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# A propagation procedure for *Cuphea aequipetala* Cav. (Lythraceae) and antioxidant properties of wild and greenhouse-grown plants

[Procedimiento para la propagación de *Cuphea aequipetala* Cav. (Lythraceae) y propiedades antioxidantes de plantas silvestres y crecidas en invernadero]

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**Abstract**
*Cuphea aequipetala* Cav. (Lythraceae) is native to Mexico and is used in folk medicine to treat tumors. An efficient protocol for *in vitro* shoot proliferation and plant acclimatization of *C. aequipetala* was developed. Total phenolic compounds and flavonoids contents were determined in methanolic extracts of roots, stems, and leaves from wild and greenhouse-grown plants. Their antioxidant properties were compared using *in vitro* assays (scavenging of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radicals, and reducing power in the phosphomolybdenum assay). This is the first report of a successful propagation procedure for *C. aequipetala*. These methods offer a viable approach for long-term *in vitro* conservation and proliferation of this species. *C. aequipetala* shoots maintained their proliferation capacity during long-term subculture (3 years). The propagated shoots can successfully acclimatize and grow to maturity, and they retain the ability to accumulate antioxidants.

**Keywords:** antioxidant activity, *Cuphea aequipetala*, flavonoids, Lythraceae, phenolic compounds, shoot proliferation, callus.

**Resumen**
*Cuphea aequipetala* Cav. (Lythraceae) es una planta nativa de México que se utiliza en la medicina tradicional para tratar tumores. En este trabajo se desarrolló un procedimiento para la proliferación de brotes y la aclimatización de plantas de *C. aequipetala*. Se determinó la concentración de compuestos fenólicos totales y de flavonoides en extractos metanólicos de raíces, tallos y hojas de plantas silvestres y crecidas en invernadero. Sus propiedades antioxidantes fueron comparadas utilizando ensayos *in vitro* (captura de radicales DPPH y ABTS y poder reductor por el ensayo de fosfomolibdeno). Este es el primer reporte exitoso sobre un procedimiento para la propagación de *C. aequipetala.* Este método ofrece una alternativa viable para la conservación a largo plazo y la proliferación de esta especie. Los brotes de *C. aequipetala* han mantenido su capacidad de multiplicación a largo plazo (tres años). Los brotes se convirtieron en plantas adultas aclimatadas, manteniendo su habilidad para acumular compuestos antioxidantes.

**Palabras Clave**: actividad antioxidante, callos, compuestos fenólicos, *Cuphea aequipetala*, flavonoides, Lythraceae, proliferación de brotes.

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## INTRODUCTION

The genus *Cuphea* (Lythraceae) comprises more than 260 species native to the Americas, and *Cuphea* species are distributed from Mexico to Brazil. *Cuphea* species are cultivated as sources of oils rich in medium-chain fatty acids (Graham and Kleiman, 1992; Phippen, 2009; 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 example, the leaves of *C. carthagenensis* are a significant source of phenolic antioxidants that may have beneficial cardiovascular effects (Schuldt *et al.,* 2004) and the leaves of *C. racemosa* are used for the treatment of urinary tract infections (Tene *et al*., 2007). *Cuphea aequipetala* Cav., known as "hierba del cancer" in Spanish or "Tozancuitlacxolli" in Nahuatl, is native to Mexico and grows in open and humid fields of pine-oak woods 2000 - 2540 m above sea level (Graham, 1991). It has been used in Mexican folk medicine since the 16$^{th}$ century to treat dermatological conditions and skin tumors (Alonso-Castro *et al.,* 2011). Teas, decoctions, or infusions are prepared from various organs of wild-harvested plants. For instance, infusions of flowers and leaves are used to relieve pain whereas decoctions of the whole plant are used to treat tumors, inflammation, and infections (Biblioteca Digital de la Medicina Tradicional Mexicana, 2011). There is also some scientific evidence for its biological activity. Aqueous extracts show activity against *Helicobacter pylori* (Castillo-Juárez *et al.,* 2009) and organic extracts have cytotoxic activity against human larynx carcinoma cells (HEp-2 cell line) (Waizel-Bucay *et al.,* 2003) and human prostate carcinoma cells (Vega-Avila *et al.,* 2004). Cytotoxic activity has been attributed to phenolic compounds such as flavonoids and tannins (Vega, 2005). Besides flavonoids, in *C. aequipetala* were found alkaloids and sesquiterpenes, which may also present biological activities (Waizel-Bucay *et al*., 2003). The ellagitannin dimer Cuphiin D1, isolated from *C. hyssopifolia,* inhibited the growth of human cervical carcinoma (Elgindi *et al.,* 2011). *C. aequipetala* is usually harvested from wild populations. Therefore, it is available only seasonally, and falling trees and fires often disrupt its habitat (Rosas *et al*., 2009). Plant cell culture is an alternative and effective source of materials for production of valuable phytochemicals. It is also useful for propagation and germplasm conservation of *C. aequipetala*, and thus, it represents a way to protect plant biodiversity. As a part of our research on the conservation and sustainable management of medicinal plant species, we have focused on several aspects of *C. aequipetala* biotechnology, including the establishment of propagation procedures and analyses of its chemical constitution and biological activities.

