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Investigation of The Effectiveness of Some Plant Compounds and Essential Oils of *Corymbia Citriodora* Against Foodborne Pathogens

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ABSTRACT

The purpose of this study was to determine the antibacterial activity of plant derived compounds and essential oils of *Corymbia citriodora* against selected Gram negative and Gram positive foodborne pathogens in broth dilution assay. The combination of compounds (cineole, terpinen-4-ol and α-terpineol; CTαT) were further tested at three different concentrations (0.2, 0.4 and 0.8%) for the killing effect against *E. coli* O157:H7 and *L. monocytogenes* in milk including whole fat and skim fat. CTαT showed antimicrobial activity against all bacteria tested at minimum inhibition concentrations (MICs) from 0.125% to 1% in broth dilution assay. Linalool was also found to be antimicrobial at MICs between 0.25% and 2%, but not for *Enterococcus casseliflavus*. Further study carried out in milk showed that CTαT at concentrations of 0.4% and 0.8% significantly reduced the population of *E. coli* O157:H7 under detection limit in skim milk, whereas it was only effective at 0.8% in whole fat milk. CTαT, on the other hand, shown to be less active towards *L. monocytogenes* as only significant effect was observed at 0.8% in skim milk. Taken together results of the present study indicate that plant derived compounds could be valuable alternatives to inactivate foodborne pathogens in milk.

Introduction

Freshly drawn milk is expected to be contaminated with many microorganisms which can be derived from the udder of the mammal, environment and even later on from the processing. Some of the microorganisms in milk present a risk to human health, although some of them might even be useful in terms of dairy technology (Demarigny et al., 1996; Langer et al., 2012). In addition, the existence of bacteria in milk is of great importance not only from the standpoint of pathogenic organisms but also for spoilage organism (Huck et al., 2008). The nutrient content of the milk and its pH value make it an excellent source of growth for most microorganisms. The bacteria of concern for human health are the following *Escherichia coli*, *Campylobacter* spp., *Salmonella* spp., *Listeria monocytogenes*, *Staphylococcus aureus* and *Shigella* spp. (Langer et al., 2012). The other pathogenic bacteria including *Bacillus cereus*, *Brucella* spp. *Coxiella burnetii* and *Yersinia* spp. are also detected in raw milk (Claeys et al., 2013). Of these bacteria, *L. monocytogenes* and *E. coli* O157/H7 so far have received the most attention because of their association with severe clinical symptoms from diarrhea and extra-intestinal diseases to even death (Kirk et al., 2015). In order to eliminate pathogenic microorganisms, heat treatment is most common and widely applied technique. Even though the number of reported outbreaks that involved milk as the known vehicle for transmission has been dramatically decreased due to the introduction of pasteurization practices, milk still continues to be a significant source for the bacteria of concern for human health (FDA, 2011, Schmid et al., 2007). According to the review done by Langer et al., (2012), there were 122 reported outbreaks associated with the consumption of contaminated dairy products between 1993 and 2006, resulting in 202 hospitalized patients and two deaths in the USA. It is also well known fact that the exact number of illnesses from consumption of contaminated milk is hard to estimate accurately due to unrecognized or sporadic cases.

It strongly appears that safety of milk have yet to be ensured in order to prevent food safety hazards. Therefore, it is prudent to find the sustainable preservative treatments in order to reduce or to eliminate pathogenic microorganisms in dairy products. The application of non-thermal strategies such as radiations, however, is not been utilized for the treatment of milk and milk products, even though there has been great interest in those as an emerging disinfectant (Farkas, 2006). In addition, preservative agents like hydrogen peroxide are not allowed to be utilized, as it has been proven to be not entirely safe for human consumption (Humberston and Krenzelok, 1990). There has been a growing demand to consume minimally and naturally processed foods

