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ADVANCES IN THE PHYTOCHEMISTRY OF *Cuphea aequipetala*, *C. aequipetala* var. *hispida* and *C. lanceolata*: EXTRACTION AND QUANTIFICATION OF PHENOLIC COMPOUNDS AND ANTIOXIDANT ACTIVITY

AVANCES EN LA FITOQUÍMICA DE *Cuphea aequipetala*, *C. aequipetala* var. *hispida* y *C. lanceolata*: EXTRACCIÓN Y CUANTIFICACIÓN DE LOS COMPUESTOS FENÓLICOS Y ACTIVIDAD ANTIOXIDANTE

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Abstract

Cuphea aequipetala and *Cuphea lanceolata* native to Mexico are used in folk medicine. Extraction procedure standardization was performed and the amount of total phenolic compounds and flavonoids was determined in methanol extracts (obtained by stirring for 24 h) from various organs of *C. aequipetala*, *C. aequipetala* var. *hispida* and *C. lanceolata*. The antioxidant properties of extracts were compared using *in vitro* free radical-scavenging assays (1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet+}$) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid ($ABTS^{\bullet+}$)) and the reducing power of phosphomolybdenum (PPM). A significant correlation was found between antioxidant activity and the amount of antioxidant components. Flowers of *C. lanceolata* showed the highest concentration of phenolic compounds (62.79±0.05 mg gallic acid equivalents (GAE)/g dry weight (DW) and the highest content of flavonoids was found in leaves of *C. aequipetala* (196.83±2.9 mg quercetin equivalents (QE)/g DW). The highest free radical-scavenging activity against $DPPH^{\bullet+}$ was found in leaves of *C. aequipetala* var. *hispida* (173.33±2.12 μmol trolox/g DW), for $ABTS^{\bullet+}$ in flowers of *C. aequipetala* (541.10±2.32 μmol trolox/g DW) and for PPM in leaves of *C. aequipetala* (1186.25±3.17 μmol trolox/g DW). Qualitative analysis indicated the presence of the flavonoid quercetin 3-β-$_D$-glucoside in all the species of *Cuphea* amongst other less polar flavonoids in *C. aequipetala* var. *hispida*. *Cuphea* spp. are prospective sources of phenolic compounds.

Keywords: antioxidant activity, *Cuphea*, phenolic compounds, free radical-scavenging, reducing power.

Resumen

Cuphea aequipetala y *Cuphea lanceolata* son especies nativas de México utilizadas en medicina tradicional. Se estandarizó el procedimiento para obtener extractos y se determinó, en extractos metanólicos (obtenidos en agitación por 24 h), el contenido de compuestos fenólicos y flavonoides totales de varios órganos de *C. aequipetala*, *C. aequipetala* var. *hispida* y *C. lanceolata*. Sus propiedades antioxidantes fueron comparadas usando métodos *in vitro* ($DPPH^{\bullet+}$ y $ABTS^{\bullet+}$) y el de poder reductor del fosfomolibdeno. La concentración más alta de compuestos fenólicos se presentó en las flores de *C. lanceolata* (62.79±0.06 mg equivalentes de ácido gálico (EAG)/g peso seco (PS); mientras que la de flavonoides en las hojas de *C. aequipetala* (196.83±2.9 mg equivalentes de quercetina (EQ)/g PS). Las hojas de *C. aequipetala* var. *hispida* presentaron la actividad de captura de radicales libres DPPH (173.33±2.12 μmol trolox/g PS), las flores de *C. aequipetala* la de captura de radicales libres ABTS (541.10±2.32 μmol trolox/g PS) mientras que el poder reductor más alto se observó en las hojas de *C. aequipetala* (1186.25±3.17 μmol trolox/g PS). Se encontró una correlación positiva significativa entre la actividad antioxidante y la concentración de compuestos antioxidantes. El análisis químico cualitativo mediante TLC indicó la prescencia del flavonoide quercetina 3-β-$_D$-glucósido en todas las especies de *Cuphea* y de otros flavonoides menos polares en *C. aequipetala* var. *hispida*. *Cuphea* spp. es una fuente natural de compuestos fenólicos.

Palabras clave: actividad antioxidante, *Cuphea*, compuestos fenólicos, captura de radicales libres, poder reductor.

