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Extracting the natural insecticide abietadiene from *P. pinaster* branches using $scCO_2$

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ABSTRACT

The interest in forest residues has increased in recent years mostly due to their potential uses as value-added products for food, cosmetic and pharmaceutical industries, as well as for other applications such as pest management strategies. Maritime pine (*Pinus pinaster*) is a native conifer to Portugal and its chain-of-value (which includes its products and residues valorization) presents relevant high economic impacts in Portugal. In this work, supercritical carbon dioxide ($scCO_2$) extraction of volatile compounds from *P. pinaster* branches was carried out using an experimental design based on distinct values of temperature and CO_2 density. Emitted volatiles from pine branches were also analyzed by solid-phase microextraction - gas chromatography/mass spectrometry (SPME-GC/MS) for comparison purposes. Extracts were collected for a total period of six hours and extraction yields were determined. Total phenolic contents, and both antioxidant and indirect insecticidal activities of the obtained extracts were determined spectrophotometrically. Extracts compositions were obtained by GC/MS. Results revealed that an increase in temperature or solvent density led to higher extraction yields (up to 6% w/w, dry basis). Abietadiene was the main volatile substance identified in the obtained $scCO_2$ extracts. This compound is the precursor of abietic acid, which is produced by conifer species as a defensive secretion against several insect and pathogen attacks. The main chemical substances emitted from pine branches included α-pinene, β-pinene, and β-caryophyllene. Extracts total phenolic contents were between 1.2% and 3.3% (mg of gallic acid equivalents/mg extract), and the DPPH assay presented IC_{50} values close to 1200 µg/mL. The obtained extracts presented potential insecticidal properties, as denoted by the acetylcholinesterase activity inhibition results: IC_{50} values between 2.8 and 10.7 mg/mL. Therefore and in conclusion, these extracts showed potential to be applied for insect control strategies.

INTRODUCTION

Plant essential oils have been gaining interest as flavors for food and pharmaceutical industries which makes them good candidates for additives/excipients. Moreover, some essential oils and emitted volatiles from plants are easily detected by insects being used in eco-friendly pest management strategies, with less impact on environment and health than synthetic insecticides [1]. Plant essential oils are mainly composed by monoterpenes and to a lesser extent by sesquiterpenes. Monoterpenes are widely known constituents of

conifers and citrus and can act as toxins, as feeding and oviposition deterrents, or as attractants to pollinators. Particular attention has been given to limonene, which is used to control ectoparasites of pet animals and which has also activity against a number of insects, mites and microorganisms, and also to linalool, which has antifungal properties and is used as food additive [2]. Trees from *Pinus sp.* are volatile oil-bearing plants whose wood processing and pruning residues have been studied as a source of value-added products including biochemicals. Moreover, studies from different pine species have reported terpenes/terpenoids, alkanes, alcohols and aldehydes as the main emitted volatiles from different parts of the tree, having relevant ecosystems functions [1, 3]. Among all pine residues, tree bark and needles have been the most intensively studied. Only a few studies have focused on the investigation of volatile oils from pine tree branches, which are an easily collected pruning residue corresponding to the needles *plus* wood *plus* bark [4].
The aim of this study is the identification of the volatile compounds of a forestry residue (*P. pinaster* branches) and the comparison of the volatiles naturally emitted (detected by SPME-GC/MS) with the ones extracted by $scCO_2$ extraction. The extractions were performed according to an experimental design and the extracts were evaluated regarding extraction yields, total phenolic contents, antioxidant and insecticidal potential activities.

MATERIALS AND METHODS

Raw material and chemicals. Maritime pine branches were collected in Coimbra (Portugal). Before being subjected to $scCO_2$ extraction, the branches were milled into a < 2 mm fraction (cross beater mill, Retsch, Germany) and then frozen. Humidity was evaluated, in triplicate, by thermogravimetric analysis (TGA, TA Instruments, model Q500). The chemicals that were used for the experiments included: carbon dioxide (≥99.5%); dichloromethane (≥99.9%, HPLC grade); chloroform (≥ 99%); ethyl acetate (≥99.9%, HPLC grade); Folin-Ciocalteu's reagent; sodium carbonate; gallic acid (≥ 98%); 1,1-diphenyl-2-picrylhydrazyl (DPPH); acetylcholinesterase (AChE, Type VI-S, 500 U/mg protein); 5,5'-dithiobis[2-nitrobenzoic acid] (≥ 98%); acetylthiocholine iodide (≥ 98%); ethanol; tris(hydroxymethyl)aminomethane (Tris Buffer) and bi-distilled water.

$ScCO_2$ extraction and characterization. Milled branches were placed in a thermostatic stainless steel cell. Extractions were performed, in duplicate, for 6 hours (in addition to a 15 min static period) in a lab-scale extractor (Separex, France) [5]. The CO_2 flow rate was around 7.5×10^{-5} kg/s and the solid-to-solvent ratio was ~1:360, w/w (dry basis, d.b.). The extracts were collected into refrigerated flasks and an adsorbent column was placed after the collection flask to recover some non-retained volatiles. Total yields were calculated as the ratio between the total extract mass (including the extract recovered in the collection flask during the 6 hours, in the adsorbent column and from the cleaning of the system) and the raw material mass, on a dry basis. The solvent density and temperature were set up according to an experimental design. Three levels were considered for temperature (35, 45 and 55 °C) and for CO_2 density (600, 750 and 900 kg/m^3). The extracts were stored at – 20 °C and protected from light, until further analysis.

