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Allelopathic potential of *Eucalyptus urograndis* on lettuce and Brazilian native forest species of *Cedrela fissilis* Vell.

Maria Gabriela Marques de Souza[1], Viviana Borges Corte[1], Elias Terra Werner[1], Hildegardo Seibert França*[2]

[1]Universidade Federal do Espírito Santo, Departamento de Ciências Biológicas, Programa de Pós-graduação em Biologia Vegetal, Vitória, ES, Brazil
[2]Instituto Federal do Espírito Santo, Campus Vila Velha, Vila Velha, ES, Brazil

***Corresponding author: hildegardo.franca@ifes.edu.br**

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Abstract: The *Eucalyptus urograndis* is widely used in Brazil, including in agroforestry systems. *Cedrela fissilis* is a potential plant species to be used in agroforestry systems or intercropped with *E. urograndis*. Therefore, it is necessary to investigate the allelopathic potential of the hybrid on this species. This work aimed to investigate the allelopathic potential of *E. urograndis* leaves and litter on the germination and growth of *Lactuca sativa* and *Cedrela fissilis* species. Leaves and litter were collected and extracted with ethanol to obtain crude extracts (CEE), which were then fractionated to obtain hexane (FHex), dichloromethane (FDCM), ethyl acetate (FAcOH), butanol (FBuOH) and aqueous (FAq) fractions. For the allelopathic potential assay, the samples were tested for percentage of germination (%G), germination speed index (GSI), shoot and root length and dry mass of seedlings using a triple-factorial design (2 x 6 x 5). Sample concentrations were of 1.25; 2.5; 5.0 and 10.0 mg/L using *L. sativa* and *C. fissilis* seeds with 5 replicates of 20 seeds for each. Gas chromatography (GC/MS) was used to analyse the most active samples. The CEE, FHex and FDCM samples of leaves and litter had a negative effect on the variables analyzed for *L. sativa* and *C. fissilis*. The FAcOH, FBuOH and FAq fractions influenced the initial growth of the plant's seeds. FDCM was more active in the reduction of germination and inhibition of initial growth of both species. *Lactuca sativa* and *Cedrela fissilis* were negatively affected by the leaves and litter of *Eucalyptus urograndis.*

Keywords: *Lactuca sativa*, allelochemicals, phytotoxicity.
Abbreviations: CEE_crude ethanolic extract; C_ Concentration; %G_Germination percentage; FAcOH_ethyl acetate; FAq_aqueous fraction; FBuOH_butanol; FDCM_dichloromethane; Fhex_hexane fractions; GC/MS_Gas chromatography coupled to mass spectrometer; GSI_germination speed index; L_Leaf; RDM_root dry mass; RL_root length; S_Samples; SDM_shoot dry mass; SL_shoot length

Introduction

The genus *Eucalyptus*, which comprises over 900 species and subspecies, is widespread throughout the world and has many uses, mainly in the production of cellulose and the extraction of bioactive compounds. The leaves are the most utilized part of the plant, with numerous biological activities (Santos, 2021). According to the IBGE (2018), eucalyptus occupies an area of 7.6 million hectares in Brazil. It is one of the most cultivated species for commercial purposes, especially for the production of cellulose and paper (Manca et al., 2020).

The widespread cultivation of eucalyptus has the potential to lead to the negative impacts associated with plantations. Observed impacts on the flora include low biodiversity, changes in floral composition compared to the native vegetation, and slow regeneration of abandoned areas, which may be related to the allelopathic potential of eucalyptus (Souza et al., 2013).

Studies have shown that eucalyptus has been used in agroforestry systems (Pezzopane et al., 2021). As a facilitator species, eucalyptus promotes native development (Alencar et al., 2011). The hybrid species *Eucalyptus urograndis*, created by crossing *E. urophylla* with *E. grandis*, could theoretically be integrated into agroforestry systems to promote other species due to its rapid growth, productivity, and resistance (Barbosa et al., 2023).

There are many native and cultivated species that can be integrated with eucalyptus in production systems. *Cedrela fissilis* Vell. is a native species widely distributed in Brazil, utilized to recovery degraded areas as well as in agroforestry systems (Melotto et al., 2019). Due to its high value, its wood is commonly used for building, making furniture and musical instrument manufacturing (CNCFlora, 2022). *Lactuca sativa* L. can be included into agroforestry gardens (Hurpia et al., 2021). It is a species sensitive to allelopathic compounds and is frequently used in this type of research (Reigosa et al., 2013; Puig et al., 2018).

Extracting compounds from plants is a fundamental step in biological research, allowing bioactive molecules to be identified and characterized. Solvents are an essential part of this process and are used for the extraction of compounds from plant organs such as leaves and roots. Non-selective solvents, such as ethanol, can extract a wide range of organic compounds regardless of polar characteristics. These resulting extracts are commonly known as 'crude extracts'. On the other hand, extraction with selective solvents is based on the polarity of the chemical compounds and results in more specific and purified extracts (Singh et al., 2021).

Table 1. Germination Percentage (%G), Germination Speed Index (GSI) measured in *Lactuca sativa* seeds submitted the crude ethanolic extract and their fractions in different concentrations from the Leaves and Litter of *Eucalyptus urograndis*.

