



## ***In vitro* Anthelmintic Activity of Chitosan Encapsulated Bromelain against Eggs, Larval and Adult Stages of *Haemonchus contortus***

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### **Authors' contributions**

This work was carried out in collaboration among all authors. Authors AH, JK and NM designed the study and developed the protocols. Author DA undertook the encapsulation work. Author KC developed the protocols for *in vitro* analysis and performed the statistical analysis. Authors AH and JK wrote the first draft of the manuscript. All authors read and approved the final manuscript.

### **Article Information**

DOI: 10.9734/JALSI/2020/v23i330151

#### **Editor(s):**

(1) Dr. Vasil Simeonov, University of Sofia, Bulgaria.

#### **Reviewers:**

(1) Ana Raquel Carneiro Ribeiro, Brasil.

(2) Francisco Avelar Pereira Júnior, Universidade Federal de Campina Grande (UFCG), Brasil.

(3) Fábio Tonissi Moroni, Universidade Federal do Amazonas, Brasil.

Complete Peer review History: <http://www.sdiarticle4.com/review-history/56200>

**Original Research Article**

**Received 22 February 2020  
Accepted 29 April 2020  
Published 23 May 2020**

### **ABSTRACT**

The objective of this study was to evaluate *in vitro* ovicidal, larvicidal and adult mortality activity of bromelain encapsulated in chitosan nanocarriers against *H. contortus*. Bromelain was isolated from peels of ripe pineapple from Kiambu County, Kenya. Isolation of bromelain was conducted with several stages of fractionations with ammonia sulphate salt and dialysis. Encapsulation of bromelain was done by use of methyl cellulose-chitosan in order to control release and activity. The

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encapsulated chitosan nanocarriers were then subjected to *in vitro* ovicidal, larvicidal and adult mortality activity according to standard procedures. The results of the assays showed that encapsulated bromelain had an IC<sub>50</sub> of 0.249 mg/ml, 0.251 mg/ml and 0.140 mg/ml on the egg hatch, larval and adult worm mortality assays, respectively. All these values showed better activity than bromelain although there was no significant difference ( $p>0.05$ ) between activities of encapsulated bromelain and bromelain. There was also a significant difference ( $p<0.05$ ), between Albendazole and the rest of the test drugs. In conclusion, this study has shown that encapsulated bromelain has anthelmintic activity on different developmental stages of *H. contortus* parasite and that it should be further investigated and developed as a novel anthelmintic drug for control of *H. contortus* and hence improve production of small ruminants.

**Keywords:** Bromelain; nanocarriers; encapsulated; anthelmintic; *Haemonchus contortus*.

## 1. INTRODUCTION

Gastrointestinal tract infections by nematodes in ruminants adversely affects productivity of the livestock industry [1], resulting in huge economic losses [2], especially in tropical and sub-tropical countries [3]. Among the nematodes, *Haemonchus contortus* is considered the main species in gastro-intestinal infections of small ruminants in terms of pathogenicity, prevalence and intensity [4]. Conventional control methods for gastro-intestinal nematodes in small ruminants have been based primarily on the use of anthelmintic synthetic compounds [5]. However, routine use and misuse has led to selected resistant strains of parasites to anthelmintic drugs [6,7].

A study conducted on 16 farms in Brazil established that the prevalence of sheep nematodes resistant to oxfendazole, levamisole and ivermectin was 88%, 41% and 59%, respectively [7]. Similarly, widespread resistance has been reported in Africa [8], Europe [9], US [10] and Asia [11]. These challenges have led to development of new drugs with different modes of action. Use of plant extracts has emerged as a possible sustainable, environmentally acceptable methods of nematode control [12]. Cysteine-proteases (CPs) are plant extracts that have been discovered to have anthelmintic properties [13] as they have catalytic sites which target peptide bonds which are present on the cuticle of nematodes [14]. Cysteine proteinases (CPs) from several plants such as pineapples and pawpaw have been demonstrated to have anthelmintic activity against nematodes of rodents, sheep and pigs [15,16].

The effects of these enzymes are due to damage of the surface of the cuticle, leading to lesions, fractures, and eventually complete destruction of the cuticle and bursting of the worms [17].

