

Activity on ixodid ticks of *Euphorbia cyparissias* extracts

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Introduction

In modern veterinary therapeutics the concept of bioterapy has become more frequent. In this context, researchers from all over the world are searching to align and to bring new information regarding the use of plants from the spontaneous flora as well as other modern means in the antiparasitary arsenal. Based on known toxicity of *quercetin-3-glucuronide* and *camphor-3-glucuronide*, the main known flavonoids from *E. cyparissias* (*Cypripis spurge*), in a previous study we have identified in plants:



► **inflorescence**, thirteen compounds: *sesquiterpenoids* were dominating, principally *elemene* (19.83%), *beta-cariophyllene* (3.31%) and its epoxidation compound, *cariophyllene-oxide* (0.58%). In lower concentrations, there were identified monoterpenoids and aromatic compounds as: *limonene* (13.3%), *ocimene* (9.58%) and *alpha-pinene* (5.81%).



► **strain** as well, but in higher concentrations were found: *elemene* (40.73%), along with *cariophyllene* (7.2%) and its *epoxide* (0.97%), as well as *selinene* and *gainene* in lower concentrations.



► **root**, seven compounds have been identified, the highest concentration being also in the favour of *sesquiterpenes*: *elemene* (64.49%), *cariophyllene* (7.2%) and its *epoxide* (1.53%), *selinene* (5.93%) and *gainene* (3.96%). Monoterpenes were identified in lower concentrations: *cymene* (6.21%), and *fenchene* (10.67%).

Aim of the study

In vitro and *in vivo* ixodid activity of *Euphorbia cyparissias* (*Cypripis spurge*) extracts based on components identified in this plant and to complete knowledge gathered, so far about ways of action of the compounds found in *Euphorbiaceae*.

Materials and methods

Tincture and glycerinated obtaining

Vegetal material of *E. cyparissias* has been harvested from Banat region, Romania and the conditionings were obtained according to the Romanian Pharmacopoeia, (1993) instructions at *Tincturae* or *Glicerolum*. *E. cyparissias* mother tincture, 20%, was diluted with 70° alcohol, obtaining through gradual dilutions the concentrations of: 10, 5, 2, 1, 0.5 and respectively 0.25%.



A. The *in vitro* activity of *E. cyparissias* tinctures

Ticks were field-collected from sheep maintained in free-grazing. The identified species, after morphological specific characteristics and according to known definition keys of *Feider, Babos* and respectively, *Estrada-Peña* were:

Dermacentor marginatus



Haemaphysalis punctata



The following concentrations of tincture were tested: 10%, 5%, 2%, 1%, 0.5%, and 0.25%.

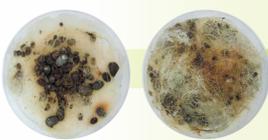
The exposure time of tinctures effectiveness on ticks was at: 30, 60, 90, respectively 120 minutes.

The monitoring was made under the stereo-microscopic magnifier MOTIC SL-47 (Microscope Services Ltd) (res. 20 x 2.5).

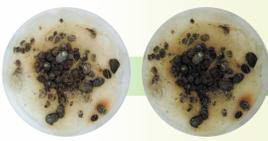
Ticks' viability was observed until exitus instalment. The following parameters were taken into consideration: *vivacity, amplitude and frequency of the locomotory appendix movement, the return from versostasis in comparison to the witness or the attached ticks, the aspect of the tarsal region, the instalment of exitus*, according to the key proposed:

Extensive movements Alert movement	Extensive movement Slow-difficult movement	Ortho or versostasis with legs movement	Versostasis Very slow legs movement	Exitus Total movement lack
++++	+++	++	+	-

The moment of statistical evaluation was considered the moment before exitus (meaning: the stage of *versostasis* +). Wetted filter sheets with 2 ml of tincture were put on Petri plates, in order to be equally moistened on its entire surface. On these plates ticks were placed, reporting 100 individuals (50/plate), for each concentration, constituting also a control lot, in the exact same conditions, (but filter sheet being moistened with diluted 10% alcohol).



Dermacentor



Haemaphysalis

Statistical Analysis

Anova statistics analyze (SPSS-7.5.) determining: *arithmetical average, standard deviation, variance and middle error of the average (standard error)* were accomplished.

