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Protein Concentration, Anthelmintic Activity, and Microbial Contamination of the Laboratory-Produced Chitosan-Encapsulated Bromelain Batches

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ABSTRACT

Bromelain has been shown to have potential as an anthelmintic for controlling livestock nematodes, such as *Haemonchus (H.) contortus*. The present study aimed to evaluate the *in vitro* quality of the laboratory-produced nanoencapsulated bromelain (NEB) and its activity against *H. contortus*. The acid-base extraction method was employed to extract four different batches of bromelain from the peels of fully ripened pineapples. It was encapsulated in chitosan to form the nano-encapsulated bromelain complex. Standard biochemical methods were employed to determine the bromelain concentration, protein concentration, *in vitro* anthelmintic activity against various stages of *H. contortus* (egg, larva, adult), and bacteria contamination for the four NEB batches. The mean concentration of extracted bromelain was 4.3 mg/ml in all four batches. There were no variations in the protein concentrations between the batches of NEB, which ranged from 1,090 mg/ml to 1.205 mg/ml. Although there were no significant differences in different batches, a variation in NEB inhibitory concentration (IC_{50}) was observed according to the different parasitic stages. The highest activity was for adult worms (LC_{50} =0.2454 ± 0.05 mg/ml), followed by the eggs (IC_{50} = 0.3 ± 0.07 mg/ml), and the larval stage (IC_{50} =0.9 ± 0.45 mg/ml). Despite the identification of certain bacterial species in the raw pineapple extract, the final product of all four batches of NEB remained free from any bacterial contamination. The current study indicated that NEB's concentration, protein concentrations, and anthelmintic activity did not vary significantly across the different batches of NEB. Additionally, the encapsulation process ensured that the final product was free of bacterial contamination and thus safe for use in animals.

Keywords: Anthelmintic activity, Bromelain, Chitosan, Nanoencapsulation

INTRODUCTION

Helminthiasis is an important tropical disease affecting livestock where it causes severe diarrhea, reduction in weight gain, impairing fertility, and extreme cases causes high mortalities (Nwoke et al., 2015). The most important nematode affecting ruminants in the tropics is *Haemonchus (H.) contortus*, which causes the livestock industry significant financial losses (Vineer et al., 2020). Proactive approaches are critical to maintaining ruminant health and productivity, and effective management tactics include rotational grazing, targeted deworming, and a focused effort to prevent drug resistance. Farmers use anthelmintics frequently to treat helminthiasis (Nwoke et al., 2015). Despite their proven benefits for animal health, many commercial treatments have some significant disadvantages. Consumers have concerns about the potential existence of synthetic drug residues in animal products and also in recent years frequent use of anthelmintics has led to the emergence of drug resistance (Wainaina Kagucia et al., 2020; Sharma et al., 2020). Therefore, there is an urgent need for innovative approaches such as the development of new drugs from plants (Wasso et al., 2020; Daiba et al., 2022).

Recent studies have demonstrated that nano-encapsulated bromelain has high anthelmintic activity (Hunduza et al., 2020; Wasso et al., 2020; Daiba et al., 2022). Bromelain is abundant in pineapple (*Ananas cosmos*, L. Merr.) fruits, stems, and peels (Eguale et al., 2007; Hunduza et al., 2020; Wasso et al., 2020). However, upon treatment, bromelain's anthelmintic action decreases due to the low pH of ruminants' abomasum and the presence of rumen microbiota (Eguale et al., 2007). Thus, studies have focused on encapsulating bromelain to enhance its activity in the gut of animals (Hunduza et al., 2020; Wasso et al., 2020; Daiba et al., 2022). All developmental phases of *H. contortus* isolated from goats can be effectively inhibited *in vitro* by nanoencapsulated bromelain (Eguale et al., 2007; Hunduza et al., 2020; Wasso et al., 2020). Studies have found that encapsulated bromelain had a greater egg hatch suppression activity than extracted (IC50 = 0.325 mg/ml) and pure bromelain (IC_{50} = 0.327 mg/ml; Hunduza et al., 2020; Mahlangu et al., 2020;

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Wasso et al., 2020; Daiba et al., 2022). In addition, *in vivo* studies have shown that nano-encapsulated bromelain has anticoccidial and antibacterial properties (Mahlangu et al., 2020; Wasso et al., 2020; Daiba et al., 2023).

