Scientia Agraria Paranaensis – Sci. Agrar. Parana.
ISSN: 1983-1471 – Online
DOI: https://doi.org/10.18188/sap.v20i3.27815

ALELOPATHIC ACTIVITY OF EXTRACTS OF *Ocotea puberula* (Rich.) Nees ON THE GERMINATION AND INITIAL GROWTH OF LETTUCE

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SAP 27815 Received: 19/07/2021 Accepted: 08/09/2021
Sci. Agrar. Parana., Marechal Cândido Rondon, v. 20, n. 3, jul./sep., p. 306-311, 2021

ABSTRACT - The interest in the exploration of compounds from the secondary metabolism is seen as a strategic alternative in agriculture, even for the control of invasive plants. The objective of this work was to measure the allelopathic activity of extracts $CHCl_3$ and AcOEt from the leaves of *O. puberula* on the germination of lettuce. The ethanolic extract was obtained from the dried and milled leaves. The crude extract was solubilized in ethanol and acidified (hydroalcoholic fraction). The C_6H_{14} fraction was obtained by liquid-liquid extraction of the hydroalcoholic fraction, the same procedure was used to extract the AcOEt fraction. The hydroalcoholic fraction was basified and extracted with $CHCl_3$. The chemical profile of the obtained fractions was analyzed by Thin Layer Chromatography using specific revelators that confirmed the presence of alkaloids as majority compounds in AcOEt and $CHCl_3$ fractions. The fractions were evaluated for allelopathic potential on lettuce cypselas at 0.8 g mL^{-1}, 0.4 g mL^{-1} and 0.2 g mL^{-1}. In germination tests the germination parameters analyzed were: germination percentage (% G) and germination speed index (IVG). The results indicated significant changes in the %G, IVG and root growth (CR) of the lettuce seeds and seedlings. The ethanolic extract of leaves of *O. puberula* showed allelopathic influence on the species *L. sativa*. Both the AcOEt and $CHCl_3$ fractions reduced the germination rates evaluated, and the latter indicated a dose-response inhibition between the treatments applied.
Keywords: allelopathy, Lauraceae, control weeds.

ATIVIDADE ALELOPÁTICA DE EXTRATOS DE Ocotea puberula (Rich.) Nees SOBRE A GERMINAÇÃO E O CRESCIMENTO INICIAL DE ALFACE

RESUMO - O interesse na exploração de compostos do metabolismo secundário é visto como alternativa estratégica na agricultura, inclusive para o controle de plantas invasoras. O objetivo desse trabalho foi avaliar a atividade alelopática de extratos $CHCl_3$ e AcOEt das folhas de *O. puberula* sobre a germinção de alface. O extrato etanólico foi obtido a partir do material seco e moído e o extrato bruto solubilizado em etanol e acidificado (fração hidroalcoólica). A fração C_6H_{14} foi obtida por extração líquido-líquido da fração hidroalcoólica, mesmo procedimento utilizado para extração da fração AcOEt. A fração hidroalcoólica foi basificada e extraída com $CHCl_3$. O perfil químico das frações obtidas foi analisado por cromatografia em camada delgada, que confirmou a presença de alcalóides, como compostos majoritários, nas frações AcOEt e $CHCl_3$. As frações foram avaliadas quanto ao seu potencial alelopático, nas concentrações 0,8 g mL^{-1}, 0,4 g mL^{-1} e 0,2 g mL^{-1}. Nos testes de germinação, os parâmetros germinativos analisados foram a porcentagem de germinação (%G) e o índice de velocidade de germinação (IVG). Os resultados indicaram alterações significativas na %G, IVG e no crescimento radicular (CR) nas sementes e plântulas de alface. O extrato etanólico de folhas de *O. puberula* mostraram influência alelopática na espécie *L. sativa*. Tanto as frações AcOEt quanto $CHCl_3$ reduziram as taxas de germinação avaliadas, sendo que esta última indicou inibição dose-resposta entre os tratamentos aplicados.
Palavras-chave: alelopatia, Lauraceae, controle plantas daninhas.

