UNIVERSIDADE FEDERAL DE SÃO CARLOS
CENTRO DE CIÊNCIAS BIOLÓGICAS E SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM ECOLOGIA E RECURSOS NATURAIS

Extração e prospecção de metabólitos secundários de folhas de *Annona glabra* L. e análise de atividade sobre o crescimento de coleóptilos de trigo e de micro-organismos.

Reginaldo Sadao Matsumoto

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“Gettin' old
Gettin' grey
Gettin' ripped off
Underpaid
Gettin' sold
Second hand
That's how it goes
Playin' in a band

It's a long way to the top
If you wanna rock 'n' roll !!”

Angus Young · Malcolm Young · Bon Scott, 1975

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Ao PPGERN, CNPQ e CAPES, pelas bolsas concedidas.

SUMÁRIO

RESUMO

O registro de substâncias de *Annonaceae* aumenta a cada ano, assim como o isolamento de compostos bioativos em espécies desta família. Atualmente há uma demanda crescente na descoberta de substâncias naturais menos tóxicas que produtos sintéticos com a função de herbicidas, algicidas ou bactericidas. Por esta razão a bioprospecção, identificação e produção de metabólitos secundários tem se tornado cada vez mais importante. Inúmeros trabalhos sobre a composição química de *Annona glabra* L. foram feitos utilizando suas sementes, frutos, caule e em menor quantidade, folhas. Entretanto, as principais atividades estudadas dizem respeito a inibição de células tumorais. Uma das grandes dificuldades de se realizar estudos é a obtenção de quantidades suficientes do produto purificado para os testes biológicos e para o seu aproveitamento econômico através da posterior síntese destas substâncias. Além da quantidade de substância isolada é também um fator importante a redução de gastos relacionados a tal processo. O presente estudo foi idealizado a partir dos resultados preliminares sobre os efeitos alelopáticos de extratos de *Annona glabra* sobre a germinação de sementes e também devido à necessidade da otimização dos processos de extração das substâncias bioativas para o seu uso como agentes algicidas e fungicidas. Foram dois processos auxiliares de extração (extração assistida por ultrassom (EAU) e extração assistida por micro-ondas (EAM)) biodirigidas em ensaios de atividade sobre coleóptilos de trigo estiolado. Após o processo de otimização, uma extração em massa (EAU) foi executada para isolar e identificar as substâncias majoritárias: **(1)** ácido (-)-Kaur-16-en-19-oico, **(2)** β-sitosterol, **(3)** Stigmasterol, **(4)** ácido ent-19-methoxy-19-oxokauran-17-oico, **(5)** ácido 16α-hydro-19-acetoxy-*ent*-kauran-17-oico (annoglabasin B), **(8)** Asimicina e substâncias minoritárias **(6)** ácido *ent*-17-hydroxykaur-15-en-19-oico e **(7)** ácido *ent*-15β,16β-epoxy-17-hydroxy-kauran-19-oico. Em que a substância **5** foi altamente inibitória frente aos coleóptilos seguidas das substâncias **4** e **8**. Os testes dos extratos acetônicos mostraram atraso no crescimento de *Selenastrum capricornutum* às concentrações de 125 a 175 mgL^{-1} e totalmente inibitórias acima de 200 mgL^{-1} e o IC_{50}, em 96h, foi de 145 mgL^{-1}. O oomiceto *Pythium aphanidermatum* foi inibido nas concentrações acima de 125 $\mu g.disc^{-1}$. Estes resultados ressaltam a importância da bioprospecção das substâncias desta espécie e abrindo oportunidades para futuros usos comerciais.

ABSTRACT

The record of Annonaceae substances increases every year, as well as the isolation of bioactive compounds in this family. Currently there is a growing demand in the discovery of natural substances less toxic than synthetic products that are herbicides, algaecides and bactericides. For this reason bio-prospection, identification and production of secondary metabolites has become increasingly important. Numerous studies on the chemical composition of *Annona glabra* L. were made using their seeds, fruits, stems and fewer leaves. However, the major activities studied are related to the inhibition of tumor cells. One of the major difficulties of conducting phytochemical studies is to obtain sufficient amounts of purified product for the biological testing and its economic exploitation through the subsequent synthesis of these substances. Besides the amount of substance isolated is also important to reduce costs related to such a process. The present study was designed from the preliminary results on the allelopathic effects of extracts of Annona glabra on seed germination and also due to the need for optimization of extraction of bioactive substances for use as algaecides and fungicides. Two procedures were assistants to extraction (ultrasound-assisted extraction (UAE) and microwave assisted extraction (MAE)) in bio-directed activity assays on wheat-etiolated coleoptiles. After the optimization process, an extraction mass (UAE) was performed to isolate and identify the major substances: **(1)** (-)-Kaur-16-en-19-oic acid, **(2)** β-sitosterol, **(3)** Stigmasterol, **(4)** ent-19-methoxy-19-oxokauran-17-oic acid, **(5)** 16α-hydro-19-acetoxy-*ent*-kauran-17-oic acid (annoglabasin B), **(8)** Asimicin and minor substances **(6)** *ent*-17-hydroxykaur-15-en-19-oic acid e **(7)** *ent*-15β,16β-epoxy-17-hydroxy-kauran-19-oic acid. Wherein the substance 5 was highly inhibitory against coleoptiles followed by the substances 4 and 8. The acetonic extract showed growth retardation of *Selenastrum capricornutum* in concentrations of 125-175 mgL-1 and fully inhibitory above 200 mgL-1 and IC50, at 96h, was 145 mgL^{-1}. The oomycete *Pythium aphanidermatum* was inhibited at concentrations above 125 $\mu g.disc^{-1}$. These results highlight the importance of bioprospecting of substances of this species and opening up opportunities for future commercial uses.

INTRODUÇÃO GERAL

A. glabra é originária da América tropical e oeste africano (PINTO *et al.*, 2005) e pode ser encontrada em locais alagadiços das regiões tropicais e subtropicais, como a Flórida (ALLEN *et al.*, 2002). A presença desta espécie em este tipo de ambiente são devidos a adaptações como a presença de raízes adventícias, aerênquima nas raízes e na base do caule, frutos flutuantes e sementes que se dispersam pela água (ZOTZ *et al.*, 1997) (NÚÑEZ-ELISEA *et al.*, 1999). A planta adulta e suas sementes também são altamente tolerantes a salinidade (MEEROW; BLACK, 1993)

Na Ásia (Sri Lanka, Tailândia, Vietnã, Península Malaia e possivelmente na China) e Austrália, é considerada uma invasora agressiva, dispersando-se facilmente em locais alagados em perturbação, seja ela de origem antrópica ou causada por fenômenos naturais. Em locais degradados, esta espécie tem potencial invasor oportunista ou colonizador como espécie pioneira (SUGARS *et al.*, 2006) e é também considerada invasora dominante em Fiji e Polinésia francesa (SPREP SOUTH PACIFIC REGIONAL ENVIRONMENT PROGRAMME, 2000)

No Brasil, *Annona glabra* distribui-se nos Estados da Bahia, Paraná, Pernambuco, Rio de Janeiro, Santa Catarina e São Paulo (PONTES *et al.*, 2004). Na Paraíba, a espécie é comumente encontrada em vegetação de mangue, restinga e matas de galeria (PONTES op. cit.). Em nossos trabalhos foi observada no litoral norte do Estado de São Paulo, no rio Massaguaçu localizado no município de Caraguatatuba, (23º37’20’’S e 54º21’25’’O) que forma um estuário cego, cuja abertura e fechamento se dão de acordo com as variações da maré e dos aportes fluviais e de chuvas. Essa variação pode chegar a mais de 2m.

A vegetação do estuário está sujeita às inundações durante o ano todo, formando bancos marginais e também no centro do rio. Nesses bancos pode-se notar o grande domínio da espécie herbácea *Crinum americanum* L. e dentre as arbustivo-arbóreas, o domínio de *Annona glabra* também é evidente, porém com indivíduos mais esparsos. Em alguns pontos ocorrem grandes acúmulos de sementes dessa espécie nos meandros de *C. americanum* (MATSUMOTO, 2009).

Em um estudo realizado com plantas medicinais foi relatada uma forte atividade biológica por parte da família Annonaceae, quando comparada a outras famílias utilizadas na pesquisa (FUJII *et al.*, 2003).

Annona glabra é usada na medicina tradicional como larvicida, sendo suas comprovadas em extratos etanólicos de casca, que mostrou atividade contra larvas

de *Aedes aegypti* (MENDONÇA *et al.*, 2005) e o pó das sementes mostrou-se tóxico para peixes (ALLEN *et al.*, 2002). Extratos etanólicos de folha e semente também mostraram atividades contra *Biomphalaria glabrata* (SANTOS, A. F. DOS; SANT'ANA, A. E. G., 2001). Extratos metanólicos de frutos frescos possuem diterpenóides que inibem significativamente a replicação do HIV em linfócitos H9 (CHANG *et al.*, 1998).

Acetogeninas isoladas de folhas demonstraram propriedades biológicas antitumorais, e isolados da casca apresentaram atividades pesticidas, bactericida, fungicida e antihelmintica (CHIU *et al.*, 2003; LIU *et al.*, 1999; PADMAJA *et al.*, 1995). Diterpenóides com atividades antitumorais também foram registrados por Zhang (2004).

Em estudos anteriores comprovamos a existência de efeitos alelopáticos de extratos aquoso e de acetato de etila, preparados com folhas de *A. glabra,* sobre a germinação e crescimento de espécies de cultivo bioindicadoras (*Lactuta sativa* e *Sesamum indicum*) e espécies infestantes de cultura (*Bidens pilosa, Ipomea grandifolia* e *Echinochloa crus-galli*) (MATSUMOTO *et al.*, 2010).

Com a comprovação das atividades biológicas descritas anteriormente, demos continuidade aos estudos de alelopatia em folhas de *Annona glabra,* para determinar as substâncias ou grupos de substâncias envolvidas neste processo. Em seguida testamos o potencial alelopático destas como inibidores de micro-organismos.

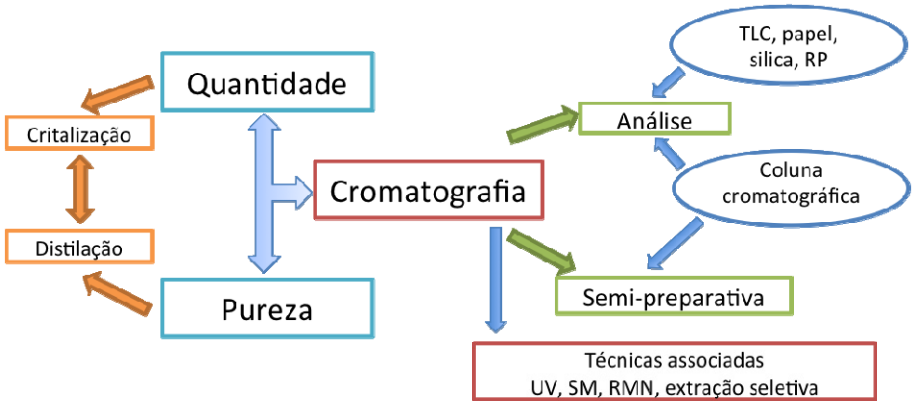

Figura 1. Plano de pesquisa seguido pelo Grupo de Alelopatía de Cádiz (GAC-Universidad de Cádiz). Figura modificada de Macías; Galindo & Galindo (2007)

Estudos de Alelopatia, sobretudo os realizados em laboratório, são baseados em testes de atividade e fitotoxidade dos compostos que possam ser extraídos da planta, isolados e identificados. Para isto, é necessário otimizar os processos de extração para se conseguir a maior quantidade possível do composto isolado (fig. 1). O

uso de Extrações Assistidas por Ultrassom (EAU) e Extrações Assistidas por Micro-ondas (EAM) podem aumentar a quantidade de substâncias a serem extraídas, com um tempo reduzido e economia de solvente (GANZLER *et al.*, 1986; KAUFMANN; CHRISTEN, 2002; VINATORU *et al.*, 1997). Nesse sentido, nossa pesquisa foi orientada para que a otimização de métodos de extração isolassem aleloquímicos, de maior atividade, em maiores quantidades para depois utilizá-los nos testes biológicos com coleóptilos de trigo. Esta etapa foi realizada no Departamento de Química Orgânica da Universidade de Cádiz (UCA) – Grupo de Alelopatia de Cádiz.

Os testes utilizando micro-organismos foram executados no Brasil, nos laboratórios de Ecologia química e Sistemática (Dep. Botânica), de Sementes (Dep. Botânica), Biotecnologia de algas (Dep. Botânica), de Plâncton (Dep. Hidrobiologia) e Laboratório de Ecologia e Microrganismos aquáticos (Dep. Ecologia e Biologia Evolutiva) da UFSCar

OBJETIVOS

1) Otimizar as condições de extração (Extração Assistida por Ultrassom e Extração Assistida por Micro-ondas) com a finalidade de produzir extratos com alta concentração em compostos bioativos.

2) Isolar e identificar as substâncias majoritárias presentes nos extratos obtidos nos processos de otimização.

3) Avaliar cada um dos compostos encontrados em relação à sua bioatividade utilizando biotestes com coleóptilos de trigo.

4) Realizar um *Screening* de atividade do extrato mais ativo sobre micro-organismos *Selenastrum capricornutum* Printz e *Pythium. aphanidermathum*.

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CAPÍTULO 1

Bioactive extracts from *Annona glabra* L. leaves using microwave assisted extraction

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Bioactive extracts from *Annona glabra* L. leaves using microwave assisted extraction.

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ABSTRACT

Annona glabra is a highly problematic invasive species in many areas around the world. As the first step in the allelopathic study of *Annona glabra*, a new microwave-assisted extraction method for the leaves was developed. The extraction method was optimized using the final bioactivity properties of the resulting extracts as target values. A Box-Behnken experimental design involving 27 different sets of extraction conditions enabled the rapid optimization of the extraction variables and also allowed a mathematical correlation to be established between the extraction parameters and the final bioactivity of the extracts. The average difference between the calculated bioactivities and the experimentally determined bioactivities was 6.8%, which indicates that the developed model fitted the experimental data. The extraction solvent was found to be the most important extraction variable. Finally, extracts were identified that showed an inhibitory activity of –60%. The microwave-assisted extraction system allowed 10 extractions to be carried out at the same time and the total extraction time was 1 min per sample.

INTRODUCTION

An extraction step is a prerequisite in allelopathic studies on plants. The experimental extraction conditions must be studied and optimized in order to produce highly

bioactive extracts. Extraction methods require significant effort, time and costs, especially when non-automated extraction methods are employed. A number of rapid and automated extraction techniques for solid samples have replaced conventional techniques such as Soxhlet extraction and these include ultrasound-assisted extraction (UAE)[1], supercritical fluid extraction (SFE)[2], pressurized liquid extraction (PLE)[3] and microwave-assisted extraction (MAE)[4]. These new assisted extraction techniques have been applied in the study of bioactive compounds and they are interesting because they reduce the consumption of solvents while increasing the sample throughput and simplifying the extraction process. Additionally, some non-regular extraction conditions can produce extracts with different compositions, either due to the higher extraction efficiency or the higher stability of bioactive compounds under the conditions used.

MAE has several advantages over traditional extraction methods as it requires shorter extraction times, smaller amounts of sample and solvents and, most importantly, it has better efficiency[5]. This kind of methodology has become increasingly important given that a single plant can contain thousands of secondary metabolites and rapid and high performance extraction is required[6].

The MAE of secondary metabolites has proven to be a fast, reliable and efficient method, as shown by its success in numerous studies[5,6,7,8]. However, references concerning the optimization of MAE methods to obtain extracts with increased allelopathic activity have not been found in the literature.

The family *Annonaceae* has 50 genera and over 2,000 species. Most of these species are shrubs and small trees. There are 119 species from the genus *Annona*[9]. *Annona glabra* L. is a shrub-tree that typically grows to a height of around 10–12 m. The trees have thin, gray trunks and sometimes grow in clumps. *Annona glabra* L. grows in swamps, is tolerant of salt water, and cannot grow in dry soil.

The flesh is sweet-scented and agreeable in flavor, but it has never attained general popular use, unlike other related fruits such as soursop (*Annona muricata* L.) and custard apple (*Annona cherimola* Mill.).

According to Cordeiro & Pinto [9], Central America, Antilles, Ecuador, Brazil and West Africa are the centers of origin of *Annona glabra*. However, nowadays this species has a wide distribution in periodically and permanently flooded habitats in the tropics and the subtropics[10]. This wide distribution is due to human activity and the invasive potential of this species. In places like North America (Florida), Asia (Sri Lanka, Thailand, Vietnam, the Malay Peninsula and possibly China) and Oceania (Australia) there are specific programs to control *A. glabra*[11,12,13].

Some biological activities have been reported for this species and these include the anticancer effects of alcoholic extracts prepared from *A. glabra* leaves, pulp and seed, which were investigated in human leukemia cell lines. Extracts were highly cytotoxic to drug-sensitive (CEM) and multidrug-resistant leukemia (CEM/VLB) cell lines[14]. Acetogenins isolated from leaves were described as being active against several tumor cells[15].

Substantial antimicrobial, antifungal and moderate insecticidal, sporicidal and cytotoxic activities were observed for the hexane extract of the stem bark of *Annona glabra* L.[16].

In traditional medicine *Annona glabra* L. is used as an antiparasitic and insecticidal agent. The aforementioned properties have been confirmed in extracts that were active against the mosquito larvae of *Aedes aegypti* L.[17,18] and the mollusk *Biomphalaria* sp.[19]. Padmaja *et al.*[16] found antimicrobial and antifungal properties in such extracts and Zhang *et al*.[20] isolated diterpenoids with anti-cancer properties from the fruits. Mendonça *et al.*[18] indicated that extracts of the caulinar cortex kill *Artemia salina* L.

