Selective and cost-effective protocol to separate bioactive triterpene acids from plant matrices using alkalinized ethanol: Application to leaves of Myrtaceae species

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ABSTRACT

Background: Triterpenes as betulinic (BA), oleanolic (OA) and ursolic acids (UA) have increasingly gained therapeutic relevance due to their wide scope of pharmacological activities. To fit large-scale demands, exploitable sources of these compounds have to be found and simple, cost-effective methods to extract them developed. Leaf material represents the best plant sustainable raw material. To obtain triterpene acid-rich extracts from leaves of Eugenia, Psidium and Syzygium species (Myrtaceae) by directly treating the dry plant material with alkalinized hydrated ethanol. This procedure was adapted from earlier methods to effect depolymerization of the leaf cutin.

Materials and Methods: Extracts were prepared by shaking the milled dry leaves in freshly prepared 2% NaOH in 95% EtOH solution (1:4 w/v) at room temperature for 6 h. Working up the product in acidic aqueous medium led to clear precipitates in which BA, OA and UA were quantified by gas chromatography.

Results: Pigment-free and low-polyphenol content extracts (1.2–2.8%) containing 6–50% of total triterpene acids were obtained for the six species assayed. UA (7–20%) predominated in most extracts, but BA preponderated in Eugenia florinda (39%). Carried out in parallel, n-hexane defatted leaves led to up to 9% enhancement of total acids in the extracts. The hydroalcoholate treatment of Myrtaceae species dry leaves proved to be a cost-effective and environmentally friendly method to obtain triterpene acids, providing them be resistant to alkaline medium. These combined techniques might be applicable to other plant species and tissues.

Key words: Alkaline extraction, cutin depolymerization, Eugenia, Psidium, Syzygium, triterpene acids

INTRODUCTION

Triterpene acids are ubiquitous lipophilic compounds found in plants throughout the vegetal kingdom. They are present in the tissues of many plant organs, particularly among the wax constituents of the leaf surface and fruit cuticles. In general, they belong to the pentacyclic lupane, oleanane or ursane series, represented by betulinic (BA), oleanolic (OA) and ursolic acid (UA), respectively [Figure 1], as well as their less abundant functional derivatives. In the last two decades, these compounds have been recognized as wide-spectrum bioactive molecules[2] and are useful ingredients in therapeutic and nutraceutical formulations.[3,4] As a result, the search for an abundant source of these compounds has intensified. Hence, innumerable plant species[5] and waste from the food and timber industries[6,7] have been reported as potential sources of triterpene acids. The presence of bioactive triterpenoids in fruit cuticles has been recently reviewed.[8]

The recovery of triterpene acids from plant tissues has been primarily achieved using methanol or ethanol as
the first extraction step, as concisely reviewed by Goulas and Manganaris.\[^9\] In addition, maceration, soxhlet,\[^9,10\] or ultrasound-assisted processes\[^9\] have been applied to diverse plant matrices for the laboratory-scale extraction of triterpene acids. Most commonly, solvents such as methanol, ethanol, chloroform, ethyl acetate or appropriate mixtures of these have been employed to obtain triterpene acids from plant tissues.\[^9,11-15\] Solvent partitions of crude extracts,\[^16\] followed by open column chromatography on silica gel\[^17\] or techniques such as countercurrent chromatography\[^18-20\] have also been utilized to isolate these compounds.

This study reports a rapid and cost-effective method to extract triterpene acids from the dry leaves of six Myrtaceae species that utilizes a 2% sodium hydroxide solution in hydrated (95%) EtOH at room temperature, applied directly to the plant matrix, followed by acid-base partitioning of the soluble fraction. The species Eugenia brasiliensis, Eugenia florida, Eugenia uniflora, Syzygium cumini, Psidium cattleianum and Psidium guajava were selected either on the basis of previous descriptions reporting their triterpene acid content\[^18\] or by chemotaxonomic correlations.\[^19\] The resulting extracts were quantitatively analyzed by gas chromatography (GC) to determine the amounts of OA, BA and UA. Furthermore, the yields and triterpene acid composition of the extracts prepared from dry or previously defatted raw material were compared.

