Bioactivity and Dereplication of Phenolic Compounds in Medicinal Plants

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ABSTRACT
Three medicinal plants with recognized anti-inflammatory potential, identified as “erva de São João” (Ageratum conyzoides), “Tanchagem” (Plantago major) and “Bardana” (Arctium lappa L.) were obtained from a medicinal herbs company located in Teófilo Otoni city (Minas Gerais State, Brazil). The dry plant material obtained in packages was submitted to the chemical procedures to prepare the crude extracts by maceration according to the Brazilian pharmacopoeia legislation. After extraction, the samples were subjected to 1H NMR, TLC and Capillary Electrophoresis analysis by co-injection of authentic patterns of phenolic acids and flavonoids to identify the major compounds and classes of secondary metabolites present in each material and then their chemical and biological potential was assessed by DPPH free radical inhibition assay and antimicrobial against E. coli. The results obtained allow us to conclude that the phytoperparation was effective in the extraction of compounds with antioxidant potential and the three species presented a high concentration of flavonoids and other phenolics that is compatible with the chemosystematic data. The screening obtained by 1H NMR spectroscopy, TLC and Capillary Electrophoresis with ultraviolet detection analysis provided us a qualitative profile of the phytochemicals present in each material. None of the extracts were active against Escherichia coli by antibacterial disk diffusion assay at concentration of 1 mg/ml.

Keywords: Medicinal plants, Phenolic compounds, Flavonoids, Antioxidant activity, Ethnopharmacology.

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INTRODUCTION
According to World Health Organization (WHO) data, around 35,000 to 70,000 plants species have been used as medicines and approximately two thirds of the world population uses plants for their primary health care. [1-3] Therefore, these data reinforce the need and
the importance of the research to prove the pharmacological activities of the medicinal plants used popularly or to discover their curative and toxicological properties. [4] Many are the species of medicinal plants recognized by the Ministry of Health that synthesize compounds of phytotherapeutic importance, however, there are few studies regarding the identification of active principles and pharmacological potential. But it is urge more studies of medicinal plants with recognized therapeutic potential from a chemical-pharmacological point of view.

Among the widely used medicinal plants are Ageratum conyzoides, Plantago major and Arctium lappa, extensively used all over the world as remedies for a wide range of diseases. “Erva de São João” (Ageratum conyzoides L.) is a plant largely used in traditional medicine of tropical and subtropical regions for its anti-inflammatory and antinociceptive properties. [3] “Bardana” (Arctium lappa Linné), a vegetable that has long been used as a diuretic, hypertension, gout, arteriosclerosis, hepatites and others inflammatory disorders. [6] A number of Plantago spp. especially P. major has been used in the treatment of diseases such as infection, inflammation and cancer. [7]

Present in all plant organisms, phenolic compounds have several pharmacological actions and their great importance is directly related to the physiological activity, the ability to capture reactive oxygen species, the ability to inhibit nitrosation and chelate metal ions, the potential of auto oxidation and the ability to modulate certain active cellular enzymes. [8] As for its functional potentialities, anticancer, anti-inflammatory, anti-arterogenic, antithrombotic, immuno-modulating, and analgesic activities have been reported, among others, besides antioxidant functions. [9]

Therefore, the present investigation aimed to evaluate the chemical composition of these three medicinal species through Capillary Electrophoresis with direct ultraviolet detection (CE-UV) by co-injection with authentic standards and analysis of their ultraviolet spectra, TLC analysis, NMR spectroscopy and to measure their antioxidant capacity in order to verify if these medicinal plants have the antioxidant effect and chemical components described in the literature and indicated in folk medicine.

**MATERIALS AND METHODS**

**Plant Material**

The dried plant material in the form of sachets was obtained from a company of herbal medicines and medicinal herbs Nature Ervas (Teófilo Otoni city, Minas Gerais state, Brazil) with appropriate botanical identification.