Concentration mg/mL	Samples	Germination			
		%G		GSI	
		Leaves	Litter	Leaves	Litter
1.25	CEE	95.0 aA	100.0 aA	21.75 aA	15.71 bB*#
	FHex	98.0 aA	97.0 aA	20.02 aA	22.02 aA
	FDCM	53.0 bA*#	21.0 bB*#	3.85 bA*#	1.27 cB*#
	FAcOH	97.0 aA	100.0 aA	21.00 aA	19.92 aA
	FBuOH	99.0 aA	99.0 aA	23.00 aA	19.75 aB
	Faq	98.0 aA	98.0 aA	21.75 aA	19.71 aA#
2.	CEE	95.0 aA	97.0 aA	17.54 bA*#	11.88 bB*#
	FHex	87.0 aB	99.0 aA	11.88 cB*#	19.49 aA*#
	FDCM	29.0 bA*#	18.0 bB*#	2.14 dA*#	1.57 cA*#
	FAcOH	96.0 aA	99.0 aA	19.5 abA*#	17.33 aB*#
	FBuOH	97.0 aA	96.0 aA	20.29 aA	17.42 aB*#
	Faq	98.0 aA	99.0 aA	20.63 aA	16.46 aB*#
5	CEE	79.0 bA*#	72.0 bA*#	10.53 cA*#	7.52 cB*#
	FHex	76.5 bA*#	58.0 cB*#	6.58 dA*#	6.04 cA*#
	FDCM	28.0 cA*#	10.0 dB*#	1.50 eA*#	0.62 dA*#
	FAcOH	98.0 aA	98.0 aA	17.25 abA*#	15.64 aA*#
	FBuOH	100.0 aA	96.0 aA	18.96 aA*#	11.00 bB*#
	Faq	100.0 aA	98.0 aA	14.63 bA*#	13.81 abA*#
10	CEE	66.0 bA*#	44.0 cB*#	5.86 cA*#	2.59 cB*#
	FHex	21.0 cA*#	7.0 dB*#	2.00 dA*#	0.81 cA*#
	FDCM	12.0 cA*#	1.0 dB*#	0.75 dA*#	0.08 cA*#
	FAcOH	93.0 aA	98.0 aA	14.46 bA*#	13.48 aA*#
	FBuOH	97.0 aA	76.0 bB*#	18.36 aA*#	7.50 bB*#
	Faq	100.0 aA	100.0 aA	13.11 bA*#	12.29 aA*#
Control Water*		98.0		23.16	
Control DMSO 0.5#		99.0		23.24	

Means followed by the same capital letter on the line compare the Leaves within the same Sample and Concentration, and lowercase letters compare the Samples within the same concentration and leaf type, do not differ statistically both according to Tukey's test at 5% of probability. Means followed by character * and/or # differ statistically from the control water and DMSO 0.5% (v/v), respectively, both by the dunnett test at 5% of probability. CEE = crude ethanolic extract; FHex = hexane fraction; FDCM = dichloromethane fraction; FAcOH = ethyl acetate fraction; FBuOH = butanolic fraction and FAq = aqueous fraction.

The fractionation of a plant extract is an additional technique that aims to the separation of the constituents into different chemical groups, usually based on their polarity or their molecular weight. The main aim of this process is to obtain more refined extracts with a more defined chemical composition, and it can be carried out on the crude extract obtained or directly from the plant. The combination of plant extraction and extract fractionation techniques allows the efficient exploitation of plant chemistry for biological applications, opening new perspectives for the development of natural products with allelopathic potential (Abubakar and Haque, 2020).

The aqueous extract of *E. urograndis* leaves and its litter have been shown to have allelopathic activity in crop species. However, studies on hybrids and their chemical composition are limited (Carvalho et al., 2015, Espinosa-García et al., 2008, Souza et al., 2018). This applies to potential negative impacts on native species. In addition, the study of the allelopathic potential of plants is essential for the identification of plant species that are suitable for agroforestry or intercropping with *Eucalyptus urograndis* to reduce the possible negative effects associated with monoculture. This study aimed to evaluate the allelopathic effect of *E. urograndis* leaves and leaf litter on the early growth and germination of *Lactuca sativa* L. and *Cedrela fissilis* Vell.

Results

Germination bioassay with Lactuca sativa

The statistical analysis of the variation in *Lactuca sativa* (Supplementary Table 1) revealed significant results for almost all observed variables within the triple interaction of leaf type (leaves and litter), samples, and concentration (L x S x C). The only exception was shoot dry mass (SDM), which exhibited a double interaction between leaf type and sample (L x S) and sample and concentration (S x C).

The effects of crude ethanolic extracts and the corresponding leaf and litter fractions on different variables were analyzed in the present study. In *L. sativa* (Table 1), FDCM significantly decreased %G in comparison with the control at all concentrations tested. The lowest mean values were observed for the litter fraction and the most active concentration was found to be 10 mg/mL (Supplementary Figure 1). At concentrations of 1.25 and 2.5 mg/mL, FDCM affected %G, whereas CEE and FHex reduced germination only at 5 mg/mL (Supplementary Figure 1A and B). FAcOH and FBuOH did not affect leaf and flask germination at any concentration. However, FBuOH (litter) significantly reduced germination compared to the control at 10 mg/mL. The germination speed index (GSI) of lettuce (Table 1) was

Table 2. Initial Growth measured in *Lactuca sativa* seeds submitted the crude ethanolic extract and their fractions in different concentrations from the Leaves and Litter of *Eucalyptus urograndis*.

