CHEMICAL COMPOSITION AND FUMIGANT EFFECT OF ESSENTIAL OIL OF *Lippia sidoides* Cham. AND MONOTERPENES AGAINST *Tenebrio molitor* (L.) (COLEOPTERA: TENEBRIONIDAE)

Composição química e efeito fumigante do óleo essencial de *Lippia sidoides* Cham. e monoterpenos sobre *Tenebrio molitor* (L.) (Coleoptera: Tenebrionidae)

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ABSTRACT

The chemical composition of *Lippia sidoides* essential oils obtained by hydrodistillation was characterized and quantified by GC/MS and their insecticidal activity by fumigation test was assayed against *Tenebrio molitor*. Moreover, the toxicity of monoterpenes carvacrol, 1,8-cineol and thymol were also evaluated when applied alone or in binary (1:1) or tertiary (1:1:1) mixture. The essential oil of *L. sidoides* has as major constituents carvacrol (31.68%), ρ-cymene (19.58%), 1,8-cineole (9.26%) and γ-terpinene (9.21%), from a 21 compounds identified, being 92.53% of total. Both compounds have insecticidal activity against *T. molitor*, being the degree of toxicity of carvacrol > 1,8-cineole > *L. sidoides* essential oil > thymol, and its respectively LC_{50} at 24h were 5.53; 5.71; 8.04 and 14.71 µL/L air. When the different mixture of carvacrol, 1,8-cineole and thymol was assayed against *T. molitor*, the synergism among them was observed. For the mixture of carvacrol:1,8-cineole LC_{50} was 5.34 µL/L air; carvacrol:thymol 7.67 µL/L air; 1,8-cineole:thymol 7.51 µL/L air and carvacrol:1,8-cineole:thymol 6.34 µL/L air. Mainly, the monoterpene thymol had a synergic effect, which increased the toxicity of carvacrol and 1,8-cineole, both in binary mixture like carvacrol:thymol and 1,8-cineole:thymol.

Index terms: “Alecrim pepper”, mealworm, natural compound, toxicity, synergism.

RESUMO

A composição química do óleo essencial de *Lippia sidoides* obtido por hidrodestilação foi caracterizada e quantificada por GC/MS, bem como sua atividade inseticida por teste de fumigação foi avaliada sobre *Tenebrio molitor*. Além disso, a toxicidade dos monoterpenos carvacrol, 1,8-cineol e timol, também foi avaliada quando esses compostos foram aplicados isoladamente, ou em misturas binárias (1:1), ou terciárias (1:1:1). O óleo essencial de *L. sidoides* tem como principais constituintes o carvacrol (31,68%), ρ-cimeno (19,58%), 1,8-cineol (9,26%) e γ-terpineno (9,21%), em 21 compostos identificados, sendo 92,53% do total. Ambos os compostos possuem atividade inseticida contra *T. molitor*, seguindo a ordem de toxicidade: carvacrol > 1,8-cineol > óleo essencial de *L. sidoides* > timol; sendo suas respectivas CL_{50} à 24 h de 5,53; 5,71; 8,04 e 14,71 µL/L ar. Quando as diferentes misturas de carvacrol, 1,8-cineol e timol foram avaliadas contra *T. molitor*, verificou-se o efeito sinérgico. Para a mistura de carvacrol:1,8-cineol a CL_{50} foi de 5,34 µL/L ar; carvacrol:timol de 7,67 µL/L ar; 1,8-cineol:timol de 7,51 µL/L ar e carvacrol:1,8-cineol:timol de 6,34 µL/L ar. Principalmente o monoterpeno timol teve efeito sinérgico, aumentando a toxicidade de carvacrol e 1,8-cineol quando em misturas binárias, tais como carvacrol:timol e 1,8-cineol:timol.

Termos para indexação: Alecrim pimenta, bicho-da-farinha, produto natural, toxicidade, sinergismo.

