Influence of dietary supplementation with *Acacia karroo* on experimental haemonchosis in indigenous Xhosa lop-eared goats of South Africa

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Abstract

The effect of dietary supplementation with fresh *Acacia karroo* on experimental haemonchosis was determined in 4-month-old castrated Xhosa lop-eared kids. The goats were randomly allotted into four treatment groups: non infected, non supplemented (NINS); infected, non supplemented (INS); infected, supplemented (IS) and non infected, supplemented (NIS). Each treatment group contained six goats. Each goat in the infected groups was exposed to a single dose of 6000 freshly cultured *L3 Haemonchus contortus* larvae. The kids in the supplemented groups received 182 g/day (on DM basis) of fresh *A. karroo* leaves, constituting 50% of their diet. Blood samples were collected every two weeks to determine haematology and serum enzyme concentrations. At the end of the experiment at day 60, all the kids were humanely slaughtered to determine the worm burdens. A significant decrease in faecal larval counts (FLC) was observed in the IS group as infection progressed while it continually increased in the INS group after Week 4 of infection. At necropsy, the INS group had significantly (P<0.05) higher worm counts than the IS group (225.5±43.75 versus 25±43.75). Highest packed cell volume (PCV) values were observed in kids in the NIS group. The INS goats had the lowest PCV values. Mean FAMACHA scores for the IS and INS groups were higher (P<0.05) than that of the NINS. Alkaline phosphatase levels (ALP) in the INS group increased as the infection progressed. On the contrary, in all other groups, ALP levels decreased up to Week 4 post infection. Supplemented goats had a significantly (P<0.05) lower mean serum glutamic pyruvic transaminase (SGPT) and serum glutamic oxaloacetic transaminase (SGOT) values than the non-supplemented group. Results from the current study suggested that consumption of fresh *A. karroo* leaves reduced infection level and subsequently the leakage of enzymes into blood. It can be concluded that consumption of fresh *A. karroo* leaves reduced the establishment of *H. contortus* in Xhosa lop-eared goats.

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Packed cell volume
Worm counts
Alkaline phosphatase
Serum glutamic oxaloacetic transaminase

1. Introduction

Sustainable goat productivity cannot be guaranteed in many communal production systems without the effective control of gastrointestinal parasites, especially *Haemonchus contortus*. *H. contortus* is endemic and causes substantial economic losses to resource-poor goat farmers in Sub-Saharan Africa (Bath, 2000; Perry et al., 2002; Torres-Acosta and Hoste, 2008; Vatta et al., 2009). In monetary terms, for example, in Kenya and South Africa annual losses due to this parasite were estimated to be between US$ 26 million and US$ 45 million (Krecek and Waller, 2006). The depressive impact of *H. contortus* on goat productivity derives not only from death and sub-optimal performance of goats, but also from the high costs of drugs for prophylaxis and treatment. The development of anthelmintic resistance also exacerbates the limitations of the use of drugs in the control of *H. contortus* (Jackson and
Coop, 2000; Nnadi et al., 2009). Moreover, use of chemotherapy has become a source of public concern due to the risk of accumulation of residues in meat products (Hoste et al., 2006).

In severe infections, H. contortus causes anaemia manifested by low packed cell volume (PCV) (Yacob et al., 2008), hypoalbuminaemia and often depressed total blood protein content in goats (Ogunsanmi et al., 1994). The damage due to haemonchosis also result in the elevation of serum enzymes such as alkaline phosphatase (ALP), acid phosphatase (ACP), serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) (Sharma et al., 2001). Haematological values and serum biochemistry measures have often been used in predicting infections, monitoring goat health and provide information regarding immunological status, toxicant exposure, and other aspects of physiological functions in goats (Ogunsanmi et al., 1994; Stein et al., 1998; Vatta et al., 2001).

