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Chemical composition and bioactivity of *Piper auritum* and *P. multiplinervium* essential oils against the red flour beetle, *Tribolium castaneum* (Herbst)

[Composición química y bioactividad de los aceites esenciales de *Piper auritum* y *P. multiplinervium* contra el escarabajo rojo de la harina, *Tribolium castaneum* (Herbst)]

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Abstract
Stored grain insects have shown resistance to synthetic insecticides, fact that has promoted the use of vegetable species for integrated pest management. *Piper auritum* and *P. multiplinervium* are two plants from the Piperaceae family present in the department of Chocó, Colombia, one of the most important hot spots of biodiversity in the world. This study was conducted to determine the repellent activity and toxicity of essential oils (EOs) isolated from these plants against *Tribolium castaneum*, using the area preference and contact toxicity methods, respectively. *P. auritum* EO presented greater repellency than *P. multiplinervium*, the first showed 100% lethality at minimum tested exposure period (24 h) whereas the second reached 16% at 72 h. EOs were analyzed by gas chromatography-mass spectrometry. *P. auritum* major components were safrole (93.2%) and miristicine (4.3%), whereas for *P. multiplinervium* were β-elemene (9.0%), trans-β-caryophyllene (5.3%) and caryophyllene oxide (4.1%). It is speculated that the repellent effect of *P. auritum* may be related to its safrole content, a known repellent. These results evidenced Piper species could be used for development of repellents against *T. castaneum*.

Keywords: Aromatic plants, repellent, toxicity, insects, bioactivity.

Resumen
Los insectos de los granos almacenados han mostrado resistencia a los insecticidas sintéticos, hecho que ha promovido el uso de especies vegetales para el manejo integrado de plagas. *Piper auritum* y *P. multiplinervium* son dos plantas de la familia Piperaceae presentes en el departamento del Chocó, Colombia, uno de los puntos de biodiversidad más importantes del mundo. En este estudio fue determinada la actividad repelente y toxicidad de los aceites esenciales (AE) aislados de estas plantas contra *Tribolium castaneum*, utilizando el método de área de preferencia y toxicidad por contacto, respectivamente. El AE de *P. auritum* presentó mayor repelencia que el de *P. multiplinervium*, el primero mostró 100% de letalidad al menor tiempo de exposición (24 h), mientras que el segundo alcanzó el 16% a las 72 h. Los AEs fueron analizados por cromatografía de gases-espectrometría de masas. Los componentes principales de *P. auritum* fueron safrol (93.2%) y miristicina (4.3%), mientras que para *P. multiplinervium* fueron β-elemene (9.0%), trans-β-cariofileno (5.3%) y óxido de cariofileno (4.1%). Se cree que el efecto repelente de *P. auritum* puede estar relacionado con su contenido de safrol, un repelente conocido. Estos resultados evidencian que las especies de Piper podrían ser utilizadas para el desarrollo de repelentes contra *T. castaneum*.

Palabras Clave: Plantas aromáticas, repelentes, toxicidad, insectos, bioactividad.

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INTRODUCTION

In a world where the use of synthetic chemicals is common in various areas of human life, and concern for sustainable product development has increased, natural resources emerge as a source of raw material solution for effective and environmentally friendly products. Nowadays, a number of synthetic insecticides and fumigants are used to protect grain storage. Infestation of storage grains is a very serious problem as various life stages of insects cause economic damage and deteriorates the quality of food grains and food products. These pests often cause extensive loss of products stored in tropical and semitropical environments (Isman, 2000). Several species of insect pests are known for attacking granaries and other food products since ancestral times. *Tribolium castaneum* is major pest food products (Mishra and Tripathi, 2011) the adult stage of *T. castaneum* is very active and can breed throughout the year in warm areas (Pugazhvendon *et al.,* 2009). They live two years or more, during which female produce nearly 1000 eggs (Shukla *et al.,* 2010). However, the use of synthetic pesticide for the management of these insect pests cause great hazards for environmental and toxic to non target livings (Mishra and Tripathi, 2011).

Over the years, pesticide use has led to a variety of problems such as environmental pollution, chemical residues in food grains, development of insecticide resistance and toxicity to non target organisms (Cosimi *et al.,* 2009; Sousa *et al.,* 2009). The increasing public awareness about pesticide safety and possible damage to the environment has resulted in greater attention being given to natural products for the control of food stored pests (Rajendran and Sriranjini, 2008). Among the group of natural molecules used to control insects, essential oils (EOs) area being considered as outstanding solutions for their effectiveness and versatility. In fact, volatility and chemical diversity makes them excellent repellents. The use EOs derived from aromatic plants as low-risk insecticides has increased considerably owing to their popularity with organic farmers and environmentally conscious consumers (Regnault-Roger *et al.,* 2012).

