SUPPLEMENTARY MATERIAL

***In vitro* antioxidant, antifungal, and antibacterial activities of five international *Calibrachoa* cultivars**

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Abstract

The total phenolic, flavonoid, and tannin contents in leaves extracts of *Calibrachoa x hybrida* (*C.h.*) (Solanaceae) international cultivars, as well as their overall antioxidant activities using DPPH and linoleic acid assays, were investigated. Furthermore, the antifungal and the antibacterial activities were examined against wide spectrum of microorganisms. DPPH and linoleic acid assays ranged from 62.1 to 80.1% and of 74.1 to 93.4%; respectively. *C. h.* Superbells[®] Trailing Rose (CHST), *C. h.* ‘Superbells[®] Frost Fire’ (CHSF), *C. h.* ‘Superbells[®] Strawberry Punch’ (CHSS), *C. h.* ‘ Superbells[®] Dreamsicle’ (CHSD), and *C. h.* ‘Superbells[®] Plum’ (CHSP) varied in their antifungal and the antibacterial activities against wide spectrum of microorganism. CHSP exhibited the highest antioxidant, antifungal and antibacterial activities followed by CHST. These activities might be attributed to the presence of phenolic, flavonoid and tannin compounds, indicating that these cultivars might be potential sources of therapeutic substances.

Keywords: *Calibrachoa*; antifungal; antibacterial; phenolics; antioxidants.

Experimental

1. Plant material and preparation of extracts

Leaf samples of *Calibrachoa x hybrida* cultivars of Superbells® Trailing Rose (CHST), *C. h.* ‘Superbells® Frost Fire’ (CHSF), ‘Superbells® Strawberry Punch’ (CHSS), ‘Superbells® Dreamsicle’ (CHSD), and ‘Superbells® Plum’ (CHSP) were collected in January 2014 from plants grown in a controlled greenhouse conditions located in Guelph, Ontario, Canada. All samples (5 of each case) were vouchered at the, Biodiversity Institute of Ontario, University of Guelph, Canada (voucher No. Hos00750-Hos00775). *Calibrachoa* extracts were obtained using methanol (Pérez-Tortosa et al. 2012) with some modifications (Elansary & Mahmoud, 2014). Dried leaves (0.25 gm) were ground then dissolved in 3 ml methanol (99%). The solution was shaken on a magnetic agitator at minimal speed, in dark condition, for 24 h at room temperature. The solution was centrifuged for 5 min (4°C) at 10000 RPM (7000 x g), the supernatant (~2.6 mL) was stored in sealed vials at -20°C. The preparation was subjected to rotary evaporator to produce semisolid extract that was kept for further analyses.

2. Chemicals and cell cultures

Analytical/HPLC grade chemicals were obtained from Sigma Aldrich, Canada. These fungi and bacteria were obtained from Henry farms Laboratories, Guelph, Canada.

3. Antioxidant capacity

The 2,2'-diphenylpicrylhydrazyl (DPPH) method was employed to determine the free radical scavenging activity of the samples (Tepe et al. 2005), the absorbance was measured at 517 nm, experiments were repeated twice in triplicate and the antioxidant activity was expressed as percentage. The β -carotene-linoleic acid assay was conducted using the method described by Tepe et al. (2005) with modifications (Elansary & Mahmoud, 2015). The mixture of β -carotene-linoleic acid was prepared by dissolving 0.5 mg β -carotene in 1 mL of chloroform, 25 μ L linoleic acid and 200 mg Tween 40. The chloroform was removed by vacuum evaporation, then 100 ml of distilled water saturated with oxygen were added (30 min 100 mL/min) and shaken vigorously. 2.5 mL of the mixture were mixed with 350 μ L of the liquid extracts, incubated for 48 h at room temperature and the absorbance was measured at 470 nm. The antioxidant activity was expressed as percentage. The BHT was used as positive control; also a blank was prepared in the same manner and the samples antioxidant capacities were compared to the BHT and the blank.

4. Total phenolic, flavonoids and tannins contents determination

The Folin-Ciocalteu colorimetric method using gallic acid as the reference was employed to determine the phenolic contents of leaf extracts, and the results was expressed as gallic acid equivalents (mg GAE / g ext.) (Singleton and Rossi, 1965; Amerine & Ough, 1988). Flavonoids determination followed the protocol of Miliauskas et al. (2004) by a pharmacopoeia method using rutin as a reference compound with modifications (Elansary & Mahmoud, 2015). Tannins were determined following the gravimetric method (Makkar et al. 1993) with modifications (Elansary & Mahmoud, 2015). All experiments were repeated twice in triplicates.