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1 Introduction

The genus *Cuphea* (Lythraceae) comprises > 260 species native to the Americas distributed from Mexico to Brazil. *Cuphea* species are cultivated as sources of oils rich in medium-chain fatty acids (Graham and Kleiman, 1992; Phippen, 2010; Tisserat *et al*., 2012) and are used in traditional ("folk") medicine for their antioxidant (Schuldt *et al*., 2004), antihypertensive (Braga *et al*., 2000), cytotoxic (Wang *et al*., 1999), antiprotozoal (Barbosa *et al*., 2007) and hypocholesterolemic activities (Biavatti *et al*., 2004). For instance, the leaves of *C. carthagenensis* represent a significant source of phenolic antioxidants that may have potentially beneficial cardiovascular effects (Schuldt *et al*., 2004). *Cuphea aequipetala* Cav., "hierba del cancer" (Spanish) or "Tozancuitlacxolli" (Nahuatl), is native to Mexico and grows in open and humid fields of pine-oak woods 2000-2540 m above sea level (m asl) (Graham, 1991). Infusion or poultice of the aerial parts (leaf and stem) of the plant is used in Mexican folk medicine to treat tumors, pounds and wounds (Aguilar-Rodríguez *et al*., 2012). Aqueous extracts of the aerial parts showed strong activity against *Helicobacter pylori* (Castillo-Juárez *et al*., 2009), whereas organic extracts of flowers, leaves and stems showed cytotoxic activity against carcinoma cells of the human larynx (HEp-2 cell line) (Waizel-Bucay *et al*., 2003) and acetone-water extracts of the whole plant showed activity against carcinoma cells of human prostate (Vega-Avila *et al*., 2004). *Cuphea lanceolata* W.T. Aiton, "Atlanchana" or "Atlancán" (Spanish) and "cigar plant" (English) is also native to Mexico. Infusion of the aerial part is recommended in folk medicine as an anti-diarrheal agent (Waizel, 2006). It grows in open fields of dry tropical forests at 2250-2500 m asl (Graham, 1991). This species has been included in breeding programs to domesticate *Cuphea*. The species of the genus *Cuphea* produce high amounts of medium chains fatty acids, which represent an alternative to coconut oil in soaps, detergents and other products or as an antimicrobial pesticide in commercial food handling (Millam *et al*., 1997; Phippen, 2010). Profiles of fatty acid are species specific (Wolf *et al*., 1983; Phippen *et al*., 2006), showing that within the genus *Cuphea* exists a high genetic variation.

There is growing interest in the use of medicinal plants as sources of natural antioxidants as potentially side effect-free alternatives to synthetic compounds (Juntachote and Berghofer, 2005; Krishnaiah *et al*., 2010) and as adjuvants in cancer therapy (Saxena *et al*., 2010). Antioxidants, including phenolic compounds (e.g., phenolic acids, flavonoids, tannins) have diverse biological effects, such as anti-inflammatory, cytotoxic and anti-tumor effects, as a result of their antioxidant activity (Vega, 2005; Krishnaiah *et al*., 2010). Quantity and quality of plant phenolics vary in response to environmental factors, such as light intensity and nutrient availability, but also on different genetic levels (between and within species and clones) and between different physiological and developmental stages (Klepacka *et al*., 2011; Estrada-Zúñiga *et al*., 2012; Naghiloo *et al*., 2012). Conversely, the activity of antioxidants may be influenced by extract preparation (Pérez-Jiménez *et al*., 2008; Jimenez *et al*., 2011; García-Márquez *et al*., 2012). Increasing interest has been focused into the procedures for extract preparation because of their influence in the yield and modification of the activity of compounds (Bolling *et al*., 2009; Turkmen *et al*., 2006).

Here, we report the standardization of the extraction procedure, the determination of the concentration of total phenolic compounds and flavonoids of various organs of wild-growing *C. aequipetala*, *C. aequipetala* var. *hispida* and *C. lanceolata*. Furthermore, the antioxidant capacity of those extracts was shown to correlate well with phytochemical content.

2 Materials and methods

2.1 Plant material

Cuphea aequipetala and *C. aequipetala* var. *hispida* were collected at the flowering stage, in order to standardize phenological stage of plants, in Lagunas de Zempoala (State of Mexico, Mexico) at 2860 m asl (latitude 19° 02' N, longitude 99° 19' W), both species grow mixed as open ground layer. *C. lanceolata* plants were collected at the flowering stage in Yautepec (State of Morelos, Mexico) at 1267 m asl (latitude 18° 53', longitude 99° 04' W) as secondary vegetation. Plants (25-30) were excised into roots, stems, leaves and flowers. They were dried at room temperature under shade for 1 week. Dry material was ground manually into a fine powder (particle size < 250 μm) using a pestle and mortar. Plants were positively identified as *C. aequipetala* Cav. (voucher numbers 21181 and 21172), *C. aequipetala* Cav. var. *hispida* Koehne (voucher numbers 21170 and 21171) and *C. lanceolata* W.T. Aiton (voucher numbers 13239,

13240 and 13238) at the Herbarium of the Universidad Autónoma del Estado de Morelos (UAEM).