The allelopathic properties of *A. glabra* are not well understood. Preliminary studies showed allelopathic effects of aqueous and ethyl acetate extracts on the germination and growth of crop seeds (*Lactuca sativa* and *Sesamum indicum*) and weed species (*Bidens pilosa*, *Ipomea grandifolia* and *Echinochloa crus-galli*)[21].

The invasive capacity of this species, together with the allelopathic effects shown by its extracts, has led to further allelopathic studies. These investigations begin with optimization of the extraction conditions in order to obtain the most active extract. In this respect, microwave-assisted extraction (MAE) was studied due to the advantages discussed above.

In a bio-guided optimization process an appropriate bioassay is required. Bioactivities during the optimization process were evaluated using the etiolated wheat coleoptile bioassay. This is a rapid (24 h) and sensitive bioassay that can be considered as a first level bioactivity indicator. The parameter measured in this bioassay is the elongation differential of coleoptile segments, a parameter that depends on the stimulation or inhibition of undifferentiated plant tissue[22,23,24]. Macias *et al*. proposed this bioassay, given its sensitivity, as a first step in evaluating potential herbicidal properties[25].

The aim of the work described here was the optimization of the MAE process to determine the best conditions to obtain active extracts of *A. glabra*. The bioactivity results from extracts were used to evaluate the extraction efficiency.

EXPERIMENTAL

Sample preparation

Dried *A. glabra* L. leaves were ground and stored in a refrigerator. Fat and wax were removed from the sample by washing 500g of powder with hexane using an ultrasound

system. The resulting extract was then filtered and dried on a rotary evaporator under vacuum to give 2.8 g of dry matter.

Etiolated wheat coleoptile bioassay

Extracts 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.

Wheat seeds (*Triticum aestivum* L. cv. Duro) were sown in 15 cm diameter Petri dishes moistened with water and grown in the dark at 25±1 ºC for 4 days[23]. 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[22].

Three extract concentrations (800, 400, 200 ppm) were studied for the bioassay along with a concentration of 0 ppm as negative control and a commercial herbicide (Logran) as a positive control.

The commercial herbicide Logran is 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%) and this was used as the internal reference as reported previously[26].

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

The coleoptiles were measured by digitalization of their images. Data were statistically analyzed using Welch's test[27]. 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.

Microwave-assisted extraction

The MAE method was developed using an ETHOS 1600 microwave extractor (Milestone, Sorisole, Italy). Extractions were performed at different power levels (250, 500, 750 Watts), using 3-6 vessels in a batch (one reference with the temperature probe and 2-5 samples). Two different solvent systems (methanol and acetone), with three percentages (10, 50, 90% methanol in acetone), different extraction volumes (25-37.5-50 mL) and temperatures (50, 75, 100 °C) were evaluated for the extraction of *Annona glabra* L. leaves. In these extractions 1 g of plant material was used.

Experimental design for the evaluation of the effects of extraction variables

Optimization of extraction variables was performed by considering the bioactivities of the various extracts using the Box-Behnken statistical methodology. The results for the 27 extractions carried out in duplicate for the different extraction variables (each variable has three levels: low, medium and high) are shown in Table 1 along with the respective responses. The results of the etiolated wheat coleoptile bioassay were used as the response variable.

The responses obtained from the various extractions were entered into to a second-order polynomial equation into which each of the various parameters was introduced. The polynomial equation is as follows:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{34} X_3 X_4 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2$$

In this equation Y is the aforementioned response, β_0 is the ordinate at the origin; X_1 [temperature (ºC)], X_2 [microwave power (Watts)], X_3 (percentage of MeOH in the extraction solvent) and X_4 [ratio solid sample (g)/extraction volume (mL)] are the independent variables; β_1, β_2, β_3 and β_4 are the linear coefficients; β_{13}, β_{14}, β_{23}, β_{24}, β_{34} and β_{12} are the cross product coefficients and β_{11}, β_{22}, β_{33} and β_{44} are the quadratic coefficients.

The analysis of data for the Box-Behnken design was carried out using Minitab 15 statistical software (Minitab, Inc. State College, PA). This software was used to estimate the effects of the variables on the final response, the variance analysis, the second order mathematical model, the optimum levels of the significant variables and the surface graphs.

RESULTS AND DISCUSSION

A Box-Behnken design was used to optimize the extraction conditions for *Annona glabra* L. leaves by microwave-assisted extraction. Four different extraction variables were studied in the following ranges: temperature values between 50 and 100 °C, microwave power between 250 and 750 Watts, solvent composition using methanol/acetone mixtures in the range between 10-90% methanol, and extraction volumes of 25-50 mL (Table 1). All experiments (27) were run in duplicate for the extraction and also in duplicate for the etiolated wheat coleoptile bioassays. Extraction time was fixed at 10 min.

Table 1. Results from the experimental design. Extraction yield and bioactivity results of etiolated wheat coleoptile bioassays for evaluation of extraction variables effects of the experimental design

°C	Power (W)	Solvent MEOH (%)	Ratio (mL)	Average bioactivity results (Measured)	Bioactivity results (Calculated)	Difference (%)
75	500	90	50	-50	-46	9
50	500	10	37.5	-58	-60	3
100	500	90	37.5	-45	-50	10
75	750	10	37.5	-57	-57	0
75	250	50	50	-45	-54	17
50	250	50	37.5	-46	-48	4
75	750	50	50	-32	-42	24
75	750	50	25	-60	-57	5
100	250	50	37.5	-63	-62	2
75	250	90	37.5	-52	-48	8
75	250	10	37.5	-46	-42	10
75	500	10	50	-49	-46	7
50	500	50	25	-53	-52	2
75	500	90	25	-41	-40	3
75	250	50	25	-43	-40	8
50	750	50	37.5	-69	-67	3
75	500	50	37.5	-54	-56	4
100	500	50	25	-58	-62	6
100	500	50	50	-52	-48	8
100	500	10	37.5	-54	-56	4
75	750	90	37.5	-37	-37	0
50	500	90	37.5	-46	-51	10
75	500	50	37.5	-54	-56	4
50	500	50	50	-73	-64	14
75	500	10	25	-53	-54	2
75	500	50	37.5	-62	-56	10
100	750	50	37.5	-53	-47	13

Bioactivity results were correlated with experimental conditions and a second-order polynomial equation was obtained. The resulting coefficients for the second-order polynomial equation from the Box-Behnken design and their significance (P-value) are presented in Table 2. Data related to the fitting properties of the resulting model, expressed as percentage difference between the experimental values for the bioactivity and the calculated ones, are presented in Table 1. The resulting average difference was 6.9%, ranging from 0% to 24%, with only four experiments showing differences greater

than 10% between experimental and calculated values, i.e. 85% of the experimental data were fitted by the model with an error of less than 10%. Therefore the resulting model explains the bioactivity results for the extracts based on the experimental conditions used during the extraction process. This means that extraction variables control the final extract composition in terms of bioactive compounds and that the extraction conditions can therefore be managed to optimize the bioactivity of the resulting extracts.

Analysis of the model clearly shows that, among the linear terms, the most influential variable was the solvent (p-value = 0.07 < 0.1). The solvent has a positive coefficient, which indicates that the use of a lower proportion of methanol (10%) gives rise to lower values for bioactive results, i.e. higher inhibitory results. The average bioactivity value for extracts in the experimental design (Table 1) obtained using 10% methanol was -53.0% whilst the average bioactivity value for extracts obtained using 90% methanol was -45.6% inhibitory effect on etiolated wheat coleoptiles. The extraction temperature, ratio and power did not have significant effects on the bioactivity, with p-values of 0.41, 0.56 and 0.81, respectively. In this way, the average bioactivity for extractions run at 50 ºC produced an inhibitory effect of -58.9%, whereas the extraction run at 100 ºC produced an inhibitory effect of -54.5%. As far as the solvent/sample ratio is concerned, the inhibitory effects were -51.5% on using the lowest ratio (25 mL of solvent/1 g of leaves) and -49.7% on using the highest ratio (50 mL of solvent/1 g of leaves). Finally, microwave power led to inhibitory effects of -49.5% on using 250 Watts and -51.7% on using 750 Watts.

Clear effects were not observed for temperature, solvent/sample ratio and power in the experimental design and interactions between these three variables and solvent compositions must be analyzed in order to determine the best values. The p-values (Table 2) from the model were used to check for interactions. Power had significant

interactions with solvent (p-value = 0.06), whilst temperature (p-value = 0.82) and ratio (p-value = 0.32) showed lower interactions.

Table 2. Coefficients for the second-order polynomial equation from the Box-Behnken design.

	Coefficient	P-value
constant	-56.833	0
temperature	1.583	0.41
power	-1.125	0.56
solvent	3.750	0.07
ratio	0.458	0.81
temperature*temperature	-3.937	0.19
power*power	4.250	0.16
solvent*solvent	6.187	0.05
ratio*ratio	3.625	0.22
temperature*power	8.375	0.02
temperature*solvent	-0.750	0.82
temperature*ratio	6.375	0.07
power*solvent	6.625	0.06
power*ratio	7.375	0.04
solvent*ratio	-3.375	0.32

The interactions between solvent composition and power level are represented in Figure 1. It can be seen that bioactivity changes quickly from values close to -40% to values around -55% on changing the power from 250 Watts to 750 Watts on using 10% methanol as the extraction solvent. It can also be seen from Figure 1 that changes are in the opposite sense and are also less marked on using high methanol percentages (90%), i.e. the higher the power the lower the resulting bioactivity (less negative values). In the same way, bioactivity values also change from close to -40% to around -55% on using 10% methanol instead of 90% methanol as the extraction solvent at higher power (750 Watts). However, an increase in the methanol percentage from 10% to 90% at low power (250 Watts) gave slightly higher bioactivity values. Therefore, as low methanol percentages produce higher bioactivity values, high power values must be used because these produced better bioactivity results for the extraction solvent with low methanol levels.

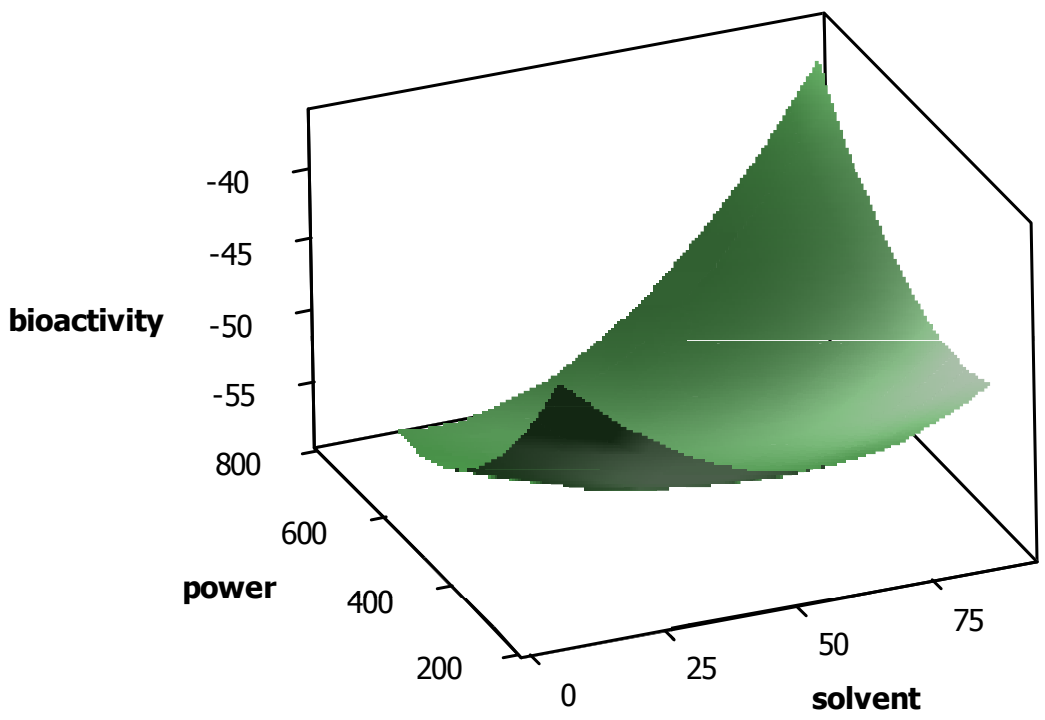

Figure 1. Surface response for solvent and power. Temperature= 75 ºC, Ratio= 37.5.

Interactions between solvent and solvent/sample ratio are presented in Figure 2. It can be seen that some of the effects are similar to those discussed above for solvent and power, albeit not as marked. Specifically, higher bioactivity results were found on changing from a ratio of 50 (extraction liquid/solid sample = 50) to 25 (extraction liquid/solid sample = 25) when low percentages of methanol were used. For high percentages of methanol the opposite effect was found, i.e. the higher the ratio the better the bioactivity results. In any case, changes in the two experiments were from -47% to -53% (10% methanol) and from -46% to -40% (90% methanol), i.e. much lower than changes found for the interactions between solvent and power.

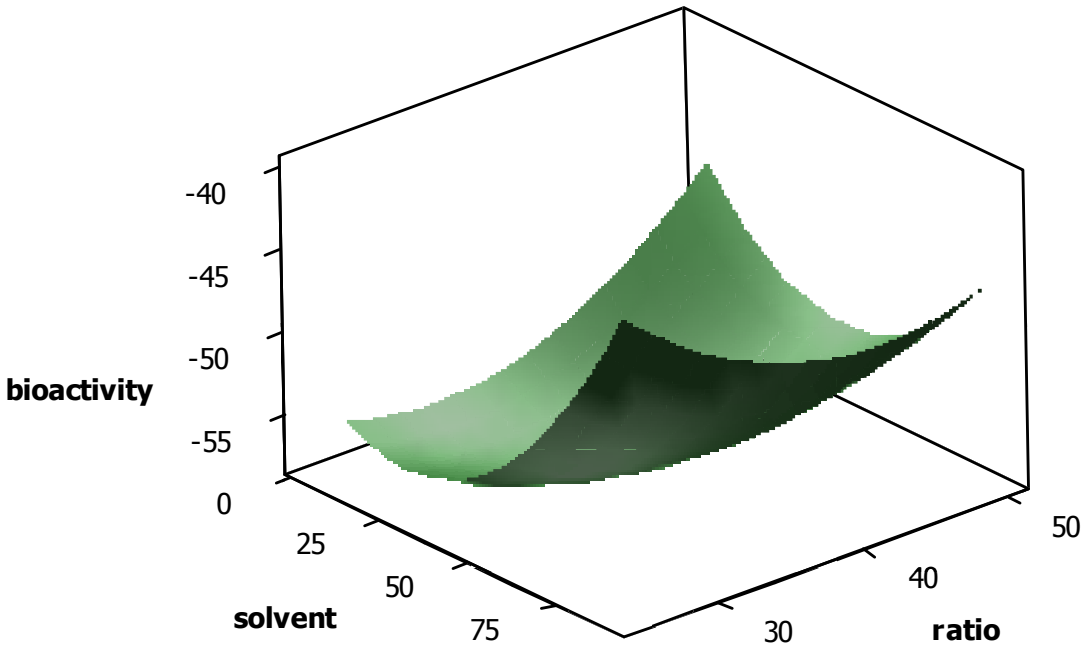

Figure 2. Surface response for solvent and ratio. Power= 500 watts, Temperature= 75 ºC.

The interaction between solvent and temperature is represented in Figure 3. It can be seen that interactions are not significant because similar changes were obtained on moving through different solvent compositions regardless of the working temperature. This finding signifies that at any temperature the evolution of the bioactivity results will be affected by the solvent composition in the same way. Additionally, the changes in the resulting bioactivities were less than 10%.

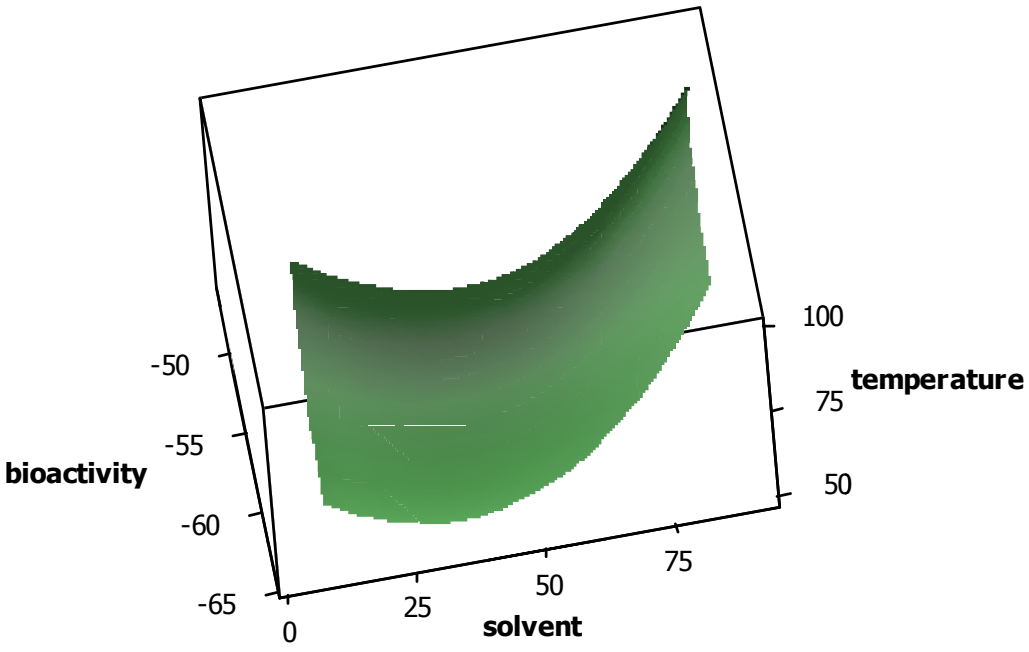

Figure 3. Surface response for solvent and temperature. Power= 500 watts, Ratio= 37.5.

