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In vitro efficacy of selected medicinal plants from Cholistan desert, Pakistan, against gastrointestinal helminths of sheep and goats

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Abstract

Gastrointestinal helminths are a major constraint to small ruminants in extensive husbandry systems of tropical regions. Yet, unavailability, high prices, side effects, and development of parasite resistance often limit the use of synthetic anthelmintics. Traditional medicinal plants might be an effective low-cost alternative. Therefore the *in vitro* anthelmintic activity of leaf extracts of the ligneous plants *Capparis decidua*, *Salsola foetida*, *Suaeda fruticosa*, *Haloxylon salicornicum*, and *Haloxylon recurvum* from Cholistan, Pakistan, was investigated against adult worms of *Haemonchus contortus*, *Trichuris ovis*, and *Paramphistomum cervi*. Various concentrations (from 7.8 to 500 mg dry matter ml^{-1}) of three extracts (aqueous, methanol, and aqueous-methanol) of each plant were tested at different time intervals for their anthelmintic activity via adult motility assay.

Plant species ($p \leq 0.01$), extract type ($p \leq 0.001$), parasite species ($p \leq 0.001$), extract concentration ($p \leq 0.001$), time of exposure ($p \leq 0.001$) and their interactions ($p \leq 0.001$) affected the number of immobile or dead helminths. The 50 % lethal concentration (LC_{50}) values indicated that the methanol and aqueous-methanol extracts of *C. decidua*, *H. recurvum*, and *H. salicornicum* as well as the methanol extract of *S. fruticosa* have the potential to be developed into plant-based remedies against the studied helminths. Further studies are needed to investigate the *in vivo* anthelmintic activity of these extracts, in order to develop effective, cheap and locally available anthelmintics for pastoralists in Cholistan and neighbouring desert regions.

Keywords: anthelmintic activity, *Haemonchus contortus*, LC_{50}, small ruminants, *Trichuris ovis*, *Paramphistomum cervi*

1 Introduction

Pakistan's livestock sector contributes approximately 55 % of the agricultural value added and 11.6 % of the national gross domestic production (Economic Survey of Pakistan, 2012). Sheep and goat keeping is an important sub-sector, given the relatively low costs of inputs including live animals, housing structures and feedstuffs, especially when compared to dairy cattle or buffaloes (Terefe *et al.*, 2012). Helminthiasis is a major constraint to small ruminant keeping in extensive rural holdings (Raza *et al.*, 2007, 2014b), reducing feed consumption and feed conversion efficiency, delaying growth, inducing weight losses, decreasing milk production and fertility, and leading to morbidity and even mortality at heavy infestation (Terefe *et al.*, 2012). In addition, helminth infestations also compromise the animal's immune status and the host becomes susceptible to other infections, eventually resulting in substantial economic losses (Garedaghi *et al.*, 2011).

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Synthetic anthelmintics are often considered the only effective way of controlling this problem but high prices, unavailability and scarcity in remote areas, side effects, chemical residues in products and environmental toxicity problems, as well as development of resistance of targeted parasites (Jabbar *et al.*, 2006a; Saeed *et al.*, 2007; Ji *et al.*, 2012) contribute to their limited use in many pastoral systems (Gilleard, 2006). Vaccination may be an alternative to control parasitic infestations, but the antigenic complexity and variation at various developmental stages of parasites has slowed the process of vaccine development (Maizels *et al.*, 1999). The search for new and more sustainable ways of controlling parasitic and other livestock diseases gave rise to studies on the efficacy of ethno-botanicals (Mathias, 2004). The use of medicinal plants is economical (Ghotge *et al.*, 2002), safe, and generally free of resistance problems; they may therefore be a valid substitute of allopathic anthelmintics (Chagas *et al.*, 2008; Tetik *et al.*, 2013). Furthermore, these remedies are easily available (Hoste & Torres-Acosta, 2011), simple to prepare and administer (Jabbar *et al.*, 2005), at low or no cost to the farmer (Chagas *et al.*, 2008; Raza *et al.*, 2014a). The history of ethno-botany is almost as old as human civilisation (Sarojini *et al.*, 2012) and people of the Indo-Pakistan subcontinent and many other regions have for centuries relied on plants for curing animal and human diseases (McCorkle, 1995; Jabbar *et al.*, 2006b). Around 87 % of the medications used against cancer, microbial and parasitic infections are derived from natural products (Newman *et al.*, 2003). This has also been acknowledged by the World Health Organisation's estimate that 80 % of the people in developing countries, or 60 % of the global human population, largely depend on plant-based remedies for the control and treatment of various human and animal diseases (World Health Organization, 2010).

Research on fields of application, use practices, doses and administration routes associated with plant resources are important for the discovery of new medicines (Kone *et al.*, 2012). Additionally, a series of detailed tests on efficacy, mode of action, safety, direct and indirect toxicities, are basic and stringent prerequisites before natural products can be used as commercial anthelmintic drugs (Street *et al.*, 2008; Hoste & Torres-Acosta, 2011). The search for medicinal plants to treat bacterial, viral, fungal or parasitic diseases is quite important in countries like Pakistan that still have an agriculture-based economy, a large proportion of (poor) rural dwellers, and are bestowed with a unique biodiversity. About 600 of the country's 6000 known plant species are considered of therapeutic value (Hamayun, 2003; Khan *et al.*, 2012).

