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Seasonal variation in the chemical composition of two chemotypes of *Lippia alba*

Angélica Ferraz Gomes[a,c], Maiara Prates Almeida[a], Mateus Freire Leite[a,d], Stefan Schwaiger[b], Hermann Stuppner[b], Maria Halabalaki[b,e], Juliano Geraldo Amaral[a], Jorge Mauricio David[c,*]

[a] *Instituto Multidisciplinar em Saúde, Universidade Federal da Bahia, 45029-094 Vitória da Conquista, BA, Brazil*
[b] *Institute of Pharmacy, Pharmacognosy, CMBI, University of Innsbruck, 6020 Innsbruck, Austria*
[c] *Instituto de Química, Universidade Federal da Bahia, 40170-280 Salvador, BA, Brazil*
[d] *Faculdade de Ciências Farmacêuticas, Universidade Federal de Alfenas, 37130-001 Alfenas, MG, Brazil*
[e] *Laboratory of Pharmacognosy and Natural Products Chemistry, School of Pharmacy, University of Athens, Greece*

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Geneposidic acid (PubChem CID: 443354)
8-*epi*-Loganin (PubChem CID:100955855)
Mussaenoside (PubChem CID: 182423)
Acteoside (PubChem CID: 5281800)
Isoacteoside (PubChem CID: 6476333)
Apigenin 7-O- glucuronide (PubChem CID: 24721579)
Luteolin 7-*O*-glucuronide (PubChem CID 528060)
Tricin 7-O-glucoronide (PubChem CID 44258271)

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ABSTRACT

Lippia alba is a popular Brazilian herb known as 'cidreira' that presents several chemotypes which exhibit different chemical profile and they are widely used as seasonings and traditional medicine. This work describes the seasonal variation of metabolites of polar extracts of carvone and linalool chemotypes, identified by GC-MS analyses of the essential oils. A methodology was elaborated in order to obtain a seasonal variation in the chemical composition of leaf employing HPLC-DAD. Acteoside, isoacteoside, geneposidic acid, 8-*epi*-loganin, mussaenoside, luteolin 7-*O*-glucoside, apigenin 7-*O*-glucuronide and tricin 7-*O*-diglucuronide have been isolated and identified for validation procedures and chromatographic analysis. Geneposidic acid was presented in all samples, in contrast to the 8-*epi*-loganin and, mussaenoside which were presented only in the carvone-chemotype. Acteoside was the major metabolite detected from July to November while tricin-7-*O*-diglucuronide was the major compound in all other months. Besides, phenylpropanoids are predominant in winter and flavonoids in summer season.

1. Introduction

Lippia alba (Verbenaceae) is a very rustic and vigorous Brazilian shrub that is widely found all over South and Central America; it is a popular herb that growing in Brazil known as 'cidreira' (Hennebelle, Sahpaz, Joseph, & Bailleul, 2008). This plant is mainly used as seasoning, drinks, infusions and as food supplement also there are many ethnopharmacological studies reporting its use for digestive disorders (Tareau, Palisse, & Odonne, 2017), to soothe vesicle ache (Zamora-Martinez & Pola, 1992), respiratory disorders (Girón, Freire, Alonzo, & Cáceres, 1991; Tareau et al., 2017) and, as a sedative and anti-hypertensive (Agra, Silva, Basílio, Freitas, & Barbosa-Filho, 2008; Matos, 1996a; Tareau et al., 2017). Besides, to date *L. alba* also presents a large amount of pharmacological studies. The subjects of experimental works dealing with this plant were performed in accordance its traditional uses such as cardiovascular (Gazola, Machado, Ruggiero, Singi, & Macedo Alexandre, 2004), neurosedative and antioxidant (Hatano, Torricelli, Giassi, Coslope, & Viana, 2012; Hennebelle, Sahpaz, Gressier, Joseph, & Bailleul, 2008; Zétola et al., 2002), analgesic and anti-inflammatory activities (Haldar et al., 2012; Viana, Vale, Rao, & Matos, 1998), antiulcerogenic activity (Pascual, Slowing, Carretero, & Villar, 2001), and anticonvulsivant action (Soares, 2001).