**MATERIALS AND METHODS**

**Plant material**

Leaves were collected and identified (1–1.5 Kg) from *E. florida* DC., *E. brasiliensis*, *Lam. E. uniflora* L., *P. cattleianum* Sabine, *P. guajava* L. and *S. cumini* (L.) Skeels. Data for the collections and voucher deposits are displayed in Table 1. The leaves (1–1.5 Kg) were oven-dried at 45°C under constant aeration (De Leo e Cia., Porto Alegre, Brazil) for 8 days. They were ground into small flakes with the aid of a kitchen blender and then properly stored in sealed glass vials until performing the extraction.

**Chemicals**

Diazomethane was prepared by treating N-methyl-N-nitroso-p-toluene sulfonamide (Diazald®, Sigma-Aldrich, St Louis, MO, USA) with sodium hydroxide (Vetec, Xerém, Brazil) in ethyl ether (Merck, Darmstadt, GE). Dichloromethane was purchased from Tedia (Fairfield, OH, USA). Ethanol p.a. absolute was obtained from Merck. UA (>90% purity, article U6753), OA (>97% purity, article O5504) and BA (90% purity, article 91466) were purchased from Sigma-Aldrich Co to be used as standards.

**Extraction**

The milled dry leaves of each species (25 or 40 g) and a freshly prepared 2% NaOH solution (100 or 180 mL) in hydrated (95%) EtOH were placed in erlenmeyer flasks, and smoothly stirred in a digital orbital shaker (IKA K5501, Leiden, NL) for 6 h at room temperature. After filtering the mixture and discarding the leaf debris, the solvent was removed from the filtrate in a rotary evaporator. To achieve an adequate homogenization, the resulting solid was suspended in methanol (10 or 16 mL) prior to the addition of distilled water (40 or 64 mL). The pH was lowered to 2–3 with 1N HCl. The suspension was stirred for an additional period (30 min) and then cooled by standing for 1 h in the refrigerator. The precipitate was filtered through filter paper (Whatman grade 4) in a Buchner funnel, left to dry at room temperature and dried overnight under high vacuum (lyophilizer Christ Mod Beta 2–16, Merrington, UK) to afford colorless to pale yellowish solids. In parallel, dry leaves of each species (150 g) were submitted to static maceration with *n*-hexane (250 mL), during 4 days at room temperature. After filtering by gravity (filter paper and a glass funnel) the leaf residue was extracted once more with the same amount of solvent at the same conditions. The filtrates were pooled and evaporated to afford the *n*-hexane extract. The defatted leaves were dried on standing in the laboratory hood, and then submitted to the alkalinized ethanol extraction as described above.

**Gas chromatography-flame ionisation detectors analysis**

**Sample preparation**

Approximately, 3 mg of the crude solid obtained from
the plant material extraction was weighed and methylated twice with excess (2 mL) diazomethane in ethyl ether. The esterified samples were dissolved in CHCl₃ (1.0 mL) before being analyzed by GC-flame ionization detectors (GC-FID).

### Quantitative gas chromatography analysis
Gas chromatography analyses were performed on an Agilent (Agilent Technologies, Santa Clara, USA) model 6890 gas chromatograph fitted with a DB-17HT column (30 m × 0.32 mm × 0.15 μm) filled with 50% phenyl-50% methylpolysiloxane (J&W Scientific, Santa Clara, USA), as previously described. The contents of BA, OA and UA methyl derivatives in the extracts were calculated with reference to the appropriate calibration curves, as previously described. All analyses were conducted in triplicate. Statistical tests were performed using Statistica 8.0 (Stat Soft Inc., Tulsa, USA). The Levene, Shapiro–Wilk and Durbin–Watson tests were used to check the linearity of the calibration curves. A confidence level of 95% was considered as statistically significant \( (P < 0.05) \).

