RESEARCH PAPER

Haemonchus contortus as a model in assessing activity of Citrullus colocynthis fruit extract to control benzimidazole-resistant parasitic nematodes
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ABSTRACT:
The present field and laboratory experimental study were designed to appraise the efficiency of crude aqueous methanol extract (CAME) of Citrullus (C.) colocynthis (bitter apple) fruits in combating benzimidazole-resistant digestive canal nematodes. To meet the requirements of the trial, the highly pathogenic gastric roundworm of ovine, albendazole-resistant Haemonchus contortus was used as a paradigm. The faecal egg count reduction test (FECRT) and egg hatch assay (EHA) revealed prevalence of resistance to the abovementioned dewormer. In reference to the RESO Computer Program, the calculated FECR% was 70.77, whilst the lower confidence interval was 49.7. The LC50 value for albendazole, calculated via probit analysis, was 2.046 μg ml⁻¹ after carrying out EHA. Antinematicidal effectiveness of C. colocynthis CAME was assessed in vivo through administration of four doses (50, 100, 150, and 200 mg/kg B.W) utilizing FECRT as an indication for their efficacies. There was a dose dependent antinematicidal potency of C. colocynthis CAME. The ovicidal activity of the plant extract was also trialed in vitro via conducting EHA. The estimated LC50 value was 0.3422 μg ml⁻¹ after performing EHA, whilst the calculated mean FECR% for the highest dose of CAME was 95.57, which is deemed effective. Moreover, administration of C. colocynthis CAME at 200 mg/kg B.W did not reveal untoward consequences in animals; accordingly, could be used as a substitute remedy in fighting antinematicidal-resistant populations of gastrointestinal nematodes.

KEY WORDS: Haemonchus contortus, bitter apple, benzimidazole dewormer, resistance, alternative therapy

INTRODUCTION:
Antinematicidal resistance (AR) is deemed a major barrier precluding the control of livestock pathogenic roundworms and a serious menace to the maintenance of population-extensive remedy policies being run to restrict rampancy of human parasitic nematodes in the under-developed countries (Waller, 2006; Gilleard, 2013). Unluckily, periodic mass therapy of humans and small ruminants utilizing synthetic antinematicidals, in particular members of benzimidazole antiparasitics has led to development of AR as a result of mutation occurrence in chemotherapeutic targets of nematodes (Beech and Silvestre, 2010; Harhay et al., 2010; Vercruysse et al., 2011). It could be said that attempts to delay prevalence of AR are too late (Waller, 2006). Thus, helminthologists now are satisfied that the conventional strategy based on allopathic (synthetic west drugs) dewormers to control pathogenic nematodes must be changed to be well-matched with the sustainability of best helmint management. In this regard, researchers and professionals in the domain of parasitology have adopted some substitutes such as immunization of vulnerable individuals, balanced foods to boost the defence system and biological control to overcome the dilemma of AR (Waller, 2006; Strain and Stear, 2001; Stear et al., 2007). Having said, these alternative approaches have not
achieved any success pragmatically and still under assessment (Waller, 2006).

On the other hand, under the tent of non-synthetic antiparasitics, ethnobotanicals are currently a promising domain of explorations expected to be reliable substitutes to contain spread of antinematicidal-resistant nematode infestations in the coming decades (Jabbar et al., 2006; Hamad et al., 2018). Thus, *Haemonchus (H.) contortus* parasite, a serious haematophagous gastric dweller nematode of ovine, resistant to the common broad-spectrum drug; benzimidazole was utilized as a paradigm to perform the trial.

Antinematicidal efficacy of aqueous methanol extract of indigenous *Citrullus (C.) colocynthis* fruits against the benzimidazole-resistant helminths were evaluated employing reliable parasitological techniques.

### 2. Materials and Methods

#### 2.1. Selection of experimental animals

Lambs (n=90) with the criteria mentioned below were chosen for the experiment:

Age: 12-24 weeks (Coles et al., 1992).

Dewormer administration record: not have been drenched for the last two months (Coles et al., 1992).

Lower eyelid paleness: checked by FAMACHA Anaemia Guide Chart. Animals in anaemic stage 3, 4 or 5 were selected (Macedo et al., 2010).

Egg per gram of feces (EPG): minimum 150 ova (Coles et al., 1992).