In products like the nano-encapsulated bromelain, before it is registered and marketed for use in animals, rigorous quality assurance is necessary to ensure the product's final safety and effectiveness. It is important to maintain consistency in batch-to-batch variations to meet quality standards and preserve product uniformity. The use of nano-encapsulation technology introduces an additional level of complexity, necessitating meticulous monitoring to avoid variations in particle size, distribution, and encapsulation efficacy (Fan et al., 2012; Nwoke et al., 2015; Lanusse et al., 2018). Furthermore, as these nanostructures may provide ideal conditions for the development of microorganisms rigorous monitoring processes are necessary to minimize the risk of microbial contamination. To guarantee that the product is safe for consumption, robust procedures for quality control should include extensive testing for microbial contaminants, such as bacteria, yeast, and mold (Dao et al., 2018). This study aimed to evaluating the quality of the laboratory-produced bromelain encapsulated (NEB) and determine its efficacy against *H. contortus* isolated from goats.

MATERIALS AND METHODS

Ethical approval

The study was approved by the Jomo Kenyatta University of Agriculture Technology (JKUAT) Institutional Animal Ethics Committee. Sampling of worms at the Ruiru slaughterhouse was approved by the meat inspector based at the facility. The protocols used for sampling and worm isolation adhered to the Kenya's Animal Diseases Act (Act No. 35 of 1984) and the University's guidelines.

Study site

The laboratory study was conducted between June and December 2023. The study was conducted at JKUAT, which is situated at latitude 1°05 S and longitude 37°00 E in Juja Sub-County, Kiambu County, Kenya. The average temperature in the area is 18.7°C with 850 mm of annual rainfall.

Batches preparation

The four different batches of extracted bromelain were produced one week apart. For all batches, the concentration of bromelain was determined after direct extraction, dialysis, and encapsulation. The extracts were stored at -20°C pending experiments on their anthelmintic properties.

Extraction of bromelain

Twenty mature pineapples (*Ananas comosus* L. Merr.) were obtained from a farm located in Kiambu County, Kenya's Gatundu Sub-County. This sub-county is located between longitudes 0° 25 and 1° 20 south and latitudes 36° 31 and 37° 15 east. It has a tropical climate with 26°C average temperature and 1200 mm of annual rainfall on average.

Bromelain was extracted using sodium acetate from pineapple peels as earlier described by Hunduza et al. (2020). The crude extract was precipitated with 40% ammonium sulfate. The resulting pellet was dissolved in 100 mM of Tris-HCl, dialyzed, and stored at -20°C.

Bromelain nano-encapsulation using chitosan

Ionic gelation method by Hunduza et al. (2020) was used to encapsulate bromelain into chitosan and this involved use of 1% sodium tripolyphosphate (STPP, Loba Chemie, India) and 1% chitosan (Sigma Aldrich, USA). After centrifugation, the pellet was recovered, washed with distilled water, dissolved in a phosphate-buffered saline (PBS) and the solution was stored at -20°C. Commercial bromelain (Jarrow Formulas, USA) was used to prepare the standard solution. The structural properties of nanoparticles were assessed using Fourier Transform Infrared Spectroscopy (FTIR), using the method outlined by Fan et al. (2012) and the result obtained was compared to other previous studies.

In vitro anthelmintic activity

Standard drug preparation

To prepare the positive control, albendazole (Sigma Aldrich, USA) was dissolved in 1% dimethyl sulfoxide (DMSO) solution (Thermo Fisher Scientific, USA). The PBS was used as a negative control at concentrations ranging from 0.125 mg/ml to 4 mg/ml.

Isolation of eggs

Mature *H. contortus* worms were obtained directly from the abomasum of goats slaughtered at the Ruiru abattoir in Kenya (Hunduza et al., 2020). The worms were subsequently placed in 7.4 pH PBS. The procedure outlined by Coles et al. (1992) was used to isolate female worms. Thereafter, five worms were added to a test tube and five milliliters of PBS

added. The worms' eggs were then gently crushed with a glass rod to release them into the solution of PBS. The mixture was then filtered to remove worm waste, and an additional 5 mL of PBS was added for homogeneity. Subsequently, 500 µL of the egg solution was applied to a McMaster slide, and the total egg concentration was counted under a microscope (Optika Microscope, Italy) at 100x magnification.