(Received in february 2, 2011 and approved in march 1, 2011)

INTRODUCTION

During the storage of food, several problems can occur due the presence of insects such as the mealworm *Tenebrio molitor* (L.) (Coleoptera:Tenebrionidae). Their presence in stored grains and bran (i.e. corn, oat and wheat), can contaminate those food with fragments of his body, feces and indirectly by saprophytic microorganism, resulting in loss of quality (GARCIA et al., 2003; PINTO JUNIOR, 2008). Control of these insects can be achieved by methyl bromide and phosphine treatment; however, several issues have been discussed in the employment of insecticides, such as residue, environment impact and toxicity to humans (GHINI et al., 2002; LEE et al., 2004).

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Therefore, one has sought for alternative defensives than showed a low impact in environment, and safe for human health, for example, natural extracts and secondary compounds derived from plants, which may be an important source of study in the pest control and simultaneously acting on the Integrated Pest Management (ISMAN, 2000). In this context, the species *Lippia sidoides* Cham. (Verbenaceae) which is popularly known as "alecrim" pepper, is a widespread plant in the northeast Brazil and showed a series of biological activities (CARTAXO et al., 2010). Their leaves have been used in alternative medicine to prevent acne, wounds, skin and scalp infections; and infusions are used for the treatment of rhinitis, vaginal diseases, mouth and throat infections (MENDES et al., 2010).

The essential oil of this species is rich in thymol and carvacrol, compounds that confer antimicrobial activity against fungi and bacteria, and they are also toxic to insects and spider mite (CAVALCANTI et al., 2004, 2010). According to Matos (2002), its leaves can present up to 4.5% of essential oils, with compounds such as thymol, carvacrol, 1,8-cineole, ρ-cymene, 4-terpineol, thymol methyl ether, trans-cariophyllene. The phytochemical extracts of leaves resulted in isolation of several other constituents, as oleanolic acid acetate, methyl-3,4-dihydroxibenzoate, lapachenol, tecomaquinone I, tectoquinone, tectol, acetylated tectol, quercetine, luteoline, glucoluteoline, lipisidoquinone, taxifolin and isolariciresinol (COSTA et al., 2002).

In this perspective, recent studies have reported a series of biological properties of essential oil, such as insecticidal activity on larvae of *Aedes aegypti* L. (CAVALCANTI et al., 2004); acaricidal activity against *Tetranychus urticae* (CAVALCANTI et al., 2010) bactericidal effect against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* (BERTINI et al., 2005; OLIVEIRA et al., 2006); *Streptococcus* spp. and *Candida albicans* (BOTELHO et al., 2007). Essential oils as well as several terpenes have been utilized in agriculture against fungi, bacteria, nematodes and pest insects. For example, the compounds thymol and 1,8-cineole are already part of some commercial formulations (REGNAULT-ROGER, 1997; ISMAN, 2006).

Due the importance to know new strategies and alternatives for insect control, and the need in find new compounds with biological activity, the aim of this research was assess the chemical composition of essential oil of *L. sidoides* as well as their toxicity against the mealworm *T. molitor*. Moreover, the toxicity of carvacrol, 1,8-cineol and thymol was assayed alone or in mixture against this mealworm.

MATERIAL AND METHODS

Essential oil extraction

The fresh leaves of *Lippia sidoides Cham*. (Verbenaceae) were collected between the months of January and March over the morning period (between 8 and 9 h) at the Medicinal Plants Garden, Federal University of Lavras (21° 14' S; 45° 00' W, 919 m of altitude and annual average temperature of 26° C). One vegetable sample was sent, processed and identified in the Biology Department of the Federal University of Lavras, being the voucher specimen deposited in the ESAL Herbarium (# 01943). The sample employed in essential oil extraction was transported in identified plastic bag to the laboratory just after collection and then performed the hydrodistillation process using a modified Clevenger apparatus, with 300 g of leaves in 1 L of water during 2.5 hours. The hydrolate collected (aqueous and organic phases) were separated by centrifugation at 900 *g* for 5 minutes and the essential oil obtained was weighted, kept in amber flask wrapped with aluminum foil and stored under refrigeration at 4° C until their use.