H. *contortus* infestation: >90% among nematode populations (Bowman et al., 2003).

The lambs chosen for the trial were marked and randomly allocated into six groups:

- **Group 1**: Albendazole resistance detection group (n=15)
- **Group 2**: *C. colocynthis* dosage (50 mg kg⁻¹ BW) drenched group (n=15)
- **Group 3**: *C. colocynthis* dosage (100 mg kg⁻¹ BW) drenched group (n=15)
- **Group 4**: *C. colocynthis* dosage (150 mg kg⁻¹ BW) drenched group (n=15)
- **Group 5**: *C. colocynthis* dosage (200 mg kg⁻¹ BW) drenched group (n=15)
- **Group 6**: infested non-drenched group (n=15)

The tentative lambs did not administer any other therapy during the trial period.

#### 2.2. General clinical examinations

The selected animals for the experiment were inspected clinically. The clinical examinations included check of temperature, pulsation, auscultation, skin inspection for external parasites, lymph node checking, oral inspection, alimentary tract status and any abnormality in respiratory system (Kahn, 2005).

#### 2.3. Preliminary tests to detect natural infection with *H. contortus* and other nematodes

- **Faecal examination**
  
  Faecal checks of all the experimental lambs were conducted during the assortment procedure (Soulsby, 1982; Coles et al., 1992; Iqbal et al., 2006a).

- **Coproculture method**
  
  Coprocultures were also carried out to evaluate the contribution of a range of parasitic roundworms in entire natural helminth infections following MAFF (1986) during the choosing procedure. Faecal samples from each group of lambs were pooled and cultured in plastic containers. Amphotericin B (5 μg g⁻¹) was added to prevent mycotic contaminations. The cultures were incubated for seven days at 27±1°C. After this time, the parasite larvae (L₃) were pulled together utilizing Baermann apparatus.

- **Baermann technique**
  
  This assay was performed to collect the parasite larvae (L₃) from the coproculture method. Approximately 15g of the incubated faeces were wrapped up in medical gauze and placed in the Baermann apparatus funnel. Lukewarm water was added to stimulate larval motility to the end of collecting tube. The “Baermann” was set up overnight and a small volume of water was collected and poured in a plastic container. Then the water sample transferred to a petridish, Lugol’s iodine was added to the culture (Iqbal et al., 2006a) and 100 larvae were counted and recognized following MAFF (1986).

#### 2.4. Antinematicidal resistance investigations

- **Fecal egg count reduction test (in vivo assay)**
  
  Albendazole 5%, a member of benzimidazole group and manufactured by Dox-AL ITALIA SpA- Italy, was purchased from market. The lambs (n=15) in group 1 were exposed to albendazole at the standard dosage (5mg kg⁻¹ BW); while group 5 kept as infested untreated control. Coproculture of experimental lambs (group 1 and control) were performed at day 0 (pre-deworming) and day 14 (post-deworming) as mentioned previously. Eggs per gram of faeces (EPG) were counted utilizing Whitlock Universal...
Egg Counting Slide (provided by JA Whitlock & Company, PO Box 51, EASTWOOD NSW 2122 AUSTRALIA). Pre-and post albendazole treatment EPG and contribution of all nematodes in the natural infestations were established. EPG were estimated by Whitlock slide utilizing the following formula:

\[ \text{EPG} = \frac{\text{Total eggs in chamber 1, 2 and 3}}{3} \times \frac{50}{2.5} \]  

(dilution factor)

Faecal egg count reduction percentage (FECR %) was calculated using the formula below:

\[ \text{FECR} \% = \left(1 - \left(\frac{\text{mean EPG treatment}}{\text{mean EPG control}}\right)\right) \times 100 \]

RESO computer program (CSIRO Animal Health Research Laboratory, Private Bag 1, Parkville, Vic. 3052, Australia) was utilized to calculate the FECR data comprising arithmetic mean, variance of counts, FECR% and 95% confidence interval. Pursuant to Coles et al. (1992), resistance is built up if (i) the FECR% is less than 95% (ii) the lower limit of 95% confidence interval is less than 90% (iii) If just one of the two norms is met resistance is suspected. On the other hand, Gill (1996) has recommended that any negative values produced from FECR% and lower limit of confidence interval were deemed equal to zero, interpreting that the resistance is widely prevalent and has reached the serious level.

