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Anthelmintic Activity of Aqueous and Ethanol Extracts of *Urtica dioica* L. and *Myrtus communis* L. Leaves on Bovine Digestive Strongyles: *In-Vitro* Study

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Abstract: The present research aimed to evaluate the in-vitro anthelmintic activity of *M. communis* and *U. dioica* against digestive strongyles in naturally infected cattle. The anthelmintic activities of the extracts were evaluated using the egg hatch assay and larval mortality assay. Leaves powder of *M. communis* and *U. dioica* was extracted by maceration. Ethanolic and water extracts were test at 0.78, 1.55, 3.1, 6.2, 12.5, 25 and 50 mg/ml. Albendazole and DMSO was used as a positive and negative control at concentration 20 mg/ml and 3%, respectively. The results of regression line indicated that LC50 of *Urtica dioica* EE and EA were very low namely 2.57, 3.02 and 3.22 mg/ml; and 4.90, 4.67 and 3.24 mg/ml for inhibition of embryonation eggs, hatching rate and L1 mortality, respectively. The mean embryonation rate maximum was 100% at 25 and 50 mg/ml concentration in AE and EE *U. dioica*; whereas *M. communis* recorded a maximum level of 57.1 ± 3.12% and 63.95 ± 3.01% at 50 mg/ml concentration for the AE and EE, respectively. The extracts of *U. dioica* leaves high effects (100%) were observed with 25 and 50 mg/ml in both extracts, whereas *M. communis* recorded a high level at 50mg/ml concentration for the AE and EE (58.05±3.69% and 65.13±3.13%, respectively). The larval mortality rate of both AE and EE from *U. dioica* showed that the extracts at and 25 and 50 mg/ml exhibited 100 % at 24h of contact. In conclusion, these findings showed that, aqueous and ethanolic extracts of *U. dioica* and *M. communis* leaves have a potential anthelmintic activity on eggs and larvae of bovine strongly parasites.

Keywords: Anthelmintic, Cattle, In-vitro, *Myrtus communis*, *Urtica dioica*.

Urtica dioica L. ve *Myrtus communis* L. Yapraklarının Sulu ve Etanolik Ekstraktlarının Sığır Sindirim Strongilozu Üzerine Antelmintik Aktivitesi: İn-vitro Çalışma

Öz: Bu araştırma, doğal olarak enfekte sığırlarda sindirim strongilozu üzerine *M. communis* ve *U. dioica* in-vitro antelmintik aktivitesini değerlendirmeyi amaçladı. Ekstraktların antelmintik aktiviteleri, yumurta tarama testi ve larva mortalite testi kullanılarak değerlendirildi. *M. communis* ve *U. dioica*'nın yaprak tozu, maserasyon ile özütlendi. Etanolik ve su ekstreleri 0.78, 1.55, 3.1, 6.2, 12.5, 25 ve 50 mg/ml'de test edildi. Albendazol ve DMSO, sırasıyla 20 mg/ml ve %3 konsantrasyonunda pozitif ve negatif kontrol olarak kullanıldı. Regresyon hattı sonuçları, *Urtica dioica* EE ve EA'nın LC50'sinin sırasıyla 2.57, 3.02 ve 3.22 mg/ml olduğundan oldukça düşük değerlere sahipti; ve embriyonasyon yumurtalarının inhibisyonu, kuluçka hızı ve L1 mortalitesi değerleri sırasıyla, 4.90, 4.67 ve 3.24 mg/ml'idi. Maksimum ortalama embriyonasyon oranı AE ve EE *U. dioica*'da 25 ve 50 mg/ml konsantrasyonda %100'iken; *M. communis*, AE ve EE için sırasıyla 50mg/ml konsantrasyonda maksimum % 57.1 ± 3.12 ve % 63.95 ± 3.01 olarak kaydedildi. *U. dioica* özlerinin yüksek etkisi (%100) her iki ekstrede 25 ve 50 mg/ml ile gözlenirken, *M. communis*'de ise AE ve EE için 50mg/ml konsantrasyonda yüksek düzeyde kaydedildi (sırasıyla %58.05±3.69 ve 65.13±3.13). *U. dioica*'nın hem AE hem de EE için larval mortalite oranı, 25 ve 50 mg/ml'deki ekstrelerin 24 saatlik temasında %100 olarak görüldü. Sonuç olarak, bu bulgular *U. dioica* ve *M. communis* yapraklarının sulu ve etanolik ekstraktlarının, sığır strongiloz parazit larvaları üzerinde potansiyel bir antelmintik aktiviteye sahip olduğunu göstermiştir.