Identification and quantification of constituents

The compounds were identified using a Shimadzu gas chromatography/mass spectrometry (GC-17A), with a selective mass detector (QP 5000). The equipment was operated with a fused silica capillary column with bonded phase (model DB5, 30 m x 0.25 mm) and helium as the carrier gas (1 mL/min). The temperatures were of 220° C in the injector and 240° C in the detector. The oven temperature was scheduled for 40 to 240° C, with additions of 3° C every minute. The compounds identification was performed by comparison the mass spectra with those in spectra library (Wiley 229) and the Kovats retention index (ADAMS, 2007). The quantification of constituents was performed in Shimadzu gas chromatograph (GC-17A) equipped with flame ionization detector and a fused silica capillary column with bonded phase (DB5, 30 m x 0.25 mm). The carrier was nitrogen at the rate 2.2 mL/min; the split rate 1:10 and injected volume of 1 µL. Initial temperature of the column was scheduled for 45 to 240° C, with increases of 3° C each minute. Temperatures in injector and detector were set at 220 and 240° C, respectively, and the column pressure at 115 KPa.

Toxicological assay

The fumigant effect of essential oil of *L. sidoides* and the monoterpenes carvacrol, 1,8-cineole and thymol (analytical grade, from Sigma Adrich, Milan, Italy) were carried out with unsexed adult of *T. molitor*, aged from two to five days. This insect was rearing with wheat/oat/corn bran (1:1:1), and maintained in wooden box (70 cm x 40 cm x 20 cm) in darkness, at 25±2°C and RH of 60±10%.

The first step of dose-response assays were performed with various concentrations (v/v) of the compounds administered alone. At the second step, to evaluate the synergism among few compounds from the essential oil, different mixture of monoterpenes carvacrol, 1,8-cineole and thymol were prepared in the same proportion in binary mixture (1:1): carvacrol:1,8-cineole, carvacrol:thymol; and 1,8-cineole:thymol, as well as the tertiary mixture (1:1:1) carvacrol:1,8-cineole:thymol. All solutions were prepared with acetone.

For the intoxication, 500 µL of each solution was applied in 2 cm^2 filter paper that was fixed into glass flask (180 mL). At the same time, each flask received six *T. molitor* adults and was immediately closed with plastic lid. For feeding insects, we used 200 mg of the same diet describe above (SAHAF et al., 2007). During the assay, all glass flasks were maintained at the same climatic conditions where the insect were reared, and the number of dead beetle was recorded at 24 and 48h.

Data analysis

The data of dead insect recorded at 24 and 48 hours after the exposure to the different treatment, was submitted to a statistical analysis using the log-logistic model available in the "drc" package (RITZ; STREIBIG, 2005) and compiled by the statistical software R® (R DEVELOPMENT CORE TEAM, 2010). The lethal concentration which causes death of 50 and 90% (LC_{50} and LC_{90}) of insects were determined (given in µL of compound/L air), as well as their confidence limits at 95%.

To determine the effect of different mixture among carvacrol, 1,8-cineole and thymol, which can have a synergistic, antagonistic or neutral effect, we adopt the model of Metcalf (1967) to estimate the synergistic ratio (SR), but with modification, as follows:

$$SR= \frac{\text{average of } LC_{50} \text{ of each compound alone}}{LC_{50} \text{ of their mixture}}$$

Following this model when the SR value is 1, the toxicity of a mixture and their compounds isolated was equal; for values exceeding 1, synergism between compounds occurs; and for lower values than 1, the mixture of compounds showed an antagonism.

RESULTS AND DISCUSSION

Chemical constitution of essential oil

All compounds found in the essential oil of *L. sidoides*, its contents and Kovats index are shown in Table 1. The hydrodistillation process used to prepare the essential oil of *L. sidoides* resulted in a transparent and slightly yellowish oil, with yield of 1.8% (w/w). The total of 21 compounds was identified, which correspond to 92.53%. Only 7.47% of compounds were not identified by GC-MS process. The major constituents of this oil were carvacrol (31.68%), ρ-cymene (19.58%), 1,8-cineole (9.26%), γ-terpinene (9.21%) and sabinene (5.26%). In minor quantities, we found thymol-methyl-ether (2.92%), thymol (2.30%) and myrcene (1.97%) among others. Below the rate of 1.90%, several others compounds were found, gonna of 1.89% for thymol acetate at 0.09% for α-humulene (Table 1).

