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Allelopathic Effects of *Psychotria viridis* Ruiz & Pavon on the Germination and Initial Growth of *Lactuca sativa* L.

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Abstract

The effects of aqueous and ethanol extracts and leaf fractions of *Psychotria viridis* Ruiz & Pavon (chacrona) at different concentrations on the germination and initial growth of *Lactuca sativa* L. were tested, and the phenolic and flavonoid compounds of these extracts and fractions were assessed. The bioassays consisted of the following treatments: crude aqueous extract (CAE) at 25, 50, 75 and 100% concentration, crude ethanol extract (CEE) and ethyl acetate, dichloromethane and methanol fractions at 6.25, 12.5, 25, 50 and 100% concentration and a control group. All treatments consisted of five replicates. The CAE, CEE and the ethyl acetate fraction of *P. viridis* caused both positive and negative effects on the seeds and seedlings of *L. sativa*. By contrast, the dichloromethane and methanol fractions only caused negative effects on *L. sativa*. The following compounds were identified in the extracts and fractions: gallic acid, chlorogenic acid, caffeic acid, ellagic acid, catechin, orientin, vitexin, quercetin, apigenin, rutin and luteolin, and the presence of the alkaloid N,N-dimethyltryptamine (DMT) has also been reported in the literature. *P. viridis* had allelopathic effects in all types of plant extracts and fractions tested, and one of these compounds or their combined action may account for these effects.

Keywords: allelochemicals, chacrona, extracts, fractions, High-Performance Liquid Chromatography (HPLC)

1. Introduction

Psychotria viridis Ruiz & Pavon (Rubiaceae), commonly known chacruna or chacrona, occurs spontaneously in the Amazon forest (Taylor, 2014), Mexico, the Antilles, Bolivia, Argentina and southeastern Brazil, and it is also grown in several regions of the world for religious purposes (Taylor, 2007). Ayahuasca, an entheogenic beverage also known as daime, caapi, yajé, Hoasca or vegetal, commonly used in religious rituals, is produced from a mixture of *P. viridis* with *Banisteriopsis caapi* (Spruce ex Griseb.) C.V. Morton (Malpighiaceae) (Schultes & Hofmann, 1993; Pépin & Duffort, 2004).

Certain plant species produce chemical substances that positively or negatively affect the germination and/or growth of other plants when released into the environment. This phenomenon is known as allelopathy, and its main function is to decrease or eliminate competition (Rice, 1984; Ferreira & Borghetti, 2004; Fujii & Hiradate, 2007).

Bagchi, Jain, and Kumar (1997) considered allelochemicals as a resource for the development of natural herbicides to be used in organic farming to minimize the environmental impact caused by commercial herbicides or as plant growth stimulants given the variety of secondary metabolite activities, especially allelopathic activity. Accordingly, the present study aimed to evaluate the allelopathic effects of leaf extracts of *P. viridis* on the germination and growth of *Lactuca sativa* L. and to chemically identify the phenolic and flavonoid compounds present therein given the presence of the alkaloid N,N-dimethyltryptamine (DMT) in *P. viridis* leaves (Quinteiro, Teixeira, Moraes, & Silva, 2006) and the reports of alkaloids, flavonoids and phenolic compounds with allelopathic action, and because the occurrence of alkaloids has already been previously studied.

2. Materials and Methods

2.1 Collection and Identification of Botanical Material

P. viridis leaves were collected in the morning period in a planting area belonging to the União do Vegetal Beneficent Spiritualist Center (*Centro Espirita Beneficente União do Vegetal*–CEBUDV), Highway Vicente Teles s/n, District of Santa Fé, municipality of Crato, Ceará state (CE), Brazil, located at 7°11′S and 39°27′W.

The plant material was collected and treated according to the usual plant collection methods, identified and sent for confirmation. The voucher specimens were deposited in the Dárdano de Andrade-Lima Herbarium of the Regional University of Cariri (*Herbário Caririense Dárdano de Andrade-Lima da Universidade Regional do Cariri*, HCDAL-URCA) under the registration number 6159.

2.2 Preparation of Extracts

A total of 200 g of fresh leaves of *P. viridis* was blended in 427 ml of distilled water using an industrial blender to prepare the crude aqueous extract (CAE). The volume of distilled water used was set based on the ratio between the fresh matter weight (FMW) and dry matter weight (DMW) (Medeiros, 1989).

