Revista Brasileira de Farmacognosia
Brazilian Journal of Pharmacognosy
21(6): 936-942, Nov./Dec. 2011

Article

Received 4 Oct 2010
Accepted 10 Jan 2011
Available online 26 Aug 2011

Keywords:
aerial parts
Ageratum fastigiatum
Asteraceae
essential oils
Eupatorieae

ISSN 0102-695X
http://dx.doi.org/10.1590/S0102-695X2011005000151

Contribution for the phytochemical studies of *Ageratum fastigiatum*

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Abstract: Organic extracts from leaves plus branches plus inflorescences of *Ageratum fastigiatum* (Gardner) R. M. King & H. Rob., Asteraceae, were fractionated through classic chromatography. The steroids stigmasterol, chondrillasterol and campesterol were isolated from hexane extract. The triterpenes lupeol, taraxasterol, α-amyrin, β-amyrin, pseudotaraxasterol, lupeol acetate and α-amyrin acetate were isolated from ethyl acetate extract. Steroids and triterpenes were identified by GC-MS. The coumarin ayapin was isolated from ethanol extract and identified by NMR. Essential oils of the fresh leaves and fresh inflorescences were obtained by hydrodistillation and analyzed for GC-MS. The main components in both essential oils were α-pinene, limonene and germacrene D.

Introduction

Species *Ageratum fastigiatum* (Gardner) R. M. King & H. Rob., Asteraceae, can be found in antropogenically disturbed areas and, more frequently, in natural areas of the plateau of Diamantina city in Minas Gerais State (Serra do Espinhaço), paths of Triângulo Mineiro, Serra da Mantiqueira, among other Brazilian places (Guimarães et al., 2002; Almeida et al., 2004).

The plant is used in popular medicine of several regions known as "matapasto" (Del-Vechio-Vieira et al., 2008). In Diamantina is also denominated "enxota" by the healers who prepare extract using leaves and branches (with or without inflorescences) fresh crushed or torn and submitted to maceration with water for some hours. The aqueous extract, after filtration, is applied topically to treat pain and inflammations (Gonçalves et al., 2007).

Phytochemical studies accomplished with this medicinal species indicated the presence of triterpenes, diterpenes, sesquiterpenes and coumarins in organic extracts from roots and aerial parts of the plant (Bohlmann et al., 1981; Bohlmann et al., 1983). The analysis of essential oils of this species revealed the sesquiterpenes prevalence; however, qualitative and quantitative differences are observed in their composition when derived from different tissues or individuals (Del-Vechio-Vieira et al., 2009a; Del-Vechio-Vieira et al., 2009b). Those variations in secondary metabolites are influenced by intrinsic factors (genetics, development, growth, vegetable organ) and extrinsic (atmospheric conditions, geographic region, soil type, presence of predators or pathogens, among other environmental factors) to plants (Lima et al., 2003; Castro et al., 2004; Gobbo-Neto & Lopes, 2007). Studies on the biological potential of essential oils and polar extracts of *A. fastigiatum* revealed antimicrobial, analgesic and anti-inflammatory activities (Del-Vechio-Vieira et al., 2007; Del-Vechio-Vieira et al., 2009a; Del-Vechio-Vieira et al., 2009b). In spite of other published works, variations in metabolism reported in these researches, and because it is a medicinal plant, stimulate the restudy of the chemical composition of *A. fastigiatum,* collected in Diamantina region.

Materials and Methods

Plant material

For the obtaining of organic extracts, aerial parts (branches with leaves plus inflorescences) of

Ageratum fastigiatum (Gardner) R. M. King & H. Rob., Asteraceae, were collected in December of 2006, in JK Campus UFVJM (S 18º12.220´ W 43º34.720´, altitude 1250 m), in the city of Diamantina-MG. Another collection of aerial parts was accomplished in April of 2008, at the same site, in the morning (before 9:00 o'clock), for extraction of essential oil. Voucher specimen was deposited in the DIA Herbarium/ UFVJM (Diamantina-MG, Brazil), under the No. 1300 and their identification was made by Dr. Carlos Victor Mendonça Filho and Dr. Fabiane Nepomuceno Costa (Departamento de Ciências Biológicas/FCBS/ UFVJM).

