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REGULAR ARTICLES

Anthelmintic effects of *Salix babylonica* L. and *Leucaena leucocephala* Lam. extracts in growing lambs

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Abstract Twenty Katahdin×Pelibuey crossbreed male lambs, 3 to 4 months of age and 24±0.3 kg of body weight, were used to study the anthelmintic effects of administering extracts of *Salix babylonica* L. (SB) and *Leucaena leucocephala* Lam. (LL). Lambs had not been treated with anthelmintics previously and were randomly allocated into four groups of five lambs each in a completely randomized design. Treatments were as follows: control (lambs fed on total mixed ration without extracts), SB (as control plus *S. babylonica* L. extract at 30 ml/day), LL (as control plus *L. leucocephala* Lam. extract at 30 ml/day), and SBLL (as control plus 30 ml/day of *S. babylonica* L. and *L. leucocephala* Lam. extracts in a 1:1 (*v*/*v*) mixture) for 63 days. Extracts were orally administered before the 8:00 a.m. feeding to each lamb. Rectal fecal samples were collected from each lamb at day 22 (P1), day 43 (P2), and day 63 (P3) of the experiment. Adult worm and egg counts were determined in each fecal sample immediately after collection. Plant secondary metabolites of total phenolics, saponins, and the aqueous fraction were 50 % lower in the SB versus LL extracts. Overall, the oral administration of extracts has improved the egg and worm count reductions in lamb feces by 54, 47, and 40 % for LL, SB, and SBLL, respectively, versus the control lambs. Reductions of worm egg counts in lamb feces were higher ($P<0.05$) in P2 and P3 versus P1. Extracts of SB and LL or possibly isolated bioactive compounds could be a promising alternative to conventional anthelmintics to treat gastrointestinal parasites of small ruminants. Such treatments could be used in control strategies against gastrointestinal nematodes in organic and conventional production systems.

Keywords Anthelmintics · Lambs · Plant extracts

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Introduction

In Mexico, small ruminant production has important social and economic functions, especially for small rural farmers who rely on these animals as a source of food and income. Production efficiency of ruminants has been limited by nutritional and sanitary problems with helminth infections being one of the major health conditions affecting humans and livestock. Exposure to nematode parasites depends on the husbandry system under which livestock are raised. In situations where Mexican farmers are almost entirely dependent on grazing, exposure to nematode larvae is continuous throughout the year.

Gastrointestinal nematode parasitism is a major cause of sheep and goat mortality in tropical Mexico (Canul-Ku et al. 2012) and other tropical countries (Carvalho et al. 2012). The impacts of gastrointestinal nematode parasitism in practical systems has stimulated research into alternative medications, such as medicinal plants or tree leaf extracts, which are used in ruminants, donkeys, camels, and humans (Wabo-Pone et al. 2009; Carvalho et al. 2012).

Naturally produced plant anthelmintics offer possible alternatives which may be sustainable and environmentally acceptable while reducing importation of drugs and boosting economic self-reliance. However, secondary metabolites (i.e., tannins,

alkaloids salts, saponins, lectins) of plant extracts are principal factors to parasite paralysis and/or their death in ruminants (Salem et al. 2011b; Carvalho et al. 2012). The mechanism of action of plant metabolites in crude extracts as anthelmintics is not completely clear, but these metabolites could cause motility inhibition, paralysis, or death of the worms in the gastrointestinal tract of ruminants.

Numerous plants are used by pastoralists and smallholder farmers as deworming agents for livestock. Engel (2007) reported that *Vernonia amygdalina* contains seven steroid glycosides as well as four sesqueterpene lactones which could kill the parasites which cause schistosomiasis, malaria, and leshmaniasis. Wynn and Fougere (2007) acknowledged that the mechanisms of action of plant metabolites may be additive, synergistic, or antagonistic by acting at a single or multiple target sites. *Myrsine africana* L. and *Rapanea melanophloeos* L. have been used as anthelmintics in humans and livestock (Desta 1995), while *Azadirachta indica* is widely used in most tropical countries against a variety of ailments, including internal parasites, in both animals and humans (El-Kamali and El-Khalifa 1999).