From Figure 1 it can be seen that on using low methanol percentages (10%) in the extraction solvent, higher power values (750 Watts) could produce higher bioactivity levels. As a result, several additional extractions were run at higher power levels. Other variables were fixed according to the previous results in the experimental design: temperature at 50 °C, extraction solvent at 25 mL of solvent mixture with 10% methanol. The bioactivity results are shown in Table 3. It can be seen that a power of 1000 Watts produced the highest bioactivity result in the etiolated wheat coleoptile bioassay. It should also be noted that high bioactivity was found for the resulting extract; an inhibition of coleoptile growth above -60% represents a very high bioactivity value given that an extract containing several different compounds was evaluated. One would expect that the isolation of pure compounds from the extract would lead to higher inhibition levels.

Table 3. Bioactivity results (mean and standard deviation) obtained using power levels outside the starting working range

°C	Power (W)	Solvent MEOH (%)	Ratio (mL)	Bioactivity results
50	750	10	25	-57 ±2.0
50	875	10	25	-52 ±1.0
50	1000	10	25	-64 ±1.0

The final extraction conditions that produced the highest bioactivity levels were as follows: solvent 25 mL of 10% methanol in acetone, temperature 50 ºC and a power of 1000 Watts. These extraction conditions require validation in order to define an extraction method to be applied in allelopathic studies. Therefore both the repeatability and reproducibility were calculated. Extractions were carried out on the same day (9 extractions) to determine repeatability and also on different days (3 days x 3 extractions per day) to determine reproducibility. The results are presented in Table 4. Significant differences were not found for extractions carried out on the same day and on different days. Relative standard deviations for both repeatability and reproducibility were around 5%. This value can be considered acceptable because it is related to both the extraction method and the bioassay results.

Table 4. Bioactivity results (mean and Standard deviation) for samples from extraction in the same day (repeatability) and in different days (reproducibility).

Extraction	Same day (9 extractions x 1 day)	Different days (3 extractions x 3 days)
1	-59	-53
2	-64	-53
3	-61	-59
4	-55	-56
5	-64	-57
6	-56	-56
7	-66	-66
8	-67	-67
9	-64	-64
Mean	-61.8	-59.0
Standard Deviation	4.29	5.38

* Values in percent, relative to controls

CONCLUSIONS

A new MAE method has been developed to obtain highly bioactive extracts from *Annona glabra* L. This represents the first step in the allelopathic study of the leaves of this plant. The MAE method produces highly bioactive extracts in a reproducible and rapid manner (able to produce up to 10 extracts at the same time in 10 min, i.e. 1 min per sample). The first step for further allelopathic studies of *A. glabra* has been completed and the applicability of MAE for allelopathic studies has been demonstrated.

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CAPÍTULO 2

Ultrasound assisted extraction of bioactive compounds from *Annona glabra* L. leaves

Matsumoto, R.S.; Varela, R.M.; Palma, M., Molinillo, J.M.G., Lima, M. I. S., Barroso, C.G, Macías, F.A.

Ultrasound assisted extraction of bioactive compounds from *Annona glabra* L. leaves

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ABSTRACT

Annona glabra is a tropical tree reported as troublesome invasive species that grows in estuaries and chokes mangrove swamps. Recently, has been reported as source of bioactive extracts such as leaf extracts with phytotoxic potential on several crop pests. To optimize the extraction of bioactive compounds, a two level fractional factorial experimental design was used for ultrasound-assisted extraction from *A. glabra* leaves. Extraction solvent was the variable with highest effect on bioactivity. Extraction time, temperature and the ultrasonic probe also showed effects on the recovery of bioactive compounds. After optimization of the extraction method, a higher scale extraction was used for isolation of compounds. Eight compounds have been isolated and identified from this extract, two steroids (β-sistosterol and stigmasterol, five diterpenes with 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*-15 β,16 β-epoxy-17-hydroxy-kauran-19-oic acid) and the acetogenin, asimicin. The most active compound, annoglabasin B, inhibited the etiolated wheat coleoptile in -95% at 10^{-3} M, -87% at 5.10^{-4} M and higher than -70% at 10^{-4} M.

INTRODUCTION

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

The fruits are edible with a pleasant taste and fragrant pulp. The spherical fruit is similar in size to an apple or bigger. Its consumption is usually local and has not reached the popularity of other fruits of the same genus. It has been reported to have anticancer[2] antimutagenic[3] and antioxidant.[4,5,6] properties

In recent years, the family Annonaceae has been studied in the research on bioactive compounds. For example, annonaceous acetogenins is a class of compound with a wide variety of biological activities as insecticidal, inhibitor of lymphocytic leukemia, carcinoma cells and mitochondrial complex I.[7,8,9]

Annona glabra L. is reported to possess parasiticide and insecticide activity since their use in traditional medicine.[10,11,12] Different parts of this species have been used for isolating bioactive compounds. Thus, fruit methanol extracts afforded diterpenes, which showed inhibition of mitochondrial, cancer cells and HIV reverse transcriptase and replication[13,14]. Ethanol extracts from seeds were found to be a potent inhibitor of complex I of the mitochondrial respiratory chain[15] and against *Biomphalaria glabrata* (mollusk) growth.[16]

Stem is also a source of bioactive substances: hexane extracts presented insecticidal, sporicidal and cytotoxic activities[17], as well as larvicidal activity against *Aedes aegypti.*[12]

Acetogenins isolated from leaves were reported to be active against several tumor cells.[11,18,19] A number of metabolites were also isolated from this species, including

monoterpenoids, diterpenoids, cyclopeptides among others, but no activity has described yet[20,21,22,23,24,25]. Despite the large number of compounds isolated from *Annona glabra*, little has been researched about the allelopathic activity of its chemical components.

In this sense, it is a highly problematic invasive species. It grows in estuaries and chokes mangroves. Seedlings cover the edges and prevent germination of other species. It affects farms, growing along fences and also invades and transforms undisturbed areas.[26] Because of that, specific programs have been developed for control.[13]

Recently, a study of *A. glabra* reported the allelopathic potential of leaf extracts against crop pests and etiolated wheat coleoptile,[27] which can be related with its invasiveness. Laboratory allelopathic studies are based on phytotoxicity assays of compounds that can be extracted, isolated and identified from a plant. And those assays depend on the available compound amount. So, before fractionation, isolation and identification, it is important to have extracts with the high concentration of the active principles.

Several advanced extraction techniques can be applied to yield bioactive extracts, like 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 soft conditions applied.[28] Cavitation is the ultrasound mechanical effect that enables greater penetration of solvent into sample.[29] Additionally, UAE based methods allow for recovery of compounds in shorter times and at lower temperatures. There are several examples reported regarding destruction of active molecules due to high extraction temperatures.[30,31,32] Therefore, UAE technique has great advantage for the extraction of heat sensitive active compounds.

The ultrasonic-assisted extraction of oils from chickpea (*Cicer arietinum* L.),[33] saponins from ginseng (*Panax quinquefolium* L.),[34] polysaccharides[35] and phenolic compounds such as corilagin from longan (*Dimocarpus longan* Lour) pericarp [36] were reported. Under the optimal conditions, 85% acidified ethanol with aid of ultrasonication has been demonstrated to obtain a higher extraction yield from longan pericarp, as compared with the conventional extraction. [36] Likewise, Zhong and Wang[37] optimized ultrasonic-assisted extraction technology to obtain polysaccharides from dried longan pulp using response surface methodology. These results demonstrate that the ultrasonic-assisted extraction is more effective than the conventional extraction in extracting bioactive compounds from longan pericarp. No previous references on the optimization of ultrasound-assisted extraction using allelopathic results as target value were found in the revised literature.

To develop an extraction process, it is important to optimize highly significant factors affecting the extraction, in order to obtain the most active extract. For that, it is necessary an effective bioassay to assess the activity during the 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, which does not allow the evaluation of interactions among different extraction variables. Experimental designs instead are chemometric techniques that can be used for both evaluation of the effects of extraction variables and evaluation of interactions among them. [38] The bioassay selected that of etiolated wheat coleoptiles, which is fast (24h) and sensitive and could be considered as a first approach for phytotoxicity where undifferentiated tissue cells[39,40,41] are used. Macías et al. have proposed it as the first step for the search of potential new herbicides[42].

In this way, the objectives of this work are the obtaining of the best conditions for UAE aiming for *A. glabra* extract with highest bioactivity; as well as the isolation and identification of active compound from the most active extract. This will allow us to identify the secondary metabolites responsible for the allelopathic activity of *A. glabra.*

EXPERIMENTAL

Sample preparation. Dried *A. glabra* leaves were grinded and stored in refrigerated environment. For elimination of fat and wax from the sample, 500 g of powder were washed with hexane using an ultrasound system (7mm diameter probe). Ultrasound parameters were fixated: amplitude at 50% and cycle at 0.5 for 15 minutes without temperature control. The raffinate was dried in laboratory oven (40 ºC) and stored for following extraction.

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

Etiolated wheat coleoptile bioassay. Wheat seeds (*Triticum aestivum* L. cv. Duro) were sown in 15 cm diameter Petri dishes moistened with water and grown in the dark at 25±1 ºC for 4 days.[41] 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.[40]

Extracts or compounds were dissolved into a buffer solution (1.1 g L^{-1} of citric acid and 2.9 g L^{-1} of calcium phosphate in distillate 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 for compounds (10^{-3}, 5.10^{-4}, 10^{-4}, 5.10^{-5}, 10^{-5} M), also 0 ppm as negative control and a commercial herbicide (Logran) as positive control were used for the bioassay. Logran is 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 comparison study reported previously.

All determinations were run in three replicates, in which, each test tube (replicate) received 2 mL of one of the treatment and 5 coleoptiles fragment. Coleoptiles were kept for 24 h in contact with the extracts. Resulting values of coleoptile growth under extract effect to negative control growth values and results are presented in form of percentage, positive values represent stimulation and negative values, inhibition, versus control (0 ppm).

The coleoptiles were measured by digitalization of their images. Data were statistically analyzed using Welch's test.[43] 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.

Extractions. A starting comparative study to select the best solvents was developed. Six different solvents were used under the same extraction conditions: hexane, chloroform, ethyl acetate, acetone, methanol and water. Extraction conditions were as it follows: 2 g of vegetal sample, 25 mL of solvent, 50% amplitude, 0.5 for cycle and 15 min as extraction time, temperature was not controlled during these extractions.

Experimental design for evaluation of extraction variables effects. A two level factorial fractional experimental design was applied. A total number of 16 instead of 128 possible extraction conditions were developed. Seven different extraction variables

were evaluated in two levels (Table 1). In these extractions 1 g of plant material was used.

General Experimental Procedures. The purities of the compounds to be tested were determined by NMR and HPLC analyses and were found to be >98%. ^{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 using a Voyager Thermoquest spectrometer. FTIR spectra were obtained on a Perkin-Elmer Spectrum BX FTIR system.

TLC – Alugram Sil G/UV_{254} plates (0.25mm thickness) were used, analyzed at UV (254 and 360 nm) and revealed by heating them 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 with a differential refractometer RI-71detector was used. Semi-preparative column (LiChrospher SiO_2, Merck, 7 and 10µ, dimensions of 10 and 250 mm) with 3 mL/min flux; Analytical Phenomenex Luna Column (10µ Silica (2) 100A) with 1 mL/min flux, were employed.

Compounds isolation. For compounds isolation a higher scale was used in the ultrasound-assisted extraction, in this way 20 g of plant sample and 500 mL acetone were used, maintaining the optimized variables. A total amount of 1 Kg of plant sample was used for extractions, recovering 8.4 g (DW) used to compose chromatographic column sample.

The most bioactive extract was select to isolate and characterize its components. A column chromatography was carried out, and monitored by TLC affording 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 (High-performance liquid chromatography) with a semi-preparative silica gel column. Hexane-ethyl acetate 15% was used affording 3 sub-fractions (2a, 2b and 2c). The major component **1**, *ent*-kaur-16-en-19-oic acid (7.2 mg) was isolated.

Fraction 3 (622 mg) – It was fractionated with HPLC semi-preparative silica gel column, using hexane-ethyl acetate 20% as eluent. Two sub-fractions (3a and 3b) were obtained, from which β-sistosterol (**2**) (5.6 mg) and stigmasterol (**3**) (1.1 mg) were isolated.

Chlorophyll was eliminated from fractions 5 to 12 using mixtures of H_2O/MeOH as eluent in a RP18 column, and finally with dichloromethane to afford the chlorophyll from the column. Free chlorophyll fraction 8 was fractionated in HPLC semi-preparative using hexane-ethyl acetate 25% and yielded 18 mg of **4** (*ent*-19-methoxy-19-oxokauran-17-oic acid).

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

Fraction 10 was subjected to HPLC semi-preparative column with hexane-ethyl acetate 60% and yielded 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 in HPLC semi-preparative column using hexane-ethyl acetate 40% and 4.3 mg of **5** annoglabasin B was obtained.

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

Bioactivity of 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**), was perfomed using wheat coleoptiles bioassay.

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.).

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

$$Y = Y_{min} + \frac{Y_{max} - Y_{min}}{1 + 10^{logIC50-X}}$$

Where *X* indicates the logarithm of concentration, Y indicates 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). The adjustment and r^2 were obtained using GraphPad Prism software v.4.00 (GraphPad Software Inc.).

RESULTS AND DISCUSSION

In the experimental design the effects of different extraction variables on the bioactivity of the resulting extracts was evaluated. Ranges for extraction variables were established to cover the most usual values including 5 and 25 ºC for temperature, 25 and 50 mL for

the extraction solvent, 5 and 15 min as extraction time, two different ultrasonic probes with different diameters were used: thin (3 mm) and thick (7 mm), 30% and 70 for ultrasound amplitude, and 0.2 and 0.8 s^{-1} for cycle. All extractions were done in duplicate. However, as a first control to extractions reliability, the resulting dry weight for the extracts was used. Therefore extraction showing relative differences higher than 20% for dry weights were discarded then repeated again before the bioactivity analyses. As it can be seen in table 1, bioactivity for the extracts ranged from 0 to -67%, it means that the ranges selected for the extraction variables produced really great differences in the recovery for the bioactive components.

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

Experiment	T. (°C)	Volume (mL)	Time (min)	Probe	Solvent	Amplitude	Cycle	Activity 800ppm
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

Graphical analysis was used to determine the effects of extraction conditions on the bioactivity values. Main effects plots by the extraction variables in the bioactivity shows clear conclusions regarding the main experimental values for that parameter. As it is showed in Fig. 1, the results from the two different solvents used showed the highest differences between the resulting bioactivity values. Experiments run using acetone

produced a -45 % of inhibitory results as average value whilst experiments run using MeOH produced -16% inhibitory results as average value. It means, changing from MeOH to acetone the inhibitory effect was threefold higher. Regarding the other extraction variables, they showed lower effects than extraction solvent, however they were also notable for extraction temperature, time and the type of ultrasonic probe. For temperature higher bioactivities results were found using 5ºC than using 25 ºC (-38% vs. -24% of inhibitory activity). Lower extraction volume (25 mL) produced higher bioactivity results than the larger ones (50 mL), -37% vs. -25% inhibitory effects were found. Shorter extraction times (5 min) also showed better bioactivity results in the extracts than the longer ones (15 min). Finally, the thinner ultrasonic probe produced better results than the thicker one, almost double inhibitory activity -41% vs. -21%.

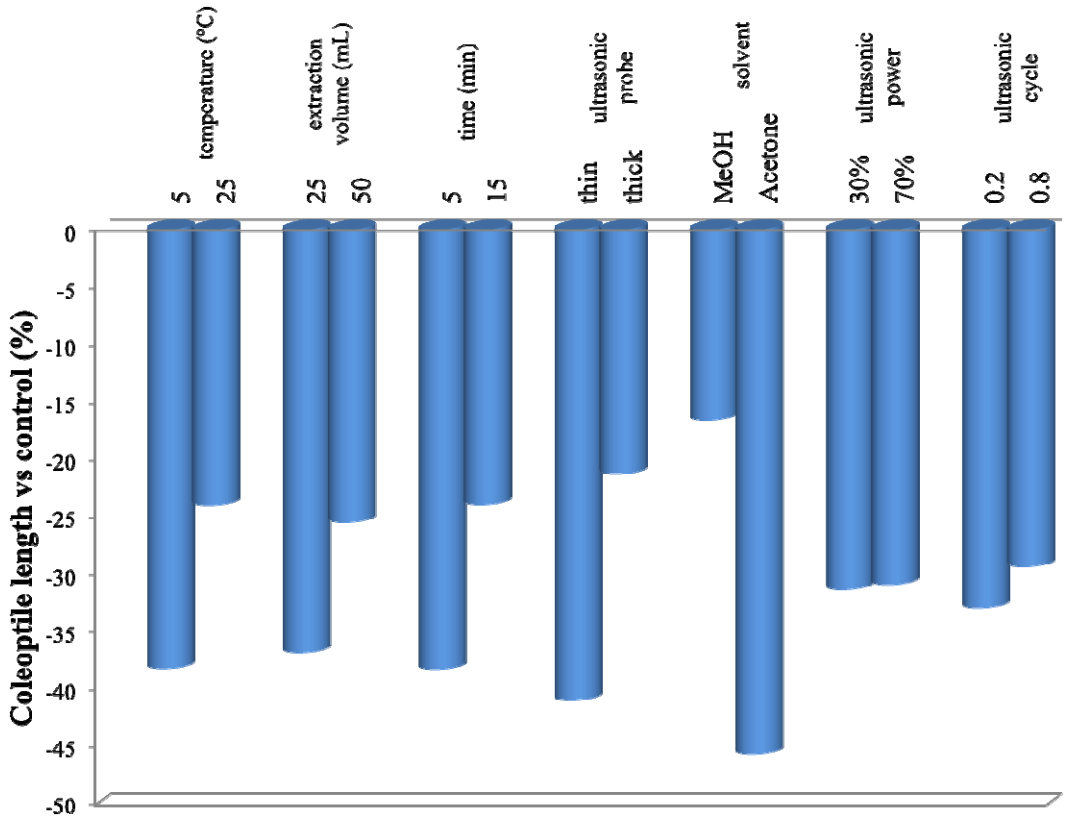

Figure 1. Main effects plot for bioactivity of extracts in the experimental design.