Previous studies already determined the anthelmintic activity of some plants indigenous to Pakistan (Lateef *et al.*, 2003; Iqbal *et al.*, 2005; Jabbar *et al.*, 2006b; Ibrar *et al.*, 2007). However, given the localized availability of these plants, we focused on the very remote and poor desert region of Cholistan, where the majority of rural dwellers rely on sheep and goat keeping. Weddings, funerals and tribal celebrations include slaughtering and exchange of animals, and traditionally wealth is assessed from the individual's herd size (Farooq *et al.*, 2008). In interviews with 120 pastoralists, five medicinal plants were reported to be effectively used against gastrointestinal parasites in sheep and goats (Raza *et al.*, 2014a). These were tested *in vitro* against three regionally very prevalent helminths (*Haemonchus contortus*, *Trichuris ovis*, and *Paramphistomum cervi*) that had been identified in a large-scale flock screening (Raza *et al.*, 2014b).

2 Materials and methods

2.1 Collection and pre-processing of plant material

Based on number of mentions and stated efficacy in a preceding survey (Raza *et al.*, 2014a) five ligneous plants were evaluated for their anthelmintic activity: *Capparis decidua* L., *Salsola foetida* L., *Suaeda fruticosa* Forssk., *Haloxylon salicornicum* (Moq.) Bunge and *Haloxylon recurvum* Bunge ex. Boiss.. Per plant, 10 kg of fresh leaves and adhering soft branches were collected as a pool from different areas of the Cholistan desert (situated between 27°42′–29°45′ N, and 69°52′–75°24′ E) during June–July 2011. The plant material was cleaned of adulterants (weeds, soil particles) and air-dried in a ventilated room. Then it was ground into fine powder using a stainless steel electrical blender. The powder was stored in a sealed cellophane bag at 4°C until use.

Standard procedures (Naumann & Bassler, 1976) were applied to determine concentrations of dry matter (DM) and organic matter (OM) of the plant material (Table 1). Concentrations of neutral detergent fibre (NDF) and acid detergent fibre (ADF) were measured in a semi-automated Ankom 220 Fiber Analyzer (ANKOM Technology, Macedon, NY, USA), following the protocol of Naumann & Bassler (1977); however no decalin or sodium sulphite was used for NDF analysis. Total phenols and total tannins were determined by the modified Folin–Ciocalteu method (Makkar, 2003)

Table 1: *Average proximate composition and concentration of phenolic compounds of five medicinal plants from Cholistan desert, Pakistan. All values are given in % of dry matter and are means of 4 replicate analyses per plant.*

Plant species	*Organic matter*	*Neutral detergent fiber*	*Acid detergent fiber*	*Total phenoles*	*Non-tannin-phenoles*	*Total tannins*	*Condensed tannins*
Capparis deciduas	89.8	57.9	41.7	0.73	0.66	0.07	0.06
Salsola foetida	60.3	26.3	21.2	1.16	0.88	0.28	0.13
Suaeda fruticosa	85.1	48.1	39.2	1.48	1.34	0.14	0.08
Haloxylon salicornicum	59.3	22.3	15.2	1.48	1.09	0.39	0.14
Haloxylon recurvum	72.5	29.9	15.9	5.90	4.31	1.59	0.19

whereby polyvinyl-polypyrrolidone was used to distinguish non-tannin phenols from tannin phenols. Condensed tannins were extracted according to Porter *et al.* (1986).

2.2 Extract preparation

Three different extracts were prepared per plant: aqueous, methanol and aqueous-methanol extract. The preparation of the aqueous extract followed the procedures described by Onyeyili *et al.* (2001): Powdered plant material (250 g air dry matter) was soaked in 1 l of water over night and then boiled for 1.5 hours. After cooling to room temperature the mixture was filtered using muslin cloth and Whatman® No.1 filter paper. The residual plant material on the filter was diluted again in 1 l of water, and the described process was repeated a second and third time. The three filtrates were pooled and water was evaporated in a force draft oven at 50°C until a volume of 50–90 ml was reached; this took 5 to 7 days.

Methanol and aqueous-methanol (30:70) extracts were prepared by cold maceration technique, modifying the method of Tabassam *et al.* (2008). Powdered plant material (250 g air dry matter) was soaked at a ratio of 1:4 in each solvent for three days. The filtrate was collected through a piece of muslin cloth and Whatman® No.1 filter paper. The residual plant material on the filter was soaked again in 1 l of solvent for a second and third time. The three filtrates were pooled and condensed to 50–70 ml volume in a forced draft oven at 40–45°C for 3 to 5 days.

The concentration of each extract was calculated on the basis of powdered dry plant matter soaked in the solvents. The concentration of the extracts was maintained at a ratio of 1 g extracted plant material per 1 ml of solvent by adding distilled water to the concentrated extract until a volume of 250 ml was reached; the thus prepared crude extracts were stored at 4°C for a maximum of three months.

