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A simple procedure for the purification of active fractions in aqueous extracts of plants with allelopathic properties

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ABSTRACT

Most studies conducted to test the allelopathic activity of plant parts have made use of water as solvent. However, the presence of polar, water-soluble substances, such as proteins and carbohydrates, tends to hamper the purification of active compounds. In this study, we present a simple purification procedure that separates the active fraction of the extract from the undesirable substances, thus facilitating the search for active molecules through standard chromatographic methods. Aqueous leaf extracts of three Cerrado species (*Caryocar brasiliense*, *Qualea parviflora* and *Eugenia dysenterica*) were prepared at 5% concentration (w/v) and stored at 4°C (crude extracts). After 24 h, these solutions were filtered and freeze-dried. The powder obtained was dissolved in methanol, filtered again, evaporated and dissolved in water for bioassays (purified extracts). For the bioassays, seedlings of *Sesamum indicum* were grown for five days in aqueous solutions prepared from crude and purified extracts at concentrations ranging from 0.1% to 1.0% (w/v). Seedling growth in distilled water was set as a control. In comparison with the control, we found that test solutions prepared from both crude and purified extracts significantly inhibited sesame seedling growth. However, solutions prepared from purified extracts were two to ten times more inhibitory to seedling growth than were those prepared from crude extracts. The inhibition of root growth ranged from 35% to 77%, depending on the plant species, at a concentration as low as 0.1%. Roots were more affected than were shoots. The effects of purified extracts on seedling morphology were similar to those observed when crude extracts were employed, indicating that the procedure of purification of crude extracts did not interfere with the mode of action of the active substances

Key words: allelopathy, cerrado, freeze-drying, leaf extract, purification

Introduction

The number of studies evaluating the allelopathic potential of the Brazilian flora has increased considerably in recent decades, and many native species have been recognized as presenting some type of allelopathic activity. Allelopathy is defined as the effect that a plant has on the development of another organism by means of the production and liberation into the environment of chemical substances that in turn affect the growth of receptive species (Rice 1984). These compounds, known as allelochemicals, can be released by the plant into the environment through lixiviation, wash-off of leaves by rain, root exudation, volatilization and even by decomposition of the organic matter.

Aqueous extracts prepared from plant parts of many Brazilian species, including *Andira humilis* Mart. ex Benth. (Periotto *et al.* 2004), *Aristolochia esperanzae* O. Kuntze (Gatti *et al.* 2004), *Campomanesia adamantium* Cambess., *Qualea parviflora* Mart., *Trembleya parviflora* (D. Don) Cogn. (Borghetti *et al.* 2005), *Solanum lycocarpum* St. Hil. (Oliveira *et al.* 2004), *Eugenia dysenterica* DC. (Pina *et al.* 2009) and *Sapindus saponaria* (Grisi *et al.* 2012) have been shown to have a strong inhibitory effect on the growth of target species such as *Lactuca sativa* (lettuce), *Raphanus sativus* (radish) and *Sesamum indicum* L. (sesame) (Periotto *et al.* 2004; Pina *et al.* 2009). However, none of those studies, which were designed to evaluate the allelopathic potential of native plant species, have actually identified the active molecules responsible for such effects. The purification and identification of the active substances involved in the observed interaction has been considered an essential step if one intends to a study to be recognized as a true allelopathy study (Blair *et al.* 2009).

The preparation of aqueous extracts of plant parts and the subsequent test for their effects on the germination and growth of the target species have been recognized as a classic procedure in the field of allelopathy (Inderjit & Dakshini 1995; Blum 2011). To a certain extent, this procedure has

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been seen as a model used to simulate, under laboratory conditions, the effect that wash-off would have on plant tissues, which would lead to the liberation of soluble substances into the environment, thereby influencing the growth of neighbor plants, (Ferreira 2004). In brief, the simplest laboratory procedure to test for allelopathic activity has been to grind plant tissues and solubilize them in water; to separate water-soluble from water-insoluble substances by filtering; to prepare solutions that are more diluted (w/v); and to test for their effects on the germination and growth of target species (Jacobi & Ferreira, 1991; Gatti *et al.* 2004; Periotto *et al.* 2004; Borghetti *et al.* 2005; Carmo *et al.* 2007; Pina *et al.* 2009; Souza Filho *et al.* 2010; Grisi *et al.* 2012). Some authors avoid grinding plant parts, because this process can dissolve cell contents that would not be released under a natural wash-off or even during tissue decomposition in soil (see Ferreira 2004). In many cases, solvents such as methanol, ethyl acetate and dichloromethane are used in the preparation of plant extracts. This procedure, although effective for the solubilization of active substances (Souza Filho *et al.* 2005; Grassi *et al.* 2005; Cunico *et al.* 2006; Lôbo *et al.* 2008; Souza Filho *et al.* 2010), has been viewed with caution in the description of allelopathic effects, given that the use of organic solvents does not reflect what would actually occur under natural conditions (Inderjit & Weston 2000; Ferreira 2004; Blum 2011).