## RESULTS AND DISCUSSION

### Extraction protocol
Affording triterpene acids from plant tissues initially requires optimization of the extraction conditions to overcome the low solubility of these compounds in most conventional solvents. In the case of fruit peels and leaf extracts, co-extraction of the residual cutin must be avoided. Cutin is a natural polymeric compound that consists of highly insoluble phenolic esters of hydroxy-fatty acids and results in an intractable consistency in the dry organic extracts. Thus, procedures that hydrolyze the polyester net structure of cutin would be useful to facilitate solvent penetration in the matrix cells and the recovery of metabolites. This procedure is known as cutin depolymerization and is usually conducted in an alkaline medium using KOH or NaOH. This treatment has been extensively employed to investigate the epoxy- and hydroxy-fatty acids originally esterified in the cutin matrix in order to establish useful correlations between the composition of cutin and its ability to protect leaf tissues. Hence, this hydrolytic treatment was employed in the earliest studies related to the isolation and identification of triterpene acids from diverse leaf and fruit organic extracts. Further examples of treating ethanol, chloroform or ether plant extracts in refluxing aqueous or alcoholic potassium hydroxide solution (3–10% w/v) to release triterpene acids from viscous plant extracts have been described. More recently, alkaline hydrolysis has also been utilized to produce workable samples for the quantitative chromatographic analyses of triterpene acids. The present study reports the effects of a 2% NaOH solution in hydrated ethanol, at room temperature, on the dry leaves of six Myrtaceae species. This method proved straightforward and rapid, and it afforded extracts with high concentrations of triterpene acids that are resistant to this extraction conditions, as the case of OA, BA and UA.

The crude leaf extract yields from treating whole or defatted dry leaves with 2% NaOH in ethanol are presented in Table 2. Higher extraction yields (2.7–2.9%) were obtained from *P. guajava, S. cumini*, and *E. florida* followed by *E. brasiliensis* and *S. cumini*.

### Table 1: General data for leaf collection and identification of *Myrtaceae* species

| Specie/collection data | Common name | Local of collection | GeoCoordinates | Voucher* |
|------------------------|-------------|---------------------|----------------|---------|
| **EB Lam. October 2009** | Brazil cherry, grumixama | Cultivate, campus State University of Campinas, SP | S: 22° 49′ 10.7″ W: 47° 04′ 27.1″ | RB 326234 |
| **EF DC October 2009** | Guamirim-cereja, pitanga preta | Wild, Guará District, city of Campinas, SP | S: 22° 47′ 29.8″ W: 47° 04′ 25.4″ | RFA 39907 |
| **EU L. July 2010** | Surinam cherry, cayenne cherry, pitanga | Campus Oswaldo Cruz Foundation, Rio de Janeiro, RJ | S: 22° 52′ 36.4″ W: 43° 15′ 01.39″ | RB361704 |
| **PC Sabine June 2010** | Araçá, araçá comum, araçá-rosa | Cultivate, urban garden, city of Campinas, SP | S: 22° 53′ 20.3″ W: 47° 02′ 39.9″ | RB 361721 |
| **PG November 2013** | Goiaba, guava | Campus Oswaldo Cruz Foundation, Rio de Janeiro, RJ | S: 22° 52′ 37.42″ WO: 43° 15′ 02.15″ | RFA 39916 |
| **SC L. Skeels July 2010** | Jambul, black plum, jambolan plum, jambolão, jameilão, azeitona-da-terra | Campus Oswaldo Cruz Foundation, Rio de Janeiro, RJ | S: 22° 52′ 34.3″ W: 41° 15′ 01.25″ | RB 380789 |

*RB=Herbarium of the Rio de Janeiro Botanical Garden, Brazil. RFA=Herbarium of the Rio de Janeiro Federal University, Brazil. EB: Eugenia brasiliensis, EF: Eugenia florida, EU: Eugenia uniflora, PC: Psidium cattleianum, PG: Psidium guajava, SC: Syzygium cumini.*
by *E. uniflora* and *E. brasiliensis* (2.3–2.5%). The lowest yield was obtained from *P. cattleianum* (1.2%). Leaves were defatted before the alkalinized ethanol extraction in an attempt to produce extracts with higher quantities of triterpene acids. This procedure was based on the enhancement of UA and OA yields in the ethanol extracts of dry apple peels in ethanol after previous treatment with *n*-hexane. The use of defatted dry leaves led to variable reductions (2.7% and 5.2%) in the crude extract yields in four species. The exceptions were the yields of *E. uniflora*, which dropped to about a quarter of the original value, and 15% for *E. brasiliensis*. In both cases, significant amounts of sesquiterpenes were present in the *n*-hexane extracts, as determined by GC-mass spectrometry (data not shown), especially in *E. uniflora* leaves, in which this metabolite class is generally predominant.

