Original Research Article

Invitro **anthelmintic activity of chitosan encapsulated bromelain against eggs, larval and adult stages of *Haemonchus contortus***

Abstract:

In livestock, routine use and misuse of the available anthelmintics has led to emergence of drug resistant nematodes and this has prompted research in natural drugs with different modes of action. Bromelain, a cysteine protease isolated from the pineapple plant is one of the emerging solutions to address resistance by nematode parasites such as *Haemonchus contortus.* The objective of this study was to evaluate *invitro* ovicidal, larvicidal and adult mortality activity of bromelain encapsulated in chitosan nanocarriers against *H. contortus* stages isolated from goats in Kenya. Bromelain was extracted from peels of ripe pineapple from Kiambu County, Kenya. The results of the assays showed that encapsulated bromelain had an IC_{50} of 0.249mg/ml, 0.251mg/ml and 0.140mg/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. 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*

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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 parasite resistance 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]. This development has led to a need to develop 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].

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Currently, nanotechnology is being applied in the formulation 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 were harvested from a farm in Gatundu sub-county, Kiambu County in Kenya. They were washed and peeled; the peels were further washed, 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 10mM Tris-HCL buffer (pH 7.4). The dissolved pellet was then put in a molecular weight cut-off (MWCO) 12kDa dialysis tubing, and then immersed in a beaker containing a mixture of 100mM 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*

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The ionic gelation method was used [20]. Thirty (30) ml bromelain (4mg/ml) was mixed with 30ml of 1% sodium tripolyphosphate (STPP) and mixed on a rotary mixer for 2 minutes. Using a syringe, 36ml of the bromelain-STPP mixture was added drop-wise to 60ml 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 and allowed to air dry 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 [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 5ml PBS, a glass rod was then used to gently crush the female worms, releasing the eggs into the PBS solution. The

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solution was then filtered using a sieve removing the worm debris. A further 5ml PBS was added to the filtrate and mixed to attain homogeneity. Using a micropipette, 500µL 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, 200µL of egg suspension containing approximately 10 *H. contortus* eggs was placed in each of the wells of a 96 well titre-plate. 200µL of encapsulated bromelain solution ranging from 0.0625mg/ml to 4mg/ml was added to the wells to bring the volume of each well to 400µL. Albendazole (Sigma Aldrich, USA) was dissolved in DMSO in the same range of concentration was used as the positive control while PBS was used as negative control. Plain extracted bromelain, bromelain (Sigma-Aldrich) and 1% chitosan were also ran 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 microscopeat 40X magnification with the help of a counter [23]. The percentage egg hatch inhibition was calculated by the following formula:

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

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

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growth of bacteria and fungi. 180µL of egg suspension was placed in each of the wells of a 96 well titre-plate and an additional 20µL of nutritive media (comprising of 1g of yeast media in 90ml normal saline and 10ml Earle's salt) was added to each well. The setup was incubated under at 28°C for 48 hours. The hatched larvae was observed under the light microscope and 200µL encapsulated bromelain solution ranging from 0.0625mg/ml to 4mg/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 ran 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 0.0625mg/ml to 4mg/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 ran 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

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were counted under dissecting microscope and recorded. 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 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_{50}) for ovicidal and effective concentration (EC_{50}) for larvicidal and adult mortality were determined using the regression line of probit according to the $\log_{10}$ of the extract concentration.

2.5 Results

2.5.1 *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 Figure 1. In the chitosan spectra, the wide and strong peak between 3500$cm^{-1}$and 3300cm^{-1}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^{-1} and one at 1317cm^{-1}belongs to the C-N stretching vibration of type I amine. In chitosan-TPP nanoparticles the tip of the peak of 3438 cm^{-1} has shifted to 3320$cm^{-1}$and became wider with increased relative intensity indicating an

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enhancement of hydrogen bonding. Also the peaks for N-H bending vibration of amine I at 1600cm^{-1}and the amide II carbonyl stretch at 1650cm^{-1}shifted to 1540cm^{-1}and 1630cm^{-1}, respectively. The bromelain-loaded chitosan also shows a P=O peak at 1170cm^{-1}. This can be attributed to the linkage between phosphoric and ammonium ions [20].

