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Sequential and fractional cold extraction of *Plantago major* and *Plantago tomentosa* seeds

Extração a frio sequencial e fracionada de sementes de *Plantago major* e *Plantago tomentosa*

S. M. S. Palavicini; R. C. Souza; M. Marco; J. L. Bernardi; N. Paroul; G. T. Backes; R. L. Cansian; C. Steffens*

Postgraduate Program in Food Engineering, URI Erechim, Av. Sete de Setembro 1621, 99700-000, Erechim-RS, Brazil.

**claristeffens@yahoo.com.br*
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This study investigated the recovery of phytochemical and biological activities in extracts from *Plantago major* and *Plantago tomentosa* seeds using different solvents. Through sequential and fractional cold extraction, using solvents with increasing polarity (n-hexane, dichloromethane, and ethanol) in the recovery of total polyphenols, flavonoids, tannins, toxicity, antioxidant, and antimicrobial activity were determined. Extracts from *P. major* presented 5.9, 1.35 and, 4.2 times more total polyphenols, flavonoids, and tannins than *P. tomentosa*, respectively. The ethanolic extract from *P. major* showed the high antioxidant activity, however in *P. tomentosa* it was observed in the dichloromethane extract. All extracts had a good minimum inhibitory concentration (MIC) against *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli*, and *Salmonella choleraesuis*. The dichloromethane and ethanol extracts, of both seeds, did not show toxicity to *Artemia salina*. The sequential and fractional cold extraction using different solvents showed an excellent alternative to obtain high biological activity from *Plantago* seeds.
Keywords: polyphenols, flavonoids, tannins.

Este estudo investigou a recuperação de atividades fitoquímicas e biológicas em extratos de sementes de *Plantago major* e *Plantago tomentosa* utilizando diferentes solventes. Por meio da extração sequencial e fracionada a frio, utilizando solventes com polaridade crescente (n-hexano, diclorometano e etanol) na recuperação de polifenóis totais, flavonóides, taninos, toxicidade, atividade antioxidante e antimicrobiana. Os extratos de *P. major* apresentaram 5,9, 1,35 e 4,2 vezes mais polifenóis, flavonóides e taninos totais do que *P. tomentosa*, respectivamente. O extrato etanólico de *P. major* apresentou alta atividade antioxidante, porém na *P. tomentosa* foi observada no extrato diclorometano. Todos os extratos tiveram uma boa concentração inibitória mínima (CIM) contra *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli* e *Salmonella choleraesuis*. Os extratos diclorometano e etanólico, de ambas as sementes, não apresentaram toxicidade para *Artemia salina*. A extração sequencial e fracionada a frio com diferentes solventes mostrou-se uma excelente alternativa para obtenção de alta atividade biológica de sementes de *Plantago*.
Palavras-chave: polifenóis, flavonoides, taninos.

1. INTRODUCTION

The use of plants and seeds has been a widespread practice in folk medicine, both in urban and rural areas, as an alternative or complementary treatment of conventional medicine [1, 2]. The genus *Plantago* (Plantaginaceae) [3], popularly known as tansagem or "Barhang" in Traditional Persian Medicine, is widely distributed worldwide, including 275 species [1]. *Plantago major* L. and *Plantago tomentosa* Lam. originate in Northern Europe and Central Asia and adapt well in tropical regions. *Plantago tomentosa* species is easily propagated by seeds that are small, with high roughness and resistance [4]. In Brazil, tansagem is found in several phytogeographic domains, especially in wetlands and temperate climate, from north to south of the country, including the biomes: Amazon, Caatinga, Cerrado, Atlantic Forests, and Pampa [5].

Plantago has potential as a food resource, being recognized as a non-conventional food plant [6]. *Plantago major* has high medicinal value [1] in popular treatment, as anesthetic, antiviral, anti-inflammatory, astringent, anthelmintic, analgesic, analeptic, antihistamine, antirheumatic,

antitumor, anti-ulcer, diuretic, expectorant, and hypotensive [7]. The plant is included in the list of notified drugs, in Resolution RDC No. 10 of the National Health Surveillance Agency [8]. Its biological activity can be highlighted [1] in extracts (aqueous fluids, including decocts and infusions, dry aqueous, dry and/or fluid hydroalcoholic and methanolic), such as polyphenols, flavonoids, sterols, tannins, carbohydrates, saponins, alkaloids, and triterpenes substances.

Different extraction methods can be employed to preserve the thermosensitive compounds, such as sequential extraction that use low temperatures. The industry demand cost-efficient extraction of biomolecules from plants. The sequential process selectively extracts the compounds according to the solvent employed, and affinity with the chemical properties. Multiple solvents can be used sequentially in order to limit the amount of analogous compounds in the desired yield [9, 10]. According to the solubility, extracts will be dissolved in different solvents, so can be used multiple solvents sequentially restricting the amount of analogous compounds in the each extract. In fractional extraction, it is possible to obtain a better yield, because the fraction of solvents avoids their saturation, reducing the extraction time and improving their performance.

Selectivity, solubility, cost, and safety should be considered in the selection of solvents. Based on the law of similarity and intermiscibility, solvents with polarity value near to the polarity of the solute are likely to perform better, and vice versa [11]. Among the solvents, the organic ones stand out for presenting great substrate/product solubilization, having a reduction of substrate/product inhibition and good displacement of chemical equilibrium. They also have good recovery from substrates/products and high concentrations of products in the isolate. The solubility of the compound to be extract depends on its hydrophilic or lipophilic characteristics, as well as the polarity impacts in the extraction efficiency. Among the used solvents in the extraction can highlight the n-hexane, chloroform, ethylacetate, acetone, etanol, and water are capable of extract the phenolic, flavonoid and terpene compounds due to similar polarity of the solvent [10].

