Biological Activity of Eucalyptus Extracts Obtained by Supercritical Carbon Dioxide

Martin Topiar*, Marie Sajfrtova, Zdenka Machalova, Jindrich Karban, Roman Pavela
E-mail: topiar@icpf.cas.cz, Fax: +420 220 920 661

ABSTRACT

Supercritical fluid extraction using several fractionation methods (sorption and series of separators) was applied on *Eucalyptus grandis* leaves in order to increase the amount of volatile compounds in extract. The yields and composition of extracts and essential oil obtained by hydrodistillation were compared. The composition of all isolates was determined using GC-MS and GC-FID. Insecticidal activity of isolates (antifeedancy, acute and chronic toxicity) was tested on larvae of Mediterranean Brocade (*Spodoptera littoralis*) and Potato Colorado Beetle (*Leptinotarsa decemlineata*). Yield of essential oil was determined at 26.7 mg/g with major analysed compounds: 1,8-cineole (42.6 % w/w), α-pinene (10.7 % w/w) and globulol (5.9 % w/w). Concentration of total volatiles in extracts varied from 2.8 to 59.5 % w/w depending on the extraction and fractionation conditions. Major volatile compounds in the isolates were 1,8-cineole (0.01 to 27.63 % w/w), globulol (0.01 to 9.84 % w/w), aromadendrene (0.01 to 11.52 % w/w) and *α*-pinene (0.02 to 3.69 % w/w). The isolate obtained at 50 °C and 9 MPa exhibited the highest acute toxicity LD_{50} = 35 μg for *Spodoptera littoralis* and LD_{50} = 38 μg for *Leptinotarsa decemlineata*. Significant differences between
the insects were observed regarding to antifeedancy when SFE extracts were more effective on the Potato Colorado Beetle.

INTRODUCTION

A great demand for natural products is observed mostly in food processing, food supplement production and in pharmaceutical industry. Bark and leaves of eucalyptus (*Eucalyptus grandis* L.), a tall tree belonging to the family Myrtaceae, have been used in traditional medicine for treatment of cold, influenza, fever, diarrhoea or toothache [1]. The eucalyptus leaves
are rich sources of essential oil, flavonoids or tanins [2], which are responsible for its insecticidal [3], repellent [4] and fumigant [5] activities assigned mostly to the content of α-pinene, 1,8-cineole and *p*-cymene. The major components of the extract from eucalyptus leaves are α-pinene, 1,8-cineole, *p*-cymene, α-terpineol, aromadendrene and guaiol [6].
Valuable substances are often obtained from plants by traditional separation techniques such as steam distillation or organic solvent extraction, although these methods are very ineffective in terms of energy consumption. In addition, use of relatively high temperatures during the process has influence on biological activity of isolates. Supercritical fluid extraction (SFE) using carbon dioxide provides high quality extracts obtained at moderate temperatures without traces of organic solvents. Composition of extracts varies depending especially on CO_2 density (varied by changes in pressure and temperature) during experiment.
Moreover, extract can be separated to several fractions with different chemical composition with help of several separation techniques. Among commonly used fractionation methods in the SFE belongs the use of series of separators maintained at different conditions. This technique with two separators has been applied to separate co-extracted waxes from volatile oils of *Foeniculum vulgare* [7], *Pistacia lentiscus* L [8] or *E. globulus* [6a], to name

a few. Another possibility how to fractionate extracts is to combine SFE with sorption. This procedure was applied to remove terpenes and other undesirable compounds from cold pressed citrus oils of mandarin [9], lemon [10], or orange peel [11]. Reverchon et al. [12] used supercritical CO_2 to desorb bergamot peel oil from silica gel. They applied operation conditions with two pressure steps, 7.5 MPa and 20 MPa, at 40 °C. The lower pressure was selective
to hydrocarbon terpenes while the oxygenated components were desorbed at the higher pressure. Commercial oil was initially adsorbed on a sorbent in most of these experiments, none of them used the SFE from plant with adsorption of extracted compounds onto a sorbent in one step.
This work is focused on the enrichment of volatile components in eucalyptus extracts by using different fractionation methods during SFE. The extracts obtained by SFE at (40-50 °C, 9-30 MPa) were compared with hydrodistillate in terms of their biological activity, yield of isolates and concentration of volatiles in them.