Although triterpene acids are poorly soluble in hydrocarbon solvents, a certain amount would eventually dissolve when using a large leaf-solvent ratio (which was not the case). Co-solvation effects resulting from the presence of other components in the extract, particularly fatty acid derivatives, may also favor this phenomenon. To examine this possibility, *n*-hexane extracts were also quantitatively analyzed for the presence of triterpene acids. As a result, the maceration with *n*-hexane at room temperature extracted between 0.5% and 1.8% of the total triterpene acid content from leaves of *E. brasiliensis*, *S. cumini* and *E. florida*. No significant losses were detected for the other three species submitted to the same procedure [Table 3a]. A higher mass loss from leaf defatting was observed for *E. florida* samples. This may be related to its high content of BA, which is the most soluble of the three acids in organic solvents. Otherwise, the losses in triterpene acids from *n*-hexane maceration were not significant, as they were <4.0% for those three species, with respect to the amount obtained by using the alcoholate protocol on the whole leaves. The low efficiency of *n*-hexane extraction of these three acids from leaves and fruit peels has been reported elsewhere. However, the increase in yield of the total triterpene acids achieved by initially macerating the leaves with nonpolar solvents may be carefully evaluated when aiming large-scale preparations. The alcoholate protocol also resulted in colorless or whitish leaf extracts in all the cases. Their low contents in polyphenol may be inferred from the acid-base partition employed, ending at pH 2. This condition should solubilize these compounds and remove them off the extracts during the final filtration and washing. Moreover, analyses by high performance liquid chromatography corroborated the absence of aromatic chromophores among the few peaks detected for all the extracts (data not shown). All this considerations point out to the high potential of the developed protocols for further purification of triterpene acids. The direct treatment of dry leaves with NaOH in ethanol skips the usual initial step for triterpene acid separation, which typically involves alcoholic extraction. There is a lack of available literature regarding the application of the depolymerization method directly to plant matrices.

The inclusion of a previous defatting procedure with the appropriate solvent should be considered for improving the cost-effectiveness of the overall process. In this context, the extraction selectivity should be further investigated in sesquiterpene-rich plant tissues. In addition, this procedure is cost-effective (consume only alcohol, sodium hydroxide and hydrochloric acid) and environmentally friendly because water and sodium chloride is the only waste products. A potential limitation of the method would be its effectiveness with plant matrices that contain metabolites susceptible to alkaline media, such as triterpene esters and lactones. This issue requires further investigation.

### Myrtaceae leaves as source of triterpene acids

The triterpene acid contents in the selected species have been reported elsewhere, including the previous isolations of UA derivatives from *E. brasiliensis* [17,18] BA from *E. florida* [19,30] and derivatives of the three acids from *P. guajava* [31] and *S. cumini*. *E. uniflora* and *P. cattleianum* were included in this group as potential targets to afford similar metabolites.

The GC chromatograms shown in Figure 2 reveal that the alkalinized ethanol extraction resulted in relatively simple extract compositions, and the signals corresponding to the triterpene acids are clearly distinguished. Furthermore, nonacidic lipophilic leaf constituents, pigments and phenols were largely minimized.

*Eugenia uniflora* extract had the lowest triterpene acid content (total <8%). In other extracts, the

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**Table 2: Extract yields after the dry leaf treatment with 2% NaOH in ethanol for the six Myrtaceae species**

| Species | EtOH/NaOH 2% extract yield (% w/w) | n-hexane extract\(^a\) % |
|---------|----------------------------------|-------------------------|
|         | Whole dry leaves | Defatted dry leaves\(^a\) (%) | |
| EB      | 2.36±0.13 | 2.00±0.26 (<15) | 1.10 |
| EF      | 2.69±0.33 | 2.55±0.15 (<5.5) | 1.10 |
| EU      | 2.47±0.10 | 1.88±0.13 (<24) | 2.03 |
| PC      | 1.19±0.12 | 1.13±0.12 (<5.0) | 0.56 |
| PG      | 2.91±0.34 | 2.83±0.09 (<2.7) | 1.23 |
| SC      | 2.78±0.22 | 2.65±0.11 (<4.7) | 1.33 |

\(^{a}\) In parentheses: amount of yield decreasing by defatting procedure, \(^{b}\) Hexane extract was generated from a single batch of dry leaves (figure 2). Except for this procedure, all other values are mean of triplicate extraction experiments. EB: Eugenia brasiliensis, EF: Eugenia florida, EU: Eugenia uniflora, PC: Psidium cattleianum, PG: Psidium guajava, SC: Syzygium cumini