Conc en mg/m L	Sample s	Initial growth							
		SL (cm)		RL (cm)		SDM (g)		RDM (g)	
		Leaves	Litter	Leaves	Litter	Leaves	Litter	Leaves	Litter
1.25	CEE	0.61abA	0.56 bA	5.55 bB	7.27 aA	0350	0.328	0.290 abA	0.318 aA
	FHex	0.53bA	0.56 abA	5.99 abA	6.39 abA	0.330	0.395	0.245 bB#	0.320 aA
	FDCM	0.37cA*#	0.42 bA*#	3.09 cA*#	3.25 cA*#	0.358	0.318	0.260 abA	0.313 aA
	FAcOH	0.61abA	0.53 bA	5.37 bA	5.75 bA	0.313	0.325	0.325 aA	0.318 aA
	FBuOH	0.62 abA	0.61 abA	6.26 abA	5.48 bA	0.353	0.370	0.335 aA	0.335 aA
	Faq	0.71 aA#	0.61 aB	6.90 aA	6.17 abA	0.338	0.373	0.323 abA	0.288 aA
2.5	CEE	0.60 abA	0.48 bB*	5.50 abA	5.69 aA	0.365	0.283	0.285 abA	0.223 bB#
	FHex	0.46 cdA*	0.50 bA	4.52 bA*#	4.70 abA*	0.363	0.390	0.223 bcB	0.303 aA
	FDCM	0.37 dA*#	0.40 cA*#	2.34 cA*#	2.93 cA*#	0.325	0.350	0.183cB*#	0.275 abA
	FAcOH	0.52 bcA	0.48 bA*	4.47bA*#	2.80 cB*#	0.315	0.268	0.263 abA	0.278 abA
	FBuOH	0.64 Aab	0.52 abB	5.52abA	3.68 bcB*#	0.360	0.383	0.320 aA	0.335 aA
	Faq	0.69 aA#	0.60 aB	5.99 aA	3.53 bcB*#	0.325	0.430	0.298 abA	0.223 bB#
5	CEE	0.46 bA*	0.40 bA*#	2.21 bA*#	1.69 bcA*#	0.363	0.370	0.278 aA	0.183 abB*#
	FHex	0.41 bA*#	0.40 bA*#	3.51 aA*#	3.47 aA*#	0.390	0.388	0.238 abA	0.233 abA
	FDCM	0.25 cA*#	0.28 cA*#	0.25 cA*#	0.82 cA*#	0.480*#	0.463#	0.168 bB*#	0.230 abA
	FAcOH	0.51 abA	0.41 bB*#	1.81 bA*#	2.21abA*#	0.343	0.380	0.178 bA*#	0.225 abA
	FBuOH	0.62 aA	0.42 abB*#	4.17 aA*#	1.25bcB*#	0.403	0.418	0.286 aA	0.253 aA
	Faq	0.50 bA	0.54 aA	3.56 aA*#	0.72 cB*#	0.365	0.518*#	0.300 aA	0.160 bB*#
10	CEE	0.34 bcA*#	0.29 bcA*#	0.55 bcA*#	0.25 aA*#	0.435	0.410	0.108 aA*#	0.123 abA*#
	FHex	0.28 cA*#	0.25 cA*#	0.93 bcA*#	1.16 aA*#	0.460#	0.420	0.153 aA*#	0.175 aA*#
	FDCM	0.00 dB*#	0.10 dA*#	0.10 cA*#	0.05 aA*#	0.000*#	0.000*#	0.130 aA*#	0.000 cB*#
	FAcOH	0.43 abA*	0.43 aA*#	0.46 bcA*#	0.29 aA*#	0.405	0.408	0.115 aA*#	0.158 abA*#
	FBuOH	0.53 aA	0.42 aB*#	2.28 aA*#	0.49 aB*#	0.473*#	0.425	0.158 aA*#	0.185 aA*#
	Faq	0.50 aA*	0.39 abB*#	1.70 abA*#	0.34 aB*#	0.468*#	0.468*#	0.138 aA*#	0.095 bA*#
Control Water*		0.62		6.38		0.36		0.31	
Control DMSO 0.5#		0,55		5.93		0.35		0.30	

Means followed by the same capital letter on the line compare the Leaves within the same Sample and Concentration, and lowercase letters compare the Samples within the same concentration and leaf type, do not differ statistically both according to Tukey's test at 5% of probability. Means followed by character * and/or # differ statistically from the control water and DMSO 0.5% (v/v), respectively, both by the dunnett test at 5% of probability. (SL: Shoot length; RL: Root length, expressed in cm/seedling; SDM: shoot dry mass; RDM: root dry mass, expressed in mg/seedling); C.= concentration; CEE= crude ethanolic extract; FHex = hexane fraction; FDCM = dichloromethane fraction; FAcOH = ethyl acetate fraction; FBuOH = butanolic fraction and FAq = aqueous fraction.

significantly affected by the highest concentrations (5 and 10 mg/mL) of CEE and its fractions, particularly FDCM.

The lettuce shoot length (SL) was lowest when exposed to a concentration of 10 mg/mL of extracts and fractions. FDCM inhibited the growth of *L. sativa* shoots at all concentrations. The mean values were significantly lower than the control values (Supplementary Figure 1 E and F; Table 2). CEE and FHex of leaves and litter caused a decrease in SL. This decrease was significantly lower than the control only from 5 mg/mL. At a concentration of 5 mg/mL, all treatments reduced the root length (RL) of *L. sativa*. At the lowest concentration tested, leaf and litter FDCM reduced RL, with means significantly lower than the control, as shown in Table 3, Supplementary Figure G and H.

Shoot dry mass (SDM) was not affected by any of the samples at 1.25 and 2.5 mg/mL, mean values were not different from controls (Table 2). FDCM from leaves and litter was highly effective in reducing SDM (Table 3 and Supplementary Figure 1I). All samples showed the greatest reduction in root dry mass (RDM) at a concentration of 10 mg/mL. At a concentration of 2.5 mg/mL, FDCM sample showed a significant reduction of RDM compared to the control (Table 2).