In literature, there are some previous extraction studies with *P. major* seeds [12, 13]. No, scientific reports have been found about bioactive compounds of *P. tomentosa* seeds. This specie is defined as *P. tomentosa* [14], and morphologic characters can be described as peremial cicle life, thickened taproot, three seeds per pyxidium, and rugose surface of seed testa [4].

Thus, considering the potential of *Plantago* species as sources of biologically active compounds, beneficial effects on human health and the lack of information about *P. tomentosa* seed, this study aimed to analyze and compare *P. major* and *P. tomentosa* extracts obtained by sequential and fractional extraction with different solvents. In the literature not is presented until this moment studies with *P. tomentosa* extracts. Also, evaluate the total polyphenols, flavonoids and tannins content, antioxidant and antimicrobial activity, and toxicity of the extracts obtained by fractional and sequential extraction with different solvents.

2. MATERIAL AND METHODS

2.1 Samples

The seeds of two species (*P. major* and *P. tomentosa*) were from 2017/2018 crop season obtained in Jacutinga municipaly (27°43’51.03”S, 52°32’00.09”W and 27°43’53.78”S, 52°32’00.70”W, respectively), Rio Grande do Sul State, Brazil. Both seeds were deposited for identification in Padre Balduino Rambo Herbarium, Erechim/RS under registration number of exsicata 12.282 and 12.285. The seeds were harvested manually and cleaned to remove all foreign materials, dried at room temperature (25°C), and stored under refrigeration (4°C).

2.2 Centesimal composition

The centesimal composition of *P. tomentosa* and *P. major* seeds were evaluated in relation to protein, lipids, ash, moisture, fiber, carbohydrate, and ash in triplicate. The analyses were determined according to Adolfo Lutz method (IAL) [15], in the Laboratory Food Research Center. Minerals: calcium (Ca), magnesium (Mg), sodium (Na), potassium (K), zinc (Zn),

copper (Cu), chromium (Cr), iron (Fe), and manganese (Mn) were determined by flame atomic absorption spectrophotometry (Varian® model spectra 55, USA) with hollow cathode lamps. Results were expressed in mg/g dry basis [15].

2.3 Extraction

The used solvents were chosen according to their polarity: n-hexane (0.0 polarity, 69ºC boiling point, and 1.375 refractive index); dichloromethane (3.1 polarity, 41ºC boiling point, and 1.424 refractive index); and ethanol (5.2 polarity, 78ºC boiling point, and 1.361 refractive index). The extraction followed the increase in polarity of the solvents, starting with n-hexane, followed by dichloromethane and ethanol.

Both species were extracted by the sequential and fractional extraction technique. The seeds were ground in a Cuisinart mixer (Cuisinart, Brazil) and the powder passed through 20-35 mesh sieves. The particles mean diameters were approximately 0.5 mm. The solvents n-hexane (95.0%, Sigma Aldrich, Germany), dichloromethane (99.5%, Sigma Aldrich, Germany), and ethanol (99.8%, Sigma Aldrich, Germany) were used sequentially in the same proportion. Each solvent being added in 3 parts of 40 mL fractions in order to minimize its saturation. The procedure was conducted using 10 g ground seed powder and 120 mL solvent, placed inside a covered glass balloon. Solvent fractions were changed every 12 h and the solids separated by filtration. When the solids were suspended in the solvent the process was repeated. All extractions were performed at controlled temperature (4±2°C) in a refrigerator (Brastemp, Brazil), protected from light during 108 h, at least in duplicate.

2.4 Yield

The solvents were used sequentially in the same material, where 10 g of ground seeds were used in 120 mL of each solvent. The extracts obtained from three successive extractions were combinate and evaporated in a rotary evaporator (Fisatom, Brazil) under reduced pressure (650 mmHg), 70 rpm and 40°C. The yields were determined by the ratio between the mass of extracts obtained (after the extraction) and the mass of the used raw material (wet basis) [16].

2.5 Total polyphenol

Total polyphenols was determined according to the Folin-Ciocalteu method [17], with modifications, using gallic acid as a reference. The reaction medium was prepared in volumetric amber flasks. The reaction mixture was composed of 0.5 mL extract; 2.5 mL Folin-Ciocalteau reagent (1:10, v/v) and 2.0 mL sodium carbonate (4% w/v). The flasks were shaked for 2 h protected from light at 25°C. The absorbance was read at 760 nm in spectrophotometer (Pró-análise, UV-1600, Brazil). Results were expressed as milligrams of gallic acid equivalents per gram of extract (mg GAE/g) using a gallic acid standard curve (2 to 100 μg/mL, R^2=0.992).

2.6 Total flavonoids

Total flavonoid content was evaluated using quercetin [18]. 0.5 mL sample extracts (concentration of 5 mg/mL), 4.3 mL ethanol (70% v/v), 0.1 mL aluminum nitrate (10% w/v), and 0.1 mL potassium acetate (10% w/v) were inserted into a test tube. The tubes were vortex (IKA®MS3 basic, USA) at 2000 rpm and stored at 25°C in the absence of light for 40 min. Then the absorbance was read in a spectrophotometer at 415 nm. The flavonoid content was obtained by linear regression analysis from the calibration curve (10-200 μg/mL).

2.7 Total tannins

The tannin content from extracts was determined by a spectrophotometric method at 760 nm [15]. Folin-Dennis reagent was used, and the concentration was calculated using a standard curve (0.1-0.9 mg/100mL) prepared with tannic acid as reference.

