In vivo nematicidal potential of camel milk on *Heligmosomoides polygyrus* gastro-intestinal nematode of rodents

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Summary

Following our previous findings on the *in vitro* anthelmintic effect of camel milk on *Haemonchus contortus*, the current study aimed at investigating its *in vivo* effect. Investigations were carried out using mice infected with *Heligmosomoides polygyrus* which is a parasite commonly used to test the efficacy of anthelmintics. Thirty six Swiss white mice of both sexes aged 5 – 6 weeks old, and weighing between 20 and 25 g were orally infected with 0.5 ml dose of 100, 1-week-old *H. polygyrus* infective larvae (L$_3$). After the pre-patent period, infected animals were randomly divided into 6 groups of 6 animals each. The nematicidal efficacy of camel milk was monitored through faecal egg count reduction (FECR) and total worm count reduction (TWCR). Four doses (8.25; 16.5; 33.0; 66.0 ml/kg body weight (bw)) for fresh camel milk and 22 mg/kg bw for albendazole were studied using a bioassay. Albendazole and 4 % dimethylsulfoxide were included in the protocol as reference drug and placebo, respectively. For all tested doses except 8.25 ml/kg bw, camel milk was effective *in vivo* against *H. polygyrus* reducing both faecal egg count and worm count ($p < 0.05$). The dose 66 ml/kg bw showed the highest nematicidal activity causing a 76.75 % FECR and a 69.62 % TWCR 7 day after initiating the treatment. These results support the possible use of camel milk in the control of gastro-intestinal helminthiasis.
Keywords: Camel milk; Faecal egg count reduction; *Heligmosomoides polygyrus*; Total worm count reduction

Introduction

The impact of gastrointestinal nematode (GIN) infection in small ruminants is linked to clinical signs associated with infection and also to subclinical economic losses (Martinez-Valladares *et al.*, 2015). Compared to other nematodes, *Haemonchus contortus* is one of the most abundant and prevalent gastrointestinal parasites in sheep and goats in Tunisia (Akkari *et al.*, 2013; Rouatbi *et al.*, 2016). The parasite can cause acute disease and high mortality in all categories of livestock. To date, the current mode of control of

gastrointestinal parasitism relies on the repeated use of synthetic anthelmintics in combination with grazing management. However, the frequent use of these anthelmintics over many years leads to the emergence of drug resistant strains of parasites (Miller *et al.*, 2012). Even with optimally timed strategic treatments, this type of control is expensive, requires efficient health delivery systems particularly in remote production areas and, in most cases, is only partially effective (Ademola *et al.*, 2004). Therefore, there is an obvious need for, and significant global interest in the development of alternative improved means of controlling parasitic nematodes

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(Britton *et al.*, 2015). In this respect, identifying therapeutically effective agents from natural products may reduce the risk of toxicity when the drug is used clinically and provide livestock farmers with environmentally friendly, easily accessible and not costly options (Lahlou, 2013).

Milk has shown positive results in controlling gastro-intestinal nematodes. Early reports suggested that milk exerted an anthelmintic effect on existing strongyle infections in foals (Leese, 1943) and on nematode infections in pigs (Spindler & Zimmerman, 1944; Spindler *et al.*, 1944; Shorb & Spindler, 1947). Calves fed entirely on milk had fewer, smaller *H. contortus* infection than calves fed "a normal diet" composed of cow's milk, alfalfa hay and grain (Porter, 1941). In addition, lower numbers of *H. placei, Cooperia* spp. and *Oesophagostomum radiatum* were found in suckled calves than in weaned counterparts (Rohrbacher *et al.*, 1958). Milk proteins, or components associated with these proteins, reduced the motility of both sheathed and exsheathed L_3 *Teladorsagia circumcincta* in *in vitro* and *in vivo* studies performed by Zeng *et al.* (2001; 2003). Camel milk is highly nutritious (Abbas *et al.*, 2013), and also has valuable medicinal and protective properties mainly due to its high concentration of immunoglobulins. Nutritional benefits of camel milk have been reported by several studies which included antihypertensive, hypoglycaemic (Agrawal *et al.*, 2003; 2011) and hypocholesterolaemic effects (Agrawal *et al.*, 2005). Moreover, camel milk is considered as an alternative to bovine milk for children who are allergic to bovine milk (Abusheliabi *et al.*, 2016; Al Haj & Al Kanhal, 2010; El-Agamy *et al.*, 2009). Scientific evidence is also building up that camel milk is unique for its therapeutic properties in terms of antioxidative factors (Kula, 2015), antibacterial (Benkarroum *et al.*, 2004), antiviral and antifungal activities (Yassin *et al.*, 2015; Abdel Galil & Alhaider, 2016).

