

Full Length Research Paper

***Plumeria rubra* (Apocynaceae): A good source of ursolic acid**

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Ursolic acid (UA) is regarded as one such compound that possesses many biological activities, such as anti-oxidation, anti-inflammatory, anticancer, and hepatoprotection, as well as the ability to induce apoptosis. Although it is present in the leaves and barks of many plants including apple peels, the traditional source to obtain large amount of this metabolite is *Plumeria rubra*. Its isolation is expensive and based on laborious extraction methods or obtained in low yield by synthetic methods. In this study, we determined the best extraction solvent of these compounds and verified the influence of season on the ursolic acid content. The High-Performance Liquid Chromatography with Diode-Array Detection (HPLC-DAD) was used to quantify ursolic acid in different months extracts. Analysis of variance (ANOVA) test followed by Turkey test were used in the statistical analysis. We demonstrated that ursolic acid was significantly more abundant in the months of May and July, and was in minor concentrations in the months of December, January, February and March. Differences in their composition during this consecutive seasons; summer (December, January, February and Mars), autumn to winter (May and July), were statistically significant. It is suggested that these differences were mainly due to the specific insects available in the three seasons. On the other hand, some authors related this to the pentacyclic triterpenes, just as 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's membranes.

Key words: *Plumeria rubra*, ursolic acid, seasonal effect, high-performance liquid chromatography with diode-array detection (HPLC-DAD).

INTRODUCTION

Triterpenoids are ubiquitous throughout the plant kingdom in the form of free acids or aglycones of saponins, and exhibit various biological activities (Liu, 2005). Already, more than 80 triterpenoids have been identified from plants, and the number of papers describing their bioactive effects has increased sharply

during the last decade (Ikeda et al., 2008). Specifically, some of them have already been used as anticancer and anti-inflammatory agents in American countries.

Ursolic acid (UA; 3- β -hydroxy-urs-12-en-28-oic acid) (Figure 1) is a natural pentacyclic triterpenoid carboxylic acid and is well known for its biological effects. In recent

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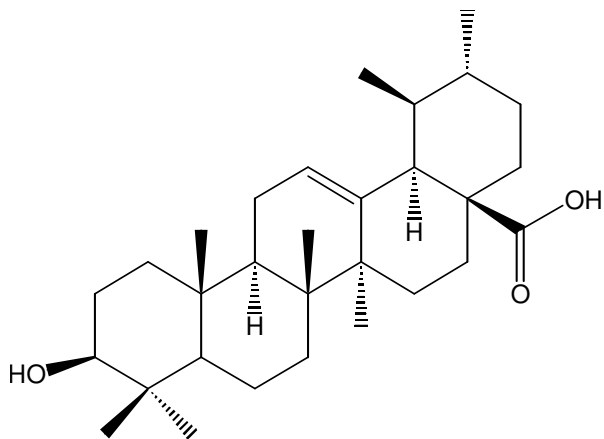


Figure 1. Ursolic acid (UA).

years, it has attracted considerable attention because of its pharmacological effects combined with a relatively low toxicity (Novotny et al., 2001). UA is regarded as one such compound that possesses many biological activities, such as anti-oxidation, anti-inflammatory, anticancer, and hepato-protection, as well as the ability to induce apoptosis (Kim et al., 2004). In the early 1990s, the antioxidative activities (Ramachandran and Prasad, 2008) of UA against free radical-induced damage were studied (Balanehru and Nagarajan, 1991, 1992). In these experiments, UA showed remarkable anti-oxidative activities by scavenging free radicals to four standard chemical (ascorbic acid, carbon tetrachloride, ADP/iron, and adriamycin) induced lipid peroxidation in isolated rat liver and heart microsome *in vitro* (Balanehru and Nagarajan, 1991, 1992). UA can serve as a starting material for synthesis of more potent bioactive derivatives, such as anti-tumor agents (Ma et al., 2005). It has been found to reduce muscle atrophy and to stimulate muscle growth in mice (Kunkel et al., 2011). Ursolic acid has potential use as a cardioprotective compound. UA was found to be a weak aromatase inhibitor ($IC_{50} = 32 \mu\text{m}$) (Gonoatto et al., 2008).