Bromelain in particular is a CP isolated from pineapple fruits and has been used as a complementary medicine to treat poultry, dogs, pigs and humans infected with intestinal parasites in developing countries [18]. However, initial investigations on the use of bromelain highlighted constraints in form of requiring multiple dosage as well as need to find the best method to administer it to target region of the gastro-intestinal tract [16]. Bromelain being enzymatic in nature is only catalytic in a narrow range of pH (5.5-8.0) and some authors have proposed that the enzyme is inactivated by low pH in the ruminant in the abomasum [16].

Currently, nanotechnology is being applied in the formulation of novel drugs, specifically encapsulation is being applied as a viable alternative to increase the stability of active compounds and to allow controlled release in target organs [19]. Chitosan-based drug delivery systems are of great interest in this study over other nanocarriers such silver nitrate, gold and zinc oxide due to their biocompatibility, biodegradability, muco-adhesive properties, prolonged drug release, and lack of toxicity [13]. The objective of this study was to evaluate the anthelmintic activity of encapsulated bromelain *in-vitro* against *H. contortus*.

## 2. MATERIALS AND METHODS

### 2.1 Extraction of Bromelain

Fresh ripe pineapples (*Ananas comosus L. Merr.*) were harvested from a farm in Gatundu sub-county, Kiambu County in Kenya. The County lies between a longitude of (36° 31' and 37° 15' east) and latitude of (0° 25' and 1° 20' south) and it also receives an average annual rainfall of 1200 mm. The harvested pineapples (*A. comosus*) were washed, packed and transported by vehicle to JKUAT botany

laboratory where it was identified by a botanist. In the laboratory, the pineapples were chopped and ground by a blender in sodium acetate extraction buffer (pH 7.4). Bromelain was extracted as previously described [16]. Briefly, the homogenate was then sieved three times to remove solid plant material. The resultant crude extract was then precipitated by adding 40% ammonium sulphate, the suspension was vigorously stirred on a magnetic stirrer for 45 minutes before incubation at 4°C for 24 hours. The suspension was then centrifuged at 10000 rpm for 10 minutes at 4°C. The pellet obtained was dissolved in 10 mM Tris-HCL buffer (pH 7.4). The dissolved pellet was then put in a molecular weight cut-off (MWCO) 12 kDa dialysis tubing, and then immersed in a beaker containing a mixture of 100 mM Tris-HCL buffer and 8% sodium chloride solution and then allowed to stand for 3 hours before the contents of the beaker were replaced with fresh buffer-sodium chloride mix and allowed to stand for 24 hours. After incubation period, the dialysis tubing was removed from the beaker, dried and the bromelain solution collected and stored at -35°C until further analysis.

## 2.2 Preparation of Encapsulated Bromelain

The ionic gelation method was used [20]. Thirty (30) ml bromelain (4 mg/ml) was mixed with 30 ml of 1% sodium tripolyphosphate (STPP) and mixed on a rotary mixer for 2 minutes. Using a syringe, 36 ml of the bromelain-STPP mixture was added drop-wise to 60 ml 1% chitosan solution under vigorous magnetic stirring followed by sonication for 45 minutes. The resultant suspension was then centrifuged at 15,000 rpm for 45 minutes [21]. The pellet obtained was washed with distilled water before being freeze-dried at -20°C. The encapsulated bromelain was characterized on a scanning electron microscope. The structural features of nanoparticles were estimated by FTIR (Fourier transform infrared) as previously described by [20]. Scanning electron microscope (SEM) analysis of encapsulated bromelain was then undertaken to describe the morphological properties and surface appearance of nanoparticles.

## 2.3 In vitro Anthelmintic Activity

*In vitro* anthelmintic activity was conducted according to World Association for the

Advancement of Veterinary Parasitology (WAAVP) guidelines [22] with slight modification on parasite collection, eggs preparation, egg hatch inhibition, larval mortality and adult worm mortality assays as described below:

### 2.3.1 Adult worm harvesting

Mature worms (*H. contortus*) were picked directly from the contents of the abomasum of infected goats at the Ruiru slaughterhouse, Kenya. The worms were put in phosphate buffered saline (PBS pH 7.4) and transported to the Pan-African University Institute for Basic Sciences Technology and Innovation (PAUSTI) laboratory. The worms were identified by viewing under the microscope and females were separated from males.