A natural alternative to synthetic anthelmintics for controlling haemonchosis in goats is the use of tannin-rich plants (Min and Hart, 2003; Paolini et al., 2003; Terrill et al., 2009). Many rangelands in the resource poor areas of South Africa are characterized by large thickets of A. karroo shrubs. Although it is considered to be an invasive plant, A. karroo contains large amounts of polyphenols, especially, condensed tannins which have anthelmintic effects on H. contortus. Consumption of Acacia leaf meal has been reported to reduce the establishment and fecundity of H. contortus (Kahiya et al., 2003). The anthelmintic properties of A. karroo have also been established in cattle (Xhomfulana et al., 2009). In addition to its potential anthelmintic activity, A. karroo is a valuable protein source with crude protein of up to 230 g/kg (Nyamukanza and Scogings, 2008) and has often been a major source of feed to browsing goats, especially during the dry season. Although they have been used in dried form in some experiments (Kahiya et al., 2003; Xhomfulana et al., 2009), fresh leaves from forages such as A. karroo are highly palatable and acceptable to browsing goats (Alonso-Diaz et al., 2008; Nyamukanza and Scogings, 2008). Nitrogen degradation is also higher in fresh forms of forages as compared with dry forms (Bonsi et al., 1994; Osuji and Odenyo, 1997).

Although, the potential for manipulating host nutrition to favour the response to nematode infections has been pointed out (Hoste et al., 2006; Marume et al., 2010), and possible synergisms between various nutritional options for helminth control have been explored (Athanasiadou et al., 2009), the question that needs attention is are there any combinative effects of proteins and tannins in A. karroo on haemonchosis in browsing Xhosa lop-eared goats. The present study was, therefore, conducted to examine the effects of consumption of A. karroo leaves on live weight, body condition, haematological and biochemical changes in indigenous Xhosa lop-eared goats experimentally challenged with haemonchosis. The hypothesis tested was that nutritional supplementation with A. karroo reduces the establishment of H. contortus in Xhosa lop-eared goats.

2. Materials and methods

2.1. Study site, experimental design and treatments

The study was conducted at the University of Fort Hare Farm, Eastern Cape Province, South Africa (32.8°S and 26.9°E). Twenty-four castrated 4-month old Xhosa lop-eared goats with an average body weights between 13.5 ± 0.23 kg (mean ± SE) were used. The experiment was a 2 × 2 (infection level × dietary level) factorial arrangement with two levels of nutrition (supplemented versus non supplemented) and two levels of Haemonchus infections (infected versus non infected). The kids were randomly allotted into four treatment groups: non infected non supplemented (NINS); infected, non supplemented (INS); infected, supplemented (IS) and non infected supplemented (NS). Each pen contained six goats. Supplemented kids were fed in individual cages.

2.2. Collection and analysis of A. karroo leaves

Fresh A. karroo leaves were hand harvested each day to feed the goats for 60 days. The fresh leaves were given to the kids from day 0 (day of infection) up to day 60, at the end of the experiment. Harvested leaves were stored in plastic sacks to reduce moisture loss. The leaves were also sprinkled with water to maintain freshness. The goats were fed individually in feeding troughs in cages. A sample of the fresh leaves was obtained and dried for the determination of proximate composition and tannin levels in the leaves. The samples of feed were analyzed for DM, crude protein (CP), crude fibre (CF) and ether extract (EE) according to Association of Official Analytical Chemists (1990). The butanol-HCl assay as described by Giner-Chavez et al. (1997) was done to determine the condensed tannins (CT) while the Folin-Ciocalteau assays described by Terrill et al. (1992b) was performed to determine the total polyphenolic content of the dried A. karroo. The proximate analysis and tannin levels of A. karroo leaves are shown in Table 1.

2.3. Goat management

From birth until weaning at approximately three months of age, the kids were raised indoors. At one month of age, the kids were separated from their dams late in the morning when the dams would be turned out to the pastures for grazing, but were allowed to be with their dams late in the afternoon and overnight for them to feed. At weaning, the kids were grouped together and housed in an open sided barn for a period of 30 days after which they were allocated to one of the four treatments. During that time the kids were offered a basal diet of 460 g/head/day (DM) of Medicago sativa hay (CP, 203 g/kg; CF, 335 g/kg; Table 1) to meet their maintenance and growth requirements for protein and energy.