B. *In vivo* activity of *E. cyparissias* extracts

The *in vivo* characterization of *E. cyparissias* tincture's activity was made on sheep and cattle belonging to the Didactic farm of USAMVBT Timisoara, maintained in free-grazing range on pastures, naturally infested with ticks.

a. *In vivo* testing of *E. cyparissias* tinctures on sheep

Animals

35 naturally infested sheep: six treatment groups of five individuals /group; one control.

Treatments

Treatment groups sprayed with dilutions of: 10, 5, 2, 1, 0.5% and 0.25%, two times: on days 0 and 7. Control lot was sprayed with 10% alcohol. Tincture was applied in every case on sternal region,

To 24 hours from application, ticks from sternal region were collected, counted and their behaviour was observed in order to establish the tinctures concentrations efficiency. To indicate a possibly long-lasting effect, a second spraying operation has been done on sheep, after seven days from the first spraying, on the same treatment groups, followed by ticks collecting after three days.

b. *In vivo* testing of *E. cyparissias* glycerinated solutions on cattle

Animals

The study, accomplished according to the WAAVP (2006) methodology, on 30 Spotted Romanian breed, heavily parasited with:

There were five treatment groups (five individuals / group / concentration) and a control one (which was sprayed with 10% glycerine). *Euphorbia* glycerinated's activity was tested through direct contact. After 24 hours from the dilutions application, all ticks from the sternal region were collected, counted and their behaviour observed in the laboratory after same methodology.

Ixodes ricinus



Results and discussions

A. *In vitro* activity of the *E. cyparissias* tinctures

Tables 1 and 2, present the evolution of average number of *D. marginatus* and *H. punctata* females which survived to the applications, representing the efficiency of the tincture, on concentrations and in relationship to the exposure time.

Testing the tincture only on female ticks was considered to be sufficient, due to the fact that are mostly responsible for the direct or indirect pathogen activity, males feeding very little, or even at all, their pathogen action being considered insignificant.

Table 1. *Dermacentor marginatus* females which survived the contact with *Euphorbia* tincture (average ± D.S. for 5 tests/dosis)

Concentration %	Exposure time (minutes)			
	30	60	90	120
10	49 (9.8±0.4)	47 (9.4±0.8)	31 (6.2±1.3)	6 (1.2±0.8)
5	49 (9.8±0.4)	46 (9.2±0.4)	37 (7.4±1.1)	14 (2.8±0.8)
2	50 (10±0)	49 (9.8±0.4)	36 (7.2±0.8)	24 (4.8±1.6)
1	50 (10±0)	49 (9.8±0.4)	32 (6.4±1.5)	24 (4.8±1.7)
0.50	50 (10±0)	50 (10±0)	39 (7.8±1.7)	26 (5.2±1.9)
0.25	50 (10±0)	50 (10±0)	32 (6.4±1.1)	30 (6.0±0.7)
Control	50 (10±0)	50 (10±0)	50 (10±0)	50 (10±0)

Table 2. *Haemaphysalis punctata* females which survived the contact with *Euphorbia* tincture (average ± D.S. for 5 tests/dosis)

Concentration %	Exposure time (minutes)			
	30	60	90	120
10	50 (10±0)	44 (8.8±0.4)	27 (5.4±1.1)	17 (3.4±1.1)
5	50 (10±0)	44 (8.8±0.4)	31 (6.2±1)	21 (4.2±1.3)
2	50 (10±0)	45 (9.0±1)	32 (6.4±2.1)	23 (4.6±0.8)
1	50 (10±0)	45 (9.0±0.7)	33 (6.6±1.1)	23 (4.6±1.3)
0.50	50 (10±0)	46 (9.2±0.8)	35 (7.0±1.2)	26 (5.2±0.8)
0.25	50 (10±0)	47 (9.4±0.5)	36 (7.2±0.8)	25 (5.2±1.2)
Control	50 (10±0)	50 (10±0)	50 (10±0)	50 (10±0)

► After 30 minutes from contact with tincture, unrelated to the used concentration, the majority of females lost their vivacity and movement capacity and were not able to come back to the normal position from *versostasis*.

► After 60 minutes, ticks exposed to the tinctures moved only in the cases when the concentrations were lower than 10%, but the great majority only moved their legs. The difference between the averages of the tick number at 30-60 minutes was only 0.6 ($p < 0.1$).