2.2 Standardization of the extraction procedure

Four different methods were tested to evaluate the efficacy of the extraction type of antioxidant compounds. For these evaluations, l00 mg of the powdered dried leaves of *C. aequipetala* were used. Following extraction methods were evaluated: 1) sequentially extraction: 100 mg of the homogenized samples were extracted with 50 mL of hexane under agitation for 24 h at room temperature, filtered through a Whatman No. 1 filter paper (Whatman, Maidstone, UK) and the supernatant collected. The pulp residues were re-extracted by the addition of 50 mL of ethyl acetate under agitation for 24 h at room temperature, filtered through a Whatman No. 1 filter paper and the supernatant collected. The pulp residues were re-extracted by the addition of 50 mL of methanol under agitation for 24 h at room temperature, filtered through a Whatman No. 1 filter paper and the supernatant collected. All supernatants were concentrated to dryness in a rotary evaporator (Büchi-490; Büchi, Switzerland). The collected supernatants were analyzed separately; 2) extraction with methanol (50 mL) under stirring for 24 h; 3) extraction with methanol (50 mL) on a water bath at 60°C for 30 min; 4) aqueous extraction (50 mL) of freshly boiled distilled water and 30 min rest and filtering through Whatman number 1 paper and rapidly cooled under tap water. All extracts were filtered and evaporated to dryness in a rotary evaporator and stored at -70°C in the dark until analyses. Methanol extracts were evaporated at 210 mbar and 40°C; aqueous extracts at 50 mbar and 60°C. For the analysis, extracts were re-suspended in 1 mL of their respective solvent. Extract yields were calculated according to the following: Extraction yield (%) = [weight of the freeze-dried extract/weight of the original sample] × 100, and were expressed as milligrams of extract per g (dry weight; DW) of leaves. Extractions were performed in triplicate. Yields of total phenols, total flavonoids, and antioxidant activity were evaluated to determine the best extraction method, which was followed for all dried organs from *Cuphea* spp.

2.3 Determination of total phenolic-compound content

Phenolic compounds in methanol and aqueous extracts were estimated using the Folin-Ciocalteu colorimetric method (Shohael *et al*., 2006). Each extract (100 μL) was mixed with 2.5 mL of deionized water, and 100 μL of Folin-Ciocalteu reagent added. The mixture was incubated at room temperature for 6 min before an aqueous solution of sodium carbonate (0.5 mL, 20%, w/v) was added, and the mixture gently mixed. A blank sample was prepared by mixing 100 μL methanol with the reagents. After 30 min, the color was fully developed and the absorbance measured at λ=760 nm. The total phenolic-compound content was determined using a standard curve prepared with gallic acid (0-25 μg/mL). Results were expressed as milligrams of gallic acid equivalent (GAE) per gram of dry weight (DW). Samples were analyzed in triplicate.

2.4 Determination of total flavonoid content

The total flavonoid content was determined using a colorimetric assay as described by Shohael *et al*. (2006). Each extract (250 μL) was mixed with 1.25 mL of de-ionized water, and 75 μL of an aqueous solution of $NaNO_2$ (5%, w/v) added. The mixture was thoroughly vortex-mixed and incubated at room temperature for 6 min. Then, 150 μL of an aqueous solution of $AlCl_3$ (10%, w/v) were added. After a further 5 min, 0.5 mL of an aqueous solution of NaOH (1 M), and 2.5 mL of de-ionized water, were added. Finally, the mixture was incubated 30 min at room temperature and the absorbance measured at 510 nm using methanol as a blank sample. Quercetin was used to create a calibration curve (0-150 μg/mL). The total flavonoid content was expressed as milligrams of quercetin equivalent (QE) per gram of DW. Samples were analyzed in triplicate.

2.5 $DPPH^{\bullet+}$

The free radical-scavenging activity of extracts was quantified by spectrophotometric means using a $DPPH^{\bullet+}$ assay (Sánchez-Moreno *et al*., 1998). A stock solution of freshly prepared $DPPH^{\bullet+}$ (3.9 mL, 60 μM) was mixed with 100 μL of each sample extract at 5 mg/mL dissolved in methanol. The mixture was shaken vigorously and incubated for 6 min at room temperature in the dark. The absorbance was immediately recorded at 515 nm. Trolox (0-15

μmol/L) was used as a reference standard. Results were expressed as micromoles of trolox per gram of DW based on a calibration curve (R^2 = 0.993). The assay was carried out in triplicate.

2.6 $ABTS^{\bullet+}$

An ABTS radical-scavenging assay was carried out using the improved ($ABTS^{\bullet+}$) method described by Re *et al*. (1999) with slight modification. Briefly, the $ABTS^{\bullet+}$ radical cation was generated by the reaction of 7 mmol/L $ABTS^{\bullet+}$ and 2.45 mmol/L potassium persulfate for 16 h at room temperature in the dark. $ABTS^{\bullet+}$ solution was diluted with methanol to an absorbance of 0.7±0.05 at 734 nm. Each extract (50 μL) was mixed with 1.9 mL of $ABTS^{\bullet+}$ solution. The mixture was incubated for 6 min at room temperature in the dark and the absorbance recorded immediately at 734 nm. Trolox solution (final concentration, 0-15 μmol/L) was used as a reference standard. The results were expressed as micromoles of trolox per gram of DW based on a calibration curve (R^2 = 0.975). The assay was carried out in triplicate.

