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Variation in phenolic constituents and antioxidant capacities of plant organs of three Cuban species of *Pluchea* Cass. (Asteraceae) under *ex vitro* and *in vitro* growth conditions

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The phenolic content and antioxidant capacity of Cuban *Pluchea* species extracts were measured by polar extraction technique with the aim to optimize antioxidant-rich extracts. Our results highlighted the variability of the phenolic content and antioxidant potential in the genus *Pluchea* according to the species and plant organs. The antioxidant capacity and phenolics were additionally examined in *Pluchea carolinensis* under various growth conditions, *ex vitro* (location, age, and harvest time) and *in vitro* (amount of added cytokinin). Some specific phenolic compounds were monitored during the harvest time. Leaves proved to be richest in antioxidants, followed by inflorescences. The antioxidant capacities of *P. carolinensis* (DPPH: 103.2 ±1.3 mg TE/g DW; ORAC: 254.4 ±14.2 mg TE/g DW) and *Pluchea rosea* (DPPH: 97.2 ±5.9 mg TE/g DW ORAC: 286.7 ±23.7 mg TE/g DW) leaf extracts were higher than that measured in material from various plant species, including fruits, vegetables and condiments. Adult *P. carolinensis* grown in a natural environment and harvested in winter showed the highest phenolic content and antioxidant capacity. A micropropagation protocol was developed for *P. carolinensis*. The presence of cytokinin in the *in vitro* culture medium increased the antioxidant capacity in leaves, but the level remained lower in natural plants.

Key words: Antioxidants, *Pluchea,* phenolic acids, flavonoids, micropropagation.

INTRODUCTION

Free radicals and antioxidants are widely discussed in the clinical and nutritional literatures. Antioxidants are needed to prevent the formation and oppose the action of damage-causing reactive oxygen and nitrogen molecules generated *in vivo*. They play an important role in preventing degenerative diseases, particularly cardiovascular diseases, cancers, and neurodegenerative diseases (Scalbert et al., 2005).

The use of natural sources of therapeutic compounds has considerably been increased in recent times, notably for the production of nutraceuticals (Han and Rhee, 2005). Many species of the botanical family Asteraceae have been identified as potential sources of antioxidants, notably certain *Pluchea* species (Fernández and Torres,

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2006; Perera et al., 2010). The aerial parts of these plants have been traditionally used as medicine. *Pluchea carolinensis* and *Pluchea odorata* are used mainly against headaches, slow digestions and as antipyretic, while *Pluchea rosea* is used as vermifuge (NapralertTM, 1975 to 2005). *P. carolinensis* is an annual shrub of 4 to 5 m, *P. odorata* is a herb of 1.5 m high and *P. rosea* is a perennial herb of 30 to 55 cm high (Alain, 1964). Potential sources of active phytochemicals have also been identified in other plant organs. For example, the methanolic crude extract of *Pluchea indica*, less roots has been noted for its antioxidant capacity (Sen et al., 2002).

Phenolic compounds contribute greatly to the antioxidant capacity of plant extracts (Tavarini et al., 2008). These phytochemicals are the most abundant secondary metabolites in plants, where they play a role in flower pigmentation and in various defense mechanisms protecting plant tissues against abiotic stresses such as UV radiation or biotic stresses such as predator and pathogen attack (Naczk and Shahidi, 2006). Not surprisingly, phenolic compounds concentration vary according to the growth conditions, some of which might be controlled in order to optimize the polyphenol content (Manach et al., 2004). The main objective of the present research was to examine how the antioxidant capacity of Cuban *Pluchea* species varies according to the plant organ and some *ex vitro* and *in vitro* growth conditions. More specific aims were to determine which plant organs and species yield the highest phenolic content and antioxidant capacity. We also investigated the impacts of *P. carolinensis* age, harvesting time, and *in vitro* growth conditions on the phenolic content and antioxidant capacity.

MATERIALS AND METHODS

Plant collection and storage

P. carolinensis specimens were collected from two western locations in Cuba: Las Cañas, Havana City *(W: 82º 22' 22'' and N: 23º 1' 12'')* and Sierra del Rosario, Pinar del Río *(W: 82º 56' 57'' and N: 22º 50' 56'')*. In both cases they were collected during the blooming stage (March). Adult cultivated specimens of *P. carolinensis* were also collected near Havana City in January, July, September, and December. Vegetative leaves from juvenile specimens having the same (bushy) appearance as the adult specimens but slightly smaller in size (about 50 cm high) and lacking floral buds were also collected in September. *P. rosea* and *P. odorata* were collected in Ciénaga de Zapata, Matanzas *(W: 87° 10'' 47'' and N: 22° 22' 89'')*. All the voucher specimens (*P. carolinensis,* HAC 41725; *P. rosea,* LS 16648; *P. odorata,* LS 17198) were authenticated and deposited at the HAC herbarium of the "Instituto de Ecología y Sistemática". The samples were dried at room temperature and the different plant organs separated and powdered.