MATERIALS AND METHODS

Materials

Eucalyptus leaves were harvested in 2012 in Portugal and supplied by Sanovia a. s. (Czech Republic). The air dried leaves (7.5 % w/w of water) were ground in a laboratory blender (Warring Commercial 8010G), sieved using an analytical sieve shaker (Fritsch, Analyssete 3 Pro) to obtain particles under 1 mm in diameter, and stored in chest freezer in closed bottles. Carbon dioxide (> 99.9 %) was purchased from Linde Gas (Prague, CR). Silica gel (60Å, 63-200 μm, technical grade, Sigma-Aldrich, Steinheim, Germany) for adsorption experiments was dried in oven (Binder, ED53) at 105 °C for 20 h and stored in a glass exsiccator. Heptane p.a. used as a solvent for GC samples and hexadecane as internal standard were purchased from Sigma (Sigma-Aldrich, Steinheim, Germany).

Supercritical fluid extraction

The SFE experiments were carried out using a 150 mL extraction column (I.D. 30 mm) filled with 40 g of dried milled plant material placed between layers of glass wool and glass beads (I.D. 2 mm) serving as solvent flow distributors. The extractor was immersed in a temperature-controlled water jacket. The compressor (NovaSwiss 560.0007) with
the pressure regulator unit (NovaSwiss 560.0009) was used to pressurize CO_2 and to control the extraction pressure. The solvent flow direction through plant material was from the top to the bottom. This direction was chosen because it often accelerates the extraction, in particular at lower Reynolds numbers and for conditions near the critical point of CO_2 where natural convection is dominant [13]. The flow rate was measured with laboratory gas meter after expansion to ambient pressure in a heated micrometer valve and adjusted to 1.8 $g.min^{-1}$. The extract separated from gaseous CO_2 was collected in a glass vial cooled by a mixture
of ethanol and dry-ice (– 78 °C) in order to reduce the escape of volatile components with CO_2. The vials with extract were weighed, sealed and stored in a freezer until GC analysis. Three types of experimental set-up (simple extraction, use of additional separator and adsorption on silica gel, see Table 1) were used to influence the concentration of volatile components in extract.
Firstly, the extraction conditions (pressure and temperature) were optimised in the simple extraction experiment where the extractor was directly connected to the micrometer valve.

Secondly, the solution flowing out of the extractor was partially depressurized before entering an additional separator where a first fraction of extract was precipitated and the rest of extract with more soluble compounds was then completely depressurized and collected in the second separator as written above.
Finally, at sorption experiments, an additional column (I.D. 10 mm, 19.5 mL) with silica gel (6.5 g) was connected between the extractor and the micrometer valve. The conditions in the extractor and the column with silica gel were equal (40°C and 12 MPa) and the compounds that passed through silica gel were collected as the first fraction. The extractor was then by-passed and the second fraction was obtained by desorption of trapped compounds with pure CO_2 at 30 MPa and 40 °C. Finally, for elution of strongly bonded compounds, the conditions of 40 °C, 30 MPa and CO_2 modified with ethanol (15 % w/w) were used.

Table 1. Summary of extraction and separation conditions and carbon dioxide density [14]

Run	p [MPa]	T [°C]	ρ_{CO2} [14] [kg·m^{-3}]	Extraction time [min]	Additional separator	Fractionation method
1	9	50	285	150	-	No fractionation
2	12	40	689	150	-	
3	30	40	910	150		
4	12	40	689	150	9 MPa/0 °C	Series of separators
5	12	40	689	150	9 MPa/50 °C	
6/1	12	40	689	200	-	Adsorption
6/2	30	40	910	300	-	Desorption
6/3*	30	40	910	100	-	Desorption

* Addition of 15% w/w ethanol as modifier

Hydrodistillation

The content and composition of essential oil in dry milled plant material were determined by hydrodistillation (HD). Eucalyptus leaves (60 g) were distilled with 600 mL of water for 3 hours, a sufficient time to complete the isolation of essential oil.

Gas chromatography

The isolates were analysed by GC-MS and GC-FID. Identification of components was based on the comparison of their mass spectra and retention indices with published results [15]. GC-MS: the analyses were performed on an Agilent 6890 gas chromatograph coupled to Agilent 5973 mass spectrometer operating in 70eV ionization mode. DB-5MS column (30 m × 0.25 mm × 0.25 μm) was used with He as carrier gas. GC-FID analyses were performed on a Hewlett Packard 5890 gas chromatograph equipped with a DB-5 column (30 m × 0.25 mm × 1 μm) and using N_2 as carrier gas.

Bioassay

Three basic insecticidal assays (antifeedancy, acute and chronic toxicity) were selected to assess biological activity of eucalyptus isolates on Mediterranean Brocade (*Spodoptera littoralis*) and Potato Colorado Beetle (*Leptinotarsa decemlineata*) larvae. To determine the acute toxicity, the isolates were dissolved in acetone and the solution was applied topically. In case of chronic toxicity, the isolates were mixed into a 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 assessed by no-choice test using leaf discs and was 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. More details on insecticidal testing were given in the previous work [16].

