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Leaf extracts of *Clusia fluminensis* Planck & Triana with allelopathic potential[1]

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ABSTRACT

Our goal was to evaluate the bioherbicide effect of ethanol extract and its fractions of increasing polarity derived from the leaves of *Clusia fluminensis*, on the germination and initial growth *of Lactuca sativa* (lettuce) and *Megathyrsus maximus* (guinea grass), as well as their activity in the catalase, peroxidase, and superoxide dismutase enzymes. For the antioxidant capacity, the DPPH, ABTS, FRAP and phosphomolybdenum tests were used, in addition to the pigment content analysis. Chemical analyses were performed by quantification of total phenol, tannin, and flavonoid contents. The ethyl acetate fraction showed the best result, at concentration 0.75mg/mL, with less influence on lettuces seeds and greater influence on guinea grass seeds. In treatments with ethyl acetate fraction, there was a significant increase in the activity of the three enzymes in lettuce seeds, up to 67% in catalase. The catalase and dismutase enzyme activity decrease in 30% and 19%, respectively in guinea grass seeds. The presence of total phenols, tannins, and flavonoids on the ethyl acetate fraction allow a correlation to the most significant antioxidant activity by the ABTS, DPPH and FRAP assays. The results, therefore, suggest that the ethyl acetate fraction from *leaves of C. fluminensis* showed phytotoxic potential.

Keywords: allelopathic effect, seed germination, antioxidant capacity, grass-colonies.

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INTRODUCTION

The production of food in an economically sustainable manner requires the use of synthetic compounds for the purpose of weed control. Weedscause losses on a global scale in the production and development of various crops, acting as competitors, pests, and pathogen hosts. Synthetic herbicides are widely used due to their high efficiency, cost-effectiveness, fast results, and easy availability. However, their uncontrolled use causes significant problems for human health and contributes to the development of herbicide-resistant weeds.[1]

In contrast, studies on botanical extracts and essential oils to evaluate allelopathic activity represent a pathway for the discovery of bioherbicides that can serve as an alternative to synthetic herbicides. There is increasing interest in secondary metabolites from different groups of substances for weeds control, with the expectation that these compounds will not cause soil and plant contamination, while exhibiting low toxicity to different life forms.[2,3]

To identify species with allelopathic effect, the family Clusiaceae, also known as Guttiferae[4] may present promising potential in the search for chemical groups of secondary metabolites. Within this family, the *genus Clusia* stands out due to its significant landscape interest, particularly in environmental management and reforestation of restinga areas.[5] Additionally, several representatives of this genus are important in folk medicine like for treating condictions such as hypertension[6] inflammatory processes, infections, and obesity control.[7] However, studies with allelopathic activity are limited, with only few descriptions of allelopathic effect of extracts obtained from *Clusia* species. The species *C. fluminensis*, native to Brazil, has been studied for its insecticidal activity[8,9] and the effects of enzymatic inhibition against snake venom,[10-12] but there are no reports on the herbicidal activity of this species against weeds.

The present work aims to verify the allelopathic potential of ethanol extract and its fractions from leaves *of Clusia fluminensis* on seed germination and seedling development of *Lactuca sativa* L. (lettuce) and *Megathyrsus maximus* (Jacq.) B.K.Simon & S.W.L.Jacobs (guinea grass), as well as the effects on the enzymes catalase (CAT), peroxidase (POX), superoxide dismutase (SOD), and chlorophyll and carotenoid pigments.

MATERIALS AND METHODS

The leaves of *Clusia fluminensis* Planck & Triana were collected from adult plants in the gardens of the Federal Institute of Espírito Santo (IFES) - Cariacica *campus* (20º19’29.557”S and 40º22’16.669”W). The species was identified and deposited in the herbarium of the Federal University of Espírito Santo (UFES) under number VIES 28316. After wash the leaves, they were dehydrated in an oven at 40ºC for 120 hours and then ground in an industrial blender.

The extract was obtained by maceration of the leaves with 96% ethanol at a ratio of 10% plant/solvent for 72 hours, followed by filtration. The ethanol extract (EEtOH) was obtained by elimination of ethanol using a rotary evaporator, and the recovered ethanol was re-added to the plant residue until the plant material was fully depleted. The crude extract obtained was stored under refrigeration at 8 ºC. A portion of the EEtOH was resuspended in a mixture of water:ethanol (2:8 v/v), and successive extractions were performed with solvents of increasing polarity, yielding the hexane (FHex), dichloromethane (FDCM), ethyl acetate (FAcEt) and butanol (FBuOH) fractions. The final residue, after separation with the respective solvents, was referred to as the aqueous fraction (Faq).

