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Global Yields, Chemical Compositions, and Antioxidant activities of extracts from *Achyrocline alata* and *Achyrocline satureioides*

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ABSTRACT - Extracts from leaves and thin branches of *Achyrocline alata* and *Achyrocline satureioides* were obtained by supercritical fluid extraction, hydrodistillation, and low-pressure ethanol extraction. The supercritical extractions were done at pressures of 100, 200, and 300 bar and temperatures of 30 and 40 oC. The chemical constituents of the extracts and volatile oil were identified by GC-MS and quantified by GC-FID. The antioxidant activity was determined using the coupled oxidation of linolenic acid and β-carotene. Larger yields were detected at 200 bar and 30^0C. Low-pressure ethanol extraction yield was 2.96% (m/m, dry basis) for *A. satureioides* and 3.98% (m/m, dry basis.) for *A. alata*. The major compounds of the extracts were trans-caryophyllene and α-humulene; the content of trans-caryophyllene in the *A. alata* extract obtained at 200 bar and 30^0 C was 5 times larger than that of the *A. satureioides* extracts. All extracts exhibited antioxidant activity stronger than β -carotene.

KEYWORDS: *Achyrocline alata, Achyrocline satureioides*, antioxidant activity, carbon dioxide, low-pressure ethanol extraction, supercritical fluid extraction, volatile oil, oleoresin

INTRODUCTION

The *Achyrocline* genus embodies several species widely spread in several Latin America countries. *A. alata* and *A. satureioides* are especially important because of flavones, flavonones, and flavonoids associated with several functional properties, such as antioxidant, anti-inflammatory, antiulcerative, antihepatoxic, vasodilatatory, antiviral, and antiplatelet activities (1 – 12). Kadarian et al (1) concluded that the treatment of mice with the aqueous extract of *A. satureioides* seemed to preserve the integrity of liver cells. The improvement in hepatic injury and liver functions by *A. satureioides* may be due to the presence of flavonoids. Other studies were carried to evaluate the biological activity of extracts from *A. satureioides*, such as cytotoxic activity against a human hepatocellular carcinoma (2), antioxidant activity (2), and glucose reduction in the blood (3).

Bohlmann et al (5) shown that the aerial parts of *A. alata* contain squalene, caryophyllene, copaene, 5,6-dihydroxy-3,7-dimethoxyflavone and the geranyl-phloroglucinols (7 and 8). Extracts of *A. alata* and *A. satureioides* contained flavonols, flavonones, caffeic acid, and esters (5, 6, 7, 8, 9, 10); the flavone apigenin was also detected in *A. alata* (7).

Polysaccharides were also identified in *A. satureioides* and associated to the anti-inflammatory activity and a strong enhancement of phagocytosis in vivo (11). Rocha et al (12) concluded that *A. satureioides* extracts might change renal ion transport, based on their observations that it affects gastrointestinal reabsorption.

Rodrigues et al (13) determined the composition of the volatile oil obtained from leaves and flowers of *A. alata*. The leaves and flowers of *A. alata* were harvested from 7 a.m. to 2 p.m., every hour. The volatile oil yield varied from as low as 2.2% (dry basis – d.b.) to as high as 12.4% (d.b.). α-pinene, 1-octen-3-ol, 1,8 cineol, β-caryophyllene, α-humulene, and bicyclogermacrene were detect in the volatile oils. α-humulene and β-caryophyllene were the two major compounds with contents varying from 19 – 25% and 12 – 17%, respectively.

Flowering of *A. alata* and *A. satureioides* occur only once a year nonetheless, the leaves can be harvested throughout the year. Therefore, the objectives of the present work were to investigate the uses of leaves and thin branches of *A. alata* and *A. satureioides*. More specifically, the objectives were (i) to determine the yield in extracts from leaves and thin branches of *A. alata* and *A. satureioides* by hydrodistillation (HD), low-pressure ethanol extraction (LPEE), and supercritical fluid extraction (SFE), (ii) to determine the influence of temperature and pressure in the content of the major constituents of the extracts obtained by SFE, and (iii) to evaluate the antioxidant activity of the various extracts.

