Seasonal evaluation and quantitative analysis of betulinic acid in ethanol extracts of *Eugenia florida* leaves through the use of HPLC-UV and GC-FID

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ABSTRACT

The betulinic acid is a known triterpenoid isolated from various organs and species of plants. This metabolite shows inhibitory activity on growth of human melanoma cells and replication of the AIDS virus. This work had as main objectives to develop a protocol for the quantification of betulinic acid present in the leaves of *Eugenia florida* by using the technique GC/MS, GC/FID and HPLC/DAD and through studies demonstrates the potential of this seasonal vegetable production as a source of natural metabolite. The betulinic acid level in the *E. florida* leaves increased significantly in the month of May, June, July, September, October and November which was mainly due to the accumulation of this compound in the tissue of the plant. Some authors reported that pentacyclic triterpenes show inhibitory activity just as betulinic acid, ursolic acid, β-amyrine and lupeol, are supposed to be toxic to insects, due to their ability to inhibit acyl chain packing in the lipid bilayers of the insect membranes. These fluctuations observed in the months may be related to the chemical ecology of *E. florida* as, for example, the attraction of pollinators or the reproductive phenology of the specimens. It is possible that the increased concentration of betulinic acid in the month of March is due to the large amount of rainfall characteristic of the Rio de Janeiro, state. However, more research is needed to determine whether other factors may be influencing the concentration of this metabolite, verify that specimens from other regions have the same or different behavior and examine whether the effect of the solvent can affect the increase in the concentration of this metabolite.

Key words: Betulinic acid, triterpenes, HPLC-UV, GC-FID.

INTRODUCTION

The betulinic acid (Figure 1) is a known triterpenoid isolated from various organs and species of plants, including flowering *Eugenia* DC (Junges, 1999). This metabolite shows inhibitory activity on growth of human melanoma cells (Pisha et al., 1995), and replication of the AIDS virus (Evers et al., 1996; Soler et al., 1996). In additional betulinic acid derivatives (Chatterjee et al., 2000; Galgón et al., 2005) induced cell apoptosis of human melanoma. This specificity in melanoma cells makes the substance compared to complex molecules such as taxol, the most promising anticancer drug (Pisha et al., 1995). However, their action is limited only to neuroblastomas and melanoma cells and is not active against other cancer cells (Chatterjee, 2000; Pezzuto et al., 1999; Pisha, 1995; Mayaux et al., 1994). The betulinic acid also has antibacterial property and inhibits the growth of colonies of *Escherichia coli* and *Staphyloccocus aureus*.

Despite all of betulinic acid pharmacological potential, it
is obtained by extraction of barks or core of some plant species or by synthetic processes, e.g. using the betulin (alcohol triterpene) as a synthetic intermediate isolated from the bark of Betula alba and Betula pendula (Galgon et al., 1999). Therefore, research is necessary to identify new natural sources, which produce large quantities of substance, easily renewable parts of the plant (leaf) thereby not affecting plant growth, development of rapid chromatographic methods which through easy manipulation studies could be used to identify the seasonal best months of collection.

As part of a program conducted in our laboratory involving search for new sources of bioactive metabolites from Brazilian plants, we investigated the leaves of E. floridea. This species belongs to the family Myrtaceae. Compounds such as flavonoids, triterpenes, tannins and especially essential oils constituted of monoterpenes and sesquiterpenes have already been isolated from the genus Eugenia (Lunardi et al., 2001). The species of this family are widely distributed in the Brazilian forests, much of it is popularly known for its edible fruits, wood, essential oils or ornamental purposes (Consolini et al., 1999; Costa et al., 2005; Siani et al., 2000). The most important genera of this family are: Melaleuca, Eucalyptus, Psidium and Eugenia (Siani et al., 2000).

Seasonal variation

Since the fourth century B.C. there are reports of procedures for the collection of medicinal plants. The executioners Greeks for example collected their samples of poison hemlock (Conium maculatum) in the morning when levels of alkaloid coniine are higher (Robinson, 1974). Temporal and spatial variations in the total content, as well as the relative proportions of secondary metabolites in plants occur at different levels and, despite the existence of a genetic control, the expression may undergo changes resulting from the interaction of biochemical processes, physiological, ecological and evolutionary (Gobbo- Lopes, 2007). In fact, the secondary metabolites represent a chemical interface between plants and the surrounding environment. Therefore, their synthesis is often affected by environmental conditions (Gobbo- Lopes, 2007).

Several factors that can coordinate or alter the rate of production of secondary metabolites in each species are genetic factors, physical environment, collection method (date, time, etc.), drying conditions and transport, storage, pH of the soil, growing conditions, nutrient soil, plant part used, interactions between plants, the presence of microorganisms etc (Silva and Vizzotto, 1996). Some factors have correlations with each other and not act alone, and may jointly affect the secondary metabolism, e.g. development and seasonality, rainfall and seasonality, temperature and altitude, among others (Gobb, Lopes, 2007). It should also be noted that, often, the changes may result from leaf development and / or appearance of new organs concomitant with constancy in the total content of secondary metabolites. This may cause decrease in the concentration of these metabolites by dilution, however, it could result in a higher total amount due to increase of biomass (Hendriks et al., 1997; Spring et al., 1987).

