STRUCTURE OF LEAVES AND PHENOLIC ACIDS
IN *Kalanchoë daigremontiana* Raym.-Hamet & H. Perrier

Mykhaylo Chernetskyy¹, Anna Woźniak², Agnieszka Skalska-Kamińska³, Beata Żuraw⁴✉, Eliza Blicharska³, Robert Rejdak², Helena Donica⁵, Elżbieta Weryszko-Chmielewska⁴

¹ Botanical Garden, Maria Curie-Skłodowska University, Sławinkowska 3, 20-810 Lublin, Poland
² Department of General Ophthalmology, Medical University of Lublin, Chmielna 1, 20-079 Lublin, Poland
³ Department of Analytical Chemistry, Medical University of Lublin, Chodzki 4a, 20-093 Lublin, Poland
⁴ Department of Botany, University of Life Sciences in Lublin, Akademicka 15, 20-950 Lublin, Poland
⁵ Department of Clinical Biochemistry, Medical University of Lublin, Staszica 16, 20-081 Lublin, Poland

ABSTRACT

*Kalanchoë daigremontiana* leaves contain phenolic compounds, which are one of the determinants of plant therapeutic properties. Light and scanning electron microscopes were used to analyse the structure of leaves. The main aims of the study included the analysis of the anatomy of leaves, localisation of phenolic compounds, and identification of phenolic acids. The thickness of the amphistomatic leaf blades, the number, the size of stomata, and the value of stomatal index, as well as the structure of the parenchyma cells have indicated that *K. daigremontiana* is adapted to arid environments. The histochemical assays revealed the presence of phenolic idioblasts in the leaf blades and petioles. The idioblasts were located in the epidermis, subepidermal layer, a deeper portion of the mesophyll, and in the sheaths of vascular bundles. The phytochemical analyses of leaves demonstrated the presence of gallic, ferulic, caffeic, p-coumaric, and protocatechuic acids in the form of esters. We carried out the research of the anatomical structure of *K. daigremontiana* leaves, which has been insufficiently documented to date. We have also revealed new localisation of phenolic compounds in the leaf tissues of this species.

Key words: Kalanchoë, tissues, phenolic idioblasts, phenolic acids

INTRODUCTION

There are over 150 species in the *Kalanchoë* Adans. genus (Crassulaceae DC.) [Descoings 2006]. *Kalanchoë daigremontiana* Raym.-Hamet et H. Perrier (syn. *Bryophyllum daigremontianum* (Raym.-Hamet et H. Perrier) A. Berger) represents the group *Bulbiliferae* Boiteau et Mannoni, section *Bryophyllum* (Salisb.) Boiteau et Mannoni. It originates from dry and hot semi-desert areas of the south-west of Madagascar [Boiteau and Allorge-Boiteau 1995]. The species was naturalised in some tropical countries, e.g. in India [Descoings 2003]. *K. daigremontiana* is an ornamental pot plant commonly cultivated in many countries [Sarwa 2001].

*Kalanchoë daigremontiana* is a leaf succulent with a life cycle dependent on environmental conditions. It is usually a biennial and sometimes perennial plant. It produces an unbranched, up to 1.5 m
high stem. The 20-cm leaves, dark green on the adaxial side, are tapered at the apex. The lamina margins are regularly serrated and exhibit purple colouration on the abaxial side. *K. daigremontiana* is characterised by vivipary. The spaces between the teeth bear numerous adventitious buds or propagules [Descoings 2003]. Propagules, i.e. the somatic embryos of *K. daigremontiana*, are a transitional form between adventitious buds and embryos [Batygina et al. 1996]. Pendulous flowers with a red or purple bell-shaped corolla form cymose panicles [Descoings 2003].

*K. daigremontiana* represents plants in which photoperiodic factors exert an effect on the formation of propagules. Upon an increase in the day length to over 13 hours, *K. daigremontiana* plants become viviparous [Chovanskaá 1970, Kopcewicz and Lewak 2005].

Many species representing the genus *Kalanchoë* have long been used in traditional medicine [Costa et al. 1995]. High pharmacological activity of *K. pinnata*, i.e. an antibacterial, antiparasitic, anti-cancer, anti-inflammatory, and cardioactive effect, has been demonstrated [Joseph et al. 2011, Pattewar 2012]. Formulations from *K. daigremontiana* sap exhibit anti-inflammatory properties as well. The leaves of this species are used in ethnomedicine to treat skin diseases and to staunch bleeding [Sarwa 2001]. *K. daigremontiana* contains bufadienolides, which reduce the proteolytic activity of thrombin [Costa et al. 1995, Kołodziejeczyk-Czepas et al. 2017].