2.7 PPM

The reducing power assay using PPM has been described by Prieto *et al*. (1999) and is based upon the reduction of molybdenum (Mo) (VI) to Mo (V) by antioxidant compounds and the formation of a green Mo complex with maximum absorption at 695 nm. The assay can be used to detect antioxidants such as ascorbic acid, phenolic compounds, and carotenoids. Each extract (100 μL; final concentration, 5 mg/mL) was incubated at 95°C with the reagent solution (sodium phosphate (28 mM) and ammonium molybdate (4 mM) in sulfuric acid 0.6 M) for 90 min. The mixture was allowed to stand at room temperature for 30 min and the absorbance recorded. Trolox solution (final concentration, 0-80 μmol/L) was used as a standard. Results were expressed as micromoles of trolox per gram of DW (Diouf *et al*., 2009) based on a calibration curve (R^2 = 0.9974). The assay was conducted in triplicate.

2.8 TLC analysis

An aliquot (15 μL) of plant extract (5 mg mL^{-1}) was carefully spotted on a 5×5 cm TLC layer (silica gel 60 F_{254}; Merck) which was then developed with a ethyl acetate:methanol:H_2O (76:16:8, v:v) solvent system. The silica plate was dried and placed upside down for 2-3 min in a 0.01 mM DPPH solution in methanol (López-Laredo *et al*., 2012). Stained silica layer revealed a purple background with yellow spots corresponding to the resolved bands with radical scavenger capacity. TLC plates were inspected also under UV light at 254 and 365 nm. Another set of TLC plates was sprayed with PEG (5%, v/v) before staining with a methanolic solution of diphenylboric acid-β-ethylamino ester (1%, p/v). Retardation factor (R_F) is a relative measure of the substance position in the chromatogram with respect to the position of the solvent front. It is the most widely used descriptor of position in TLC and was calculated according to the following equation: $R_F = z_i/(z_i - z_0)$, where z_i is the migration distance of substance, z_f is the migration distance of front measured from the immersion line, and z_0 is distance between immersion line and sample application (Reich and Schibli, 2007).

2.9 Statistical analyses

Statistical analyses were performed by two-way ANOVA. The all-pairwise multiple comparison procedure of Duncan was used to determine statistically different values at $P < 0.05$. Pearson correlation coefficients and P values were used to show correlations and significance at $P < 0.05$. SigmaPlot for Windows version 11.0 (Systat Software Inc., San Jose, CA, USA) was used to carry out statistical analyses.

3 Results and discussion

3.1 Standardization of extraction procedures

Extraction yields, total content of phenolic compounds, total flavonoids, and antioxidant activity were evaluated to determine the best extraction method (Table 1). The amount of extractable components ranged from 30 mg/g DW (hexane extract) to 324 mg/g DW (methanol in a water bath). The amount of total phenol compounds increased in the order methanol/water bath > methanol/stirring > methanol/sequential extraction > water extract > ethyl acetate > hexane ($P < 0.05$) (Table 1). All fractions were rich in flavonoids except ethyl acetate and hexane fractions, and the highest concentration was observed in the methanol/stirring method (196.14±2.93 mg QE/g DW, $P < 0.05$).

Table 1. Extraction yield, content of total phenolic compounds and flavonoids and, antioxidant properties of extracts from leaves of *C. aequipetala* prepared by different procedures[a]

Extraction procedure	Yield[b]	TPC[c]	Flavonoids[d]	Free-radical scavenging activitye		Reducing power[e]
				DPPH$^{\bullet+}$	ABTS$^{\bullet+}$	
Sequential extraction (24 h for each fraction obtained)						
Hexane fraction	30	0	0	0	0	0
Ethyl acetate fraction	272	0.75 ± 0.01^{D}	0	24.33 ± 1.4^{E}	47.72 ± 0.8^{E}	0
Methanol fraction	218	38.04 ± 0.021^{C}	134.00 ± 2.28^{C}	503.62 ± 13.3^{C}	1062.43 ± 15.2^{C}	3736.7 ± 29.3^{B}
Methanol/Stirring for 24 h	283	55.42 ± 0.50^{B}	196.14 ± 2.93^{A}	597.06 ± 6.6^{A}	1743.21 ± 27.3^{A}	4182.8 ± 9.9^{A}
Methanol/Water bath at 60°C for 30 min	324	61.86 ± 0.33^{A}	198.66 ± 3.57^{A}	520.99 ± 7.9^{B}	1346.09 ± 55.1^{B}	3782.8 ± 46.6^{B}
Hot water extraction for 30 min	259	38.17 ± 0.41^{C}	143.32 ± 1.98^{B}	411.38 ± 1.9^{D}	654.06 ± 37.2^{D}	2759.7 ± 25.6^{C}