RESULTS

Isolate yield and composition

The total yield of isolates and composition of volatiles varied depending on used fractionation method as shown in Figure 1.

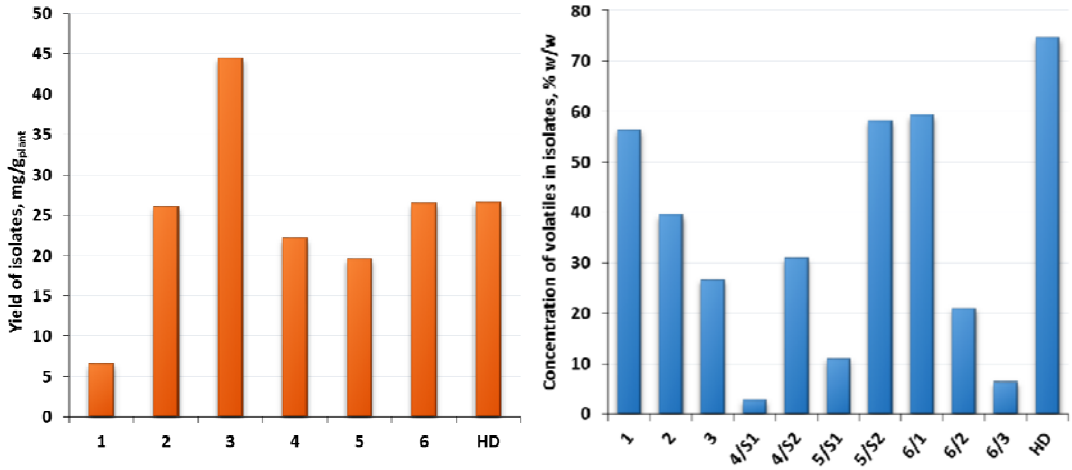

Figure 1. Yield of isolates (■) and concentration of GC-detected compounds in isolates (■) obtained by simple extraction (1-3), SFE with additional separator (4-5), sorption on silica gel during SFE (6) and hydrodistillation (HD).

The content of essential oil in dry eucalyptus leaves was 26.7 mg/g. In the simple extraction experiment (run 1-3), the extract yield increased with increasing CO_2 density but the selectivity towards volatiles was radically decreasing. The use of additional separator at conditions 9 MPa and 50 °C led to increased concentration of volatiles in extract by 20 % w/w compared to simple extraction. In the sorption experiment, volatile compounds were concentrated in the first two fractions whereas most of the non-volatile compounds were eluted with CO_2 modified by ethanol into the third sample.

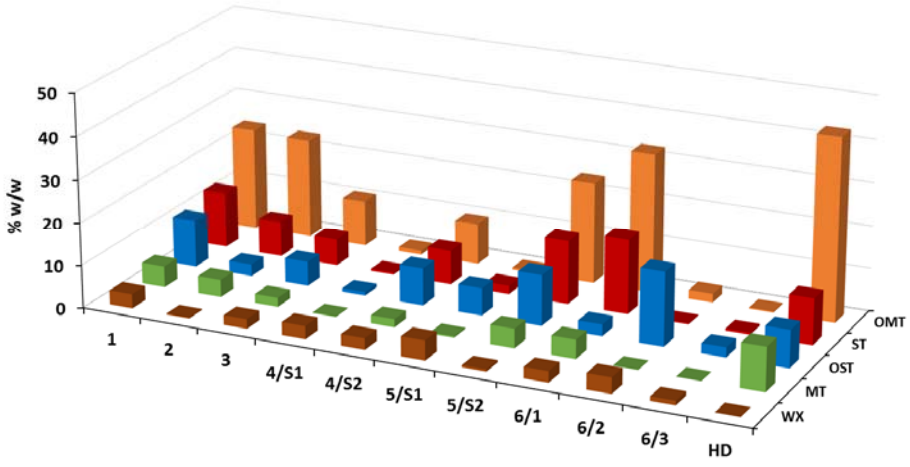

Figure 2. Concentration (% w/w) of major compounds groups in isolates obtained by different methods: simple extraction (run 1-3), use of additional separator (run 4-5), sorbent fractionation (run 6) and hydrodistillation (HD). MT: monoterpenes, OMT: oxygenated monoterpenes, ST: sesquiterpenes, OST: oxygenated sesquiterpenes, WX: waxes.