2.8 Antioxidant Activity

The free radical scavenging activity was evaluated using the 1,1-diphenyl-2-picrylhydrazil (DPPH) (Sigma Aldrich, Germany) according to Miranda and Fraga (2006) [19], with modifications. The reaction consisted of 500 μL of 0.1 mM DPPH ethanolic solution with 500 μL extract containing decreasing concentrations (10-0.0025 mg/mL). The control solution was prepared with 500 μL DPPH and 500 μL ethanol. In the white solution, only the ethanol solvent (without sample and DPPH) was used. After 30 min at room temperature, protected from light, the absorbance values were measured at 515 nm. The uptake percentage of the DPPH radical was obtained in terms of the percentage of antioxidant activity (AA%). After evaluating the ideal concentration range, was calculated the inhibitory concentration (IC_{50}) of the extract needed to inhibit 50% of the DPPH radicals obtained from from the linear regression curve [20].

2.9 Antimicrobial activity - Minimum inhibitory concentrations (MIC)

MIC was performed using the indirect method of bacterial growth by optical density in liquid culture medium [21], with modifications. The evaluation of antibacterial activity against Gram-positive (*Staphylococcus aureus* - ATCC 6538 and *Listeria monocytogenes* - ATCC 7644) and Gram-negative bacteria (*Salmonella choleraesuis* - ATCC 10708 and *Escherichia coli* - ATCC 25922). The bacteria were previously grown in Luria Bertani broth (LB –10 g/L tryptone, 5 g/L yeast extract, 5 g/L sodium chloride) at 37°C for 24 h. After, 10 μL of active cultures (10^8 CFU/mL) were inoculated into microplate wells containing LB broth and different concentrations of extract. Each concentration was evaluated in triplicate and as blank and control, containing only culture medium with the microorganism, respectively. Subsequently, the microplates were incubated (Fanem®, model 320-SE, Brazil) at 37°C for 24 h. Before and after the incubation period, 0 and 24 h, respectively, was reading using the automated microplate reader (Bio-Tek Instruments Inc, USA) with pre-set wavelength at 490 nm. The MIC values were determined as the lowest concentration of extract that inhibited the bacterial growth, obtained by the difference between after the incubation period and initial time.

2.10 Toxicity

Toxicity measurement was carried out according to Meyer et al. (1982) [22], with modifications. *Artemia salina* cysts were placed in a glass botle with artificial saline solution (10 g marine salt, 1 L deionized water, and 0.7 g sodium bicarbonate) with artificial illumination, under aeration, at 30°C during 24 h incubation for the hatching.

After, the larvaes were exposed to 20 mL of each different extract concentration (10 to 1000 μg/mL) for 24 h, using test tubes, each one containing at least 40 nauplii of *A. salina*; and as control, containing only culture medium. A control experiment (white) was performed to be sure that mortality observed was due toxicity of the compounds and not from the restriction of food. Before and after the incubation (0 and 24 h) was reading the alive and dead nauplii, being considered alive those who showed any type of movement when observed near a light source for 10 s. The tests considered valid were those with the mortality equal to or less than 10% of the population. The lethal concentration capable of causing 50% mortality (LD_{50}) was determined employing a non-linear regression model between the concentrations and observed mortality.

2.11 Statistical analysis

The results of the assays (triplicate) were submitted to ANOVA followed by *Tukey* and T Student test with 95% confidence using the Statistic software 5.0.

3. RESULTS AND DISCUSSION

3.1 Centesimal composition of the seeds

Table 1 shows the centesimal composition of the *P. major* and *P. tomentosa* seeds. Statistical difference ($p<0.05$) was observed between the two species, where *P. major* had high moisture and fiber content, while the *P. tomentosa* showed higher protein, lipid, ash, and carbohydrate contents.

Table 1. *Centesimal composition of Plantago major and P. tomentosa seeds.*

Analysis		*P. major*	*P. tomentosa*
Moisture (g/100g)		$10.505^{a} \pm 0.005$	$9.465^{b} \pm 0.015$
Protein (g/100g)		$16.790^{b} \pm 0.010$	$17.435^{a} \pm 0.005$
Lipid (g/100g)		$0.100^{b} \pm 0.001$	$0.110^{a} \pm 0.001$
Ash (g/100g)		$2.960^{b} \pm 0.010$	$3.855^{a} \pm 0.005$
Fiber (g/100g)		$10.780^{a} \pm 0.005$	$10.040^{b} \pm 0.015$
Carbohydrate (g/100g)		$58.870^{b} \pm 0.005$	$59.080^{a} \pm 0.015$
Minerals	**Ca**	$0.829^{a} \pm 0.003$	$0.752^{b} \pm 0.043$
	Mg	$0.505^{a} \pm 0.026$	$0.337^{b} \pm 0.005$
	Na	$1.539^{a} \pm 0.165$	$1.424^{a} \pm 0.085$
	K	$0.819^{a} \pm 0.060$	$0.622^{b} \pm 0.019$
	Zn	$0.079^{a} \pm 0.001$	$0.060^{b} \pm 0.009$
	Cu	$0.030^{a} \pm 0.001$	$0.020^{a} \pm 0.004$
	Fe	$0.013^{a} \pm 0.001$	$0.013^{a} \pm 0.001$
	Mn	$0.031^{a} \pm 0.005$	$0.034^{a} \pm 0.001$

Values are means ± SD of three measurements. Means in the same line with different letters are significantly different ($p < 0.05$) by T *student* test.