MATERIALS AND METHODS

Raw material characterization and preparation

The leaves and thin branches from *A. alata* and *A. satureioides* were obtained from cultivars developed in a breeding program conducted at CPQBA and cultivated in the experimental field (Campinas, Brazil). The plants were in the 6^{th} generation of massal selection, aiming to produce not dormant seeds, erect and vigorous plants and good flowering yields. The leaves and thin branches were collected in September 18, 2003 at 9 a.m. and dried in a tray drier with air circulation (Fabber, model 170, Piracicaba, Brazil) at 40°C for 24 hours. The dried leaves and thin branches were packed in plastic bags and stored at –16°C. Before use, the dried leaves and thin branches were cut with a scissor. The leaves and thin branches' humidities were 10.1% for *A. alata* and 10.2% for *A. satureioides* and were determined using a microwave humidity analyzer (CEM, Model Smart 5, Matthews, NC).

Extraction techniques: SFE, HD, and LPEE

The total amount of soluble material (X_o) at given a temperature and pressure was determined using a Speed SFE system (Applied Separations, Inc., model 7071, Allentown, PA) equipped with a 5 mL extraction cell (Thar Designs, Pittsburgh, PA). Approximately 2.00 g of *A. alata* and *A. satureioides* were used; the beds average apparent densities were 382 kg/m^3 and 366 kg/m^3, respectively. The CO_2 (99.8%, Gama Gases Industriais, Campinas, Brazil) was used was admitted into the system at 7×10^{-5} kg/s. The extraction time was 1 hour and the assays were replicated. The amount of the CO_2-soluble material was calculated as the ratio of the total mass of extract to the total initial dry mass of *A. alata* or *A. satureioides*. The experiments were run at 30 and 40 °C and pressures of 100, 200 and 300 bar. The assays were replicated. The hydrodistillation (HD) procedure was described by Rodrigues et al (13): Approximately 4.5 g (d.b.) of comminuted leaves and thin branches and 150 mL of distilled water were added to the micro hydrodistillation apparatus, the volatile oil was collected in 25 mL of dichloromethane (Merck, purity ≥ 99.5%, lot K24900450 809, Darmstadt, Germany). After 1 hour of distillation, the organic phase was separated and extracted with 20 mL of dichloromethane and dried with anhydrous sodium sulfate (Synth, 99.0% purity, Lot 26072, Diadema, Brazil). This solution was concentrated using a rotovap (Heidolph Instruments, model Laborota 4001, Germany) with a vacuum controller (Heidolph Instruments, model Rotavac Control, Germany) at 40°C.

The low-pressure ethanol extraction (LPEE) was done using 0.5 g of dried leaves and thin branches and 4 mL of ethanol (Merck, purity ≥ 99.8%, lot K3186583 312, Darmstadt, Germany). The methodology of LPEE was described by Pesek et al (14): The dry raw material and solvent were centrifuged during 5 min at 2000 rpm. Then the extracts were static during 30 min. The solvent was removed using a rotovap (Heidolph Instruments, model Laborota 4001, Germany) with a vacuum controller (Heidolph Instruments, model Rotavac Control, Germany) at 40°C.

Characterization of SFE extracts (GC-EM and GC-FID)