***Germination bioassay with* Cedrela fissilis**

In the statistical analysis of variation for *Cedrela fissilis*, significant results were observed for almost all variables within the triple interaction of leaf type (leaves and litter), samples and concentration (L x S x C) (Supplementary Table 2).

It was found that the seed germination of *C. fissilis* was significantly lower with FDCM and FAcOH samples from both leaf types at all concentrations (Supplementary Figure 2A and B) in comparison to the control (Table 4). At the concentration of 10 mg/ml, CEE and all leaf fractions significantly reduced % G (Supplementary Figure 2A). The FBuOH and FAq fractions of litter samples had no effect at any concentration tested (Table 4 and Supplementary Figure 2B).

The FDCM from leaves significantly reduced the GSI of *C. fissilis* at all concentrations (Supplementary Figure 2C). Similarly, the FDCM from litter reduced the GSI starting from 2.5 mg/mL (Supplementary Figure 2D). In both cases (Supplementary Figure 2C and D), the highest activity was observed at a concentration of 10 mg/mL.

The shoot length of *C. fissilis* was significantly lower in both leaves and litter at all concentrations when treated with FDCM in comparison to the control (Table 5, Supplementary

Table 3. Shoot dry mass (SDM) measured in *Lactuca sativa* seeds submitted the crude ethanolic extract and their fractions from the Leaves and Litter and in different Concentrations of *Eucalyptus urograndis*.

	Initial growth - SDM					
			Concentrations (mg/mL)			
Samples	Leaves	Litter	1.25	2.5	5.0	10
CEE	0.378 abA	0.347 cA	0.338 a	0.323 ab	0.366 c	0.422 a
FHex	0.385 abA	0.398 bA	0.362 a	0.376 a	0.388 bc	0.44 a
FDCM	0.290 cA	0.282 dA	0.337 a	0.337 ab	0.471 a	0.00 b
FAcOH	0.343 bA	0.345 cA	0.318 a	0.291 b	0.361 c	0.406 a
FBuOH	0.396 aA	0.398 abA	0.361 a	0.371 a	0.41 abc	0.448 a
Faq	0.373 abB	0.446 aA	0.355 a	0.377 a	0.441 ab	0.467 a

Leaf: Means followed by the same capital letter on the line compare the Leaves within the same Sample, and lowercase letters compare the Samples within the same leaf type, do not differ statistically both according to Tukey's test at 5% of probability. Concentrations: Means followed by the same lowercase letters compare the Samples within the same concentration, do not differ statistically according to Tukey's test at 5% of probability. CEE= crude ethanolic extract; FHex = hexane fraction; FDCM = dichloromethane fraction; FAcOH = ethyl acetate fraction; FBuOH = butanolic fraction and FAq = aqueous fraction.

Table 4. Germination Percentage (%G), Germination Speed Index (GSI) measured in *Cedrela fissilis* seeds submitted the crude ethanolic extract and their fractions in different concentrations of leaves and litter of *Eucalyptus urograndis*.

Concentrations (mg /mL)	**Germination**				
		%G		GSI	
	Samples	Leaves	Litter	Leaves	Litter
1.25	CEE	95.00 aA	100.00 aA	3.32 aA*#	2.85 aB*#
	FHex	98.00 aA	97.00 aA	2.91 aA*#	2.92 aA*#
	FDCM	37.00 bA*#	36.00 bA*#	1.60 bA	1.22 bB*#
	FAcOH	50.00 Ba*#	47.00 bA*#	1.70 bA	1.61 bA
	FBuOH	99.00 aA	99.00 aA	3.04 aA*#	2.94 aA*#
	Faq	98.00 aA	98.00 aA	3.40 aA*#	2.93 aB*#
2.5	CEE	95.00 aA	97.00 aA	3.16 aA*#	2.63 aB*#
	FHex	87.00 aB	99.00 aA	2.29 bB	2.90 aA*#
	FDCM	28.00 cA*#	37.00 bA*#	1.23 cA*#	1.16 bA*#
	FAcOH	54.00 bA*#	47.00 bA*#	1.23 cA*#	1.57 bA
	FBuOH	97.00 aA	96.00 aA	2.91 aA*#	2.80 aA*#
	Faq	98.00 aA	99.00 aA	3.36 aA*#	2.85 aB*#
5	CEE	79.00 bA	72.00 bA	2.45 aA	1.86 bB
	FHex	66.00 bA	58.00 bcA*#	1.63 bA	1.43 bcA#
	FDCM	27.00 dA*#	31.00 dA*#	0.73 cA*#	1.01 cA*#
	FAcOH	46.00 cA*#	47.00 cdA*#	1.63 bA	1.52 bcA
	FBuOH	100.00 aA	96.00 aA	2.95 aA*#	2.54 aB*
	Faq	37.00 cdB*#	98.00 aA	1.10 bcB*#	2.75 aA*#
10	CEE	66.00 aA*#	40.00 dB*#	1.78 aA	1.06 cdB*#
	FHex	21.00 deA*#	7.00 eB*#	0.51 cA*#	0.17 eA*#
	FDCM	12.00 eA*#	21.00 eA*#	0.42 cA*#	0.66 deA*#
	FAcOH	47.00 bcA*#	57.50 cA*#	1.55 abA	1.49 bcA
	FBuOH	59.00 abB*#	76.00 bA	1.55 abB	1.93 bA
	Faq	35.00 cdB*#	100.00 aA	1.08 bB*#	2.74 aA*#
Control Water*		82.50		1.93	
Control DMSO0.5#		83.00		2.04	

Means followed by the same capital letter on the line compare the Leaves within the same Sample and Concentration, and lowercase letters compare the Samples within the same concentration and leaf type, do not differ statistically both according to Tukey's test at 5% of probability. Means followed by character * and/or # differ statistically from the control water and DMSO 0.5% (v/v), respectively, both by the dunnett test at 5% of probability. CEE= crude ethanolic extract; FHex = hexane fraction; FDCM = dichloromethane fraction; FAcOH = ethyl acetate fraction; FBuOH = butanolic fraction and FAq = aqueous fraction.

