IN VITRO CHARACTERIZATION OF ANTIOXIDANT PROPERTIES OF CUBAN ENDEMIC VARIETIES OF *Erythroxylum alaternifolium* A. Rich. ISOLATION OF TWO FLAVONOL GLYCOSIDES

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ABSTRACT

The total antioxidant capacity from leaves of three Cuban endemic varieties of *Erythroxylum alaternifolium* was measured using three techniques: 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS⁺) and Oxygen radical absorbance capacity (ORAC). The highest values of antioxidant capacity and total phenols were measured in polar *n*-butyl alcohol extracts. Among the varieties of *E. alaternifolium* assayed, the var. *parvifolium* showed the strongest antioxidant capacity: ABTS (6.49 ± 0.05 mg TE/g dw); DPPH (11.16 ± 0.01 mg TE/g dw) and ORAC (35.1 ± 1.5 mg TE/g dw). Additionally, *n*-butyl alcohol extract of *E. alaternifolium* var. *parvifolium* also showed the highest content in phenolics with 284.2 ± 7.3 mg CAE/g dw and in flavonols with skeleton quercetin, kaempferol and myricetin with 471 μ g/g dw. Moreover, two flavonol glycosides: ombuin-3-*O*-rutinoside and quercetin-3-*O*-rutinoside were isolated for the first time from *n*-butyl alcohol extract of *E. alaternifolium* var. *alaternifolium*.

Keywords: *Erythroxylum*; Antioxidant assays; Flavonols aglycone; Ombuin-3-*O*-rutinoside; Quercetin-3-*O*-rutinoside.

INTRODUCTION

Erythroxylaceae Kunth is a tropical family comprising four genera and 260 species and it is well known as "The Coca Family". The genera are *Aneulophus* Benth. (two species), native to tropical Africa, *Nectaropetalum* Engl. (six species), in the tropics of South Africa, *Pinacopodium* Exell & Mendoca (two species) in Africa and *Erythroxylum* P. Browne, the most species-rich genus, with 250 described species. The *Erythroxylum* species are shrubs distributed in the tropical regions of South America, Africa, Southeast Asia and Australia of which 22 are found in Cuba (16 endemics). The leaves of *Erythroxylum* have been used in traditional medicine as anti-inflammatory and anti-bacterial agents, tonics and stimulants, among other uses¹. Pharmacological screening has also identified the *Erythroxylum* genus as a promising source of antiviral drugs². The protective effect of crude polar leaf extracts from *E. minutifolium* Griseb. var. *minutifolium* and *E. confusum* Britt. on the oxidative damage induced by model toxicants in rat hepatocyte cultures have also been studied³. In addition, the antioxidant capacity of *E. coca* Lam. has been recently reported⁴.

On the other hand, many chemical studies have been carried out with the genus *Erythroxylum*. It was shown that alkaloids based on the tropane skeleton are the most widely distributed secondary metabolites in the genus. The species *E. coca* and *E. novogranatense* (Morris) Hieron. are well known as natural sources of cocaine. In Cuba, some novel tropane alkaloids have also been isolated from *E. rotundifolium* Lunan (currently considered as a synonym of *E. suave* O. E. Schulz) and *E. alaternifolium* A. Rich var. *alaternifolium*⁵. However, terpenoids⁶ and flavonoids⁷ are other groups of metabolites widespread in the tissues of *Erythroxylum* species. The most frequent flavonoids are flavonols often glycosylated at position 3 of the flavan skeleton¹.

Kaempferol and quercetin are the main aglycone flavonols in *Erythroxylum*⁸, and the occurrence of flavonols has been useful as a chemotaxonomic marker in some taxa⁸⁻¹¹. In Cuba, three flavonoid glycosides with a quercetin skeleton were isolated from endemic *E. minutifolium* Griseb. var. *minutifolium* and quantified in other Cuban species: *E. areolatum* Linn., *E. confusum* Britt. and *E. suave*¹. Flavonoids are a group of phytochemicals with a wide arsenal of biological properties, which include free radical scavenging, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory action, among other biological properties¹².