Salem et al. (2011b) detected 60 chemical constitutes that may have anthelmintic effects in ruminants, formed by a mixture of *Salix babylonica* L. extract with *Leucaena leucocephala* Lam. extract, and these were very different from those in individual extracts of both species. There is a little information on the use of *S. babylonica* L. or *L. leucocephala* Lam. (Kabore et al. 2012; Ríos-de Álvarez et al. 2012) extracts as anthelmintics in ruminants. Our aim was to evaluate the potential anthelmintic impacts of *S. babylonica* L. or *L. leucocephala* Lam. extracts, or their (1:1, *v*/*v*) mixtures, on some parasite species in growing lambs.

Material and methods

Animals, treatments, and housing

Twenty Katahdin×Pelibuey crossbreed male lambs 3 to 4 months of age and 24±0.3 kg of body weight were used in a completely randomized design experiment. Lambs had not been previously treated with anthelmintics and were randomly allocated into four experimental groups. After 2 weeks of adaptation consuming a total mixed ration (TMR), lambs were weighed and distributed individually into four treatments of five lambs each: control group TMR (ingredients and composition in Table 1), SB group (as control plus *S. babylonica* L. (SB) extract at 30 ml/day), LL group (as control plus *L. leucocephala* Lam. extract at 30 ml/day), and SBLL group (as control plus 30 ml/l of *S. babylonica* L. and *L. leucocephala* Lam. (LL) extracts in a 1:1 (*v*/*v*) mixture).

Extracts of *S. babylonica* and *L. leucocephala* contained in g/kg DM of leafs: 9.6 and 24.8 total phenolics, 12.8 and 13.2 saponins, 76.8 and 116.5 aqueous fraction of lectins, polypeptides, starch (Cowan 1999), respectively.

Table 1 Ingredients and chemical composition of the growing lamb's diet[1]

Diet	g/kg DM
Ingredient composition	
Soyabean, meal	220
Alfalfa, hay	150
Sorghum, grain	550
Fish, meal	35
Mineral/vitamin, premix[2]	25
Salt	20
Chemical composition	
Organic matter	911.3
Crude protein	218.6
Ether extract	119.0
Neutral detergent fiber(om)	141.2
Acid detergent fiber(om)	59.1
Lignin(sa)	21.0

[1] Adapted from Salem et al. (2011a)

[2] Mineral/vitamin premix (25) (Vitamin A (12 000 000 IU), Vitamin D3 (2 500 000 IU), Vitamin E (15 000 IU), Vitamin K (2.0 g), Vitamin B1 (2.25 g), Vitamin B2 (7.5 g), Vitamin B6 (3.5 g), Vitamin B12 (20 mg), Pantotenic acid (12.5 g), Folic acid (1.5 g), Biotin (125 mg), Niacin (45 g), Fe (50 g), Zn (50 g), Mn (110 g), Cu (12 g), I (0.30 g), Se (200 mg), Co (0.20 g)

A weekly stock volume (2 l each) of individual extracts as well as their 1:1 mixture was prepared for daily administration. The 1:1 extract mixture was also prepared weekly by mixing the SB and LL extracts (50:50, *v*/*v*) before administration to the animals. Lambs in the four groups were fed ad libitum a TMR formulated to meet all of their nutrient requirements (NRC 1985). Extracts were orally administered daily before the 8:00 a.m. feeding to each lamb. Samples of rectal feces were collected from each animal at day 22 (P1), day 43 (P2), and day 63 (P3) of the experiment for identification of worms and eggs of parasite species. Freshwater was always available.

Preparation of extracts

Extracts of SB and LL leaves were prepared according to Salem et al. (2011a). Briefly, tree leaves were collected randomly from several young and mature trees during summer, chopped into 1- to 2-cm lengths, and immediately extracted at 1 g leaf/8 ml of solvent mixture. The mixture of solvents contained 10 ml methanol, 10 ml ethanol, and 80 ml distilled water. Plant materials were individually soaked and incubated in solvent in the laboratory at 25 to 30 °C for 48 to 72 h in closed jars of 20 l. After incubation, the jars were heated at 30 °C for 1 h and then immediately filtered. Filtrates were

collected and stored under 4 °C for use in the daily oral administrations to the lambs.

