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Conservation strategy for *Pelargonium sidoides* DC: Phenolic profile and pharmacological activity of acclimatized plants derived from tissue culture

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ABSTRACT

Ethnopharmacological relevance: *Pelargonium sidoides* DC (Geraniaceae), a popular medicinal plant used in folk medicine in the treatment of respiratory-related infections has gained international prominence due to its usage in several herbal formulations. This has led to high demand and the subsequent decimation of wild populations.
Aim of the study: Using plant tissue culture techniques, *Pelargonium sidoides* plants were cloned *in vitro*, acclimatized under greenhouse conditions and evaluated for their phytochemical content and pharmacological activity.
Methods: Phenolic content in extracts of *in vitro*-derived, greenhouse-acclimatized and wild *Pelargonium sidoides* plants were analyzed using UPLC-MS/MS. The oxygen radical absorbance capacity (ORAC), 2,2-diphenyl-1-picryl hydrazyl (DPPH) radical scavenging activity and minimum inhibitory concentration (MIC) of the extracts against bacterial and fungal strains were evaluated.
Results: Similarities in phenolic profiles were identified confirming the chemical signatures that characterize *Pelargonium sidoides* plants. Extracts of greenhouse-acclimatized and wild plants exhibited comparable antimicrobial and antioxidant properties.
Conclusions: Overall, the study highlights the potential of integrating plant tissue culture technologies in conservation strategies of medicinal plants. In particular, the results strongly suggest the feasibility of both large-scale cultivation and plant part substitution as alternative solutions to the current destructive overharvesting practices of wild *Pelargonium sidoides* populations.

1. Introduction

The growth of the complementary medicine system continues to escalate against the background of dwindling wild plant populations. The envisaged health benefits of natural medicinal remedies continue to stimulate more interest and demand, particularly in developed countries. However, harvesting of medicinal plants from the wild remains the main source of raw materials (Canter et al., 2005). This has generated debate on the feasibility of large scale cultivation of medicinal plants to supply the seemingly insatiable pharmaceutical market for natural plant-derived products. The pharmaceutical industry is faced with many challenges favouring the use of natural products instead of the conventional chemo-clinical drugs (Khateeb et al., 2012). Domestic cultivation of medicinal plants in controlled environments offers the opportunity to overcome challenges associated with variability and possible instability of bioactive compounds (Canter et al., 2005). However, seed propagation of some medicinal plant species may be limited by their inherent low seed viability and germination potential. In light of these limitations, biotechnology applications such as plant tissue culture are likely to play an important role in the mass propagation of genetically uniform clones with

Abbreviations: AAPH, 2,2′-Azobis(2-methylpropionamidine) dihydrochloride; ANOVA, Analysis of variance; DMRT, Duncan's multiple range test; DPPH, 2,2–Diphenyl-1-picryl hydrazyl; MemTR, 6-(-3-Methoxybenzylamino)-9-β-D-ribofuranosylpurine; MIC, Minimum inhibitory concentration; MS, Murashige and Skoog medium; ORAC, Oxygen radical absorbance capacity; PGR, Plant growth regulator; PPF, Photon flux density; TE, Trolox equivalents; UPLC, Ultra performance liquid chromatography.

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potential to produce highly consistent bioactive compounds (Albrecht et al., 2012). This is more important for endangered and/or rare plant species as well as those that are difficult to propagate using conventional methods.

Pelargonium sidoides DC (Family: Geraniaceae), a plant species predominantly found in the Eastern Cape Province of South Africa, is renowned for its pharmacological properties in treating tuberculosis, bronchitis, cough, fever and other respiratory-related ailments (Kayser and Kolodziej, 1997; Matthys et al., 2007; Brendler and van Wyk, 2008) as well as respiratory viral infections (Schnitzler et al., 2008; Michaelis et al., 2011; Tahan and Yaman, 2013). However, *Pelargonium sidoides*, classified as 'declining' on the Red Data list of South African plants (2009) is fast becoming endangered due to overharvesting of its tubers from wild populations for the production of phytopharmaceuticals for the local and expanding international export markets (Colling et al., 2010). In addition, the problem of low seed viability and germination of *Pelargonium sidoides* remains a major challenge (Lewu et al., 2006). Thus, the use of genetically uniform *in vitro*-derived propagules will be a vital component in large scale cultivation of *Pelargonium sidoides*. The pharmacological activity of *Pelargonium sidoides* is partly attributed to highly oxygenated coumarins (7-hydroxy-5, 6-di-methoxycoumarin; 6,8-dihydroxy-5,7-dimethoxycoumarin), gallic acid-derivatives, flavonoids, phenolic and hydroxycinnamic acid-derivatives (Kayser and Kolodziej, 1997; Kolodziej, 2007; Colling et al., 2010), which are the main constituents of commercial products such as Umckaloabo® (EPs®7630) and Linctagon-C™. These characteristic phytochemical signatures are ideal biomarkers in assessing the quality and quantity of cultivated medicinal plants and their standardization (Albrecht et al., 2012). The objectives of the current study were to evaluate the quality and quantity of phenolic constituents (as biomarkers) in acclimatized *Pelargonium sidoides* plants using ultra performance liquid chromatography (UPLC-MS/MS) analysis and to compare their respective pharmacological activity with that of wild plants. In addition, the phytochemical and pharmacological properties of aerial parts were compared to the predominantly used underground tubers for both acclimatized and wild plants.