In a preliminary study, our group demonstrated for the first time the *in vitro* nematicidal effect of camel milk against *H. contortus*, a gastrointestinal nematode of ruminants (Alimi *et al.*, 2016) and we were not aware of any published work investigating the *in vivo* anthelmintic effects of camel milk.

Therefore, the current study aimed to assess the *in vivo* nematicidal potential of camel milk against the rodent nematode, *Heligmosomoides polygyrus*. *H. polygyrus* belongs to the superfamily *Trichostrongiloidea* as do most nematodes of veterinary importance (Githiori *et al.*, 2003a; 2003b) and its biological cycle is easily maintained in the laboratory mouse (*Mus musculus*). *H. polygyrus* is a standard experimental model used for routine screening of potential drug candidates (Githiori *et al.*, 2003a).

Materials and Methods

Experimental Animals
Mice
Albino Swiss mice (*n* = 36), of both sexes age 5 to 6 weeks and weighing between 20 to 25 g, were used. Animals were obtained from the animal house of the Higher Institute of Biotechnology of Beja (University of Jendouba, Tunisia). Mice were housed in polypropylene cages with steel wire tops in an air conditioned room (22 ± 1 °C, 45 – 75 % relative humidity) maintained in a controlled atmosphere of 12 h light/12 h dark cycle. Food and water were provided *ad libitum.*

Helminth parasite
Infective third stage larvae (L_3) of *H. polygyrus* were generously provided by Dr. Rick Maizels, University of Edinburgh, UK. The parasite was cultured from the egg to L_3 stage in Petri dishes containing wet filter paper. Briefly, egg-containing faecal materials were macerated in the wet filter paper and incubated till they hatch into the first larval (L_1) stage, which underwent several stages of moulting before emerging as the third stage infective larvae (Adiel *et al.*, 2013).

Table 1. Faecal egg count (FEC) and % reduction of FEC at days 3, 5 and 7 after treatment with 4 % dimethylsulfoxide (DMSO), albendazole and different doses of camel milk.

Group	Dose	D_0	D_3	D_5	D_7
DMSO (4%)	-	11000 ± 9899	19000 ± 4242 (0.0)	16590 ± 1254 (0.0)	23870 ± 2440 (0.0)
Albendazole (mg/Kg bw)	22	26500 ± 2687	6161 ± 1328[c] (67.57)	13275 ± 4631 (19.98)	2940 ± 226[c] (87.68)
Camel milk (mL/Kg bw)	8.25	6403 ± 855	15111 ± 5727[a] (18.42)	13360 ± 1966 (19.83)	19420 ± 3012[a,b] (18.73)
	16.5	8705 ± 714	11187 ± 2022 (42.11)	12325 ± 318 (25.32)	15440 ± 636[a,b] (34.23)
	33	14300 ± 990	13203 ± 424 (30.79)	11905 ± 1124 (28.24)	10180 ± 318[a,b] (56.57)
	66	16910 ± 523	8022 ± 566[b] (57.78)	5350 ± 424[b] (67.75)	5550 ± 537[a,b] (76.77)

[a] p<0.05 comparison with positive control group (Albendazole)
[b] p<0.05 comparison with negative control group (DMSO 4 %)
[c] p<0.05 positive control group *vs.* negative control group

Preparation of the Albendazole solution

For the reference drug, albendazole (99.8 % pure standard reference, Médivét, S.A., Tunisia), 50 milligrams were diluted with 0.8 ml of DMSO and then distilled water was added to obtain the final volume of 50 ml. The obtained solution had a concentration of 1 mg/ml. A unique dose of 22 mg/kg bw of albendazole was administered. The 4 % DMSO was used in the *in vivo* assay as placebo (negative control) (Yondo *et al.*, 2013).

