



King Saud University

Saudi Journal of Biological Sciences

www.ksu.edu.sa
www.sciencedirect.com


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 Skłodowska-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 IC₅₀ 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.

© 2016 The Authors. Production and hosting by Elsevier B.V. on behalf of King Saud University. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

* 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.



Production and hosting by Elsevier

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 (O₂⁻), peroxy (ROO·), alkoxy (RO·), hydrogen

<http://dx.doi.org/10.1016/j.sjbs.2016.01.037>

1319-562X © 2016 The Authors. Production and hosting by Elsevier B.V. on behalf of King Saud University.

This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Please cite this article in press as: Bogucka-Kocka, A. et al., Phenolic acid content, antioxidant and cytotoxic activities of four *Kalanchoë* species. Saudi Journal of Biological Sciences (2016), <http://dx.doi.org/10.1016/j.sjbs.2016.01.037>

peroxide (H₂O₂), 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), anti-inflammatory (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 donors, 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.-Hamet & H. Perrier, and *Kalanchoe nyikae* Engl. (Crassu-

laceae) 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 Chernetsky, Botanical Garden, University of Maria Skłodowska-Curie, Lublin, Poland (Descouings, 2003; Chernetsky, 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 cm² (Becton, Dickinson & Company) at a concentration of 1 × 10⁶ cells/mL. All cultures were incubated in humidified atmosphere supplemented with 5% CO₂, 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 µg/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),

phosphate buffer, pH 7.4 (to superoxide and nitric oxide scavenging assays) or ethanol (to cytotoxic and LC–MS/MS assays), and transferred to a 10 mL graduated flask.

2.4. Maceration (*K. daigremontiana* (KDM), *K. pinnata* (KPM), *K. milloti* (KMM), *K. nyikae* (KNM))

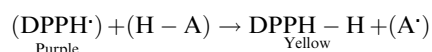
100 g of fresh plant materials were homogenized with 100 mL of 70% ethanol, and then transferred quantitatively to a beaker, rinsing the homogenizer three times with 100, 50 and 30 mL of 96% ethanol. The macerates were placed on a shaker for 60 min and then filtered through hard filter paper. Residues in the filter paper were extracted with 400 mL of 96% ethanol and shaken on a shaker for 3 h, then filtered. Maceration was carried out once again, using 400 mL of 96% ethanol. Collected extracts were concentrated using a vacuum evaporator to dryness. The residues were dissolved in HPLC-grade water (to DPPH assay), phosphate buffer, pH 7.4 (to superoxide and nitric oxide scavenging assays) or ethanol (to cytotoxic and LC–MS/MS assays), and transferred to a 10 mL graduated flask.

2.5. Superoxide radical scavenging activity

Antiradical activity was determined spectrophotometrically in a Multiskan Ascent plate reader, by monitoring the effect of the extracts on the reduction of NBT to the blue chromogen formazan induced by O_2^- , at 560 nm. Superoxide radicals were generated by the NADH/PMS system according to a described procedure (Fernandes et al., 1999). All components were dissolved in phosphate buffer 19 mM, pH 7.4.

2.6. DPPH radical scavenging activity

The scavenging reaction between (DPPH \cdot) and an antioxidant (H–A) can be written as:



The antiradical activity of the extracts was determined spectrophotometrically in a Multiskan Ascent plate reader, by monitoring the disappearance of DPPH \cdot at 515 nm, according to a published procedure (Silva et al., 2004). The reaction mixtures in the sample wells consisted of 25 μ L of extract and 200 μ L of DPPH, dissolved in methanol. The reaction was conducted at room temperature.

2.7. Nitric oxide (NO \cdot)-scavenging activity

The ability of the extracts to scavenge nitric oxide radical was determined spectrophotometrically in a Multiskan Ascent plate reader according to a described procedure (Sumanont et al., 2004), with some modifications. The reaction mixtures in the sample wells consisted of extract and SNP dissolved in saline phosphate buffer, pH 7.4. The plates were incubated at 25 °C for 60 min under light. Afterwards, Griess reagent (1% sulphanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2% H_3PO_4) was added and the absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthylethylenediamine was measured at 540 nm.

2.8. Trypan blue assay

In vitro cytotoxicity was assessed using the trypan blue assay. Cell lines in concentrations of 1×10^6 cells/mL were treated with different concentrations of testing extracts and incubated 24 h at 37 °C in humidified atmosphere supplemented with 5% CO_2 . At the end of this period, the medium from each plate was removed by aspiration. Next, the cells were washed with PBS and centrifuged at 800 rpm for 10 min, and then PBS was removed by aspiration. Then, 10 μ L of cell suspension were incubated for 5 min with the 10 μ L of 0.4% trypan blue solution (Bio-Rad) (Hafid-Medheb et al., 2003). Thereafter, in TC20TM Cell Automated Counter (Bio-Rad) cells were counted.

The assessment of life span was carried out using trypan blue dye which selectively migrates to the cytosol of cells, the membranes of which, due to occurring changes, become permeable for this dye (dead or dying cells). Cells which are not dyed are regarded as alive and properly metabolising.