[a]Values correspond to mean ±standard error ($n = 3$). Within each column, different capital letters indicate significant difference according to Duncan test ($\alpha = 0.05$). [b]Extract yields expressed as milligrams of extract per g DW of leaves. [c]TPC: total phenolics compounds; data expressed as mg of Gallic Acid Equivalent (GAE) per g DW. [d]Data expressed as mg of Quercetin Equivalent (QE) per g DW. [e]Data expressed as μmoles of Trolox per g DW.

This extraction procedure allowed obtaining the highest antioxidant activity (free-radical scavenging activity and phosphomolybdenum reducing power). Aqueous extract presented a similar content of phenolic compounds (38.17±0.41 mg GAE/g DW) but a higher content of flavonoids (143.32±1.98 mg QE/g DW) than that of the methanol fraction result of the sequential extraction. Free-radical scavenging of the aqueous extract (411.38±1.9 μmol trolox/g DW and 654.06±37.2 μmol trolox/g DW) and reducing power (2759.7±25.6 μmol trolox/g DW) was less than that of the methanol extracts regardless of the procedure followed for their preparation. Hexane fraction did not present antioxidant activity whereas ethyl acetate fraction presented low free-radical scavenging activity against DPPH and ABTS (24.33±1.4 and 47.72±0.8 μmol trolox/g DW, respectively). Variations in the quantity of total phenolic compounds and flavonoids in the extracts with different solvents and extraction methods have been reported. For instance, in the methanol extracts of *Lythrum salicaria* (Lythraceae), the content of phenolic compounds and flavonoids was lower than that in water extracts (Tunalier *et al*., 2007). In contrast, the content of phenolic compounds in methanol and water extracts of *Lawsonia inermis* (Lythraceae) was similar (Guha *et al*., 2011). The amount of extracted phenolic compounds and/or flavonoids depends on the temperature and time extraction, but also on the polarity of used solvents and on the species/organ specific content of less or more polar compounds (Serrano-Maldonado *et al*., 2011; García-Márquez *et al*., 2012). In the present study, the analyses shown below were undertaken by extraction using methanol and stirring for 24 h due to significantly higher values of phenols, flavonoids and antioxidant activity.

3.2 Total phenolic compounds and flavonoids content

In general, phenolic compounds are responsible for many interactions between plants and their biotic and abiotic environment. These compounds have an organ specific distribution and accumulate differentially during ontogenesis and under the influence of environmental factors (Hutzler *et al*., 1998; Ayan *et al*., 2007). Moreover, phenolic compounds biosynthesis is subcellularly compartmentalized and the role of cell compartments has not been totally elucidated. For instance, L-phenylalanine (precursor), quercetin and kaempferol glycosides (flavonoids) are synthesized in the chloroplasts but other cell compartments such as cytosol, vacuole or endoplasmic reticulum are involved in the biosynthesis of precursors, intermediates and end products (Santiago *et al*., 2000; Zhao and Dixon, 2010). At this level, they fulfill three major functions: substrates, energy sources and regulators (Kefeli *et al*., 2003). In the aerial parts phenolic compounds play a role as part of the defense system of plants against pest and pathogens (Lattanzio *et al*., 2006) but also as pigments to attract pollinators (Buer *et al*., 2010). Furthermore,

in roots flavonoids are involved as signal molecules in the symbiosis plant-bacteria (Buer *et al*., 2010).

References show that organ specific accumulation of phenolic compounds is species specific (Bhatt *et al*., 2012; Liu *et al*., 2012). For the genus *Cuphea*, seems to be the aerial parts where the higher levels of phenolic compounds are found (Calzada, 2005; Krepsky *et al*., 2012). Results of present work show that the highest content of total phenolic compounds was found in the leaves of *C. aequipetala* and *C. aequipetala* var. *hispida* (55.62±0.50 and 60.74±0.23 mg GAE/g DW, respectively), while for *C. lanceolata* flowers showed the highest amount of these compounds (62.79 ±0.05 mg GAE/g DW) (Fig. 1A). In the three species, the stem contained the lowest level of phenolic compounds. Similarly, flavonoids were more abundant in the leaves of *C. aequipetala* and *C. aequipetala* var. *hispida* (196.83±2.94 and 124.74±1.28 mg QE/g DW, respectively) and in the flowers of *C. lanceolata* (135.81±1.55 mg QE/g DW) (Fig. 1B). According to statistical analyses, the total content of phenolic compounds was specific to species ($P < 0.05$), variety ($P < 0.05$), and plant organ (roots, stems, leaves or flowers, $P < 0.05$). For total phenolic compounds, flavonoid concentration was highly dependent upon the species ($P < 0.05$), variety ($P < 0.05$), and plant organ ($P < 0.05$).

