Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
www.entomoljournal.com
JEZS 2020; 8(2): 790-794

Received: 19-01-2020
Accepted: 21-02-2020

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Larvicide activity of two chemotypes of *Hyptis suaveolens* (Lamiaceae) poit, 1806 and alphacypermethrin on larvae of *Rhipicepalus* (Boophilus) *microplus* (Can., 1887) (Acari: ixodidae)

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Abstract
Hyptis suaveolens (Lamiaceae) Poit, 1806 is a well-known herbal medicine with a variety of useful properties, including its acaricidal effect. This experiment was carried out to study the bioacaricidal activity of two chemotypes of *H. suaveolens* essential oil (EO) against larval of *Rhipicephalus* (*Boophilus*) *microplus* (Canistrini, 1887) (Acari; Ixodidae). For this purpose, five serial concentrations (0.15625%; 0.625%; 1.25%; 2.5%; and 5.00% w/v) of each chemotype of *H. suaveolens* were used for the larval immersion test (LIT). Larvae between 14 and 21 days old were fasted and placed in each envelope. Bioassays were performed at 27 ± 1 °C, RH 80%. Larval mortality was observed 24 h after treatment and showed 1.55–66.49% for the *H. suaveolens* Chemotype 1,8-cinéole (Eucalyptol) and 90.72- 99.24 of the *H. suaveolens* Chemotype *β*-caryophyllène. The LIT showed that the LC_{50} and LC_{95} were (45.0–138.2) mg.mL-1 and (0.0068 - 3.2) mg.mL-1 respectively. A positive correlation between *H. suaveolens* Chemotype *β*-caryophyllène EO concentration and tick control, was observed by the strong acaricidal effects against *R.* (*B.*) *microplus*. Our results showed that the *β*- caryophyllène is a promising candidate as an active raw material in the formulation of bio-acaricides against resistant strains of *R.* (*B.*) *microplus*.

Keywords: Chemotypes, *Hyptis suaveolens*, Larvicide, tick, *Boophilus microplus*

1. Introduction
Rhipicephalus (Boophilus) *microplus*, Ixodidae, known as the cattle tick, is an ectoparasite endemic to tropical and subtropical regions of the world [22, 15]. This tick was included in the genus Rhipicephalus after molecular and morphological studies showing the phylogenetic relationship between Rhipicephalus and Boophilus [3, 17]. This parasite is a major problem for livestock breeders, establishing a very efficient host-parasite relationship. This has resulted in enormous losses due to blood loss, damage to the animals' skin, reduced weight gain and the transmission of pathogens such as Babesia bovis, Babesia bigemina and Anaplasma marginale, which cause "Tristeza Parasitária" disease, mainly in calves [11]. But also an increase in expenditure on acaricide chemicals to control the infestation. Thus, the control of this parasite is done at farm level and acaricide products are frequently administered on a monthly basis throughout the year [4].
The development of resistance of R. (B.) microplus to chemical acaricides in several regions of Benin [9, 16, 27], as well as the well-documented damage these compounds cause to the environment and the food chain, has led to a global trend to reduce their use as much as possible. An alternative to the control of livestock ticks with a lower environmental impact would be the use of active ingredients from plants with acaricidal properties, based on the encouraging results of experiments with certain plants [6].
These observations motivated the present study whose objective was to evaluate the activity of two chemotypes of *H. suaveolens* as a potential control measure for R. (Boophilus) microplus larvae. The aim is to verify whether the difference in chemical composition is related to biological activity.

2. Materials and Methods

2.1 Leaf harvesting

The leaves of *H. suaveolens* (L.), constitute the plant material. It's a medium aromatic weedy shrub found in the tropics and subtropical region. In Benin, it is abundantly distributed as aggressive annual weedy species. The genus *Hyptis* is well known for its traditional application as an anticancer, antibacterial, antifungal and anticonvulsant agent [19]. They were harvested early in the morning in their natural environments in Benin's main climatic zones during September and October 2017. The harvests were successively made in the communes of Sème-Podji, Dassa and Kérou respectively in the southern, central and northern climatic zones.

2.1.1 Extraction of essential oils and analysis of chemical constituents

The harvested leaves were transported to the laboratory where they were dried at a temperature of 17 °C away from sunlight. Samples were identified and certified under number AA6726/HNB at the Benin National Herbarium using the analytical flora of Akoègninou *et al.* (2006) [2].

The essential oils were extracted by simple distillation using a Clevenger hydrodistiller [5] at the Laboratory of Enzymology and Protein Biochemistry of the Institute of Applied Biomedical Sciences (ISBA). The dry leaves of *H. suaveolens* and distilled water were boiled for 2 to 3 hours, then the plant extracts condensed in a water cooler were recovered at the end of the process in a decanting tank.

