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RESEARCH ARTICLE

In Vitro Anthelmintic Efficacy of *Haloxylon salicornicum* Leaves Extract using Adult *Heamonchus contortus* Worms

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A B S T R A C T

Herbal dewormers always remained a constant and consistent source of curing various health constraints including parasitic infestations. In this study, anthelmintic efficacy of aqueous methanolic and ethyl acetate extract of *Haloxylon* (*H.) salicornicum* was investigated against *Heamonchus contortus*. Four serial dilutions (25, 12.5, 6.25 and 3.125mg/mL) were tested to evaluate the highest effective dose of both extracts. Levamisol (0.55mg/mL) in case of adult motility assay and oxfendazole (25, 12.5 and 6.25µg/mL) in egg hatch assay were used as standard drugs. After administration of extracts, a highest mean paralysis (10.00±0.00) of all adult worms of *H. contortus* was exhibited only in 8 and 10 hours post exposure respectively, at highest tested dose of 25mg/ml. Ethyl acetate and aqueous-methanol extracts were found to inhibit egg hatching up to 91 and 63% respectively, at dose of 25mg/mL. Hence, the results of current study exhibited a strong anthelmintic potential of *H. salicornicum* leaves against haemonchosis. Further *in vivo* studies would be needed to determine the optimal non-lethal dose to maximize the anthelmintic potential of *H. salicornicum* against this parasite in livestock.

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INTRODUCTION

Gastrointestinal parasites are major restraint to livestock production by reducing their feed conversion capability, growth rate, weight gain and milk production (Terefe *et al*., 2012; Rehman *et al*., 2016; Rehman *et al.*, 2021). Among the gastrointestinal diseases that affect the growth and fertility of small ruminants including sheep and goat, heamonchosis ranks at highest on a global index (Zaman *et al.*, 2014; Štrbac *et al*., 2021; Tabassum *et al.*, 2022). The causative agent *Heamonchus* (*H.*) *contortus* is estimated to be responsible of loss of 10 billion USD to farmer community (Roeber *et al.*, 2013). Haemonchosis leads to hematological and biochemical alterations, protein depletion, inefficient digestion and poor reproductive performance in both sheep and goat (Bachaya *et al*., 2006; Qamar *et al*., 2021). Moreover, the infection also affects the immune status of the animal, thus exposing the host to other secondary infections leading to substantial economic losses (Raza *et al*., 2016).

For the control of haemonchosis, the available anthelmintics are quite costly, not easily available or even scarce and have various side effects or toxicity issues (Rashid *et al.*, 2022). Moreover, the evolution of resistance in various parasites against these anthelmintics contributes to the limited use of synthetic drugs in many pastoral systems (Raza *et al*., 2016). Many parasitic nematodes of veterinary interest have genetic traits that support the development of anthelmintic resistance (Kaplan, 2004). In the same context, *H. contortus* has been recognized as multi-resistant parasite against all broad range anthelmintics including benzimidazole, ivermectin and imidazothiazole (Devi *et al*., 2014). To overcome this issue, the search for alternate and more reliable ways of

controlling these parasitic infections prompted the exploration of anthelmintic potential of medicinal plants especially those are available locally (Baz *et al*., 2021; Moryani *et al*., 2021; Dahab *et al.,* 2022; Degla *et al*., 2022; Nawaz *et al.,* 2022). The use of medicinal plants is an economically safe mode of medication that have no toxic effects and therefore could be an effective substitute of synthetic drugs (Alkenani *et al*., 2021; Murtaza *et al*., 2021, Srisanyong *et al.,* 2021; Ahmad *et al*., 2022; Jamil *et al.,* 2022). Furthermore, these remedies are easily available and sometimes are free of cost, quite simple in preparation and application (Sayyar *et al*., 2021; Wajiha and Qureshi, 2021). Green nanoparticles are being investigated for their anthelmintic efficacy (Umair *et al.,* 2022).