Egg hatch assay

The egg hatch assay (EHA) was carried out using the Coles et al. (1992) method. Briefly, 10 *H. contortus* eggs were suspended in 200 µL of egg solution and added to each well of a 96-well ELISA plate. Next, each well received 200 µL of an encapsulated bromelain solution, with concentrations ranging from 0.125 mg/ml to 4 mg/ml. After that, the plate was incubated for 48 hours at 28°C in a humid atmosphere. To stop the hatching process, Lugol's iodine was added to each well. The number of hatched eggs and larvae was counted under the microscope. A formula was used to determine the percentage inhibition of egg hatch (Coles et al., 1992).

$$\%\ \text{Egg inhibition} = \frac{\text{Total number of eggs} - \text{number of hatched larvae}}{\text{Total number of eggs}} x100$$

Larval and adult worms' mortality assay

The larval mortality assay (LMA) was conducted in accordance with the methodology described by Coles et al. (1992), Amphotericin B (5 g/ml, Affy Parenterals, India) was added to the egg suspension to inhibit the growth of bacteria and fungi. In each well of a 96-well titer plate, 180 µL of egg suspension was added, along with an extra 20 µL of nutrient media. The nutrient media consisted of one gram of yeast medium in 90 mL of normal saline and 10 mL of Earle's salt solution. The plate was then incubated at a temperature of 28°C for a duration of 48 hours. After this, the hatched larvae were observed using a light microscope at a magnification of 100x. An encapsulated bromelain solution (200 µL), with concentrations ranging from 0.125 mg/ml to 4 mg/ml, was then introduced into the wells. The numbers of dead and viable larvae were subsequently determined using a microscope set at a magnification of x100.

The adult worm's mortality assay (AWMA) was conducted following the procedure described by Eguale et al. (2007) and Hunduza et al. (2020). Ten actively motile adult worms obtained from the Ruiru slaughterhouse were placed in Petri plates containing NEB solutions of varying concentrations (0.125 mg/ml to 4 mg/ml). The worms were then observed for 24 hours. They were then placed in lukewarm fresh PBS for 30 minutes. A microscope was used to count the number of live and dead worms/larvae. The mortality rate for each concentration of extract was calculated using the following formula (Hunduza et al., 2020).

$$\text{Worm/larva mortality}\ (\%) = \frac{\text{Number of dead worms/larva}}{\text{Total number ofworms/larva}} \text{x}100$$

Determination of microbial contamination

Bacterial presence in the final product was evaluated as earlier described by Omorotionmwan et al. (2019). Briefly, crude extract of bromelain and NEB samples were inoculated onto nutrient agar (HiMedia Laboratories, India) and incubated at 37°C for 48 hours. The total plate count enumeration was done. After examination of the morphology of the colonies, the isolated bacteria were then gram-stained and further identified using biochemical tests, including catalase, Simmons-Citrate, methyl red / Voges-Proskauer (MR-VP), and urease.

Statistical analysis

The obtained data was entered and analyzed using Microsoft Excel (Microsoft, USA). The FTIR graphs were prepared using OriginPro 2023 (OriginLab, Germany version 10.0.5.157), while the anthelmintic activity graphs were prepared using GraphPad Prism (Dotmatics, United Kingdom, version 9.5.1). The statistical analyses were undertaken using analysis of variance (ANOVA) with a significance level set at $p < 0.05$.

RESULTS

Bromelain concentration

The concentration of bromelain after extraction from pineapples (Figure 1) was 4.3 ± 0.04 mg/ml. However, the concentrations decreased to 3.0 ± 0.31 mg/ml after dialysis and then to 1.2 ± 0.04 mg/ml after nanoencapsulation. The concentrations of bromelain did not differ significantly between the various batches ($p > 0.05$).

Fourier Transform Infrared Spectra of Nano-Encapsulated bromelain and Nano-encapsulated commercial bromelain

The effectiveness of the ionic gelation method in creating bromelain-loaded chitosan nanoparticles and the nature of interactions between bromelain and chitosan were evaluated using Fourier Transform Infrared (FTIR) analysis. The FTIR spectra of the chitosan matrix and chitosan nanoparticles loaded with bromelain are shown in Figure 2. The

chitosan spectrum exhibited an apparent and wide peak within the range of 3500 cm-1 to 3300 cm-1, which can be attributed to the stretching vibration of hydrogen-bonded O-H groups. The primary amines and the IA-type amide's N-H stretching peaks overlapped in the same area. The A-type amine's C-N stretching vibration is represented by the peak at 1317 cm-1, whereas the asymmetric C-O-C stretching peak is situated at roughly 1150 cm-1. There was no variation between peaks NEB in the various batches. The batches had the same transmittance as the standard (commercial bromelain).

