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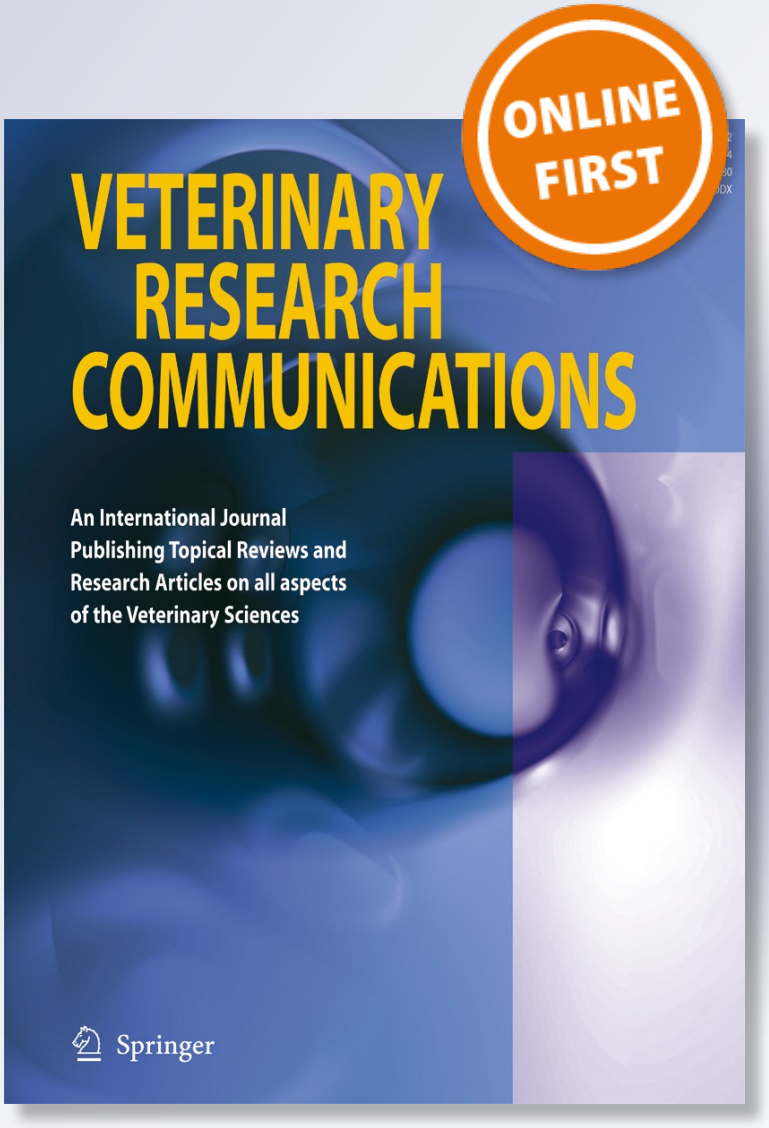

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REVIEW ARTICLE

In vitro evidence that the pastoral *Artemisia campestris* species exerts an anthelmintic effect on *Haemonchus contortus* from sheep

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Abstract Occurrence of anthelmintic resistant strains of helminths is increasing. The aim of this study was to evaluate the in vitro anthelmintic activity of *Artemisia campestris* in comparison to albendazole against *Haemonchus contortus* of sheep. In this respect, in vitro anthelmintic activities of crude aqueous and crude ethanolic extracts of aerial parts of *A. campestris* were investigated on eggs and adults of *Haemonchus contortus*. Chemical analyses revealed that overall profile of both extracts samples were dominated by flavonoids among them quercetin and apigenin derivatives were the most abundant phenolics constituents. Both extract types completely inhibited egg hatching at a concentration close to 2 mg/ml. Lethal concentration 50% of *A. campestris* ethanolic and aqueous extracts were 0.83 and 1.00 mg/ml respectively ($p<0.05$). The ethanolic extract showed better in vitro activity against adult parasites than the aqueous extract in terms of the paralysis and/or death of the worms at different hours post-treatment. Dose dependent activity was also observed for both extract. After 8 and 24 h of exposure, the ethanolic extract induced 91.3 and 100% mortality at the highest tested concentration respectively, while the aqueous extract induced 3.22 and 70.96% at the same concentration respectively.