For the crude ethanol extract (CEE) preparation, 500 g of fresh leaves of *P. viridis* was ground and soaked in 3L of ethanol P.A. (99.3%) and stored in glass containers for seven days. That mixture was then filtered and the solvent was evaporated using a rotary vacuum evaporator and concentrated using a water bath. The ethanol extracte was vaporated fully.

The extract was fractionated by vacuum filtration using solvents of increasing polarity to prepare the fractions. Thus, 10.9 g of ethanol extract from *P. viridis* leaves was used for this purpose generating the following yields: hexane fraction: 0.42 g; dichloromethane fraction: 1.03 g; ethyl acetate fraction: 1.25 g and methanol fraction: 7.01. The hexane fraction was discarded because it failed to show sufficient yield to perform the bioassays. The dichloromethane (DCMF), ethyl acetate (EAF), and methanol (MF) fractions were used in the bioassays.

The (CEE) and fractions were dissolved in 66% ethanol in a ratio of 1:1, where 100 mg of the crude ethanol extract and 100 ml of ethanol were obtained, thus obtaining the stock solution of 100%, while the concentrations of 6.25, 12.5, 25, 50% were obtained by dilution (Mazzafera, 2003).

2.3 Bioassays

The aqueous extract bioassays consisted of four treatments at the concentrations of 100, 75, 50 and 25% and one control group at 0% (distilled water). The ethanol extract and the dichloromethane, ethyl acetate and methanol fractions were at concentrations of 100, 50, 25, 12.5 and 6.25%. Each treatment consisted of five replicates with 20 *L. sativa* seeds. The experimental design used was completely randomized (CRD).

According to Souza, Cattelan, Vargas, Piana, Bobrowski, and Rocha, (2005), the main advantage of using lettuce as the target of allelopathic studies lies in the sensitivity of the seeds of the species, therefore, even in low concentrations of allelochemicals its process of Germination can be compromised. In addition, germination is rapid in approximately 24 h, has linear growth, is insensitive to pH differencesIn wide range of variation and the osmotic potentials of the solutions (RICE, 1984).

The experiments were conducted in Petri dishes with two filter paper disks moistened with 3 ml of the extract and fractions in different extract concentrations. Conversely, the control was moistened in 3 ml of distilled water. The experiments were conducted in a biological oxygen demand (BOD) seed germination chamber at a temperature of 25 °C and photoperiod of 12 hours for seven days. The plates were left open for 48 hours to evaporate the alcohol completely for the bioassays with ethanol extract and fractions (Mazzafera, 2003).

The pH of the extracts and fractions at different concentrations was analyzed using a pH meter. The osmolarity of the crude aqueous extract at different concentrations was also analyzed using an osmometer. Solutions with pH higher than 6.0, which is the range considered optimal by Macias, Gallindo, and Molinillo (2000), were adjusted using 0.1 N KOH and 5% HCl solutions.

2.4 Parameters Assessed

The following parameters were assessed: germination percentage (GP), germination speed index (GSI; assessed every 24 hours) and hypocotyl and radicle length (evaluated after seven days of sowing). Five seedlings were used per replicate to measure the length of *L. sativa* hypocotyls and radicles, totaling 25 seedlings per treatment.

2.5 Statistical Analysis

The statistical analysis consisted of analysis of variance and regression analysis using the software ASSISTAT version 7.7 beta. The plots were disregarded for the regression equations with $R^2 \leq 0.7$. Data were transformed into ($X = 1/X$) or ($X = 1/\sqrt{X}$) where necessary.

2.6 Chemical Auantification by High-Performance Liquid Chromatography (HPLC)

The analyses were performed under gradient conditions, at were phenomenex C18 columns (4.6 mm × 250 mm) packed with particles 5 µm in diameter were used for the reverse phase chromatographic analyses. for the extract aqueous, the mobile phase was water containing 2% formic acid (A) and methanol (B), and the gradient composition was: 17% B to 10 min 40 with change; 20, 30, 50, 70 and 10% B at 20, 30, 40, 50 and 60 min, respectively, following the method described by Klimaczewski et al. (2014). For the extract etanolic, the mobile phase water containing 2% of acetic acid (A) and methanol (B) was used, and the composition of the gradient was: 5% of (B) to 2 min with change; 25% (B) to 10 min; 40, 50, 60, 70 and 80% (B) every 10 minutes; Following the method described by Barbosa Filho et al. (2014). And for de fractions elution binary eluent gradient A (0.05% trifluoroacetic acid in water) and eluent B (100% acetonitrile) was used for the detection of the compounds. The elution schedule was set as follows: 5% B between 0-5 minutes, 12% B between 5-50 minutes, 30% B between 50-51 minutes, 90% B between 51-56 minutes, and 5% B between 56-70 minutes (COLPO et al., 2014).