### 2.3.2 Egg liberation and count estimation

Approximately 5 worms were put in a small test tube containing 5 ml PBS, a glass rod was then used to gently crush the female worms, releasing the eggs into the PBS solution. The solution was then filtered using a sieve removing the worm debris. A further 5 ml PBS was added to the filtrate and mixed to attain homogeneity. Using a micropipette, 500 µL of the egg suspension was picked and dropped on a McMaster slide, the number of eggs was then counted under the microscope and the total egg concentration estimated and recorded.

### 2.3.3 Egg hatch assay

The egg hatch test (EHA) was performed based on the methodology described by [22]. Briefly, 200 µL of egg suspension containing approximately 10 *H. contortus* eggs was placed in each of the wells of a 96 well titre-plate. 200 µL of encapsulated bromelain solution in a concentration ranging from (0.0625 mg/ml to 4 mg/ml) was added to the wells to bring the volume of each well to 400 µL. Albendazole (Sigma Aldrich, USA) was dissolved in DMSO in the same range of concentration as bromelain was used as the positive control while PBS was used as negative control. Plain extracted bromelain, bromelain (Sigma-Aldrich) and 1% chitosan were also run as parallel tests. Each test was done in triplicate. The setup was incubated in humidified environment at a temperature of 28°C and allowed to stand for 48 hours. After the 48 hours, a drop of Lugol's iodine was added to each well to stop the hatching process. The number of hatched larvae

and eggs was counted on a microscope at 40X magnification with the help of a counter [23]. The percentage egg hatch inhibition was calculated by the following formula:

$$\% \text{ Egg hatch inhibition} = \frac{\text{Total number eggs} - \text{number hatched larvae}}{\text{Total number of eggs}} \times 100$$

### 2.3.4 Larval mortality assay

Larval Mortality Assay was conducted according to World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines [22]. Collection of adult parasite, egg recovery and concentration of eggs was done just as indicated in section 2.3.2. Amphotericin B (5 µg/ml) from Sigma-Aldrich Germany, was added to the egg suspension to control growth of bacteria and fungi. 180 µL of egg suspension was placed in each of the wells of a 96 well titre-plate and an additional 20 µL of nutritive media (comprising of 1 g of yeast media in 90 ml normal saline and 10 ml Earle's salt) was added to each well. The setup was incubated under at 28°C for 48 hours. The hatched larvae were observed under the light microscope and 200 µL encapsulated bromelain solution ranging from 0.0625 mg/ml to 4 mg/ml was placed in the wells. Albendazole (Sigma Aldrich, USA) was dissolved in DMSO in the same range of concentrations was used as the positive control while PBS was used as negative control. Plain extracted bromelain, commercially bought bromelain and 1% chitosan were also run as parallel tests. Each test was done in triplicates, the setup was further allowed to stand for 24 hours under the same conditions. The number of dead and live larvae was counted under a microscope and recorded. The percentage of mortality of larvae was calculated using the following formula:

$$\% \text{ Larval mortality} = \frac{\text{Number of dead larvae}}{\text{Number of larvae in culture}} \times 100$$

### 2.3.5 Adult worm mortality assay

Adult mortality assay was conducted according to the procedure by [2]. Adult *H. contortus* were collected and prepared as described in section 2.3.1. Ten actively moving worms were placed in petri dishes with encapsulated bromelain solution ranging from 0.0625 mg/ml to 4 mg/ml. Albendazole (Sigma Aldrich, USA) was dissolved in DMSO in concentrations ranging from 0.0625 to 4 mg/ml was used as a positive control while PBS was used as negative control. Plain

extracted bromelain, bromelain (Sigma Aldrich) and 1% chitosan were also run as parallel tests. Each test was done in triplicates, the setup was allowed to stand for 24 hours under humidified conditions at 28°C. The motility of each worm was observed after 24 hours and the treated worms were kept for 30 minutes in the lukewarm fresh PBS to observe the recovery of motility. Finally, the number of live and dead worms were counted under a microscope and recorded as described by [17]. Lack of motility was an indicator of parasite mortality. Percentage mortality of worms was calculated for each extract concentration using the formula:

$$\% \text{ Mortality} = \frac{\text{Number of dead worms}}{\text{Total number of worms}} \times 100$$