To evaluate allelopathic potential, the germination bioassay was performed using seeds of a dicotyledon, *Lactuca sativa*, and a monocotyledon, *Megathyrsus maximus* (Tanzania cultivar). The seeds were distributed in four Petri dishes with 12 cm diameter lined with two sheets of filter paper Whatman nº.1 moistened with 5.0 mL of EEtOH and the fractions FHex, FDCM, FAcEt, FBuOH and Faq, at a concentration of 1.0mg/mL. Twenty seeds were distributed per plate, with five replications, and the plates were kept in a germination chamber (BOD type) at 20 ºC *for L. sativa and* 25ºC for *M. maximus* with constant light.[13] Germination counting was performed every 24 hours for seven days, and measurements of radicle and aerial lengths were taken seven days after the start of the experiments. Two stages of development were evaluated for allelopathic activity: germination and initial growth.

For the germination, the following were analyzed: germination percentage (%G)[14], germination speed index (GSI)[15], mean germination time (MGT)[16], germination speed percentage (GS%) and allelopathy index (AI)[17]. For initial growth test, root length (RL), shoot length (SL), fresh root mass (FRM) and fresh shoot mass (FSM)[18]. Fresh mass was obtained from of 10 randomly selected samples among the seedlings that had root and epicotyl length evaluated. The root and aerial part were weighed

separately, and the results were expressed in grams per shoot and grams per root. To this end, the aerial and root parts were subjected to weight measurement on an analytical balance both prior to and following their drying in an oven with air circulation at 40 °C. The allelopathic bioassay was repeated for the sample that presented the best activity, but in concentrations of 1.0mg/mL, 0.75 mg/mL, 0.50 mg/mL, and 0.25 mg/mL.

The antioxidant capacity was measured using the DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2′-azino-di-(3-ethylbenzthiazoline sulfonic acid), FRAP (Ferric Reducing Antioxidant Power) and phosphomolybdenum tests with solutions of EEtOH, FHex, FDCM, FAcEt, FBuOH and Faq at concentrations of 1.0, 0.75, 0.50 and 0.25 mg/mL. The scavenging of the free radical was performed by adding 1mL of DPPH solution at 1mM and 3mL of sample, and then incubating the mixture for 30 minutes at room temperature and in the dark before measurement. For the standard curve, 4mL of DPPH solution was used at concentrations of 10 µM, 20 µM, 30 µM, 40 µM, 50 µM and 60 µM, diluted in methanol. The absorbance was measured by a spectrophotometer (Biospectro SP-220) at 515 nm, and the tests were carried out in triplicate. Trolox was used as antioxidant standard, and the results were expressed in terms of the antioxidant capacity of the compound equivalent to Trolox (µM trolox/g of extract), expressed as TEAC (Trolox Equivalent Antioxidant Capacity) value.[19]

To quantify the total amount of ABTS radical scavenged by extract, and it fractions, a solution was prepared by reaction of 7 mM ABTS with 140 mM potassium persulfate. To stabilize the ABTS radical, the solution was kept in the dark at room temperature for 16 hours. Afterward, the ABTS solution was diluted in ethanol until an absorbance of 0.7 ± 0.05 to 734 nm was achieved. The calibration curve of the Trolox standard was made at concentrations of 100, 500, 1000, 1500 and 2000 µmol. In the dark, an aliquot of 30 µL of each sample or standard solution were added to test tubes, followed with 3.0 mL of the ABTS radical solution. The absorbances were measured at 734 nm after 6 min of reaction, using ethanol as the blank. The results were expressed in terms of the antioxidant capacity of the compound equivalent to Trolox, reported as the TEAC (Trolox Equivalent Antioxidant Capacity) value.[19]

In the FRAP test, 900 µL of FRAP reagent (freshly prepared and heated at 37 °C) were mixed with 90 µL of distilled water and 30 µL of the samples at concentrations of 1.0, 0.75, 0.50 and 0.25 mg/mL, as well as a blank. The measurements were performed after 30 minutes at 595 nm using a Beckman DU-640 spectrophotometer (Beckman Instruments Inc., Fullerton, CA, USA) equipped with a self-thermostat cell holder. The temperature was maintained at 37 °C. The FRAP reagent is prepared by mixing 2.5 mL of a 10 mM TPTZ solution in 40 mM HCl, 2.5 mL of 20 mM $FeCl_3.6H_2O$, and 25 mL 0.3 mM acetate buffer, pH 3.6. The calibration curve for the Trolox standard was made at concentrations of 0.5, 0.75, 1.0, 1.25, 1.5, 1.75 e 2.0 µmol. The antioxidant results were expressed in µmol of Trolox per gram of samples.[20]

The total antioxidant capacity (TAC) of ethanol extract and its fractions FHex, FDCM, FAcEt, FBuOH and Faq was evaluated by the phosphomolybdenum method.[21] An aliquot of 300 µL of samples at concentrations of 1.0, 0.75, 0.50 and 0.25 mg/mL was added in 3 mL of containing solution 0.6M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate. The mixture was placed in a water bath at 95 °C for 90 min. Absorbance was measured at 695 nm with UV/Vis. spectrophotometer. Total antioxidant capacity was expressed in ascorbic acid equivalents (µg EAA/g of samples).