**Egg hatch assay (in vitro assay)**

This assay was executed according to the standardized protocol that was accepted by World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P) (Coles et al., 1992) to diagnose resistance against albendazole. Briefly, eggs were extracted from faeces, suspended in water and estimated. Albendazole 5% was dissolved in 0.3% Dimethylsulfoxide (DMSO) and stock solution was prepared as 50 µg ml⁻¹. The stock solution was serially diluted (0.0244-50 µg ml⁻¹) in a multiwell plate. The control well received merely 1 ml solvent (0.3%DMSO). One ml (approximately 150 eggs ml⁻¹) of egg suspension was added to each well including control well. Plate was incubated at 27°C ±1 for 48 hours and 70% relative humidity. After incubation, two drops of Lugol’s iodine was added. At least 100 of the unhatched eggs (dead and embryonated) and hatched larvae were counted to calculate the hatching inhibition percentage (Coles et al., 1992). The following formula was used for evaluation of hatching inhibition (%):

\[ \text{Hatching inhibition} \% = \left(\frac{\text{P test}}{\text{P total x}100}\right) \]

P test: number of unhatched or embryonated eggs. P total: number of unhatched or embryonated eggs + Larvae (L₁).

LC₅₀ values were calculated for the eggs by probit analysis. Eggs with an LC₅₀ value in excess of 0.1 µg ml⁻¹ were deemed as an indication of antinematicidal (albendazole) resistance as suggested by Coles et al. (1992).

2. 5. Evaluation of plant extracts against resistant *Haemonchus contortus*

**Extraction of Citrullus colocynthis**

*C. colocynthis* fruits were harvested naturally from Mamzainer plain, Kandinawa district, South of Hawler. After the separation of seeds, the tissues were dried in shade at room temperature. The herbaceous tissues were crushed to a powder using an electric grinding machine. The crushed tissues were solved in 70% aqueous methanol by cold maceration at 25-30°C and the mixed materials were mixed 2-3 times every day by a stirrer. After 72 hours, the materials were filtrated through a piece of porous cloth and the filtrate was collected in a container. The abovesaid procedure was recurred thrice. The extract was evaporated to dryness at room temperature (Gilani et al., 2004). The crude aqueous-methanol extracts (CAME) was kept at 4°C until employed towards the parasitic nematodes.

**Fecal egg count reduction test (in vivo assay)**

The animals in group 2, 3, 4 and 5 were exposed to CAME of *C. colocynthis* at doses; 50, 100, 150 and 200 mg kg⁻¹ BW correspondingly, whilst, group 6 exploited as infested non-drenched control. Fecal examinations and coprocultures of the tentative lambs were performed at day 0 (pre-therapy) and day 14 (post-therapy) as mentioned previously. Pre- and post- CAME therapy EPG and parasite infestations were recorded. FECR % was estimated utilizing the undermentioned formula:

\[ \text{FECR} \% = \left(1 - \left(\frac{\text{mean EPG treatment}}{\text{mean EPG control}}\right)\right) \times 100 \]

**Egg hatch assay (in vitro assay)**

This technique was conducted to assess the inhibitory potencies of different dosages of the CAME on egg hatching of the parasitic nematode. The test was carried out according to the protocol explained by Coles et al. (1992) with slight...
changes by a number of investigators to be appropriate for evaluation of medicinal botanicals (Macedo et al., 2010). One gram of CAME was suspended in 10 ml of 70% acetone and this was deemed as mother solution (100 mg ml⁻¹) which was serially diluted in a 24 multiwell plate. The ova specimens were treated with 12 concentrations (100-0.048 mg ml⁻¹) of the extract. For positive control, 0.025 mg ml⁻¹ of albendazole5% was dissolved in 0.3% DMSO. The well of negative control got merely 1ml of 70% acetone. LC50 was determined utilizing probit analysis. The rest procedure was similar as pursued in studies on antinematicidal resistance.

