http://dx.doi.org/10.5935/0103-5053.20140286

J. Braz. Chem. Soc., Vol. 26, No. 2, 350-358, 2015.
Printed in Brazil - ©2015 Sociedade Brasileira de Química
0103 - 5053 $6.00+0.00

Article

Seasonal Effects on HPLC-DAD-UV and UPLC-ESI-MS Fingerprints and Analgesic Activities of *Vernonia Condensata* Baker Extracts

Sabrina Afonso,[a] Aldair C. de Matos,[a] Vitor A. Marengo,[b] Estefânia G. Moreira,[b] Daniely X. Soares,[c] Héctor Henrique F. Koolen[c] and Ieda S. Scarminio*[,a]

[a]Departamento de Química and [b]Departmento de Ciências Fisiológicas, Universidade Estadual de Londrina, Rodovia Celso Garcia Cid, PR 445, Km 380, 86051-980 Londrina-PR, Brazil

[c]Institute of Chemistry, University of Campinas, CP 6154, 13083-970 Campinas-SP, Brazil

Vernonia condensata Baker leaves have different uses in Brazilian folk medicine, including as analgesic and anti-inflammatory agents. The purpose of this study was to evaluate the seasonal effects on their high performance liquid chromatography with a diode array detector (HPLC-DAD-UV) and ultra-performance liquid chromatography coupled to a mass spectrometer with an electrospray interface (UPLC-ESI-MS) fingerprints, as well as their analgesic activities in mice. There were significant seasonal effects on the relative abundances of the metabolites of the *V. condensate* leaves as well as on their activities. Analgesic activities in the writhing test were observed with the polar fraction of the leaf extracts collected in autumn, winter and summer (400 mg kg^{-1}); and with the intermediate fraction of leaves collected in autumn (25 and 400 mg kg^{-1}) and in the summer (100 mg kg^{-1}). In conclusion, the results confirm peripherally-mediated anti-inflammatory and analgesic activities for *V. condensata* leaves and suggest that these are influenced by the harvesting season. *N*-oxides alkaloids as well as vernonioside play important roles in determining this activity.

Keywords: *Vernonia condensata*, fingerprint, **s**easonal effect, analgesic activity, anti-inflammatory

Introduction

Chemical fingerprinting is an important procedure for showing chemical information of medicines in the form of spectra, chromatograms and other graphs obtained by analytical techniques.[1-2] In practice, fingerprints can be chromatographic or spectral profiles which show characteristics of the herbal medicine investigated. The chemical components of the extract may be pharmacologically active, or serve as a marker, or a pattern indicating the presence of a specific molecule. High performance liquid chromatography (HPLC), gas chromatography (GC), thin layer chromatography (TLC) and capillary electrophoresis (CE) can all be applied for fingerprinting.[3] One of the main advantages of HPLC is the possibility to make hyphenation alternatives with different detectors such as the diode array detector (DAD) and mass spectrometry (MS) for herbal fingerprint.

*e-mail: ieda@qui.uel.br

Peumus boldus, popularly known as "boldo do chile" is a Chilean tree traditionally employed in folk medicine in various countries.[4] It can be found in pharmacies in extract form or in capsules containing leaf powder as well as being marketed as teas. It is recommended primarily for the treatment of diseases of the digestive and hepatobiliary systems.[5] Boldine alkaloid is the major active ingredient in this plant species and is responsible for its analgesic[6] and antioxidant[7] properties. In Brazil, *Vernonia condensata* Baker (Asteraceae = Compositae), known as "boldo-baiano", "figatil" and "alum",[8] is used in folk medicine as tea to replace *Peumus boldus*. *V. condensata* is listed in RENISUS (National List of Medicinal Plants of Interest to Health System provided by the Ministry of Health), a document that contains 71 herbal species and/or genus traditionally used in folk medicine in Brazil. The Ministry of Health considers this plant a possible phytomedicine but it still lacks the scientific support necessary to be prescribed and regulated by the governmental health program (Relação Nacional de Plantas Medicinais de Interesse

ao Sistema Único de Saúde – RENISUS).[9] Experimental studies have suggested that the aqueous extract of *V. condensata* leaves have analgesic/antiinflamatory activity.[10,11] Literature studies have attributed the activities of *Vernonia* species to the vernolide, a class of sesquiterpene lactones.[10,12-15]

In phytochemical studies, it is recognized that the extractor system directly influences the amount of compounds that are extracted and, consequently, the pharmacological activity of phytocomplexes. In our previous work we used an experimental mixture design to choose the best extractor system to evaluate the antinociceptive activity of the *V. condensata* in mice.[11] This methodology resulted in the determination of useful mathematical models for describing the effects of extraction mixture compositions on antinociceptive activities. The reduction in the average relative percentage of abdominal contractions induced by acetic acid in mice was shown to be a function of ethanol, dichloromethane and acetone proportions.

In this work high performance liquid chromatography with a diode array detector (HPLC-DAD-UV) and ultra-performance liquid chromatography coupled to a mass spectrometer with an electrospray interface (UPLC-ESI-MS) fingerprints for *V. condensata* were used to compare the profile compounds probably responsible for the analgesic/antiinflammatory effect in fractionated extracts of *V. condensata* leaves. HPLC-DAD-UV spectra were compared with the spectrum of the boldine authentic standard. In order to investigate seasonal influence on pharmacological activity, this study was conducted with plant material collected in three different seasons: summer, autumn and winter. The extracts were fractionated by liquid-liquid extraction.[16,17] The mean lethal dose (LD_{50}) was also estimated to indicate acute toxicity.