Anahtar Kelimeler: Antelmintik, In-vitro, *Myrtus communis*, Sığır, *Urtica dioica*.

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INTRODUCTION

Helminthiasis is one of the most widespread infectious diseases affecting mostly in cattle, sheep and goat. In the recent study conducted in Algeria, cattle were shown to be parasitized by several species namely Eimeria spp, digestive strongles, Fasciola hepatica, Paramphistomum daubneyi, Strongyloides papillosus, Moniezia benedeni and *Toxocara vitulorum* (1). The grazing animals are constantly exposed to parasites and are thus re-infected, as well as delaying it and causing the development of immunity in young people. Despite the use of anthelmintics, the appearance of resistance to common drugs is observed in animals (2,3).

The cattle breeding in the world is subjected to the different constraints, among them the diseases of the gastrointestinal parasitisms. Endoparasites such as digestive strongyles cause significant economic losses such as reduced milk production, reduced weight and mortality (4). Helminths cause damage to the host in several ways, which include damage of gastrointestinal wall and suckling of blood and nutrients.

Medicinal plants are being widely used, either as a single drug or in combination delivery system. The herbal plants are considered as a valuable source of natural products and drugs against various disorders and diseases and also for development of industrials products (5). Because of its bioactive products, phytotherapy based on the use of preparation of medicinal plant remains the most serious alternative to treat or control of many health problems and infections, as an antimicrobial, antioxidant, anti-inflammatory, antipyretic, immunostimulant (6). Traditional use of medicinal plants as anthelmintics activities of various botanical have been reported, *e.g. Artemisia brevifolia* (7), *Asimina trilobal* (8), *Adhatoda vasica* (9), *Leonotis ocymifolia* (10), *Leuceana leucocephala* (11), and *Murrabium vulgare* (12) *Urtica dioica* (Urticaceae) and *Myrtus communis* (Myrtaceae) are plants of Mediterranean and Middle East origin especially in North Africa, *e.g.* Algeria (13). These plants are used in traditional medicine for the treatment of various diseases. Note that the biological activities of *U. dioica* and *M. communis* are due to bioactive contents such as flavonoids, diterpenoids and phenylethanoid glycosides (14,15). *U. dioica* is known for its antihyperglycaemic activity (16), hepatoprotective effect (17), anti-inflammatory activity (18), antioxidant and antibacterial activities (19). Similarly, *M. communis* have an antioxidant properties (15), antibacterial activity (20), antihyperglycemic activity (21), growth performance, hematological and biochemical parameters (22).

To our knowledge, no work using anthelmintic activity of plant extracts from *M. communis* and *U. dioica* growing in Algeria was reported. The present research aimed to evaluate *in-vitro* the anthelmintic activity of *M. communis* and *U. dioica* against digestive strongyles in naturally infected cattle.

MATERIALS and METHODS

Ethics committee approval was received for this study from the scientific committee of Faculty of Nature and Life Sciences, University of Bejaia (Report of Faculty Scientific Council N 07 dated December 14, 2014).

Plant Materials

The leaves of *U. dioica* and *M. communis* were collected in March 2015 in the Aokas locality (Bejaia province, Northern Algeria) (36° 36' N, 4° 41' E). The scientific authentication of plants was carried out at the Ecology Laboratory (University of Bejaia, Algeria). After cut in small pieces, both plants were put to drying at room temperature for one week. Thereafter, plant material was pounded using coffee grinder resulting in a fine powder that kept in dark until the use for *in-vitro* anthelmintic test.