On the basis of the ethno medicinal and pharmacological uses described in some species of the genus *Erythroxylum* and their reported content in flavonoids we decided to evaluate the antioxidant capacity of non-polar and polar leaf extracts from three endemic varieties of *E. alaternifolium* using three different assays. The concentration of total phenols and flavonoids were also determined and three aglycone flavonol forms were quantified using HPLC. Two flavonol glycosides were isolated from the *n*-BuOH leaf extract of the *E. alaternifolium* var. *alaternifolium*.

EXPERIMENTAL

Plant material

The varieties of *E. alaternifolium* were collected in two western localities of Cuba. *E. alaternifolium* var. *alaternifolium* was collected in Loma de La Coca, Havana while *E. alaternifolium* var. *parvifolium* and *E. alaternifolium* var. *suborbiculare* were collected in Cajalbana, Pinar del Río. MSc. Ramona Oviedo Prieto, specialist of Cuban *Erythroxylaceae*, performed the taxonomic identification of the varieties of *E. alaternifolium* and the voucher specimens were kept in the herbarium HAC of the "Instituto de Ecología y Sistemática".

Extraction

First, 50 g of dried and powdered leaves of the three varieties of *E. alaternifolium* were extracted with 200 mL of *n*-hexane during 60 min using reflux. The remaining material was re-extracted successively with 200 mL of chloroform, 200 mL of ethyl acetate and eventually with 200 mL of *n*-butanol (*n*-BuOH), using the same extraction conditions. The resulting non-polar and polar solutions were filtered and dried at low pressure. 1 mg/mL solutions in methanol were prepared before analyses. All extractions were performed in duplicates.

Determination of total phenolic compounds

The amount of total phenolic compounds in the different crude extracts was determined spectrophotometrically using the Folin-Ciocalteu's reagent¹³, using chlorogenic acid as standard (0 to 500 mg/L). 3.6 mL of diluted crude extracts and 0.2 mL of Folin-Ciocalteu's reagent were mixed for 3 min. After addition of 0.8 mL of Na₂CO₃ solution (20 % w/v), absorbance at 750 nm was recorded. Results were expressed in milligrams of chlorogenic acid equivalents per gram of leaf dry weight (mg CAE/g dw).

Estimation of total flavone and flavonol content

The flavones and flavonol content in crude extracts was determined spectrophotometrically using a method based on the formation of a flavonoid-aluminium complex, having a maximum absorbance at 430 nm. Quercetin was used to make the calibration curve ($0.25 \cdot 10^{-2}$ to $5.0 \cdot 10^{-2}$ μ g/mL). 1 mL of the sample and 1 mL of the solution of AlCl₃·6H₂O (2 % in methanol) were mixed. After 10 min the absorbance was recorded at 430 nm¹⁴. Results were expressed in milligrams of quercetin equivalents per gram of leaf dry weight (mg QE/g dw).

HPLC quantitative flavonol analyses

For each crude extract, solutions of 0.5 mg/mL in methanol were prepared and hydrolyzed with chlorhydric acid¹⁵. Elution was carried out through a reverse phase RP C-18 column (250 × 4.6 mm, 5 μ m) at 30 °C, using a HPLC Merck Hitachi LaChromElite chromatograph equipped with a UV-Visible

L-2450 diode array detector. Mobile phase A, consisted in 5 % CH₃CN in water and mobile phase B was 50 % CH₃CN in water. Both solvents were adjusted at pH 1.8 with 70 % perchloric acid. The flow rate was 1.2 mL/min and the gradient was the following: 0-6 min (60 % of B), 6-10 min (100 % of B). Optical density was monitored at 365 nm. Quercetin, kaempferol and myricetin were used as aglycone flavonol standards. Results were expressed in micrograms of flavonol per gram of leaf dry weight (mg of flavonol/g dw).

Antioxidant capacity using three different techniques

(±)-6-hidroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was used as standard (0 to 0.1 mM) for each assay. The extracts were diluted in methanol (DPPH and ABTS⁺ assays) or phosphate buffer (ORAC assay) and measured at a concentration in the linear part of the calibration curve. Results were expressed as milligrams of Trolox equivalents per gram of leaf dry weight (mg TE/g dw). Each sample was analyzed in triplicate.

DPPH

The radical scavenging capacity was examined by the reduction of free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) in methanol¹⁶. The solution of the free radical was prepared by stirring 75 mg of DPPH in 1 L of methanol overnight. 0.75 mL of diluted extract or standard, and 1.5 mL of DPPH solution, were mixed. After 5 min, the absorbance at 517 nm was recorded.