The analysis of the chemical constituents of essential oils and the determination of their relative centesimal compositions were carried out in the LEXVA-ANALYTIC laboratory by gas chromatography (GC) equipped with a flame ionization detector (CG-FID) and by coupled gas chromatography and mass spectrometry (GC-SM) according to the following operating conditions (Table 1).

Table 1: Operating conditions

Gas chromatograph: GC/MS 7890/5975C	- Apolar column: DB5 MS: 40 m 0.18 mm 0.18 µm ; - Temperature programming: 50 °C for 5min - 50 °C/min up to 300 °C - Carrier gas: He : He : 1 ml/min ; - Sample: 4% in solution in acetone or hexane; - Injection volume: 2 µl ; - Injector: 280 °C with 1/100 divider; - Mass range: 33 to 550; - The oil compounds are identified by a combined search for retention times (laboratory library) and mass spectra (NIST library 225 000 spectra)
Gas chromatograph: GC/FID 7890	- Apolar column: DB5 MS: 40 m 0.18 mm 0.18 µm ; - Temperature programming: 50 °C for 5min - 50 °C/min up to 300 °C - Carrier gas: He : He : 1 ml/min ; - Sample: 4% in solution in acetone or hexane; - Injection volume: 2 µl ; - Injector: 280 °C with 1/100 divider; - Percentages (%) are calculated from the peak areas given by the GC/FID without the use of a correction factor.

2.2 Tick larvae production

Engorged females of *Rhipicephalus microplus* were collected on animals from the Kpinnou rearing Farm (N6.56828 E1.78623) in Athiémé, a Benin Municipality. These animals did not receive any acaricidal treatment no less than 30 days before the sampling.

They were transported in an isothermal box to the National Laboratory of Veterinary Parasitology (LNPV) where they were identified using OPTIKA binocular magnifiers at X 10 magnification, according to the dichotomous identification key of Walker *et al.* (2003) [26]. Females of *R.* (*B.*) *microplus* were laid at laboratory room temperature (28°C ± 1). The eggs were then carefully separated from the females and placed in soft bags at a rate of 0.1 g/sac for incubation. The bags were also kept at laboratory room temperature (28°C ± 2) with a relative humidity of 85-90% until hatching, which took place after 21-23 days.

At hatching, F1 larvae take about 15 days to complete their chitinization. Once chitinization is complete, the larvae ascend to the upper edge of the pockets where they gather in clusters (swarms). This behaviour is similar to that observed in nature where larvae climb to the end of grass stems to await the passage of a potential host in order to take the blood meal and initiate the parasite phase. Swarmed larvae, i.e. larvae capable of taking the blood meal, were handled during this study.

2.3 Larval immersion test (LIT)

The immersion test was used to determine the effectiveness of the plant extracts on the larvae of *R.* (*Boophilus*) *microplus* [24] modified by Rosado-Aguilar *et al.* (2010) [22]. Tween-80, an emulsifier, was diluted at 2% in distilled water to serve as control 0% (0.00 mg/mL). This control solution was used to prepare a set of five solutions of concentrations, 0.15625% (1.5625 mg/mL); 0.625% (6.25 mg/mL); 1.25% (12.5 mg/mL); 2.5% (25 mg/mL); and 5.00% (50 mg/mL) for each of the three essentials oils of *H. suaveolens*.

The standard test packets of larvae [8] were used. About, Approximately 100 to 200 larvae were delicately placed with an N°4 brush in each packet, which was immediately sealed with third clasp. The envelopes containing the larvae were then immersed in the petri dishes' solutions for 10 minutes [22]. After immersion, the packets of larvae are then placed in other Petri dishes and kept in room temperature (28 ± 2°C) with 85 to 90% relative humidity for 24 hours. Three repetitions are made for each concentration and for each essential oil of *H. suaveolens*. The packets were previously labelled with the date and the time of immersion, the chemotype of *H. suaveolens*, the date and time at the end of the experiment. To end up, other larvae was immersed into a chemical acaricide, alphacypermethrin, at similar concentrations like those used for the leave extracts of the tested plants, with the aim of making a comparison.

At the end of the test, the packets were opened, and the containing larvae were counted using a gluing tip stick through a magnifying glass. The number of living and dead larvae was recorded. Only those larvae capable of movement were considered alive. The larval mortalities were not corrected by the formula of Abott [1] recommended by the Food and Alimentation Organization [8], because, the mortalities recorded from the control groups were all lower or equal to 5%.

2.4 Statistical analysis
The mean mortality and standard deviation of data from the bioassay were calculated using R Core® Team software (version 3.5.1-2019) with a 5% error. The LC_{50} and LC_{90} values and their 95% confidence interval (CI) were then estimated by probit analysis.