From an analytical perspective, the use of water as a solvent for the solubilization of active substances can hamper subsequent stages in the purification process of allelochemicals, because water also solubilizes molecules that are polar but undesirable in this type of study, such as carbohydrates, amino acids and nucleic acids. Once they are in the solution, these substances make it more difficult to search for and purify the active molecules by conventional methods, such as column chromatography and high-performance liquid chromatography. However, the removal of water by freeze-drying and the resuspension of the powder in organic solvents (such as chloroform and ethyl acetate) can render solutions that are easier to handle in the subsequent procedures of purification of the active fractions (Macias *et al.* 2004). Although this procedure should be avoided in an allelopathic study (Inderjit & Weston 2000; Ferreira 2004), the use of methanol rather than water for the solubilization of plant extracts facilitates subsequent chromatographic purification procedures (Souza Filho *et al.* 2005; 2010) in the search for the allelochemicals that are more polar. As an alternative, extracts to be prepared from plant tissues should first be solubilized in water. If allelopathic activity is detected, the aqueous extracts could then be dried to a powder and resuspended in solvents of lower polarity to facilitate subsequent analyses. Therefore, it becomes necessary to evaluate whether such sample preparation procedures alter the allelopathic activity of the crude aqueous extracts. In view of this, the objective of this paper was to present a laboratory procedure, starting from an aqueous crude extract, that separates the active fractions from the inactive ones through simple purification techniques such as freeze-drying, powder resuspension and filter-paper filtration. Our hypothesis was that this procedure would not interfere with the mode of action of the plant extracts under investigation.

Material and methods

Preparation of the extract solutions

Caryocar brasiliense A. St.-Hil. (Caryocaraceae), *Qualea parviflora* Mart. (Vochysiaceae) and *Eugenia dysenterica* DC. (Myrtaceae) were the Cerrado species selected for this study. Previous studies have described the allelopathic effects that leaf extracts of these species have on the growth of sesame (Borghetti *et al.* 2005; Pina *et al.* 2009). Mature leaves were collected from at least five individuals of each species standing on the campus of the University of Brasilia (15°46'13" S and 46°52'07" W), in Brasília, Brazil. In the laboratory, the leaves were oven-dried (NT-516; Nova Técnica, Piracicaba, Brazil) at 50°C for 24 h. They were then lightly triturated in an electric blender (Skymsen; Siemsen, Brusque, Brazil) and immersed in distilled water in order to obtain stock solutions in a concentration of 5% (w/v). The stock solutions were stored at 4°C for 24 h in darkness for the solubilization of active compounds (Borghetti *et al.* 2005; Pina *et al.* 2009). Subsequently, they were vacuum-filtered through a qualitative paper filter (no. 1; Whatman, Clifton, NJ, USA) to obtain a crude leaf solution of each species (crude extract). Part of each crude solution was diluted to 1.0%, 0.5% and 0.1% with distilled water to test for its phytotoxic activity on the growth of sesame. The remaining crude solutions were stored at −18°C and subsequently freeze-dried in a rotary evaporator (MA-120; Marconi, Piracicaba, Brazil) until a soft white powder was obtained. The powder was weighed and dissolved in methanol (Merck P.A.), after which the solution was filtered through a Whatman no. 1 qualitative paper filter. Each solution was evaporated (MA-206; Marconi), and the powder thus obtained was weighed again. The powder was then dissolved in distilled water to obtain a 5% concentration solution (w/v), the purified extract, which was diluted to 1.0%, 0.5% and 0.1% with distilled water in order to test for its phytotoxic activity on the growth of sesame.

Bioassays

The bioactivity of the crude and purified extracts was tested on the growth of sesame seedlings. For this purpose, seeds of sesame (*Sesamum indicum* L. - Pedaliaceae) were firstly germinated in distilled water at 30°C in a growth chamber (MA-403; Marconi), and seedlings with a radicle size of approximately 2 mm were selected for bioassays. The

selected seedlings were immediately placed for growth on 150 × 10 mm Petri dishes (ten per plate) lined with a Whatman no. 1 qualitative paper filter moistened with 10 ml of each solution to be tested. The bioassays were conducted in the same growth chambers at 30°C, on a 12/12-h light/dark cycle (white light) for five days. The concentrations ranged from 0.1% to 1.0% (w/v), depending on the extract (crude or purified). The control of the experiments consisted of seedlings placed in distilled water to grow. After five days of incubation, the roots and shoots of the seedlings were measured with a caliper (Mitutoyo, Aurora, IL, USA) for comparisons among treatments. To calculate the percentage of growth inhibition of the treated seedling in relation to the control, the root and shoot growth of the seedlings incubated in distilled water (controls) was taken as 100%.

Experimental design and statistical analysis

At least three replicates of 10 seedlings each were employed for each treatment. The experiment was conducted in a completely randomized design (with the deviance information criterion), consisting of 4 treatments (extract concentration) for each extract (crude or purified) and for each species. The average length of roots and shoots of the seedlings was compared among the treatments through ANOVA followed by *a posteriori* Student's t-test (Statistical package BIOSTAT 2.0, Ayres *et al.* 1998). The significance level was 0.05.