► After 90 minutes leg movements were slower and the number of the ticks which manifested them was lower. The difference of the averages between 30 and 90 minutes was significantly higher 3.28 ($p < 0.001$), and between 30 and 120 minutes it was 5.65 ($p < 0.001$).

Between 60 and 90 minutes from the exposure, averages difference was also significant 2.68 ($p < 0.001$), as well as the one between 60 and 120 minutes 5.05 ($p < 0.001$).

The survival rate was lower after 90 and 120 minutes after the exposure.

In the case of the readings at 90 and 120 minutes, significant differences were found between the averages 2.36 ($p < 0.001$).

That is why we have considered as sufficient the examination up to 120 minutes.

In restrained space, all ticks died after approximately three hours from the exposure, in the cases of the 10%, 5% and 2% concentrations. Female ticks which have fed were much resistant than the hungry ones. In every situation, the anti-parasitic effect appeared after 30 minutes from the exposures and manifested itself through the loss of the movement capacity in the most of cases.

Statistically, there are differences regarding concentration which is used ($F = 6.51$, $df = 5$, $p < 0.001$).

B. The *In vitro* activity of the *E. cyparissias* extracts

a. *In vivo* testing of *E. cyparissias* tinctures on sheep

Identified species were *D. marginatus* and *H. punctata*, with prevalence of the individuals belonging to the first species. Decrease of vivacity as well as the incapacity of coming back out of *versostasis*, with changes of cuticle aspect in the tarsal area (colour and consistency), were observed by comparison to the ticks from control lot, or ticks which attached after the experimental sprayings. Tincture *in vivo* efficiency after 24 hours varied between 1% and 23% for *D. marginatus* and for *H. punctata* between 7% and 27% (Table 3).

Table 3. Ticks collected from sheep treated with *E. cyparissias* tincture after 24 hours from the first application

Concentration %	Species	Ticks Number	Alive	Dead	Efficiency %
10	<i>D. marginatus</i>	304	236	68	23
	<i>H. punctata</i>	11	8	3	27
5	<i>D. marginatus</i>	332	297	35	11
	<i>H. punctata</i>	31	24	7	23
2	<i>D. marginatus</i>	448	376	72	16
	<i>H. punctata</i>	14	12	2	14
1	<i>D. marginatus</i>	389	339	50	13
	<i>H. punctata</i>	12	10	2	16
0,5	<i>D. marginatus</i>	381	362	19	5
	<i>H. punctata</i>	9	8	1	11
0,25	<i>D. marginatus</i>	282	278	4	1
	<i>H. punctata</i>	14	13	1	7
Control	<i>D. marginatus</i>	478	478	0	0
	<i>H. punctata</i>	213	213	0	0

At the second spraying, after seven days, on the sheep initially treated with 10, 5 and respectively 2%, tincture concentrations, no satiated ticks were found, the number of fixed ticks being lower, 0 up to 25 ticks, but hungry, which means that they weren't fixed from a long time. At the concentrations of 1, 0.5 and respectively 0.25%, presence of almost satiated female ticks (23-30) was observed, denoting an older presence on the host (Table 4).

Table 4. Ticks collected from sheep treated with *E. cyparissias* tincture at 72 hours from the first application

Concentration %	Species	Ticks
10	<i>D. marginatus</i>	3
	<i>H. punctata</i>	0
5	<i>D. marginatus</i>	9
	<i>H. punctata</i>	1
2	<i>D. marginatus</i>	25
	<i>H. punctata</i>	4
1	<i>D. marginatus</i>	20
	<i>H. punctata</i>	3
0,5	<i>D. marginatus</i>	32
	<i>H. punctata</i>	2
0,25	<i>D. marginatus</i>	38
	<i>H. punctata</i>	3
Control	<i>D. marginatus</i>	176
	<i>H. punctata</i>	47

After second tincture application, when ticks were collected after three days, revealed a ticks' number reduction, but in some situations not proportional with used concentration. It was also noticed that *H. punctata* seemed to be more sensitive to all tincture concentrations used, in comparison to *D. marginatus*. The same effect was noticed to sheep examination after 72 hours from the second spraying.