The enzymatic antioxidant activity of catalase (CAT), peroxidase (POX) and superoxide dismutase (SOD) was measured only for the sample that showed the best allelopathic effect for *M. maximus*. The crude enzymatic extract was prepared by homogenizing 0.3g of seeds and roots of guinea grass, previously macerated in liquid N_2, followed by the addition of 2.0 mL of the potassium phosphate buffer (0.1M, pH 6.8), 0.1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride (PMSF) and 1% (w/v) polyvinylpolypyrrolidone (PVPP).[22] The mixture was centrifuged at 12.000 *xg* at 4 ºC for 15 minutes. The supernatant was used as enzyme sample for of CAT, POX, and SOD analysis.

The catalase activity (CAT) the was calculated using the molar extinction coefficient of 36 $M.cm^{-1}$[23] and the result expressed in $\mu mol.min^{-1}.mg^{-1}$ protein. For peroxidase activity (POX), the enzymatic activity was calculated using the molar extinction coefficient of 2.47 $mM.cm^{-1}$[24,25] and the result was expressed in $\mu mol.min^{-1}.mg^{-1}$ protein. To determinate superoxide dismutase (SOD) activity, one unit of enzyme activity was defined as the amount of the enzyme required to inhibit, by photoreduction, 50% of nitro blue tetrazolium chloride (NBT)[26,27]. All experimental were realized in four replicates with duplicates.

The sample with best allelopathic effect was analyzed for chloroplastidics pigments contents (chlorophyll *a*, chlo-

rophyll *b and* carotenoids) in *M. maximus* seedlings. For this, 4 mg of seedlings were crushed with 2 mL of acetone at 20%, then homogenized and centrifuged by 15,000 *g*. for 10 min at a temperature of 4 °C. The supernatant was removed, and chlorophylls *a*, *b* and carotenoids were quantified using UV-Vis spectrophotometer in 470, 646 and 663 nm. Pigment concentrations were determined by equations:

Chlorophyll *a* = (12.25 A_{663}) – (2.79 A_{646}); Chlorophyll *b* = (21.50 A_{646}) – (5.10 A_{663}); Total Chlorophyll = Chlorophyll *a* + Chlorophyll *b*; Carotenoids = [(1000 A_{470}) – (1.82 Clorofila *a*) – (85.02 Clorofila *b*)]/198. Where: A_{470} = absorbance at 470 nm; A_{663} = absorbance at 663 nm; A_{646} = absorbance to 646 nm.

The results were presented in mg per gram of dry mass (mg.g^{-1} DM) as described.[28]

For the quantification of total phenolic content, 0.2 mL of each 1.0 mg/mL sample, 0.5 mL of 10% (v/v) Folin-Ciocalteu solution, and 1.0 mL of 7.5% (w/v) sodium carbonate solution were added to 8.4 mL of water. The mixture was incubated for 30 minutes in the dark, then the absorbance was measured using a spectrophotometer at 760 nm. Methanol was used as the blank. Standard solutions of gallic acid at the concentrations of 100; 50; 25; 12.5; 6.25; 3.125; 1.56 and 0.78 µg/mL were used to make the standard curve. The results were expressed in gallic acid equivalent per milligram (µg GAE/mg).[29]

The total tannins content was performed by the casein precipitation method[30], which consisted of the contact of 1 g of casein powder with 6 mL of the samples at 1.0 mg/mL, and 12 mL of water under constant agitation for three hours at room temperature (25 ºC). Afterward, the samples were filtered through Whatman filter paper, the volume was adjusted to 25 mL, and an aliquot of this solution (5mL) was removed. Then, total phenolics were determined by the Folin-Ciocalteu method previously described. The tannins content was calculated as the difference between the value found in this reading and that the obtained in the total phenolic content. The result was expressed in gallic acid equivalent per milligram (µg GAE/mg).

The total flavonoid content of the ethanol extract of *C. fluminensis* leaves and its fractions was determined by the colorimetric method using aluminum chloride.[31] A volume of 1.5 mL of 2% $AlCl_3$ solution in ethanol was mixed with 1.5 mL of sample solution (1.0 mg/mL). After incubation for 10 minutes at room temperature, absorbance was measured at 415 nm with a UV/Vis spectrophotometer (Thermo, Waltham, MA, USA). Ethanol was used as the blank. Quercetin was used as a standard to make the calibration curve, at concentrations of 100; 50; 25; 12.5; 6.25; 3.12; 1.56 and 0.78 µg/mL and the results were expressed as quercetin equivalents (µg QE/mg).

