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### Original Article

## ***In-Vitro* Assessment of the Acaricidal Properties of *Artemisia annua* and *Zataria multiflora* Essential Oils to Control Cattle Ticks**

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### ABSTRACT

**Background:** The aim of this study was to investigate the 'acaricidal effect' of *Zataria multiflora* and *Artemisia annua* essential oils on *Rhipicephalus (Boophilus) annulatus*.

**Methods:** This study was carried out in 2009 in the Laboratory of Parasitology of the Faculty of Veterinary Medicine of Shahrekord University, west central Iran. Six dilutions (5, 10, 20, 40, 60 and 80  $\mu\text{L}/\text{cm}^3$ ) of both essential oils were used against engorged female *R. (Boophilus) annulatus* ticks using an in vitro immersion method. The mortality rates for each treatment were recorded 6, 15 and 24 hours post inoculation (hpi). Mortality rate was analyzed using Repeated Measures Analysis of Variance, and comparison of means was carried out using General Linear Models Procedure.

**Results:** The mortality rate caused by different dilutions of *Z. multiflora* essential oil ranged from 26.6% (using 10  $\mu\text{L}/\text{cm}^3$ ) to 100% (using 40  $\mu\text{L}/\text{cm}^3$ ) and for *A. annua* essential oil it was 33.2 to 100% (using 20 and 80  $\mu\text{L}/\text{cm}^3$ , respectively) by the end of the experiment (36 hpi). No mortality was recorded for the non-treated control group or for dilutions less than 5 and 10  $\mu\text{L}/\text{cm}^3$  using *Zataria* and *Artemisia* essential oils, respectively. For *Z. multiflora* mortality peaked at 15 hpi for all concentrations other than 20  $\mu\text{L}/\text{cm}^3$  and took 24 h to achieve its maximum effect while for *A. annua* the two highest concentrations needed 24 hpi to reach their full effect. In addition, essential oils applied at more than 20 and 60  $\mu\text{L}/\text{cm}^3$  caused 100% egg-laying failure in engorged female ticks by *Zataria* and *Artemisia*, respectively while no failure was observed for the non-treated control group. The mortality rate in both botanical acaricides was dose-dependent.

**Conclusion:** Both these medicinal plants have high potential acaricidal effects on the engorged stage of *R. (Boophilus) annulatus* in vitro.

**Keyword(s):** *Artemisia Annua*, *Botanical Acaricidal Agents*, *Essential Oil*, *Rhipicephalus (Boophilus) Annulatus*, *Zataria Multiflora*

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## Introduction

**R***hipicephalus annulatus* (*Boophilus*) is a one-host tick, which feeds on cattle around the Mediterranean Sea, Asia Minor, and Central America (1). It is one of the most important bovine tick species in the northern part of Iran, serves as a vector for the agents of some human diseases, and plays an important role as a vector of bovine babesiosis, an important disease of cattle. Tick control throughout the world is based mainly on the repeated use of chemical acaricides. Indiscriminate use of these tick-controlling chemicals has resulted in problems related to environmental pollution, milk contamination, and the development of resistance in target species with a subsequent increase in cost (1,2).

In view of these problems, there has been an increasing interest in searching for alternative sustainable control methods of ticks in recent years. Numerous pathogens and predators of ticks have been known for decades, but few biocontrol programs have been developed for ticks (3). Some studies have used herbal medicine such as *Margaritaria discoidea* plant extracts against the ticks *Rhipicephalus appendiculatus* and *Hyalomma varigatum* (4) or *Matricaria chamomile* flower extract against the adult stage of *R. (B.) annulatus* (5).

As the first step towards using plant essential oils as acaricidal agents, in the present study we determined, for the first time, the tick mortality caused by two botanical acaricides (*Zataria multiflora* and *Artemisia annua*) against the adult stage of *R. (B.) annulatus* in Iran. *Z. multiflora* is a thyme-like plant that grows wild in central and southern Iran. It is used in traditional folk remedies for its antiseptic, analgesic (pain-relieving) and carminative (anti-flatulence and intestine-soothing) properties. Despite its impressive array

of medical applications, little research had been carried out on the acaricidal effect of this plant for controlling ticks. *A. annua* is available commercially in China and Vietnam as an antimalarial drug and is efficacious against drug-resistant strains of *Plasmodium* (6). However, the plant grows wild and become naturalized in many countries, including Europe and America. It is cropped on a large scale in China, Vietnam, Turkey, Iran, Afghanistan, Australia, Argentina, Bulgaria, France, Hungary, Romania, Italy, Spain, and the former Yugoslavia (7-10). *Artemisia* (Asteraceae, Compositae family) has 34 species in Iran with a vast cover, density, and distribution. It is one of the most important genera after *Astragalus*.