In vitro propagation of *Pluchea carolinensis*

Seeds of *P. carolinensis* collected from the western region of Cuba were germinated *in vitro* on MS medium (Murashige and Skoog, 1962). Disinfection of the seeds was carried out by successive rinsing, first with distilled water and Tween (2 to 3 drops) over 5 min, then with $HgCl_2$ (0.1%) for 1 min, and finally with sodium hypochlorite (3%) for 30 min. After disinfection, the material was rinsed with distilled water. Stems of plantlets obtained after two months of seed germination were cut into small pieces of about 1.5 cm and grown *in vitro* on three different media: MS without growth regulator, MS supplemented with 0.1 mg/L benzylaminopurine (BAP), and MS with 0.3 mg/L BAP. Leaves were submitted to two drying methods*:* room temperature (7 days) and freeze drying.

Extraction procedure

Five grams of dried, powdered material (leaves, stems, inflorescences, or roots of *P. carolinensis*, *P. odorata,* or *P. rosea*) was extracted by four successive 72 h EtOH: H_2O (7:3 v/v) macerations: 4×50 ml for leaves and stems; 4×100 ml for inflorescences and roots (Perera et al. 2010). In addition, 5 g leaves of adult cultivated *P. carolinensis* specimens were also extracted with 50 ml EtOH: H_2O (7:3 v/v) by reflux for 2 h and with EtOH (98%) at room temperature. All extracts were filtered and evaporated under reduced pressure and freeze dried. Between 0.1 and 0.3 g leaves of *in-vitro*-grown plants were dried by means of two different protocols (drying at room temperature and freeze drying). The same extraction and maceration conditions were used as for natural specimens. The hydroalcoholic extracts were dried. All extractions from plant organ specimens were performed in duplicate.

Determination of the antioxidant capacity and phenolic concentration

The antioxidant capacity of each crude extract was expressed in milligrams of Trolox equivalents per gram of dried plant organ weight (mg TE/g DW) and the phenolic content in milligrams of gallic acid equivalents per gram of dried plant organ weight (mg GAE/g DW).

Total phenolics

Total phenolics in the different crude extracts were determined with Folin-Ciocalteu's reagent, using gallic acid as standard (10 to 50 µg/ml) (ISO 14502-1: 2005). The absorbance at 765 nm was recorded.

DPPH˙ scavenging assays

The radical-scavenging capacity was examined by reduction of the $DPPH^{\cdot}$ free radical in methanol (Lo Scalzo, 2008). As standard, (±)-6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox), 50 to 100 µM was used. The absorbance at 520 nm was recorded after 5 min.

Oxygen radical absorbance capacity (ORAC)

150 µL fluorescein (4. 10^{-6} M) and 25 µL diluted sample prepared in 75 mM phosphate buffer (pH 7.4) were mixed. The phosphate buffer was used as blank and Trolox as standard for each run. The reaction was started by adding 25 µL of 173 mM AAPH and incubating at 37°C. Fluorescence was recorded at 538 nm, with an excitation wavelength of 485 nm (Wu et al., 2004), every two minutes for 3 h.

High performance liquid chromatography

One mg/ml of the samples were run through a Grace Davison

GraceSmart RP C-18 column (250 × 4.6 mm, 5 µm) at 30°C on an HPLC Merck Hitachi LaChromElite chromatograph equipped with an L-2130 pump, a L-2200 autosampler, and a UV-Visible L-2450 diode array detector. Results were expressed in milligrams of phenolic compound per gram of dry extract.

Flavonoid analyses

H_2O/TFA/AcN from 99.95:0.05:0 to 34.98:0.02:65 in 40 min were used at 1 ml/min flow rate and the wavelength was fixed at 360 nm. Twenty two flavonoids were used as standards: daidzin, taxifolin, rutin, hyperoside, quercetin-3-O-glucoside, genistin, kaempferol-3-O-rutinoside, quercetagetin, quercitrin, spiraeoside, myricetin, ononin, daidzein, luteolin, quercetin, herbacetin, naringenin, genistein, kaempferol, apigenin, isorhamnetin and casticin.

Phenolic acid analyses

H_2O/CH_3COOH from 98:2 (v/v) to H_2O/CH_3COOH/AcN (0:0:100) in 73 min. The flow rate was 0.5 ml/min and the wavelengths were fixed at 260, 275 and 325 nm. Twelve phenolic acids were used as standards: gallic acid, gentisic acid, hydroxybenzoic acid, vanillic acid, syringic acid, salicylic acid, *p*-coumaric acid, ferulic acid, *o*-coumaric acid, sinapic acid, ellagic acid and rosmarinic acid.

Caffeoylquinic acid derivatives

TFA/AcN from 100:0 to TFA/AcN 35:65 in 45 min. The flow rate was 1.0 ml/min and the wavelength was fixed at 328 nm. Eight caffeoylquinic derivative acids were used as standard: caffeic acid, 3-caffeoylquinic acid, cynarine, 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, 3,4,5-tricaffeoylquinic acid and 1,3,4,5-tetracaffeoylquinic acid.

Statistical analyses

For all antioxidant capacity ($DPPH^{\cdot}$ and ORAC) and phenolic content determinations, data were compared by ANOVA, using Tukey's HSD post hoc test $P < 0.05$, $n = 3$.