**Crude extracts preparation**

For preparation of the crude extract by maceration, the plant material passed through the process of maceration in 25 mL of 70% hydroalcoholic solution for three days at room temperature. All materials obtained were dried and the yield (%) was calculated.

**Analysis by \(^1\)H NMR and TLC**

\(^1\)H-NMR (500 MHz) spectra of the samples (CD3OD) were obtained on Varian Inova 500\(^*\) Spectrometer. All samples were analyzed by TLC eluted in Hexane and AcOEt mixtures in order to indicate the presence of compounds that could act as potential antioxidants and obtain a chemical profile of each plant material.

**Analysis by Capillary Electrophoresis**

In the electrophoretic analysis, an Agilent Technologies Model 7100 CE device was equipped with a high voltage source (± 30 kV), a Photodiode Array Detector (PDA), a temperature control inside the cartridge by air passage and Agilent ChemStation - Rev. B.04.03 (Model 7100) Data Control, Acquisition and Data Processing Program. Capillary without melted silica coating (Polymicro Technologies) with a total length of 48.5 cm in diameter. The operating parameters of the CE were: detection at 254 nm, capillary at 25°C and applied voltage of +20 kV. The buffer used was 100 mmol L\(^{-1}\) of Tris-HCl, pH 8.5. The new capillary was activated and conditioned on the first day of use by washing with a pressure of 1000 mbar with 1.0 mol L\(^{-1}\) NaOH solution for 30 minutes followed by 10 minutes of water. At the beginning of the analysis, the capillary was conditioned for 5 minutes NaOH 1 mol L\(^{-1}\), followed by 5 minutes water and 10 minutes electrolyte, injection: 50 mbar during 5s, preconditioning between analyzes: 2 min. The cleaning conditioning between the running was 5 minutes with the running electrolyte.

**Antioxidant activity**

For the evaluation of antioxidant activity, the in vitro photocolorimetric method of free radical DPPH (2,2-diphenyl-1-picrylhydrazyl) described by Mensor et al. (2001). [10] For the analysis of the samples, 20 μg ml\(^{-1}\) of the methanolic solution of the diluted extracts was added to 220 μg ml\(^{-1}\) of a free radical methanolic DPPH solution. After 20 minutes of reaction, the reading was carried out at 518 nm using a UV-Vis Shimadzu UV 1601 spectrophotometer.

All readings were performed in triplicate and the average of the obtained data was calculated, the percentage of the antioxidant activity of the extracts was calculated by the following formula:

\[% \text{AA} = 100 - \left(\frac{(Aa-Ab) \times 100}{Ac}\right)\]

Where % AA = percentage of antioxidant activity; Aa = absorbance of the sample; Ab = white absorbance; Ac = control absorbance.

**Antimicrobial activity**

The phytochemical extracts from three medicinal plants were evaluated for their antibacterial potential against Escherichia coli ATCC 25922 by the disc diffusion method at 1 mg ml\(^{-1}\) concentration in aqueous solution. The methodology of diffusion in agar per disc, performed according to CLSI recommendations. [11] The antimicrobial Meropenem 10μg (CENTERLAB) was used as a positive control.

**RESULTS**
Evaluation of the antioxidant and antibacterial activity from medicinal herbs

Concerning the antioxidant activity, the samples obtained by maceration showed antioxidant activity varying between 59-71% inhibition of the DPPH free radical (Table 1). The species Ageratum conyzoides showed high potential of DPPH Free-Radical Scavenging with 75% free radical inhibition.

Table 1: % inhibition values of DPPH free radical in plant samples of three medicinal species obtained by maceration.

<table>
<thead>
<tr>
<th>Medicinal herbal</th>
<th>DPPH Free-Radical Scavenging Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ageratum conyzoides</td>
<td>75%</td>
</tr>
<tr>
<td>Plantago major</td>
<td>61%</td>
</tr>
</tbody>
</table>

Concerning the antibacterial activity, none of the extracts from three medicinal plants were active against Escherichia coli by antibacterial disk diffusion assay at concentration of 1 mg ml⁻¹.