To our knowledge, these results depict for the first time that *A. campestris* possesses in vitro anti-*Haemonchus contortus* properties.

Keywords *Artemisia campestris* · Anthelmintic · *Haemonchus contortus* · Tunisia

Introduction

Helminthoses represent a major constraint to the development of ruminant production, as they cause important financial losses particularly in areas where extensive grazing is practiced (Waller 1997). Gastrointestinal nematodes impair animal health welfare and productivity since they induce a decrease in meat production and reproduction and increase death rate (Hoste et al. 2005; Jabbar et al. 2006).

Commercial anthelmintics have been used for decades throughout the world to reduce the impact of helminths. Despite huge amounts of money spent worldwide to control these parasites, animal industry has not considerably benefited from the use of anthelmintics. Indeed, resistant parasite populations to several commercial anthelmintics are increasing, hence threatening field control of parasites (Jabbar et al. 2006). In several developing countries, small farmers have limited access to commercial anthelmintics and veterinary services due to their non-availability and/or to their high cost. These stockowners rely on the ethno-veterinary medicine as an alternative and a sustainable control option readily adaptable (Hussain et al. 2008; Al-Shaibani et al. 2009; Deeba et al. 2009; Sindhu et al. 2010) and such use underlines a rich background of indigenous knowledge that needs to be captured (Landau et al. 2014). Screening and proper assessment of the claimed effect on parasites and other animal diseases of

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some plant species could offer sustainable, accessible and environmentally acceptable alternatives.

Haemonchus contortus is one of the major gastrointestinal pathogens of small ruminants (O'Connor et al. 2006). This species was used by several authors to evaluate the anthelmintic effects of various medicinal plant species (Alawa et al. 2003; Assis et al. 2003; Hounzangbe-Adote et al. 2005; Eguale et al. 2006; 2007). Several methods are commonly used for testing nematicidal activity of both chemical and plant extract drugs. Amongst them, in vitro assays are relevant and cheaper than in vivo methods. Egg hatch assay (EHA) (Hubert and Kerboeuf 1984) is currently used for the detection of anthelmintic resistance in gastrointestinal nematodes (GIN) (Timothy et al. 2012). The adult worms motility (AWM) assays allow a more realistic evaluation of the in vivo nematicidal activity (Hounzangbe-Adote et al. 2005). These two tests are based on the hypothesis that in vitro nematicidal activity is indicative of a potential in vivo activity.

Artemisia campestris, commonly known as "tgouft" in Tunisia, is a perennial aromatic herb belonging to asteraceae family; it is widespread in Northern Africa and other similar Mediterranean agro-ecological zones and is commonly used as an herbal medicine. The leaves of this species are collected in summer and used in traditional medicine as decoction for their antispasmodic, anti-inflammatory, anti-rheumatic, antimicrobial, anthelmintic and anti-venin properties (Le floc'h 1983; Kotb 1985). Moreover, Ahmed et al. (2011) showed that ethanol; water infusion extracts and essential oil from *A. campestris* inhibit in vitro human adenocarcinoma cells. The phytochemical assessment of this species revealed the presence of tannins, polyphenols, flavonoids, saponosides and essential oils (Akrout 2005; Sefi et al. 2010). Different compounds have been isolated from the solvent (chloroform, hexane, and alcohol) extracts of this species such as flavonoids, chromones, and acetophenones (Tarhouni 1996; Vasconcelos et al. 1996). They are suspected to be the origin of the observed biological activities (Aniya et al. 2000; Memmi et al. 2007).

To our knowledge, there have been no published reports on the anthelmintic effects of this species. This study therefore aimed to test the in vitro anthelmintic efficacy, against *H. contortus*, of crude aqueous and ethanolic extracts of *A. campestris* aerial parts.

Materials and methods

Plant material and extracts preparation

Fresh leaves and stems of *A. campestris* were collected at Elhania, Sidi Bouzid governorate (Central Tunisia) during the spring season. The plant material was rinsed under running tap water then air dried at 50° C in a ventilated oven, until a constant weight was reached and finally ground up to a fine powder.

