Ultrasonics Sonochemistry 21 (2014) 1578–1584

Contents lists available at ScienceDirect

Ultrasonics Sonochemistry

journal homepage: www.elsevier.com/locate/ultson

Bio-guided optimization of the ultrasound-assisted extraction of compounds from *Annona glabra* L. leaves using the etiolated wheat coleoptile bioassay

Sadao Matsumoto [a], Rosa M. Varela [b], Miguel Palma [c,*], José M.G. Molinillo [b], Inês S. Lima [a], Carmelo G. Barroso [c], Francisco A. Macías [b]

[a] Department of Botany, Federal University of São Carlos, CP 676, CEP 13565-905 São Carlos, São Paulo, Brazil
[b] Department of Organic Chemistry, Faculty of Science, Agrifood Campus of International Excellence (ceiA3), University of Cádiz, 11510 Puerto Real, Cádiz, Spain
[c] Department of Analytical Chemistry, Faculty of Science, Agrifood Campus of International Excellence (ceiA3), University of Cádiz, 11510 Puerto Real, Cádiz, Spain

ARTICLE INFO

Article history:
Received 22 November 2013
Received in revised form 6 January 2014
Accepted 25 January 2014
Available online 2 February 2014

Keywords:
Bio-guided extraction
Ultrasound-assisted extraction
Annona glabra
Allelopathic activity

ABSTRACT

A bio-guided optimization of the extraction of bioactive components from *Annona glabra* leaves has been developed using the etiolated wheat coleoptile bioassay as the control method. The optimization of an ultrasound-assisted extraction of bioactive compounds using allelopathy results as target values has been carried out for the first time. A two-level fractional factorial experimental design was applied to optimize the ultrasound-assisted extraction. The solvent was the extraction variable that had the most marked effect on the resulting bioactivity of the extracts in the etiolated wheat coleoptile bioassay. Extraction time, extraction temperature and the size of the ultrasonic probe also influenced the bioactivity of the extracts. A larger scale extraction was carried out in the next step in the allelopathic study, i.e., the isolation of compounds from the bioactive extract and chemical characterization by spectroscopic techniques, including NMR. Eight compounds were isolated and identified from the active extracts, namely two steroids (β-sistosterol and stigmasterol), five diterpenes with the kaurane skeleton (*ent*-kaur-16-en-19-oic acid, *ent*-19-methoxy-19-oxokauran-17-oic acid, annoglabasin B, *ent*-17-hydroxykaur-15-en-19-oic acid and *ent*-15β,16β-epoxy-17-hydroxy-kauran-19-oic acid) and the acetogenin asimicin.

The most active compound was annoglabasin B, which showed inhibition with values of −95% at 10^{-3} M, −87% at 5×10^{-4} M and greater than −70% at 10^{-4} M in the etiolated wheat coleoptile bioassay.

1. Introduction

Annona glabra is a tropical fruit tree from the family Annonaceae and it is in the same genus as the Soursop (*Annona muricata*) and Cherimoya (*Annona cherimola*) [1]. The tree is native to Florida in the United States, the Caribbean, Central and South America, and West Africa. *A. glabra* grows in swamps, is tolerant of saltwater, and cannot grow in dry soil.

The fruit of *A. glabra* is edible and it has a pleasant taste and fragrant pulp. The spherical fruit is similar in size to an apple or it may be slightly larger. The consumption of this fruit is usually local and it has not achieved the popularity of other fruits of the same genus. The fruit has been reported to have anticancer [2], antimutagenic [3] and antioxidant properties [4–6].

In recent years, the family Annonaceae has been studied in the search for bioactive compounds. For example, annonaceous acetogenins represent a class of compound with a wide variety of biological activities, including insecticidal behavior and inhibition of lymphocytic leukemia, carcinoma cells and mitochondrial complex I [7–9].

A. glabra L. is reported to have parasiticidal and insecticidal activity and it is used in traditional medicine [10,11]. Bioactive compounds have been isolated from different parts of this species. For example, fruit methanol extracts afforded diterpenes that inhibited mitochondrial cells, cancer cells and HIV reverse transcriptase and replication [12,13]. Ethanol extracts from seeds were found to be potent inhibitors of complex I of the mitochondrial respiratory chain [14] and were active against *Biomphalaria glabrata* (mollusc) growth [15]. The stem is also a source of bioactive substances: hexane extracts presented insecticidal, sporicidal and cytotoxic activities [16] and also showed larvicidal activity against *Aedes aegypti* [10].

* Corresponding author. Tel.: +34 956 01 63 60; fax: +34 956 01 64 60.
E-mail address: miguel.palma@uca.es (M. Palma).

http://dx.doi.org/10.1016/j.ultsonch.2014.01.024
1350-4177/

Acetogenins isolated from leaves were reported to be active against several tumor cells [11,17]. A number of metabolites were also isolated from this species, including monoterpenoids, diterpenoids, and cyclopeptides among others, but the activities of these compounds have not been reported to date [18–22]. Despite the large number of compounds isolated from *A. glabra*, very little research has been carried out on the allelopathic activity of its chemical components.

As mentioned above, *A. glabra* is a highly problematic invasive species that typically grows in estuaries and chokes mangroves. Seedlings cover the edges of mangroves and prevent the germination of other species. *A. glabra* affects farms as it grows along fences and also invades and transforms undisturbed areas [23]. As a result of these problems, a number of specific programs have been developed to control this species [13]. Recently, a study of *A. glabra* showed the allelopathic potential of leaf extracts against crop pests and etiolated wheat coleoptiles [24] and these results can be related to the level of invasiveness. Allelopathic studies carried out in the laboratory are based on phytotoxicity assays of compounds that can be extracted, isolated and identified from a plant. These assays depend on the amount of compound available and are carried out prior to fractionation, isolation and identification. It is therefore important to obtain extracts in which the concentrations of the active components are high.