The few reports on *in vitro* culture of *Cuphea* are consistent with results obtained for other species. That is, genotype plays an important role in morphogenic responses, and therefore, specific protocols must be developed for each genotype of interest (Rita and Floh, 1995; Millam *et al.,* 1997). To develop techniques for *in vitro* conservation of *C. aequipetala* and to provide a source of cultivated plant material, a protocol to regenerate shoots from micro-cuttings of wild individuals was established (Salcedo *et al.,* 2009). Here, we report procedures for proliferation of *C. aequipetala* shoots *in vitro*, and for acclimatization of this valuable medicinal plant. We also determined total phenolic compounds and flavonoids contents and determined antioxidant activity of methanolic extracts from wild and greenhouse-grown plants using *in vitro* models. The antioxidant capacity was positively correlated with the amount of total phenolic compounds and flavonoids.

## MATERIALS AND METHODS

### *Plant material*

*Cuphea aequipetala* Cav. (Lythraceae) plants were collected at the flowering stage from wild populations in Zempoala, State of Mexico, Mexico, at 2860 m above sea level (latitude 19°02′ N, longitude 99°19′ W) in July and August, 2008. The plants were positively identified as *C. aequipetala* at the Herbarium of the Universidad Autónoma del Estado de Morelos (UAEM). The voucher number of the deposited specimen is 13238.

We used 28-day-old *in vitro* plantlets of *C. aequipetala* that were derived from buds with leaves of wild-grown individuals as sources of explants (Salcedo *et al.,* 2009). Briefly, 2-cm buds with leaves were washed with commercial soap solution (1%, *w/v*) for 10 min and then washed three times with tap water. Then, the buds were surface sterilized with 1% (*v/v*) sodium hypochlorite for 5 min with agitation (manually) in a laminar flow cabinet. Buds were washed three times (2 min each) with distilled sterile

water and soaked in ethanol 70% (*v/v*) for 2 min. Finally, the buds were immersed in a solution of the fungicide benomyl (0.1%, *w/v*). Buds were placed on shoot induction medium consisting on Murashige and Skoog salts (MS, Murashige and Skoog, 1962) with 3% sucrose (*w/v*), thiamine (0.9 mg/L), folic acid (0.5 mg/L), biotin (0.05 mg/L), the plant growth regulators (PGRs) 1.0 µM 6-benzyladenine (BA) and 0.5 µM α-naphthalene acetic acid (NAA), and 0.8% agar (*w/v*). After the medium had solidified, 100 µL of a solution of the antibiotic ciprofloxacin (50 mg/L) was sprayed onto the medium in each dish. Once induced, shoots were subcultured every 3 weeks onto fresh MS medium containing 3% sucrose (*w/v*), 1 µM BA, and 0.5µM indole-3-butyric acid, and 0.8% agar (*w/v*). The pH of all media was adjusted to 5.8 before sterilization. The cultures were maintained in a growth room at 25 ± 2° C under a 16-h light/8-h dark photoperiod with illumination of 103 µmol/$m^2$/s provided by four 75 W cool-white fluorescent lamps (Osram SL5938, Mexico).

***Influence of type of explant and auxin***

For shoot and callus induction, we evaluated the effects of explant and auxin type. Nodal segments (0.2 cm), internode segments (0.3 cm), and leaf tissue (0.3 cm) were placed on MS medium supplemented with 2.5 µM BA and 2.5 µM of either 2,4-dichlorophenoxyacetic acid (2,4-D) or indole 3-butyric acid (IBA). A control without PGRs was included. All media were supplemented with 3% sucrose (*w/v*), 0.8% agar (*w/v*), and the pH was adjusted to 5.8 prior to sterilization. Three explants were placed in each vessel, and a total of 15 explants were used for each treatment. The explants were incubated under the climatic conditions described above. The percentage of explants that produced new shoots or callus, the shoot proliferation rate (number of harvested shoots/number of explants), and the average shoot length were recorded after 28 days. The experiment was repeated twice.

***Rooting and plant acclimatization***

Six-week-old *in vitro* plantlets (2 cm in height) were removed from jars and gently washed with distilled water to remove adhering medium. Then, they were planted in 200-pot trays filled with a sterilized mixture of peat moss:agrolite:vermiculite (60:20:20, *v/v*) adjusted to pH 5.8 ± 0.3 and moistened with tap water (15 mL/pot). The trays were covered with plastic foil for 30 days to maintain a relative humidity (RH) of ≥ 90% and incubated under cool-white fluorescent tubes (four 55 W lamps, SL5928 Osram, Mexico) at a light intensity of 77 µmol/$m^2$/s under a 16-h light/8-h dark photoperiod at 20 ± 3 ºC. The plants were irrigated manually with tap water each week. After 30 days, single plants (6 cm in height) were transferred to pots (15 × 12 cm) filled with potting mix and were acclimatized by gradually opening and finally removing the plastic foil over a period of 6 weeks (RH approx. 55%). The plants were kept under controlled environmental conditions at 20 ± 3 ºC and a 16-h light/8-h dark photoperiod with light provided by cool-white fluorescent tubes (four 55 W lamps, SL5928 Osram, Mexico) at an intensity of 77 µmol/$m^2$/s. The plants were irrigated manually with tap water when necessary. After 4 weeks, the plants were moved to a greenhouse with mean day/night temperatures of 23/28 °C and a maximum natural solar irradiance of 1750 µmol/$m^2$/s (measured in the middle of sunny cloudless days). The plants were irrigated manually with tap water when necessary to maintain a substrate moisture content of 30 - 40% (*v/v*). Full flowering occurred at this stage. Plant survival and average height were recorded periodically. These experiments were repeated twice, and 200 plants were cultivated each time.

