IDENTIFICATION OF TRITERPENES AND STEROLS FROM *PTEROGYNE NITENS* (FABACEAE-CAESALPINIOIDEAE) USING HIGH-RESOLUTION GAS CHROMATOGRAPHY

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ABSTRACT

As part of our ongoing bioprospecting studies on secondary metabolites in plants of Cerrado and Atlantic Rain Forest of São Paulo State (Brazil), we describe the identification of triterpenes and sterols present in the hexane extracts and foliar epicuticular wax of *Pterogyne nitens* Tulasne (Fabaceae-Caesalpinioideae) using high resolution gas chromatography. Compounds detected in these extracts include *β*-amyrin (**1**), taraxerol acetate (**2**), lupenone (**3**), *β*-amyrenone (**4**), germanicone (**5**), campesterol (**6**), stigmasterol (**7**), and *β*-sitosterol (**8**). The method reported herein is shown to be a rapid, sensitive and reproducible tool for describing triterpenes and sterols present in non-polar extracts.

Keywords: *Pterogyne nitens*; Fabaceae-Caesalpinioideae; sterols; triterpenes; gas chromatography.

INTRODUCTION

Nature is an extremely rich source of highly diverse and innovative chemical structures, which have played a significant role in the process of drug discovery and design.[1,2] In this context, there has been renewed alert in the rapid rate of plant species extinction[3], which reduces the time left to explore the remaining resources of natural compounds. Urgent efforts are thus mandatory to speed up collection and selection of plants for chemical-biological investigation and possible conservation for future beneficial use of humankind.[4]

Among legumes of the Caesalpinioideae subfamily, *Pterogyne nitens* Tul. popularly named "bálsamo", "cocal", "amendoim-bravo", "yvi-raró" is the sole member of the genus, and has been submitted to a strong anthropogenic impact, which has led to its numerical population retraction, and classification into the category of species in critical risk. It is found only in small non-protected areas in South America, and is therefore, subjected to the prompt possibility of extinction[5-7]. Regarding ethnopharmacological information, this legume is used for treatment of parasitic infestations, mainly *Ascaris lumbricoides*.[8] In previous chemical reports, the chromatographic fractionation of the extract from the leaves furnished cytotoxic guanidine alkaloids.[9,10] Recent works on the leaves and fruits led to a series of flavonols and flavones which exhibited myeloperoxidase inhibitory and radical scavenging properties.[11-14]

In this work, we have employed high-resolution gas chromatography (HRGC) to investigate the sterols and triterpenes from *P. nitens*, as a promising tool to our bioprospecting activities within program BIOTA-FAPESP (The Biodiversity Virtual Institute Program), which aims to inventory and characterize the biodiversity of São Paulo State, and explore mechanisms for its conservation and sustainable use.

EXPERIMENTAL

Plant material

The leaves, stems, fruits and flowers of *Pterogyne nitens* were collected in the Botanic Garden of São Paulo, São Paulo State, Brazil, in May 2003 and identified by Dr. Inês Cordeiro (IBt-SMA). A voucher specimen (SP204319) has been deposited in the herbarium of the Botanic Institute (São Paulo, SP, Brazil).

Preparation of the hexane extracts and foliar epicuticular wax

Each plant material (50.0 g) was dried at 40 ºC, powdered and extracted exhaustively at room temperature with hexane (200 mL, 5 days x 3). The solvent was removed under reduced pressure in a rotatory evaporator (< 40 ºC) to yield dried hexane extracts from leaves (3.1 g), stems (1.8 g), fruits (2.5 g) and flowers (1.7 g).

Fresh leaves (100.0 g) were individually dipped for *ca*. 5 s into 50 mL dichloromethane. After filtration to remove plant debris, the solvent was evaporated using a stream of gaseous nitrogen affording a foliar epicuticular wax (87.0 mg) which was immediately analyzed by HRGC.