The crude aqueous and ethanolic extracts were used in the present trial. For this, 100 g of *A. campestris* powdered aerial parts were sequentially extracted by maceration in distilled water at room temperature (20–25° C). The brew was collected and filtered with Whatman number one filter paper then lyophilized. For the ethanolic extract, 100 g of powdered plant were added to 500 ml of 95° ethanol then incubated at room temperature and frequently mixed. The solution was filtered through Whatman number one filter paper and finally, the solvent was evaporated in Rotavapor. The whole process was repeated 3 times (24 h for each). All extracts were concentrated, dried and kept in dark flask at +4° C until used.

Analyses of aqueous and ethanolic plant extract composition

The chemical compositions of both *A. campestris* aqueous and ethanolic extracts were carried out by chromatography/mass spectrometry (HPLC/MS) analysis. HPLC–MS separation (series 1100, Agilent, Waldbronn, Germany) was performed using an Agilent C18 reversed-phase column (150 × 4.6 mm) maintained at 33° C with a direct injection of 25 µl of the extract, 100 bars pressure and 0.25 ml/min flow rate. A programmable variable wavelength UV detector was used for the analytic detection. Elution was performed by gradient mode using two mobile phases (A and B). Phase A solution consisted of water and acetic acid (999/1 v/v respectively), whilst phase B solution was acetonitrile. The gradient programme was chosen as follows: phase B was set at 5% during the 5 first minutes, increased linearly to 100% at 65 min, remained at 100% for three minutes and decrease to 5% at 69 min. Chromatographic peaks were integrated by Mass Lynx Software. The Mass spectroscopy (MS) was performed using a Micro mass Quattro Ultima PT MS model. The ion trap detector with electro-spray ionization (ESI) source was used for quantification in negative ionization mode under specific operating conditions (capillary voltage (KV): 3.20; capillary temperature (C): 300; multiplier (V): 550 cone gas flow (L/h): 60).

The identity of the components was assigned by comparison of their retention indices HPLC–MS spectra with commercially available standards from a homemade library or reported in the literature.

In vitro anthelmintic assays

The anthelmintic efficacy tests of the two plant extracts on *H. contortus* were performed using tow different procedures. For each assay, the eggs or adult worms were obtained from faeces and abomasum of Barbarine donor lambs aged 4 to 6 months and experimentally infected by oral administration of 6.000 *H. contortus* third stage larvae (L3).

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Egg hatch assay

The eggs used in the present assay were collected from previously mentioned donor sheep according to the guidelines of the World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) (Timothy et al. 2012). After crushing the faeces in water, and successive siftings (300, 150, 70, and 38 μm sieves), eggs were collected and centrifuged for 10 min at 2,500 rpm. The supernatant was removed and a sodium chloride solution (density 1.2) was added. After homogenization, the mixture was centrifuged for 15 min at 3,000 rpm. The floating eggs were then extracted by pouring the supernatant on a 38 μm sieve and abundant washing with distilled water.

Freshly collected eggs were incubated with different extracts in quadruplets. Leaves and stems *A. campestris* aqueous and ethanolic extracts were used as test treatment. Untreated eggs in Phosphate Buffered Saline (PBS) with Dimethyl sulfoxide (DMSO) (0.5%) solution were used as negative control. Whilst, albendazole, reference drug (99.8% pure standard reference, Médivét, S.A., Tunisia) was dissolved in DMSO and diluted at three concentrations (0.25; 0.5 and 1 μg/ml); the latest served as positive control. For each extract concentration, approximately, 200 eggs in 1 ml of PBS were placed in each test tube. Aqueous extracts at different concentrations (2.0, 1.0, 0.5 and 0.25 mg/ml) in 1 ml PBS were used. Ethanolic extracts, at the same concentrations, in a volume of 1 ml in PBS with DMSO (0.5%) were used. The test tubes were covered and incubated at 27 °C for 48 h. Hatched larvae (dead or alive) and unhatched eggs were counted under dissecting microscope at 40 x magnification.