Optimization of the highest influent extraction variables – In view of the main effects of temperature and solvent volume, it was decided exploring categories for these variables that were not included in the initial experimental design. Extraction time would be optimized later after evaluating the kinetic for the extraction and there were not more options for the ultrasonic probe time, then 5 min and thin probe were fixed as extraction variables for those parameters. Four different extraction volumes (10, 15, 20, 25 mL) at the temperature of 5°C were used.

Temperature was fixed because of values less than 5°C showed few bioactivity variations of the extracts, meaning no interesting results. Additionally there is an increase in time for stabilization of the extraction system caused by this distinct temperature from the environment. Different volumes also produced weak variation on bioactivity (Table 2), so the initial value (25 mL) was chosen because extracts can be more easily filtered using 25 mL instead of lower volumes.

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

Therefore, the most suitable extraction variables were: 25 mL Acetone, 5 °C, 2 mm probe, cycle of 0.2, 30% amplitude for 20 minutes, using 1 g plant sample.

Establishment of extraction kinetics – Extractions were developed at several length of time (1, 2.5, 5, 7.5, 10, 15, 20 min). Longer times than 20 min were not used due to operational point of view.

The extraction time of 20 minutes was the most appropriate, since presented the higher values on extraction mass (Kruskal-Wallis with Dunn's post-test) and bioactivity results on the coleoptile bioassay (Table 3).

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	-54 ±0.5[a]
2.5	-56 ±1.5 [a]
5	-52 ±1.5 [a]
7.5	-57 ±6.5 [ab]
10	-55 ±1.0 [ab]
15	-58 ±8.5 [ab]
20	-62 ±2.0 [b]

Analytical properties of the extraction method

Reproducibility and repeatability – 9 extractions were done in the same day to determine repeatability and 9 extractions (3 extractions per day in 3 different days) were run to determine reproducibility.

No significant differences of mean values of bioactivity results between extractions developed in the same day or those made in different days, according to Kruskal-Wallis analysis with Dunn's post-test P 0.05 (table 4)

Table 4. Bioactivity results (mean and Standard deviation) for extracts obtained in the same day (repeatability) and in different days (reproducibility).

Extraction	Same day (9 extractions x 1 day)	Different days (3 extractions x 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

* Values in percent, relative to controls

Therefore, the developed method is appropriate to obtain extracts in a reproducible way through time, in terms of bioactivity, enabling an optimization for future studies of allelopathy. Additionally it has to be noted that high bioactivity values (around -60%) were found for the extracts obtained under the optimized extraction conditions.

After the optimization of the extraction method, a higher scale extraction was used to obtain enough amount of extract to be able to follow with the next step in the allelopathic study, i.e. isolation of compounds from the bioactive extract. Eight compounds have been isolated and identified from this extract (fig. 2), two steroids (β-sistosterol (**2**)[44] and stigmasterol (**3**)[45], five diterpenes with kaurane skeleton (*ent*-kaur-16-en-19 oic acid (**1**)[22], *ent*-19-methoxy-19-oxokauran-17-oic acid (**4**),[46] annoglabasin B (**5**)[14], *ent*-17-hydroxykaur-15-en-19-oic acid (**6**)[47] and *ent*-15 β,16 β-epoxy-17-hydroxy-kauran-19-oic acid (**7**)[48,49] and the acetogenin asimicin (**8**)[50] whose spectroscopic data are identical to those previously reported.

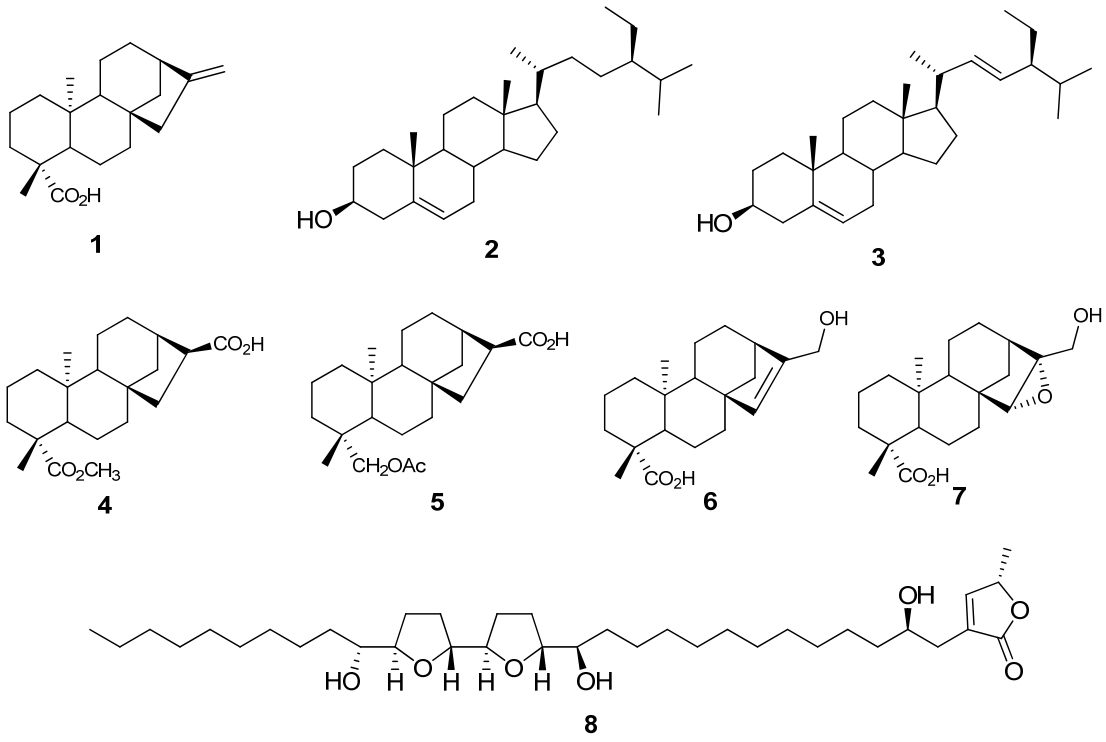

Figure 2. Chemical structures of isolated compounds from *Annona glabra* leaves.

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 were assayed in the etiolated wheat coleoptiles bioassay (fig. 3). The compounds that sowed higher activity were the kauranic diterpenes **1**, **4** and **5** and the acetogenin **8**.

The most active one, annoglabasin B (**5**), showed strong inhibition, reaching -95% at 10^{-3} M, -87% at 5.10^{-4} M and higher than -70% at 10^{-4} M. Activity decreased with dilution but even at the lowest concentrations tested showed an activity close to -60% at $5 \cdot 10^{-5}$ M and above -30% at 10^{-5} M. Its IC50 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 of activity for this compound is that described as weak activity against HIV replication in H9 lymphocyte cells.[14]

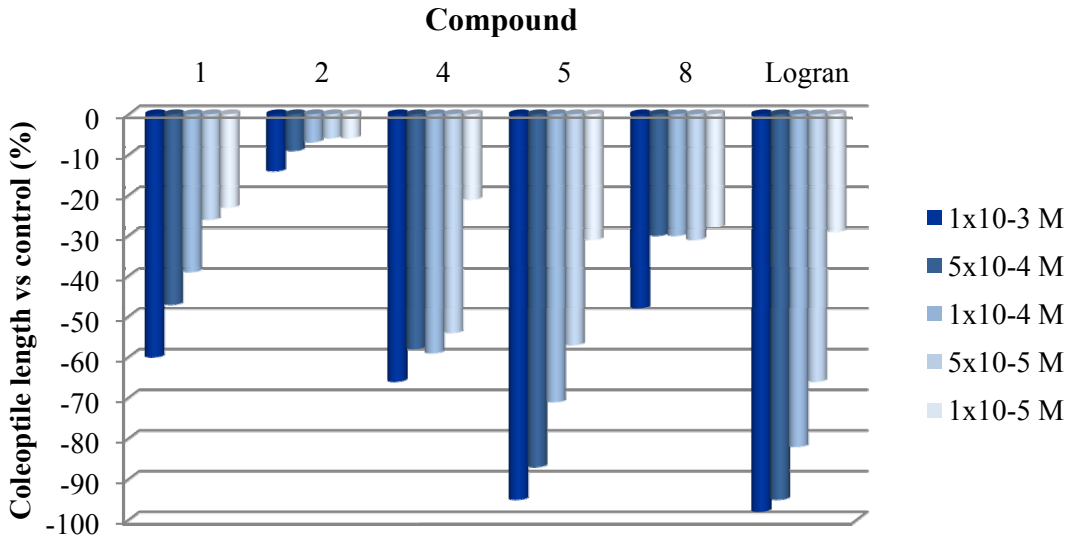

Figure 3. Bioactivity results for pure compounds from *Annona glabra* leaves.

The next most active compound was 4 with an IC50 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 remains with dilution except at the lowest concentration (10^{-5} M), keeping their

inhibition values above -50%. Previously, it has been reported for *ent*-19-methoxy-19-oxokauran-17-oic acid (**4**) cytotoxic selectivity for PC3 (prostate cancer) cells but with weak potencies.[46]

The third active compound is *ent*-kaur-16-en-19 oic acid (**1**) with an IC50 733,2 μM (r^2 0.9174). This also showed inhibition, -60% a 10^{-3} M, close to -50% and about -40% at 5.10^{-4} M and 10^{-4} M respectively. Previously, e*nt*-kaur-16-en-19-oic acid (**1**) has been described as an active compound, with MIC values of 10 μg mL^{-1} against the following microorganisms: *Streptococcus sobrinus, Streptococcus mutans, Streptococcus mitis, Streptococcus sanguinis* and *Lactobacillus casei*; and was proposed as a prototype for the discovery of new effective anti-infection agents against microorganisms responsible for caries and periodontal diseases.[51] It has also shown antiplatelet aggregation,[52] analgesic,[53] antifungal,[54,55] smooth muscle relaxant,[56] hypoglycemic,[57] cytotoxic and embryotoxic effects.[58]

The last tested compound that showed activity was asimicin (**8**) with values of -48% at 10^{-3} M and an IC50 of 2013 μM. while β-sitosterol (**2**) showed no 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. [50] Although asimicin (**8**) has been active in this bioassay, its activity is lower than that shown by diterpenes from *Annona glabra*, in particular annoglabasin B (**5**) is the most active compound assayed.

The results of the optimization of the extraction by ultrasound show how this study has led to the conditions for obtaining the most active extract. Isolation and characterization of the components of this extract has identified the metabolites that could be responsible for the activity that *Annona glabra* shows. From them, the diterpene annoglabasin B (**5**) was the most active followed by *ent*-19-methoxy-19-oxokauran-17-oic acid (**4**). Both

have a carboxylic acid function at position 17, and an ester function on carbon 19 and appear to have a role in the defense mechanisms of this species.

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CAPÍTULO 3

Toxicity of *Annona glabra* L. extracts on *Selenastrum capricornutum* Printz

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207 words (altogether, title, abstract)

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Abstract

Annona glabra is a semi-deciduous tree that contains several active substances, including secondary metabolites, some of which with antimicrobial and antifungal activity. However, only a few studies investigated the allelopathic activity of these substances or their toxicity to algae. A consequence of environmental nutrient enrichment is explosive growth of phytoplankton, which leads to fast eutrophication and a decrease in water quality. In the search for eco-friendly algaecides, we analyzed the effects of acetone extracts of *A. glabra* leaves on the algae *Selenastrum capricornutum*. We evaluated ten extract concentrations ranging from 0 to 400 mgL^{-1}, in 72 and 96 h of acute ecotoxicity testing. The results showed no effect on *S. capricornutum* up to 75 mgL^{-1}, but a significant inhibitory effect at 125 mgL^{-1} and above, which reduced the growth rate as well as the final biomass of the algae. Extract concentrations above 200 mgL^{-1} were completely inhibitory. The half maximal inhibitory concentration (IC_{50}) for 72 h (135.6 mgL^{-1}) and 96 h of exposure (145 mgL^{-1}) to our crude extracts are comparable to those obtained with commercial fungicides and herbicides used in aquatic ecosystems. The inhibitory effects of *A. glabra* extracts on algal growth bring about potential applications for the isolation and identification of algaecide substances.

Key words: allelopathy, algal growth, inhibition, leaf extract.

Introduction

Annona glabra L. is a semi-deciduous plant adapted to flooded environments, including brackish- and saltwater. It is considered invasive in Asia and Oceania (Yáñez-Espinosa & Terrazas 2001; Mielke et al. 2005; Sugars, Charleston & Doak 2006). This species forms clusters along coastal wetlands and its fruits produce several seeds that germinate better in sunny conditions (Mata & Moreno-Casasola 2005; Setter, Setter, Graham & Vitelli 2008).

A. glabra has been reported to have parasiticide and insecticide activity, which supports its use in traditional medicine (Liu, McLaughlin 1999; Bobadilla, Zavala, Sisniegas, Zavaleta, Mostacero, et al. 2005; Mendonça, Silva, Santos, Ribeiro Júnior & Sant'Ana 2005). Some of its isolated compounds have antimicrobial, antifungal, and anticancer properties (Padmaja, Thankamany, Hara, Fujimoto & Hisham 1995; Zhang, Peng, Xia, Wang & Han 2004). Previous studies in our laboratory showed an allelopathic potential of the leaf extracts of *A. glabra* to the germination of weed species and etiolated wheat coleoptile (Matsumoto, Ribeiro, Takao & Lima 2010).

Submerged leaves of aquatic angiosperms have no stomata, reduced cuticles, and few tight cell connections, which facilitates the release of organic compounds. *A. glabra* has glabrous leaves, which may favor the leaking and leaching of intracellular compounds (Gross 2003). Several studies on allelopathy in aquatic ecosystems pointed to an influence of macrophytes on algae through *in situ* and laboratory experiments (Macías, Galindo, García-Díaz & Galindo 2007; Wu, Gao, Wang, Liu & Zhou 2009; Zhu, Liu, Wang, Gao & Wu 2010; Zhang, Sun, Ge, & Wu, 2011).

An excess of nutrients in water bodies stimulates plant growth, accelerates eutrophication, and may lead to explosive phytoplankton growth with negative environmental impacts. As immediate consequences, we observe a decrease in water quality and a reduction of light penetration. In addition, depending on the dominant algae species, toxins may be released, affecting the biota (Hu & Hong 2008). Thus, the search for specific, natural products from plants that reduce microalgae growth is important for the control of phytoplankton blooms in the environment. Plants and their secondary metabolites are good candidates (Zhu et al. 2010).

Some of the advantages of using natural compounds to control phytoplankton populations are that they do not have toxic metals, pro-oxidants, and organic amines in

their composition, in contrast to the chemical algaecides available in the market. Chemical algaecides have broad toxicity to aquatic organisms and can persist in the environment (Hu et al. 2008), whereas natural products may be more specific and less toxic to higher trophic levels. According to Hu et al. (2008) and Duke, Cantrell, Meepagala, Wedge, Tabanca et al. (2010), natural toxins are environmentally and toxicologically safer molecules than synthetic toxins, because they don't have potentially dangerous substances in their composition.

Traditionally, studies on plant allelopathy and toxicity to aquatic organisms (e.g., microalgae and cyanobacteria) use more frequently aquatic plants (e.g., macrophytes) than tree species as sources. The present research aimed at evaluating the effect of leaf extracts of *Annona glabra* on *S. capricornutum* Printz., used as a test organism. The results showed that the extracts were effective against the Chlorophyceae.

Materials and methods

We obtained *Annona glabra* L. leaves at different stages of maturation from trees in the Massaguaçu River Estuary, Brazil (23º37'20''S and 54º21'25''W). We deposited the voucher specimen 7503 in the herbarium of Departamento de Botânica, at Universidade Federal de São Carlos. After collection, we dried the leaves in a greenhouse at 45 °C for 48 h, ground them, and stored the powder in a freezer at -20 °C until extraction.

We extracted active compounds of *A. glabra* from 1.0 g of ground and dried samples, using an ultrasound assisted extraction (UAE) procedure, following Firdaus, Izam & Rosli (2010). The extraction method recovers organic compounds, including those with allelopathic potential, such as alkaloids and flavonoids. The UAE conditions

were: 25 ml acetone, 2 mm probe, cycle of 0.2, 30 % amplitude of 200 W, and instrument potency of 24 Hz . These conditions were applied during 20 minutes at 5 °C.

To evaluate the effects of leaf extracts of *A. glabra* on *S. capricornutum*, we performed toxicity tests with extract concentrations ranging from 25 to 400 mg L^{-1} (25, 50, 75, 125, 150, 175, 200, 300, and 400). The control had no extract addition, but 50 ml of acetone. We made three replicates for each treatment. We used *S. capricornutum* as a test organism. We kept stock cultures in LC Oligo culture medium (AFNOR 1980) under laboratory-controlled conditions of light intensity (130 $\mu Em^{-2}s^{-1}$), light/dark cycle (16:8 h), and temperature (23 ± 2 ^{o}C).

