Effect of Extraction Methods on Botanical Insecticide Activity

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Supercritical fluid extraction (SFE) was used for isolation of volatile components from savory (*Satureja hortensis* L.) and thyme (*Thymus vulgaris* L.). Three types of extracts were prepared using the benefit of variable solvent power of supercritical carbon dioxide under different experimental conditions. Composition and product activities of the CO_2 extracts were compared with hydrodistillation (HD) and Soxhlet extraction. The composition of volatile compounds in the isolates was determined by gas chromatography, and the toxicity and antifeedant effects of the extracts on larvae of Colorado potato beetle (*Leptinotarsa decemlineata*) were evaluated.
Strong insecticidal effects of all isolates against larvae were observed, but significant differences between the particular isolates and plants were found. The most effective isolates were the essentials oils which showed the lowest LD_{50}. The efficiency of extracts of S. hortensis (SFE2 and SFE3) was comparable with that of hydrodistilate and higher than the efficiency of other extracts, while their extraction yield was by 73 % higher than the yield of hydrodistillate.

INTRODUCTION

Many aromatic plants produce biologically active secondary metabolites which are isolated using various extraction techniques. The obtained products are used in food, cosmetic, perfume and detergent industries, as well as in pharmacology for their antibacterial, antifungal, antioxidant and anticarcinogenic properties and also in agriculture in plant protection area for their pesticidal activity.
It was confirmed that the number of insect and mite species resistant to synthetic pesticides has continued to rise, apart from risks associated with the use of these chemicals [1, 2]. Therefore, there is the urgent need to develop safer, environmentally friendlier and efficient alternatives that have potential to replace synthetic pesticides and are convenient to use. In the search for alternatives to conventional pesticides, essential oils extracted from aromatic plants have been widely investigated. Their toxicity and repellent effects to stored-product insects and greenhouse pests have been of special interest during the last decade [3, 4]. In particular, terpenes and terpenoids belong to active components of essential oils [5, 6].
Traditional extracts of aromatic plants are obtained by the hydrodistillation (essential oil) or by the liquid extraction with organic solvents. Recently, much attention has been directed to the use of near critical and supercritical carbon dioxide (SFE) as the solvent, particularly in food, pharmaceutical and perfume industries. SFE is more selective than the extraction with commonly used solvents, which extract unwanted components as well [7]. Due to low temperatures used by SFE, no decrease occurs in biological activity of extracted components against harmful organisms, such as microbes, viruses, fungi and also pests [8, 9].
The aim of this study was a comparison of the insecticidal activity and the chemical composition of the extracts from savory (*Satureja hortensis*) and thyme (*Thymus vulgaris*) obtained using SFE, hydrodistillation and Soxhlet extraction.
Savory and thyme are herbaceous aromatic plants traditionally used for their beneficial biological activities. Some components of their essential oils are responsible for antifeedant or repellent effects on pests [10, 11]. Supercritical CO_2 extraction of essential oil from these herbs has been studied [12,

13] whereas the extraction efficiency and the extract composition were compared with other isolation methods such as hydrodistillation [13, 14] or subcritical water extraction [15]. For the extraction of savory and thyme oils with supercritical carbon dioxide, the optimum operating conditions have been described, but biological properties of the extracts were not tested except antioxidant activity of savory extract [16, 17].

MATERIALS AND METHODS

Materials

The air-dried savory and thyme were stored in dark in closed bottles at room temperature. The plant material was ground before each experiment. Carbon dioxide (>99.9%) was purchased from Linde Technoplyn (Prague, CR). Ethanol for spectroscopy (Lachema Neratovice, CR) was used as the entrainer.

The chromatographic standards of essential oil components and hexane p.a. used as a solvent for GC were purchased from Sigma (Sigma-Aldrich, Steinheim, Germany). Hexane (Lach-Ner, CR) and technical ethanol (Chemopetrol, Litvínov, CR) were used as solvents for Soxhlet extraction.