The differences found in this study, when compared to some others in literature, may be explained by the edaphoclimatic effects as well as the analytical procedures employed. For example, Cavalcanti et al. (2004, 2010) found thymol as major compound in *L. sidoides* essential oils. However, only the first authors not found the presence of carvacrol. Following Botelho et al. (2007), who studied the microbial activity of the same essential oil, they found that the major constituents among others were thymol (56.70%) and carvacrol (16.70%), wich diverges with the results reported in this research, mainly the percentage of content.

For example, the difference in composition of essential oil of *L. sidoides* was described by Sousa et al. (2002) as a variation according to the extraction method, that may be performed by pressurized carbon dioxide or pressurized fluids (steam or ethanol). By the method employed, alterations in concentrations as well as in quantification can occur. Therefore, these differences justify the objective of developing research specific to the species found in one locality and not to generalize data from one location to another.

Toxicological assay

At high doses of essential oil of *L. sidoides*, carvacrol, 1,8-cineole and thymol, we verified greater mortality in adults of *T. molitor* (Table 2). According to data reported in Table 2 at 24 hours, carvacrol and 1,8-cineole showed largest toxicity, with LC_{50} of 5.53 and 5.71 µL/L air, respectively. The lethal concentration (LC_{50}) of the essential oil of *L. sidoides* against *T. molitor* was 8.04 µL/L air and for thymol 14.71 µL/L air. Similar behavior was observed in all LC_{50} values at 48 hours. An exception occurred when *T. molitor* was poisoned with 1,8-cineole and carvacrol at 48 hours. The lowest LC_{50} average was carvacrol (4.77 µL/L air) followed by 1,8-cineole (5.27 µL/L air), however for LC_{90}, the more toxic was 1,8-cineole with average of 6.83 µL/L air and not carvacrol (7.23 µL/L air) (Table 2). In general and time-independent, the range of toxicity against *T. molitor* was carvacrol > 1,8-cineole > *L. sidoides* essential oil > thymol.

Table 1 – Chemical composition, Kovats index and percentage of compounds of the essential oil of *Lippia sidoides*.

Compound	Kovats retention index	Content (%)
α-humulene	1455	0.09
β-selinene	1488	0.14
δ-terpineole	1170	0.15
Terpinolene	1084	0.18
α-terpineol	1195	0.19
1-octen-3-ol	976	0.38
α-thujene	927	0.66
caryophyllene oxide	1582	1.00
α-phelandrene	1006	1.28
4-terpineol	1181	1.39
α-terpinene	1016	1.50
(E)-caryophyllene	1419	1.50
thymol acetate	1364	1.89
Myrcene	987	1.97
Thymol	1290	2.30
thymol-methyl-ether	1229	2.92
Sabinene	968	5.26
γ-terpinene	1058	9.21
1,8-cineole	1033	9.26
ρ-cymene	1025	19.58
Carvacrol	1302	31.68
Total identified		92.53
Total unidentified		7.74

As the use of pure compounds (i.e. carvacrol, 1,8-cineole and thymol) for pest control is expansive, the result obtained with the essential oils of *L. sidoides* showed that it can be used like a powerful insecticide. As the essential oil is a complex mixture, where the different compounds are in suffering some kind of interaction among them, we can affirm in this case that occur a synergism, which play an important role in insect toxicity.

The Table 3 shows the importance of interaction among the compounds existent in a complex mixture of essential oils, which is directly related to toxicity to insects. In all situations, the different mixture (binary and tertiary) of carvacrol, 1,8-cineole and thymol has a similar behavior concerning the toxicity against *T. molitor*, and in most cases they showed a short value of lethal concentration, when applied alone compared (Table 3). In this experiment, the lowest value was observed in carvacrol:1,8-cineole mixture with LC_{50} average at 24 hours of 5.34 µL/L air, followed by tertiary mixture of carvacrol:1,8-cineole:thymol (6.34 µL/L air); thymol:1,8-cineole (7.51 µL/L air) and carvacrol:thymol (7.67 µL/L air). For 48 hours, we found the same behavior of toxicity that was found in 24 hours.

