Parasitol Res (2018) 117:59–65
https://doi.org/10.1007/s00436-017-5662-0

ORIGINAL PAPER

Seasonal analysis and acaricidal activity of the thymol-type essential oil of *Ocimum gratissimum* and its major constituents against *Rhipicephalus microplus* (Acari: Ixodidae)

Aldilene Silva Lima[1] · Marlise Neves Milhomem[2] · Odair Santos Monteiro[3] · Alana Carynne Pereira Arruda[1] · Joaquim Antônio Martins de Castro[3] · Yan Michel Lopes Fernandes[3] · José Guilherme Soares Maia[4] · Lívio Martins Costa-Junior[1]

Received: 23 January 2017 / Accepted: 19 October 2017 / Published online: 19 November 2017

Abstract The tick *Rhipicephalus microplus* affects cattle health, with production loss in tropical and subtropical regions. Moreover, the use of commercial acaricides has been reduced due to the resistance of this parasite. Although alternatives such as plant bioactive molecules have been sought, essential oils present variations in their chemical constituents due to environmental factors, which can interfere with their acaricidal activity. The objective of the present study was to evaluate the seasonal influence of the essential oil of *Ocimum gratissimum* and its major constituents on acaricidal activity against *R. microplus* larvae. A high-yield essential oil of *O. gratissimum* and its major constituents were used, and a plant with a thymol-type oil was selected for seasonal analysis and acaricidal activity against *R. microplus*. Gas chromatography (GC) and GC-mass spectrometry (MS) were employed to identify 31 oil constituents (average yield of 6.26%). The main compounds were found to be thymol (33.4 to 47.9%), γ-terpinene (26.2 to 36.8%), and *p*-cymene (4.3 to 17.0%). Concerning acaricidal activity, the December (LC_{50} 0.84 mg/mL) and September (LC_{50} 1.58 mg/mL) oils obtained in the dry season were the most active, and assays performed with commercial standards revealed LC_{50} values of *p*-cymene, thymol, and γ-terpinene of 1.41, 1.81, and 3.08 mg/mL, respectively. Overall, lower acaricidal activities were found for oils produced from plants harvested in the rainy season. The results showed that seasonal variation in the chemical composition of the *O. gratissimum* essential oil influences its acaricidal activity. The seasonal variations in the thymol-type essential oil of *O. gratissimum* can represent an important strategy for the control of *R. microplus*.

Keywords Natural product · Thymol · γ-Terpinene · *p*-Cymene · Tick · Acaricidal activity

✉ Lívio Martins Costa-Junior
livio.martins@ufma.br

[1] Departamento de Patologia, Universidade Federal do Maranhão, São Luís, MA, Brazil

[2] Programa de Pós Graduação em Ciências da Saúde, Universidade Federal do Maranhão, São Luís, MA, Brazil

[3] Programa de Pós-Graduação em Química, Universidade Federal do Maranhão, São Luís, MA, Brazil

[4] Programa de Pós-Graduação em Recursos Naturais da Amazônia, Universidade Federal do Oeste do Pará, Santarém, PA, Brazil

Introduction

Cattle are significantly affected by the tick *Rhipicephalus microplus* Canestrini, 1888 (Acari: Ixodidae), which affects animal health and causes production losses, in tropical and subtropical areas (Fao 2004; Grisi et al. 2014). Although the use of chemical pesticides has had good efficacy against this parasite, their misuse, high cost, and decreased efficiency due to increased resistance of *R. microplus* populations as well as residues in the environment that are harmful to animals and humans have led to the search for new control alternatives (Graf et al. 2004; Lem et al. 2014; Kumar et al. 2015; Banumathi et al. 2017). Bioactive molecules from plants used in ethno-veterinary applications can be an alternative to synthetic chemicals for tick control (Cruz et al. 2013; Benelli et al. 2016; Pavela et al. 2016; Banumathi et al. 2017).