Figure 2 E and F). In addition, FBuOH leaves significantly reduced shoot length compared to control at all concentrations, while FBuOH litter demonstrated a reduction at 2.5 mg/mL. Most treatments reduced SL at the highest concentration, except FAcOH litter and leaf FAq.

Supplementary Figure 2 G and H show that a concentration of 10 mg/mL resulted in the greatest reduction in root length (RL). Most samples had a significant reduction in RL, except for CEE and FHex from litter and FAq from leaves. At all concentrations, the FAcOH leaf mean was lower than the control. However, at 2.5 mg/mL, FDCM from litter and FBuOH from leaves and litter also showed a reduction in RL (Table 5).

Most of the 10 mg/mL concentration treatments affected the root dry mass (RDM) of *C. fissilis*. However, there was no

Table 5. Initial Growth measured in *Cedrela fissilis* seeds submitted the crude ethanolic extract and their fractions in different concentrations of leaves and litter of *Eucalyptus urograndis*.

C mg /mL	Samples	Initial growth							
		SL (cm)		RL (cm)		SDM (g)		RDM (g)	
		Leaves	Litter	Leaves	Litter	Leaves	Litter	Leaves	Litter
1.25	CEE	5.55 aA	5.39 abA	3.33 bB	4.21 aA	13.38	12.61	2.57 abA	2.10 abA*#
	FHex	5.55 aA	5.55 aA	3.96 abA	4.36 aA	13.29	11.61*#	2.16 bA*#	1.88 bA*#
	FDCM	4.30 bA*#	4.25 bA*#	3.11 bA	2.66 bA#	13.46	13.32	2.15 bA*#	2.08 abA*#
	FAcOH	5.85 aA	4.92 abB	4.63 aA*	3.01 bB	13.77	12.90	3.34 aA	2.55 abA
	FBuOH	4.18 bB*#	4.99 abA	3.12 bA	3.50 abA	13.81	13.39	2.55 abA	2.99 aA
	Faq	5.93 aA	5.02 abB	3.65 bA	3.10 bA	13.78	12.16	2.97 abA	2.19 abA*#
2.5	CEE	5.11 abA	5.02 abA	3.20 abA	3.77 aA	14.27	12.23	2.59 aA	2.38 abA*#
	FHex	5.20 abA	5.34 aA	3.68 aA	3.74 aA	12.59	12.39	2.08 aA*#	1.97 abA*#
	FDCM	2.89 cB*#	3.84 bcA*#	2.53 bA#	2.93 abA	13.80	12.39	2.19 aA*#	1.60 bA*#
	FAcOH	5.08 abA	5.06 aA	2.30 bA*#	2.81 abA	12.93	13.84	2.19 aB*#	2.85 aA
	FBuOH	4.22 bA*#	3.46 cA*#	2.62 bA#	2.63 bA#	14.79	13.15	2.45 aA*#	2.51 abA#
	Faq	5.54 aA	4.65 abB	3.19 abA	2.82 abA	13.66	13.47	2.54 aA#	2.14 abA*#
5	CEE	5.05 aB	4.31 aA*	2.88 abA	3.42 abA	13.43	13.22	2.53 aA#	2.17 aA*#
	FHex	4.87 abA	4.59 aA*	3.13 aB	3.99 aA	13.10	11.76*#	2.42 aA*#	1.99 aA*#
	FDCM	2.97 cA*#	3.32 bA*#	2.42 abA*#	2.57 bcA#	13.88	13.94	1.43 bA*#	1.88 aA*#
	FAcOH	4.92 abA	4.39 abA*#	2.16 bA*#	2.22 cA*#	13.42	13.55	2.14 abA*#	2.59 aA
	FBuOH	3.84 bcA*#	3.69 abA*#	1.96 bA*#	2.56 bcA#	13.88	12.09*	2.19 abA*#	2.60 aA
	Faq	5.18 aA	4.76 aA*	3.13 aA	2.82 bcA	13.92	13.25	2.80 aA	2.28 aA*#
10	CEE	3.36 bcB*#	4.31 aA*#	2.43 abA*#	2.91 abA	13.00	13.34	1.92 abA*#	2.39 aA*#
	FHex	3.84 bA*#	4.25 aA*#	1.81 bcB*#	3.65 aA	13.43	15.27	1.85 abA*#	2.49 aA#
	FDCM	2.20 cA*#	2.28 bA*#	1.43 cA*#	1.79 cdA*#	16.16	13.27	1.08 bA*#	1.05 bA*#
	FAcOH	4.44 abA*#	5.12 aA	2.25 abcA*#	2.59 bcdA#	13.64	13.16	2.59 aA	3.14 aA
	FBuOH	3.60 bA*#	2.76 bB*#	2.10 abcA*#	1.67 dA*#	14.02	11.60*#	2.35 aA*#	1.07 bB*#
	Faq	5.10 aA	4.47 aA*#	2.91 aA	2.67 bcA#	13.43	12.58	2.61 aA	2.34 aA*#
Control Water*		5.96		3.57		15.07		3.51	
Control DMSO0.5#		5.81		3.83		14.80		3.57	