Camel milk

For 6 consecutive days in November 2016, camel milk samples were collected early morning from a camel farm located in the district of Sidi Bouzid (central Tunisia). Milk was recovered by hand milking. Samples were collected in sterile screw bottles and kept in cooling boxes (4 °C) until transported to the laboratory for immediate use.

Experimental design

Animals were screened for helminth parasites and subsequently treated with 7.5 mg/kg bw of albendazole to eliminate any roundworm infection. Then, the mice were randomly allocated into cages and allowed to acclimatize for 1 week. They had access to food and water *ad libitum.* In all studies, a dose of ≈100, 1-week-old *H. polygurus* infective larvae (L_3) was used to infect the mice, contained in 0.5 ml of distilled water. The mice were infected orally using an oral gavage needle (0.6 × 0.9 mm).

After the pre-patent period (9 to 11 days) (Smyth, 1996), infected mice were randomly allocated into 6 groups of 6 individuals each and treated as follows:

Group **1**: 4 % DMSO (negative control);

Group **2**: 22 mg of Albendazole kg^{-1} bw (positive control);

Group **3**: 8.25 ml of camel milk kg^{-1} bw;

Group **4**: 16.5 ml of camel milk kg^{-1} bw;

Group **5**: 33 ml of camel milk kg^{-1} bw;

Group **6**: 66 ml of camel milk kg^{-1} bw;

Groups 3 to 6 were treated orally with fresh camel milk at the different studied doses for 6 consecutive days (Day 9 to Day 14), while group 2 was treated with a single dose of albendazole (22 mg/kg bw) as standard anthelmintic (positive control) on Day 9.

Mice faecal samples

From Day 9 to Day 16 mice were isolated in individual cages to collect faecal pellets. For each mouse, a sample of faecal material was collected early in the morning before administration of the treatment on Days 9, 12, 14 and also on Day 16. Faecal pellets were immediately collected with a teaspoon, and placed in labelled Petri dishes containing 0.5 – 1 ml distilled water to prevent faecal materials from drying out. Faecal egg count was calculated as eggs per gram (EPG) of the faecal material according to the McMaster technique (Thienpont *et al.*, 1979). In brief, 2 g of this specimen was weighed, homogenized in a porcelain mortar and sus-

pended in 60 ml saturated salt solution (0.4 % NaCl) (Thienpont *et al.*, 1979). Aliquots were mixed thoroughly with a Pasteur pipette and an equal volume of the suspension was introduced quickly under each of the two McMaster chambers (Hawksley, England) and viewed under a light microscope (10 x magnification).

The EPG was calculated according to the equation: (number of eggs counted x total volume)/ (volume counted x weight of faecal material).

The faecal egg count reduction (FECR) was determined by the following formula (Coles *et al.*, 1992): FECR (%) = 100 (1- T/C); T: means of FEC in the treated groups; C: means of FEC in the control groups.

Worm recovery

On Day 16 (8 days after the start of the treatments), mice were humanely euthanized using chloroform, and the body cavity was opened to remove the small intestine. This organ was placed in labelled Petri dishes containing 20 – 30 ml of distilled water and opened longitudinally with small scissors. The intestine was passed through the arm of a small forceps and the exudate containing parasites was washed in water (Githiori, 2004). The percentage of total worm count reduction (TWCR) was calculated by the method described by Enriquez (1993): TWCR (%) = 100 × (Total worm count in control group - Total worm count in treated group)/ Total worm count in control group.

Statistical analysis

The statistical analysis was done using STATVIEW v.5.0.1 software (SAS Institute, Cary, NC). The comparisons of means for FEC and TWC were done using analysis of variance (ANOVA) followed by Fisher's PLSD and all data were reported as mean± standard deviation. Differences were considered to be statistically significant when the p-value was less than 0.05.

