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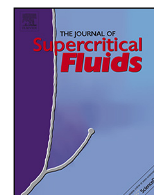

Allelopathic properties of the fractions obtained from sunflower leaves using supercritical carbon dioxide: The effect of co-solvent addition

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ABSTRACT

The work described here is a continuation of a previous study centered on the extraction, using supercritical carbon dioxide, of bioactive substances from sunflower leaves of the *Helianthus annuus* L. variety *Arianna*. In this study the addition of 9% of ethanol as co-solvent was analyzed. The extraction was carried out (P = 100/400 bar, T = 35/55 °C, ethanol = 9%) in order to analyze the influence of pressure, temperature and sample pre-treatment on the extraction yield and bioactivity of the extracts. The addition of 9% of ethanol to the supercritical solvent enhanced both the extraction yield and the biological activity of the extracts. The best conditions were a pressure of 400 bar and a temperature of 55 °C. In an effort to improve the bioactivity of the extract, a cascade fractionation of the extracts was carried out and this gave different results in terms of biological activities and extraction yields. The phytochemical compositions of the extracts were analyzed by thin layer chromatography. The fractionation that gave the best results was carried out at 90 bar and 40 °C in the first separator. Finally, the effect of extracts on the growth of seeds from different plants was analyzed.

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1. Introduction

The current interest in pollution-free agriculture has led to the prohibition or restriction of several herbicides and this has encouraged the search for natural herbicides. Vegetable products such as composite allelochemicals constitute an attractive source of active compounds, not only due to their chemical diversity but also for their biological action as pesticides and their less harmful nature for the environment and human health. This latter advantage arises because such compounds are easily biodegradable and many of them are safe and clean. The number of functional compounds recovered from plant raw material depends on both the plant type and the method used for the extraction. The sunflower is undoubtedly one of the most useful plants both in terms of the amount of plant matter that can be obtained from a widespread crop and the large number of metabolites that it contains, principally in the leaves of the plant [1,2]. Extraction with supercritical fluids (SFE) has been used as an alternative to conventional methods of extraction and/or fractionation. The addition of modifiers or co-solvents to CO_2 improves the efficiency of the extraction by increasing the solubility of the solute and the extraction yields [3]. Among the organic solvents, ethanol has been widely used as a co-solvent in the extraction of bioactive compounds due to its low toxicity in comparison to other alternatives [4,5].

One of the easiest ways to assess allelopathic properties is to employ bioassays, in which the germination or emergence of seedlings is quantified and the length of the root or stem is measured. Various types of bioassay have been reported in the literature, including wheat coleoptile bioassays [1,6] and others related to phytotoxicity [7] and hydroponic conditions [8].

The bioactive properties of supercritical extracts have been reported in numerous papers [9–15]. The supercritical extraction of sunflower leaves to give an extract with a high bioactive capacity has been studied recently. The effects of pre-treatment, pressure, temperature and co-solvent addition have been analyzed and good results have been obtained [3,9–11]. Nevertheless, in these studies the bioactivity of the extract was only assessed in a general bioassay and allelopathic properties were not analyzed apart from the results in the previous publication in this series [12]. In the previous work we extracted bioactive substances from the leaves of

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three varieties of sunflower (*Stella*, *Hilian* and *Arianna*) using pure CO_2 as solvent. The extractions were carried out at temperatures of 35 °C and 55 °C and pressures of 100 bar and 400 bar. The results indicated that the best extraction yields and activity profiles were obtained with the *Arianna* variety at a pressure of 400 bar and a temperature of 55 °C. The results also showed that fractionation of the resulting extracts improves their biological activity, but the potential allelopathic properties of the fractionated extracts are not comparable to those of a commercial herbicide (Logran®).

In an effort to improve on the previous results, the objective of the present study was to increase the extraction yields through the addition of 9% (v/v) ethanol to carbon dioxide and to increase the biological activity of the extracts obtained. The fractionation process was investigated in cascade using ethanol as co-solvent under different conditions of pressure and temperature. The allelopathic properties of the resulting extracts were analyzed using three bioassays: (i) general activity bioassays on wheat coleoptiles, (ii) a phytotoxicity bioassay carried out on tomato, lettuce, onion and watercress seeds, and (iii) a bioassay carried out under hydroponic conditions. The results were compared with those obtained with a synthetic commercial herbicide (Logran®) and with the results reported in the previous paper, where extracts were obtained with carbon dioxide alone as solvent [12].

2. Materials and methods

2.1. Raw material

The raw material used in this study consisted of sunflower leaves (*Helianthus annuus* L.) of the variety *Arianna*. The leaves were cultivated at the 'Rancho de la Merced' of the Andalusia Institute of Agricultural and Fishery Research and Training (Junta of Andalusia, Jerez de la Frontera, Spain). The leaves were collected manually in June 2009 during the third growth stage of the plant (plant with flowers, around 1 m in height), since it has been found that at this stage the plant shows greater phytotoxicity [2]. One portion of the leaves was congealed at −25 °C and the other portion was air-dried on blotting paper to constant weight.

