Short communication: An in vitro assessment of the antibacterial activity of plant-derived oils

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ABSTRACT

Nonantibiotic treatments for mastitis are needed in organic dairy herds. Plant-derived oils may be useful but efficacy and potential mechanisms of action of such oils in mastitis therapy have not been well documented. The objective of the current study was to evaluate the antibacterial activity of the plant-derived oil components of Phyto-Mast (Bovinity Health LLC, Narvon, PA), an herbal intramammary product, against 3 mastitis-causing pathogens: *Staphylococcus aureus*, *Staphylococcus chromogenes*, and *Streptococcus uberis*. Plant-derived oils evaluated were *Thymus vulgaris* (thyme), *Gaultheria procumbens* (wintergreen), *Glycyrrhiza uralensis* (Chinese licorice), *Angelica sinensis*, and *Angelica dahurica*. Broth dilution testing according to standard protocol was performed using ultrapasteurized whole milk instead of broth. Controls included milk only (negative control), milk + bacteria (positive control), and milk + bacteria + penicillin-streptomycin (antibiotic control, at 1 and 5% concentrations). Essential oil of thyme was tested by itself and not in combination with other oils because of its known antibacterial activity. The other plant-derived oils were tested alone and in combination for a total of 15 treatments, each replicated 3 times and tested at 0.5, 1, 2, and 4% to simulate concentrations potentially achievable in the milk within the pre-dry-off udder quarter. Thyme oil at concentrations $\geq 2\%$ completely inhibited bacterial growth in all replications. Other plant-derived oils tested alone or in various combinations were not consistently antibacterial and did not show typical dose-response effects. Only thyme essential oil had consistent antibacterial activity against the 3 mastitis-causing organisms tested in vitro. Further evaluation of physiological effects of thyme oil in various preparations on mammary tissue is recommended to determine potential suitability for mastitis therapy.

Key words: mastitis, plant-derived oils, alternative to antibiotics, organic

Short Communication

Organic dairy products represented almost 6% of the entire US dairy market in 2010 (Organic Trade Association, 2011). Because organic dairies are prohibited from using synthetic antibiotics in cattle (Electronic Code of Federal Regulations, 2013), a need exists for effective alternatives to synthetic antibiotics to treat mastitis. Dairies with a 6-mo average bulk tank SCC over 250,000 cells/mL had significantly higher prevalence of *Staphylococcus aureus*, *Streptococcus* spp., CNS, and *Streptococcus agalactiae* in organic herds compared with conventional herds (Pol and Ruegg, 2007a). In contrast, organic and conventional herds in North Carolina had similar prevalence of *Staph. aureus*, *Streptococcus* spp., CNS, and *Corynebacterium* spp. (Mullen et al., 2013).

Organic dairies have been reported to use a wide variety of nonantibiotic treatments for mastitis, from *Aloe vera* to vitamin supplements (Pol and Ruegg, 2007b). Peer-reviewed clinical efficacy studies evaluating those alternatives are scarce (Ruegg, 2009), but anecdotal reports exist of the efficacy of plant essential oils for improvement of milk quality in dairy cattle (Karreman, 2007). Plant-derived oils are the main ingredients in one intramammary product, Phyto-Mast (Bovinity Health LLC, Narvon, PA), labeled for improvement of milk quality and approved for use in organic production by the Ohio Ecological Food and Farm Association (Columbus). Phyto-Mast has some effectiveness in curing infections during the dry period when used as dry cow therapy (Mullen et al., 2014). Presence of the essential oil of *Thymus vulgaris* (thyme) in the formula may account for antibacterial action. Essential oil of thyme has strong antibacterial activity (Cowan, 1999; Kalembe and Kunicka, 2003) and contains a phenolic molecule called thymol that has strong activity against gram-negative bacteria (Helander et al., 1998) and common mastitis-causing pathogens (Ananda Baskaran et al., 2009).

