

POLYPHENOLS IN YERBA MATE SEEDS: POTENTIAL INHIBITORS OF GERMINATION

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Abstract

Due to its economic, social, and ecological potentials, yerba mate can be a source of employment and income in the southern region of Brazil. The objectives of the present study was to (1) quantify the total polyphenols in fresh yerba mate seeds (FS), seeds dehydrated for 70 d (SD), and seeds stratified in sand for 180 d (SS); (2) evaluate the potential germination and growth inhibitory effects of water, ethanol + water (1:1), and ethanol (99%) extracts of yerba seeds (FS, SD, and SS) using lettuce seedlings; and (3) verify the presence of polyphenols in fresh seeds of yerba mate through histochemical methods. In the bioassay, extracts of yerba mate seeds were used in the germination of lettuce seeds. For the histochemical analysis, sections of yerba mate seeds were stained with specific dyes. The results revealed that yerba mate fresh seeds contain higher concentration of phenolic compounds, which decreased in dehydrated seeds, and almost no phenolic compound was observed in stratified seeds. The bioassay results showed that the polyphenols present in the extracts of yerba mate seeds resulted in phytotoxic effect on the roots of lettuce seedlings. The histochemical observations revealed the presence of "lignified rough barrier" and phenolic compounds between the endocarp and endosperm of the yerba mate seeds.

Keywords: Allelopathy, Aquifoliaceae, yerba mate seed.

Resumo

Polifenóis em sementes de erva-mate: potenciais inibidores da germinação. Devido aos seus potenciais econômicos, sociais e ecológicos, a erva-mate pode ser uma fonte de emprego e renda na região sul do Brasil. Os objetivos do presente estudo foram (1) quantificar os polifenóis totais em sementes frescas de erva-mate (FS), sementes desidratadas por 70 d (DP) e sementes estratificadas em areia por 180 d (SS); (2) avaliar os potenciais efeitos inibitórios da germinação e do crescimento de água, etanol + água (1: 1) e etanol (99%) de sementes de erva-mate (FS, SD e SS) usando mudas de alface; e (3) verificar a presença de polifenóis em sementes frescas de erva-mate por meio de métodos histoquímicos. No bioensaio, extratos de sementes de erva-mate foram utilizados na germinação de sementes de alface. Para a análise histoquímica, cortes de sementes de erva-mate foram corados com corantes específicos. Os resultados revelaram que as sementes frescas de erva-mate contêm maior concentração de compostos fenólicos, que diminuíram nas sementes desidratadas, e quase nenhum composto fenólico foi observado nas sementes estratificadas. Os resultados do bioensaio mostraram que os polifenóis presentes nos extratos das sementes de erva-mate resultaram em efeito fitotóxico nas raízes das plântulas de alface. As observações histoquímicas revelaram a presença de "barreira rugosa lignificada" e compostos fenólicos entre o endocarpo e o endosperma das sementes de erva-mate.

Palavras-chave: Alelopatia, Aquifoliaceae, semente de erva-mate.

INTRODUCTION

Ilex paraguariensis A. St. Hil., popularly known as yerba mate, belongs to the family Aquifoliaceae. It is a typical tree of the Mixed Ombrophilous Forest and native to the regions of Paraguay, Brazil, and Argentina (CARVALHO, 2006). It is consumed as a beverage (chimarrão, tererê, and mate tea) in Brazil and in countries of natural occurrence (BARZOTTO; ALVES, 2013); however, in the international market it is used for phytotherapeutic (RIL *et al.*, 2011), aesthetic, and culinary purposes (BARZOTTO; ALVES, 2013). Yerba mate is the main non-timber product of the Brazilian agribusiness, and owing to its economic, social, and ecological potentials, this crop is one of the best sources of employment and income.