ABTS⁺

The ABTS⁺ radical was prepared by mixing a solution of ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) with K₂S₂O₈, to obtain a final concentration of 7 mM ABTS and 2.45 mM K₂S₂O₈. Before use, the ABTS⁺ solution was diluted in ethanol 96 % to get an absorbance at 734 nm of 0.700 ± 0.020 at room temperature. 5 µL of the diluted extract or standard were mixed with 1 mL of the ABTS⁺ solution, and the absorbance was recorded at 734 nm after 4 min¹⁷. A blank was recorded before each sample.

ORAC

ORAC assays were carried out using a Fluoroskan Ascent FL, ThermoLabsystems fluorometer and the Ascent Software Version 2.6 for processing the data. The temperature of the incubator was set to 37°C. 150 µL of fluorescein (4 · 10⁻⁶ M) and 25 µL of diluted sample were mixed. Phosphate buffer was used as a blank and Trolox as a standard during each run. The reaction was started by the addition of 25 µL of 173 mM AAPH (2',2'-azobis(2-amidinopropane)dihydrochloride). Fluorescence was recorded every two minutes for 3 hours at 538 nm with an excitation wavelength of 485 nm. The ORAC value refers to the net protection area under the quenching curve of fluorescence in the presence of antioxidants¹⁸.

Purification of flavonols glycosides and structural elucidation

600 g of dry powdered leaves of *E. alaternifolium* var. *alaternifolium* were extracted using solvents of increasing of polarity, as described above. 1 g of the *n*-BuOH crude extracts was dissolved in methanol and loaded on a Sephadex LH-20 column (2 cm × 80 cm). Elution was carried out in methanol (1.7 L). A total of 67 fractions were collected and analyzed by thin layer chromatography (TLC) on silica gel with methanol: ethyl acetate (EtOAc): H₂O (1:4:0.5 v/v/v) as phase mobile. 20 mL of each fractions were collected and after were pooled in 12 subfractions according to the similarity of the chromatographic profile on TLC: f.1 (35.3 mg); f.2 (169.0 mg); f.3 (37.5 mg); f.4 (19.3 mg); f.5 (39.0 mg); f.6 (32.4 mg); f.7 (133.6 mg); f.8 (90.4 mg); f.9 (29.6 mg); f.10 (87.5 mg); f.11 (97.1 mg); f.12 (73.6 mg). Compound E.A-F1 and E.A-F2 were purified from fractions f.3 and f.5 respectively after successive crystallizations with ethanol.

The structures of these compounds was established by using the following spectroscopic methods: UV-visible spectra recorded in methanol in a Ultraspec 2100 spectrophotometer (Amersham Biosciences); NMR spectra were recorded in a spectrometer Varian at 500 MHz respectively, using DMSO-*d*₆ and TMS as internal reference; mass spectra were recorded on a spectrometer using electrospray ionization, positive mode.

Statistical analyses

Data of antioxidant capacity, as well as total phenolic content were treated by analysis of variance (ANOVA) and computed using StatGraphic software version 5.0. Differences among means were determined by the least significant difference, Tukey HSD's post test with significance defined at *P* < 0.05.

The Spearman correlation coefficients (*r*²), among total phenolic and flavonoid content vs. antioxidant capacity (DPPH and ABTS⁺) respectively were also estimated using GraphPad InStat software version 3.00.

RESULTS AND DISCUSSION

Dry powdered leaf material was extracted successively in *n*-hexane, chloroform, ethyl acetate and *n*-BuOH. The different extraction yields for each non-polar and polar leaf extracts of the three varieties of *Erythroxylum alaternifolium* are shown in Table 1. The highest levels of phytochemicals were obtained from the *n*-BuOH extracts of the three varieties.

Table 1. Extraction yield (in % dry weight) obtained by reflux from 50 g of dried leaves of the three endemic varieties of *Erythroxylum alaternifolium*.