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**Note:**

[11,21] The triterpene acid contents in the selected species have been reported elsewhere, including the previous isolations of UA derivatives from *E. brasiliensis* [17,18]. BA from *E. florida* [19,30] and derivatives of the three acids from *P. guajava* [31] and *S. cumini*. [32] *E. uniflora* and *P. cattleianum* were included in this group as potential targets to afford similar metabolites. [19] The inclusion of a previous defatting procedure with the appropriate solvent should be considered for improving the cost-effectiveness of the overall process. In this context, the extraction selectivity should be further investigated in sesquiterpene-rich plant tissues. In addition, this procedure is cost-effective (consume only alcohol, sodium hydroxide and hydrochloric acid) and environmentally friendly because water and sodium chloride is the only waste products. A potential limitation of the method would be its effectiveness with plant matrices that contain metabolites susceptible to alkaline media, such as triterpene esters and lactones. This issue requires further investigation.

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content ranged from 11% in *P. guajava* to 50% in *E. florida* [Table 3b]. These levels were increased in the extracts from defatted leaves, approximately 4% in the case of *E. brasiliensis* and *P. cattleianum* and 9–12% in the other four species [Table 3c]. This increase results from the minor amount of fatty acids, alcohols and hydrocarbons in the extracts of defatted leaves regardless of the qualitatively similar chromatograms observed from both extraction protocols (data not shown). Except for *E. florida*, which afforded an extract rich in BA (39%), UA was the predominant triterpene acid in the leaf extracts, particularly in *E. brasiliensis* (≈15%) and *P. cattleianum* (≈20%). Considering the minor levels of the two other acids, these two extracts are good candidates for the further purification of UA.

Few quantitative studies on triterpene acids in *Myrtaceae* species have been reported in the literature. Subjecting the defatted leaves of *E. florida* to soxhlet extraction with ethanol resulted in extracts containing up to 30% BA, depending on seasonal conditions. More selective extraction of BA was achieved using chloroform or ethyl acetate and varying the extraction method; although total extract yield was significantly lower. Isolation from the ethanol extract by high-speed countercurrent chromatography yielded 17% of the pure BA, after solvent partitioning and open silica gel column chromatography. Compared with these studies, the method in the present study resulted in extracts containing higher amounts of BA and UA, thus demonstrating that these two *Myrtaceae* species are viable sources for these compounds.

**CONCLUSION**

Workable, pigment-free and low-polyphenol content extracts were obtained by treating directly dry leaves of selected plants with a solution of 2% NaOH in ethanol at room temperature. This procedure may be useful for producing triterpene acid-based bioactive extracts as well
as initializing the purifications of such compounds. The inclusion of a previous leaf defatting procedure with the appropriate solvent should be regarded at the light of the process cost-effectiveness.

Once applied to leaves, this method also stands out for its sustainability aspect, since it is one of the main processes in the plant being a renewable source of vegetal raw material. From selecting the proper species and aiming the production of valuable triterpene acids, this method is also useful to purify any of the three compounds assayed in this study. Due to the previous knowledge on the contents of triterpene acids, the leaves of Myrtaceae species were good matrices to verify the protocol efficiency. However, the scope of the method indeed excludes the limits of any botanical group, since it may be applied to general plant substrates containing cutin (or suberin). Moreover, the method is cost-effective and environmentally friendly, and can be applied to recover triterpene acids that are resistant to the extraction conditions, as is the case of OA, BA and UA. In spite of this fact, molecules that would be more susceptible, as triterpene lactones and esters cannot be immediately excluded, since substrates containing them have not been assayed yet.

Table 3: Content of triterpene acids in Myrtaceae leaf extracts after extraction with alkaline ethanol