Chemical Profile by TLC and NMR Spectroscopy from Herbal Medicines

The results from chemical composition obtained by 1H NMR and TLC analysis from Ageratum conyzoides, Arctium lappa and Plantago major can be briefly describe in Table 2. Some of the compounds can be found in all three species, like phenolic compounds and terpenes. The results obtained are in agreement with the chemosystematic data of the related taxa.

Table 2: Chemical profile of each species studied by 1H NMR and TLC.

<table>
<thead>
<tr>
<th>Species studied</th>
<th>Chemical profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ageratum conyzoides</td>
<td>Phenolic acids, coumarin, pyrones, tannins, flavonoids, lactones, terpenes, saponins and chromenes.</td>
</tr>
<tr>
<td>Arctium lappa</td>
<td>Flavonoids, phenylpropanoids, phenolic acids, sugars, lactones, lignans, terpenes, steroids and fatty acids.</td>
</tr>
<tr>
<td>Plantago major</td>
<td>Flavonoids, terpenes, steroids, aromatic acids, fatty acids and alkaloids.</td>
</tr>
</tbody>
</table>

Concerning the yield, all the materials obtained showed great yield by the evaluated method.

Table 3: Phenolic compounds and their respective pKa, migration time on CE-UV analysis and UV λmax.

<table>
<thead>
<tr>
<th>Compound</th>
<th>pKa</th>
<th>Migration Time</th>
<th>UV λmax (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeic acid</td>
<td>4.62</td>
<td>5.60</td>
<td>235</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>4.40</td>
<td>6.00</td>
<td>273</td>
</tr>
<tr>
<td>Quinic acid</td>
<td>3.46</td>
<td>2.74</td>
<td>255</td>
</tr>
<tr>
<td>Galacturonic acid</td>
<td>3.24</td>
<td>2.77</td>
<td>230</td>
</tr>
<tr>
<td>Hydroxybenzoic acid</td>
<td>4.54</td>
<td>7.89</td>
<td>250</td>
</tr>
<tr>
<td>Kojic acid</td>
<td>7.66</td>
<td>3.58</td>
<td>225</td>
</tr>
<tr>
<td>Quercetin</td>
<td>6.44</td>
<td>4.25</td>
<td>253 (369)</td>
</tr>
<tr>
<td>Genistein</td>
<td>6.55</td>
<td>4.51</td>
<td>270 (340)</td>
</tr>
<tr>
<td>Chrysirin</td>
<td>6.64</td>
<td>4.43</td>
<td>269 (313)</td>
</tr>
<tr>
<td>Narinigenin</td>
<td>7.91</td>
<td>4.49</td>
<td>250 (330)</td>
</tr>
</tbody>
</table>

Dereplication of phenolic compounds by Capillary Electrophoresis from medicinal herbal

The flavonoid and phenolic acid analyzed from medicinal herbs extracts samples were identified by comparison with migration time and UV spectra of standards injected into the CE system, separately. Then, 100μL of each standard was added to a vial to prepare the standard mixture for co-injection experiments. Table 3 shows the compounds that were used as standards in these analysis as well as their respective pkas, migration time and maxima wavelength absorption bands (λmax) presented in the UV spectra.

The analysis by CE-UV from Ageratum conyzoides allowed us to verify the presence of many phenolic compounds. The presence of 13 flavonoids and 7 phenolic acids were identified, which justifies the antioxidant potential observed by this extract, which inhibited 75% of the DPPH free radicals in the
evaluated by the electropherogram of these samples showed that the two major compounds belong to the class of phenolic acids and the other compounds were present in this phytochemical extract possibly belong to the class of flavonoids. Other classes of compounds were also observed, as quinones, but in low concentrations. The results are shown in Table 4. A survey conducted in the databases indicated that the results obtained are in agreement with the expected results for the target species of this study. It is observed a predominance of flavonoids in the three species and an expressive concentration of phenolic acids and chemically related compounds.