Experimental

Chemicals

HPLC grade acetonitrile and methanol were purchased from Vetec Química Fina (Rio de Janeiro-RJ, Brazil). Mobile phase mixture preparations were made using water prepared with the Millipore Milli-Q purification system (São Paulo-SP, Brazil). Ethanol, dichloromethane, acetone, methanol, chloroform and sulfuric acid were also purchased from Vetec. Ammonium hydroxide, potassium bromide, acetic acid and boldine were purchased from Merck, Synth, Biotec and Sigma-Aldrich, respectively. All reagents were of analytical grade. Tween 80 Polyoxyethylene and Ibuprofen (Spidufen® 400) were purchased from Fischer Scientific Company and Zambon Switzerland Ltda., respectively.

Plant material

The plant material was collected at the State University of Londrina (UEL), Londrina-PR, Brazil, during each season of the year, autumn (April, 24), winter (July, 10), spring (October, 20) of 2009 and summer (January, 19) of 2010. A voucher specimen authenticated by the biologist, Mr. Manuel Paiva, Department of Animal and Vegetal Biology, UEL, is deposited at the institutional herbarium, under number 38694.

Characterization of plant material

For moisture percentage 2.0 g of powdered leaves were left in an oven for 2 h at 105 ºC. For total ash percentage 3.0 g of powdered leaves were left in a muffle furnace for 12 h at 450 ºC. For both analyses the samples were cooled in a desiccator with silica crystals and then weighed. The procedure was repeated until constant weight and performed in duplicate.

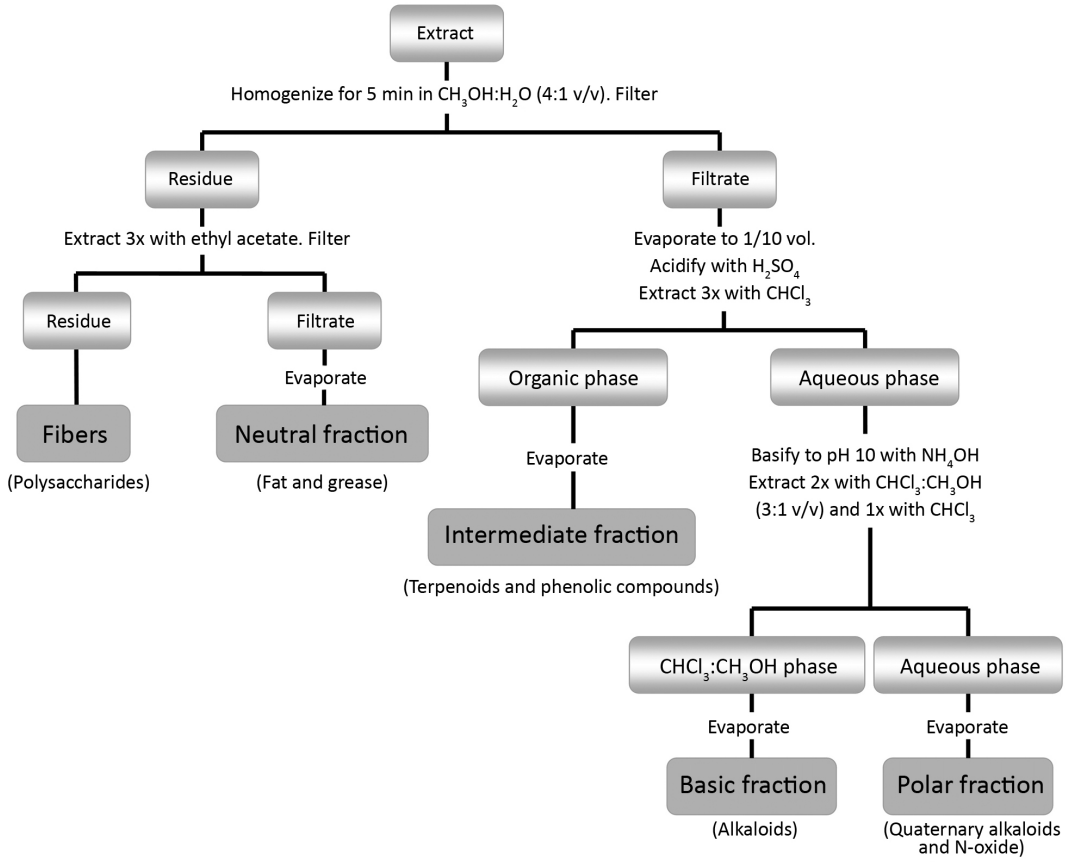
Extract preparation and fractionation

Each extract was prepared with 10 g of powdered leaves and extracted with 100 mL of 60:30:10 (v:v:v) acetone:dichloromethane:ethanol mixture and subjected to maceration in an ultrasound bath for 30 min. This proportion was chosen based on the results of our previous work.[11] The material was filtered and the solvent was evaporated under reduced pressure to obtain the crude extract.

The extracts were fractionated by liquid-liquid extraction resulting in fibers (polysaccharides) and four fractions: neutral (fat and grease), intermediate (terpenoids and phenolic compounds), basic (alkaloids) and polar (quaternary alkaloids and *N*-oxides), Scheme 1.[15] HPLC analysis and biological evaluations were conducted with the polar and intermediate fractions of each extract. The other fractions were not evaluated due to their very low yields.