2.3 Determination of anthelmintic activity

For each test lasting 24 hours, adult and motile helminths were freshly collected from 20–30 sheep and/or goats slaughtered at the abattoir of Multan, Pakistan, and pooled. Helminth species were identified based on the morphological characteristics described by Soulsby (1982), Kaufmann (1996), and Urquhart *et al.* (1996). *Haemonchus contortus* was collected from abomasal contents, *Trichuris ovis* from the large intestine and *Paramphistomum cervi* from rumen contents. The three species had been found most prevalent in pastoral sheep and goat flocks in Cholistan (Raza *et al.*, 2014b). Collected parasites were washed in phosphate buffered saline (PBS) solution (Alawa *et al.*, 2003) and directly used for evaluation of anthelmintic activity via adult motility assay (Singh *et al.*, 1985; Iqbal *et al.*, 2012; Lone *et al.*, 2012).

For this assay, 10 individual helminths per species were counted into a Petri dish and, at an ambient temperature of 25–30°C, were exposed to seven different concentrations (see below) of the three extract types (aqueous, methanol and aqueous-methanol); each treatment was replicated three times. For each combination of plant and extract type, the dilution series was as follows: 500, 250, 125, 62.5, 31.25, 15.62 and 7.81 mg dry plant material per ml solvent. Levamisole (0.55 mg ml^{-1}) and oxyclozanide (34 mg ml^{-1}) served as positive control, and pure PBS served as negative control in each dilution series.

Inhibition of motility or death of individual helminths were indiscriminately used as indicator for anthelmintic activity. Motility loss / mortality were observed at 2, 4, 6, 8, 10, 12 and 24 hours after adding the extract to the Petri dishes, using a convex lens magnifying glass (5X). At the end of each observation interval, the treated helminths were kept for five minutes in lukewarm fresh PBS to test recapture of motility. The number of immobile/dead individuals was recorded for each combination of plant species and extract type at the specific concentration and time interval.

2.4 Statistical analysis

Data were analysed using Microsoft Excel 2007 and SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). The effect of plant species (n=5), extract type (n=3), concentration of extract (n=7) and time of exposure (n=7) on motility/mortality of the three helminth species was determined using the Kruskal-Wallis test, since the residuals of the dependent variable (number of dead parasites) were not normally distributed (Kolmogorov-Smirnov test). A p value $\leq$ 0.05 was considered significant.

The 50 % lethal concentration (LC_{50}), that is the concentration of plant extract required to kill 50 % of the adult parasites, was calculated using Probit analysis (Probit transformation of percentage mortality and natural logarithmic transformation of dose according to Robertson & Preisler (1992) as given below:

$$\log LC_{50} = \log LC_p - (probit\ p\text{-}5)/b \qquad (1)$$

where LC_{50} is the dose lethal to 50 % of the exposed helminths; p is the proportion of death helminths; LC_p is the dose lethal to p of the exposed helminths; b is the probit dose-response slope; and *probit p*-5 is the probability for 50 % mortality.

3 Results

In all tests, 100 % mortality of *H. contortus* and *T. ovis* was obtained by the positive control (levamisole) within 2 hours of exposure, while 100 % mortality of *P. cervi* was observed with oxyclozanide within 4 hours of exposure. No mortality of helminths was observed in PBS (negative control). Across all treatments the highest and lowest anthelmintic activity occurred at a concentration of 500 and 7.8 mg ml^{-1}, respectively, and at 12 and 2 hours of exposure (Tables 2–7). The aqueous and aqueous-methanol extracts of *C. decidua* and the methanol extract of *H. salicornicum* killed a maximum of *H. contortus* (7.1, 6.6, and 7.7 out of 10 individuals, respectively) at 500 mg ml^{-1} (Table 2). In case of *T. ovis*, the aqueous extract of *C. decidua* and the methanol and aqueous-methanol extracts of *H. recurvum* were potent at 500 mg ml^{-1} (Table 3), whereas the aqueous extract of *S. foetida* as well as the methanol and aqueous-methanol extracts of *H. recurvum* killed the highest mean number of *P. cervi* (Table 4).

At 500 mg ml^{-1} concentration, aqueous extracts of *C. decidua* and *S. foetida*, methanol extracts of all plants and aqueous-methanol extracts of *C. decidua*, *H. salicornicum* and *H. recurvum* killed all *H. contortus* (10 of 10 individuals) after 12 hours of exposure (Table 5). Similarly, at 500 mg ml^{-1} and 12 hours of exposure, the aqueous extracts of *C. decidua* and *S. fruticosa*, the methanol extracts of all plants and the aqueous-methanol extracts of *S. foetida*, *H. salicornicum* and *H. recurvum* killed all *T. ovis* (Table 6). In case of *P. cervi*, no aqueous plant extract killed all parasites, whereas the methanol extracts (at 500 mg ml^{-1}) of *H. salicornicum* and *H. recurvum* and the aqueous-methanol extract (at 500 mg ml^{-1}) of *H. recurvum* killed all helminths after 12 hours of exposure (Table 7).

Across the whole series of tests, plant species ($p \leq 0.01$), extract type ($p \leq 0.001$), parasite species ($p \leq 0.001$), extract concentration ($p \leq 0.001$), time of exposure ($p \leq 0.001$) and their interactions ($p \leq 0.001$) had significant effects on the number of immobile/dead helminths (Table 8).