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The study objective was to investigate antimicrobial activity of oils from each herbal component of Phyto-Mast in milk against common mastitis-causing pathogens cultured in vitro.

Bacterial Cultures

Single isolates of *Staph. aureus*, *Staphylococcus chromogenes*, and *Streptococcus uberis* were obtained from clinical mastitis cases on North Carolina dairy farms by the Milk Quality and Mastitis Laboratory at the College of Veterinary Medicine at North Carolina State University (Raleigh). Isolates were plated on Trypticase soy agar with 5% sheep blood (**TSA**; Becton, Dickinson and Co., Franklin Lakes, NJ). Purity of each culture was confirmed by Gram stain, morphology on mannitol salt agar (*Staph. aureus* and *Staph. chromogenes*), and morphology on blood agar (*Strep. uberis*). Bacteria were grown on TSA plates for 18 h in preparation for testing, and then kept refrigerated for the duration of the experiment. For each replication of each treatment tested, 1 colony was removed from the refrigerated plate and placed into 3 mL of Mueller-Hinton broth. Bacteria were grown to the midpoint of the log phase [6 h for *Staph. aureus* and *Staph. chromogenes* (Fujikawa and Morozumi, 2006), and 3 h for *Strep. uberis* (Almeida and Oliver, 1993)] before beginning the experiment.

Herbal Oils

Canola oil extractions of 4 different herbs and essential oil of thyme were obtained from Herbal Vitality Inc. (Sedona, AZ). The 4 herbs included *Angelica dahurica*, *Angelica sinensis*, *Gaultheria procumbens* (wintergreen), and *Glycyrrhiza uralensis* (Chinese licorice). Plant oils were kept refrigerated in brown glass bottles to prevent light degradation or volatilization of the oils.

Experimental Design

Herbal oils were tested at concentrations of 0.5, 1, 2, and 4% (vol/vol). Those concentrations would theoretically be achievable in the pre-dry-off udder quarter, provided that milk production was approximately 2.45 kg/quarter per day (5.4 lb/quarter per day). Because of the known antibacterial activity of thyme essential oil, it was tested by itself at concentrations of 1, 2, and 3% (vol/vol). The other 4 herbal oils were tested alone and in 15 combinations at each test concentration (0.5, 1, 2, and 4%), for a total of 60 combinations. Combination treatments were prepared by mixing equal (vol/vol) amounts of each of the oils to a total volume of 5 mL in a 10-mL vial: 6 mixes of 2 oils each at 2.5 mL, 4 mixes of 3 oils each at 1.67 mL, and 1 mix of 4 oils

each at 1.25 mL. Vials containing oil preparations were kept sealed and refrigerated when not in use. All oil-containing vials were vortexed for 15 s before beginning each trial.

Several controls were included in every testing of each treatment. Milk was cultured alone as a negative control to ensure that pasteurization was successful. An antibiotic control included milk + bacteria + penicillin-streptomycin at 1 and 5% concentrations, using the same antibiotic control as in a dry cow study (Mullen et al., 2014). The positive control was milk + bacteria, to document growth of bacteria in the milk. Phyto-Mast was also tested at 1, 2, 3, and 4% concentrations. Canola oil was tested at 1 and 70% concentrations to determine if it had an antibacterial effect without the herbal extract. For each bacterial species, 3 replicates of every treatment were tested at every concentration and control. Replicates were randomized by date, bacterium, and treatment to minimize experimental bias.

Antibacterial Activity Testing

Herbal oils were tested using a modified protocol for broth dilution testing (CLSI, 2008). Whole UHT pasteurized and homogenized organic milk was purchased from a grocery store and used instead of Mueller-Hinton broth as the growth medium. Vials were prepared for testing each control and treatment by first adding a calculated volume of milk, and then adding the volume of treatment solution to reach the percentage by volume (0.5, 1, 2, or 4%) tested. Following addition of treatments to milk, vials were vortexed for 90 s. Ten microliters of the inoculated Mueller-Hinton broth was added, and then the test vials were vortexed for another 15 s and placed in an incubator at 37°C for 24 h. Test vials contained a total of 1 mL of liquid.