Preparation of Extracts

Leaves powder of *U. dioica* and *M. communis* were extracted by maceration technique with ethanol by shaking (Corning® PC-400D hot plate). Briefly, leaves powder was incubated with ethanol 70% and water in the ration 1:10 W/V for 24h and then homogenized through polytron homogenizer (23). The combined ethanolic and water extracts were evaporated to dryness to give crude ethanolic and water extracts. Also, the resulting supernatants were filtered through Whatman paper number 1. The combined ethanolic and water extracts were evaporated at 50 °C to dryness to give crude ethanolic and aqueous extracts (EE and AE, respectively). In order to improve solubility in water, the extract was dissolved in dimethyl sulfoxide solution (DMSO, 3%). A total volume (100 ml) was obtained which produced a stock solution at a concentration of 50 mg/ml from which a series of dilutions were made to obtain solutions at different concentrations 0.78, 1.55, 3.1, 6.2, 12.5, 25 and 50 mg/ml.

Recovery of Eggs from Digestive Strongyles

Fresh eggs were obtained from the faeces of naturally infected cattle according to Chollet *et al*. (24). Briefly, 3g of faeces were collected, homogenized in a mortar, suspended in saturated salt solution (NaCl, d=1.2) and filtered through two sieves (1 and 0.15 mm). 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 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 Petri dish (16 cm diameter).

Evaluation of the Ovicidal Activity

The ovicidal efficacy test of the different extracts was performed using two procedures described by Coles et al. (25). 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 giving 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 block eggs hatching. The number of embryonated eggs per well was counted using stereo microscope (at 40x magnification). The percentage of embryonation (EM %) was determined as follows (26).

$$\text{EM } (\%) = \frac{\text{Number of embryonated eggs}}{\text{Number of eggs nature}} \text{x}100$$

After 24h incubation, to assess the effects of the plant extract in the second experiment, all embryonated eggs and first stage larvae (L1) were counted using a microscope (at 40x magnification). The hatching rate (E %) was estimated using the following formula (27).

$$\text{EM}(\%) = \frac{\text{Number of larvae 1}}{\text{Number of embryonated eggs in culture}} \text{x}100$$

Recovery of Nematode Larvae

Eggs were cultured using the technique described by Smyth (28). Briefly, 3ml of the eggs suspension was poured on 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, L1 larvae were observed in Petri dish and were collected with a Baermann apparatus.

Evaluation of the Larvicidal Activity

To assess the effects of the extracts on larvae (L_1), 1ml of a solution containing about 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 incubated in at temperature for 24h. The number of dead or immobilized larvae was assessed under a microscope (at 40x magnification). The corrected mortality rate (cMR %) was determined using the following formula (29).

$$\text{cMR}\ (\%) = \frac{\text{Mce} - \text{Mt}}{100 - Mt}\text{x}100$$

Where cMR is the corrected mortality rate (%), Mce is the mortality obtained during the test and Mt is the mortality registered in the negative controls wells. If the mortality rate in the later wells is lower than 5%, cMR=Mce.

Statistical Analysis

The results (mean ± SD) were expressed as percentage (%). The different rates embryonation (EM %), eclosability (E %) and mortality (cMR %) due to the plant extracts were compared using the chi-square test at 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 summarize the extracts of *Urtica dioica* and *M. communis*LC_{50} values of *in vitro* anthelmintic activity test. The results of regression line indicated that LC_{50} of *Urtica dioica*EE and EA were very low namely 2.57, 3.02 and 3.22 mg/ml; and 4.90, 4.67 and 3.24 mg/ml for inhibition of embryonation eggs, hatching rate and L_1mortality, respectively. On the other hand, EE and AE of *M. communis*showed important values of LC_{50} for inhibition of embryonation eggs (28.18 and 31.62 mg/ml, respectively), hatching rate (20.42 and 30.9 mg/ml, respectively) and L_1mortality (24.55 and 31.84 mg/ml, respectively).