2.2. Supercritical fluid extraction

The first part of the study into extraction conditions (concerning the influence of pressure and temperature on the extraction yields obtained from dried and congealed samples) was carried out in equipment supplied by Thar Technology (Pittsburgh, PA, USA, model SF100). The equipment used included an extraction vessel (capacity of 100 mL) and two double piston pumps with a maximum flow rate of 50 g/min, one for CO_2 and the other for co-solvent. A thermostatic jacket allowed control of the extraction temperature. The cyclonic separator allowed the periodic discharge of the extracted material during the SFE process. The procedure used is the same as that described in the previous publication [12] but with the addition of ethanol (99.9% purity, Panreac) as co-solvent. Extracts were dried at the same temperature as applied in the extraction process in a rotary evaporator under vacuum.

The second part of the study (fractionation process) was carried out in a Thar Technology SF2000 system equipped with an extractor (2 L capacity), two cyclonic separators (500 mL each) connected in series, and with two high pressure pumps; one for CO_2 (P200 model), capacity of 200 g/min, and the other for the co-solvent (P50 model).

2.2.1. Extraction and fractionation conditions

The extractions were conducted at temperatures of 35 and 55 °C and pressures from 100 to 400 bar on congealed and dried samples. The flow rate measured for the supercritical fluid was 20 g/min of CO_2 and 2 g/min of ethanol during 3 h. This amount of co-solvent was selected because it is the minimum amount of ethanol that can be supplied by the pump. In previous studies with other systems, we concluded that a minimum amount of co-solvent gives better bioactivity results [3].

The same solvent system, *i.e.* a mixture of CO_2 and 9% (v/v) ethanol, was used for the fractionation. The best extraction conditions were used in the extractor (55 °C and 400 bar) and four fractionation conditions were tested. These conditions are listed below:

- Extraction 1 (E1) – conditions in separator 1: 200 bar and 45 °C, atmospheric conditions in separator 2.
- Extraction 2 (E2) – conditions in separator 1: 100 bar and 40 °C, atmospheric conditions in separator 2.
- Extraction 3 (E3) – conditions in separator 1: 90 bar and 40 °C, atmospheric conditions in separator 2.
- Extraction 4 (E4) – conditions in separator 1: 70 bar and 40 °C, atmospheric conditions in separator 2.

2.3. Bioassays

Three different bioassays were employed to test the extracts obtained. The methodology involves the preparation of solutions with different concentrations of the extract (1000 ppm, 500 ppm, 250 ppm, 125 ppm, 75 ppm for the coleoptile bioassay and 800 ppm, 400 ppm, 200 ppm for phytotoxicity tests in Petri dishes and hydroponic bioassay conditions) and the addition of coleoptiles or seeds. The length of the coleoptiles or the length of the root or stem is then evaluated. A more detailed description of the bioassays is presented in a previous publication [12].

Coleoptile bioassay: This is a relatively simple test used as a step prior to the evaluation of bioactive agents. The test involves analysis of the elongation of apical zones of cereals (wheat in this case) in the presence of liquid media containing the potentially allelopathic agents.

Phytotoxicity tests in Petri dishes: This assay reproduces the natural conditions for the performance of products to be tested on the seeds of selected plant species. The range of concentrations used allows an evaluation of the relationship between the phytotoxic activity of the product and its concentration. The selection of species is detailed in the bibliography [7]. The species tested were as follows: monocot *Allium cepa* L. (onion) and the dicots *Lepidium sativum* L. (watercress), *Lactuta sativa* L. (lettuce) and *Lycopersicum esculentum* L. (tomato).

Hydroponic bioassay conditions: Researchers have used this technique to study the secondary metabolites produced by plants grown under hydroponic conditions with a nutrient solution used as support [8]. This assay is a higher step to examine the activity of the selected statement and determine the potential of metabolites as a basis for the future design of model natural herbicides.

2.4. Thin layer chromatography

Thin layer chromatography (TLC) was carried out on Alugram Sil G/UV254 (Macherey Nagel) 0.25 mm thick plates. The detection of the different compounds was carried out by exposure to UV light (254–365 nm) and plates were developed chemically with oleum prepared with sulfuric acid (Panreac 98%), water and acetic acid (Panreac 100%) (1:4:20) with subsequent heating at 150 °C. Mixtures of hexane/acetone (60:40 (v/v)) were used as the eluent.

2.5. Cluster analysis

Statistical treatments were performed using the program STATGRAPHICS Plus 4.0 (Statistical Graphics Corp. 1994–1999).

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