Due to its diverse applications, yerba mate has aroused the interest of world markets. Its export in August 2017 increased by 1.05% compared with that during the same period in 2016 in countries such as

Germany, Chile, Uruguay, and the United States (MDIC, 2017). Due to its economic importance and demand, its cultivation has been resumed. In this milieu, the prospect of renewal of old yerba mate plantations is relevant.

The most commonly used mode to propagate yerba mate is the seedlings produced from seeds. However, its mature fruits have approximately 99% immature embryos, making its germination irregular, slow, and often low (FOWLER *et al.*, 2007). In order to promote the embryonic development and the degradation of the lignified endocarp, nurserymen use seed stratification between two layers of sand for six months (ZANON, 1988).

In this context, different chemical compounds in the seeds determine the vigor of the seedlings during germination, as some can be mobilized in the reserve tissues and used as energy fuel, whereas others can inhibit or delay seed germination (MARCOS-FILHO, 2015). Some of these inhibitors, called allelochemicals, derived by secondary plant metabolism, reflect the genotype and environment interaction. Metabolites, such as phenols, terpenes, alkaloids, tannins, coumarins, and flavonoids, might exhibit phytotoxic potential, capable of causing cellular damage. These damages occur due to the changes in the permeability and selectivity of cell membranes or modifications at the hormonal and photosynthetic levels, thus, inhibiting the germination of seeds and reducing the initial growth of seedlings (AUMONDE *et al.*, 2013).

Studies on two species of common holly (*Ilex rotunda* Thunb. and *I. latifolia* Thunb.) in China and Japan, cultivated as ornamentals, which produce seeds with difficulty in germination, reported the presence of inhibitory substances of germination in the embryo and endosperm (TEZUKA *et al.*, 2013). Thus, the low percent of germination of *I. paraguariensis* seeds can be attributed to the same factors, which probably influence the entire germination process, but have not been investigated in yerba mate.

Therefore, histochemical studies are important to elucidate the cellular composition and organization. However, there are only a few studies regarding the histochemical aspects of forest seeds and their correlation with the germination process (CORTE *et al.*, 2008). Therefore, during the detection of germination inhibitors, the use of lettuce seeds (*Lactuca sativa* L.) in bioassays has been pointed out as an efficient method, as its seeds and seedlings are efficient indicators of the phytotoxic effect owing to their sensitivity to several allelochemicals (AUMONDE *et al.*, 2013).

The objectives of this study was to (1) quantify the total polyphenols in yerba mate fresh seeds (FS), seeds dehydrated for 70 d (SD), and seeds stratified in sand for 180 d (SS); (2) evaluate the potential germination and growth inhibitory effects of the water, ethanol + water (1:1), and ethanol (99%) extracts of yerba mate seeds (FS, SD and SS) using lettuce seedlings; and (3) verify the presence of polyphenols in yerba mate fresh seeds by histochemical methods.

MATERIAL AND METHODS

The experiment was conducted between March and September 2017 at the Non-Timber Forest Technology Laboratory, Embrapa Forestry, Colombo (state of Paraná, Brazil) (25° 20' S and 49° 14' W, altitude of 950 m). Yerba mate seeds used to obtain extracts included: fresh (FS), dehydrated in a chamber with a temperature of 10 °C and 25% relative humidity for 70 d (SD), and stratified between two layers of sand for 180 d (SS). The seeds were obtained from Ivaí (state of Paraná, Brazil) (25° 1' 8.64" S and 50° 49' 13.29" W, altitude of 750 m) and Colombo (state of Paraná, Brazil) (25° 20' S and 49° 14' W, altitude of 950 m).

To quantify the phenolic compounds, 5 g of seeds crushed in a bench blender (Britannia, 600W), mixed with 25 mL of each solvent (ethanol, ethanol + water (1:1), and water at 80 ± 2 °C), and maintained in dark at temperature of 25 °C for 24 h; the extract was then filtered and added to the respective solvents in volumetric flasks.

