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

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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].

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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

presented as percentage differences from control. Thus, zero represents the control, positive values represent stimulation of the studied parameter and negative values represent inhibition.

2.4. Extractions

An initial comparative study was carried out to select the best solvents. Six different solvents were used under the same extraction conditions: hexane, chloroform, ethyl acetate, acetone, methanol (MeOH) and water. The extraction conditions were as follows: 2 g of plant sample, 25 mL of solvent, 50% amplitude, 0.5 s^{-1} for cycle and an extraction time of 15 min. Temperature was not controlled during these extractions.

2.5. Experimental design for the evaluation of extraction variables

A two-level factorial fractional experimental design was applied. A total number of 16, rather than the possible 128, sets of extraction conditions were employed. Seven different extraction variables were evaluated in two levels (Table 1). In these extractions 1 g of plant material was used.

2.6. General experimental procedures

The purities of the compounds to be tested were determined by NMR and HPLC and they were found to be >98% pure. ^{1}H and ^{13}C NMR spectra were recorded using $CDCl_3$ as solvent on a Varian INOVA spectrometer at 399.99 and 100.577 MHz, respectively. The resonance of residual chloroform was set to δ 7.25. The solvent peak for ^{13}C was set to δ 77.00 (chloroform) and this was used as the internal reference. UV–vis spectra were obtained using a Varian Cary 50 BIO spectrophotometer with chloroform as the solvent. Mass spectra (EIMS) were recorded on a Voyager Thermoquest spectrometer. FTIR spectra were obtained on a Perkin–Elmer Spectrum BX FTIR system.

TLC – Alugram Sil G/UV_{254} plates (0.25 mm thickness) were used. The plates were analyzed with UV light (254 and 360 nm) and revealed by heating at 150 °C. Three revealers were used: oleum – sulfuric acid, H_2O and acetic acid (1:4:20); anisaldehyde – anisaldehyde, sulfuric acid, acetic acid, ethanol (25:25:1:450); and vanillin – vanillin, sulfuric acid, ethanol (1:1:18).

HPLC (High-performance liquid chromatography) – A Hitachi L-6020 system with a differential refractometer RI-71 detector was used. Columns: Semi-preparative column (LiChrospher SiO_2, Merck, 7 and 10 μm, 10 and 250 mm column length) with 3 mL min^{-1} as flow rate; Analytical Phenomenex Luna Column (10 μm Silica (2) 100A) with 1 mL min^{-1} as flow rate.

2.7. Compound isolation

For the isolation of compounds a large scale ultrasound-assisted extraction was carried out. In this process 20 g of plant sample and 500 mL acetone were used in conjunction with the optimized variables. A total of 1 kg of plant sample was used for extractions, with 8.4 g (DW) of material recovered and used to prepare the sample for column chromatography.

The extract with the highest bioactivity was selected and its components were isolated and characterized. Purification by column chromatography was monitored by TLC and this process afforded 17 fractions. The eluents used were hexane, hexane/acetone (5%, 10%, 15%, 20%, 40%, 60% and 80%), acetone and methanol.

Fraction 2 (257.3 mg) – This fraction was subjected to HPLC with a semi-preparative silica gel column. Hexane/ethyl acetate 15% was used as eluent to afford the major component 1, *ent*-kaur-16-en-19-oic acid (7.2 mg).

Fraction 3 (622 mg) – This was fractionated on a semi-preparative silica gel HPLC column, using hexane/ethyl acetate 20% as eluent. β-Sistosterol (**2**) (5.6 mg) and stigmasterol (**3**) (1.1 mg) were isolated.

Chlorophyll was removed from fractions 5–12 using mixtures of H_2O/MeOH as eluent on an RP18 column and dichloromethane used to elute the chlorophyll from the column. Free chlorophyll (fraction 8) was further fractionated by semi-preparative HPLC using hexane/ethyl acetate 25% to yield 18 mg of **4** (*ent*-19-methoxy-19-oxokauran-17-oic acid).

Fraction 9 provided 6.1 mg of **5**, 16α-hydro-19-acetoxy-*ent*-kauran-17-oic acid (annoglabasin B).

Fraction 10 was subjected to a semi-preparative HPLC column with hexane/ethyl acetate 60% to yield 1.3 mg of **6** (*ent*-17-hydroxykaur-15-en-19-oic acid) and 1.6 mg of **7** (*ent*-15β,16β-epoxy-17-hydroxy-kauran-19-oic acid).