The extracts were analyzed in a GC-MS system (Hewlett Packard 5980, Palo Alto, CA) equipped with a fused silica capillary column (30 m × 0.25 mm × 0.25 μm, HP-5, Palo Alto, CA). The electron impact technique (70 eV) was used. The carrier gas was helium (1mL/min; 99.99% purity, White Martins Gases Industriais, Campinas, Brazil); split 1:40 was used. The temperatures of the injector and detector were 220 and 250°C, respectively. The temperature program was 60°C to 240°C at 3°C/min. One microliter of the samples was injected (10 – 15 mg/mL of extract). The identification of the substances was based on (i) comparison of substance mass spectrum with GC-MS system data bank (Wiley Library); (ii) comparison of mass spectra with data in literature (*15*), and (iii) retention index (16). All extracts were analyzed in a GC–FID system (Shimadzu, QP- 5000, Kyoto, Japan), equipped with a fused silica capillary column DB-5 (30 m × 0.25 mm × 0.25 μm, J & W Scientific, Folsom, CA). The carrier gas was helium (1.7 mL/min.; 99.99% purity, White Martins Gases Industriais, Campinas, Brazil); a sample split ratio of 1:20 was used. The temperatures of the injector and detector were 240 and 230ºC, respectively. The column was programmed

at 3ºC/min. 60°C to 240°C. One microliter of the samples was injected (5 μL of extract diluted in 1 mL ethyl acetate of chromatographic grade, EM Science, lot 3903991).

Antioxidant activity

The antioxidant activity (AA) of extracts was evaluated using the coupled oxidation of linolenic acid and β-carotene. The methodology of Hammerschmidt and Pratt (17) was used with the required modifications 18. The reaction substrate was prepared using 10 mg of β-carotene (Acros, 99%, lot 40415-0010, New Jersey, NJ), 10 mL of chloroform (Merck, 99.0 – 99.4% PA, lot K31503045 301, Darmstadt, Germany), 60 mg of linolenic acid (Sigma - Aldrich, 99%, lot L2376, St. Louis, MO), and 200 mg of Tween 40 (Sigma - Aldrich, 99%, lot 032K0104, St. Louis, MO). This solution was concentrated using a rotovap (Heidolph Instruments, model Laborota 4001, Germany) with a vacuum controller (Heidolph Instruments, model Rotavac Control, Germany) at 50°C and afterwards diluted with 50 mL of distilled water. The oxidation reaction was conducted using the following procedure: to 1 mL of substrate was added 2 mL of distilled water and 0.05 mL of extract diluted in ethanol (Merck, 99.8% PA, lot K31865683, Darmstadt, Germany) (0.02 g of extract in 1 mL of ethanol). The mixture was set into a water bath (Tecnal, TE 159, Piracicaba, Brazil) at 40°C, and the reaction product was monitored using a spectrophotometer (Hitachi, U-3010, Tokyo, Japan) at 0, 1, 2, and 3 h of reaction, by taking absorbance readings at 470 nm. The antioxidant activity was calculated by Eq. 1.

$$AA = 100 \times \left(1 - \frac{abs_{extract}^{t=0} - abs_{extract}^{t}}{abs_{control}^{t=0} - abs_{control}^{t}} \right)$$

where *abs*: absorbance at 470 nm (Equation 1)

RESULTS AND DISCUSSION

The 30 and 40°C global yields for the systems *A. alata* + CO_2 and *A. satureioides* + CO_2 are in Table 1. Both systems showed the same behavior; the maximum yields were obtained at 30°C at the pressure of 200 bar. The global yields (X_o) from *A. alata* were systematically larger than that of *A. satureioides*. For the range of conditions used, the inversion pressure is above 250 bar. The global yield is closely related to the solubility of a solute (the mixture that forms the extracts) in the supercritical solvent (19). Therefore, the effects of the solvent density over the solubility of the solute in the solvent will be important at pressures below 250 bar, while, above this value, the effects of the solute's vapor pressure will predominate. Considering the yield, at 30°C the best pressure of extraction was 200 bar and at 40°C it was 300 bar. The Analysis of Variance (ANOVA) indicated that the effects of temperature, pressure, and the interaction of these variables explained 91.2 and 82.1% of the variations in the global yields for *A. alata* and *A. satureioides*, respectively; the standard deviation estimated by the model was 0.4 for both systems. The effects of temperature, pressure, and the interactions of these variables on the global yields were significant (p_{value} = 0.01, 0.003, 0.03, respectively) for *A. alata*. For *A. satureioides*, the effects of pressure on global yields were significant (p_{value} = 0.01) while the effects of temperature and the interactions of temperature and pressure were not significant (p_{value} = 0.16 and 0.22, respectively) while. LPEE produced 2.96 % (m/m dry basis) for *A. satureioides* and 4.29 % (m/m dry basis) for *A. alata* extracts. Trans-caryophyllene and α-humulene were the major compounds of the SFE, HD and LPEE extracts; other high molecular mass substances not identified with retention times of 56 to 59 min were detected in the LPEE extracts. Tables 2 and 3 show the chemical composition (GC-MS) of volatile oil or HD extracts of *A. alata* and *A. satureioides*. Trans-caryophyllene and α-humulene were the major constituents of the volatile oil. In the A. alata volatile oil three substances with retention times of 27.234, 31.934, and 32.587 min. and molecular masses of 204, 222, and 222 were not identified. In the A. satureioides volatile oil two compounds with retention times of 31.92 and 32.544 min. and molecular mass of 222 were not identified.