INTRODUCTION

The term allelopathy can be considered as any direct or indirect effect that a plant or microorganism exerts on another plant, through the production of chemical compounds released into the environment (SOUZA; FURTADO, 2002). Thus, the International Society of Allelopathy, established that the term allelopathy is defined as the interference of secondary metabolites produced by plants, algae, bacteria and fungi, which act on the development and growth of other organisms (REIGOSA et al., 2013).

The allelopathic action can occur in several ways, it can be in an allelochemical way, by the leaching process in plants, volatilization and exudation of roots, as well as by microbial activity and decomposition of dry residues (FAROOQ, et al. 2011). In this context the allelopathic interaction can influence seed germination and seedling development, nutrient assimilation, photosynthesis, root growth, pest and disease control, among others (SILVA et al., 2013; SILVA et al., 2015; FONSECA et al., 2015; PINHEIRO et al., 2016).

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These compounds released into the environment are called allelochemicals or secondary metabolites. Each plant species can synthesize more than one type of secondary metabolite (FERREIRA, 2004). Among the best-known secondary metabolites are substances belonging to the group of phenolic acids, coumarins, terpenoids, flavonoids, alkaloids, cyanogenic glycosides, benzoic acid derivatives, tannins and complex quinones (MAULI et al., 2009). Among the secondary metabolites, the alkaloids, generally defined as natural molecules for presenting nitrogen in its structure, are abundant in plants and represent one of the most widespread classes of compounds and have multiple and varied pharmacological properties (ZANIN et al., 2007).

Despite the increase in research on the allelopathic potential of native species that has occurred in recent decades, current knowledge is still considered scarce. Several researches have been conducted with different plants, including those belonging to the genus *Ocotea*, aiming the isolation and characterization of chemical compounds. Among the identified plant metabolites, the benzylisoquinolinic and aporphinic alkaloids, the lignans and neolignans, the monoterpenes, the sesquiterpenes and the phenylpropanoids stand out (FARAGO et al., 2005). Studies conducted with *O. puberula* showed allelopathic potential on the germination of invasive species (SILVA; AQUILA, 2006). However, it should be noted that these species do not present visual indications of the occurrence of allelopathy in the field.

The use of herbicides is the main resource in the control of invasive plants, but it is not always effective, because it only eliminates more susceptible species, causing an increasing selection pressure on resistant or tolerant species. Thus, there is a clear need to search for new management alternatives to control resistant weeds, as well as methods to prevent the occurrence of resistance in new areas. With new technologies and recent advances in the chemistry of natural products through modern techniques of extraction, isolation, purification and identification at the molecular level, the allelopathic interactions between plants can become an available management alternative and become an efficient method for the control of resistant weeds (SILVEIRA, 2021).

The objective of this work was to evaluate the allelopathic potential of chemical fractions extracted from leaves of *Ocotea puberula* (Rich.) Nees (*O. puberula*), a native species of Brazilian flora, verifying its action on germination and initial growth of the bioindicator species of allelopathy, lettuce *(L. sativa* L.).

MATERIAL AND METHODS

Leaves of the species *O. puberula* (Rich.) Nees were collected in August 2017, in a Relevant Ecological Interest Area (REIA), located in Santa Helena (PR), under geographic coordinates of 24°51'05.3 "S and 54°21'06.1 "W. The leaves were carefully separated and dried in an air oven at a constant temperature of 60°C for 24 h and then processed in a knife mill (Micro Mill type Willye TE-648), with a stainless-steel sieve (30 mesh).

To obtain the ethanolic extract, the methodology described by Fongaro et al. (2017) was used, with adaptations. Weighed 300 g of the dried and ground plant material. 600 mL of ethanol was added to this material, keeping it in an ultrasonic bath for 60 min. After this period, a vacuum filtration was performed and the whole procedure in triplicate. The solvent resulting from the three extractions was collected and evaporated at a rotary evaporator at reduced pressure, where the yield of the crude extract calculated in relation to the initial dry biomass resulted in 32 g (10.6%).