3. Results and Discussion

3.1 GP and GSI

The crude aqueous extract of *P. viridis* leaves stimulated the germination of *L. sativa* seeds at concentrations of 25% and 50% and inhibited it at 75% and 100% compared to the control (Figure 1). Conversely, the ethyl acetate fraction stimulated germination at 6.25% but caused inhibition at 50 and 100% (Figure 2). Such findings may result from the joint or isolated action of allelochemicals present in the extract and fraction. Reigosa, Sánchez-Moreiras, and González (1999) reported that the effects of allelochemicals on different plant physiological processes most likely depend on the concentration and usually act by promoting activation at low concentrations and inhibition at high concentrations.

Seeds subjected to the methanol fraction at 6.25, 12.5 or 50% concentration experienced inhibition of their germination. This result has been observed in other Rubiaceae species. Frescura (2012) tested the allelopathic effects of *Psychotria brachypoda* (Müll. Arg.) Briton and *Psychotria birotula* Smith (Rubiaceae) on *Eruca sativa* Mill. noting that the *P. brachypoda* extract significantly inhibited the germination rate and GSI of *E. sativa* at a concentration of 20 mg/L, corroborating the results found in the present study.

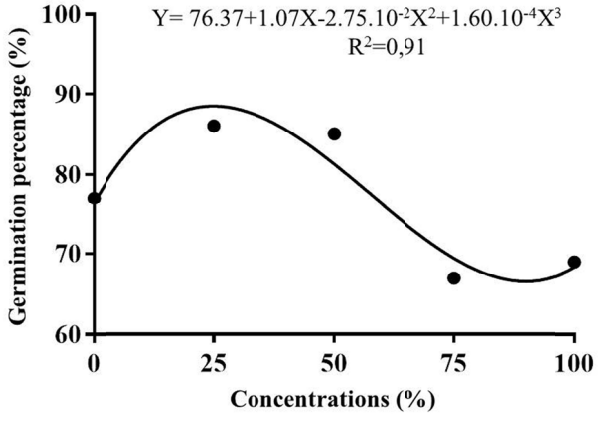

Figure 1. Germination Percentage (GP) of *L. sativa* (lettuce) subjected to different concentrations of crude aqueous extract (CAE) of *P. viridis* leaves

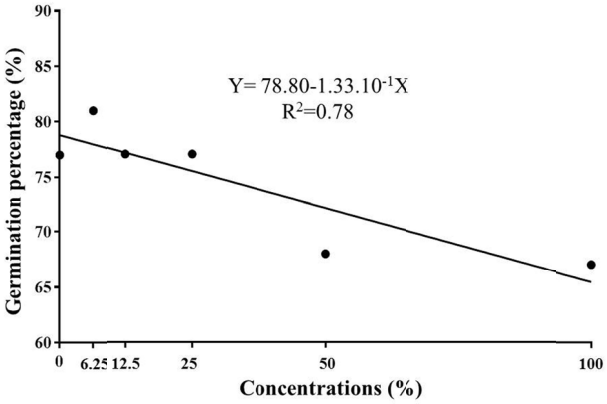

Figure 2. Germination percentage (GP) of *L. sativa* subjected to different concentrations of the ethyl acetate fraction from *P. viridis* leaves

The germination speed index (GSI) was reduced by the CAE and by the dichloromethane fraction (DCMF), decreasing with increasing extract concentrations (Figures 3 and 4).

Similar results were found by Pires et al. (2010), wherein the germination rate and the GSI of *Calopogonium mucunoides* Desv., *Stylosanthes capitata* Vogel and *L. sativa* were negatively affected by a high concentration of aqueous extract of dried *Coffea arabica* L. (Rubiaceae) leaves.