An important characteristic is the potentiation of certain compounds when mixed, commonly known as synergism. In this case, only the mixture of carvacrol:1.8-cineole at 48 hours have a antagonistic effect compared when are applied alone, with SR of 0.97; where the others were strengthened at different ratio (Table 3). The short effect of synergism was observed when applied a binary mixture of carvacrol:1,8-cineole, that has a SR average of 1.05, being most advantageous the use of isolate compound in detriment a mixture. For the tertiary mixture, we observed a average of SR of 1.23, being than the best

Table 2 – Lethal concentration (24/48 hours; µL essential oil /L air) in fumigation test of the chemical standards and the essential oil of *Lippia sidoides* to adults of *Tenebrio molitor*.

Treatments	Parameters					
	24 hours		48 hours		D.F.	χ^2
	LC_{50} (CI 95 %)	LC_{90} (CI 95 %)	LC_{50} (CI 95 %)	LC_{90} (CI 95 %)		
L. sidoides	8.04 (7.52 – 8.60)	12.47 (11.06 - 14.07)	7.04 (6.58 - 7.53)	10.56 (9.50 - 11.73)	80	75.63
carvacrol	5.53 (5.21 – 5.87)	8.78 (7.83 - 9.84)	4.77 (4.48 - 5.08)	7.23 (6.58 - 7.94)	92	65.38
1,8-cineole	5.71 (5.33 – 6.12)	7.79 (7.12 - 8.52)	5.27 (4.88 - 5.70)	6.83 (6.28 - 7.43)	102	72.62
thymol	14.71 (13.77 - 15.71)	17.21 (16.09 - 18.42)	12.69 (11.05 - 14.58)	16.77 (15.17 - 18.55)	90	80.03

LC_{50} or LC_{90} (CI 95 %) – lethal concentration and confidence limits at 95%.
D.F. – degrees of freedom. χ^2 – Chi-square value. N = 288 insects.

Table 3 – Lethal concentration (24/48 hours; µL compound/L air) of different ratio of chemical standards against *T. molitor* and synergistic ratio.

Treatments	Parameters[1]						
	24 hours		48 hours		N	D.F.	χ^2
	$LC_{50\ (CI\ 95\ \%)}$	$LC_{90\ (CI\ 95\ \%)}$	$LC_{50\ (CI\ 95\ \%)}$	$LC_{90\ (CI\ 95\ \%)}$			
carvacrol:1,8-cineole	5.34 (5.11 – 5.59)	8.02 (7.37 – 8.73)	4.45 (4.21 – 4.70)	7.27 (6.63 – 7.98)	288	152	112.87
carvacrol:thymol	7.67 (7.12 – 8.27)	10.14 (9.30 – 11.05)	7.29 (6.73 – 7.89)	9.52 (8.73 – 10.38)		102	116.42
1,8-cineole:thymol	7.51 (6.84 – 8.24)	10.73 (9.62 – 11.97)	7.05 (6.42 – 7.74)	9.36 (8.40 – 10.44)		90	96.959
carvacrol:1,8cineole:thymol	6.34 (5.84 – 6.88)	10.69 (9.29 – 12.29)	5.42 (4.97 – 5.92)	9.23 (8.07 – 10.55)		80	86.452

Treatments	Synergistic Ratio[2]							
	24 hours				48 hours			
	LC_{50}	Effect	LC_{90}	Effect	LC_{50}	Effect	LC_{90}	Effect
carvacrol:1,8-cineole	1.05	synergism	1.03	synergism	1.13	synergism	0.97	antagonism
carvacrol:thymol	1.32	synergism	1.28	synergism	1.23	synergism	1.24	synergism
1,8-cineole:thymol	1.36	synergism	1.16	synergism	1.27	synergism	1.26	synergism
carvacrol:1,8cineole:thymol	1.36	synergism	1.05	synergism	1.40	synergism	1.11	synergism

LC_{50} or $LC_{90\ (CI\ 95\ \%)}$ - lethal concentration and confidence limits at 95%. [1] Parameters estimated in dose-response assay (see subitem 2.2).
N - number of insects. D.F. - degrees of freedom.
χ^2 - Chi-square value. [2] Synergistic Ratio estimated according Metcalf (1967).

value of synergism were found when did a mixture of carvacrol:thymol (average of SR 1.27) and 1,8-cineole:thymol (average of SR 1.26). With this result it can be suggested that thymol has a good role like synergist in mixture of carvacrol or 1,8-cineole (Table 3).

