In-vitro Anthelmintic Effects of Aqueous and Ethanolic Extracts of *Marrubium vulgare* Leaves Against Bovine Digestive Strongyles

Marrubium vulgare (karaderme) Yapraklarının Sulu ve Etanolik Ekstraktlarının Sığır Sindirim Strongilozuna Karşı *in-vitro* Antelmintik Etkileri

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ABSTRACT

Objective: The aim of the present study was to evaluate *in vitro* the anthelmintic activity of *Marrubium vulgare* L. growing in Algeria against digestive strongyles in naturally infected bovine.
Methods: The anthelmintic activities of the extracts were evaluated using the egg hatch assay and larval mortality assay. Leaves powder of *M. vulgare* as extracted by maceration. Ethanolic (EE) and aqueous extracts (AE) were tested at 0.78, 1.55, 3.1, 6.2, 12.5, 25, and 50 mg/ml. Albendazole and dimethyl sulfoxide were used as positive and negative controls at concentrations20 mg/ml and 3%, respectively.
Results: The mean embryonation rate was maximum in AE and EE (48.4±3.47% and 54.2±2.87%, respectively) of *M. vulgare* leaves. The extracts of *M. vulgare* leaves high effects were observed with 50 mg/ml, but the lowest reduction on parasite eggs hatchability was observed in cultures exposed to 0.78 mg/ml to both extracts. The larval mortality rate of both AE and EE from *M. vulgare* showed that the extracts at 50 mg/ml exhibited 45.8±1.99% and 51±2.53%, respectively, at 24h.
Conclusion: The findings of the present study showed that AE and EE of *M. vulgare* leaves have a potential anthelmintic activity on eggs and larvae of bovine strongyles parasites *in vitro*.
Keywords: *Marrubium vulgare*, anthelmintic effects, Bovine, in vitro
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ÖZ

Amaç: Bu çalışmanın amacı Cezayir'de yetişen *Marrubium vulgare* yapraklarının, doğal yollarla enfekte olmuş sığırlarda sindirim strongilozuna karşı in vitro antelmintik etkisini değerlendirmektir.
Yöntemler: Ekstraktların antelmintik aktiviteleri yumurtadan çıkma testi (egg hatch assay) ve larva mortalite testi (larval mortality assay) kullanılarak değerlendirildi. *M. vulgare* yaprak tozları maserasyon ile ekstrakte edildi. 0.78, 1.55, 3.1, 6.2, 12.5, 25 ve 50 mg/mL ölçülerinde etanolik (EE) ve sulu ekstraktlar (AE) test edildi. Sırasıyla 20 mg/mL ve 3% konsantrasyonlarında albendazol and dimetil sülfoksit, pozitif ve negatif kontroller olarak kullanıldılar.
Bulgular: *M. vulgare* yapraklarının ortalama embriyonlaşma oranı AE ve EE'de maksimum idi (sırasıyla %48,4±3,47 ve %54,2±2,87). *M. vulgare* ekstraktlarının yapraklarının 50 mg/mL ile yüksek etki gösterdiği gözlenmiştir, ancak her iki ekstrakta da, 0,78 mg/mL'ye maruz bırakılan kültürlerde en düşük parazit yumurta kuluçka randımanı gözlenmiştir. *M. vulgare*'den elde edilen AE ve EE'nin larva mortalite oranları, 24 saatte 50 mg/mL'deki ekstraktlar için sırasıyla %45,8±1,99 ve %51±2,53 olarak gözlenmiştir.
Sonuç: Bu çalışmanın bulguları, *M. vulgare* yapraklarının AE ve EE'sinin, *in vitro* olarak sığır strongil parazitlerinin yumurtaları ve larvaları üzerinde potansiyel bir antelmintik etkiye sahip olduğunu göstermiştir.
Anahtar Kelimeler: *Marrubium vulgare*, antelmintik etkiler, sığır, *in vitro*
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INTRODUCTION

Parasitic diseases are among the factors that limit ruminant production worldwide, accounting for large economic losses. Digestive strongyles are parasitic diseases caused by strongylid nematodes that include a number of parasitic species, (such as *Haemonchus, Ostertagia, Trichostrongylus, Cooperia, Nematodirus, Bunostomum, Oesophagostomum*, etc.) and are among the bovine parasites most commonly encountered in temperate regions. Helminthes are the most common infections in cattle, affecting almost all bovine

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breeds. The negative impact of helminth infections in livestock productivity in some countries has been established, such as retarded growth, weight loss, reduced food consumption, lower milk production, impaired fertility, and high mortality rates in cases of massive infections (1). A recent study in Algeria (Bass Kabylie region) demonstrates that digestive strongyles are the most prevalent species in cattle (2). This infection is generally controlled by synthetic drugs; however, excessive use of such drugs has led to widespread resistance.

