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In vitro Antimicrobial Activity of Nanoencapsulated Bromelain against Bacteria Isolated from Milk of Dairy Goats with Sub-clinical Mastitis in Thika East Sub-county, Kenya

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Authors' contributions

All authors conceived and designed the study. Authors MP and KJ wrote the protocols and executed the experiment. Author MN performed the extraction and nanoencapsulation. All authors analyzed data, wrote the manuscript and approved this manuscript.

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ABSTRACT

Mastitis in dairy goats is managed by a variety of antibiotics. Due to the emergence of antibiotic resistance, there is need for development of new antimicrobial agents. In the current study, the *in vitro* activity of nanoencapsulated bromelain, using bromelain extracted from the pineapple fruit, *Annanus comosus* was investigated against bacteria isolated from milk of dairy goats with sub-clinical mastitis. Nanoencapsulation of bromelain was done using the ionic gelation method of chitosan nanoparticles with sodium trypolyphosphate as the cross linking agent. In this study, the agar well diffusion method was used to test for antimicrobial activity while the broth microdilution

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method was used to test for the Minimum Inhibitory Concentration (MIC). The isolates used were *Staphylococcus aureus*, Coagulase Negative *Staphylococci, Serratia* spp., *Klebsiella* spp., *Enterobacter* spp., *Citrobacter* spp. and *Escherichia coli* isolated from milk of dairy goats with sub-clinical mastitis in Thika East Sub-county, Kenya. The agar well diffusion method showed that bromelain and nanoencapsulated bromelain had antimicrobial activity. All of the tested bacteria were sensitive to extracted bromelain at 5 mg/ml and less. The tested bacteria were less sensitive to commercial bromelain (57.1%) at 5 mg/ml and less. The MIC of nanoencapsulated bromelain against *Enterobacter* spp., *Citrobacter* spp., *Serratia* spp. and Coagulase Negative *Staphylococci* was 25 µg/ml, while that of *Escherichia coli* was 50 µg/ml. The MIC of nanoencapsulated bromelain against *Klebsiella* spp. and *Staphylococcus aureus* was 200 µg/ml. The low MICs recorded in this study shows that nanoencapsulated bromelain has high antimicrobial potential which warrants further *in vivo* studies in dairy goats to determine its efficacy against sub-clinical mastitis.

Keywords: In vitro; efficacy; bromelain; mastitis; Staphylococci; E. coli.

1. INTRODUCTION

Mastitis in dairy goats is an economically important disease associated with inflammation of the mammary gland and is characterized by changes in the physical characteristics of the udder or milk [1]. Mastitis leads to milk yield reduction, lowers the hygienic value of milk and affects the sensory quality and fatty acid profile of the by-products like cheese [2,3]. The prevalence of sub-clinical dairy goat mastitis in Kenya ranged between 28.7% and 61% [4,5,6,7]. In Tanzania [8], a prevalence of 76.7% was recorded, in Pakistan [9], a prevalence of 38% was noted while in Bulgaria [10], a prevalence of 44.2% was found. These studies show that mastitis is a disease of economic concern in Kenya and worldwide.

The treatment of mastitis is usually by the usage of antibiotics. However, the use of antibiotics has limitations which include the emergence of antibiotic resistance. In a recent study in dairy goats in Kenya, there was widespread occurrence of antibiotic resistance in bacteria isolated from goats having sub-clinical mastitis [7]. Further, the extensive use of antibiotics has implications in human health as the antibiotic resistance strains may enter the food chain through such means as consumption of raw milk and its by-products. Thus, there is need for the development of antibiotic agents and for goats, ideally a drug which may be taken as in-feed for the treatment of mastitis.

Medicinal plants are believed to be important source of new chemical substances with potential therapeutic effects and recently, the use of medicinal products containing enzymes has increased due to their broad therapeutic potential. These natural products are usually non toxic, devoid of side effects, easily available and affordable [11]. The use of secondary metabolites such as enzymes as therapeutic agents is limited by their biochemical properties. Nanoencapsulation of these enzymes is important to protect these enzymes from degradation before they reach the site of action. Nanoencapsulation of drugs involves forming drug-loaded particles with diameters ranging from 1 to 1000 nm [12].

Bromelain is a general name for a family of sulfhydryl-containing proteolytic enzymes obtained from *Ananus comosus* [13]. The primary component of bromelain is a sulfhydryl proteolytic fraction. It also contains peroxides, acid phosphatase, several protease inhibitors and organically bound calcium [13]. Bromelain has antimicrobial properties and has been shown to have bactericidal properties. Bromelain is a cysteine protease which cleaves gylcyl, alanyl and leucyl bonds [13].