Following incubation, vials were vortexed for 15 s. Serial dilution was used to determine bacterial counts, using a 0.1-mL aliquot from the vortexed vial and sterile 0.85% saline solution to create eight 10-fold dilutions. Dilutions were plated on eighths of a TSA plate and incubated for 24 h at 37°C. Colony-forming units of all dilutions were recorded. Colony counts from the lowest readable dilution were used in the analysis (Miles and Misra, 1938).

Analyses

Results are reported as growth of bacteria in the treatment sample relative to the growth of the milk + bacteria control, as a percentage of the growth of the control. Treatments were considered successful at reducing bacterial growth if all 3 replications resulted in reduction of bacterial growth compared with the

control. The ability of each treatment to completely eliminate bacterial growth was assessed using PROC MIXED in SAS (version 9.2; SAS Institute Inc., Cary, NC). The model was $Y = \mathbf{X}\beta + \varepsilon$, where Y represents the number of colony-forming units present after treatment, averaged across all 3 replications; β is a vector of fixed effects, including treatment and concentration with known design matrix $\mathbf{X}$; and ε is the error term. In other words, the average number of colony-forming units present in the sample following treatment was modeled using treatment and concentration as explanatory variables. Each treatment was evaluated to determine if the mean percentages of bacterial growth for each treatment were significantly different from 0. If this test did not yield a significant P -value ($P < 0.05$), the treatment was considered to have the ability to completely eliminate bacterial growth.

Control Observations

Both 1 and 5% concentrations of penicillin-streptomycin were bactericidal for all replications of the 3 bacteria tested (Table 1). Every test run of milk alone also resulted in no bacterial growth for all replications of all bacteria. The positive control, milk + bacteria, had bacterial growth for all replications of all bacteria. All replications with canola oil at both 1 and 70% had no effect on bacterial growth.

Efficacy Against *Staphylococcus aureus*

Numbers of colony-forming units per milliliter present in the milk + bacteria controls for *Staph. aureus* were 8×10^8 for replication 1, 2×10^8 for replication 2, and 4.7×10^8 for replication 3. Thyme oil killed all bacteria present at concentrations of 2 and 3% and was the only herbal treatment with the ability to completely eliminate bacterial growth (Table 1). No other oils or oil combinations consistently reduced bacterial growth

(Table 2), and variation in results for all concentrations were similar to those presented in Table 2.

Efficacy Against *Staphylococcus chromogenes*

Numbers of colony-forming units present in the milk + bacteria controls for *Staph. chromogenes* were 1.1×10^8 for replication 1, 1.5×10^8 for replication 2, and 1.3×10^8 for replication 3. Thyme oil at 2 and 3% was bactericidal, preventing *Staph. chromogenes* from multiplying and killing all bacteria that were inoculated into the thyme-containing vials. Thyme oil was the only herbal treatment with the ability to completely eliminate growth of *Staph. chromogenes* (Table 1). All herbal treatments reduced bacterial growth of *Staph. chromogenes* as compared with the control, but not significantly so (Table 2).

Efficacy Against *Streptococcus uberis*

Numbers of colony-forming units present in the milk + bacteria controls for *Strep. uberis* were 4×10^8 for replication 1, 4.7×10^8 for replication 2, and 4×10^8 for replication 3. Thyme oil at 2 and 3% was bactericidal versus *Strep. uberis* and the 1% concentration also reduced growth of *Strep. uberis* significantly (Table 1). Thyme oil was the only herbal treatment with the ability to completely eliminate growth of *Strep. uberis*.