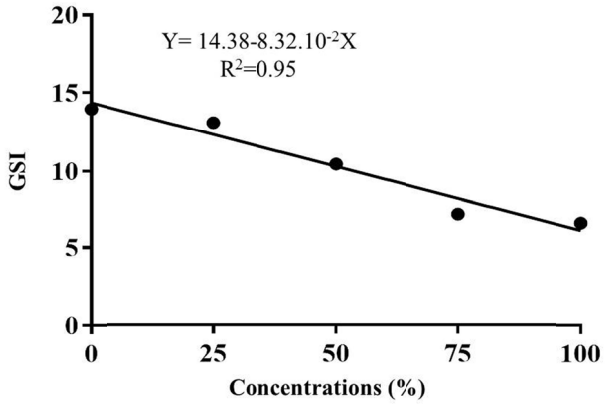

Figure 3. Germination speed index of *L. sativa* (lettuce) seeds subjected to different concentrations of crude aqueous extract of *P. viridis*

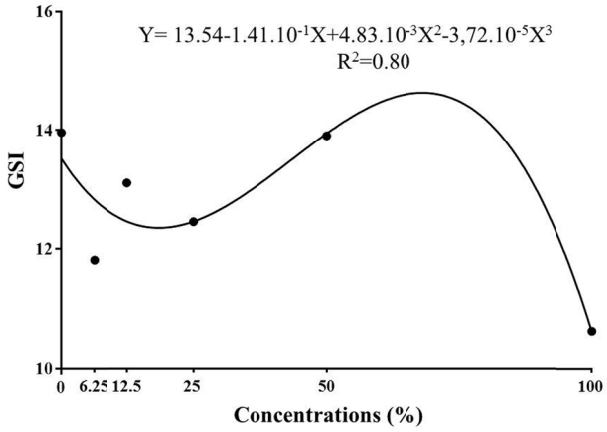

Figure 4. Germination speed index (GSI) of *L. sativa* seeds subjected to different concentrations of the dichloromethane fraction from *P. viridis*

The changes in the germination pattern, according to Ferreira (2004), can be resulting from the action of secondary metabolites on membrane permeability, transcription and translation of DNA, respiration, sequestration of oxygen (phenols), enzyme and receptor conformation, or the combination of these factors. Souza Filho (1997) points out that the same substance can perform several functions, depending on its concentration and form of translocation.

3.2 Hypocotyl and Radicle Biometrics

The hypocotyl growth of *L. sativa* seedlings was stimulated by the crude aqueous extract at 25% and 50% concentration and reduced at 75% and 100% (Figure 5). The crude ethanol extract also caused increased hypocotyl growth at 6.25, 50 and 100% concentration, but it caused decreased growth at 12.5 and 25% (Figure 6).

Seedling growth stimulation is often reported in studies related to allelopathy, and this process may be related to the effects of extracts on the phyto-hormone production of the target species or increased sensitivity of its tissues (Rice, 1984). The greater seedling growth at lower concentrations of extract may be a mechanism of protection according to Hong, Xuan, Eiji, and Khanh, (2004). Conversely, that may be related to the fact that a given chemical compound has an inhibitory or stimulating effect depending on its concentration in the environment according to Goldfarb, Pimentel, and Pimentel (2009).

Treatment with the dichloromethane fraction or methanol fraction inhibited *L. sativa* hypocotyl growth at all concentrations tested (Figures 7 and 8). Maraschin-Silva and Aqüila (2006) tested the allelopathic potential of five Brazilian native species. However, *Psychotria leiocarpa* Cham. & Schltdl. was the only species able to reduce the hypocotyl length parameter of lettuce seedlings, also inhibiting radicle length and causing a frail and brittle appearance.

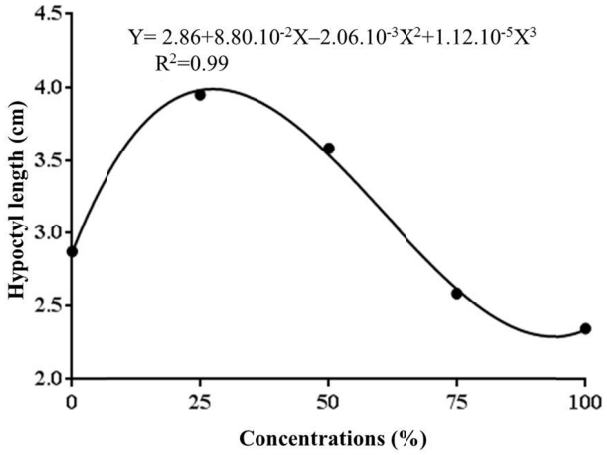

Figure 5. Mean length of *L. sativa* (lettuce) hypocotyls under the effect of different concentrations of crude aqueous extract of *P. viridis*