3. Results and Discussion
3.1 Chemical analyzes of essential oils of *H. suaveolens*
The different essential oils obtained are pale yellow in color and have a very strong odor. Chromatographic analyses made it possible to identify the different compounds with the database (Table 2).

Table 2: The main compounds identified in essential oils of *H. suaveolens* according to climatic zones.

Tr	Chemical Constituents	climatic zones of origin of the leaves of *H. suaveolens*		
		South	Center	North
		% FID		
13.80	Sabinene	7.180	3.340	7.700
15.96	Eucalyptol	12.112	0.151	10.924
17.70	Terpinolene	-	6.698	2.528
17.73	Linalool Trans-Oxide	6.557	-	-
17.89	Fenchone	11.812	2.780	4.719
18.93	Fenchol*	0.894	1.127	11.810
27.75	*β*-Caryophyllene	10.338	20.691	12.457
29.63	Bicyclogermacrène	5.755	9.783	3.375
31.65	Dehydro-Isolongifolene*	2.465	8.432	2.354

Tr: Retention time; * Unidentified isomer; - Absent

Monoterpenic hydrocarbons	**38.087**	**19.236**	**27.37**
Oxygenated monoterpenes	3.567	3.555	15.162
Monoterpenic oxydes	18.687	0.456	11.405
Sesquiterpenic hydrocarbons	27.98	51.865	29.191
Oxygenated sesquiterpenic	2.048	8.7	4.01
Terpenic ketones	0.977	0.256	0.289
Alcohol	0.362	3.096	0.786
Aldehydes	0.028	0.079	0.21
Esters	0.557	0.689	0.31
Diterpenes	3.028	2.363	5.742
TOTAL	95.321	90,295	94,475

In this table, the essential oil obtained from the leaves of *H. suaveolens* harvested in the South is predominantly monoterpenic (38.08%) with a relatively high content of Eucalyptol (1.8-Cineole) (12.11%) followed by fenchone (11.81%). While, those extracted from the leaves harvested respectively in Central and Northern Benin are predominantly sesquiterpenic rich in *β*-caryophyllene (20.69 - 12.45%). Two chemotypes therefore emerge from this analysis of chemical compounds.

There is also analogy in the chemical composition of the essential oils of the leaves harvested in the southern and northern climatic zones. Indeed, when the content of *β*-Caryophyllene is low, Eucalyptol is relatively high and vice versa (Table 2).

A comparison with the data in the literature confirms our results. Noudogbessi *et al.* (2013) [20] on seven samples of *H. suaveolens* collected from localities in Benin showed that they were mainly composed of 1,8-cineole (eucalyptol), sabinene, and *β*-caryophyllene. On the other hand, the essential oil analyzed by Kossouoh *et al.* (2010) [13] is rather rich in *β*-caryophyllene, trans- α-bergamotene, caryophyllene oxide and bicyclogermacene. These different profiles are also those obtained in Nigeria [7], Côte d'Ivoire [25] and Dakar [18]. This difference depends on ecotypes because the variation in chemical composition in plants is related to climatic factors, geographical origin, age of the plant but also ecological and pedological factors. Concentrations of these substances may also vary according to seasonality, circadian rhythm and plant development [10].

3.2 Larvicidal activity of essential oils of *H. suaveolens*
Laboratory tests carried out to determine the efficacy of two chemotypes of *H. suaveolens* essential oil on *R.* (*B.*) *microplus* larvae are summarized in Table 3.

Table 3: Larvicidal activity of essential oil of *H. suaveolens* against larvae of *R.* (*B.*) *microplus* between two chemotypes and alphacypermethrin

Concentrations (mg/mL)	*H. suaveolens Ct* 1,8-cinéole (Eucalyptol)	*H. suaveolens Ct β*-caryophyllène (20.69%)	*H. suaveolens Ct β*-caryophyllène (12.45%)	Alphacyperthrin
	Mortality percentage			
0.00	$0,84^{a} \pm 1,16$	$0,84^{a} \pm 1,16$	$0,84^{a} \pm 1,16$	$0,84^{a} \pm 1,16$
1.5625	$1,55^{a} \pm 2,13$	$90,72^{b} \pm 3,61$	$0,21^{a} \pm 0,41$	$4,93^{c}$ 2,84
6.25	$0.00^{a} \pm 0.00$	$98,94^{c} \pm 1,18$	$15,02^{b} \pm 3,76$	$100^{c} \pm 0.00$
12.5	$0.00^{a} \pm 0.00$	$99,52^{c} \pm 0,92$	$39,62^{b} \pm 4,94$	$100^{c} \pm 0.00$

25	2,54[a] ± 2,84	96,66[b] ± 1,93	97,75[b] ± 1,44	100[c] ± 0.00
50	66,49[a] ± 6,64	99,24[c] ± 0,84	95,13[b] ± 2,02	100[c] ± 0.00

Mortality rates followed by different letters within the same line are significantly different at the 5% threshold (p<0.05).