We found that the concentrations of compounds varied depending on the genotype (intra-varietal and intra-specific), and that they were distributed non-uniformly among plant organs. The leaves of *C. aequipetala* and *C. aequipetala* var. *hispida* and the flowers of *C. lanceolata* were the best source of total phenolic compounds (Fig. 1A) and flavonoids (Fig. 1B). Several reports have shown differences in the concentrations of phytochemicals among varieties and species within a genus, which may also be influenced (among other factors) by different growing conditions (e.g., soil, altitude, temperature, nutrition) (Gesch *et al*., 2010; Kim *et al*., 2011; Zheljazkov *et al*., 2011) or harvest time (He *et al*., 2010). For instance, total phenolic compounds and flavonoid content was shown to vary significantly in the leaves of six species of Artemisia (Asteraceae) (Carvalho *et al*., 2011), whereas the concentration of the bioactive compounds in *Salvia miltiorrhizae* (Labiatae) was affected mainly by genetic factors (He *et al*., 2010). The differences in phenolic compound and flavonoid concentration among *Cuphea* species may be the result of adaptation processes of plants to environmental conditions. Several authors have explained that light increases flavonoid concentrations in certain organs/tissues of the plant (Wang *et al*., 2009; Ghasemzadeh *et al*., 2010; Martz *et al*., 2010). Plants growing at higher altitudes are exposed to higher light intensities, so they must develop mechanisms to prevent damage caused by photo-destruction. *C. lanceolata* grows at lower elevations, agreeing with the notion of a lesser need of protection against light, whereas *C. aequipetala* and *C. aequipetala* var. *hispida* grow at higher altitudes where light intensities are more important. However, the results of present work demonstrate that flowers don't follow this behavior, showing that the genetic fixed information play an important role in the determining of secondary metabolites amounts/profiles.

The highest concentration of total phenolic compounds and flavonoids found in the present study for *Cuphea* spp. (62.79 mg GAE/g DW and 196.83 mg QE/g DW) were higher than those reported for other

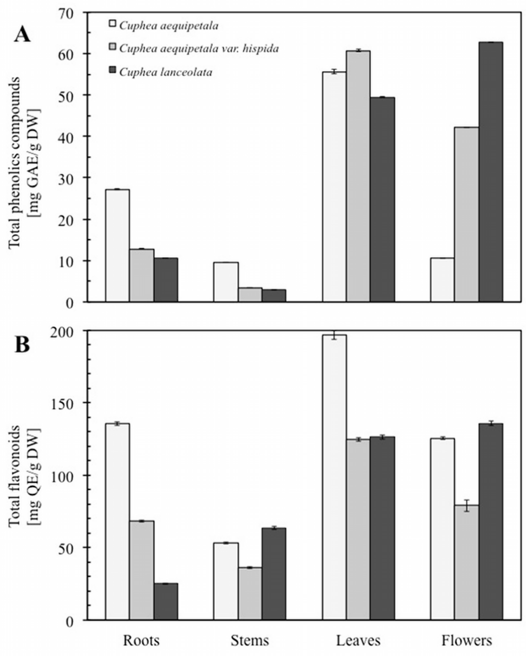

Fig. 1. Total phenolics (A) and flavonoids (B) concentration in different tissues from *C. aequipetala* (□), *C. aequipetala* var. *hispida* (■) and *C. lanceolata* (■). Values expressed are mean ($n = 3$) ± standard error.

medicinal plants considered as high in antioxidant compounds such as *Sargentodoxa cuneata* Redh Et Wils (Sargentodoxaceae) (52.35 mg GAE/g DW)

or *Fraxinus rhyncophylla* Hance (Oleaceae) (40.27 GAE/g DW) (Li *et al*., 2008) but lower than the contents reported for other Lythraceae used for medicinal purposes. Methanol extracts from the bark of *Lafoensi pacari* (Lythraceae) contained 141 mg GAE/g dry matter (Solon *et al*., 2000), whereas those from the whole plants of *Lawsonia innermis* Linn. (Lythraceae) contained 238-310 mg GAE/g dry matter (Guha *et al*., 2011).