We placed approximately 50 ml of the extract in 150-ml Erlenmeyer flasks, and vacuum dried the sample at 35 ^{o}C to volatilize the solvent. After complete dryness, when only the tested organic compound remained, we added 50 ml of exponentially growing *S. capricornutum* culture. Hence, in the beginning of the experiment, each Erlenmeyer flask contained the dried extract and $5x10^4$ cells ml^{-1}. Bioassays were incubated under the same growth conditions as the algae stock cultures. Daily aliquots (0.5 ml) were obtained up to 96 h of exposure to estimate algae biomass (cell ml^{-1}). We counted algae cells in an Improved Neubauer chamber under optical microscope. We obtained growth rates by plotting the natural log of cell ml^{-1} against experimental time and calculating a linear regression for the exponential part of the growth curve. Growth rate values were calculated as the slopes of linear regressions.

We estimated the inhibitory concentration that caused 50% (IC_{50}) of decrease in *S. capricornutum* biomass at 72 and 96 h of exposure. The final biomass of the treatments at 96 h of exposure were contrasted through an ANOVA with a Tukey post-hoc test. We used the F statistics to compare growth rates.

Results

Figure 1 shows cell density at 96 h of exposure as a function of extract concentration of *A. glabra*, as well as IC50 values for 72 h (135.6 mgL^{-1}) and 96 h of exposure (145 mgL^{-1}). The results show that up to 75 mgL^{-1} no effects of the leaf extract of *A. glabra* were detected on *S. capricornutum*. Above this concentration, inhibition increased gradually with extract concentration, until reaching null microalgal growth at 200 mgL^{-1} and higher. No statistical difference was detected for the three highest concentrations tested.

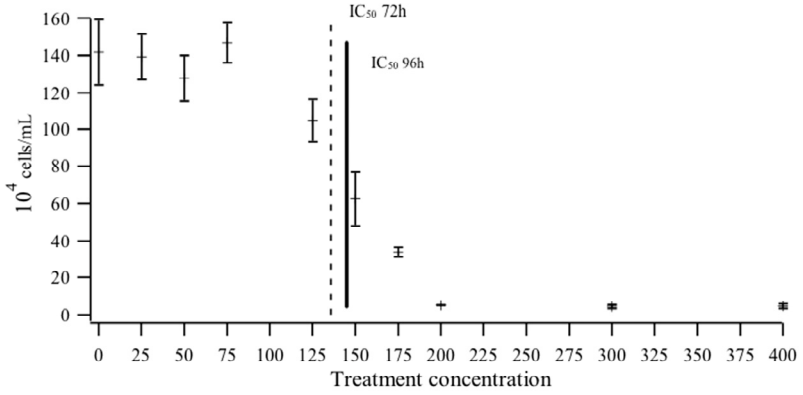

Figure 1. *S. capricornutum* culture density (cell ml^{-1}) at 96 h of exposure as function of extract concentration of *Annona glabra* (mg L^{-1}). The bold line represents the IC_{50} values for 96 h of exposure and the dashed line represents the IC_{50} for 72 h of exposure.

Figure 2 shows cell density as a function of experiment duration. Since no significant difference (F test) was detected in the growth rates among replicates, the data could be pooled together and a single slope was calculated for each treatment. Figure 2 shows that at the extract concentrations of 125, 150, and 175 mgL^{-1} there was a delay in the population growth of *S. capricornutum*, analogous to a *lag* phase in standard batch cultures. So, we calculated linear regressions for the growth curves of the treatments after 24 h of exposure for 125 and 150 mgL^{-1}, and after 48 h of exposure for 175 mgL^{-1}. For the treatments at the concentrations of 200, 300, and 400 mgL^{-1}, no growth was

observed and cell densities (~ $5x10^4$ cel ml^{-1}) were statistically similar (ANOVA $p>0.05$).

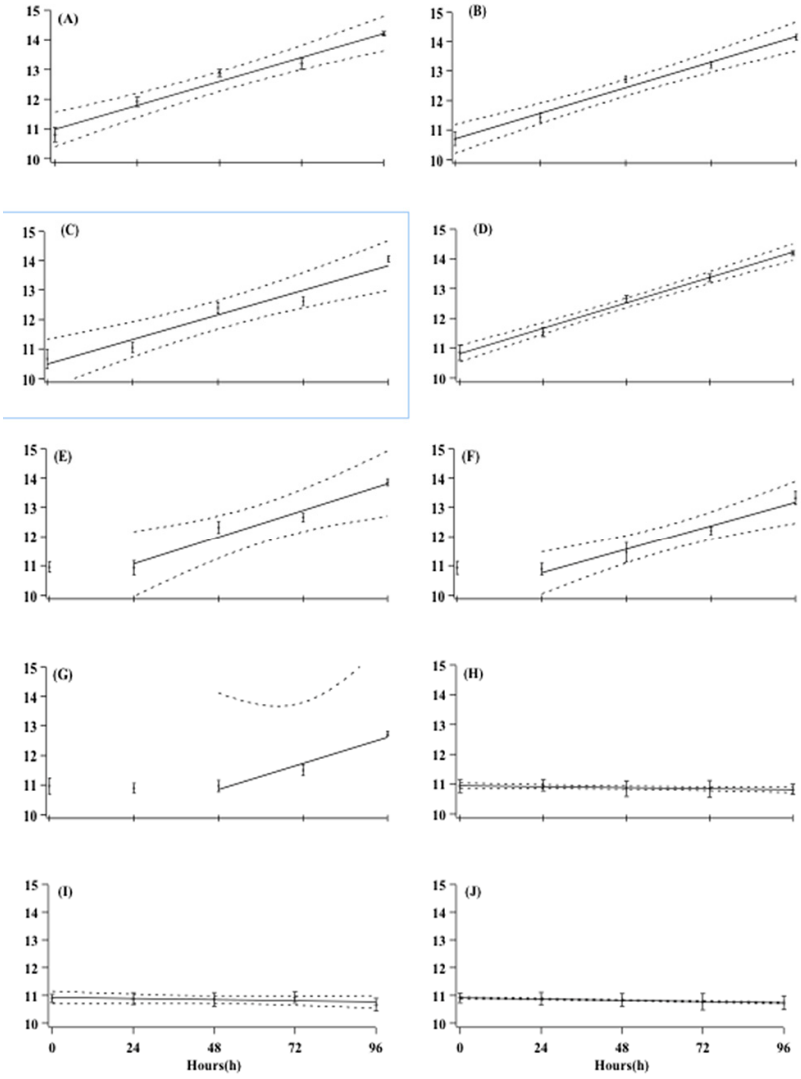

Figure 2. *S. capricornutum* density (cell ml^{-1}, ln) as a function of exposure time (h) for each treatment. Values represent average (± SD) of ln (cells ml^{-1}). Growth rate values are 0.81 ± 0.02 for the control (A); 0.87 ± 0.01 for 25 mgL^{-1} treatment (B); 0.85 ± 0.03 for 50 mgL^{-1} treatment (C); 0.85 ± 0.03 for 75 mgL^{-1} treatment (D); 0.91 ± 0.03 for 125 mgL^{-1} treatment (E); 0.80 ± 0.04 for 150 mgL^{-1} treatment (F); 0.88 ± 0.05 for 175 mgL^{-1} treatment (G); -0.03 ± 0.02 for 200 mgL^{-1} treatment (H); -0.04 ± 0.02 for 300 mgL^{-1} treatment (I); -0.05 ± 0.02 for 400 mgL^{-1} treatment (J). Dashed lines represent 95 % of confidence intervals.

Discussion

Our results show that extracts of the plant *A. glabra* at concentrations from 125 to 175 mgL^{-1} delayed the growth of the alga *S. capricornutum*, resulting in lower cell density at the end of the experiment, though the extracts did not affect its growth rate.

At concentrations from 200 to 400 mg L^{-1} algae cells were unable to divide. At concentrations below 125 mg L^{-1}, there was no significant difference compared to the control. These results are consistent with previous studies. Ridge, Walters & Street (1999) showed that the leaf litter of *Quercus robur* L. inhibited the growth of *Chlorella vulgaris* Beyerinck [Bejjerinck] 1890. These authors also studied bales of barley (*Hordeum vulgare* L.), a terrestrial plant historically used in the management of undesired algal growth in shallow lakes. They showed that *H. vulgare* affected interactions in the phytoplankton community in the natural environment, reducing cyanobacteria dominance at a concentration of 38-50 mg L^{-1}.

Our results are also consistent with those of Cantrell, Schrader, Mamonov, Sitpaeva, Kustova et al. (2005), who showed that *Haplophyllum sieversii* Fish., a terrestrial herbaceous plant, inhibited completely the growth of *S. capricornutum* at concentrations above 100 mg L^{-1}. In our study, a complete inhibition of *S. capricornutum* by the extract of *A. glabra* was obtained at concentrations above 200 mg L^{-1}.

Several *in situ* and laboratory studies showed that macrophytes can act as algae growth inhibitor (Donk & Bund 2002; Xian, Chen, Zou, & Yin 2006; Hilt 2008; Toporowska, Pawlik-Skowrońska & Wojtal 2008; Zhu et al. 2010). A study on the effects of aqueous extracts of five plant species on *S. capricornutum*, including Papaveraceae macrophytes, obtained IC_{50} at concentrations above 21 mg L^{-1}. *Stylophorum lasiocarpum* was the fourth most active species, with IC_{50} at the concentration of 114 mg L^{-1} (Jančula, Suchomelová, Jakub, Smutná, Maršálek et al. 2007). By comparing our results to those of Jančula et al (2007), we can assume that the extracts of *A. glabra*, which are toxic at the concentration of 145 mgL^{-1}, have a strong inhibitory effect. However, ethyl acetate fractions of the macrophytes *Potamogeton malaianus* and *P. maackianus* were toxic to the cyanobacteria *Microcystis aeruginosa* at

the concentration of 60 mg L^{-1}, and reduced its biomass in ~ 54 % and 58 %, respectively (Wang, Cheng, Zhang, & He, 2010).

The IC_{50} of extracts of *A. glabra* on *S. capricornutum* in the present study was 135.6 mgL^{-1} and 145 mgL^{-1} for 72 h and 96 h of exposure. Hence, the plant *Annona glabra* is a promising species for the isolation of compounds with algaecide properties. Table 1 summarizes the action of some extracts and pure products in tests on *S. capricornutum.*

Table 1. Comparison of the half maximal inhibitory concentration (IC_{50}) of extracts and compounds on *Selenastum capricornutum* Printz in toxicity assays.

Donor Species/ Product type	Substances	Inhibition	mg L^{-1}	Reference
Annona glabra	Acetone extract	IC_{50} 72h	135.6	our results
		IC_{50} 96h	145	
Haplophyllum sieversii	Crude extracts	100%	>100	Cantrell et al. 2005
	Flindersine	IC_{50} 96h	17.8	
	Haplamine	IC_{50} 96h	15.9	
Myriophyllum spicatum	Pyrogallic acid (PA)	IC_{50} 72h	5.45-8.45	Zhu et al. 2010
	Gallic acid		4.78-8.99	
	Ellagic acid		10.06-14.69	
	(+)-catechin		7.45-11.75	
Chelidonium majus	Aqueous extracts	IC_{50} 96h	60.87	Jančula et al. 2007
Dicranostigma lactucoides			21.27	
Macleaya microcarpa			868.09	
Sanguinaria canadensis			23.9	
Stylophorum lasiocarpum			114.1	
Fungicide	FLC+PCH SC 62,5+625 G	IC_{50} 72h	100	®Bayer 2010
Herbicides	Lawn weedkiller ready to use2	IC_{50} 72h	270	®Bayer 2012
	Glyphosate	IC_{50} 48h	270	Cedergreen & Streibig 2005
	Triasulfuron		0.405	
	Metsulfuron-methyl		0.677	
	Terbuthylazine		0.055	
	Bentazone		13.6	
	Mesotrione		6.78	
Algaecide		IC_{50} 72h	0.04	®Lonza 2004

Several commercial herbicides and fungicides bring risks to the environment. Harmful and long-term effects on aquatic organisms have been related to exposure to commercial herbicides. The IC_{50} at 72 h of exposure of some of these particular products on *S. capricornutum* are between 270 mg L^{-1} and 100 mg L^{-1} (®Bayer 2010; Bayer 2012). An assessment of the impacts of herbicides on non-target aquatic plants and algae, including *S. capricornutum*, showed IC_{50} values ranging from 0.055 to 270 mg L^{-1} (Cedergreen & Streibig 2005). Commercial algaecides as Barquat low foam presents IC_{50} of 0.04 mg L^{-1} against *S. capricornutum* (®Lonza 2004).

Gao & Tam (2011) compared the responses of *Chlorella vulgaris* and *S. capricornutum* to nonyphenol stress. They obtained higher tolerance of *C. vulgaris* in relation to *S. capricornutum* and assumed it was due to a better acclimation of the former. By acclimation, the authors meant more efficient and rapid responses to nonyphenol and/or higher nonyphenol biodegradation or transformation ability in *C. vulgaris*.

Our results are consistent with several studies that observed delayed growth when the microalgae were exposed to toxic agents. Mulderij, Donk & Roelofs (2003) observed a decrease of 7% in the growth rate of *S. capricornutum* and an extended lag phase in cultures of *Chlorella minutissima* exposed to charophyte (*Chara globularis* var. *globularis* Thuillier, and *Chara contraria* var. *contraria* A. Braun).

According to *NRA* (2001), *S. capricornutum* presented lower growth rate and delayed growth when exposed to 10 mg L^{-1} semduramicin (anticoccidial), whereas a total growth inhibition was observed at 39 mg.L^{-1}. In our study, a similar delay was observed before the beginning of the exponential growth phase when the algae were exposed to concentrations of 125 - 175 mg L^{-1} of the extract of *A. glabra*. However, after such a lag phase, the two highest growth rates were observed. This may be related

to a survival strategy under a stressing situation, with the algae adjusting its metabolism to the presence of the toxic agent (Stebbing 1982; Stebbing 1987). This author linked a similar behavior to what is known as the *hormesis effect*, with the toxicant stimulating the growth. In our study, statistically higher growth rates were obtained at the concentrations of 25, 125, and 150 mg L^{-1}, but not at the concentration of 75 mg L^{-1} of *A. glabra* extract, suggesting that a regulatory over-correction mechanism may have occurred. Nevertheless, more concentrations and higher exposure time should be tested to confirm the *hormesis* hypothesis for the organisms that we have tested.

Our results show that *A. glabra* extracts at concentrations up to 75 mg L^{-1} have no effect on final algae biomass or growth rate. However, we obtained a significant delay in the exponential growth phase and a reduction in population density at the concentration of 125 mg L^{-1}. This is evidence of the potential of *A. glabra* extracts for the isolation of natural substances that can be used in phytoplankton control.

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CAPÍTULO 4

Activity of *Annona glabra* L. leaf extract on the Oomycete *Pythium aphanidermathum* (Edson) Fitzp.

Reginaldo Sadao Matsumoto, Maria Augusta Ferraz Machado Miranda, Maria Inês Salgueiro Lima

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ABSTRACT

Phytopathogenic strains of the genus *Pythium* causes billion dollar losses all over the world on natural and crop species. It is known that long term application of synthetic chemicals causes environmental contamination and increase of resistant strains. Thus, the searches for new and safer substances are concentrated in natural sources such as medicinal plants. *Annona glabra* is a small tree used in traditional medicine and reported to posses a great number of compounds, but most of with few bioactivities described. Ten concentrations of *A. glabra* extracts were prepared with a 9 serial two-fold dilution starting with 1000 $\mu g.disc^{-1}$. Extracts were tested on *P. aphanidermatum* with a disc diffusion method. *A. glabra* extracts inhibited *P. aphanidermatum* in upper concentrations of 125 $\mu g.disc^{-1}$ and compared to the following studies which used different concentrations and target species, strong effects are observed even when compared with fungicides as Cycloheximide and Bifonazole. (-)-kaur-16-en-19-oic acid, Stigmasterol and Sitosterol are identified substances isolated from this extracts are described in literature as antifungals. These substances could explain part of the anti-oomycete activity. Our results highlight the importance of the bioprospection of substance of this organisms.

INTRODUCTION

Oomycetes has been recently separated form *Fungi* kingdom, and contains several plants pathogens. *Phytophthora* and *Pythium*are are examples of some of the world's most destructive genera (WEST, VAN *et al.*, 2003).

Pythium species are a filamentous organism that causes severe diseases in many plant species and are responsible for huge economic losses in agriculture worldwide.This genus had more than 120 recognized species with a wide host range, from grass to trees species, and most of phytopathogenic *Pythium* species often lives in

wet or flood soil conditions and causes fruit, root or collar rot and seedlings damping-off (NECHWATAL; MENDGEN, 2007).

Natural and crop species are suffering with pathogenicity of *Pythium*, leading to significant economic losses of fruit trees, sugarcane (HENDRIX; CAMPBELL, 1973), rice (EBERLE *et al.*, 2007), soybean and corn (BRODERS *et al.*, 2007), carrots (BARR; KEMP, 1975) and more specifically, some hydroponic crop culture, as lettuce and tomato are commonly hosted by *Pythiumaphanidermatum* (Edson) Fitzp. (PATEKOSKI *et al.*, 2010;HENDRIX; CAMPBELL, 1973).