To obtain the fractions, 15 g of crude extract was solubilized in 80 mL of ethanol and 160 mL of acidified water (pH = 2.0) was added, providing the hydroalcoholic fraction. The hexanic fraction (C6H12) was obtained by liquid-liquid extraction of the hydroalcoholic fraction by adding 80 mL of hexane and the whole procedure was performed in triplicate. The C6H12 was evaporated at a rotary evaporator at reduced pressure, resulting in 4.3g (28.7%) of this fraction.

The ethyl acetate (AcOEt) fraction was obtained using the same procedure as the hexanic fraction, yielding 1.9g (12.7%). To obtain the chloroformic fraction ($CHCl_3$), the hydroalcoholic fraction was basified at pH = 7.0 (NaOH 5%). Subsequently, liquid-liquid extraction was performed, adding 80 mL of CHCl3 to the basified hydroalcoholic fraction, performing this procedure in triplicate. The $CHCl_3$ was evaporated at a rotary evaporator at reduced pressure, resulting in 0.36 g (2.4%) of this fraction. All crude extracts and fractions obtained were stored under refrigeration.

The chromatographic profile of the crude extract and fractions was analyzed in silica gel chromatoplates (60 UV/254 nm, Macherey-Nagel®), using the following mobile phases: $CHCl_3$/AcOEt (1:9) and AcOEt/MeOH (8:2). The sample compositions were compared semiquantitatively, with 5 µL, of sample solutions at 10 mg mL^{-1} being applied using capillaries. The chromatoplates were visualized under ultraviolet light at a wavelength of 254 nm and revealed with a reagent specific for the class of alkaloids, with Dragendorff reagent.

The germination tests were conducted in the Botany Laboratory of the Federal Technological University of Paraná (UTFPR), Santa Helena *Campus*. For the germination evaluations, the seeds used in the study were placed in Petri dishes, previously prepared, containing two germitest paper disks each. As plant material, seeds of lettuce cultivar GrandRapids were used, placed on the germ paper, moistened with the treatment solution.

The Petri plates were moistened with 5 mL of each solution, each solution corresponding to a treatment: water (control) and the fractions of 0.2, 0.4 and 0.8 g mL^{-1} of AcOEt and $CHCl_3$. Each experimental unit consisted of four plates containing 25 seeds and 2 repetitions, resulting in 56 plates and 1400 seeds in total. After placing the seeds, the plates were transferred to a BOD growth chamber (LimaTec® brand), with a 12 h photoperiod, controlled temperature of 25ºC and 70% relative humidity, until the end of the experiment.

During the experiment the temperature and photoperiod were monitored daily and the germinated seeds were counted and removed, that is, those that presented radicle protrusion (±2mm) (FERREIRA and AQUILA, 2000). To calculate the germination percentage (%G) and germination speed index (GVI), Equations 1 and 2 were used, respectively:

$$\%G = \left(\frac{N}{25}\right) 100 \qquad \text{Equation 1}$$

Where:
N = final number of germinated seeds.

$$IVG = \Sigma \left(\frac{ni}{ti}\right) \qquad \text{Equation 2}$$

Where:
ni = number of seeds that germinated at time 'i',
ti = time after test installation and
i = 1 → X days (FERREIRA, 2004).

Seedlings were obtained by pre-germinating lettuce seeds in distilled water under the conditions described in the previous item. The growth was measured after seven days of incubation, using millimeter paper to measure the length of the primary root. The experimental design was entirely randomized, with three Petri dishes, 25 seeds, and two repetitions. The data obtained were submitted to ANOVA and the means were compared using the Scott-Knott test ($p>0.05$). Data analysis was performed using the SISVAR program (FERREIRA, 2000).