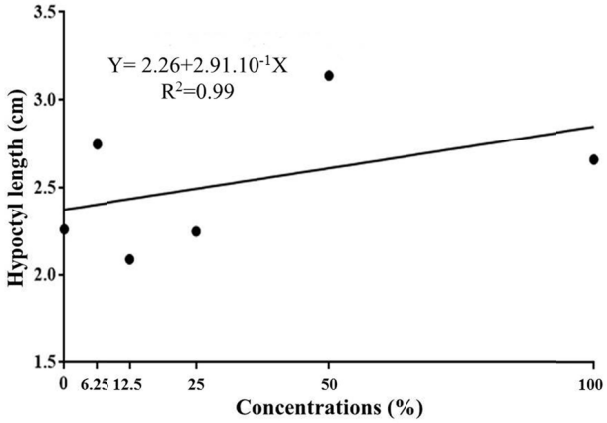

Figure 6. Hypocotyl length of *L. sativa* seedlings subjected to different concentrations of crude ethanol extract (CEE) of *P. viridis*

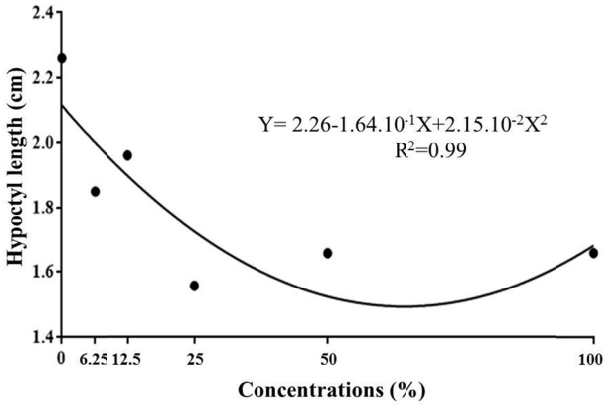

Figure 7. Hypocotyl length of *L. sativa* seedlings subjected to different concentrations of the dichloromethane fraction from *P. viridis*

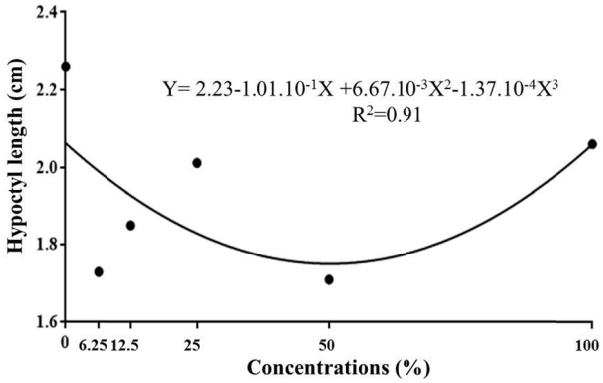

Figure 8. Hypocotyl length of *L. sativa* seedlings subjected to different concentrations of the methanol fraction from *P. viridis*

The radicle length of *L. sativa* seedlings was inhibited by all treatments from the lowest concentration tested (Figures, 9, 10, and 11). In general, roots are more sensitive to substances present in extracts and fractions than other seedling structures (Chon, Coutts, & Nelson, 2000). That results from the fact that roots are in direct and prolonged contact with allelochemicals compared to the other seedling structures (Chung, Ahn, & Yun, 2001) and/or from physiological differences between the structures (Aquila, Ungaretti, & Michelin, 1999).