Healthy cows and those with intramammary infections when fed bromelain have been shown to have low levels of milk somatic cell counts, which are used as an indicator of lack of sub-clinical mastitis [14]. Further, in dairy goats fed with bromelain, there was no case of sub-clinical mastitis [15]. However, in the latter study, the authors did not ascertain whether the lack of mastitis in the goats was due to the antimicrobial activity of bromelain. Although the *in vitro* antimicrobial activity of bromelain has been reported [16,17,18], the *in vivo* use can be limited by the inactivation by the low pH found in the stomach [19]. There is therefore need for encapsulating the bromelain with nanoparticles such as those from chitosan. Chitosan is one of the most abundant polysaccharides in nature and has shown some antimicrobial properties.

Further, chitosan is non-toxic, biodegradable, biocompatible and has low allerginicity [20]. Chitosan nanoparticles are engineered from chitosan by cross-linking using sodium tripolyphosphate (TPP) [21]. In this study, the antimicrobialactivity of nanoencapsulated bromelain was evaluated against bacteria isolated from milk of dairy goats with sub-clinical mastitis.

2. MATERIALS AND METHODS

2.1 Isolation of Bacteria

The bacteria were isolated from milk of dairy goats with sub-clinical mastitis from Thika East Sub-County, Kenya. One isolate was randomly selected from the seven major groups of isolated bacteria namely *Staphylococcus aureus*, Coagulase Negative *Staphylococci, Serratia* spp., *Klebsiella* spp., *Enterobacter* spp., *Citrobacter* spp. and *Escherichia coli.* The isolation and characterization of these bacteria was described in a previous study [7].

2.2 Bromelain Extraction and Nanoencapsulation

Fresh pineapple fruits from a farm near Thika Town in Kenya were used. Bromelain was extracted from peels of the fresh pineapple [22]. A solution of 4 mg/ml bromelain was prepared. The nanoparticles were prepared by ionic gelation method [23,24]. Low molecular weight chitosan (Sigma, U.S.A) was dissolved in 1.5%v/v acetic acid solution to give a 1% chitosan solution. Equal amounts of the bromelain and 1% w/v sodium tripolyphosphate (sttp) were then mixed. This was added drop-wise to the chitosan in a ratio of 3:5. The nanoparticles solution was centrifuged and the resultant nanoparticles were washed in distilled water and air dried at room temperature [25]. The resultant nanoparticle consisted of bromelain and chitosan in the ratio 3:5 respectively. To make a solution of 100 mg/ml, 2.5 g of the nanoencapsulated bromelain was dissolved in 25 ml of 1,5% V/V acetic acid.

2.3 *In vitro* Antimicrobial Activity of Nanoencapsulated Bromelain

Antibiotic sensitivity was tested using the agar well diffusion method [26]. For the agar well diffusion method, six different concentrations, in triplicate were tested. The test compounds were initially diluted to a set concentration and serial dilution done until the last concentration: [commercial bromelain (B4882-Sigma, U.S.A)(5000µg/ml-156.25µg/ml), extracted bromelain (5000µg/ml-156.25µg/ml), encapsulated bromelain (200µg/ml -6.25µg/ml)].

Mueller Hinton Agar plates were inoculated by spreading 100 µl of the bacterial inoculum. Four holes, each 8mm in diameter, were aseptically made in the media using a micropipette tip. Fifty (50) µl of the six different concentrations the test compound was introduced into each of the wells. The plates were left to stand for two hours to allow the extracts to sink into the media before incubation at 35ºC for 16 hours. The zones of inhibition were then measured. The zones of inhibition were compared to the Streptomycin values [27] to determine susceptibility or resistance. An isolate was graded as sensitive if it had a zone of inhibition of 15 or higher and not sensitive if it had a zone of inhibition of 14 and below [27].

2.4 Minimum Inhibitory Concentration Determination

The broth microdilution method was used to determine the Minimum Inhibitory Concentration (MIC) [26]. 50 µl of test reagent was introduced to the wells 2-12 of the 96 well microtitre plates. The tests were done in triplicate and 100 µl of the test reagent was added into well 1.Serial dilution was done up to the 11^{th} well. The 12^{th} well was used as a control. Bacterial suspensions were standardized to 0.5 MacFarland's and diluted 1:150 in Mueller Hinton Broth and then 50 µl of inoculum was transferred into each well. Streptomycin (Sigma, USA) was used as the standard drug. The plates were incubated at 35˚C for 16-18 hours. The MIC was then determined as the last well where there was no visible bacterial growth in natural light.