Samples clean-up

Samples of each crude extract (10 mg) were dissolved in chloroform (3.0 mL) and percolated through chromatographic column over celite:norit (1:1, 100.0 mg) + silica gel (200.0 mg) eluted with chloroform (10.0 mL). After evaporation to dryness at room temperature, the eluate was dissolved in hexane: ethyl acetate (7:3) and analyzed by HRGC in three replicates.

Gas chromatographic conditions

All the extracts were analyzed by HRGC on a Varian model CP-3800 gas chromatograph equipped with split injector [initial splitless; 0.75 min (1:50); 2.00 min (1:20)] at 260 ºC, and flame ionization detector at 310 ºC for SPB-5 column and 290 ºC for SPB-50 column. The injected volume was 2.0 µL. SPB-50 (cross-linked 50% phenyl-methyl-siloxane, 30 m x 0.25 mm x 0.25 µm) and SPB-5 (cross-linked 5% phenyl-methyl-siloxane, 30 m x 0.25 mm x 0.25 µm) capillary columns were employed. In the case of SPB-50, the column temperature was 280 ºC (isotherm), whereas for SPB-5, a column temperature program was employed in which the initial temperature was 250 ºC, held for 12 min, followed by a temperature increase of 6 ºC min^{-1} to 280 ºC, then held for additional 30 min. Nitrogen was employed as carrier gas at an average linear velocity of 1 mL/min. Triterpenes and sterols were identified by comparison of the relative retention (RR) of the samples with the RR of the standard sterols and triterpenes. Cholesterol was used as internal standard.

Authentic standard sterols and triterpenes

Triterpenes and steroids used as standards were previously isolated from Lauraceae and Rubiaceae species, including *Alibertia macrophylla*, *A. sessilis*, *Licaria rodriguesii*, *L. subbulata*, and *Aniba parviflora*. Their molecular structures were identified by ^{1}H NMR, ^{13}C NMR and MS techniques.[15-17]

RESULTS AND DISCUSSION

Hexane extracts from leaves, fruits, flowers and stems of *P. nitens*, in addition to the foliar epicuticular wax were analyzed by HRGC using a method which does not require pre-derivatizations. The GC-FID method used in this study employed two different columns: SPB-5 and SPB-50, which were used due to their low polarity, inertness towards organic compounds and high temperature limit. Such features make them ideal for the analysis of underivatized semi-volatile plant extracts.

The chromatogram obtained in SPB-50 column for the hexane extract from *P. nitens* stems evidences good resolution of peaks and thus proper identification of major components by comparison of RR (relative retention) values with those of authentic samples (Figure 1A). The presence of each triterpene or sterol was additionally confirmed by co-injection with authentic standards to avoid misclassifications due to overlapping of retention zones.

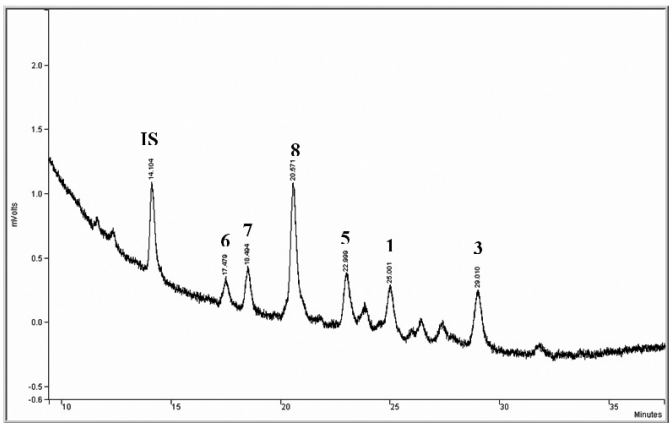

Figure 1A. Gas chromatogram (HRGC) on a SPB-50 column of the extract from *Pterogyne nitens* stems [**IS**- internal standard (cholesterol) (R_T= 14.104 min); **6**-campesterol (R_T = 17.479 min); **7**- stigmasterol (R_T = 18.494 min); **8**-*β*-sitosterol (R_T = 20.571 min); **5**-germanicone (R_T = 22.999 min); **1**- *β*-amyrin (R_T = 25.001 min); **3**-lupenone (R_T = 29.010 min)]