Infrared analysis

The data of Fourier transform infrared analysis (FTIR) were treated using the entire infrared spectra. 1.0 mg of the crude extract was weighed with 0.2 g of dry solid KBr that was then homogenized in an agate mortar with a few drops of chloroform. The spectra were recorded in the 4000-400 cm^{-1} region, with 4 cm^{-1} resolution and 32 scans, using a Shimadzu FTIR-8300 spectrophotometer.



Scheme 1. Scheme for fractionation of the crude extract.

HPLC analysis

Solvents for HPLC analysis were chosen according to the solvent selectivity triangle proposed by Snyder *et al.*,[19] 5 mg of the sample were dissolved in 3 mL of extractor solvent and, after 1 h, filtered through 0.22 µm nylon filters (Millipore Millex) and kept at −5 ºC until analysis. HPLC analysis was conducted on a SPD M10AV Finnigan Surveyor liquid chromatography (Thermo-Electron Corporation) with a photodiode array detector (PDA). A quaternary Thermo Electron Corporation pump was used with a C_{18} column Kinetex 2.6 µm of HILIC 100 Å Phenomenex (150 × 4.6 mm) under the following conditions: injection volume 20 µL, flow rate of mobile phase at 1.0 mL min^{-1}. HPLC analysis was carried out in the isocratic mode and nine mobile phases with different proportions of acetonitrile, water and methanol were tested. The choice of these nine mobile phases was based on an experimental design of solvent mixtures according to Delaroza and Scarminio.[20] The compounds were monitored at 210, 240 and 254 nm (running time of 15 min) and UV spectra from 200 to 600 nm were recorded. Satisfactory separation was reached at 210 nm. The data were processed using ChromQuest 4.2 software.

UPLC-ESI-MS analysis

Chromatographic separations were performed using an Acquity UPLC chromatographic system (Waters, Millford, MA) equipped with a binary pump system as well as an Acquity BEH C_{18} column (2.1 mm × 150 mm, 1.7 µm particle size), also from Waters Corporation. The UPLC system was coupled to a Quattro Micro API triple quadrupole (Waters, Manchester, UK) mass spectrometer (MS) using a Z-spray electrospray ionization (ESI) source. The mobile phase consisted of water with 0.1% formic acid (A) and pure acetonitrile (B) using the following gradients: 0-2.5 min, 5-30%; 2.4-5.7 min, 30-70%; 5.7-7.0 min, 70-100% with a (B) mobile phase; 7.0-7.5 min was held isocratically at 100% of the (B) mobile phase and finally 7.5-8.0 min before returning to the initial conditions (5% of B mobile phase). The MS was operated in the positive mode owing to the basic natures of the target compounds in the polar extract of *Vernonia condensata* plant. The data were acquired in the scan mode using an *m/z* range of 250 to 600 Da. The ionization source conditions were: 3 kV capillary voltage, 150 °C source temperature, 80 L h^{-1} cone gas flow, 800 mL h^{-1} dissolution gas flow and 350 °C dissolution temperature. The nitrogen nebulization

gas was 99% pure. The data were processed using the MassLynx v4.1 software.

Animals

Male Swiss mice (25-30 g) from the institutional colony, were acclimatized for 7 days in a temperature and light controlled room under a 12:12 h L:D cycle (lights on at 06:00 h). They had free access to tap water and food, except for a fasting period of 3 h before the experiments in order to decrease interference of gastrointestinal content in the absorption process. The experimental procedures were approved by the State University of Londrina Animal Ethics Committee (CEEA, 78 / 09).

Evaluation of analgesic activity

Median lethal dose (LD_{50})

Oral LD_{50} was estimated according to guideline 423 from the Organization for Economic Cooperation and Development.[21] The starting dose was 2000 mg kg^{-1}.

Writhing test

Polar fractions were dissolved in distilled water and the intermediate fraction in 2% Tween 80 solution at the desired concentration just before use. The method described by Qin *et al*.,[22] was used with a few modifications. Briefly, mice (8-10 animals in each dose group) were gavaged with 25, 100 or 400 mg kg^{-1} of each fraction or vehicle (VEH, 2% Tween 80). A positive control group received ibuprofen (IBU, 200 mg kg^{-1}, gavage), a standard anti-inflammatory and analgesic drug. After 30 min, all the animals were injected intraperitoneally (ip) with 0.8% acetic acid in a volume of 10 mL kg^{-1}. Immediately after this injection, the mice were allocated in individual plexiglass cages and the number of writhes (contraction of the abdominal muscle together with a stretching of the hind limbs) was counted during 20 min.

Data are presented as percentage inhibition of writhes induced by each extract as compared to its concurrent control and was determined as follows:

$$\% \text{ inhibition} = 100 - \left(\frac{\text{writhes in each treated animal}}{\text{mean writhes in vehicle group}} \times 100 \right)$$

Hot plate test

In this test, only the polar fractions were evaluated since they provided better results in the writhing test. Mice (8 animals in each dose group) were gavaged with 25 or 400 mg kg^{-1} of the polar fractions or distilled water (VEH) and after 30 min they were placed individually on the hot plate at 58 °C. The reaction time (animal raising and/or paw licking) was recorded and a cut-off time of 60 seconds was adopted.[23]

Statistical analysis

Data from the characterization of plant material were analyzed by one-way ANOVA complemented with Tukey's test. Data from the writhing test were analyzed by the Kruskal-Wallis procedure and complemented with Dunn's test. Data from the hot plate test were analyzed by one-way ANOVA. Differences among groups were considered significant if $p < 0.05$.