EOs have repellent, insecticidal, and growth-reducing effects on a variety of insects (Rajendran and Sriranjini, 2008; Ukeh *et al.,* 2009; Mondal and Khalequzzaman, 2010).

The demand for naturally active compounds such as EOs has move forward the necessity to search these chemicals in hot spots for biodiversity. Colombia is a country with approximately 45.000 plant species, and it is among the first countries throughout Latin America and the world in terms of biodiversity heritage. However, little is known about its richened and the enormous possibilities for innovation and sustainable development from this natural resource (Stashenko *et al.,* 2010). At the same time, as people depend on synthetic repellents to control pests of stored products, EOs are exported without any added value, reducing the income for many families, and the ability to create jobs through the manufacture of products derived from these natural resources.

The bioactivity of a large number of essential oils and their constituents have been evaluated against *T. castaneum* (Jahromi *et al.,* 2012; Amin *et al.,* 2012; Zandi-Sohani *et al*., 2013; Liang *et al*., 2013). In Colombia, EOs from *Cananga odorata*, *Lippia alba, L. orignanoides, Cymbopogon citratus, C. martinii*, *C. flexuosus, Eucalyptus citriodora,* and *Citrus sinensis* have been tested as repellents using this insect (Olivero-Verbel *et al*., 2009, 2010; Caballero-Gallardo *et al*., 2011, 2012). However, species from the Piperaceae family have not been studied. In this context, the aim of this paper was to evaluate the repellent and insecticidal properties of EOs isolated from *Piper auritum* and *P. multiplinervium* cultivated in Chocó, Colombia against *T. castaneum* Herbst, one of the most far-flung and destructive stored-product pests throughout the world (Zapata and Smagghe, 2010).

MATERIALS AND METHODS

Plant Material

Two plant species of the genus Piper were used in the assays, *P. auritum* and *P. multiplinervium* (Figure 1). Plant material was collected from Chocó, Colombia, at the municipalities of Quibdó and Lloró, respectively). The taxonomic identification of the plants was carried out in the Colombian National Herbarium (COL) of the Institute of Natural Sciences, Faculty of Sciences of the National University of Colombia (Bogotá) and stored with voucher specimens COL No. 512209 and COL No. 519977 for *Piper auritum* and *P. multiplinervium*, respectively.

Figure 1

Studied species from Chocó, Colombia.

Extraction of essential oils

Essential oils were extracted from fresh leaves by microwave-assisted hydrodistillation (MWHD) as previously described by Stashenko *et al*. (2004). The process used a Clevenger type hydrodistillation equipment placed inside a domestic microwave LG microwave MS- 1242 ZK model, set at 2450 MHz, 800 W. The extraction was conducted in 10 intervals; 10 min for each one with 5 min rest time each one. The oil was dried using anhydrous sodium sulphate.

Gas chromatography–mass spectrometry (GC–MS)

An aliquot of each essential oil (20 µL) was dissolved in dichloromethane to a final volume of 1 mL, and then transferred to vials for GC–MS. The chromatographic analysis was performed using an Agilent Technologies 6890 Plus (Palo Alto, CA) GC coupled to an Agilent Technologies MSD 5975 selective detector mass equipped with a split/splitless injector port (1:50 split ratio), an automatic injector Agilent 7863, and a data system HP ChemStation. One microliter of solvent was injected into the GC–MS equipment for the corresponding chromatographic analysis. A 60m×0.25mm i.d.×0.25µm with 5% phenyl poly(methylsiloxane) stationary phase. The oven temperature was set at 45 °C for 5 min, then increased 4 °C/min up to 150°C for 2 min, then to 5 °C/min up to 250 °C for 5 min, and finally at 10°C/min up to 275°C. Helium was used as a carrier gas with 16.47 psi column head pressure and 1 mL/min linear velocity. The components identification were based on Kováts indices (Ik) and by comparison of the mass spectra fragmentation patterns with those found in databases or libraries (NIST02, Adams, Wiley7n) (Adam, 2004).