Preparation of organic extracts

Aerial parts of *A. fastigiatum* were dried in the shade at room temperature until constant weight and later triturated. The dried powdered material (500 g) was macerated successively with *n*-hexane, ethyl acetate and ethanol (3:1 w/v - solvents Vetec analytical degree) at room temperature for three days in each solvent. Each macerate was separated by filtration and concentrated under vacuum in a rotatory evaporator Fisatom 801 (below 40 °C) to give the dried crude extracts: 7.03 g (hexane extract), 20.10 g (ethyl acetate extract) and 16.12 g (ethanol extract).

General experimental procedures for isolation of constituents from the organic extracts

In the processes of classic column chromatography (CCC) was used as stationary phase the silica gel 60 (35-70 mesh - Vetec) and as mobile phase the *n*-hexane, ethyl acetate, ethanol or methanol (Vetec or Dinâmica) and mixtures of those solvents in a growing gradient of polarity.

The processes of fractionation of the crude extracts, as well as their fractions, were monitored by comparative thin layer chromatography (TLC) having as objectives the observation of the purity degree of products of fractionation and detection of fractions with similar chromatographic profiles, for its subsequent combination. For both, were used chromatoplates of aluminum covered with silica gel 60 with fluorescence indicator (Macherey-Nagel - UV_{254}), several eluents systems and different revealing agents (Wagner & Bladt, 1996).

Isolation and identification of constituents from the organic extracts

The hexane extract (4.50 g) was fractioned through CCC giving 164 fractions of 30 mL, that were combined into seventeen fractions (Ia-XVIIa). The fraction IXIa (87 mg), obtained by grouping of fractions 53 to 59 (eluted with hexane/ethyl acetate 8:2) was submitted to CCC. From the preparative chromatography resulted 49 fractions of 5 mL, pooled into fifteen fractions (Ib-XVb). Of those fractions, Xb (9 mg), resulting from the combination of fractions 10 and 11 (eluted with hexane/ethyl acetate 7:3), was analyzed through a chromatography system in gaseous phase coupled to a mass spectrometry (GC-MS). The analysis was performed using Shimadzu-QP2010 equipment, with capillary column DB-5MS Agilent (30 m x 0.25 mm, film thickness 0.25 µm), under the following conditions: Helium was used at pressure of 182.20 kPa, with flow of 1.50 mL/min; temperature in the injector was 260 °C; the initial temperature of the column was 250 °C staying for 12 min, increasing to 280 °C (6 °C/min) and being conserved by 20 min. The ionization mode used was the electronic impact at 70 eV. The 5-α-cholestane (Sigma) was used as internal standard, being calculated the relative retention (RR) of each constituent of the analyzed mixture (McNair & Bonelli, 1969). The constituents of the sample were identified through the analysis of their mass spectra and for comparison of their RR with RR standards of steroids (Supelco). The quantification of each constituent of the mixture was accomplished through the relative area of the chromatogram peaks.

The ethyl acetate extract (20.10 g) was suspended in 400 mL of a mixture of methanol/distilled water 4:1, being filtered and being obtained a solid residue (5.12 g). The hydroalcoholic filtrate was submitted to partition with 3 x 100 mL *n*-hexane (Vetec) to separate non-polar compounds that could be present in it. Soon, the hydroalcoholic phase was partitioned with 3 x 100 mL chloroform (Vetec). Thus were obtained the hexane, chloroform and hydroalcoholic phases. The chloroform and hexane phases were concentrated in rotatory evaporator (around 40 °C) obtaining dried material: 721 mg and 11.18 g, respectively. The hydroalcoholic phase was concentrated in rotatory evaporator at 40 °C (the methanol was removed) and then it was lyophilized (lyophilizer L101/Liotop), obtaining 3.08 g of dried material. Ten grams of chloroform phase were chromatographed (CCC), resulting in 143 fractions of 40 mL, pooled into nine fractions (Ic-IXIc). The fraction VIc (431 mg), resulting from the combination of the fractions 76 to 81 (eluted with hexane/ethyl acetate 2:8), was fractioned by CCC, resulting in 105 fractions of 15 mL. The chromatographic analyses (TLC) of those 105 fractions resulted in the grouping into eight fractions (Id-VIIId). The fraction VId (25 mg), resulting from the combination of the fractions 60 to 76 (eluted with ethyl acetate), was analyzed by GC-MS. The analysis was performed in Shimadzu-QP2010 equipment, with capillary column DB-17 Agilent (30