## 2.4 Data Analysis

Results obtained from the study were analysed using Microsoft office excel (Windows Version, 2016, Microsoft) and Statistical Packages for Social Science (SPSS, Microsoft, USA) software version 23. Mean percentage egg hatch inhibition rates, larval and adult mortality from encapsulated bromelain, Albendazole (ALB) extracted and commercially bought bromelain at different concentrations and ratios were compared using paired sample T-test at  $p < 0.05$  significant levels. The concentration required to inhibit 50% (IC<sub>50</sub>) for ovicidal and effective concentration (EC<sub>50</sub>) for larvicidal and adult mortality were determined using the regression line of probit according to the log<sub>10</sub> of the extract concentration.

## 3. RESULTS

### 3.1 FTIR Spectral Analysis

The ability of the ionic gelation process forming bromelain-loaded chitosan nanoparticles was assessed using FTIR to determine bromelain-chitosan interactions. The FTIR spectra of chitosan matrix, bromelain loaded chitosan nanoparticles are shown in Fig. 1. In the chitosan spectra, the wide and strong peak between 3500 cm<sup>-1</sup> and 3300 cm<sup>-1</sup> area is due to hydrogen-bonded O-H groups stretching vibration. The peaks of N-H stretching from primary amine and type II amide are overlapped in the same region [24]. The peak for the asymmetric stretch of C-O-C is at around 1150 cm<sup>-1</sup> and one at 1317 cm<sup>-1</sup> belongs to the C-N stretching vibration of type I amine. As described

by [20], chitosan-TPP nanoparticles showed a broad peak of  $3438\text{ cm}^{-1}$  to  $3320\text{ cm}^{-1}$  which indicated an enhancement of hydrogen bonding. The N-H bending was observed at  $1600\text{ cm}^{-1}$  which showed the presence of amine I. The peaks obtained at  $1650\text{ cm}^{-1}$  to  $1540\text{ cm}^{-1}$  indicated the presence of amide II carbonyl stretch. The bromelain-loaded chitosan also shows a P=O peak at  $1170\text{ cm}^{-1}$ . This can be attributed to the linkage between phosphoric and ammonium ions [20].

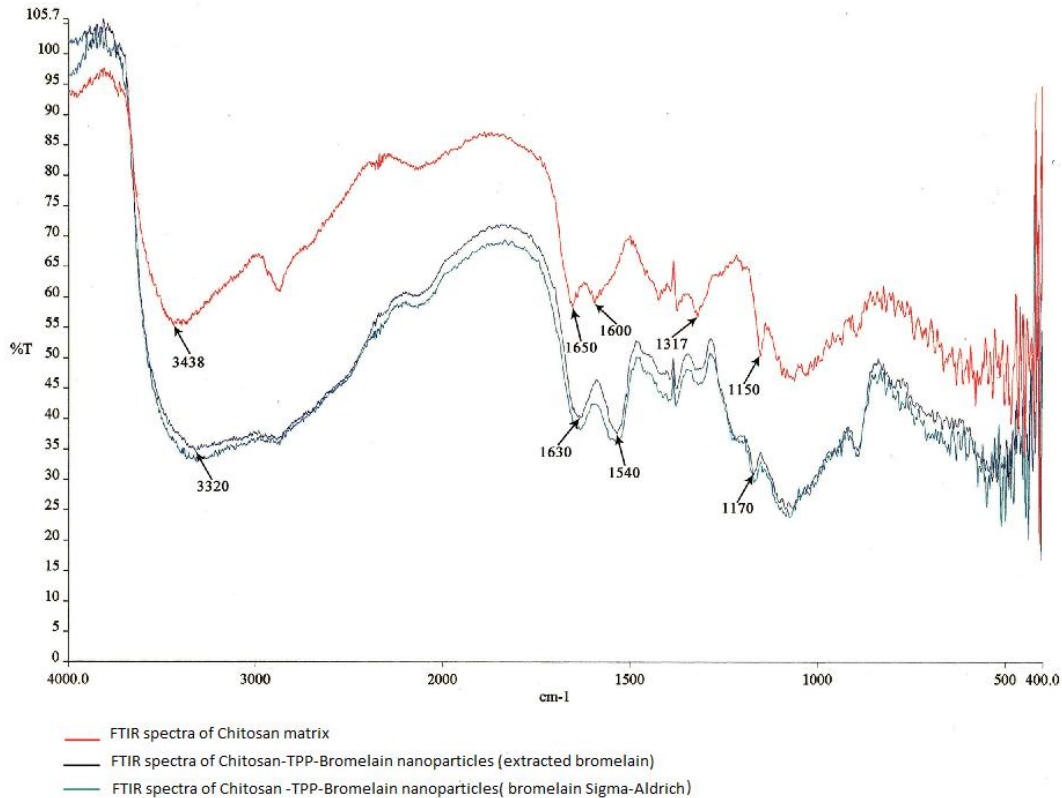
### 3.2 Scanning Electron Microscopy Analysis