Parasitology test

Fecal samples from each lamb within each experimental group were collected rectally in the morning before feeding, with ova counted using the McMaster procedure (Ojeda-Robertos et al. 2008), at day 22 (P1), day 43 (P2), and day 63 (P3) of the experiment. These samples were evaluated for the presence of worm eggs by a salt floatation technique (MAFF 1979), and the eggs were counted by the McMaster method. Fecal pellets were collected and weighed, and 60 ml of saturated salt solution was added per gram of feces. The pellets were broken up using a mechanical stirrer and then strained in a sieve with an aperture of 250 μm. Ten milliliters of the strained solution was used for the determination of fecal egg counts using a two-chamber McMaster slide with a limit of detection of 200 eggs per gram of feces. Identification of nematode eggs in the feces was done according to MAFF (1979).

Individual contents of feces were prepared with up to 500 ml of saturated salt solution. Five aliquots of fecal content (1 g of fresh feces) from each lamb were used to identify the worm species in the subsample by counting using a stereoscope (×40). Fecal cultures were prepared in each experimental period as five replicates of pooled samples from each lamb as described by Terrill et al. (2004) to allow counting and identification of parasite nematode larvae to species. The average egg accounts and identified worms from each lamb within each experimental treatment were used for statistical comparisons among experimental groups.

Diet and secondary metabolite analysis

Samples of TMR were analyzed according to the AOAC (1997) for DM (#934.01), ash (#942.05), N (# 954.01), and ether extract (#920.39). Neutral detergent fiber (Van Soest et al. 1991), acid detergent fiber, and lignin (AOAC 1997, #973.18) analyses used an $ANKOM_{200}$ Fibre Analyzer unit (ANKOM Technology Corporation, Macedon, NY, USA).

As described in Salem et al. (2011a), secondary metabolites were determined in each plant extract (i.e., LL and SB). Extracts, 10 ml, were fractionated by funnel separation with a double volume of ethyl acetate to determine total phenolics by drying and quantifying total phenolics layer in the funnel. After total phenolics separation, a double volume of *n*-butanol was added to fractionate saponins. The remaining solution was considered to be the aqueous fraction.

Statistical analyses

The experimental design was completely randomized with repeated measures in time, where lambs were the experimental units. Data were analyzed using the MIXED procedure of SAS (2002) for repeated measures (Littell et al. 1998). The structure of the variance–covariance error matrix employed was unstructured, based on Bayesian criteria observed with several alternative structures. Terms in the model were diet (i.e., control, SB, LL, SBLL), experimental period (i.e., P1, P2, P3), and diet×period, with lamb (lambs 1 to 5 within each treatment) included as random effects. The repeated term was period, with lamb within diet the subject. Results reported in tables and in text are least square means of fixed effects with their corresponding standard errors. Tests of simple effects were used to partition (slice) interaction effects by diet in order to test the effects of period separately for each diet (
SAS 2002). The statistical model used for the analysis was

$$y_{ijk} = \mu + d_i + a(d)_{j(i)} + p_k + (dp)_{ik} + \varepsilon_{ijk}$$

where y_{ijk} is the value measured at period k on the jth lamb assigned to the ith diet (extract), μ is the overall mean effect, d_i is the ith fixed diet (extract) effect, $a(d)_{j(i)}$ is the random effect of the jth lamb within the ith diet, p_k is the fixed kth period (time) effect when the measurement was taken, $(dp)_{ik}$ is the fixed interaction effect between diet and period, and ε_{ijk} is the random error associated with the jth lamb assigned to the ith diet at period k.

Results

Secondary metabolites (i.e., total phenolics, saponins, and aqueous fraction) of individual extracts were 50 % lower in SB versus LL extracts. There were no extract×experimental period interactions for any worm species. Oral administration of extracts improved the reduction of identified worm species, overall, in lamb feces by 54, 47, and 40 %, respectively, for LL, SB, and SBLL. The reduction of all identified adult worms was much higher ($P<0.05$) in P2 and P3 versus P1 except *Bonostomum* sp., *Strongiloides papillosus*, and *Nematodirus spathiger*. The potential anthelmintic impact differed among identified adult worms. Extracts of SB, LL, or SBLL reduced ($P<0.05$) *N. spathiger* by 100 % while individual extracts of SB and LL reduced ($P<0.05$) worms of *Nematodirus battus* by 82 % compared to the SBLL extract or control lambs (Table 2).