3.3 Antioxidant properties

The antioxidant activity of organs of *C. aequipetala* and *C. aequipetala* var. *hispida* using the DPPH assay varied from 19.19±0.1 to 169.33±2.1 and 18.48±0.13 to 173.33±2.1 μmol trolox/g DW, respectively (Fig. 2A). For both varieties, antioxidant activity using DPPH could be ranked in descending order: leaves > flowers > roots > stems. *Cuphea lanceolata* exhibited mean DPPH activities between 5.31±0.1 and 159.50±0.6 μmol trolox/g DW, and this could be ranked in organs in descending order as flowers > leaves > roots > stems (Fig. 2A). Meanwhile, scavenging activity evaluated by ABTS of *C. aequipetala* and *C. aequipetala* var. *hispida* extracts was between 106.71±0.3 to 541.10±2.32 and 14.42±0.2 to 336.23±0.8 μmol trolox/g DW, respectively; with the leaves and flowers being the most active (Fig. 2B). Mean ABTS values for *C. lanceolata* ranged between 20.07±0.1 and 275.60±3.9 μmol trolox/g DW, with the flowers being the most active. According to statistical analyses, *C. aequipetala* exhibited higher free radical-scavenging activity ($P < 0.05$) than *C. aequipetala* var. *hispida* and *C. lanceolata*, and the activities were dependent on the plant organ ($P < 0.05$). The highest values of free-radical scavenging against ABTS found for *Cuphea* spp. in the present study are higher than those reported for other medicinal plants; for instance, *Sargentodoxa cuneata* Rehd. Et Wils (265.43±4.62 μmol trolox/g DW), *Fraxinus rhynchophylla* Hance (166.09±0.34 μmol trolox/g DW) or *Paeonia suffruticosa* Andr (221.10±0.34 μmol trolox/g DW) (Li *et al*., 2008).

The reducing power of *C. aequipetala* and *C. aequipetala* var. *hispida* was, on average, between 93.11±0.2 to 1186.25±3.2 and 107.83±0.8 to 341.52±1.2 μmol trolox/g DW, respectively (Fig. 3). For both varieties, reducing power in organs could be ranked in the descending order: leaves > flowers > roots > stems. Within *C. lanceolata*, the flowers and leaves were more active (553.06±0.6 and 195.03±6.3 μmol trolox/g DW, respectively) than roots and stems (101.43±0.8 and 51.23±0.3 μmol trolox/g DW, respectively). The species ($P < 0.05$), variety ($P < 0.05$) and the organ ($P < 0.05$) were found to significantly influence the reducing power of the extracts. Reducing power presented by the leaves of *Cuphea aequipetala* is close to that reported for bark extracts of *Populus tremuloides* Michx (1406.74 μmol trolox/g DW) but higher than the reported for the synthetic antioxidant *tert*-butyl-4-hydroxy-toulene (BHT) (686.79 μmol trolox/g) which is used in food processing (Diouf *et al*., 2009).

The extracts of *Cuphea* spp. presented free-radical scavenging activity and reducing power. For most all samples, PPM values were higher than $ABTS^{\bullet+}$ and $DPPH^{\bullet+}$ values. These differences in the capacity of the extracts to scavenge $ABTS^{\bullet+}$/$DPPH^{\bullet+}$ radicals and to reduce PPM are in accordance with previous observations (Marwah *et al*., 2007; Pasko *et al*., 2009).

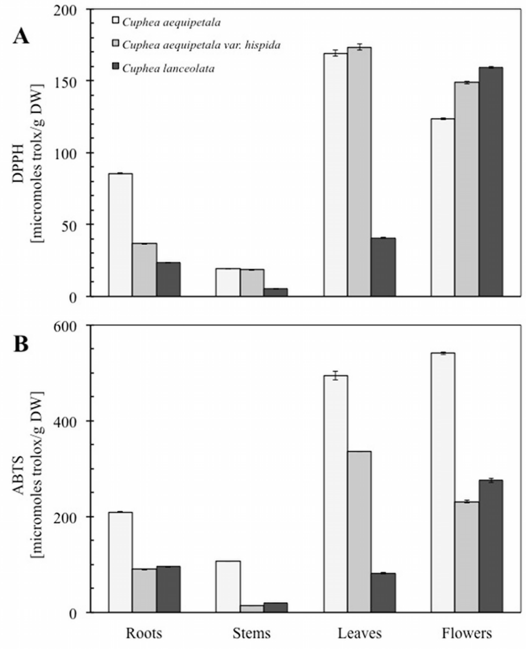

Fig. 2. Free-radical scavenging activity in different organs of *C. aequipetala* (■), *C. aequipetala* var. *hispida* (■) and *C. lanceolata* (■) as determined by DPPH (A) and ABTS (B) assays. Values expressed are mean ($n = 3$) ± standard error.

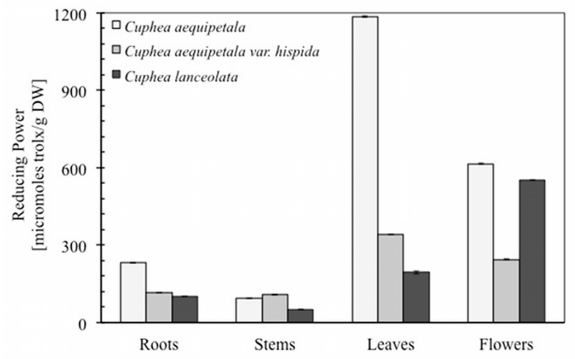

Fig. 3. Reducing power in different organs of *C. aequipetala* (□), *C. aequipetala* var. *hispida* (■) and *C. lanceolata* (■) as determined by phosphomolybdenum assay. Values expressed are mean (n = 3) ± standard error.