2.6. Statistical analysis
The mean EPG for resistance detection in treatment and control groups (after 10-14 days of therapy with albendazole 5%) was analyzed through the RESO computer program. The degree of resistance prevalence was evaluated by this program via estimating FECR% and calculating lower limit of confidence interval. The data procured from EHA for different concentrations of albendazole and C. colocynthis CAME to assess their ovicidal efficacy against H. contortus eggs, one way ANOVA was applied using Graph Pad Prizm (version 7). Tukey as multiple comparison test was utilized to compare among doses. All obtained data were expressed as Mean±SE. For computation of LC50 (µg ml⁻¹) at 95% confidence interval for avoiding 50% of egg hatching, probit analysis of LC50 value on the EHA was applied. For analysis of the data recovered from FECRT and evaluation the impact of various doses of C. colocynthis CAME on reduction of EPG, one way ANOVA was applied followed by application of Tukey test for comparison between doses.

3. Results
3.1. Identification of H. contortus infections in experimental animals
The larvae (L3) of H. contortus were recognized following conduction of pre-treatment coproculture and Baermann apparatus (Picture 1).

3.2. Composition of natural roundworm infestations in tentative lambs
The lambs comprised in the trial were principally infested with H. contortus; however, other parasitic nematodes were involved. Pre-treatment coproculture followed by Baermann apparatus demonstrated that H. contortus, through identification of L3, as the main contributor to the EPG (>90%) of the tentative groups of lambs (Table 1 & 2).

![Picture 1 Haemonchus contortus larvae (L3) after performing coproculture and Baermann technique](image)

Table 1 Pre-treatment and post-treatment proportion of Haemonchus contortus larvae (L3) and other nematode species in the lambs chosen for resistance studies based on pooled faecal specimens of group 1 and 6 (control)

| Groups                  | H. contortus L3 (%) | L3 (%) of other nematodes |
|-------------------------|---------------------|---------------------------|
| Group 1 (Albendazole5% group) | 95                  | 5                         |
| Group 6 Control (untreated) | 93                  | 7                         |
### Table 2 Pre-treatment proportions of Haemonchus contortus larvae (L₃) and other nematode species in the lambs chosen for assessment of Citrullus colocynthis based on pooled faecal specimens of group 2, 3, 4, 5 and 6 (control)

| Groups                        | H. contortus L₃ (%) | L₃ (%) of other nematodes |
|-------------------------------|---------------------|---------------------------|
| Group 2 (50mg kg⁻¹ BW)        | 94                  | 6                         |
| Group 3 (100 mg kg⁻¹ BW)      | 93                  | 7                         |
| Group 4 (150 mg kg⁻¹ BW)      | 95                  | 5                         |
| Group 5 (200 mg kg⁻¹ BW)      | 96                  | 4                         |
| Group 6 Control (untreated)   | 92                  | 8                         |

### 3.3. Study of albendazole resistance

**Coproculture**

As apparent from the obtained data of pre-therapy coproculture of pooled faecal specimens of all experimental lambs, the albendazole and control groups had 95 and 93% *H. contortus* infestation, respectively, while infestation with other parasitic roundworms was 5 and 7% for albendazole and control groups, respectively (Table 1). Post-therapy (after 14 days), the albendazole and control groups had 92 and 96% *H. contortus* infestation, respectively, whilst infestation with other parasitic roundworms was 8 and 4% for albendazole and control groups, respectively (Table 1). Accordingly, it is an indication of development of resistance by the parasite against albendazole.

**FECRT**

Mean EPG reduction, FECR% and lower confidence interval 95%, on day 14 post-treatment with albendazole depended on statistical analysis and RESO computer program, were 224.40 (control mean EPG= 767.80), 70.77 and 49.7, respectively. The aforementioned data had confirmed that the resistance was developed against the above drug.

**EHA**

The LC₅₀ of albendazole was calculated to be 2.046 μg ml⁻¹ (range 1.572-2.568), which was in excess to 0.1 μg ml⁻¹ signifying development of resistance towards *H. contortus* (Coles *et al*., 1992). Correlation between influences of various concentrations of albendazole and hatching inhibition (%) via carrying out EHA is demonstrated in figure 1.

### 3.4. Antinematicidal potency of *C. colocynthis* CAME towards resistant *H. contortus*

**Coproculture**

Post-therapy coproculture of pooled faecal specimens of all the lambs (four groups) exposed to different doses of *C. colocynthis* CAME is presented in table 3.