Ethical Approval and/or Informed Consent

Mice were housed and maintained in a pathogen-free environment at the Department of Comparative Medicine. All experiments were performed according to the protocol No (NIH publication 86-23 revised 1985) USA, approved by (National Ethic Committee of Tunis University) IACUC.

Results

Faecal egg count reduction (FECR)

At D_0 of the administration of the treatment (corresponding to Day 9 of the experiment), mean FEC varied from 6403 ± 855 to 26500 ± 2687 (Table 1). For the lowest two doses of camel milk (8.25 and 16.5 ml/kg bw) and for the negative control (4 % DMSO) group, the mean FEC increased throughout the treatment period. This increase was highly significant (p < 0.05) when compared with groups that received fresh camel milk and albendazole. Treat-

Table 2. Mean worm intensity and % reduction of TWC at day 7 after treatment with 4 % dimethylsulfoxide, albendazole and different doses of camel milk.

Group	Dose (mg/kg)	Mean worm intensity after treatment ± standard deviation	% reduction of total worm count (TWCR)
DMSO	-	79.33±12.5[a]	0
Albendazole (mg/kg bw)	22	19.25±4[b]	**75.95**
Camel Milk (mL/kg bw)	8.25	70 .5±7.8[a,c]	**11.39**
	16.5	61.8 ±10.7[c]	**22.78**
	33	37.8±4.26[d]	**53.16**
	66	24.33±4.1[b]	**69.62**

a, b, c, d, numbers with the same letter in the same column are not significantly different at p < 0.05.
TWC: total worm count; TWCR: total worm count reduction; DMSO: dimethylsulfoxide

ment with albendazole was associated with a significant reduction in FEC (p < 0.05) starting day 3 post treatments, but this reduction was not significant on day 5. In this assay, albendazole was more active in comparison to the tested camel milk, but this commercial anthelmintic failed to show complete effectiveness (87.68 %) in infected mice. The dose rate 66 ml/kg bw for camel milk showed a nematicidal activity of (76.75 %). FECR was dose dependent.

Effects of camel milk and albendazole on the parasitic intensity of the nematode/Total worm count reduction (TWCR)
Albendazole was the most effective, causing a reduction of 75.95 % in TWC, while camel milk produced 69.62 % reduction at 66 ml/kg bw (Table 2). Results from albendazole and the highest dose of camel milk were not different (p > 0.05). Reduction of the TWC by camel milk was dose dependent (Table 2); the lowest dose rate (8.25 ml/kg bw) was associated with a TWC not different from the negative control (4 % DMSO), i.e. 0 % of TWCR (Table 2).

Discussion

In a preliminary study, *in vitro* tests have been undertaken and camel milk showed a nematicidal effect against *H. contortus,* a gastro-intestinal nematode of sheep, reducing egg hatching and adult worm motility by 100 % at a concentration close to 100 mg/ml (Alimi *et al*., 2016). The current study was performed to validate the anthelmintic activity of camel milk *in vivo* using *H. polygyrus.*
Our study revealed that, fresh camel milk significantly reduced the FEC and the TWC of *H. polygyrus*. This activity was more visible at the dose 66ml/kg bw by day 7 post-treatment, and resulted in a 76.75 % reduction of FECR and 69.62 % reduction of TWCR. This activity was dose and time dependent. We thought that, camel milk affect both the reproduction system of the worm and the infra-population. Also, our findings clearly demonstrated a reduction of parasite burdens in mice receiving camel milk; the reduction being evident 3 days after the start of the treatment. In fact, this reduction