![Diagram showing the concentration of flavonoids and phenolic acids in the three species](image)

**DISCUSSION**

Concerning the antioxidant activity, the samples obtained by maceration showed antioxidant activity varying between 59-71% inhibition of the DPPH free radical. It was also found a positive correlation between antioxidant activity and the variable concentration of phenolic compounds, that is, as the contents increased in the samples, there was a percentage increase of the antioxidant activity. This result confirms data from the literature that antioxidant activity is directly correlated with phenol content. Phenolic compounds are capable of intercepting the free radical oxidation chain through the hydrogen donation of their phenolic hydroxyls. The confirmation of the antioxidant activity in these extracts can contribute to validate the medicinal use of the evaluated species, since the use as remedy of the products of these herbs is part of the habits of many communities.

The chemical composition of Ageratum conyzoides has not been yet accurately established. The 1H-NMR spectrum of the hydroalcoholic extracts of the A. conyzoides species showed signals in the region of 6-7 ppm indicative of α-oxygenated aromatic hydrogens. Also, aromatic hydrogens were observed around 3.0 ppm, possibly of peri-carbonylic hydrogens and, also singlets around 9.0 which may be related to the presence of nitrogen from aromatic alkaloids, which may be related to the presence of toxic alkaloids of pyrrolizidine already described in this species by Faqueti et al. (2017). Many signs of alpha-oxygenated aromatic hydrogens have also been observed, which may be derived from compounds such as phenolic acids, coumarin, benzopyrones, chlorogenic acid, coumaric acid, tannins and other phenols already reported in this species, as well as several aromatic methoxy hydrogens compounds such as polymethoxyflavones. The presence of carboxylic, ester and aliphatic hydrogens observed in the spectrum suggest compounds such as sesquiterpene lactones described in the species. However, free sugars and heterosides such as flavones and glycosylated flavonoids described in the species were found in higher concentration, the presence of which can be detected in the 1H NMR spectrum by the high intensity of the signals and multiplets between 3.0-4.5 ppm and by aromatics at 6-8 ppm. These extracts also showed the presence of terpenes, saponins by the signals at 0.8-2.9 ppm and other compounds reported in the species as a large number of terpenoids, chromenes and phytol - a diterpene alcohol. Chromones are the main constituents of low polarity found in the leaves of these species according to literature data evaluated.

Regarding the 1H NMR spectra analysis from Arctium lappa species, in the region of aromatic hydrogens around 6 ppm indicated phenolic compounds with a high substitution pattern such as polyphenols and flavonoids, phenylpropanoids (Gao et al., 2013), phenolic acids as derivatives of caffeic acids - such as dicaffeoylquinic acid, etc and other aromatic compounds such as arctiin - a bioactive lignin component of Arctium lappa, as well as signals close to 8 and 9 ppm from an aromatic system with little oxygen substitution. High concentrations of sugars were also observed for the signals at 3.1-4.3 ppm and anomic hydrogens at 5.1 and 5.4 ppm, whose signals may be free sugars or heterosides as glycosylated, glycosylated lactones and butyro lactone lignans reported in the species. The sugar molecules can be derived from glycosylated flavonoids, saponins or sugars, which is compatible with the chemosystematic of the species. The signs of aliphatic hydrogens may be from terpenes, steroids, and fatty acids such as the amide derived from the fatty acid isolated from this species.