The Scanning Electron Microscopy (SEM) images showed the morphological properties and surface appearance of nanoparticles. The nanoparticles (red-arrow pointed) (Fig. 2) have nearly spherical shape, smooth surface and have a poly-dispersed size distribution, ranging from 200-700 nm. There were also a few bigger particles (blue-arrow pointed), as a result of agglomeration of particles.

### 3.3 Egg-Hatch Inhibition (EHI) Assay

Encapsulated bromelain, extracted bromelain, bromelain (Sigma-Aldrich) and Albendazole (Sigma-Aldrich) in concentrations ranging from 0.0625-4 mg/ml were tested for their anthelmintic activity and results are presented in Fig. 3. Higher drug concentration resulted in higher egg hatch inhibition highlighting direct proportionality between drug concentration and anthelmintic activity.

At every concentration, Albendazole had the highest egg hatch inhibition, followed by encapsulated bromelain. There was no significant ( $p>0.05$ ) difference in egg hatch inhibition between encapsulated bromelain and bromelain and between extracted bromelain and commercially bought bromelain. However, there were significant ( $P<0.05$ ) differences in egg hatch inhibition between Albendazole and encapsulated bromelain. The concentrations required to inhibit 50% (IC<sub>50</sub>) of the four drug tests are presented in Table 1.



**Fig. 1. Fourier-transformed infrared spectroscopy spectra for chitosan matrix and chitosan-TPP-bromelain nanoparticles**