The concentration of total polyphenols in the yerba mate seed extracts was determined by the redox reaction using Folin-Ciocalteu reagent, which reacts with the hydroxyls present in the polyphenols. The results were expressed as mg.g⁻¹ equivalent in gallic acid. To quantify the total polyphenols, the adapted Folin-Ciocalteu method was used, with the modifications of Frizon *et al.* (2015) for weed seeds. Gallic acid (Sigma) was used as a standard. The calibration curve was constructed using different aliquots of this standard solution—150, 200, 300, 400, 500, 600, 700, 800, 900, and 1000 µL ($r^2 = 0.9994$). The equation of line was used to calculate the amount of total polyphenols in the samples of yerba mate seeds.

To volumetric flasks, different aliquots of the standard solution, 500 µL of the Folin-Ciocalteu reagent, and 2 mL of 15% of sodium carbonate solution (Anidrol) were added. The samples were homogenized and maintained in dark, and after 2 h the absorbance of the solution was determined by UV-vis spectrophotometry (SHIMADZU, UV-1800) at the wavelength of 760 nm. Thus, to determine the absorbance of the extracts of yerba mate seeds, the same procedure was followed, replacing the standard solution of gallic acid with 100 µL of the respective extracts (ethanolic, ethanol + water (1:1), and aqueous extract). Absorbance of the solution was read in triplicates.

For the bioassay, Grand Rapids garden lettuce seeds purchased from the local trade were used. The extracts were prepared with fresh (FS), dehydrated (SD), and stratified seeds (SS) in solvents (ethanol, ethanol + water (1:1), and water). The effect of the three different extracts was compared with that of the distilled water, as control.

The percent of germination was calculated using Equation 1.

$$(1) \quad \%G = \frac{NG \times 100\%}{NT}$$

In which: NG: number of seeds germinated; and NT: number of seeds placed to germinate.

The germination rate was calculated using Equation 2.

$$(2) \quad GSI = \frac{G1}{N1} + \frac{G2}{N2} + \frac{G3}{N3} + \dots + \frac{Gn}{Nn}$$

In which: GSI: germination speed index; G1: number of seeds germinated, and N1: number of days after sowing.

The germination test was conducted in dark using gerbox plastic boxes with four replicates of 50 seeds, placed on filter paper, and moistened with different extracts, of volume equivalent to 2.5 times the weight of two sheets of paper-filter (BRASIL, 2009). The gerboxes were conditioned in a biochemical oxygen demand germination chamber, at 25 °C in dark for 7 d. The daily counts were obtained at the same time, every 12 h, from the day of emergence of radicle of length at least 2 mm (MACIAS *et al.*, 2000). On the seventh day after sowing, the seedlings were evaluated, classified as normal or abnormal, and the length (cm) of the hypocotyl and radicle was measured using a ruler.

For the histochemical analyses, we followed the method of Ventrella *et al.* (2013). One hundred yerba mate seeds previously hydrated in water for 24 h at 35 °C were manually sectioned using a blade. Subsequently, the sections were stained with safranin and astra blue, for double staining of cellulosic wall (blue) and lignified or suberified wall (red); and ferric chloride solution (III), hydrochloric vanillin, and phloroglucinol acid to stain phenolic compounds (brown). The stained sections were then mounted on semi-permanent slides and the material was observed under a microscope (Carl Zeiss AxioLab. A1) and photographed with a digital camera (Sony Cyber Shot Lens G). The experimental design adopted for all the tests was completely randomized. The experiment regarding the quantification of total polyphenols was conducted in a 3 x 2 x 2 factorial scheme and the bioassay in a 3 x 4 x 2 factorial scheme. The treatment variances were evaluated for homogeneity by Bartlett's test and the variables that presented significant differences by F-test, with the means compared by Tukey's test at a level of 5% of probability.