***Preparation of extracts***

Three-month-old (flowering stage) greenhouse-grown plants were used for analyses. We also analyzed wild-grown plants for comparison. Plants were separated into roots, stems and leaves. Each plant part was freeze-dried and ground into a fine powder (particle size < 250 µm) using a pestle and mortar. Chemical compounds were extracted by stirring 100 mg lyophilized tissue in 50 mL methanol for 24 h, and then extracts were filtered through Whatman #l paper and concentrated using a Büchi rotary evaporator (Büchi-490; Büchi, Switzerland) under reduced pressure (210 mbar) at 40 °C and 50 rpm. The residue was re-suspended in 10 mL water and lyophilized.

***Quantification of phenolic compounds***

Phenolic compounds in extracts were estimated using the Folin-Ciocalteu colorimetric method (Shohael *et al.*, 2006). An aliquot (100 µL) of the extract was mixed with 2.5 mL deionized water, and 100 µL Folin-Ciocalteu reagent was 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 was 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 are expressed as milligrams of gallic acid equivalent (GAE) per gram dry weight (DW). Samples were analyzed in triplicate.

***Quantification of total flavonoids***

The total flavonoid content was determined by a colorimetric assay as described by Shohael *et al.* (2006). An aliquot (250 μL) of the extract was mixed with 1.25 mL de-ionized water, and 75 μL of an aqueous solution of $NaNO_2$ (5%, *w/v*) was added. The mixture was thoroughly mixed with a vortex mixer and incubated at room temperature for 6 min. Then, 150 μL of an aqueous solution of $AlCl_3$ (10%, *w/v*) was added. After a further 5 min, 0.5 mL NaOH (aq) (1 M) and 2.5 mL de-ionized water were added. Finally, the mixture was incubated for 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 is expressed as milligrams of quercetin equivalents (QE) per gram DW. Samples were analyzed in triplicate.

***Determination of free-radical scavenging activity***

***2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical-scavenging assay:*** The free radical-scavenging activity of extracts was quantified spectrophotometrically using a DPPH assay (Sánchez-Moreno *et al.,* 1998). A stock solution of freshly prepared DPPH (3.9 mL, 60 μM) was mixed with 100 μL sample extract (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. The results are expressed as micromoles trolox per gram DW based on a calibration curve ($R^2$ = 0.993). The assay was carried out in triplicate.

***2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) free radical-scavenging assay:*** An $ABTS^{\bullet+}$ radical-scavenging assay was carried out using the improved ABTS method described by Re *et al.* (1999), with slight modifications. 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. The $ABTS^{\bullet+}$ solution was diluted with methanol to an absorbance of 0.7 ± 0.05 at 734 nm. The diluted sample (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 are expressed as micromoles trolox per gram DW based on a calibration curve ($R^2$ = 0.975). The assay was carried out in triplicate.

***Reducing power assay with phosphomolybdenum***

The reducing power assay using phosphomolybdenum 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 sample (100 μL; final concentration, 5 mg/mL) was incubated at 95 °C with the reagent solution (28 mM sodium phosphate and 4 mM ammonium molybdate in 0.6 M sulfuric acid) 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 are expressed as micromoles trolox per gram DW (Diouf *et al.,* 2009) based on a calibration curve ($R^2$ = 0.9974). The assay was conducted in triplicate.

***Thin layer chromatography (TLC) bioautography analysis***

An aliquot (18 μL) of plant extract (10 mg/mL) was carefully spotted on a 5×5 cm TLC plate (silica gel 60 $F_{254}$; Merck) which was then developed with an 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 0.01 mM DPPH• solution in methanol (Anandjiwala *et al.,* 2007). The stained silica layer revealed a purple background with yellow spots corresponding to resolved bands with radical scavenger capacity. TLC plates were also inspected under UV light at 254 nm 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*). The retardation factor ($R_F$) is a relative measure of the position of a compound on a chromatogram relative to the position of the solvent front. It is the most widely used descriptor of the position of compounds in TLC, and was calculated as follows (Reich and Schibli, 2007):

$$R_F = \frac{z_i}{(z_f - z_0)}$$

Where $z_i$ is the migration distance of the substance, $z_f$ is the migration distance of the solvent front measured from the immersion line, and $z_0$ is the distance between the immersion line and the site of sample application.

