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Chemical composition of the essential oil from *Ageratina jocotepecana* and its repellent effect on Drywood termite *Incisitermes marginipennis*

[Composición química del aceite esencial de *Ageratina jocotepecana* y su efecto repelente
en termitas de madera seca *Incisitermes marginipennis*]

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Abstract: Essential oils obtained from new plant species with metabolomes unexplored or poorly known are a natural resource to find molecules with deterrent (irritant) effect. The aim of this study was to evaluate the chemical composition and the termite repellent activity of the essential oils from *Ageratina jocotepecana*. The repellent effect was determined by the pine drywood termite *Incisitermes marginipennis* behavior of to sense the contact of the tunnel wall in the wooden colony in the presence of an irritant obstacle caused by essential oils. Gas chromatographic analysis of the essential oils from flower, leaf, and stem showed quantitative and qualitative differences in components. Twenty-eight volatile components were identified by their mass spectra (MS). β-caryophyllene, carvacrol, spathulenol, and terpinen-4-ol were the four major components, of them in relation 0.1 M citronellol, the 0.1 M carvacrol was the best repellent of the termite. Essential oils from *A. jocotepecana* exhibited a termite repellent effect due to their major components. Additionally, more research about the termite repellent action of carvacrol is still needed.

Keywords: *A. jocotepecana*, *I. marginipennis*, Essential oils, Repellency, Carvacrol

Resumen: Los aceites esenciales obtenidos de nuevas especies de plantas con metabolomas inexplorados o poco conocidos son un recurso natural para encontrar moléculas con efecto disuasivo (irritante). El propósito del estudio fue evaluar la composición química de los aceites esenciales de *Ageratina jocotepecana* y su actividad repelente de termitas. El efecto repelente fue determinado por el comportamiento de las termitas de la madera seca de pino *Incisitermes marginipennis* de sentir el contacto de la pared del túnel en la colonia de madera en la presencia de un obstáculo irritante causado por los aceites esenciales. El análisis de cromatografía de gases de los aceites esenciales de flores, hojas y tallo mostró diferencias cuantitativas y cualitativas en componentes. Veintiocho componentes volátiles fueron identificados por sus espectros de masas (MS). β-cariofileno, carvacrol, spathulenol y terpinen-4-ol fueron los cuatro componentes mayoritarios, de ellos en relación con 0,1 M citronelol el control positivo, el carvacrol 0,1 M fue el mejor repelente de la termita. Además, más investigación sobre la acción repelente de termitas de carvacrol se necesita realizar.

Palabras clave: *A. jocotepecana*, *I. marginipennis*, Aceite esencial, Repelencia, Carvacrol

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INTRODUCTION

Currently, the problems of pollution and pest resistance are evident, as well as, establishment of a strict environmental regulation in relation to use of conventional pesticides. As a consequence, safe plant products are being tested around the world as alternative pest control agents, *e.g*. termite repellent compounds. *Incisitermes marginipennis* (Latreille) is a drywood termite that deteriorates pine drywood in service, is its only food source. It is considered a pest when the number of individuals increases and competes against cellulosic products utilize by humans. But, it is also a beneficial insect within the ecosystem due to its important role in soil fertilization. *I. marginipennis* is involved in the process of bio-recycling of lignocellulose and other polysaccharides (Ohkuma, 2003). By its social organization and cryptic lifestyle is difficult to control it. Therefore, it is necessary to control it with safe plant dissuasive molecules. Historically, the essential oils have been used for their natural properties, *e.g*. antimicrobial and insecticidal activities. Furthermore, essential oils have the advantage over other chemical compounds to be accepted as GRAS substances (USEPA, 1993). Termite management requires a green approach not aggressive that including botanical insecticides or plant components with deterrent effects such as repellent molecules.

On America continent one of the areas with the greatest plant biodiversity is located in the central plain between the coasts of the Pacific Ocean and the Gulf of Mexico. In the area, plants abound including those that are considered endemic. The species of the genus *Ageratina* Spach, a member of the Asteraceae family are interesting examples (Rodríguez Jiménez & Espinosa Garduño, 1995; Calderón de Rzedowski & Rzedowski, 2001). The *Ageratina* genus has increased the number of its members by some reclassifications and also by the discovery of new plant species (Villaseñor *et al*., 2004; García Sánchez *et al*., 2011). So far, there are 265 plant species of *Ageratina* genus that have been successfully accepted within this classification (The Plant List, 2015). One hundred forty three species of *Ageratina* genus have been reported on this area. Even, the first report was of *A. macvaughii* King & Rob in 70s of XX century (Turner, 1996).