Although it is present in the leaves and barks of many plants including apple peels, the traditional source to obtain large amount of this metabolite (~10%) is *Plumeria rubra*. Its isolation is expensive and based on laborious extraction methods. Therefore the necessity to discover this new source with high yields of UA which occur preferably in the leaves (Liobikas et al., 2011; Ikeda et al., 2008), and an accurate quality control is necessary because the chemical composition and concentration of active or inactive components of a medicinal herb can vary considerably according to the soil, light, water supply, environmental cultivation conditions, and the season when it is harvested (Silva, 1996; Gobbo Neto and Lopes, 2007). The study focused on the factors that could interfere with the concentrations of UA profile of *Plumeria rubra* extracts, such as harvesting at different

seasons and many solvents described for the extraction procedure with the aim of contributing to determine the most suitable conditions for achieving the best season's productivity of this triterpenoid and the establishment of the *Plumeria rubra* extract, with a potential source of this economically important metabolite for the chemical industries. Furthermore we determined the best solvent for acquisition and optimizing of the production of the extracts, and verified whether UA concentrations from the leaves extracts was dependent on the season and also what months the concentrations tend to be higher.

MATERIALS AND METHODS

General experimental procedures

Gas chromatography-Flame ionization detector (GC-FID) analysis was performed on an HP 6890 model (Agilent Technologies, Network series) coupled to a mass detector HP 5971 model, equipped with library spectra Wiley (software 59943B). We used a capillary column HP-5 (30 m \times 0.25 mm \times 0.25 mm liquid phase). Oven temperature program of 290 to 350°C at 5°C/min; carrier gas: helium 11.3 L/min; split mode of (5:1) and finally held for 30 min. The mass spectrometer unit was performed with the same conditions as the GC analysis. In GC-FID experiments, 1 mg of extract and standard (Carlo Erba, Italy) were transferred into glass vial and submitted through methylation with CH_2N_2 , with 100% yield. The samples were dissolved in CH_2Cl_2 in concentrations of 1.4 mg/mL and injected for GC-FID and Gas chromatography-Magnetic separation (GC-MS) analysis. The identification of UA methylated from extract was done with the use of Wiley and NBS peak matching library search system. Authentic standard of the UA and data reported in the literature were also used for further identification as described.

High performance liquid chromatography (HPLC) grade acetonitrile was purchased from Sigma Aldrich (Steinheim Germany); H_3PO_4 from Carlo Erba (Milan, Italy). Water was purified by Milli-Q_{plus} system from Millipore (Milford, MA, USA). UA was purchased from Sigma Aldrich (98.5%).

High-Performance Liquid Chromatography with Diode-Array Detection (HPLC/DAD) analysis was performed on a SPD-10APV instrument with diode array detector managed by C-R6A Chromatopac (Shimadzu) and UV-vis spectra was recorded at 210 nm. The eluent was MeOH: H_2O (9:1) adjusted with 0.32 mL of H_3PO_4 . Flow elution was 1 mL min^{-1} , 10 μL samples and standard were injected (Wang et al., 2008; Liang et al., 2009). HPLC analyses were performed on each sample after dilution of 4 mg of dichloromethane extracts were dissolved in 0.5 mL Dimethyl sulfoxide (DMSO) and the volume was completed with 1.0 mL of mobile phase (Wang et al., 2008; Liang et al., 2009).

A Supelcosil LC-18 (5 μm , 25 cm, 4.60 mm), maintained at 25°C was used. Identification of UA was performed by HPLC-DAD comparing the retention time and UV spectrum. The purity of peak constituent was checked by diode array detector coupled to the HPLC system comparing each peak with authentic sample of UA (Sigma Aldrich) and examination of UV spectra. Calibration curve was carried out using calibration graph with five points. Calibration graph for HPLC was recorded with UA amounts ranging from 0.66 to 5.34 mg/mL. Data acquisition and processing were performed with Ezchrom Elite software. All solvent used were of HPLC grade.