Fraction 11 was fractionated on a semi-preparative HPLC column using hexane/ethyl acetate 40% to give 4.3 mg of annoglabasin B (**5**).

Fractions 13 and 14 were combined and subjected to column chromatography using hexane/acetone mixtures of increasing polarity to give 11 sub-fractions (13A to 13K). Fraction 13F was fractionated by column chromatography with hexane/ethyl acetate mixtures from 20% to ethyl acetate 100% as eluent to give 7 new sub-fractions. The major sub-fraction was submitted to a semi-preparative HPLC column with hexane/ethyl acetate 90% to give compound **8** (asimicin, 36.1 mg).

The bioactivities of the major compounds *ent*-kaur-16-en-19-oic acid (**1**), β-sistosterol (**2**) *ent*-19-methoxy-19-oxokauran-17-oic acid (**4**), annoglabasin B (**5**) and asimicin (**8**) were evaluated using the wheat coleoptile bioassay.

Table 1
Experimental conditions for the 16 extraction in the experimental design and respective activity on coleoptile bioassay (mean values in percentage and standard deviation).

Experiment	T. (°C)	Volume	Time (min)	Probe	Solvent	Amplitude	Cycle	800 ppm
1	5	25	5	Thin	MeOH	30	0.2	−56 ± 6.5
2	25	25	5	Thin	Acetone	30	0.8	−57 ± 4.5
3	5	50	5	Thin	Acetone	70	0.2	−67 ± 2.0
4	25	50	5	Thin	MeOH	70	0.8	−26 ± 1.0
5	5	25	15	Thin	Acetone	70	0.8	−57 ± 3.0
6	25	25	15	Thin	MeOH	70	0.2	−7 ± 5.5
7	5	50	15	Thin	MeOH	30	0.8	19 ± 0.5
8	25	50	15	Thin	Acetone	30	0.2	−36 ± 2.0
9	5	25	5	Thick	MeOH	70	0.8	−21 ± 4.5
10	25	25	5	Thick	Acetone	70	0.2	−45 ± 2.0
11	5	50	5	Thick	Acetone	30	0.8	−32 ± 0.5
12	25	50	5	Thick	MeOH	30	0.2	0 ± 12.5
13	5	25	15	Thick	Acetone	30	0.2	−49 ± 1.0
14	25	25	15	Thick	MeOH	30	0.8	0 ± 5.5
15	5	50	15	Thick	MeOH	70	0.2	−2 ± 3.0
16	25	50	15	Thick	Acetone	70	0.8	−20 ± 1.5

2.8. Calculation of IC_{50}

The activity data were fitted to a sigmoidal dose–response model (constant slope) by employing the GraphPad Prism v.4.00 software package (GraphPad Software Inc., La Jolla, CA, USA).

Statistical analysis. IC_{50} values were obtained after the activity data had been adjusted to concentration (logarithmic scale) to give a constant slope sigmoidal dose–response curve defined by the equation

$$Y = Y_{min} + \frac{Y_{max} - Y_{min}}{1 + 10^{\log IC_{50} - X}}$$

where X is the logarithm of concentration, Y is the response (activity) and Y_{max} and Y_{min} are the maximum and minimum values of the response, respectively. Goodness of fit is described by the determination coefficient (r^2).

3. Results and discussion

In an effort to identify the compounds responsible for the bioactivity properties of *A. glabra* leaves, the ultrasound-assisted extraction method was optimized using a factorial experimental design to highlight the most important variables that affect the bioactivity of the extracts. The etiolated wheat coleoptile bioassay was the method used to determine the bioactivity. The extraction variables that showed the highest effects on the bioactivity were subsequently studied. Finally, the extraction process was scaled-up to produce extracts with high levels of bioactivity. The components were isolated and their chemical structures were elucidated by spectroscopic techniques to identify the compounds present in the extracts.

3.1. Optimization of the extraction method

A two-level factorial fractional experimental design was applied using results from the etiolated wheat coleoptile bioassay as target values to be maximized. In the experimental design the effects of different extraction variables on the bioactivity of the resulting extracts were evaluated. The ranges for the extraction variables were established to cover the most common values; 5 and 25 °C for temperature, 25 and 50 mL for the extraction solvent volume, 5 and 15 min for extraction time, two different ultrasonic probes with different diameters [thin (3 mm) and thick (7 mm)], 30% and 70% ultrasound amplitude, and 0.2 s^{-1} and 0.8 s^{-1} for cycle. All extractions were carried out in duplicate. However, the resulting dry weight of the extracts was considered as an initial parameter to assess the reliability of extractions. As a result, extractions that gave rise to relative differences of greater than 20% in terms of dry weights were discarded and then repeated prior to analysis of the bioactivity.

