INSECTICIDAL ACTIVITY OF ETHYL ACETATE EXTRACT OF *Borago officinalis* L. (BORAGINACEAE) AGAINST *Ctenocephalides felis* And *Archaeopsylla erinacei* (SIPHONOPTERA, PULLICIDAE)

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Résumé : Les maladies à transmission vectorielle sont responsables de plus de 17% des maladies infectieuses, et provoquent plus d'un million de décès chaque année. Cependant, l'utilisation abusive des insecticides chimique a entrainé la résistance des insectes. Ce travail, porte sur l'évaluation de l'activité insecticide de l'extrait d'acétate d'éthyle de la Bourrache (*Borago officinalis* L.), plante largement répandue au Nord de l'Algérie, sur la puce de chat *Ctenocephalides felis* et la puce du Hérisson *Archaeopsylla erinacei*, véhiculant des maladies systémiques est vectoriels graves. A cette fin, des spécimens adultes des deux espèces de puces *C. felis* et *A. erinacei* ont été exposé à une série de concentration de l'extrait végétal (50 mg/ml, 25 mg/ml, 12.5 mg/ml and 6.25 mg/ml) en utilisant des bandelettes de papier wattman préalablement imprégnées. L'extrait végétal est obtenu par fractionnement en utilisant des solvants appropriés, il est ensuite caractérisé par une chromatographie sur couche mince à haute performance (HPTLC). Le rendement d'extraction était de 4,87 % avec une concentration en flavonoïdes de 120,99 ± 0,81 µg eq Q/g d'extrait. La caractérisation chromatographique par HTPLC révèle la présence de la rutine, la rhamnétine et la cyanidine-3-glucoside dans cet extrait. Les résultats obtenus montrent que l'extrait d'acétate d'éthyle de la Bourrache réduit considérablement la population des deux espèces de puces *A. erinacei* et *C. felis* après 48 heures de contacte. Les valeurs de CL_{50} étaient de 9,74 mg/ml et 11,24 mg/ml respectivement. Les résultats de la cinétique de mortalité présentent des valeurs de TL_{50} de 7,29h et 8,51h surles mêmes espèces respectivement.

Mots clés : Activité insecticide, Puces, HPTLC, Bourrache, Maladies à transmission vectorielle

Abstract: Vector-borne diseases account for more than 17% of infectious diseases, and cause more than one million deaths each year. However, the misuse of chemical insecticides has resulted in insect resistance. This work concerns the evaluation of the insecticidal activity of the ethyl acetate extract of Borage (*Borago officinalis* L.), a plant widely distributed in northern Algeria, on the cat flea *Ctenocephalides felis* and the flea of the Hedgehog *Archaeopsylla erinacei*, carriers of serious vector diseases. For that, adult flea specimens of *C. felis* and *A. erinacei* were exposed to a series of concentrations of plant extracts. (50 mg / ml, 25 mg / ml, 12.5 mg / ml and 6.25 mg / ml) using pre-impregnated wattman paper strips. The plant extract was obtained by fractionation using appropriate solvents; it was then characterized by high performance thin layer chromatography (HPTLC). The extraction yield was 4.87% with a flavonoid concentration of 120.99 ± 0.81 µgeq Q / g extract. Chromatographic characterization by HTPLC reveals the presence of rutin, rhamnetin and cyanidin-3-glucoside in this extract. The results obtained show that the ethyl acetate extract of Borage greatly reduces the population of the two species of flea *A. erinacei* and *C. felis* after 48 hours of contact. The LC_{50} values were 9.74 mg / ml and 11.24 mg / ml respectively. The mortality kinetics results show TL_{50} values of 7.29h and 8.51h on the same species respectively.