It is known that *Ageratina* sp commonly grow in this area, but, even though there are reports of recollection a few species have studies about their biology, chemistry, and pharmacological relevance. Other, they are species recently discovered in the area or scarcely reported which their chemistry and biology fundamental is unknown (Calderón de Rzedowski & Rzedowski, 2001; Ramírez López *et al*., 2010). One of them is *Ageratina jocotepecana* B. L. Turner, there is limited information about it, with only three reports that include it and all of them have been made in the same area in approximately 40 years of botanical exploration. At present, the origin, geographical distribution, basic biology, and phytochemistry of *A. jocotepecana* are still unknown.

Therefore, this work represented an opportunity to put together the previously known concepts, partial knowledge of the plant metabolome, and the possibility of discover new plant species that allow us to build a phytochemical database of the genus *Ageratina*, especially, *A. jocotepecana*. The utility of the data base will be importantly diverse. It could be used as a reference for plant chemotaxonomy or even as a source in the search of plant bioactive molecules. Hence, the aim of this study was to evaluate the chemical composition and the termite repellent activity of the essential oils obtained from *A. jocotepecana*.

MATERIAL AND METHODS

Biological specimens

Ageratina jocotepecana B.L. Turner specimens were collected in Michoacán, México at the 51 Km of the Zacapu-Morelia highway (national highway 15) (N 19°42'219", O 101°35'953") at the 2234 meters above sea level (m.a.s.l.). *A. jocotepecana* was taxonomically classified by Professor B.L. Turner at the Texas University. One plant specimen is at the herbarium of the Instituto de Ecología A.C. Mexico (IE-BAJIO) in Pátzcuaro, Michoacán, Mexico with a voucher number 188459.

Colonies of pine drywood termites were collected from infested pinewood in service at Morelia, Michoacán at 1921 m.a.s.l (N 19°42'16", O 101°11'30"). The insect species were determined by Professor Amalia Ojeda Aguilera of the Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP, a Mexican government agency), in accordance with the taxonomic keys for the morphology of the soldier termite (Krishna & Weesner, 1969; Nickle & Collins, 1988). A colony of drywood termites of the species *Incisitermes* *marginipennis* Latreille (Isoptera: Kalotermitidae) were cultivated on pinewood probes (5x5x30 cm) inside a container (40x50x150 cm) under regulated environmental conditions, at temperature of 25 ºC and in the dark during a whole month for their acclimatization prior to the repellency test.

The insect and plant species utilized for this research were carefully manipulated in the laboratory following the official Mexican standards for natural resource management (Secrertaría de Desarrollo Social de México, 1994).

Equipment utilized

One µl of each essential oils was analyzed using an HP-5MS capillary column (30 m x 0.25 mm ID x 0.25 µm film thickness) (5% phenyl methylsiloxane) in an Hewlett Packard brand model 5890 Series II Plus gas chromatograph coupled to an Hewlett Packard model 5989 mass selective detector.

The injection was split (60:1) at 240 °C. Helium was used as carrier gas at a constant flow of 1.0 ml/min. Electronic flow control (EFC) was used to maintain a constant helium carrier gas flow of 1 ml per min. The GC oven temperature was held at 50 °C, and then increased by 20° C per min to 200 °C, which was maintained for 5 min. Then to 260 °C at 20 per min to with a final hold time of 5 min. The MS interface was 260 °C and the ion trap worked at 200 °C. The mass spectra were taken at 70 eV (in EI mode) with a scanning speed of 1 scan 1, from m/z 50 to 500. Data were collected and integrated using software from Hewlett Packard. The total run time for this essay was 30 min in reference of the selected method that was followed (Damián Badillo *et al*., 2008).

Essential oil analysis

Steam distillation was carried out by passing steam into a 5-liter round-bottomed flask containing the fresh plant material (195-230 g) for 180 min. The condensate (water and oil) was collected in a round-bottomed flask. The condensate was subsequently extracted three times using dichloromethane until extract was a pure essential oil mix. Then, sodium sulfate was added to the dichloromethane to remove any moisture. Finally, the dichloromethane was removed through rotary evaporation and stored in sealed vials at 4 °C in the dark until analyzed and tested. The yields of essential oils from flower, leaf and stem were 0.8, 1.3, and 0.7%, respectively. The individual identification of compounds was based upon the comparison of their retention indexes (RI) obtained by using *n*-alkanes (C6-C32) as a standard and by checking their retention times. The essential oil components were also confirmed by comparing their mass spectra with the mass spectra of the pure chemicals (Sigma Chemical Co.) and by comparison with the NIST02 library and previous literature data (Adams, 2007). Relative percentage amounts were calculated from TIC by the computer. Spathulenol was purified from aerial part (flower, leaf and stem) hexane extract by chromatographic techniques and its structure was determined by GC/MS and RMN consecutively. Carvacrol, terpineol, citronellol, terpinen-4-ol, and *β*-caryophyllene were purchased from Sigma Aldrich with 95% of minimum purity.