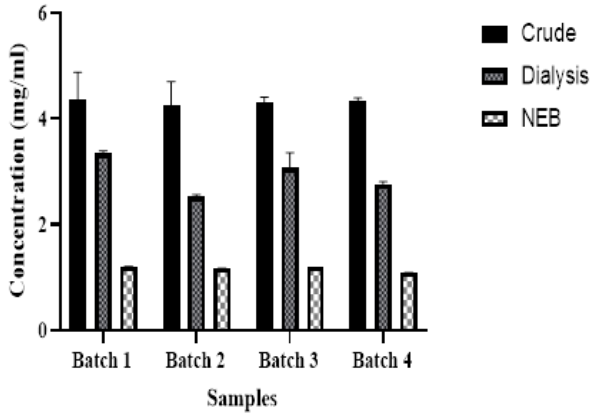

Figure 1. Concentration of bromelain from pineapple peels in the crude extract, after dialysis and nanoencapsulation of bromelain with Chitosan. NEB: Nanoencapsulated Bromelain

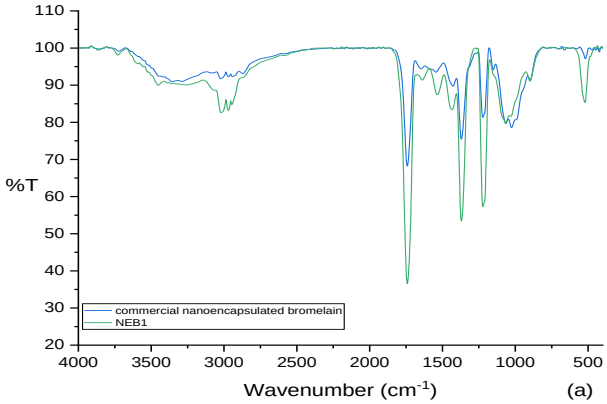

Figure 2. Fourier transform infrared spectra of the nano-encapsulated commercial bromelain and encapsulated extracted bromelain. Fourier transform infrared was prepared using OriginPro 2023. NEB: Nano-encapsulated bromelain. T: Transmittance

Anthelmintic activity of nano-encapsulated bromelain

Egg hatch assay

The results of the EHA are shown in Figure 3. The IC_{50} of the Albendazole was 0.25 ± 0.2 mg/ml, while the IC_{50} for the batches of commercial encapsulated bromelain (NCB) was 1 ± 0.5 mg/ml. The IC_{50} of the batches of NEB was 0.5 ± 0.3 mg/ml. There were no differences ($p > 0.05$) in the IC_{50} values of commercial nano-encapsulated bromelain and NEB. Further, there were no statistically significant differences ($p > 0.05$) in the IC_{50} of the various batches.

Larval mortality assay

The results of the Larval mortality assay **(**LMA) are presented in Figure 4. The IC_{50} of the Albendazole was 0.25 ± 0.3 mg/ml. The IC_{50} of NEB was 1.3 ± 0.5 mg/ml while that of NCB was 0.6 ± 0.3 mg/ml. There was no variation in the IC_{50} among NEB and NCB batches ($p > 0.05$).

Adult worm mortality

Figure 5 shows the results on the mortality of adult worms in *H. contortus*. The IC_{50} of Albendazole was 0.3 ± 0.2 mg/ml. The batches of NCB had an IC_{50} of 0.9 ± 0.05 mg/ml, while that of NEB was higher (IC_{50} = 0.245 ± 0.05 mg/ml, $p < 0.05$). The IC_{50} of the different batches did not show any statistically significant difference ($p > 0.05$).

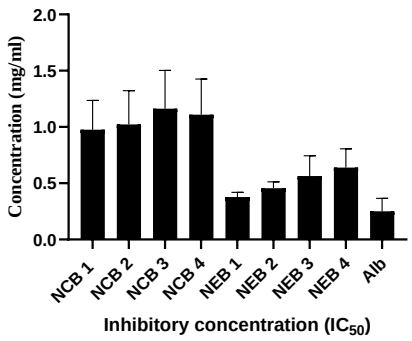

Figure 3. Inhibitory concentration (IC_{50}) in mg/ml of albendazole, nanoencapsulated bromelain (NEB), and commercial non-encapsulated bromelain (NCB) on eggs of *Haemonchus contortus.* The drugs were prepared in 4 batches (NCB 1, 2, 3, 4; NEB 1, 2, 3, 4). NEB: nano encapsulated bromelain; NCB: commercial nano encapsulated bromelain; Alb: Albendazole