The protein and carbohydrate contents found in the present study are higher in relation to those previously found in *P. major* seeds (15.01% protein, 1.45% lipid, 7.14% ash, 26.9% fiber and 45.87% carbohydrates) [12]. These differences in constituents may be due to differences in genotype and environmental conditions during the grain development, ripening, crop, and local of production. Nevertheless, the *Plantago* seeds evaluated have high potential for food products enrichment and for human consumption as sources of protein, mineral, and fiber.

It can be observed that the Na, Cu, Fe, and Mn mineral contents did not differ ($p>0.05$) between species. Ca, Mg, K, and Zn showed significant differences ($p<0.05$), with higher values in *P. major*. The seed mineral values are in general low than those found on the leaf, as reported by Stef et al. (2010) [23] in *P. major* leaves for K (1.295 mg/g), Ca (3.763 mg/g), Mn (0.484 mg/g), and Na (0.099 mg/g). In addition, for *Plantago coronopus* leaf Pereira et al. (2016) [24] found 14.0 mg/g Ca; 6.34 mg/g Mg; 50 mg/g Na; 0.05 mg/g Zn; 0.41 mg/g Fe; and 0.01 mg/g Mn. *Plantago* species (*P. major*, *P. lanceolata*, and *P. media*) are in general valuable sources of amino acids and minerals for human nutrition.

3.2 Yield and biological activity

The highest yield was obtained in *P. major* extract using n-hexane solvent (Table 2). Comparing the species, a higher yield was obtained for *P. major* extract in n-hexane, while for *P. tomentosa* was in dichloromethane, showing a significant difference ($p<0.05$) between samples. However, in the ethanol extracts, no significant difference ($p>0.05$) was observed between the samples. The results indicate that *P. major* seeds have a high amount of low

polarity compounds and a low amount of intermediate to high polarity compounds. On the other hand, *P. tomentosa* seeds have a high amount of compounds with low to intermediate polarity.

This difference in the yields between the extract samples may be attributable to the difference in solvent polarities which increase the solubility of phytochemical compounds [25]. In the maceration process was used the fractional solvent (solvent fractions were changed three times), that helped the mass transfer to the solvent solubilize the chemical constituents inside the cells and diffuse them in the solvent, avoiding the equilibrium set up and saturation of the solvent. The high yield obtained for n-hexane can be due to the components in the free state migrate first from the material to the solvent. Also, can be related to the complexity of each matrix.

According to Kumoro et al. (2009) [26] plant matrices contain several molecules of different functional groups, so it is difficult to predict their solubility in a specific solvent. Solubility of substances are due to chemical affinity between species in a system in relation to polarity (strongly interaction with other polar molecules). Since maceration extraction was performed under the same temperature and time conditions, differences in the yield may also occur due to the presence of non-soluble particles [27].

In the total yield of the extracted compounds from the seeds, the *P. tomentosa* presented 12.92% and *P. major* 11.02%. This yield obtained in the seeds can be considered high when compared to leaves of *P. lanceolata* in Soxhlet extraction with organic solvents, with yield of 7.97% for petroleum ether [28]. The low yields obtained in the extract and the fractions are due to the low temperature (4ºC) used in the cold maceration. This can be due the surface tension and viscosity of the solvents remain relatively high, difficult the access of soluble matrix compounds and reduce yield [29].

In the extraction process solvent used in fractions, helped the mass transfer, solubilizing the chemical constituents and separating them, avoiding the equilibrium set up and saturation. The high yield obtained in n-hexane can be due to that the components in the Free State migrate first to the solvent, and to the complexity of each matrix.

Table 2. *Yield, total polyphenols, flavonoids, tannins and antioxidant activity for the extracts obtained from Plantago major and P. tomentosa seeds.*

Solvent	*P. major*	*P. tomentosa*
	Yield (%)	
n-hexane	$7.506^{a.A} \pm 0.806$	$4.877^{a.B} \pm 1.012$
Dichloromethane	$1.730^{b.B} \pm 0.132$	$5.617^{a.A} \pm 0.602$
Ethanol	$1.789^{b.A} \pm 0.145$	$1.806^{b.A} \pm 0.604$
Total yield	**11.025 ± 0.704**	**12.923 ± 0.969**
	Total Polyphenols (mg EAG/g)	
n-hexane	$25.107^{b.A} \pm 3.390$	$15.116^{cB} \pm 0.562$
Dichloromethane	$12.794^{c.B} \pm 3.662$	$27.981^{a.A} \pm 3.883$
Ethanol	$340.085^{a.A} \pm 25.064$	$21.193^{b.B} \pm 0.021$
Total	**377.986 ± 65.966**	**64.290 ± 8.169**
	Total Flavonoids (mg EQ/g)	
n-hexane	$43.009^{c.A} \pm 7.648$	$37.866^{b.A} \pm 3.439$
Dichloromethane	$54.352^{b.B} \pm 0.418$	$87.287^{a.A} \pm 8.661$
Ethanol	$114.063^{a.A} \pm 11.075$	$31.551^{b.B} \pm 6.624$
Total	**211.423 ± 31.711**	**156.704 ± 8.046**
	Total tannins (g/100g)	
n-hexane	$0.993^{c.A} \pm 0.004$	$0.170^{c.B} \pm 0.007$
Dichloromethane	$1.871^{a.A} \pm 0.017$	$0.231^{b.B} \pm 0.020$
Ethanol	$1.726^{b.A} \pm 0.008$	$0.687^{a.B} \pm 0.002$
Total	**4.590**	**1.088**
	IC_{50} (mg/mL)*	
n-hexane	$32.965^{a.A} \pm 2.984$*	$30.189^{a.A} \pm 6.448$
Dichloromethane	$2.534^{b.A} \pm 0.519$	$0.139^{c.B} \pm 0.015$
Ethanol	$0.019^{c.B} \pm 0.001$	$0.169^{b.A} \pm 0.009$

Values are means ± SD of three measurements. The same line with different lowercase letters and same column with uppercase in the same analysis are significantly different ($p<0.05$) by T *student* test and Tukey test, respectively.