<table>
<thead>
<tr>
<th>Component</th>
<th>Medicago sativa</th>
<th>Acacia karroo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture content (fresh leaves)</td>
<td>91.5</td>
<td>91.9</td>
</tr>
<tr>
<td>Dry matter</td>
<td>20.3</td>
<td>23.2</td>
</tr>
<tr>
<td>Crude protein</td>
<td>33.5</td>
<td>25.9</td>
</tr>
<tr>
<td>Crude fibre</td>
<td>48.3</td>
<td>50.2</td>
</tr>
<tr>
<td>Neutral detergent fibre</td>
<td>41.2</td>
<td>28.9</td>
</tr>
<tr>
<td>Acid detergent fibre</td>
<td>2.5</td>
<td>3.6</td>
</tr>
<tr>
<td>Ether extract</td>
<td>1.4</td>
<td>2.3</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>0.8</td>
<td>0.08</td>
</tr>
<tr>
<td>Ash</td>
<td>6.9</td>
<td>5.1</td>
</tr>
<tr>
<td>CT (Butanol-HCl assay)</td>
<td>–</td>
<td>2.1</td>
</tr>
<tr>
<td>Total phenolics (Folin Ciocalteau assay)</td>
<td>–</td>
<td>0.5</td>
</tr>
</tbody>
</table>
(80 g/day CP and 5.69 MJ/day ME), according to the National Research Council standards (NRC, 2007). The goats had ad libitum access to water. After they were allocated into the four different treatment groups, the kids in the supplemented groups received an additional 200 g per head per day of fresh A. karroo leaves collected each day for two months. The fresh A. karroo leaves were given to the goats individually in feeding troughs in two equal portions both in the morning and afternoon. The kids in the non-supplemented groups continued to receive 500 g/day of lucerne. A state veterinarian examined the kids every week throughout the trial and also assured that the welfare standards were met.

2.4. Infection protocol

Infective larvae (L3) of H. contortus (95% pure) were obtained from donor sheep infected with a pure strain of nematode at Onderstepoort Veterinary Institute (South Africa). On day 0 of the experiment, each of the goats in the infected groups was exposed to a single dose of 6000 L3 H. contortus larvae according to infection protocols used by Yacob et al. (2008) and Sharma et al. (2001). The experiment was conducted for 60 days.

2.5. Measurements

2.5.1. Body weight and condition scores

After every two weeks, the kids were weighed using the commercial goat weigh scale in the morning at 0800 h, faecal samples collected for faecal cultures and blood samples taken for haematological examination. Concurrently, body condition scores (BCS) were assessed by one researcher throughout the experiment using the 5 point scale (1 = very thin to 5 = obese) (Aumont et al., 1994).

2.5.2. Faecal cultures, larval counts

Faecal cultures were prepared for each kid in each treatment for faecal larval counts (FLC). Approximately 5 g of fresh faecal samples were obtained directly from the rectum of the kids at the beginning of the experiment and fortnightly thereafter. The faecal matter was spread into a plastic pan at less than 3 cm deep. Faecal matter was then covered with peat moss and misted with tap water to a moist consistency and covered with lid. Pans were incubated for 7 – 10 days at 27 °C. The temperature was maintained throughout the experiment to reduce discrepancies. The faecal cultures were then placed into a Baermann apparatus to extract larvae according to the Baermann method (Sloss et al., 1994). Larvae were counted using a dissection scope at 7.5 magnification and averaged over three counts to determine the final concentration.

2.5.3. FAMACHA scores

Parallel to the blood and faecal sample collection, the colour of the ocular mucous membranes of each of the kids was examined and classified into one of five categories according to the FAMACHA® eye colour chart: 1 = red, non-anaemic; 2 = red-pink, non-anaemic; 3 = pink, mildly-anaemic; 4 = pink-white, anaemic; 5 = white, severely anaemic (Bath, 2000).

2.5.4. Haematology

Packed cell volume was measured by the microhaemato-crit method, while the differential leucocyte count (TLC), mean corpuscular volumes (MCV) and mean cell haemoglobin concentrations (MCHC) were determined at the National Health Laboratory Services (NHLS), East London, South Africa.

2.5.5. Serum enzymes

Quantitative estimation of alkaline phosphatase (ALP), serum glutamic pyruvic transaminase (SGPT) and serum glutamic oxaloacetic transaminase (SGOT) were done within 24 h of collection at the National Health Laboratory Services (NHLS) (South Africa). The enzymes were analyzed using a clinical chemistry analyzer (Gilford Impact, 404IE, Ciba Coming Diagnostic Corp., Gilford Systems, Oberlin, OH 44774). Enzyme assays were all performed on an UV-vis spectrophotometer (SPECTORD 50 PC, Analytik Jena AG) using respective commercial kits following the protocol described previously by Ogunsanmi et al. (1994).