Supercritical fluid extraction

The SFE experiments were carried out using the 150 mL extraction column (I.D. 30 mm) filled with 30-40 g of plant particles placed between layers of glass beads serving as solvent flow distributors. The extractor was immersed in a temperature-controlled water bath. CO_2 was pressurised by compressor NovaSwiss 560.0007 and controlled by the pressure regulator unit NovaSwiss 560.0009 to operating pressure. In experiments with entrainer, the stream of CO_2 was before entering the extractor mixed with ethanol pumped at a constant flow rate by high-pressure pump LCP 4020.3 ECOM, CR. The solution leaving the extractor at its bottom was depressurised across the heated micrometer valve to atmospheric pressure and the extract was collected in pre-weighed glass traps cooled by ethanol-dry ice mixture to prevent the escape of volatile compounds. The amount of gaseous solvent leaving the trap was measured using a gas meter. The extract was weighed and the closed trap was stored in a refrigerator. Before the biological tests, the extract was homogenized by dissolving in acetone (1:1) and small part of the solution was separated for need of analysis.

Three types of extracts were prepared:

- the oleoresin extracted by CO_2 at 28 MPa and 50 °C (SFE1),
- the extract rich in essential oil, extracted by CO_2 at 12 MPa and 50 °C (SFE2),
- the extract enriched with polar components, extracted by CO_2 modified with ethanol (4.3 wt. %) at 28 MPa and 50 °C (SFE3). To reduce ethanol condensation the separator was not cooled.

The carbon dioxide flow rate was adjusted to 1.42 g min^{-1}. The direction of solvent flow in the extractor was selected with respect to the finding that the down-flow of fluid accelerates extraction rates, in particular at lower Reynolds numbers and for conditions near the critical point of CO_2, where natural convection is dominant [18].

Soxhlet extraction

Dry plant material (10-12 g) was extracted with 250 ml ethanol (SE) or hexane (SH) in Soxhlet apparatus for 7 h. The solvent was removed from the extract using a vacuum evaporator.

Hydrodistillation procedure

The content of essential oil in savory and thyme was determined by hydrodistillation (HD). Dried plant (30 g) was distilled for 5 h, which was sufficient to complete the essential oil isolation. The oil was collected via a side arm. The amount of oil recovered was measured gravimetrically

GC analysis

The isolates were analyzed by GC-MS and GC-FID. Identification of compounds was based on the comparison of their mass spectra and retention indices with published results [19] and where possible with authentic compounds.

GC-MS: the analyses were performed on an 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 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.

Insecticidal activity

Antifeedant activity of the extracts against the 4th instar larvae was investigated through no-choice test using leaf discs. Test solutions were prepared from the stock extract solution by further dilution in acetone to produce three different concentrations: 0.02, 0.2 and 2 % (w/v). Leaf discs (28 mm in diameter) were prepared from potato leaves using a cork borer and weighed before the test. Each disc was dipped in the test solution for 10 sec. Control leaf discs were dipped in acetone for the same period of time. All discs were left at room temperature for 5 min to evaporate the solvent. In the no-choice test, each arena contained only one treated leaf disc (n = 20 for each treatment). Meanwhile, a group of 20 arenas with one larva and one control disc in each was set up for control. After 4 h, the remnants of leaf discs were collected and dried separately at 60°C to a constant weight. The amount of food consumed was calculated depending on the initial fresh weight of each disc and the dry weight of its remnants, using a standard curve of the relation between fresh weight and dry weight of different sized leaf pieces. The antifeedant index (AFI) was calculated from the formula:

$$AFI = [(C-T)/(C+T)] \times 100 \text{ (in \%)},$$

where C is the consumption of control discs and T is the consumption of treated discs. In the no-choice tests, the food consumed by the 20 animals that were given control discs was averaged, and the mean was used as C for the calculations of the AFI for each observed T. The experiment was carried out at 25±1°C, RH 65 ± 5% and light regime. The antifeedant indexes at different treatments were compared using an analysis of variance (ANOVA) followed by Tukey test ($P<0.05$) for multiple-comparison where significant differences were observed.

Acute toxicity of extracts, measured as mortality after 24 hours, was determined by topical application to early fourth instars larvae *Leptinotarsa decemlineata.* The extracts were dissolved in acetone as a carrier and each larva received 1μL of the solution per treatment, with acetone alone as the control. The range of five doses that were used to establish the lethal doses was determined by preliminary screening. Four replications of 20 larvae or adults were tested per dose. The doses were applied to the dorsum using a repeating topical dispenser attached to 100 μL syringes. All treated larvae from each replicate were transferred to relevant diet in plastic boxes (10 × 10 × 7 cm). The boxes were placed for 24 hours in a growth chamber (L16:D9, 25 °C). Death was recorded when the larvae or adults did not respond to prodding with forceps.