Kalembe and Kunicka (2003) noted that herbal essential oils are promising alternatives to synthetic antibiotics because of availability, biodegradability, and probable low risk of side effects. The current study leads us to conclude that 4 of the plant-derived oils tested were not effective as antimicrobial agents for mastitis treatments, although they may have antiinflammatory or analgesic effects as documented in other species (Poppenga, 2002; Abe et al., 2003; Kai et al., 2003; Kang et al., 2008; Genovese et al., 2009). Only thyme oil had antibacterial activity against all patho-

Table 1. Plant-derived oils and antibiotics and their effect on growth of mastitis-causing organisms in milk in vitro

Mastitis-causing organism	Treatment	Growth of bacteria relative to control at various concentrations (vol/vol) of treatment ¹ (%)			
		5%	3%	2%	1%
<i>Staphylococcus aureus</i>	<i>Thymus vulgaris</i> ²		0 ± 0	0 ± 0	137 ± 108
	Penicillin-streptomycin	0 ± 0			0 ± 0
<i>Staphylococcus chromogenes</i>	<i>Thymus vulgaris</i>		0 ± 0	0 ± 0	35 ± 15
	Penicillin-streptomycin	0 ± 0			0 ± 0
<i>Streptococcus uberis</i>	<i>Thymus vulgaris</i>		0 ± 0	0 ± 0	2 ± 2
	Penicillin-streptomycin	0 ± 0			0 ± 0

¹Growth of bacteria are presented as growth relative to the milk + bacteria control, averaged over 3 replications and presented as mean ± SE. For example, at 1% concentration, *Thymus vulgaris* had 137% of the bacterial growth of the control, whereas no growth was observed at 2 and 3% concentrations of *Thymus vulgaris* essential oil.

²*Thymus vulgaris* = essential oil of thyme.

Table 2. Plant-derived oils and combinations of oils and their effect on growth of mastitis-causing organisms in milk in vitro

Treatment ¹	Growth of bacteria relative to control at 4% concentration (vol/vol) of treatment ¹ (%)		
	<i>Staphylococcus aureus</i>	<i>Staphylococcus chromogenes</i>	<i>Streptococcus uberis</i>
<i>Angelica dahurica</i>	88 ± 27	52 ± 31	102 ± 31
<i>Angelica sinensis</i>	139 ± 34	42 ± 24	143 ± 52
<i>Gaultheria procumbens</i>	108 ± 32	65 ± 43	159 ± 58
<i>Glycyrrhiza uralensis</i>	52 ± 31	51 ± 25	138 ± 67
<i>A. dahurica</i> , <i>A. sinensis</i>	108 ± 47	42 ± 24	138 ± 49
<i>A. dahurica</i> , <i>Ga. procumbens</i>	218 ± 86	45 ± 18	119 ± 55
<i>A. dahurica</i> , <i>Gl. uralensis</i>	130 ± 65	43 ± 16	139 ± 36
<i>A. sinensis</i> , <i>Ga. procumbens</i>	160 ± 70	47 ± 21	104 ± 46
<i>A. sinensis</i> , <i>Gl. uralensis</i>	149 ± 76	53 ± 31	139 ± 36
<i>Ga. procumbens</i> , <i>Gl. uralensis</i>	157 ± 61	34 ± 13	114 ± 38
<i>A. dahurica</i> , <i>A. sinensis</i> , <i>Ga. procumbens</i>	136 ± 50	50 ± 25	125 ± 42
<i>A. dahurica</i> , <i>A. sinensis</i> , <i>Gl. uralensis</i>	85 ± 22	47 ± 16	154 ± 29
<i>A. dahurica</i> , <i>Ga. procumbens</i> , <i>Gl. uralensis</i>	113 ± 44	55 ± 38	115 ± 44
<i>A. sinensis</i> , <i>Ga. procumbens</i> , <i>Gl. uralensis</i>	77 ± 16	59 ± 31	147 ± 69
<i>A. dahurica</i> , <i>A. sinensis</i> , <i>Ga. procumbens</i> , <i>Gl. uralensis</i>	131 ± 13	37 ± 7	133 ± 63
Phyto-Mast ³	237 ± 107	47 ± 16	114 ± 58

¹Growth of bacteria are presented as growth relative to the milk + bacteria control, averaged over 3 replications and presented as mean ± standard error. Treatments were also tested at 2, 1, and 0.5% concentration (vol/vol) and had large standard errors similar to those values reported here.