Plant matrices contain several molecules of different functional groups, it difficult to predict the solubility in a specific solvent [26]. Solubility of substances is due to chemical affinity between species in a system. Since extraction was performed under the same temperature and time conditions, differences in the yield may also occur due to the presence of non-soluble particles.

The high concentration of polyphenols in the *P. tomentosa* and *P. major* were obtained in dichloromethane, and ethanol extracts, respectively (Table 2). The lipophilicity of biological active compounds is one of the most important pharmacological features, and interactions with cell membranes play an essential role in their biological activity. It penetration through the lipid membrane depends on structure [30]. Polyphenols are generally more hydrophilic than lipophilic owing to their phenolic nature.

The results of total polyphenols determined by Folin-Ciocalteu method, obtained in ethanol extracts for *P. major* presented higher concentration (340.08 mg/g), with significant difference ($p<0.05$) of the n-hexane and dichloromethane extracts (Table 3). For *P. tomentosa* the high concentration of polyphenols (27.98 mg/g) was obtained in the dichloromethane extract, differing statistically ($p<0.05$) from the n-hexane and ethanol extracts. These high concentrations can be due phenolic substances have a high affinity for polar solvents.

Comparing the species, *P. major* showed a highest concentration of polyphenols in the ethanol and n-hexane extract, and *P. tomentosa* in the dichloromethane extract, presenting significant difference ($p<0.05$) between the samples. The lipophilicity of biological active compounds is an important pharmacological features, and interactions with cell membranes play an essential role in their biological activity. Their penetration through the lipid membrane depends on their structure, where planarity is preferred. The relative lipophilicity of polyphenols depends on the number of contained 3-hydroxyl group [30]. Polyphenols are generally more hydrophilic than lipophilic owing to their phenolic nature. Thus polyphenols, including aglycones, glycosides, and oligomers, are extracted using water, polar organic solvents such as methanol, ethanol, acetonitrile, acetone, or their mixture [31].

The amount of total polyphenols in *P. major* is 5.9 times higher in relation to *P. tomentosa*. This content found in *P. major* can be compared with a study performed by Dudonné et al. (2009) [32] in 30 different plants, where reported that aqueous extracts with highest polyphenolic content (300-400 mg/g) were the oak (*Quercus robur*), pine (*Pinus maritima*) and cinnamon (*Cinnamomum zeylanicum*), obtained by maceration using the Folin-Ciocalteu method, and considered potential sources of natural antioxidants.

In addition, the total polyphenol content in *P. major* seeds is superior to the best results found in leaves (13.05 mg/g) and seeds (7.43 mg/g) in ethanol extracts obtained by maceration method [12]. Souri et al. (2008) [33] reported that the polyphenol content obtained by maceration in *P. major* seeds were 6.73 mg/g and *P. ovata* of 2.49 mg/g, where classified in three groups: high (>3 mg/g), moderate (1-3 mg/g) and low (<1 mg/g). Polyphenols can be classified in three groups depending on content: high (>3 mg/g), moderate (1-3 mg/g) and low (<1 mg/g). According to the classifications, all results of total polyphenols can be considered high.

The total flavonoid content in ethanol extract of *P. major* had the highest concentration (Table 2). In *P. tomentosa* the highest flavonoid content (87.29 mg/g) was obtained in the dichloromethane extract. Isoflavones, flavanones, methylated flavones and flavonols as less polar flavonoids are extracted with organic compounds like chloroform, dichloromethane, diethyl ether or ethyl acetate. Flavonoid glycosides and more polar aglycones are extracted using alcohols or alcohol-water mixtures [34].

The total flavonoid content in *P. major* seeds was 1.35 times higher than in *P. tomentosa*. The high concentration of tannins (1.871 g/100g) was observed for *P. major* in dichloromethane extract, differing statistically ($p<0.05$) from n-hexane and ethanol extracts. In *P. tomentosa* the high content (0.687 g/100g) was observed in the ethanol extract, differing statistically ($p<0.05$) from the n-hexane and dichloromethane extracts (Table 2). Comparing the species, the high tannin concentration was observed in *P. major* for both solvents, statistically differing ($p<0.05$) from *P. tomentosa*. *Plantago major* had 4.2 times more total tannins than *P. tomentosa* (Table 2). Low-molecular-weight hydrolyzable tannins are soluble in different aqueous and organic solvents. The high-molecular-weight condensed and hydrolyzable tannins are insoluble, and when tannins form complexes with protein or cell wall polysaccharides, they remain insoluble.

The advantage of sequential extraction is the solvent affinity. Different polarity creating a natural selection (cleaning), leaving the desired compounds. Also, in the fractional and sequential extraction, high yield was obtained because the fractionation avoided the saturation, improving the performance.

Tansagem seed extracts presented low amount of tannins, since species known for their high tannin content have up to 20 g/100g total tannins, such as guava (*Psidium guajava* - 13 to 17 g/100g), guava pitanga (*Psidium ruum* - 20g /100g), and black acacia (*Acacia mearnsii* – 14 g/100g) with high potential for industrial use in tannin production [35].