The experiments were conducted in a completely randomized design. Analysis of variance (ANOVA) was performed, followed by Tukey´s multiple comparison test ($p \leq 1$ and 5%) for *C. fluminensis* samples. A 5% *t*-test of significance was also used for the enzymatic and pigment analyses.

RESULTS AND DISCUSSION

The allelopathic potential of the extract and fractions was performed against *L. sativa* and *M. maximus* seeds, and results are showed in Table 1. The germination percentage (%G) of lettuce seeds decreased with the treatments with EEtOH, FHex, FDCM, and FBuOH compared to the control. On the other hand, the FAcEt and Faq samples did not differ from the control, maintaining high germination percentages of 95.72% and 98.66%, respectively. The germination speed index (GSI) and germination speed (GS) decreased for all samples analyzed, with FHex showing the lowest value (13.00), indicating a slower germination process compared to the control (18.60). Similarly, the germination speed (GS) was affected across all samples, with FBuOH showing the most pronounced reduction (63.64%) compared to the control (100%). The mean germination time (MGT) of lettuce seeds increased in treatments submitted to EEtOH, Fhex, and FBuOH. All samples exhibited allelopathic effect in accords the allelopathy index (AI) in lettuce seeds. For the initial growth parameters (RL, SL, FRM and FSM), a decrease in RL was observed for all treatments, except for the Faq sample, while SL decrease significantly in the EEtOH, FDCM, FBuOH and Faq samples. The FRM and FSM parameters decreased for all samples examined.

The *M. maximus* seeds were more sensitive for FAcEt treatment across all variables of germination (%G, GSI, MGT, GS and AI) and initial growth (RL, SL, FRM and FSM) compared to lettuce seeds (Table 1). The FAcEt treatment reduced %G to 80.55%, while the control maintained 100%. GSI decreased across all treatments, with FAcEt showing the lowest value (4.48). MGT was highest in FBuOH (85.83 hours) and FHex (94.82 hours), indicating delayed germination.

Table 1. Germination and initial growth analysis *of L. sativa and M. maximus* seeds, submitted to ethanol extract (EEtOH) and hexane (FHex), dichloromethane (FDCM), ethyl acetate (FAcEt), butanol (FBuOH) and aqueous (Faq) fractions at the concentration of 1.0 mg/mL. Distilled water was used with control

Lactuca sativa									
Samples	**%G**	**GSI**	**MGT (hours)**	**GS (%)**	**AI**	**RL**	**SL**	**FRM**	**FSM**
Control	100.00 a	18.60 a	25.51 d	100.00 a	0.00 a	4.68 a	0.84 a	0.40 a	0.096 a
EEtOH	92.03 bc	15.46 b	31.10 bc	83.29 b	-0.17 b	3.43 c	0.56 d	0.024 c	0.041 d
FHex	83.00 d	13.00 c	36.15 b	69.94 c	-0.32 c	4.20 b	0.80 ab	0.013 f	0.030 f
FDCM	86.39 cd	15.67 b	27.42 cd	84.52 b	-0.15 b	3.85 b	0.74 bc	0.014 e	0.033 ef
FAcEt	95.72 ab	16.74 b	29.06 cd	90.10 b	-0.10 b	4.21 b	0.87 a	0.024 c	0.058 c
FBuOH	85.73 cd	11.80 c	47.17 a	63.64 c	-0.36 c	3.26 c	0.71 c	0.020 d	0.036 e
Faq	98.66 a	16.65 b	29.93 cd	89.62 b	-0.10 b	4.76 a	0.72 bc	0.030 b	0.062 b
Megathyrsus maximus									
Samples	**%G**	**GSI**	**MGT (hours)**	**GS (%)**	**AI**	**RL**	**SL**	**FRM**	**FSM**
Control	100.00 a	5.16 a	91.00 ab	100.00 a	0.00 a	2.88 d	2.18 b	0.0123 a	0.025 b
EEtOH	90.52 b	4.86 b	87.79 bc	90.88 bc	-0.09 c	3.33 bc	1.54 d	0.0042 cd	0.015 e
FHex	95.82 ab	4.89 a	94.82 a	91.56 b	-0.08 b	3.42 bc	1.91 c	0.0044 bc	0.016 de
FDCM	91.60 bc	4.87 a	88.55 bc	91.17 b	-0.09 b	3.20 cd	1.87 c	0.0039 d	0.018 d
FAcEt	80.55 d	4.48 b	85.52 cd	83.78 c	-0.16 c	2.40 e	1.92 c	0.0041 cd	0.020 c
FBuOH	90.48 c	4.96 a	85.83 cd	92.73 b	-0.07 b	3.66 ab	2.08 b	0.0041 bc	0.018 d
Faq	89.06 c	5.09 a	83.19 d	95.36 ab	-0.05 ab	3.82 a	2.38 a	0.0048 b	0.028 a