The TC20TM Cell Automated Counter uses multi-facial plane analysis to assess cell viability. Upon insertion of a counting slide each cell was analysed using images acquired from multiple focal planes during the focusing step. The cell viability was assessed via trypan blue exclusion. Each combination of the experiment was repeated in triplicate, and the IC_{50} was determined for each sample.

2.9. LC–ESI–MS/MS analysis of phenolic acids

The amount of phenolic acids was determined by LC–ESI–MS/MS (electrospray ionization mass spectrometry). For this purpose an Agilent 1200 Series HPLC system (Agilent Technologies, USA) equipped with a binary solvent pump, an autosampler, a degasser, and a column oven connected to a 3200 QTRAP Mass spectrometer (ABSciex, USA) was used. Chromatographic separations were carried out at 25 °C on a Zorbax SB-C18 column (2.1 \times 50 mm, 1.8- μ m particle size; Agilent Technologies, USA). The binary mobile phase consisted of solvent A [10 mM ammonium acetate in acetonitrile–water–acetic acid 95:3:2 (v/v/v), pH4.7] and B [10 mM ammonium acetate in acetonitrile–water–acetic acid 50:48:2 (v/v/v), pH4.7]. Injection volume was 10 μ L. The flow rate was 300 μ L/min and the gradient was as follows: 0–1.0 min – 10% B; 5–12 min – 30% B; 13–15 min – 95%; 15.1–18 min – 10% B.

The QTRAP–MS system was equipped with electrospray ionization source (ESI) operated in the negative-ion mode. ESI conditions: capillary temperature 600 °C, curtain gas at 0.17 MPa, nebulizer gas at 0.41 MPa, negative ionization mode source voltage –4500 V. Nitrogen was used as curtain and collision gas. Negative ion mode was performed with multiple-reaction-monitoring (MRM). The data were acquired and processed using Analyst 1.5 software (ABSciex, USA). The analytes were identified by comparing the retention times and m/z values obtained by MS and MS/MS with the mass spectra from corresponding standards tested under the same conditions. The calibration curves obtained in the MRM mode were used for quantification of all analytes. The identified compounds were quantified on the basis of their peak areas and comparison with a calibration curve obtained with the corresponding standards. Linearity ranges for calibration

curves were specified. The limit of detection (LOD) and quantification (LOQ) for phenolic acids were determined at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a dilution series of known concentrations.

3. Results and discussion

For all tested fractions, IC_{50} ($\mu\text{g/mL}$) values were determined, indicating the concentration of each fraction required to reduce 50% of the radicals under the test conditions or causing mortality of half of the cells in culture. Based on the obtained values, all tested fractions possessed antioxidant activity. The obtained IC_{50} values ($\mu\text{g/mL}$) ranged from $51.3 \pm 0.6 \mu\text{g/mL}$ to $1457 \pm 8 \mu\text{g/mL}$ (Tab. Tab1), indicating a large variation in the activity of the analysed extracts. Not surprisingly, all *Kalanchoe* extracts were capable of scavenging DPPH \cdot radicals to some extent. From the estimated IC_{50} values, *K. milloti* extract emerged as the most potent scavenger, followed by *K. pinnata* > *K. daigremontiana* (maceration) > *K. nyikae* > *K. daigremontiana* ASE (Fig. 1). Moreover, all extracts

showed antioxidant potential against nitric oxide. The most active extract was that obtained from *K. pinnata*, followed by *K. milloti* > *K. daigremontiana* (maceration) > *K. nyikae* > *K. daigremontiana* (ASE) (Fig. 2). Protective capacity against superoxide was established for extracts from all species, but the most pronounced effect was observed for the extract obtained from *K. pinnata* ($IC_{50} = 51.3 \pm 0.6 \mu\text{g/mL}$), followed by *K. daigremontiana* (maceration) > *K. daigremontiana* (ASE) > *K. milloti* > *K. nyikae* (Fig. 3).

Antioxidant properties of various species of *Kalanchoe* had been reported in previous studies, but to the best of our knowledge none of these studies also covered the species analysed in the present paper (except from *K. pinnata*) (Asiedu-Gyekye et al., 2012; Bhatti et al., 2012; Harlalka et al., 2007; Mohan et al., 2012; Sharma et al., 2014). For example, the antioxidant and radical scavenging activities of *K. pinnata* aqueous leaf extract were evaluated *in vitro* (DPPH, nitric oxide, and lipid peroxidation) (Harlalka et al., 2007). The study revealed that *K. pinnata* extract possesses significant antioxidant and oxidative radical scavenging activities. In the DPPH method EC_{50}

