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Flavonol Glycosides and Gallic Acid from Flowers of *Kalanchoe delagoensis*

Flavonóis Glicosídicos e Ácido Gálico em Flores de Kalanchoe delagoensis

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Kalanchoe delagoensis (also known as *Bryophyllum delagoensis* and *Kalanchoe tubiflora*) is a Crassulaceous plant used for healing wounds and dermatitis. The species showed antitumor potential in recent pharmacological surveys. The present study aimed to isolate the phenolic compounds from the aqueous extract of *K. delagoensis* flowers. Here, we report the occurrence of the flavonoids corniculatusin 3-*O*-β-glucopyranoside **1** (8-methoxyquercetin 3-*O*-glucoside), kaempferol 3-*O*-β-glucopyranoside **2**, quercetin 3-*O*-β-glucuronopyranoside **4**, and gallic acid **3**. Our original results contribute to the knowledge of the chemical composition of *K. delagoensis*.

Keywords: Corniculatusin 3-*O*-glucoside; miquelianin; astragalin; gallic acid; flavonoids; Crassulaceae

1. Introduction

Kalanchoe delagoensis Eckl. & Zeyh. (also known as *Bryophyllum delagoensis*; *Kalanchoe tubiflora*) is a succulent herbaceous plant belonging to the Crassulaceae family and originally from Madagascar.[1,2] It has cylindrical leaves and produces terminal branched inflorescences, with numerous showy, orange-red flowers. The species is popularly known as the chandelier plant or mother of thousands. The last name is due to the rapid vegetative propagation of the plant through the apical buds of leaves.[2,3] This characteristic led to the spread of *K. delagoensis* throughout many parts of the world, facilitated by its cultivation for ornamental purposes. In some countries (e.g., China, South Africa, Mexico), it is considered an invasive species.[4] *K. delacoensis* is also found in Brazil, where it is well adapted to regions with extreme conditions, such as intense solar irradiation, drought, and heat.[2,5]

The whole *K. delagoensis* plant or its leaves are used to treat dermatitis and wound healing in southern Uganda and in the southern regions of Brazil, respectively.[6,7] Some *Kalanchoe* species have been used in traditional medicine, and various species have been studied with focus on their pharmacological activities.[8-13] The most important chemical constituents found in the genus are flavonoid glycosides and bufadienolides.[9,14–16] The main secondary metabolites reported in *K. delagoensis* belong to the bufadienolide and cardenolide chemical classes.[17–20] Phenolic acids and flavonoids were also reported.[18,21]

This plant has been the object of studies, mainly concerning its antitumor potential. The growth inhibition of some cancer cell lines was demonstrated for a butanol-soluble fraction of *K. delagoensis* aerial parts.[22] Additionally, Hsieh and collaborators (2016) showed that an aqueous fraction of *K. delagoensis* has *in vitro* inhibitory effect against A549 cells as well as the tumor growth in A549-xenografted nude mice.[23] Moreover, bufadienolides isolated from the whole plant have been shown to inhibit lung and melanoma cell lines.[17,24]

The genus *Kalanchoe* has been studied in our laboratories in an interdisciplinary program in order to search for phenolic bioactive substances.[9,12,13,25–28] The leaves are the focus of most reports on the chemical and biological activities of *Kalanchoe* species, and to date, there are scarce reports on the phenolic composition of the flowers of these plants. The earliest study refers to the flavonoids from the flowers of *K. spathulata*.[29] Two decades later, Nielsen and collaborators studied the phenolic composition of flowers of *K. blossfeldiana*—a potted plant species of great commercial value due to the wide ornamental use—leading to the isolation of a series of flavonoids.[29,30] A few years later, we demonstrated that the flowers of *K. pinnata* are a source of T-cell suppressive flavonoids.[26] Additionally, anthocyanins were tentatively identified on flowers of *K. daigremontiana.*[31]

The great potential of the genus *Kalanchoe* as a promising source of new drugs encouraged us to investigate the phenolic composition of the aqueous extract from *K. delagoensis* flowers.

2. Materials and Methods

2.1. General experimental procedures

The ^{1}H- and ^{13}C-NMR spectra (DMSO-d_6 or CD_3OD signals as internal reference) were recorded on a Bruker DRX-300 (^{1}H: 300.13 MHz; ^{13}C: 75.48 MHz; Billerica, United States) at Instituto de Química (UFRJ), on a Varian Mercury 300 (^{1}H: 300.13 MHz; ^{13}C: 75.48 MHz; Palo Alto, United States) at IMA (UFRJ), on a Bruker Avance III 800 MHz (^{1}H: 800.50 MHz; ^{13}C: 200.12 MHz; Billerica, United States) at CNRMN (UFRJ), or on a Varian NMRSYS-500 (^{1}H: 499.77 MHz; ^{13}C: 125.68 MHz; Palo Alto, United States) spectrometer at LAMAR (UFRJ). Mass spectra (electron spray ionization – ESI) were recorded on a Waters Micromass Q-TOF Micro (Milford, United States) spectrometer at the Instituto de Química (UFRJ) or on a BRUKER MicroTOF-II mass spectrometer (Billerica, United States) at IPPN (UFRJ).

