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Interconverting flavanone glucosides and other phenolic compounds in *Lippia salviaefolia* Cham. ethanol extracts

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ABSTRACT

Four interconverting flavanone glycosides [(2*R*)- and (2*S*)-3′,4′,5,6-tetrahydroxyflavanone 7-*O*-β-D-glucopyranoside, and (2*R*)- and (2*S*)-3′,4′,5,8-tetrahydroxyflavanone 7-*O*-β-D-glucopyranoside], in addition to eight known flavonoids [naringenin, asebogenin, sakuranetin, 6-hydroxyluteolin 7-*O*-β-D-glucoside, (2*R*)- and (2*S*)-eriodictyol 7-*O*-β-D-glucopyranoside, aromadendrin and phloretin], three phenylpropanoid glycosides [forsythoside B, alyssonoside and verbascoside] and the epoxylignan lariciresinol 4′-*O*-β-D-glucopyranoside were isolated and identified in the EtOH extract of the aerial parts of *Lippia salviaefolia* Cham. The phytochemical study herein was guided by preliminary antioxidant tests, namely, β-carotene protection and 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity. The crude extracts, their active fractions and the isolated compounds were assayed against intracellular reactive oxygen species (ROS) and human embryonic kidney HEK-293 and human melanoma M14 cancer cell growth. Aromadendrin and phloretin were able to counteract elevation of ROS induced by the oxidant *t*-butylhydroperoxide (*t*-BOOH) in HEK-293 cells, whereas phloretin strongly protected HEK-293 cells from ROS damage at 1 μM. Additionally, phloretin exhibited a significant growth inhibitory effect at 20–40 μM in both HEK-293 and M14 cells and induced a concentration dependent apoptosis at 20 μM in M14 cells, suggesting a selective action towards malignant cells. Due to their equilibria, the four interconverting flavanone glycosides were studied using 1D and 2D NMR, HPLC–CD–PDA and HRMS analyses.

1. Introduction

The *Lippia* genus is distributed in South and Central Americas and in tropical Africa with ca. 70% of all known *Lippia* species found in Brazil (Viccini et al., 2006). Species from this genus are widely used in folk medicine for gastrointestinal and respiratory disorders and hypertension (Pascual et al., 2001). Additionally, *Lippia alba* (Mill.) N.E.Br and *Lippia sidoides* Cham. infusions of the aerial parts are widely used in Brazil as a tranquilizer and an antiseptic for general use, respectively (Fontenelle et al., 2007; Lemos et al., 1990; Lorenzi and Matos, 2002; Mors et al., 2000). A limited number of chemical and pharmacological studies have also been reported for various *Lippia* species, mainly focusing on the antimicrobial, insect repellent, and larvicidal effects of their essential oils. Oxidative stress is known to have a central role in degenerative processes related to aging and in several diseases (Bernonville et al., 2010; Masella et al., 2005). The search for natural antioxidant products may therefore represent a strategy for the discovery of new drugs (Hostettmann et al., 2003). The investigation of antioxidant agents in the EtOH extracts of leaves and stems of *Lippia salviaefolia* Cham., a native Brazilian plant from the Cerrado biome, was guided by β-carotene bleaching and free radical scavenging with 2,2-diphenyl-1-picrylhydrazyl (DPPH). The effects of the isolated compounds on intracellular reactive oxygen species (ROS) and human embryonic kidney HEK-293 and human melanoma M14 cancer cell growth inhibition were then evaluated, as previous reports have indicated as strong correlation of antioxidant properties and apoptosis induction on several cancer cell types, including MCF-7 and M07e cell lines (Dozio et al., 2010; Maraldi et al., 2010).