Key words: Insecticidal activity, Fleas, HPTLC, Borage, Vector-borne diseases

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Introduction

Fleas are hematophagous insects that parasitize mammals and rarely birds, some of which can sometimes bite humans. The presence of fleas is noted throughout the year, especially with the phenomenon of global warming and district heating systems. Peak infestation and maximum risk of contamination are often from late March to late October **[1]**. The ability of these parasites to transmit infectious disease agents during the blood meal gives them greater importance in human public health, the plague is the most known and the most feared, but fleas are also associated with other diseases in particular, murine typhus, flea-borne rickettsioses or bartonellosis such as cat scratch disease **[2].**

Plague is considered a re-emerging disease worldwide, as was recently the case in Oran, Algeria **[3]**. These recent outbreaks have shown that the plague can reappear in areas that have remained silent for a long time **[4]**. In addition, in recent years, we have also seen the emergence of the flea-borne spotted fever, *Rickettsia felis* transmitted to humans by the cat flea *C. felis***[5]** and can be found around the world **[6].** Thus, **[7],** using molecular biology techniques, detected for the first time in Algeria the flea contamination by *Rickettsia typhi* responsible for murine typhus after having identified by the same technique their contamination by *Rickettsia felis* in 2006.

Facing this scourge, several control methods: physical, chemical and biological have been developed **[8]** but chemical control continues to be the most used because of its effectiveness on the target. However, marketed insecticides are often harmful to health and the environment because of their persistence **[9].** Most of these molecules act primarily on the neurological system (neuro inhibitor). In addition, it also exists in vertebrates (central nervous system), hence the possibility of exerting a toxic action after the passage of the blood-brain barrier. In addition, the inappropriate use of these insecticides has resulted in insect resistance **[10, 11].**

Biological control takes different forms, but the use of natural substances of plant origin as insecticides is currently the subject of several studies **[12]**. These natural substances with a broad spectrum of action in pharmacology, such as bactericides, fungicides, etc., can be presented as an effective alternative to synthetic insecticides **[13].** This work fits into this context; it reports the evaluation of the insecticidal effect of the ethyl acetate extract of the aerial parts of *Borago officinalis* L. against the adults of the cat flea (*C. felis*) and the Hedgehog flea (*A. erinacei*).

Material and methods

Plant material

The plant *B. officinalis* L. was harvested at the stage of full bloom (April) from Boumerdes area, a Mediterranean region located at 45 km east of Algiers, Algeria. The plant was identified and authenticated by a botanist at Boumerdes University.

Fleas

Two flea species present in Algeria and in the Mediterranean area were used for the insecticidal test.These are the cat's flea (*C. felis*) and the hedgehog flea (*A. erinacei*). These species were collected alive on their preferred hosts during different

entomological exits to perform this study. The specimens of fleas collected were identified and sorted according to the key of identification of fleas proposed by **Duchemin (2003) [14].** They are then put in breeding according to the method described by **Ratovonjato *et al*. (2000) [15]** using an insectarium which maintains the appropriate physical conditions for the proper development of fleas, ie 22 to 27°C of temperature and 75 to 80% relative humidity.

Extract preparation

The collected plant was cleaned with running water and reduced to small pieces, then dried at room temperature for two weeks. The dried aerial parts (stem, leaf and flowers) were powdered in a plant sample grinder (Philips, France). The powder obtained (100 g) was extracted according to the protocol described by **Bruneton (1999) [16]**. This extraction comprises a maceration step in methanol and a liquid-liquid extraction step using ethyl acetate. The resulting extract was evaporated to dryness using a rotavapor (Stuart RE300DB, UK).

Dosage of flavonoids

The flavonoid assay was performed according to the aluminum trichloride methods **[17]**. This technique consists in mixing 1 ml of the plant extract with 1 ml of the AlCl3 solution at 2%. The absorbance was read subsequently using a spectrophotometer (Optizen 2120 UV / Vis, Korea) after 10 minutes of reaction. The calibration curve of quercetin (0-350 µg/ml) was used to deduce the concentration of the extract in flavonoids, and the results are expressed in microgram equivalent of quercetin per gram of extract.

High performance thin layer chromatography.