2.6. Slaughter procedures

At day 60, all the goats were humanely slaughtered at a local abattoir for worm recovery from the abomasa. A day before slaughter, the goats were deprived of feed for 24 h. Clean water was provided ad libitum. The electrical stunner was used to stun the goats and then slaughtered using standard procedures. Skinning, evisceration and washing procedures were completed while the carcasses were on the overhead rail. The abomasa were removed and tied at both ends for worm recovery at the University laboratory.

2.7. Worm counts at necropsy

At necropsy, the abdominal cavity was opened and abomasa were removed. The abomasa were opened and the contents emptied and washed with tap water into a bucket to make up 5 l. After thorough stirring, a 500 ml aliquot (10%) was collected into labelled plastic bottles and then examined to estimate the size of the worm populations. After settling for 5-6 h, approximately 50 ml was decanted and replaced with approximately 50 ml formalin as a preservative. The washed abomasa of each goat was soaked in water over night at room temperature and re-washed in 5 l of water. Subsequently, soak samples were collected for worm recovery, identification and enumeration (Fakae et al., 1999).

2.8. Statistical analyses

The differences in FLC, PCV, MCV, eosinophil counts, TLCA, MCHC, BCS, body weights and FAMACHA scores, ALP, SGOT and SGPT between the treatment groups were analysed using mixed model procedures for repeated measures of SAS (2003) according to Littell et al. (1996). Faecal larval counts and worm count data were log transformed [ln (x + 10)] and the resulting transformed variables were tested for normality using probability plots, skewness and kurtosis. The transformed data were reported as back-transformed means. Worm count data were analysed using the RANDOM statement in the mixed model procedure. Only INS and IS treatments
supplemented kids maintained body weights between Week 0 and Week 6 post infection, sharply declining thereafter. In all groups, a gradually decline in BCS was observed up to the 4th week post infection coming to a constant thereafter. The decline in BCS was however, more pronounced in the INS group. The INS group had the lowest (P < 0.05) BCS than the other groups throughout the experiment.

3.2. Faecal larval counts

Infection by *H. contortus* was expected to become patent in the infected groups three weeks post infection. Infection, diet and their interactions significantly (P < 0.05) affected faecal larval counts (FLC). An increase in FLC was observed in both infected groups up to Week 4 post infection (Fig. 1). The increase, however, was gradual in the IS treatment group but more pronounced in the INS group. The FLC decreased significantly (P < 0.05) in the IS treatment group after Week 4. On the contrary, FLC in the INS treatment group continued to increase until the end of the experiment.

3.3. Worm counts

At necropsy, the INS group had significantly higher (P < 0.05) worm counts than the IS group (225.5 ± 43.75 versus 25 ± 43.75). Mature worms constituted approximately 97% of the total worm counts at necropsy in the two infected groups.

3.4. FAMACHA scores

Diet and diet × infection interaction significantly (P < 0.05) affected FAMACHA scores. Throughout the study period, FAMACHA score for both the IS and INS groups were higher (P < 0.05) than that of the uninfected group (NINS) (Table 2). The NINS

Figure 1. Mean changes in faecal larval count (FLC), packed cell volume (PCV), body condition score (BCS) and body weight (BWT) of goats in different dietary and infection groups.
Infection, diet and their interaction significantly (P<0.05) affected SGOT values. The infected goats had significantly higher (P<0.05) SGOT values than the non infected group (Fig. 2). The NIS groups had the lowest SGOT values throughout the experiment. In the INS group, a sharp increase in SGOT levels was observed while in the IS group, a gradual increase in SGOT levels was observed after Week 6 post infection.