***Statistical analyses***

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

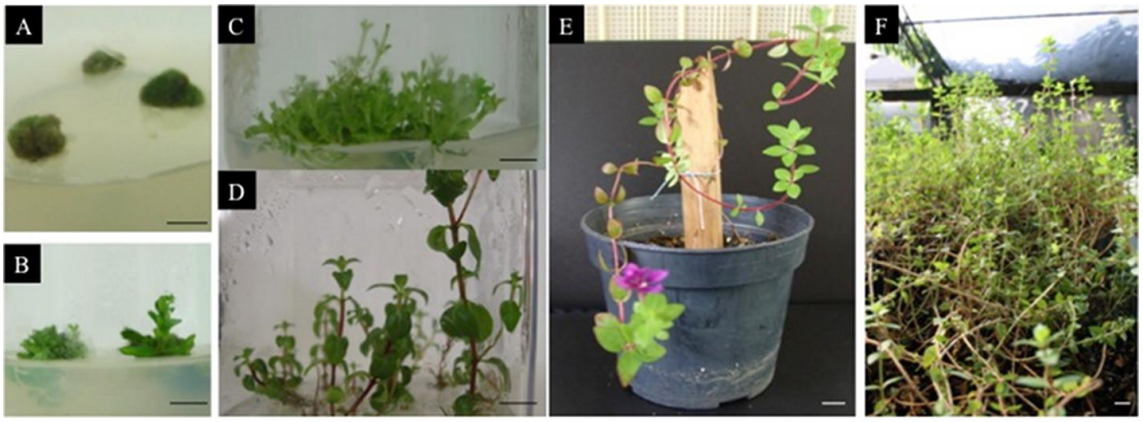


**Figure 1**

**Callus induction, shoot proliferation, and plant acclimatization of *Cuphea aequipetala*. Leaves-derived callus after 28 days on MS medium supplemented with 2.5 μM 2,4-D+2.5 μM BA (A). Shoot induction from nodes (B) and shoot multiplication on solid MS medium containing 2.5 μM BA at 28 days (C). *In vitro* plantlets 28 days after culture on solid MS medium with 2.5 μM BA (D). Acclimatized plants 6 weeks after culture in pots (E) and growing in the greenhouse (F).**

## RESULTS

***Influence of explant and auxin type on callus induction and shoot proliferation***

All three explant types (leaf, internode, and nodal segments) formed callus and shoots, usually within the first 10 days. Leaf and intermodal segments mainly formed callus (Figure 1A), whereas nodal segments mainly formed shoots (Figure 1B and C). The highest callus induction rate was obtained from leaf segments on medium containing BA+2,4-D (93.3%) or BA+IBA (83.3%) followed by nodal segments on medium with BA+2,4-D (56.7%) (Table 1). On medium with BA+IBA, calli were friable (easily disaggregated) and reddish. The highest shoot induction rate was obtained from nodal segments in medium with BA alone (70%) or BA+IBA (66.7%). These combinations of PGRs also resulted in the highest yields of shoots per explant (7 and 5 shoots/explant, respectively) ($P < 0.05$) (Table 1). These shoots elongated to an average length of 9 mm after 28 days of culture and developed between 4 and 6 leaves. Shoots that developed from tissues cultured with BA alone formed roots after 28 days of culture and were used for the acclimatization stage. On media without PGRs, shoots elongated up to 15 mm but yields were significantly lower (1 - 2 shoots/explant) (Table 1).

**Table 1**
**Influence of two auxins (2.5 µM) and 2.5 µM BA on shoot and callus induction from different explants of *C. aequipetala* after 28 days of culture on solid MS culture medium.**

| Explant | PGR | Callus induction (%) | Shoot induction (%) | No. shoots/explant | Shoot length (mm) | No. leaves/explant |
|---|---|---|---|---|---|---|
| Leaf | PGR-free medium | 0 | 3.3 | 1 ± 0.1 | 15.5 ± 0.1 | 6 |
| | BA | 47.8 | 3.3 | 1 ± 0.1 | 0.8 ± 0.1 | 2 |
| | BA + IBA | 83.3 | 10.0 | 3 ± 0.3 | 5.8 ± 0.1 | 2 |
| | BA + 2,4-D | 93.3 | 0 | 0 | 0 | -- |
| Internode | PGR-free medium | 29.2 | 6.7 | 1 ± 0.1 | 11.3 ± 0.1 | 7 |
| | BA | 41.1 | 6.7 | 2 ± 0.2 | 5.1 ± 0.1 | 2 |
| | BA + IBA | 40.0 | 13.3 | 3 ± 0.4 | 3.1 ± 0.1 | 3 |
| | BA + 2,4-D | 36.7 | 0 | 0 | 0 | 0 |
| Node | PGR-free medium | 0 | 63.3 | 2 ± 0.4 | 8.5 ± 0.3 | 6 |
| | BA | 20.8 | 70.0 | 7 ± 3.4 | 8.9 ± 0.3 | 4 |
| | BA + IBA | 25.0 | 66.7 | 5 ± 2.1 | 8.5 ± 0.2 | 4 |
| | BA + 2,4-D | 56.7 | 0 | 0 | 0 | 0 |

Data represent mean ± standard error for 2 replicates (ten explants each)

## PLANT ACCLIMATIZATION

At 30 days after transfer from MS medium with BA to trays with potting mix, the survival rate of acclimated plants was 52% and the plants were 5.69 ± 0.54 cm in height. The average root length was 0.3 cm. In the next stage, the plants reached 11.94 ± 2.81 in height after 40 days and the survival rate increased significantly (100%). All of the *C. aequipetala* plants transferred to the greenhouse survived and flowered (Figure 1E and F).