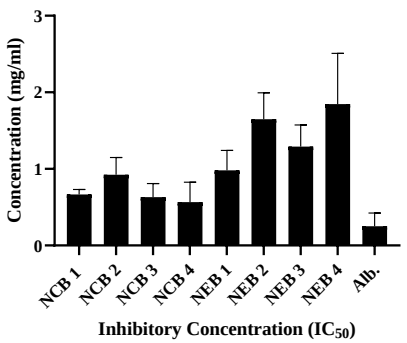

Figure 4. Inhibitory concentration (IC_{50}) in mg/ml of albendazole, nano encapsulated bromelain (NEB), and commercial nonencapsulated bromelain (NCB) on mortality of *Haemonchus contortus* larva. The drugs were prepared in 4 batches (NCB 1, 2, 3, 4; NEB 1, 2, 3, 4). NEB: nano encapsulated bromelain; NCB: commercial nano encapsulated bromelain; Alb: Albendazole \

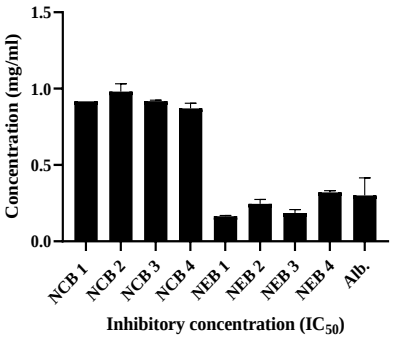

Figure 5. Inhibitory concentration (IC_{50}) in mg/ml of albendazole, nanoencapsulated bromelain (NEB), and commercial nonencapsulated bromelain (NCB) on adult *Haemonchus contortus* mortality. The drugs were prepared in 4 batches (NCB 1, 2, 3, 4; NEB 1, 2, 3, 4). NEB: nano-encapsulated bromelain; NCB: commercial nano-encapsulated bromelain; Alb: Albendazole

Microbial contamination

The total plate counts of the bacterial contamination in the crude pineapple extract batches ranged between 2000 CFU/ml and 7500 CFU/ml. The bacteria present in the crude extracts are shown in Table 1 and include *Proteus mirabilis, Klebsiella pneumoniae, Escherichia coli, Citrobacter freundii, Enterobacter cloacae Enterobacter aerogenes, Proteus vulgaris, Enterococcus faecalis, Staphylococcus aureus, Salmonella enterica, Serratia marcescens, Enterococcus faecium,* and *Klebsiella oxytoca*. The final product did not have any microbial contamination.

Table 1. Species of bacteria isolated from the crude extract of pineapples using biochemical methods in 4 batches isolated in Kenya

Isolated bacteria	Batch 1	Batch 2	Batch 3	Batch 4
Proteus mirabilis	✓			
Klebsiella pneumoniae	✓			✓
Escherichia coli	✓	✓	✓	✓
Enterobacter aerogenes	✓			
Enterococus cloaceae		✓		✓
Citrobacter freundii		✓		
Staphilococcus aureus		✓		
Enterococcus faecalis			✓	
Proteus vulgaris	✓		✓	
Enterococcus faecium			✓	
Serratia marcescens				✓
Klebsiella oxytoca		✓		✓
Salmonella enterica	✓			✓

DISCUSSION

The present study assessed the quality and efficacy of the laboratory-produced chitosan-encapsulated bromelain (NEB) compared to the nano-encapsulated commercial bromelain (NCB) against different parasitic stages of *H. contortus*. There were minimal variations in the concentrations of bromelain across the various batches which suggests some degree of reproducibility and the enzyme's relative stability during production. Similar concentration of bromelain were obtained by Daiba et al. (2022) in the crude extract and after encapsulation of bromelain. Omotoyinbo and Sanni, (2017) found that after 70% ethanol precipitation, total enzyme activity and total protein content also decreased. However, these authors showed that recovery rates for pineapple peel bromelain remained constant while specific activity and yield increased.

The FTIR spectra obtained for all batches of NEB and commercial nano-encapsulated bromelain (NCB) were similar. This additionally confirms the consistency and reliability of the observed spectral patterns. Devakate et al. (2009) reported similar FTIR spectra for extracted bromelain from the pineapple fruit. The different organic groups (O-H at 3500-3300 cm^{-1} for hydrogen bond, the amide N-H of type I'A and the amine of type A of the C-N observed at 1317 cm^{-1}, and the asymmetric groups C-O-C at 1150 cm^{-1}) obtained are similar to the results obtained by Wasso et al. (2020) and Daiba et al. (2022).