The bioactive molecules of the plant extract was performed by high performance thin layer chromatography (CAMAG, Switzerland). The samples were deposited on a TLC plate coated with layer of silica gel 60 F254 (10 x 20 cm with 0.2 mm of thickness, Germany) in strip form. The operation was carried out using an automatic injection syringe (CAMAG, Switzerland). After injection of the extract (20 µl) and standard solutions, the TLC plate was transferred to a glass chamber CAMAG previously saturated with migration solvent for 30 minutes. The mobile phase consists of acetone, toluene, ethanol and NH3 (45%, 45%, 7% and 3%). After migration, the plate was removed from the chamber, discharged onto a hot plate CAMAG , and analyzed under UV light at 200 nm, using a CAMAG TLC scanner operated by version 4.03 of the winCATS software.

Preparation of dilutions and impregnation of the strips

Strips of Whatman paper (5 cm x 1.5 cm), one of ends that was pointed have been prepared aseptically and protected from any insecticide.The latter were impregnated with different concentrations of the plant extract (50 mg / ml, 25 mg / ml, 12.5 mg / ml and 6.25 mg / ml), at the rate of three strips per concentration. Each strip receives 400 µl of extract spread over the entire surface using a micropipette. The strips used as a negative control receive 400 µl of distilled water. The impregnated strips were left in the open area for about 30 minutes, to avoid creating high humidity in the tubes, which could lead to a falsely high mortality rate.

Insecticidal toxicity test

10 flea specimens were transferred to each test tube, for a total of 150 individuals for each species, divided into 15 test tubes. The strips were subsequently introduced very quickly into the tubes containing the fleas. The order of the concentrations was respected starting with the control group, then going from the least concentrated batch to the most concentrated. The fleas have been put in darkness, the exposure time is measured by means of a timer and the counting starts as soon as the first impregnated strip is introduced into the first tube.

The number of dead fleas is counted, after an exposure time of 30 min, 1h, 4 h, 24h and 48 h. All fleas that were unable to jump or stand on their feet were considered dead. The mortality rate was calculated by comparing the number of dead fleas in the treated group compared to the control group. It is then corrected according to Abbott's (1925) formula to eliminate natural mortalities that may occur at the time of handling.

Statistical analysis

The results of the insecticidal activity of the ethyl acetate extract of B. officinalis L. were expressed as mean ± SD. An ANOVA test was performed for the statistical comparison between the groups using the statistical presentation system, Statistica version 6. A value of $P < 0.05$ was considered statistically significant. Post-hoc tests, namely the Sheffe test, the Tukey-Kramer test and the Neuman-Keuls test were used to better explore the results obtained.

Probit analysis was used to calculate the lethal concentrations LC50 and LC90 and lethal times TL50 and TL90 in a 95% confidence interval (CI).

Results

The plant extract obtained has a gelatinous appearance with a brown coloration. The extraction yield was 4.87%. The content of flavonoids obtained was 120.99 ± 0.81µg equivalent of quercetin/g of the extract.

Chromatographic analysis of the ethyl acetate extract of the aerial parts of B. officinalis L. by the HPTLC technique reveals the presence of flavonoids monoglycosides. The compounds identified in this extract are rutin, rhamnetin and cyanidin-3-glucoside. **(Figure 1).**

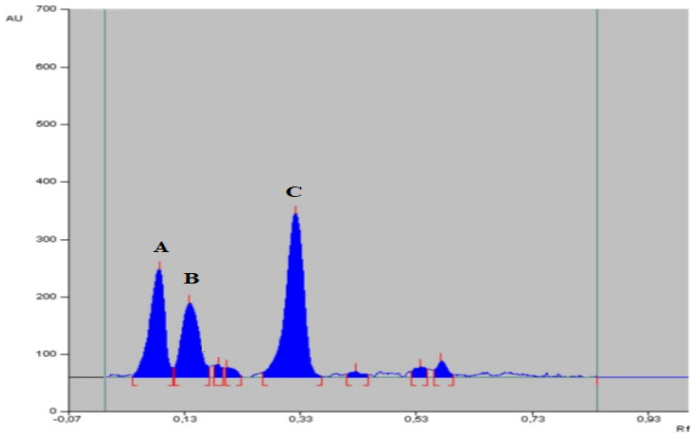

Figure 1. - Chromatogram analysis by HPTLC of ethyl acetate extract of the areal parts from *B. officinalis* L. at wavelength 200 nm with acetone, toluene, ethanol, NH3 (45%, 45%, 7%, 3%).
A: rhamnetin; **B**: cyanidin-3-glucoside; **C**: rutin