The amounts of trans-caryophyllene in the *A. alata* extracts were larger than the amounts of α-humulene for every SFE condition as well as for HD and LPEE. For the *A. satureioides* extracts, the amounts of these substances were approximately equal. The amount of trans-caryophyllene in the *A. alata* SFE extracts at 30°C increased from 100 to 200 bar while at 40°C it increased from 200 to 300 bar. Only traces of trans-caryophyllene were detected in the *A. satureioides* SFE extracts except at 30°C and pressures of 200 and 300 bar. α-humulene was not detected in *A. alata* and *A. satureioides* extracts at 30 and 40°C at the pressures of 100 and 200 bar. This behavior can be related to the fact that more than one inversion pressure can be present in systems formed by a mixture of terpenoids (20). Figures 1 and 2 show the antioxidant activities of *A. alata and A. satureioides* extracts obtained by HD, LPEE and SFE.

Table 1- Global yields isotherms* (%, dry basis - d.b.) for the systems: A. alata + CO_2 and A. satureioides + CO_2.

			Global yield, X_o (%, d.b.)			
Pressure, bar	ρ_{CO2}, kg / m^3 20		*A. alata*		*A. satureiodes*	
	30 °C	40 °C	30 °C	40 °C	30 °C	40 °C
100	772	629	2.6	1.3	1.4	0.5[a]
150	847	780	-	3.6	-	-
200	891	840	4.2	2.6	2.4	2.6
300	948	910	3.3	3.7	2.0	2.2

** The bed average apparent density was 366 kg/m^3 for A. satureioides and 382 kg/m^3 for A. alata and the solvent flow rate was 7 ×10-5 kg/s. The experimental error estimated as the standard deviation of the model (ANOVA), was ± 0.4.*

aWithin experimental error.

Table 2 - Chemical composition of volatile oil of HD extract of A. alata identified by GC-MS.

Rt (min)	RI	Compounds	% area
13.362	1164	borneol	0.8
14.440	1189	α-terpineol	0.8
22.541	1375	α-copaene	2.4
24.715	1425	Trans-caryophyllene	72.7
25.952	1455	α-humulene	8.5
26.842	1476	γ-muurolene	0.8
27.234	1485	MM 204	1.1
27.608	1494	α-selinene	1.1
27.830	1499	α-muurolene	0.8
28.397	1513	γ-cadinene	0.7
28.823	1523	δ-cadinene	2.9
30.679	1569	caryophyllene alcohol	2.0
31.193	1582	caryophyllene oxide	1.1
31.934	1600	MM 222	0.6
32.587	1617	MM 222	2.3
33.494	1640	epi-α-cadinol	0.8
		Not identified	0.6

aRt: retention time; RI: retention index.

Table 3 - Chemical composition of volatile oil (HD extract) of A. satureioides identified by GC-MS.