Keeping in view above discussion, project was designed to estimate the anthelmintic efficacy of *Haloxylon (H.) salicornicum* against *H. contortus*. *H. salicornicum* belongs to the family Chenopodiaceae. The plant is a common shrub in desert areas of Pakistan and generally known as "Khar" in local language (Shafi *et al*., 2001). It is a perennial shrub, considered as most auspicious species for re-seeding and sand dune fixation. It is quite palatable, succulent, semi halophytic plant and generally well adapted to survive under harsh conditions of desert. Moreover, it has been reported to possess anti-diabetic and anticoagulant (Ajabnoor *et al*., 1984), anti-inflammatory (Al-shanawani, 1996), anti-cancer and anti-plasmodial (Sathiyamoorthy *et al*., 1999), antibacterial and hepato-protective activities (Ahmad and Eram, 2011), also act as a fly repellant (Farooq *et al*., 2008) and used to treat intestinal ulcers (Shafi *et al*., 2001).

H. salicornicum has been documented by Farooq *et al*., (2008) previously to be used in treating helminths. The preliminary anthelmintic activity of *H. salicornicum* was reported by using its aqueous, methanol and combined aqueous-methanol extracts against gastrointestinal parasites i.e. *Trichuris ovis* and *paramphistomum cervi* (Raza *et al*., 2013). Furthermore, the anti-tick activity of the methanolic extract of the plant against *Hyalomma dromedarii* was also studied and found a complete sterility and cessation of motility of the parasite just after one hour of application (Khaled *et al*., 2015). To extend our understanding, the current study was designed to investigate the *in vitro* anthelmintic potential of this plant against *H. contortus* to develop most effective and cheaper anthelmintic that could be available locally for the herdsman in the desert environment.

MATERIALS AND METHODS

Study area: The plant material was collected from the Cholistan desert; seventh largest desert of the world. It is situated adjacent to districts of Bahawalpur, Rahim Yar Khan and Bahawalnagar in the southern border of Punjab province, Pakistan. It extends over an area of 6.2 million hectors between the longitudes of 69º52´ to 75º 24´E and latitudes of 27º42´ to 29º45´N. The climate of the sandy desert is hot and arid with mean summer (18-48ºC) and winter (-2–30ºC) temperatures and an average annual rainfall ranging from 100-250mm (Farooq *et al*., 2008; Ali *et al*., 2017). Cholistan is a poorly developed region where the majority of rural dwellers rely on livestock especially sheep and goat production as a source of their income. To treat various diseases like rheumatism, gastrointestinal tract (GIT) and respiratory disorders in their animals, the native ethnic tribes utilize the majority of desert plants having various therapeutic properties (Shafi *et al*., 2001).

Collection and processing of plant material: The fresh leaves of *H. salicornicum* adhering soft branches were collected (5kg). The leaves were air dried in shade for 15 days at room temperature and then chopped and grinded into fine powder using mechanical grinder. Powdered plant material was then stored at 4°C in airtight bottle until further processing. Preparation of extract of *H. salicornicum* and its anthelmintic study against *H. contortus* was carried out as previously cited and briefly described underneath (Iqbal *et al.,* 2012).

Preparation of plant extracts: Plant powder (40g) was soaked into 70% aqueous methanol using cold extraction technique at room temperature with periodic stirring for three days. After the time, the extract was filtered through muslin cloth. Plant material on muslin cloth was again mixed in solvent for three days, again filtered and the process was repeated. Filtrate collected after three filtrations was subjected to evaporation through rotary evaporator at temperature 40°C. At the end of evaporation, extract was collected in the form of syrup which was further dried in water bath and stored at 4°C until used for parasitological studies. The same procedure was adopted for the preparation of ethyl acetate extract (Rehman *et al.,* 2021).

High-Performance Liquid Chromatography methods: Ethanolic and ethyl acetate extracts were subjected to HPLC for estimation of their phytochemical constituents. CSW32-Chromatography station was used, and the graphs were developed using the Data Apex ® 2001 software. Shim-Pak CLC-ODS (C-18), 250mm x 46cm, 5um columns were used for the chromatography. Flow rates were adjusted @ 1mL/min in an ultraviolet-visible detector at a wavelength of 280nm.