The results obtained show that the ethyl acetate extract showed a statistically significant efficacy on the adults of the two species of fleas studied ($p < 0.01$). This efficiency was directly proportional to the concentrations used. In fact, the extract at a concentration of 50 mg / ml resulted in a greater efficacy, reducing the C. felis population by 71.43 ± 4.08% after 24h and by 96 ± 2.89% after 48 hours. of contact. At the lowest concentration (6.25 mg / ml), the extract had a low mortality of 3.57 ± 2.89% after 24 hours and 16 ± 8.66% after 48 hours of exposure. **(Table 1).**

Table 1. Percentage of corrected mortality of the *C. felis* flea treated with ethyl acetate extract from the aerial parts of *B. officinalis* L. expressed as mean ± SD

	(6,25 mg/ml)	(12,5 mg/ml)	(25 mg/ml)	(50 mg/ml)	Control
30 min	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	3,33 ± 2,89	0,00 ± 0,00
1h	0,00 ± 0,00	0,00 ± 0,00	3,33 ± 2,89	6,67 ± 2,89	0,00 ± 0,00
4h	0,00 ± 0,00	3,33 ± 2,89	10,00 ± 5,00	13,33 ± 2,89	0,00 ± 0,00
24h	3,57 ± 5,00	25,00 ± 5,00	50,00 ± 2,89	71,43 ± 2,89	6,67 ± 2,89
48h	16,00 ± 8,66	28,00 ± 8,66	84,00 ± 5,77	96,00 ± 2,89	16,67 ± 2,89

The statistical analysis of the results showed that the mortalities recorded in the treated group at the lowest concentrations (6.25 and 12.5 mg / ml) did not differ significantly from the negative control ($p > 0.5$). . However, from the mean concentration (25 and 50 mg / ml), the extract acted in a highly significant manner ($P < 0.01$).

This extract acted in a similar way towards the adults of the hedgehog fleas, resulting in a mortality directly proportional to the concentrations used. Indeed, the plant extract at the concentration of 50 mg/ml caused in a significant mortality (73.08 ± 5.77 after 24 h and 96 ± 2.89% after 48 h of contact). In addition, the extract at a concentration of 6.25 mg/ml reduced the population of this species by 3.85 ± 2.89 % after 24 h and by 24 ± 7.64% after 48 h of exposure. **(Table 2).**

Table 2. Percentage of corrected mortality of the *A. erinacei* flea treated with ethyl acetate extract from the aerial parts of *B. officinalis* L. expressed as mean ± SD

	(6,25 mg/ml)	(12,5 mg/ml)	(25 mg/ml)	(50 mg/ml)	Témoin
30 min	0,00 ± 0,00	0,00 ± 0,00	3,33 ± 2,89	6,67 ± 2,89	0,00 ± 0,00
1h	0,00 ± 0,00	0,00 ± 0,00	3,33 ± 2,89	10,00 ± 0,00	0,00 ± 0,00
4h	0,00 ± 0,00	10,00 ± 0,00	13,33 ± 2,89	16,67 ± 2,89	0,00 ± 0,00
24h	3,85 ± 2,89	26,93 ± 2,89	53,85 ± 5,00	73,08 ± 5,77	13,33 ± 2,89
48h	24,00 ± 7,64	36,00 ± 7,64	88,00 ± 5,00	96,00 ± 2,89	16,67 ± 2,89

Statistical analysis of the mortality results observed after 48 hours of contact showed a significant difference of the mortalities in the treated groups from the 12.5 mg / ml concentration (P <0.05). It should be noted that the group treated at the lowest concentration (6.25 mg/ml) did not show a significant difference compared to the negative control. A similar result was also observed between the mean and the high concentration (25 and 50 mg / ml, P> 0.5).