The purification of the extract was performed by column chromatography on silanized silica (RP-2, 70–230 mesh or RP-18, 40–63 µm, Merck, Kenilworth, United States), gel Sephadex LH-20-100 lipophilic (25–100 µm; Sigma, Saint Louis, United States), or gel Sephadex G-10 (40–120 µm; Pharmacia Fine Chemicals, Uppsala, Sweden). The eluates were monitored by thin layer chromatography (TLC) on silica 60 F_{254} (Merck, Kenilworth, United States) using n-butanol/acetic acid/water (BAW; 8:1:1). The chromatograms were visualized under UV light at 254 nm and 365 nm and revealed with ceric sulphate solution to detect phenolic substances followed by heating (50 °C) on a hot plate. After development with ceric sulphate, spots corresponding to flavonoids showed a yellow-orange color. Solvents used in chromatographic procedures were from Tedia Brazil (Rio de Janeiro, Brazil).

2.2. Plant material and extraction

In this study, we used two batches of flowers collected from specimens growing in two different localities in the Rio de Janeiro State. Flowers of *K. delagoensis* were collected by the seaside at Rio das Ostras, RJ, Brazil and on the slope of a hill in Arraial do Cabo (Figure 1), RJ, Brazil. A voucher specimen (RFA39965) was deposited at the herbarium of the Institute of Biology (UFRJ, Brazil). Flowers from Rio das Ostras (batch 1; 990 g) were cut into small pieces, crushed in a blender, and submitted to an infusion with boiling distilled water at 40% (w/v). The infusion was filtered (2450 mL) and concentrated in a waterbath until the volume reached 250 mL. The concentrated extract (KDL-A) was frozen and lyophilized (dry extract = 41.9 g). Flowers from Arraial do Cabo (batch 2; 126 g) were extracted using the same infusion procedure (40% w/v). The infusion (290 mL) was concentrated in a water bath until it reached the volume of 165 mL. The concentrated extract (KDL-B) was submitted to lyophilization (dry extract = 5.2 g).

Figure 1. *Kalanchoe delagoensis* flowers (Arraial do Cabo,RJ, Brazil). Source: author's own collection

2.3. Isolation of phenolic substances

The crude extract KDL-A (41.9 g) was dissolved in distilled water and two parts of ethanol were added to the final resulting solution. The precipitate was removed by filtration, and the supernatant was lyophilized after the evaporation of residual ethanol. The dry supernatant was dissolved in distilled water and extracted successively with dichloromethane, ethyl acetate, and *n*-butanol. Each organic fraction was dried, separately, affording 0.36 g (CH_2Cl_2), 1.07 g (AcOEt), and 3.84 g (BuOH). The AcOEt fraction (1.07 g) was dissolved in distilled water and injected into an RP-2 column (7.5 x 1.5 cm), which was eluted with a water/ethanol gradient. Three fractions were obtained: KDL-A1 (223.5 mg, 100% H_2O), KDL-A2 (580.5 mg, 100% and 70% H_2O), and KDL-A3 (187.9 mg; 70%, 50%, 30% H_2O). The second fraction showed a rich flavonoid profile based on TLC. KDL-A2 was analyzed on an RP-2 column (35 x 1.0 cm) using a water/ethanol gradient. Similar fractions were pooled together according to their TLC profile: KDL-A2-a (276.5 mg, 100% H_2O), KDL-A2-b (190.3 mg, 100% H_2O), and KDL-A2-c (60.9 mg, 90% and 80% H_2O). KDL-A2-b (190.3 mg) was injected into a Sephadex LH-20 column (18 x 0.7 cm, methanol) affording: KDL-A2-b-1 (14.4 mg, 100% H_2O), KDL-A2-b-2 (149.3 mg, 90% H_2O), and KDL-A2-b-3 (26.3 mg, 70% H_2O). KDL-A2-b-2 (149.3 mg) was purified on an RP-2 column (27 x 7 cm) in a water/methanol gradient, affording: KDL-A2-b-2-a (80.9 mg, 100%, 90% and 70% H_2O), KDL-A2-b-2-b (27.50 mg, 70% H_2O), and KDL-A2-b-2-c (32.7 mg, 70%