RESULTS

The equation of line $y = 1.1009x - 0.135$ and the value of $R^2 = 0.9994$ were obtained using the data obtained from the calibration curve. From the equation of line and the absorbance values of each extract, the content of total polyphenols present in the yerba mate seeds was determined. The ethanolic extracts had the highest content of total polyphenols, ranging from 0.2165 (FS sample) to 0.0281 mg.g⁻¹ (SS sample) in the seeds from Ivaí and from 0.2084 (FS sample) to 0.0232 mg.g⁻¹ (SS sample) in the seeds from Colombo. Whereas, the ethanol + water (1:1) and aqueous extracts had the lowest content of total polyphenols.

The results of the analysis of variance revealed that there was a significant difference among the extracts investigated in terms of total polyphenol content. The ethanolic extract of all samples, except SD sample from Colombo, presented the highest content of total polyphenols, and the lowest content of 0.0549 mg.g⁻¹ was obtained in the aqueous extract. The SS samples had the lowest concentration of total polyphenols in all the extracts compared with that of the other samples (FS and SD). The lowest concentration of polyphenols was observed in the aqueous extract, with only 0.0136 mg.g⁻¹.

The histochemical analyses revealed the presence of rough barriers between the endocarp and the integument in the yerba mate seeds (Figure 1A), and staining showed that the barriers are lignified.

The highlighted region in Figure 1B, between the endocarp and endosperm, more specifically along the integument, presented a brown coloration due to staining with corresponding specific dyes; a large amount of phenolic compounds in the region was adjacent to the endocarp.

Table 1. Content of total polyphenol (mg.g⁻¹) in the extracts of fresh yerba mate seeds (FS), seeds dehydrated for 70 d (SD) and seeds stratified for 180 d (SS).

Tabela 1. Teores de polifenóis totais (mg.g⁻¹), referentes a três diferentes extratos elaborados com sementes de erva-mate frescas (SF), sementes desidratadas durante 70 dias (SD) e sementes estratificadas por 180 dias (SE), oriundas de Ivaí e Colombo (PR).

Samples	Treatment Origin	Ethanolic extract	Ethanol + water (1:1)	Aqueous extract	CV%
FS	Colombo	0.2084 A a	0.1870 B a	0.1883 B a	12.2
	Ivaí	0.2165 A a	0.1488 C b	0.1520 B b	19.2
	CV%	17.4	13.6	12.4	-
SD	Colombo	0.0472 A a	0.0374 B b	0.0549 A a	16.3
	Ivaí	0.0647 A a	0.0560 B a	0.0626 A a	15.6
	CV%	12.3	18.5	18.8	-
SS	Colombo	0.0232 A a	0.0175 B b	0.0146 C b	14.1
	Ivaí	0.0281 A a	0.0195 B b	0.0136 C c	11.5
	CV%	13.3	17.2	16.3	-

CV%: Coefficient of variation. Mean followed by the same uppercase (row) and lowercase (column) letters did not differ statistically by Tukey's test at the 5% of probability level.