The volatile fraction that was naturally emitted by *P. pinaster* branches was determined by SPME-GC/MS (headspace mode). The desorption occurred in the injection port of the GC/MS for 1 min at 250 °C and the separation was achieved on a DB-5MS fused silica capillary column, using helium as the carrier gas, at a 1 mL/min flow rate. Identification of compounds was based on the comparison between the mass spectra of the substances

with the data bank of Mass Spectra Libraries (NIST and Flavors and Fragrances of Natural and Synthetic Compounds (FFNSC2.L)). The identified compounds were then compared with those obtained by $scCO_2$ extraction. Essential oils obtained by $scCO_2$ extraction were analyzed by GC/MS in ethyl acetate (1 mg/mL) with an injection volume of 0.2 µL.

Total phenolic contents were determined by the Folin-Ciocalteu method and the results were expressed as gallic acid equivalents in percentage (mg GAE/mg extract × 100). For the antioxidant evaluation, the DPPH scavenging technique was used and the results were expressed as IC_{50} values. Insecticidal potential of the extracts was determined *in vitro* by the acetylcholinesterase (AChE) activity inhibition assay, and these results were presented as IC_{50} values [6]. It should be noted that the mechanism of toxicity of most insecticides is based on the inhibition of AChE [7].

RESULTS

Humidity of milled branches (Figure 1B) was close to 45% (w/w).

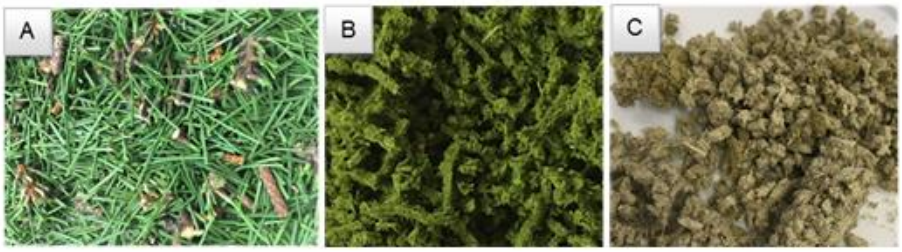

Figure 1. Pictures of the *P. pinaster* branches after being cut into portions of 2-4 cm (A); milled (< 2 mm) (B); and processed by $scCO_2$ extraction (C).

Total yield values ranged from 0.77 ± 0.04% to 5.8 ± 0.7% (w/w, d.b.) (Figure 2), with higher yields achieved for higher density and temperature values. These findings are in accordance with literature results for $scCO_2$ extraction from several plant materials, which report higher extraction yields for CO_2 densities above 700 kg/m^3 [5]. These yield values are in agreement with the ones of $scCO_2$ extracts obtained from *P. pinaster* bark (0.97 – 1.37%) [8] and from *P. nigra* needles (1.60%) [9].

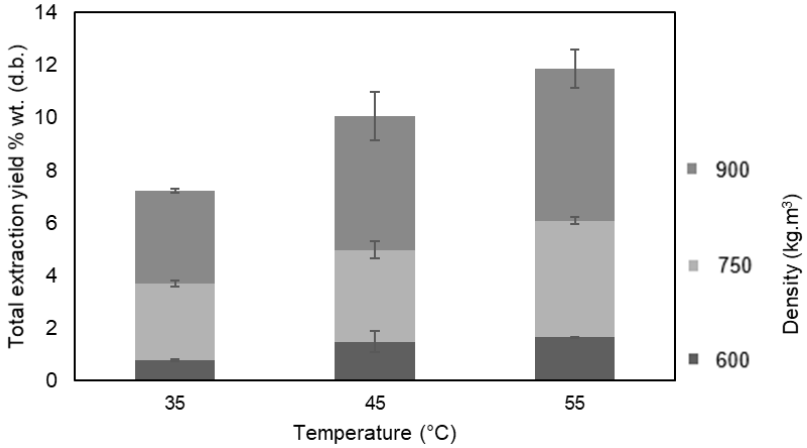

Figure 2. Total yield for extracts obtained at distinct temperatures (°C) and solvent densities (kg/m^3).

Regarding the analysis of the volatile fraction naturally emitted by the *P. pinaster* branches (SPME-GC/MS), many terpenes were identified, including: 1. α-pinene; 2. β-pinene; 3. β-myrcene; 4. limonene; and 5. β-caryophyllene; * peak attributed to the fiber (Figure 3).