An extract×experimental period interaction ($P<0.01$) of worm egg reduction only occurred for *S. papillosus* (SB (highest reduction)>LL=SBLL>control (lowest reduction), $P<0.05$). Eggs of *Strongiloides* sp., *S. papillosus*, and *Eimeria* were lowest ($P<0.05$) in P2 and P3 than in P1. Individual extracts improved (SB>LL, $P=0.05$) the egg count reduction of *Strongiloides* sp., *S. papillosus*, and *Eimeria* (SB>LL, $P<0.05$), while the reduction was higher ($P<0.05$)

Table 2 Effect of *S. babylonica* L. and *L. leucocephala* Lam. extracts as well as their mixture (1:1, *v/v*) on the identified worm species and egg and larvae counts per gram of feces in growing lambs after 22 (P1), 43 (P2), and 63 (P3) days of the experiment ($n=5$ lambs)

	Extract				SEM	Period			SEM	*P* value		
	Control	SB	LL	SBLL		P1	P2	P3		Extract	Period	Extract×Period
Worm species counts												
Haemonchus sp.	27.2 a	13.4 c	19.4 b	22.8 ab	1.77	25.9 a	19.9 b	16.3 b	1.53	0.001	0.007	0.855
Ostertagia sp.	12.3 a	8.4 b	6.7 b	8.7 b	0.88	11.9 a	8.6 b	6.6 b	0.76	0.001	0.002	0.085
Oesophagostomum spp.	11.0 a	8.2 b	7.6 b	8.6 b	0.75	10.8 a	8.8 b	6.9 c	0.65	0.019	0.001	0.438
Cooperia sp.	8.0 a	5.2 b	4.1 b	5.3 b	0.62	7.6 a	6.4 a	3.0 b	0.54	0.002	<.001	0.068
Bonostomum sp.	4.8 a	3.8 a	2.1 b	3.7 a	0.49	4.0	3.9	2.8	0.42	0.007	0.117	0.989
N. battus	6.0 a	1.1 b	1.7 b	4.8 a	0.59	4.5 a	3.3 ab	2.4 b	0.51	<.001	0.026	0.967
Chaberita sp.	5.4 a	3.6 b	4.0 b	4.3 ab	0.40	4.9 a	4.8 a	3.3 b	0.34	0.017	0.006	0.799
S. papillosus	5.2 a	3.1 b	2.1 c	2.1 c	0.27	3.6 a	3.0 ab	2.8 b	0.23	<.001	0.074	0.275
N. spathiger	0.33 a	0.00 b	0.00 b	0.00 b	0.100	0.08	0.08	0.08	0.08	0.040	1.000	1.000
Egg and larvae counts												
Strongiloides sp.	66.3 a	40.0 c	49.9 bc	55.4 ab	4.93	62.9 a	48.9 b	46.9 b	4.27	0.008	0.028	0.855
S. papillosus	28.8 a	13.9 c	26.9 b	26.2 b	2.45	28.0 a	24.5 ab	19.3 b	2.12	0.001	0.027	0.002
Trichuris spp.	11.1	9.0	8.2	7.9	4.39	16.1	13.3	12.8	3.80	0.231	0.804	0.996
Nematodirus sp.	17.6	17.3	17.6	15.4	2.56	21.8 a	16.7 ab	12.5 b	2.21	0.922	0.024	0.165
D. filaria (larvae)	19.9 a	18.6 a	17.7 a	5.4 b	1.52	18.2	15.3	14.2	1.32	<.001	0.110	0.367
M. capillaris (larvae)	12.2 b	25.3 a	11.6 b	15.1 b	3.42	19.4	14.4	14.3	2.96	0.032	0.395	0.749
Fasciola hepatica	19.3	13.4	11.7	11.8	2.03	14.3 a	12.0 ab	8.4 b	1.76	0.564	0.080	0.987
Eimeria	87.4 a	65.0 b	46.9 c	57.1 bc	5.24	72.8 a	65.3 ab	54.2 b	4.54	0.001	0.026	0.736

Mean values of tree extract species or experimental periods with different letters differ ($P< 0.05$)

in the extract mixture (SBLL, $P<0.05$) than in individual extracts of eggs of *Dictyocaulus filaria* and *Muellerius capillaris* larvae. Reduction of egg worm counts in lamb feces was higher ($P<0.05$) in P2 and P3 versus P1 (Table 2).