4. Discussion

The study intended to evaluate the efficacy of the use of fresh *A. karroo* leaves as an alternative approach in the control of haemonchosis in goats. One key element in the use of fresh Acacia leaves as both supplement and anthelmintic are their palatability and acceptability to goats. Goats do not prefer dried leaves (Kahiya et al., 2003). The goats used in the current study had no browsing experience. As such, the high consumption of the Acacia leaves throughout period confirmed the Acacia leaves as highly acceptable and palatable. Various studies also showed similar observation (e.g. Alonso-Diaz et al., 2008; Nyamukanza and Scogings, 2008). *Acacia karroo* is widespread in the drought-prone Eastern Cape province of South Africa and has been one of the major sources of feed for browsing goats. Xhosa lop-eared goats are more of browsers than grazers (Bakare and Chimonyo, 2010). It has higher body frame than other indigenous goats and long front legs making it possible to reach out to leaves of tree branches. Responses to fresh *A. karroo* leaves could be useful in making strategies on its management and control, as it is generally regarded as an undesirable plant that reduces grazing capacity of the veld.

The marked decrease in FLC and low worm counts at necropsy in goats that consumed the Acacia leaves highlight that they possess anthelmintic properties. *A. karroo* leaves contain various types of secondary plant metabolites, including condensed tannins (Hoste et al., 2006; Kahiya et al., 2003). The ability of condensed tannins to bind to proline- and hydroxyproline-rich structures that form the body and internal organs of the nematode and to change their physical and chemical properties makes them potential inhibitors of Haemonchus infections (Anthony et al., 2005; Brunet et al., 2007; Kahiya et al., 2003). In the current study, the reduction in the FLC could have been associated with the tannin-induced reduction in fecundity of the worms. Condensed tannins may interfere directly with the biology of various nematode stages or indirectly, by improving the host nutrition through protection of the diet proteins from ruminal degradations thereby modulating worm biology (Paolini et al., 2003; 2005). Contact with tannin extracts affects the establishment of third-stage larvae, either by disturbing the exsheathment (Brunet et al., 2007) or the association of parasite with the mucosae (Brunet et al., 2008). In addition to the widely held view that Xhosa lop-eared goats are resistant to haemonchosis (Marume et al., 2010), further studies need to be conducted to establish the interaction of genes for resistance and effect of tannins.

The subdued increase in initial body weights in the NIS and IS groups compared to the NINS during the first 2 weeks of the trial was expected. Consumption of high concentrations of condensed tannins (>7% of dry matter) has often been associated with a reduction in feed intake, growth inhibition and interference with the morphology and the proteolytic activity of microbes in the rumen [Min et al., 2003; Waghorn and McNabb,
matophagous nature of worm eggs at the commencement of the experiment. The haemostasis had not been established, indicating that the goats had no infection. However the initial increase in PCV towards was expected. This period coincided with the full establishment of the infection. Therefore, the suppression of infection elicited by the Acacia leaves suggests a combinative effect of tannins and proteins. Similar reports have been made for cattle (Mapiye et al., 2010). In the current study, as the experiment progressed, the initial increase in PCV in the case of NIS and NINS group and the suppression of the infection by the Acacia diet in the case of the IS group. Changes in the levels of MCV, MCH and MCHC are expected to indicate fluctuations in erythropoietic activities due to infection. The infection is manifested by increased blood loss and low levels of haemoglobin in erythrocytes. The leucocyte differential aids in the assessment of the leucocytosis. Leucocytes have an important role in the resistance against nematode infection. In the current study, the TLCA in the IS group were higher than that of the uninfected groups (NINS and NIS).

The decrease in PCV in the infected groups from Week 4 onwards was expected. This period coincided with the full establishment of the infection. However the initial increase in PCV in the IS group is difficult to explain. It is probable that the infection had not been established, indicating that the goats had no worm eggs at the commencement of the experiment. The haematophagous nature of H. contortus is associated with anaemia and, hence, reduction in PCV. In animals, PCV changes are linked to the intensity of H. contortus infection (Marume et al., 2010; Yacob et al., 2008). Goats in the IS group maintained PCV levels, probably due to the suppression of the infection by the condensed tannins in the Acacia leaves. To support the impact of tanniferous forages, goats fed on sericea lespedeza hay showed lower FEC and a sustained PCV after 35 days of infection (Moore et al., 2008; Shaik et al., 2006). High PCV values observed in the NIS group could be due to the effect of the A. karroo leaves on boosting the immune system and providing additional proteins to the goats. The high PCV values in this group are also consistent with the observed high body weights compared to the other groups. High MCV values obtained in the IS, NIS and NINS are consistent with the absence of infection, in the case of NIS and NINS group and the suppression of the infection by the A. karroo diet in the case of the IS group. Changes in the levels of MCV, MCH and MCHC are expected to indicate fluctuations in erythropoietic activities due to infection. The infection is manifested by increased blood loss and low levels of haemoglobin in erythrocytes. The leucocyte differential aids in the assessment of the leucocytosis. Leucocytes have an important role in the resistance against nematode infection. In the current study, the TLCA in the IS group were higher than that of the uninfected groups (NINS and NIS).