### *Total contents of phenolic compounds and flavonoids*

We quantified total phenolic compounds in extracts from *C. aequipetala.* Wild-grown plants contained from 9.60 ± 0.1 (stems) to 55.62 ± 0.5 (leaves) mg GAE/g DW, and propagated plants contained from 6.89 ± 0.1 (roots) to 14.98 ± 0.1 (leaves) mg GAE/g DW (Figure 2). According to the statistical analysis, the total phenolics content was significantly affected by the source of the plant material (wild or greenhouse-grown; $P < 0.05$) and the plant organ (roots, stems, or leaves; $P < 0.05$). On average, wild-grown plants contained three-fold more phenolics (30.81 mg GAE/g DW) than greenhouse-grown plants (9.66 mg GAE/g DW). The leaves from wild-grown plants showed the highest phenolics contents and the stems the lowest. Similarly, the amount of flavonoids in wild-grown plants ranged from 53.38 ± 0.7 (stems) to 196.83 ± 2.94 (leaves) µg QE/g DW whereas the average flavonoids content in greenhouse-grown plants was 21.59 µg QE/g DW. In wild-grown plants, these compounds were significantly more abundant in leaves and roots than in stems (Figure 2). In contrast, in greenhouse-grown plants, flavonoids were more abundant in the stems and leaves. Like the phenolic compounds, the flavonoids content was significantly affected by material source ($P < 0.05$) and plant organ ($P < 0.05$).

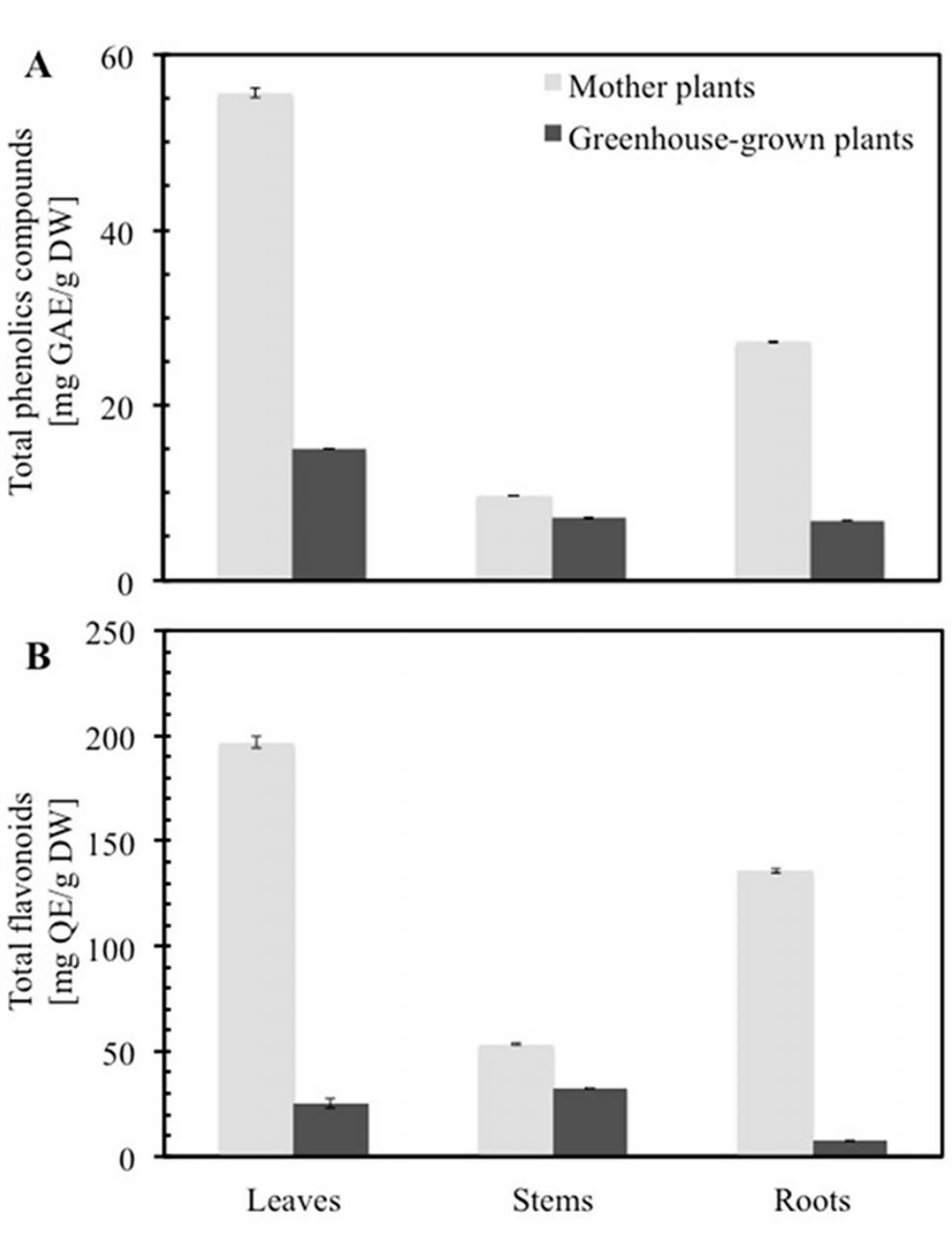


**Figure 2**
**Total phenolic compounds and flavonoids contents in different tissues of *Cuphea aequipetala* mother plants collected in Zempoala, State of Morelos (Mexico), and in propagated plants after 3 months of cultivation in the greenhouse.**

***Antioxidant properties***

In the DPPH assay, the free-radical scavenging activity of extracts from wild-grown plants ranged from 19.19 ± 0.1 to 169.33 ± 2.1 μmol trolox/g DW (Table 2). Leaf extracts showed the highest activity, followed by root extracts, and then stem extracts. In the ABTS assay, the activity of these same extracts ranged from 106.71 ± 0.3 to 494.37 ± 8.6 μmol trolox/g DW and the leaf extracts showed the highest activity. The greenhouse-grown plants had average DPPH activity between 21.86 ± 0.3 to 87.83 ± 0.8 μmol Trolox/g DW; leaf extracts showed the highest activity, followed by root extracts, and then stem extracts (Table 2). Average ABTS values ranged from 43.38 ± 0.1 to 119.50 ± 0.3 μmol Trolox/g DW; leaf and stem extracts had higher activity than root extracts. According to the statistical analysis, wild-grown plants showed higher free-radical scavenging activity against both DPPH ($P < 0.05$) and ABTS ($P < 0.05$) than greenhouse-grown plants, and activity differed among the various organs.