In the present study, we examined the acaricidal potential of the Essential Oils obtained from the *Artemisia annua* and *Zataria multiflora* against the Cattle Ticks to elucidate its ability as a cattle fever tick biocontrol agent.

## Material and Methods

### *The essential oils*

The study was carried out in the Parasitology Laboratory of the Faculty of Veterinary Medicine of Shahrekord University, west central Iran, 2009. For the experiment, concentrated 100% (pure) essential oils of *Zataria* and *Artemisia* were purchased from Barij Essence Co., Kashan-Iran. According to information provided by the producer, the plant essential oils were prepared by hydro-distillation of sterilized plant materials (leaves and stems) using a Clevenger-type apparatus as follows: The aerial parts were steam distilled for 60 min in a full glass apparatus. The extraction was carried out for 120 min in 500 ml of water. The oils were kept in the dark at 4°C before use.

### **Tick rearing**

Adult ticks were collected from naturally infested cattle in Mazandaran Province in the northern part of Iran. Female ticks were selected morphologically (Full engorged) and maintained in the laboratory at 26°C and 80% relative humidity (RH) in test tubes for laying eggs and the males were discarded. After the eggs hatched, tick larvae were fed on healthy 1-3 months-old Holstein calves. Fully engorged female ticks between 18 and 22 days-old were collected from experimentally infested cattle and selected morphologically based on body size for the experiment. Ticks were taken to the laboratory within 3-4 hours to perform subsequent experiments.

### **Bioassay on ticks**

Dilutions (from 5 up to 80  $\mu\text{L}/\text{cm}^3$ ) of *A. annua* and *Z. multiflora* essential oils in three replicates were prepared with the use of 60% ethanol (EtOH) as solvent to determine the acaricidal effect of each dilution. For this purpose, a total of 420 engorged female *R. (B.) annulatus* were washed with 70% EtOH for 1 min. Thirty ticks were used for each dilution (six dilutions for *Z. multiflora*, six dilutions for *A. annua* and 2 control groups containing 30 engorged ticks for each treatment). After drying, 10 females per replicate were immersed in each plant dilution and in the control solution (EtOH 60%) for 1 min. The experiment was carried out in triplicate. Ticks were then transferred to Petri dishes containing moist filter paper and incubated for 24 h at 26°C and 80% RH in the dark. For each essential oil, one control group of 10 ticks was also treated in the same manner except that they were immersed in 60% EtOH. Ticks were studied with a dissecting microscope and mortality rates of groups were recorded. The mortality rate was recorded 6, 15 and 24 h post inoculation (hpi) by counting dead ticks; all cadavers were placed into separate Petri dishes to observe

the effects of essential oils on the ticks. Dead ticks were diagnosed based on three criteria: signs of cuticle darkness and hemorrhagic skin lesions, leg movement, halted Malpighian tube movement. The main criterion to diagnose the death of ticks was the lack of Malpighian tube movement, which is very clear in *R. (B.) annulatus*. The second criterion was the lack of movement of legs tested with a paintbrush with ticks placed in an inverted position under a stereomicroscope lamp. The third criterion was a change of cuticle color and hemorrhagic skin lesions demonstrated by dead ticks. Failure in egg laying was then directly observed by eye and calculated. All groups, including treatments and controls, were checked every 9 h and data were recorded. Any changes in ticks such as egg laying were added to the data set (11).

### **Statistical analysis**

Mortality was expressed as the mean  $\pm$  SEM using Repeated Measures Analysis of Variance, and comparison of means were carried out using General Linear Models Procedure, Least Squares Means. Egg-laying failure (ELF) and weight of eggs produced were analyzed with General Linear Models Procedure. A value of  $P < 0.05$  was considered significant. Mean concentration dose (LD/LC50) values were calculated using a Probit test analysis (EPA Probit Analysis Program Used For Calculating LC/EC Values Version 1.5) (12, 13).

## **Results**

An initial qualitative assay based on morphological observation of ticks treated and/or non-treated with these botanical agents showed that there was increased mortality rate and inhibited egg laying. The cuticle of dead ticks was dark and these ticks became immotile. Malpighian tube move-

ment also stopped and haemorrhagic swelling lesions appeared on the skin surface. Treated ticks could not lay eggs, especially at higher concentrations of the botanical acaricides. In contrast, engorged non-treated ticks were able to lay approximately 2000 eggs (Fig. 1), the colour of their cuticle was normal and motile Malpighian tubes were easily visible.