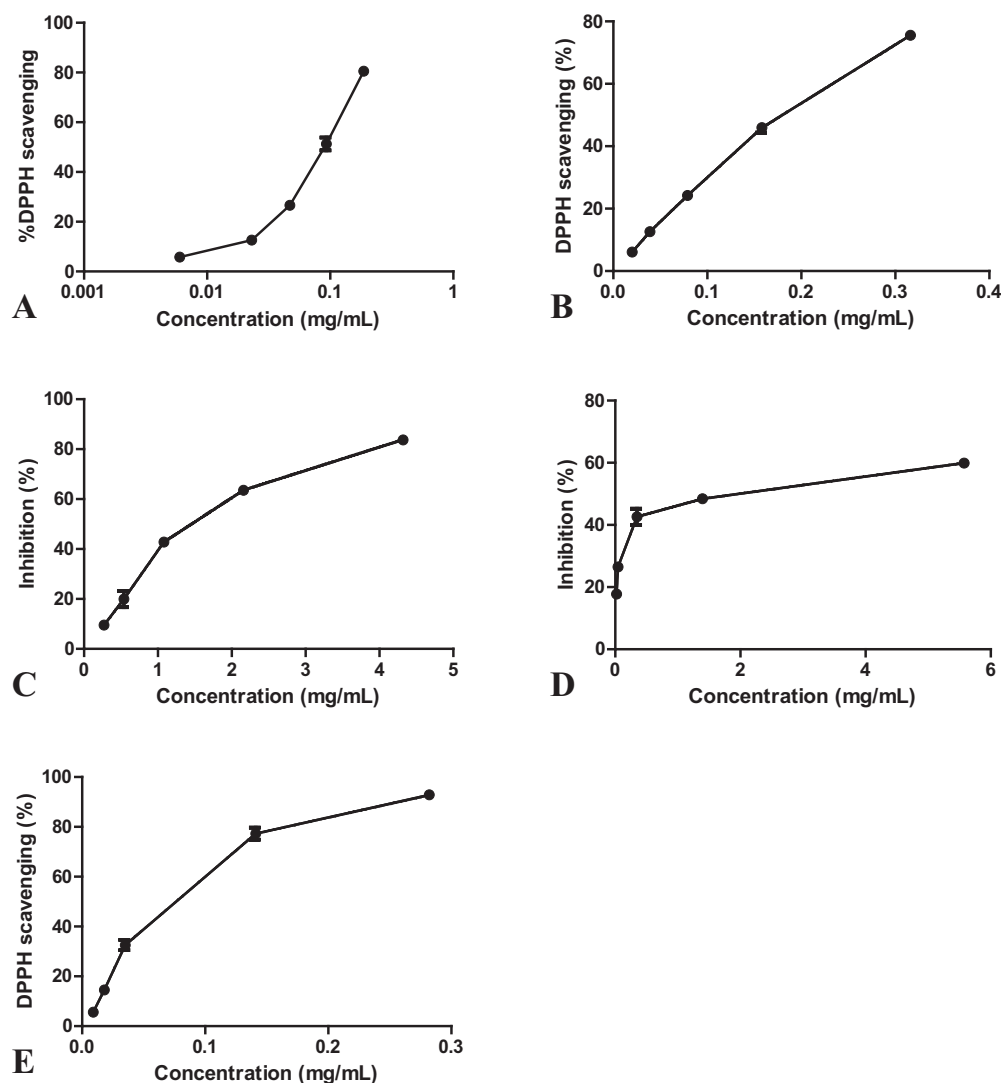


Figure 1 Effect of examined extract on DPPH reduction. Values show mean \pm SE from 3 experiments performed in triplicate. (A) *K. pinnata*, (B) *K. daigremontiana* ASE, (C) *K. daigremontiana* maceration, (D) *K. nyikae*, (E) *K. milloti*.