Repellency test

The repellent activity test was done in accordance to next procedure: The essential oils (65 µg/ml stock dissolution), 0.1M isolated compound and 0.1 M citronellol (purity ≥ 95%) a well-known insect repellent and plant metabolite. They were dissolved in ethanol and then applied on disks of paper filter No. 3 previously placed in petri dish. The outer edge of the paper disk was impregnated with 30 µL of serial concentrations (10^0 to 10^4 dilutions) of plant essential oils, isolate compounds or with a commercially bought citronellol used as positive control. As well as, with a mixture was prepared in accord to the amount of the major compound detected in the leaf extract: caravacrol, *β*-caryophyllene, spathulenol, and terpinen-4-ol; 0.19: 1.96: 0.61: 0.66 M, respectively. All disks were air dried to remove the solvent. Ten worker termites were released on impregnated paper disk in the petri dish. This system was put in isolate chamber in the dark. Drywood termites were distributed randomly in the petri dish, each one of them with a tendency to be in contact with the vertical wall of the petri dish. Perhaps, this behavior is due to the possibility that they need the “security of the tunnel” and their contact with the tunnel wall in the wooden colony. Essential oils act as an irritant obstacle between the termite and the vertical wall petri dish. The repellent activity of plant essential oils was determined by recording the number of termites in the center of the paper disc every 10 min.

Data analysis

The data were collected from three independent experiments each one with three replicates. They are expressed by mean ± standard error mean (s.e.m.) and were analyzed for statistical significance using one way analysis of variance (ANOVA) followed by Tukey multiple comparison test or Student`s t-test depending on the case. The tests were performed using Statistica 7 for Windows. The difference between groups were considered significant when $\alpha = 0.01$.

RESULTS AND DISCUSSION

Repellent effect of essential oils on drywood termite

If the essential oils act as an irritant obstacle between the termite and the vertical wall petri dish is interpreted that exhibits a repellent effect. In this work the repellent effect of the essential oils from aerial parts (the yields were: flower, leaf and stem were 0.8, 1.3, and 0.7%, respectively) of *A. jocotepecana* on *I. marginipennis* was determined, see Table 1.

All essential oils at high concentration were significantly similar in their repellent effect. However, the flower and leaf essential oils at low concentration exhibited a significantly higher repellent effect than the effect observed by stem essential oils and the well-known repellent compound the citronellol. This data reveal that essential oils from *A. jocotepecana* containing components or mixture of them with repellent effect of the drywood termite.

Table 1
Repellent effect of essential oils from *A. jocotepecana* in drywood termite *I. marginipennis*.

Dilution	**Repellency (%)**			
	Flower	**Leaf**	**Stems**	**Citronellol**
10^0	**91.3 ± 0.9Aa**	**93.3 ± 0.8Aa**	**86.0 ± 1.3Aa**	**93.3 ± 3.2Aa**
10^1	**85.7 ± 1.2Aa**	**87.0 ± 2.1Aa**	**77.3 ± 0.7Ba**	**78.3 ± 2.2Ba**
10^2	**79.3 ± 1.9Aa**	**83.0 ± 1.5Aa**	**74.7 ± 0.3Aa**	**59.5 ± 4.2Bb**
10^3	**59.0 ± 0.7Ab**	**69.3 ± 1.2Ab**	**38.0 ± 1.9Bb**	**23.4 ± 1.5Bc**
10^4	**46.0 ± 1.4Ab**	**41.3 ± 0.4Ac**	**20.3 ± 0.6Bc**	**18.9 ± 4.6Bc**

Initial concentrations of the Flower, Leaf and Stems essential oils were 65 μg/ml. Values are means ± s.e.m. Means with the same uppercase letter in the same row lowercase letters in the same column showed no significant difference, Tukey test; $\alpha = 0.01$.

Chromatographic analysis of essential oils from *A. jocotepecana*

The chemical composition of the essential oils from the fresh aerial parts of *A. jocotepecana* is in Table 2. Twenty-eight compounds were identified by GC-MS.