The ethanolic extract from *P. major* showed the best antioxidant activity (Table 2). The results of both extracts can be compared with commercial antioxidants, such as ascorbic acid (IC_{50}=0.002 mg/mL) and butylated hydroxytoluene (IC_{50}=0.005 mg/mL). The IC_{50} can be divided into 3 groups depending on the content: high (IC_{50}<0.02 mg/mL), moderate (0.02 mg/mL<IC_{50}<0.075 mg/mL) and low (IC_{50}>0.075 mg/mL). Therefore, the ethanol extract from *P. major* had high antioxidant activity. The excellent antioxidant activity can be due to the use of sequential and fractional cold extraction, where n-hexane and dichloromethane solvents may have extracted compounds with high affinity of no and intermediate polarity. Thus, the sequential extraction cleans and extract only the compounds with high affinity for ethanol and has high antioxidant activity in *P. major* extract.

In *P. tomentosa* the highest antioxidant activity was obtained in the dichloromethane extract. *Plantago* species could be considered as a source of natural antioxidants. The compounds that have property against DPPH is probably the hydroxyl groups. Another chemical component responsible for sequestering activity is flavonoids. A β ring occupied with 3′,4′-dihydroxy and/or 3-OH group can attract and decrease oxygen radicals in neutralized substances such as water [12].

3.3 Antimicrobial activity

Both extracts evaluated were effective against the studied bacteria, with similar MIC values (Table 3). The dichloromethane extract from *P. major* showed better efficacy against Gram-negative bacteria, followed by n-hexane in both Gram-positive and Gram-negative bacteria. The lowest MIC was obtained in n-hexane extract from *P. tomentosa* (*L. monocytogenes*), followed by ethanol (*S. aureus* and *S. choleraesuis*). The high antibacterial activity was attributed to the high polyphenol content. In this sense, both extracts evaluated showed good potential for bacterial inhibition and can be used as a natural antibacterial agent.

Özkan et al. (2012) [36] investigates the antibacterial activity (*E. coli, B. cereus, B. subtilis, S. epidermidis, S. aureus, P. aeruginosa, K. pneumonia, S. Enteritidis,* and *P. mirabilis*) in leaves of *P. major* in the acetone and methanol extract using the macrodilution liquid method. The authors observed that the ethanol extract was effective only against *E. coli* and *B. cereus*, and acetone extract showed effect against all investigated bacterial species. Uzun et al. (2004) [37], using the Soxhlet extraction in ethanolic extract of *P. major* leaves obtained MIC values of 0.01952 mg/mL in *S. aureus* and 0.01250 mg/mL in *E. coli.* Sharifa et al. (2012) [38] tested the whole *P. major* plant macerated in methanol and ethanol for MIC in *B. subtilis, S. aureus, C. albicans, Candida tropicalis*, and *E. coli*. The results showed that methanol and ethanol extracts showed bactericidal activity against Gram positive and Gram negative bacteria at concentrations of 100-200 mg/mL. In this sense, both extracts of *P. major* and *P. tomentosa* seeds evaluated showed good potential for bacterial inhibition and can be used as a natural antibacterial agent.

Table 3. MIC values of Plantago major, and P. tomentosa extracts.

Bacteria	MIC (mg/mL)		
	n-hexane	Dichloromethane	Ethanol
P. major			
S. aureus	1.288	2.479	2.126
E. coli	1.288	1.239	2.126
L. monocytogenes	1.288	3.746	2.126
S. choleraesuis	1.288	1.239	2.126
P. tomentosa			
S. aureus	3.249	3.615	1.765
E. coli	2.436	1.807	2.354
L. monocytogenes	1.624	1.807	2.354
S. choleraesuis	2.436	1.807	1.765

3.4 Toxicity

The toxicity results showed that only n-hexane extract from *P. tomentosa* showed 100% mortality at 250 µg/mL. Concentrations 10-150 µg/mL have 19.32 to 92.09% of mortality, indicating toxicity against *A. salina* nauplii. The mortality rate observed in *P. major* at 1000 µg/mL was 73.13% (Table 4).

Table 4. *Toxicity values against Artemia salina nauplii of Plantago major and P. tomentosa extracts obtained in different solvents.*

Concentration (µg/mL)	Mortality (%)					
	n-hexane	Dichloromethane	Ethanol	n-hexane	Dichloromethane	Ethanol
	P. major			***P. tomentosa***		
0	0	0	0	n.a.	0	0
10	0.84	n.a.	n.a.	19.32	n.a.	n.a.
25	5.63	n.a.	n.a.	43.45	n.a.	n.a.
50	18.62	n.a.	n.a.	64.53	n.a.	n.a.
75	21.93	n.a.	n.a.	n.a.	n.a.	n.a.
100	30.71	1.19	0	86.90	3.18	1.91
250	34.58	0	0	100.00	n.a.	n.a.
500	44.44	0.80	0	n.a.	4.57	4.78
750	54.76	2.25	0.84	n.a.	2.59	1.99
1000	73.13	17.17	5.53	n.a.	5.92	3.18
Blank					0.50	

n.a. - Concentration not evaluated.

In dichloromethane extract, the mortality was between 1.19-17.17% and 3.18-5.92% in *P. major* and *P. tomentosa*, respectively. These results did not show toxicity, since mortality higher than 50% (LC_{50}) was obtained for *A. salina* nauplii. Ethanol extract showed the lowest mortality among the studied solvents, thus not demonstrate toxicity. In the absence of extract, no mortality was observed (white).

The toxicity results of the n-hexane extract (LC_{50}) against *A. salina* nauplii that presented mortality above 50% was 58.89 µg/mL *P. tomentosa* and 621.75 µg/mL *P. major*. Thus, the *P. tomentosa* have high toxicity with maximum mortality rate (100%) at the concentration of 200 µg/mL.