Rt (min)	RI	Compounds	% area
8.181	1030	1,8 cineole	1.5
13.367	1164	borneol	8.2
14.437	1189	α-terpineol	6.0
16.819	1244	---	4.3
24.407	1418	Trans-caryophyllene	27.7
25.842	1452	α-humulene	9.5
28.788	1523	δ-cadinene	2.1
30.649	1568	caryophyllene alcohol	2.5
31.92	1600	MW 222	2.9
32.544	1616	MW 222	3.6
33.14	1631	---	1.3
33.488	1640	epi-α-cadinol	2.8
34.001	1654	α-cadinol	2.0
		Not identified	25.6

aRt: retention time; RI: retention index.

Table 4 shows the contents (GC-FID) of trans-caryophyllene, α-humulene, and other high molecular mass substances not identified with retention times of 54.3, 56.3, and 59.2 min for the LPEE and SFE extracts.

Table 4 - Content (area percent) of trans-caryophyllene (t-C), α-humulene (α-H) and unidentified substances ($C_{NI,1}$, $C_{NI,2a}$, $C_{NI,2s}$, and $C_{NI,3}$) determined by GC for SFE, LPEE and HD extracts of A. alata and A. satureioides.

SFE Conditions		*A. alata*				*A. satureioides*			
P, bar	**T, °C**	t-C	α-H	$^{b}C_{NI,1}$	$^{c}C_{NI,2a}$	t-C	α-H	$^{d}C_{NI,2s}$	$^{e}C_{NI,3}$
100	30	12.3	[a]tr	41.8	62.2	tr	tr	nd	18.3
100	40	24.4	5.0	nd	29.0	tr	tr	21.8	nd
200	30	25.7	1.9	27.1	50.8	4.2	4.4	60.0	nd
200	40	17.9	tr	nd	39.4	tr	tr	52.8	nd
300	30	18.9	4.2	16.1	53.1	9.5	6.7	51.4	nd
300	40	31.9	6.6	nd	42.1	tr	tr	67.3	nd
HD		72.7	8.5	nd		27.6	9.5	nd	nd
LPEE		30.3	3.9	5.1	55.6	3.0	2.6	51.1	0.9

atr: relative proportion smaller than 2.6%
bCNI, 1: Substance not identified with retention time of 54.3 min.
cCNI,2a: Substance not identified with retention time of 56.3 min.
dCNI,2s: Substance not identified with retention time of 56.3 min.
eCNI,3: Substance not identified with retention time of 59.2 min.
nd: not detected.

Figures 3 - 6 relate the antioxidant activities of the extracts with the content of trans-caryophyllene and α-humulene and the SFE conditions. The antioxidant activity of SFE and LPEE extracts did not show straightforward relation with the contents of trans-caryophyllene and α-humulene. Other compounds maybe related with the antioxidant activity of these extracts; possibly, the high molecular mass compounds not identified that were detected in the SFE and LPEE extracts, but not in the HD extracts.

This study showed that extracts from leaves and thin branches of *A. alata* and *A. satureioides* obtained by SFE, LPEE, and HD contain two major volatile compounds (trans-caryophyllene and α-humulene); high molecular mass substances with retention times of 56 to 59 min. were present in the SFE and LPEE extracts but not in the HD extracts. The contents of the trans-caryophyllene and α-humulene in the extracts obtained by hydrodistillation were greater than that in the SFE and LPEE extracts. The antioxidant activity of SFE and LPEE extracts were

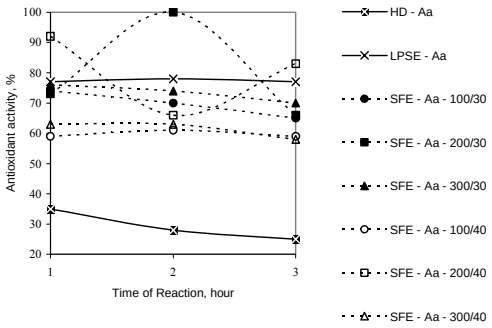

Figure 1: Antioxidant activities of A. alata SFE extracts as a function of reaction time.