**FECRT**

The results of antinematicidal efficacy of *C. colocynthis* CAME (four different doses) against albendazole-resistant parasite in the tentative lambs naturally infected with the predominant *H. contortus* (>90%) besides comparison between impacts of various doses of CAMEs on the egg reduction mean is statistically analyzed in table 4. There was a significant difference (P<0.05) between all doses. As evident from the procured data of coproculture and FECRT (Table 3 and 4), the two groups (4 & 5) exposed to effective doses *C. colocynthis* CAME (FECR% >80), very few *H. contortus* larvae were detected.
contortus larvae (L₃) and other nematode larvae (group 4) and no larvae of this parasite and other parasitic roundworms (group 5) were detected.

**EHA**
The data obtained from analysis of variance (ANOVA) of EHA regarding ovicidal efficacy of various concentrations of *C. colocynthis* extract through computation of hatching inhibition (%) had demonstrated different effects of various concentrations (dose-dependent ovicidal efficacy) which shown in figure 2. The estimated LC₅₀ was 0.3422 µg ml⁻¹ (range 0.2733- 0.4275) at the level of 95% confidence interval.

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**Figure 1** Correlation between the impacts of various concentrations of albendazole 5% and hatching inhibition (%)
Figure 2 Correlation between the impacts of various concentrations of *Citrullus colocynthis* CAME and egg hatching inhibition (%)

Table 3 Post-treatment proportions of *Haemonchus contortus* larvae (L₃) and other nematode species on day 10-14 in the lambs chosen for assessment with different doses of *Citrullus colocynthis* CAME based on pooled faecal specimens

| Groups                  | *H. contortus* L₃ (%) | L₃ (%) of other nematodes |
|-------------------------|-----------------------|---------------------------|
| Group 2 (50mg kg⁻¹ BW)  | 92                    | 8                         |
| Group 3 (100 mg kg⁻¹ BW)| 94                    | 6                         |
| Group 4 (150 mg kg⁻¹ BW)| poorly recovered      | poorly recovered           |
| Group 5 (200 mg kg⁻¹ BW)| not found             | not found                 |
| Group 6 (control)       | 95                    | 5                         |

Table 4 Egg per gram of faeces and percentages of faecal egg count reduction in animals on day 10-14 post-treatment with different doses of *Citrullus colocynthis* CAME

| Groups                  | Mean EPG±SE     | Mean FECR%  |
|-------------------------|-----------------|-------------|
| Group 2 (50 mg kg⁻¹ BW) | 345.3±14.51C    | 55.07       |
| Group 3 (100 mg kg⁻¹ BW)| 214.2±13.59 BC | 72.13       |
| Group 4 (150 mg kg⁻¹ BW)| 113.2±8.46 AB  | 85.28       |
| Group 5 (200 mg kg⁻¹ BW)| 34.4±8.26 A    | 95.57       |
| Group 6 (control)       | 767.8±72.95 D   | -           |

Means sharing similar letters are statistically non-significant (P>0.05)

4. Discussion

Undeniably, AR has been emerged among parasitic nematodes of livestock in every country around the world (Waller, 2006; Hamad, 2014; Hamad *et al*., 2014; Hamad *et al*., 2017; Hamad *et al*., 2018). This problem has started to threaten public health as well (Beach and Silvestre, 2010; Harhay *et al*., 2010). Having said, benzimidazoles are among the major broad-spectrum antinematicidals that human and animal roundworms have developed resistance against them seriously as compared to other classes of synthetic dewormers (Kaplan, 2004; Vercruysse *et al*., 2011). Hence, in this study, resistance to albendazole was detected among *H. contortus* populations in naturally infected sheep utilizing RESO computer program. The computed FECR% was (70.77), whilst the lower confidence interval was (49.7) which signify that the AR was emerged (Coles *et al*., 1992). LC₅₀ value of albendazole (2.046 μg ml⁻¹) estimated by probit analysis after conduction of EHA also verified development of AR among the populations *H. contortus*. In this regard, Coles *et al*. (1992) have reported that LC₅₀ value higher than 0.1 μg ml⁻¹ is an indication of benzimidazole group resistance. It can be concluded that AR level among the nematode individuals in ovine of the study area is above 25% and the nematodes are resistant to albendazole. It is evident that the AR could not be diagnosed by these traditional parasitological approaches if the resistance level is below 25% among the gastrointestinal roundworm individuals (Martin *et al*., 1989).
The livestock raisers are adapted to drench their animals 3-4 times with benzimidazole annually in the investigation zone (Veterinarian file in the investigation site). In this regard, Barnes et al. (1995) and Waller et al. (1995) have mentioned that recurrent annual administrations of an anthelmintic are closely related to the emergence and rampancy of resistance among gastrointestinal nematodes. This will conducive to incidence of mutation (loss of drug binding) at β-tubulin isotype 1 (drug target) (Beech and Silvestre, 2010).