RESULTS

Extraction yields and preparation of crude extracts

Leaves, stems, inflorescences, and roots of *P. carolinensis*, *P. odorata,* and *P. rosea* were subjected to extraction with 70 or 98% ethanol. Extraction yields are shown in Table 1. *P. carolinensis* specimens collected from different locations, at different ages and at different times over a period of several months were investigated. Two extraction methods were also tested (maceration and reflux). The extraction yield was slightly higher after maceration (20%) than after reflux (19.1%). In almost all experiments, the highest extraction yields were obtained from leaves, followed by inflorescences, stems, and roots. Extraction yields for *P. carolinensis* material varied according to plant age, the location, and the harvest time. For leaves harvested in March, the highest extraction yield was obtained from adult natural specimens of *P. carolinensis* (25.5%), followed by adult cultivated specimens (20%) and juvenile cultivated specimens (18.9%). In cultivated juvenile plants, stems showed the highest phytochemical content (23.5%). In *P. carolinensis*, the amount of phytochemicals having accumulated in the leaves was found to vary over time: the best extraction yield was obtained from leaves harvested in December (24.8%).

Variation in the phenolic contents and antioxidant capacities in different plant organs of *Pluchea* species

First, the antioxidant capacity of leaf extracts from *P. carolinensis* was evaluated through $DPPH^{\cdot}$ assay by using two different extraction methods. Maceration and reflux in 70% ethanol were compared during March. The $DPPH^{\cdot}$ assay showed no significant difference between the two extraction methods: the antioxidant capacity after maceration was 44.1 ± 1.6 mg TE/g DW while after reflux extraction was 44.0 ± 0.8 mg TE/g DW. As maceration in 98% ethanol yielded a much lower antioxidant capacity (8.8 ± 0.1 mg TE/g DW) than maceration in 70% ethanol (44.1 ± 1.6 mg TE/g DW), the latter extraction procedure was used for comparing the antioxidant capacities of *Pluchea* species plant organ extracts (Figure 1).

Similar trends were observed with all three species: leaf extracts showed the highest antioxidant capacity, followed by inflorescences, stems, and roots. The highest $DPPH^{\cdot}$ and ORAC values were measured for *P. carolinensis* (103.2 and 254.4 mg TE/g DW, respectively, equivalent to 412.5 and 1016.5 µmol TE/g DW) and *P. rosea* (97.2 and 286.7 mg TE/g DW equivalent to 388.2 and 1145.4 µmol TE/g DW).

The phenolic contents and antioxidant capacities of collected *P. carolinensis* specimens at two locations: Sierra del Rosario, Pinar del Río (wild natural specimens) and Las Cañas, Havana City (cultivated specimens) were also compared. The highest antioxidant levels were found in the natural *P. carolinensis* specimens (Figure 2).

Average temperatures and precipitations were checked over the harvest month (March) at both locations. The temperature and precipitations in Sierra del Rosario, Pinar del Río were 22.4 ºC and 66 mm, respectively, while in Las Cañas, Havana City, they were 22.9°C and 39 mm (Table 2).

Seasonal variation of *P. carolinensis* leaf antioxidant capacity

The phenolic content and antioxidant capacity of leaf extracts from adult cultivated *P. carolinensis* specimens were monitored five times over a year (Figure 3). The lowest antioxidant capacities were found at the flowering stage (March) and in July. The highest $DPPH^{\cdot}$ antioxidant capacities were detected in January (56.8 mg TE/g DW), September (55.4 mg TE/g DW), and December (52.6 mg

Table 1. Phytochemical extraction yields from five grams of dried plant organs of *Pluchea* species.

Species	Month	Plant organ	Extract (mg) ± SD *	Yield (%)
P. carolinensis [1]	March (Flowering)	Leaves	999 ± 12	20.0
		Inflorescences	873 ± 32	17.5
		Stems	396 ± 11	7.9
		Roots	342 ± 22	6.8
		+Leaves	956 ± 12	19.1
		++Leaves	451 ± 21	9.0
	January	Leaves	943 ± 46	18.9
	March		999 ± 12	20.0
	July		1079 ± 15	21.6
	September		1082 ± 10	21.6
	December		1238 ± 18	24.8
P. carolinensis [2]	March (Flowering)	Leaves	1275 ± 32	25.5
		Inflorescences	1080 ± 33	21.6
		Stems	767 ± 31	15.3
		Roots	351 ± 23	7.0
P. carolinensis [3]	September	Leaves	943 ± 28	18.9
		Stems	1176 ± 29	23.5
		Roots	756 ± 7	15.1
P. odorata	May (Flowering)	Leaves	1142 ± 15	22.8
		Inflorescences	790 ± 4	15.8
		Stems	451 ± 14	9.0
		Roots	350 ± 8	7.0
P. rosea	May (Flowering)	Leaves	975 ± 10	19.5
		Inflorescences	717 ± 8	14.3
		Stems	418 ± 11	8.4
		Roots	359 ± 22	7.2

Extractions performed in 70% ethanol, except where was indicated. *P. carolinensis* [1]: adult cultivated specimens collected in Las Cañas, Habana City. *P. carolinensis* [2]: adult natural specimens collected in Sierra del Rosario, Pinar del Río. *P. carolinensis* [3]: juvenile cultivated specimens collected in Las Cañas, Ciudad de La Habana. ┼ Reflux extraction; ┼┼ Reflux extraction performed with 98 % ethanol. * Results are expressed as the means of the weight of the crude extracts ± standard deviation (*n=2*).