It can be seen from the results in Table 1 that the bioactivities of the extracts ranged from 0% to −67% and this shows that the ranges selected for the extraction variables produced large differences in the recovery of the components responsible for bioactivity.

Graphical analysis was used to determine the effects of extraction conditions on the bioactivity values. The main effects of the extraction variables on the bioactivity are represented in plots and clear conclusions can be drawn regarding the main experimental values for a given parameter. It can be seen from Fig. 1 that the results obtained for the two different solvents show the greatest differences between the resulting bioactivity values. Experiments carried out with acetone produced an average inhibition of −45% whereas experiments carried out with MeOH produced an average inhibition of −16%. In other words, the change from MeOH to acetone led to a three-fold increase in the inhibitory effect. The other extraction variables showed less marked effects than extraction solvent, although the effects were also notable for extraction temperature, extraction time and the size of the ultrasonic probe. As far as temperature is concerned, higher bioactivities were obtained on using 5 °C than 25 °C (−38% versus −24% inhibitory activity, respectively). A lower extraction volume (25 mL) led to higher bioactivity results than the larger volume (50 mL), with inhibitory effects of −37% and −25%, respectively. A shorter extraction time (5 min) also gave rise to better bioactivity results for the extracts in comparison to the longer extraction time (15 min). Finally, the thinner ultrasonic probe produced better results than the thicker probe and resulted in almost double the inhibitory activity (−41% vs −21%).

Optimization of the most influential extraction variables. In view of the notable effects of temperature and solvent volume, it was decided to explore different values for these variables that were not included in the initial experimental design. It was planned to optimize the extraction after evaluating the kinetics

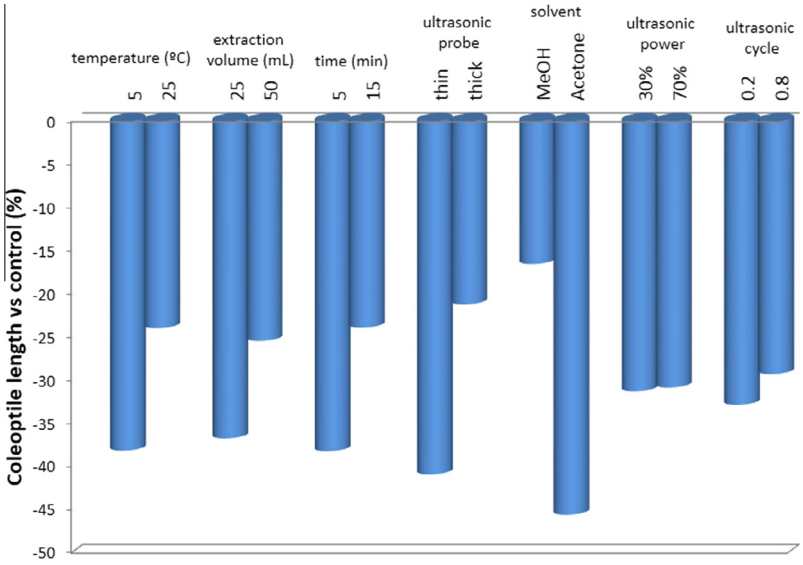

Fig. 1. Main effects plot for bioactivity of extracts in the experimental design.

for the extraction and for this purpose the ultrasonic probe time was fixed at 5 min with the thin probe. Four different extraction volumes (10, 15, 20, 25 mL) were employed at a temperature of 5 °C.

In these experiments the temperature was fixed because values below 5 °C led to very little variation in the bioactivity of the extracts and these results could not easily be differentiated. In addition, the time required for stabilization of the extraction system increased. The use of different volumes also produced weak variations in bioactivity (Table 2) and, as a consequence, the initial solvent volume (25 mL) was chosen because extracts could be filtered more easily on using 25 mL rather than the lower volume.

The most suitable extraction variables were as follows: 25 mL acetone, 5 °C, 2 mm probe, cycle of 0.2 s^{-1}, 30% amplitude for 20 min, using 1 g of plant sample.

Evaluation of extraction kinetics. Extractions were carried out for different times (1, 2.5, 5, 7.5, 10, 15, 20 min). Longer times than 20 min were not used for operational reasons. Any extraction time in the range from 7.5 to 20 min was appropriate as they gave higher values for the extraction mass (Kruskal–Wallis with Dunn's post-test) and better bioactivity results in the coleoptile bioassay (Table 3).