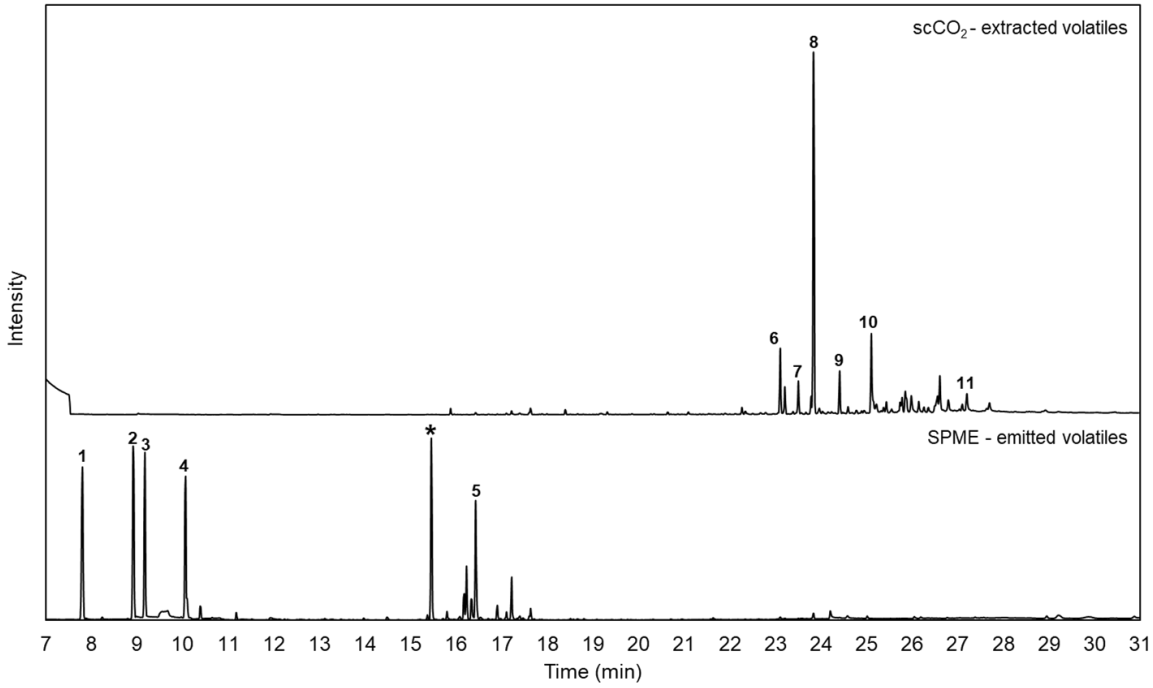

Figure 3. Composition profile of $scCO_2$ extracts (35 °C and 600 kg/m^3) and emitted volatiles from *P. pinaster* branches.

The main compounds identified from the GC/MS analysis of the $scCO_2$ extracts are also represented in Figure 3 and include, among others: 6. rimuene; 7. dehydroabietane; 8. abietadiene; 9. abieta-(8(14),13(15)-diene) 10. labda-8(17),13Z-dien-15-ol; and 11. abietic acid. Interestingly, the $scCO_2$ was selective for the compounds that were identified between 22 and 28 min. Moreover, the abietadiene appears as the main volatile for all the $scCO_2$ extraction conditions. This compound is the precursor of abietic acid, which is produced by conifer species as a defensive secretion against insect and pathogen attack [10]. These findings are in accordance with the results already obtained by other authors, since abietadiene was also an important component of the *P. pinaster* essential oils from wood, cones and needles [11].

Concerning the results achieved for the extracts characterization, the DPPH assay revealed IC_{50} values close to 1200 µg/mL, a reduced antioxidant activity when compared to $scCO_2$ extracts obtained from aerial parts of other plants, which presented IC_{50} values between 186.34 and 397.93 µg/ml [12]. Therefore, the antioxidant activity of these extracts should be confirmed by other methods based on different mechanisms of action.

Total phenolic contents ranged from 1.2 ± 0.1 to 3.3 ± 0.1% (mg GAE/mg extract, d.b.). These low contents may be related to the higher polarity of phenolic compounds when compared to the one of $scCO_2$. Higher phenolic contents may be obtained by adding a co-solvent in the extraction process, such as ethanol.

The AChE assay presented IC_{50} values ranging from 2.83 ± 0.02 to 10.7 ± 0.4 mg/mL. These IC_{50} values are lower than the ones obtained for two commercial insecticides (IC_{50} ~13 mg/mL), which reveal the insecticidal potential of the extracts. These findings may be attributed to the main volatile compound of the extracts, abietadiene. To confirm the insecticidal activity of this compound, it should be isolated and then evaluated through the AChE assay.

CONCLUSIONS

Volatile oils were successfully extracted from *P. pinaster* branches using $scCO_2$, a green solvent. Both temperature and solvent density favored extraction yields. AChE assay results highlighted the potential of these extracts to be applied as natural insecticides on forest pest management strategies. Abietadiene, the main volatile compound of these extracts, may have an important role in this insecticidal activity, but additional studies should be performed to isolate this compound and confirm this hypothesis. The addition of a co-solvent such as ethanol may enhance the extracts phenolic contents and consequently their antioxidant activity.

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