In these assays, the transfer of hydrogen and electrons occurs at different redox potentials and is also dependent upon the structure of the antioxidant (Marwah *et al*., 2007). Factors such as the presence of pigments (e.g., anthocyanins, carotenoids) (Dykes *et al*., 2005), the solubility of the extract in the testing systems or the solvent in which the reaction takes place, have been reported to affect the capacity of the extracts to react with different radicals (Cai *et al*., 2004; Adedapo *et al*., 2008). The ability of extracts of *Cuphea* spp. to scavenge different free radicals in different systems may be an advantage for therapeutic agents to treat radical-related diseases (Adedapo *et al*., 2008; Sucontphunt *et al*., 2011). Furthermore, the antioxidant properties of *Cuphea* spp. may be associated with its traditional use to treat conditions consistent with radical-related diseases (e.g., tumors).

3.4 Qualitative chemical analysis by TLC

Qualitative analysis by TLC using DPPH was performed to detect the chemical components of *Cuphea* responsible of the free-radical scavenging activity and as an initial study of their chemical constitution. This method enables rapid detection and localization of active compounds in a complex extract (Gu *et al*., 2008). For this, the extracts from leaves of the three species of *Cuphea* were analyzed. In all cases, the extracts resolved at least one band with antiradical activity (R_F = 0.60), which was also observed under UV light at 264 and 365 nm and gave a yellow fluorescence after reaction with diphenylboric acid-β-ethylamino ester (data not shown).

Table 2. Pearson correlation coefficient values for total phenolics compounds (TPC), flavonoids free-radical scavenging (DPPH and ABTS) and reducing power by PPM

	TPC	Flavonoids	DPPH	ABTS
TPC	-	0.733	0.794	0.468
Flavonoids	0.733	-	0.742	0.753
DPPH	0.794	0.742	-	0.831
ABTS	0.468	0.753	0.831	-
PPM	0.553	0.733	0.794	0.856

This band agrees with that of the flavonoid quercetin 3-β-$_D$-glucoside. TLC profile of the leaves of the three species was similar in revealing fluorescent zones at 365 nm that reacted with diphenylboric acid-β-ethylamino ester, giving yellow fluorescence suggesting the presence of other flavonoids (Wagner and Bladt 1996). The leaves of *C. aequipetala* presented additional bands corresponding to flavonoids of less polarity than *C. aequipetala* var. *hispida* and *C. lanceolata*. In the aerial parts of *Cuphea carthagenensis* the main flavonoids are quercetin-5-O-β-glucopyranoside, quercetin-3-O-α-arabinofuranoside and quercetin-3-sulfate, which have been suggested as chemical markers of this species (Krespsky *et al*., 2012). These results are under further investigation.

3.5 Correlation analyses

We undertook correlation and regression analyses to determine the relationship between the phytochemical content and antioxidant capacity of the extracts. Total phenol content was significantly correlated ($P < 0.05$) with radical-scavenging activity based on the reduction of $DPPH^{\bullet+}$ (r=0.794) and $ABTS^{\bullet+}$ (r=0.468) as well as with reducing activity (r = 0.553). Similarly, flavonoid concentration was significantly correlated ($P < 0.05$) with scavenging activity against $DPPH^{\bullet+}$ (r=0.742), $ABTS^{\bullet+}$ (r=0.753) and reducing power (r=0.733) (Table 2). Phenolic compounds (e.g., phenolic acids, flavonoids, tannins) are considered to be the major contributors to the antioxidant activity of plants. The antioxidant capacity of phenolic compounds is attributed to their redox properties, which allow them to act as reducing agents, hydrogen donators, singlet-oxygen quenchers and metal-chelators (Rice-Evans and Miller 1996; Vermerris and Nicholson 2008). We found that the antioxidant activity of *Cuphea* spp. (free radical-scavenging and reducing power) was

strongly correlated with total phenolic and flavonoid content (Table 2). However, the highest correlation coefficients were for flavonoids, suggesting that these types of compounds are the major contributors to the antioxidant properties of *Cuphea* spp. In related species, such as *Lawsonia inermis* Linn. (Lythraceae), antioxidant activity was also found to correlate strongly with total polyphenol content (Guha *et al*., 2011).

Conclusions

Cuphea spp. could be used as sources of natural antioxidants, such as phenolic compounds. Total phenolic compound and flavonoid concentrations were dependent upon the species, varieties and organs of plants.

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