H_2O). KDL-A2-b-2-b exhibited one yellow spot (R*f* = 0.56; BAW 8:1: 1) corresponding to a flavonoid (**1**), which was obtained as an amorphous yellow powder (27.5 mg; R*f* 0.56; BAW 8:1:1). The chromatography of KDL-A2-c (60.9 mg) on Sephadex LH-20 (14 x 0.7 cm; methanol) followed by purification on a RP-18 column (140.65 cm; water/methanol) afforded an enriched flavonoid fraction (37.5 mg; Rf = 0.71, BAW 8:1:1) that was finally chromatographed on an RP-18 column (17 x 0.4 cm; gradient water/ethanol). The material obtained was a light-yellow powder (3 mg; Rf = 0.71, BAW 8:1:1) shown to be a flavonoid (**2**).

The second extract, KDL-B (5.2 g), was purified following the same procedure described for KDL-A. The partition with dichloromethane, ethyl acetate, and butanol afforded 1 mg, 192 mg and 636 mg, respectively, of each organic fraction after lyophilization. An aliquot of 133 mg of the ethyl acetate fraction was dissolved in distilled water and injected into an RP-2 column (12.3 x 1 cm). Four fractions were eluted with a water/methanol gradient: KDL-B1 (18 mg, 100% H_2O), KDL-B2 (16 mg; 100% H_2O), KDL-B3 (20 mg, 90% H_2O), and KDL-B4 (59 mg; 70%, 50%, 30% H_2O). KDL-B2 was enriched in a phenolic substance (R*f* 0.90), while KDL-B3 was enriched in a polar flavonoid (R*f* 0.30). This polar flavonoid was also present in KDL-B4 in a mixture with other substances. KDL-B2 (18 mg) was injected in a Sephadex G-10 column (25 x 0.4 cm) and eluted in a water/ethanol gradient, (100%, 80%, and 50% water) affording a phenolic substance (**3**) (R*f* 0.90) as a pale beige powder (7 mg).

KDL-B4 (59 mg) was injected in an RP-2 column (19.5 x 1 cm) and eluted with a water/methanol gradient. This column afforded a fraction enriched in the polar flavonoid (R*f* 0.30): KDL-B4-b. KDL-B4-b (4 mg) was pooled together with KDL-B3 (20 mg) in order to purify this flavonoid. This pool (24 mg) was injected in a Sephadex G-10 column (31 x 0.5 cm) eluted in a water-ethanol gradient (100%, 80%, and 50% water), which afforded 6.5 mg of a flavonoid-enriched fraction (KDL-B3-4-b; 100%, 80%, and 50% water). KDL-B3-4-b (6.5 mg) was purified in a Sephadex LH-20 column (38.5 x 0.8 cm; water/20% ethanol), affording 3.8 mg of a flavonoid (**4**) (R*f* 0.30). NMR and Mass Spectrometry data of the isolated substances are shown in Supplementary Information.

3. Results and Discussion

In the present study, the combined processes of precipitation, organic partition, and column chromatography of aqueous extracts of *K. delagoensis* flowers obtained by infusion (40% w/v) led to the isolation of three flavonoids and a phenolic acid (Figure 2).

Compound **1** (27.5 mg) was identified as corniculatusin 3-*O*-β-glucopyranoside (or 8-methoxyquercetin 3-*O*-β-glucopyranoside), based on ^{1}H- and ^{13}C-NMR, COSY ^{1}H-^{1}H, HMQC, and HMBC data obtained in CD_3OD.[32] This

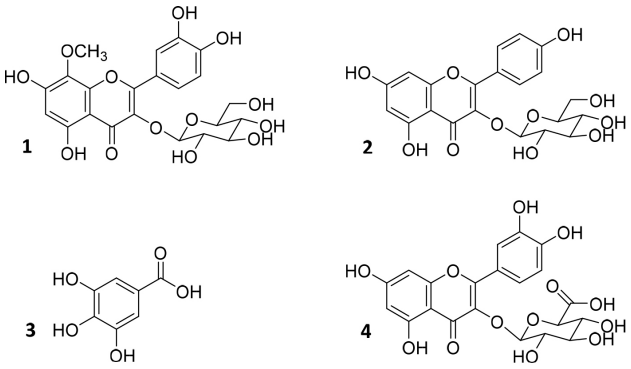

Figure 2. Phenolic substances isolated from *Kalanchoe delagoensis* flowers: corniculatusin 3-*O*-glucoside (**1**), astragalin (**2**), gallic acid (**3**), and miquelianin (**4**)