Essential oils are crude extracts obtained by steam distillation that contain volatile bioactive compounds derived from plant secondary metabolism and are mainly composed of monoterpenes, sesquiterpenes, phenylpropanoids, and coumarins. The production of such essential oils may be related to three different factors: genetics, the environment, and cultivation techniques (Maffei 2010; Pavela and Benelli 2016; Piątkowska and Rusiecka-Ziółkowska 2016). In addition to environmental parameters, temperature and atmospheric precipitation have been identified as factors that influence the composition and content of essential oils in aromatic plants (Suhr and Nielsen 2003; Cruz et al. 2014; Santos et al. 2016).

Ocimum gratissimum L. (Lamiaceae) is an herbaceous plant of Asian origin and naturalized in the Brazilian territory with the common name "Alfavaca" (Maia et al. 2001). The essential oils of *O. gratissimum* are used as a flavoring, analgesic, anticonvulsant, antimicrobial, antifungal, antioxidant, insecticide, and leishmanicidal (Dubey et al. 2000; Kéita et al. 2001; Adebolu and Oladimeji 2005; Faria et al. 2006; Freire et al. 2006; Koba et al. 2009). The acaricidal effect of the oil of *O. gratissimum* against *R. microplus* was previously reported, with varying results according to the oil origin and chemical type (Hue et al. 2015). Thus, *O. gratissimum* can produce different chemical types (Benitez et al. 2009), at least four of which are known: eugenol, thymol and geraniol, and ethyl cinnamate as the major compounds (Dubey et al. 2000; Vieira et al. 2001). Acaricidal activity has been reported for chemotypes thymol and eugenol (Hue et al. 2015).

Previous studies have shown seasonal quantitative fluctuation in the composition of essential oils (Medini et al. 2009; Ennajar et al. 2011; Evergetis et al. 2016). For example, a seasonal study was carried out to analyze the composition of the thymol-type oil of *O. gratissimum* according to the rainy and dry seasons in the Oriental Brazilian Amazon, and the results showed important differences in antimicrobial activity (Castro 2015). Although the acaricidal activity of *O. gratissimum* thymol-type oil has already been reported (Hue et al. 2015), to our knowledge, the present study is the first to evaluate relationships between the seasonal variation and acaricide effect of these oils.

Materials and methods

Plant and chemical material

O. gratissimum plants were cultivated at the Active Germplasm Bank (Berta Lange de Morretes) of Federal University of Maranhão (UFMA), São Luís, Brazil (2° 33′ 13″ S 44° 18′ 19″ W). A voucher specimen was deposited at the MAR Herbarium of UFMA, under the number 5150. Leaves were collected at 8 a.m. in February, April, July, September, and December 2014. The material was dried in a cool and ventilated room for 5 days and then ground before oil extraction. Climatic data were collected from Agritempo Database (https://www.agritempo.gov.br/agritempo/index.jsp). Thymol, γ-terpinene, and *p*-cymene standards were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Plant processing and essentials oil extraction

Leaves were air-dried, ground, and subjected to hydrodistillation using a Clevenger-type apparatus (100 g, 3 h). The oils were dried over anhydrous sodium sulfate, and percentage contents were calculated according to plant dry weight. The moisture content of fresh leaves (2 g) was determined using an infrared moisture analyzer at 115 °C (three replicates).

Oil composition analysis

Analysis of oils was carried out by gas chromatography-mass spectrometry (GC-MS) using a Thermo Focus DSQ II (Thermo Fisher Scientific, MA, USA) under the following conditions: DB-5-ms (30 m × 0.25 mm; 0.25-mm film thickness) fused-silica capillary column; programmed temperature, 60–240 °C (3 °C/min); injector temperature, 250 °C; carrier gas, helium, adjusted to a linear velocity of 32 cm/s (measured at 100 °C); injection type, split (1.0 μL), from 1:1000 hexane solution; split flow adjusted to yield a 20:1 ratio; septum sweep constant at 10 mL/min; EIMS, electron energy, 70 eV; temperature of the ion source and connection parts, 200 °C. Quantitative data regarding the volatile constituents were obtained by peak area normalization using a FOCUS GC/FID (Thermo Fisher Scientific) operated under conditions similar to those used for GC-MS, except that the carrier gas was nitrogen. The retention index was calculated for all volatile constituents using a homologous series of *n*-alkanes (C_8–C_{32}, Sigma-Aldrich), according to Van den Dool and Kratz (1963).