| Species | OA      | BA      | UA      | Total acids |
|---------|---------|---------|---------|-------------|
|         | A. Triterpene acid content in the n-hexane extract (%) |
| EB      | 0.4±0.0 | 0.1±0.0 | <QL     | 0.5±0.2     |
| EF      | 2.0±0.1 | 0.4±0.1 | 0.7±0.1 | 1.3±0.2     |
| EU      | <QL    | <QL    | <QL    | -           |
| PC      | <QL    | <QL    | <QL    | -           |
| PG      | 0.2±0.0 | 0.4±0.1 | <QL     | 0.7±0.1     |
| SC      | <QL    | <QL    | <QL    | -           |
|         | B. Triterpene acid content from whole leaves (%) |
| EB      | 4.3±0.2 | 1.5±0.1 | 14.8±0.2 | 20.6±0.3    |
| EF      | 2.4±0.5 | 3.8±0.3 | 9.0±0.1 | 50.2±0.3    |
| EU      | 1.7±0.0 | <QL    | 4.7±0.2 | 6.4±2.4     |
| PC      | 5.7±0.1 | <QL    | 20.4±1.2 | 26.1±1.3    |
| PG      | 2.2±0.1 | 1.5±0.1 | 7.3±0.2 | 11.0±0.3    |
| SC      | 4.8±0.4 | 3.5±0.1 | 8.8±1.0 | 17.1±1.5    |
|         | C. Triterpene acid content from defatted leaves (%) |
| EB      | 4.2±0.3 | 1.3±0.1 | 16.0±0.9 | 21.5±1.2    |
| EF      | 2.5±0.0 | 42.9±0.3 | 9.5±0.1 | 54.8±0.3    |
| EU      | 1.6±0.1 | <QL    | 5.4±0.0 | 7.0±0.1     |
| PC      | 5.7±0.2 | <QL    | 21.4±1.7 | 27.1±1.8    |
| PG      | 2.2±0.0 | 1.9±0.2 | 8.0±0.1 | 12.1±0.3    |
| SC      | 5.5±0.4 | 3.9±0.2 | 10.0±1.3 | 19.4±1.9    |

*All values are mean of extraction experiments in triplicate and averaged from three sample injections. EB: Eugenia brasiliensis, EF: Eugenia florida, EU: Eugenia uniflora, PC: Psidium cattleianum, PG: Psidium guajava, SC: Syzygium cumini, OA: Oleanolic acid, BA: Betulinic acid, UA: Ursolic acid. QL: Quantitation limit