TE/g DW). The highest ORAC assay measurements were obtained in January (190.1 mg TE/g DW), followed by September (162.0 mg TE/g DW) and December (172.0 mg TE/g DW). Although the dry extract yield was lowest in January (18.9%, Table 1), the ORAC antioxidant capacity was almost twice as high in that month than in March (92.4 mg TE/g DW), with a 20% extraction yield. In general, a correlation between total phenolic content and antioxidant capacity was observed for all months except March.

High Performance Liquid Chromatography (HPLC) analysis

Several standard phenolic compounds were monitored in leaf extracts of cultivated *P. carolinensis* during the selected months (Table 3). Only 1,3,4,5-tetracaffeoylquinic acid derivatives was previously isolated from *P. carolinensis* (Perera, 2012). Two flavonols and eight phenolic acids were identified as the major phenolic compounds. However, a few phenolics were not detected during some months i.e. caffeic acid (January), quercitrin (March) and 3-caffeoylquinic acid (March and July). The most prominent phenolic compounds were rosmarinic acid and 4,5-dicaffeoylquinic acid. Total content in phenolics identified were higher in January, September, and December than March and July. Additionally, the highest values of antioxidant capacity were also measured in January, September and December.

Antioxidant capacity variations with plant age

The antioxidant capacities (DPPH¨) and phenolic contents of leaf, stem and root extracts from adult and juvenile cultivated specimens of *P. carolinensis* collected

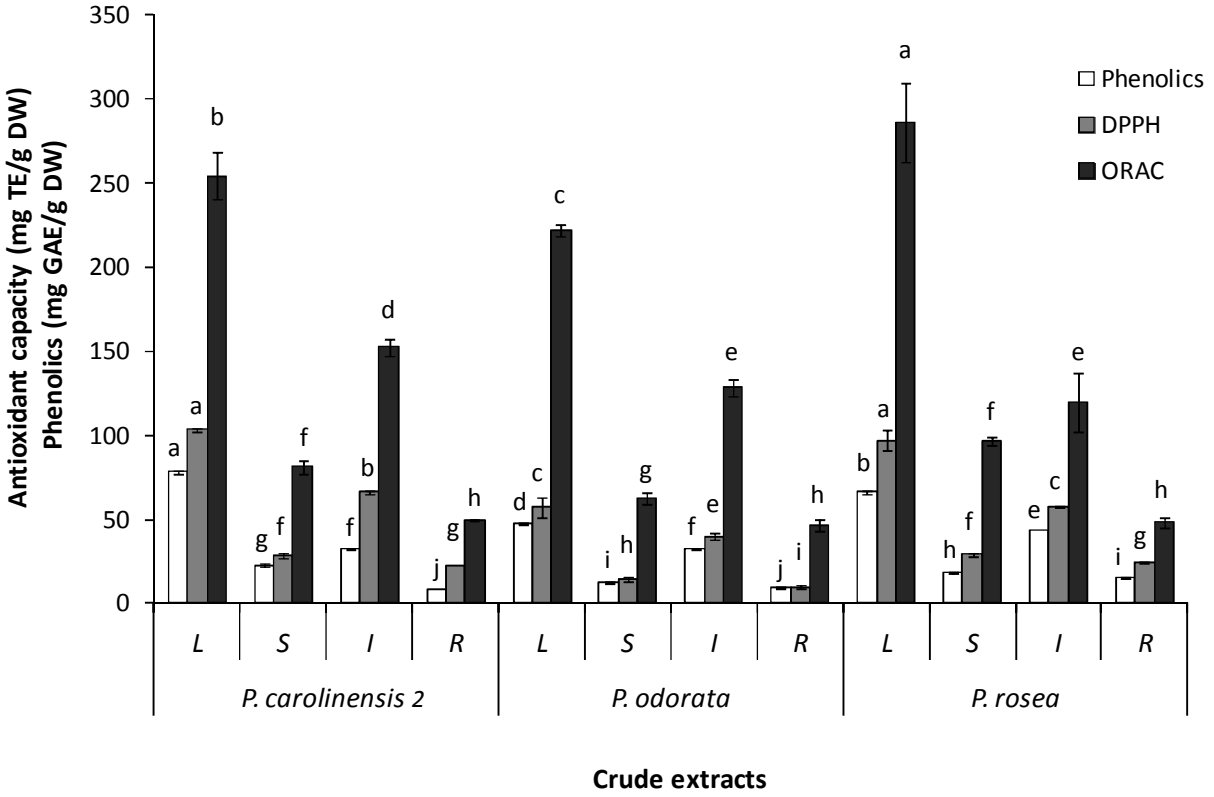

Figure 1. Antioxidant capacity (DPPH· and ORAC) and total phenolic content of plant organs of *Pluchea* species harvested in March. L: leaves; S: stems; I: inflorescences; R: roots. Different letters indicate significant statistical differences, Tukey HSD's post hoc test $p < 0.05$, $n = 3$. Coefficients of variance for total phenolics and the antioxidant capacity were lower than 10% and 15% respectively.

in September from the same location: Las Cañas, Havana City were compared (Figure 4). Leaf extracts from adult plants showed higher antioxidant capacity than juvenile plants leaf extracts. In addition, there appeared no statistically significant difference in antioxidant capacity between leaf and stem extracts from juvenile plants. Also, stems of juvenile plants showed a higher antioxidant capacity than those of adult specimens.