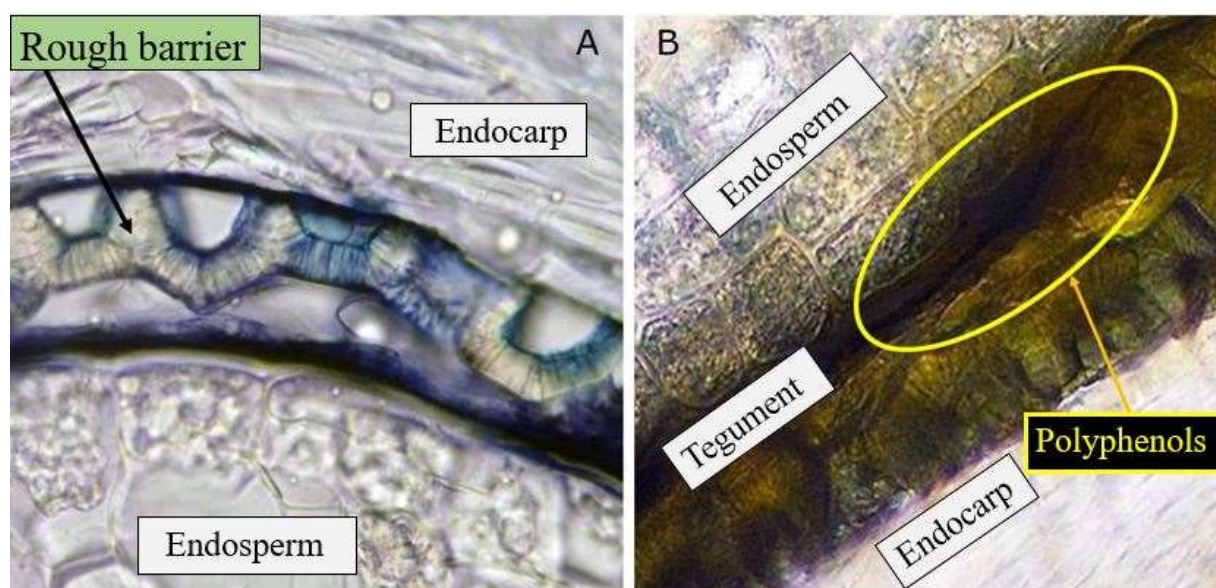


Figure 1. Cross-section of yerba mate seeds, indicating: (A) lignified rough barrier; (B) presence of polyphenols. Photo: Silva (2017).

Figura 1. Corte histológico transversal em sementes de erva-mate, indicando: (A) barreira rugosa lignificada; (B) presença de polifenóis. Foto: Silva (2017).

For the bioassay, a pre-test was carried out with 100% crude extract, obtained from 5 g of seeds (FS, SD, and SS) in 25 mL of solvent (ethanol, ethanol + water (1:1), applied in lettuce seeds. From the results of this test, we decided to dilute the three extracts, each with its solvent (ethanol, ethanol + water (1:1), and water) to 50% concentration and use them in the bioassay with lettuce seeds.

The results of the GSI test (Table 2) indicated that there was no difference between the extract types (ethanol, ethanol + water (1:1), and aqueous extract) in the germination speed of lettuce seeds. The ethanolic extract, compared with that of the other two extracts (extract ethanol + water (1:1) and aqueous extract), presented the lowest GSI. The ethanolic extract of different samples (FS, SD, and SS) and seeds from different sources (Ivaí and Colombo) did not differ statistically, i.e., their germination rate varied between 6.28 and 6.80. The same was observed for the extracts ethanol + water (1:1) and aqueous extract, when compared with each other, and different samples and sources.

Table 2. Germination speed index (GSI) of lettuce seeds treated with extracts of fresh yerba mate seeds (FS), seeds dehydrated for 70 d (SD), and seeds stratified for 180 d (SS), with distilled water as control. The yerba mate seeds were collected from Ivaí and Colombo (state of Paraná, Brazil).

Tabela 2. Índice de velocidade de germinação (IVG) das sementes de alface submetidas a três diferentes extratos, elaborados com sementes de erva-mate frescas (SF), sementes desidratadas durante 70 dias (SD) e sementes estratificadas por 180 dias (SE), provenientes de Ivaí e Colombo (PR), com água destilada como controle.

Origin	Treatment	Ethanol extract (*ns)	Ethanol + water (1:1) (*ns)	Aqueous extract (*ns)	Control
	Sample				
Ivaí	FS	6.28	7.17	8.50	13.32 A a
	SD	6.52	7.40	8.66	13.56 A a
	SS	6.72	7.74	8.78	13.92 A a
Colombo	FS	6.50	7.11	8.02	13.30 A a
	SD	6.67	7.36	8.11	13.64 A a
	SS	6.80	7.90	8.54	13.88 A a

ns: not significant by the Tukey's test at 5% probability level. Mean followed by the same uppercase (row) and lowercase (column) letters did not differ statistically by Tukey's test at the 5% probability level.