Long term application of synthetic chemicals for pest control cause food and environmental contamination due to their residues and slow degradation, but this toxicity effects can be overcome by searching for new and safer alternative measures (eco-friendly and effective natural substances) to control plant fungal diseases as well (LU *et al.*, 2008)(PAWAR, 2011)(SALAMCI *et al.*, 2007).

Addressed for medicine or agriculture purposes, many efforts have been made to extract bioactive compounds from different types of sources,but mainly from medicinal plants (KHAN; KHAN, 2010).

Annona glabra L. from *Annonaceae* family live in wet estuary areas and has been used, since traditional medicine, for many purposes (anti-cancer, antibacterial, insecticidal, ant parasitic) by its biological activity (PINTO *et al.*, 2005). Despite of many studies about isolating chemical compounds from *A.glabra,* most of it, were extracted from fruits and stem and less from leaves (ALALI *et al.*, 1999)(CHANG *et al.*, 2000)(HSIEH *et al.*, 2004).

Little is known about chemical compounds biological activities, with the exception of extensive studies on the Annonaceous acetogenins, that despite their diverse bioactivity, efforts are concentrated on studies of antitumor activity (GALLARDO *et al.*, 1998; ZENG *et al.*, 1996;ALALI *et al.*, 1999).

By its properties, this species is a good candidate in the search for bioactive compounds. In this work, the inibitory activity of acetone extract from *Annona glabra* leaves was evaluated against *Pythium* ssp. strains through disc diffusion method.

MATERIAL AND METHODS

Pure isolate of *Pythium aphanidermatum*stra in CCMA 243 (Laboratório de Microbiologia Ambiental - Embrapa MeioAmbiente - CNPMA) was cultured in

PDA (potato dextrose agar). Plates were disposed in culture chambers at 25 ± 2°C, 12h photoperiod,untilthe growth reached the edge of an 85 mm Petri dish plate.

Annona glabra leaves were collected in summer season (2011) in Massaguaçu river estuary (23º37'20''S e 45º21'25''W) and oven-dried at 40°C for 48h. Samples were grinded, bagged and stored in freezer (-20°C). A solution was prepared with 20g ofleaf powderand 500 mL acetone using an Ultrasound Assisted Extraction, filtrated (14 μm mesh) and dried it out at vacuum rota evaporator. The stock extract, 25 $\mu g.\mu L^{-1}$, was prepared with acetone, filtered (0.2μm mesh) and eleven concentrations were prepared in a twofold serial dilution.

Disc diffusion test was used to evaluate the anti-oomycete activity. A 5mm diameter plug of the PDA with *P. aphanidermatum* was transferred to the centre of a Petri dish plate with PDA.

Six millimetres paper disks were soaked with 40 μL of the extract, dried and disposed equidistant from the centre of the plate (23 mm).Three groups of plates were established (A, B and C). Each group received 4 discs with different concentrations of the extract (A- 0, 0.97, 1.95, 3.9 $\mu g.disc^{-1}$; B- 7.8, 15.6, 31.2, 62.5 $\mu g.disc^{-1}$; C- 125, 250, 500, 1000$\mu g.disc^{-1}$). The 0μg disc was soaked with 40 μL of acetone and used as negative control. Four group replicates were applied.Three PDA plates with anoomycete plug on the centre were prepared as growth control.

The *P. aphanidermatum* growth and inhibition zones (radius) were measured every 24h for 15 days. All plates were cultured as the same conditions. *P.aphanidermatum*growth was fitted in Boltzmann equation and curves were compared with ANOVA. Inhibition radiuses were compared with ANOVA with Tukey'spost-test.

RESULTS

The fitted curves of control and the tested groups (A, B and C) present no difference, showing similarfinal growth and growth rate of *P.aphanidermatum*. No inhibition zones were noticed on A and B groups. The C group began to show inhibition zones on day 7,when the oomycete reached the discs and zones were measured to day10.The radius data from inhibition zones were pooled to calculate the average and standard deviation for ANOVA comparison. From day 11 to the end of the experiment, the oomycete has recovered and grew overthe discs and vanishing the inhibition zones, then occupying the whole plate.

ANOVA comparisons showed differences among the four concentrations (125, 250, 500, 1000µg.disc^{-1}) of C group, except between 500 and 1000, which present similar inhibition zones of 15.95 ±1.01 and 16.38 ± 1.07 mm (figure 1).

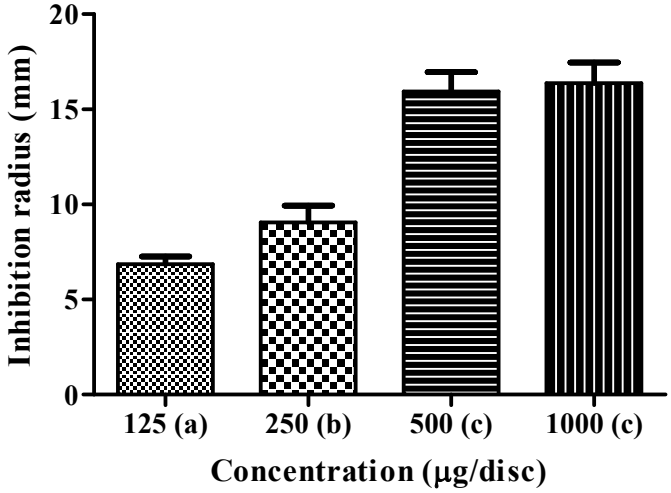

Figure 1. Average inhibition zones (in millimetres) and standard deviations of the C group discs (125, 250, 500, 1000µg.disc^{-1}). Different letters, after concentration numbers represent significant differences by ANOVA with Tukey's post-test.

DISCUSSION

Oomycetes were long considered a class within kingdom *Fungi*, but fundamental differences in physiology, biochemistry and genetics separates both groups (LATIJNHOUWERS *et al.*, 2003) . For that reason, there are no specific anti-oomycetes substances developed. Asas an alternative, fungicides are used to control these organisms.

Microorganisms, including fungal strains, to tree species Quais ??? have been used as biocontrol agents and for bioprospection of substances with anti-oomycetes activity (KIM *et al.*, 2007;ISLAM; TAHARA, 2005).

The Bryophytes *Plagiochasma appendiculatum, Thuidiumdelicatulum*, *Thuidiumcymbifolium, Bryumcellulare, Bryumargentium* and *Racomitriumcrispulum* were effective against fungal strains (BODADE *et al.*, 2008).

The herbaceous plant *Verbascum thapsus* is also a medicinal plantand its extracts were inhibitory to phytoathogenic fungi *Fusarium graminearum* and *Macrophomina phaseolina* (VOGT *et al.*, 2010). Extracts of *Verbascum georgicum* Bentham (leaves, flower and steam) at 300 µg/disk did not presented effect against four

fungal species (*Alternaria alternate, Aspergillus flavus, Fusarium oxysporum, Penicillium* spp.) on the disc diffusion method (ŞENGUL *et al.*, 2005)

A.glabra extracts inhibited *P.aphanidermatum* in upper concentrations of 125 μg.disc^{-1} and seems to have an oomycetestatic effect. Compared to the following studies which used different concentrations and target species,strong effects are observed even when compared with fungicides as Cycloheximide and Bifonazole.

Despite the use of a different methodology (broth culture), a wide range of plant species inhibited the growth of oomycetes. The survey tested the activity 24 crude plants extracts against *Saprolegnia australis*, a parasitic oomycete on fish, and the best 12 extracts reduced growth at 100 mg.L^{-1} and *Atractylodes macrocephala*, *Zingiber officinale* seed, *Chrysanthemum* ssp. and *Yucca* sp. were as effective as 10 mg.L^{-1} malachite green slowing growth (CARUANA *et al.*, 2012).

Jagtap *et al.*(2012) showed the effects of extracts of *Allium sativum* at 10% concentration against the phytopathogenic *Phytophtora nicotianae*, inhibiting 47% the mycelial growth in plate assay.

In comparison to the activity of extracts from tree species, crude root extracts from Mexican avocado trees (*Persea americana*) were screened for anti-oomycete activity against the phytopathogenic *Phytophthora cinnamomi*. The root extracts inhibited 50% of mycelial growth using a similar disc diffusion method (SÁNCHEZ-PÉREZ *et al.*, 2009).

More similar to our study, other surveys have also used crude extracts against *Pythium* species. Methanol extracts from *Tagetes patula* presented a dose dependent inhibition on *Pythiumultimum* growth (MARES *et al.*, 2004).

Ours results showed that less concentrated *A. glabra* extracts (125 – 1000 μg.disc^{-1})were more effective against *P.aphanidermatum* (6 – 16 mm inhibition zones radiuses) when compared to crude methanolic extracts of seven species from the Centaurea genus, *C. solstitidis, C. iberica, C. depressa, C. virgata, C. glaucus,* and *C. behen*, tested for antifungal activity. In a similar methodology, a 5 mm plug of *P.aphanidermatum* and a 6 mm diameter paper disk impregnated with 50 μL (5 mg.disc^{-1}) of the extract was transferred to potato dextrose agar (PDA)(ABBASI, 2012). All species except *C. iberica* were inhibited by the extracts (6.2 to 8.9 mm radius inhibition zone), also weaker results compared to ours.

Leaf extracts from five medicinal plants (*Aloe vera* Mil, *Alternanthera sessilis* L, *Murraya koenigii* L, *Pithecellobium dulce* Benth and *Vitex negundo* L) were

tested in a disc diffusion method at 10 µg.disc^{-1}concentration. Methanol extracts from all species inhibited the *Pythium debaryanum* growth and *V. negundo* had the major radius inhibitory zone (5 mm radius) (GOMATHI *et al.*, 2011).

Other studies of medicinal plants activity also used leaf extracts against *Pythium* species. These 15 medicinal plants (*Lawsoniainermis, Mimosa pudica, Phyllanthus niruri, Tephrosia purpurea, Vinca rosea, Pongamia piñata, Aegle marmelos, Azadirachta indica, Brassica campestris, Piper nigrum, Euphorbia tirucalli, Vitex negundu, Ageratum conyzoides, Tagetes patula, and Zigiphus jujube*) inhibited *Pythium debaryanum* mycelia growth (AMBIKAPATHY, 2011;PATTNAIK *et al.*, 2012).

(-)-Kaur-16-en-19-oic acid isolated from hexane extracts from *Annona glabra* L. bark presented antifungal activity against *Aspergillus niger*, *Penicillium notatum, Trichophyton mentagrophytes, Microsporum gypseum*, *Candida albicans, Microsporum gypseum* and *Epidermophyton floccosum* (PADMAJA *et al.*, 1995).Stigmasterol and Sitosterol isolated from *Bulbinenatalensis* Baker (Asphodelaceae) inhibited *Aspergillus flavus, Penicillium digitatum* and *Fusarium verticilloides* strains (MBAMBO *et al.*, 2012). But there are no specific anti-oomycetesactivities recorded for these substances.

Since that *A. glabra* and oomycetes share flooded habitats, these antioomycete activity from acetone extract are an important evidence plant pathogen interaction and a potential species for the prospection of new substances for oomycete control. Historically, fungicides are used to oomycete control, and these isolated substances, and others, must be involved in the activity observed in this study.

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CONSIDERAÇÕES FINAIS

As extrações utilizando micro ondas (EAM) foi altamente eficaz, já que a bioatividade dos extratos brutos atingiram 60% de inibição sobre os coleóptilos de trigo e as melhores condições de extração foram: 25 mL de solvente, 10% metanol em acetona, à 50 ºC e 1000 watts de potência.

A otimização nas extrações assistidas por ultrassom (EAU) também chegaram a 60% de inibição. As melhores condições das variáveis foram: 25 mL de acetona, 5 °C, sonda de 2 mm, ciclo de 0.2, 30% de amplitude por 20 minutos, utilizando 1 g de material vegetal.

Os extratos acetônicos da EAU propiciou o isolamento e identificação de seis substâncias majoritárias **(1)** ácido (-)-Kaur-16-en-19-oico, **(2)** β-sitosterol, **(3)** Stigmasterol, **(4)** ácido ent-19-methoxy-19-oxokauran-17-oico, **(5)** ácido 16α-hydro-19-acetoxy-*ent*-kauran-17-oico (annoglabasina B), **(8)** Asimicina e duas substâncias minoritárias **(6)** ácido *ent*-17-hydroxykaur-15-en-19-oicoe **(7)** ácido *ent*-15β,16β-epoxy-17-hydroxy-kauran-19-oico.

A annoglabasina B (ácido 16α-hydro-19-acetoxy-*ent*-kauran-17-oico) provocou a maior inibição do alongamento dos coleóptilos de trigo, com resultados comparáveis ao herbicida comercial, Logran. Seguido pela substância **4**, atingindo quase 70% de inibição. Estas duas substâncias tinham poucas atividades descritas, por esta razão se destacaram no presente trabalho provocando alta inibição.

Os testes dos extratos acetônicos mostraram atraso no crescimento de *Selenastrum capricornutum* às concentrações de 125 a 175 mgL^{-1} e totalmente inibitórias em concentrações a partir de 200 mgL^{-1} e o IC_{50}, em 96h, foi de 145 mgL^{-1}. O fungo *Pythium aphanidermatum* foi inibido nas concentrações acima de 125 $\mu g.disc^{-1}$.

Estes resultados mostram o potencial dos extratos de *Annona glabra* para a inibição de algas e fungos. É possível que essas substâncias represente um tipo defesa desta espécie em relação aos micro-organismos existentes nas áreas alagadas em que vive.

ANEXO 1

ISOLATION AND IDENTIFICATION OF BIOACTIVE COMPOUNDS FROM LEAF EXTRACT OF *ANNONA GLABRA* L.

MATERIAL AND METHODS

Sample preparation – We dried *A. glabra* L. leaves, grinded and stored them in refrigerated environment. For elimination of fat and wax from the sample, we submitted 500g of powder to a hexane washing procedure with the support of an ultrasound system (7mm diameter (**Ø**) probe). We fixated ultrasound parameters: amplitude at 50% and cycle at 0.5 for 15 minutes without temperature control. We dried the plant material into laboratory oven at 50°C and stored it at refrigeration (-20°C) as sample for following extraction.

Extraction – According to a study on Optimization of ultrasound assisted extraction (UAE) of *Annona glabra*, the best conditions to higher recovery of bioactive extract was using 1g of vegetal sample, 25mL of acetone, temperature of 5°C, 2mm **Ø** probe, cycle of 0.2 and amplitude of 30%, for 20 minutes. We used a high intensity probe ultrasound generation system of 200W, 24Hz. (model UP 200S from Dr.Hielscher GmbH). Its amplitude controller allows setting any desired level between 10–100% ranges. Also the cycle controller allows setting the duration of the application in a 0.1–1.0 range (fraction of a second).

For higher scale, we employed a larger probe (7mm **Ø**), and for each extraction we used 20g of plant sample, 500ml acetone, maintaining the optimized variables. We extracted a total of 1Kg of plant sample, recovering 8.4g (DW) used to compose chromatographic column sample.

Etiolated wheat coleoptile bioassay – We evaluated the activity of extracts using this fast (24h) and sensitive bioassay, which could be considered as first level's in general bioactivity evaluation on undifferentiated tissue cells (Nitsch, 1956; Hancock *et al*, 1964).

We prepared the concentrations diluting the dry extracted into a buffer solution (1.1 g/L of Citric acid and 2.9 g/L of Calcium phosphate in distillate water at pH 5.6) containing 0.5µL/mL DMSO (Dimethyl sulfoxide) and 20 g/L of Sucrose.

The experimental design comprehends five extract concentrations (10^{-3}, $5*10^{-4}$, 10^{-4}, $5*10^{-5}$, 10^{-5} M and buffer solution as negative control) and a commercial

herbicide (Logran) as positive control. We prepared all treatments in three replicates, in which, each test tube (replicate) received 2mL of one treatment and 5 coleoptiles fragment. We sealed and mounted them to a horizontal rotor into germination chambers at 25°C, in absence of light.

TLC – We used Alugram Sil G/UV_{254} plates (0.25mm thickness) analyzed them at UV (254 and 360 nm) and revealed and heated them at 150°C. We used three visualization reagents: Oleum –sulfuric acid, H_2O and acetic acid (1:4:20); Anisaldehyde – anisaldehyde, sulfuric acid, acetic acid, ethanol (25:25:1:450); Vanillin – vanillin, sulfuric acid, ethanol (1:1:18).

HPLC (High-performance liquid chromatography) – We used a Hitachi L-6020 and a differential refractometer RI-71; semi-preparative column (LiChrospher SiO_2, Merck, 7 and 10µ, dimensions of 10 and 250 mm) used with 3 $mL.min^{-1}$ flux; Analytical Phenomenex Luna Column (10µ Silica (2) 100A) with 1 $mL.min^{-1}$ flux.

EXPERIMENTAL

Column chromatography - We elected the solvents used in column chromatography driven by TLC (Thin Layer Chromatography), that results in a sequence of increasing polarity solvents: Hexane, Hexane:Acetone (95:5), Hexane:Acetone (90:10), Hexane:Acetone (85:15), Hexane:Acetone (80:20), Hexane:Acetone (60:40), Hexane:Acetone (40:60), Hexane:Acetone (20:80), Acetone and Methanol for column cleaning process.

We acquired 17 major fractions (1-17), with the following yields (dry weight expressed in milligrams): 1 (1364.7), 2 (257.3), 3 (622.5), 4 (141.1), 5 (382.7), 6 (174), 7 (372.6), 8 (235.9), 9 (361.6), 10 (244.5), 11 (381.1), 12 (271.5), 13 (554.9), 14 (589.9), 15 (297.4), 16 (152.5), 17 (176.5), Acetone (136.3), Methanol (1464.3), totaling (8190) presenting little retention and lost in the column.