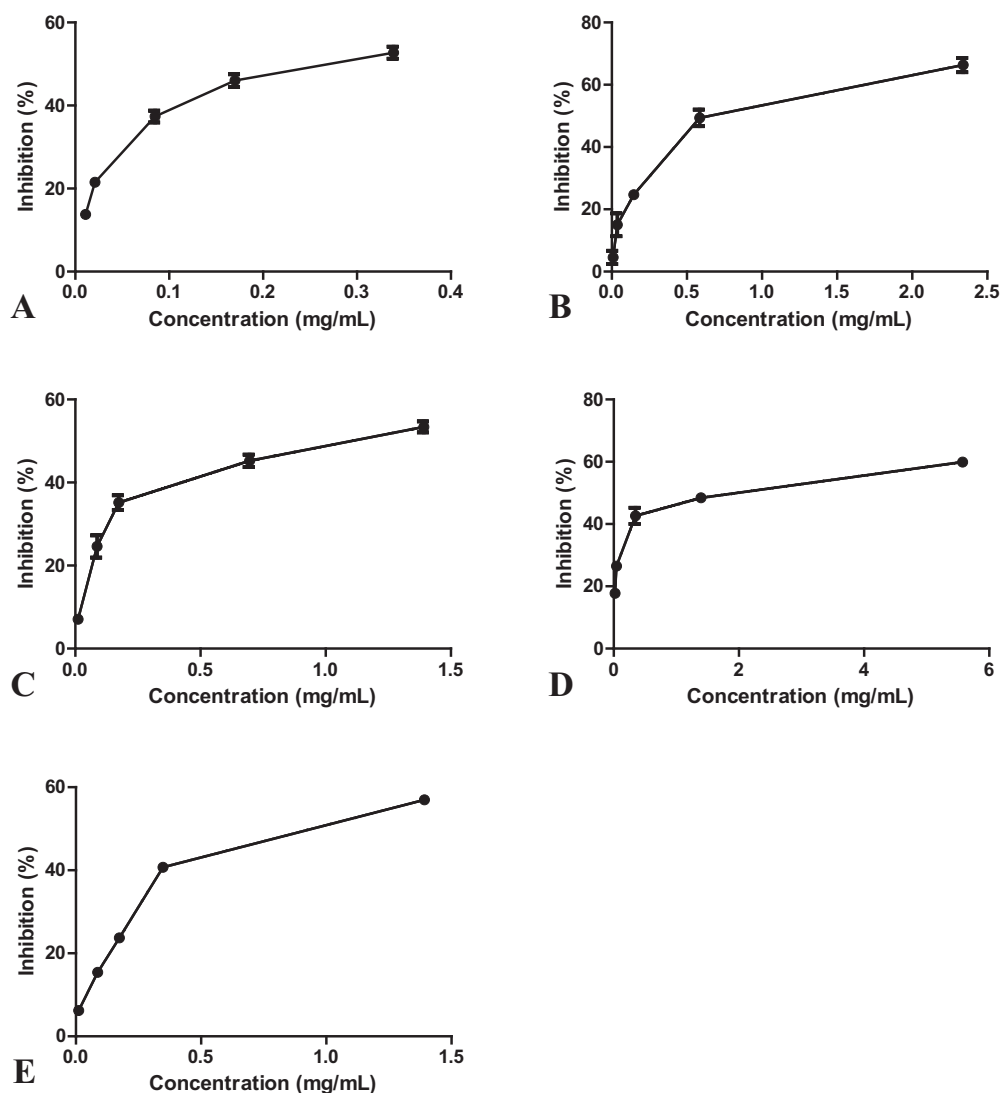


Figure 2 Antioxidant activity of examined extract against nitric oxide. Values show mean \pm SE from 3 experiments performed in triplicate. (A) *K. pinnata*, (B) *K. daigremontiana* ASE, (C) *K. daigremontiana* maceration, (D) *K. nyikae*, (E) *K. milloti*.

value for leaf extract was found to be 116.25 $\mu\text{g/mL}$ and 90 $\mu\text{g/mL}$ for nitric oxide radical – scavenging activity. The result in the DPPH assay is comparable to that obtained in our studies (90.6 $\mu\text{g/mL}$). Lai et al. (2011) tested the methanolic extract and fractions of the stems of *Kalanchoe gracilis* for their antioxidant capacity using the DPPH and the reducing power assays. The study revealed that chloroform fractions of *K. gracilis* exhibited good antioxidant activities ($\text{IC}_{50} = 0.64 \pm 0.08 \text{ mg/mL}$) and this result was comparable to that obtained in our study for methanol extract of *K. milloti*.

The KPM fraction had the most potent cytotoxic activity towards cells of both cell lines tested. In contrast, the KNM fraction showed the weakest activity against the J45.01 cell line. The KDM fraction had the weakest cytotoxic activity to cells of normal lymphocytes of the H9 line. All obtained results for cytotoxicity are summarized in Table 1.

The obtained results show that the KDA fraction had similar cytotoxic effect on the cells of the both investigated cell lines. Mortality of cells of the J45.01 and H9 cell lines was similar at the three mid-range concentrations. However, at the

highest concentration of the KDA fraction, the mortality of cells of J45.01 cell line increased and was about 20% higher than the mortality of cells of the H9 cell line. The mortality of the J45.01 cell line exposed to the tested range of concentrations of the KDM fraction was in the range from 25% to 80%. In the case of the H9 cell line, mortality was in the range of 35–50%. Higher concentrations also resulted in a stronger cytotoxicity towards the J45.01 line. Of all the studied fractions, the KDM fraction showed the weakest cytotoxic activity against normal lymphocyte cell line H9 ($\text{IC}_{50} = 957 \mu\text{g/mL}$), a fact which would be beneficial in the treatment of cancer.

Based on the obtained results, the sensitivity of cell lines was in a similar range but with a slightly higher cytotoxicity towards the J45.01 cell line. The average difference in mortality against the different cell lines was 7.34%. The obtained results showed large differences in cytotoxic activity towards the cell lines tested. In the tested concentration range, the mortality of cell line H9 was after exposure the KNM fraction in the range between 21% and 30%, while the mortality of the J45.01 cell line was in the range from 42% to 54%. This

fraction showed the weakest cytotoxic activity against cell line J45.01 among all studied fractions ($IC_{50} = 846 \mu\text{g/mL}$) and an equally weak cytotoxic activity against the normal lymphocyte H9 cell line ($IC_{50} = 508 \mu\text{g/mL}$). The results also revealed the

relatively high cytotoxic activity of the KPM fraction as compared to the other tested extracts. Based on IC_{50} values, this fraction showed the strongest cytotoxic activity of all tested fractions against both cell lines tested – H9 ($IC_{50} = 116 \mu\text{g/mL}$)