In vitro propagation of *P. carolinensis* and antioxidant capacity of vitroplants

After one month of culture, the number of leaves produced per plantlet was highest when the medium was supplemented with growth regulator, but the leaves were smaller in this case. All cultured shoots developed roots two weeks after culture, independently of the medium used. DPPH· and ORAC antioxidant capacities of leaf *in vitro* crude extracts by using two different drying methods are shown in Figure 5.

The drying method had no significant effect on the antioxidant capacity determined by the ORAC assay, but plants grown on MS+BAP showed a slightly higher DPPH· value after freeze-drying than after room temperature drying. Whatever the assay method, leaf extracts from plants grown in MS medium without the growth regulator showed the lowest antioxidant capacities.

DISCUSSION

In our previous studies, the antioxidant capacity of leaf extracts from three Cuban *Pluchea* species was reported (Perera et al., 2010). Therefore, in the present research, we studied for the first time how the antioxidant capacity and phenolic compounds varied on different plant organs, plant ages, seasonal collection and *in vitro* culture.

The highest antioxidant capacities were measured in leaf extracts, followed by inflorescences, stems, and roots. These results are in agreement with those obtained by Falleh et al. (2008) and Wenguo et al. (2012), as leaf extracts from *Cynara cardunculus* L. and *Houttuynia cordata* Thunb. showed higher antioxidant capacities than stem, flower or root extracts.

The ORAC values for leaves of these Cuban *Pluchea* species were higher than those measured for leaves of several endemic plants of the Macaronesian islands (c. a 250- 620 µmol TE/g DW) (Tavares et al., 2010). In addition, the antioxidant capacity of the Cuban *Pluchea*

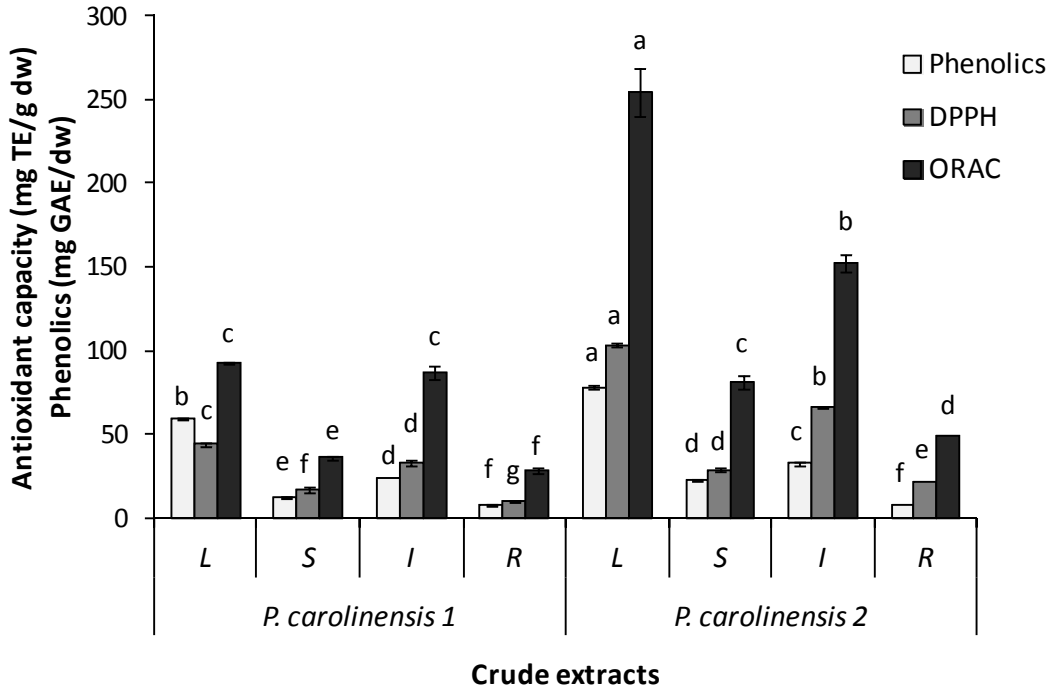

Figure 2. Antioxidant capacity (DPPH$^{\cdot}$ and ORAC) and total phenolic content of plant organs of *Pluchea carolinensis* harvested in March in two locations. *P. carolinensis 1*: cultivated specimens of Las Cañas, Havana city; *P. carolinensis 2:* wild specimens of Sierra del Rosario, Pinar del Rio. Different letters indicate significant statistical differences, Tukey HSD's post hoc test $p < 0.05$, $n = 3$. Coefficients of variance for total phenolics and the antioxidant capacity were lower than 10% and 15% respectively.