Validation of anthelmintic activity

Adult motility assay: Adult *H. contortus* worms were collected from the abomasum of freshly slaughtered sheep and kept in phosphate buffer saline (PBS). Minimum ten adult worms were exposed to separate petri plates containing each of the four serial dilutions of either plant extract at room temperature (25-30ºC). In addition, two petri dishes were also used for positive (Levamisol®) and negative (PBS) controls. Following treatments were used and each was replicated in thrice:

i Ethyl acetate extract @ 25, 12.5, 6.25, 3.12mg/mL
ii Aqueous methanol extract @ 25, 12.5, 6.25, 3.12 mg/mL
iii Levamisol @ 0.55mg/mL
iv PBS @ 20 mL per petri dish

The motility of the worms was observed at time intervals of 0, 2, 4, 6, 8, 10 and 12 hours under stereomicroscope. On each observation, motile worms were counted and worms did not show any signs of movement were drained out. These separated worms were kept in PBS for 10 minutes and considered alive in case of recovery in motility, otherwise recorded as dead for that specific treatment.

Egg hatch assay: For egg hatch assay, female worms were collected in a mortar containing small amount of PBS and crushed slightly with pestle allowing them to release the eggs. The obtained solution was filtered through mesh sieve of about 80µm and eggs were collected in a petri dish. Collected fluid containing eggs was diluted until a concentration of 200eggs/mL. Two-fold serial dilutions of both aqueous methanol and ethyl acetate extracts were prepared as mentioned earlier. Oxfendazole (serially diluted in distilled water) and PBS (pH=7.4) were used as positive and negative controls respectively. Different treatments were as under:

i Ethyl acetate extract @ 25, 12.5, 6.25, 3.125mg/mL
ii Aqueous methanol extract @ 25, 12.5, 6.25, 3.125 mg/mL
iii Oxfendazole @ 25, 12.5, 6.25µg/mL
iv PBS @ 1mL per well

The eggs (n=200/well) were left in contact with one of the above treatments in 24 well plates at 28°C in an incubator for 48 hours. After the incubation period, hatched larvae (alive or dead) and unhatched eggs per each well were counted under an inverted microscope.

Statistical analysis: The collected data of adult motility assay were statistically analyzed using SPSS software while that of egg hatch assay were analyzed using probit analysis. The data were expressed as mean ± standard error of mean (S.E.M.). $P<0.05$ was considered as statistically significant level.

RESULTS

Phytochemical analyses: Phytochemical analyses of extract through HPLC confirmed presence of quercetin, gallic acid, vanillic acid, benzoic acid, synirgic acid, M-crumeric acid and cinamic acid (Table 1 and Fig. 1).

Adult motility assay: The highest and lowest anthelmintic activity was observed at a concentration of 25mg/mL and 3.125mg/mL respectively. Highest tested concentration of 25mg/mL of aqueous methanol (Table 2) and ethyl acetate (Table 3) extracts paralyzed all the worms at 8 and 10 hours post-exposure respectively. Ethyl acetate extract was found relatively less effective (10 vs 8 hours) compared to aqueous methanol extract in inhibition of motility of worms. Levamisol exhibited 100% inhibition of motility of all the worms just after 4 hours of exposure. While no inhibition of motility of worms was observed in negative control (PBS) until 12 hours post-exposure.

Egg hatch assay: Aqueous methanol extract induced maximum inhibition of egg hatching of 63% at dose of 25 mg/mL after 48 hours of exposure (Table 4). While ethyl acetate extract inhibited hatching of 91% eggs at dose of 25mg/mL at 48 hours post-exposure (Table 5). Oxfendazole caused maximum inhibition of 71% at the dose rate of 25µg/mL and minimum inhibition of 45.67% at the dose rate of 3.125µg/mL. The aqueous-methanol extract was found to be less effective as compared to that of ethyl acetate extract in inhibiting hatching of Haemonchus eggs.

