Journal of Applied Botany and Food Quality 95, 154 - 166 (2022), DOI:10.5073/JABFQ.2022.095.020

[1]Laboratório de Anatomia Vegetal, Departamento de Botânica, Instituto de Biologia, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Brazil
[2]Instituto de Pesquisas de Produtos Naturais, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Brazil

Kalanchoe fedtschenkoi R. Hamet & H. Perrier, a non-conventional food plant in Brazil: HPLC-DAD-ESI-MS/MS profile and leaf histochemical location of flavonoids

Jamile Marques Casanova[1], Luana Beatriz dos Santos Nascimento[1], Livia Marques Casanova[2], Silvana Daflón Castricini[2], Jacqueline Elis de Souza[2], Raíssa Mara Kao Yien[2], Sônia Soares Costa[2*], Eliana Schwartz Tavares[1*]

(Submitted: May 11, 2022; Accepted: September 19, 2022)

Summary

Kalanchoe fedtschenkoi (Crassulaceae) is a non-commercial food species in Brazil, whose leaves are used in several aqueous preparations. Their morphology and potential nutraceuticals (e.g., antioxidant phenolics) have been poorly studied. Anatomical features are useful for the correct identification of plants, avoiding their misuse. We describe the anatomy and tissue flavonoid location of *K. fedtschenkoi* leaves, as well as the phenolic composition and the antioxidant activity of their aqueous extracts. The succulent leaves have a unistratified epidermis with anisocytic or helicocytic stomata, collateral vascular bundles, and phenolic idioblasts throughout the leaf blade. NP-reagent tests suggested the presence of kaempferol derivatives (yellow fluorescence) in the regular epidermis cells, subsidiary cells, and leaf mesophyll. An orange color, assigned to quercetin derivatives, was detected in stomata guard-cells and below the epidermis. The HPLC-DAD-ESI-MS/MS evidenced the presence of flavonoids, mostly kaempferol glycosides (especially sagittatin A), some of them firstly described as constituents of *K. fedtschenkoi*. The extracts also showed high antioxidant activity. We report, for the first time, the complete anatomical description of *K. fedtschenkoi* leaves, the tissue location of flavonoids, and the flavonoid composition of the extracts. The high flavonoid content and antioxidant activity of *K. fedtschenkoi* leaves can be an advantage for their consumption as a food, in addition to being a possible source of nutraceuticals.

Keywords: Leaf Anatomy; Flavonoids; Nutraceuticals; 2-aminoethyl diphenylborinate; HPLC-DAD-ESI-MS/MS; DPPH free-radical assay.

Introduction

According to the Food and Agricultural Organization of the United Nations (FAO), there are around 250 thousand plant varieties available for agriculture. However, only about 120 are cultivated for human consumption, with a few crops (mainly rice, corn, and wheat) supplying over 75 percent of the global plant-derived energy intake (FAO, 2010; JACOB and ALBUQUERQUE, 2021).

Nevertheless, noncommercial edible crops are important for the nutrition and subsistence of people in developing nations, being cultivated in backyards, especially in small cities and rural areas. Many of these plants are underused but have nutritional value, as well as the potential to prevent and treat diseases (FAO 2010; JACOB and ALBUQUERQUE, 2021).

Furthermore, several studies have shown an overlap between food and the medicinal uses of a set of neglected edible species (JACOB and ALBUQUERQUE, 2021), which have gained the interest of researchers in Brazil, to encourage the sustainable exploitation of the biodiversity of food sources, as well as their diversification and enlargement (KINUPP and LORENZI, 2014; FERREIRA-OTERO and RIBEIRO, 2019).

In addition to nutritional compounds, such as lipids, carbohydrates, and amino acids, several of these food plant species are rich in bioactive molecules. Among them, phenolics stand out as nutraceutical compounds, including resveratrol and numerous flavonoids (CALDERÓN-OLIVER and PONCE-ALQUICIRA, 2018).

Flavonoids are abundant in several food and beverages, including onions, soy, cocoa products, wine, and green tea. These compounds have a vast array of pharmacological activities, such as anticancer, antimicrobial, immune system promoting, anti-inflammatory, and anti-diabetic, most of which relate to their antioxidant potential (YAO et al., 2004). In fact, the consumption of greater amounts of antioxidant molecules might help to protect against diseases, and this consumption depends mainly on the composition of the diet (CALDERÓN-OLIVER and PONCE-ALQUICIRA, 2018). Therefore, together with well-established food plants rich in flavonoids, neglected edible plant species can be a potential alternative source of these antioxidant compounds.

Kalanchoe is a genus of the Crassulaceae family, which has around 145 species (STEVENS, 2017), some of which are used as food in Brazil, mainly as salads or juices (KINUPP and LORENZI, 2014). The genus is native to tropical Africa and has been introduced throughout the tropics (BOITEAU and ALORGE-BOITEAU, 1995). Besides their use as food ingredients, the *Kalanchoe* species are especially known for their medicinal properties, with an exponential number of published studies in recent years. Our group has contributed to the chemical and pharmacological knowledge of some of them (e.g., *Kalanchoe pinnata* (Lam.) Pers., *Kalanchoe thyrsiflora* Harv., and *Kalanchoe daigremontiana* Raym.-Hamet & H. Perrier), with an emphasis on their flavonoid constitution and therapeutic value (COSTA et al., 2008; FERREIRA et al., 2014; ÜRMÉNYI et al., 2016).