Fraction 2 (257.3 mg) – We purified this fraction in HPLC (High-performance liquid chromatography) with semi-preparative normal phase column. We used Hexane-Ethyl acetate 15% for separation, resulting in 3 sub-fractions (2a, 2b and 2c). We purified a majoritarian compound **1** (7.2mg) from fraction 2b and identified it with NMR (Nuclear Magnetic Resonance) spectroscopy as (-)-Kaur-16-en-19-oic acid (figure 1).

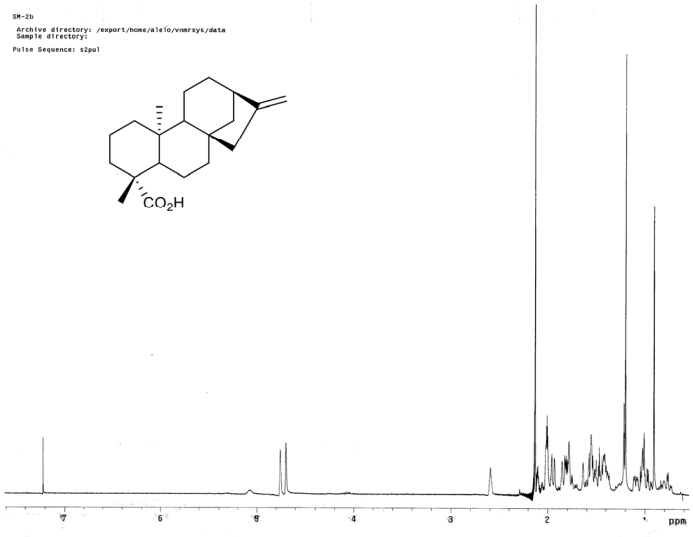


Figure 1. [1]H NMR Spectra from fraction 2b and structure identified as (-)-Kaur-16-en-19-oic acid.

Fraction 3 (622.5 mg) – We fractionated it with HPLC semi-preparative normal phase column, using Hexane-Ethyl acetate 20% as eluent. We obtained two sub-fractions (3a and 3b), in which, was identified two substances from 3b fraction: **(2)** β-sitosterol and a minor part of **(3)** Stigmasterol (figures 2 and 3). We fractionated only a part of the fraction 3, for identification of the substances, recovering 5.6 and 1.1 mg, respectively

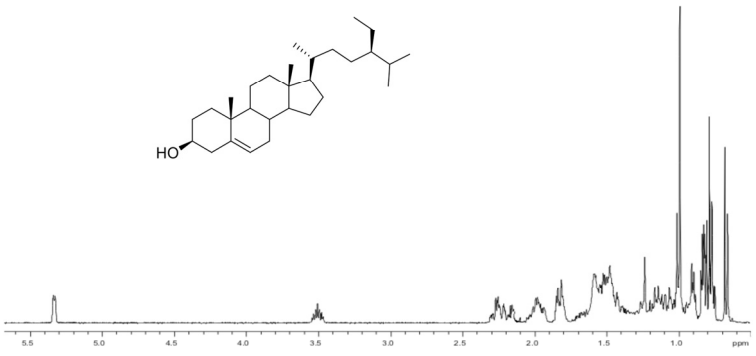


Figure 2. [1]H NMR Spectra from fraction 3b and structure identified as β-sistosterol.

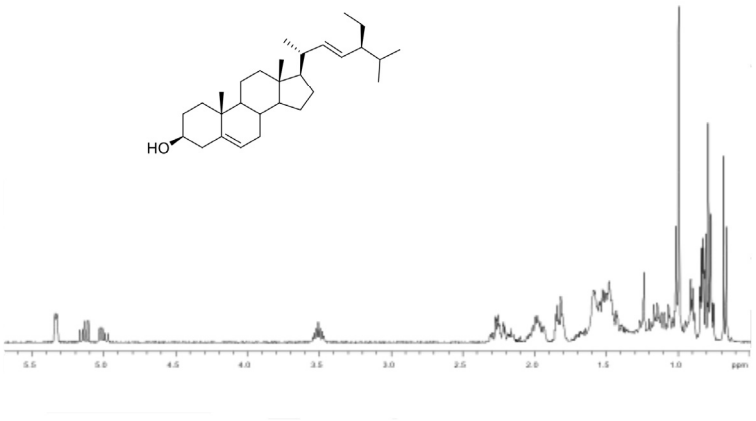


Figure 3. ^{1}H NMR Spectra from fraction 3b and structure identified as Stigmasterol

We submitted the fractions 5 to 12 to a reverse phase (RP18) column (Methanol) for chlorophyll elimination. This process generated a reference code (a1, b1 c1) to fraction numbers that indicates increasing gradients of chlorophyll in the fractions. As example: fraction 8a1.

We submitted the 8 to 11 chlorophyll free fractions, through a cleaning process of base line with SPE (solid phase extraction) syringe. The solvent mixture (Hexane/Ethyl acetate) that generates clean fraction for HPLC use, received a code referent to polarity of solvents.

In this particular case, the analysis of TLC showed that fraction 8b1 could be united to 8a1 vial and submitted to SPE with Hexane/Ethyl acetate 5% and receives a code as 8a1-5.

Fraction 8a1-5 (60.9mg) – We fractionated it in HPLC semi-preparative column with Hexane/Ethyl acetate 25% and 10% acetic acid. We obtained 7 fractions titled as 8a1-5a to 8a1-5g, in which, 8a1-5c (figure 4) was the majoritarian fraction (18mg) and identified as ent-19-methoxy-19-oxokauran-17-oic acid **(4)**.

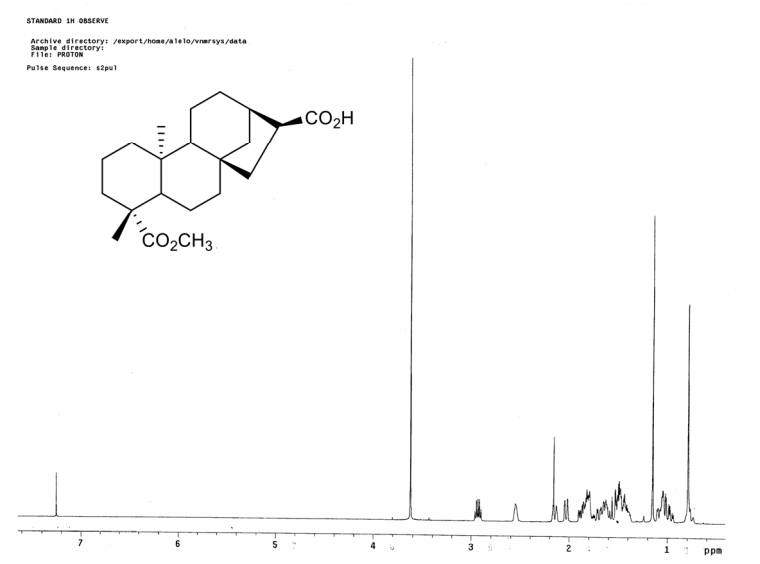


Figure 4. [1]H NMR spectra from fraction 8a1-5c identified as ent-19-methoxy-19-oxokauran-17-oic acid.

The fraction 8a1-5d (1.4mg) is a diterpenoid class substance identified as **(5)** 16α-hydro-19-acetoxy-*ent*-kauran-17-oic acid (annoglabasin B). NRM and structure are represented in figure5.

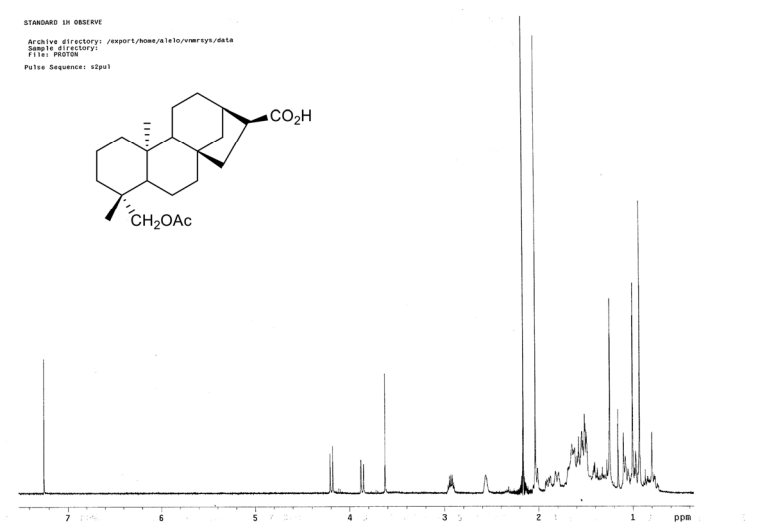


Figure 5. [1]H NMR spectra from fraction 8a1-5d and structure identified as 16α-Hydro-19-acetoxy-19-al-ent-kauran-17-oic acid (annoglabasin B).

Fraction 9a1-5 and 9b1-5 (139mg) – We merged both fractions, due to analysis of their TLC, in 9a1-5 flask and submitted it directly to [1]H NMR, the spectra

presented a similar pattern as 8a1-5d **(5)** but no purification at HPLC was made (figure 6).

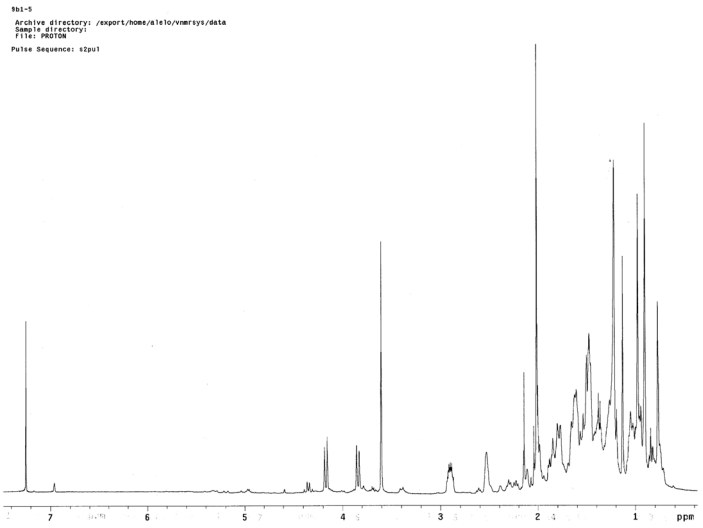


Figure 6. ^{1}H NMR spectra from fraction 9b1-5.

Fraction 10a1-15 (22.7mg) – We submitted the fraction to HPLC semi-preparative column with Hexane/Ethyl acetate 60%. We obtained 9 sub-fractions, 10a1-15a to 10a1-15i. 10a1-15a is the major substance **(5)** and is represented in figure 7 and the fraction 10a1-15b **(6)** (*ent*-17-hydroxykaur-15-en-19-oic acid) and 10a1-15d **(7)** *ent*-15β,16β-epoxy-17-hydroxy-kauran-19-oic acid are minor substances similar to the diterpenoid of fraction 10a1-15a, however, with different structure (figure 8 and 9).

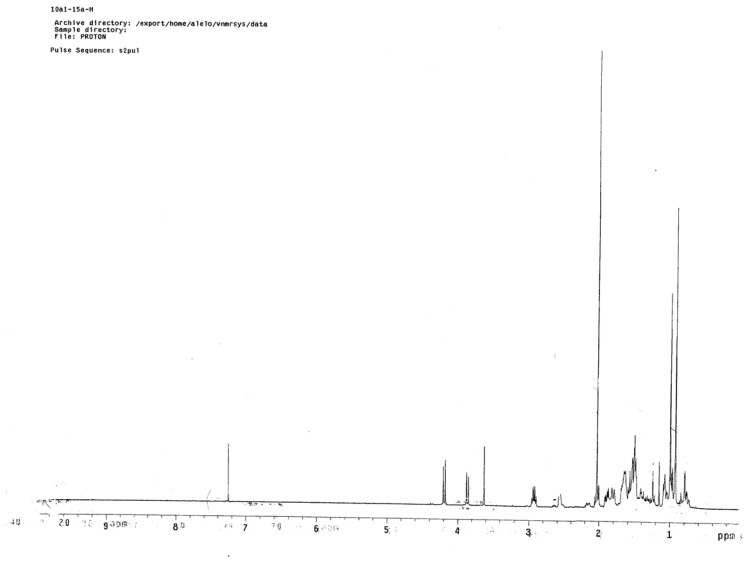


Figure 7. ^{1}H NMR spectra from fraction 10a1-15a (9.1mg), was the majoritarian substance. Same spectra pattern to diterpenoid of fraction 8a1-5d.

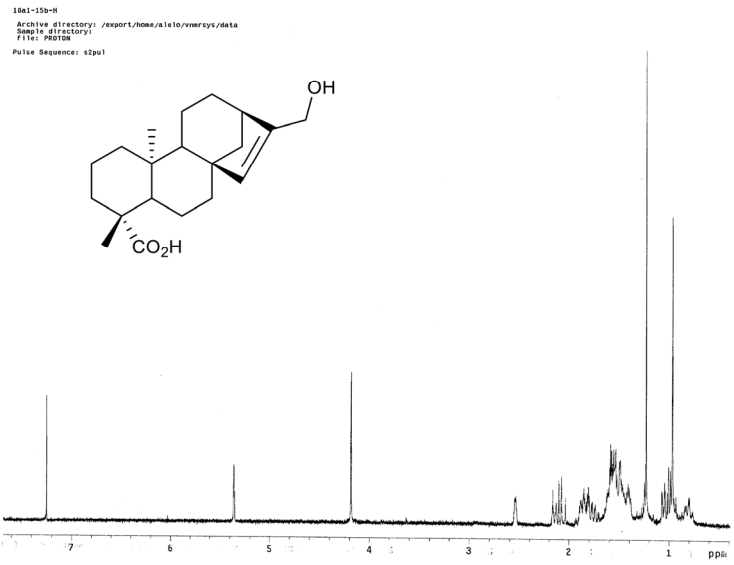


Figure 8. ^{1}H NMR spectra from fraction 10a1-15b (1.3mg) and structure identified as **(6)** *ent*-17-hydroxykaur-15-en-19-oic acid.

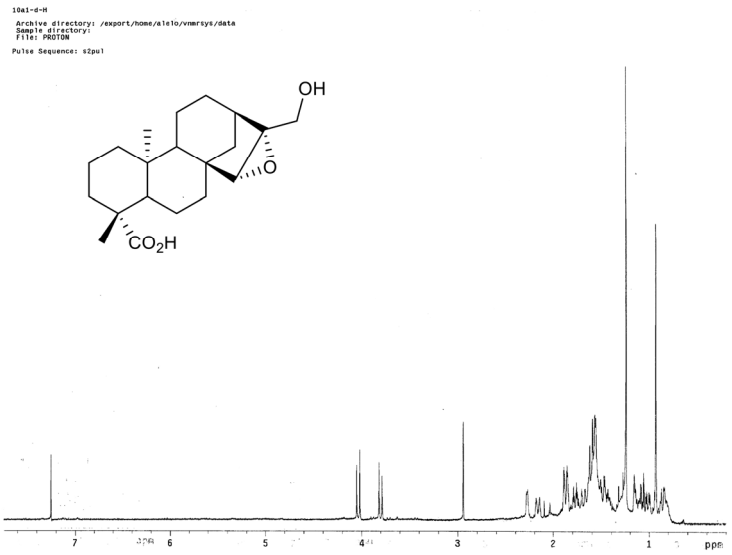


Figure 9. ^{1}H NMR spectra from fraction 10a1-15d (1.6mg) and structure identified as **(7)** *ent*-15β,16β-epoxy-17-hydroxy-kauran-19-oic acid.

Fraction 11a1-15 (14mg) – We fractionated it in HPLC semi-preparative column with Hexane/Ethyl acetate 40%. We obtained 4 sub-fractions 11a1-15a to 11a1-15d. The majoritarian fraction 11a1-15b (figure 10) is the same diterpenoid of 8a1-15d **(5)**.

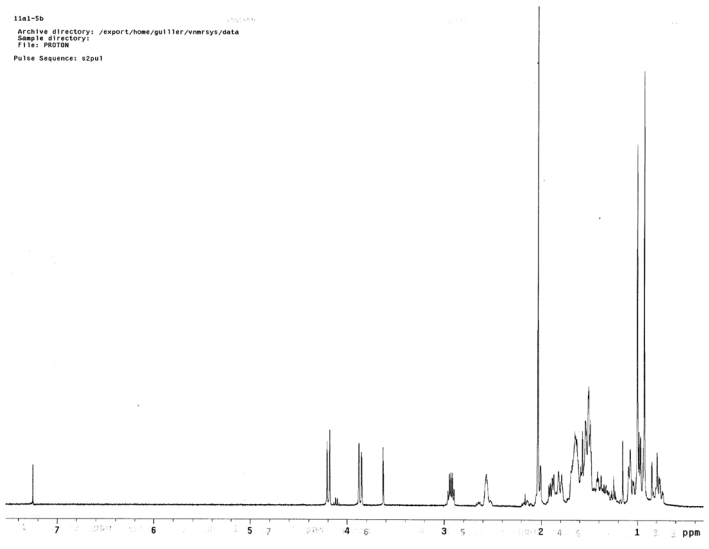


Figure 10. ^{1}H NMR spectra from fraction 11a1-15b (2.8mg). Similar to diterpenoid of fraction 8a1-5d.

Fraction 11b1 and 11c1 (119mg) – We merged both fractions, due to their TLC, in 11b1 flask. After cleaning process, we submitted fraction 11b1-15 to

HPLC semi-preparative column with Hexane/Ethyl acetate 40%, Fraction 11b1-30 were appropriate to HPLC procedure as well. We obtained 7 sub-fractions, 11b1-15a to 11b1-15g. The fraction 11b1-15c is the majoritarian substance and wasn't totally isolated, however the estimated weight is 10.81 mg from the initial 119 mg and was identified as the same compound 8a1-5d (5).