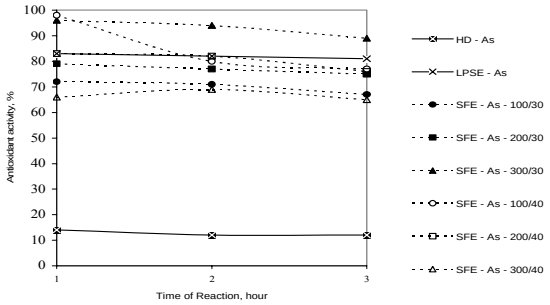

Figure 2: Antioxidant activities of A. satureioides SFE extracts as function of reaction time

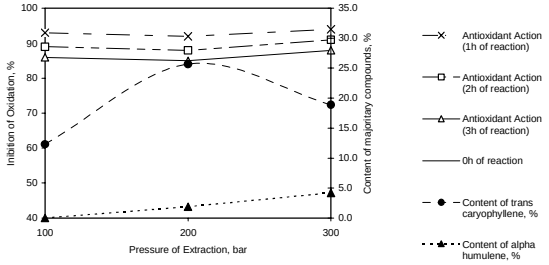

Figure 3: Antioxidant activities as a function of trans-caryophyllene and α-humulene content and the extraction pressure for the A. alata SFE extracts obtained at 30 °C.

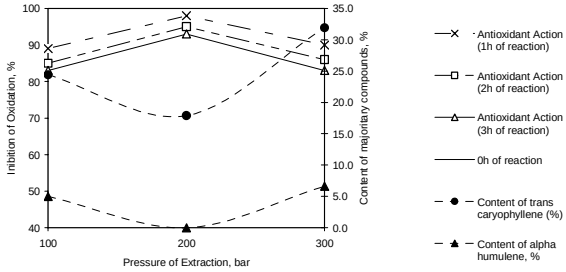

Figure 4: Antioxidant activity as a function of trans-caryophyllene and α-humulene contents and extraction pressure for the A. alata SFE extracts obtained at 40 °C.

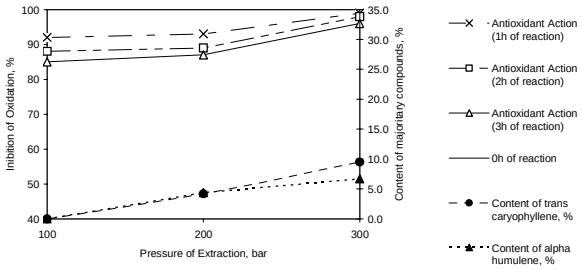

Figure 5: Antioxidant activity as a function of trans-caryophyllene and α-humulene contents and extraction pressure for the A. satureioides SFE extracts obtained at 30^{0}C 30 °C.

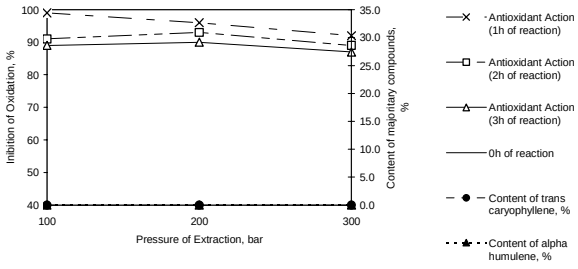

Figure 6: Antioxidant activity as a function of trans-caryophyllene and α-humulene contents and the extraction pressure for the A. satureioides SFE extracts obtained at 40 °C.

twice larger than that of the volatile oil (HD), this maybe associated with other phenolic compounds such as flavonoids. The extracts of *A. alata* and *A satureioides* exhibited antioxidant activity stronger than β-carotene; nonetheless, the correlation between antioxidant activity and content of trans-caryophyllene and α-humulene could not be established.

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