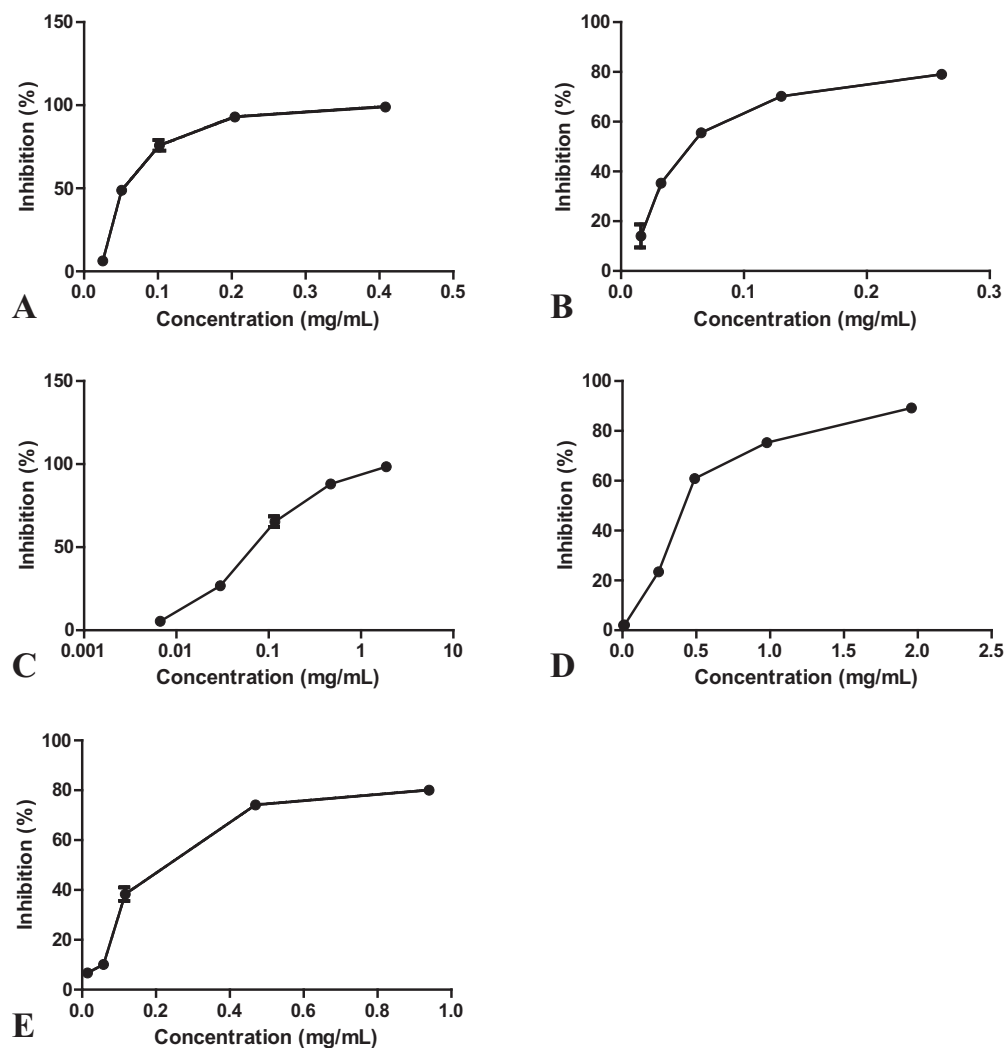


Figure 3 Effect of the examined extract on NBT reduction induced by superoxide radical generated in a NADH/PMS system. Values show mean \pm SE from 3 experiments performed in triplicate. (A) *K. pinnata*, (B) *K. daigremontiana* ASE, (C) *K. daigremontiana* maceration, (D) *K. nyikae*, (E) *K. milloti*.

Table 1 Comparison of inhibitory concentration (IC_{50}) values for each plant extract; expressed in $\mu\text{g/mL}$.

Species	IC_{50} ($\mu\text{g/mL}$)				
	DPPH	Superoxide	Nitric oxide	H9	J45
<i>K. daigremontiana</i> ASE	1457 \pm 8	72.6 \pm 1.5	1102 \pm 7	844.5	712.5
<i>K. daigremontiana</i> maceration	180 \pm 1	56.2 \pm 0.8	650 \pm 3	956.5	359.4
<i>K. pinnata</i>	90.6 \pm 1.1	51.3 \pm 0.6	270 \pm 1	115.8	264.8
<i>K. milloti</i>	61.5 \pm 1.0	213 \pm 1	586 \pm 2	560.5	503.5
<i>K. nyikae</i>	341 \pm 2	419 \pm 2	974 \pm 3	507.6	846.1

The colour values mean the most active extracts.

Table 2 LC–ESI–MS/MS analytical results of phenolic acids investigated in *Kalanchoe* extracts, including retention times, mass-to-charge ratio (m/z) and fragments obtained with given collision energy. Compounds confirmed by comparison with authentic standards.

Compound	Peak No.	T_R (min)	m/z experimental	Fragments	Collision energy (eV)
Gallic acid	1	0.31	168.8	125	–14
				78.8	–34
Chlorogenic acid	2	0.41	352.8	190.9	–22
				85	–56
γ -Resorcylic acid	3	0.55	152.9	109.1	–12
<i>p</i> -Coumaric acid	4	1.13	162.8	119	–16
				93	–48
Synapic acid	5	1.39	222.8	148.9	–20
				120.9	–44
Ferulic acid	6	1.5	192.8	177.9	–12
				133.9	–16
Caffeic acid	7	4.39	178.7	134.9	–16
				88.9	–14
Vanillic acid	8	5.38	166.8	122.9	–12
				107.9	–20
β -Rosorcylic acid	9	5.48	153	108.9	–12
Syringic acid	10	6.37	196.8	182	–12
				122.9	–24
Protocatechuic acid	13	7.79	152.9	107.8	–36
				80.9	–14

Table 3 Analytic parameters of LC–MS/MS quantitative method; data for calibration curves, limit of detection (LOD) and limit of quantification (LOQ) values for each analysed phenolic acids.

