Saudi Journal of Biological Sciences (2016) xxx, xxx–xxx

King Saud University

Saudi Journal of Biological Sciences

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ORIGINAL ARTICLE

Phenolic acid content, antioxidant and cytotoxic activities of four *Kalanchoë* species

Anna Bogucka-Kocka [a], Christian Zidorn [b,1], Małgorzata Kasprzycka [a], Grażyna Szymczak [c], Katarzyna Szewczyk [a,*]

[a] *Chair and Department of Pharmaceutical Botany, Medical University of Lublin, 1 Chodźki Street, 20-093 Lublin, Poland*
[b] *Institut für Pharmazie der Universität Innsbruck, Abteilung Pharmakognosie, Innrain 80/82, A-6020 Innsbruck, Austria*
[c] *Botanical Garden, University of Maria Sklodowska-Curie, 3 Sławinkowska Street, 20-810 Lublin, Poland*

Received 8 August 2015; revised 6 December 2015; accepted 15 January 2016

KEYWORDS

Kalanchoe;
Antioxidant activity;
Cytotoxicity;
Phenolic acids;
LC–MS

Abstract Phenolic acid composition, antioxidant, and cytotoxic activities in leaves of four *Kalanchoe* (Crassulaceae) species were evaluated. Determination of phenolic acid contents were conducted by an optimized LC–ESI-MS/MS method. The results show that *Kalanchoe daigremontiana* Raym.-Hamet & H. Perrier (using ASE extraction) and *Kalanchoe pinnata* (Lam.) Pers. contain the highest amounts of phenolic acids, while *Kalanchoe nyikae* Engl. the lowest ones. Among phenolic acids ferulic, caffeic and protocatechuic acids were occurring in the highest quantities in the analysed species. The greatest amounts of ferulic and protocatechuic acids were found in *K. daigremontiana* and *K. pinnata*. Moreover, the antiradical and cytotoxic activities of *Kalanchoe* extracts were investigated. All tested extracts possessed antioxidant activity. The obtained IC_{50} values (μg/mL) ranged from 49.9 μg/mL to 1410 μg/mL, indicating a large variation of the activity of the analysed extracts. Cytotoxicity assays revealed dose-dependent effects in the cells lines tested. Only *K. pinnata* extract showed a high cytotoxicity against the H-9 human T cell line. Other extracts (*K. daigremontiana*, *Kalanchoe milloti*, *K. nyikae*) showed more pronounced cytotoxicity towards J45.01 cells (human acute lymphoblastic leukaemia T cells).

The present study demonstrated that *Kalanchoe* extracts have significant antioxidant and cytotoxic effects. This suggests that these species can be used as new sources of natural antioxidants and potential anticancer compounds.

* Corresponding author.
E-mail address: k.szewczyk@umlub.pl (K. Szewczyk).
[1] Current address: Istituto di Chimica Biomolecolare – Consiglio Nazionale delle Ricerche, Via Campi Flegrei, 34, 80078 Pozzuoli (NA), Italy.

Peer review under responsibility of King Saud University.

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1. Introduction

A growing amount of research in biology and medicine is being devoted to reactive oxygen species (ROS). There is now considerable evidence that ROS induce oxidative damage in biomolecules. This damage causes cancer and several other diseases. Some of the relevant ROS are hydroxyl (·OH), superoxide anion ($O_2^{\cdot-}$), peroxyl (ROO·), alkoxy (RO·), hydrogen

http://dx.doi.org/10.1016/j.sjbs.2016.01.037
1319-562X

peroxide (H_2O_2), and hypochloride (HOCl). Besides these, reactive nitrogen species like nitric oxide ('NO) are also important. Antioxidants, which scavenge free radicals, are known to play important roles in preventing reactive species-induced diseases (Halliwell et al., 1995).

The genus *Kalanchoe* (Crassulaceae) encompasses succulent perennial plants. The species of *Kalanchoe* are mainly found in Madagascar, South and East Africa, Arabia and South-East Asia, tropical America, and Australia (Asiedu-Gyekye et al., 2012; Sharker et al., 2012). Many species have been used as medicinal plants and were used by many ethnicities to treat a variety of illnesses. In traditional medicine, *Kalanchoe* species have been used to treat ailments such as infections, rheumatism, and inflammation (Nayak et al., 2010). Moreover, these plants are used for the treatment of earache, burns, ulcers, diarrhoea, and insect bites (Okwu and Nnamdi, 2011). The best known representative of the genus is *Kalanchoe pinnata* [syn. *Bryophyllum pinnatum* (Lam.) Kurz]. *K. pinnata* contains polyphenolic compounds such as flavonoids and phenolic acids (e.g. *p*-hydroxycinnamic, caffeic, *p*-coumaric, ferulic, *p*-hydroxybenzoic, protocatechuic acids, quercetin, kaempferol, luteolin, astragalin, rutin, and patuletin) (Asiedu-Gyekye et al., 2012; Mohan et al., 2012; Muzitano et al., 2006). This species showed various pharmacological activities such as antidiabetic (Ojewole, 2005), antibacterial (Okwu and Nnamdi, 2011; Sharker et al., 2012; Quazi et al., 2011), antiinflammatory (Ojewole, 2005), anticonvulsant (Nguelefack et al., 2006), antioxidant (Harlalka et al., 2007; Mohan et al., 2012; Sharker et al., 2012), antinociceptive (Ojewole, 2005), hepatoprotective (Yadav and Dixit, 2003), antitumour (Supratman et al., 2001), and nephroprotective activities (Harlalka et al., 2007).