5. Antifungal activities

Seven fungi were used: *Aspergillus flavus* (ATCC 9643), *Aspergillus ochraceus* (ATCC 12066), *Aspergillus niger* (ATCC 6275), *Candida albicans* (ATCC 26555), *Penicillium funiculosum* (ATCC 56755), and *Penicillium ochrochloron* (ATCC 48663). The cultures were kept on malt agar at 4°C then subcultured every month. Spore suspension concentration of (1.0×10^5) was used and the minimum inhibitory (MIC) and minimum fungicidal (MFC) concentrations assays were performed using the microdilution method (Espinel-Ingroff 2001) by preparing a serial of dilutions in 96-well microtiter plates. Leaf extracts were diluted in 5% of DMSO (1 mg/mL and 10 mg/mL), then added to microplates containing broth Malt medium with inoculum and incubated for 72 h at 28°C on a rotary shaker. MIC was defined as the lowest concentration that inhibits fungi growth at the bionocular microscope level. MFC was defined as the lowest concentration with no visible growth indicating 99.5% killing of the original inoculum. MIC was determined by serial sub-cultivations of 2 µl of stem bark extract and isolated compounds dissolved in a medium. The sub-cultivations were incubated for 72 h in microtiter plates that contain 100 µl broth and inoculum, then incubated for 72 h at 28°C. Triplicates were used in all experiments and repeated each experiment twice. The fungicides fluconazole (FLZ) and ketoconazole (KLZ) were used as positive controls (1–3500 µg/ml).

6. Antibacterial activities

We performed experiments using Gram + and Gram – bacteria. The Gram-positive bacteria were *Bacillus cereus* (clinical isolate), *Staphylococcus aureus* (ATCC (American type culture collection) 6538) and *Micrococcus flavus* (ATCC 10240) and the Gram-negative bacteria were *Escherichia coli* (ATCC 35210), *Pseudomonas aeruginosa* (ATCC 27853), and *Enterobacter cloacae* (ATCC 35030). The microdilution method (Espinel-Ingroff 2001) was used to determine the minimum inhibitory (MIC)

and minimum bactericidal (MBC) concentrations. The bacterial suspensions were adjusted with sterile saline to a concentration of 1.0×10^5 CFU/mL and stored at 4°C. Inocula were screened for contamination by culturing on a solid medium. Stem bark extract and examined compounds were added (1 and 10 mg/mL) to 100 µl Tryptic Soy broth (TSB) with a bacteria inoculum (1.0×10^4 CFU per well), reaching the desired concentration in a microtitre plate to measure the MICs and MBCs. The mixtures in microplates were incubated for 24 h at 37 °C in a rotary shaker. The MIC was defined as the lowest concentrations without visible growth (at the binocular microscope). The MBC was defined as the lowest concentration showing no visible growth, indicating killing of 99.5% of the inoculum, and was determined by serial sub-cultivations of 2 µL into microtitre plates containing 100 µL of TSB for each well and incubation for 24 h. Using a microplate manager, the optical density was measured at 655 nm, and experiments were in triplicates and repeated three times. The results was compared to positive controls (streptomycin and ampicillin, 1 mg/mL), and negative control (5% DMSO).

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Table S1. *Calibrachoa* cultivars leaves methanolic extracts DPPH and β -carotene-linoleic acid assay results as well as total phenolics, flavonoids and tannins contents. Values are expressed as mean of triplicate determinations $\pm$ sd.

	DPPH free radical scavenging activity %	β -Carotene-linoleic acid assay %	Total phenolic content (mg GAE g ⁻¹ ext.)	Flavonoids (mg/ g ⁻¹ ext.)	Tannins (mg/ g ⁻¹ ext.)
<i>Calibrachoa</i> cultivars					
CHST	75.6 $\pm$ 0.5	87.3 $\pm$ 1.4	5.1 $\pm$ 0.3	0.8 $\pm$ 0.1	0.3 $\pm$ 0.04
CHSF	62.1 $\pm$ 0.6	74.1 $\pm$ 1.5	4.3 $\pm$ 0.8	0.5 $\pm$ 0.1	0.1 $\pm$ 0.01
CHSS	72.4 $\pm$ 0.7	83.2 $\pm$ 2.1	4.8 $\pm$ 0.7	0.6 $\pm$ 0.1	0.2 $\pm$ 0.01
CHSD	71.3 $\pm$ 0.1	83.1 $\pm$ 2.3	4.7 $\pm$ 1.2	0.6 $\pm$ 0.1	0.2 $\pm$ 0.02
CHSP	80.1 $\pm$ 0.5	93.4 $\pm$ 2.4	5.4 $\pm$ 0.5	0.9 $\pm$ 0.1	0.3 $\pm$ 0.01

Notes: *C. h.* Superbells[®] Trailing Rose (CHST), *C. h.* ‘Superbells[®] Frost Fire’ (CHSF), *C. h.* ‘Superbells[®] Strawberry Punch’ (CHSS), *C. h.* ‘ Superbells[®] Dreamsicle’ (CHSD), and *C. h.* ‘Superbells[®] Plum’ (CHSP).