The seasonal variation (collection of plants at different times or seasons), is a very significant factor in the percentage and production of secondary metabolites (Mitscher et al., 2000; Navarro et al., 2002). Due to its importance, it is necessary a study of seasonality be put into consideration when working with medicinal plants (Mitsche et al., 2000; Navarro et al., 2002).

This work had as main objectives to develop a protocol for the quantification of betulinic acid present in the leaves of Eugenia floridea by using the technique GC/MS, GC/FID and HPLC/ DAD and through studies demonstrates the potential of this seasonal vegetable production as a source of natural metabolite.

MATERIAL AND METHODS

General experimental procedures

1H and 13C NMR spectra were recorded on a Brucker (AM-200 and 500 MHz), chemical shifts are given in d values referred to internal tetramethysilane (TMS), EIMS (MS Agilent 5973; 70 eV) and Infrared (IV) spectra were recorded on a Nicolet spectrophotometer with Fourier transform Model Magna–IR 760 and wavelengths are expressed in reciprocal centimeter (cm⁻¹).

HPLC analysis were performed at room temperature using the following system: second pump system (Shimadzu LC10AD model, Japan) a photodiode array detector (Shimadzu, SPD10ADVP model), an auto injector (Shimadzu SIL10ADVP model), an oven (Shimadzu, CTO 8 A model) and under the following conditions: Shimpack C-8 (1 cm x 4.6 mm i.d.) guard column and C-18 column (25
Plant material

Healthy leaves of *E. florida* and adults were collected during 12 months (August 2009 to July 2010) on the campus Oswaldo Cruz Foundation, State of Rio de Janeiro. The species identification was carried out by biologist Sergio Monteiro of Oswaldo Cruz Foundantion [Laboratory of Production and Processing of Raw Plant (LPBMPV)], and a voucher specimen was deposited in the Herbarium of the Botanical Garden of Rio de Janeiro with the number RB 328061.

Methylation

A solution of diazomethane (CH$_2$N$_2$) in ether was prepared and added (excess) in drops to the solutions of extracts, EF-1 and standard (1 mg) CHCl$_3$ or MeOH. The resulting solutions were allowed to stand for 12 h and the ether removed by passing a stream of N$_2$ (Leonard et al., 1995).

Betulinic acid quantification

GC analysis was performed on 6890N (Agilent Technologies, Network series) equipped with a HP-5 column (30 x 0.25 mm; 0.25 µm liquid phase). Oven temperature program of 70 - 300°C at 5°C/min; carrier gas: helium 1.13L/min; split mode of (20:1) and finally held for 30 min. The mass spectrometer unit was performed with the same conditions with the GC analysis. The calibration curve of the GC/FID was made in triplicate from different concentrations of esterified betulinic acid standard (0.1 to 1µg.mL$^{-1}$) and the curve was constructed using the average values of the detector response. The detector response was linear to the concentration internal of 0.1 to 1µg.mL$^{-1}$ ($r^2 = 0.999$, Figure 2a).

HPLC grade acetonitrile was purchased from TEDIA (Brazil); 0.05% TFA (Trifluoroacetic acid) from Vetec (Brazil). Water was purified by Milli-Qplus system from Milipore (Milford, MA, USA). Betulinic acid was purchased from Carl Roth (Karlsruhe, Germany with 99%). The 30 mg ethanol extracts were then dissolved in 5 mL of mobile phase. The mobile phase consisted of a gradient of 0.05% aqueous trifluoroacetic acid: acetonitrile delivered at a 1.0 mLmin$^{-1}$ as follows initial (t= 0 min) 30:70, linear gradient over 20 min to 15:85, linear gradient over 10 min to 100:0 and a new linear gradient over 20 min (30:70); 40 min was the total time of analysis. Flow rate was 1 mL.min$^{-1}$. Quantification was performed using the detector set at a wavelength of 210 nm. Injection volume was 30 µl. The peak of betulinic acid was identified in each chromatogram from of the ethanol extracts monthly (twelve months) with the help of injection of the standard solution of betulinic acid or comparison of the UV spectrum. The calibration curve of the HPLV-UV was made in triplicate from different concentrations of betulinic acid standard (0.1 to 1µg.mL$^{-1}$) and the curve was constructed using the average values of the detector response. The detector response was linear to the concentration internal of 0.1 to 0.5 µg.mL$^{-1}$ ($r^2 = 0.999$, Figure 2b).

RESULTS AND DISCUSSION

Extraction of betulinic acid

The leaves of *E. florida* (17.1 kg) were dried at 400°C, ground and subjected to soxhlet extraction with ethanol. The diluted extract was removed under reduced pressure (4.7 g). An aliquot of the methanol extract (200 mg) was dissolved in methanol (20 ml) and recrystallized using mixtures of CHCl$_3$ and MeOH. Recrystallization occur and a white crystal was obtained (EF ~ 1; 50 mg).