FAMACHA scores, PCV, body weight changes and body condition have been used to evaluate subclinical haemonchosis and also resistance of goats to parasites (Baker et al., 1998; Van Wyk and Bath, 2002). In the current study, the FAMACHA scores for the uninfected goats were lower than in infected groups, as expected. However, no significant difference was observed between the IS and NINS groups. On the contrary, the FAMACHA scores for the INS were significantly higher than those of the other three treatment groups. High FAMACHA scores in the INS revealed the effects of the infection. In addition, the high FAMACHA scores observed in this group are consistent with the high FLC, worm counts at necropsy, low body weight gain and low PCV observed in this group. The FAMACHA system has been extensively tested in South Africa with some excellent results and has been used in identifying infected animals for treatment (Kaplan et al., 2004; Van Wyk and Bath, 2002). Its use in goats has been validated by Vatta et al. (2001).

Serum biochemistry measures have often been used in predicting infections, monitoring goat health and provide information regarding immunological status, toxicant exposure, and other aspects of physiological functions in goats (Ogunsanmi et al., 1994; Stein et al., 1998; Vatta et al., 2001). Serum enzyme
levels are likely to change in different clinical conditions (Sharma et al., 2001). Information obtained from serum biochemistry measures in infected and non infected goats can therefore provide excellent basis for judgement with respect to the nature of the disease and the extent of tissue and organ damage (Tibbo et al., 2008). Although serum enzymes are of value in detecting liver diseases, they can also be used to establish some disruptive activities of haemonchosis and other parasites in the organs of their origin or altered membrane permeability through traumatic damage to the lining of the abomasal mucosa (Al-Zubaidy et al., 1987).

The low ALP values in the IS groups compared to the NIS goats suggest the role played by A. karroo supplementation in reducing the damage caused by H. contortus. The ALP values for the IS group were comparable to the NIS and the NINS goats. Generally, the haemolytic activity of H. contortus culminates in the invasion and damage to the abomasal mucosa, causing the enzymes to be leaked into blood. This was confirmed by the high values for ALP observed in the INS group. Similar observations were made by Sharma et al. (2001) in Barbary goats, and in parasitized West African Dwarf goats by Mbuh and Mbwaye (2005). Although protein supplementation of parasitised goats has been shown to reduce the level of haemonchosis (Hoste et al., 2006; Phengvichith and Ledin, 2007), the effect of condensed tannins in forage legumes has received little attention. The ALP in the IS, NINS and NIS were lower than those reported by Mbuh and Mbwaye (2005), but comparable to those reported by Sharma et al. (2001).

Elevated levels of these enzymes normally indicated an increase in haemolytic activities of H. contortus in infected goats (Sharma et al., 2001). The effect of A. karroo on serum biochemical response to haemonchosis is also highlighted by the low SGOT values obtained in the IS group compared with the INS group. Siddiqua et al. (1990), Chakraborty and Lodh (1994) and Sharma et al. (2001) reported an increase in SGOT levels in goats infected with haemonchosis. In the current study, A. karroo reduced the haemolytic activity of H. contortus resulting in low SGOT levels, as also observed with the ALP in the IS group. Serum glutamic oxaloacetic transaminase levels in the infected and non infected goats were also comparable to those reported elsewhere (Mbuh and Mbwaye, 2005; Sharma et al., 2001). Together with acid phosphates, ALP, SGOT and SGPT are some of the enzymes that are used as tools in diagnosis of clinical disease condition.

5. Conclusions

Consumption of fresh A. karroo leaves reduced infection establishment, resulting in an increase in PCV and a reduction in enzyme leakage into blood Xhosa lop-eared goats supplemented with fresh A. karroo leaves. Therefore, A. karroo can be regarded as an important feed resource that mitigates the effects of haemonchosis on goat productivity.

Conflict of interest statement

All authors have ready through the document and had no objections to the publication of the manuscript.