Results and Discussion

Characterization of vegetal material

Table 1 shows the results for moisture and ash contents of the *V. condensata* leaves in the four seasons studied. Its material characterization was carried out because it is a plant of high popular consumption that has been little studied. The results show that moisture and ash contents for the summer and spring are slightly higher than leaves collected in the autumn and winter. According to the Brazilian Pharmacopeia[24] the maximum value accepted for moisture percentage and total ash percentages is 10%, showing that these results are above the accepted limits.

Table 1. Means and standard deviations of moisture and total ash percentages of *Vernonia condensata* Baker leaves collected in the summer, autumn and winter

Season	Mean	
	Moisture percentage	Total ash percentage
Summer	12.386 ± 0.086^{b}	10.964 ± 0.074^{b}
Autumn	11.165 ± 0.165^{a}	10.683 ± 0.005^{a}
Winter	11.269 ± 0.067^{a}	10.922 ± 0.033^{ab}
Spring	11.790 ± 0.039^{c}	11.024 ± 0.093^{b}

[a,b,c]Means with same letters within the same column are not significantly different at $p > 0.05$ as determined by ANOVA and Tukey tests.

Effect of seasonality on yields

Data for the effects of seasonality on yields are shown in Table 2 that contains the average and standard deviations of the crude extract and fraction yields for each season. As can be seen yields of crude extracts and fractions varied depending on the season. The highest yield of polar fractions was obtained during the autumn, followed by summer, spring and then winter, whereas the intermediate fraction summer yield is higher than the autumn one with

Table 2. Yields and standard deviations of the crude extracts and their fiber, neutral, intermediate, basic and polar fractions

Sample	Mass / g			
	Autumn	Winter	Summer	Spring
Crude	0.599 ± 0.010[a]	0.475 ± 0.014[b]	0.519 ± 0.001[c]	0.657 ± 0.006[d]
Fiber	0.018 ± 0.001[a]	0.014 ± 0.000[b]	0.011 ± 0.000[c]	0.011 ± 0.000[c]
Neutral	0.065 ± 0.003[a]	0.070 ± 0.000[a]	0.059 ± 0.008[ab]	0.045 ± 0.003[b]
Intermediate	0.243 ± 0.003[ac]	0.189 ± 0.001[b]	0.257 ± 0.001[a]	0.212 ± 0.021[bc]
Basic	0.027 ± 0.000[a]	0.029 ± 0.000[ac]	0.014 ± 0.004[b]	0.036 ± 0.001[c]
Polar	0.234 ± 0.001[a]	0.069 ± 0.004[b]	0.113 ± 0.008[c]	0.098 ± 0.000[c]

[a,b,c,d]Means with same letters within the same row are not significantly different at $p > 0.05$ as determined by ANOVA and Tukey tests.

the lowest yield in the winter. For all crude extracts the intermediate fractions has the highest average yield for each season. The yields of the other fractions were too low to allow biological testing. Literature shows that alkaloid *N*-oxides[6] of *Peumus boldus* ("boldo do Chile") and terpenoids (vernonioside)[12] of *Vernonia condensata* were found in the polar and intermediate fractions, respectively, thus only the polar and intermediate fractions were used for biological evaluation and chromatographic analysis.

It should be noted that the sums of the fractional yields are smaller than their corresponding crude extract yields for all the seasons. This is due to crude extract material that is not soluble in the different solvent media used in fractionation. Interestingly the autumn crude extract is only 0.012 g higher than the sum of its fractionation extracts.

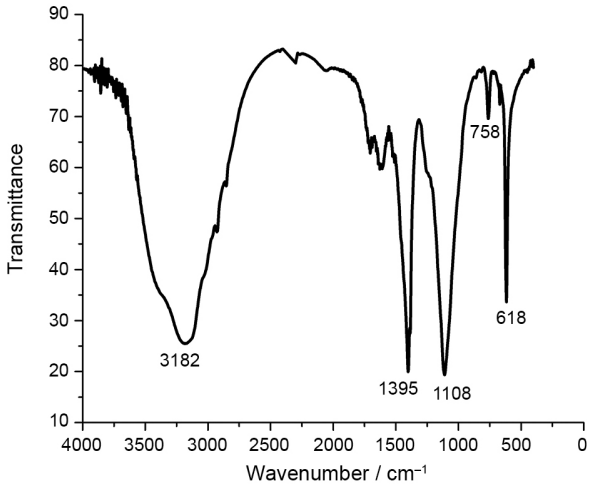


Figure 1. FTIR spectrum of the autumn polar fraction extract of *V. condensata* leaves.

Fingerprints analysis

Figure 1 shows the FTIR spectrum of the polar fraction, similar for the others seasons. This spectrum shows bands characteristic of heterocyclic compounds containing an N-H group, such as in the *N*-oxide alkaloids like pyridines, pyrazines, pyrroles and furans.[25] The N-H stretching band is observed at the 3182 cm^{-1}. Heterocyclic compounds also show characteristic band patterns in the 1600-1300 cm^{-1} ring stretching region and between the 800-600 cm^{-1} interval that can be attributed to the out-of-plane C-H bending vibrations. *N*-oxide alkaloids are known to be hepatotoxic.[26] These features are not found in the spectra of the intermediate fraction.