The concentrations of selected groups of compounds obtained by use of mentioned separation techniques are represented by Figure 2. The use of additional separator at conditions 9 MPa and 50 °C resulted into the increase in sesquiterpene concentration in extract by 6.6 % w/w and oxyxenated sesquiterpene concentration by 9 % w/w, compared to simple extraction. The adsorption was effective in terms of separation of monoterpenes, oxygenated monoterpenes and sesquiterpenes in the first fraction, whereas the oxygenated sesquiterpenes represented mostly by globulol were desorbed from silica gel with pure supercritical carbon dioxide
at conditions 30 MPa and 40 °C.

Insecticidal activity

Only essential oil and CO_2 extract obtained at conditions 9 MPa and 50 °C were effective in terms of acute toxicity for both kinds of insects, the toxicity of the extract was slightly higher (Mediterranean Brocade: LD_{50}=38 µg, Colorado Potato Beetle: LD_{50}=35 µg). The chronic toxicity and antifeedancy results for Mediterranean Brocade and Colorado Potato Beetle
are shown in Figure 3. Although CO_2 extracts contained less volatiles than essential oil, their chronic toxicity and antifeedancy were up to four times, respectively ten times higher. This phenomenon could be caused by synergic effect of non-volatile compounds which together with volatiles can increase insecticidal activity of extracts. As regards the antifeedancy, Colorado Potato Beetle is more sensitive to eucalyptus isolates than Mediterranean Brocade.

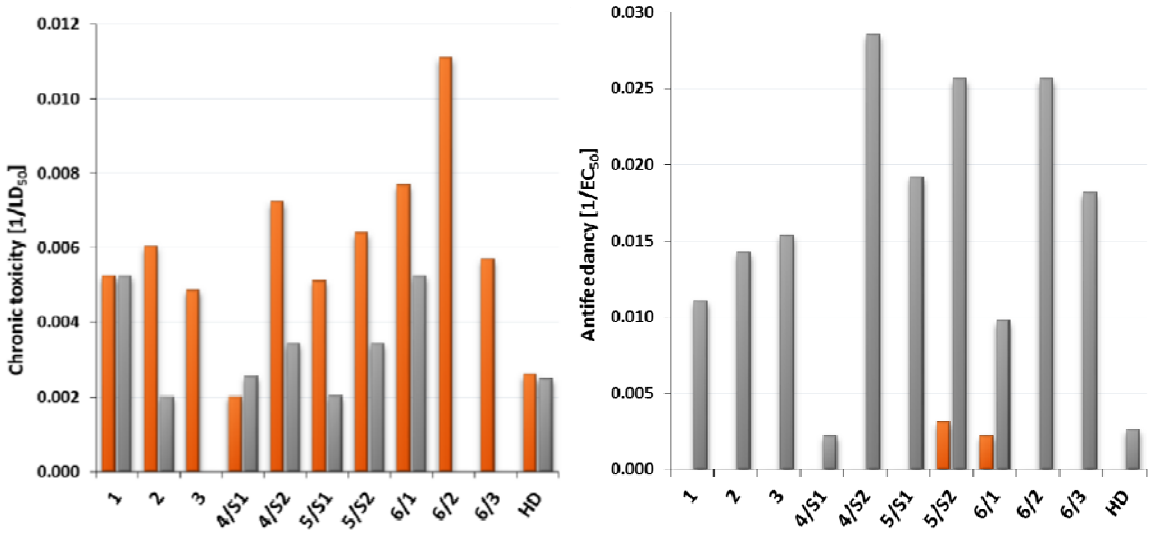

Figure 3: Insecticidal activity (antifeedancy [$cm^2.\mu g^{-1}$], chronic toxicity [$cm^2.\mu g^{-1}$]) of isolates obtained by different methods (1-3 simple extraction, 4-5 use of additional separator, 6 sorbent fractionation, HD hydrodistillation) against Mediterranean Brocade (■) and Colorado Potato Beetle (■).

CONCLUSION

Combination of SFE together with sorption on silica gel led to the concentration of volatile compounds into the first fraction which contained 1,8-cineole (27.6 % w/w), aromadendrene

(11.52 % w/w), and α-pinene (3.69 % w/w) while the second fraction contained globulol (9.85 % w/w).

The use of additional separator led to the separation of monoterpenes, oxygenated monoterpenes and sesquiterpenes from other less volatile and non-volatile components of extract.

Acute toxicity was observed only at essential oil and CO_2 extract obtained at 9 MPa and 50 °C. CO_2 extracts exhibited the stronger insecticidal activity than essential oil in terms of chronic toxicity for Mediterranean Brocade and antifeedancy for Colorado Potato Beetle which can be caused by synergic effect of non-volatile components in the extracts of *E. grandis*.

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