Insects and rearing conditions

All experiments were conducted in the laboratory using long-established colonies of *T. castaneum.* The strain was kept in glass containers covered with a plastic mesh. Insects were reared on a diet of whole oat flour and kept at 26 ± 2 °C, with a relative humidity of 70 - 85%, and a 10:14 h light:dark photoperiod. Red flour beetle adults of both sexes were collected, and those with size between 3.0 and 4.5 mm, and 7-10 days old, were employed in the experiments (Caballero-Gallardo *et al.*, 2011).

Repellency tests

The repellent activity was measured using the area preference method (Tapondjou *et al.*, 2005; Olivero *et al.*, 2009; Olivero-Verbel *et al.*, 2010; Caballero-Gallardo *et al.*, 2011; Lü and Shi, 2012). The solutions of EOs were prepared in acetone, and in all cases, a volume of 0.5 mL was evenly applied to a half-filter paper disk to obtain the desired oil volume per area unit of 0.00002, 0.0002, 0.002, 0.02, and 0.2 µL/cm^2. The other half of the filter paper was treated with an equal volume of acetone as a vehicle control. Test areas consisted of 9 cm Albet DP 597125 filter paper cut in half (31.8 cm^2). A formulation of IR3535 [ethyl 3-(N-acetyl-N-butylamino) propionate], a synthetic

repellent (Licciardi *et al.*, 2006; Faulde *et al.*, 2010) which has been approved in the USA for skin applications (World Health Organization, 2001), was employed as a positive control, utilizing the same experimental conditions as the oils. The treated and control half disks were air-dried for 10 min to remove the solvent, re-attached with adhesive tape, and kept in 90 mm glass Petri dishes. Twenty adults of *T. castaneum* of both sexes were released at the center of each filter paper disk. Dishes were covered and placed in darkness at 26 ± 2 °C and relative humidity of 70–85%. The numbers of *T. castaneum* specimens on treated and untreated portions of the experimental paper halves were counted for each dish after 2 and 4 h exposure. Percentage repellency (PR) for a given treatment time was obtained using the formula:

$$PR = [(Nc-Nt)/(Nc+Nt)] \times 100$$

Where Nc and Nt are the number of insects on the untreated (control) and treated areas, respectively. Three replicates were used for each tested concentration of EO, and each assay was repeated twice.

Contact toxicity on filter papers

The contact toxicity on filter papers was conducted using filter paper discs (Albet DP 597125, 9 cm diameter) (Tapondjou *et al.*, 2005). Oils were dissolved in acetone at concentrations of 0.2, 0.5, 0.9 and 1.2 μL/cm^2, and 1 mL of each solution was uniformly distributed on the surface of the paper that was then placed in glass Petri dishes. After 10 min, once the solvent had been evaporated, 20 unsexed adults were transferred into each disc and stored in darkness at 26 ± 2 °C and 75 ± 10 % RH (Olivero *et al.*, 2009; Olivero-Verbel *et al.*, 2010; Caballero-Gallardo *et al.*, 2011). Three replicates were employed for each treatment, repeating each assay twice. Mortality was recorded after 24, 48 and 72 h. IR3535 was utilized as a positive control. Insects were considered dead when no leg or antennal movements were recorded.

Data analysis

The paired t-test was utilized to compare mean number of insects on the treated and untreated area of the filter paper. Repellency or attractancy was established if significant differences occurred for positive or negative percentage repellency, respectively. Normal distribution and equality between variances were checked by Kolmogorov-Smirnov and Bartlett's tests, respectively. Comparisons between mean PRs for evaluated EOs were obtained using ANOVA, with Dunn's post-test used to compare treated with control-vehicle groups. Statistical analysis was performed with GraphPad 3.00. The mean repellence concentration (RC_{50}) values were calculated using probit analysis (Finney, 1971). For all purposes, significance was set at $P < 0.05$.

RESULTS

The results of repellency assays for tested EOs are presented in Table 1. Data showed that at tested concentrations, both EOs were strongly repellent against *T. castaneum.* At the lowest assayed concentration (0.00002 μL/cm^2), the EOs isolated from *P. auritum* and *P. multiplinervium* showed attractant activity at both exposure times (PR: -35 ± 9%, -30 ± 17% and -5 ± 10%, -8 ± 13%, respectively). The commercial repellent IR3535 was less effective than the two tested EOs, when the organisms were exposed to 0.02 and 0.2 μL/cm^2.

Significant differences were found for the PR between the essential oil of *P. auritum* and the commercial repellent (IR3535) at higher concentrations (0.02 and 0.2 μL/cm^2). However, for the essential oil isolated from *P. multiplinervium*, no such differences were recorded.