m x 0.25 mm, film thickness 0.25 μm), under the following conditions: Helium was used at pressure of 114.10 kPa, with flow of 1.40 mL/min; the temperature in the injector was of 260 °C; the initial temperature of the column was 120 °C, rising to 260 °C to 20 °C/min (staying 5 min), increasing to 280 °C to 2 °C/min (being conserved by 9 min), arriving up to 290 °C to 2 °C/min (staying for 20 min). The ionization mode used was the electronic impact at 70 eV. The 5-α-cholestane (Sigma) was used as internal standard. The constituents of the sample that were identified through the analysis of their mass spectra and by comparison of their RR calculated with RR standards of triterpenes (isolated from extracts of several plants which were identified through NMR^{1}H and ^{13}C presented good degree of purity). The quantification of each constituent of the mixture was accomplished through the relative area of the chromatogram peaks.

The preparative chromatography (CCC) of the ethanol extract (9,30 g) yielded 222 fractions of 40 mL, grouped into eighteen fractions (Ie-XVIIIe). The fraction VIIIe (129 mg), resulting from combination of the fractions 60 to 68 (eluted with hexane/ethyl acetate 7:3), was submitted to recrystallization with hexane and drops of ethyl acetate and ethanol. From fraction VIIIe were obtained 30 mg of colorless crystals, analyzed by NMR. The NMR^{1}H (400 MHz) and NMR^{13}C (100 MHz) spectra were recorded on Brucker-Advance DPX400 spectrometer, with DMSO-d_6 (Aldrich) as solvent and TMS (Merck) as internal reference.

Essential oil extraction and GC-MS analysis

Fresh leaves (483.45 g) and fresh inflorescences (296.26 g) were submitted separately to hydrodistillation and cohobation in Clevenger apparatus for 3.5 h. After extraction, each essential oil was separate from water by decantation and was desiccated through slow percolation in simple filtration system containing Na_2SO_4 anhydrous (Synth). The essential oils were maintained under low temperature and protected from light until the moment of analysis.

The GC-MS analyses were carried out on a Shimadzu GC-MS-QP2010 gas chromatography-mass spectrometer equipped with capillary column DB-5-MS Agilent (30 m x 0.25 mm, film thickness 0.25 μm) under the following conditions: Helium was used as the carrier gas at pressure of 81.90 kPa, with flow of 1.33 mL/min; the temperature in the injector was 250 °C; the temperature of the oven progressed from 60 to 240 °C to 3 °C/min. The ionization mode used was the electronic impact at 70 eV. Later, under the same experimental conditions, each oil was coinjected with a homologous series of linear hydrocarbons (C_9-C_{25})-Alltech, to accomplishment calculations of the retention index (RI) of each constituent of the samples applying Van Den Dool & Kratz Equation (1963). The compounds identification was performed by analysis and comparison of the mass spectra with database of Wiley 7 library and by comparison of RI with those of the literature (Adams, 1995; Tellez et al., 1999; Araujo et al.; 2003; Bin Ahmad & Bin Jantan, 2003; Choi, 2003; Hognadottir & Rouseff, 2003; Kariot et al., 2003; Priestap et al., 2003; El-Sayed et al., 2005; Sylvestre et al., 2006; Machado et al., 2007; Morteza-Semnani et al., 2007; Torres et al., 2008; Brasil et al., 2009; Chaichana et al., 2009; Moghtader, 2009; Lamien-Meda et al., 2010). The relative quantification of the components of each sample was obtained through the relative area of the peaks in the chromatograms.