Table 1. The EE and AE fifty lethal concentration (LC_{50}, mg/ml) of *U. dioca* and *M. communis* on inhibiting embryonation, hatchability eggs and mortality L_1 of bovine digestive strongyles.

Tablo 1. U. dioca ve M. communis'ün EE ve AE'nin letal konsantrasyonu ($LC_{50,}$ mg/ml)'nun sığır sindirim strongilozunun L1 mortatilesi, kuluçkalama ve embriyonasyonu üzerindeki inhibisyonu.

Extractplants		Inhibiting embryonation	Inhibiting hatching	Mortality L_1
U. dioica	Ethanolic	2.57	3.02	3.22
	Aqueous	4.90	4.67	3.24
M. communis	Ethanolic	28.18	20.42	24.55
	Aqueous	31.62	30.9	31.84

The results indicated than the both extract of *U. dioica*leaves seems to be more efficient against gastrointestinal strongyles in different tested stages than the extracts *M. communis*. The variation of mean embryonation rate of digestive strongyles eggs according to the concentrations of extract of *U. dioica* and *M. communis* leaves is shown in Figure 1A and 1B, respectively. The inhibition effect was proportional to the extract concentration. The mean embryonation rate maximum was 100% at 25 and 50 mg/ml concentration in AE and EE *U. dioica*; whereas *M. communis* recorded a maximum level of 57.1 ± 3.12% and 63.95 ± 3.01% at 50mg/ml concentration for the AE and EE, respectively. The mean embryonation rate of AE and EE was very low in distilled water and 3% DMSO. Both extracts inhibition of embryonation rate were significantly lower than positive control ($P<0.05$).

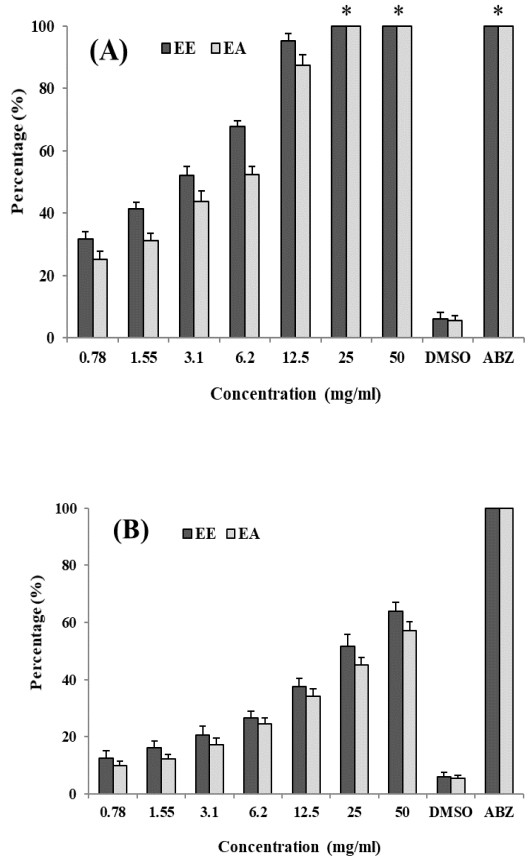

Figure 1. Mean embryonation (EM%, ± SD) of leaves extract of *U. dioca* (A) and *M. communis* (B) at different concentrations against bovine digestive strongyles.

Şekil 1. Sığır sindirim strongilozuna karşı farklı konsantrasyonlardaki U. dioca (a) ve M. communis (B) ekstrakt yapraklarının ortalama embriyonasyonu (EM%, ± SD).

*Values by asterisk superscripts in negative and positive control (DMSO and ABZ, respectively) compared with each extract treatment are not statistically different (P>0.05).