²*Gaultheria procumbens* = wintergreen; *Glycyrrhiza uralensis* = Chinese licorice.

³Phyto-Mast (Bovinity Health LLC, Narvon, PA) contains all of the listed plant oils and thyme essential oil (*Thymus vulgaris*) and is an intramammary herbal preparation used for improving milk quality.

gens tested, and was consistently effective at concentrations of 2% or greater.

The broth dilution method is often used to assess antibacterial efficacy of different essential oils (Hood et al., 2003) but milk was used instead of broth in the current experiment to simulate the environment of the udder. Lipids found in whole milk may have hydrophobic properties that could bind or decrease the antimicrobial properties of essential oils against mastitis-causing pathogens (Burt, 2004). This potential association between herbal oils and milk fat, as well as other lipophilic molecules in milk, is why whole milk was chosen as the in vitro model for evaluating antibacterial activity in the current study.

The testing method used resulted in large variations in bacterial growth within replications of the experiment and across the concentration levels. Although only the results at the 4% concentration of all plant oils were reported, the variation and inconsistency in results was also seen at the other tested concentrations. Replications were conducted on different days and a new carton of whole milk was used for each replication, both of which could have added variability to the experiment. The milk production level at dry-off that was assumed in this study is low (9.8 kg/d, or 21.6 lb/d) for conventional herds, but cows are often at or below such milk yields at dry-off in organic herds (K. A. E.

Mullen, personal observation). Cows with higher milk production would likely need higher doses to achieve desired concentrations in milk.

The proprietary composition of Phyto-Mast was not available to us for this research, and concentrations of ingredients tested may not be representative of the full formula of Phyto-Mast. Since the current study was completed, the formula of Phyto-Mast was altered to include more thyme essential oil (H. Karreman, Bovinity Health LLC, Narvon, PA; personal communication). Neither Phyto-Mast nor plant essential oils have been approved by the US Food and Drug Administration for intramammary use in cattle.

Essential oil of thyme at 2 and 3% had consistent antibacterial activity against the 3 mastitis-causing pathogens tested (*Staph. aureus*, *Staph. chromogenes*, and *Strep. uberis*), whereas none of the other oils or combinations of oils tested in this experiment showed evidence of antibacterial activity. Further investigations of in vivo effects of thyme oil alone or in various preparations on mastitic organisms would be of interest.