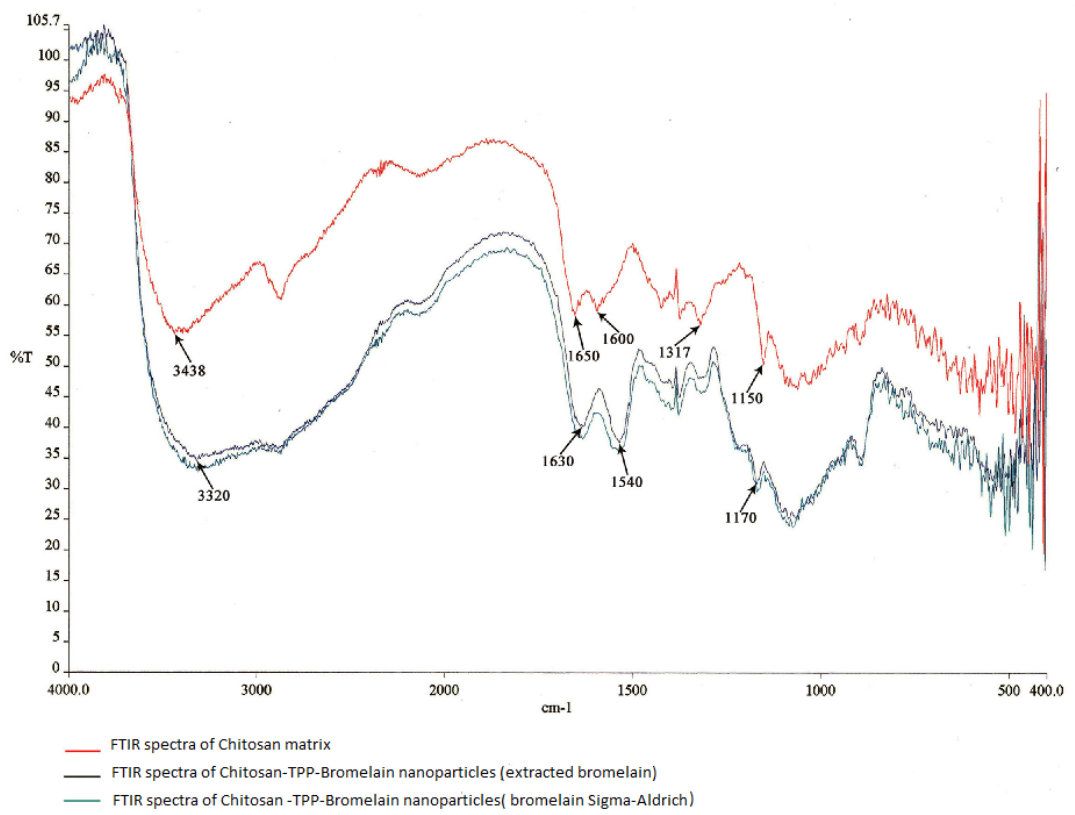

FIGURE 1: FTIR Spectra for chitosan matrix and chitosan-TPP-bromelain nanoparticles

2.5.2 *SEM analysis*

The SEM images showed the morphological properties and surface appearance of nanoparticles. The nanoparticles (red-arrow pointed) (Figure 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