in egg count is an indication of reduced fecundity.
The possible explanation for such a decrease may be attributed to high amounts of proteins and peptides such as lysozyme (LZ), lactoferrin (LF), lactoperoxidase (LP), short peptidoglycan recognition protein (PGRP) all present in camel milk (Zeng *et al.*, 2001; 2003).
Camel milk is gaining popularity because of scientific reports of its high nutritional qualities and therapeutic value (Abusheliabi *et al.*, 2016). As such, camel milk composition has been widely studied throughout the world (Abbas *et al.*, 2013; Abu-Lehia, 1989; Alimi *et al.*, 2016; Asres & Yusuf, 2014; Konuspayeva *et al.*, 2009; Yadav *et al.*, 2015). The findings of the present study confirm the therapeutic activity of fresh camel milk on *H. polygyrus*, a nematode parasite infecting mice.
There are unfortunately no similar results in the literature using camel milk with which our results can be compared. Nevertheless, studies in sheep (Zeng *et al.*, 2001), cattle (Rohrbacher *et al.*, 1958; Satrija *et al.*, 1991), rabbits (Rohrbacher *et al.*, 1958), horses (Leese, 1943), and pigs (Shorb & Spindler 1947) have all demonstrated lower worm burdens in young mammals fed milk than in those weaned to solid feed or grass. Nevertheless, none of the previous studies tested camel milk.
Arguments to support the involvement of various components in milk have been adduced in some previous work; such benefits could accrue through a direct effect of milk on the nematode or indirectly through enhancement of the host immune response or of host resilience to the pathological effects of infection (Zeng *et al.*, 2003).
Direct effects could operate through specific effects of milk components, for example, of oligosaccharides on the adhesion of pathogens to host mucosa (Hakkarainen *et al.,* 2005), or of milk proteins and components associated with milk proteins on motility of nematode larvae (Zeng *et al.* 2003). However, indirect effects could operate via the superior amount and quality of proteins supplied by milk, which are protected from degradation in the rumen by the

esophageal groove reflex, promoting greater or more rapid development of host immunity or greater host resilience to the pathogenic effects of infection; such effects were tested and confirmed in earlier works (Bown *et al.* 1991; Sykes & Coop, 2001).

Another indirect effect which has been put forward regarding the resistance to parasitism of milk-fed animals is the high pH of milk which was suggested to protect against nematodes. Indeed, high pH of milk has been suggested as a possible contributing factor to low worm burdens in milk-fed calves (Rohrbacher *et al.*, 1958) and is involved in increasing gut motility, hence causing expulsion of nematodes from skim-milk-fed pigs (Spindler *et al.*, 1944).

With regards the more specific anthelmintic effect of camel milk, Agrawal *et al.* (2002; 2005) put forward the hypothesis that high content of lactoferrin in camel milk, acts as a prebiotic having a strong physiological activity in the gastrointestinal tract. It has also been suggested that lactoferrin possesses antiparasitic activity towards a broad spectrum of species, such as *Pneumocystis carinii*, *Toxoplasma gondii* and *Tritrichomonas vaginalis*. (Cirioni *et al.*, 2000; Omata *et al.*, 2001). The antiparasitic effect of lactoferrin is predominantly linked to iron sequestration and destabilization of the parasite membrane (Elbarbary *et al.*, 2014).

The anthelminthic effects of camel milk may also be attributed to its antioxidant activity (Al-Humaid *et al.*, 2010). Camel milk possesses high levels of vitamins (B_2, C, and E) and is rich in mineral content (sodium, potassium, copper, magnesium, and zinc) (Al-Humaid et al., 2010; Nagy et al., 2013). Camel milk concentration in vitamin C is 3 to 5-fold higher than in bovine milk (Haddadin *et al.*, 2008; Salwa & Lina, 2010) and beyond its nutritional role; vitamin C exerts a powerful antioxidant activity (Abdel Galil *et al.*, 2016). In addition, the high minerals content in camel milk (Nagy *et al.*, 2013) may act as antioxidant, and thereby removes free radicals (Powell, 2000; Kumar *et al.*, 2015).

Conclusion

This study has demonstrated the *in vivo* anti-parasitic effect of camel milk using the intestinal parasite *H. polygyrus* and its monogastric host the mouse with an observed reduction of faecal egg count by over 76 %. Our findings are backed by previous results from our laboratory on the *in vitro* anthelmintic effects of camel milk on *H. contortus.* While the *in vivo* anthelmintic effects of camel milk needs to be proven using ruminant species, current results may have important implications for the control of gastrointestinal parasites. Additional work is suggested (i) to identify camel milk components responsible of reducing the parasite burden, (ii) to elucidate their mechanism of action and (iii) to test their efficacy against a broader spectrum of helminth classes like trematode, cestode and nematodes.

Conflict of Interest

All authors declare no conflict of interest.

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