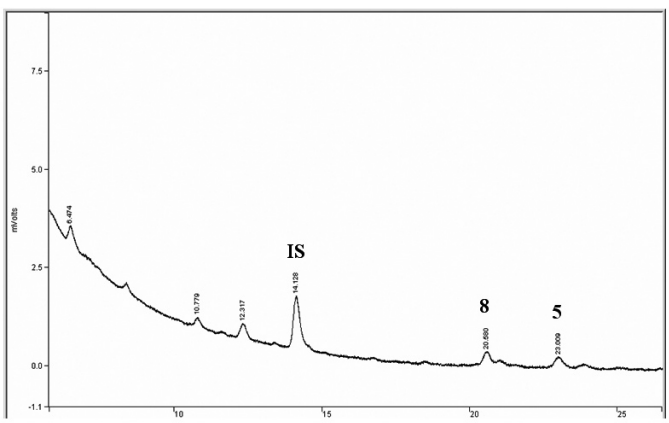

Figure 1B. Gas chromatogram (HRGC) on a SPB-50 column of the extract from *Pterogyne nitens* leaves [**IS**- internal standard (cholesterol) (R_T = 14.128 min); **8**-*β*-sitosterol (R_T = 20.580 min); **5**-germanicone (R_T = 23.009 min)]

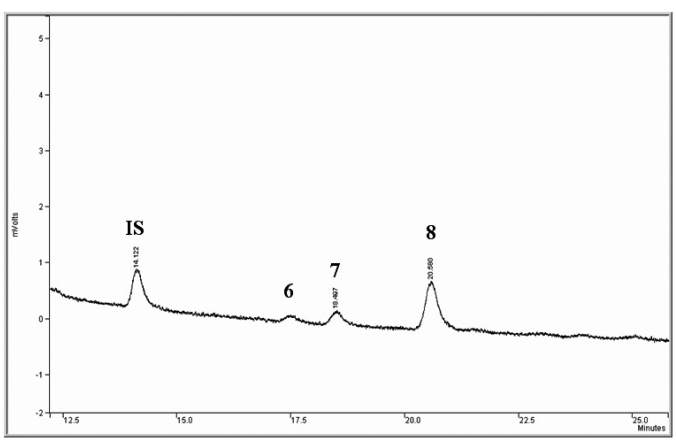

Figure 1C. Gas chromatogram (HRGC) on a SPB-50 column of the extract from *Pterogyne nitens* fruits [**IS**- internal standard (cholesterol) (R_T = 14.122 min); **6**-campesterol (R_T = 17.495 min); **7**- stigmasterol (R_T = 18.497 min); **8**-*β*-sitosterol (R_T = 20.580 min)]

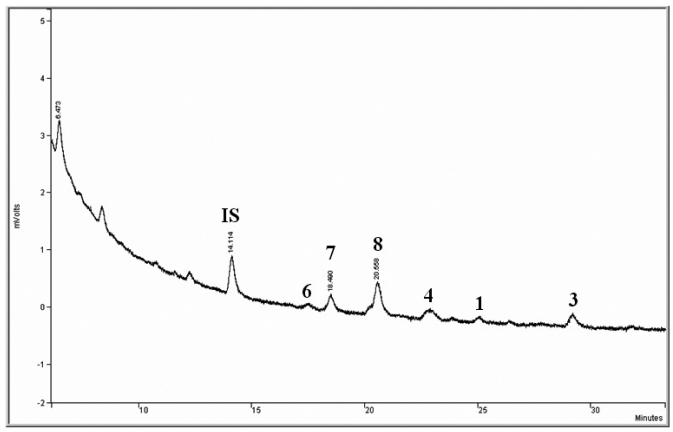

Figure 1D. Gas chromatogram (HRGC) on a SPB-50 column of the extract from *Pterogyne nitens* flowers [**IS**- internal standard (cholesterol) (R_T = 14.114 min); **6**-campesterol (R_T = 17.579 min); **7**- stigmasterol (R_T = 18.490 min); **8**-*β*-sitosterol (R_T = 20.558 min); **4**- *β*-amyrenone (R_T = 23,123 min); **1**- *β*-amyrin (R_T = 25.103 min); **3**-lupenone (R_T = 29.115 min)]