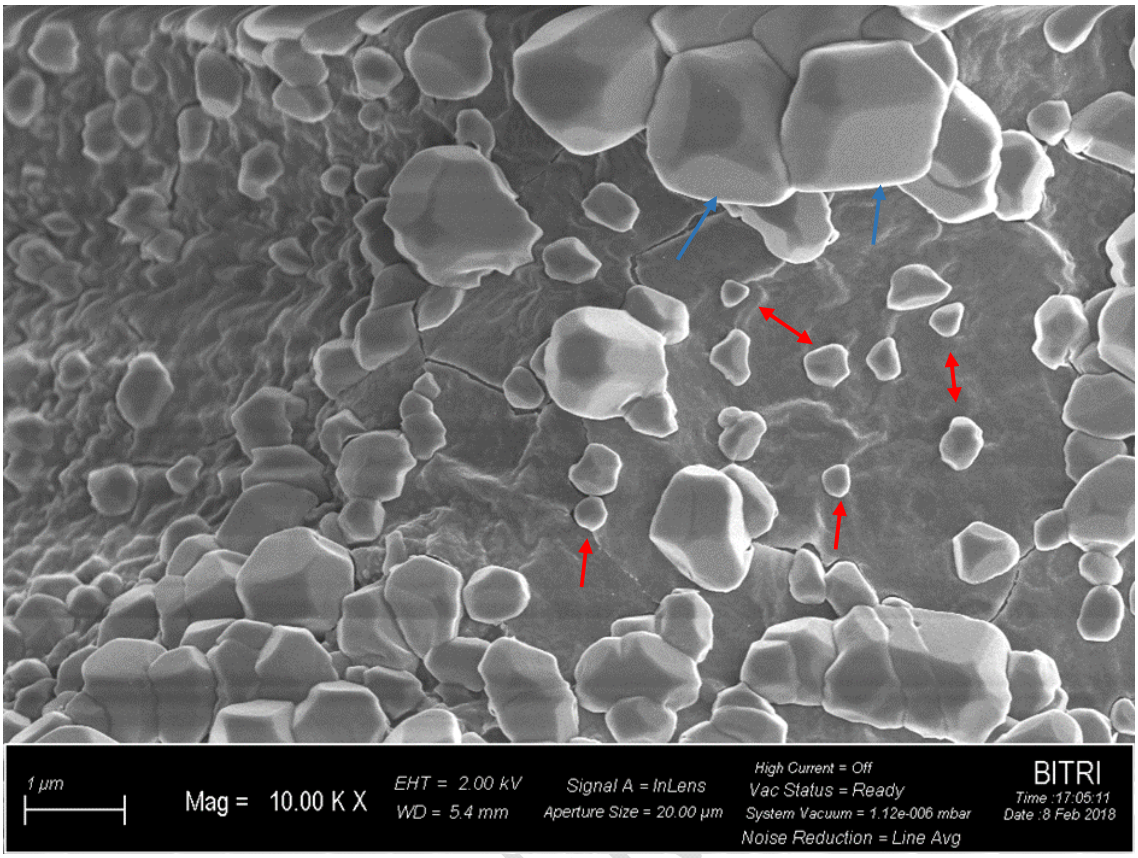

FIGURE 2: SEM images of bromelain loaded chitosan nanoparticles.

2.5.3 Egg-hatch inhibition (EHI) assay

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

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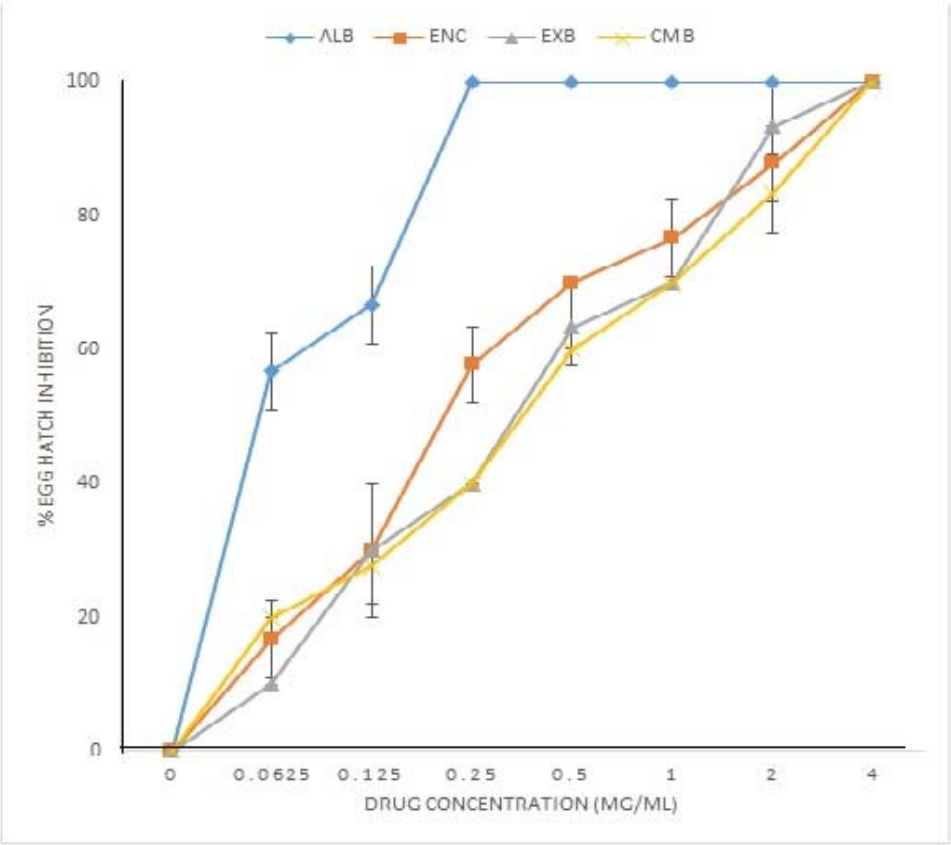

FIGURE 3: Mean hatch inhibition for eggs of *Haemonchus contortus* exposed to Albendazole, encapsulated bromelain and bromelain

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

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% (IC50) of the four drug tests are presented in Table 1.

