Pesticidal Activity of CO_2 Extracts from *Lippia javanica*

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ABSTRACT

In this study, conditions of supercritical fluid extraction (SFE) of volatile compounds from *Lippia javanica* were optimized to improve the extraction yields and selectivity. The composition and pesticidal activity of the CO_2 extracts were compared with the isolates obtained by hydrodistillation, maceration and Soxhlet extraction. The GC/MS and GC/FID techniques were used to determine the composition of the isolates. The insecticidal activity (acute and chronic toxicity and antifeedancy) and antifungal activity of isolates were tested on the larvae of *Spodoptera littoralis* and pathogenic, toxinogenic fungi (*Fusarium oxysporum, Penicillium expansum and Aspergillus fumigatus*). The maximum concentration of the volatiles (72% w/w) in CO_2 extract was achieved at 9 MPa and 50 °C, however with lowest yield. In this perspective, the optimum SFE conditions were 12 MPa and 50 °C with the concentration of volatiles in extract 53% w/w and yield of extract 16 mg. g^{-1} of plant. The essential oil from HD exhibited stronger acute toxicity and antifeedancy than other isolates. In case of CO_2 extracts, a good acute toxicity (LD_{50} = 62 μg) of the extract obtained at 9 MPa and 50 °C was observed. High chronic toxicity (LD_{50} = 48 μg) of extract was achieved at 12 MPa, 40 °C with additional separator.

INTRODUCTION

Due to increasing pest resistance against synthetic pesticides and negative impact of synthetic pesticides on the environment, new approaches are needed to manage pest attacks on agricultural crops. Botanical pesticides based on plant extracts appear to be a promising alternative, which can be also used in the field of bio-organic food industry.[1] Essential oils from aromatic plant contain a large amount of biologically active terpenes and terpenoids, which often exhibit pesticidal activities.[2]

Lippia javanica (Burm.f.) Spreng (Verbenaceae), grown in the South Africa, is an erect shrub with strongly aromatic leaves. The indigenous people in South Africa use *L. javanica* infusions and tea for treatment of various ailments: primarily, respiratory diseases, headaches and fever.[3] Moreover, many studies reported the antimicrobial, antifungal, insecticidal and repellent activity of its essential oil [4-7]. These biological activities are probably caused by the high content of monoterpenes (limonene, p-cymene, myrcene) and oxygenated monoterpenes (myrcenone, piperitenone, linalool, 6,7- epoxymyrcene) in aerial parts of *L. javanica* [8].

Traditionally, essential oils from aromatic plants are isolated by hydrodistillation (HD) and extracts are obtained by organic solvent extraction (Soxhlet extraction, maceration). These methods can cause thermal degradation of some compounds that can lead to the decrease of biological activity.

To avoid the use of high temperature and the use of organic solvents, supercritical fluid extraction with carbon dioxide (SFE) is applied. On the contrary to essential oil from HD, the CO_2 extracts contain also non-volatile substances wherein the composition of extracts depends on the extraction conditions. To increase the volatile concentration in CO_2 extracts,

the extraction and separation conditions have to be optimized. The moderate conditions (9–12 MPa and 35–50 °C) of SFE are suitable to achieve extract with high concentration of volatiles. [9]
The SFE has been previously used to isolate insecticidal active compounds of *Tanacetum parthenium (L.)*. [10] By optimization of SFE conditions, three different extracts were obtained at 50 °C: oleoresin at 28 MPa, volatile compounds-rich extract at 12 MPa and extract enriched with polar components at 28 MPa with CO_2 modified by 4.3% of acetone. In this case, the CO_2 extracts obtained at 28 MPa and 50 °C and with modifier were more effective in terms of antifeedancy and growth inhibition effect against model insect than pure essential oil.
The aim of this study is: (i) the optimization of SFE conditions of *L. javanica* in order to improve the yield and concentration of volatiles in extracts and (ii) the comparison of the concentration of volatiles and pesticidal activity of CO_2 extracts with isolates obtained by maceration, Soxhlet extraction and hydrodistillation, and finally (iii) finding the relationship between the composition and pesticidal activity of isolates.