including dairy products among people. One of the most important and popular natural antimicrobial source is extracts and compounds of plants which have been known for almost a hundred years to possess natural biological (Kubeczka, 2016). Essential oils (EOs) are natural volatile compounds extracted through steam distillation from the non-woody part of the plants and EOs make up a complicated mixture in which there are mainly terpenoids, flavonoids, and phenols (Franz and Novak, 2016). For the last two decades, there, thus, have been great attentions being paid to identify biologically active plant derived compounds and many of these studies reported a number of biological activities such as antimicrobial, antiviral, insecticidal, antifungal and antiparasitic (Buchbauer and Bohusch, 2016). Moreover, anti-inflammatory, antioxidant, antiulcerogenic and wound healing activities of the plants and plant derived compounds have also been shown (Lassak and McCarthy, 2001). With regard to increasing interest, the use of EOs in the food industry has been extensively evaluated due to the high activity against pathogenic contaminants *in vitro* and *in vivo* experiments. So that, the objectives of this study were to determine the antimicrobial activity of EOs from *Corymbia citriodora* (formerly called as *Eucalyptus citriodora*) and compounds including eugenol, linalool and blend of compounds (cineole, terpinen-4-ol and α-terpineol named as CTαT for this study) against Gram negative and Gram positive foodborne pathogens. Survival of *E. coli* O157:H7 and *L. monocytogenes* in milk containing a combination of CTαT and stored at 4°C was also evaluated.

Material and Methods

Bacterial Strains and Test Agents

The following test organisms were used; *Salmonella* Typhimurium ATCC 14028, *S. aureus* ATCC 25923, *L. monocytogenes* N7144, *Enterococcus casseliflavus* ATCC 700327, *E. coli* ATCC 25922, *E. coli* O145, O26, O111, O103, O104 and O157:H7 (ATCC 43895). EOs obtained from *C. citriodora*, linalool, eugenol and CTαT (mixture of cineole, terpinen-4-ol and α-terpineol) used in this study were all provided from Alan Twomey (BioAust, Australia).

MIC Determination of Test Agents

To determine the *in vitro* antibacterial activity, minimal inhibitory concentration (MIC) was determined using broth microdilution method in liquid culture (Wiegand et al., 2008). Briefly, stock cultures of the organism at -80°C were inoculated on Tryptone Soya Agar (TSA) and incubated for 24 h at 37°C. Subsequently, colonies were cultured in Tryptone Soya Broth (TSB) at 37°C for 18–24 h. The bacterial inoculum was prepared from an overnight culture. Stock solutions of each test agents were serially diluted in ethanol/ethoxylated castar oil (1/1 v/v). Each bacterial suspension (190 µL) and diluted test agents (10 µl) were then mixed in each well of 96-well plate. Bacterial cell density (about 1-2 x 10^6 cfu/ml) was adjusted using Mcfarland 0.5 turbidity standards and the final concentrations of agents ranged from 2 to 0.001 %, v/v in each well of the plate. Solvent served as growth control and its concentration used in the assay was 2%. The last wells of plate treated with compounds without microorganism were used as a sterility control. The microplates were incubated at 37ºC for 24 h under aerobic condition. Following incubation, MICs was determined using iodonitrotetrazolium chloride by exactly as described previously (Kurekci et al., 2013) and experiment was carried out three times for reproducibility.

Inoculation and Analysis of Milk

Ultra-high-temperature (UHT) sterilized whole (3% fat) and skim milk (no fat) were purchased from a supermarket and the sterility of milk samples were assessed by surface plating (0.1 ml) of milk onto TSA, followed by incubation at 37°C for 48 h. The milk samples were spiked with inoculum of either *E. coli* O157:H7 or *L. monocytogenes* and 10 ml of spiked milk samples were subsequently dispensed into each tube. In order to determine the initial counts of *E. coli* O157:H7 or *L. monocytogenes* in milk serially diluted spiked milk samples (0.1 ml) were plated onto TSA. Plates were incubated at 37°C for 24 h and then colonies were counted. CTαT was added into each tube at three different concentrations (0.2, 0.4 and 0.8%). Sterile water and solvent were also added into tubes for negative and positive control, respectively. For each treatment including controls five replicate tubes were used. The samples were mixed by vortex and refrigerated at 4°C and surviving bacterial populations were determined after 24 h by serial dilutions prepared and then plated on TSA. For each dilution, the plates were incubated at 37°C for 24 h and then colonies grown on the plates were counted.

Statistics

Microbial counts were converted into $\log_{10}$ cfu/mL and the data were presented as means with standard deviations of five replicate. Differences in the number of viable bacteria between control and treatments were determined by using one-way ANOVA (SPSS 21) with Tukey's multiple comparisons test. Values with $P<0.001$ were considered significantly different.