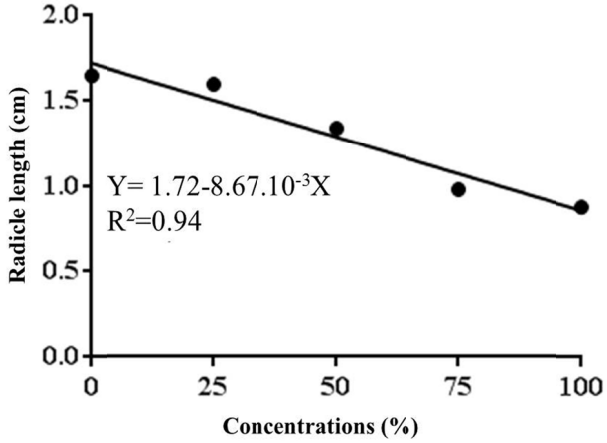

Figure 9. Mean length of *L. sativa* (lettuce) radicles under the effect of different concentrations of crude aqueous extract of *P. viridis*

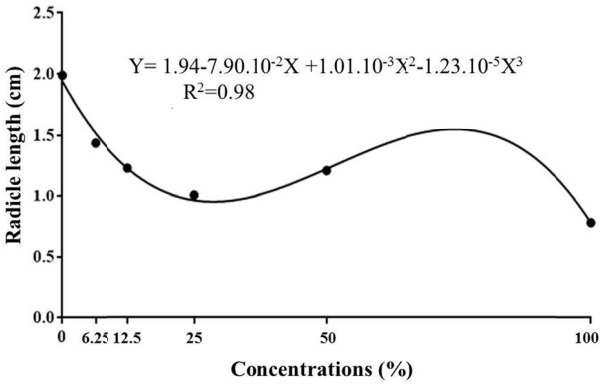

Figure 10. Radicle length of *L. sativa* seedlings subjected to different concentrations of crude ethanol extract (CEE) of *P. viridis*

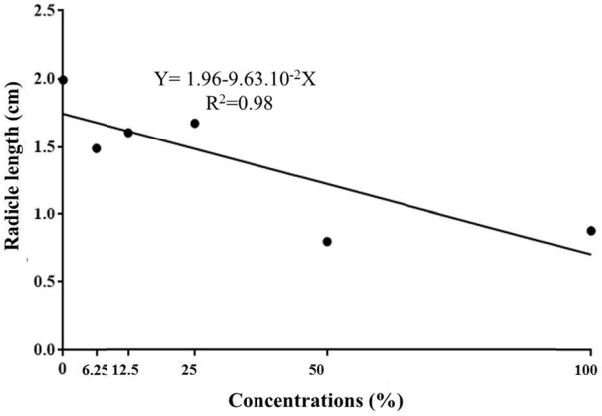

Figure 11. Radicle length of *L. sativa* seedlings subjected to different concentrations of ethyl acetate fraction of *P. viridis*

3.3 Osmolarity and pH

The pH values of Aqueous and Ethanol Extracts and Fractions were above the optimal range for seed germination and seedling growth as shown in Tables 1 and 2, which were adjusted to 6.0 or near that value.

Table 1. Physico-chemical characteristics of the crude aqueous extract of *P. viridis* leaves

Concentration	Normal pH	Adjusted pH	Osmolarity
25%	4.67	6.13	-0.063 MPa
50%	4.74	6.05	-0.093 MPa
75%	4.73	6.17	-0.143 MPa
100%	4.72	6.23	-0.181 MPa

Table 2. pH values according to the concentration of the ethanol extract and fresh leaf fractions of *P. viridis*

Treatment	Concentration (%)	Normal pH	Adjusted pH
Ethanol Fraction	6.25%	5.6	6.3
	12.5%	6.0	6.0
	25%	6.4	6.4
	50%	5.2	6.5
	100%	5.1	6.6
Dichloromethane Fraction	6.25%	6.1	6.1
	12.5%%	5.8	6.1
	25%	5.9	6.1
	50%	5.7	6.0
	100%	5.4	6.3
Ethyl Acetate Fraction	6.25%	5.5	6.0
	12.5%	5.8	6.0
	25%	7.0	6.7
	50%	6.2	6.2
	100%	5.4	6.7
Methanol Fraction	6.25%	4.9	6.4
	12.5%	4.9	6.3
	25%	5.0	6.6
	50%	5.3	6.3
	100%	4.5	6.3

Macias et al. (2000) recommend adjusting the pH of aqueous extracts to 6.0 because this is the optimal pH range for seed germination and observation of allelopathic effects. Both seedling germination and growth are affected when the pH is extremely alkaline or extremely acid (Roy, 1986), with deleterious effects observed under pH conditions below 4 and above 10 (Eberlein, 1987). An extract may contain solutes, including sugars, amino acids and organic acids, that may mask the allelopathic effect of the extract because they affect the pH according to Ferreira and Áquila (2000).