Means followed by the same capital letter on the line compare the Leaves within the same Sample and Concentration, and lowercase letters compare the Samples within the same concentration and leaf type, do not differ statistically both according to Tukey's test at 5% of probability. Means followed by character * and/or # differ statistically from the control water and DMSO 0.5% (v/v), respectively, both by the dunnett test at 5% of probability. (SL: Shoot length; RL: Root length, expressed in cm/seedling; SDM: shoot dry mass; RDM: root dry mass, expressed in mg/seedling); C.= concentration; CEE= crude ethanolic extract; FHex = hexane fraction; FDCM = dichloromethane fraction; FAcOH = ethyl acetate fraction; FBuOH = butanolic fraction and FAq = aqueous fraction.

difference between the controls. FDCM showed the highest activity from both leaves (Supplementary Figure 2 I and J).

Chemical composition identification by gas chromatography coupled to mass spectrometer (GC/MS)

GC/MS analysis of CEE, FDCM and FHex revealed the presence of various allelochemicals, including monoterpenes, sesquiterpenes, triterpenes and fatty acid derivatives (Table 6). Among the identified monoterpenes, α-terpineol was found in all fractions analysed. The α-terpineol acetate was present in the crude ethanolic extract and the FHex of the litter. In addition, the allelochemical eucalyptol was identified in the EEC of both leaf types and in the litter FHex and FDCM.

In the FDCM, spathulenol was the only sesquiterpene identified in the litter, while the viridiflorol derivatives were identified in the leaves. In contrast, calamenene, caryophyllene oxide, and cubenol were identified exclusively in the litter FHex, while flavesone, germacrene B, and cubeban-11-ol were characterized in the leaves. Aromadendrene was found in CEE and FHex from both litter and leaves. Spathulenol showed a similar distribution. The coniferyl alcohol sesquiterpene was found exclusively in the CEE of *E. urograndis* leaves. The triterpene β-Sitosterol was found in all litter extracts, with a higher concentration in the hexane extract of leaves.

Discussion

The allelopathy of the genus *Eucalyptus* is already well known and is associated with the low natural diversity in the understorey of plantations (Zhang et al., 2022). Therefore, the evaluation of the allelopathic potential *in vitro* is of utmost importance to increase the success in the implementation of agroforestry systems or intercrops with *Eucalyptus*, with the aim of selecting species that are more tolerant to the released allelochemicals.

The species analysed in the present study were negatively affected by the crude extracts and their fractions from both leaf types of *E. urograndis*, suggesting that they may not be suitable for cultivation with the hybrid in question. These results for percent germination and germination speed index (GSI) in lettuce and *C. fissilis* are consistent with previous studies in which a decrease in percent germination and GSI was observed in several cultivated species, including *Pennisetum glaucum* (Sousa et al., 2018) and *Urochloa decumbens* and *Panicum maximum* forages (Carvalho et al., 2015), when exposed to aqueous extracts of *E. urograndis*.

E. urograndis releases allelochemicals into the soil and litter that inhibit the germination and growth of certain cultivated species (such as beans, maize, cucumber, and melon). This finding supports the previous discovery of the allelopathic activity of *E. urograndis* on various plant species (Espinosa-García et al., 2008; Song et al., 2019). This was also observed in the present study. The results obtained for the variable GSI of *L. sativa* and *C. fissilis* show that the tested samples can

Table 6. Compounds found in crude ethanolic extract (CEE), hexane (FHex) and dichloromethane (FDCM) fractions (more activity in allelopathic tests) from *Eucalyptus urograndis* leaves and litter through gas chromatography coupled to mass spectrometer (GC/MS).

			CEE (%AREA)[1]		FHEX (%AREA)[1]		FDCM (%AREA)[1]	
RT (MIN)	Identified compound	KI	Litter	leaves	Litter	leaves	Litter	leaves
7.474	Decane	1000	-	-	-	+(0.33)	-	-
8.107	*o*-Cymene	1022	-	-	+(1.37)	-	-	-
8.278	Eucalyptol	1028	+(2.82)[2]	+(2.71)	+(7.00)	-	+(1.16)	-
12.123	endo-Borneol	1162	-	+(1.25)		+(0.69)	+(0.78)	+(1.17)
12.87	*α*-Terpineol	1188	+(1.32)	+(4.24)	+(0.73)	+(1.66)	+(1.37)	+(2.13)
17.275	*α*-Terpinyl acetate	1349	+(2.99)	-	+(3.91)	-	-	-
17.992	Menth-6-en-2,8-diol	1375	-	+(1.06)		-	-	-
19.04	Caryophyllene	1416	+(1.85)	+(1.86)	+(1.4)	+(2.47)	-	-
20.103	Aromadendrene	1458	+(1.20)	-	+(1.03)	+(0.92)	-	-
21.66	Calamenene	1521	-	-	+(1.11)	-	-	-
22.179	Flavesone	1543	-	-		+(1.18)	-	-
22.672	Germacrene B	1563	-	-		+(1.01)	-	-
22.936	Spathulenol	1578	+(2.06)	-	+(2.68)	+(2.21)	+(1.18)	-
23.061	Caryophyllene oxide	1580	-	-	+(2.62)	-	-	-
23.087	Viridiflorol derivatives	1581	+(1.57)	+(1.54)		+(4.04)	-	+(0.83)
23.263	Viridiflorol	1588	-	-	+(1.24)	+(2.77)	-	+(0.87)
23.32	Cubeban-11-ol	1590	-	-		+(1.39)	-	-
24.13	Cubenol	1626	-	-	+(0.94)	-	-	-
26.522	Coniferyl alcohol	1733	-	+(4.11)		-	-	-
31.181	Hexadecanoic acid	1960	-	+(2.17)		-	-	+(2.12)
31.856	Ethyl Palmitate	1994	+(3.73)	-	+(4.64)	-	-	-
34.035	Phytol	2112	-	+(1.67)		+(7.56)	-	-
34.932	Linoleic acid ethyl ester	2162	+(1.69)	-	+(2.15)	-	-	-
35.047	Ethyl linolenate	2169	+(3.35)	-	+(4.17)	-	-	-
44.677	Tocopherol	3150	+(7.24)	-	+(4.22)	-	-	-
45.803	*β*-Sitosterol	3356	+(3.24)	-	+(5.64)	+(10.05)	+(1.28)	-

