.1042 |
| | Chlorhexidine & cetrimide | 14 | 0.39 (0.19) | 0.00 (0.00) | 100 | 0.0630 |
| | All dipping | 42 | 0.40 (0.21) | 0.02 (0.02) | 32 | 0.0850 |
| COL[4] | Control | 16 | 0.13 (0.07) | 0.21 (0.19) | - | 0.6794 |
| | Guava Leaf Extract | 13 | 0.35 (0.19) | 0.03 (0.03) | 27 | 0.0976 |
| | Iodine | 15 | 0 (0.00) | 0 (0.00) | NA | NA |
| | Chlorhexidine & cetrimide | 14 | 0.26 (0.23) | 0.00 (0.00) | 100 | 0.2792 |
| | All dipping | 42 | 0.21(0.19) | 0.01 (0.01) | 18 | 0.2960 |

[1]Total bacterial count; [2]Staphylococcal count; [3]Streptococcal count; [4]Coliform count; NA=not assayed, *significant difference when P ≤ 0.05

## DISCUSSION

The present study found that the aqueous methanolic extract of guava leaf possessed *in vitro* antibacterial activity against clinically important mastitis-causing bacteria isolated from milk samples. These findings were in agreement with other studies reported previously that the guava leaf extract had a potent antibacterial activity against various bacteria including *S. aureus* [18], *Streptococcus* spp., and *E. coli* [15, 22]. Additionally, the antibacterial activities were more potent against gram-positive than gram-negative bacteria [18, 23]. The *in vitro* antibacterial activities against bacteria by the crude extract could be due to presence of some active phyto chemicals in guava leaves such as phenolic compounds, flavonoids, terpenoids, triterpene, tannins, essential oils, saponins, glycosides, carotenoid, and a number of other fixed substances [15, 16, 18, 24-28]. These phyto chemicals are known to exhibit useful biological activities and a variety of medicinally important effects, including antibacterial activities and they may have acted alone or in combination to affect the bacterial organisms [24, 26]. The phyto chemical constituents in the guava extract may differ attributable to the biochemical variations within species, geographical locations, extraction methods, and solvent used [18, 28]. However, in the present study, the active ingredients of the guava leaf extract were not analyzed because of available well-established identifications of phyto chemical.

In this study, we found that the numbers of teat-end bacteria counts were statistically decreased, particularly for TBC and STA, after teat dipping with all disinfectants. All the teat disinfectant were dipped after routine udder sanitization using chlorinated water wash and dry wiping with a single use towel. These practices were the standard procedure recommended for smallholder dairy farms worldwide, especially in most developing countries. However, the results also revealed that no statistical reductions of all bacterial counts were detected in the control, suggesting that the standard practices for udder sanitization could not completely remove all bacteria from teat skin. These results were concurrent with previous studies [4, 6], indicating that the use of additional disinfectants is essential in order to reduce the remaining bacteria on teat-end. Generally, teat dipping is a simple, effective, and economical procedure for reducing bacteria populations on the teat skin. Disinfection with an appropriate germicidal solution is the most effective tool for the mastitis control in order to prevent new intramammary infections in dairy cows. Efficacy of a pre-milking teat disinfectant had been estimated to reduce new intramammary infection comparing to without disinfection at approximately 50% [8, 12]. The bacteria on teat skin could not only be causative of the intramammary infection during milking but also be the possible source of contamination into raw milk.

The present study found that teat dip containing guava leaf extracts possessed potential efficacy in reduction of both TBC and STA, indicating that it had the beneficial effect on the decrease of bacterial loads on the teat skin. All the possible confounding factors were examined, but none of the significant confounder was detected, therefore, the reduction could be impliedly due to the potency of guava leaf extract. The result was in agreement with the previous study [6] reported that pre-milking teat dipping was advantageous for smallholder dairy farms in the reduction of numbers of bacteria at teat tip area of healthy milking cows. These findings indicated that use of guava leaf extracts as the additional teat dip following routine udder sanitizing procedure has an advantage on reduction of both mastitis-causing pathogen, *S. aureus* indicated by reducing in STA, and other bacteria causing poor milk hygiene indicated by reduce in TBC. However, the efficacy of guava leaf extract in reducing teat-end bacterial counts was relatively lower than that of using the other 2 chemical disinfectants, *i.e.* iodine and combined chlorhexidine-cetrimide. Previous studies have likewise shown that use of chemical disinfectants during teat preparation before milking can reduce the numbers of staphylococcal and streptococcal pathogens on teat skin [4, 11, 12, 29]. In this study, the concentration of guava leaf extract used for teat dipping was merely relying on the *in vitro* MBC, however, increasing its concentrations should be considered not only to reduce mastitis-causing bacteria but also to decrease bacterial contamination into raw milk. It is worth noting that pre-milking teat dipping was tested immediately after udder sanitization using chlorinated water as the routine practice recommended for dairy farmers. Therefore, all the bacterial counts presented here were only the number of the remaining viable bacteria on the teat skin of the cow.

Furthermore, there was no any irritation to teat-end and/or teat skin of all the cows during the present study, indicating that teat dips containing these disinfectants including guava leaf extract are useful and safe for cow's udder health [30]. Also, use of the guava leaf crude extracts as teat dip has advantages over the chemicals that are harmless for milker's health and nontoxic to the milk consumers. Recently, the reviews have shown that both leaf and fruit of guava are safe without any adverse effects in mice and other animal models as well as controlled human studies [17, 28]. Guava is the native plant grown worldwide, especially in the tropical countries and has been used traditionally as a medicinal plant throughout the world. This is the first report of the application of teat dip originated from the extract of guava leaves on the teat-end bacterial counts in the field experiment.

## CONCLUSION

The 50% aqueous methanolic guava leaf extracts could be used as an alternative of pre-milking teat disinfectant for reduction of teat-end bacterial loads, particularly for the total bacterial count and staphylococcal count. This may lead to lower chemical uses, which may promote more hygienic, safe milk for consumers, and decrease costs of mastitis risk control, especially in the developing and the third world countries where guava is native and easy to obtain. However, the efficacy of guava leaf extracts as the disinfectant against other bacteria either causing bovine mastitis or contributing to low milk quality needs to be further investigated.

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## CONFLICTS OF INTERESTS

The authors have no conflict of interests regarding the publication of the present article.

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