LC_{50} values strongly depended on parasite species ($p \leq 0.01$) and extract type ($p \leq 0.01$; Table 9). Among the aqueous extracts, *C. decidua* (at 142.5 mg ml^{-1}) and *S. fruticosa* (at 191.8 mg ml^{-1}) showed a clear anthelmintic activity against *H. contortus* and *T. ovis*, respectively, while the aqueous extract of *S. foetida* (at 239.2 mg ml^{-1}) was effective against *P. cervi*. Among the methanol extracts, those of *H. recurvum* were most potent against all three helminths. Among the aqueous-methanol extracts, the one of *C. decidua* (at 162.8 mg ml^{-1}) was most effective against *H. contortus*, whereas that of *H. recurvum*, at 104.8 and 181.2 mg ml^{-1}, respectively, was most effective against *T. ovis* and *P. cervi* (Table 9).

When ranking extract types for their effectiveness against a certain helminth, the aqueous extract of *C. decidua* (142.5 mg ml^{-1}) was most potent against *H. contortus*, followed by *S. foetida* (177.9 mg ml^{-1}) and *S. fruticosa* (191.1 mg ml^{-1}). Among methanol extracts, *H. recurvum* (43.5 mg ml^{-1}) was more effective than *S. fruticosa* (61.5 mg ml^{-1}) and *S. foetida* (134.2 mg ml^{-1}), and among aqueous-methanol extracts *C. decidua* (162.8 mg ml^{-1}) killed most *H. contortus* followed by *S. foetida* (198.9 mg ml^{-1}) and *H. recurvum* (274.8 mg ml^{-1}).

To affect *T. ovis* with an aqueous extract, *S. fruticosa* (191.8 mg ml^{-1}) was most potent, followed by *C. decidua* (209.9 mg ml^{-1}) and *S. foetida* (264.5 mg ml^{-1}); concerning methanol extracts, *H. recurvum* (117.1 mg ml^{-1}) was more potent than *S. fruticosa* (153.1 mg ml^{-1}) and *S. foetida* (153.4 mg ml^{-1}), and with aqueous-methanol extracts, *H. recurvum* (104.8 mg ml^{-1}) gave best results followed by *H. salicornicum* (157.6 mg ml^{-1}) and *C. decidua* (206.4 mg ml^{-1}).

To control *P. cervi* with aqueous extract, *S. foetida* (239.2 mg ml^{-1}) was most effective, followed by *C. decidua* (411.9 mg ml^{-1}) and *S. fruticosa* (611.1 mg ml^{-1}); with methanol and aqueous-methanol extract *H. recurvum* (103.5 and 181.2 mg ml^{-1}, respectively) was most potent, followed by *H. salicornicum* (221.8 and 288.4 mg ml^{-1}) and *C. decidua* (352.6 and 315.6 mg ml^{-1}).

4 Discussion

Parasitic infestations and related disorders and diseases are considered a major health threat to extensively kept sheep and goats (Iqbal *et al.*, 2005). Among different parasitic diseases, helminth infections are not only rampant (Saeed *et al.*, 2007) but the parasites are also developing resistance against the commonly used synthetic drugs throughout the globe (Jabbar *et al.*, 2006a; Canul-Ku *et al.*, 2012; Holm *et al.*, 2014). Considering these serious problems along with sometimes relatively high costs of synthetic anthelmintics and their various side effects, the exploration of alternative plant-based medication of traditional healthcare systems is indicated (John *et al.*, 2006; Cala *et al.*, 2012; Chagas *et al.*, 2008). It is quite difficult to precisely estimate the proportion of the global population that uses herbal medicines. Healthcare approaches based on traditional medicines have been transmitted for generations, with continuous change of recipes and doses, and are still in use globally (Tetik *et al.*, 2013). Of the circa 422,000 known flowering plants (Govaerts, 2001) more than 50,000 are estimated to be used for therapeutic purposes (Schippmann *et al.*, 2002). The majority of humans still rely on such products given (i) hundreds of years of credence and observations, (ii) regional or temporal unavailability of modern drugs, or (iii) deliberate preference of herbal remedies (Aburjai *et al.*, 2007; Tetik *et al.*, 2013). Even though we are aware of the debate whether conservation trough protection or conservation through use is more appropriate to secure populations of (endangered) plant (and animal) species (Berkes, 2007), this aspect is only relevant once a herbal remedy derived from wild plant stands has been proven to be effective.

Table 2: *Mortality (n) of adult Haemonchus contortus at decreasing concentrations of aqueous, methanol and aqueous-methanol extracts of five medicinal plants after 12 hours of exposure. Values are means of three replicates per treatment with 10 adult parasites per replicate.*

Extract	*Plant species*	*Concentration (mg ml^{-1})*						
		500.0	*250.0*	*125.0*	*62.5*	*31.3*	*15.6*	*7.8*
Aqueous extract	*C. decidua*	7.11	6.44	5.33	4.00	2.44	1.39	0.72
	S. foetida	6.61	5.89	4.72	3.67	2.33	0.72	0.17
	S. fruticosa	6.22	5.67	4.72	3.89	2.17	1.11	0.44
	H. salicornicum	3.39	2.39	1.83	1.28	0.72	0.39	0.22
	H. recurvum	3.39	2.67	2.39	1.44	0.67	0.33	0.17
	SEM	0.575	0.592	0.544	0.532	0.399	0.231	0.138
Methanol extract	*C. decidua*	6.33	5.78	5.28	3.72	2.50	1.28	0.67
	S. foetida	6.28	5.78	5.28	3.83	2.72	1.50	0.89
	S. fruticosa	7.06	6.39	5.89	5.44	4.00	2.94	2.22
	H. salicornicum	7.67	7.33	6.28	5.39	4.28	3.33	2.33
	H. recurvum	7.39	7.06	6.50	5.61	4.94	3.72	2.89
	SEM	0.528	0.565	0.593	0.559	0.518	0.442	0.371
Aqueous methanol extract	*C. decidua*	6.61	6.00	4.83	3.83	2.22	1.44	0.78
	S. foetida	6.22	5.44	4.56	3.94	1.94	0.94	0.11
	S. fruticosa	5.61	4.61	3.67	2.61	1.72	0.72	0.33
	H. salicornicum	5.67	4.33	3.56	2.17	1.06	0.61	0.22
	H. recurvum	5.44	4.89	3.94	2.72	1.44	0.78	0.33
	SEM	0.498	0.515	0.486	0.451	0.323	0.239	0.130