Adult worms motility assay

This test was performed according to Hounzangbe-Adote et al. (2005). Adult worms were collected from a lamb, at week 6 post-experimental infection. Immediately after being slaughtered, the abomasum was removed, opened and placed at 37° C, 9‰ sodium chloride solution. The collected parasites were then washed and kept in a PBS solution. Five to ten actively moving worms were placed in Petri dishes filled with 2.0, 1.0, and 0.5 mg/ml of *A. campestris* aqueous or ethanolic extracts in PBS or PBS with DMSO (0.5%) respectively in a volume of 4 ml. Albendazole dissolved in DMSO then in PBS at a final concentration of 0.5 mg/ml was used as positive control. PBS with DMSO (0.5%) was used as negative control. All the tests were performed in triplicates. Inhibition of worm motility was the criteria of anthelmintic activity. The required delays for larvae paralysis and/or complete immobility were recorded at 0; 4; 8 and 24 h. To test if the larvae could retrieve their motility after 24 h, they were washed with distilled water and resuspended in PBS for 30 min. Worms' death was ascertained by the absence of motility during an observation period of 5–6 s. The immobility index was calculated as follow:

Immobility index (%)=100 × (number of dead worms per Petri dish/total number of worms per Petri dish).

Statistical analyses

The statistical analyses were performed with SPSS-10.0 software package for Windows. Lethal concentration 50% (LC_{50}) for egg hatch inhibition was calculated by probit analysis. Regression was used for evaluation of dose–response relationship using Minitab® Release 14. The result of the worm motility inhibition was expressed as mean±standard error of mean (S.E.M). Means of anthelmintic efficacy were compared by *Student's* test. A probability of 0.05 was used as a threshold for statistical significance.

Results

Chemical analysis of *Artemisia campestris* aqueous and ethanolic extracts

According to HPLC-MS analysis, a large part of phenolic compounds were assigned. Quercetin derivatives were the most abandon phenolic component in ethanolic extract. While, apigenin derivatives were the most abandon phenolic compounds identified in the aqueous extract (Table 1).

Egg hatch assay

Albendazole inhibited 92.05% of egg hatching at 1 μg/ml (LC_{50}=0.314 μg/ml). Crude ethanolic extract (LC_{50}=0.827

Table 1 Clause of major compounds in aqueous and ethanolic *Artemisia campestris* extracts (ranked in decreasing order) identified by Liquid Chromatography/Mass Spectrophotometry

Type of extract	Chemical component
Aqueous extracts compounds	Quercetin
	3 methyl- quercetin
	7 methyl- quercetin
	7 methyl - taxifolin
	7,3 methyl- kaempferol
Ethanolic extracts compounds	Apigenin-6,8- di C-glucoside
	Eupafolin- glucoside
	Acethyl- luteolin- glucuronide
	Apigenin-7- glucoronide
	Hesperidin
	Luteolin

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mg/ml) showed higher inhibitory effects than crude aqueous extract (LC_{50}=1.00 mg/ml) on egg hatching. The two extracts showed a statistically significant difference in their dose-dependent ovicidal activity ($p<0.05$) (Fig. 1; Table 2). The maximum concentration required to induce total (100%) egg hatch inhibition for aqueous and ethanolic extracts was 2 mg/ml (Fig. 1)

Adult worm motility

Artemisia campestris crude ethanolic extract inhibited more worms than the aqueous extract in all tested concentrations. Dose dependent activity was also observed for both extracts. After 8 and 24h of exposure, the ethanolic extract induced 91.3 and 100% mortality at the highest tested concentration respectively, while the aqueous extract induced 3.22 and 70.96% at the same concentration respectively (Table 3). In addition, motility was higher with the crude aqueous extract treatment, as the worms survived for a significantly longer period in the presence of aqueous extract. This indicated that ethanolic extract has greater anthelmintic activity than the aqueous extract. There was 78% mortality of worms in albendazole within 8 h post-exposure. However, the worms in DMSO negative control solution showed neither paralysis nor mortality. Finally, no worm recovered motility in the PBS revival test.