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TABLE 1: IC_{50} values of Albendazole, encapsulated bromelain and bromelain on *H. contortus* eggs

Test Drug	IC_{50} 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)

2.5.4 ***Larval mortality assay***

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

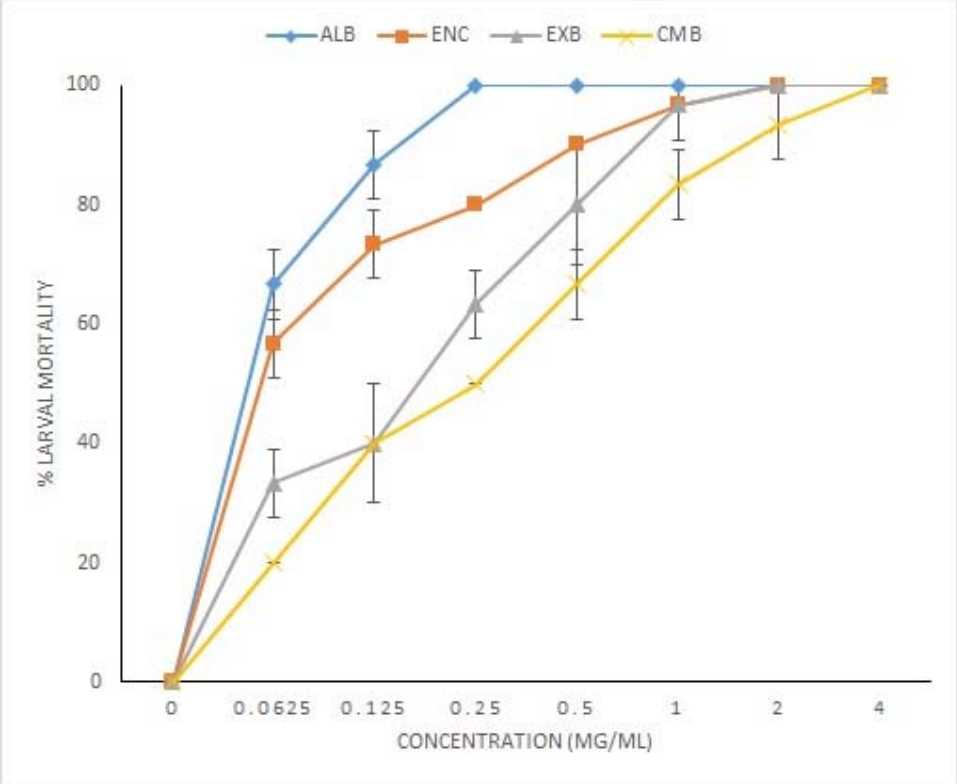

FIGURE 4: Mean larval mortality of *Haemonchus contortus* exposed to Albendazole, encapsulated bromelain and bromelain.
Key: ALB-Albendazole; ENC-encapsulated bromelain; EXB-extracted bromelain; CMB-bromelain (Sigma Aldrich)

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.

TABLE 2: EC_{50} values of Albendazole, encapsulated bromelain and bromelain on *H. contortus* larvae.

Test Drug	EC_{50} 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)

2.5.5 *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 Figure 5 and Table 3 respectively. At all concentrations, Albendazole had the greatest effect on the worms and required 0.25mg/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.