As other species from this genus, *K. daigremontiana* has considerable antioxidant activity related to the content of phenolic acids [Bogucka-Kocka et al. 2016]. Phenolic acids are an important group of substances with a variety of pharmacological anti-inflammatory and fungistatic activities [Nowak and Rychlińska 2012]. Many methods have been reported in analyses of phenolic compounds: HPLC, capillary electrophoresis, voltammetric [Tyszczuk et al. 2011], gas chromatography. Planar chromatography was also used [Wójcik-Kosior 2006]. The antioxidant properties are also assigned to vitamin E present in this species, whose novel form containing tocopherols was detected by Kruk et al. [2011]. Additionally, *K. daigremontiana* leaf tissues contain sterols [Costa et al. 1995] and triterpenoids [Maarseveen and Jetter 2009].

Previous anatomical investigations of *Kalanchoë* species conducted by other authors demonstrated phenolic idioblasts located in various leaf zones. In *K. crenata*, *K. daigremontiana*, *K. gastonis-bonnieri*, *K. laciniata*, *K. pinnata*, and *K. pumila*, phenolic idioblasts formed a regular or irregular layer below epidermis and were evenly spaced in mesophyll [Balsamo and Uribe 1988, Chernetskyy and Weryszko-Chmielewska 2008, Legramandi 2011, Moreira et al. 2012, Brzezicka et al. 2015]. In the leaves of *K. crenata*, *K. gastonis-bonnieri*, *K. laciniata*, *K. pinnata*, and *K. pumila*, the phenolic idioblasts were irregularly distributed around vascular bundles and hydathodes [Chernetskyy and Weryszko-Chmielewska 2008, Leal-Costa et al. 2010, Legramandi 2011, Moreira et al. 2012, Brzezicka et al. 2015]. Phenolic compounds located in epidermal cells near stomata were found only in *K. crenata* [Moreira et al. 2012].

Since phenolic compounds are an important group of biologically active compounds contained in *Kalanchoë* plant organs and their localisation and the anatomy of *K. daigremontiana* leaves have not been sufficiently explored, the aim of the study was: (i) to show the leaf anatomy, (ii) to localise phenolic compounds in tissues, and (iii) to perform qualitative analysis of phenolic acids in fresh *K. daigremontiana* leaves.

**MATERIALS AND METHODS**

**Plant material**

The material for the anatomical study consisted of typical *K. daigremontiana* leaves sampled from the middle part of the stems of five biennial plants growing in the collection of greenhouse plants of the Botanical Garden, Maria Curie-Skłodowska University in Lublin. The plants grew in plastic pots in a collection of succulents located in a well-lit greenhouse. The cultivation conditions were characterised by an average annual air temperature of 23°C and 64%
humidity. In summer, the temperature during the day reached 39°C. Leaves for anatomical analyses were collected in June and July.

For phytochemical analysis, the leaves of a two-year old *K. daigremontiana* plant were collected in July from the garden of the Faculty of Pharmacy, Medical University in Lublin. Six fully developed leaves were taken from one plant for the investigations.

**Anatomical analysis**

The morphological characteristics of the leaf blade margins, adventitious buds, and propagules were observed under a stereoscopic microscope *Olympus* SZX 12.

The anatomical/histological examination of leaf tissues was carried out with the use of a *Nikon* SE light microscope with a scale in the ocular. Semisolid glycerol sections were made from fresh and fixed in 70% ethanol leaves. Hand-made cross-sections of the leaf and paradermal sections from the upper part of fresh leaves were prepared. The following morphological features were analysed: the density of epidermal cells and stomata (per 1 mm²), the location and length of stomata. The following anatomical characteristics of leaves were analysed: the leaf blade thickness in its middle part on the midrib and between the midrib and the margin, the height of epidermal cells and the thickness of their outer walls, the mesophyll structure and diameter of parenchyma cells, the location of phenolic idioblasts, and the distribution of vascular bundles.

For morphometric analyses, two mature leaves were collected from each of the five examined plants. Next, semi-fixed preparations were made and 3 measurements for each trait were performed. The measurements were performed for 30 stomata.

To visualise lignified cell walls, some leaf cross sections were treated with 5% phloroglucine (ca. 1 min.) and 15% hydrochloric acid (HCl, rinsed in redistilled water and sealed in a 70% glycerol solution. Potassium dichromate [Gabe 1968] or toluidine blue [Ramalingam and Ravindranath 1970] were used as histochemical assays for localisation of phenolic compounds in the leaf tissues. Phenolic compounds stained orange or dark brown when treated with potassium dichromate, whereas green or blue colour was obtained after toluidine blue treatment.

Based on the number of epidermal cells and stomata per leaf unit area, the stomatal index (I) was calculated; it expresses the percent ratio of stomata to the number of epidermal cells with stomata in the analysed area:

\[ I = \frac{S \times 100}{S + E} \]  

\( S \) – number of stomata per 1 mm²;  
\( E \) – number of epidermal cells per 1 mm².