With respect to the percent of germination of lettuce seeds (Table 3), a significant difference was observed between the samples and between the treatments. In general, the ethanolic extract significantly inhibited the germination of lettuce seeds compared with that of the control. This inhibitory effect was low when treated with other samples (SD and SS) from both the sources. Furthermore, the aqueous extract of the SS sample (Colombo) exhibited the least inhibitory effect on the germination of lettuce seeds.

Table 3. Percent of germination (%G) of lettuce seeds treated with extracts of yerba mate seeds (FS), seeds dehydrated for 70 d (SD), and seeds stratified for 180 d (SS), with water as control (C). The yerba mate seeds were collected from Ivaí and Colombo (state of Paraná, Brazil).

Tabela 3. Porcentagem de germinação (%G) das sementes de alface submetidas a três diferentes extratos, elaborados a partir de sementes de erva-mate frescas (SF), sementes desidratadas durante 70 dias (SD) e sementes estratificadas por 180 dias (SE), provenientes de Ivaí e Colombo (PR), com água como controle (C).

Origin	Treatment	Ethanol extract	Ethanol + water (1:1)	Aqueous extract	Control
	Sample				
Ivaí	FS	22.0 B b	14.0 C c	40.0 A b	82.0 A a
	SD	26.0 B b	28.0 B b	42.0 A b	84.0 A a
	SS	34.0 C a	34.0 B a	50.0 B a	86.0 A a
Colombo	FS	24.0 B b	20.0 B b	44.0 A c	83.0 A a
	SD	32.0 B a	22.0 C a	54.0 A b	85.0 A a
	SS	34.0 B a	24.0 C a	56.0 A a	88.0 A a

Mean followed by the same uppercase (row) and lowercase (column) letters did not differ statistically by Tukey's test at the 5% of probability level.

The evaluation of the phytotoxic effect of the different extracts on lettuce seedlings (Table 4) showed that in relation to the hypocotyl, there was no significant difference between the treatments and between the samples.

The data regarding the length of the radicle revealed that there was no significant difference between the extract treatments. However, the average root length of lettuce seedlings treated with all the FS extract (ethanol, ethanol + water (1:1), and aqueous extract) was 0.8 cm, which was significantly different from that of the control treatment (3.1 cm).

Among FS, SD, and SS samples, there was a significant difference in the root length of lettuce seedlings in all the treatments. The percent of abnormal seedlings was 88% in the treatment with the ethanolic extract of FS samples from Ivaí and 2% in the control treatment with extracts of SS samples from the same source.

Table 4. Phytotoxic effect of extracts of fresh yerba mate seeds (FS), seeds dehydrated for 70 d (SD), and seeds stratified for 180 d (SS), with distilled water as control (C), was determined based on the length (cm) of hypocotyl (H) and radicle (R) of lettuce seedlings, and percent (%) of abnormal seedlings (AS).

Tabela 4. Avaliação do efeito fitotóxico de três diferentes extratos, elaborados com sementes de erva-mate frescas (SF), sementes desidratadas durante 70 dias (SD) e sementes estratificadas por 180 dias (SE), provenientes de Ivaí e Colombo (PR), com água como controle (C), para as variáveis comprimento (cm) do hipocótilo (H), radícula (R) das plântulas de alface e percentual (%) de plântulas anormais (PA).

Origin	Treatment	Ethanol extract			Ethanol + water (1:1)			Aqueous extract			Control		
		H (ns)	R	AS ^(ns)	H (ns)	R	AS ^(ns)	H (ns)	R	AS ^(ns)	H (ns)	R	AS ^(ns)
Ivaí	Sample												
	FS	3.5	0.8 B c	88.0	3.5	0.8 B c	82.0	3.6	0.8 B c	79.0	3.3	3.1 A a	6.0