Discussion

The major finding was the high effectiveness of the extract from SB versus LL in reducing parasites in the host based on fecal egg and adult worm counts in lambs which had been naturally infected by parasites during the grazing period before the experiment. The reduction of fecal egg counts and the adult worms suggests that these extracts could have an additional effect on the fecundity of the female worms. Increasing the period of extract administration led to a greater reduction of worm and egg counts due to continued administration of low doses (i.e., 30 ml/lamb/day) of crude plant extract to parasites, which could lead to development of helminths. However, oral administration of tree leaf extracts showed no obvious side effects on the lambs during the experimental period of the 63 days (e.g., edema or anemia), and this was confirmed by improved average daily gain of lambs that received the SB, LL, and SBLL extracts compared with control (Salem et al. 2011a).

Secondary metabolites of the extracts could have caused their effects through binding to a specific building block, beta tubulin, to prevent its incorporation into microtubules which are essential for worm energy metabolism (Schoenian 2008). Paralysis of worm tissues makes them unable to feed, thereby leading to death. The higher efficacy of the SBLL extract mixture on egg counts than the individual SB or LL extracts could be due to new chemicals slowly released by the mixture of the two (Salem et al. 2011b) and their effect depending on the saturation of target receptors. It is likely that at higher concentrations of chemical constitutes, all binding receptors on the worms were occupied, thus leading to hyperpolarization of membranes to limit excitation and impulse transmission thereby causing flaccid paralysis of worm muscles, an observation similar to that by Wasswa and Olila (2006).

Phenolic compounds, a type of secondary metabolites, have the capacity to bind to proteins and could function via several mechanisms. Méndez-Ortíz et al. (2012) reported a 58.8 % reduction in *Haemonchus contortus* when using *Havardia albicans*, possibly due to the higher concentration of condensed tannins in that experiment. Phenolics could bind to the cuticle of larvae, which is high in glycoprotein in the wells of larvae, and reduce nutrient availability which could have resulted in larvae starvation and death (Thompson and Geary 1995). The direct effects involve the capacity of tannins

to bind with nutritional proteins, intestinal mucosa proteins and cuticle glycoproteins (Hoste et al. 2006), or fecal egg proteins of larvae. These processes could reduce larval growth and development, or inhibit egg hatching, consequently resulting in larval death (Hoste et al. 2006). It is also likely that alkaloids in the plant extracts could also have contributed to the paralysis and consequent worm death. The nematocidal activity of alkaloids had also been demonstrated by Satou et al. (2002) when they used two rat nematodes, *Strongyloides ratti* and *Strongyloides venezuelensis* models for human nematodes. Alkaloid salts in contrast are competitive antagonists at muscarinic acetylcholine receptor sites, prevent binding of acetylcholine, and are reportedly physiologically active with sedative and analgesic properties in addition to leading to excitation of cells and neurological dysfunction (Tarnopolsky and Beal 2001). However, saponins in the crude extracts of SB or LL could have caused feed refusal and starvation of the parasites, leading to their death thereby explaining the variation in potency. There is, however, no information on SB or LL anthelmintic study for comparison with our findings. Similar views on saponin effects on feeding were held by Francis et al. (2002), who also reported that saponins kill protozoans and mollusks. It is also probable that in vivo paralysis leads to loss of grip of parasites on the gut wall, leading to spontaneous expulsion of parasites in the feces. Ríos-de Álvarez et al. (2012) extracted lectins from *L. leucocephala* and *Gliricidia sepium*, studied their anthelminthic properties on sheep gastrointestinal nematodes such as *Teladorsagia circumcincta*, *H. contortus*, and *Trichostrongylus colubriformis*, and found that both *G. sepium* and *L. leucocephala* had a dose-dependent effect on larval feeding leading to the conclusion that lectins may contribute to the anthelmintic properties of some tropical forage plant extracts.