Preparation of test ticks

Engorged *R. microplus* females of the resistant jaguar strain (Reck et al. 2014) were collected directly from artificially infested animals. Engorged females without structural damage were selected and maintained at 27 °C and ≥ 80% relative humidity until oviposition was completed. Eggs were collected and incubated in an oxygen demand biochemical incubator for hatching. Randomly selected larvae aged 14–21 days were used in larval immersion tests. This study was approved by the UFMA ethics committee, under number 23115018061/2011-01.

Larval immersion test

The larval immersion test was performed according to Klafke et al. (2006). Oils and purified terpenes (standards) were diluted in a solution containing 1.0% ethanol and 0.02% Triton X-100.

The tests were conducted with eight concentrations, ranging from 0.66 to 5.0 mg/mL, and the experiment was carried out with four replicates for each treatment. A control group with a 1.0% ethanol and 0.02% Triton X-100 solution was included. Approximately 500 larvae were immersed for 10 min in each concentration and then transferred to a filter paper to dry. Approximately 100 larvae were transferred to a clean dry filter paper (8.5 × 7.5 cm) that was folded and closed with clips. The packets were incubated at 27 ± 1 °C with relative humidity ≥ 80% for 24 h. After incubation, dead and live larvae were counted.

Statistical analysis

Lethal concentrations were calculated by probit regression carried out with GraphPad Prism 6.0 software (GraphPad Software Inc., La Jolla, CA, USA). Significant differences in the average efficiency of each oil or the purified terpenes were considered when there was no overlap between the 95% confidence limits of the LC_{50} values (Roditakis et al. 2005).

Results

Yield and oil composition

The seasonal analysis of the oil composition of *O. gratissimum* leaves collected in February and April (rainy season), July (transitional period), and September and December (dry season) is displayed in Table 1. Individual constituents were identified by comparison of both mass spectrum and GC retention data with authentic compounds previously analyzed and stored in the GC-MS system and also with the aid of commercial libraries containing retention indices and mass spectra of compounds commonly

Table 1 Yield and seasonal chemical evaluation for the essential oil of *Ocimum gratissimum*