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REFERENCES

1. Bringe K, Schumacher CF, Schmitz-Eiberger M, Steiner U, Oerke EC. Ontogenetic variation in chemical and physical characteristics of adaxial apple leaf surfaces. Phytochemistry 2006;67:161-70.
2. Liu J. Oleanolic acid and ursolic acid: Research perspectives. J Ethnopharmacol 2005;100:92-4.
3. Jäger S, Winkler K, Pfüller U, Scheffler A. Solubility studies of oleanolic acid and betulinic acid in aqueous solutions and plant extracts of Viscum album L. Planta Med 2007;73:157-62.
4. Xi J, Chang Q, Chan CK, Meng ZY, Wang GN, Sun JB, et al. Formulation development and bioavailability evaluation of a self-nanoemulsified drug delivery system of oleanolic acid. AAPS PharmSciTech 2009;10:172-82.
5. Jäger S, Trojan H, Kopp T, Laszczyk MN, Scheffler A. Pentacyclic triterpene distribution in various plants - rich sources for a new group of multi-potent plant extracts. Molecules 2009;14:2016-31.
6. Frithegott RT, Welinder RM, Nigro EM, Frithetto N, Sianic AC. Isolation of ursolic acid from apple peels by high speed counter-current chromatography. Food Chem 2008;106:767-71.
7. Domingues RM, Sousa GD, Silva CM, Freire CS, Silvestre AJ, Pascoal-Neto C. Eucalyptus globulus biomass residues from pulping industry as a source of high value triterpenic compounds. Ind Crops Prod 2009;31:65-70.
8. Szakiel A, Paczkowski C, Pensec F, Bertsch C. Fruit cuticular waxes as a source of biologically active triterpenoids. Phytochem Rev 2012;11:263-84.
9. Poulas V, Mangananis GA. Towards an efficient protocol for the determination of triterpenic acids in olive fruit: A comparative study of drying and extraction methods. Phytochem Anal 2012;23:444-9.
10. Guinda A, Rada M, Delgado T, Gutiérrez-Adánez J, Castellano JM. Pentacyclic triterpenoids from olive fruit: A comparative study of drying and extraction methods. Phytochem Anal 2010;21:9685-91.
11. Ellgardt K. Triterpenes in Apple Cuticle of Organically and IP Programme. Sveriges Alnarp, Sweden: Lantbruksuniversitet; 2006. p. 1579-652.
12. Banki RM, Pandey DK. Optimizing conditions for oleonic acid extraction from Lantana camara roots using response surface methodology. Ind Crops Prod 2008;27:241-8.
13. Schneider P, Hosseiny SS, Szczotka M, Jordan V, Schlitter K. Rapid solubility determination of the triterpenes oleandric acid and ursolic acid by UV-spectroscopy in different solvents. Phytochem Lett 2009;2:85-7.
14. Tarvainen M, Suomela J, Kallio H, Yang B. Triterpenes in Plantago major: Identification, quantification and comparison of different extraction methods. Chromatographia 2010;71:279-84.
15. Siani AC, Nakamura MJ, Dos Santos DS, Mazzei JL, do Nascimento AC, Valente LM. Efficiency and selectivity of triterpene acid extraction from decoctions and tinctures prepared from apple peels. Pharmacogn Mag 2014;10:S225-31.
16. Jeong W, Hong SS, Kim N, Yang YT, Shin YS, Lee C, et al. Bioactive triterpenoids from Callistemon lanceolatus. Arch Pharm Res 2009;32:845-9.
17. Magina MD, Dalmarco EM, Dalmarco JB, Colla G, Pizzolatti MG, Brighente MC. Bioactive triterpenes and phenolics of leaves of *Eugenia brasiliensis*. Quim Nova 2012;35:1184-8.
18. Frighetto N, Welendorf RM, Silva AM, Nakamura MJ, Siani AC. Aplicação de cromatografia centrífuga de contracorrente na purificação de ácido ursólico das folhas de *Eugenia brasiliensis* Lam. Braz J Pharmacogn 2005;15:338-43.
19. Frighetto N, Welendorf RM, Pereira da Silva AM, Nakamura MJ, Siani AC. Purification of betulinic acid from *Eugenia florída* (Myrtaceae) by high-speed counter-current chromatography. Phytochem Anal 2005;16:411-4.
20. Maurya A, Srivastava SK. Preparative-Scale separation of anticancer triterpenes from *Eucalyptus* hybrid by centrifugal partition chromatography. Sep Sci Technol 2011;46:1189-94.
21. Lima AM, d’Avila LA, Siani AC. Comparison between methyl and trimethylsilyl ester derivatives in the separation and GC quantification of triterpene acids in *Eugenia brasiliensis* leaf extract. Chromatographia 2014;77:629-35.
22. Jin IJ, Ko YI, Kim YM, Han SK. Solubilization of oleanolic acid and ursolic acid by cosolvency. Arch Pharm Res 1997;20:269-74.
23. Huelin FE. Studies in the natural coating of apples. IV. The nature of cutin. Aust J Biol Sci 1959;12:175-80.
24. Kolattukudy PE. Biopolyester membranes of plants: Cutin and suberin. Science 1980;208:990-1000.
25. Holloway PJ, Deas AH. Epoxyoctadecanoic acid in plant cutins and suberin. Phytochemistry 1973;12:1721-35.
26. Sando CE. Ursolic acid. J Biol Chem 1931;90:477-95.
27. Pereira SI, Freire CS, Pascoal Neto C, Silvestre AJ, Silva AM. Chemical composition of the epicuticular wax from the leaves of *Eucalyptus globulus*. Phytochem Anal 2005;16:364-9.
28. Costa DP, Santos SC, Seraphin JC, Ferri PH. Seasonal variability of essential oils of *Eugenia uniflora* leaves. J. Braz Chem Soc 2009;20:1287-93.
29. Zhao A. Process of extracting ursolic acid from haw. Univ. Qingdao, assignee. Patent CN 100543030 C; 23 Sep, 2009.
30. Nóbrega AN. Padronização de Extratos de Eugenia Florida DC. e Seu Estudo Toxicológico Para o Desenvolvimento de um Fitoterápico ou Fitofármaco. MSc Thesis, Faculty of Pharmacy, Fluminense Federal University, Niterói, Brazil; 2012.
31. Ghosh P, Mandal A, Chakraborty P, Rasul MG, Chakraborty M, Saha A. Triterpenoids from *Psidium guajava* with Biocidal Activity. Indian J Pharm Sci 2010;72:504-7.
32. Gupta GS, Sharma DP. Triterpenoid and other constituens of *Eugenia jambolana* leaves. Phytochemistry 1974;13:2013-4.

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