These plant metabolites may have worked alone or in combination to cause motility inhibition as paralysis or death of the worms that was achieved in all our crude extracts. Wynn and Fougere (2007) acknowledged that plant metabolite action may be additive, synergistic, or antagonistic in manner and acts at a single or multiple target sites. Thus, it is likely that a number of compounds could have contributed to the anthelmintic activity in both our plant extracts. Indeed, Salem et al. (2011b) detected 60 chemical constitutes formed by a mixture of *S. babylonica* L. and *L. leucocephala* Lam. extracts. The main compounds in the extract mixture were 9,12,15-octadecatrienoic acid-ethyl ester with a concentration of 294 mg/g followed by 9,12-octadecadienoic acid-methyl ester (112 mg/g) and hexadecanoic acid-methyl ester, a saturated fatty acid, with a concentration 90 mg/g. Hexacontanoic acid (21 mg/g), 5,8,11,14,17-eicosapentaenoic acid-methyl ester (20 mg/g), and octadecanoic acid-16-methyl-methyl ester (10 mg/g) were the major fatty acids. However, there was 129 mg/g of 1,3-dioxane-4-(hexadecyloxy)-2-pentadecyl, and the aliphatic hydrocarbon in the tritetracontane form was 32 mg/g. These chemicals were very different from those in the individual extracts of SB and LL and could have anthelmintic activity in growing lambs. Carvalho et al. (2012) evaluated the anthelmintic activity of some plant extracts of *Piper tuberculatum*, *Lippia sidoides*, *Mentha piperita*, *Hura crepitans*, and *Carapa guianensis. P. tuberculatum* contains piperamides as (Z)-piplartine, (E)-piplartine, 8,9-di as well as hydropiplartine, piperine, 10,11-dihydropiperine, 5,6-dihydropiperlongumine, and pellitorine. The major oil compounds were thymol for *L. sidoides*, menthol for *M. piperita*, and oleic acid for *C. guianensis*. The authors concluded that the extracts of *P. tuberculatum*, *L. sidoides*, and *M. piperita* had activity when tested in vitro, but that doses of *P. tuberculatum* extracts had no effect when used in vivo. Kabore et al. (2012) evaluated six concentrations of aqueous extracts of *L. leucocephala* and *G. sepium* to investigate egg hatching and larval development of *H. contortus* parasites in goats in order to estimate their anthelminthic properties, and reported that only the dose of 50 mg/ml of *G. sepium* extract had an inhibitory effect on parasite egg hatching compared to control.

Differences among adult worm and egg count reductions in response to the extracts that we used may be due to differences in their susceptibility to secondary metabolites in the extracts. The highest susceptibility, or reduction, of *S. papillosus* and *N. spathiger* compared to other parasite species by the present extract administration of SB, LL, or SBLL was probably due to a negative impact of extracts on larval development per se compared with other worm species, but gave clear evidence that it affected the viability of infective larvae which had developed under exposure to extracts. The higher reduction of adult worm counts than egg counts may be because the plant extracts in our study were more effective against adult parasites than larvae which are considered to be at a highly resilient stage of the life cycle (Wharton 1986).

Conclusions

Results evidence an anthelmintic action of individual and mixed extract of *S. babylonica* and *L. leucocephala*, depending on their concentrations and types of bioactive compounds. These extracts could be a promising alternative to conventional anthelmintics and indicate that their utilization as an alternative control of gastrointestinal parasites of small ruminants has promise. However, more studies with an economic evaluation are needed to develop a commercial product based on *S. babylonica* and *L. leucocephala* extracts which could be used in large-scale sheep production systems. Further research is needed to study their bioactive constituents, as well as the reproducibility, dosage, application regime, toxicity, and effectiveness of *S. babylonica* and *L. leucocephala* extracts in other

host species and against other economically important gastrointestinal nematode species.

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