The average values for reducing power of extracts from wild-grown plants ranged from 93.11 ± 0.2 to 1186.25 ± 3.2 μmol trolox/g DW (Table 2). Leaf extracts showed the greatest reducing power, followed by root extracts, and then stem extracts. For the greenhouse-grown plants, leaf extracts showed the highest activity (329.47 ± 0.6 μmol trolox/g DW) and root extracts the lowest (89.10 ± 0.3 μmol trolox/g DW). Here also, both the source of the plant material ($P < 0.05$) and the organ ($P < 0.05$) significantly affected the reducing power of the extracts.

**Table 2**
**Free radical-scavenging activity and reducing power of extracts from different organs of wild and greenhouse-grown plants of *C. aequipetala*.**

| | Free radical scavenging (μmol trolox/g DW) | | Reducing power (μmol trolox/g DW) |
|---|---|---|---|
| | DPPH | ABTS | |
| Wild plants | | | |
| Leaves | $169.33 \pm 2.10^{A}$ | $494.37 \pm 8.6^{A}$ | $1186.25 \pm 3.2^{A}$ |
| Stems | $19.19 \pm 0.10^{E}$ | $106.71 \pm 0.3^{C}$ | $93.11 \pm 0.2^{E}$ |
| Roots | $85.62 \pm 0.48^{B}$ | $209.38 \pm 1.2^{B}$ | $231.04 \pm 0.4^{C}$ |
| Greenhouse-grown plants | | | |
| Leaves | $87.83 \pm 0.8^{B}$ | $119.50 \pm 0.3^{C}$ | $329.47 \pm 0.6^{B}$ |
| Stems | $21.86 \pm 0.3^{D}$ | $117.74 \pm 0.2^{C}$ | $111.56 \pm 0.4^{D}$ |
| Roots | $43.28 \pm 0.2^{C}$ | $43.38 \pm 0.1^{D}$ | $89.10 \pm 0.3^{E}$ |

**Values are mean (n = 3) ± standard error**
**Within each column, values with different capital letter are significantly different at $P<0.05$ (Tukey's test)**

***TLC bioautography analysis***

A TLC bioautography method using DPPH was performed to detect the components of *C. aequipetala* extracts with free-radical scavenging activity (Figure 3). This method enables rapid detection and localization of active compounds in a complex extract (Gu *et al.*, 2008). In this assay, the DPPH scavenging activity was observed as yellow spots on a purple background. The same stained TLC plates were inspected under UV light at 254 nm (Figure 3B) and 365 nm (Figure 3C) to reveal the profile of phenolic compounds and flavonoids in the extracts. Every part

of the wild-grown and greenhouse-grown plants showed at least one band with free-radical scavenging activity, as observed by bleaching of DPPH (data not shown). Figure 3A shows that the extract from leaves of greenhouse-grown plants resolved at least one band with antiradical activity, which was also observed at 254 (Figure 3B) and 365 nm (Figure 3C). The TLC profile of wild-grown and greenhouse-grown plants of *C. aequipetala* revealed blue and red fluorescent zones at 365 nm (Figure 3C) suggesting that DPPH scavenging is due to flavonoids (Wagner and Bladt, 1996). This was confirmed by the reaction of these bands with diphenylboric acid-β-ethylamino ester, giving yellow fluorescence. The extracts from wild-grown and greenhouse-grown plants showed a band with similar $R_F$ (0.60) to that of the flavonoid, quercetin 3-β-$_D$-glucoside.

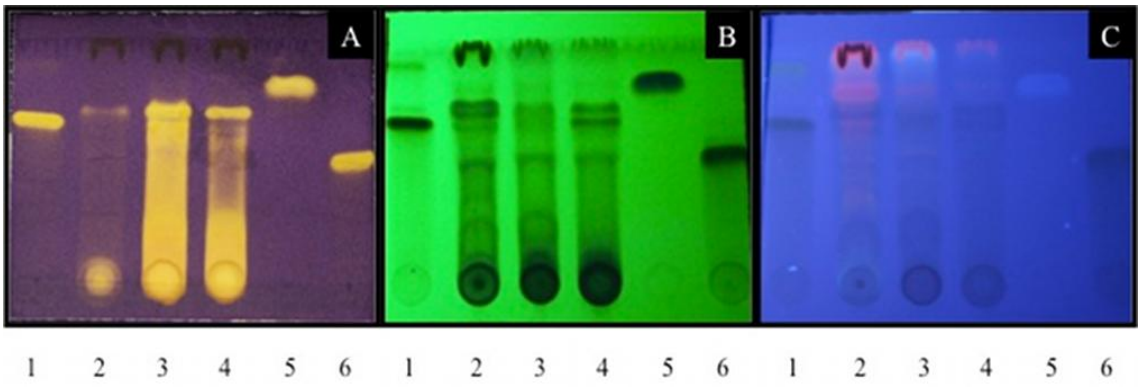


**Figure 3**

**TLC plates stained with a 0.01 mM DPPH• solution in methanol and visualized under visible light (A), under ultraviolet light at 254 nm (B), and 365 nm (C). An aliquot (18 μL) of extract (10 mg/mL) from leaves of *in vitro* plantlets (2), wild-grown (3) and greenhouse-grown (4) plants of *C. aequipetala* was applied to the TLC plate and separated with ethyl acetate:methanol:$H_2O$ (76:16:8, *v/v*). Standards: (1) quercetin 3-β-$_D$-glucoside; (5) ferulic acid; (6) quercetin 3-β-$_D$-rutinoside.**