Several advanced extraction techniques can be applied to yield bioactive extracts and these include ultrasound-assisted extraction (UAE), supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE). UAE is a very common extraction technique for the recovery of active components, mainly due to the mild extraction conditions applied [25]. Cavitation is the ultrasound mechanical effect that enables greater penetration of solvent into the sample [26]. Additionally, UAE methods allow the recovery of compounds in shorter times and at lower temperatures. Several examples have been reported for other techniques in which the destruction of active molecules occurs due to high extraction temperatures [27–29]. Therefore, the UAE technique is advantageous for the extraction of heat-sensitive active compounds.

The ultrasound-assisted extraction of oils from chickpea (*Cicer arietinum* L.) [30], saponins from ginseng (*Panax quinquefolium* L.) [31] and polysaccharides [32] and phenolic compounds such as corilagin from longan (*Dimocarpus longan* Lour) pericarp [33] have been reported. Under the optimal conditions, i.e., 85% acidified ethanol with the aid of ultrasonication, a higher extraction yield from longan pericarp has been obtained in comparison to the conventional extraction approach [33]. Likewise, Zhong and Wang [34] optimized an ultrasound-assisted extraction process to obtain polysaccharides from dried longan pulp using a response surface methodology. The results obtained demonstrate that ultrasound-assisted extraction is more effective than conventional techniques for the extraction of bioactive compounds from longan pericarp. A review of the literature showed that the optimization of ultrasound-assisted extraction using allelopathy results as the target values has not been reported previously.

When developing an extraction process it is important to optimize highly significant factors that affect the extraction in order to obtain the most active extract. In this respect, it is necessary to carry out an effective bioassay to assess the activity during the extraction process. The classical approach of changing one variable at a time and studying the effect of the variable on the response is a complicated technique that does not allow the evaluation of interactions between different extraction variables. Experimental design provides techniques that can be used for both the evaluation of the effects of extraction variables and interactions between them [35]. The bioassay selected in this study was the etiolated wheat coleoptile bioassay, which is both rapid (24 h) and sensitive. Furthermore, this bioassay can be considered as an initial assessment of phytotoxicity in which undifferentiated tissue cells are used [36–38]. This technique was proposed by Macías et al. as the first step in the search for potential new herbicides [39].

The aims of the work described here were (i) to define the best UAE conditions to obtain *A. glabra* extracts with the highest bioactivity and (ii) to isolate and identify the active compounds from the most active extract. This study would allow the identification of the secondary metabolites responsible for the allelopathic activity of *A. glabra*.

2. Materials and methods

2.1. Sample preparation

Dried *A. glabra* L. leaves were ground and stored in a refrigerator. Fat and wax were removed from the sample by washing 500 g of powder with hexane using an ultrasound system (7 mm diameter probe). Ultrasound parameters were fixed as follows: amplitude at 50% and cycle at 0.5 s^{-1} for 15 min without temperature control. The raffinate was dried in a laboratory oven (40 °C) and stored at 4 °C prior to extraction.

2.2. Ultrasound-assisted extractions

A high intensity probe ultrasound generation system of 200 W and 24 Hz (model UP 200S from Dr. Hielscher GmbH) was used for the extractions. The amplitude controller allowed the use of any power level in the range 10–100%. The cycle controller allowed the use of any cycle in the range 0.1–1.0 (fraction of a second). Two different probes were available: 2 and 7 mm diameter.

2.3. Etiolated wheat coleoptile bioassay

Wheat seeds (*Triticum aestivum* L. cv. Duro) were sown in 15 cm diameter Petri dishes moistened with water and they were grown in the dark at 25 ± 1 °C for 4 days [37]. The roots and caryopses were removed from the shoots. The latter were placed in a Van der Weij guillotine and the apical 2 mm were cut off and discarded. The next 4 mm of the coleoptiles were removed and used for bioassays. All manipulations were performed under a green safelight [38].

Extracts or compounds were dissolved in a buffer solution (1.1 g L^{-1} of citric acid and 2.9 g L^{-1} of calcium phosphate in distilled water at pH 5.6) containing 0.5 mL L^{-1} DMSO (dimethyl sulfoxide) and 20 g L^{-1} of sucrose. Three extract concentrations (800, 400, 200 ppm) and five concentrations of compounds (10^{-3}, 5×10^{-4}, 10^{-4}, 5×10^{-5}, 10^{-5} M) were used in the bioassay along with 0 ppm as a negative control and a commercial herbicide (Logran) as a positive control.

The commercial herbicide Logran, a combination of *N*-(1,1-dimethylethyl)-*N*-ethyl-6-(methylthio)-1,3,5-triazine-2,4-diamine (terbutryn, 59.4%) and 2-(2-chloroethoxy)-*N*-{[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl}benzenesulfonamide (triasulfuron, 0.6%), was used as the internal reference in accordance with a previously reported comparative study [39].

All determinations were run in three replicates, in which each test tube (replicate) was charged with 2 mL of one of the treatments and 5 coleoptile fragments. Coleoptiles were kept in contact with the extracts for 24 h. The resulting values for coleoptile growth in the presence of extract, along with negative control growth values, are presented as percentages, with positive values representing stimulation and negative values inhibition versus the control (0 ppm).

The coleoptiles were measured by digitalization of their images. Data were statistically analyzed using Welch's test [40]. Data are