The osmotic potential of the aqueous extract was -0.06, -0.09, -0.14 and -0.18 MPa at concentrations of 25, 50, 75 and 100%, respectively. Such parameters constitute acceptable standards for seedling germination and growth in tests with potential allelopathics. Research studies, including those conducted by Mano (2006) and Gatti, Perez & Lima, (2004), have shown that those values are acceptable for allelopathic tests with seed germination.

3.4 Compound Quantification by HPLC

The presence of gallic acid (tR = 11.85 min; peak 1), catechin (tR = 16.27 min; peak 2), chlorogenic acid (tR = 20.63 min; peak 3), caffeic acid (tR = 23.81 min; peak 4), rutin (tR = 32.19 min; peak 5) and quercetin (tR = 42.05 min; peak 6; Figure 12) was detected in the aqueous extract of *P. viridis*.

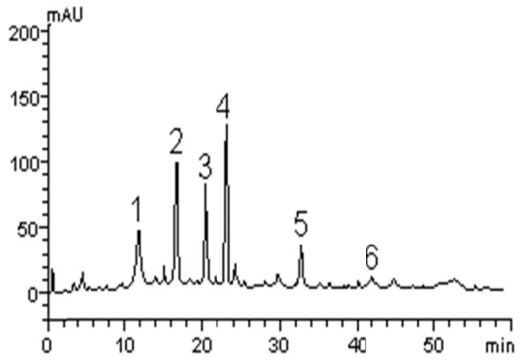

Figure 12. Phenolic and flavonoid compounds present in the aqueous extract of *P. viridis*

Note. Gallic acid (peak 1), catechin (peak 2), chlorogenic acid (peak 3), caffeic acid (peak 4), rutin (peak 5) quercetin (peak 6).

The presence of gallic acid (tR-10.67 min, peak 1), chlorogenic acid (tR = 20.07 min, peak 2), caffeic acid (tR = 24.91 min, peak 3), orientin (tR = 27.86 min, peak 4), vitexin (tR = 43.27 min, peak 5), quercetin (tR = 49.11 min, peak 6) and apigenin (tR = 62.73 min, peak 7; Figure 13) was detected in the crude ethanol extract.

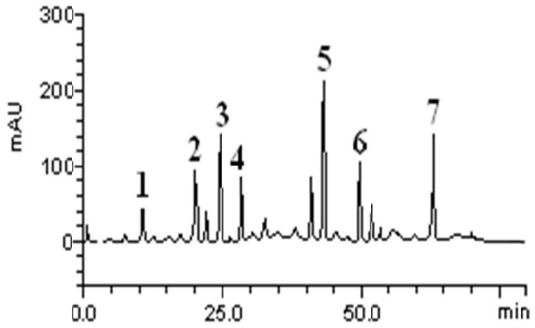

Figure 13. Phenolic and flavonoid compounds detected in the ethanol extract of *P. viridis*

Note. Gallic acid (peak 1), chlorogenic acid (peak 2), caffeic acid (peak 3), orientin (peak 4), vitexin (peak 5), quercetin (peak 6) and apigenin (peak 7).

Moreover, the presence of gallic acid (retention time (tR) = 10.73 min, peak 1), catechin (tR = 17.04 min, peak 2), chlorogenic acid (tR = 22.19 min, peak 3), caffeic acid (tR = 26.53 min, peak 4), ellagic acid (tR = 29.45 min, peak 5), rutin (tR = 38.91 min, peak 6), quercetin (tR = 49.11 min, peak 7), luteolin (TR = 54.30 min, peak 8) and apigenin (tR = 59/78 min, peak 9) was detected in the methanol, ethyl acetate and dichloromethane fractions (Figures 14).