Negative control (phosphate buffered saline): no dead parasite; Positive control (Levamisole, 0.55 mg ml^{-1}): all parasites dead at 2 h after exposure.
For significant effects of plant species, extract type and extract concentration, respectively, on parasite mortality please refer to text.
SEM: standard error of the mean.

Table 3: *Mortality (n) of Trichuris ovis at decreasing concentrations of aqueous, methanol and aqueous-methanol extracts of five medicinal plants, after 12 hours of exposure. Values are means of three replicates per treatment with 10 adult parasites per replicate.*

Extract	*Plant species*	*Concentration (mg ml^{-1})*						
		500.0	*250.0*	*125.0*	*62.5*	*31.3*	*15.6*	*7.8*
Aqueous extract	*C. decidua*	6.28	5.22	4.50	3.33	2.39	1.39	0.61
	S. foetida	5.72	5.06	4.11	3.28	1.78	0.94	0.22
	S. fruticosa	6.11	5.56	4.22	3.22	2.11	0.94	0.39
	H. salicornicum	3.06	2.22	1.61	1.06	0.72	0.22	0.17
	H. recurvum	3.50	2.61	2.17	1.39	0.611	0.28	0.11
	SEM	0.524	0.544	0.513	0.459	0.349	0.228	0.121
Methanol extract	*C. decidua*	5.67	5.33	4.61	3.22	2.22	1.28	0.72
	S. foetida	6.61	5.78	4.78	3.67	2.50	1.33	0.56
	S. fruticosa	6.33	5.72	4.78	4.00	2.72	1.39	0.56
	H. salicornicum	6.28	5.44	4.72	3.94	2.72	1.94	0.89
	H. recurvum	6.67	5.83	5.56	5.17	3.61	2.28	1.17
	SEM	0.586	0.634	0.669	0.629	0.517	0.411	0.258
Aqueous methanol extract	*C. decidua*	6.17	5.11	4.11	3.11	2.28	1.11	0.50
	S. foetida	5.89	4.67	3.50	2.83	1.72	0.89	0.22
	S. fruticosa	5.72	5.06	4.17	3.17	2.22	1.44	0.78
	H. salicornicum	6.94	6.22	5.39	4.17	3.22	1.83	0.89
	H. recurvum	7.33	6.06	5.67	4.61	3.67	2.50	1.17
	SEM	0.500	0.533	0.553	0.528	0.475	0.355	0.214

Negative control (phosphate buffered saline): no dead parasite; Positive control (Levamisole, 0.55 mg ml^{-1}): all parasites dead at 2 h after exposure.
For significant effects of plant species, extract type and extract concentration, respectively, on parasite mortality please refer to text.
SEM: standard error of the mean.

Table 4: *Mortality (n) of Paramphistomum cervi at decreasing concentrations of aqueous, methanol and aqueous-methanol extracts of five medicinal plants, after 12 hours of exposure. Values are means of three replicates per treatment with 10 adult parasites per replicate.*

Extract	*Plant species*	*Concentration (mg ml^{-1})*						
		500.0	*250.0*	*125.0*	*62.5*	*31.3*	*15.6*	*7.8*
Aqueous extract	*C. decidua*	5.44	4.67	4.06	2.94	1.94	1.00	0.44
	S. foetida	5.56	5.22	4.33	3.17	2.06	0.61	0.33
	S. fruticosa	4.50	3.72	2.83	2.17	1.39	0.67	0.28
	H. salicornicum	3.06	1.94	1.67	1.22	0.72	0.39	0.17
	H. recurvum	3.06	2.28	1.83	1.00	0.72	0.44	0.11
	SEM	0.532	0.551	0.500	0.417	0.324	0.187	0.105
Methanol extract	*C. decidua*	5.28	4.22	3.06	2.06	1.17	0.61	0.44
	S. foetida	3.28	2.50	1.33	0.78	0.50	0.28	0.06
	S. fruticosa	3.72	3.11	2.06	1.33	0.78	0.33	0.11
	H. salicornicum	6.11	5.28	4.22	2.83	2.00	1.00	0.22
	H. recurvum	7.39	6.67	6.06	4.67	2.83	2.11	1.39
	SEM	0.528	0.548	0.549	0.479	0.372	0.279	0.198
Aqueous methanol extract	*C. decidua*	4.83	4.28	3.44	2.61	1.78	1.06	0.33
	S. foetida	4.22	3.83	3.33	2.33	1.67	0.83	0.28
	S. fruticosa	2.89	1.61	0.83	0.56	0.22	0.11	0.00
	H. salicornicum	5.28	4.44	3.78	2.50	1.72	0.89	0.44
	H. recurvum	6.67	6.06	5.33	4.11	1.39	0.89	0.50
	SEM	0.488	0.515	0.498	0.435	0.325	0.227	0.130

Negative control (phosphate buffered saline): no dead parasite; Positive control (Oxyclozanide, 34 mg ml^{-1}): all parasites dead at 4 h after exposure.
For significant effects of plant species, extract type and extract concentration, respectively, on parasite mortality please refer to text.
SEM: standard error of the mean.