[1] %Area: indicates the % area of each compound found in the GC/MS chromatogram; [2] + : indicates the presence of the compound in plant extract; RT: retention time; KI: Kovats index

cause a decrease in the vigour of the plants, as evidenced by the lower values of GSI in the treatments as compared to the control.

The study confirms the phytotoxicity of *E. urograndis* as it significantly inhibited the initial growth of both *L. sativa* and *C. fissilis*. These results are particularly important due to the lack of studies on *C. fissilis*. The allelochemicals present were responsible for inhibiting the initial growth in both plants. Allelochemicals can inhibit length extension and cell division, resulting in reduced radicle and shoot length and subsequent reduced mass accumulation (Li et al., 2023, Xu et al., 2023). These chemicals have a direct impact on germination and initial growth by affecting crucial plant processes, including cell division, growth, plasmalemma permeability, photosynthesis, respiration, enzyme activity, and protein synthesis (Cheng and Cheng, 2015, Scavo and Mauromicale, 2021).

The terpenoids found in the nonpolar samples have also been detected in essential oils of other plant species with allelopathic and/or cytotoxic activity (Araniti et al., 2018). The presence of different allelochemicals with known allelopathic activity in the leaf and litter FDCM suggests that this fraction was the most active among those studied.

Therefore, the phytotoxic activity observed after testing CEE, Hex and DCM fractions may be due to these compounds. The presence of terpenes in the leaves of *E. urograndis* is a possible reason for the low diversity of both plants and animals in the understory of eucalyptus plantations. Terpenes are diffused into the surrounding medium when volatilized from plant parts and, being heavier than air, settle on the soil and affect other species (Rizvi et al., 1999; Song et al., 2019; Xu et al., 2023).

The analysis revealed that both leaf types had a negative impact on the variables, emphasising the significance of the hybrid as a potential source of allelochemical release. The allelopathic potential of litter has not been extensively researched, and this study demonstrates that the leaves of the hybrid, even after senescence, effectively release allelochemicals into the environment, primarily into the soil. This is consistent with previous studies on species of the *Eucalyptus* genus (Li et al., 2023, Song et al., 2019).

The litter results highlighted its allelopathic potential and suggested that its removal in the field could improve the performance of other plant species intercropped with the hybrid. Removing the litter could reduce the concentration of allelochemical compounds in the soil, which would benefit the development of other species. The concentration of allelochemicals is a decisive factor in the observed effect (Luo et al., 2023).

This *in vitro* study demonstrates the allelopathic potential of *E. urograndis*, as the compounds found in its leaves and litter inhibit the germination and initial growth of the receiving species. Further field studies are needed to investigate intercropping between *E. urograndis*, *L. sativa* and *C. fissilis*.

Materials and methods

Preparation of ethanol extracts and their fractionation

To evaluate the allelopathic potential of the *Eucalyptus urograndis* hybrid, two types of leaf material were collected separately: Leaves (mature, fully developed leaves in the canopy) and litter (old leaves from the surface layer deposited in the soil at a depth of 1 cm). A total of 1 kg of each type of leaf was collected at 10 different points in the

plantation. Leaves from the canopy and leaves that had fallen to the ground were collected at each point.

The plant material (was collected in a 4-year-old clonal plantation (clone PL 3335) of the company Fibria, Aracruz, Espírito Santo, Brazil (19°80'92.7"S; 40°15'90.5"W) in December/2018. The area receives annual fertilization and soil corrections and chemical weeding, the last of which was carried out on 09/2015. The reainfall in the municipality of Aracruz – ES, was 151-200 mm and the temperature ranged from 24°C to 26°C (INCAPER, 2018). The species selected to test the allelopathic activity was *Lactuca sativa* L. seeds of the cultivar 'Cinderela' (ISLA Sementes Ltda, Porto Alegre, Brasil, Batch: 0077301610000220; val: 11/2020) and *Cedrela fissilis* Vell. seeds (2018 harvest; val:12/2019, Arbocenter Comércio de Sementes Ltda, São Paulo, Brasil).

The leaves and litter were dried in a forced air oven at 55°C for 72 hours. After drying, 263g of leaves and 537 g of litter were obtained. The leaves and litter were weighed separately and extracted with commercial ethanol at a ratio 1:10 (w/v of leaves: ethanol) using a turbolization process and macerated for approximately 72 hours. The material was then filtered to separate the plant residue from the extracts (Filho et al. 2023).