Constituents (%)	RI_{Calc}	RI_{Lit}	Rainy season		Transitional	Dry season	
			Feb	Apr	Jul	Sep	Dec
α-Thujene	920	924	1.6	2.1	2.8	2.2	4.0
α-Pinene	929	932	0.4	0.7	0.7	0.5	0.3
Camphene	944	946	0.1	0.1	0.1	0.1	0.1
Sabinene	966	969	0.3	0.5	0.4	0.4	0.4
Myrcene	981	988	1.6	2.4	1.9	1.8	4.4
α-Phellandrene	1002	1002	0.2	0.4	0.4	0.4	0.2
α-Terpinene	1013	1014	1.8	2.4	2.2	2.4	1.9
p-Cymene	1019	1020	*4.3*	*11.9*	*17.0*	*6.9*	*15.8*
(*Z*)-β-Ocimene	1030	1032	0.1	–	–	–	0.1
(*E*)-β-Ocimene	1042	1044	–	0.2	0.1	0.1	–
γ-Terpinene	1052	*1054*	*36.8*	*31.3*	*30.3*	*35.1*	*26.2*
(*Z*)-Sabinene hydrate	1065	1065	0.5	0.6	0.9	0.8	0.6
Terpinolene	1083	1086	0.3	0.4	0.5	0.4	0.4
(*E*)-Sabinene hydrate	1095	1098	0.2	0.3	0.3	0.3	0.2
p-Mentha-1,3,8-triene	1107	1108	0.1	0.1	–	0.1	–
Borneol	1165	1165	0.1	0.2	0.1	0.1	0.1
Terpinen-4-ol	1174	1174	0.3	0.5	0.5	0.5	0.4
p-Cymen-8-ol	1178	1179	0.1	0.1	0.1	0.1	0.1
α-Terpineol	1185	1186	–	0.1	0.1	0.1	0.1
Thymol methyl ether	1227	1232	0.2	0.5	0.5	0.4	0.6
Thymol	1285	1289	*47.9*	*39.5*	*33.4*	*39.1*	*37.7*
Carvacrol	1294	1298	0.8	0.9	1.2	1.0	0.9
β-Elemene	1384	1389	0.1	0.1	0.2	0.2	0.1
(*E*)-Caryophyllene	1411	1417	0.7	1.1	1.7	2.3	1.2
α-Humulene	1447	1452	0.1	0.2	0.2	0.2	0.1
Germacrene D	1478	1484	0.2	0.2	0.2	0.2	0.1
β-Selinene	1485	1489	0.9	1.5	1.8	2.1	1.8
Viridiflorene	1491	1496	0.2	0.5	0.6	0.7	0.4
β-Curcumene	1507	1514	0.1	0.2	0.2	0.2	0.1
Spathulenol	1568	1577	–	0.2	0.3	0.2	0.1
Caryophyllene oxide	1578	1582	–	0.3	–	–	–
Monoterpene hydrocarbons			47.5	52.4	56.4	50.3	53.8
Oxygenated monoterpenes			50.2	42.8	37.1	42.5	40.7
Sesquiterpene hydrocarbons			2.2	3.8	4.9	5.9	3.8
Oxygenated sesquiterpenes			0.1	0.5	0.3	0.2	0.1
Total			99.9	99.5	98.7	98.9	98.4
Yield (%)			6.4	6.7	6.4	6.0	5.8

RI_{Calc}, retention index calculated; *RI_{Lit}*, retention index from literature (Adams, 2007); –, not detected; Perceptual in Italic correspond at major compound.

Table 2 Efficacy of the oils of *Ocimum gratissimum* and their major constituents against the *Rhipicephalus microplus* larvae

Month oils	LC_{50} (mg/mL)	95% CI	LC_{90} (mg/mL)	95% CI	r^2
February oil	2.57	2.49–2.66	4.23	3.72–4.82	0.98
April oil	2.02	2.00–2.05	2.45	2.31–2.62	0.98
July oil	2.15	2.06–2.15	3.19	2.71–3.77	0.96
September oil	1.58	1.53–1.64	2.42	2.18–2.70	0.98
December oil	0.84	0.80–0.88	1.37	1.18–1.59	0.97
Standards					
Thymol	1.81	1.78–1.83	2.13	2.03–2.24	0.96
γ-Terpinene	3.08	2.80–3.38	5.54	5.04–6.09	0.99
p-Cymene	1.41	1.38–1.44	1.83	1.75–1.92	0.99

LC_{50}, concentration (mg/mL) at which 50% of the *R. microplus* larvae died; *95% CI*, confidence interval at 95% probability; r^2, coefficient of determination

found in essential oils (NIST 2005; Adams 2007). Thirty-one components were identified in the oils, comprising an average of 99% of the total composition. The monoterpene class was the most represented, both hydrocarbons (47.5 to 56.4%) and oxygenated (37.1 to 50.2%) forms. The major identified compounds were thymol (33.4 to 47.9%), γ-terpinene (26.2 to 36.8%), and *p*-cymene (4.3 to 17.0%). The leaf distillation process provided oil yields of 6.4 and 6.7% in February and April, respectively (rainy season), 6.4% in July (transitional period), and 6.0 and 5.8% in September and December, respectively (dry season).