Note: The parameters analyzed were germination percentage (%G); germination speed index (GSI); mean germination time (MGT), in hours; germination speed, in percentage (GS%); allelopathy index (AI), in %; root length (RL), in centimeters (cm), shoot length (SL), in centimeters (cm); fresh root mass (FRM), in grams, and fresh shoot mass (FSM), in grams. The means with the same letter in a column, equal treatment, and compared with the control, do not differ statistically from each other. The results obtained were submitted to variance analysis (ANOVA) and the means analyzed by the Tukey test at 5% probability.

The allelopathy index (AI) was negative in all treatments, with FBuOH (-0.20) showing the strongest inhibitory effect. Initial growth parameters, such as RL and SL, were significantly reduced, with the lowest values observed in FAcEt (RL of 1.92 cm and SL of 1.29 cm). FRM and FSM also decreased in all treatments, with FBuOH resulting in the lowest FSM (0.016 g) compared to the control (0.025 g).

Therefore, regarding the mechanism of action in the studied seeds, only the FAcEt sample was used for allelopathic assays, as it is considered the most promising. For this, the FAcEt sample was tested at concentrations of 0.25; 0.50; 0.75 and 1.0 mg/ml, with distilled water used as a control.

The results for the germination tests of *L. sativa* showed that FAcEt affected only the MGT in all concentrations tested. In the initial growth analyses, the most significant effects were observed on RL and SL at concentrations 0.50 and 1.0 mg/mL (Table 2).

For *M. maximus*, the FAcEt at 0.75mg/mL had the greatest inhibitory effect on %G, GSI, GS, and AI. In terms of initial growth, only the RL showed no significant alterations at the concentration 0.75mg/mL (Table 2). Among the concentrations tested, 0.75mg/mL was chosen as the lowest concentration with a significant allelopathic effect.

In this study, lettuce (*Lactuca sativa*) seeds and guinea grass (*Megathyrsus maximus*) were used to evaluate the effects of ethanol extract and fractions of different polarities of *C. fluminensis* on their germination development. The fractional extraction was performed to lead samples with different yields of bioactive compounds. After evaluated all samples, the 1mg/mL of ethyl acetate fraction did not influence %G, MGT and SL of lettuce, unlike in guinea grass, where all these germination variables were affected along with GSI, GS and AI. Jmii et al.[32] (2020) studied the phytotoxicity of three extracts of *Thapsia garganica* from organic solvent with increasing polarity, finding that lettuce was less sensitive compared to weeds, with polar fractions showed more activity than no-polar fractions. In our study, the FAcEt sample demonstrate activity at a minimum inhibition concentration of 0.75 mg/mL for both seeds.

The non-interference in the lettuce germination and the greater interference in guinea grass seeds suggest that the ethyl acetate fraction may act as a modulator, that promotes

a smaller grass population without interfering with lettuce. In addition, slower germination seeds can produce smaller seedlings, which influences the process of competition for resources, making these plants more susceptible to stresses and predation, and, consequently, reducing their chances of survival.

The ABTS, DPPH, FRAP and TAC-phosphomolybdenum tests exhibited considerable dose-dependent antioxidant capacity of the samples studied, comparable to Trolox and ascorbic acid as standard reference (Table 3). The FAcEt fraction showed highest antioxidant capacity in the DPPH, ABTS and FRAP assays, reaching 645.22 µg/mL, 1527.65 µg/mL, and 60.04 µg/mL, respectively, at 1.00 mg/mL. In contrast, the hexane fraction (FHex) showed the lowest activity across these assays. However, the FHex fraction was more activity in the phosphomolybdenum assay at all concentration tested (135.98 µg/mL at 1.00 mg/mL).

The antioxidant enzymatic assay was performed only with FacEt fraction due to greater effect in allelopathic tests for *L. sativa* and *M. maximus* seeds. The enzymatic activities were measured in *µ*mol/min/mg protein and observed an increase in CAT, POX and SOD activities in lettuce seeds treated with the FAcEt sample. On the other hand, a decrease in CAT and SOD activity were detected in guinea grass seeds, while POX activity showed no statistical difference compared to the control (Figure 1).