The analysis of the leaf surface micromorphology was carried out using a BS 300 *Tesla* scanning electron microscope (SEM). Fragments of the leaf blade between the midrib and margin were collected from 5 leaves. Leaf fragments were fixed in 2% glutaraldehyde with 2.5% paraformaldehyde in 0.075 M phosphate buffer at pH 6.8 and 4°C for 12 h. Next, they were washed twice in the buffer each time for 15 minutes and in redistilled water for the same time. Subsequently, the samples were dehydrated in the increasing acetone series (30, 50, 70, 90, and 100%) for 30 minutes in each change. After dehydration, the material was critical point dried in liquid CO₂ and sputtered with gold using a CS 100 *Sputter Coater*.

The preparations were assessed for the morphological and location of stomata, the shape of anticlinal walls and the surface of the outer walls of epidermis, the presence of wax structures, and the sculpture of the cuticle surface.

**Qualitative phytochemical analysis by thin-layer chromatography – densitometric method**

Phenolic acid standards of the highest grade, listed in Table 1, were purchased from Sigma (St. Louis, MO, USA) and prepared as 0.1% solutions in methanol.

Caffeic acid for the spectrophotometric assay was prepared as a stock solution at a concentration of 0.1% of methanol. The range of standard concentrations from 0.005 to 0.08% was prepared to construct a calibration graph.
Composition of mobile phases in phenolic acid planar chromatography analysis

<table>
<thead>
<tr>
<th>Kind of stationary phase</th>
<th>Composition of mobile phase</th>
<th>Development program</th>
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</thead>
<tbody>
<tr>
<td>Si 60</td>
<td>tertbutyl-methyl ether + hexane + acetic acid + metanol; 3.5 : 5.0 : 0.5 : 0.2 (v/v)</td>
<td>Number of development cycles 2, Distance of development (mm) 85</td>
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<tr>
<td>Si 60 DIOL</td>
<td>tertbutyl-methyl ether + heptane + formic acid + metanol + toluene; 3.0 : 4.0 : 0.5 : 0.3 : 2.0 (v/v)</td>
<td>Number of development cycles 3, Distance of development (mm) 85</td>
</tr>
<tr>
<td></td>
<td>tertbutyl-methyl ether + heptane + formic acid + toluene; 3.0 : 4.0 : 2.0 : 2.0 (v/v)</td>
<td>Number of development cycles 1, Distance of development (mm) 85</td>
</tr>
<tr>
<td></td>
<td>tertbutyl-methyl ether + heptane + formic acid + metanol + toluene; 3.0 : 4.0 : 2.0 : 2.0 (v/v)</td>
<td>Number of development cycles 3, Distance of development (mm) 20</td>
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All solvents used in the experiments and for sample preparation purchased from Polish Reagents (POCh, Gliwice, Poland) were pro analysis grade.

In the present publication, high performance thin layer chromatography with densitometry was applied for qualitative analysis of phenolic acids in K. daigremontiana methanol extract of fresh leaves of the plant.

The method for extraction and purification of phenolic acids was based on literature data with some modifications [Tyszczuk et al. 2011]. For sample preparation, triple exhaustive extraction (15 min each) in an ultrasonic bath (at 55°C) (Sonorex Type RK 102 HB Bandelin, Berlin, Germany) was conducted in three replicates. 10 g of fresh, crushed plant leaves and three portions (50 mL) of the extractant (methanol) were taken each time.

The extracts were pooled and evaporated to dryness in a rotary evaporator (HB Basic RV 05-ST, IKA, Łódź, Poland) under reduced pressure.

The dry residue was washed with portions of hot distilled water (about 30 mL in total) at 4°C for 24 h. Then the solution was filtered with the use of a paper filter and defatted by shaking out twice in a separator with petroleum ether (10 mL each time). Subsequently, the water extract solution was extracted ten times with portions (10 mL) of diethyl ether in the separator. The ether extracts were combined and shaken ten times with a 5% water solution of NaHCO3 (10 mL each time) to receive water-soluble phenolic acid salts. The extract was next acidified up to pH 3 (with 36% HCl) and extracted ten times with 10 mL portions of diethyl ether in the separator. The ether solution of free phenolic acids was dried with the use of Na2SO4 anhydrite, filtered, and evaporated. The dry residue was dissolved in 5 mL of methanol and used in chromatographic experiments.
Fig. 1. Densitogram of setting the analytical lambda for chosen phenolic acids
Chromatography was performed on 10 cm × 10 cm HPTLC F254 plates coated with silica gel and silica gel modified with DIOL groups (Merck, Darmstadt, Germany). First, the most common [Sowa et al. 2013] silica gel with high performance quality was used. The multiply gradient development (MGD) technique with an experimentally chosen composition of mobile phases (tab. 2) was applied. The plates were washed with methanol and acetone and dried in a stream of hot air before use. Samples (8 μL) and standards (3 μL) were applied to the plates as 5 mm bands by means of a Hamilton syringe. Chromatograms were developed in horizontal Teflon DS chambers (Chromdes, Lublin, Poland). The development programs are presented in Table 2. The spots were detected and preliminarily identified by UV illumination at λ = 245 nm and λ = 366 nm and documented with the use of a digital camera. The HPTLC-Diol plates were also derivatized with the use of a reagent specific to phenolic compounds: a mixture of sulfanilic acid with a 5% solution of sodium nitrate in equal parts, which gives phenolic acids yellow to brown colour of spots.