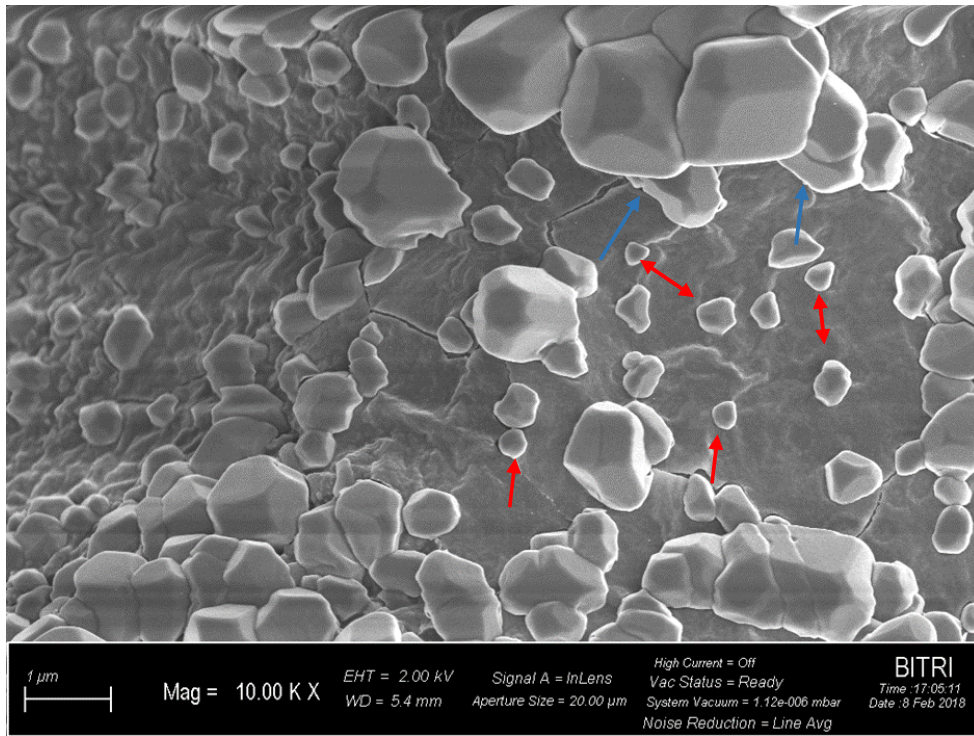


Fig. 2. SEM images of bromelain loaded chitosan nanoparticles

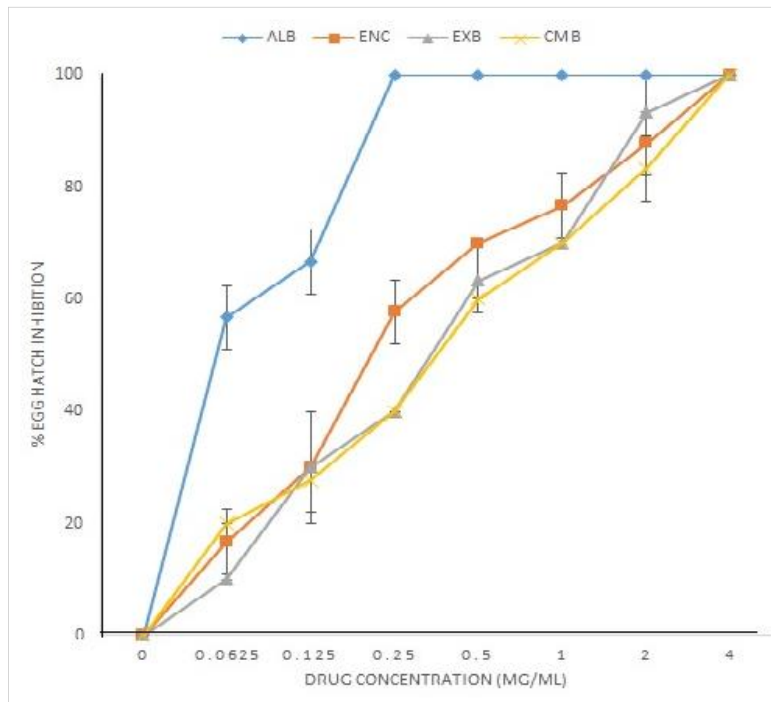


Fig. 3. Mean hatch inhibition for eggs of *Haemonchus contortus* exposed to Albendazole, encapsulated bromelain and bromelain

Key: ALB-Albendazole; ENC-encapsulated bromelain; EXB-extracted bromelain; CMB- bromelain (Sigma Aldrich)

**Table 1. IC50 values of Albendazole, encapsulated bromelain and bromelain on *H. contortus* eggs**

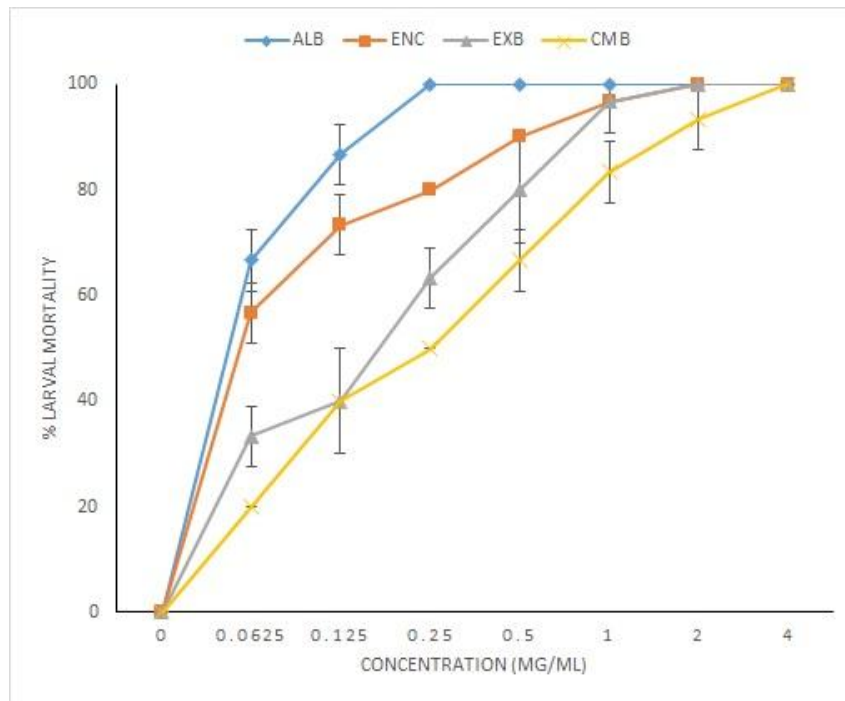
Test drug	IC50 values (mg/ml)		
	Mean	Lower boundary*	Upper Boundary*
Albendazole	0.064	0.026	0.090
Encapsulated bromelain	0.249	0.174	0.340
Extracted bromelain	0.325	0.233	0.442
Bromelain	0.327	0.225	0.459