The worldwide interest in herbal products has grown significantly. A large number of medicinal plants have been used to treat parasitic infections in humans and animals. Herbal medicine can increase profits by reducing the use of conventional anthelmintics and extending the useful life of the limited number of anthelmintics available. Plants and their bioactive products are reported to have multiple medicinal applications in the treatment or control of many health problems and infections, including its use as antimicrobial, antioxidant, anti-inflammatory, antipyretic, and immunostimulant (3).

Marrubium vulgare L. (*Lamiaceae*) is native to North Africa, Central and Western Asia, and Southern Europe. This plant is used in folk medicine for the treatment of a variety of diseases. *M. vulgare* is currently used by traditional healers alone or combined with other herbs to treat bronchitis, coughs, and colds. Moreover, it is traditionally used for its antioxidant, antibacterial (4), analgesic, and hypoglycemic effects (5). It is essentially rich in phenolic compounds among other phytochemicals and is widely used in traditional medicine. Recently, Amessis-Ouchemoukhet al. (4) demonstrated the potential role of this plant in the inhibition of cyclooxygenase1 and acetylcholinesterase activities. The important biological activities of *Marrubium* are attributed to its different bioactive compounds, such as flavonoids, diterpenoids, and phenylethanoid glycosides (6).

It is important to establish a successful program and to search for the development of alternative, safer, and environmentally friendly anthelmintic agents. There is also urgency in the use of medicinal plants to treat cases of parasitism in ruminants, and much of the success has been achieved against a variety of parasites. To our knowledge, no study using the anthelmintic activity of plant extracts from *M. vulgare* L. was reported in Algeria. The aim of the present study was to evaluate in vitro the anthelmintic activity of *M. vulgare* L. growing in Algeria (area of Béjaïa) against digestive strongyles in naturally infected bovine.

METHODS

The experimental protocol was approved by the Scientific Council of the Faculty of Life and Nature Sciences, University of Abderrahmane Mira, Béjaïa, Algeria.

Plant material and extraction

The leaves of *M. vulgare* were collected in March 2015 from the Aokas locality (Béjaïa Province, North Algeria; 36° 36′ N, 4° 41′ E. The species identification was performed at the laboratory of ecology (University of Béjaïa, Algeria). The plant was cut into small pieces and dried at room temperature (25-30 °C) for 1 week. Thereafter, the plant material was pounded using a coffee grinder resulting in a fine powder and was kept in the dark.

Leaves powder of *M. vulgare* was extracted by maceration with ethanol by shaking (Corning® PC-400D hot plate). Briefly, leaves powder was extracted with 70% ethanol and water in a 1:10 W/V ratio for 24h and then homogenized using a Polytron homogenizer (Brinkmann Instruments,Inc., Westbury, NY, USA) (7). The combined ethanolic extract (EE) and water extract were evaporated to dryness to yield crude EE and water extract. In addition, the resulting supernatants were filtered through Whatman paper no. 1. The combined EE and water extract were evaporated at 50 °C to dryness to yield crude EE and aqueous extract (AE). In order to improve solubility in water, the extract was dissolved in dimethyl sulfoxide solution (DMSO, 3%).A total volume (100 mL) was obtained that produced a stock solution at a concentration of 50 mg/ml from which a series of dilutions was made to obtain solutions at different concentrations of 0.78, 1.55, 3.1, 6.2, 12.5, 25, and 50 mg/mL.

Recovery of nematode eggs

Fresh eggs were obtained from the feces of naturally infected cattle according to Chollet et al. (8). Briefly, 3g of feces was collected, homogenized in mortar, suspended in saturated salt solution (NaCl, d=1.2), and filtered through two sieves (1 and 0.15 mm). The content was centrifuged at 1000 g for 5 min. The supernatant was poured through a 45 µm sieve. The retained material on the sieve containing the eggs was washed with tap water to remove the salt solution. It was then turned, and the opposite side was washed with tap water. Finally, the eggs were collected in a Petri dish (16 cm diameter).

Evaluation of ovicidal activity

The ovicidal efficacy test of different extracts was performed using two procedures as described by Coles et al. (9). An egg suspension of 0.2 ml was distributed in a flat-bottomed microtiter plate containing approximately 100 eggs/well and mixed with the same volume of plant extract yielding the following final concentrations: 0.78, 1.55, 3.1, 6.2, 12.5, 25, and 50 mg/mL. The eggs were incubated at room temperature for 24h.