An initial study was performed to investigate the mobile phases to obtain more information about the HPLC-DAD-UV fingerprint for each season. The best separation of chemical compounds from the fractions was obtained with a mobile phase composed of acetonitrile:water:methanol (26:51:23, v:v:v) at 210 nm.

HPLC-DAD fingerprints obtained in the chromatographic analysis of the polar fractions of the leaves collected during the different seasons showed two metabolites being more abundant (Figure 2). As the metabolites are not known, HPLC-DAD-UV spectra were employed to compare the relative abundance of two main metabolites presented in these chromatograms. As can be seen, in each chromatogram the spectra profile is very similar and present three absorbance peaks: 227, 289 and 325 nm and 216, 278 and 328 nm. The relative abundance increases from autumn to winter is greater than the spring and summer.

To explore these chemical markers, UPLC-ESI-MS analysis was based on the skeleton of the aporphine alkaloids. These alkaloids make up a large group including boldine. The total ion chromatogram of the polar fraction (UPLC-ESI-MS fingerprint) is presented in Figure 3. This figure, compared with the fingerprint of the polar fraction of the aqueous extract, shows only two common metabolites whose retention times (Rt) are 1.77 min and 1.78 min (aqueous and organic phase, respectively) and 2.77 min, being that the first showed greater relative abundance than any other (2.77 min). The *m/z* of protonated species are Rt

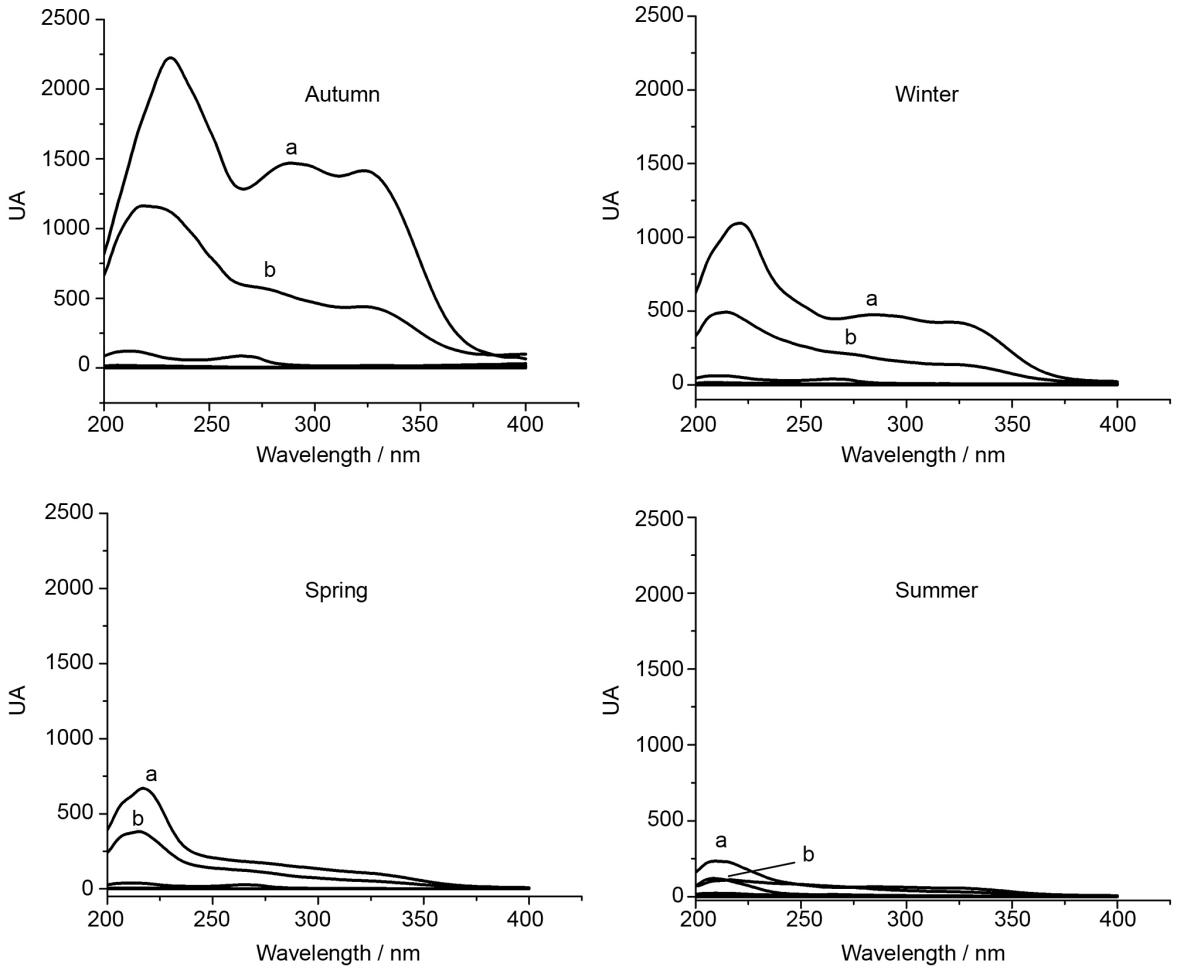


Figure 2. Chromatographic fingerprint and HPLC-DAD-UV spectra of the polar fraction extracts for each season of *V. condensata* leaves. The mobile phase was composed of acetonitrile:water:methanol (26:51:23, v:v:v) at 210 nm.