Plant material and extraction

Healthy leaves of *P. rubra* were collected during 12 months

Table 1. Quantification (w/w \pm standard deviation; n = 3) of Ursolic acid content as measured by HPLC during the different sampling times.

Months (weight of dried plant material =10 g)	Weight of dichloromethane extract (g)	Temperature (°C)/Hours	Ursolic acid (%)
January	1.69	25/15:30	5.22 \pm 0.47
February	1.57	27/15:20	8.74 \pm 0.83
March	0.75	30/14:30	0.27 \pm 1.27
April	1.5	26.7/15:00	5.74 \pm 0.42
May	1.75	29/15:30	15.78 \pm 0.04
Jun	0.616	26/15:00	11.79 \pm 0.05
July	1.5	28/15:22	16.40 \pm 0.36
August	1.4	27/14:00	8.46 \pm 0.37
September	1.9	20/15:10	13.71 \pm 0.24
October	1.7	26/14:30	12.82 \pm 0.11
November	1.6	26/14:40	4.68 \pm 0.69
December	0.616	28/14:00	8.57 \pm 0.04

(January to December of 2010). The leaves of *P. rubra* L. were collected in the Campus Foundation, Rio de Janeiro state, Brazil. Voucher specimen was deposited at the Herbarium of UFRJ (University Federal do Rio de Janeiro) with the number 24346 and its identity confirmed by research of this institution. The leaves (200 g) were dried, pulverized and maintained under -20°C until it was ready to be analyzed. Six samples of the leaves (10 g) were extracted separately in a soxhlet apparatus at a temperature of 60°C for 48 h, with hexane, ethyl acetate, dichloromethane, chloroform, ethanol and methanol to determine the best extraction solvent. These solvents were selected in compliance with literature date for the extraction of UA (Junges, 2000; Frighetto, 2008; Rauter, 2007). The solvents were removed under vacuum to yield 1.0 g of hexanic extract, 1.4 g of ethyl acetate extract, 1.6 g dichloromethane extract, 1.7 g chloroformic extract, 1.8 g ethanolic extract and 2.0 g methanolic extract from the leaves. The crude extracts (1 mg) and authentic sample of UA (1 mg) were methylated with ethereal solution of diazomethane previously prepared from diazald.

These methylated crude extracts and ursolic ester were analyzed by GC and GC/MS. Identification of UA was performed by GC and GC/MS analysis comparing the retention and mass spectrum of peaks in the crude extracts with ursolic ester. The GC and GC/MS showed that the efficiency of the UA extraction does not depend on the type of solvent. The extraction yield and extraction efficiency of UA were similar, therefore the dichloromethane extract presented the minor quantity of the interferent. After these results, all crude extracts utilized to study seasonal variation were extracted with dichloromethane. The dichloromethane extracts (200 mg) was dissolved in hot methanol. The cooling of methanol provoked the precipitation of significant amounts of crude UA which was further crystallized in methanol to afford amounts of very pure acid (20 mg). Identification of UA was performed by spectroscopic methods [(mass spectrometry and Carbon nuclear magnetic resonance (¹³C-RMN)].

Ursolic acid (UA) quantification

The leaves of *P. rubra* were collected during different months (Table 1) and extract was prepared as described. Ten microliters of sample solution were injected into the HPLC system. The quantification of compound was carried out with the application of

UA (Sigma Aldrich) as a standard. Standard solutions containing 0.66, 1.34, 2.67, 4.00 and 5.34 mg/mL of UA were made up to create a calibration curve (Figure 2). The relationship between peak areas (detector response) and amount of UA was linear with $r^2 = 0.996$. To evaluate the repeatability of the injection integration the UA standard solution and all extracts and standard were injected three times and the relative standard derivation values were calculated and showed in Table 1. Data were expressed as means standard (SD). Differences between months were analyzed in different days in triplicate (Table 1).