Varieties of <i>E. alaternifolium</i>	Yield (%)				
	<i>n</i> -hexane	chloroform	EtOAc	<i>n</i> -BuOH	Total
<i>alaternifolium</i>	0.39	0.5	0.24	1.14	2.3
<i>parvifolium</i>	0.28	0.29	0.82	1.90	3.3
<i>suborbiculare</i>	0.41	0.94	0.5	1.15	3.0

Antioxidant capacity

Several kind of free radicals are known for inducing oxidation of biological molecules e.g amino acids, proteins among other. Thus, the antioxidant capacity was assayed in all extracts using three *in vitro* techniques widely used for these analyses. (Fig 1)

Fig 1. Antioxidant capacity levels (DPPH, ABTS⁺ and ORAC) after polar leaf extractions from *E. alaternifolium* varieties. var. *alaternifolium* (Era); var. *parvifolium* (Erp); var. *suborbiculare* (Ers). Different letters indicate statistical differences among varieties for each assay at *P* < 0.05 (*n* = 3).

The antioxidant capacity was evaluated in all non-polar and polar crude extracts prepared. The antioxidant capacity of the *n*-hexane, chloroform (not showed in the figure 1) and EtOAc extracts were very low or undetectable when DPPH and ABTS assays were used. However, the *n*-BuOH extracts contained important levels of antioxidants. Antioxidant capacity as evaluated by the ABTS assay was not different between the three varieties. Results obtained through the DPPH and the ORAC assays were significantly higher for the *parvifolium* variety with 11.2 and 35.1 mg TE/g dw respectively. These results suggest intra-specific variation of antioxidant capacity in *E. alaternifolium*. Antioxidant screening performed in other Cuban species also showed the highest antioxidant capacity in *n*-BuOH extracts¹⁸. These results are in concordance with those obtained from other Cuban plant species, fruits and vegetables^{15,19}. The antioxidant capacity of some fruits and vegetables varied in the range of 6- 68 µmol TE/g dw (DPPH) and 16-212 µmol TE/g dw (ORAC). The variety *E. alaternifolium* var. *parvifolium* showed 45 µmol TE/g dw (DPPH) and 140 µmol TE/g dw (ORAC). Thus, the most promising variety showed antioxidant values in the range of oranges (29.1 µmol TE/g dw) and strawberries (54.6 µmol TE/g dw) for DPPH and green grapes (136.6 µmol TE/g dw) and apricot (143.8 µmol TE/g dw) for ORAC.

Estimation of phenolic and flavone plus flavonol contents

The levels of phenolic compounds ranged from 1.3 to 284.0 mg CAE/g dw while flavones plus flavonol content ranged from 0.02 to 0.21 mg QE/g dw (Fig 2). The highest levels of both families of compounds were measured in the polar *n*-BuOH extracts, especially in *E. alaternifolium* var. *parvifolium*.

Fig 2. Total phenol (A) and flavones plus flavonol content (B) in non-polar and polar extracts of *E. alaternifolium* var. *alaternifolium* (Era); var. *parvifolium* (Erp); var. *suborbiculare* (Ers). Different letters indicate statistical differences among varieties at $P < 0.05$ ($n=3$).

The values of total phenolics and total flavones and flavonols are in the range of those reported to other Cuban plant species¹⁹. The total phenolic contents in the EtOAc extracts are also in the range of those observed in some fruits and vegetables (12–116 mg CAE/g dw). Moreover, highest content in phenolic compounds has been measured in the *n*-BuOH crude extracts of the *Erythroxylum* varieties (Figure 2A). On the other hand, the highest levels in flavones and flavonols were measured in the *n*-BuOH extracts (Figure 2B). This spectrophotometric technique is able to estimate specifically the content in flavonoids with an hydroxyl group at position 5 mainly⁷. Other flavonoids like anthocyanins, flavanones and flavan-3-ols do not form complex with aluminum²⁰.

Flavonol content after EtOAc and *n*-BuOH extractions

In the genus *Erythroxylum* it is known that glycosylated flavonols are more abundant than aglycones. The three more important plant aglycone flavonols were quantified in the polar EtOAc and *n*-BuOH extracts by HPLC after hydrolysis of the samples (Table 2). An acidic hydrolysis was performed on both polar extracts to obtain aglycones.

Table 2. Quantification of three flavonol aglycones after hydrolysis of EtOAc and *n*-BuOH leaf extracts from three varieties of *E. alaternifolium*.