Compound	LOD [ng/mL]	LOQ [ng/mL]	R^2	Linearity range [ng/mL]
Gallic acid	50	100	1.000	100–10,000
Chlorogenic acid	100	200	0.9986	200–50,000
γ -Resorcylic acid	5	10	1.000	25–25,000
<i>p</i> -Coumaric acid	10	25	0.9982	50–2500
Synapic acid	7	25	0.9993	25–5000
Ferulic acid	10	25	0.9997	25–5000
Caffeic acid	50	100	0.9957	100–5000
Vanillic acid	100	200	0.9972	200–50,000
β -Rosorcylic acid	10	20	0.9991	20–700
Syringic acid	50	100	0.9973	100–50,000
Protocatechuic acid	10	20	1.000	25–25,000

and J45.01 ($IC_{50} = 265 \mu\text{g/mL}$). KPM is the only one fraction among studied extracts which was characterized by a pronounced cytotoxicity against the H9 cell line.

In the course of our studies, it was found that all extracts induce dose – dependent apoptosis in cells lines tested. Only the extract obtained from *K. pinnata* showed a greater cytotoxicity towards the H9 line (a clone of normal human T lymphocytes). Other extracts (*K. nyikae*, *K. daigremontiana* (maceration), *K. daigremontiana* (ASE), and *K. milloti*) showed more pronounced cytotoxicity towards the J45.01 line, a human acute lymphoblastic leukaemia T cell line. The present study showed a positive correlation between antioxidant and

cytotoxic activities of the fractions of *K. pinnata*, the extract for which the lowest IC_{50} values were obtained.

To the best of our knowledge, this is the first study conducted of the cytotoxic effects on the human cell lines – H-9 and J45.01 for extracts of *K. daigremontiana*, *K. milloti*, *K. nyikae*, and *K. pinnata*. Earlier studies on different *Kalanchoe* species reported cytotoxic effects of bufadienolides from leaves of *K. pinnata* and *K. daigremontiana* \times *tubiflora* against Epstein–Barr virus early antigen (EBV-EA) activation in Raji cells induced by the tumour promoter 12-O-tetradecanoylphorbol-13-acetate (Supratman et al., 2001). Extracts of stems of *K. gracilis* (Lai et al., 2011) and kalanchosides A–C from the aerial parts of this taxon (Wu et al., 2006) showed cytotoxic activity against a panel of human tumour cell lines. Aqueous and alcoholic extracts of the leaves of *Kalanchoe thrysiflora* and *Kalanchoe marmorata* were tested for their cytotoxic activity against the MCF7 breast carcinoma cell line (Sibgab et al., 2012). A methanol extract of *Kalanchoe hybrida* also showed significant cytotoxicity towards the MCF7 and NCI-H460 cell lines (both large cell carcinoma of lung cell lines) (Kuo et al., 2008).

The next step in our study was the quantitative determination of phenolic acids in *Kalanchoe* extracts. Phenolic acids are a group of compounds exhibiting strong antioxidant activity. The antiradical potency of phenolic compounds strongly and positively correlates with the number of hydroxy groups bound to the aromatic rings. Moreover hydroxy groups in *ortho* and *para* position to other hydroxy groups enhance the anti-oxidative and antiradical activity of phenolic acids (Sroka, 2005). Determinations of phenolic acid contents were conducted using the optimized LC–ESI–MS/MS method for nine acids: gallic, chlorogenic, β - and γ -resorcylic, *p*-coumaric, ferulic, caffeic, syringic, and protocatechuic acid. Results of the optimization of conditions of LC–ESI–MS/MS analysis are displayed in Tables 2 and 3. Our analyses revealed the presence of benzoic acid derivatives (gallic, protocatechuic,

Table 4 Phenolic acid contents in *Kalanchoe* expresses in µg per g of dry weight (DW) of plant material. Abbreviations: “–”, not detected; trace, trace amounts. Mean values of three replicate assays with standard deviation.

Species	Phenolic acid content [µg/g DW]									
	Gallic	Chlorogenic	γ-Resorcylic	Ferulic	Caffeic	β-Rosorcylic	Syringic	p-Coumaric	Protocatechuic	
<i>K. nyike</i>	3.8 ± 0.01	0.07 ± 0.01	0.4 ± 0.00	0.7 ± 0.001	Trace	0.77 ± 0.11	0.28 ± 0.00	1.2 ± 0.03	2.39 ± 0.02	
<i>K. daigremontiana</i> MAC	0.9 ± 0.12	0.16 ± 0.01	0.2 ± 0.01	15.1 ± 0.14	8.2 ± 0.10	0.12 ± 0.05	0.56 ± 0.02	1.3 ± 0.02	12.68 ± 0.06	
<i>K. daigremontiana</i> ASE	1.1 ± 0.07	0.97 ± 0.00	0.21 ± 0.06	72.1 ± 0.28	16.5 ± 0.06	0.22 ± 0.01	7.46 ± 0.03	1.3 ± 0.10	24.82 ± 0.17	
<i>K. pinnata</i>	1.2 ± 0.01	–	0.44 ± 0.01	34.8 ± 0.43	25.8 ± 0.21	0.14 ± 0.02	2.37 ± 0.06	–	14.36 ± 0.03	
<i>K. millotii</i>	1.1 ± 0.02	Trace	0.22 ± 0.07	1.6 ± 0.05	37.0 ± 0.57	0.13 ± 0.001	1.2 ± 0.00	1.1 ± 0.14	5.69 ± 0.10	