Larvicidal activity assay

The lethal concentration of the *O. gratissimum* oils exhibited variation according to the collection month, which was also observed for the commercial standards of the main constituent thymol, γ-terpinene, and *p*-cymene (Table 2). The oils obtained from leaves harvested in December (LC_{50} 0.84 mg/mL, 95% CI 0.80 to 0.88, r^2 0.97) and September (LC_{50} 1.58 mg/mL, 95% CI 1.53 to 1.64, r^2 0.98), i.e., the dry season, were the most active (Fig. 1). Conversely, the lowest lethal concentration was observed for the February oil (LC_{50} 2.57 mg/mL, 95% CI 2.49 to 2.66, r^2 0.98), i.e., from plants collected in the rainy season (Fig. 1). Concerning the assays conducted with commercial standards of the main oil constituents, the highest acaricidal activity was observed for *p*-cymene (LC_{50} 1.41 mg/mL, 95% CI 1.38 to 1.44, r^2 0.99), followed by thymol (LC_{50} 1.81 mg/mL, 95% CI 1.78 to 1.83, r^2 0.96) and γ-terpinene (LC_{50} 3.08 mg/mL, 95% CI 2.80 to 3.38, r^2 0.99) (Table 2).

Discussion

It has been reported that environmental factors such as precipitation, temperature, light incidence, and day length may quantitatively alter the composition of the oils of various aromatic plants (Figueiredo et al. 2008; Mossi et al. 2012; Kiazolu et al. 2016; Pavela and Benelli 2016). The constituents identified in the oils of *O. gratissimum* showed variation in their percentage according to the month of collection, confirming the influence of the season on the plant. This change can be observed in the sum of the percentages of thymol, γ-terpinene, and *p*-cymene, the main constituents of the oils, in the various plant collection months: February (89.0%), April (82.7%), July (80.7%), September (81.1%), and December (79.7%). Another approach to observing variation in oil constituents is to add the percentage values of monoterpenes (hydrocarbons and oxygenated) for each month: February (97.7%), April (95.2%), July (93.5%), September (92.8%), and December (94.5%).

At the same time, plants under climatic factor stress may present variability in the production of secondary metabolites, differentiating them and thus affecting their bioactivity (Sampaio et al. 2016). For instance, oil production by plants of the Lamiaceae family is affected by rainfall, as the organs that accumulate these oils are located on the leaf surface (Blank et al. 2011); thus, a lack of rain contributes to increasing constituents with acaricidal action. The acaricidal activity of the *O. gratissimum* oil

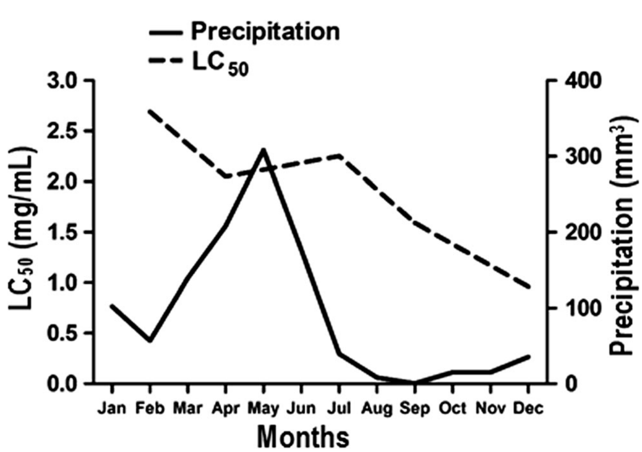

Fig. 1 Lethal concentration 50% (LC_{50}) for the oils of *Ocimum gratissimum* extracted on different months against *Rhipicephalus microplus* larvae and local precipitation during the collection

showed different values according to the collection month, with the December oil (LC_{50} 0.84 mg/mL) being the most active.