MATERIALS AND METHODS

Materials

The air-dried aerial parts of *Lippia javanica* (South Africa, harvested in 2013) were supplied by Matoušek CS a. s. and stored in closed bottles in refrigerator to prevent escape of volatiles. Before each experiment, the plant material was ground in a laboratory blender (Warring Commercial 8010G) and sieved. The particles with diameter under 1 mm were used for experiments.
Carbon dioxide (> 99.9 %) was purchased from Linde Technoplyn, CR. The solvents for maceration were hexane (p.a., Lach-Ner, CR) and acetone (pure, Lachema Neratovice, CR).
The solvent for the samples for gas chromatography, *n*-heptane, p.a., and internal standard, hexadecane, were purchased from Sigma-Aldrich, Steinheim, Germany.

Supercritical fluid extraction

The extraction column (150 mL, I.D. 30 mm) was equipped with temperature-controlled water jacket. The grounded plant material (40 g) was placed in extraction column between layers of glass beads in order to facilitate solvent distribution. The compressor (NovaSwiss 560.0007) with the pressure regulator unit (NovaSwiss 560.0009) were used to pressurize CO_2 and to regulate extraction pressure. Experiments were performed at different combinations of pressures and temperatures (see Table 1). Additional separator was used in experiment 5 to achieve high concentration of volatiles in extract. To assess the possible synergic effect of polar compounds, the polar modifier (acetone) was added by high pressure pump (ECOM Praha, CR) to CO_2 at different concentration.
The solution leaving the extractor or the additional separator was depressurized across a heated micrometer valve. The extract was collected in pre-weighed glass vials cooled by ethanol-dry ice mixture (-78 °C) to prevent the escape of volatile compounds (run 1-6) from extract.
In experiments with modifier, the acetone was evaporated under N_2 atmosphere. The vials with the extract were weighed, tightly closed, and stored in a refrigerator. The amount of gaseous solvent leaving the vial was measured using a gas meter.

Table 1: Summary of SFE conditions and calculated carbon dioxide density[11]

Run	Pressure [MPa]	Temperature [°C]	Density of CO_2 [kg.·m^{-3}]	Solvent
1	9	50	285	CO_2
2	9	40	487	CO_2
3	12	50	586	CO_2
4	12	40	714	CO_2
5*	12	40	714	CO_2
6	30	40	910	CO_2
7	30	40	910	CO_2 + 5% w/w acetone
8	30	40	910	CO_2 + 10% w/w acetone

*additional separator: 8 MPa; 50 °C (5/S1)

Soxhlet extraction

Dry plant material (10-12 g) was extracted with 250 mL ethanol (S-AC) in Soxhlet apparatus for 6 h. The solvent was removed from the extract by a rotary vacuum evaporator.

Maceration

The plant material was placed in a flask together with the solvent in the ratio 1:10. Hexane (M-HE) and acetone (M-AC) were used as solvents of different polarity to produce macerates. The maceration was carried out for 48 hours at room temperature and solution was shaken. The plant material was separated from the macerate by filtration and the solvent was gently evaporated using vacuum evaporator.

Hydrodistillation

Hydrodistillation (HD) was used to isolate essential oil from dry plant. The ground plant material (60 g) was distilled with 600 mL of water. The distillation was carried out for 3 hours, a sufficient time to complete the isolation of essential oil.

Bioassay

Larvae of *Spodoptera littoralis*, an important polyphagous pest, were chosen as a model insect to test the insecticidal activity. To determine the acute toxicity, the isolates were dissolved in acetone and the solution was applied topically on larvae *S. littoralis*. In case of chronic toxicity, isolates were mixed into the semi-synthetic diet. and after 5 days the mortality was evaluated. Acute and chronic toxicity were evaluated as lethal doses (LD_{50}) of the isolates that cause 50% mortality of larvae after 24 h and 5 days, respectively.