syringic) and cinnamic acid derivatives (caffeic, *p*-coumaric, ferulic) in *Kalanchoe* extracts (Table 4). The results showed that *K. daigremontiana* (ASE) contained the highest amounts of phenolic acids (124 µg/g of dry weight), while *K. nyike* contained the lowest amounts (9.61 µg/g of dry weight). Among phenolic acids ferulic and caffeic acids were those occurring in the highest quantities in *K. daigremontiana* ASE and *K. pinnata*. The largest amounts of protocatechuic acid were found for *K. daigremontiana* ASE, 24.8 ± 0.2 µg/kg of dry weight).

Moreover, this study yielded additional information about differences resulting from extraction procedures and also between different species from the genus *Kalanchoe*. Overall, the most pronounced antioxidant activity was displayed by *K. pinnata*. The extract obtained from *K. daigremontiana* by maceration showed better antioxidant properties than the extract prepared from the same species by the ASE method.

4. Conclusions

The present study focused on antioxidant and cytotoxic potential of four *Kalanchoe* species and determinations of their chemical composition in terms of phenolic compounds. The obtained extracts possess significant and dose-dependent antioxidant capacities *in vitro*, as well as moderate cytotoxic activity. The findings of this study revealed that *Kalanchoe* extracts could be used as a readily accessible source of natural antioxidants, and as such may be used as crude material in the pharmaceutical industry.

Acknowledgements

The authors wish to thank Professor Paula C.B. de Andrade, Professor Patrícia Valentão, and Dr. Carla Sousa, Laboratório de Farmacognosia, Faculdade de Farmácia Universidade do Porto, Portugal for their valuable suggestions and technical support.

References

- Asiedu-Gyekye, I.J., Antwi, D.A., Bugyei, K.A., Awortwe, C., 2012. Comparative study of two *Kalanchoe* species: total flavonoid, phenolic contents and antioxidant properties. *Afr. J. Pure Appl. Chem.* 6 (5), 65–73.
- Bhatti, M., Kamboj, A., Saluja, A.K., Jain, U.K., 2012. *In vitro* evaluation and comparison of antioxidant activities of various extracts of leaves and stems of *Kalanchoe pinnatum*. *Int. J. Green Pharm.* 6 (4), 340–347.
- Chernetskyy, M., 2007. Kseromorficzne przystosowania struktury liści wybranych gatunków z rodzaju *Kalanchoe adans* (Crassulaceae DC.) (Doctoral dissertation). Akademia Rolnicza w Lublinie, Lublin, Poland (in Polish).
- Chernetskyy, M., 2012. The role of morpho-anatomical traits of the leaves in the taxonomy of Kalanchoideae Berg. subfamily (Crassulaceae DC.). *Mod. Phytomorphol.* 1, 15–18.
- Descoings, B., 2003. *Kalanchoe*. In: Egli, U. (Ed.), *Illustrated Handbook of Succulent Plants – Crassulaceae*. Springer-Verlag, Berlin-Heidelberg, New York (143–181).
- Fernandes, E., Borges, F., Milhazes, N., Carvalho, F.D., Bastos, M.L., 1999. Evaluation of superoxide radical scavenging activity of gallic acid and its alkyl esters using an enzymatic and a non-enzymatic system. *Toxicol. Lett.* 109, 42.
- Hafid-Medheb, K., Augery-Bourget, Y., Minathy, M.N., Hanania, N., Robert-Lézénès, J., 2003. Bcl-XL is required for heme synthesis