The highest acaricidal activity for the December oil may be related to the increase in the *p*-cymene content (15.8%, dry season), which was influenced by low rainfall (below 50 mm, see Fig. 1). Among the commercial standards tested, *p*-cymene displayed the highest acaricidal activity (LC_{50} 1.41 mg/mL). This monoterpene hydrocarbon with acaricidal activity is found in several essential oils, and it may have contributed to the highest acaricide action found in for the *O. gratissimum* oil (Cruz et al. 2013; Lage et al. 2013). Thymol, the most volatile component identified in the oils of *O. gratissimum* (average of 39.5%), is an oxygenated monoterpene with a high acaricidal activity against *R. microplus* and resistant strains, as well as against *Rhipicephalus sanguineus* Latreille, 1806, *Ixodes ricinus* L., 1758 and *Amblyomma cajennense* Fabricius, 1787 (Daemon et al. 2009; Scolarick et al. 2012; Cruz et al. 2013; Lage et al. 2013; Araújo et al. 2015; Hue et al. 2015; Costa-Júnior et al. 2016; Soares et al. 2016; Tabari et al. 2017). The commercial standard of thymol tested against *R. microplus* larvae exhibited an LC_{50} value of 1.81 mg/mL, slightly higher than that obtained for *p*-cymene. These results for the isolated compounds thymol and *p*-cymene indicate that these components are more toxic than the monoterpenes S-(+)-carvone, R-(+)-limonene and citral, with an LC_{50} value ≥ 31.2 mg/mL (Peixoto et al. 2015).

Among the analyzed *O. gratissimum* oils, γ-terpinene showed the second highest abundance percentage, 26.2% in the December oil, which presented the greater acaricide action. The LC_{50} value of the commercial standard of γ-terpinene was 3.08 mg/mL, that is, lower compared with *p*-cymene and thymol. Nonetheless, γ-terpinene of a *Satureja thymbra* L. (Lamiaceae) oil presented an acaricide effect against the tick *Hyalomma marginatum* Koch, 1844 (Cetin et al. 2010). In addition, γ-terpinene of *Lippia gracilis* Schauer and *Lippia sidoides* Cham oils harvested in Bahia and Sergipe, Brazil, respectively, was reported to having a synergistic effect on the observed acaricidal activity, together with *p*-cymene and carvacrol, a positional isomer of thymol (Cruz et al. 2013; Soares et al. 2016).

In our study, the oil obtained from plants harvested in December showed the highest acaricidal activity when compared to the commercial standards of its main constituents (Table 2). This difference can be attributed to the synergistic effect of other components, which exist at smaller percentages in the oil. However, the oil produced from leaves harvested in December displayed with lowest LC_{50} when compared to purified components. Considering that an essential oil is a complex mixture of secondary constituents, these components may present synergistic or antagonistic effects that may or may not contribute to their bioactivity (Pandey et al. 2014; Lima et al. 2016).

Thymol, *p*-cymene, and carvacrol also co-occur as major constituents in some traditional oils, such as *Satureja hortensis* L. (savory) and *Thymus vulgaris* L. (oregano) (Krstev et al. 2009; Borugă et al. 2014). Thus, it is no coincidence that these same aromatic monoterpenes are found in the oil of *O. gratissimum*. All these constituents are generated by the same biosynthetic process of plants and derived from γ-terpinene, a cyclohexadiene also present in the oil (Poulose and Croteau 1978). The median sum of thymol, γ-terpinene, *p*-cymene, and carvacrol in the analyzed oils was 83.6%.

The results of the present study indicate that the thymol-type essential oil of *O. gratissimum* may represent a significant biological alternative for the control of *R. microplus*. The results also showed that the dry season, with higher acaricidal activity found for the extracted oil, is the most appropriate collection time for this plant. The present study extends existing knowledge of this essential oil for practical applications.

Acknowledgments The authors wish to thank CNPq (The Brazilian National Council for Scientific and Technological Development) for awarding a fellowship to L.M. Costa-Júnior, CAPES (Brazilian Federal Agency for support and evaluation of graduate education) and FAPEMA (Maranhão State Research Foundation) for the scholarships to A.S. Lima and M.N. Milhomem, respectively. We also thank CNPq and FAPEMA for their financial support and Dr. JRS Martins for donating the *R. microplus* strain.

Compliance with ethical standards This study was approved by the UFMA ethics committee, under number 23115018061/2011-01.

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