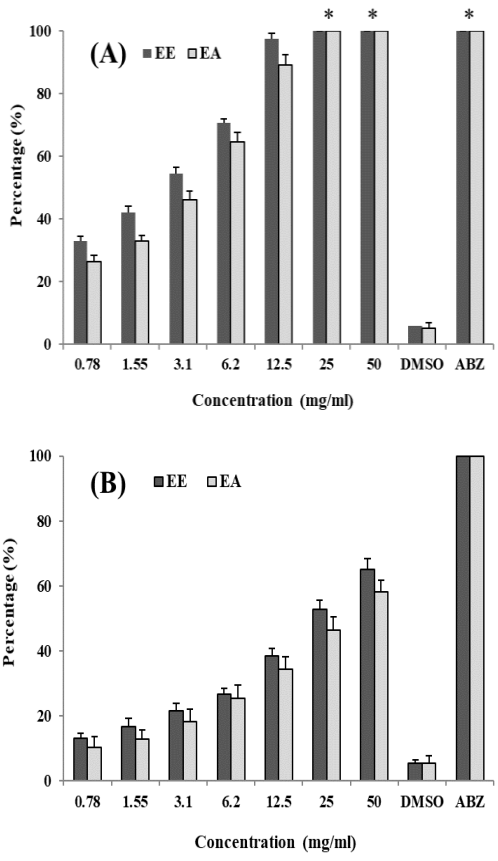

Figure 2. Mean hatchability rate (E%, ± SD) of leaves extract of *U. dioca* (A) and *M. communis* (B) at different concentrations against bovine digestive strongyles.

Şekil 2. Sığır sindirim strongilozuna karşı farklı konsantrasyonlardaki U. dioca (A) ve M. communis (B) ekstrakt yapraklarınının ortalama kuluçkalama oranı (E%, ± SD).

*Values by asterisk superscripts in negative and positive control (DMSO and ABZ, respectively) compared with each extract treatment are not statistically different (P>0.05).

The variation of the mean hatching rate eggs of *U. dioica* and *M. communis*according to the concentration of AE and EE is illustrated in Figure 2A and 2B, respectively. The effect *U. dioica* and *M. communis* varied according to the concentration of both extracts concentration. The extracts of *U. dioica* leaves high effects (100%) were observed with 25 and 50 mg/ml in both extracts, whereas *M. communis* recorded a high level at 50 mg/ml concentration for the AE and EE (58.05±3.69% and 65.13±3.13%, respectively).

The effects of different extracts of *U. dioica* and *M. communis*on on L_1 larvae of gastrointestinal strongyles after 24h of contact are depicted in Figure 3A and 3B, respectively. The larval mortality rate of both AE and EE from *U. dioica* showed that the extracts at and 25 and 50 mg/ml exhibited 100 % at 24h of contact. At concentration of 3.1 mg/ml, the mean larval mortality rates were similar in AE and EE of *M. communis* (14.4±2.55 and 14±2.1%, respectively). Generally, larval mortality rate was concentration dependent in both extract. Distilled

water and 3% DMSO in both AE and EE were 5.8±1.45 and 6.6±1.27%, respectively. On the other hand, *M. communis* recorded 58.05 ± 2.19% and 65.13 ± 2.07% for AE and EE at a 25 and 50 mg/ml concentration, respectively.

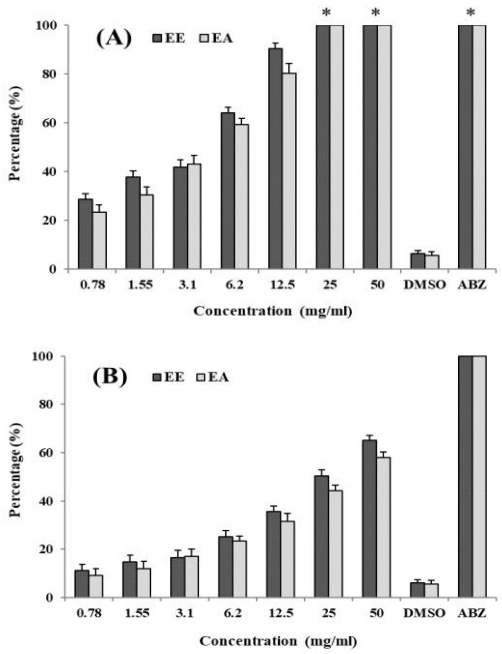

Figure 3. Mean rate of mortality (cMR%, ± SD) of leaves extract of *U. dioca* (A) and *M. communis* (B) at different concentrations against bovine digestive strongyles.