Results

Results obtained in the current study revealed that all agents displayed antimicrobial activity at variable concentrations in broth dilution assay (Table 1). Among the test agents evaluated for their antimicrobial activity, CTαT were found to be the strongest with MICs ranging from 0.125 % to 1% for all tested organisms. Linalool also showed activity with the MICs ranging from 0.25% to 2% towards all organisms except *E. casseliflavus*. Eugenol showed antimicrobial activity against *L. monocytogenes*, *S. aureus* and some *E. coli* strains at concentration of 2%, but did not display activity against *S.* Typhimurium and *E. casseliflavus* at concentration of 2%, which was the highest concentration used in the

current study. The poorest overall antimicrobial activity was observed with the EO obtained from *C. citriodora* which only displayed activity towards *L. monocytogenes*, *E. casseliflavus* and *E. coli* ATCC and *E. coli* 0157/H7, while did not show activity against *S.* Typhimurium, *S. aureus* and other *E. coli* strains tested.

Table 2 shows the survival of *E. coli* O157:H7 and *L. monocytogenes* at 4°C in skim and whole milk treated with different concentrations of CTαT. The sterility of UHT milk samples were confirmed before spiking. After spiking, the initial population of *E. coli* in whole and skim milk were 6.82 ± 0.06 and 6.56 ± 0.07 $\log_{10}$ cfu/mL, respectively and the number of *L. monocytogenes* were 6.70 ± 0.04, 6.76 ± 0.02 $\log_{10}$ cfu/mL in whole and skim milk respectively. As shown in Table 2, the addition of CTαT at concentration 0.4% and 0.8% significantly decreased the *E. coli* O157:H7 population to the under detection limit compared with negative and positive controls (P<0.001). However, the addition of CTαT at 0.8% was sufficient to reduce *L. monocytogenes* below the detection limit in skim milk (P<0.001). When whole milk samples were treated with CTαT at 0.8% and held at 4°C for 24 h, the *E. coli* O157:H7 inactivation was more than 2.5 log cfu/mL when compared to controls (P<0.001). The number of *L. monocytogenes* in whole milk samples was, however, not affected by CTαT treatment.

Discussion

The current study investigated the antimicrobial properties of EOs from *C. citriodora*, as well as some plant compounds, against Gram negative and Gram positive pathogenic bacteria including those very important for food industry. All test agents exhibited a variable degree of antimicrobial activity towards major foodborne pathogenic bacteria tested with different MIC values. Linalool had a MIC value of 0.25-0.5%, 0.5%, 0.5% or 2% against *E. coli* strains, *S.* Typhimurium, *S. aureus* and *L. monocytogenes*, respectively. These results were found to be slightly higher than those of Carson and Riley (1995). These researchers found 0.25% and 0.06% MIC values for linalool against *S. aureus* or *E. coli*, respectively. In addition, it was observed that eugenol had antimicrobial activity against *S. aureus*, *L. monocytogenes* and some *E. coli* serovars at concentration of 2%, which was in accord with earlier studies on the *in vitro* effects of eugenol against *Helicobacter pylori* (Ali et al., 2005). This observation is also consistent with similar findings of Sanla-Ead et al., (2012) who reported MICs values for eugenol against several foodborne pathogens at a concentration between 12.5 and 50 µl/mL. Nevertheless, our results for eugenol are contrast to results obtained by other authors who found much lower MIC values for several foodborne pathogens including *Salmonella* Typhi (0.0125%), *E. coli* (0.05%) and *S. aureus* (0.1%) (Devi et al., 2010; Walsh et al., 2003).

There are many studies undertaken to explore the biological activities, content and composition of extracts and EOs obtained from *C. citriodora* (Cimanga et al., 2002; Lis-Balchin and Deans, 1997). In the current study, *C. citriodora* displayed weak antimicrobial activities against particularly Gram positive bacteria (*L. monocytogenes* and *E. caseliflour*) and as well as some *E. coli* serovars. Similarly, Luqman et al., (2008) reported that EOs of *C. citriodora* had more stronger activity towards Gram positive bacteria than Gram negative bacteria at MIC values of 10 or >10 mg/mL. In addition, some studies have revealed that cineole, α-terpinene and terpinen-4-ol had remarkable antimicrobial activity against foodborne pathogens (Kurekci et al., 2013; Thomsen et al., 2013). The results of many studies also indicated that the EOs are more effective than the major compounds derived from them (Cox et al., 2001; Loughlin et al., 2007). The development of resistance to complex mixture of pure compounds appears to be unlikely due to a complex activity of these compounds (Hammer et al., 2012). Therefore, we evaluated the antimicrobial efficacy of CTαT and found that CTαT had a remarkable activity against a variety of foodborne pathogens tested.