EF-1 was analyzed by spectrophotometer 1H and 13C NMR (Bruker AC 200, 200 MHz) using as solvent chloroform (CDCl$_3$) and methanol (CD$_2$OD) deuterated at a ratio of 9:1 to tetramethylsilane (TMS) as internal reference standard. An aliquot of EF-1 (5 mg) was methylated with diazomethane and subjected to mass spectrometry (MS; Agilent Technologies). The spectral data obtained were compared with the results of Oliveira et al. (2002).

The substance showed an EF-1 in the form of white crystals and the IR spectrum showed a broad band at 3450 cm$^{-1}$ showing characteristic of hydroxyl groups and acid, a broad band at 2942 cm$^{-1}$ one of alkyl groups and bands at 1686 and 1639 cm$^{-1}$ corresponding respectively to the axial deformation of carbonyl acid and alkene.

The information that led to elucidation of the structure was obtained from experiments nuclear magnetic resonance
Figure 2. Calibration curve of betulinic acid, A: GC-FID and B: HPLC-UV.

spectra [DEPT, HMQC, 1H-1H COSY (homonuclear correlation spectroscopy) and HMBC experiment] which indicate a known pattern of the terpenes series lupanos (Nick et al., 1994; Mahato and Kundu 1994; Budzikiewicz et al., 1964). The 1H NMR spectrum showed two signals of multiplet in δH 4.69 and 4.58, referring to vinyl hydrogen (H-20), δH 1.66 a signal corresponding to the methyl group bonded to carbon and fifth signals sp² corresponding to the methyl tertiary (δH 0.74; 0.85, 0.94, 0.96 and 1.00). The 13C NMR spectrum confirmed the presence of signals in vinyl 152.02 and 110.15 ppm (double bond), carbonyl acid in 180.03 ppm and secondary alcohol in 79.69 ppm (Nick et al., 1994; Mahato and Kundu, 1994).

The methylation EF-1 with diazomethane promoted the removal of hydrogen from the carboxyl acid and incorporation of a methyl group from the diazomethane, leading to formation of an ester, molecular weight 470. The derivatization and the formation of the ester are ideal possible to decrease the molecular interactions between the sample and a chromatographic column and thus decrease the retention time. An aliquot of esterified EF-1 (1.4mg) was subjected to MS electron impact (70 eV). The MS spectrum of esterified EF-1 confirmed the presence of a terpene class of lupanos due to the absence of peaks m/z 218 and m/z 203 characteristic of the series oleanane and ursane (rearrangement retro Diels-Alder ring C). The presence of the methyl ester group at C-28 is confirmed by the ion m/z 262 (10%). Other peaks were obtained m/z 208 (5%), m/z 190 (10%) and m/z 189 (100%) from the break ring C and the molecular ion m/z 470 [5% (Budzikiewicz et al., 1964)]. The spectral data obtained from the EF-1 and the ester data were similar to those observed in the literature to betulinic acid (Nick et al., 1994; Mahato and Kundie, 1994; Budzikiewicz et al., 1964).

After calibration with standard of betulinic acid, the monthly extracts from leaves of E. florida were analyzed. Those extracts were analyzed in triplicate and the average areas corresponding to betulinic acid was calculated. From these average areas, percentage composition of the betulinic acid in the extract were calculated using the linear equation generated during calibration of betulinic acid (Figure 2) carried out in HPLC-UV and GC-FID (Table 1).

Conclusions

Several activities are being attributed to betulinic acid, however, despite all of its potential pharmacological characteristics, it is still obtained by extraction of the bark and heartwood of some (Soler, 1996), synthetic processes (Evers et al., 1996) and by biotransformation (Galgon, 2005). Unlike these traditional species whose income was less than 3%, we found that betulinic acid was present in all extracts analyzed (Table 1), with yields well above those
found in past researches. The betulinic acid level in the *E. florida* leaves increased significantly in the month of May, June, July (autumn - winter), September, October and November (winter) which was mainly due to the accumulation of this compound in the tissue of the plant. Rodriguez et al. (1997) and Prades et al. (2011) reported that pentacyclic triterpenes inhibit the growth of certain human cells just as betulinic acid, ursolic, acid, β-amyrine and lupeol, are supposed to be toxic to insects, due to their ability to inhibit acyl chain packing in the lipid bilayers of the insect membranes.

These fluctuations observed in the months described in Table 1 may be related to the chemical ecology of *E. florida*, for example, the attraction of pollinators or the reproductive phenotype of the specimens.

It is possible that the increased concentration of betulinic acid in the month of March is due to the large amount of rainfall characteristic of the Rio de Janeiro state. However, more research is needed to determine whether other factors may be influencing the concentration of this metabolite, verify that specimens from other regions have the same or different behavior and examine whether the effect of the solvent can affect increase in the concentration of this metabolite.

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