2. Materials and methods

2.1. Chemicals

Trolox (vitamin E), fluorescein sodium salt, and 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) were purchased from Fluka® Analytical and Aldrich Chemistry, Steinheim, Germany. Benlate® (antifungal compound) and the agar bacteriological (gelling agent) were purchased from Du Pont de Nemours Int., South Africa and Oxoid Ltd., Basingstoke, Hampshire, England, respectively. Deuterium-labelled 4-hydroxybenzoic (2,3,5,6-D4) and salicylic (3,4,5,6-D4) acids were purchased from Cambridge Isotope Laboratories (Andover, MA, USA) while formic acid and methanol used for preparing mobile phases were from Merck (Darmstadt, Germany). *Meta*-methoxytopolin riboside [6-(-3-methoxybenzylamino)-9-β-D-ribofuranosylpurine (M*em*TR)] was provided by the Laboratory of Growth Regulators, Palacký University and Institute of Experimental Botany AS CR, Olomouc, Czech Republic. All chemicals used in the assays were of analytical grade.

2.2. Plant material, culture initiation and in vitro growth conditions

The primary explants were leaf petioles obtained from mature *Pelargonium sidoides* DC plants growing in the University of KwaZulu-Natal (UKZN) Botanical Gardens, Pietermaritzburg, South Africa but were originally obtained from Lesotho. A voucher specimen (Moyo 08 NU) was identified by Dr C.J. Potgieter and deposited in The Bews Herbarium, UKZN. Aseptic *in vitro* cultures were established from leaf petioles as previously described by Moyo et al. (2012). All experiments were done using shoot-tip explants maintenance culture that were continuously produced on a Murashige and Skoog (MS, 1962) basal medium supplemented with 1.0 μM M*em*TR.

Murashige and Skoog (1962) basal medium supplemented with vitamins, sucrose (30 g/l), myo-inositol (0.1 g/l) and plant growth regulators (PGRs) was used for all the experiments. The medium was adjusted to pH 5.8 with either 1.0 M KOH or HCl before adding the gelling agent (8 g/l agar) and autoclaving at 121 °C and 15 KPa for 20 min. Filter-sterilised ascorbic acid (180 mg/l) was added after autoclaving and cooling to approximately 50 °C. Cultures were maintained at a temperature of 25 ± 2 °C under a 16 h photoperiod. A constant photosynthetic photon flux density (PPF) of 40–50 μmol m^{-2} s^{-1} provided by cool white fluorescent lamps (Osram L 58W/640, Germany) was used.

2.3. Acclimatization of in vitro-derived plants

In vitro plants growing on multiplication medium supplemented with 1.0 μM M*em*TR were inoculated to PGR-free MS medium for the regeneration of rooted shoots. After 6 weeks the shoots were transferred and established *ex vitro* under greenhouse conditions in vermiculite:soil (1:1) medium. The acclimatization process included a 3-day transition in the misthouse. The *in vitro*-derived plants were successfully acclimatized under greenhouse conditions and harvested after 12 months for phytochemical and pharmacological analysis.

2.4. Preparation of plant extracts

Wild, greenhouse-acclimatized and tissue culture-derived plants were seperated into aerial and underground parts, freeze-dried and lypholized. The ground materials (100 mg/mL) were homogenized with 80% methanol in a 2 mL Eppendorf, using an oscillation ball mill (MM 301, Retsch, Haan, Germany) at a frequency of 27 l/s for 3 min. The resultant extracts were centrifuged for 10 min at 20 000 rpm and the supernatant was retained for subsequent analysis.

2.5. Ultra performance liquid chromatography (UPLC-MS/MS) analysis of phenolic acids

Phenolic acid compounds including protocatechuic acid, 4-hydroxybenzoic acid, vanillic acid, caffeic acid, *p*-coumaric acid and ferulic acid in tissue cultured, acclimatized and wild *Pelargonium sidoides* plant extracts were quantified using UPLC-MS/MS as described by Gruz et al. (2008). Deuterium-labelled internal standards were added to the extraction solvent prior to plant material homogenization. The supernatants were filtered through 0.45 μm nylon membrane filters (Alltech, Breda, Netherlands) and then analyzed using ACQUITY Ultra Performance LC™ (UPLC) system (Waters, Milford, MA, USA) linked to a Micromass Quattro micro™ API benchtop triple quadrupole mass spectrometer (Waters MS Technologies, Manchester, UK).

2.6. 2,2-Diphenyl-1-picryl hydrazyl (DPPH) radical scavenging activity

The DPPH assay for the determination of free radical scavenging activity was done as outlined by Moyo et al. (2010). Methanolic DPPH (100 μM) was prepared freshly before the assay. Decrease in the purple colouration of the reaction mixtures was read at 517 nm in a Cary 50 UV–vis spectrophotometer. Ascorbic acid was used as a standard antioxidant. The assay was done using