Table 1 Minimal inhibitory concentration (%) of *C. citriodora* EO and compounds using broth microdilution assay*

Test organisms	Test Compounds (MIC %)			
	C. citriodora	Eugenol	Linalool	CTαT
E. coli O157/H7	2	NA	0.5	0.25
E. coli O145	NA[a]	2	0.5	0.125
E. coli O104	NA	NA	0.25	0.125
E. coli O103	NA	NA	0.5	0.125
E. coli 026	NA	2	0.5	0.125
E. coli O111	NA	2	0.5	0.125
E. coli	2	2	0.25	0.125
S. typhimurium	NA	NA	0.5	0.25
S. aureus	NA	2	0.5	0.5
L. monocytogenes	1	2	2	1
E. casseliflavus	2	NA	NA	1

[a]NA; No activity at highest concentration tested (2%)

Table 2 Survival of *E. coli* and *L. monocytogenes* (log_{10} cfu/ml) in milk containing CTαT [*]

Treatments	*E. coli*		*L. monocytogenes*	
	Skim Milk	Whole Milk	Skim Milk	Whole Milk
P-Control	6.77 ± 0.03^{a}	6.79 ± 0.14^{a}	6.66 ± 0.07^{a}	6.72 ± 0.03
N-Control	6.47 ± 0.49^{a}	6.65 ± 0.16^{a}	6.69 ± 0.05^{a}	6.66 ± 0.08
CTαT (0.8%)	ND^{b}	3.95 ± 0.13^{b}	ND^{b}	6.92 ± 0.27
CTαT (0.4%)	ND^{b}	6.31 ± 0.12^{a}	6.44 ± 0.14^{a}	6.77 ± 0.06
CTαT (0.2%)	6.30 ± 0.22^{a}	$6.75 \pm 0{,}10^{a}$	6.74 ± 0.06^{a}	6.78 ± 0.08

[*]Values are means of five replicates. ND: No colonies detected on the agar plates., [a, b, c] Means with different superscripts on the column differ significantly (Tukey, P<0.001).

Apart from the *in vitro* antimicrobial activities, we also investigated the application of CTαT to reduce the *E. coli* O157:H7 and *L. monocytogenes* population in whole and skim milk. CTαT was selected for this study as effectiveness of these compounds has already been proved against a wide variety of microorganisms *in vitro*. Our work showed that CTαT at concentration of 0.8% and 0.4% caused a significant reduction in the cell counts of *E. coli* O157:H7 whereas only 0.8% resulted in the same effect towards *L. monocytogenes* in skim milk. This result is in agreement with our results obtained in broth dilution assay. Similarly, Dikici et al. (2013) and Shah et al. (2013) have reported that *L. monocytogenes* was much more resistant to eugenol when compared to *E. coli* O157:H7 in çiğ köfte and milk. The antimicrobial concentration of eugenol in this study was much lower than those previously reported (Dikici et al., 2013; Shah et al. 2013; Yoon et al. 2011). Shah et al. (2013) reported that *L. monocytogenes* and *E. coli* O157:H7 were both completely inhibited in all milk samples (whole, reduced fat and skim) when eugenol concentration was increased to 6.5 g/L. However they found that eugenol at the level of 1.0, 1.5 and 2.0 g/L failed to inactivate both bacteria.

It is noteworthy to report herein that the fat content of milk appears to have a significant effect on the antimicrobial activity of CTαT against tested bacteria. Similarly, Shah et al. (2013) and Yoon et al. (2011) informed that the inhibitory activity of plant derived compounds was decreased by the fat content of milk. It was suggested that fat globules can attached to the surface of bacterial cells resulting in the formation of barrier disabling the penetration of EO/compounds through the cell wall (Farbood et al., 1976). As noted earlier, the possible binding of milk fat with EO or compounds undoubtedly plays a role in limiting the effectiveness of these agents (Juven et al., 1994).

Conclusion

Milk and dairy products unfortunately continue to be one of the major sources of the bacteria of concern for human health even though the heat-treatment is often used to eliminate pathogen microorganisms present in raw milk. With increasing demands for organic foods, there has been also growing interest to explore for natural antimicrobial additives which are not harmful for human health and as well not altering the organoleptic properties of foods. According to the data obtained from the current study, significant reduction in the population of *E. coli* O157/H7 and *L. monocytogenes* was observed in skim milk stored at 4°C for 24 h. Taken together, the data obtained from studies in both *in vitro* and model food system suggest that freshly drawn milk might be treated with natural plant derived compounds prior to the introduction of heat treatment.

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