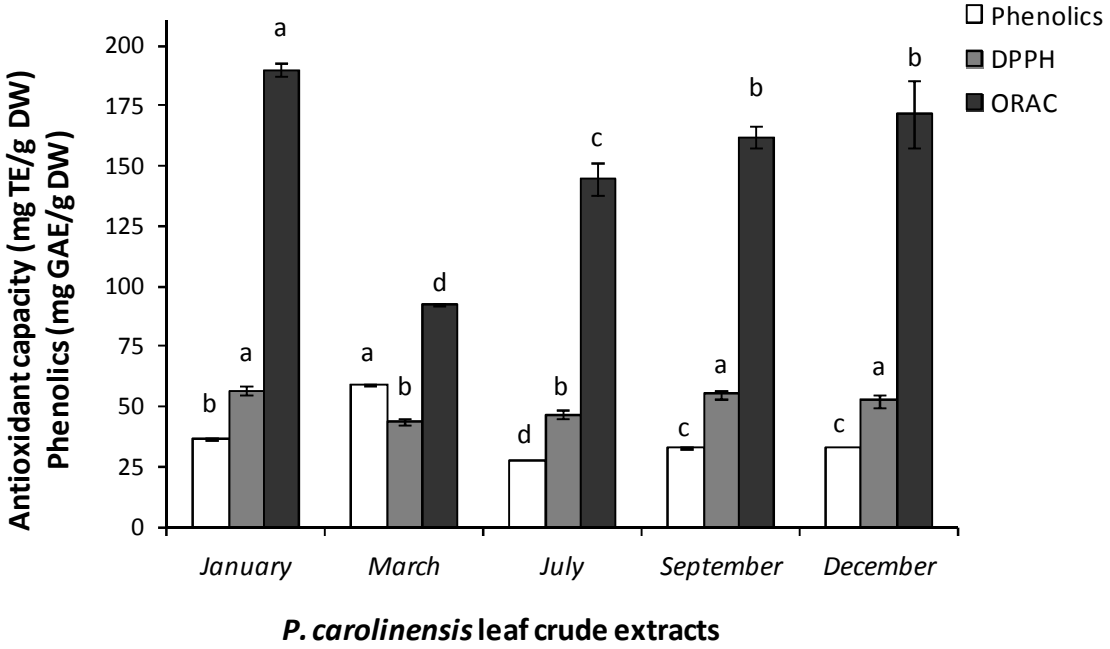

Figure 3. Antioxidant capacity (DPPH$^{\cdot}$ and ORAC) and total phenolic content in crude leaf extracts of adult cultivated specimens of *P. carolinensis* on different months of a year. Different letters indicate significant statistical differences, Tukey's HSD post hoc test $p < 0.05$, $n = 3$. Coefficients of variance for DPPH$^{\cdot}$ and total phenolics were lower than 5% and lower than 10% for ORAC.

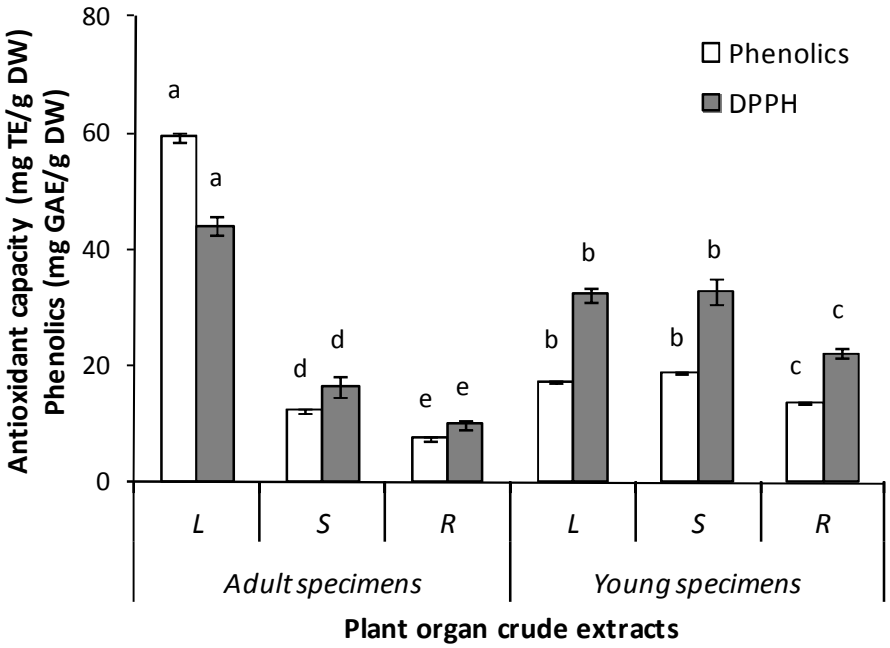

Figure 4. Antioxidant capacity (DPPH˙) and total phenolic content from adult and juvenile cultivated specimens of *P. carolinensis* respectively, collected both in September, Las Cañas, Havana city. (L: leaves; S: stems; R: roots). Different letters indicate statistical differences Tukey's HSD post hoc test $p < 0.05$, $n = 3$. Coefficients of variance for DPPH˙ and total phenolics were lower than 15% and 5% respectively.