RC_{50} values are shown in Table 2. Repellent action was highly dependent upon oil concentration and exposure time. *P. auritum* essential oil was the most active with a RC_{50} value of 0.002 μL/cm^2.

The results for the contact lethality on filter paper for the examined EOs are shown in Table 3.

The EO from *P. auritum* had greater toxicity than that from *P. multiplinervium.* After 24 h exposure, the first EO caused 81±2% mortality at the lowest tested concentration (0.2 μL/cm^2), but at 0.9 μL/cm^2 196 this was 100%. However, for *P. multiplinervium*, the maximum tested concentration (1.2 μL/cm^2) and exposure time (72 h) only produced 16±4% lethality.

The results obtained to repellent and mortality of essential oils against *T. castaneum* were analyzed using Student's t-test. In the case of the repellent activity were no significant differences were observed between the two essential oils, whereas significant differences were observed to essential oil *P. auritum* (Table 3).

The IR3535 presented very little effect on the survival of *T. castaneum* adults. Chemical composition of EO isolated from the two *Piper* species, as resulted from GC-MS analysis, is presented in Table 4. The

results revealed quite different compositions between the two species. The essential oil of *P. auritum* was characterized by high phenylpropanoid content (97.5%), in which safrole (93.2%) was found to be the major constituent and miristicine the second (4.3%). Among monoterpene hydrocarbons, γ-terpinene was found at low concentration (0.3%). The *P. multiplinervium* oil presented a high content of sesquiterpene hydrocarbons (67%) with β-elemene (8.7%), trans-β-caryophyllene (5.3%) α-selinene (3.8%) and β-selinene (3.4%) as major components, as well as oxygenated sesquiterpenes (7.1%), with caryophyllene oxide (4.1%) and trans-nerolidol (3.0%) as representative compounds.

DISCUSSION

Different plant products, especially EOs, have considerable potential as insecticides and repellents, and they are gaining tremendous importance for the management of stored products, as these mixtures are considered biodegradable and ecologically safe. The red flour beetle, *T. castaneum*, is one of the most widespread and pernicious destructive stored-product pests throughout the world (Zapata et al., 2010; El-Sayed and Genan, 2012). Results presented here have shown that essential oils obtained from Piper auritum and *P. multiplinervium* possess good bioactivity toward this insect. In addition, these oils have similar or better repellent properties than the commercial repellent used as a positive control (IR3535).

Properties of EOs are directly related to their chemical composition (Ben Marzoug et al., 2011), that in turn, are a function of the plant part extracted, time of collection and growth environment conditions, among others (Zapata and Smagghe, 2010). The number and concentration of chemicals present in an EO may vary dramatically, even within the same species (Zapata and Smagghe, 2010). In this study, *P. multiplinervium* and *P. auritum* had different biological activities, reflecting their distinct chemical nature. *P. multiplinervium* does not contain a major component representing at least 50% of the oil composition, whereas *P. auritum* is mainly composed by safrole (93.2%). This chemically based difference may explain the acute and low-dose toxicity observed for *P. auritum* (100% mortality after 24 h exposure, and RC_{50}= 0.002 μL/cm^2).

The bioactivity of the EOs results from interaction among structural components, particularly the major constituents, although, the other compounds in the oil may also have a vital function (Intirach *et al*., 2012). In this study the principal compound of *P. auritum* was safrole (93.2%), a methylenedioxy compound (Maul *et al*., 2011), reported to have toxic properties derived from its structure as benzene derivative (Tripathi *et al*., 2009). The metabolism of this naturally occurring chemical leads to the formation of safrole 2',3'-oxide, an electrophilic compound that produces DNA adducts in vitro and in vivo (Shen *et al*., 2012), probably explaining its reported ability to induce liver tumors in mice and rats (Rietjens *et al*., 2005). Several studies have been published about safrole being the main component in several Piper species (Abreu *et al*., 2002; Protti *et al*., 2005; Sauter *et al*., 2012; Souto *et al*., 2012), and used alone, it has been reported to be effective against *T. castaneum* adults (Huang *et al*., 1999).

This work has clearly revealed that *Piper* chemotypes can vary considerably in their composition and biological properties; and therefore, their presence in Colombian flora is an important potential for promissory bioactive molecules, in particular as repellents, as it has been also demonstrated for *P. aduncum*, a species from the Brazilian biodiversity that has been shown to work as a repellent against the ant *Solenopsis saevissima* (Smith) (Hymenoptera: Formicidae) (Souto *et al*., 2012).