All means of data obtained from the various toxicity bioassays were corrected with Abbott´s formula [20]. Probit analysis [21] was used to determine LD50, LD90, and the corresponding 95% confidence intervals.

RESULTS

Extract yields and compositions

The yields and composition of the isolates obtained by supercritical fluid extraction under different conditions, the conventional extraction with organic solvents (hexane and ethanol) and the hydrodistillation of savory and thyme were compared. As shown in **Tables 1** and **2**, for both herbs the maximum extraction yield was obtained by Soxhlet extraction with ethanol and the lowest one was

observed for hydrodistillation. The extraction yields obtained with supercritical carbon dioxide, irrespective of extraction conditions, varied between 3 - 4 % for *S. hortensis* and 1.6-3.3 % for *T. vulgaris* extracts and were comparable to the quantity of extract obtained by hexane. When a lower density of CO_2 at the SFE2 was used, the extraction yield was lower than in case of the SFE1, because the extraction of substances less soluble than essential oil components was suppressed.

Table 1: Yields of extract (wt. %) and concentration of major components in extract (mg g^{-1}) of savory obtained using different methods of isolation

Component:	Method of Isolation					
	HD	SFE1	SFE2	SFE3	SH	SE
Extract	1.69	3.98	2.9	3.72	4.01	8.76
α-Terpinene	23.6	5.8	9.8	0.5	-	-
p-Cymene	31.8	16.3	20.2	5.0	1.5	-
γ-Terpinene	247.1	128.1	135.9	39.3	8.7	-
Thymoquinone	-	9.5	4.7	14.3	10.1	-
Carvacrol	323.9	282.0	268.9	319.5	199.9	125.6
β-Caryophyllene	5.0	3.5	3.9	3.5	2.0	-
β-Bisabolene	11.3	8.2	10.0	8.4	6.3	0.8
Others*	31.2	11.9	11.31	6.5	0	1.8

*α-Thujene, α-pinene, β-pinene, myrcene, α-phellandrene, limonene, β-phellandrene, terpinen-4-ol, thymol, thymohydro quinone.

Table 2: Yields of extract (wt. %) and concentration of major components in extract (mg g^{-1}) of thyme obtained using different methods of isolation

Component:	Method of Isolation					
	HD	SFE1	SFE2	SFE3	SH	SE
Extract	0.78	3.29	1.64	3.27	2.34	7.05
p-Cymene	191.4	19.3	25.6	4.2	15,5	-
γ-Terpinene	116.5	25.7	17.8	2.1	14,8	-
Linalool	20.3	4.1	4.2	1.0	1.7	-
Borneol	15.3	3.5	5.3	1.0	-	-
Thymol	405.9	142.5	153.1	93.2	108.5	34
Carvacrol	17.5	5.7	7.5	4.2	4.8	-
β-Caryophyllene	15.1	6.7	7.7	5.1	5.5	-
Others*	95	16.1	14.6	3.1	2.5	-

*α-Thujene, α-pinene, camphene, 1-octen-3-ol, myrcene, α-phellandrene, α-terpinene, limonene, β-phellandrene, 1,8-cineole, *cis*-sabinene hydrate, terpinolene camphor, terpinen-4-ol, α-terpineol, thymol methyl ether, carvacrol methyl ether, thymoquinone, bornyl acetate.

The major components in all savory isolates were carvacrol and, except for the Soxhlet extracts, γ-terpinene (**Table 1**). The major components in thyme extracts were thymol, γ-terpinene and p-cymene (**Table 2**).

Using HD, pure essential oil was obtained with maximum concentration of volatile components. When supercritical CO_2 was used as solvent, more volatile components were obtained under conditions of SFE1 and SFE2. It was observed that some of volatile components escaped if the separator was not cooled at the SFE3 conditions. The low content of terpenic components in the SH extracts was caused by their loss during the solvent evaporation from the extract. The alcohol extract contained almost no

volatile components but a large amount of polar components that could have a synergistic effect with the terpenic components.