2.5 Data Analysis

Data of zones of inhibition was entered into MS Excel (Microsoft, USA). The data was statistically analysed to give the mean of three plates/ isolate. The means were used to compare with the CLSI values. The data was presented as tables.

3. RESULTS

3.1 *In vitro* Antimicrobial Activity of Extracted and Commercial Bromelain Using the Agar Well Diffusion Method

The activity of extracted bromelain was dose dependent where all (100%) the different bacteria species were sensitive to extracted bromelain at 5 mg/ml. *Enterobacter* spp. and *Klebsiella* spp. were also sensitive to extracted bromelain at 2.5 mg/ml while the rest were not sensitive at this concentration. *Klebsiella* spp. was the only bacteria sensitive to extracted bromelain at 1.25 mg/ml (Table 1).

Only four bacterial isolates; *Enterobacter* spp., *Klebsiella* spp., *Serratia* spp. and *S. aureus*, were sensitive to commercial bromelain at 5 mg/ml, but *Citrobacter* spp., *E. coli* and Coagulase Negative *Staphylococci* were not sensitive at this concentration. *Enterobacter* spp., *Klebsiella* spp. and *Serratia* spp. were also sensitive at 2.5 mg/ml. Only *Klebsiella* spp. was sensitive at 1.25 mg/ml (Table 1).

3.2 *In vitro* Antimicrobial Activity of Nanoencapsulated Bromelain Using the Agar Well Diffusion Method

All the 7 (100%) isolates were sensitive to nanoencapsulated bromelain at 200 µg/ml and 100 µg/ml. All the other bacteria except *Citrobacter* spp. were sensitive to nanoencapsulated bromelain at 50 µg/ml. *Klebsiella* spp, *E. coli*, Coagulase Negative *Staphylococci* and *S. aureus* were sensitive to nanoencapsulated bromelain at 25µg/ml while all the isolates were not sensitive at 12.5µg/ml and 6.25 µg/ml (Table 2).

3.3 Minimum Inhibitory Concentration

The MIC of Streptomycin was 22.2µg/ml for *Citrobacter* spp., *Klebsiella* spp., *E. coli*, *Staphylococcus aureus*, *Enterobacter* spp., *Serratia* spp. isolates. The MIC of Streptomycin was 44.4 µg/ml for Coagulase Negative *Staphylococci.* Extracted bromelain and commercial bromelain did not show any inhibition in all the isolates tested, showing that the MIC of commercial and extracted bromelain was higher than tested 5 mg/ml (Table 3).

Table 1. *In vitro* antimicrobial activity of bromelain against bacteria isolated from milk of dairy goats with subclinical mastitis using the agar well diffusion method

	Bromelain (mg/ml) sensitivity to specific bacteria	
Bacteria	**Extracted Bromelain**	**Commercial Bromelain**
Enterobacter spp.	2.5	2.5
Citrobacter spp.	5	>5
Klebsiella spp.	1.25	1.25
Escherichia coli	5	>5
Serratia spp.	5	2.5
*CNS	5	>5
Staphylococcus aureus	5	5*

Key: CNS-Coagulase negative staphylococci, 5*-highest concentration tested*

Table 2. *In vitro* antimicrobial activity of nanoencapsulated bromelain against bacteria isolated from milk of dairy goats with subclinical mastitis using the agar well diffusion method

Bacteria	Nanoencapsulated bromelain (µg/ml)
Enterobacter spp.	50
Citrobacter spp	100
Klebsiella spp.	25
Escherichia coli	25
Serratia spp.	50
*CNS	25
Staphylococcus aureus	25

*Key: *CNS = Coagulase negative staphylococci*

Table 3. Minimum Inhibitory Concentration of bromelain and Streptomycin against bacteria isolated from milk of dairy goats with sub-clinical mastitis

Isolate	MIC (µg/ml)			
	Commercial bromelain	Extracted bromelain	Nanoencapsulated bromelain	Streptomycin
Enterobacter spp.	>5000*	>5000*	25	22.2
Citrobacter spp.	>5000*	>5000*	25	22.2
Klebsiella spp.	>5000*	>5000*	200	22.2
Escherichia coli	>5000*	>5000*	50	22.2
Serratia spp.	>5000*	>5000*	25	22.2
*CNS	>5000*	>5000*	25	44.4
Staphylococcus aureus	>5000*	>5000*	200	22.2

*Key: CNS-Coagulase Negative Staphylococci, *Highest Concentration Tested*

The MIC of nanoencapsulated bromelain for *Enterobacter* spp., *Citrobacter* spp., *Serratia* spp. and Coagulase Negative *Staphylococci* was 25 µg/ml. The MIC of nanoencapsulated bromelain for *E. coli*was 50 µg/ml and 200 µg/ml for *Klebsiella*spp. and *S. aureus*. (Table 3).