Table 5: *Time effects on the mortality (n) of Haemonchus contortus in aqueous, methanol and aqueous-methanol extracts of five medicinal plants at a concentration of 500 mg ml^{-1}. Values are means of three replicates per treatment with 10 adult parasites per replicate.*

Extract	*Plant species*	*Time (h) of exposure*					
		2	*4*	*6*	*8*	*10*	*12*
Aqueous extract	*C. decidua*	2.00	4.67	7.00	9.00	10.00	10.00
	S. foetida	2.00	4.33	6.00	8.00	9.33	10.00
	S. fruticosa	2.00	4.00	6.00	7.00	8.67	9.67
	H. salicornicum	0.00	1.00	3.00	4.00	5.00	7.33
	H. recurvum	0.33	1.00	3.00	4.00	5.00	7.00
	SEM	0.452	0.823	0.837	1.030	1.082	0.672
Methanol extract	*C. decidua*	2.00	3.00	5.00	8.00	10.00	10.00
	S. foetida	1.67	2.67	5.00	8.33	10.00	10.00
	S. fruticosa	3.00	4.67	6.67	8.00	10.00	10.00
	H. salicornicum	4.00	5.67	7.33	9.00	10.00	10.00
	H. recurvum	4.00	5.00	6.67	8.67	10.00	10.00
	SEM	0.488	0.583	0.478	0.194	0.000	0.000
Aqueous methanol extract	*C. decidua*	3.00	4.00	6.00	7.67	9.00	10.00
	S. foetida	2.67	4.00	6.00	7.00	8.33	9.33
	S. fruticosa	1.67	3.33	5.00	6.00	8.00	9.67
	H. salicornicum	2.00	3.00	5.00	6.33	7.67	10.00
	H. recurvum	1.33	3.00	4.33	6.00	8.00	10.00
	SEM	0.309	0.226	0.323	0.324	0.226	0.133

Negative control (phosphate buffered saline): no dead parasite; Positive control (Levamisole, 0.55 mg ml^{-1}): all parasites dead at 2 h after exposure.
For significant effects of plant species, extract type and extract concentration, respectively, on parasite mortality please refer to text.
SEM: standard error of the mean.

Table 6: *Time effects on the mortality (n) of Trichuris ovis in aqueous, methanol and aqueous-methanol extracts of five medicinal plants at concentration of 500 mg ml^{-1}. Values are means of three replicates per treatment with 10 adult parasites per replicate.*

Extract	*Plant species*	*Time (h) of exposure*					
		2	*4*	*6*	*8*	*10*	*12*
Aqueous extract	*C. decidua*	2.00	4.00	5.33	7.67	8.67	10.00
	S. foetida	1.67	3.33	5.33	6.33	8.00	9.67
	S. fruticosa	2.67	3.67	4.67	6.67	9.00	10.00
	H. salicornicum	0.00	1.00	2.67	3.67	5.00	6.00
	H. recurvum	0.67	1.67	3.00	4.00	5.33	6.33
	SEM	0.476	0.591	0.534	0.782	0.847	0.915
Methanol extract	*C. decidua*	0.00	2.00	4.00	8.00	10.00	10.00
	S. foetida	3.00	4.33	5.33	7.33	9.67	10.00
	S. fruticosa	2.67	3.67	4.67	7.67	9.33	10.00
	H. salicornicum	2.00	3.33	4.336	8.00	10.00	10.00
	H. recurvum	2.67	3.67	5.00	8.67	10.00	10.00
	SEM	0.542	0.386	0.236	0.221	0.133	0.000
Aqueous methanol extract	*C. decidua*	2.67	3.67	5.33	7.00	8.67	9.67
	S. foetida	2.00	3.33	5.33	6.67	8.00	10.00
	S. fruticosa	2.00	3.33	4.33	6.67	8.33	9.67
	H. salicornicum	2.67	4.67	6.00	8.33	10.00	10.00
	H. recurvum	4.00	5.00	6.33	8.67	10.00	10.00
	SEM	0.365	0.350	0.343	0.429	0.422	0.817

Negative control (phosphate buffered saline): no dead parasite; Positive control (Levamisole, 0.55 mg ml^{-1}): all parasites dead at 2 h after exposure.
For significant effects of plant species, extract type and extract concentration, respectively, on parasite mortality please refer to text.
SEM: standard error of the mean.