Flavonols	EtOAc extracts			<i>n</i> -BuOH extracts		
	Era	Erp	Ers	Era	Erp	Ers
kaempferol	nd	nd	nd	Nd	nd	2 ± 1
myricetin	34 ± 2	123 ± 3	70 ± 1	127 ± 2	292 ± 2	137 ± 3
quercetin	14 ± 1	49 ± 8	37 ± 1	72 ± 2	179 ± 1	88 ± 1
Total	48	172	107	139	471	225

The results were expressed in mg of flavonol/g dw. nd: no detected. var. *alaternifolium* (Era); var. *parvifolium* (Erp); var. *suborbiculare* (Ers).

Myricetin and quercetin were found in all polar extracts of the three tested varieties while kaempferol only appeared in the *n*-BuOH extract of *E. alaternifolium* var. *suborbiculare* at very low concentration.

The highest concentrations of myricetin and quercetin were observed in the *n*-BuOH polar extracts. Myricetin was the most abundant flavonol in the three varieties. The highest concentrations of myricetin and quercetin were observed in *E. alaternifolium* var. *parvifolium* with 292 mg/g dw and 179 mg/g dw respectively.

Correlation between phenolic content and the antioxidant capacity

A correlation between antioxidant capacity and phenolic content of *n*-BuOH extracts was looked after. DPPH and ABTS⁺ vs. total phenols showed significant correlations, $r^2=0.8951$ and $r^2=0.9371$ respectively. These correlations are in agreement with those obtained on different fruits and vegetables^{15,21,22} and those obtained for other plant species^{19,23}. These results suggest that phenolic compounds are responsible for a large part of the antioxidant capacity of *n*-BuOH polar extracts of the *E. alaternifolium* varieties. In addition, the Spearman correlation coefficients for DPPH[•] and ABTS⁺ vs. flavones/flavonols were 0.8956 and 0.8461 respectively. Weak correlations were found between ORAC and phenolic or flavones/flavonols content.

Thus in comparison with *n*-hexane, chloroform and EtOAc extracts, leaf crude *n*-BuOH extracts of the three varieties of *E. alaternifolium* showed the highest content of phenolics and flavonoids. Among the three varieties evaluated in this study, var. *parvifolium* proved to be the best candidate as source of antioxidants, showing the highest antioxidant capacity, as well as the highest content of total phenolics and aglycones flavonols. However, as the two varieties *parvifolium* and *suborbiculare* are not well distributed in Cuba, further chemical studies were carried out on *E. alaternifolium* var. *alaternifolium*.

Structural elucidation of glycosylated flavonols

Compounds isolated from *E. alaternifolium* var. *alaternifolium* were identified by spectroscopic analyses (UV-Visible, mass spectrometry and NMR) as well as comparison with literature reports.

Compound E.A-F1 (13 mg) and **E.A-F2** (6.3 mg) (Figure 4) were obtained as a yellow crystalline solid, and molecular formulas were respectively determined as $C_{29}H_{34}O_{16}$ and $C_{27}H_{30}O_{16}$ by mass spectrometry.

Figure 4. Isolated compounds from *E. alaternifolium* var. *alaternifolium*. Ombuin-3-rutinoside (E.A-F1): $R_1, R_2 = OCH_3$; quercetin-3-rutinoside (E.A-F2): $R_1, R_2 = OH$

Quercetin-3-*O*-rutinoside and ombuin-3-*O*-rutinoside have already been identified in leaves of other Cuban species e.g. *E. areolatum*, *E. confusum*, *E. suave*, *E. minutifolium*. Hence these compounds could be found in the most Cuban *Erythroxylum* species. The antioxidant capacity using different techniques has already been reported for quercetin-3-*O*-rutinoside²⁴. Quercetin-3-*O*-rutinoside could be one of the metabolites responsible of the antioxidant capacity detected in the *n*-BuOH extract of *E. alaternifolium* var. *alaternifolium*.

CONCLUSIONS

The antioxidant potential of three endemic Cuban varieties of *E. alaternifolium* was assayed. The polar *n*-BuOH extracts followed by EtOAc showed the strongest antioxidant capacity and highest phenolic and flavonol contents. The most promising variety, *E. alaternifolium* var. *parvifolium* showed an antioxidant capacity similar to some fruits like oranges, strawberries and apricot. Two glycosylated flavonols: quercetin-3-*O*-rutinoside and ombuin-3-*O*-rutinoside were isolated from *E. alaternifolium* var. *alaternifolium*.

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