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2. Results and discussion

2.1. Fractionation guided by preliminary anti-oxidative tests and structural identifications

The *n*-BuOH (FBu1) and EtOAc (FAc1) fractions, obtained from liquid–liquid extraction of the EtOH extract of leaves of *L. salviaefolia* (EELLs), inhibited 93.0 ± 2.2% and 47.3 ± 0.4% of DPPH free radicals at 10 μg/ml, respectively, whereas the EELLs showed 33.5 ± 5.7% inhibition. Additionally, the β-carotene bleaching test on TLC plates evidenced strong orange spots, indicating the protection of β-carotene against air oxidation in both fractions, prompting further fractionation in order to identify the components of interest. Among the FAc1 subfractions, A2 was the most active, scavenging 85.1 ± 1.8% of DPPH free radicals at 33.3 μg/ml, and displaying several antioxidant spots between R_f 0.2–0.8 in the β-carotene test. Fractionation of A2 yielded 17 fractions (B1–B17). Naringenin (**1**) (Venturella et al., 1980), asebogenin (**2**) (Hermoso et al., 2003), sakuranetin (**3**) (Hurabielle et al., 1982; Mizuno et al., 1987), 6-hydroxyluteolin 7-*O*-β-D-glucoside (**4**) (Lu and Foo, 2000), the mixture of (2*R*)- and (2*S*)-eriodictyol 7-*O*-β-D-glucopyranoside (**5a** and **5b**) (Pan et al., 2008), aromadendrin (**6**) (Han et al., 2007) and phloretin (**7**) (Hufford and Oguntimein, 1980) were isolated from fractions B9, B10, B13 and B16 based on the β-carotene test and identified by 1D and 2D NMR spectroscopic experiments. Additionally, the chromatographic profiles obtained using the TLC β-carotene test, HPLC–UV and HPLC–ESI-MS of the *n*-BuOH fractions obtained from the EtOH crude extracts of leaves (EELLs) (FBu1) and stems (EESLs) (FBu2) were compared. Both fractions presented similar qualitative chromatographic profiles (data not shown) and similar spots on TLC β-carotene test; however, FBu2 showed higher yields for the more polar compounds, and was therefore selected for further fractionation by Size Exclusion Chromatography (SEC). Three of the SEC active fractions (G1–G3) were submitted to HPLC–UV analysis and afforded phenolic compounds **8**–**13**. The phenylpropanoid glycosides forsythoside B (**8**) (Delazar et al., 2005), alyssonoside (**9**) (Çaliş et al., 1992) and verbascoside (**11**) (Wu et al., 2004) and the epoxylignan lariciresinol 4′-*O*-β-D-glucopyranoside (**10**) (Shoeb et al., 2004) were isolated from G1 and G2, while G3, provided the new compounds (2*S*)-3′,4′,5,6-tetrahydroxyflavanone 7-*O*-β-D-glucopyranoside (**12a**), (2*R*)-3′,4′,5,6-tetrahydroxyflavanone 7-*O*-β-D-glucopyranoside (**12b**), (2*S*)-3′,4′,5,8-tetrahydroxyflavanone 7-*O*-β-D-glucopyranoside (**13a**) and (2*R*)-3′,4′,5,8-tetrahydroxyflavanone 7-*O*-β-D-glucopyranoside (**13b**). The ^{1}H NMR spectrum of fraction G3 was typical of a mixture of flavanone glycosides, with overlapping signals at δ_H 5.31/5.30 and 5.41/5.40 (Fig. 1A), corresponding to H-2 of flavanones, and multiplets at δ_H 2.76–2.85 and 3.14–3.19 (H-3a and H-3b region) (Fig. 1B) suggesting a mixture of two epimer pairs of flavanone glycosides.