of 1.77 min and 1.78 min, *m/z* 328.3 $[M + H]^+$ and Rt of 2.77 min, *m/z* 356.3 $[M + H]^+$. This result was assigned to the components, boldine (*m/z* 328.3 $[M + H]^+$) and Xylopine or 4-hydro-palmatine (*m/z* 356.3 $[M + H]^+$), that could not be differentiated and present the same *m/z.*

The fingerprints were compared by DAD-UV spectra against an authentic standard boldine. Figure 4 shows the spectrum of standard boldine which also has three peaks of absorbance at 203, 282 and 310 nm. The boldine DAD-UV spectrum was not consistent with those presented in Figure 2, because it exhibits different UV absorption maxima under the same chromatographic condition. Whereas in the HPLC analysis the two major metabolites of the organic fraction eluted before boldine, therefore it can be concluded that they are slightly more polar than those of standard boldine.

HPLC-DAD-UV spectra of the main peaks obtained in the chromatographic analysis for the intermediate fractions of the leaves collected during the different seasons were also examined. Figure 5 shows the DAD-spectra of the

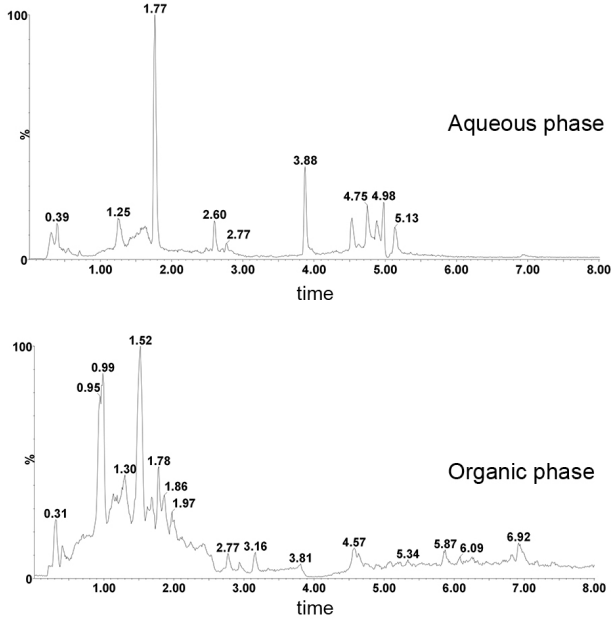


Figure 3. Total ions chromatogram in the aqueous and organic polar phases of the *V. condensata* leave extracts.

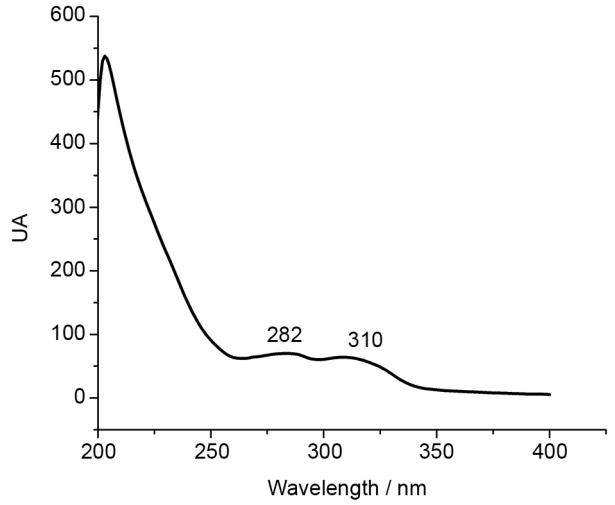


Figure 4. HPLC-DAD-UV spectrum of standard boldine, mobile phase composed of acetonitrile:water:methanol (26:51:23, v:v:v) at 210 nm.

chromatographic peaks of the extract prepared from leaves collected in the summer for the intermediate fraction. For these spectra we conclude that the extract contains six steroidal glycosides with similar spectral profiles, whose absorptions vary between 233-235, 241-244 and 250-252 nm. These results are supported by the literature, where the spectra of vernonioside A2, A3, B1, B2, D and E were found in *Vernonia* genus and present the same absorption peaks.[27] *In vivo* experiments have shown that vernonioside B2 identified in *V. condensata* also possesses an antinociceptive effect.[12]

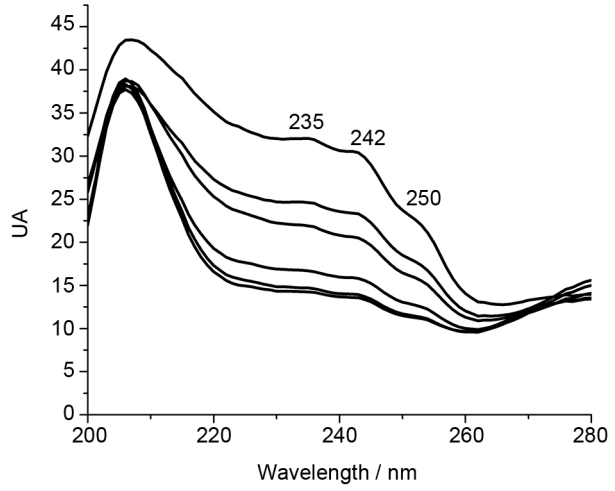


Figure 5. HPLC-DAD-UV spectra of various chromatographic peaks for one summer intermediate fraction extract of *V. condensata* leaves. The mobile phase was composed of acetonitrile:water:methanol (26:51:23, v:v:v) at 210 nm.