Antifeedancy of the isolates was tested by no-choice test using leaf discs and characterized by the feedant deterrent index (FDI). The extracts dissolved in acetone were applied to the upper side of the leaf disc. After 24 h, the leaf residues were dried at 60°C to the constant weight. The relation between fresh weight and dry weight of different sized leaves was used to determine the amount of food consumed. The effective concentrations causing 50% of feeding deterrence (EC_{50}) were evaluated using probit analysis.

Antifungal activity was tested on model pathogenic and toxinogenic fungi *Fusarium oxysporum*, *Penicillium expansum* and *Aspergillus fumigatus*. Inhibitory effect of isolates on mycelial radial growth of fungi was assessed by the agar dilution method and minimum inhibitory concentration (MIC) of isolates was determined. The detailed description of the biassay can be found in the previous papers [10,12, 13].

GC analysis

The isolates were analyzed by GC-MS and GC-FID. GC-MS: the analyses were performed on Agilent 6890 gas chromatograph coupled to Agilent 5973 mass spectrometer operating in 70 eV ionization mode. DB-5MS column (30 m x 0.25 mm x 0.25 μm) was used with He as a carrier gas. GC-FID: the analyses were performed on a Hewlett Packard 5890 gas chromatograph equipped with a DB-5 column (30 m x 0.25 mm x 1 μm) and using N_2 as a carrier gas.

RESULTS

Yield and composition of isolates

Figure 1 compares the yields and concentration of the volatiles in the isolates obtained by different separation methods.

The essential oil was obtained by HD with the yield of volatiles 6.6 mg. g^{-1} of plant.

The yield of CO_2 extracts increased with the CO_2 density and the amount of polar modifier added to CO_2. Therefore, the highest SFE yield, 28 mg. g^{-1} of plant, was obtained at 30 MPa and 40°C (run 6). The yield and concentration of volatiles in CO_2 extract obtained at 9 MPa and 50 °C (run 1) was very similar to the results for essential oil. At extraction conditions 40 °C and 12 MPa (run 4), the concentration of volatiles in extract was 41 % w/w. Using the same extraction conditions with additional separator set at 50 °C and 8 MPa (run 5), the concentration of volatiles in extract increased to 67 % w/w. When acetone as a CO_2 modifier was used, the concentration of the volatiles in the extracts decreased (run 7, 8). This was caused primarily by the co-extraction of more polar compound and/or by loss of the volatile compounds during evaporation of acetone from extract.

The yields of volatiles in CO_2 extracts reached similar or higher values than the yield of essential oil from hydrodistillation. The optimal conditions with respect to both yield and concentration of volatiles in extract were at the CO_2 density from 487 to 586 kg·m^{-3} (run 2, 3).

The yield of the other isolates strongly depends on the used method and solvent (Figure 1). The extraction with hexane was more selective than extraction with acetone.