### Quantification of tick inhibition

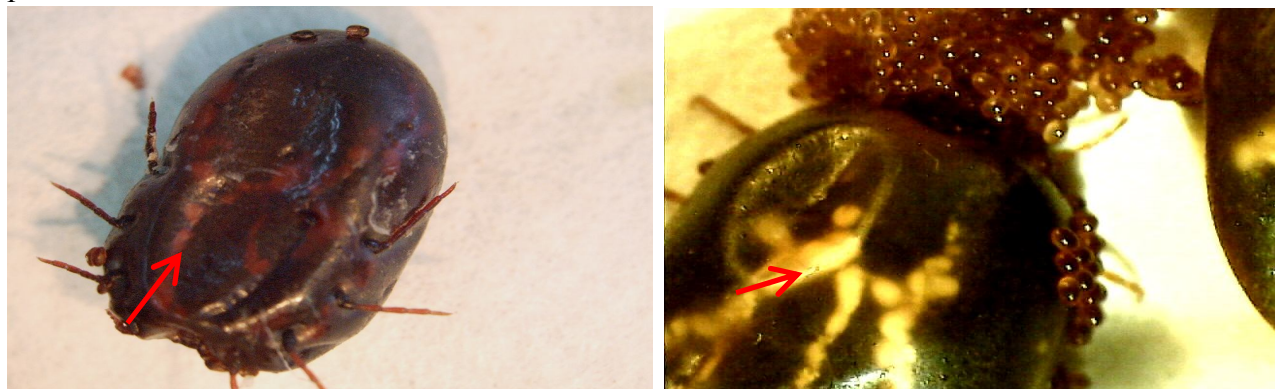
#### Mortality rate

Table 1 shows a high acaricidal effect caused by higher concentrations of both botanical agents. At 60 and 80  $\mu\text{L}/\text{cm}^3$  15 hpi 100% mortality was observed for *Zataria* essential oil while 100% mortality was observed for *Artemisia* essential oil at 80  $\mu\text{L}/\text{cm}^3$  24 hpi. No mortality was observed in the control group. The first acaricidal effects were observed as early as 6 hpi. These were dose-dependent and decreased in subsequent days. There were significant differences between treated and control groups for both oils, especially at higher concentrations. There was no considerable mortality at lower dilutions (10 and 20  $\mu\text{L}/\text{cm}^3$ ) for both plant oils. The LC/LD50 was 2.16  $\mu\text{L}/\text{cm}^3$  for *Zataria* and 3.73  $\mu\text{L}/\text{cm}^3$  for *Artemisia*, indicating that these plant acaricides had a considerable acaricidal

effect against engorged *R. (B) annulatus in vitro*. Only 5  $\mu\text{L}/\text{cm}^3$  for *Artemisia* could not inhibit egg production. At least *in vitro* these essential oils could be used as effective acaricidal agents in the control of *R. (B) annulatus*.

#### Failure in egg laying (mass of produced eggs)

The acaricidal efficacies of different concentrations of *A. annua* and *Z. multiflora* essential oils are reported in Table 2, which shows severe failure in egg laying and mass of produced eggs compared with the non-treated control. All ticks in the control group could lay eggs but some treated ticks were killed before they could lay eggs. The application of *Zataria* essential oil at  $> 10 \mu\text{L}/\text{cm}^3$  fully suppressed egg laying; the same effect could only be achieved for *Artemisia* essential oil at 80  $\mu\text{L}/\text{cm}^3$ . Dilutions  $> 10 \mu\text{L}/\text{cm}^3$  and caused 100% ELF compared with the control using *Zataria* and *Artemisia* essential oils, respectively (Table 2). There was an understandably concomitant decrease in mass of eggs produced as ELF increased for both essential oils (14).



**Fig. 1:** Comparison of a *Zataria*-treated dead tick (left) with a non-treated live tick (right). Tick inoculated with *Zataria* essential oil shows major signs of death including a dark cuticle, immobilized Malpighian tubes, and haemorrhagic swelling skin lesions (arrow). In the non-treated live tick, the colour of the cuticle

is normal and motile Malpighian tubes are easily visible (arrow). Egg-laying has stopped in the dead tick (obviously; left), but it is normal for the live control tick (right).

**Table 1:** Mortality rate (mean±SEM; n=30) of *Rhipicephalus (B) annulatus* exposed to different concentrations of *Z. multiflora* and *A. annua* essential oils

Dose (µL/cm <sup>3</sup> )	hpi exposed to <i>Zataria. multiflora</i>			hpi exposed to <i>A. annua</i>		
	6 hpi	15 hpi	24 hpi	6 hpi	15 hpi	24 hpi
0.0 (control)	0.0±0.188a	0.00±0.20	0.0±0.1a (0%)	0.0±0.16ea	0.0±0.07a	0.0±0.15ea (0%)
5	0.00±0.462da	0.00±0.40	0.0±0.24a (0%)	0.0±0.42ea	0.0±0.17a	0.0±0.39e (0%)
10	2.0±0.462b	2.66±0.40	2.66±0.24b (26.6%)	0.0±0.42a	0.0±0.17a	0.0±0.39a (0%)
20	3.32±0.462b	3.98±0.40	4.66±0.24c (46.6%)	3.32±0.42b	3.32±0.17a	3.32±0.39b (33.2%)
40	8.68±0.462c	10±0.40	10.00±0.24d (100%)	4.0±0.42b	4.0±0.17a	4.00±0.39b (40%)
60	9.34±0.462c	10±0.40	10.00±0.24d (100%)	7.34±0.42c	7.34±0.17b	8.66±0.39c (86.60%)
80	9.34±0.462c	10±0.40	10.00±0.24d (100%)	9.32±0.42d	9.32±0.17a	10.00±0.39d (100%)