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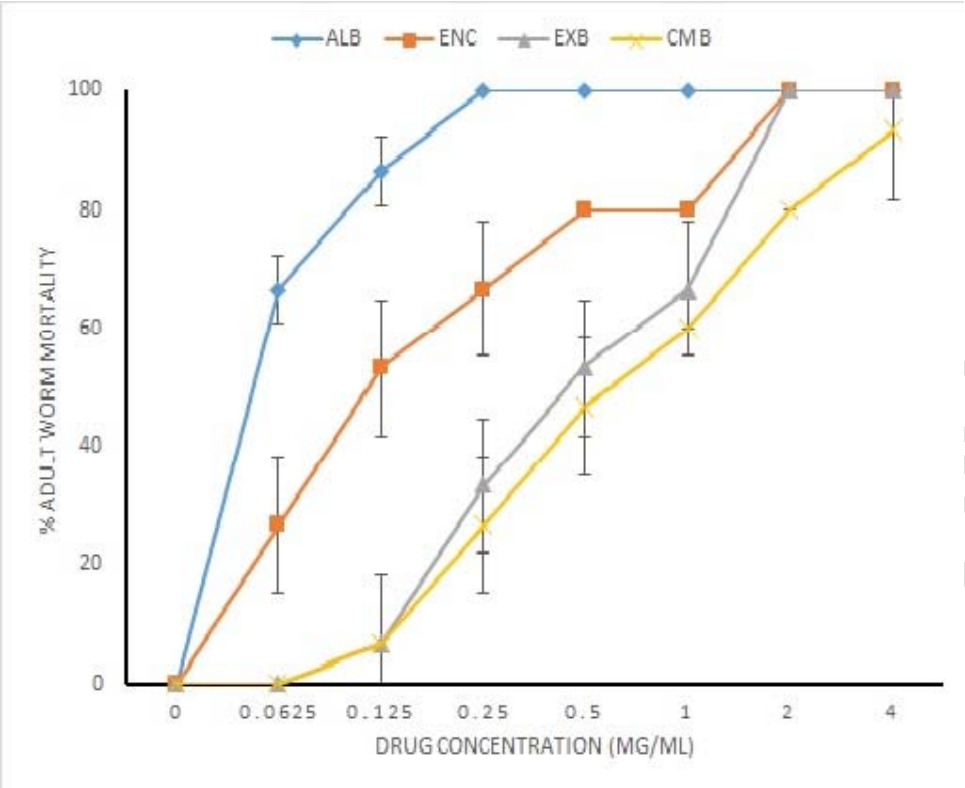

FIGURE 5: Mean adult worm mortality of *H. contortus* exposed to Albendazole, encapsulated bromelain and bromelain
Key: ALB-Albendazole; ENC-encapsulated bromelain; EXB-extracted bromelain; CMB-bromelain (Sigma Aldrich)

TABLE 3: EC_{50} values of Albendazole, encapsulated bromelain and bromelain on *H. Contortus* adult worms.

	EC_{50} values (mg/ml)		
Test Drug	Mean	Lower boundary*	Upper Boundary*
Albendazole	0.048	0.032	0.058
Encapsulated bromelain	0.140	0.069	0.221
Extracted bromelain	0.464	0.326	0.661
Bromelain	0.659	0.574	0.758

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

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3. 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 tripolyphosphoric 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 200-700nm; 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 IC_{50} values obtained for Albendazole (IC_{50}-0.064mg/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 $1C_{50}$ value of 0.249mg/ml as compared to the two bromelain samples with 0.325 and 0.327mg/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

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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, (IC_{50} of 0.251mg/ml in larval mortality and 0.140mg/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 is well developed in adults [17].

4. **Conclusions**

The findings of this study clearly show that bromelain-loaded chitosan nanoparticles have anthelmintic activity on eggs, larvae and adult worms of *H. contortus* parasite. It is postulated that bromelain is responsible for the anthelmintic activity and therefore encapsulated bromelain should be further assessed through *in-vivo* studies in order to develop a novel anthelmintic drug for control of *H. contortus* and other nematodes infecting livestock and man. Further studies on the loading capacity of chitosan for bromelain together with the toxicity analysis should be carried out in order to ascertain the feasibility of using encapsulated bromelain as an alternative anthelmintic drug.

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.

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5. References

[1] J. M. L. dos Santos, J. P Monteiro, W. L. C. Ribeiro, I. T. F Macedo, A. L. F. Camurça-Vasconcelos, L. da S. Vieira, C. M. L. Bevilaqua, "Identification and quantification of benzimidazole resistance polymorphisms in Haemonchus contortus isolated in Northeastern Brazil," Veterinary Parasitology, vol. 199(3–4), 2014.

[2] T. Eguale, G. Tilahun, A. Debella, A. Feleke, E. Makonnen, "In vitro and in vivo anthelmintic activity of crude extracts of Coriandrum sativum against Haemonchus contortus," Journal of Ethnopharmacology, vol. 110(3), pp. 428–433, 2007.