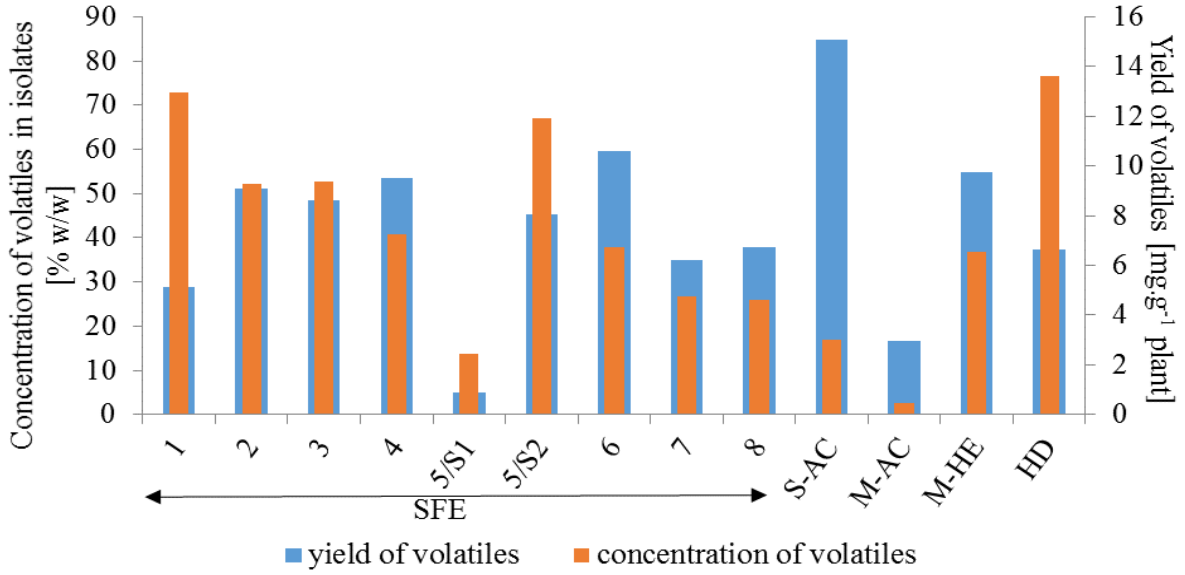

Figure 1. Yield of volatiles (mg. g^{-1} of plant) and concentration of volatiles (% w/w) obtained by supercritical fluid extraction (run 1-8), Soxhlet extraction with acetone (S-AC), maceration with acetone (M-AC) and hexane (M-HE), and hydrodistillation (HD).

The main volatile components in the isolates are compared in the Figure 2. The CO_2 extracts contained considerably high concentration of myrcenone up to 44% w/w (run 5/S2). In comparison to CO_2 extracts, the essential oil contained on the average three times lower concentration of myrcenone. Moreover, much higher concentration of *(E)*-ocimenone was observed in essential oil than in other isolates.
The significant difference in the composition of HD and SFE isolates was probably caused by the thermal degradation of myrcenone to *(Z)*, *(E)*-ocimenone during hydrodistillation. A similar phenomenon was observed in chamomile extracts (chamomile essential oil) by Reverchon et al.[14].
Besides, the extracts contained also non-volatile components that caused decrease in the concentration of main volatiles in isolates.

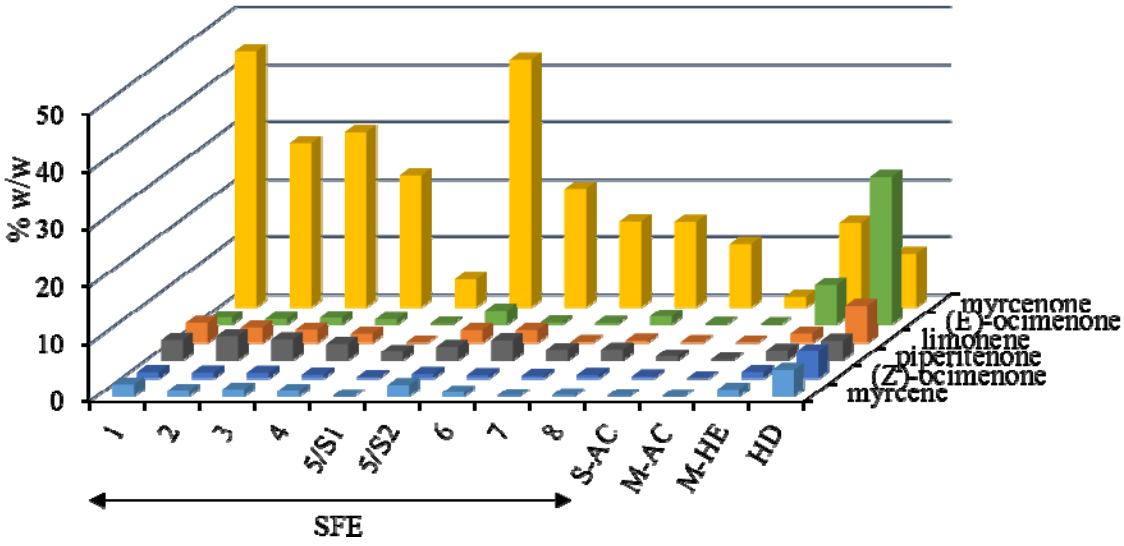

Figure 2. Concentration (% w/w) of major components in isolates obtained by different methods: supercritical fluid extraction (run 1-8), Soxhlet extraction with acetone (S-AC), maceration with acetone (M-AC) and hexane (M-HE), and hydrodistillation (HD).