At a first sight the slow way of plant extracts action could be considered a disadvantage. It is to note that, *in vivo* the effect of the *Euphorbia* tincture is not a spectacular or a „knock-down” type, its action being slow and death occurring later, after 48-72 hours from the initial applications in most situations, the hungry or poorly fed ticks being more sensitive than the satiated ones, this observation being made as well in the *in vitro* testing case.

b. *In vivo* testing of *E. cyparissias* glycerinated solutions on cattle

In all situations, ticks' viability modification has been noticed as well as reduction of movement capacity, vivacity, of incapacity to come back from *versostasis*, modification of the cuticle aspect (colour and consistency) in comparison to the ticks from the control lot or the ones attached after the application of the tinctures. The mortality percentage of ticks after 24 hours varied between 2% and 53%, the difference from a case to another being given by the number of ticks fixed in the sternal region (Table 5).

The ixodid effect of used glycerinated solutions was visible and easy to monitor. The action was considered slow, the death of ticks occurring late, in most cases after 24-48 hours from the application, being linked to the concentration in every case. Results obtained in the experiment, suggest that the good efficiency of the glycerinated solutions in comparison to tinctures is probably due to the better adhesion and easier penetration of the parasite's cuticle. Recommendable concentrations for glycerinated from the efficiency point of view, in our opinion are 5% and 10%.

Table 5. Ticks collected from cattle treated with *E. cyparissias* glycerinated after 24 hours after first application

Concentration %	Species	Number of ticks	Alive	Dead	Efficiency %
10	<i>Ixodes ricinus</i>	410	194	216	53
	<i>Ixodes ricinus</i>	299	297	112	37
5	<i>Ixodes ricinus</i>	398	376	69	17
	<i>Ixodes ricinus</i>	459	339	44	10
0,5	<i>Ixodes ricinus</i>	327	362	21	6
	<i>Ixodes ricinus</i>	385	278	8	2
Control	<i>Ixodes ricinus</i>	366	478	0	0

At the second spraying with the glycerinated solutions, done after seven days, on cattle subjected to treatment with 10, 5, 2 and respectively 1% concentrations, no satiated ticks were identified, the number of ixodides being low and all of them hungry. At lower concentrations we have identified satiated female ticks (Table 6).

Table 6. Ticks collected from cattle treated with *E. cyparissias* glycerinated after 72 hours after second application

Concentration %	Species	Harvested ticks
10	<i>Ixodes ricinus</i>	0
5	<i>Ixodes ricinus</i>	2
2	<i>Ixodes ricinus</i>	7
1	<i>Ixodes ricinus</i>	13
0,5	<i>Ixodes ricinus</i>	28
0,25	<i>Ixodes ricinus</i>	43
Control	<i>Ixodes ricinus</i>	201

Discussion

The present study proved us the importance of comparing the *in vitro* to *in vivo* results, which can be significantly different. If in the case of the *in vitro* tests, ticks generally died after one or two hours of contact, regardless of the used concentration, but in the case of field applications on animals the situation was completely different.

In vivo, the contact time between ticks and tincture is much shorter (being an alcoholic solution it quickly evaporates thus making the contact short). That is why, when interpreting the results, it is compulsory to take into account the way in which the ixodid - acaricidal contact was done, meaning *in vivo* or *in vitro* conditions. This fact must be analyzed closer from the perspective of making the right choice of excipient basis from the point of view of a greater remanence, especially for the parasites' tarsal-cuticle area, the contact area with highest therapeutic significance in our case. Also an unforeseen aspect, but important for future studies, is that the effect of the extracts on: specific capacity of food conversion, reproductive index and larvae hatching should be followed.

Conclusions

Tincture obtained from *E. cyparissias* could be used with results as an ecologic alternative management method in the ticks' control, being a cheap solution, with a sizeable role in the reducing use of synthetic and other harming ixodidical conditionings.

The alcoholic extract from the strain and inflorescence of *Euphorbia cyparissias* has a significant *in vitro* acaricidal effect, even in lower concentrations (1%), but only *in vitro* conditions this being dependent to the exposure time ($p < 0.001$) and dosis used ($p < 0.001$).

There are no significant differences of acaricide effect of *E. cyparissias* tincture regarding the tick species, but in *in vivo* conditions, the treatments' efficiency being only low-moderate.

The glycerinated solutions from *Euphorbia cyparissias* have an ixodidical effect when the 5 and 10% concentrations are used and ixodifugal effect at 2%.