[3] C. Marley, R. Cook, R. Keatinge, J. Barrett, N. Lampkin, "The effect of birdsfoot trefoil (Lotus corniculatus) and chicory (Cichorium intybus) on parasite intensities and performance of lambs naturally infected with helminth parasites," Veterinary Parasitology, vol. 112(1–2), pp. 147–155, 2003.

[4] R. C.Krecek, P. J. Waller, "Towards the implementation of the "basket of options" approach to helminth parasite control of livestock: Emphasis on the tropics/subtropics," Veterinary Parasitology, vol. 139(4), pp. 270–282, 2006.

[5] P. Mendoza de Gives, J. Flores Crespo, D. Herrera Rodriguez, V. Vazquez Prats, E. Liebano Hernandez, G.E. Ontiveros Fernandez, "Biological control of Haemonchus contortus infective larvae in ovine faeces by administering an oral suspension of Duddingtonia flagrans chlamydospores to sheep," Journal of Helminthology, vol. 72(4), pp. 343, 1998.

[6] M. Schnyder, P. Torgerson, M. Schönmann, L.H.H. Kohler, "Multiple anthelmintic resistance in Haemonchus contortus isolated from South African Boer goats in Switzerland," Veterinary Parasitology, vol. 128(3–4), pp. 285–290, 2005.

[7] A. C. F. L. Melo, C. M. L. Bevilaqua, I.F. Reis, "Ciência animal brasileira.Ciência Animal Brasileira," Universidade Federal de Goiás, Escola de Veterinária, vol.10, 2000.

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[8] M. Ejlertsen, S.M. Githiqia, R.O. Otieno, S.M. Thamsborg, "Accuracy of an anaemia scoring chart applied on goats in sub-humid Kenya and its potential for control of Haemonchus contortus infections," Vet. Parasitol, vol. 141, pp. 291–301, 2006.

[9] K. Stafford, G.C. Coles, "Nematode control practices and anthelmintic resistance in dairy calves in the south west of England," Vet. Rec, vol. 144, pp. 659–661. 1999.

[10] L.C. Gasbarre, L.L. Smith, J.R. Lichtenfels, P.A. Pilitt, "The identification of cattle nematode parasites resistant to multiple classes of anthelmintics in a commercial cattle population in the US," American Association of Veterinary Parasitologists. Phildelphia, vol.49, 2004.

[11] S.L. Deore, S.S. Khadabadi, K.S. Kamdi, V.P. Ingle, N.G. Kawalkar, P.S. Sawarkar, U.A. Patil, A.J. Vyas, "In vitro Anthelmintic activity of Cassia tora," International Journal of ChemTech Research, 2009.

[12] W. L. C. Ribeiro, I.T.F. Macedo, J.M.L. dos Santos, E.F. de Oliveira, A.L.F. Camura-Vasconcelos, H.C.B. de Paula, C.M.L. Bevilaqua, "Activity of chitosan-encapsulated Eucalyptus staigeriana essential oil on Haemonchus contortus," Experimental Parasitology, vol. 135(1), pp. 24–29, 2013.

[13] M. De Aquino Mesquita, E, Silva Jr, A.M. Panassol, E.F. De Oliveira, A.L.C.F. Vasconcelos, H.C.B. De Paula, C.M.L. Bevilaqua, "Anthelmintic activity of Eucalyptus staigeriana encapsulated oil on sheep gastrointestinal nematodes," Parasitology Research, vol. 112(9), pp. 3161–3165, 2013.

[14] K. Chobotova, A.B. Vernallis, F.A.A. Majid, "Bromelain's activity and potential as an anti-cancer agent: Current evidence and perspectives," Cancer Letters. Elsevier, 2010.

[15] J.M. Kagira, P.W.N. Kanyari, N. Maingi, S.M. Githigia, J.C. Ng'ang'a, J.W. Karuga, "Characteristics of the smallholder free-range pig production system in western Kenya," Tropical Animal Health and Production, vol. 42(5), pp. 865–873, 2010.

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[16] D.J. Buttle, J.M. Behnke, Y. Bartley, H.M. Elsheikha, D.J. Bartley, M.C. Garnett, I.R. Duce, “Oral dosing with papaya latex is an effective anthelmintic treatment for sheep infected with Haemonchus contortus,” Parasites & Vectors, vol. 4(1), pp. 36, 2011.