RESULTS AND DISCUSSION

The chemical profile analysis of the crude extract and fractions of *O. puberula* leaves by CCD indicated the presence of alkaloids in the $CHCl_3$ and AcOEt fractions with the revelation of Dragendorff reagent, specific for this class of secondary metabolites. The mobile phase AcOEt/MeOH 8:2 demonstrated the best separation of the compliant compounds. Allelopathic test on lettuce seeds (standard target species) was employed to evaluate the bioactivity of *O. puberula* leaf extract on germination and initial growth.

It was found that the leaves of *O. puberula* showed allelopathic activity, varying the potential according to the treatment and sample, and most samples significantly reduced parameters of germination and initial growth of lettuce seeds. The $CHCl_3$ fraction, which showed a higher degree of purity compared to the AcOEt fraction by CCD analysis, stands out. Chemical study conducted with *O. puberula* demonstrated the presence of aporphinic alkaloids in seedlings and adult plants, with greater accumulation in leaves, followed by stems and roots (ZANIN et al., 2011).

All concentrations of the $CHCl_3$ extract significantly reduced %G and IVG when compared to the control (Table 1), with the highest concentration (0.8 g mL^{-1}) causing a 50% reduction in %G and 80% reduction in IVG. The AcOEt fraction did not significantly inhibit the germination of lettuce seeds. However, the IVG had a significant reduction in all concentrations evaluated. Candido et al. (2016) evaluating the allelopathic effects of *O. pulchella* leaf extract, found that the AcOEt fraction of this extract inhibited by 45% and 65% the germination of lettuce and tomato, respectively.

TABLE 1 - Germination percentage (%G) and germination speed index (GVI) of lettuce (*Lactuca sativa*) in *Ocotea puberula* leaf fractions and concentrations of the solutions.

Leaf fractions *Ocotea puberula*	Concentrations (mg L^{-1})	%G	IVG
$CHCl_3$	0.0	99 a*	13.48 a
	0.2	85 b	7.43 b
	0.4	72 c	3.87 c
	0.8	50 c	2.64 c
CV(%)		10.9	13.63
AcOEt	0.0	99 a	14.10 a
	0.2	95 a	8.68 b
	0.4	94 a	7.52 b
	0.8	91 a	5.01 c
CV(%)		4.44	9.02

*Means followed by equal letters in the column do not differ statistically, by Scott-Knott test, at 5% probability of error.

Allelopathic compounds can cause changes in germination pattern and may result from effects on cell division, membrane permeability, enzyme activation, oxygen sequestration (phenols) among others (ROSADO et al., 2011). Often the allelopathic effect is not manifested on the percentage of germination, but on the speed index of seed germination. This factor may have an ecological significance, because plants that germinate more slowly may be smaller in size. And as a consequence, they may be more susceptible to stresses and have less chance in competition for resources (SILVEIRA et al., 2021). Therefore, this variable was also analyzed.

Regarding the GVI, Table 1 shows that there was a significant difference between the treatments and the control, in both extracts tested. Among the extracts tested in the bioassay, $CHCl_3$, reduced up to 80% in the concentration of 0.8 g mL^{-1}, providing greater reduction in seed germination time and promoting delayed germination.

Although it did not cause a negative effect on germination, the AcOEt fraction of the tested extract inhibited, by approximately 65%, the GVI of lettuce seeds.

Studies show that, although the final germination percentage may not be significantly affected by the action of allelochemicals, the germination pattern can be modified, verifying differences in the speed and synchrony of germination of seeds subjected to such compounds (SANTANA et al., 2006). The importance of this methodology is due to the fact that seeds constitute biological units through which ecological processes, such as intra- and interspecific competition, the invasion of new niches by non-native species, the colonization of new habitats and the regeneration of native vegetation, among others, can be triggered.