Most of the antioxidant potential in plants is due to the redox properties of phenolic compounds which act as reducing agents, hydrogen donators, and singlet oxygen quenchers. Antioxidant activity of polyphenols is exerted through various mechanisms. These compounds act as reducing agents, have an ability to scavenge free radicals and chelate metal ions, act as cofactors of enzymes catalysing oxidative reactions, inhibit oxidases, terminate radical chain reactions, and stabilize free radicals (Rice-Evans et al., 1997; Szewczyk and Zidorn, 2013).

Many medicinal herbs, such as *Kalanchoe* species exhibiting significant antioxidant activities have been employed as natural antioxidants. The effectiveness of plant extracts and natural compounds of high antioxidant activity in the prevention of many cancer types is well documented but the use of antioxidant agents in adjunctive cancer therapy is still controversial because of conflicting findings (Johnson, 2001).

The aim of this paper was to verify the cytotoxicity of *Kalanchoe* extracts against various tumour cell lines and to evaluate the antioxidant activity of extracts prepared from all species investigated. In addition, phenolic acids in the studied species were quantified by LC–MS/MS.

2. Materials and methods

2.1. Materials and chemicals

The leaves of *Kalanchoe daigremontiana* Raym.-Hamet & H. Perrier, *K. pinnata* (Lam.) Pers., *Kalanchoe milloti* Raym.-Hamlet & H. Perrier, and *Kalanchoe nyikae* Engl. (Crassulaceae) were used. The plant material was collected from the glasshouse of the Botanical Garden of Maria Curie-Skłodowska University (coordinates N 51°16′; E 22°30′) in Lublin, in May 2010. Voucher specimens (KD-0510; KP-0510; KM-0510; KN-0510) were deposited in the Department of Pharmaceutical Botany, Faculty of Pharmacy, Medical University of Lublin. The identity of plants was confirmed by Dr. Mykhaylo Chernetskyy, Botanical Garden, University of Maria Sklodowska-Curie, Lublin, Poland (Descoings, 2003; Chernetskyy, 2007, 2012).

All standards were purchased from Sigma Aldrich (Steinheim, Germany). HPLC-grade methanol, acetonitrile, water, acetic acid, and ammonium acetate were purchased from J.T. Baker (Netherlands). 1,1-Diphenyl, 2-picryl hydrazyl (DPPH), beta-nicotinamide adenine dinucleotide (NADH), phenazine methosulphate (PMS), nitroblue tetrazolium chloride (NBT), sulphanilamide, phosphoric acid, naphthylethylenediamine, dimethyl sulphoxide (DMSO), and sodium nitroprusside (SNP) were obtained from Sigma–Aldrich (St. Louis, MO). Other chemicals used for preparation of the extracts were of analytical grade, and obtained from Polish Reagents (POCH, Gliwice, Poland).

2.2. Cell lines and culture medium

Human cell lines – H-9 (human T cell from the European Collection of Cell Cultures, ECACC cat. No. 85050301) and J45.01 (human acute lymphoblastic leukaemia T cell, cat. No. 93031145 by ECACC) grown in aggregates in suspension, were cultured according to ECACC protocols in 24-well plates, growth area 2 cm^2 (Becton, Dickinson & Company) at a concentration of 1×10^6 cells/mL. All cultures were incubated in humidified atmosphere supplemented with 5% CO_2, for 24 h at 37 °C in an incubator (Biotech). The growing medium consisted of: RPMI 1640 medium, 10% heat-inactivated foetal calf, 2 mM glutamine and antibiotics [penicillin in a concentration of 100 U/mL, streptomycin in a concentration of 100 μM/mL, and amphotericin B in a concentration of 2.5 μg/mL (Gibco, Carlsbad, USA)]. One day after seeding, the cells were exposed to the examined ethanol extracts; the final concentration of ethanol was thereby reduced to 1% in the assays. This concentration of ethanol did not affect cell viability. All tests were performed in triplicate. Cells were observed using a BX41 Olympus light/fluorescence microscope. Data were processed employing the MultiScan software.

2.3. Accelerated solvent extraction (ASE) (K. daigremontiana; KDA)

100 g of fresh leaves of *K. daigremontiana* were homogenized with diatomaceous earth in a ratio of 1:2. The plant material was placed in the stainless-steel cell of a Dionex (UK) ASE 200 accelerated solvent extractor, and extracted with 70% ethanol. When the sample cells were loaded into the carousel of the ASE 200 system [Dionex (UK)], extractions were performed by filling the cell with solvent before heating (pre-fill method). For performing extraction, approximately 15–30 mL of solvent was used. Extraction was performed at 100 bar and at 40 °C, for 10 min (1 cycle). The obtained extracts were concentrated under reduced pressure, dissolved in small portion of HPLC-grade water (to DPPH assay),