After isolation of **12a**, **12b**, **13a** and **13b** by HPLC–UV, interconversions of these isomers were observed, i.e., the purified compounds displayed ^{1}H NMR signals indicative of the four isomer mixture, similar to that obtained for the G3 starting fraction. Additional HPLC–PDA experiments were performed on G3, with collection of one chromatographic peak per run followed by reinjection of the collected peak after 20 min and 8 h, without concentration and protecting samples from light and heat (in freezer). Initial interconversions were observed for the four isomers in the first reinjection (20 min). As this study demonstrated the instability of the isolated isomers, complementary 1D and 2D NMR spectroscopic experiments in CD_3OD and DMSO-d_6 of mixture G3 were carried out. Additionally, HPLC–CD–PDA experiments of G3 confirmed the presence of two diastereoisomeric epimer pairs distributed in four peaks (Fig. 2A and B), since each peak pair (eluted at 62 and 64 min, and at 94 and 102 min, respectively) had the same UV spectra and opposite Cotton effects in CD (Fig. 2C and D). A positive Cotton effect (CE) at 376 nm ($n \rightarrow \pi^*$ band) and a strong negative CE at 294 nm ($\pi \rightarrow \pi^*$ band) established the 2*S* configuration of **12a**, whereas opposite CE's established the 2*R* configuration for **12b**. A negative CE at 376 nm and a positive CE at 294 nm (Fig. 2D) defined the 2*R* of **13b**, whereas opposite CE's established the 2*S* configuration for **13a** (Gaffield, 1970; Slade et al., 2005).

A series of selective 1D NOESY and 1D TOCSY experiments allowed at first differentiation between signals of constitutional isomers (**12a**/**12b** from **13a**/**13b**) and, in a second step, between epimers. Irradiation of the overlapping signals at δ_H 5.30/5.31 (H-2) in the 1D NOESY experiment showed spatial correlation in the aromatic region just with resonances at δ_H 6.95 (H-2′) and 6.81 (H-5′/H-6′) (for both **12a** and **12b**), whereas irradiation at δ_H 5.40/5.41 (H-2) evidenced spatial correlations only with signals at δ_H 7.00 (H-2′) and 6.87 (H-6′) (for both **13a** and **13b**). At this point, splitting resonances of H-2 for each epimer pair could be clearly identified. Furthermore, COSY and 1D TOCSY experiments showed correlations which led to the complete assignment of the B-ring protons for the epimeric pairs **12a**/**12b** and **13a**/**13b** (Tables 1 and 2).

Since molar extinction coefficient ε of the epimers are the same (indeed, the UV spectra obtained on HPLC-PAD analysis were identical for each pair of epimers, with λ_{max} 286, 361 nm for **12a** and **12b**, and 287, 362 nm for **13a** and **13b**) the most intense H-2 signal in the ^{1}H NMR spectrum (Fig. 1A) was assigned to the compound with the most intense peak at 62 min (Fig. 2A and B), (2*S*)-3′,4′,5,6-tetrahydroxyflavanone 7-*O*-β-D-glucopyranoside (**12a**). For this isomer, pseudo-equatorial orientation (α-orientation) of the C2–C1′ bond leads to the thermodynamically favored

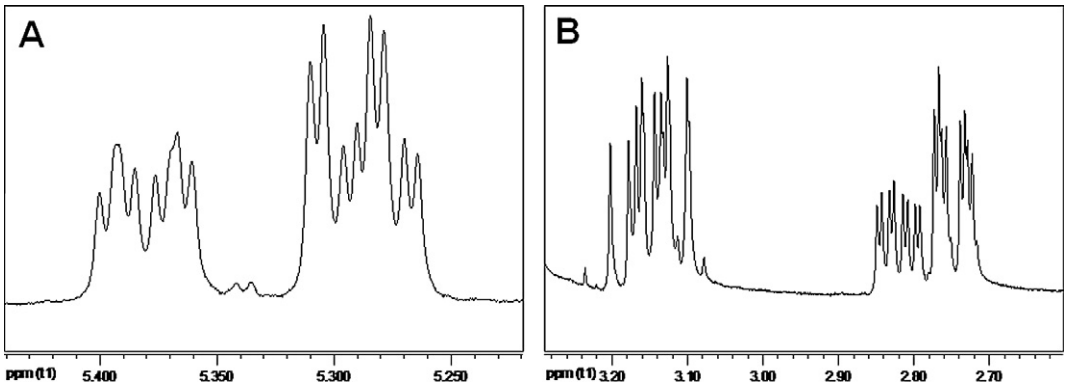

Fig. 1. Partial ^{1}H NMR spectrum of fraction G3 (**12a**/**12b** and **13a**/**13b**) expanded in the H-2 (A) and H-3 (B) regions.