4. DISCUSSION

The findings of the current study show that the extracted bromelain may be more potent against bacteria than commercial bromelain. The difference in potency has been attributed to the loss of activity that happens in the production, purification and standardization of commercial bromelain. As bromelain is a protease, the secondary structure may be distorted by changes in pH and temperature during processing, making it lose some of its activity.

Bromelain has been shown to be effective against multidrug resistant *Pseudomonas aeruginosa* [28], *E. coli, Shigella sonnei* and *Salmonella paratyphi* [29], *S. aureus*, *P. aureginosa*, *E. coli* and *Streptococcus pneumonia* [30], *Proteus* spp. [31] and *Acetinobacter* spp. [32] isolated from food products.

In the current study, where bacteria were isolated from dairy goats having sub-clinical mastitis, bromelain was potent against both gram-positive (*Staphylococcus aureus* and Coagulase Negative *Staphylococci* and gram negative bacteria (*Serratia* spp., *Enterobacter* spp., *E. coli*, *Citrobacter* spp. and *Klebsiella* spp.). This shows that bromelain can be used in management of sub-clinical mastitis caused by either gram positive or gram negative bacteria. However, the mode of action needs to be investigated.

In the current study, most (85.7%) of the bacterial isolates were sensitive to nanoencapsulated bromelain up to 50µg/ml. There was no significant difference in sensitivity of gram negative bacteria and gram positive bacteria to nanoencapsulated bromelain. The MIC of nanoencapsulated bromelain ranged from 25µg/ml to 200µg/ml for different isolates. Similar studies [19] in bacteria causing mastitis in cattle recorded an MIC of 8µg/ml chitosan-chitooligosaccharide and bromelain combination which was lower than found in this study. This may be due to the differences in the acetylation of chitosan used as this plays a key factor [21].

The MIC of both commercial and extracted bromelain against the seven bacterial species could not be ascertained. Bromelain has catalytic activity at the active site which contains the sulfyhydryl group [33]. Although the exact mode of action of bromelain is not known, it has been hypothesized that it hydrolyses some peptide bonds present in the bacterial cell wall, eventually causing cell death [17]. The MIC for bromelain in this study was thus assumed to be higher than 5 mg/ml.

The MIC of nanoencapsulated bromelain was determined for all the bacterial isolates. In this present study, the *in vitro* activity of bromelain was enhanced by nanoencapsulating bromelain with chitosan nanoparticles. In a similar study [34], the anti-inflammatory potential of bromelain was concluded to have been enhanced by encapsulating bromelain in Katira gum nanoparticles. This was attributed to enhanced absorption due to reduction in particle size or protection of bromelain from acid proteases. Chitosan nanoparticles have been shown to have antimicrobial properties [19]. They have also been shown to have toxic effects against methicillin resistant *S. aureus* and *Klebsiella*

pneumonia [35]. The low MIC of nanoencapsulated bromelain against bacteria obtained in this study may be due to the combined antibacterial effects of the bromelain and the chitosan nanoparticles against the bacteria.

The differences noted in the antimicrobial activity between bromelain and nanoencapsulated bromelain obtained in this study shows that nanoencapsulation can increase the antibacterial activity of bromelain.

5. CONCLUSION

The present study showed that bromelain was effective against gram positive and gram negative bacteria which cause mastitis in dairy goats. Encapsulation of bromelain with chitosan nanoparticles increased the antimicrobial potential of bromelain. Nanoencapsulated bromelain should thus be tested for *in vivo* safety and efficacy against mastitis in dairy goats. This will eventually help in the harnessing of bromelain extraction to minimise losses in the pineapple industry by extracting bromelain from pineapple stems, leaves and crowns which are usually agricultural and industrial waste.

ETHICAL APPROVAL

Animal Ethic committee approval has been collected and preserved by the author(s).

DATA AVAILABILITY

The data used to support the findings of this study are available from the corresponding author upon request.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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