The ethanolic extract was concentrated in a rotary evaporator until the complete elimination of ethanol, which, after its recovered, was added again to the plant residue to continue the extraction process until the plant material was exhausted (Lima, 2022). The crude ethanolic extract (CEE) obtained from the leaves and litter were stored in glass vial under refrigeration at a temperature of -6 °C. Part of the concentrated CEE of the leaves and litter was resuspended, separately in a water:ethanol mixture (2:8 – v/v) and liquid-liquid extractions were performed with solvents of increasing polarity, obtaining hexane (FHex), dichloromethane (FDCM), ethyl acetate (FAcOH), butanol (FBuOH) and aqueous fractions (FAq). After fractionation, the solvent was removed to obtain concentrated samples of leaves and litter for testing (Lima, 2022).

Germination bioassay with* Lactuca sativa *L and* Cedrela fissilis *Vell.

To evaluate the allelopathic activity of ethanolic extracts and their fractions, germination tests were carried out with *L. sativa* and *C. fissilis* seeds. Ethanolic extracts of leaves and litter were diluted in distilled water and the fractions (FHex, FDCM, FAcOH, BuOH and Aq) were solubilized with 0.5% dimethylsulfoxide (DMSO) solution in water to give solutions at concentrations of 1.25; 2.5; 5.0 and 10.0 mg /mL. Negative controls were distilled water and 0.5% (DMSO) solution in distilled water (Filho et al. 2023). In each treatment, 5 replicates of 20 seeds each were used, totaling 100 seeds of *L. sativa* L and 100 seeds of *C. fissilis* Vell. . Then, the seeds were placed for germination in 5 Petri dishes of 7 cm diameter, with a double sheet of filter paper soaked with 3 mL of samples, 3 mL of 0,5% DMSO or 3 mL of distilled water (Filho et al. 2023).

Petri dishes were kept in a germination chamber (BOD) in constant light at 20 °C for *L. sativa* (Ahmed et al., 2020) and at 25 °C with 12h/12h photoperiod for *Cedrela fissilis* Vell. (Santos et al 2015). Germination counts were made every 24h for 7 days for *L. sativa* and 21 days for *C. fissilis*. The germination percentage [G = (N/A) x 100, where: N = total germinated seeds; A = total seeds (Labouriau & Valadares 1976], germination speed index [$GSI = (G1/N1) + (G2/N2) + \ldots (Gn/Nn)$ where: G1 = seeds germinated at the first count; N1 = days elapsed until first count; G2 = seeds germinated in the second count; N2 = days elapsed until second count; Gn = seeds germinated in the last count and Nn = days elapsed until last count (Maguire, 1962) and initial growth were evaluated.

To analyze the initial growth, root and shoot length, and dry mass were measured at the end of the germination bioassay, 7 days for *L. sativa* and 21 days for *C. fissilis*. Root lenght (RL) and shoot lenght (SL) were measured with a caliper and expressed in centimeters per seedling (cm/seedling). After measurement, roots and shoots were kept at 60°C for 72h and then weighed to obtain root dry mass (RDM) and shoot dry mass (SDM). The mass was weighed on an analytical balance and expressed in milligrams per seedling (mg/seedling) (Pereira et al., 2019).

Gas chromatography coupled to mass spectrometer (GC/MS) analysis.

The chemical characterization of the samples with the best activity was analyzed by gas chromatography coupled to a mass spectrometer (GC/MS). The equipment used was an Agilent (7890 B) with model 5977A MSD mass detector (Agilent Technologies, USA) and 70eV electron impact. A 30 m x 250 µm x 0.25 µm HP-5 column was used for separation (Adams 2017). The injector temperature was set at 290 °C and the detector at 310°C. The elution system was a heating ramp starting at 40 °C with a heating rate of 5 °C/min up to 280 °C, followed by a heating rate of 15 °C/min up to 310 °C. Alkane standard from C10 to C40 was used to calculate the Kovats index (KI) to characterize the chemical compounds. Substances identification was performed by comparing NIST (National Institute of Standards & Technology) library mass spectra with retention indices and mass spectra similarity with literature data (Adams 2017, Nist Chemistry WebBook 2018).

Statistical analysis

The experiment was conducted in a 2x6x5 factorial scheme, being 2 materials (leaves and litter); 6 types of samples (CEE, FHex, FDCM, FAcOH, FBuOH and FAq); and 5 concentrations (0; 1.25; 2.5; 5.0 and 10.0 mg /mL), in a completely randomized design, each treatment consisted of 5 replicates of 20 seeds. The data for all variables were subjected to a three-factor analysis of variance (ANAVA) using the F test with a 5% probability level of significance. The homogeneity of variances and normal distribution were analyzed using the Shapiro-Wilk test. When significant interaction between the qualitative factors (leaves and samples) was detected, one-way ANAVA was performed for leaves within each sample and concentration, and samples within each concentration and leaf type. The means of the variables were compared using Tukey's test at a level of 5% probability. When significant interaction in quantitative factor (concentrations) was detected, linear regression analysis was performed for variables, and the linear model were chosen based on the significance of the regression coefficient and determination coefficient (R2), adopting the levels of 5% of probability, using the F test, considering the biological phenomenon under study. To compare water and DMSO controls with each treatment, the Dunnett test was used at a level of 5% probability. All analyses were performed with R (version 4.1.1; http://www.r-project.org) in Rstudio (version 2021.09.0 + 351).

Conclusion

The *E. urograndis* leaves and leaf litter showed allelopathic activity, inhibiting germination and initial growth of *Lactuca sativa* and *Cedrela fissilis*. The DCM fraction of leaves and litter was the most active. CEE, FHex and FDCM have terpenes in their phytochemical composition with allelopathic effects. For the use of *C. fissilis* and *L. sativa* in agroforestry systems or consortia with *E. urograndis*, additional studies are necessary.

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