Germination is less sensitive to allelochemicals than seedling growth (FERREIRA, 2004), but experimental quantification is much simpler, because, for each seed, the phenomenon is discrete, germinating or not. In this context, allelopathic substances can induce the appearance of abnormal seedlings, being root necrosis one of the most common symptoms. All extracts of *O. puberula* leaves caused decreased growth of the root system and the greatest effects were seen in seedlings submitted to the $CHCl_3$ fractions (Table 2). The activity profile of the $CHCl_3$ leaf extract showed a dose-response relationship in which increasing concentration from 0.2 to 0.8 g mL^{-1} resulted in an inhibition of approximately 75% in lettuce root length.

Even not being observed statistical difference between the AcOEt of the extracts of the leaves of *O. puberula* on the germination of the lettuce seeds, the growth of these was negatively affected by all the evaluated concentrations, however, without presenting dose-response relation. It can be inferred that the activity presented by the AcOEt fraction may be influenced by other metabolites present in this fraction as observed by CCD analysis. In a study conducted by Araújo et al., (2015), the fractions $CHCl_3$, AcOEt and hydroalcoholic remainder of the leaves of *O. pulchella* showed toxic activity capable of causing mortality of the microcrustacean, suggesting bioactivity. A study using the fruits of the species *O. heterochroma* (CUCA et al., 2009) showed activity for the crude ethanolic extract, butanolic fraction and with the benzene fraction being considered with good bioactivity.

Several species of the genus *Ocotea* are being studied for their antioxidant and antibacterial potential. Among them are *O. nutans* (BETIM et al., 2021) and *O. paranaensis* (GRUBNER et al., 2020). The essential oils extracted from some species are also sources of study of this genus of plants. The essential oil of *O. nutans* was considered efficient regarding antibacterial activity, larvicidal and antioxidant capacity (BETIM et al., 2018). However, the action of these secondary compounds in other plant species is still poorly referenced.

TABLE 2 - Root length (cm) of lettuce (*Lactuca sativa*) seedlings in *Ocotea puberula* leaf fractions and concentrations of the solutions.

Leaf fractions *Ocotea puberula*	Concentrations (mg L^{-1})	Root length (cm)
$CHCl_3$ (FCl)	0.0	2.72 a*
	0.2	1.85 b
	0.4	1.12 c
	0.8	0.71 d
CV(%)		8.65
AcOEt (FAc)	0.0	2.84 a
	0.2	1.91 b
	0.4	1.74 b
	0.8	1.66 b
CV(%)		7.32

*Means followed by equal letters in the column do not differ statistically, by the Scott-Knott test, at 5% probability of error.

The herbicidal potential of the genus *Ocotea* was identified in a pioneering study by Borges et al. (1993), in which they evidenced that ethanolic and aqueous extracts of the leaves of *O. pretiosa* significantly reduced the growth of hypocotyl and radicle of lettuce seedlings. Prichoa et al. (2013), demonstrated the interference of *O. odorifera* extract in reducing the germination percentage, germination speed index and root length, as well as by the structural abnormalities found in *L. sativa* seedlings. Allelopathy can be considered a viable alternative in weed management, due to its excellent interaction potential and ecological importance, as well as the possibility of providing new chemical structures for the production of bioactives that combat pests or invasive plants and are less harmful to the environment.

Thus, new studies should be conducted to evaluate the allelopathic effect of *O. puberula* extracts on the germination and initial growth of invasive species, through bioassays with different solvents and concentrations. Specific molecules already known and isolated from extracts of this same plant can also be used to evaluate its allelopathic potential. It is worth mentioning that the results obtained in the laboratory for allelopathy may not be confirmed under natural conditions, since the simultaneous occurrence of biotic and abiotic factors may interfere in the results.

CONCLUSIONS

The ethanolic extract of *O. puberula* leaves showed allelopathic influence on *L. sativa*.

Both the AcOEt and $CHCl_3$ fractions reduced the germination rates evaluated, the latter indicating a dose-response inhibition among the treatments applied.

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