Results and Discussion

Eleven compounds were obtained from organic extracts from aerial parts (leaves plus branches plus inflorescences) of *A. fastigiatum*: three compounds from

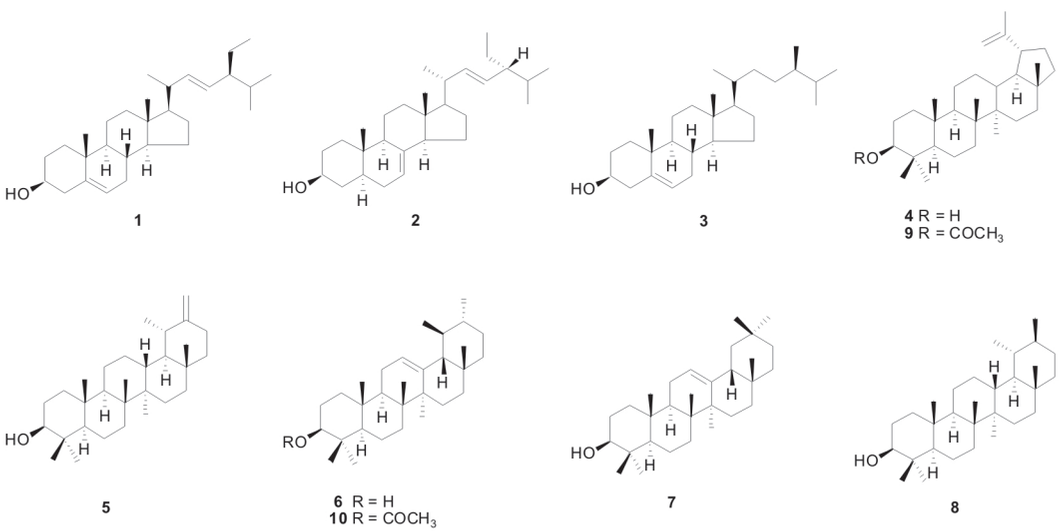


Table 1. Relative retention (RR) of steroids standards, relative retention and relative abundance of steroids isolated from the hexane extract of the aerial parts of *Ageratum fastigiatum.*

Steroid	RR of steroids standards[a]	t_R (min)[b] of steroids isolated from *A. fastigiatum*	RR of steroids isolated from *A. fastigiatum*[a]	Relative abundance (%) of steroids isolated from *A. fastigiatum*
campesterol	1.641	19.667	1.641	0.42
stigmasterol	1.691	20.324	1.695	55.92
chondrillasterol	1.806	21.686	1.809	39.64

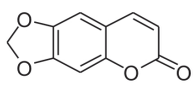
[a]internal standard used to calculate RR: 5-α-cholestane, with t_R=11.984 min. [b]t_R: retention time in minutes.

hexane extract, seven compounds from ethyl acetate extract and one compound from ethanol extract.

The fractionation of the hexane extract led to the isolation and the identification of three steroids in mixture, **1-3**. The stigmasterol **1** was the majority constituent (55.92%), following for the chondrillasterol **2** (39.64%) and by campesterol **3** (0.42%). RRs of the components of the mixture compared RRs of steroids standards found in the Table 1. In the hexane extract were identified 95.98% of the mixture constituents. This is the first study which identifies steroids in organic extract of *A. fastigiatum*.

The chloroform phase, obtained from the partition of the ethyl acetate extract, after successive preparative chromatography yielded a mixture of seven triterpenes **4-10**, identified through GC-MS and RR obtained by GC (Table 2). The lupeol **4**, major component of mixture (51.06%), was previously identified in extracts of aerial parts of the plant (Bohlmann et al., 1981; Bohlmann et al., 1983), as well as the taraxasterol **5** (Bohlmann et al., 1983) that in our study presents relative quantification of 2.01%. All other identified triterpenes in the ethyl acetate extract were not detected in previous studies accomplished with that vegetable species: α-amyrin **6** (30.39%), β-amyrin **7** (5.75%), pseudotaraxasterol **8** (0.71%), lupeol acetate **9** (0.38%) and α-amyrin acetate **10** (0.21%). In the ethyl acetate extract were identified 90.51% constituents of the mixture. The triterpenes found are characteristic of the Asteraceae family which presents in all the tribes those metabolites with oleane skeletons (such as β-amyrin), ursane (α-amyrin, taraxasterol, pseudotaraxasterol) and lupeane (lupeol) (Hegnauer, 1977; Zdero & Bohlmann, 1990).