The synergism among monoterpenes is found in many essential oils and this effect was studied by Hummelbrunner and Isman (2001), who stood out that (E)-anetol acts synergistically with thymol, citronelal and α-terpineol against the caterpillars *Spodoptera litura* (Fab.) (Lepidoptera:Noctuidae). As shown in this study, a large number of papers reports evidence the toxicity of essential oils against pests from stored products. Ho et al. (1994, 1996, 1997) demonstrated the toxicity of essential oils to insects, that have mainly cinnamic aldehyde, á-pinene, αnethol, thymol and eugenol. According to Lee et al. (2003), the monoterpenes that may be volatiles and lipophylic, can penetrate through breathing and quickly intervene in physiological functions of insect. These compounds can also act directly as neurotoxic compounds, affecting acetylcholinesterase activity or octopamine receptors (ISMAN, 2000).

As this work, the inseticidal activity of *L. sidoides* was reported by Furtado et al. (2005) against *A. aegypti*, which was effective to kill the larvae. Botelho et al. (2007) found that essential oil of *L. sidoides* from Northeast Brazil has as major components thymol and carvacrol and Cavalcanti et al. (2004) also verified that this compound are toxic against *A. aegypti* larvae. Acording Regnault-Regnault-Roger and Hamraoui (1995), the monoterpenes carvacrol and thymol among others, have insecticidal activity against *Acanthoscelides obtectus* (Say.) (Coleoptera: Bruchidae), as well as them affect the reproduction of this coleopter. More recently, Choi et al. (2006) showed the toxicity of several monoterpenes against *Lycoriella mali* (Fitch) (Diptera: Sciaridae), as demonstrated in this research on carvacrol, 1,8-cineole and thymol had a good insecticidal activity.

The potential of essential oils can be shown not only in insects, but also in controlling mites. According Adamczyk et al. (2005), there are several thymol-based commercial insecticides to control *Varroa destructor* (Acari: Mesostigmata) in honeybee colony, as well as Novelino et al. (2007) found that this compound have toxic effect against the tick larvae *Boophilus microplus* (Canestrini) (Acari: Ixodidae), causing 100% of mortality when applied at the ratio of 1% in DMSO. According to these authors, the efficiency of control is similar to that used in a synthetic mixture of clorfenvinfos+diclorvos. Although studies have demonstrated the power of essential oils against insect, Isman (2000, 2006) highlights that the toxicity of these compounds is directly linked to insect species, developmental phase and experimental conditions, requiring the achievement of a good experimental design to obtain satisfactory results.

CONCLUSION

In conclusion, the results showed that essential oil of *L. sidoides* presented as major constituents carvacrol, ρ-cymene, 1,8-cineole and γ-terpinene, being identified 92.53% of all constituents. By the toxicity assay, was verified that the essential oil of *L. sidoides* has insecticidal activity against *T. molitor*, as well as monoterpenes, carvacrol, 1,8-cineole and thymol. Moreover, these compounds may be used like a new tool in Integrated Pest Management. The synergistic effect of thymol can be used to increase the toxicity of carvacrol and 1,8-cineole, mainly in binary mixture as thymol:carvacrol or thymol:1,8-cineole.

ACKNOWLEDGEMENTS

We would like to thank the Laboratório de Análises e Sínteses de Agroquímicos, Departamento de Química, Universidade Federal de Viçosa; the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior and Fundação de Amparo à Pesquisa do Estado de Minas Gerais to according the doctoral scholarship to the first author and other resources, as well as PhD. Priscila Cintra-Socolowski for the critical review.

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