A Lugol iodine solution was added to prevent the eggs from hatching. The number of embryonated eggs per well was counted using a stereo microscope (40× magnification). The percentage of embryonation (EM%) was determined as follows (10): E (%) = Number of embryonated eggs X 100/Number of eggs nature.

After 24h of incubation, all embryonated eggs and first stage larvae (L_1) were counted using a microscope (40× magnification) to assess the effects of the plant extract in the second experiment. The hatching rate (E%) was estimated using the following formula (11): E (%) = Number of Larvae 1 X 100/Number of embryonated eggs in culture.

Recovery of nematode larvae

The eggs were cultured using the technique described by Smyth (12). Briefly, 3ml of the eggs suspension was poured on a filter paper covering the bottoms of one Petri dish, then covered to maintain a high relative humidity (65%-67%) to prevent the dish from drying out, and stored at +24°C. After 3 days of incubation, L_1 larvae were observed in a Petri dish and were collected using a Baermann apparatus.

Evaluation of larvicidal activity

For assessment of the effects of the extracts on larvae (L_1), a 1mL solution containing approximately 15-20 L_1 was distributed in each

well of a flat-bottomed microtiter plate and mixed with the same volume of a specific extract. The flat-bottomed microtiter plate was covered, and the larvae were incubated at room temperature for 24h. The number of dead or immobilized larvae was assessed under a microscope (40× magnification). The corrected mortality rate (%) was determined using the following formula (13): cMR (%) = (Mce - Mt) X 100/100 - Mt.

Where cMR is the corrected mortality rate (%), Mce is the mortality obtained during the test, and Mt is the mortality registered in the negative control wells. If the mortality rate in the latter wells is <5%, cMR=Mce.

Statistical Analysis

The results (mean±SD) were expressed as percentage (%). The different rates of embryonation (EM%), eclosability (E%), and mortality (cMR%) due to the plant extracts were compared using the chi-square test at a 5% significance level. The lethal concentration 50 (LC_{50}) was determined using the regression lines of the SAS Probit according to the decimal logarithm of the concentration. All tests were repeated five times for each treatment and control. Albendazole (ABZ) was used as a positive control at 20 mg/mL concentration.

RESULTS

Table 1 summarizes the extracts of *M. vulgare* LC_{50} values of in vitro anthelmintic activity test. Regression analysis indicated that LC_{50} valueswere 39.72, 38.66, and 42.38 mg/ml for the inhibition of embryonation eggs, hatching rate, and L_1 mortality of *M. vulgare* EE, respectively. However, the LC_{50} value of AE of *M. vulgare* was >50 mg/mL for all tests.

The results indicated that EE of *M. vulgare* leaves appears to be more efficient against gastrointestinal strongyles in different tested stages than AE. Table 2 shows the variation of the mean embryonation rate of digestive strongyles eggs according to the concentrations of the extract of *M. vulgare* leaves. The inhibition

Table 1. Lethal concentration (LC_{50}, mg/mL) of AE and EE *M. vulgare* on inhibiting embryonation, hatchability eggs, and mortality L_1 of bovine digestive strongyles

Extract	Inhibiting embryonation	Inhibiting hatching	Mortality L1
Ethanolic	39.72 mg/mL	38.66 mg/mL	42.38 mg/mL
Water	>50 mg/mL	>50 mg/mL	>50 mg/mL

Table 2. Mean embryonation (EM%, ±SD) of leaves extract of *M. vulgare* at different concentrations against bovine digestive strongyles

Concentration (mg/mL)	Percentage of embryonation (EM%, ±SD)	
	AE	EE
0.78	8.4±2.02	10.6±2.16
1.55	10.4±2.01	13.8±2.41
3.1	14.6±2.16	17.4±2.56
6.2	20.8±2.40	22.6±2.78
12.5	29±2.73	31.8±3.08
25	38.2±3.08	43.8±3.08
50	48.4±3.47	54.2±2.87
DMSO	5.8±1.45*	6.4±2.01*
ABZ	100±0*	100±0*

*Negative and positive controls (DMSO and ABZ, respectively) compared with each extract treatment are statistically different in the same column ($p<0.05$).
AE: aqueous extracts; EE: ethanolic extracts; DMSO: dimethyl sulfoxide solution; ABZ: Albendazole