Şekil 3. Sığır sindirim strongilozuna karşı farklı konsantrasyonlardaki U. dioca (A) ve M. communis (B) ekstrakt yapraklarının ortalama mortalite oranı (cMR%, ± SD)

*Values by asterisk superscripts in negative and positive control (DMSO and ABZ, respectively) compared with each extract treatment are statistically different (P> 0.05).

The mean embryonation rate of AE and EE was very low in distilled water and 3% DMSO. Both extracts inhibition of embryonation rate were significantly lower than positive control (P<0.05).

DISCUSSION and CONCLUSION

For decades, plants extracts have been used in alternative veterinary medicine for the treatment of helminth infections and they constitute today a part of therapy in traditional practice particularly in the African countries (30). Due to the development of populations resistant to all of the drug families currently available (31) and concerns regarding drug residue in food and in the environment have stimulated the search for alternative control strategies (32). In the attempt to reduce the use of chemicals due to the concern about human health and environmental toxicology, new and safer food control approaches such as the use of natural compounds are nowadays being developed (33). Previous studies showed significant biological activity of the *U. dioica* and *M. communis* extract. The goal of this research is to evaluate the anthelmintic effect of *U. dioica* and *M. communis* extract propose a new therapy alternative against digestive strongyles in cattle. The results of this investigation demonstrated a noticeably inhibitory *in-vitro* effect leaves extracts of *U. dioica* and *M. communis* on the development of eggs and larvae digestive strongyles in naturally infected cattle. Our results support the hypothesis of a direct effect of the active compounds on eggs and larvae with the plant extracts (34). Previous studies reported that *U. dioica* and *M. communis* extracts are rich in bioactive compounds such as flavonoids, diterpenoid, tannins and phenylethanoid glycosides (14,15,35). Moreover, investigations have used *in-vitro* test to screen the individual or combined anthelmintic effect of some plant compounds in bovine (36).

Our results are in agreement with those reported by Al-Shaibani et al. (37) and Moussouni et al. (12) which it presented a remarkable anthelmintic activity of AE and EE of *U. dioica* and *M. communis* leaves. Likewise, various published results indicate that that increased concentrations of plant extracts led an increase anthelmintic activity (12,38). In our *in-vitro* evaluation, DMSO (3%) and ABZ (20 mg/ml) were used as respectively negative and positive control for any direct effects on digestive strongyles and to compare with plant extracts. There was very little inhibitory effect of DMSO on the embryo development and egg hatching, while positive control completely prevented it.

Our results revealed that EE of both extracts gives more satisfactory results than with AE on inhibition of embryonated and egg hatching in bovine digestive strongyles; this in agreement with the results previously described (12,39). These results may be due to the addition of water in the ethanol solution which increased the polyphenols solubility modeling of the organic solvent polarity, whereas water given more amount of yield but only is not good to extract polyphenols (40). In addition, Bimakr et al. (41) explained that the higher concentrations of more bioactive flavonoid compounds were detected with ethanol 70 % due to its higher polarity than pure ethanol. Noted that organic solvent extracts more bioactive compounds from plants than the aqueous extract (42).