flavonoid was originally isolated from *Lotus corniculatus* (Fabaceae).[33] As far as we know, it seems that this flavonoid has not been reported for *Kalanchoe* species. Corniculatusin 3-*O*-β-glucopyranoside is not frequent in nature, having been reported in these few species: *Drosera binata* (Droseraceae),[34] *Erica cinerea* (Ericaceae),[35] *Epimedium* spp. (Berberidaceae),[36] *Geraea canescens* (Asteraceae),[37] and *Persicaria mitis* (Polygonaceae),[38] according to data from a structure-based search in the SciFinder database and keyword-based search in other databases. There are some reports on the aglicone corniculatusin (8-methoxyquercetin) in the genus *Sedum*, from the Crassulaceae family. [39,40] This skeleton is less frequent than its isomer patuletin (6-methoxyquercetin). It was reported that corniculatusin 3-*O*-β-glucopyranoside is able to attract insects and stimulate oviposition in the desert sunflower *Geraea canescens.*[37] To date, no other biological activity has been reported for this flavonoid.

Compound **2** (4 mg) was identified as kaempferol 3-*O*-glucopyranoside (astragalin) based on ^{1}H- and ^{13}C-NMR, COSY ^{1}H-^{1}H, HMQC, and HMBC data obtained in DMSO-d_6 and ESI/Q-TOF mass spectrometry.[41,42] The flavonoid astragalin was described early in *Kalanchoe pinnata* and more recently, in *Kalanchoe thyrsiflora* and *Kalanchoe prolifera.*[12,43,44] This flavonol glycoside is known to have antiallergic,[45,46] anti-asthmatic,[46,47] antileukemic,[48] anti-inflammatory,[49,50] and antidiabetic properties[51] and antitumor activity in various cell lines,[52,53] among others.[53]

Compound **3** (7 mg) was identified as gallic acid with a basis on ^{1}H-NMR, HSQC, and HMBC data obtained in DMSO-*d*6.[54] This substance was previously reported in leaves of *K. pinnata* and *K. thyrsiflora*.[12,55] The antitumor activity of gallic acid is well documented, as reviewed by Verma et al. (2013). Our group reported gallic acid as the main antileukemia compound from *Kalanchoe thyrsiflora* leaf extract in a study with lymphocytic leukemia cell (Jurkat cells).[12] Additionally, various activities were reported for this hydroxyphenolic acid such as antiviral, antibacterial, antidiabetic, anti-inflammatory, and antihypertensive.[55,57–62]

Compound **4** (3.8 mg) was identified as quercetin

3-*O*-β-glucuronopyranoside (quercetin 3-*O*-glucuronide or miquelianin) based on ^{1}H NMR, HSQC, and HMBC data obtained in DMSO-d_6 and ESI/Q-TOF mass spectrometry.[63,64] This flavonol glucuronide was previously described in *Kalanchoe pinnata* flowers,[26] presenting T-cell suppressive activity, and the potential to prevent and treat gastritis and esophagitis.[26,65–67] Miquelianin also showed antidepressant activity, being considered one of the active substances in the antidepressant herbal medicine, *Hypericum perforatum.*[68–70] Recently, antitumor effects in human breast cancer cells were reported for this flavonoid.[71]

Studies about the chemical composition of the flowers from *K. delagoensis* led to the isolation of cardenolides and bufadienolides.[19,20,72] The toxicity from flowers of *K. delagoensis* for cattle was attributed to bufadienolide cardiac glycosides by McKenzie et al. (1987).[73] More recently, new cardenolides, bufadienolides glycosides, and a megastigmane were reported from an ethanol extract of the whole plant.[17,24,74,75] Some phenolic acids, such as gallic acid, cinnamic acid, and *trans*-ferulic acid, were also reported.[74] Recently, five phenolic acids, including gallic acid, and seven flavonoids were identified in a methanol extract of leaves from *K. delagoensis* by means of HPLC-DAD and comparison with commercial standards. The flavonoids detected were the flavonol aglicones kaempferol and quercetin, as well as their glycosides kaempferol-7-*O*-rhamnoside, trifolin (kaempferol-3-*O*-galactoside), robinin (kaempferol-3-*O*-robinoside-7-*O*-rhamnoside), isoquercitrin (quercetin-3-*O*-glucoside), and quercitrin (quercetin-3-*O*-rhamnoside).[21] Interestingly, none of those flavonoids were isolated from the flowers of *K. delagoensis* in our study.

4. Conclusions

This is the first study of the phenolic constituents from the flowers of *K. delagoensis*. Our findings contributed to the knowledge of the chemical composition of a plant species potentially useful as a source of bioactive compounds.

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