The lethal median concentration (LC_{50}) is the dose required a given substance to kill 50% of the test organism. Substances with a LC_{50} below 1000 µg/mL in *A. Salina* are considered bioactive/toxic [22]. Low LC_{50} indicates that the extract is highly lethal and the plant extract contains potent cytotoxic compounds. So, the n-hexane extract for *P. tomentosa* is considered toxic against *A. salina* nauplii.

4. CONCLUSION

Plantago major extracts obtained by sequential and fractional process showed high levels of total polyphenols, total flavonoids, tannins, and antioxidant activity. The highest amount of total polyphenols, flavonoids and antioxidant activity were observed for *P. major* in ethanolic extract. The opposite behavior was observed for tannins, with highest values for *P. major* in dichloromethane extract and for *P. tomentosa* in ethanolic extract. All extracts showed good MIC, and did not present toxicity to *A. salina* nauplii using dichloromethane and ethanol extracts. The use of sequential and fractional cold extraction with solvents of different polarities presented a great alternative for the extraction process. Phytochemicals from these seeds can be used by the food industry to substitute synthetic antioxidants.

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6. REFERÊNCIAS BIBLIOGRÁFICAS

1. Samuelsen AB. The traditional uses, chemical constituents and biological activities of *Plantago major* L. A review. J Ethnopharmacol. 2000;71(1-2):1-21. doi: 10.1016/s0378-8741(00)00212-9
2. Mello JC, Gonzalez MVD, Moraes VWR, Prieto T, Nascimento OR, Rodrigues T. Protective effect of *Plantago major* extract against t-BOOH-induced mitochondrial oxidative damage and cytotoxicity. Molecules. 2015;20(10):17747-59. doi: 10.3390/molecules201017747
3. Spring MA. Ethnopharmacologic analysis of medicinal plants used by laotian hmong refugees in minnesota. J Ethnopharmacol. 1989;26(1):65-91. doi: 10.1016/0378-8741(89)90114-1
4. Hassemer G. *Plantago tomentosa* (Plantaginaceae), not *P. virginica* naturalised in South Africa: First records of this species outside South America. South African J Bot. 2020 Jul;131:56-63. doi: 10.1016/j.sajb.2020.02.012
5. Mota JH, de Melo EP, Soares TS, Vieira MC. Phosphatic and nitrogenous fertilization effects on tansagem medicinal plant (*Plantago major* L.) growth. Cienc e Agrotecnologia. 2008;32(6):1748-53. doi: 10.1590/S1413-70542008000600011
6. Bahadori MB, Sarikurkcu C, Kocak MS, Calapoglu M, Uren MC, Ceylan O. *Plantago lanceolata* as a source of health-beneficial phytochemicals: Phenolics profile and antioxidant capacity. Food Biosci. 2020 Apr;34:100536. doi: 10.1016/j.fbio.2020.100536
7. Zubair M, Ekholm A, Nybom H, Renvert S, Widen C, Rumpunen K. Effects of *Plantago major* L. leaf extracts on oral epithelial cells in a scratch assay. J Ethnopharmacol. 2012;141(3):825-30. doi: 10.1016/j.jep.2012.03.016
8. Brasil. Agência Nacional de Vigilância Sanitária (ANVISA). Formulário de fitoterápicos da Farmacopéia Brasileira. Brasília (DF): Anvisa, 2011.
9. Azmir J, Zaidul ISM, Rahman MM, Sharif KM, Mohamed A, Sahena F, et al. Techniques for extraction of bioactive compounds from plant materials: A review. J Food Eng. 2013;117(4):426-36. doi: 10.1016/j.jfoodeng.2013.01.014
10. Altemimi A, Lakhssassi N, Baharlouei A, Watson DG, Lightfoot DA. Phytochemicals: Extraction, isolation, and identification of bioactive compounds from plant extracts. Plants. 2017;6(4):42. doi: 10.3390/plants6040042
11. Zhang QW, Lin LG, Ye WC. Techniques for extraction and isolation of natural products: A comprehensive review. Chinese Med (United Kingdom). 2018;13:1-20. doi: 0.1186/s13020-018-0177-x
12. Kobeasy M, Osama A-F, El-Salam S, El-Ola Z. Biochemical studies on *Plantago major* L. and *Cyamopsis tetragonoloba* L. Int J Biodivers Conserv. 2011;3:83-91. doi: 10.5897/IJBC.9000014
13. Mazzutti S, Riehl CAS, Ibañez E, Ferreira SRS. Green-based methods to obtain bioactive extracts from *Plantago major* and *Plantago lanceolata*. J Supercrit Fluids. 2017;119:211-20. doi: 10.1016/j.supflu.2016.09.018
14. Rahn K. *Plantago* section Virginica; a taxonomic revision of a group of American plantains, using experimental, taximetric and classical methods. Dansk Botanisk Arkiv. 1974;30:1-180.
15. Instituto Adolfo Lutz (IAL). Métodos físico-químicos para análise de alimentos. 4. ed. São Paulo: Intituto Adolfo Lutz; 2008.