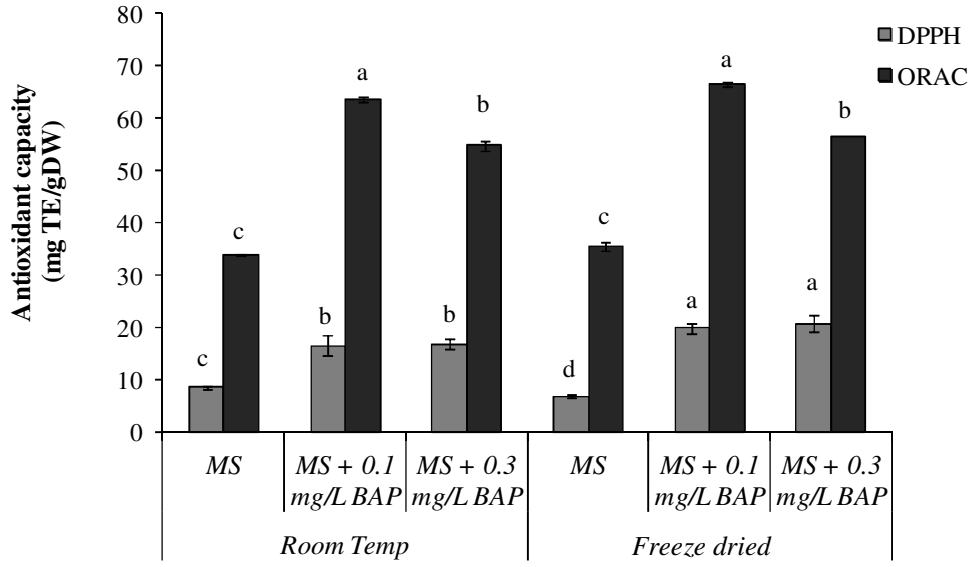

Figure 5. Antioxidant capacity (DPPH and ORAC) of crude leaf extracts of *P. aarolinensis* from in *vitro* plantlets. Three different culture media (MS, MS+ 0.1 mg/mL and MS+ 0.3 mg/mL of BAP). Different letters indicate statistical differences Tukey HSD's post hoc test $P < 0.05$, $n = 3$. Coefficients of variance for DPPH and ORAC were lower than 10%.

species was higher than that recorded for a large number of popular fruits, vegetables, and condiments (Kevers et al., 2007). This suggests that Cuban *Pluchea* species could be used as rich sources of antioxidants.

The phytochemical composition and antioxidant capacity of Tartaric buckwheat (*Mentha puligium*) is also

Table 2. Nutrients and organic material contents in soils of two Western Cuba locations.

Soil	Nutrients (mg/kg)						Organic material (%)
	N	P	K	Na	Ca	Mg	
SR	5.04	8.59	46.83	32.65	10.19	8.54	4.37
LC	3.06	0.57	14.96	22.51	29.35	6.58	0.83

SR: Sierra del Rosario, Pinar del Río; LC: Las Cañas, Habana City.

Table 3. Level of specific phenolic compounds in crude leaf extracts of adult cultivated specimens *P. carolinensis* collected at different dates of a year.

Phenolic	Months				
	January	March	July	September	December
Qt	3.81±0.08	Nd	4.14±0.22	5.25±0.54	5.94±0.59
L	4.93±0.34	4.53±0.63	5.38±0.74	6.6±0.33	7.32±0.09
Ra	11.52±0.35	8.49±0.39	6.96±0.47	10.25±0.36	11.95±0.75
Ca	Nd	0.03 ±0.01	0.11 ±0.05	0.07 ±0.01	0.12 ±0.02
3-CQA	0.05 ±0.01	Nd	Nd	0.06 ±0.03	0.05 ±0.01
3,4-diCQA	3.23 ±0.03	2.31 ±0.33	1.52 ±0.04	4.58 ±0.02	1.89 ±0.01
3,5-diCQA	2.14 ±0.01	1.87 ±0.52	1.82 ±0.02	4.20 ±0.02	1.84 ±0.01
4,5-diCQA	11.65 ±0.03	10.41 ±0.25	6.53 ±0.02	18.6 ±0.02	7.29 ±0.27
triCQA	1.87 ±0.01	0.76 ±0.02	2.03 ±0.03	5.42 ±0.06	1.99 ±0.10
tetraCQA	1.54 ±0.04	0.61 ±0.02	2.75 ±0.07	4.35 ±0.2	2.49 ±0.10
Total	**40.7**	**29.0**	**31.2**	**59.4**	**40.9**

Analyses were conducted in triplicate and results were expressed in milligrams of phenolic compounds per gram of dry weight. Quercitrin (Qt), Luteolin (L), Rosmarinic acid (Ra), cafeic acid (Ca), Chlorogenic acid (3-CQA), 3,4-dicaffeoylquinic acid (3,4-diCQA), 3,5-dicaffeoylquinic acid (3,5-diCQA), 4,5- dicaffeoylquinic acid (4,5-diCQA), 3,4,5-tricaffeoylquinic acid (triCQA), 1,3,4,5-tetracaffeoylquinic acid (tetraCQA).

reported to vary according to the harvest site (Guo et al., 2011) and developmental stage (Karray-Bouraoui et al., 2010). As plant secondary metabolites are described to be influenced by both abiotic and biotic stress (Björkman et al., 2011).