The presence of phenolic acids in the extract of *K. daigremontiana* was confirmed with the use of densitometric measurements. silica gel plates were scanned for each identified compound to set the analytical lambda (fig. 1). By multivalve scan of standards’ spots, the analytical lambda with maximum absorbance was set (fig. 1). After chromatogram development, the plates were subjected to densitometric scanning with the use a Desaga CD 60 densitometer (Desaga, Heidelberg, Germany) controlled with a Pentium computer, in which the peaks of the standards of the investigated phenolic acids corresponded with the ones from the extract (figs 6 A, 6 B).

Then, the extract and the standard were scanned in the established conditions. The chromatogram on DIOL plates with the best separation parameters was densitometrically scanned at 254 nm.

**Quantitative spectrometric analysis.** An extract of the plant with methanol was prepared for spectrophotometric analysis. 10 g of fresh leaves were extracted three replicates, with 50 mL of the extractant each time, in a boiling water bath EkoTerm TW12 (Julabo, Sellbach, Germany) under reflux. The combined methanol extracts were filtrated with the use of filter paper and evaporated to dryness in a rotary evaporator HB Basic RV 05-ST (IKA, Łódź, Poland) under reduced pressure. The dry residue was dissolved in 20 mL of hot water and left in a refrigerator for 12 hours. After filtration, the water solution was diluted up to 100 mL in a volumetric flask.

The content of phenolic acids, calculated as caffeic acid, was determined using the spectroscopic method with the Arnov’s reagent (sodium molybdate (10 g) and sodium hydroxide (10 g) dissolved in water in a 100 mL volumetric flask) described in The Polish Pharmacopoeia IX [2011]. Five different volumes of the stock solution were used for preparation of the calibration graph. 5 mL of distilled water, 1 mL of a hydrochloric acid solution (0.5 mol L⁻¹), 1 mL of Arnov’s reagent and, after 6 min., 1 mL of sodium hydroxide solution (1 mol L⁻¹) were also added to five 10 mL volumetric flasks. The flasks were filled up with distilled water to the mark. Measurements of absorbance at 490 nm (Spectrophotometer Genesis, Thermo Scientific, Chicago, USA) were done for each concentration exactly after 3 minutes from the last reagent addition in a glass cell of 1 cm. A mixture of all reagents without the caffeic acid standard was used as a reference. Measurements of the extract were performed in the same conditions (glass cell, reference sample composition). Six samples of the investigated extract were added instead of the caffeic acid standards to the reagent mixture and measured at λ = 490 nm.

**RESULTS**

**Leaf anatomy.** The fleshy *K. daigremontiana* leaves are characterised by a cylindrical petiole (fig. 2 A, B) and a bifacial, peltate leaf blade (fig. 2 A, C) with a mean thickness of 3820.0 μm in the midrib and 1913.3 μm between the margin and midrib (tab. 3). The leaf blade margin and the abaxial side of the leaf are purple (fig. 2 B–F). The apex of the teeth in the lamina margin bears hydathodes (fig. 2 D, F), and adventitious buds (fig. 2 D, E), from which propagules are formed (fig. 2 C, D), develop between the teeth on lingulate appendages.
K. daigremontiana leaves are covered by single-layered epidermis (figs 3 A, B, 4 C). The epidermal cells on the adaxial side of the leaves are approx. 1.3-fold higher than the cells of the abaxial epidermis (tab. 3). In the top view, they have irregular isodiametric or slightly elongated shapes (fig. 3 A, B). On the abaxial surface, they are nearly 2-fold smaller than on the adaxial surface, as evidenced by their number per unit area (tab. 3). The cells of the petiole epidermis have a prosenchymatic shape (fig. 3 C). The anticlinal walls of the epidermal cells are usually undulating in their outlines (figs 3 A, B). The outer walls of these cells are thickened and covered by a cuticle (fig. 4 C) providing the leaves with a shiny coating (fig. 2 A, C). The surface of the outer walls is slightly convex (figs 3 A, D, 4 C) and the cuticle is smooth (fig. 3 A) or undulating (fig. 3 A, D). The cuticle bears striated ornamentation only on subsidiary cells (fig. 3 A). Wax patches are present on the cuticle surface (fig. 3 A, D).