The sub-fractions 8a1-5d, 9b1-5 (not totally purified), 10a1-5a, 11a1-15b and 11b1-15c represent same diterpenoid 16α-Hydro-19-acetoxy-19-al-ent-kauran-17-oic acid totaling 163.11 mg.

Fraction 13 and 14 – We merged both fractions in the same fraction 13 flask, totalizing 1148mg. We performed a glass column chromatography (30cm x 2.5cm Ø) started with Hexane till Hexane:Acetone (80:20%), resulting in 11 sub-fractions (13A to 13K).

We proceeded fractioning 13F (262.9mg) in glass column (30cm x 2.5cm Ø) started with Hexane:Ethyl acetate (80:20%) till Ethyl acetate 100%, resulting in 7 sub-fractions (13F1-13F7). This last fraction was the majoritarian (126mg) and was submitted to HPLC semi-preparative column with Hexane/Ethyl acetate 90% generating 6 fractions 13F7 a-f.

On the fraction 13F7e (figure 11) we isolated a majoritarian substance **(8)** acetogenin (36.1mg) identified as Asimicin.

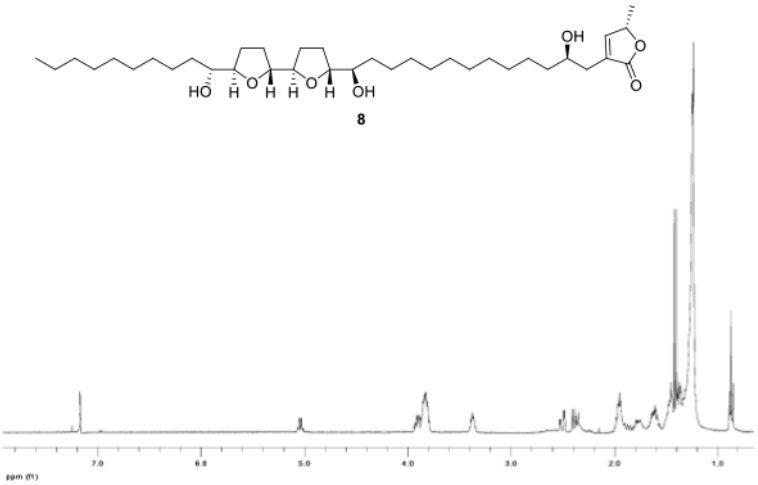


Figure 11. ^{1}H NMR spectra from fraction 13F7e and structure identified as Asimicin.

To a fast evaluation of bioactivity of the majoritarian analytes: 8a1-5c, 8a1-5d and 13F7e, we submitted them to a wheat coleoptile bioassay and results are represented in figure 12. In the statistical analisys we compared all substances within the same concentration with ANOVA and Tukey post-test.

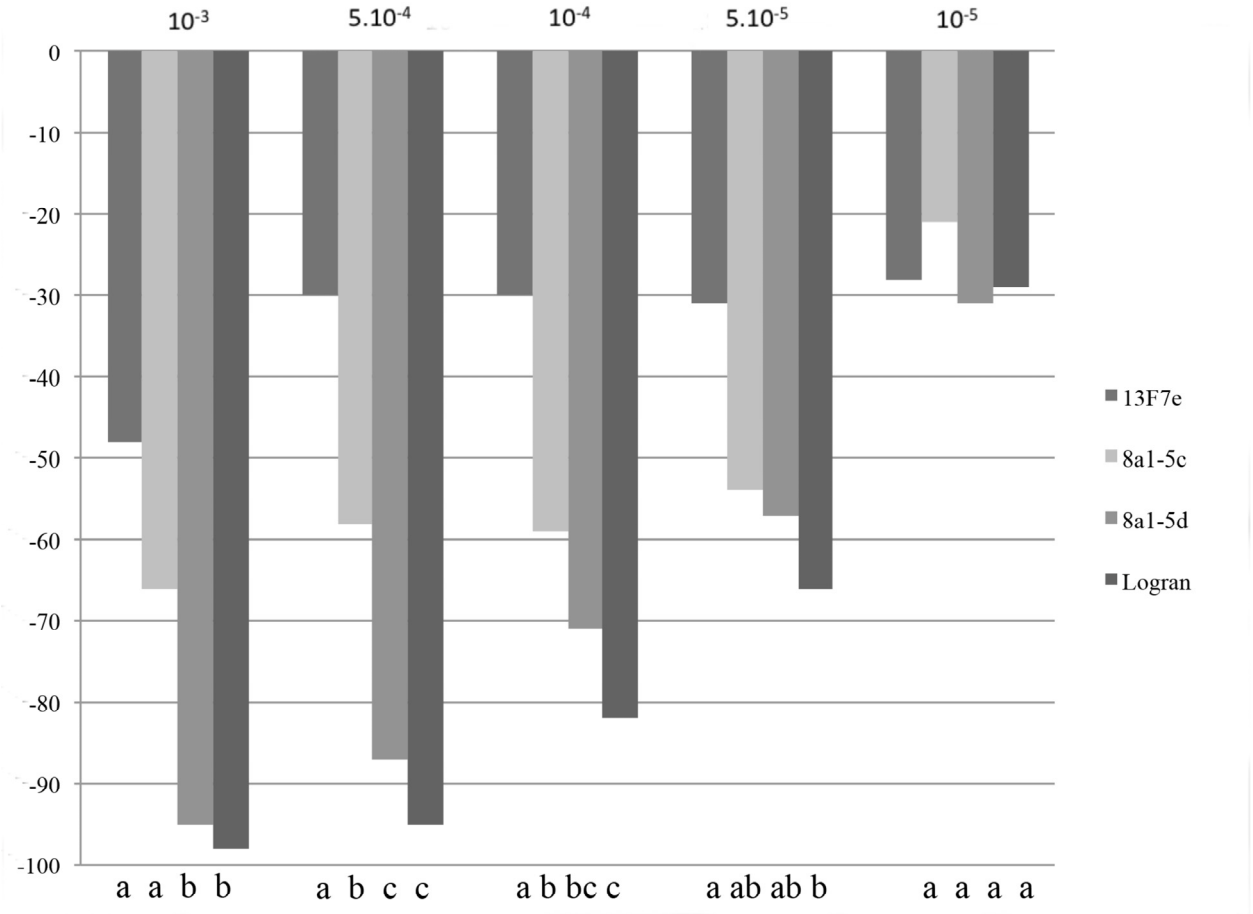
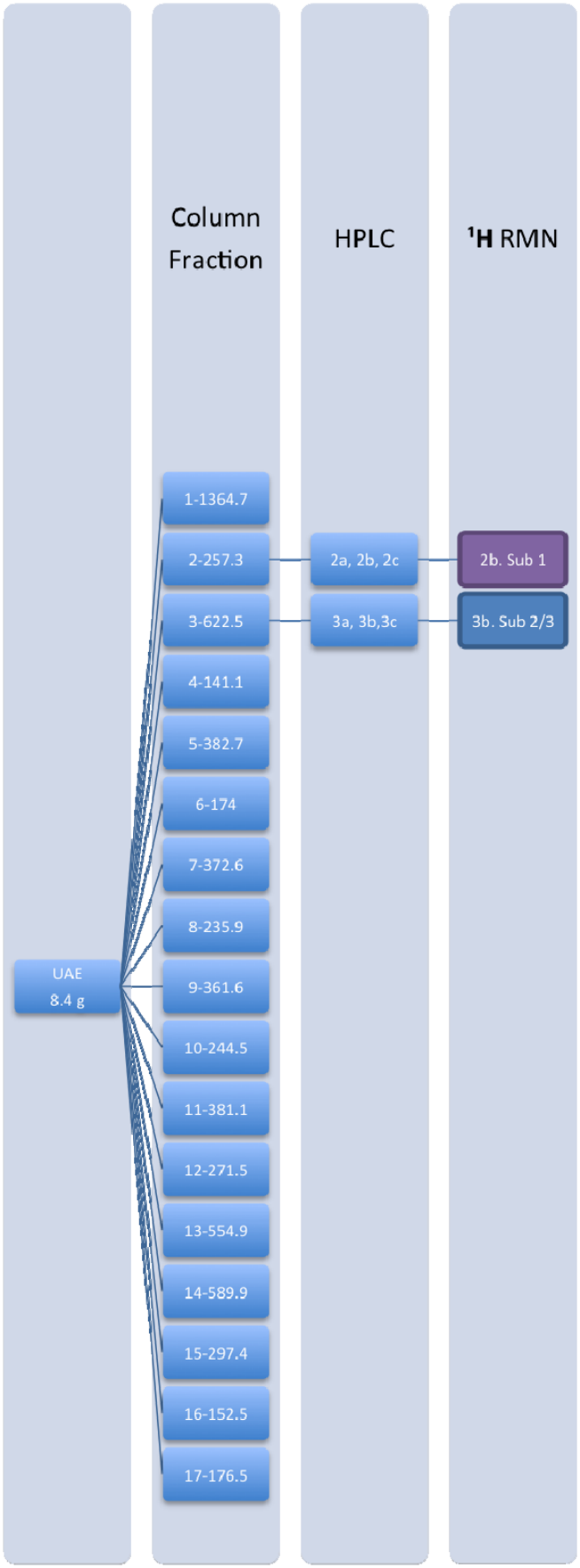
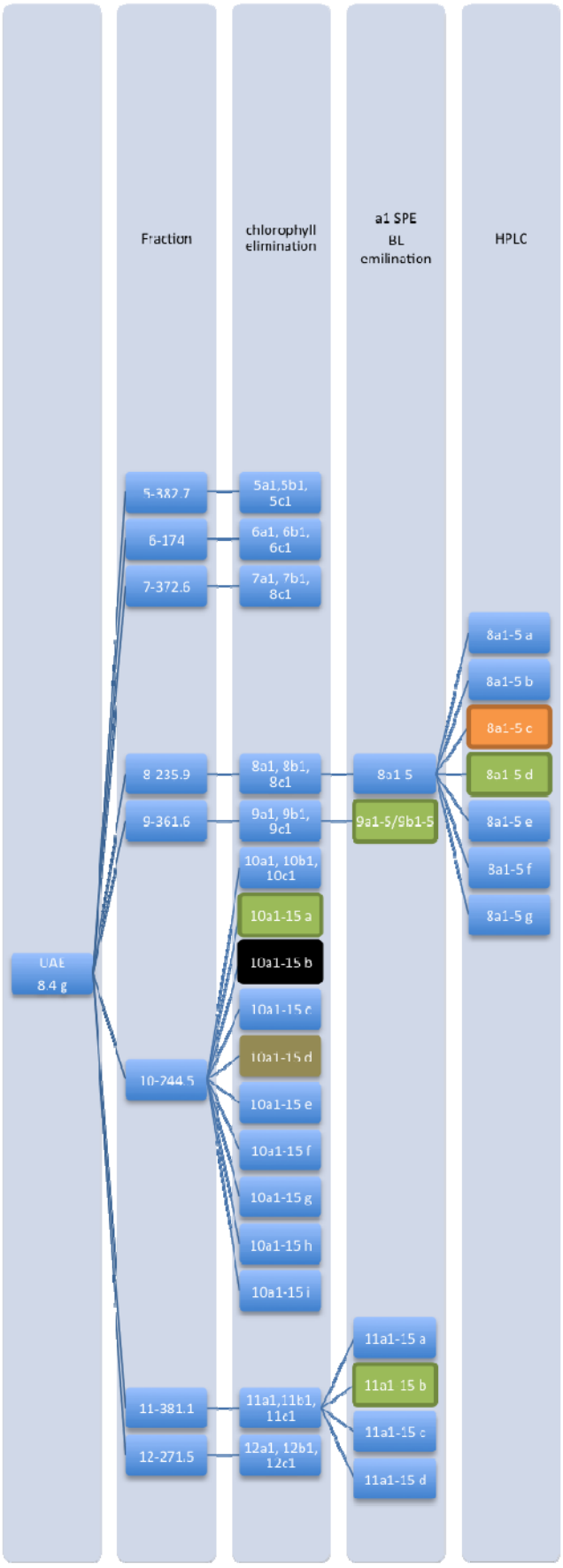


Figure 12. Bioactivity results from 8a1-5c **(4)** (ent-19-methoxy-19- oxokauran-17-oic acid), 8a1-5d **(5)** (16α-Hydro-19-acetoxy-19-al-ent-kauran-17-oic acid) and 13F7e **(8)** (Asimicin). Within each concentration, same letters represent no significant difference and different letters, significant difference in ANOVA and Tukey post-test.

The inhibition rates of substance 8a1-5d (16α-Hydro-19-acetoxy-19-al-ent-kauran-17-oic) had no significant differences, in all concentrations, to industrial herbicide Logran highlighting its activity. 8a1-5c shows intermediate activity and 13F7e (asimicin) had the weakest inhibition. In a sinergic point of view this substances could explain the allelopathic pattern found in Acetone raw extract.

Flowcharts of the process of isolation of compounds from acetone leaf extract.




Fraction
chlorophyll elimination
a1 SPE BL emilination
HPLC
UAE 8.4 g
5-382.7
6-174
7-372.6
8 235.9
9-361.6
10-744.5
11-381.1
12-271.5
5a1,5b1, 5c1
6a1, 6b1, 6c1
7a1, 7b1, 8c1
8a1, 8b1, 8c1
9a1, 9b1, 9c1
10a1, 10b1, 10c1
10a1-15 a
10a1-15 b
10a1-15 c
10a1-15 d
10a1-15 e
10a1-15 f
10a1-15 g
10a1-15 h
10a1-15 i
11a1,11b1, 11c1
12a1, 12b1, 12c1
8a1 5
9a1-5/9b1-5
11a1-15 a
11a1 15 b
11a1-15 c
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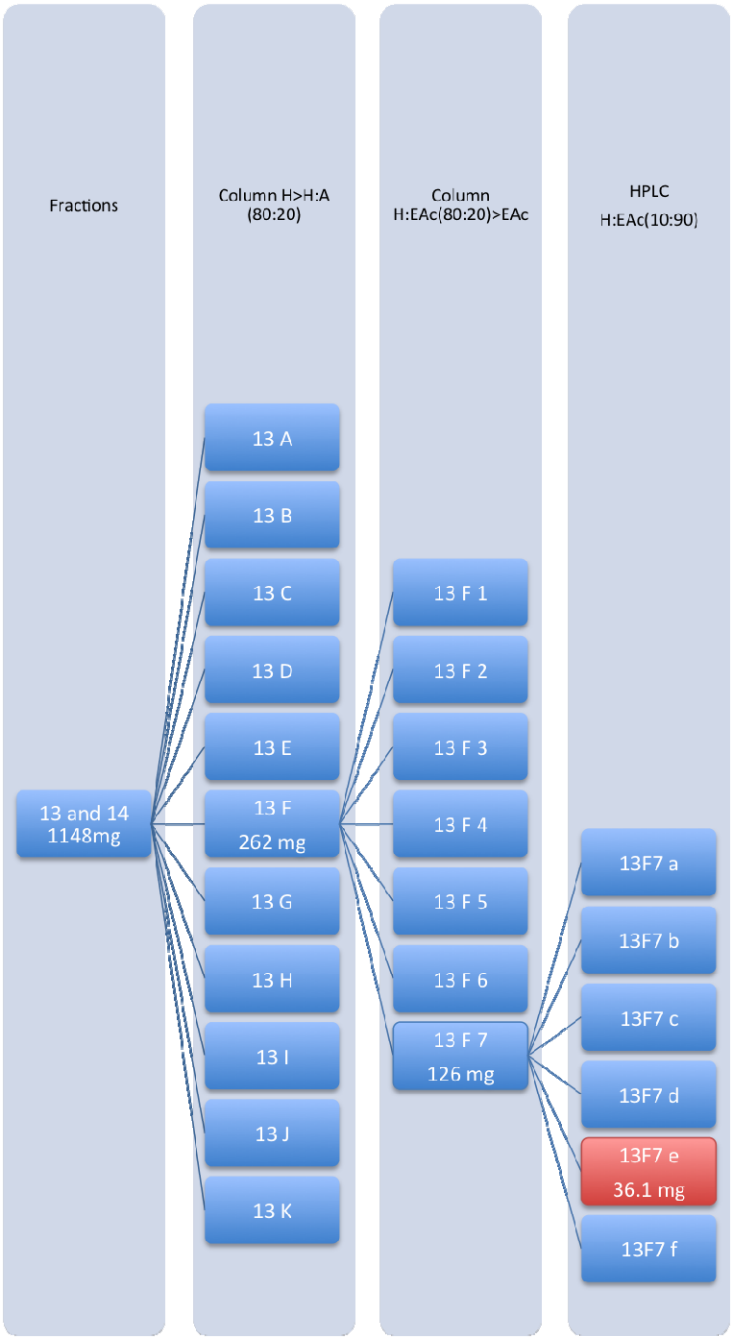
8a1-5 f
8a1-5 g



Substance 1 (-)-Kaur-16-en-19-oic acid

Substance 2 β-sistosterol

Substance 3 Stigmasterol

Substance 4 ent-19-methoxy-19-oxokauran-17-oic acid

Substance 516α-Hydro-19-acetoxy-19-al-ent-kauran-17-oic acid (annoglabasin B)

Substance 6 *ent*-17-hydroxykaur-15-en-19-oic acid

Substance 7 *ent*-15β,16β-epoxy-17-hydroxy-kauran-19-oic acid

Substance 8 Asimicin