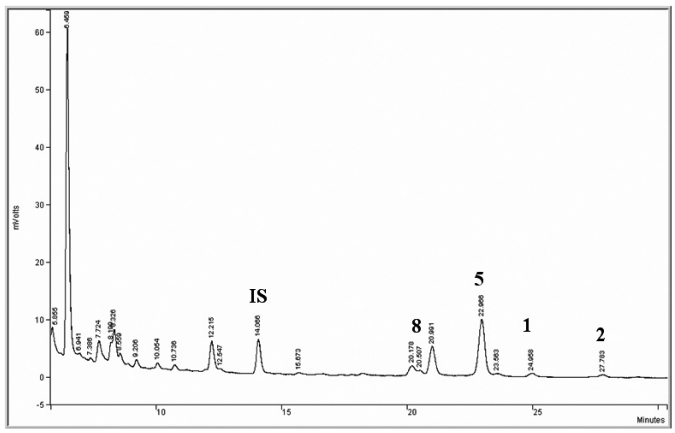

Figure 1E. Gas chromatogram (HRGC) on a SPB-50 column of foliar epicuticular wax from *Pterogyne nitens* [**IS**- internal standard (cholesterol) (R_T = 14.066 min); **8**- *β*-sitosterol (R_T = 20.507 min); **5**- germanicone (R_T = 22.966 min); **1**- *β*-amyrin (R_T = 24.958 min)] **2**- taraxerol acetate (R_T = 27.783 min)]

Table 1 summarizes the results from the HRGC analysis, indicating the chemical composition of foliar epicuticular wax and hexane extracts from *P. nitens*. The presence of such low-polarity compounds was evidenced by comparison with the relative retention values of authentic standards obtained for SPB-5 and SPB-50 columns.

Table 1. Chemical composition of the hexane extracts and foliar epicuticular wax of *Pterogyne nitens* from HRGC analysis (SPB-5 and SPB-50 columns)

Compound	Leaves	Fruits	Stems	Flowers	Epicuticular wax	RR[a]	RR[b]
α-amyrin	-	-	-	-	-	1.326	2.006
β-amyrin	-	-	+	+	+	1.290	1.774
lupeol	-	-	-	-	-	1.326	2.163
taraxerol	-	-	-	-	-	1.261	1.710
α-amyrin acetate	-	-	-	-	-	1.544	2.156
β-amyrin acetate	-	-	-	-	-	1.464	1.928
lupeol acetate	-	-	-	-	-	1.544	2.239
bauerenyl acetate	-	-	-	-	-	1.704	2.511
taraxerol acetate	-	-	-	-	+	1.427	1.934
friedelanoyl acetate	-	-	-	-	-	1.785	2.887
friedelin	-	-	-	-	-	1.550	2.750
lupenone	-	-	+	+	-	1.325	2.011
α-amyrenone	-	-	-	-	-	1.325	1.937
β-amyrenone	-	-	-	+	-	1.265	1.725
germanicone	+	-	+	-	+	1.256	1.648
campesterol	-	+	+	+	-	1.124	1.239
stigmasterol	-	+	+	+	-	1.167	1.314
β-sitosterol	+	+	+	+	+	1.246	1.457

RR = relative retention related to the internal standard (cholesterol): [a]SPB-5 column; [b]SPB-50 column. (+) presence of the compound; (-) absence of the compound.