Table 2 Dose-effect regression equations and determination coefficients of *Artemisia campestris* extracts on *Haemonchus contortus* egg hatching

Treatment	LC50	Regression equations and determination coefficients (R^2)
Ethanolic extract	0.827 mg/ml	$y1.869e^{0.810x}$, R^2=0.902
Aqueous extract	1.00 mg/ml	$y=4.392e^{0.606x}$, R^2=0.924
Albendazole	0.314 µg/ml	$y=4.385\times+5{,}213$, R^2=0776

Discussion

The current study is the first report of the anthelmintic activity of *A. campestris* species against *H. contortus* reducing egg hatching and adults' worm motility. The plant is an important pastoral species in arid rangelands and has several medicinal virtues.

A large number of medicinal plants have been used to control parasitic infections in humans and animals (Luciana et al. 2013; Landolsi et al. 2013; Jyotsna et al. 2014). During preliminary screening of their anthelmintic activity, the majority of the researchers prefer in vitro tests including, larval (Perrett and Whitfield 1995) and adult (Parveen 1991) paralysis tests as well as egg hatching inhibition assays (Alawa et al. 2003), or biochemical tests (Khunkitti et al. 2000).

Artemisia campestris aqueous and ethanolic extracts totally inhibited *H. contortus* egg hatching at the concentration of 2 mg/ml. *Thymus capitatus* (another important pastoral species form arid Tunisia) aqueous and ethanolic extracts totally inhibited *H. contortus* egg hatching at 2 mg/ml concentration (Landolsi et al. 2013). Eguale et al. (2007) reported that aqueous and hydro-alcoholic extract of *Hedera helix* induce complete (100%) egg hatch inhibition at the same concentrations. Lower activity was observed for *Spigelia anthelmia* extracts on *H. contortus* egg hatching; it was effective at the concentration of 50 mg/ml (Assis et al. 2003). At the concentration of 10 mg/ml, the seed ethanol extract of *Mangifera indica* revealed 91% of effectiveness on *H. contortus* eggs (Costa et al. 2002). The aqueous extracts of *Annona senegalensis* seeds showed low *H. contortus* egg hatching inhibition activity (11.5%) at the concentration of 7.1 mg/ml (Alawa et al. 2003).

Significant anthelmintic effects of both *A. campestris* extracts on adults *H. contortus* were observed in terms of worms'

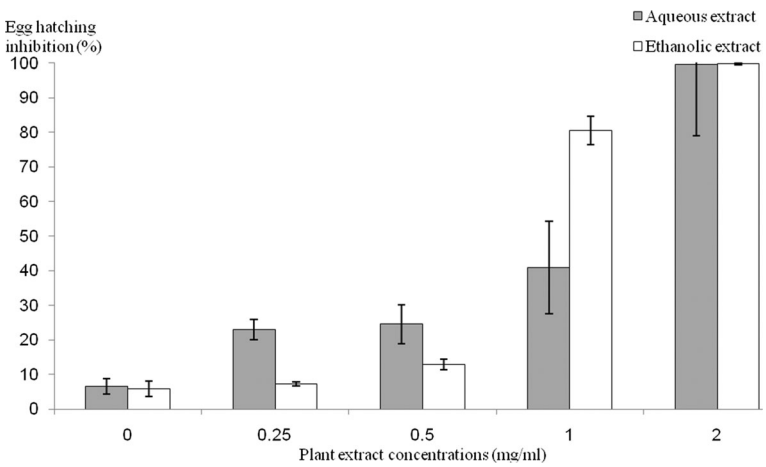

Fig. 1 Dose-dependent profile of the percent hatching egg of *Haemonchus contortus* submitted to increasing concentrations of plant extracts (0; 0.25; 0.5; 1 and 2 mg/ml)

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Table 3 In vitro anthelmintic efficacy of *Artemisia campestris* crude aqueous and ethanolic extracts on *Haemonchus contortus*