DISCUSSION

In vitro methods being applied, and rational approaches are extensively used in veterinary parasitology to determine the anthelmintic potential of various plants by measuring the motility index of adult and larval worms as well as inhibition of egg hatching of parasites (Hounzangbe-Adote *et al*., 2005; Vasconcelos *et al*., 2007). These *in vitro* results have directed the way to develop innovative deworming strategies regarding time and cost effectiveness. The plant selected for the current study has been documented in ethno-botanical survey conducted by the botanists of Cholistan desert to be used as an anthelmintic by farmers (Farooq *et al*., 2008). The results of our study showed that both methanolic and ethyl acetate extracts of *H. salicornicum* possess strong anthelmintic potential against *H. contortus*. Hence, in the present study, statistically significant association has been observed between graded concentrations of the *H. salicornicum* extracts and the exposure time. Its anthelmintic activity has also been previously explored by using its aqueous, methanol and aqueous methanol extracts on gastrointestinal parasites i.e., *Trichuris ovis* and *Paramphistomum cervi* (Raza *et al*., 2013). Wherein, it was found that methanol and aqueous methanol extracts of the plant killed all the worms after 12 hours of exposure at concentration of 500mg/mL. While all adult worms were killed in this study at much lower dose i.e. 25mg/mL just after 8 and 10 hours of exposure respectively. Hence, the aqueous methanol extract of *H. salicornicum* revealed its strongest anthelmintic potential against the motility of adult *H. contortus*. This variation in anthelmintic potential of extract may be attributed to different solvents used (Malu *et al.*, 2009), those have varying capacity to absorb the compounds and metabolites from solid material of the plant, hence affecting the efficacy of the plant extracts (Ncube *et al.*, 2008). The most significant point in this study is that the aqueous-methanol extract of *H. salicornicum* has strongest anti-haemonchus potential as compared to all other extracts that have been used so far for their anthelmintic activity against this parasite. In the same context, previously it was found that anthelmintic effects of this plant using its methanolic extract against *Hylomma dromedarii* resulted in complete sterility and inhibition of motility of *Hyalomma dromedarri* just after one hour (Khaled *et al*., 2015).

The phytochemical screening of *H. salicornicum* indicated the presence of major metabolites including saponins, alkaloids, tannins and glycosides in both stem and leaves (Ashraf *et al*., 2013). Moreover, the chemical analysis revealed the presence of condense tannins that demonstrated the anthelmintic efficacy of *H. salicornicum* against the parasites by two mechanism. Firstly, the irreversible binding of the compound alters the chemical and physical properties of the protein surface, cuticle and alimentary canal of the parasite. So that the organisms lose the ability to grip into the gastrointestinal mucosal epithelium and hence, are ejected from the body of the host (Athanasiadou *et al*., 2001; Cenci *et al*., 2007). Secondly, the interaction of tannins with free dietary proteins reduces the availability of nutrients to the parasites that affects their life stages and induces death of worms by malnourishment (Hoste *et al*., 2006; Shaukat *et al*., 2019).