Although, other factors such as bad quality of dewormers, use of insufficient dosages by owners and storage conditions in developing countries have been incriminated as well (Coles et al., 1995). On the other hand, unavailability of reliable alternatives to synthetic chemotherapeutics nowadays, ethnobotanicals could be appropriate substitutes to control nematodiasis in humans and livestock particularly in under-developed countries (Jabbar et al., 2006; Sindhu et al., 2014). In continuation of previous limited herbological studies, albendazole-resistant $H. \text{contortus}$ individuals were exposed to the indigenous medicinal plant, $C. \text{colocynthis}$ fruit CAME utilizing $in \text{ vivo}$ and $in \text{ vitro}$ assays. Evidently, a number of bioactive phytochemical ingredients from bitter apple fruits such as alkaloids, glycosides, fatty acids, flavonoids and essential oils have been extracted (Rahuman et al., 2008; Hussain et al., 2014). Moreover, $C. \text{colocynthis}$ fruit extracts have been investigated widely for their broad range pharmacological efficacies and medicinal uses, which comprise mosquito larvicidal activity against the early fourth instar larvae of $Culex \text{quinquefasciatus}$ (Diptera: Culicidae) (Rahuman et al., 2008), antibacterial and anticandidal (Marzouk et al., 2009; Rasool and Jahanbakhsh, 2011), antioxidant and anti-inflammatory/analgesic (Saba and Oridupa, 2010) and other activities such as antidiabetic, antilipidemic, anthelmintic, anticancer...etc (Hussain et al., 2014).

It is noteworthy mentioning that the potency of $C. \text{colocynthis}$ fruit extracts has not been studied $in \text{ vitro}$ and $in \text{ vivo}$ against antinematicidal-resistant alimentary tract roundworms of humans and livestock elsewhere, hence it could be said that this study is an original investigation in this field. The FECR% results post-administration of 50, 100, 150, and 200 mg kg$^{-1}$ BW of the CAME were 55.07, 72.13, 85.28 and 95.57 respectively. In this connection, it should be pointed out to the recommendations of W.A.A.V.P (second edition) edited by Wood et al. (1995) proposing that any anthelmintic with FECR% (98) is reckoned highly effective; FECR% (80) and above is effective; whilst FECR% less than (80) is not recommended for employment. Accordingly, the two doses 150 and 200 mg kg$^{-1}$ BW of the extract are effective. The study has also revealed that the highest doses were more efficacious as compared to the lowest doses. This therapeutic activity phenomenon of medicinal plants was reported by many researchers in the field of phytotherapy (Iqbal et al., 2001; Iqbal et al., 2006b; Hamad et al., 2013; Hamad, 2018).

5. Conclusions

In accordance of the results of this research work, it may be extracted that the resistance percentage is above 25% among $H. \text{contortus}$ communities to albendazole in sheep in Khabat district, Hawler governorate, where the study conducted. The most probable reasons behind the prevalence of albendazole resistance in the above area are the random and frequent annual use of benzimidazole members. On the other hand, the effective doses of $C. \text{colocynthis}$ fruit CAME were 150 and 200 mg kg$^{-1}$ BW Consequently which enhanced in reducing EPG with percentage 85.28 and 95.57, respectively., these two doses could be employed in treating sheep carrying benzimidazole-resistant $H. \text{contortus}$ individuals. Furthermore, the EHA has revealed that the CAME of $C. \text{colocynthis}$ fruit can prevent egg hatching but not embryonations.

Acknowledgements

We would like to express our honest gratefulness to Dr. Mohammad Mahmmod Kadim, the director of veterinary dispensary at Khabat district for the generous facilities he offered us through connection with local shepherds for allowing us access to the animals to fulfill our field investigations. Also thanks go to the deanship of college of science for offering the fund to carry out this study.

Conflict of interest

The researchers acknowledge that there is no conflict of interest concerning contents of the present research article.

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