RESULTS AND DISCUSSION

The extractive efficiency of triterpenoids compounds from plant material is greatly depended on the solvent. In our research, we observed that the efficiency of the UA extraction did not depend on the type of the solvent. The extraction yield and extraction efficiency of UA were similar in the solvents used (hexane, ethyl acetate, dichloromethane, chloroform, ethanol and methanol). Therefore, the dichloromethane extract presented the minor quantity of the interferent. On the other hand, many authors (Junges et al., 2000; Frighetto et al., 2008; Rauter et al., 2007) reported that ethanol was the best solvent for extraction of the UA from plant.

The triterpene (UA) was quantified based on the area \times mg calibration curve, using Ursolic acid (Sigma Aldrich) external standard and UV correction. The method utilized in this work was based on Wang et al. (2008). The calibration curve was constructed in the range of 0.66 to 5.34 mg/ml. The method showed a linear relationship between peak areas and known concentrations of UA between 0.66 to 5.34 mg/ml. Analysis of this standard on five different points yielded the following mean calibration curves for UA ($y = 22833x + 10468$) ($r^2 = 0.996$).

Strong seasonal variations in twelve months dichloromethane extracts were observed. The UA level in the *P. rubra* leaves increased significantly in the May, June, July

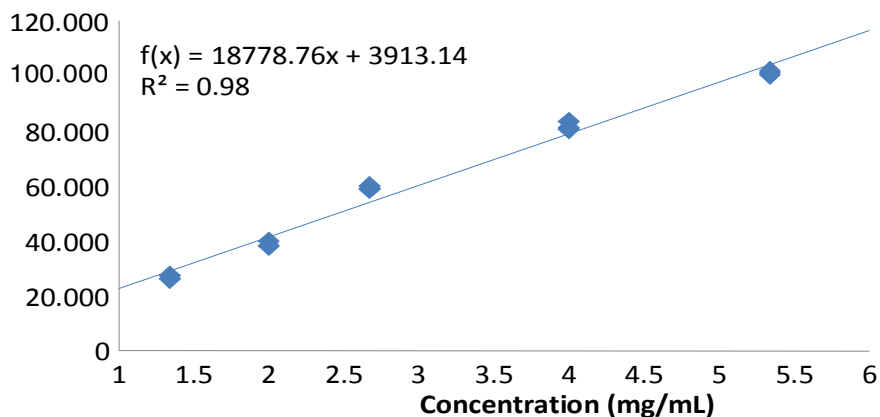


Figure 2. Calibration curve of ursolic acid.

(autumn to winter) and, September and October (winter), which was mainly due to the accumulation of this compound in vegetal tissue. Differences in their composition during two consecutive seasons, summer (December, January, February and March), autumn to winter and spring (September and October) of 2010, were statistically significant. It is suggested that these differences were mainly due to the specific insects available in the three seasons. Some authors stated that the leaves are rich in metabolites, principally terpenoids and these constitutes the first line of defense towards various biotic and abiotic stressors (Batovska et al., 2008). Rodriguez et al. (1997) related this to the pentacyclic triterpenes, just as 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.

Conclusion

Several activities are being attributed to ursolic acid, however despite all of their potential pharmacological, it is still obtained by extraction of the apple peels. Unlike these traditional species whose income was less than 10%, we found that triterpenoid was present in all extracts analysed (Table 1), with yields well above those found in literature. Table 1 shows the results obtained after quantitative analyses of twelve months dichloromethane extracts. These fluctuations observed in the months described in Table 1 may be related to the chemical ecology of *P. rubra* as, for example, the attraction of pollinators or the reproductive phenology of the specimens. It is possible that increased concentration of ursolic acid in the months of May, June, July (autumn to winter), and September and October (winter) are due to the large amount of rainfall characteristic of Rio de Janeiro state. However, more research is needed to determine whether other factors may be influencing the concentration of this metabolite, and check within a

population of species of *P. rubra* producing specimens if the concentrations are greater than those of ursolic acid in this study.

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