- during the chemical induction of thyroid differentiation of murine erythroleukemia cells independently of its antiapoptotic function. *Blood* 101, 2575–2583.
- Halliwell, B., Aeschbach, R., Lörliger, J., Aruoma, O.I., 1995. The characterization of antioxidants. *Food Chem. Toxicol.* 33, 601–617.
- Harlalka, G.V., Patil, C.R., Patil, M.R., 2007. Protective effect of *Kalanchoe pinnata* Pers. (Crassulaceae) on Gentamycin-induced nephrotoxicity in rats. *Indian J. Pharmacol.* 39 (4), 201–205.
- Johnson, I.T., 2001. Antioxidants and antitumor properties. In: Pokorny, J., Yanishlieva, N., Gordon, M. (Eds.), *Antioxidants in Food*. CRC Press, Boca Raton, FL, p. 100.
- Kuo, P.C., Kuo, T.H., Su, C.R., Liou, M.J., Wu, T.S., 2008. Cytotoxic principles and α -pyrone ring-opening derivatives of bufadienolides from *Kalanchoe hybrida*. *Tetrahedron* 64, 3392–3396.
- Lai, Z.R., Ho, Y.L., Huang, T.H., Lai, S.C., Tsai, J.C., Wang, C.Y., Huang, G.J., Chang, Y.S., 2011. Antioxidant, anti-inflammatory and antiproliferative activities of *Kalanchoe gracilis* (L.) DC stem. *A. J. Chin. Med.* 39 (6), 1275–1290.
- Mohan, S.C., Balamurugan, V., Salini, S.T., Rekha, R., 2012. Metal ion chelating activity and hydrogen peroxide scavenging activity of medicinal plant *Kalanchoe pinnata*. *J. Chem. Pharm. Res.* 4 (1), 197–202.
- Muzitano, M.F., Tinoco, L.W., Guette, C., Kaiser, C.R., Rossi-Bergmann, B., Costa, S.S., 2006. The antileishmanial activity assessment of unusual flavonoids from *Kalanchoe pinnata*. *Phytochemistry* 67, 2071–2077.
- Nayak, B.S., Marshall, J.R., Isitor, G., 2010. Wound healing potential of ethanolic extract of *Kalanchoe pinnata* Lam. leaf – a preliminary study. *Indian J. Exp. Biol.* 48, 572–576.
- Nguelefack, T.B., Nana, P., Atsamo, A.D., Dimo, T., Watcho, P., Dongmo, A.B., Tapondjou, L.A., Njamen, D., Wansi, S.L., Kamanyi, A., 2006. Analgesic and anticonvulsant effects of extracts from the leaves of *Kalanchoe crenata* (Andrews) Haworth (Crassulaceae). *J. Ethnopharmacol.* 106 (1), 70–75.
- Ojewole, J.A.O., 2005. Antinociceptive, anti-inflammatory and antidiabetic effects of *Bryophyllum pinnatum* (Crassulaceae) leaf aqueous extract. *J. Ethnopharmacol.* 99 (1), 13–19.
- Okwu, D.E., Nnamdi, F.U., 2011. Two novel flavonoids from *Bryophyllum pinnatum* and their antimicrobial activity. *J. Chem. Pharm. Res.* 3 (2), 1–10.
- Quazi, M.A., Nazim, S., Afsar, S., Siraj, S., Patel, M.S., 2011. Evaluation of antimicrobial activity of roots of *Kalanchoe pinnata*. *Int. J. Pharmacol. Bio. Sci.* 5 (1), 93–96.
- Rice-Evans, C.A., Miller, N.J., Paganga, G., 1997. Antioxidant properties of phenolic compounds. *Trends Plant Sci.* 2 (4), 152–159.
- Sharker, S.M., Hossain, M.K., Haque, M.R., Chowdhury, A.A., Kaiser, A., Hasan, C.M., Rashid, M.A., 2012. Chemical and biological studies of *Kalanchoe pinnata* (Lam.) growing in Bangladesh. *Asia Pac. J. Trop. Biomed.*, S1317–S1322
- Sharma, A., Bhot, M., Chandra, N., 2014. *In vitro* antibacterial and antioxidant activity of *Bryophyllum pinnatum* (Lam.) Kurz. *Int. J. Pharm. Pharm. Sci.* 6 (1), 558–560.
- Silva, B.M., Andrade, P.B., Valentão, P., Ferreres, F., Seabra, R.M., Ferreira, M.A., 2004. Quince (*Cydonia oblonga* Miller) fruit (pulp, peel and seed) and jam: antioxidant activity. *J. Agric. Food Chem.* 52, 4705–4712.
- Sibgab, A.N., El-Ahmady, S., Saad, R.M.S., 2012. *Kalanchoe thrysiflora* Harv. and *Kalanchoe marmorata* Baker; DNA profiling, biological guided fractionation of different extracts; isolation and identification of cytotoxic compounds. *J. Appl. Pharm. Sci.* 02, 215–220.
- Sroka, Z., 2005. Antioxidative and antiradical properties of plant phenolics. *Z. Naturforsch.* 60c, 833–843.
- Sumanont, Y., Murakami, Y., Tohda, M., Vajragupta, O., Matsumoto, K., Watanabe, H., 2004. Evaluation of the nitric oxide radical scavenging activity of manganese complexes of curcumin and its derivative. *Biol. Pharm. Bull.* 27 (2), 170–173.
- Supratman, U., Fujita, T., Akiyama, K., Hayashi, H., Murakami, A., Sakai, H., Koshimizu, K., Ohigashi, H., 2001. Anti-tumor promoting activity of bufadienolides from *Kalanchoe pinnata* and *K. daigremontiana x tubiflora*. *Biosci. Biotechnol. Biochem.* 65 (4), 947–949.
- Szewczyk, K., Zidorn, C., 2013. Etnobotany, phytochemistry, and bioactivity of the genus *Turnera* (Passifloraceae) with a focus on damiana – *Turnera diffusa*. *J. Ethnopharmacol.* 152 (3), 424–443.
- Wu, P.L., Hsu, Y.L., Wu, T.S., Bastow, K.F., Lee, K.H., 2006. Kalanchosides A–C, new cytotoxic bufadienolides from the aerial parts of *Kalanchoe gracilis*. *Org. Lett.* 8, 5207–5210.
- Yadav, N.P., Dixit, V.K., 2003. Hepatoprotective activity of leaves of *Kalanchoe pinnata* (Pers.). *J. Ethnopharmacol.* 86 (2–3), 197–202.