***Correlation analysis***

To determine the relationship between the phytochemical content and the antioxidant capacity of the extracts, we performed correlation and regression analyses. Total phenol content was significantly ($P < 0.05$) positively correlated with radical scavenging activity based on the reduction of DPPH (r = 0.949) and ABTS (r = 0.982) as well as with reducing power (r = 0.952). Similarly, flavonoid concentration was significantly ($P < 0.05$) positively correlated with the scavenging activity against DPPH (r = 0.813) and ABTS (r = 0.926) and with reducing power (r = 0.828) (Figure 4).

**DISCUSSION**

In this study, we investigated the effects of BA and two auxins (IBA and 2,4-D, 2.5 μM) on callus and shoot induction from different explants (nodal segments, internodes, and leaves) of *Cuphea aequipetala* Cav. (Lythraceae). We developed an efficient protocol for acclimatization of *C. aequipetala* plants and investigated the antioxidant activity of extracts from different organs (roots, stems, and leaves) of wild-grown and greenhouse-grown plants of *C. aequipetala*. We also analyzed the correlation between antioxidant activity and the concentrations of phenolic compounds and flavonoids. We found that nodal segments were competent for callus and shoot induction whereas leaf and internode segments were competent for callus induction (Table 1). Morphogenic responses differ among explant types in other species of *Cuphea* (Rita and Floh, 1995; Millam *et al.*, 1997). For instance, the formation of callus was the only response from internodal sections of eight different species of *Cuphea* including *C. laminuligera, C. wrightii, C. lutea,* and *C. leptopoda.* For the latter three species, leaf explants showed high rates of shoot formation whereas *C. laminuligera* produced shoots

only from internodal tissues (Millam *et al.*, 1997). In the absence of PGRs, *C. aequipetala* nodal segments formed long shoots (8.5 mm in average), but with a poor proliferation rate (Table 1). The other explants formed longer shoots (up to 15 mm) but also with a poor proliferation rate. This may reflect the development of preexisting meristems into shoots (Singh and Chaturvedi, 2010; Garcia *et al.*, 2011). BA alone or combined with IBA induced shoot formation from nodal segments of *C. aequipetala* with a high proliferation rate. In contrast, no shoots formed on medium containing BA and 2,4-D. The highest callus induction rate was in leaf explants on medium containing BA and 2,4-D. Different effects of auxins on morphogenic responses have been reported elsewhere; for instance, nodes of *Eupatorium triplinerve* (Asteraceae) developed shoots (8.1 shoots per node) on MS medium containing BA (8.87 μM) and IBA (2.46 μM). There are few reports on *in vitro* culture of plants in the Lythraceae. Previously, using micro-cuttings from wild-grown *C. aequipetala*, we obtained shoot induction rates of 27% with yields of 8 to 10 shoots per explant (Salcedo *et al.*, 2009) on medium containing BA (1 μM) and IBA (0.5 μM). For *C. laminuligera*, the best results were obtained using leaf segments as explants on MS medium supplemented with BA (8.8 μM) and NAA (2.2 μM), yielding 12 shoots per leaf explant (Millam *et al.*, 1997). Similarly to *C. aequipetala*, the best callus induction in *C. wrightii* and *C. tolucana* was observed from leaf segments on MS medium containing BA (0.04 μM) plus 2,4-D (0.05 μM) (Przybecki *et al.*, 2001). For other closely related species such as *Lawsonia inermis* (Lythraceae), shoots were regenerated from apical and axillary meristems on medium containing only BA (1-4.4 μM), yielding 1 to 3 shoots per explant (Rout *et al.*, 2001). The reddish coloration of callus may be because of the accumulation of anthocyanins (Phippen, 2009). *Cuphea aequipetala* shoots developed roots *in vitro* in the same shoot induction medium (containing BA) before being transferred to potting mix, and the resulting plants successfully acclimatized and reached maturity. For *C. ericoides*, the survival rate was 20% when plants were grown in a mixture of sterile soil, sand, and vermiculite.