Larval mortality obtained from this experiment was high and varied significantly between the two chemotypes of *H. suaveolens* (Table 3). The *β*-caryophyllene chemotype appears to be more effective on *R.* (*B.*) *microplus* larvae than the 1,8-cineol (eucalyptol) chemotype. The 24-hour mortality rate recorded with the lowest concentration (1.5625 mg/mL) of this chemotype was 90.72 ± 3.61%, whereas even the highest concentration (50 mg/mL) of the 1,8-cineol chemotype caused only 66.49 ± 6.64% mortality (p=0.000 significantly different). This difference is also confirmed by the relatively low mortality rates obtained with the essential oil in which *β*-caryophyllene and 1,8- cineole (eucalyptol) are relatively in the same proportions (12.42% and 10.92% respectively). The chemotype *β*-caryophyllene also obtained the lowest LC_{50} and LC_{95} values, (0.0068 mg.mL-1) and (3.2 mg.mL-1) respectively (Table 4). The mortality of the negative control was less than 5% and that of the positive control was greater than 90%, demonstrating the quality of the test.

Table 4: Lethal concentration LC_{50} and LC_{95} of two chemotypes of *Hyptis suaveolens* and alphacypermethrin for *R.* (*B.*) *microplus* and respective confidence intervals (CI), calculated starting from the interpolation of the larval Immersion test (LIT) by Probit analysis

	LC_{50} (mg/mL) (95% CI)	**L_{95} (mg/mL) (95% CI)**
H. suaveolens Ct 1,8-cinéole (Eucalyptol)	45.0 (42.7 - 47.5)	138.2 (11.95 - 15.99)
H. suaveolens Ct β-caryophyllène (20.69%)	0.0068 (0.0015 - 0.029)	3.2 (2.3 - 4.3)
H. suaveolens Ct β- caryophyllène (12.45%)	12.5 (12.2 – 12.8)	36.0 (34.3 - 37.7)
Alfacyperméthrine	2.04 (2.00 - 2.08)	2.67 (2.45 - 3.01)

LC_{50}: lethal concentration 50%; LC99.9: lethal concentration 95%; CI: confidence intervals.

The average acaricidal activity obtained in the present study may be linked to the content of this oil in sesquiterpenes. In fact, according to literature, several terpene derivates are mentioned to be toxic against the cattle tick. Sesquiterpenes (hydrocarbons and oxygenated) have demonstrated acaricidal activity and synergistic effect against *R.* (*B.*) *microplus*

For example, Kosgei *et al.* (2014) [12] observed that for the essential oil of *Lippia kituiensis*, which contained high levels of sesquiterpenes (56.57%), caused 100% mortality of the larvae of *Rhipicephalus sanguineus* at a concentration of 4.5 mg/ml. *Ocotea diospyrifolia*, had the same effect (95%) in the larval immersion test (LIT) at a concentration of 40%. Eleven sesquiterpene compounds were identified in l'huile essentielle of de cette plante [23]. Ribeiro *et al.* (2008) [21] also evaluated the larvicidal effect of *Drimys brasiliensis* essential oil, which contained predominantly sesquiterpenes (30.4% cyclocolorenone), on the larvae of *R.* (*B.*) *microplus*. One hundred percent of the larvae were killed at concentrations of 2.5%, 1.25% and 0.625%.

We believe that the sesquiterpene (*β*-caryophyllene), the major constituent of the essential oil, contributed fundamentally to the larvicidal activities of *H. suaveolens* against *R.* (*B.*) *microplus* in the present study. However, minor components present in the oil may also have contributed to the biological activity of the oil [14]. The bioactive compounds of essential oils are known to have disruptive effects on the basic metabolic, biochemical, physiological and behavioural functions of ticks.

4. Conclusion

Chemical composition and larvicidal activity of two chemotypes of essential oil from leaves of *H. suaveolens* on bovine tick *R.* (*B.*) *microplus* were investigated. This study showed that sesquiterpene hydrocarbon-dominant essential oils presented the best result as acaricidal activity for *R.* (*B.*) *microplus* larvae, in contrast to the 1,8-cineole (eucalyptol) chemotype. Formulations with *H. suaveolens* oil or with *β*-caryophyllene should be developed and evaluated to find better compounds for the control of cattle ticks in the future. However, as the yield of oil extraction from *H. suaveolens* is really low, it would be interesting to carry out genetic selection to obtain genotypes suitable for commercial production of this species by improving the production of *β*-caryophyllene.

5. Acknowledgements

The authors are thankful to the Government of Benin for financial support through the Scholarship fellowship initiated by the Ministry of Higher Education and Scientific Research.

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