Several studies have been conducted to determine the anthelmintic effects of some plants (12,43,44). In this study, LC_{50} value of both extracts of *U. dioica* showed low activity on inhibiting embryonation, hatchability eggs and mortality L_1 of bovine digestive strongyles compared to AE and EE of *M. communis*. Cela and co-authors (45) reported low LC_{50} values extract of *M. azedarach* fruits and methanol extract of *T. claussenii* leaves (0.7 and 1.1 µg/ml, respectively). On the other hand, the relatively high values LC_{50} of *Securidaca longepedunculata* and *Melia azedarach* was recorded (46,47). The LC_{50} is considerably low indicating that the extract has a wide margin of safety compared to extracts from several medicinal plants. In general, the EE and AE effects of *U. dioca* on inhibiting embryonation, hatchability eggs and mortality L_1 of bovine digestive strongyles from 12.5 to 50 mg/ml concentrations exhibited high activities in our investigation. The observation of aqueous extract of *A. maricata*mentioned by Ferreira et al. (44) were very high (84.91% at 50 mg/ml) compared to AE and EE *M. communis*on inhibiting embryonation, hatchability eggs and mortality L_1 of bovine digestive strongyles. Recently, Moussouni et al. (12) reported similar results of aqueous and ethanolic extract of *M. vulgare* on embryonic inhibition (48.4 and 54.2, respectively). Also, Hernandez-Villegas et al. (43) provided an important ethanolic extract effect of *Phytolacca icosandra* on inhibition of hatching (97.5% to 3.6 mg/ml) and L1 mortality (89% at 4 mg/ml). The variations observed could be explained by the differences in the composition and content of different biochemical components of plant extracts. In another study, Brunet et al. (48) highlighted the role of tannin-rich plants such as sainfoin on the development of larvae L1 by affecting the drawdown kinetics of *H. contortus* L3. Reduction of nematode egg excretion and worm burden have been also recorded in goats and sheep fed with tanniferous plants (49,50). This observation supports the hypothesis that the chemical structure of condensed tannins is one of the factors modulating their antiparasitic efficacy (51). Equally, Ademola et al. (52) demonstrated the effect of flavonoids and tannins in blocking the migration of L3 larvae of *H. contortus*. In numerous research related that biochemical activity of flavonoids have been attributed to their anti-oxidative and anti-bacterial properties (53). The anthelmintic effects found in our study may be attributed to tannins and saponins penetration into inside the egg and prevent the segmentation of blastomeres (54,55) and blocking the cuticle post-synaptic receptors consequently, paralyzing larval formation (56). It is noted also that active compounds may have induced the release of gamma aminobutyric acid (GABA) which blocked transmission of nerve impulses or decoupling the oxidative phosphorylation reaction which can lead to the exhortion of the energy of larvae (57). In addition, one of the specificities of tannins is their ability to bind to many macro-molecules (58,59) such the enzymes, blocking their activity (60). The founding of this study could be explained by the function inhibition and alteration of some vital process of digestive strongyles such nutrition and penetration into the host tissues.

Our results support reports from the literature in various countries that relationship effects of plant extracts and extract concentration on of embryo eggs

inhibition, hatching rate and larvae mortality. Indeed, the low content of bioactive compounds of *M. communis* induced the weak effect obtained on digestive strongyles when compare to ABZ activity. As regard *U. dioica* extract, the results was similar to positive control, this could be explained by the fact that plant extracts are rich in bioactive compounds A study conducted by Athanasiadou et al. (61) suggests that the inhibitory effect of S*chinopsis quebracho-colorado* extracts on the settlement of *H. cortocus* depended directly on active phytochemical content.

The findings of this study showed that, aqueous and ethanolic extracts of *U. dioica* and *M. communis* leaves have a potential anthelmintic activity on eggs and larvae of bovine strongly parasites. However, the both extracts of *U. dioica*seem to be more efficient against gastrointestinal strongyles in different tested stages than the extracts *M. communis*. In order to test its efficacy, it would be interesting to test the bioactive compounds of both plant extracts. Also, the investigations are necessary to evaluate the toxicity level of *U. dioica* and *M. communis* extracts in laboratory rats.

Conflict of interest

The authors declare that they have no conflict of interest.

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