[17] G. Stepek, A.E. Lowe, D.J. Buttle, I.R. Duce, J.M. Behnke, “In vitro and in vivo anthelmintic efficacy of plant cysteine proteinases against the rodent gastrointestinal nematode, Trichuris muris,” Parasitology, vol. 132(Pt 5), pp. 681–9, 2006.

[18] J,M. Behnke, D.J. Buttle, “Developing novel anthelmintics from plant cysteine proteinases,” Parasitesandvectors.biomedcentral, 2008 pp.?.

[19] F. Leimann, O. Gonçalves, “Antimicrobial activity of microencapsulated lemongrass essential oil and the effect of experimental parameters on microcapsules size and morphology,” Elsevier, 2008.

[20] W. Fan, W. Yan, Z. Xu, “Formation mechanism of monodisperse, low molecular weight chitosan nanoparticles by ionic gelation technique,” Elsevier, 2012.

[21] Q. Gan, T. Wang, C. Cochrane, P. McCarron, “Modulation of surface charge, particle size and morphological properties of chitosan-TPP nanoparticles intended for gene delivery,” Colloids and Surfaces B: Biointerfaces, vol. 44(2–3), pp. 65–73, 2005.

[22] G,C. Coles, C. Bauer, F.H.M. Borgsteede, S. Geerts, T.R. Klei, M.A. Taylor, P.J. Waller, “World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) methods for the detection of anthelmintic resistance in nematodes of veterinary importance,” Veterinary Parasitology, vol. 44(1–2), pp. 35–44, 1992.

[23] K. Cheruiyot, L.H. Kutima, G.P. Kareru, F.N. Njonge, “In vitro Ovicidal Activity of Encapsulated Ethanolic Extract of Prosopis juliflora Against Haemonchus contortus Eggs,” Researchgate, 2015.

[24] Y. Xu, Y. Du, “Effect of molecular structure of chitosan on protein delivery properties of chitosan nanoparticles,” International Journal of Pharmaceutics, vol. 250(1), pp. 215–

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226, 2003.

[25] M.V. Maciel, S.M. Morais, C.M.L. Bevilaqua, A.L.F. Camurça-Vasconcelos, C.T.C Costa, C.M.S. Castro, “Ovicidal and larvicidal activity of Melia azedarach extracts on Haemonchus contortus,” Veterinary Parasitology, vol. 140(1–2), pp. 98–104, 2006.

[26] G, A. E. Stepek, D.J. Lowe, D. Buttle, I. R. Duce, J. M. Behnke, “The anthelmintic efficacy of plant-derived cysteine proteinases against the rodent gastrointestinal nematode, Heligmosomoides polygyrus, in vivo,” The University of Nottingham repository, 2007.

[27] W. Luoga, F. Mansur, D.J. Buttle, I.R. Duce, M.C. Garnett, A. Lowe, J.M. Behnke, “The relative anthelmintic efficacy of plant-derived cysteine proteinases on intestinal nematodes,” Journal of Helminthology, vol. 89(2), pp. 165–174, 2015.

[28] G.D. Altun, S.A. Cetinus, "Immobilization of pepsin on chitosan beads," Food Chemistry, vol. 100(3), pp. 964–971, 2007.

[29] W. Luoga, "Factors affecting the anthelmintic efficacy of cysteine proteinases against GI nematodes and their formulation for use in ruminants," Nottingham Repository, 2013.

[30] O. Ngaradoum, J.M. Kagira, S.M. Karanja, N. Maina, "In Vitro Ovicidal and Larvicidal Activity of Aqueous and Methanolic Extracts of Ziziphus Mucronata Barks Against Haemonchus Contortus," European Journal of Experimental Biology, vol. 7(1), 2017.

[31] S.K. Kahiro, J.M. Kagira, N. Maina, S.M. Karanja, F.N. Njonge, "Enzymatic Activity of Bromelain from Crude Extracts of Crown, Peels and Stem of Pineapples from Different Agro-ecological Zones of Thika Region, Kenya," Asian Journal of Biotechnology and Bioresource Technology, 2017.

[32] P. Bhatnagar, S. Patnaik, A.K. Srivastava, M.K.R. Mudiam, Y. Shukla, A.K. Panda, K.C. Gupta, "Anti-Cancer Activity of Bromelain Nanoparticles by Oral Administration," Journal of Biomedical Nanotechnology, vol. 10(12), pp. 3558–3575, 2014.

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