The present study showed that NEB had good activity against egg hatching, and the values obtained were close to those obtained by previous studies (Hunduza et al., 2020, Wasso et al., 2020). Anthelmintic activity has been investigated in various plant extracts. In a study conducted by Yongwa et al. (2020) and Thuo et al. (2017), extracts from *Albizia gummifera* and *Zanthoxylum usambarense* displayed significant anthelmintic activity against nematode eggs of *Haemonchus* spp., *Trichostrongylus* spp., and *Oesophagostomum* spp. For the study by Thuo et al. (2017), the inhibitory action (IC_{50}) of NEB on *H. contortus* eggs was 0.5 ± 0.3 mg/ml which is close to that reported in the present study. Further, the NEB activity obtained in the present study was comparable to those reported by Yongwa et al., (2020) in their study on *Senna italica* where they observed an IC_{50} of 0.69 mg/mL on inhibiting hatching of *H. contortus* eggs.

In the present study, the results obtained indicated that NEB has anthelmintic activity comparable to commercial bromelain in causing mortality of *H. contortus* larvae. Products that are able to limit nematode larval growth as well as cause mortality can be used to effectively control helminthiasis. Further in the current study, NEB exhibited high activity against adult worms which is similar to the results obtained by Hunduza et al. (2020) who showed that a concentration of

NEB at 0.2 mg/ml is required to kill adult worms. The activity of encapsulated bromelain in causing adult worm mortality was higher than that of inhibiting egg hatching and larval mortality. The variation in activity against adult and larval stages could be due to differences in the cuticle composition of the two stages (Hunduza et al., 2020). Previous studies have demonstrated that the cuticles of adult worms, which are fully grown, are the primary focus of bromelain activity (Daiba et al., 2022; 2023).

The adult worms' stage of *H. contortus* consume blood, leading to anemia in the host. In addition, the use of the encapsulated bromelain (NEB) can cause the death of adult worms, which in turn disrupts the parasite's life cycle by limiting egg production and decreasing environmental contamination.

The results of the current study showed that the crude extract of the pineapples had a high number of viable bacteria, whereas, in the final NEB product, there was no bacteria. This is similar to the study by Omorotionmwan et al. (2019), which isolated a variety of bacteria from pineapple peel. This could be due to contamination emanating from the production and harvesting of the pineapples. The primary bacteria observed in this study were *E. coli, S. aureus, E. faecalis, E.faecium, P. mirabilis, P. vulgaris, K. pneumoniae, P. oxytoca,* and *S. enterica*. In a study conducted by Omorotionmwan et al. (2019), it was observed that pineapple peels and pulp contained *Staphylococcus aureus, Streptococcus faecalis*, several *Bacillus* species, and *Clostridium* species. Dao et al. (2018) noted that the raw materials used in the production of pharmaceutical and cosmetic products frequently contained contamination derived from different bacteria. Therefore, the removal of these bacteria is necessary during the production process in order to guarantee the safety and quality of the product. Despite the initial high number of bacteria present in the crude extract of pineapples, the absence of viable bacterial cells in the final NEB product suggests successful bacteria elimination during the production process. The successful elimination of these bacteria in the final product illustrates that the production process effectively eliminates the bacteria.

CONCLUSION

The results of the current study indicated that the laboratory-produced NEB has anthelmintic activity against all stages of *H. contortus*, but it is more active against the adult worm stages. The laboratory production process demonstrates high levels of efficiency and consistency, particularly demonstrated by the consistent concentration levels of bromelain and the observed anthelmintic activity in the four batches of NEB. In addition, the method of encapsulating ensures the safety of the final product, thereby demonstrating high-quality assurance of laboratory-produced NEB. There is a need to undertake further development and commercialization of the product as an herbal anthelmintic drug for use in livestock.

DECLARATIONS

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Availability of data and materials

Inquiries regarding data availability should be directed to the respective authors.

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Authors' contributions

This work was completed with the assistance of all authors. The methods and design of the study were developed by authors Maanicus Bez-bang, Naomi Maina, and John Kagira. The author Maanicus Bez-bang undertook the encapsulation work, the *in vitro* analysis, and the statistical analysis. The authors Maanicus Rodolpher Bez-bang Kotangou, John Kagira, and Naomi Maina wrote the first draft of the manuscript. All the authors read and approved the final manuscript.

Competing interests

The authors have not declared any conflict of interest.

Ethical considerations

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by all the authors.

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