\*95% confidence limits for concentration (mg/ml)

**3.4 Larval Mortality Assay**

Results of larval mortality and effective concentrations required to inhibit 50% of larvae are presented in Fig. 4 and Table 2, respectively.

There was significant difference ( $p < 0.05$ ), between Albendazole and the rest of the test drugs and no significant difference ( $p > 0.05$ ) between the encapsulated bromelain and the bromelain test samples.



**Fig. 4. Mean larval mortality of *Haemonchus contortus* exposed to Albendazole, encapsulated bromelain and bromelain**

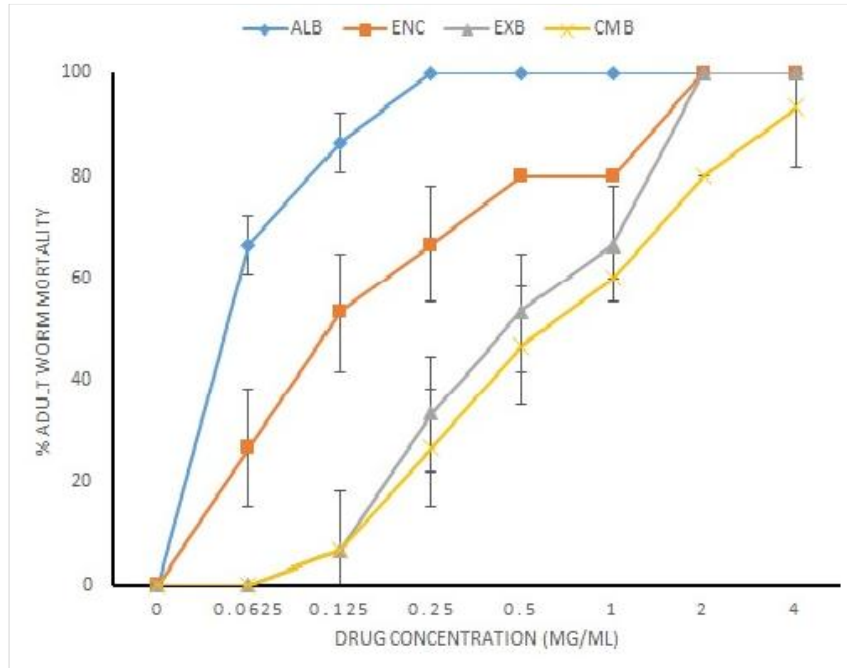
Key: ALB-Albendazole; ENC-Encapsulated bromelain; EXB-Extracted bromelain; CMB- Bromelain (Sigma Aldrich)

**Table 2. EC50 values of Albendazole, encapsulated bromelain and bromelain on *H. contortus* larvae**

Test drug	EC50 values (mg/ml)		
	Mean	Lower boundary*	Upper Boundary*
Albendazole	0.048	0.034	0.058
Encapsulated bromelain	0.251	0.184	0.319
Extracted bromelain	0.343	0.250	0.440
Bromelain	0.421	0.335	0.511

\*95% confidence limits for concentration (mg/ml)





**Fig. 5. Mean adult worm mortality of *H. contortus* exposed to Albendazole, encapsulated bromelain and bromelain**

Key: ALB-Albendazole; ENC-encapsulated bromelain; EXB-extracted bromelain; CMB- bromelain (Sigma Aldrich)