Extract activities

As shown in **Table 3**, differences were found between the plants and methods of isolation by the evaluation of insecticidal activities of the savory and thyme extracts obtained using different methods.

Table 3: Contact toxicity and antifeedant activity (AFI in %) of the oil (HD), supercritical extracts (SFE1-3) and extracts with organic solvents (SH and SE) of *Thymus vulgaris* and *Satureja hortensis* against larvae of *Leptinotarsa decemlineata*

Mean values with the same superscript letter are not significantly different (P< 0.05).

Isolates	Satureja hortensis					Thymus vulgaris				
	Contact toxicity		Antifeedant activity			Contact toxicity		Antifeedant activity		
	LD_{50}	LD_{90}	2%	0.2%	0.02%	LD_{50}	LD_{90}	2%	0.2%	0.02%
SFE1	60	103	100.00^{a}	33.69^{c}	23.39^{bc}	220	432	100.00^{a}	65.95^{ab}	58.48^{a}
SFE2	28	85	100.00^{a}	21.85^{c}	13.14^{c}	201	416	100.00^{a}	45.69^{ab}	43.26^{ab}
SFE3	30	86	100.00^{a}	37.09^{bc}	20.47^{c}	240	498	95.29^{a}	28.76^{c}	30.92^{bc}
SH	44	102	96.97^{ab}	57.69^{b}	49.21^{a}	260	>500	75.80^{b}	37.80^{b}	23.99c
SE	78	240	92.91^{b}	72.67^{a}	50.29^{a}	475	>500	70.56^{b}	57.38^{ab}	31.54^{bc}
HD	22	52	100.00^{a}	87.45^{a}	29.50^{b}	29	55	100.00^{a}	76.22^{a}	67.74^{a}

Strong deterrent effects of all isolates against larvae were observed. In the no-choice test with leaf discs, the consumption of control food by the fourth instars larvae *L. decemlineata* was significantly higher than the consumption of treated food in the treatment concentrations (P<0.05). The AFI declined depending on the concentration. The maximum antifeedant effect was observed after the application of 2% solutions of the isolates from both plants; the isolates obtained by standard extraction methods showed slightly lower efficiencies compared to the isolates obtained by HD and SFE methods (**Table 3**). At lower concentrations (0.2% and 0.02%), significant differences among the isolates obtained using different methods were observed. While the most active extracts from *S. hortensis* were those obtained by Soxhlet extraction (SE and SH), the extracts obtained by HD, SFE1 and SFE2 from *T. vulgaris* possess a higher activity than those obtained from the plant by Soxhlet extraction.

All isolates from both *S. hortensis* and *T. vulgaris* exhibited good insecticidal activity against the larvae of *L. decemlineata*. Generally, the savory isolates were more efficient in topical application. The mortality within 24 h after the application is assumed to be caused mostly by monoterpenes, which are known to exhibit fast contact and fumigant effects. The present results confirm the assumption because the monoterpene levels in savory isolates (**Table 1**) were much higher than in thymus isolates (**Table 2**). In accordance with the assumption, the highest percentage of monoterpenes was found in the oil and the lowest percentage was in the isolates obtained by the standard Soxhlet method. The activity of the oils obtained by hydrodistillation from both plants was similar, and their lethal doses were smaller than lethal doses of the samples obtained by other extraction methods. The supercritical extracts from savory, and particularly the extracts SFE2 and SFE1, showed biological activity almost as high as the oil. Thus, taking into account the lower energetic consumption and higher extraction yield of SFE compared to hydrodistillation, supercritical fluid extraction can be recommended as a suitable method for obtaining savory extracts of high potential for the preparation of botanical insecticides.

CONCLUSION

The SFE extracts of *S. hortensis* and *T. vulgaris* showed toxicologic and antifeedeant effects against larvae of Colorado potato beetle. Therefore it can be suggested that these isolates and/or the plant oils can be used as new insecticidal agents against *L. decemlineata*. The development of natural or biological insecticides should help to decrease the negative effects of synthetic chemicals such as residues in products, insect resistance and environmental pollution. However, further studies need to be conducted to evaluate the cost and safety of these reagents.

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