K. daigremontiana leaves are amphistomatic. There are many anisocytic stomatal complexes in the epidermis of both lamina surfaces (fig. 3 A, B), with a 2-fold higher number on the abaxial than adaxial side (tab. 3). Subsidiary cells surrounding the stomata have a different shape and are smaller than the other epidermal cells (fig. 3 A–C). The length of the leaf blade stomata on the adaxial and
abaxial surfaces is in the range of 28–30 µm (tab. 3). The stomata are located at the level of the other epidermal cells. The stomatal cuticle forms thickened, convex outer cuticular ledges (fig. 3 A, D). Based on the number of stomata and epidermal cells (per 1 mm² of the leaf blade area), the stomatal index calculated for both surfaces of the *K. daigremontiana* leaves differs only by 1% (tab. 3). Some stomatal complexes are present in the leaf petiole epidermis as well (fig. 3 C).

Fig. 3. Surface of *Kalanchoë daigremontiana* leaf epidermis – A, C, D (SEM), B (LM): A – fragment of the adaxial leaf blade surface with stomata (arrows), wax patches (double arrows) and thickened anticlinal walls of epidermal cells; B – fragment of the epidermis on the abaxial surface of the leaf blade with stomata (arrows) and undulating anticlinal cell walls; C – elongated epidermal cells with a stoma (arrow) on the leaf petiole surface; D – stomata (arrows) and wax patches (double arrows) on the adaxial leaf blade epidermis. Scale bars: A–D = 20 µm
Table 3. Mean measurement results and some structural traits of Kalanchoë daigremontiana leaf blade (n = 30)

<table>
<thead>
<tr>
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<th>Mean ± Standard Deviation</th>
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<tbody>
<tr>
<td>Leaf blade thickness</td>
<td></td>
</tr>
<tr>
<td>In the midrib (µm)</td>
<td>3820.0 ± 13.47</td>
</tr>
<tr>
<td>Between the margin and the midrib (µm)</td>
<td>1913.3 ± 10.79</td>
</tr>
<tr>
<td>Epidermis traits</td>
<td></td>
</tr>
<tr>
<td>Height of cells (µm)</td>
<td>ad 34.10 ± 1.70</td>
</tr>
<tr>
<td></td>
<td>ab 26.70 ± 1.13</td>
</tr>
<tr>
<td>Number of cells per 1 mm² area</td>
<td>ad 227.2 ± 1.64</td>
</tr>
<tr>
<td></td>
<td>ab 425.3 ± 1.89</td>
</tr>
<tr>
<td>Length of stomata (µm)</td>
<td>ad 30.04 ± 0.53</td>
</tr>
<tr>
<td></td>
<td>ab 28.42 ± 0.73</td>
</tr>
<tr>
<td>Number of stomata per 1 mm² area</td>
<td>ad 21.2 ± 0.84</td>
</tr>
<tr>
<td></td>
<td>ab 45.3 ± 1.09</td>
</tr>
<tr>
<td>Stomatal index (%)</td>
<td>ad 8.54</td>
</tr>
<tr>
<td></td>
<td>ab 9.62</td>
</tr>
<tr>
<td>Diameter of mesophyll cells</td>
<td></td>
</tr>
<tr>
<td>Subepidermal mesophyll (µm)</td>
<td>ad 78.3 ± 15.47</td>
</tr>
<tr>
<td></td>
<td>ab 39.7 ± 13.05</td>
</tr>
<tr>
<td>Middle part of the mesophyll (µm)</td>
<td>max. 160.0 ± 19.32</td>
</tr>
<tr>
<td></td>
<td>min. 123.6 ± 17.89</td>
</tr>
</tbody>
</table>

ad – adaxial surface of the leaf blade
ab – abaxial surface of the leaf blade
max. – maximal diameter
min. – minimal diameter

The chlorenchymatic tissue in the K. daigremontiana leaves is not differentiated into the palisade and spongy parts; it forms a small-celled mesophyll under the epidermis and a large-celled water-bearing parenchyma in the middle part of the leaf (fig. 4 A, B). The subepidermal mesophyll in the leaf blades usually consists of one or several layers of small, closely adherent cells (fig. 4 B, C).

The cells of the subepidermal mesophyll on the adaxial side of the leaf blade are generally 2-fold larger than such cells on the abaxial side. In the cross section of the leaf blades, the diameter of the cells of these mesophyll layers is approx. 39.7 µm (abaxial layer) and 78.3 µm (adaxial layer) (tab. 3). They are 2–4-fold smaller than the cells of the water-bearing chlorenchyma in the middle part of the leaf. The cells of the water-storing chlorenchyma in the middle part of the leaf have diameters of 160.0 × 123.6 µm (tab. 3); their shape factor is in the range of 1.2–2.0, with a mean value of 1.3 and determines the ellipsoidal shape of the cells of this tissue.