Table 7: *Time effects on the mortality (n) of Paramphistomum cervi in aqueous, methanol and aqueous-methanol extracts of five medicinal plants at concentration of 500 mg ml^{-1}. Values are means of three replicates per treatment with 10 adult parasites per replicate.*

Extract	*Plant species*	*Time (h) of exposure*					
		2	*4*	*6*	*8*	*10*	*12*
Aqueous extract	*C. decidua*	1.00	3.00	5.00	6.33	7.67	9.67
	S. foetida	1.67	3.33	4.67	6.00	8.00	9.67
	S. fruticosa	0.67	1.67	3.33	5.00	7.00	9.33
	H. salicornicum	0.00	1.00	2.00	3.33	4.67	7.33
	H. recurvum	0.00	1.00	2.67	3.67	4.67	6.33
	SEM	0.316	0.494	0.573	0.602	0.726	0.688
Methanol extract	*C. decidua*	1.67	3.00	4.33	6.00	7.67	9.00
	S. foetida	0.67	1.67	2.33	3.33	5.33	6.33
	S. fruticosa	1.00	2.00	3.00	4.33	5.33	6.67
	H. salicornicum	2.00	3.33	5.33	7.33	8.67	10.00
	H. recurvum	3.00	5.33	7.00	9.00	10.00	10.00
	SEM	0.408	0.645	0.833	1.017	0.921	0.799
Aqueous methanol extract	*C. decidua*	2.00	3.00	4.33	5.00	6.33	8.33
	S. foetida	1.00	2.67	3.67	5.00	6.00	7.00
	S. fruticosa	1.00	1.67	2.33	3.00	4.00	5.33
	H. salicornicum	2.00	3.33	4.00	5.00	7.67	9.67
	H. recurvum	1.67	3.67	6.67	8.33	9.67	10.00
	SEM	0.226	0.343	0.704	0.859	0.939	0.865

Negative control (phosphate buffered saline): no dead parasite; Positive control (Oxyclozanide, 34 mg ml^{-1}): all parasites dead at 4 h after exposure.
For significant effects of plant species, extract type and extract concentration, respectively, on parasite mortality please refer to text.
SEM: standard error of the mean.

Table 8: *Effect* * *of plant, treatment* †*, extract type, parasite species, extract concentration and time of exposure on the number of dead parasites.*

Independent variable	*df*	χ^2	$p \leq$
Plant species	4	15.193	0.004
Treatment	6	330.37	0.001
Extract type	2	116.31	0.001
Parasite species	2	119.67	0.001
Extract concentration	6	1979.91	0.001
Time of exposure	5	2346.83	0.001
Concentration × plant	21	1517.77	0.001
Concentration × extract type × plant	38	1279.75	0.001
Concentration × extract type × parasite	25	1514.31	0.001

* Kruskal-Wallis test: data were non-normally distributed.
† Treatment: the five plants plus the positive and negative control (see footnotes of Tables 2–7).
df: degree of freedom
χ^2: Chi-square value

Table 9: LC_{50} *values of different plant extracts, and statistical effect*[1] *of plant, parasite and extract type on* LC_{50} *values.*

Plant species	*Extract type*	LC_{50} (mg ml^{-1})		
		Haemonchus contortus	*Trichuris ovis*	*Paramphistomum cervi*
Capparis decidua	A	142.5	209.9	411.9
	M	139.6	296.9	352.6
	AM	162.8	206.4	315.6
Salsola foetida	A	177.9	264.5	239.2
	M	134.2	153.4	917.8
	AM	198.9	248.5	378.8
Suaeda fruticosa	A	191.1	191.8	611.1
	M	61.5	153.1	576.9
	AM	275.4	243.2	986.0
Haloxylon salicornicum	A	2449.1	2414.9	3102.2
	M	360.6	199.1	221.8
	AM	294.5	157.6	288.4
Haloxylon recurvum	A	952.9	917.5	2706.1
	M	43.5	117.1	103.5
	AM	274.8	104.8	181.2

Independent variable	*df*	χ^2	*p=*
Plant species	4	1.49	0.828
Parasite species	2	9.16	0.010
Extract type	2	7.99	0.018
Plant × extract type	3	20.01	0.029
Plant × parasite	10	11.35	0.331
Extract type × parasite	5	9.02	0.108

The grey highlights indicate the most effective concentration for each type of plant × extract combination against the specific parasite.
[1] Kruskal-Wallis test: data were non-normally distributed.
Extract type: A: aqueous, M: methanol, AM: aqueous-methanol.
df: degree of freedom
χ^2: Chi-square value

Recent investigations demonstrated that some medicinal plants have poisonous effects; therefore, caution must be exercised when administering unknown ethnobotanical remedies (Elgorashi *et al.*, 2003; Barlow & Schlatter, 2010). Therefore, we assessed the anthelmintic efficacy of plants that are frequently used against gastrointestinal disorders by local livestock keepers (Raza *et al.*, 2014a) through *in vitro* trials as a first step. Such tests prevent probable toxic effects on animals but allow for a rapid screening of their anthelmintic potential (Aremu *et al.*, 2012). To assess the *in vitro* anthelmintic activity of remedies, different researchers used adult worms (Iqbal *et al.*, 2004, 2005, 2006; Reddy & Seetharam, 2009), larvae (Githiori *et al.*, 2002; Bachaya *et al.*, 2009), eggs (Alawa *et al.*, 2003) or several developmental stages (Eguale *et al.*, 2007; Tadesse *et al.*, 2009) of various helminths. Based on literature review we decided to perform the adult motility assay since (i) this method is reliable, practicable under field conditions and cheap, (ii) parasites can be collected easily from animals at slaughterhouses, and (iii) only small amounts of plant extract are required for *in vitro* testing. Furthermore, pastoralists mostly treat helminth infestations in their animals when adult parasites are present in the faeces.