Insecticidal activity

Figure 3 illustrates the insecticidal activity of the isolates obtained by different separation methods. The trend dependencies of the insecticidal activity of isolate on the isolation methods varied according the type of tests. The essential oil from HD exhibited strong acute toxicity and antifeedancy with low values of LD_{50}=43 µg and EC_{50}=85 µg. cm^{-2}, respectively. The acute toxicity of the CO_2 extracts was positively influenced by increasing concentration of volatiles in extracts and the highest acute toxicity (LD_{50}=62 µg) of extract was achieved at 9 MPa, 50 °C. The hexane macerate was most active in terms of chronic toxicity (LD_{50}=39 µg) followed by CO_2 extract obtained at 12 MPa, 40 °C with additional separator (run 5/S1) with LD_{50}=48 µg. In case of chronic toxicity, other components in extracts cause synergic effect with volatiles. Antifeedancy of the extracts was negligible in comparison with essential oil, only runs 2 and 3 caused a feedant deterrence.

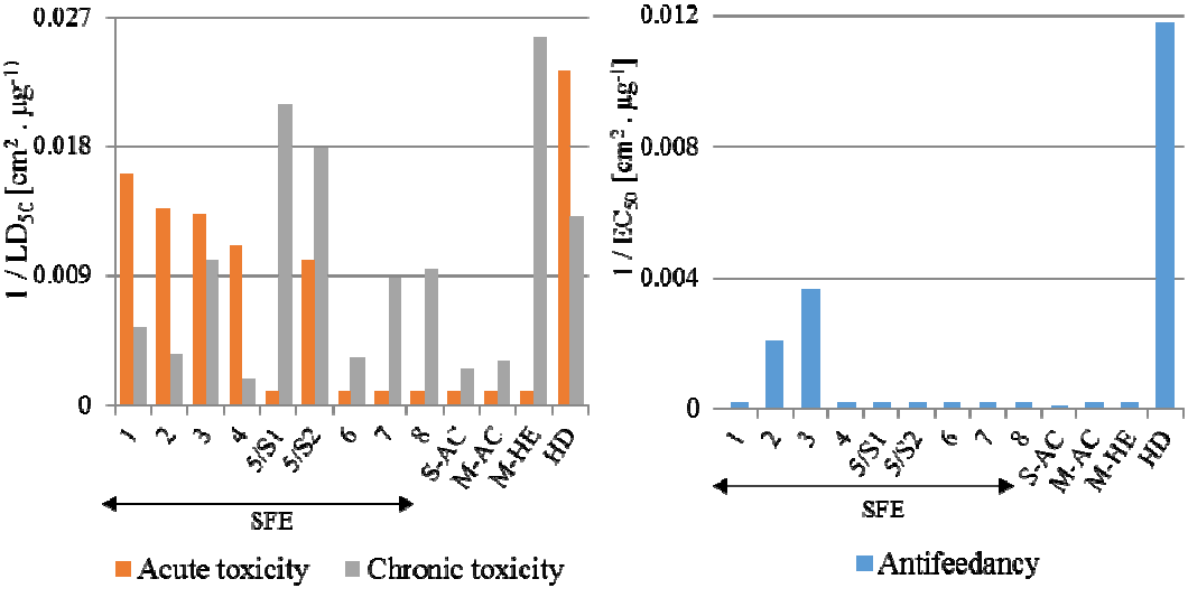

Figure 3: Insecticidal activity of isolates obtained by different methods: supercritical fluid extraction (run 1-8), Soxhlet extraction with acetone (S-AC), maceration with acetone (M-AC) and hexane (M-HE), and hydrodistillation (HD).

Antifungal activity

Figure 4 shows the inhibition effect on fungal mycelium growth of the isolates obtained by different methods.