The central vascular bundles in the leaves are surrounded by perivascular sheaths composed of tiny mesophyll cells (fig. 4 D).

Between the epidermis and mesophyll in the K. daigremontiana leaf petioles, there are 1–3 layers of compact angular collenchyma (fig. 4 C). The number of collenchyma layers in the petiole clearly decreases from its base to the lamina. The subepidermal angular collenchyma is fragmentarily distributed on the abaxial side of the leaf blade below the vascular bundle. The size of the collenchyma cells is similar to that of the small mesophyll cells.

The sclerenchyma in the leaves is located around larger vascular bundles in the form of fibres constituting a mestome sheath. It is the best-developed tissue in the leaf petioles and near the main vascular bundle in the leaf blades (fig. 4 D). The vascular bundles represent the closed collateral type. In the central veins in the petiole and the lamina veins located on their extension are visible three large bundles (fig. 4 A). The cross sections show fine lateral vascular bundles surrounding large bundles in the petioles and leaf blades (fig. 4 A, B).
Fig. 4. Tissues of Kalanchoë daigremontiana leaves in cross sections (LM): A – cross section of the leaf petiole base with visible vascular bundles (VB); B – fragment of a cross section of the leaf blade with visible vascular bundles (VB); C – fragment of a cross section of the petiole; layers of angular collenchyma cells (Co) present under a single-layered epidermis (E) with thickened outer cell walls; D – cross section of the main vascular bundle of the leaf blade with visible xylem (X), phloem (Ph), and sclerenchyma (Sc); E, F – cross section of a fragment of leaf blade epidermis treated with toluidine blue. Visible content of phenolic compounds in vacuoles; G–J – phenolic idioblasts (ID) dispersed in the mesophyll (G, H) and surrounding vascular bundles (I, J); fresh leaves treated with potassium dichromate. Scale bars: A, B = 1 mm, C, D, G–J = 50 µm, E, F = 10 µm
The different tissues of the *K. daigremontiana* leaf contain numerous phenolic idioblasts accumulating phenolic compounds in their vacuoles. The localisation of phenolic idioblasts was confirmed with the use of the histochemical assays. Phenolic compounds are present in epidermal cells (fig. 4 E, F) and in the subepidermal layer. Phenolic idioblasts are dispersed in the parenchyma as single cells or form multicellular aggregates (fig. 4 G, H). They are also located in close proximity to the vascular elements (fig. 4 I, J).

There are few phenolic idioblasts around the large vascular bundles in the leaf petioles (fig. 5 A). They surround the smaller vascular bundles partially (fig. 5 B) or completely (fig. 5 C). 1–3 layers of these cells are visible near some bundles in the leaf petioles (fig. 5 D).

**Identification of phenolic acids.** For preliminary identification of phenolic acids, comparison of retention factor ($R_F$) values with $R_F$ of standard substances was conducted. A combination of two stationary phases was applied for separation of the extract components. In the described conditions, three phenolic acids: protocatechuic, p-coumaric, and ferulic in the *K. daigremontiana* extract were separated and identified (figs 6 A, 6 B). Simultaneously, the presence of salicylic and vanillic acid was not confirmed. Given the doubts about chlorogenic, caffeic, and syringic acid, sequential experiments were carried out. Satisfactory separation of two phenolic acids, caffeic and gallic, was achieved on silica gel modified with DIOL groups (fig. 7). Additionally, the presence of...

Fig. 6 A. Densitometric identification of phenolic acids in the methanol extract of fresh Kalanchoë daigremontiana leaves
chlorogenic acid, which was supposed to be detected on silica gel plates, was eliminated. No content of vanillic, chlorogenic, salicylic, and syringic acid from the group of free phenolic acids was confirmed in the K. daigremontiana methanol extract.

**Visualisation of phenolic acids.** For better identification of phenolic acids on DIOL plates, the method of plate derivatization with sulfuric acid and sodium nitrate was used. Visualisation of spots under light of 320 nm and sulfuric acid derivatization confirmed the presence of gallic acid in the extract. Consequently, the spots of caffeic and protocatechuic acids had the same values and colour. Identification of caffeic acid was conducted in UV light at $\lambda = 254$ nm in plates without the derivatization reagent. The colour of the spot was light blue.

With the use of two stationary phases and a combination of three visualization techniques, five phenolic acids in the methanol extract of fresh leaves of K. daigremontiana were separated and identified.

The presence of ferulic, gallic, caffeic, p-coumaric, and protocatechuic acid in the extract was confirmed. Selected chromatograms are presented in Figs 7 and 8.
Fig. 7. Chromatogram of phenolic acids on a DIOL-silica plate in UV light at $\lambda = 254$ nm. Numbers refer to the phenolic acid names shown in Table 1.