Our results indicate that *β*-sitosterol (**8**) is present in all the analyzed extracts. Additional widely occurring sterols: campesterol (**6**) and stigmasterol (**7**), were also characterized in all analyzed hexane extracts, except from the leaves. The pentacyclic triterpenes *β*-amyrin (**1**) and lupenone (**3**) were detected in the stems and flowers, whereas germanicone (**5**) was identified in the leaves and stems; and *β*-amyrenone (**4**) was detected in the flowers extract. Further GC-FID analyses of foliar epicuticular wax showed the presence of **1**, **5** and, taraxerol acetate (**2**). These results clearly demonstrate that the hexane extracts from stems and flowers present higher chemodiversity than the others. On the other hand their chemical profile indicated common major constituents concerning sterols and triterpenes in the analyzed samples evidencing great similarity among the studied plant part extracts.

Altogether, these results corroborate differences in the secondary metabolites production by different plant tissues, which can be relevant from the point of view of the chemical interface between plants and the surrounding environment.[18] In this context, several studies indicated variable production of secondary metabolites by medicinal plants.[19] Zangerl and Bazzaz reported different levels of furanocoumarins from *Pastinaca sativa* (Apiaceae) leaves and roots.[20] Likewise, Rees described flowers of *Hypericum hirsutum* (Clusiaceae) contain five to ten times more hypericin that the leaves.[21] Considering that young leaves and other growing plant parts are generally better protected by secondary compounds than mature tissues, young leaves of rosette plants of *Cynoglossum officinale* (Boraginaceae) were found to contain up to 53 times higher levels of pyrrolizidine alkaloids than older leaves.[22]

In the case of non-polar extracts, bioactivity has been often associated with complex mixtures of triterpenoid and/or steroid compounds[23-26]. However, no further analysis/identification of the mixture components is subsequently performed in most cases due to two reasons: Firstly, traditional phytochemical procedures, which include isolation of the components from isomeric mixtures, may be tedious, time-consuming and relatively expensive. Secondly, due to their structural complexity the analysis of triterpenes and sterols by spectroscopic techniques has been shown to be difficult in many cases. Gas chromatography coupled with mass spectrometry (GC-MS) has emerged as the best technique for characterization of low-polarity compounds, including triterpenes, sterols, glycerols, waxes, and derivatized fatty acids. However, this hyphenated analytical technique is still inaccessible to many research groups around the world. On the other hand, GC is more readily available and might also be employed as a preliminary procedure for rapid, simple, and relatively cheap characterization of non-polar compounds from plant extracts, including epicuticular waxes. The present phytochemical screening using HRGC showed to be reliable and useful for the identification of triterpenes and sterols in complex matrices such as hexane extracts and foliar epicuticular wax from *P. nitens* (Figure 2). Other interesting applications of this technique include the selection of plant extracts containing bioactive non-polar compounds for further phytochemical and pharmacological investigations, which may represent a crucial step in bioprospecting studies and corroborates its importance as a tool for conducting phytochemical studies.

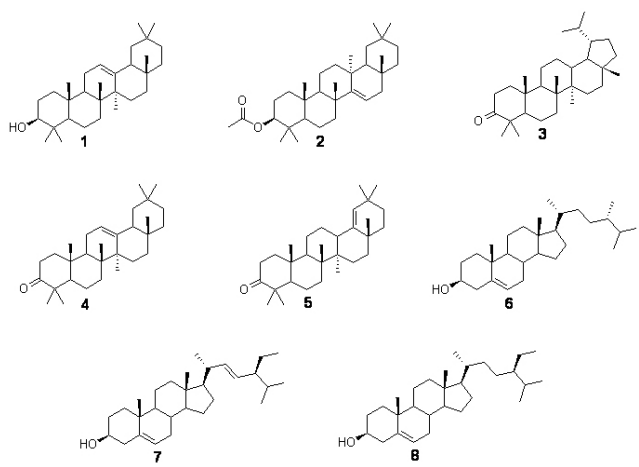

Figure 2. Structures of triterpenes (**1**–**5**) and sterols (**6**–**8**) from *Pterogyne nitens.*

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