**Table 3. EC50 values of Albendazole, encapsulated bromelain and bromelain on *H. contortus* adult worms**

Test drug	EC50 values (mg/ml)		
	Mean	Lower boundary*	Upper Boundary*
<b>Albendazole</b>	0.048	0.032	0.058
<b>Encapsulated bromelain</b>	0.140	0.069	0.221
<b>Extracted bromelain</b>	0.464	0.326	0.661
<b>Bromelain</b>	0.659	0.574	0.758

\*95% confidence limits for concentration (mg/ml)

### 3.5 Adult Worm Mortality Assay

The results of the Adult Worm Mortality assay and the concentrations required to eliminate 50% of *H. contortus* are shown in Fig. 5 and Table 3 respectively. At all concentrations, Albendazole had the greatest effect on the worms and required 0.25 mg/ml to achieve 100% mortality. There was significant difference ( $p < 0.05$ ) between Albendazole and all the other test samples, however there was no significant difference ( $p > 0.05$ ) between encapsulated bromelain and the bromelain samples.

### 4. DISCUSSION

In tropics, haemonchosis causes massive livestock and economic losses [25]. Due to the

widespread occurrence of drug resistant *H. contortus* [26] strains, plant products such as bromelain has been suggested as a possible anthelmintic [27]. The current study sought to encapsulate bromelain due to the numerous advantages inferred by the process as described by [28] and to assess the efficacy of encapsulated bromelain against *H. contortus*. The study showed that changes in the positions of the peaks in the FTIR spectral analysis which could be attributed to the linkage between phosphoric and ammonium ions. So we infer that the triphosphoric groups of TPP were successfully linked with ammonium groups of chitosan. The inter- and intra-molecular hydrogen bonds are also enhanced in bromelain-loaded chitosan nanoparticles [20,24]. Further, the SEM images showed that the particles had a poly-



dispersed size distribution ranging from 200-700 nm; a more uniform size distribution can be achieved by increasing the sonication period and intensity during nano-formulation [20]. There were also some bigger particles as a result of agglomeration of the smaller one as the samples were imaged more than a month after formulation. The current study evaluated the efficacy of encapsulated bromelain against egg, larval and adult stages of *H. contortus*. The egg hatch assay has been used for screening for plants compounds with anthelmintic activity [29,30]. In this study, the IC50 values obtained for Albendazole (IC50-0.064 mg/ml) which was used as the positive control, are comparable to the values in the study carried out by others using different plant extracts [23,31]. Encapsulated bromelain had an IC50 value of 0.249 mg/ml as compared to the two bromelain samples with 0.325 and 0.327 mg/ml respectively for extracted and commercially bought bromelain and this could be attributed to the fact that encapsulation process stabilizes the bromelain protein structure and activity [32]. There was no significant difference between encapsulated bromelain and either of the bromelain samples possibly due to the fact that even in encapsulated bromelain the efficacy is derived from the bromelain enzyme.

Similar to the egg hatch inhibition assay, encapsulated bromelain exhibited better efficacy, (IC50 of 0.251 mg/ml in larval mortality and 0.140 mg/ml in adult worm mortality assay) as compared to either of the bromelain test samples in the respective assays. On comparison, the efficacy of encapsulated bromelain in adult worm mortality assay was better than that in either larval mortality and egg hatch assays and this could be due to the difference in the cuticle in adult worms and in larvae and the outer coat of eggs of *H. contortus* [2]. Previous studies have shown that bromelain activity mainly targets the cuticles of the worms which are well developed in adults [17].

## 5. CONCLUSION

From this study, bromelain-loaded chitosan nanoparticles have shown anthelmintic activity on eggs, larvae and adult worms of *H. contortus* parasite. The activity could be related to the presence of bromelain. Therefore, encapsulation of bromelain maybe evaluated further as novel anthelmintic drug for control of *H. contortus* in ruminants and thus *in-vivo* studies are recommended to ascertain its efficacy.

## DATA AVAILABILITY STATEMENT

The raw data [Anthelmintic Activity of Chitosan Encapsulated Bromelain against *Haemonchus contortus*] used to support the findings of this study are available from the corresponding author upon request.

## ACKNOWLEDGEMENTS

The authors are grateful to Pan-African University of Institute of Basic Science, Technology and Innovation (PAUSTI) and Jomo Kenyatta University of Agriculture and Technology (JKUAT) for financially sponsoring this research.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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