Table 2
Bioactivity results (mean values in percentage and standard deviation) for extracts obtained using different solvent volumes.

Volume (mL)	Bioactivity (%)
25	−62.0 ± 0.0
20	−62.0 ± 1.0
15	−61.0 ± 2.0
10	−63.5 ± 0.5

Table 3
Bioactivity results (mean values in percentage and standard deviation) for extracts obtained using different extraction. In the column, same superscript means non-significant differences (*P*-level 95%).

Extraction time (min)	Bioactivity (%)
1.0	−54 ± 0.5[a]
2.5	−56 ± 1.5[a]
5.0	−52 ± 1.5[a]
7.5	−57 ± 6.5[ab]
10.0	−55 ± 1.0[ab]
15.0	−58 ± 8.5[ab]
20.0	−62 ± 2.0[b]

[a,b] Values followed by the same letter are indicated as not significant different (α = 0.05).

Table 4
Bioactivity results (values in percent, relative to controls) for extracts obtained in the same day (repeatability) and in different days (reproducibility).

Extraction	Same day (9 extractions × 1 day)	Different days (3 extractions × 3 days)
1	−64	−63
2	−61	−59
3	−63	−62
4	−59	−59
5	−63	−58
6	−54	−52
7	−56	−61
8	−59	−61
9	−62	−59
Mean	−60.1	−59.3
Standard deviation	3.4	3.2

3.2. Analytical properties of the extraction method

Reproducibility and repeatability – 9 extractions were carried out on the same day in order to determine repeatability and 9 extractions (3 extractions per day on 3 different days) were carried out to determine reproducibility.

Significant differences were not found in the mean values of bioactivity results between extractions carried out on the same day or on different days, according to the Kruskal–Wallis analysis with Dunn's post-test *P* 0.05 (Table 4).

Therefore, the extraction method developed is appropriate to obtain extracts in a reproducible way in terms of bioactivity, thus enabling an optimization for future allelopathy studies. It should also be noted that high bioactivity values (around −60%) were found for the extracts obtained under the optimized extraction conditions.

3.3. Isolation of active compounds

Having optimized the extraction method, a larger scale extraction was carried out in order to obtain sufficient extract for the next step in the allelopathic study, i.e., isolation of compounds from the bioactive extract. Eight compounds were isolated and identified from this extract (Fig. 2), two steroids (β-sistosterol (**2**) [41] and stigmasterol (**3**)) [42], five diterpenes with the kaurane skeleton (*ent*-kaur-16-en-19 oic acid (**1**) [20], *ent*-19-methoxy-19-oxokauran-17-oic acid (**4**) [43], annoglabasin B (**5**) [12], *ent*-17-hydroxykaur-15-en-19-oic acid (**6**) [44,45] and *ent*-15 β,16 β-epoxy-17-hydroxy-kauran-19-oic acid (**7**) [46,47]) and the acetogenin asimicin (**8**) [48]. The spectroscopic data for these compounds are identical to those reported previously.

The major compounds were *ent*-kaur-16-en-19-oic acid (**1**), β-sistosterol (**2**), *ent*-19-methoxy-19-oxokauran-17-oic acid (**4**), annoglabasin B (**5**) and asimicin (**8**) and these were assayed in the etiolated wheat coleoptile bioassay (Fig. 3). The compounds that showed the highest activities were the kauranic diterpenes **1**, **4** and **5** and the acetogenin **8**. The most active compound, annoglabasin B (**5**), showed strong inhibition, with values of −95% at 10^{-3} M, −87% at 5×10^{-4} M and more than −70% at 10^{-4} M. The activity decreased with dilution but, even at the lowest concentrations tested, activities of close to −60% at 5×10^{-5} M and above −30% at 10^{-5} were obtained. The IC_{50} value for this compound was 30.4 μM (r^2 0.8639). In general, these results are very similar to those obtained for the commercial herbicide Logran. The only precedent for the activity of this compound concerns weak activity against HIV replication in H9 lymphocyte cells [12].

