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Research Article

Antimicrobial Activity of a Commercially Available Plant-Based Product

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Abstract

In the US, organic dairy cattle cannot be treated with antibiotics and maintain their organic status. There are no alternate products approved by US FDA to treat bovine mastitis on organic farms. Therefore, herbal treatments must be investigated for mastitis control. National organic dairy regulations allow the use of herbal products to prevent and manage bacterial mastitis. Phyto-mast is one botanical phytoceutical, composed of five plant extracts in a canola oil base, commonly used on organic dairy farms to manage mastitis and maintain organic status.

Unfortunately, there are no data regarding in vitro bacterial activity of Phyto-mast®; therefore, five different potential mastitis-causing bacteria were tested for their susceptibility to Phyto-mast® in vitro according to the Clinical Laboratory Standards Institute techniques. Based on our results Phyto-mast® appears bacteriostatic to *E. coli* and *S. agalactiae,* but is ineffective in decreasing the growth of *Staphylococcus* species and *S. bovis*.

ABBREVIATIONS

ATCC: American Type Culture Collection; CO: Canola Oil; CFU: Colony Forming Units; FDA: United States Food and Drug Administration; MANOVA: Multiway Analysis of Variance; PM: Phyto- Mast®; USDA: United States Department of Agriculture

INTRODUCTION

Bovine mastitis is inflammation of the mammary gland, usually associated with bacterial infection, that occurs in dairy cattle during lactation or drying off [1]. Due to the cost of withtreating mastitis, scrutiny by consumers, and increased demand for organic products, many producers (especially organic farms or those transitioning to organic status) use herbal products to treat and prevent mastitis [2,3]. However, with the use of herbal extracts, which are unapproved compounds by FDA, testing safety and effectiveness of these phytoceuticals is necessary to protect both human and animal health [4].

As organic milk demand has increased, FDA-approved herbal remedies and alternative treatments are necessary to provide acceptable treatment options for these animals [5]. Unfortunately at this time, there are no FDA-approved herbal remedies to treat organic dairy cattle. Therefore, only teat sealants and topical herbal remedies can be legally used for prevention of mammary infections under US federal law. Furthermore, antibiotic treatments disqualify treated animals from organic status in the United States, and therefore those animals are no longer eligible for organic milk production [2,6]. In order to maintain USDA organic status of the animal, there must be other options for organic dairy producers.

Currently, alternate and scientifically untested products are used to treat and prevent mastitis [2,3]. Several products, often in combination, are used for this treatment. While some documentation on individual herbal compounds is available, few studies have attempted to discern the effects of combinations of herbal products. One combination therapy that is currently being used on organic farms is Phyto-mast®, a trademarked phytoceuticals [3]. This product is compounded from several plant oil extracts including *Thymus vulgaris*, *Gaultheria procumbens*, *Glycyrrhizauralensis*, and *Angelica sinensis* in a canola oil vehicle (product label). It is a phytoceutical, meaning it is made entirely from a plant base and is currently used to maintain udder health and prevent mastitis [7]. Only a few studies have been completed on organic farms to examine the clinical outcome of Phyto-mast® use [8, 9] despite its popularity in the southeastern US.

Despite the recommended use of many herbal extracts as anti-mastitis agents, inadequate studies have been conducted to evaluate their antimicrobial activity against potential mastitis-causing bacteria. Although individual plant oil derived molecules, such as thymol, have been evaluated for their effectiveness as antimicrobials against mastitis-causing species [10-12], only one other *in vitro* study has examined the efficacy of these herbals in combination for antimicrobial activity in different bacterial species [13].

Therefore, we evaluated the *in vitro* antimicrobial activity of a combination of herbal extracts in canola oil (available as Phyto-mast®) at approximately a 9% v/v concentration in a controlled environment to simulate an intramammary infusion. A total of 9% of herbal product to milk concentration is likely the peak obtainable level in mammary tissue and is the maximum concentration that we could emulsify into solution. This study design was used to evaluate the ability of a combination of herbal extracts as an antibiotic substitute (PM) to inhibit the growth of five bacterial species. The following bacteria were chosen as they represent each of the following types of mastitis-causing pathogens: the opportunistic environmental pathogen, *Escherichia coli* and *Streptococcus bovis*, and contagious mastitis pathogens, *Staphylococcus aureus* and *Streptococcus agalactiae*. The fifth species, *Staphylococcus epidermidis,* is an example of a coagulase negative *Staphylococcus*, which are commonly found on the teat but less likely to cause clinical mastitis [14].