16. Piva GS, Weschenfelder TA, Franceschi E, Cansian RL, Paroul N, Steffens C. Linseed (*Linum usitatissimum*) oil extraction using different solvents. Food Technol Biotechnol. 2018;56(3):366-72. doi: 10.17113/ftb.56.03.18.5318
17. Singleton, Rossi. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am J Enol Vitic. 1965;16:144-68.
18. Garrido G, Ortiz M, Pozo P. Fenoles y flavonoides totales y actividad antioxidante de extractos de hojas de *Lampaya medicinalis* F. Phil. J Pharm Pharmacogn Res. 2013;1(1):30-8.
19. Miranda A, Fraga C. Free radical scavenger activity: determination of the antioxidant profile of bioactive substances. In: Monge A, Ganellin CR, editors. Practical studies for medicinal chemistry. Genebra (CH): IUPAC. 2006.
20. Silvestri JDF, Paroul N, Czyewski E, Lerin L, Rotava I, Cansian RL, et al. Chemical composition and antioxidant and antibacterial activities of clove essential oil (*Eugenia caryophyllata* Thunb). Rev Ceres. 2010;57(5):589-94. doi: 10.1590/S0034-737X2010000500004
21. Pierozan MK, Pauletti GF, Rota L, dos Santos ACA, Lerin LA, di Luccio M, et al. Chemical characterization and antimicrobial activity of essential oils of *Salvia* L. species. Ciência e Tecnol Aliment. 2009;29(4):764-70. doi: 10.1590/S0101-20612009000400010
22. Meyer BN, Ferrigni NR, Putnam JE. Brine shrimp: A convenient general bioassay for active plant constituents. Planta Med. 1982;45(5):31-4. doi: 10.1055/s-2007-971236
23. Stef D, Gergen L, Hărmănescu M, Pop C, Drugă M, Bujancă G, et al. Determination of the Macro Elements Content of Some Medicinal Herbs. Sci Pap Anim Sci Biotechnol. 2010;43(1):122-6.
24. Pereira CG, Custódio L, Rodrigues MJ, Neng NR, Nogueira JMF, Carlier J, et al. Profiling of antioxidant potential and phytoconstituents of *Plantago coronopus*. Brazilian J Biol. 2016;77(3):632-41. doi: 10.1590/1519-6984.02416
25. Naima R, Oumam M, Hannache H, Sesbou A, Charrier B, Pizzi A, et al. Comparison of the impact of different extraction methods on polyphenols yields and tannins extracted from Moroccan *Acacia mollissima* barks. Ind Crops Prod. 2015;70:245-52. doi: 10.1016/j.indcrop.2015.03.016
26. Kumoro AC, Hasan M, Singh H. Effects of solvent properties on the Soxhlet extraction of diterpenoid lactones from Andrographis paniculata leaves. ScienceAsia. 2009;35(3):306-9. doi: 10.2306/scienceasia1513-1874.2009.35.306
27. Oliveira VB, Zuchetto M, Oliveira CF, Paula CS, Duarte AFS, Miguel MD, et al. Efeito de diferentes técnicas extrativas no rendimento, atividade antioxidante, doseamentos totais e no perfil por clae-dad de *Dicksonia sellowiana* (Presl.). Hook, dicksoniaceae. Rev Bras Plantas Med. 2016;18(1 suppl 1):230-9. doi: 10.1590/1983-084X/15_106
28. Fayera S, Neelaiah B, Dekebo A, Bogale Y. Phytochemical investigation and antimicrobial study of leaf extract of *Plantago lanceolata*. Nat Prod Chem Res. 2018;6(2):100031. doi: 10.4172/2329-6836.1000311
29. Mezzomo N, Mileo BR, Friedrich MT, Martínez J, Ferreira SRS. Supercritical fluid extraction of peach (*Prunus persica*) almond oil: Process yield and extract composition. Bioresour Technol. 2010;101(14):5622-32. doi: 10.1016/j.biortech.2010.02.020
30. Hendrich AB. Flavonoid-membrane interactions: Possible consequences for biological effects of some polyphenolic compounds. Acta Pharmacol Sin. 2006;27(1):27-40. doi: 10.1111/j.1745-7254.2006.00238.x
31. Tsao R. Chemistry and biochemistry of dietary polyphenols. Nutrients. 2010;2(12):1231-46. doi: 10.3390/nu2121231
32. Dudonné S, Vitrac X, Coutiére P, Woillez M, Mérillon JM. Comparative study of antioxidant properties and total phenolic content of 30 plant extracts of industrial interest using DPPH, ABTS, FRAP, SOD, and ORAC assays. J Agric Food Chem. 2009;57(5):1768-74. doi: 10.1021/jf803011r
33. Souri E, Amin G, Farsam H, Barazandeh Tehrani M. Screening of antioxidant activity and phenolic content of 24 medicinal plant extracts. Daru. 2008;16(2):83-7.
34. Andersen ØM, Markham KR. Flavonoids. Andersen OM, Markham KR, editors. Flavonoids: Chemistry, biochemistry and applications. Boca Raton (US): CRC Press; 2005.
35. Pansera MR, Iob GA, Atti-Santos AC, Rossato M, Atti-Serafini L, Cassel E. Extraction of tannin by *Acacia mearnsii* with supercritical fluids. Brazilian Arch Biol Technol. 2004;47(6):995-8. doi: 10.1590/S1516-89132004000600019
36. Özkan O, Metiner K, Seyyal A. Antibacterial effects of ethanol and acetone extract of *Plantago major* L. on gram positive and gram negative bacteria. Kafkas Univ Vet Fak Derg. 2012;18(3):503-5. doi: 10.9775/kvfd.2011.5824
37. Uzun E, Sariyar G, Adsersen A, Karakoc B, Ötük G, Oktayoglu E, et al. Traditional medicine in *Sakarya province* (Turkey) and antimicrobial activities of selected species. J Ethnopharmacol. 2004;95(2-3):287-96. doi: 10.1016/j.jep.2004.07.013
Sharifa AA, Jamaludin J, Kiong LS, Chia LA, Osman K. Anti-urolithiatic terpenoid compound from *Plantago major* Linn. (Ekor Anjing). Sains Malaysiana. 2012;41(1):33-9.