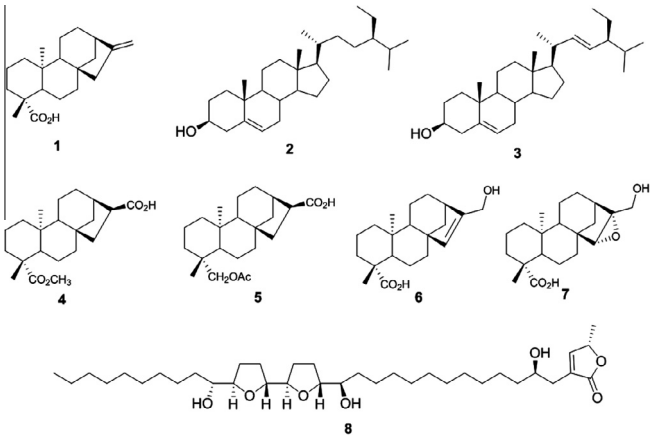

Fig. 2. Chemical structures of isolated compounds from *Annona glabra* leaves.

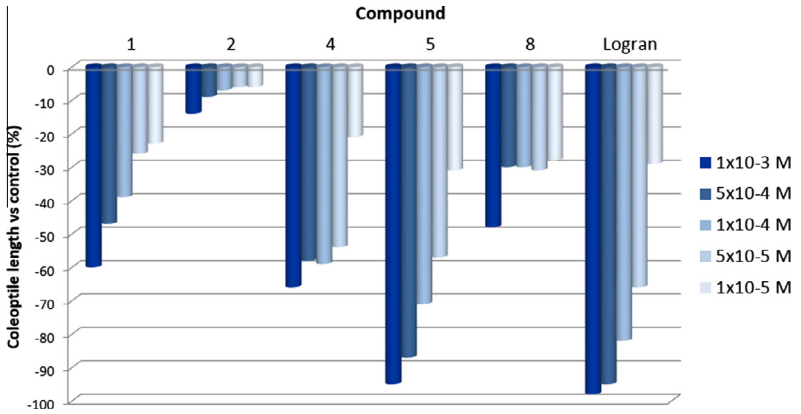

Fig. 3. Bioactivity results for pure compounds from *Annona glabra* leaves.

The second most active compound was **4**, which had an IC_{50} value of 33.3 μM (r^2 0.9892). This compound shows inhibition values above −65% at the highest concentration tested (10^{-3} M) and close to −60% at 5×10^{-4} M. A remarkable feature of this compound is that its activity is retained upon dilution, except at the lowest concentration (10^{-5} M), and the inhibition values remain above −50%. It has previously been reported that *ent*-19-methoxy-19-oxokauran-17-oic acid (**4**) shows cytotoxic selectivity for PC3 (prostate cancer) cells, albeit with weak potencies [43].

The third most active compound was *ent*-kaur-16-en-19 oic acid (**1**), which has an IC_{50} of 733.2 μM (r^2 0.9174). This compound also showed inhibition of −60% a 10^{-3} M and close to −50% and −40% at 5×10^{-4} M and 10^{-4} M, respectively. Compound **1** (*ent*-kaur-16-en-19-oic acid) was previously described as an active compound, with MIC values of 10 μg mL^{-1} against the microorganisms *Streptococcus sobrinus*, *Streptococcus mutans*, *Streptococcus mitis*, *Streptococcus sanguinis* and *Lactobacillus casei*. This compound was also proposed as a prototype for the discovery of new effective anti-infection agents against microorganisms responsible for caries and periodontal diseases [49]. Compound **1** has also shown antiplatelet aggregation [50], analgesic [51], antifungal [52,53], smooth muscle relaxant [54], hypoglycemic [55], cytotoxic and embryotoxic [50] effects.

The last compound that showed activity was asimicin (**8**) and this gave values of −48% at 10^{-3} M and an IC_{50} of 2013 μM. In contrast, β-sitosterol (**2**) did not show any significant activity. Asimicin (**8**) is extremely cytotoxic and shows promising pesticidal activities against mosquito larvae, spider mites, aphids, the Mexican bean beetle, striped cucumber beetle, blowfly larvae, and nematodes [48]. Although asimicin (**8**) was found to be active in this bioassay, its activity was lower than that shown by diterpenes from *A. glabra* – in particular annoglabasin B (**5**), which is the most active compound assayed.

4. Conclusions

The optimization of the ultrasound extraction process led to the best conditions to obtain the most active extract. Isolation and characterization of the components from this extract enabled the identification of the metabolites that could be responsible for the activity shown by *A. glabra*. Of these, the diterpene annoglabasin B (**5**) was the most active and this was followed by *ent*-19-methoxy-19-oxokauran-17-oic acid (**4**). Both of these compounds have a carboxylic acid function at position 17 and an ester function on carbon 19 and these appear to play a role in the defense mechanisms of this species.

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