MATERIALS AND METHODS

Antimicrobial susceptibility assays

Five bacterial species were chosen to evaluate the antimicrobial activity of Phyto-mast® (PM), a botanical phytoceutical, commonly used on some organic dairy farms to treat mastitis and encourage udder health (3). The following American Type Culture Collection (ATCC) strains of bacteria were chosen: *Escherichia coli* (25922), *Staphlyccoccus aureus* (25923), *Streptococcus bovis* (33317), and *Streptococcus agalactiae* (13813), obtained from Fisher Scientific (Pittsburgh, PA) or Presque Isle Cultures (Erie, PA). *Staphylococcus epidermidis* was obtained from Carolina Biological (Burlington, NC). The bacteria were grown on Tryptic Soy agar at 37°C under aerobic conditions except for *Streptococcus sp.* which were grown in a candle jar using Tryptic Soy agar with 10% sheep's blood.

The Clinical Laboratory Standards Institute method for antimicrobial susceptibility for broth dilution was used with modifications to allow for the analysis of essential oils [15,16]. All trials were run in triplicate for a minimum of five trials per bacterium. Samples for each bacterium included growth in Canola Oil (CO) and treatment with PM. In brief, nutrient broth was used in all experiments as appropriate emulsification of PM and CO did not occur in Mueller Hinton Broth (as per CLSI recommendation). PM or CO was emulsified into nutrient broth using Tween 80 as follows: 98 µL of Tween 80 was added to 3.443 mL of nutrient broth and vortexed for 2 minutes; 360 µL of either PM or CO was added and the mixture was vortex for an additional 6 minutes. The emulsification was an opaque solution that prevented traditional visual inspection of broth dilution assays for determining minimum inhibitory concentration. This technique is similar to previously reported methods of testing essential oils [15].

One bacterial colony was inoculated into 10mL nutrient broth and grown for 24 hours. A subsample of each bacterial species was diluted to a 0.5 McFarland standard in 5 mL nutrient broth. Following emulsification, 100 µL of each bacterial strain (previously prepared to the McFarland standard) was inoculated into the test and control tubes and gently mixed. The final volume was 4 mL with 2.45 % Tween 80 and either 9 % PM or 9 % CO. These solutions were incubated for 24 hours at 37°C with shaking. Serial dilutions (10^{-3} – 10^{-8}) were performed and samples plated under aforementioned growth conditions and colony forming units (CFUs) were counted.

Statistical analysis

Five (or more) bacterial trials were run in triplicate to confirm antimicrobial susceptibility results for each bacterial species. Statistical tests were run using JMP® Statistical Discovery Software (version 10) available through SAS (Cary, NC).

Multivariate MANOVA analysis with repeated measures to account for sample replicates and the differences across 10^{-5} to 10^{-7} serial dilutions was performed at 24 hours after colony growth to compare CO and PM. For *S. epidermidis*, only 10^{-5} and 10^{-6} dilutions were included while *E. coli* had only 10^{-6} and 10^{-7} dilutions included. These modifications were due to the lack of countable plates at the excluded dilutions. MANOVA included blocking BY each bacterial species with factors of Trial #, Treatment (PM or CO) and Replicates to predict the dependent variable (Y): bacterial CFU/mL on plates 10^{-5} to 10^{-7}, except where noted above. The analysis was used to determine if there was a statistically significant difference in the CFUs/mL for bacterial grown with (PM) or without treatment (CO). Results were considered statistically significant at a p-value of 0.05.