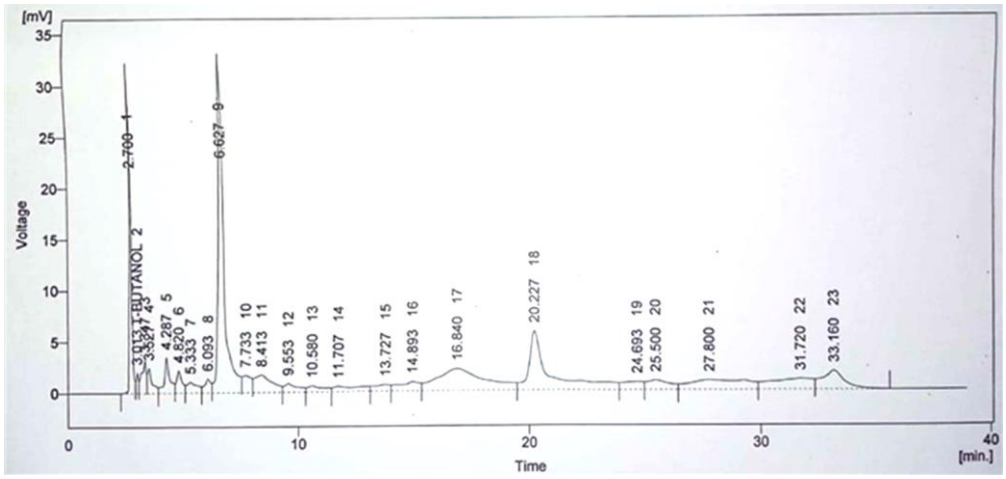

Fig. 1: HPLC diagram of ethanolic extract of *Haloxylon salicornicum*.

Table 1: Phytochemical composition of ethanolic extract of *Haloxylon salicornicum* plant achieved through HPLC

Sr. No	Retention time	Area (mV. s)	Compounds	Concentration (ppm)
1	2.700	1644.20	Quercetin	9.76
2	4.287	159.209	Gallic Acid	2.08
3	13.727	29.550	Vanillic Acid	1.83
4	14.893	53.901	Benzoic Acid	5.71
5	16.840	303.455	Synirgic acid	7.58
6	20.227	354.117	M-crumeric Acid	4.24
7	24.693	46.211	Cinamic acid	1.61

Table 2: Adulticidal efficacy of various concentrations of aqueous methanol extract of *H. salicornicum* against *H. contortus*

	Time passed after starting treatments						
Treatments	0 hour	2 hour	4 hour	6 hour	8 hour	10 hour	12 hour
Levamisole 0.55 mg/mL	0.00±0.00^{i}	5.66±0.33^{d}	10.00±0.00^{a}	10.00±0.00^{a}	10.00±0.00^{a}	10.00±0.00^{a}	10.00±0.00^{a}
PBS	0.00±0.00^{i}	0.00±0.00^{i}	0.00±0.00^{i}	0.00±0.00^{i}	0.00±0.00^{i}	0.00±0.00^{i}	0.00±0.00^{i}
3.125 mg/mL	0.00±0.00^{i}	2.66±0.33^{g}	5.00±0.00^{de}	8.00±0.00^{b}	8.00±0.00^{b}	9.66±0.33^{b}	10.00±0.00^{a}
6.25 mg/mL	0.00±0.00^{i}	0.00±0.00	2.66±0.66^{g}	4.33±0.33^{e}	6.66±0.33^{c}	8.33±0.33^{b}	10.00±0.00^{a}
12.5 mg/mL	0.00±0.00^{i}	1.66±0.33^{h}	3.33±0.88^{f}	8.00±0.00^{bc}	10.00±0.00^{a}	10.00±0.00^{a}	10.00±0.00^{a}
25 mg/mL	0.00±0.00^{i}	4.33±0.33^{e}	6.66±0.33^{c}	8.66±0.66^{b}	10.00±0.00^{a}	10.00±0.00^{a}	10.00±0.00^{a}

Table 3: Adulticidal efficacy of various concentrations of ethyl acetate extract of *H. salicornicum* against *H. contortus*