There were ten monoterpenes, eight sesquiterpenes, six hydrocarbons, three phenolic compounds, and a diterpene. The essential oils obtained from flower, leaf, and stems had different composition. Besides, the quantity of the compounds in each of the tissue was as follow: 16 in flower, 23 in leaf, and 7 in stems. Some of them were commonly detected into all evaluated plant tissues but in different concentrations although roots were not analyzed in this study. The major components identified in the flower were carvacrol, spathulenol, terpinen-4-ol, and β-caryophyllene. In leaf, the major compounds found were carvacrol, spathulenol, thymol, terpinen-4-ol, nerodiol, cubebene, β-caryophyllene, and fenchene.

For stem, the major components were carvacrol, spathulenol, and terpinen-4-ol. The presence of the major components of the essential oils of *A. jocotepecana* in other species of *Ageratina* spp. is variable, *e.g.* carvacrol was identified in *A. ibaguensis*; methyl carvacrol, β-caryophyllene and sptahulenol were identified in *A. adenofora* (Sanabria *et al.*, 1999; Padalia *et al.*, 2010). Recently, spathulenol and terpinen-4-ol are reported as minor components in *A. jahnii* but not identified in *A. pichinchensis* and the carvacrol and the β-caryophyllene are missing in both plant species (Torres-Barajas *et al.*, 2013). Essential oils from *Ageratina* spp. show variability in the chemical composition both intra as inter species. This chemical variability is related to ecosystem, its life history, its basic biology, methods of obtaining of essential oils and detection of the components.

Table 2

Major compounds identified in essential oils from *A. jocotepecana*.

Compound	RI	Relative content (%)		
		Flower	Leaf	Stem
***α*-fenchene**	**956**	**0.46**	**1.84**	**1.79**
***m*-cresol**	**1080***	**1.0**	**-**	**-**
Linalool	**1107***	**0.49**	**-**	**-**
***cis-β*-terpineol**	**1114**	**0.61**	**0.29**	**-**
***cis-2*-menthenol**	**1118**	**0.93**	**-**	**-**
***cis*-verbenol**	**1140***	**-**	**0.65**	**-**
(+/-)-terpinen-4-ol	**1175***	**10.11**	**2.08**	**3.36**
***cis*-piperitol**	**1192**	**0.87**	**0.21**	**-**
bornyl acetate	**1287**	**-**	**0.26**	**-**
Thymol	**1289**	**-**	**5.85**	**-**
Carvacrol	**1305***	**30.08**	**32.5**	**50.32**
(+/-)-*α*-cubebene	**1343**	**-**	**2.0**	**-**
***2,3,5,6*-tetramethyl phenol**	**1355**	**0.5**	**0.2**	**-**
(+/-)-*β*-bourbonene	**1389**	**1.52**	**0.8**	**0.89**
***β*-caryophyllene**	**1467***	**4.4**	**1.86**	**1.62**
Alloaromedrene	**1475**	**-**	**0.94**	**-**
***3,4*-diethyl phenol**	**1534***	**-**	**0.5**	**-**
Nerodiol	**1537**	**-**	**2.32**	**-**
(+/-)-*β*-caryophyllene oxide	**1597**	**1.06**	**-**	**0.47**
(+/-)-spathulenol	**1630***	**13.50**	**9.79**	**30.40**
***13*-tetradecanolide**	**1643**	**-**	**1.07**	**-**
Octadecane	**1800**	**0.16**	**0.86**	**-**
***2,6,10,14*-tetramethyl heptadecane**	**1894**	**1.15**	**-**	**-**
Eicosane	**2000**	**-**	**0.2**	**-**
Manool	**2050***	**0.16**	**0.18**	**-**
Docosane	**2200***	**-**	**0.15**	**-**
Neptacosane	**2700**	**-**	**0.24**	**-**
***n*-hentriacontane**	**3100**	**-**	**0.21**	**-**

***Spectrum compared with pure compound**

Repellent activity of carvacrol on I. marginipennis

A supposition is that the major components in essential oil determine its biological properties. It was observed that the essential oils from each plant part exhibited a notable repellent effect on *I. marginipennis*. Carvacrol, *β*-caryophyllene, spathulenol and terpinen-4-ol, were the major components detected in all plant essential oils studied. They were used in isolated form in a repellent assay (Table 3).

Table 3
Major compounds of essential oils from *A. jocotepecana* and their repellent effect against drywood termite *I. marginipennis*.