From the preparative chromatography of the ethanol extract a coumarin was isolated and identified as ayapin or 6,7-methylene-dioxide coumarin **11**. The structure of compound **11** was elucidated as coumarin using NMR ^{1}H and ^{13}C spectral data compared with the literature (Sun et al., 2007; Canuto et al., 2010). The ayapin was previously isolated from extracts of aerial parts and roots of the plant (Bohlmann et al., 1983). In fact, chemosystematic studies indicate that coumarins presents wide occurrence in Asteraceae family, and ayapin is found in Eupatorieae tribe (Hegnauer, 1977; Zdero & Bohlmann 1990; Ribeiro & Kaplan, 2002).



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The essential oils of leaves and of inflorescences of *A. fastigiatum* obtained by hydrodistillation presented yields of 0.04 and 0.07% (m/m), respectively. That difference of 42.86% in the yield can be associated to variations in biosynthesis and/or in storage of secondary metabolites by cells of different tissues. This has been observed in some vegetable species in relation to their essential oils from

Table 2. Relative retention (RR) of triterpenes standards, relative retention and relative abundance of triterpenes isolated from the ethyl acetate extract of aerial parts of *Ageratum fastigiatum.*

Triterpene	RR[a] of triterpenes standards	t_R (min)[b] of triterpenes isolated from *A. fastigiatum*	RR[a] of triterpenes isolated from *A. fastigiatum*	Relative abundance (%) of triterpenes isolated from *A. fastigiatum*
β-amyrin	2.181	30.796	2.185	5.75
α-amyrin	2.366	33.498	2.376	30.39
lupeol	2.387	33.883	2.404	51.06
α-amyrin acetate	2.475	34.941	2.493	0.21
lupeol acetate	2.506	35.364	2.509	0.38
pseudotaraxasterol	2.664	37.627	2.669	0.71
taraxasterol	2.722	38.416	2.725	2.01

[a]internal standard used to calculate RR: 5-α-cholestane, with t_R = 14.093 min. [b]t_R: retention time in minutes.

Table 3. Retention index (RI) and relative percentage composition of the essential oil from leaves and of essential oil from inflorescences of *Ageratum fastigiatum*.

Constituents[a]	RI literatura[b]	Essential oil from leaves		Essential oil from inflorescences	
		$RI_{calculated}$	Relative amount (%)	$RI_{calculated}$	Relative amount (%)
α-thujene	923, 931[c], 933	924	0.2	931	0.18
α-pinene	927, 933, 934, 939[c], 941	932	51.27	937	41.18
camphene	952, 953[c], 954	948	0.25	948	0.18
sabinene	965, 973, 976[c], 977	971	1.80	970	0.90
β-pinene	980[c], 981, 984	977	3.23	976	5.01
myrcene	991[c], 992, 993, 994, 995	987	1.92	987	0.90
limonene	1031[c], 1036, 1039	1027	17.18	1027	16.51
trans-β-ocimene	1050[c], 1051	1043	4.68	1043	4.93
linalool	1096, 1098[c], 1104, 1107, 1112, 1127	-	-	1099	0.16
lavandulol	1166[c]	-	-	1161	0.46
terpin-4-ol	1177[c], 1178, 1181, 1198	1178	0.24	1178	0.39
α-terpinyl acetate	1350[c], 1352, 1354	-	-	1356	1.61
α-copaene	1353, 1375, 1376[c], 1377, 1391	1372	0.66	1372	1.17
trans-β-caryophyllene	1414, 1418[c], 1421, 1422, 1428, 1437, 1467	1414	3.18	1414	5.78
α-humulene	1437, 1452, 1454[c], 1472	1450	1.35	1450	1.72
germacrene D	1480[c], 1482, 1485, 1486, 1497	1476	9.09	1476	8.23
α-muurolene	1499[c], 1502, 1505	-	-	1494	0.19
germacrene A	1502, 1503[c], 1560	1502	0.10	-	-
α-amorphene	1485, 1506	-	-	1506	0.27
γ-cadinene	1512, 1513[c], 1516, 1521	1513	0.45	1508	0.11
spathulenol	1552, 1575, 1576[c], 1579	1571	0.11	1575	0.34
viridiflorol	1590[c], 1593, 1615	1615	2.33	1615	4.56
allo-aromadendrene epoxide	1641, 1642, 1646	-	-	1632	0.92