Fig. 8. Chromatogram of phenolic acids on a silica plate in UV light at $\lambda = 254$ nm after derivatization with sulfuric acid and sodium nitrate. Numbers refer to the phenolic acid names shown in Table 1.
Spectroscopic analysis. The spectroscopic method with the Arnov’s reagent was used for quantitative determination of free phenolic acids from *K. daigremontiana*. In the spectroscopic analysis, the calibration graph of the dependence between the absorbance and concentration of caffeic acid was drafted. The total content of phenolic acids is 2 µg·10 g⁻¹ in fresh *K. daigremontiana* leaves.

DISCUSSION

Leaf structure. Stomatal complexes in representatives of the genus *Kalanchoë* belong to the anisocytic type characterised by the presence of three subsidiary cells surrounding the stomata [Metcalfe and Chalk 1957]. There are usually greater numbers of stomata on the abaxial than adaxial side of leaves [Sharma and Lewis 1987], which was also shown in this study. The abaxial side of the *K. daigremontiana* leaves had smaller stomata than those located on the adaxial side. As reported by Sharma and Lewis [1987], the number of stomata and undulation of the anticlinal walls of epidermal cells in representatives of the genus *Kalanchoë* has a diagnostic importance.

Other authors reported that the number of stomata per unit area in *Kalanchoë blossfeldiana*, *K. daigremontiana*, and *K. tubiflora* leaves reached up to 50 mm⁻², likewise in other succulents and is several times lower than that observed in mesophytes [Strobel and Sunberg 1983–1984]. A similar range of the number of stomata was demonstrated in the *K. daigremontiana* analysed in the present study and in previously investigated species from this genus [Chernetskyy 2006, Chernetskyy and Weryszko-Chmielewska 2008]. The number of stomata per 1 mm² area in *K. orgyalis* and *K. tomentosa* ranged from 54 to 92 depending on the side of the leaf [Chernetskyy 2006]. The author of the latter study suggests that the density of stomata, small sizes of epidermal cells, presence of trichomes, and reduced sizes of stomata in the leaves of these species may be a result of advanced xeromorphism associated with adaptation to arid environments. In *K. daigremontiana*, besides fully mature stomata, underdeveloped stomata in different stages of development were observed, which may limit transpiration in adverse conditions.

Sharma and Dunn [1968] reported that a greater number of stomata and greater cuticle thickening with granular texture and waxy coating were observed in *K. fedtschenkoi* leaves in dry and hot conditions than in wet habitats. The number and structure of cells surrounding the stomata were unchanged, regardless of the conditions. The authors found that the stomatal index was not a stable diagnostic feature for this taxon, as it changes in different ecological conditions. The highest stomatal index was calculated for control plants and specimens growing in desert conditions, while the lowest value was reported for plants growing in wet conditions.

The assimilation tissue in *K. daigremontiana* leaves is not differentiated into palisade and sponge mesophyll but is divided into a small-celled subepidermal mesophyll and a large-celled water-bearing CAM-type mesophyll in the middle part of the leaf. Similar mesophyll differentiation was reported in previous studies of this and other species from the genus *Kalanchoë* [Balsamo and Uribe 1988, Chernetskyy and Weryszko-Chmielewska 2008, AbdelRaouf 2012, Brzezicka et al. 2015]. In the anatomical characteristics of the family Crassulaceae, Metcalfe and Chalk [1957] report that palisade tissue is rarely present in the leaves of the taxa of this family.

In the species analysed in this study, the cells of the large-celled mesophyll contain chloroplasts and are involved in the photosynthesis process as well as water storage. The main adaptation trait in leaf succulents is accumulation of water in the parenchyma of deeper leaf layers, which is associated with the presence of large vacuoles in the cells. In highly specialised leaf succulents (e.g. *Aloë*, *Haworthia*, *Lithops*, *Salsola*, and some *Peperomia*), the central part of leaves is composed of a specialised water-bearing tissue, whose large cells only occasionally contain chloroplasts [Troll 1959, Kaul 1977, Chauser-Volfson et al. 2002, Nuzhyna and Gaydarzhy 2015]. Similarly to other genera from the family Crassulaceae, this tissue in genus *Kalanchoë* representatives...
is replaced by water-bearing mesophyll with smaller cells containing many chloroplasts [Lyubimov et al. 1986].