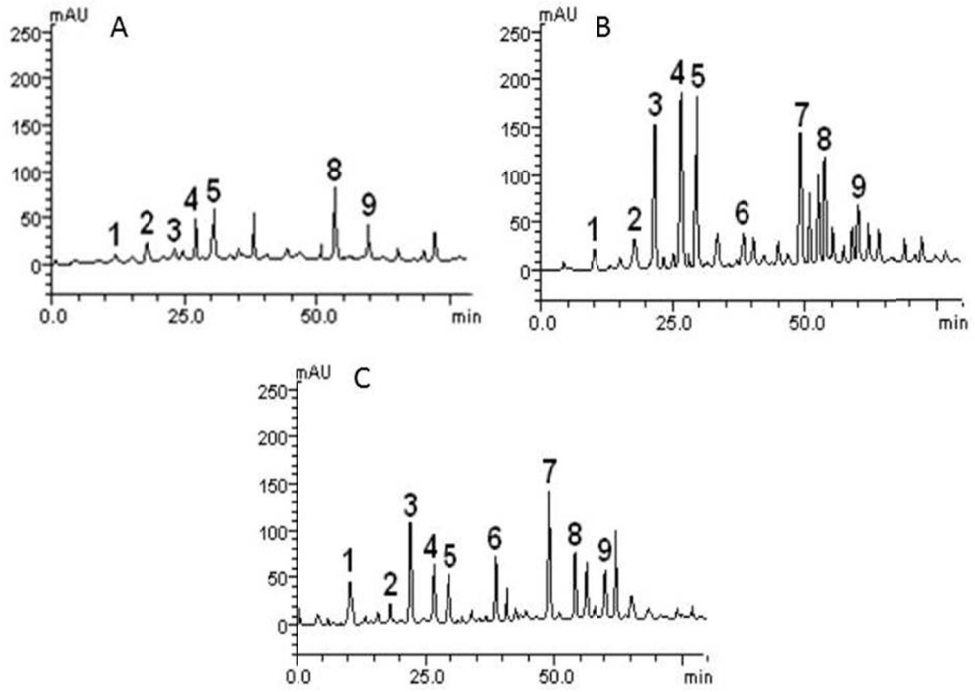

Figure 14. Phenolic and flavonoid compounds detected in the following fractions: A: dichloromethane, B: ethyl acetate and C: methanol

Note. Gallic acid (peak 1), catechin (peak 2), chlorogenic acid (peak 3), caffeic acid (peak 4), ellagic acid (peak 5), rutin (peak 6), quercetin (peak 7), luteolin (peak 8) and apigenin (peak 9).

Several functions have been attributed to flavonoids, including plant protection against incident ultraviolet and visible rays; protection against insects, fungi, viruses and bacteria; animal attraction for pollination; antioxidant action; plant hormone action control; allelopathic action and enzyme inhibition (Simões, Schenkel, Gosmann, Mello, & Mentzz, 2010).

The involvement of phenols including hydroquinone, ellagic acid and gallic acid esters in plant defenses and their involvement in interrelationships between animals and plants with different activities, including seed germination inhibition, fungal growth and plant growth in general has been highlighted in chemical ecology (Simões et al., 2010).

The gallic acid found in all *P. viridis* extracts and fractions is considered a special secondary metabolite, widespread in the plant kingdom, that shows various biological activities including an allelopathic effect on other plants (Woodson, Ames, Selassie, Hansch, & Weinshilboum, 1983; Souza Filho et al., 2006; Li, Wang, Ruan, Pan, & Jiang, 2010). Similarly, caffeic acid has been detected with abundant occurrence in soil, and its inhibitory effect on the germination and growth of various plants has been proven under laboratory conditions (Inderjit, 1995).

This was the first chemical quantification of phenolic and flavonoid compounds in *P. viridis* because most studies performed using that species have investigated the occurrence of the alkaloid N, N-dimethyltryptamine in its composition. Given that the focus of the present study was to examine the allelopathic performance of *P. viridis,* these findings are of great value to knowledge regarding the species' ecology and its allelopathic potential, and they may support future research in pursuit of a bio-herbicide as a biological alternative with specific action that is less harmful to the environment.

4. Conclusions

The findings indicate that *P. viridis* has an allelopathic effect on seed germination and seedling growth of *L. sativa*, both positively and negatively, varying according to the concentration, and the phenolic and flavonoid compounds identified in extracts from the species under study, or even the alkaloid present in its leaves, which may act as allelochemicals together or alone, may account for these effects.

Acknowledgements

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Abbreviations

AI, alternate furrow irrigation; FI, fixed furrow irrigation; CI, conventional furrow irrigation; AN, alternate nitrogen supply; FN, fixed nitrogen supply; CN, conventional nitrogen supply; UP, under the plant; SP, south of the plant; NP, north of the plant; V_6, V_{12}, VT, R_2 and R_6 represents 6 collars, 12 collars, tasseling, filling and maturity of maize development stage, respectively.