Different letters within a column indicate significant differences between treatments ( $P<0.05$ )

hpi : hours post inoculation

**Table 2:** Failure in egg laying (mean±SEM; n=30) and mass of produced eggs of *Rhipicephalus (B) annulatus* engorged females exposed to different concentrations of *Z. multiflora* and *A. annua* essential oils

Dose (µL/cm <sup>3</sup> )	Ticks exposed to <i>Z. multiflora</i>		Ticks exposed to <i>A. annua</i>	
	MPE (g)	ELF (%)	MPE (g)	ELF (%)
0.0 (control)	1.22±0.01a	0.0	1.22±0.01ad	0.0
5	1.16±0.04a	4.92	1.14±0.042d	0.0
10	0.58±0.04b	52.46	1.22±0.042a	6.56
20	0.43±0.04d	65.58	0.36±0.042b	70.50
40	0.0±0.04e	100	0.32±0.042b	73.78
60	0.0±0.04e	100	0.46±0.042c	96.23
80	0.0±0.04e	100	0.00±0.042c	100.00

Different letters within a column indicate significant differences between treatments ( $P<0.05$ ).

hpi : hours post inoculation

MPE: mass produced eggs

ELF: egg laying failure

## Discussion

Over the past few decades, plant extracts and essential oils have been widely used against phytophagous pests and mosquitoes (15-17), have potential in tick control since some of them are selective, and have little or no harmful effect on non-target organisms (18). Many essential oils are known to possess various bio-efficacies such as acaricidal, ovicidal, repellent, anti-feeding and biocidal activities against various arthropod pests (14, 18, 19).

Despite an impressive array of medical applications of *Zataria* and *Artemisia*, to our knowledge no research had ever been carried out on their acaricidal effects in Iran. Bioassays on ticks showed that both *Zataria* and *Artemisia* caused significantly high mortality rates of engorged ticks. *Z. multiflora* is an aromatic plant that grows wild in central and southern regions of Iran and has considerable chemical composition. *Artemisia* species have numerous favorable characteristics from a medical plant view point. Based on the report of Rabie et al. artemisia ketone (14.31%), 1,8-cineole (9.78%), pinocarvone (9.07%), camphor (8.11%) and *trans*-pinocarveol were the main constituents of the aromatic parts of *Artemisia annua* (20). GC-MS data of *Z. multiflora* extract showed that it contained thymol (52.4%),  $\gamma$ -terpinene (13.6%), *p*-cymene (13.2%), carvacrol (6.1%) and  $\alpha$ -terpinenyl acetate (5.4%) as main constituents (21). Our protocol was not designed to address the mode of action of *Z. multiflora* essential oil whose anti-bacterial property has already been reported (21). Their results showed that the oil contained a high percentage of antimicrobial components such as thymol (52.4%) and carvacrol (6.1%). The high biological activity of this oil may also be the reason for its high acari-

cidal effects. In another report (22) the bioactivity of essential oils from *Z. multiflora* against *Staphylococcus aureus* was tested, revealing a high antimicrobial effect. Biological activities reported for the compounds isolated from *Artemisia annua* are antimalarial, antimicrobial, in which artemisinic acid, a well-known precursor for semi-synthesis of artemisinin, has shown antibacterial activity (23, 24). It has been shown that artemisia ketone and thymol are the main constituents of both essential oils, which have anti-microbial and anti-protozoan activity (23-25). It is yet unknown if the acaricidal activity of such oils against *R. (B.) annulatus* could also be attributed to artemisia ketone and thymol. In conclusion, although selected essential oils are not standardized but the results obtained in this study show that these botanical agents with high acaricidal activity could potentially provide new therapeutic products of commercial importance for the bio-agritech industry, provided they can be formulated appropriately. The results obtained from this experiment successfully show that *Zataria* and *Artemisia* essential oils are effective against the adult stages of *R. (B.) annulatus* in Iran, although formulations, chemical and photostability and application routes is needed to support this statement, these results, the first, show promise for these botanicals to be considered as biocontrol agents of cattle fever ticks under field conditions.

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authors declare that they have no conflicts of interest.

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