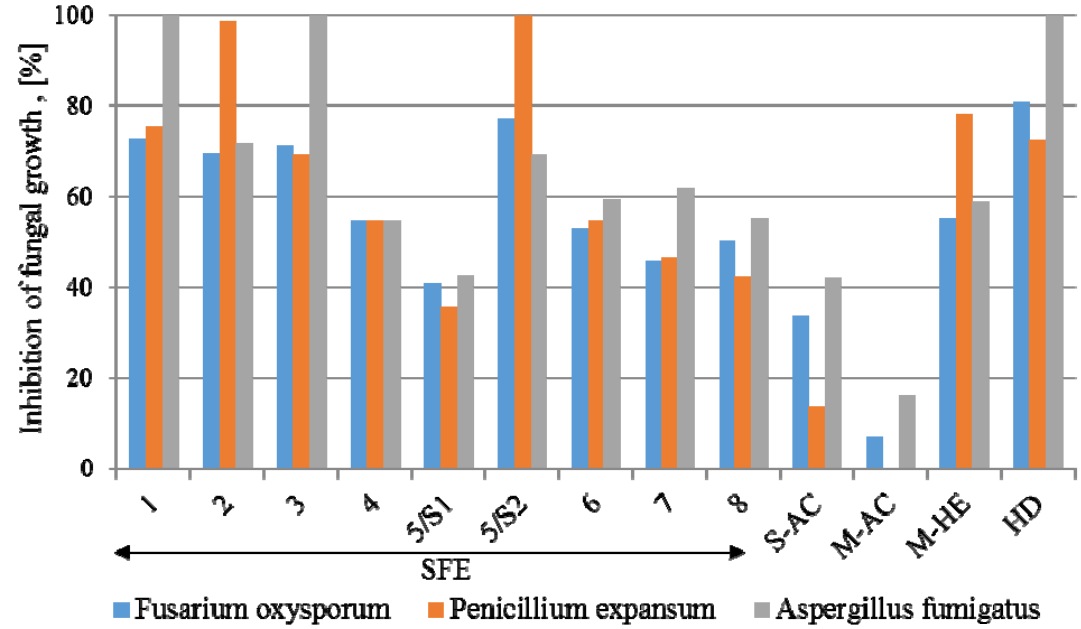

Figure 4. Percent inhibition of fungal mycelium growth of isolates (at 1mg/ml) obtained by supercritical fluid extraction (run 1-8), Soxhlet extraction with acetone (S-AC), maceration with acetone (M-AC) and hexane (M-HE), and hydrodistillation (HD).

The antifungal activity varied in dependence on the isolation method and kind of fungi. CO_2 extracts obtained at 50 °C and pressures 9 MPa and 12 MPa (run 1, 3) and essential oil from HD showed the strongest effect against *Aspergillus fumigatus*. On the contrary, the mycelium growth of *Penicillium expansum* was totally inhibited by the CO_2 extracts obtained at 40 °C, 9 MPa and 40 °C, 12 MPa with additional separator (runs 2 and 5/S2). All isolates exhibited lower antifungal effect against *Fusarium oxysporum*. The most significant antifungal activity showed the isolates with higher concentration of volatiles.
The relationship between concentration of volatiles and inhibition effect on mycelium growth of fungi is demonstrated in the Figure 5.