Evaluation of analgesic activity

Spectroscopic data associated with principal component analysis (PCA) showed that samples collected in the spring and summer show no difference. Therefore, to reduce the number of animals used in biological testing, the samples collected in the spring were not tested.

Average lethal dose

The toxicity of the extract was evaluated by the lethal 50 dose. Oral LD_{50} of the polar fraction of the aqueous extract and the polar and intermediate fractions of the organic extract were estimated as 5000, 2500 and 500 mg kg^{-1}, respectively.

Writhing test

Kruskal-Wallis complemented by Dunn's test demonstrated that all positive control groups (IBU) were different from the negative control groups (VEH) validating our experimental conditions. Figure 6 shows the percentage of abdominal writhe inhibitions in relation to the VEH group induced by IBU and by the different doses of the evaluated fractions. The polar fractions prepared with leaves collected during the autumn, winter and summer, at the higher evaluated dose, i.e., 400 mg kg^{-1}, were as effective as IBU. Moreover, the polar fraction prepared with leaves from summer also presented a significant analgesic effect at the 100 mg kg^{-1} dose level (Figure 6a). On the other hand the efficacy of IBU was lower in the summer followed by autumn and then winter. The results indicate that the percentage of inhibition does not appear to be correlated with the two main metabolites in the polar fractions (Figure 6a), because in summer they showed lower concentrations and the percentage inhibition relative to autumn was higher when compared to that of IBU.

The intermediate fraction of organic extracts prepared with leaves from different seasons resulted in heterogeneous data and even though some results had statistical significance (autumn: 25 and 400 mg kg^{-1}; summer: 100 mg kg^{-1}), these fractions were not as effective as IBU in inhibiting acetic acid-induced writhes (Figure 6b).

One-way ANOVA demonstrated that the polar fractions prepared with leaves collected during the autumn were ineffective in increasing the reaction time in the hot plate test (ANOVA, $p > 0.05$). Means and standard errors of the mean for reaction time in seconds for the control was 11.6 ± 1.3, whereas these values for the polar fraction extracts of the different dosages were 12.1 ± 1.2 (25 mg kg^{-1}) and 11.7 ± 1.5 (400 mg kg^{-1}).

Even though there were seasonal variations, the present results showed that polar fractions of the extracts from *Vernonia condensata* Baker leaves were as effective as

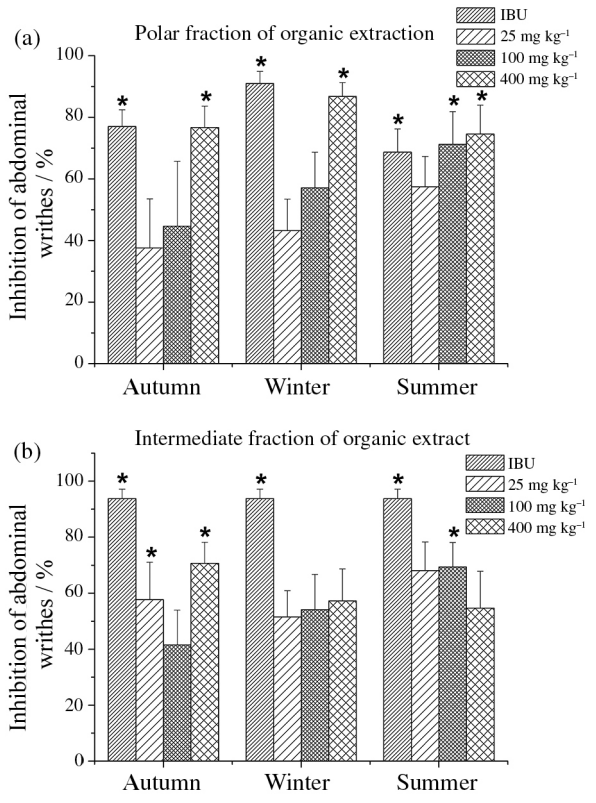


Figure 6. Efficacy of ibuprofen (IBU, 200 mg kg^{-1}) and different doses: (a) the polar fraction of organic extract and (b) the intermediate fraction of organic extract of *V. condensata* leaves collected in autumn, winter and summer.

ibuprofen in inhibiting abdominal writhing in mice, a model for inflammatory pain. The lack of effect for the polar fractions in the hot plate test confirms its peripheral action as has been previously suggested by Frutuoso *et al*..[10] In the writhing test polar fractions were effective at higher doses (100 or 400 mg kg^{-1}). This result possibly reflects the low yield of the bioactive compounds.

Conclusion

No direct correlation was observed between yield and anti-inflammatory and analgesic activity which is not surprising since crude yield is not an indicator of the yield of specific constituents that may be responsible for biological activity. Chemical analyses conducted in this study seem to support the absence of boldine and/or its derivatives in the fractions extracted from *V. condensata* leaves. The identification and isolation of these bioactive metabolites are promising for further studies. The results from the present study confirm that the autumn polar fraction and summer intermediate fraction extracts of *V. condensata* leaves display greater effects on peripherally-mediated anti-inflammatory and analgesic activity. The results suggest also that the *N*-oxide alkaloids play an important role in the activity of the polar fraction extract while other constituents such as vernonioside metabolites play a role on the intermediate fraction. This latter presented some signs of anti-inflammatory activity even though they are less consistent and more heterogeneous than those for the polar fraction. The harvesting season has a significant effect on the relative abundance of the metabolites of the *V. condensata* leaves analyzed.

