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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Submitted: 19-07-2014 Revised: 13-08-2014 Published: 10-07-2015

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 florida* (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

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Website: www.phcog.com

DOI: 10.4103/0973-1296.160453

Quick Response Code:

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.[1] 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, innumerous 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

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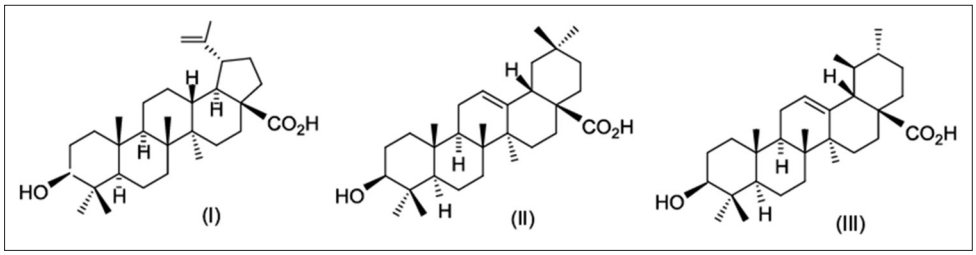

Figure 1: Triterpene structures of the triterpene acids. I = betulinic (3 β-hydroxyursan-12-en-28 oic) acid; II=oleanolic (3 β-hydroxyolean-12-en-28-oic) acid, III = ursolic (3 β-hydroxy-lup-20(29)-en-28-oic) acid

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 ethanol 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*-toluenesulfonamide (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

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

Specie/ collection data	Common name	Local of collection	GeoCoordinates	Voucher[a]
EB Lam. October 2009	Brazil cherry, *grumixama*	Cultivate, campus State University of Campinas, SP	S: 22° 49" 10.7″ W: 47° 04" 27.1″ Alt.: 604 m	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″ Alt.: 583 m	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″ Alt.: 15 m	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″ Alt.: 625 m	RB 361721
PG L. November 2013	Goiaba, guava	Campus Oswaldo Cruz Foundation, Rio de Janeiro, RJ	S: 22° 52" 37. 42″ WO: 43° 15" 02. 15″ Alt.: 13 m	RFA 39916
SC L. Skeels July 2010	Jambul, black plum, jambolan plum, *jambolão, jamelão, azeitona-da-terra*	Campus Oswaldo Cruz Foundation, Rio de Janeiro, RJ	S: 22° 52" 34.3″ W: 41° 15" 01.25″ Alt.: 15 m	RB 380789

[a]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*

the plant material extraction was weighed and methylated twice with excess (2 mL) diazomethane in ethyl ether. The esterified samples were dissolved in CH_2Cl_2 (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.[21] 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.[3,13,22] 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.[23] 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[24] 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.[25] 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.[26] 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.[23,25] More recently, alkaline hydrolysis has also been utilized to produce workable samples for the quantitative chromatographic analyses of triterpene acids.[27] 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

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[b] %
	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 (<5.2)	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]*n*-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*

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.[11] 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.[28]

Although triterpene acids are poorly soluble in hydrocarbon solvents,[22] 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.[3] 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.[11,21] 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.[29] 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*.[32] *E. uniflora* and *P. cattleianum* were included in this group as potential targets to afford similar metabolites.[19]

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

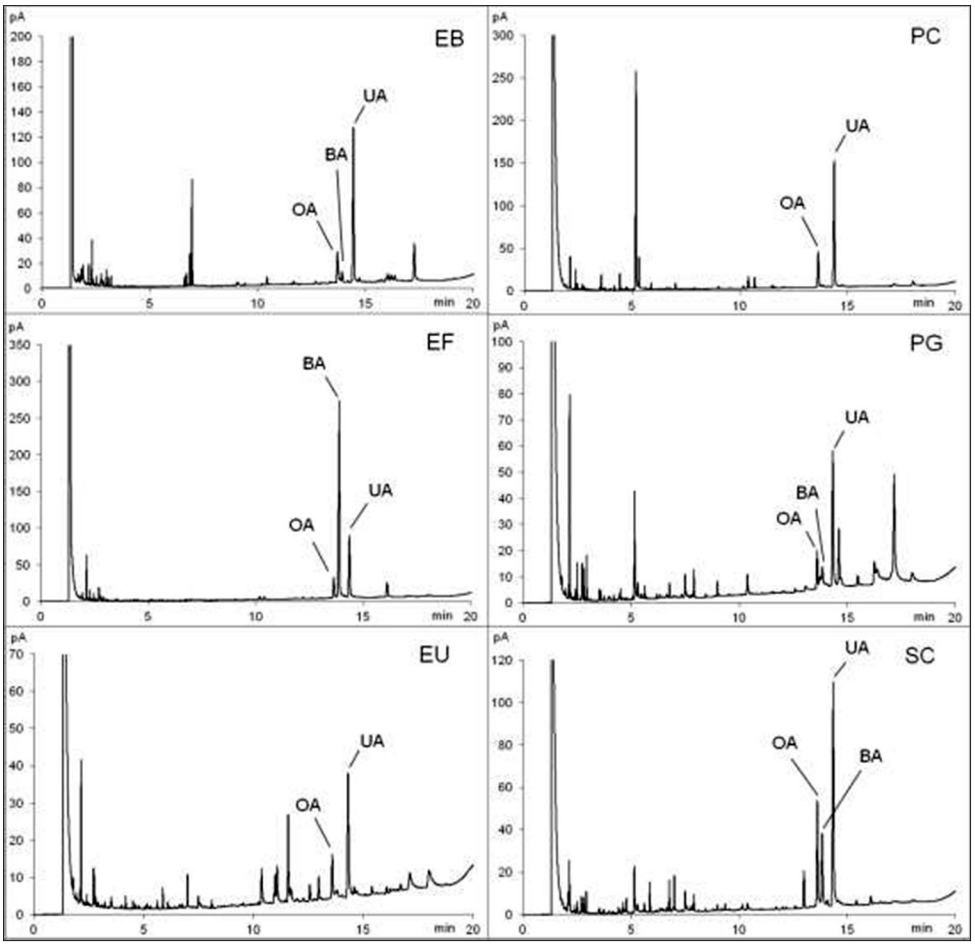

Figure 2: Typical chromatograms of the extracts from the six Myrtaceae species obtained from dry leaves treatment with NaOH 2% in ethanol. OA=Oleanolic acid; BA=Betulinic acid; UA=Ursolic acid. EB=*Eugenia brasiliensis*; EF=*Eugenia florida*; EU=*Eugenia uniflora*; PC=*Psidium cattleianum*; PG=*Psidium guajava*, SC=*Syzygium cumini*

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.[30] Isolation from the ethanol extract by high-speed countercurrent chromatography yielded 17% of the pure BA.[19] UA was purified in low yield (0.22%) from the ethanol extract of *E. brasiliensis* after solvent partitioning and open silica gel column chromatography.[18] 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

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	0.2±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	38.8±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 (%)[a]			
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

[a]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

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 this part of the plant plays 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 exceeds 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.

ACKNOWLEDGMENTS

We are indebted to the biologist Sérgio S. Monteiro for helping with botanical authentication and vouchers, and to Dr. André L. F. Sampaio for providing part of the commercial standards.

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Cite this article as: Lima AB, Siani AC, Nakamura MJ, D'Avila LA. Selective and cost effective protocol to separate bioactive triterpene acids from plant matrices using alkalinized ethanol: Application to leaves of Myrtaceae species. Phcog Mag 2015;11:470-6.

Source of Support: Nil, **Conflict of Interest:** None declared.