The chemical profile of Plantago major showed the presence of phenolic compounds highly oxygenated by the signals in 5.8, 6.2 to 6.9 ppm that can be derived from flavonoids and phenolic acids as derivatives of hydroxycinnamic acids: chlorogenic and neochlorogenic acids isolated from this species, low concentration of aliphatic compounds such as terpenes and steroids and derivatives and high concentration of sugars. The presence of few signs of anomic hydrogens suggests a mixture of sugars with other oxygenated aliphatic compounds such as Tartaric, Citric, Malic, and Malonic and Succinic Acid already reported in the species. The presence of phenolic...
heterosides may be of the class of flavonoids such as glycosylated flavanone \[25\] and flavones luteolin 7-glucoside and luteolin 7-glucuronide \[26\] or of glycosylated phenolic acid such as verbascoside isolated from this species. \[27\] The presence of few signs in the aliphatic region that can be attributed to terpenes and steroids suggests fatty acids such as stearic acid, oleic acid, pentadecanoic acid, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), which are quite common in this species. \[28\] No signs of aromatic alkaloids were observed. The leaves of this plant have been previously found to contain the glycoside aucubin, vitamin K, ascorbic acid, polygalacturonide pectin, bitter and tannin compounds, carotene, and a small amount of alkaloids. \[29\]

TLC analysis using chemical and physical developers (such as UV/Vis light and I\(_2\)) allowed us to infer the class of Natural Products present in each material. The results were compared with the data obtained from NMR analysis and comparison with chemosystematic data.

Flavonoids have UV absorption spectrum characteristic determined by the common benzopyrone nucleus, with two absorption maxima: one occurring between 240-285 nm (band II) due to the existence of ring A and the other between 300-400 nm (band I) due to the existence of ring B. Simple phenols, specifically those derived from benzoic acid (C\(_6\)C\(_1\)) have wide distribution in the plant kingdom and various pharmacological properties, such as antioxidant activity, whose antioxidant capacity is directly associated with an increase in the group of hydroxylation and decreased glycosylation. \[30\]

The analysis by CE-UV from Ageratum conyzoides allowed us to verify the presence of many phenolic compounds. The presence of 13 flavonoids and 7 phenolic acids were identified, which justifies the antioxidant potential observed by this extract, which inhibited 75% of the DPPH free radicals in the evaluated method. Analysis from peak area on electropherogram of these sample showed that the two major compounds belong to the class of phenolic acids, whose information is in agreement with the data observed in the 1\(^{H}\) NMR spectrum and the chemosystematic of the plant family. The other compounds expressive in this phytochemical extract possibly belong to the class of flavonoids. Other classes of compounds were also observed, as quinones, but in low concentrations.

Concerning the qualitative analysis of the electrophoretic profile from Arctium lappa, they showed a variety of phenolic compounds, the major constituents being the phenolic acids and flavonoids, based on the areas of the electropherogram peaks. The flavonoids were found in low concentrations in this extract and by the analysis of the UV spectra, possibly belong to the class of flavones and flavonoids. The chemical profile obtained by CE from Plantago major extract showed to be mostly composed of phenolic acids and other simple phenols, since the UV spectra from the peaks showed absorption bands below 280 nm.

Although preliminary, this work contributed important information about the preliminary quality control of medicinal herbs used by population. More important information should be investigated to ensure the effectiveness and quality of plant material marketed as medicinal and widely used in folk medicine. Although the selected species are used worldwide for medicinal purposes, their bioactivity and micromolecular profile have not been elucidated in order to guarantee the safety of the consumption of their phytopreparations. This study showed that the species had good potential for DPPH free radical inhibition and the analysis of TLC and 1H NMR spectra provided the chemical profile consistent with the literature data and the electrophoretic profile showed the variety of flavonoids and other phenolic compounds. In this way, this work contributed with phytochemical information of herbs marketed as medicinal and widely used by the population for the treatment and prevention of diseases. The data obtained show that the medicinal plants targeted by this study have chemical constituents responsible for the pharmacological action indicated for the species and showed an efficient method for the analysis of phenolic molecules of plant matrices by capillary electrophoresis.

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REFERENCES