RESULTS AND DISCUSSION

Repeated measures MANOVA demonstrated that PM appeared to be effective in inhibiting the growth of *E. coli* and *S. agalactiae* at a p-value <0.05 for each (Table 1, Figure 1) even when accounting for differences across replicates and trials. There was no difference between CO and PM for the negative control. A visual comparison of the data is included in Figure 1. Figure 1 shows CFU/mL counts for all bacterial species based on their 10^{-6} dilution sample replicates. Only *E. coli* and *S. agalatiae* were statistically significant, due to the large variability of growth of the different species of bacteria (Figure 1). CFU/mL across all trials and replicates for all 10^{-6} dilution plates in canola oil and Phyto-mast are included in Figure 1with error bars for ease of comparison. PM did not demonstrate a statistically significant

Table 1: Results of multivariate MANOVA comparing PM to CO.

Bacterial Species	p-value
E. coli	0.0065*
S. agalactiae	0.0315*
S. aureus	0.3152
S. bovis	0.3018
S. epidermidis	0.0847

*p-values indicates a significant difference between Phyto-mast®(PM) and canola oil (CO) control where Phyto-mast® has lower colony forming units than Canola Oil.

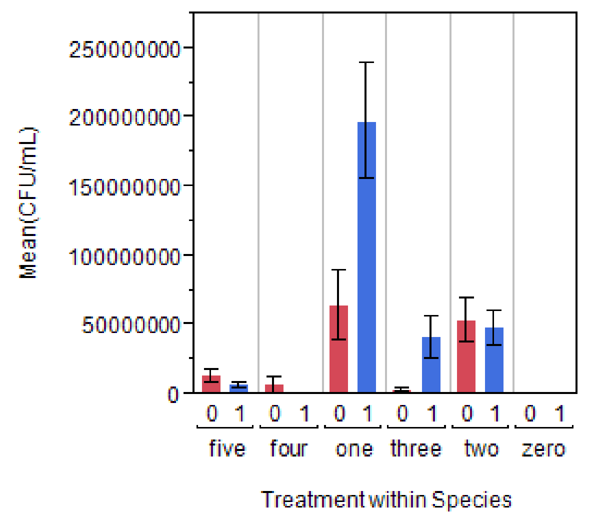

Figure 1 Overall growth of all bacterial species in CFU/ mL for Phyto-mast® (0) and Canola oil (1) from 10^{-6}dilutions. Error barre presents one standard error for the mean of all samples. From this graph, bacterial control samples one and three show much higher growth compared to Phyto-mast ®.Five is ***S.** epidermidis*; Four is *S. bovis*; Three is *S. agalactiae*; Two is *S. aureus*; One is *E. coli;* Zero is no bacterial exposure*.*

effect on the growth of *S. aureus, S. epidermidis, or S. bovis.* Table 1 shows the p-values for all bacterial comparisons, with *E.coli* and *S. agalactiae* having significant p-values of 0.0065 and 0.0315, respectively. Despite some significant differences across trials and replicates for *E. coli* but not *S. epidermidis;* the model was still able to demonstrate that PM was effective compared to the control.

PM may be an important addition to the treatment options at organic dairy farms because it appears to inhibit the growth of susceptible strains of *S. agalactiae* and *E. coli*. However, PM would not be a first choice phytoceutical on some of the more persistent mastitis cases because PM did not decrease the growth of *S. aureus* and some other bacteria tested in this study.

Based on the effect of PM in data above, we recommend that organic dairy farmers intending to use PM to treat mastitis in their cattle perform a bacterial culture at initiation of treatment to determine the causative agent(s). In many cases, using PM without a culture could result in a worsening infection and delayed optimal treatment which may result in decreased milk production. All of the species of bacteria we used were highly susceptible strains; therefore we emphasize that appropriate clinical judgment be used in evaluating mastitis prior to initiation of treatment with PM. Furthermore, if a withProducer knows that *S. aureus* is problem on his farm, based on our research, the use of PM would be inappropriate. Other essential oils or oil combinations may be more beneficial including cinnamon and oregano for these infections [11, 12].

CONCLUSION

Organic dairy products in the US may be more expensive in some markets than conventional products, which make improved treatment options essential for those farms. Combination herbal remedies such as PM may prove useful in the arsenal against mastitis infections in organic dairy cattle. PM appeared to be effective in inhibiting the growth of *E. coli* and *S. agalactiae,* but not against *S. aureus, S. epidermidis or S. bovis* in *in vitro* experiments*.* It is important that further testing be done to explore which causative agents of mastitis might be susceptible to PM or other combination phytoceuticals.

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