The composition of the *L. alba* essential oils are very variable, suggesting the existence of a high number of chemotypes and for this reason, many authors have tried to classify *L. alba's* chemotypes according to the major compound present in their essential oils (Hennebelle et al., 2008; Matos, 1996b). In Brazil, there are at least three main chemotypes named as citral, carvone and linalool types (Yamamoto, 2006). According to Matos (1996b) each different chemotype has a specific biological activity, for example, myrcene-citral

* Corresponding author.
E-mail address: jmdavid@ufba.br (J.M. David).

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chemotype is related with the tranquilizer, analgesic and, antispasmodic activities similarly, limonene-citral chemotype which showed also anxiolytic, sedative and, antispasmodic activities. On the other hand, limonene-carvone is related with mucolytic action and for gastrointestinal disorders.

Besides the chemical variation present in the different chemotypes, other factors can also interfere with the chemical variation of secondary metabolites of plants such as temperature, humidity, luminosity, altitude, pluviometry, ultraviolet radiation, soil and nutrient conditions, seasonality, circadian cycle, method of collection, drying and part of the plant used (Gobbo-Neto & Lopes, 2007; Petropoulos et al., 2018). Meanwhile, most studies dealing with chemical variability of plants have been concentrated to in commercial species from temperate regions (Gobbo-Neto & Lopes, 2007). In Brazil, there is a much smaller number of studies about native species for this purpose and there is a need to expand the study on the behavior of native species in relation to those factors.

Moreover, there are a few studies describing the polar constituents chemical variation of different *L. alba*'s chemotypes (Timóteo, Karioti, Leitão, Vincieri, & Bilia, 2015). Further, seasonality is another important factor that could be interfere the chemical composition and, consequently, their biological activities. To date, there are many publications describing the seasonal variation of the *L. alba's* essential oil (Barros, Zambarda, Heinzmann, & Mallmann, 2009; Castro, Ming, & Marques, 2002) but the seasonal variation of the compounds present in the polar extracts has not been reported yet. Bearing in mind the possible variation in terms of secondary metabolites content, this work describes possible alterations in the chemical content influenced by seasonal factor. In order to perform this study a methodology based on HPLC-DAD was developed to afford validated qualitative and quantitative data and, this method was employed to analyze the samples from the seasonal studies of non-volatile secondary metabolites *L. alba* of the linalool and carvone chemotypes.

2. Material and methods

2.1. Plant material, cultivation of seedlings and leaf extraction

The plant material used for isolation as well as analysis was cultivated at the campus of the University of Bahia, campus Vitória da Conquista and the respective voucher specimens (# 1536 specimen) are deposited at the Herbarium Mongoyós of the same university. The authors have authorization of access to Genetic heritage management council (CGEN) (# 010806/2015–6). All of the chemotypes were identified by their essential oil composition by GC–MS analyses. In order to facilitate the identification of the chemotypes in this work different letters will be used to identify them: carvone-limonene (A), linalool-eucalyptol (B).

Plant seedlings of *L. alba* linalool-eucalyptol (A) and carvone-limonene (B) chemotypes had been planted in 2013 and three individuals of each chemotype were selected to conduct the seasonal study and they are named LA 7, LA 10 and, LA 15 for specimens of chemotype-A and LA 3, LA 4 and, LA 12 to chemotype-B. Leaf samples of these individuals were obtained monthly from December 2014 to December 2015 and their respective extracts were prepared using fresh leaves with 80% ethanol as solvent using maceration method at the room temperature. The extracts were evaporated to dryness using rotary evaporator under reduced pressure and then dried with the aid of nitrogen gas up to them get constant weight. The dry extract was conditioned in desiccator with use of vacuum to avoid the absorption of water. Solution 80% ethanol was employed as solvent extractor selected due previous studies (Zétola et al., 2002) who demonstrated that *L. alba* extracts prepared at 80% ethanol solution showed the most significant sedative and myorelaxant effects among a range of hydroethanolic solutions. Furthermore, the extracts prepared with 80 and 90% ethanol solution showed the highest anticonvulsant activity at 200 mg kg^{-1} in the convulsion induced by PTZ (Pentylenetetrazol) model in mice (Soares, 2001). Ethanol also has less toxicity and causes less damage to the environment than other solvents. Therefore, the extracts prepared with 80% ethanol solution could be a suitable concentration in order to obtain a satisfactory activity.