The five tested plants expressed anthelmintic activity against the three selected helminth species; yet, their efficacy varied with respect to the parasite species and with respect to the solvent used for extract preparation (Nyong *et al.*, 2009). The latter has to be attributed to differences in the nature and concentration of the plant secondary metabolites responsible for killing the parasites (Eguale *et al.*, 2007). Increasing the extract concentration and lengthening time of exposure enhanced parasite mortality, pointing to dose- and time-dependent anthelmintic properties. Several other studies on the *in vitro* and *in vivo* activity of plant-based anthelmintics also reported dose- and time-dependent effects (Costa *et al.*, 2011; Ahmed *et al.*, 2012; Cala *et al.*, 2012; Ji *et al.*, 2012; Moreno *et al.*, 2012; Nalule *et al.*, 2013).

Although only of limited explanatory power, the chemical analysis revealed the presence of condensed tannins and other phenolic compounds in all five plants. Especially tannins exhibit anthelmintic activity by two mechanisms. Firstly, through irreversible binding they can change the chemical and physical properties of the parasites' protein surfaces, such as cuticle, oral cavity, oesophagus, cloaca and vulva. In consequence, helminths lose their grip onto the host's gastrointestinal epithelium and are expelled from the body (Athanasiadou *et al.*, 2001; Cenci *et al.*, 2007). Secondly, interaction of tannins with free dietary proteins may reduce the availability of nutrients to the parasite, affect its life cycle and result in death by starvation (Athanasiadou *et al.*, 2001; Hoste *et al.*, 2006).

The LC_{50} values of the five plants varied according to parasite and solvent. This is most probably due to the different chemical components extracted by the solvents and their biological effects on the parasites (Eloff, 1998). Of *C. decidua*, the methanol extract was most potent against *H. contortus*, indicating that the active components responsible for this effect were lipophilic in nature. On the other hand, the aqueous-methanol extract of *C. decidua* was most effective against *T. ovis* and *P. cervi*, suggesting that against these two parasites a combination of lipophilic and hydrophilic compounds were jointly effective. Of *S. foetida*, the methanol extract was most effective against *H. contortus* and *T. ovis*, while the aqueous extract was most effective against *P. cervi*. This points to lipophilic secondary metabolites being active against *H. contortus* and *T. ovis*, and hydrophilic compounds affecting *P. cervi*. Of *S. fruticosa*, the methanol extract was most potent against all three helminths, indicating that the active components responsible for the anthelmintic activity of this plant are lipophilic in nature. In case of *H. salicornicum*, combinations of lipophilic and hydrophilic compounds were effective against *H. contortus* and *T. ovis*, while lipophilic compounds were active against *P. cervi*. Lipophilic compounds of *H. recurvum* were killing *H. contortus* and *P. cervi*, while a combination of lipophillic and hydrophilic compounds were effective against *T. ovis*. Although detailed secondary metabolite profiles of the plants used in this study are still to be determined, they all contain water-soluble phenols (see Table 1), saponins (water-soluble), glucosinolates (water-soluble), tannins (Table 1; water- and fat-soluble), carotenoids, phytosterols and alkaloids (all fat-soluble), which all exhibit a certain anthelmintic activity. The anthelmintic activity of alkaloids, for example, has been demonstrated against *Strongyloides ratti* and *S. venezuelensis* (Satou *et al.*, 2002). The above-mentioned secondary metabolites may have worked individually or caused synergistic effects against the helminths (Briskin, 2000).

Overall, the methanol and aqueous-methanol extracts exhibited stronger anthelmintic activity than aqueous extracts. Anthelmintic drugs reach target sites in helminths either by oral route or by diffusion through their cuticle. Several studies showed that trans-cuticle diffusion is the common entry pathway of non-nutrient and non-electrolyte substances, and most broad-spectrum anthelmintics affect parasites by this route (Debella, 2002; Eguale *et al.*, 2007). Thereby, lipophilic anthelmintics have a higher capability to cross the external surface of helminths than hydrophilic compounds (Geary *et al.*, 1999). The higher effectiveness of methanol and aqueous-methanol extracts as compared with aqueous extracts might therefore have been due to their better trans-cuticle absorption (Eloff, 1998).

5 Conclusions

This *in vitro* study revealed that methanol and aqueous-methanol extracts of *C. decidua*, *H. recurvum* and *H. salicornicum* as well as the methanol extract of *S. fruticosa* are effective against *H. contortus*, *T. ovis*, and *P. cervi*. In a next step *in situ* safety studies are needed to examine the activity of the extracts under on-station experimental conditions, as some of the tested plants may be poisonous. This should be followed by *in vivo* bioactivity experiments under typical management conditions for small ruminants, to account for the impact of digestion and absorption processes, and particularly the interaction of the remedies with ingested feed. Although it is still necessary to isolate and identify the active compounds responsible for the anthelmintic activity, the present results indicate that some of the locally very frequently used medicinal plants have the potential to be developed into effective low-cost anthel-

mintics for systematic curative treatment of small ruminants of pastoralists in the Cholistan desert and neighbouring arid regions of Sindh province, Pakistan, and probably even Rajasthan, India.

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