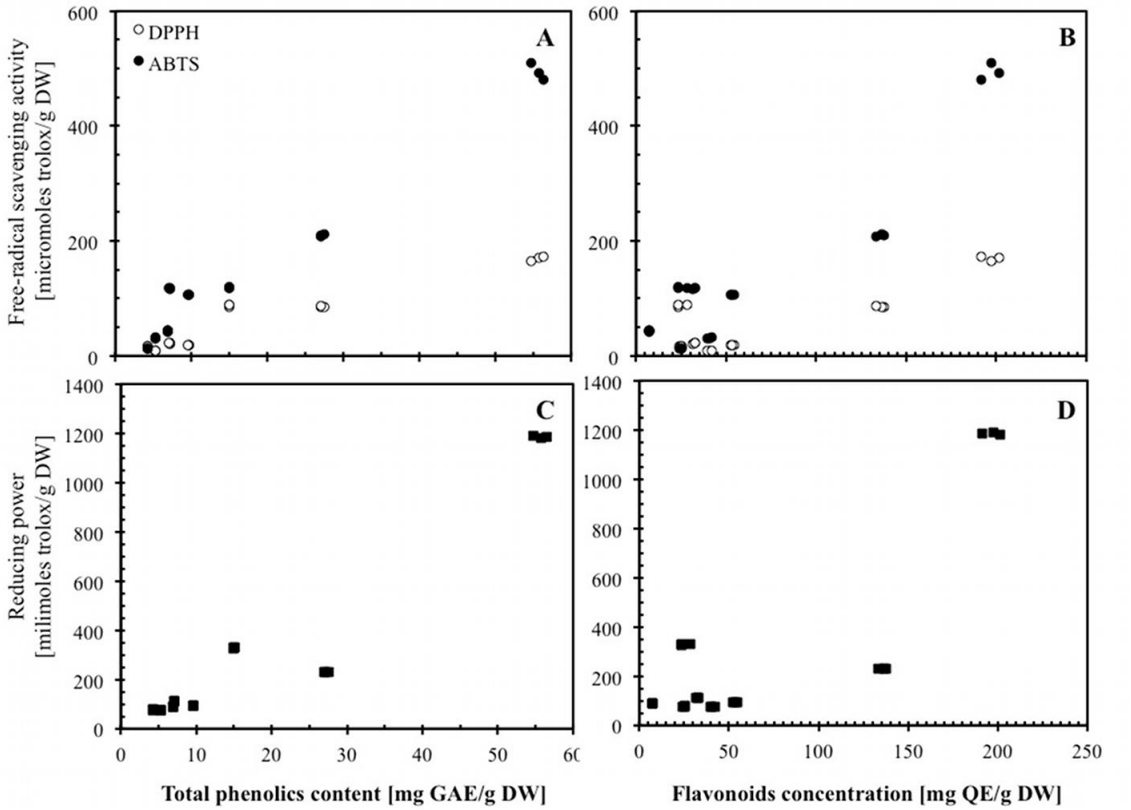


**Figure 4**
**Correlation between total phenolic and flavonoid contents, and antioxidant activity**

The concentrations of total phenolic compounds and flavonoids varied depending on the source of *C. aequipetala* (wild vs. greenhouse-grown plants) and the organ type (leaf, stem, or root) (Figure 2). Several reports show differences in the concentrations of phytochemicals that may be attributed to growth conditions such as nutrients, light (Mora-Izquierdo *et al.,* 2011), or harvest time (Aslam *et al.,* 2010). Furthermore, spatial distribution of phytochemicals has been reported elsewhere. For instance, in *Equisetum giganteum* (Equisetaceae), the concentrations of phenolic compounds, tannins, flavonoids, and hydroxycinnamic acids were higher in the lateral branches than in the main stems (Ricco *et al.,* 2011). The highest concentrations of total phenolic compounds and flavonoids found in the present study exceeded those reported for *C. carthaginensis* (Schuldt *et al.,* 2004) but were lower than those reported for other members of the Lythraceae used for medicinal purposes. Methanolic extracts from the bark of *Lafoensia pacari* (Lythraceae) contained 141 mg GAE/g dry matter (Solon *et al.,* 2000), whereas extracts from whole plants of *L. inermis* (Lythraceae) contained 238 - 310 mg GAE/g dry matter (Guha *et al.,* 2011).

The extracts of the different organs of wild and greenhouse-grown *C. aequipetala* showed free radical-scavenging activity against DPPH and ABTS as well as reducing power in the phosphomolybdenum assay (Table 2). For most of the samples, the values were higher in the ABTS assay than in the DPPH assay, and the highest values were in the phosphomolybdenum assay. These differences in the capacity of the extracts to scavenge ABTS/DPPH radicals and to reduce phosphomolybdenum are consistent with previous observations (Marwah *et al.,* 2007; Pasko *et al.,* 2009). The ability of extracts of *C. aequipetala* 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 *C. aequipetala* may be associated with its traditional use to treat conditions consistent with radical-related diseases (e.g., tumors). Phenolic compounds (e.g., phenolic acids, flavonoids, tannins) are considered as 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 donors, singlet-oxygen quenchers, and metal chelators (Rice-Evans and Miller, 1996; Vermerris and Nicholson, 2008). We found that the antioxidant activity of *C. aequipetala* (free radical-scavenging and reducing power) was strongly correlated with total phenolic and flavonoids contents (Figure 3). In related species such as *L. inermis* (Lythraceae), antioxidant activity was also found to correlate strongly with total polyphenol content (Guha *et al.,* 2011).

## CONCLUSIONS

This is the first report of a successful propagation procedure for *C. aequipetala,* offering a viable approach for long-term *in vitro* conservation and proliferation of this species. *C. aequipetala* shoots maintained their proliferation capacity during long-term subculture (3 years). They were able to successfully acclimatize and grow to maturity, and they retained their ability to accumulate antioxidants. The antioxidant properties of *C. aequipetala* may be associated with its traditional use to treat conditions consistent with radical-related diseases (e.g. tumors). Cultivated plants of *C. aequipetala* are an alternative to wild plants as a source of natural antioxidants.

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