2.2. Chemicals standard solutions

All solvents used for standards isolation were purchased from VWR International (Darmstadt, Germany). Sephadex LH-20 was purchased from Pharmacia Biotech, Uppsala, Sweden. Solvents for HPLC and thin-layer chromatography (TLC) silica gel 60 F254 plates were obtained from Merck (Darmstadt, Germany). Ultrapure water was produced by a Sartorius Arium® 611 UV water purification system (Göttingen, Germany). NMR (Nuclear Magnetic Resonance) solvents, containing 0.03% TMS (Tetramethylsilane) as internal standard, were purchased from Euriso-Top (Gif-sur-Yvette, France). Analytical standards acteoside, isoacteoside, geneposidic acid, 8-*epi*-loganin, mussaenoside, luteolin 7-*O*-glucoside, apigenin 7-*O*-glucuronide and tricin 7-*O*-diglucuronide (tricin 7-*O*-glucuronosyl-(1- > 2)-glucuronide) were isolated with a purity of 95% (HPLC) from the extracts using Silica CC (Column Chromatography), Sephadex-LH 20CC and, semi-preparative HPLC. Purities of compounds were evaluated by HPLC–DAD-ESI-MS and NMR (1D and 2D) analysis confirmed the identification.

2.3. Isolation and identification of analytical markers

Part of the extracts with A and B chemotypes (approximately one gram of each) were jointed and then solubilized in methanol. Then that this solution was centrifuged and the soluble portion was submitted to size exclusion chromatography on a Sephadex LH-20 column, using pure methanol as mobile phase. The fractions from this column were combined by TLC (10:1:1:2.5 of ethyl acetate–acetic acid–formic acid–water as mobile phase using UV-light and spray reagent vanillin/H_2SO_4 at 5%) and HPLC–DAD–ESI-MS. The fractions which containing the compounds of interest were selected for further fractionation. For the subsequent columns were used methanol–water system (1:1) and pure methanol as mobile phase depending on the solubility of the fractions used. The compounds geneposidic acid (12.0 mg), acteoside (15.0 mg), isoacteoside (8.9 mg), luteolin 7-*O*-glucoside (5.8 mg), and tricin 7-*O*-diglucuronide (22.0 mg) were isolated and purified using Sephadex column.

Apigenin 7-*O*-glucuronide (4.0 mg), 8-*epi*-loganin (7.1 mg) and, mussaenoside (18.0 mg) were isolated by semi-preparative HPLC (Dionex system model UltiMate 3000 – semipreparative HPLC system with autosampler, UV detector and fraction collector managed by a software Chromeleon®). The flavonoids were isolated from the extracts by HPLC employing a semi-preparative column C-18 (Phenomenex Synergy 4μ Polar RP 80A 250 × 10.00 mm) whose oven temperature was set to 45 °C. The mobile phase consisted of a mixture of 0.2% acetic acid solution and acetonitrile 75:25, respectively. An isocratic elution mode with flow rate 2.5 ml min^{-1}, injection volume was 100 mL and the detection at λ = 254 nm was employed. The sample was solubilized at the concentration of 1 mg/mL and injected several times, collecting the major peak. For the iridoids it was used the same parameters except the mobile phase that consisted of water and acetonitrile (83:17) and the wavelength UV. In this case, two peaks were collected which the first one corresponded to 8-*epi*-loganin and the second one to mussaenoside.

2.4. Instrumental conditions

2.4.1. NMR experiments

For structure elucidation 1- and 2D NMR experiments were carried out by NMR Bruker Ultrashield plus 600 (Bruker Biospin, Rheinstetten, Germany) operating at 600.19 MHz (^{1}H) and 150.91 MHz (^{13}C) at