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REFERENCES

- Abe, M., F. Akbar, A. Hasebe, N. Horiike, and M. Onji. 2003. Glycyrrhizin enhances interleukin-10 production by liver dendritic cells in mice with hepatitis. *J. Gastroenterol.* 38:962–967.
- Almeida, R. A., and S. P. Oliver. 1993. Growth curve, capsule expression and characterization of the capsular material of selected strains of *Streptococcus uberis*. *Zentralbl. Veterinärmed. B* 40:697–706.
- Ananda Baskaran, S., G. W. Kazmer, L. Hinckley, S. M. Andrew, and K. Venkitanarayanan. 2009. Antibacterial effect of plant-derived antimicrobials on major mastitis pathogens in vitro. *J. Dairy Sci.* 92:1423–1429.
- Burt, S. 2004. Essential oils: Their antibacterial properties and potential applications in foods—A review. *Int. J. Food Microbiol.* 94:223–253.
- CLSI (Clinical and Laboratory Standards Institute). 2008. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; approved standard—Third Edition. Volume 28. Document M31-A3. CLSI, Wayne, PA.
- Cowan, M. M. 1999. Plant products as antimicrobial agents. *Clin. Microbiol. Rev.* 12:564–582.
- Electronic Code of Federal Regulations. 2013. Title 7: Agriculture Part 205—National Organic Program. Accessed May 4, 2013. http://www.ecfr.gov/cgi-bin/text-idx?c=ecfr&SID=f942f8fe8cf4407c29e50053befcc89a&tpl=/ecfrbrowse/Title07/7cfr205_main_02.tpl.
- Fujikawa, H., and S. Morozumi. 2006. Modeling *Staphylococcus aureus* growth and enterotoxin production in milk. *Food Microbiol.* 23:260–267.
- Genovese, T., M. Menegazzi, E. Mazzon, C. Crisafulli, R. Di Paola, M. Dal Bosco, Z. Zou, H. Suzuki, and S. Cuzzocrea. 2009. Glycyrrhizin reduces secondary inflammatory process after spinal cord compression injury in mice. *Shock* 31:367–375.
- Helander, I. M., H.-L. Alakomi, K. Latva-Kala, T. Mattila-Sandholm, I. Pol, E. J. Smid, L. G. M. Gorris, and A. von Wright. 1998. Characterization of the action of selected essential oil components on gram-negative bacteria. *J. Agric. Food Chem.* 46:3590–3595.
- Hood, J. R., J. M. Wilkinson, and H. M. Cavanaugh. 2003. Evaluation of common antibacterial screening methods utilized in essential oil research. *J. Essent. Oil Res.* 15:428–433.
- Kai, K., K. Komine, K. Asai, T. Kuroishi, Y. Komine, T. Kozutsumi, M. Itagaki, M. Ohta, Y. Endo, and K. Kumagai. 2003. Anti-inflammatory effects of intramammary infusions of glycyrrhizin in lactating cows with mastitis caused by coagulase-negative staphylococci. *Am. J. Vet. Res.* 64:1213–1220.
- Kalemba, D., and A. Kunicka. 2003. Antibacterial and antifungal properties of essential oils. *Curr. Med. Chem.* 10:813–829.
- Kang, O.-H., H.-S. Chae, Y.-C. Oh, J.-G. Choi, Y.-S. Lee, H.-J. Jang, J.-H. Kim, Y. C. Kim, D. H. Sohn, H. Park, and D.-Y. Kwon. 2008. Anti-nociceptive and anti-inflammatory effects of *Angelica dahuricae radix* through inhibition of the expression of inducible nitric oxide synthase and NO production. *Am. J. Chin. Med.* 36:913–928.
- Karreman, H. J. 2007. Treating Dairy Cows Naturally: Thoughts & Strategies. Acres USA, Austin, TX.
- Miles, A. A., and S. S. Misra. 1938. The estimation of the bactericidal power of blood. *J. Hyg. (Lond.)* 38:732–749.
- Mullen, K. A. E., L. G. Sparks, R. L. Lyman, S. P. Washburn, and K. L. Anderson. 2013. Comparisons of milk quality on North Carolina organic and conventional dairies. *J. Dairy Sci.* 96:6753–6762.
- Mullen, K. A. E., S. P. Washburn, and K. L. Anderson. 2014. Effect of two herbal intramammary products on milk quality and quantity compared with conventional and no dry cow therapy. *J. Dairy Sci.* 97:3509–3522.
- Organic Trade Association. 2011. U. S. organic industry overview. Accessed Sep. 1, 2013. <http://www.ota.com/pics/documents/2011OrganicIndustrySurvey.pdf>.
- Pol, M., and P. L. Ruegg. 2007a. Relationship between antimicrobial drug usage and antimicrobial susceptibility of gram-positive mastitis pathogens. *J. Dairy Sci.* 90:262–273.
- Pol, M., and P. L. Ruegg. 2007b. Treatment practices and quantification of antimicrobial drug usage in conventional and organic dairy farms in Wisconsin. *J. Dairy Sci.* 90:249–261.
- Poppenga, R. H. 2002. Herbal medicine: Potential for intoxication and interactions with conventional drugs. *Clin. Tech. Small Anim. Pract.* 17:6–18.
- Ruegg, P. L. 2009. Management of mastitis on organic and conventional dairy farms. *J. Anim. Sci.* 87:43–55.