Acknowledgements

The authors are grateful to CAPES, CNPq and FUNDAÇÃO ARAUCÁRIA for financial support.

References

1. Bourguignon, B.; de Aguiar, P. F.; Thorre, K.; Massart, D. L.; *J. Chromatogr. Sci*. **1994**, *32* 144.
2. Ding, Y.; Chin, J.; *Appl. Prob. Stat*. **1986**, *2*, 153.
3. Choi, D. W.; Kim, J. H.; Cho, S. Y.; Kim, D. H.; Chang, S. Y.; *Toxicology* **2002**, *581*, 181.
4. O'Brien, P.; Carrasco-Pozo, C.; Speisky, H.; *Chem.-Biol. Interact.* **2006**, *159*, 1.
5. Srivastava, A.; Tandon, P.; Ayala, A. P.; Jain, S.; *Vib. Spectrosc.* **2011**, *56*, 82.
6. Backhouse, N.; Delporte, C.; Givernau, M.; Cassels, B. K.; Valenzuela, A.; Speisky, H.; *Agents Actions* **1994**, *42*, 114.
7. Hidalgo, M. E.; Alarcón, M. G.; Ojeda, J. R.; Fernández, E. C.; Sobarzo-Sánchez, E. M.; De La Fuente, J. R.; *J. Braz. Chem. Soc.* **2010**, *21*, 2205.
8. Lorenzi, H.; Matos, F. J. A.; *Plantas Medicinais no Brasil: Nativas e Exóticas*, 1st ed.; Instituto Plantarum: Nova Odessa, 2002.
9. http://portalsaude.saude.gov.br/images/pdf/2014/maio/07/renisus.pdf, accessed in December 2014.
10. Frutuoso, V. S.; Gurjão, M. R. R.; Cordeiro, R. S. B.; Martins, M. A.; *Planta Med.* **1994**, *60*, 21.
11. Risso, W. E.; Scarminio, I. S.; Moreira, E. G.; *Indian J. Exp. Biol.* **2010**, *48*, 811.
12. Valverde, A. L.; Cardoso, G. L. C.; Pereira, N. A.; Silva, A. J. R.; Kuster, R. M.; *Phytother. Res*. **2001**, *15*, 263.
13. Siedle, B.; García-Piñeres, A. J.; Murillo, R.; Schulte-Mönting, J.; Castro, V.; Rüngeler, P.; Klaas, C. A.; Costa, F. B.; Kisiel, W.; Merfort, I.; *J. Med. Chem*. **2004**, *47*, 6042.
14. Sakamoto, H. T.; Gobbo-Neto, L.; Cavalheiro, A. J.; Lopes, N. P.; Lopes, J. L. C.; *J. Braz. Chem. Soc.* **2005**, *16*, 1396.
15. Monteiro, M. H. D.; Gomes-Carneiro, M. R.; Felzenszwalb, I.; Chahoud, I.; Paumgartten, F. J. R.; *J. Ethnopharmacol*. **2001**, *74*, 149.
16. Soares, P. K.; Bruns, R. E.; Scarminio, I. S.; *J. Sep. Sci.* **2009**, *32*, 644.

17. Souza, E. B. R.; Silva, R. R.; Afonso, S.; Scarminio, I. S.; *J. Sep. Sci.* **2009**, *32*, 4176.
18. Di Stasi, L. C.; *Plantas Medicinais: Arte e Ciência - Um Guia de Estudo Interdisciplinar*, 1ª ed.; Fundação Editora UNESP: São Paulo, 1996.
19. Snyder, L. R.; Carr, P. W.; Rutan, S. C.; *J. Chromatogr. A.* **1993**, *656*, 537.
20. Delaroza, F.; Scarminio, I. S.; *J. Sep. Sci.* **2008**, *31*, 1034.
21. OECD (Organization for Economic Cooperation and Development) *Guideline for Testing of Chemicals: Acute Oral Toxicity – Acute Toxic Class Method*, Guideline 423: Paris, 2001.
22. Qin, X.-q.; Yuan, Y; Liu, C.-s.; Wang, Q.-y.; Shen, X; Yang, B.-c.; *Acta Pharmacol Sin.* **2007**, *28*, 1851.
23. Lisboa, S. F. S.; Oliveira, P. E.; Costa, L. C.; Venâncio, E. J.; Moreira, E. G.; *Pharmacology* **2007**, *80*, 49.
24. BRASIL, Agência Nacional de Vigilância Sanitária - *Farmacopéia Brasileira: Monografias*, 5ª ed.; Brasília, 2010.
25. Stuart, B. H. *Infrared Spectroscopy: Fundamentals and Applications*, John Wiley & Sons Ltda: Chichester, 2004.
26. Bosi, C. F.; Rosa, D. W.; Grougnet, R.; Lemonakis, N.; Halabalaki, M.; Skaltsounis, A. L.; Biavatti, M. W.; *Rev. Bras. Farmacogn.* **2013**, *23*, 425.
27. Luo, X.; Oyugi, D. A.; Lin, C.; Izevbigie, E. B.; Lee, K. S.; *Exp. Biol. Med.* **2010**, *235*, 1472.

Submitted: September 17, 2014
Published online: December 5, 2014

FAPESP has sponsored the publication of this article.