Table 3. Mean hatchability rate (E%, ±SD) of leaves extract of *M. vulgare* at different concentrations against bovine digestive strongyles

Concentration (mg/mL)	Hatchability rate (E%, ±SD)	
	AE	EE
0.78	8.8±1.45	11±1.57
1.55	11±1.36	14.2±2.40
3.1	15.4±2.02	18.2±2.27
6.2	21.6±2.16	23.6±1.68
12.5	29.8±2.41	32.6±2.30
25	39.4±3.20	44.8±2.14
50	49.2±3.08	55.2±2.41
DMSO	5.8±1.45*	6.2±1.45*
ABZ	100±0*	100±0*

*Negative and positive controls (DMSO and ABZ, respectively) compared with each extract treatment are statistically different in the same column ($p<0.05$).
AE: aqueous extracts; EE: ethanolic extracts; DMSO: dimethyl sulfoxide solution; ABZ: Albendazole

Table 4. Mean rate of mortality (cMR%, ±SD) of leaves extract of *M. vulgare* at different concentrations against bovine digestive strongyles

Concentration (mg/mL)	Percentage of mortality (Mc%, ±SD)	
	AE	EE
0.78	7.8±1.99	9.6±2.02
1.55	10.2±2.28	12.6±2.30
3.1	14.4±2.55	14±2.1
6.2	19.8±1.82	21.4±2.67
12.5	26.8±3.8	30.2±2.41
25	37.6±3.2	42.6±2.56
50	45.8±1.99	51±2.53
DMSO	5.8±1.45*	6.6±1.27*
ABZ	100±0*	100±0*

*Negative and positive controls (DMSO and ABZ, respectively) compared with each extract treatment are statistically different in the same column ($p<0.05$).
AE: aqueous extracts; EE: ethanolic extracts; DMSO: dimethyl sulfoxide solution; ABZ: Albendazole

effect was proportional to the extract concentration. The mean embryonation rate was maximum in AE and EE (48.4±3.47%and 54.2±2.87%, respectively). The lowest concentration of AE and EE of *M. vulgare* leaves (0.78 mg/ml) did not affect parasite embryonation. At 3.1 mg/ml, the embryonation rates were moderate in the solution containing both extracts. The mean embryonation rate of AE and EE (5.8±1.45% and 6.4±2.01%, respectively) was very low in distilled water and 3% DMSO. Both extracts of inhibition of embryonation rate were significantly lower than positive control ($p<0.05$).

Table 3 illustrates the variation of the mean hatching rate eggs of *M. vulgare* according to the concentration of AE and EE.*M. vulgare* effect varied according to the concentration of extracts. The extracts of *M. vulgare* leaves high effects were observed with 50 mg/ml, but the lowest reduction on parasite eggs hatchability was observed in cultures exposed to 0.78 and 1.55 mg/ml to both extracts. With regard to DMSO diluted in water (3%), its activity on the hatchability rate was lower than that shown by both extracts at minimal concentration. The ABZ-treated cultures (positive control) were significantly higher ($p<0.05$) than the tested concentration of AE and EE.

Table 4 shows the effects of different extracts on L_1 larvae of gastrointestinal strongyles after 24h of contact. The larval mortality rate of both AE and EE from *M. vulgare* showed that the extracts at 50 mg/ml exhibited 45.8±1.99% and 51±2.53%, respectively, at 24h. At a concentration of 3.1 mg/mL, the mean larval mortality rates were similar in AE and EE (14.4±2.55% and 14±2.1%, respectively). Generally, the larval mortality rate was concentration dependent in both extracts. Distilled water and 3% DMSO in both AE and EE were 5.8±1.45% and 6.6±1.27%, respectively. Significantly different ($p<0.05$) from tested concentrations of AE and EE and from ABZ-treated cultures were observed.

DISCUSSION

The present study was conducted in order to find a phytotherapic product to help control gastrointestinal parasites in bovines and to provide an alternative use of plant extracts. These tests measured the effect of anthelmintic activity directly on the processes of hatching, development, and motility of endoparasites. The results of our study have demonstrated that extracts of *M. vulgare* leaves have an inhibitory effect on the eggs and larval development of bovine digestive strongyles parasite in vitro. As previously described, *M. vulgare* possesses different bioactive compounds, such as flavonoids, diterpenoids, tannins, and phenylethanoid glycosides (4). Moreover, plants with anthelmintic activities contain phytochemicals, such as polyphenols, tannins, flavonoids, and saponins. This anthelmintic activity may be attributed to an individual or a combined effect of the bioactive compounds (14).