The difference in rainfall might be partly responsible for the observed variations in phenolic content and antioxidant capacity, but this should be checked by comparing a larger number of harvest sites over several years. The soil collected at Sierra del Rosario, Pinar del Río was richer in organic material and nutrients (except calcium) than that collected at Las Cañas, Havana City. This could be another cause of differences in phenolic content and antioxidant capacity. In the field, on the other hand, complex plant-animal interactions occur continuously. Plants have developed a broad range of mechanisms to reduce herbivore attack, including specific responses which, by activating various metabolic pathways, considerably alter plant chemical and physical properties. Phenolic compounds constitute the most common group of metabolites that plants use for defense (Harborne, 1999). Overproduction of methyl jasmonate in response to herbivore attack has been reported (Choonkyun et al., 2003). In addition, plant secondary metabolites constitute a powerful arsenal against attacking herbivores. Collected wild specimens are normally more prone to herbivore attack than cultivated specimens. We observed higher levels of phenolics and higher antioxidant capacities in natural plants than in cultivated ones. Such differences have been reported for bilberries grown under different environmental conditions (Martz et al., 2010).

P. carolinensis was used in further experiments for monitoring the antioxidant capacity, total phenolic compounds as well as some specific flavonoids and phenolic acids by HPLC over five months of a year. Both, antioxidant capacities and phenolic constituents were higher in January, September and December than in March and July.

Antioxidant capacity and phenolic compounds were also compared during two plant ages. Leaf extracts from juvenile plants showed a lower antioxidant capacity than leaf extracts from adult plants. Ascorbic acid is the most abundant, powerful and water-soluble antioxidant in plants, where it acts to prevent or minimize damage caused by radicals. The ascorbic acid content is reported to be highest in mature leaves with fully developed chloroplasts and the highest chlorophyll contents (Mingjun et al., 2010; Sarvajeet and Narendra, 2010). Thus, plant age is another important characteristic to be

taken into account in order to optimize the antioxidant compound yield.

We also study the variation of antioxidant capacities when *P. carolinensis* was *in vitro* cultivated with and without growth regulator. Leaf extracts from MS with BAP showed highest antioxidant capacities than those obtained from MS culture. In experiments conducted on *in vitro Salvia officinalis* L. plants, Santos-Gomes et al. (2002) showed that increasing the concentration of the cytokinin kinetin from 1.5 mg/L to 4.0 mg/L variably affected the concentrations of phenolic compounds such as phenolic acids, flavonoids, and phenolic diterpenes, causing the levels of caffeic acid, 3-caffeoylquinic acid and rosmarinic acid to increase but some levels of phenolic diterpenes decreased.

The ORAC assay measurements were likewise higher for leaf extracts from *ex vitro* plants (highest activity measured: 66.5 mg TE/g DW in the presence of 0.1 mg/ml BAP) than for extracts from *in vitro* plants. In $DPPH^{\cdot}$ assays, leaf extracts from *in vitro* plants grown in the presence of BAP showed an antioxidant capacity of only 20.8 mg TE/g DW, as compared to 44.1 mg TE/g DW for leaf extracts from *ex vitro* adult plants and 32.3 mg TE/g DW for leaf extracts from ex vitro juvenile plants.

Possibly relevant to this finding is the observation that plantlets in *in vitro* culture are exposed to various stresses such as high concentrations of nutrients, high humidity, ethylene accumulation, the addition of growth regulators, etc. (Kevers et al., 2004). The production of some polyamines and proline, a traditional stress marker in plants, has notably been found to increase in *in vitro* plantlets of *Prunus avium* L. exposed to hyperhydric stress (Franck et al., 2004).

CONCLUSIONS

We have obtained crude extracts from Cuban *Pluchea* species and measured their antioxidant capacity. Leaves proved richest in antioxidants, followed by inflorescences. The antioxidant capacities measured in leaf extracts of the most promising species (*P. carolinensis* and *P. rosea*) were significantly higher than those measured in material from other plant species, including fruits, vegetables, and condiments. Cuban *Pluchea* species having emerged as an important reservoir of antioxidant molecules, we examined how (*ex vitro* and *in vitro*) growth conditions and harvest timing affect the antioxidant capacity of *P. carolinensis*. The results indicate that the best season for collecting this plant is during the winter. Furthermore, adult *P. carolinensis* plants harvested in their natural environment display a higher antioxidant capacity than adult or juvenile specimens cultivated under *ex vitro* or *in vitro* conditions. Finally, we have developed a successful method of *P. carolinensis in vitro* propagation that should allow future sustainable exploitation without affecting natural populations of this species.

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