	Time passed after starting treatments						
Treatments	0 hour	2 hour	4 hour	6 hour	8 hour	10 hour	12 hour
Levamisole 0.55mg/mL	0.00±0.00^{k}	7.66±0.33^{d}	10.00±0.33^{a}	10.00±0.00^{a}	10.00±0.00^{a}	10.00±0.00^{a}	10.00±0.00^{a}
PBS	0.00±0.00^{k}	0.00±0.00^{k}	0.00±0.00^{k}	0.00±0.00^{k}	0.00±0.00^{k}	0.00±0.00^{k}	0.00±0.00^{k}
3.125mg/mL	0.00±0.00^{k}	0.33±0.33^{j}	2.33±0.67^{h}	3.66±0.33^{g}	6.00±0.57^{e}	9.66±0.33^{ab}	10.00±0.00^{a}
6.25mg/mL	0.00±0.00^{k}	0.00±0.00^{k}	2.33±0.33^{h}	4.33±0.33^{f}	6.33±0.33^{e}	9.66±0.33^{ab}	10.00±0.00^{a}
12.5mg/mL	0.00±0.00^{k}	1.66±0.88^{i}	4.00±0.57^{f}	6.00±0.58^{e}	8.33±0.66^{c}	10.00±0.00^{a}	10.00±0.00^{a}
25mg/mL	0.00±0.00^{k}	2.33±0.33^{h}	4.66±0.33^{f}	7.00±0.00^{de}	9.00±0.00^{b}	10.00±0.00^{a}	10.00±0.00^{a}

Values carrying same letters as superscript have no significant statistical difference (P<0.05) at CI 5%.

Table 4: Egg hatch inhibition of aqueous methanol extract of *H. salicornicum* against the eggs of *H. contortus*

Treatments	Concentrations	Percent Egg hatch Inhibition
PBS	1ml/well	41.00±0.02
Oxfendazole (µg/mL)	25µg/mL	59.00±0.03
	12.5µg/mL	45.67±0.02
	6.25µg/mL	40.00±0.02
Aqueous methanol	25mg/mLl	63.00±0.04
extract (mg/ml)	12.5mg/mL	57.00±0.02
	6.25mg/mL	55.00±0.01
	3.125mg/mL	53.00±0.01

Table 5: Egg hatch inhibition of ethyl acetate extract of *H. salicornicum* against the eggs of *H. contortus.*

Treatments	Concentrations	Percent Egg hatch Inhibition
PBS	1 ml per well	40.00±0.02
Oxfendazole (µg/mL)	25µg/mL	71.00±0.05
	12.5µg/mL	56.33±4.94
	6.25µg/mL	45.67±4.70
Ethyl acetate	25mg/mL	91.00±0.00
extract (mg/mL)	12.5mg/mL	81.67±0.01
	6.25mg/mL	76.30±0.00
	3.125mg/mL	72.00±0.01

Furthermore, tannins not only improve the protein nutrition and immune system of the host but also react directly by attaching with parasites skin (Min *et al.*, 2003) and also affect the oxidative phosphorylation leading to blockage of ATP synthesis in the parasites (Martin, 1997). Other scientists also demonstrated a very complex reaction occurred between tannins of plant extracts and cuticle of parasitic nematodes causing paralysis and even death of these worms (Vidyadhar *et al.*, 2010). Thus, a correlation has been demonstrated between the parasitic cuticular changes caused by condensed tannins of plant extracts to study the anthelmintic efficacy of walnut extract against the adult worms of *Trichostrongylus colubriformis* (Hoste *et al.*, 2006). Moreover, it has also been shown that cuticular changes in the adult worms of *H. contortus* inhibit the parasites motility and disturb their food intake, eventually leading to death due to under-nutrition (Martínez-Ortíz-de-Montellano *et al.*, 2013; Yoshihara *et al.*, 2015). From the above information, it could be concluded that the tannins

of *H. salicornicum* might be the responsible factor for its anthelmintic efficacy. Further, *in vivo* studies of *H. salicornicum* would be conducted to determine the optimal non-lethal concentration required to maximize its anthelmintic potential against *H. contortus* in livestock.

Conclusions: It can be concluded that *H. salicornicum* has potential of herbal dewormer. However, *in vivo* anthelmintic activity of *H. salicornicum* needs to be determined.

Authors contribution: FAA**,** TR, KJI & SSIB conceived the idea. SM & TR executed research. AZF performed statistical analyses. FAA, SM & TS wrote manuscript; TS, MO & RR reviewed manuscript.

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