[a]Constituents ordered in agreement with the retention time in column DB-5MS [b]RI found in the literature, obtained in non-isothermal analyses, in columns DB-5MS or DB-5, under several analytical conditions. [c]RI are the same ones observed in Adams (1995).

different tissues (Koketsu et al., 1997; Botrel et al., 2009; Del-Vechio-Vieira et al., 2009b). In the specific case of essential oil of leaf origin, the yield obtained in our work is low when compared to other studies, that presented yields between 0.40 to 1.20%, also using hydrodistillation as extractive procedure (Del-Vechio-Vieira et al., 2009a; Del-Vechio-Vieira et al., 2009b). However, the variations observed among the yields might have happened because the different population and geographical origins (the vegetable was collected in São João del Rei-MG, in previous researches) and different collection periods (month and year) of materials vegetables. There aren't previously chemical studies accomplished with essential oil extracted from the inflorescences this vegetable species.

The chemical components identified in each sample of essential oil as well as their retention indexes and their relative amounts are in the Table 3. Were identified 98.04% and 95.70% of components of the essential oil of leaves and of essential oil from inflorescences, respectively. Significant quantitative differences were not observed between the major constituents of both essential oils: α-pinene, limonene, germacrene D, *trans*-β-caryophyllene, β-pinene and β-ocimene. However, qualitative differences among the minor constituents of essential oils can be observed: linalool, lavandulol, α-terpinyl acetate, α-muurolene, α-amorphene and *allo*-aromadendrene epoxide in essential oil of inflorescences, not detected in the leaves; and, the germacrene A, only observed in the essential oil of the leaves.

When the our results are compared with previous studies it can be verified the qualitative and quantitative differences in the chemical composition of

essential oils of leaves.

In research accomplished by Del-Vechio-Vieira et al. (2009a) the essential oil was obtained from dried leaves, being identified thirty chemical components, of which the majority ones were germacrene D (24.15%), the α-humulene (11.15%), the β-cedrene (10.63%) and α-pinene (9.50%). In the current study the β-cedrene was not detected, the α-humulene appear in small amount (1.35%) and the germacrene D was not majority terpene (9.09%), being the α-pinene (51.27%) the main component observed. More abundant components in our research (*trans*-β-caryophyllene, β-pinene and β-ocimene) were not detected by Del-Vechio-Vieira et al. (2009a). Among the factors that could influence those differences between the two researches we can mention the use of fresh material in our study and the use of dry leaves on the previous work.

In the second study of Del-Vechio-Vieira et al. (2009b) with essential oil extracted from fresh leaves were identified 20 terpenoids, being the majority ones of germacrene D (20.00%), β-caryophyllene (19.60%), 1,10-di-epi-cubenol (15.40%), α-humulene (4.90%). These sesquiterpenes, we find in our work the germacrene D (9.09%), β-caryophyllene (3.18%) and α-humulene (1.35%), not representing, however, the major components. Still, the most abundant components in our essential oil, such as the α-pinene (51.27%) and the limonene (17.18%), were not observed by these authors. Differences between vegetable populations and geographic origin and time for collection of leaves might have contributed to the differences observed in chemical composition of essential oils studied, among other factors.

Therefore, quantitative and qualitative chemical variations were observed in different studies of *A. fastigiatum* performed with individuals from different populations, collected in different regions and seasons. However, there is also certain constancy, at least qualitative, in the chemical composition of essential oils of leaves from this vegetable species analyzed in our work and in other studies (Del-Vechio-Vieira et al., 2009a; Del-Vechio-Vieira et al., 2009b), for example, the presence of β-caryophyllene, α-copaene, α-humulene, germacrene D, spathulenol, among others. These last three sesquiterpenes were also found in organic extract of the aerial parts of *A. fastigiatum* collected in Goiás (Bohlmann et al., 1983). Taraxasterol, lupeol and ayapin were also isolated from extracts of this vegetable species (Bohlmann et al., 1981; Bohlmann et al., 1983), which coincides with our results.

Acknowledgments

The authors are grateful to Prof. Dr. Antonio Gilberto Ferreira (Departamento de Química - UFSCar) for obtaining the NMR spectra and FAPEMIG for financial support (CDS-1645/05).

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