Results and discussion

In this study, we have demonstrated that freeze-drying of the aqueous leaf extracts followed by resuspension in methanol does not affect their allelopathic potential (Tab. 1). Root growth was significantly inhibited by these extracts, in a dose-dependent manner, and the roots were more affected than were the shoots (Tab. 1). This greater inhibition of the root in respect to the shoot growth has been observed in many allelopathic and phytotoxic studies employing aqueous extracts obtained directly from plant tissues (Jacobi & Ferreira 1991; Oliveira *et al.* 2004; Borghetti *et al.* 2005; Maraschin-Silva & Áquila 2006; Carmo *et al.* 2007; Pina *et al.* 2009; Grisi *et al.* 2012).

In comparison with their respective crude extracts, the inhibition of sesame growth was greater when purified extracts were utilized; at concentrations as low as 0.1%, the root growth of seedlings incubated in purified extracts was inhibited by approximately 34% for *Qualea parviflora* and by approximately 77% for *Eugenia dysenterica* (Tab. 2). For the three Cerrado species, the morphological effects of the crude and purified extracts on the seedlings were similar; there was a reduction in the number of lateral roots and in the number of root hairs, as well as reversion of root gravitropism at higher concentrations. The major difference between the seedlings treated with the crude extract and those treated with the purified extracts was the length of the roots and shoots, indicating that the mode of action of the bioactive substances is not affected by the procedure described here. This method also contributes to increasing the biological activity of the extracts, probably because filtration of the methanol-dissolved solution removes insoluble and inactive substances. In fact, the inhibitory effect that the purified extracts had on seedling growth was found to be 2- 10 times greater than was that achieved with the original, crude solutions (Tab. 2).

Table 1. Average growth of *Sesamum indicum* L. seedlings incubated in aqueous leaf extracts of *Caryocar brasiliense* A. St.-Hil., *Qualea parviflora* Mart. and *Eugenia dysenterica* DC. Three replicates of ten seedlings were used per treatment.

Purified extract	*Caryocar brasiliense*		*Qualea parviflora*		*Eugenia dysenterica*	
	Roots (cm)	Shoots (cm)	Roots (cm)	Shoots (cm)	Roots (cm)	Shoots (cm)
0.0%	5.01±1.86 a	2.56±0.75 a	5.27±1.60 a	2.28±0.63 a	5.27±1.60 a	2.28+0.63 a
0.1%	2.71±0.88 b	2.00±0.40 a	3.44±0.86 b	1.97±0.36 a	1.16±0.18 b	2.43+0.37 a
0.5%	0.61±0.24 c	1.37±0.31 b	0.55±0.30 c	0.90±0.33 b	0.63±0.21 c	1.20+0.37 b
1.0%	0.28±0.05 d	0.74±0.41 c	0.35±0.11 c	0.55±0.29 b	0.33±0.17 d	0.48+0.27 c

Values in the same column followed by the same letter do not differ significantly, according to the ANOVA and *a posteriori* Student's t-test (p=0.05).

Table 2. Proportional growth inhibition of *Sesamum indicum* L. seedlings incubated in aqueous leaf extracts of *Caryocar brasiliense* A. St. Hil., *Qualea parviflora* Mart. and *Eugenia dysenterica* DC. Number of replicates (of ten seedlings) per treatment is in parentheses. For comparisons among treatments, the root and shoot size of the seedlings incubated in water was taken as 100%.

Type of extract	Concentration	*Caryocar brasiliense*		*Eugenia dysenterica*		*Qualea parviflora*	
		Roots	Shoots	Roots	Shoots	Roots	Shoots
Crude	0.5%	53.68% (n = 6)	3.04% (n = 6)	-	-	41.27% (n = 3)	19.89% (n = 3)
	1.0%	76.22% (n = 9)	30.06% (n = 9)	84.80% (n = 12)	9.84% (n = 12)	71.23% (n = 3)	22.16% (n = 3)
Purified	0.1%	55.16% (n = 6)	3.12% (n = 6)	77.99% (n = 3)	0% (n = 3)	34.72% (n = 3)	13.60% (n = 3)
	0.5%	91.00% (n = 6)	38.47% (n = 6)	88.05% (n = 3)	47.37% (n = 3)	34.72% (n = 3)	13.60% (n = 3)
	1.0%	95.03% (n = 3)	62.18% (n = 3)	93.74% (n = 3)	78.51% (n = 3)	93.36% (n = 3)	75.88% (n = 3)

When the proposal of a study is to reveal some kind of allelopathic interaction taking place in the field, the use of organic solvents in the preparation of extracts should be avoided (Blum 2011). Instead, the use of water has been recommended in allelopathic studies because it reflects more closely what would happen under natural conditions (Ferreira 2004). However, once the allelopathic activity of a species has been demonstrated, the solubilization of the active fractions in organic solvents may be desirable because it facilitates the subsequent steps of separation and identification of the active molecules (Lôbo *et al.* 2006; Macias *et al.* 2004). The procedure described here allows the purification of allelochemicals that are more polar, because it helps to remove from the aqueous solutions insoluble and undesirable substances that could hamper further chromatographic analysis, a useful procedure if one intends to delve further into the study of allelopathy.

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