In short, reported data revealed, for the first time, the repellent and insecticidal activity of essential oils extracted from *P. auritum* and *P. multiplinervium* grown in the department of Chocó, Colombia, and this property could be related to the presence of some particular compounds.

Table 1

Essential oils	Time									
	2 Hours Concentration (µL/cm^2)					4 Hours Concentration (µL/cm^2)				
	0.00002	0.0002	0.002	0.02	0.2	0.00002	0.0002	0.002	0.02	0.2
P. auritum	-35 ± 9[ab]	30 ± 7[a]	32 ± 11[a]	82 ± 3[ab]	90 ± 4[ab]	-30 ± 17	38 ± 10[a]	45 ± 9[a]	55 ± 12[a]	87 ± 3[a]
P. multiplinervium	-5 ± 10	7 ± 13	42 ± 11[a]	70 ± 8[a]	82 ± 6[a]	-8 ± 13	10 ± 16	45 ± 11[a]	70 ± 9[a]	80 ± 4[a]
IR3535[c]	7 ± 13	17 ± 9	37 ± 15	50 ± 4[a]	72 ± 4[a]	5 ± 14	12 ± 8	42 ± 15[a]	50 ± 7[a]	72 ± 6[a]

Percentage Repellency[1] (PR) after two exposure times for two essential oils against *T. castaneum*

[1]Values are mean (SE of six replicates).

[a]Significant difference between the number of the organisms on both the treated and untreated halves, using a paired t test (*P* < 0.05).

[b]Significant difference between essential oils and the positive control (IR3535), using ANOVA, with Dunn's post-test.

[c]. Commercial repellent.

Table 2

RC_{50} (95% Confidence intervals) values of the essential oils tested against *T. castaneum*

Essential oils	Exposure time (h)	RC_{50} (µL/cm^2)	Regression parameters*	
			R	*P*
P. auritum	2	0.002 (0.001-0.005)	0.940	0.0602
	4	0.002 (0.001-0.008)	0.920	0.0798
P. multiplinervium	2	0.008 (0.004-0.016)	0.967	0.0334
	4	0.007 (0.003-0.015)	0.966	0.0336
IR3535[a]	2	0.015 (0.005-0.048)	0.997	0.0002
	4	0.016 (0.006-0.045)	0.983	0.0027

***. From probit analysis.**

[a]. Commercial repellent.

Table 3

Essential oils	Concentration (µL/cm^2)	Mortality (%) 24 h	48 h	72 h
Piper auritum	0	0 ± 0	0 ± 0	0 ± 0
	0.2	81 ± 2*	90 ± 4*	96 ± 2*
	0.5	92 ± 2*	98 ± 1*	99 ± 1*
	0.9	100 ± 0*	100 ± 0*	100 ± 0*
	1.2	100 ± 0*	100 ± 0*	100 ± 0*
Piper multiplinervium	0	0 ± 0	0 ± 0	0 ± 0
	0.2	0 ± 0	3 ± 2	9 ± 4
	0.5	4 ± 2	7 ± 2	12 ± 1
	0.9	5 ± 2	8 ± 2	15 ± 5
	1.2	6 ± 2	10 ± 3	16 ± 4
IR3535*	0	0 ± 0	0 ± 0	0 ± 0
	0.2	0 ± 0	0 ± 0	2 ± 1
	0.5	0 ± 0	0 ± 0	3 ± 1
	0.9	0 ± 0	1 ± 1	3 ± 1
	1.2	1 ± 1	2 ± 1	4 ± 2

Mortality rates in *T. castaneum* exposed to EOs and IR3535
***. Significant difference between the two EOs, using Student's t-test ($P < 0.05$).**
[a]. Commercial repellent.

Table 4

KIDB5[a]	Compound	Chemical Class	*Relative Composition (%)* *Piper auritum*	*Piper multiplinervium*
1065	γ–terpinene	Monoterpene hydrocarbons	0.3	
1290	Safrole	Phenylpropanoids	93.2	
1520	Miristicine		4.3	
1398	β-elemene	Sesquiterpene hydrocarbons		8.7
1420	trans-β-caryophyllene			5.3
1490	β- selinene			3.4
1497	α- selinene			3.8
1566	trans-nerolidol	Oxygenated sesquiterpenes		3.0
1584	Caryophyllene oxide			4.1

Chemical composition (%) of tested essential oils
[a]Kovats index on DB-5 column

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