It appears from the results of the analysis of probits as a function of the logarithms of the concentrations, that the ethyl acetate extract from the aerial parts of *B. officinalis* L. exhibited a significant insecticidal activity compared to the two species of fleas studied (*C. felis* and *A. erinacei*). The LC50 values noted are 9.74 mg / ml for *A. erinacei* and 11.24 mg / ml for *C. felis*. These two values are between the two low concentrations tested (6.25 and 12.5 mg / ml). For LC90, the values observed are approximately 74 mg / ml, i.e. 73.49 mg / ml (*A. erinacei*) and 73.86 mg / ml (*C. felis*) **(Table 3).**

The result of the mortality kinetics of the fleas treated with different concentrations of ethyl acetate extract, shows the rapid action of this extract on the fleas studied. Thus, the values of TL50 are observed from the seventh hour (7.29h) for *A. erinacei* and at the end of the ninth hour (8.51h) for *C. felis*. The extract also showed TL90 more spread out in time with 35.22 h for *A. erinacei* and 37.87 h for *C. felis*.

Table 3. - Lethal concentrations of ethyl acetate extract of *B. officinalis* L. in adults of *A. erinacei* and *C. felis*

LC	[1]LC_{EAE} (mg/ml)	
	C. felis	***A. erinacei***
LC_{10}	4,00	3,21
LC_{20}	5.70	4.70
LC_{50}	11.24	9.74
LC_{75}	19.32	17.41
LC_{90}	31.63	29.56
LC_{99}	73.86	73.49

[1]: Values of lethal concentrations of ethyl acetate extract from the aerial parts of B. officinalis L. calculated by probit analysis from the interpolation of mortality results. $_{EAE}$:ethyl acetate extract

Discussion

The bioactive compounds of plants such as phenolic compounds, terpenoids, coumarins, flavonoids and alkaloids constitute a rich and varied group of bioactive molecules, which have a broad spectrum in pharmacology as insecticide, bactericide, mycocide, etc. **[8]**. The pharmacological and medical use of these natural substances has recently particularly interested scientists, in particular with the phenomenon of resistance observed by arthropod vectors to chemical insecticides **[18, 11]**. This resistance is the result of indiscriminate and uncontrolled use of chemical molecules, which can even affect human and animal health, but also cause possible contamination of the environment by persistent chemical residues **[19]**.

In this study, the extraction yield was 4.87 %, with a flavonoid concentration of 120.99 ± 0.81 µg equivalent of uercetin /ml of the extract. This result is lower than that reported by **Afif Chaouach *et al.* (2014) [20]**, obtaining a yield of 16.10% of the ethyl acetate extract, by working on the same plant harvested in Tizi Ouzou (Algeria). This variation is probably due to the influence of abiotic ecological factors on the synthesis of these secondary metabolites **[21]**.

Similarly, **Karimi *et al.* (2017) [22]**,confirmed the presence of salicylic acid as the majority phenolic compound and myricetin as the majority flavonoid compound in the methanolic extract of Borage collected in April in Iran. These authors using the RP-HPLC analysis technique also confirmed the presence of gallic acid, pyrogallol, caffeic acid, rutin and daidzein in this plant **[22]**. Some of the biomolecules compound described in this work are associated with a range of biological activities already described in the literature, such as rutin which is endowed with antibacterial and antioxidant activities **[20]**.

Recently, very little work has been done on the pulicidal activity of plant extracts, unlike work on their insecticidal effects against other hematophagous arthropods. These bioactive molecules are an essential means in the resistance of plants to insects, in particular phenolic compounds which are considered as one of the important defenses against insects. However, their specific mode of action is not yet clearly known **[23]**. Thus, researchers believe that the toxic effect of these biomolecules can be caused by penetration of the cuticle (contact effect), in the respiratory system (fumigant effect) or the digestive system (ingestion effect) **[24, 25]**.