Plant growth, development and productivity are influenced by various environmental stresses, which often disturb homeostasis and induce osmotic stress, leading to the accumulation of reactive oxygen species (ROS). Antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and peroxidases (POX), are associated with ROS scavenging in plants.[33]

The FAcEt sample of 0.75mg/mL of *C. fluminensis* increased CAT, POX and SOD activity by 66.76%, 44.29%, and 40.09%, respectively, in lettuce seeds. The SOD catalyzes the dismutation of superoxide anion ($^{\bullet}O2^{-}$) to form H_2O_2 and O_2, which is the first step in the defense against ROS. The CAT and POX work in close synchrony with SOD to prevent formation of more harmful ROS, as H_2O_2 is the substrate for both enzymes.[33]

The guinea grass presented a different response when expose to the 0.75mg/mL FAcEt sample, with decrease of SOD, CAT and POX activities. The CAT activity decreased by 30%, while SOD and POX activities were reduced by 18.79% and 1.41%, respectively, compared to the control. Allelochemicals could inhibit various enzymes, including proteases, catalases, peroxidases, phosphorylases and cellulases allowing ROS to act on the cells of guinea grass seeds.[34]

This situation contrasts with the high exogenous antioxidant potential observed in the DPPH, ABTS, FRAP, and phosphomolybdenum assays, suggesting that while

Table 2. Germination and initial growth analysis of *L. sativa* and *M. maximus*, treated with ethyl acetate fraction (FAcEt) at different concentrations. Distilled water was used as a control

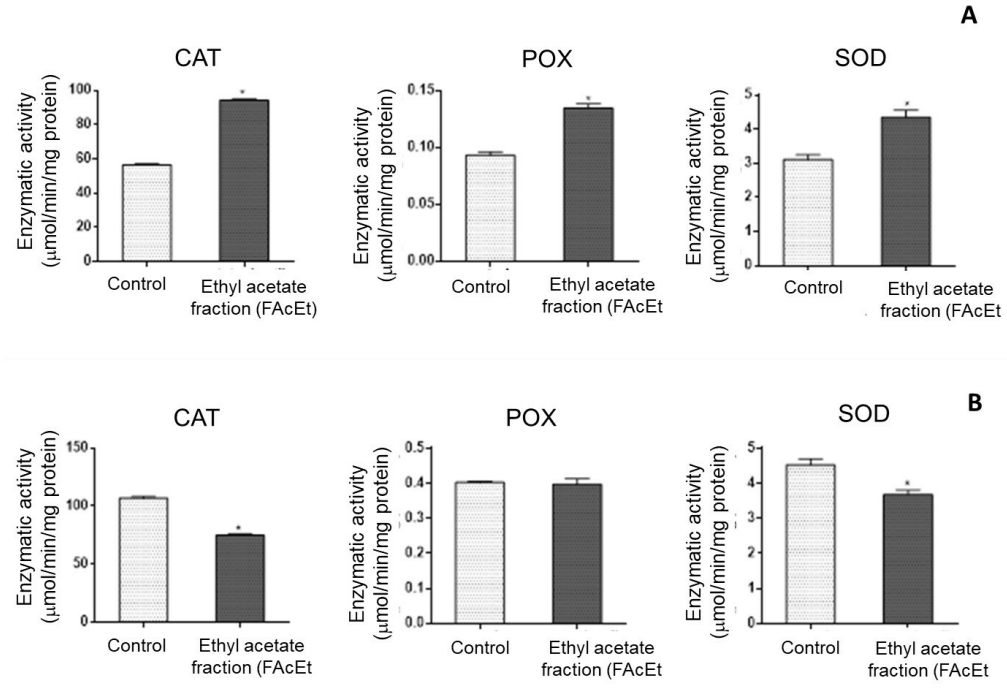
Lactuca sativa									
mg/mL	**%G**	**GSI**	**MGT (hours)**	**GS (%)**	**AI**	**RL**	**SL**	**FRM**	**FSM**
0.0	100.00a	18.60a	25.50c	100.00a	0.0a	4.68a	0.84a	0.039a	0.096a
0.25	94.95a	16.57a	28.99a	89.21a	-0.11 a	2.46c	0.74c	0.022c	0.074b
0.50	93.78a	16.08a	30.40a	86.59a	-0.13 a	5.12a	1.00ab	0.028cb	0.051c
0.75	94.95a	16.30a	30.05a	87.78a	-0.12 a	3.38b	0.76c	0.016d	0.030d
1.0	94.95a	16.13a	30.37a	86.93a	-0.13 a	5.40a	1.02a	0.017d	0.036d
Megathyrsus maximus									
mg/mL	**%G**	**GSI**	**MGT (hours)**	**GS (%)**	**AI**	**RL**	**SL**	**FRM**	**FSM**
0.0	100.00a	5.04a	90.39 a	100.00 a	0.00 a	2.88a	2.18a	0.124a	0.025a
0.25	85.13b	4.88a	81.81 b	91.07 a	- 0.09 a	2.64a	2.14ab	0.003b	0.024a
0.50	82.97b	4.77a	82.57 ab	88.99 a	- 0.11 a	2.32ab	2.02ab	0.002b	0.021ab
0.75	70.48c	3.88b	87.06 ab	72.95 b	- 0.27 b	2.28ab	1.86b	0.002b	0.018bc
1.0	64.15c	3.5b	85.75 ab	65.91 b	- 0.34 b	1.86 b	1.40 c	0.001c	0.013c