	SD	2.7	1.4 B b	80.0	2.7	1.5 B b	79.0	2.8	1.5 B b	74.0	3.3	3.2 A a	3.0
	SS	3.2	2.5 B a	78.0	3.3	2.6 B a	75.0	3.3	2.7 B a	70.0	3.4	3.3 A a	2.0
	FS	3.3	0.8 B c	87.0	3.4	0.8 B c	81.0	3.4	0.8 B c	77.0	3.6	3.9 A a	5.0
Colombo	SD	2.3	1.5 B b	79.0	2.3	1.5 B b	78.0	2.4	1.5 B b	75.0	3.6	3.9 A a	4.0
	SS	3.0	2.6 B a	77.0	3.2	2.6 B a	76.0	3.3	2.7 B a	71.0	3.6	3.9 A a	3.0

(ns): not significant. Mean followed by the same uppercase (row) and lowercase (column) letters did not differ statistically by Tukey's test at 5% probability level.

DISCUSSION

With respect to germination, Bewley and Black (1994) suggested that the contact of seeds with a wet substrate reduces the influence of inhibitors. The method used to promote physiological and metabolic changes in yerba mate seeds in order to improve germination is sand stratification, in which the seeds are arranged in a humid and aerated environment for 180 d. Therefore, it is assumed that the contact of yerba mate seeds with sand (a substrate with high porosity, causing great permeability of water) during the stratification process allows the leaching of these inhibitors.

In the present study, a comparison of the extracts of FS and SD samples revealed a reduction in the content of phenolic compounds in the extract of SD due to the dehydration conditions (exposed in open trays in a regulated environment at 10 °C and 25% relative humidity (RH) for 70 d), thus verifying that low temperatures (10 °C) contribute to the reduction in these compounds. Similarly, Vieira *et al.* (2000), while evaluating the seeds of *Oryza sativa* L. (rice), observed that under cold and dry conditions (10 °C and 50% RH), there was a reduction in the total polyphenol concentration. Similarly, in the seeds of *Sorghum vulgare* Pers (sorghum), Oliveira *et al.* (2011) observed a reduction in the content of phenolic compounds due to their oxidation during their storage in a cold chamber (10 °C and 50% RH).

In the present study, the results of the histochemical analyses revealed the presence of polyphenols, as well as a rough structure located between the endocarp and endosperm, which was a "lignified rough barrier". It is believed that this barrier, due to its composition (lignin), hinders the expansion of embryonic cells and spreading of phenolic compounds, which inhibit germination.

In the bioassay, a reduction in the germination of lettuce seeds indicated the potential inhibitory effect of the extracts of yerba mate seeds in the mobilization of nutrients to the radicle and hypocotyl. The appearance of abnormal lettuce seedlings, with atrophied and defective primary roots, and the absence of secondary root and root necrosis correlated with the action of yerba mate seed extracts, indicating the allelopathic effect of the extracts, as the roots of lettuce are sensitive to phytotoxic actions.

The results of the present study indicated that the extracts of yerba mate seeds can affect the metabolism of lettuce, mainly to the process of germination and root growth. This information will be useful to future research in order to elucidate the different components in the extracts and their role in the inhibition of germination of yerba mate seeds.

To the best of our knowledge, the present study is the first to verify the presence of polyphenols in the seeds of *I. paraguariensis*. However, further studies are needed to elucidate the different polyphenols present in yerba mate seeds. The information will help identify phenolic compounds that inhibit germination, in order to understand the underlying mechanisms and develop solutions to promote efficient germination, as only approximately 20% of yerba mate grass seeds germinate.

CONCLUSIONS

- In fresh yerba mate seeds, there was a high concentration of phenolic compounds, which decreased in dehydrated seeds, and almost no phenolic compound was observed in stratified seeds.
- The polyphenols in the extracts of yerba mate seeds resulted in phytotoxic effect on the roots of lettuce seedlings.
- The histochemical observation revealed the presence of a lignified rough barrier and phenolic compounds between the endocarp and the endosperm of yerba mate seeds.

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