AE and EE of leaves of *M. vulgare* at high concentration showed a significant anthelmintic activity, in agreement with those reported by Al-Shaibani et al. (15). The results of the present study also correspond with those published previously, which demonstrated that the anthelmintic activity resulted with increasing the plant extract concentrations (16). More interesting, the present study also considered the positive and negative controls of both extract tests, which give a more tangible comparison in the experiment. As expected, the low concentration of DMSO (3%) examined did not have any effect on hatching, whereas ABZ completely prevented egg hatching at the standard dose used (20 mg/ml). In addition, the results obtained for the inhibition of embryonated and egg hatching, as well as for the larvae mortality, are slightly higher with EE than with AE in bovine digestive strongyles. A similar observation was reported by Tiwari et al. (17); in their study, EE possesses a high amount of polyphenols than AE. In addition, the higher concentrations of more bioactive flavonoid compounds were detected with 70% ethanol due to its higher polarity than pure ethanol (18). Hence, organic solvent extracts were more bioactive compounds from plants than AE (19).

Previous studies have used this test to screen the anthelmintic activity of some plant compounds (20). In the present study, AE showed practically similar activity with an LC_{50} value of 50 mg/mL than EE with an LC_{50} value of 49.7 mg/ml. Recently, Ngaradoum et al. (21) showed that methanolic extract of *Ziziphus mucronata* barks has a significantly ($p<0.05$) higher activity with an inhibition concentration 50 (IC_{50}) value of 3.9 mg/mL than AE with an IC_{50} value of 14.7 mg/mL when tested against *Haemonchus contortus* egg. In addition, Zabré et al. (22) reported that *Acacia raddiana* is effective with IC_{50}=1.58 mg/mL for AE and IC_{50}=0.58 mg/mL for acetonic extract for egg hatch inhibition. These differences may be attributed to the used concentration and the composition of plant extracts. Our results on parasite eggs suggested that EE of *M. vulgare* appears to be active than AE on embryonation mechanism and hatching egg. This could be attributed to the difference in proportions of the secondary metabolite components and the greatest facility of extracts diffusion through the egg shell that were responsible for anthelmintic activity. It has been reported that tannins or saponins can possibly penetrate the different layers of the egg and inhibited the formation of larva by affecting the *morula* (23).

Moreover, several studies had demonstrated that tannins and saponins could stop the larval formation and hatching process of *H. contortus* eggs (24). In this research, the ovicidal activities of the extracts could be due to the fact that the active compounds passed through the egg shell and stopped the segmentation of blastomeres or paralyzed the larvae inside the embryonated egg (25). In addition, the larvicidal activity of plant extract may be attributed to tannins capacity to bind free protein available in the tubes for larval nutrition, therefore leading to larval starvation or decrease in gastrointestinal metabolism directly through the inhibition of oxidative phosphorylation, causing larval death (26).

The biochemical and pharmacological activities of flavonoids have been attributed to their antioxidative and antibacterial properties (4) and act by penetrating the cuticle blocking the postsynaptic receptors and consequently paralyzing effects on larvae (27). In addition, Chartier et al. (28) noted that active compounds may have induced the release of gamma-aminobutyric acid that blocked the transmission of nerve impulses or decoupling the oxidative phosphorylation reaction that can lead to the exhortation of the energy of larvae.

Some studies have shown that some local plants that contain tannins can impair different key biological processes of the parasitic life cycle, namely the eggs development into larvae, the excretion

of eggs by adult worms, and the establishment of the infective L_3 larvae (29).The findings of the present study showed that increasing the concentration of the plant extracts resulted in increased inhibition of embryo nation eggs, hatching rate, and mortality L_1 effect of bovine strongyles parasites that could be related to a supplement of different active compounds. Moreover, the activity level of the different concentrations of *M. vulgare* extract was significantly lower than the ABZ activity. This difference can be explained by the presence of small concentrations of the active ingredient in the plant extracts. It is likely that at higher concentration, all binding receptors on the larvae were occupied, thus leading to hyperpolarization of membranes limiting excitation and impulse transmission causing flaccid paralysis of larvae muscles, a similar observation also made by a previous study (30).

CONCLUSION

The findings of the present study showed that AE and EE of *M. vulgare* leaves have a potential anthelmintic activity on eggs and larvae of bovine strongyles parasites. Further studies geared toward isolation, identification, characterization, and purification of bioactive compounds from this plant should be conducted in order to test its efficacy. In addition, *in vivo* investigations are required to warrant the ethno medicinal application of *M. vulgare* as anthelmintic agent.

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