The present study indicates that, in the *K. daigremontiana* leaves, cells containing phenolic compounds were present in the epidermal and subepidermal tissue, forming a continuous or discontinuous layer. They were also located singly near the vascular elements or surrounded the entire bundles as 1–3 layers. We also found phenolic idioblasts dispersed singly or in groups in the mesophyll. In turn, Balsamo and Uribe [1988] reported that phenolic idioblasts in *K. daigremontiana* leaves were located only in the subepidermal layer and were evenly spaced throughout the mesophyll. Our research has provided new data concerning the location of phenolic idioblasts in this species, as we found them in the epidermis and around the vascular bundles, where they even formed multi-layer bundle sheath. The presence of tannin-containing cells was detected in leaves of other species from the genus *Kalanchoë* [Chernetskyy and Weryszko-Chmielewska 2008, Legramandi 2011, Chernetskyy 2012, Brzezicka et al. 2015]. The content of phenolic compounds was also observed in some cells of the stalks of non-glandular trichomes in *K. millotii* [Weryszko-Chmielewska and Chernetskyy 2005].

Chernetskyy and Weryszko-Chmielewska [2008] found well visible chloroplasts in the cytoplasm of *K. pumila* phenolic idioblasts, which were several fold smaller than chloroplasts contained in mesophyll cells. Similarly, there were chloroplasts in *K. daigremontiana* cells accumulating phenolic compounds. Single starch grains and spherical osmophilic structures were noted in *K. pumila* cells. The electron-dense central vacuoles of such cells exhibited dark flocculent content. Such features of the structure of phenolic idioblasts have been shown in other plant species as well [Bačić et al. 2004].

The content of phenolic compounds in plant cells is an important adaptive trait, as the compounds play a protective role by absorption of UV radiation, which is unfavourable to plant organs, and protect plants from pathogenic agents and damage caused by entomofauna [Oleszek et al. 2001, Kopcewicz and Lewak 2005].

**Phenolic compounds**

Some of *Kalanchoë* species are supposed to have many positive effects on the human organism [Scholtsik et al. 1986, Muzitano et al. 2006, Kamboj and Saluja 2009]. The main group of illnesses treated by *Kalanchoë* originates from oxidative-stress. The effectiveness of plants in healing the diseases comes from the high amount of phenolic derivatives. The presence of phenolic compounds determines the anti-inflammatory, wound healing, and free radical scavenging activity of the plant [Sazhina et al. 2014]. Although *K. daigremontiana* is well known for medical applications, its exact chemical composition is documented very poorly. The concentration of phenolic acids in a free form in a plant was described by Bogucka-Kocka et al. [2016] as 124 µg·g⁻¹ of dry weight. The authors of the cited study found the highest content of ferulic, protocatechuic, and caffeic acids in the analysed *K. daigremontiana* material. Fresh leaves were subjected to two kinds of extraction: accelerated solvent extraction and maceration with ethanol. In the studies reported by these authors, quantification of phenolic acids with the HPLC method was conducted after extract concentration and filtration.

The presented publication describes the qualitative content of phenolic acids in a hydrolysed methanol extract. Phenolic acids described in our publication occur in the plant in the form of esters. Exhaustive extraction with sonication was used. A combination of two stationary phases and gradient elution was used in the HPTLC method.

The content of phenolic acids presented in this study is partially similar to previous investigations conducted by other authors. We confirmed the presence of gallic, ferulic, caffeic, p-coumaric, and protocatechuic acids. We did not detect chlorogenic and syringic acids, which were found with the HPLC method by Bogucka-Kocka et al. [2016]. We also investigated salicylic and vanillic acid but their presence was not confirmed.

The presented quality investigations are the first step in the analysis of phenolic acids from *K. daigremontiana*. So far, we have determined the sum of phenolic acids from esters with organic acids. The knowledge of the content of basic compounds in

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*R. Kamboj and E. Weryszko-Chmielewska*.

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K. daigremontiana with confirmed medical activity is still very poor; therefore, future investigations are planned e.g. quantification analysis of particular phenolic acids and hydrolysis of glycosides.

CONCLUSIONS

1. We have demonstrated in the study that the fleshy Kalanchoë daigremontiana leaves are characterised by bifacial, amphistomatic leaf blades with the abaxial epidermis bearing an approximately 2-fold greater number of stomatal complexes than the adaxial surface. The leaf parenchyma is differentiated into a small-celled subepidermal mesophyll and a large-celled mesophyll located in the central part of the leaf. Both mesophyll types contain chloroplasts.

2. The results of the histochemical assays, indicate the content of phenolic compounds in various lamina and petiole tissues, i.e. in the cells of the epidermis, subepidermal and perivascular parenchyma (1–3 layers), and in the cells scattered in the leaf mesophyll, which is partly new information about this species. The phenolic compounds in the different leaf tissues are contained at substantial levels, which are probably reflected in the therapeutic activity.

3. The phytochemical analyses have evidenced the presence of gallic, ferulic, caffeic, p-coumaric, and protocatechuic acids representing phenolic compounds in the ester form in Kalanchoë daigremontiana leaves.

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