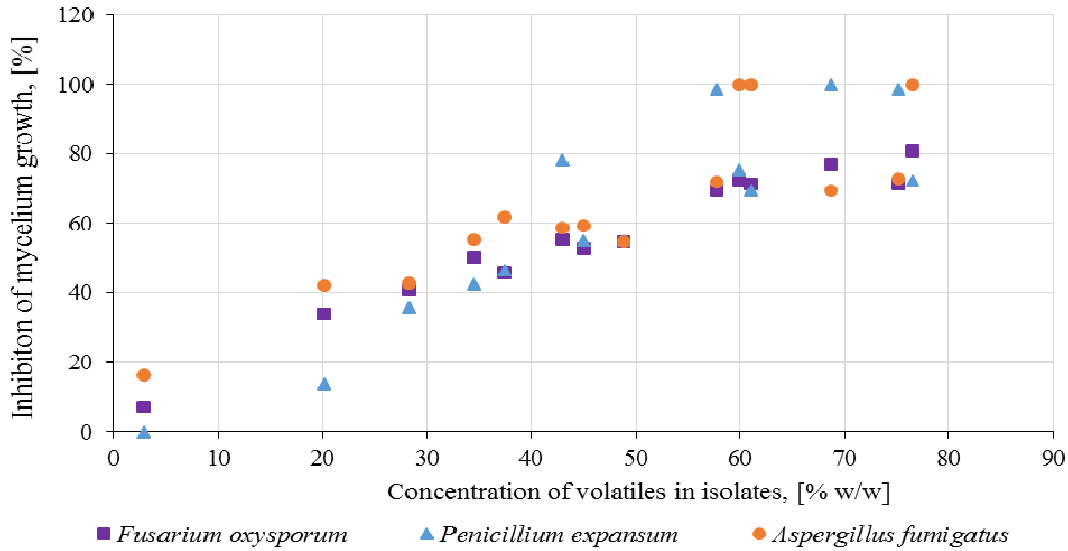

Figure 5. Relationship between inhibition of mycelium growth and the content of volatiles in isolates.

CONCLUSION

The supercritical fluid extraction was used for the isolation of volatiles from *Lippa javanica* to reach maximal pesticidal activity of extracts. The optimum relation between the yield and concentration of volatiles in extracts was achieved at SFE conditions 9 MPa, 40°C and at 12 MPa and 50 °C.
The strongest insecticidal activity in terms of acute toxicity and antifeedancy was exhibited by the essential oil from hydrodistillation. On the other hand, hexane macerate and CO_2 extracts obtained at 12 MPa, 40 °C with additional separator showed significant chronic toxicity caused by synergic effect of non-volatile components in the extracts of *L. javanica*.
The antifungal activity increased with the concentration of the volatile components in isolates. Whereas the CO_2 extracts obtained at 40 °C and pressures 9, 12 MPa totally inhibited the mycelium growth of *Penicillium expansum*, the essential oil from HD and the CO_2 extracts obtained at 50 °C and pressures 9, 12 MPa showed strongest effect against *Aspergillus fumigatus.*

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REFERENCES

[1] DAYAN, F. E., CANTRELL, C. L., *et al.*, Bioorganic & Medicinal Chemistry, Vol. 17, **2009**, p. 4022.
[2] ISMAN, M. B., Crop Protection, Vol. 19, **2000**, p. 603.
[3] PASCUAL, M. E., SLOWING, K., *et al.*, J Ethnopharmacol, Vol. 76, **2001**, p. 201.
[4] OMOLO, M. O., OKINYO, D., *et al.*, Phytochemistry, Vol. 65, **2004**, p. 2797.
[5] KATSVANGA, C. A. T. A. S. C., Tropical and Subtropical Agrosystem, Vol. 4, **2004**, p. 101.
[6] THEMBO, K. M., VISMER, H. F., *et al.*, J Appl Microbiol, Vol. 109, **2010**, p. 1479.
[7] NGASSAPA, O., RUNYORO, D. K. B., *et al.*, Flavour Fragrance J., Vol. 18, **2003**, p. 221.
[8] VILJOEN, A. M., SUBRAMONEY, S., *et al.*, J Ethnopharmacol, Vol. 96, **2005**, p. 271.
[9] REVERCHON, E., PORTA, G. D., *et al.*, Journal of Agricultural and Food Chemistry, Vol. 43, **1995**, p. 1654.
[10] PAVELA, R., SAJFRTOVÁ, M., *et al.*, Industrial Crops and Products, Vol. 31, **2010**, p. 449.
[11] ANGUS, S., REUCK, K. M. D., *et al.*, *International Thermodynamic Tables of the Fluid State Carbon dioxide*. **1976**, Pergamon. p. 37.
[12] PAVELA, R.,VRCHOTOVÁ, N., Industrial Crops and Products, Vol. 43, **2013**, p. 33.
[13] ZABKA, M., PAVELA, R., *et al.*, Industrial Crops and Products, Vol. 30, **2009**, p. 250.
[14] REVERCHON, E.,SENATORE, F., Journal of Agricultural and Food Chemistry, Vol. 42, **1994**, p. 154.