In this study, a promising result was found, by testing the insecticidal activity of ethyl acetate extract from the aerial parts of *B. officinalis* L. against the cat flea and that of the hedgehog. The results of this study agree with those reported by **Collart and Hink (1986) [26]**, working on the effect of D-limonene on the flea of the cat *C. felis*. These authors revealed a statistically significant difference in mortality between fleas treated with D-limonene and untreated fleas. The mortality rate was 50.8% after 24 hours and 85.5% after 48 hours of contact ($P < 0.05$). Thus, **Hink and Fee (1986) [27]** found a toxicity of D-limonene at all the biological stages of the cat flea, *C. felis*. In fact, the LC_{50} value obtained for the contact exposure test for adults was 160 µg / cm^2 compared to 259 µg / cm^2 for the vapor exposure test. In addition, these authors revealed a mortality of 100% in the larvae treated at the concentration of 650 µg / cm^2 after 24 h of contact, and 100% of mortality of the eggs at the concentration of 65 µg / cm^2, with high toxicity in nymphs (LC50 = 376 µg / cm2).

Dolan *et al.* (2007) [28], found the susceptibility of adults of the rat flea *Xenopsylla cheopis* to three essential oils extracted from cedar wood incense, Port-Orford cedar and western juniper. The best result found is attributed to cedar wood incense (LC_{50} = 0.24 µg / ml; LC_{90} = 0.31 µg / ml), followed by western juniper (LC_{50} = 0.31 µg / ml; LC_{90} = 0.93 µg / ml) and Port-Orford cedar (LC_{50} = 1.21 µg / ml; LC_{90} = 1.85 µg / ml). In addition, **Kilonzo *et al.* (2001) [29]** found that the aqueous seed extract of *Azadirachta indica* significantly reduced the infestation of goats by the flea *C. felis* ($P < 0.05$). Indeed,

the average density of the insect population was significantly lower in treated goats than in their untreated counterparts.

In another study by **Lans*et al.* (2008) [30]**, on ethnoveterinary drugs used by organic breeders in British Columbia (Canada). These authors report the insecticidal effect of some herbal remedies against fleas in cats and dogs. Indeed, the plants *Artemisia vulgaris* L. (Asteraceae), *Juniperus communis* L., *Lavandula officinalis* L. (Labiatae) and *Melissa officinalis* L. (Lamiaceae) used in the form of infusion, decoction and spray have been endowed with an insecticidal effect. In addition, **Mehlhorn *et al.* (2005) [31]** found the repellent effect of the CO_2 extract from the seeds of the Mediterranean plant *Vitex agnus-castus* (monk pepper) used as a spray. This extract at a concentration of 3% repelled adults of *C. felis* from the treated animals for at least 6 h.

Similar results have been reported by **Panella *et al.* (2005) [32]**, working on the insecticide and acaricidal activity of essential oil components in Alaskan yellow cedar wood. Indeed, fifteen bioactive molecules were tested separately. Carvacrol showed a biocidal activity against ticks, fleas and mosquitoes with LC_{50} values after 24h of 0.0068, 0.0059 and 0.0051%, respectively. While, nootkatone exhibited the greater biocidal activity against the *X. cheopis* flea with an LC_{50} of 0.0029% and an LC_{90} of 0.008%.

A recent study by **Cortés Salazar and Romero Serrato (2017) [33]**, using shampoos prepared with different concentrations of the decoctions of *Artemisia vulgaris* (Asteraceae) shows that this plant has an important insecticidal effect against *C. felis* and *C. canis*.

Indeed, the preparation at the concentration of 50%, 70% and 90% caused an average mortality of 69.7%, 82.7% and 87% of the fleas respectively after two weeks of exposure.

Conclusion

The ethyl acetate extract from the aerial parts of B. officinalis L. has shown toxicity against fleas*C. felis* and *A. erinacei*. At a concentration of 50 mg/ml, the extract considerably reduces the population of these ectoparasites eliminating almost all of these insects after 48 h of contact. This toxicity is associated with the bioactive compounds of Borage, in particular rhamnetin, cyanidin-3-glucoside and rutin. This extract can present an ecological and effective alternative to the control of cat fleas and Hedgehogs.

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