Note: The parameters analyzed were germination percentage (%G); germination speed index (GSI); mean germination time (MGT), in hours; germination speed, in percentage (GS%); allelopathy index (AI), in %; root length (RL), in centimeters (cm), shoot length (SL), in centimeters (cm); fresh root mass (FRM), in grams, and fresh shoot mass (FSM), in grams. The means with the same letter in a column, equal treatment, and compared with the control, do not differ statistically from each other. The results obtained were submitted to variance analysis (ANOVA) and the means analyzed by the Tukey test at 5% probability.

Table 3. Antioxidant capacity of ethanol extract (EEtOH) of leaves *of Clusia fluminensis* and its hexane (FHex), dichloromethane (FDCM), ethyl acetate (FAcEt), butanol (FBuOH) and aqueous (Faq) fractions

Antioxidant assay	Concentration of extracts (mg/mL)	Extracts and Antioxidant Activity					
		EEtOH	FHex	FDCM	FAcEt	FBuOH	Faq
DPPH	0.25	218.89cC	160.28dD	289.50cB	375.16dA	258.44dB	270.17cB
	0.50	235.22cD	199.94cE	283.61cC	441.06cA	325.44cB	291.28cC
	0.75	290.67bE	230.61bF	359.56bD	554.50bA	421.33bC	475.50bB
	1.00	332.00aD	291.67aE	470.11aC	645.22aA	487.67aBC	511.06aB
ABTS	0.25	71.31dC	62.09 dC	159.11dB	381.91dA	180.23dB	171.51dB
	0.50	142.62cD	124.17cD	318.22cC	763.82cA	360.45cB	343.02cB
	0.75	213.93bD	186.26bD	477.33bC	1145.74bA	540.68bB	514.53bB
	1.00	285.24aE	248.34aF	636.45aD	1527.65aA	720.68aB	686.04aC
FRAP	0.25	1.59cD	1.15cD	5.18dC	17.63dA	9.16dB	7.61dBC
	0.50	4.31bD	3.02bcD	10.40cC	26.82cA	13.18cB	11.50cBC
	0.75	5.86bD	3.88bD	15.43bC	35.68bA	22.26bB	16.37bC
	1.00	13.31aD	10.23aE	29.25aC	60.04aA	38.26aB	31.61aC
MOLI	0.25	47.37dB	61.04dA	39.04dC	44.55dB	44.75bB	42.72dB
	0.50	84.21cA	88.95cA	45.91cC	73.47cB	51.55cC	50.98cCD
	0.75	107.21bB	126.77bA	75.96bE	87.63bD	83.65bD	101.75bC
	1.00	118.22aB	135.98aA	100.99aD	94.89aE	92.81aE	105.39aC

Note: The antioxidant assays performed were ABTS, DPPH, FRAP and MOLI (phosphomolybdenum). The extract and its fractions were tested at concentrations 0.25; 0.50; 0.75 e 1.0 mg/mL. The results for the ABTS, DPPH and FRAP assays were expressed in Trolox equivalent (µM trolox g^{-1}). The results for the phosphomolybdenum assay were expressed as ascorbic acid equivalent (µM ascorbic acid g^{-1}). The statistical analysis was performed in a factorial experiment with Anova variance analysis. The means followed by the same letter do not differ statistically from each other, with uppercase letters comparing the same row and lowercase in the same column. The Tukey Test was applied at the 5% probability level.



The asterisk (*) indicates significant results of the sample in relation to the control, using the *T test.*

Figure 1. Enzymatic activity of CAT, POX, and SOD, in *Lactuca sativa* seeds (**A**) and *Megathyrsus maximus* (**B**), submitted to control treatment and ethyl acetate fraction, at concentration 0.75mg/mL.

C. fluminensis exhibits strong external antioxidant activity, it may disturb the endogenous antioxidant machinery responsible for oxidative neutralization in *M. maximus*. These results may explain the greater loss of germination in grass seeds, a phenomenon not observed in lettuce, which consequently resulted in growth inhibition in *M. maximus*.

The FAcEt sample had a significant effect on the increase of all chloroplast pigments analyzed, except in the chlorophyll *a*/*b* ratio. (Table 4).