Dilution	***Repellency (%)**					
	Carvacrol 0.1 M	***β*-caryophyllene 0.1 M**	**Spathulenol 0.1 M**	**Terpinen-4-ol 0.1 M**	**Mixture**	**Citronellol 0.1 M**
10^0	**87 ± 2.6 A**	**16 ± 4.5 B**	**10 ± 2.9 B**	**31 ± 11.1 B**	**84.0 ± 4.5 A**	**95 ± 1.3 A**
10^1	**57 ± 8.3 B**	**3 ± 1.6 C**	**11 ± 3.3 C**	**9 ± 5.5 C**	**84 ± 3.1 A**	**91 ± 0.9 A**
10^2	**33 ± 5.6 B**	**2 ± 1.3C**	**0 ± 0 C**	**4 ± 2.9 C**	**44± 3.8 B**	**80 ± 1.6 A**

***Values are means ± s.e.m. Means with the same letter in the same row had no significant differences. Tukey test; α = 0.01**

Carvacrol, *β*-caryophyllene, spathulenol, terpinen-4-ol, and thymol had insecticidal and repellent activities (Bakkali *et al*., 2008). Carvacrol and thymol exhibited insecticidal activity against the insect *Sithophilus granarius* L. on stored wheat (Rozman *et al*., 2007). It has been reported that *β*-caryophyllene acts as a larvicidal toward vector mosquito *Aedes aegyptii* (L) (Albuquerque *et al*., 2004). Terpinen-4-ol has insecticidal effect towards the adult insect *Sithophilus zeamais* (Jirovetz *et al*., 2005), while *β*-bourbonene, nerodiol, cubebene, and fenchene are associated with antioxidant, microbial, and antiprotozoal activities; they are also used as flavor ingredients in cosmetic and food products (Machado *et al*., 2010).

It is important to mention that the minor concentration components of essential oils may contribute to the termite repellency since some of them also exhibit insecticidal and repellent activities. It is possible that they contribute to enhance the repellent effect of flower and leaf essential oils at low concentration. But, in many cases, the mixture of compounds found in essential oils at different concentration ratios may have a synergistic or antagonistic effect (Miyazawa *et al*., 2001; Orhan *et al*., 2008). For instance, linalool exhibited strong fumigant activity on *S. zeamais* (Perez *et al*., 2010; Wang *et al*., 2011). Also, terpineol mixed with eugenol and cinnamic acid (3 blends) act as neuro-insecticide against carpenter ants *Camponotus pennsylvanicus* De Geer and German cockroaches *Blatella germanica* (Enan, 2001).

Interestingly, carvacrol exhibited a strong repellent effect on drywood termite and was at a lower concentration than the other three components assayed into mixture. Carvacrol is known as an acetylcholinesterase inhibitor which causes an overstimulation of neurons in insects such as mosquito *Aedes aegypti*, the tick *Dermacentor variabilis*, and the cockroach *Periplaneta americana* (López *et al*., 2010; Anderson & Coats, 2012). In the drywood termite, carvacrol might have more than one mechanism of action. One of them may be the same as described for organophosphate and carbamate insecticides (Bisset *et al.*, 2009). Another may be that the carvacrol is acting as a positive allosteric modulator of termite GABA receptors, such as, the effect observed of carvacrol in arthropods where it enhanced the binding of the radiotracer [^{3}H]-*t*-butyl bicycle-*o*-benzoate ([^{3}H]-TBOB) to membrane preparation of house fly head. Also, carvacrol significantly increased the $^{36}Cl^-$ uptake induced by GABA in membrane micro sacs prepared from American cockroach ventral nerve cords (Tong & Coats, 2010). Another possibility is the binding of the carvacrol to drywood termite octopamine receptor such in the case of American cockroach octopamine receptor (Enan, 2001). Finally, there is no way to know if carvacrol turns any of these mechanisms involved in the irritability, escape and survival responses of drywood termite *I. marginipennis*. This brings forward a significant number of unanswered questions that should be focus in future research.

CONCLUSIONS

The results presented in this study contribute to increase and improve the previous knowledge of the *Ageratina* genus. The results also show, for the first time, the essential oil composition of *A. jocotepecana* and its repellent effect against drywood termites *I. marginipennis*. All three essential oils from the aerial parts (flower, leaf, and stems) of *A. jocotepecana* have some significant potential as drywood termite repellents and should be considered in future testing. Furthermore, carvacrol represents a potential agent to be used to protect the pinewood due to its repellent effect against drywood termite *I. marginipennis*. Finally, in spite of all this new information, further research about the termite repellent action of carvacrol is still needed.

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