Treatment	Concentrations (mg/ml)	Mortality rate (%) of *Haemonchus contortus* worms post-exposure to various treatments (mean±SEM)			
		0h	4h	8h	24h
Crude aqueous extract	0.5	0±0.00	0±0.00	2.85±0.06	74.28±0.00
	1	0±0.00	0±0.00	3.22±0.07	70.96±0.00
	2	0±0.00	0±0.00	3.22±0.07	70.96±0.27
Crude ethanolic extract	0.5	0±0.00	0±0.00	62.96±0.21	100±0.00
	1	0±0.00	0±0.00	74.19±0.16	100±0.00
	2	0±0.00	0±0.00	91.30±0.14	100±0.00
Positive control (albendazole)	0.5	0±0.00	12.50±0.05	78.04±0.06	100±0.00
Negative control (PBS with DMSO 0.5%)		0±0.00	0±0.00	0±0.00	16.12±0.07

paralysis and/or death at different post-treatment intervals and even low concentrations (91.3 and 3.2 % after 8 h post-exposure respectively in ethanolic and aqueous extract at 2 mg/ml). Several studies revealed that the effect of plant extracts on adult worms can occur only at high concentrations. For example, *Euphorbia helioscopia* methanolic extract induces highest nematode immobility (98%) at 50 mg/ml concentration (Bashir et al. 2012) while methanol extract of *Artemisia brevifolia* at concentration of 25 mg/ml is needed to induce significant in vitro anthelmintic activity on adult *H. contortus*. Aqueous extracts had no significant effect (Iqbal et al. 2004). In view of all these results in the literature, we put forward the statement that *A. campestris* was highly active against *H. contortus* egg hatching and worms' immobility.

It is generally considered that the plant extracts' anthelmintic proprieties are related to their content in condensed tannins (Akkari et al. 2008a, b; Hoste et al. 2006). Nevertheless, in vitro assays suggest that some other flavonoid molecules, e.g. flavonols or flavanols, might also possess anthelmintic properties (Molan et al. 2003, 2004; Barrau et al. 2005; Brunet and Hoste et al. 2006). The dose dependent relationship between the concentration of tannins and/or flavonoid compounds and the anthelminthic activity has been repeatedly demonstrated in both in vitro (Brunet and Hoste et al. 2006; Molan et al. 2002) and in vivo essays (Hoste et al. 2006; Terrill et al. 2009). It has been suggested that antioxidant flavonoids might have an anthelmintic activity (Ferreira 2011).

In the case of our study, it was revealed that profiles of both extracts samples were dominated by flavonoids; quercetin, apigenin and kempherol derivatives were the most abundant phenolic constituents (Table 1). Apigenin is one of important flavonoid, having a chemopreventive (Ruelia- de-Sousa et al. 2010) and antimicrobial activities (Palacios et al. 1983). Kaempferol and quercetin have a wide range of pharmacological activities including antioxidant, anti-inflammatory, antimicrobial and antiallergic activities (Shaik et al. 2006; Calderon-Montana et al. 2011). In addition, Lasisi and Kareem (2011) reported that quercetin present in *Bridelia ferruginea* stem barks have an anthelmintic activity.

The results indicated that quercetin, apigenin and Kempherol are the probable active molecules of both plant extracts, but the considerable activity suggests a possible additive or synergistic relationship between these major components and the other plant extract components. Indeed, both extracts are a complex mixture of compounds that can interact with multiple molecular targets in various developmental parasites' stages.

The greater anthelmintic activity of crude ethanolic extract compared to crude aqueous extract could be due to a high concentration of alcohol soluble active anthelmintic molecules in *A. campestris*. Furthermore, the greater anthelmintic activity of the crude ethanolic extract in the current study could be due to easier and rapid transcuticular absorption of the ethanolic extract into the worms owing to the lipid soluble nature of the ethanolic extracts (Eguale et al. 2007).

Based on the results of the present study, it can be concluded that *A. campestris* crude aqueous and ethanolic extracts aerial parts showed significant in vitro dose-dependent activity against sheep *H. contortus* as ascertained by worm motility inhibition and egg hatching inhibition. Prior to in vivo trials, further in vitro trials at different concentrations against different worm species and stages are required to determine a more global anthelmintic activity of this plant species and its potential use in controlling small ruminants' gastrointestinal nematodes.

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