The increase in chlorophyll *a*, chlorophyll *b* and carotenoids content could be a response to the acclimatization of *M. maximus* to the stress promoted by the ethyl acetate fraction of *C. fluminensis*, which may be responsible for the elevation of carotenoid levels. Chloroplasts and mitochondria are the two primary sites of ROS generation, and the production and accumulation of ROS in the plants leads to the destruction of cell organelles and functions. This process causes membrane lipid peroxidation, resulting in damage to cell membranes, degradation of biological macromolecules, and ultimately, cell death.[33,35]

The results for the of total phenolic, tannins, and flavonoids content of the ethanol extract of *Clusia fluminensis* leaves and their hexane, dichloromethane, ethyl acetate, butanol and aqueous fractions are presented in Table 5.

The samples with the highest total phenolic content were FAcEt, Faq, FDCM, and FBuOH with values of 576.16; 530.91; 499.83; and 498.76 µg GAE / mg, respectively, without statistical differences between them. These were followed by FHex samples with 193.56 and EEtOH with 173.51 µgGAE/mg. The more polar samples presented a higher tannin content compared to the no-polar samples, with Faq presenting 510.72 µgGAE/mg. The flavonoid content was observed in the FHex samples with 5.12 µgQE/mg, followed by EEtOH with 4.07 µgQE/mg and the lowest concentration was in the Faq, with 0.55 µgQE/mg.

The phenolic compounds and their derivatives were evaluated across all samples, and the FAcEt contained highest amounts of total phenolic, while the tannins and flavonoid contents were not the highest. In a previous study by da Silva and Paiva[36] (2012), on the quantification of total flavonoids in *C. fluminensis*, the most non-polar extracts presented higher percentages than the methanolic extracts.

In vitro antioxidant tests demonstrated that FAcEt had the greatest effect in the DPPH, ABTS, and FRAP assays. Although FAcEt did not have the highest tannin or flavonoid content, the presence of certain chemical groups likely contributed to its pronounced antioxidant effect compared to the other fractions tested. It is suggested that coumarins

Table 4. Chloroplastidic pigment content in *Megathyrsus maximus* cuttings treated with control and ethyl acetate extract at 0.75mg/mL

Variables	Control	FAcEt
Chlorophyll *a* (mg.g^{-1} MS)	4.94 ± 0.00 a	5.73 ± 0.02 b
Chlorophyll *b* (mg.g^{-1} MS)	2.58 ± 0.00 a	3.00 ± 0.07 b
Total Chlorophyll (mg.g^{-1} MS)	7.52 ± 0.00 a	8.73 ± 0.00 b
Carotenoides (mg.g^{-1} MS)	1.50 ± 0.00 a	1.66 ± 0.03 b
Chlorophyll a/b	1.91 ± 0.00 a	1.91 ± 0.00 a
Total chlorophyll/carotenoids	5.02 ± 0.00 a	5.26 ± 0.00 b

The results were submitted to the T test with 5% probability. Letters in the same line indicate statistically significant results

Table 5. Total phenolic, tannins, and flavonoids contents in the *Clusia fluminensis* samples

Samples	Phenolic content	Tannin content	Flavonoid content
	(µg EAG/mg)	(µg EAG/mg)	(µg QE/mg)
EEtOH	173.51b	92.82E	4.07b
FHex	193.56b	173.86d	5.12A
FDCM	499.83a	413.90c	3.83bc
FAcEt	576.16a	478.59b	3.38bc
FBuOH	498.76A	498.76ab	3.00C
Faq	530.91a	510.72A	0.55D

EEtOH = ethanol extract; FHex = hexane fraction; FDCM = dichloromethane fraction; FAcEt = ethyl acetate fraction; FbuOH = butanol fraction; Faq= aqueous fraction; EAG = gallic acid equivalent; QE = quercetin equivalent. The means followed by the same letter do not differ statistically from each other, with uppercase letters comparing the same row and lowercase in the same column.

and lignans may be responsible for this activity, along with other phenolic compounds. Coumarins and lignans are secondary metabolites classified as simple phenolic compounds with known antioxidant capacity.[37] In a previous study on other *Clusia* species, the ethanolic crude extract of stems showed an EC50 value of 3.462±0.301 g extract/g DPPH.[38]

CONCLUSION

Leaf extracts of *C. fluminensis* demonstrated significant allelopathic potential, particularly in the germination and initial growth of *M. maximus*. Negative effects were observed in nearly all analyzed variables, with the ethyl acetate extract showing the most pronounced activity. This extract also exhibited high antioxidant capacity, alterations in enzymatic antioxidant activity, and changes in pigment content. These findings, combined with the rich phytochemical and metabolic composition of the extracts - especially the ethyl acetate fraction - suggest that this extract is the most promising for further allelopathy studies. Additionally, it shows potential as a natural alternative for biological control, providing an option to replace conventional chemical herbicides.

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