Here, we focus our interest on *Kalanchoe fedtschenkoi* R. Hamet & H. Perrier (syn. *Bryophyllum fedtschenkoi*), a common species in Brazil, whose leaves are used not only for the preparation of juices and salads, but also for homemade jams, creams, and pickled leaves (KINUPP and LORENZI, 2014). This succulent plant differs from others of the genus, mainly by its natural grayish to brownish colored leaves, which also explains its use for ornamental purposes. Despite the leaves being used as a food, there are few reports on its flavonoid characterization, tissue location, and anatomical description (COSTA et al., 2008; ABDEL-RAOUF, 2012; CHERNETSKYY and WERYSZKO-CHMIELEWSKA, 2012), which would provide a better understanding of the leaf flavonoid composition of a given nutraceutical product. In addition, studies that contribute to the correct identification of plants are relevant to the quality control of the raw material. This is an important aspect to be considered in the food production chain, as well as in other industries that employ plants (NASCIMENTO and BARBOSA, 2007). In this sense, anatomical features are useful for identification. Unfortunately, few plants have been anatomically characterized,

* Corresponding authors

even though their misidentification and consequent inappropriate use can cause casualties (Tilney and Van Wyk, 2010). It is noteworthy that a preliminary study by our group confirmed the presence of the following glycosyl flavonoids in aqueous leaf extracts of *K. fedtschenkoi*: kaempferol 3-*O*-β-D-xylopyranosyl (1→2)-*O*-α-L-rhamnopyranoside 7-*O*-α-L-rhamnopyranoside (sagittatin A), kaempferol 3-*O*-β-D-xylopyranosyl (1→2)-*O*-α-L-rhamnopyranoside, and kaempferol 3-*O*-β-D-glucopyranoside 7-*O*-α-L-rhamnopyranoside. Among them, sagittatin A was identified as the major flavonoid in the leaf extract (Castricini, 2004; Costa et al., 2008). Recently, the presence of other phenolics, such as caffeic acid, kaempferol, and quercetin, has also been reported in the extracts of different parts of *K. fedtschenkoi*. These extracts were shown to inhibit the growth of some multidrug-resistant bacteria (Richwagen et al., 2019). However, despite the existence of such reports, a detailed characterization by LC-MS/MS of the flavonoids present in the *K. feldtschenkoi* leaf extracts was not conducted so far.

Based on the above, the aims of this study were (*i*) to describe the leaf anatomy of *K. fedtschenkoi*, (*ii*) to localize flavonoids in the leaf tissues, (*iii*) to provide a qualitative phenolic profile of aqueous leaf extracts, and (*iv*) to determine their *in vitro* antioxidant activity.

Materials and methods

Plant material

Five different specimens of *K. fedtschenkoi* R. Hamet & H. Perrier at the vegetative stage, grown under sunny conditions (758.1 ± 69.4 µmol m^{-2} s^{-1}) at the Federal University of Rio de Janeiro, UFRJ, Rio de Janeiro, Brazil (Lat.: 22º 51’ 32.8’’, Long.: 43 13’ 50.5’’), were used for this study. A voucher specimen (RFA 39965) was deposited at the Federal University of Rio de Janeiro Herbarium.

Anatomical studies

Fully expanded leaves were collected from the five plants, immediately fixed in FAA (Formaldehyde 37%, Glacial Acetic Acid, Ethanol 70%, 1:1:18; Sigma-Aldrich, Missouri, EUA) 70% (Johansen, 1940) for 48 h, and then preserved in ethanol 70%. Paradermal sections were mechanically made, and the fragments were stained with hydroalcoholic safranin (Johansen, 1940). Cross sections were made with a Ranvier microtome at the base, middle third, and the apex of the leaf blade and the proximal, median, and distal regions of the petiole. The sections were stained with a mixture of Astra blue and safranin (Sigma-Aldrich, Missouri, EUA) (Bukatsch, 1972), mounted in 50% glycerin, and observed and photographed with an Olympus CH30 light microscope.

Histochemical test using NP reagent

Fresh material was used to perform the histochemical analysis. Leaf fragments were treated with a 1% w/v 2-aminoethyl diphenylborinate (NP; Sigma-Aldrich, Missouri, EUA) methanolic solution and observed under a fluorescence Leica DMLB microscope (filter emission at 340-380 nm bandpass, and barrier filter at 425 nm) (Schnitzler et al., 1996; Casanova et al., 2020). In order to detect the autofluorescence of some constituents (e.g., lignin, suberin, cutin, chlorophyll, and secondary metabolites) (Talamond et al., 2015), untreated material was also analyzed.

Extraction and HPLC-DAD-ESI-MS/MS analysis

Pooled leaves (1.2 kg) obtained from five plants of *K. fedtschenkoi* were cut into small pieces, crushed, and infused (20% p/v) with distilled water. The extract was filtered on cotton and paper, and then lyophilized (43.3 g of extract dry weight).

HPLC-ESI-MS/MS analyses were carried out on a Thermo Scientific LCQ FLEET UHPLC system (Thermo Fisher Scientific) (Faculty of Pharmacy, UFRJ). All reagents used for HPLC analysis were HPLC grade, obtained from Tedia Brazil (Rio de Janeiro, Brazil). The extracts were diluted in water/acetonitrile (5:1) to a final concentration of 2 mg/mL. The following flavonoids, previously isolated by our group, were used as standards at 0.1 mg/mL: kaempferol 3-*O*-β-D-xylopyranosyl (1→2)-*O*-α-L-rhamnopyranoside 7-*O*-α-L-rhamnopyranoside (sagittatin A) from *K. fedtschenkoi* (physical and spectroscopic data provided as a Supplementary file), kaempferol 3-*O*-β-D-xylopyranosyl (1→2)-*O*-α-L-rhamnopyranoside from *K. daigremontiana* (Ürményi et al., 2016), and kaempferol 3-*O*-β-D-glucopyranoside 7-*O*-α-L-rhamnopyranoside from *Sedum dendroideum* (De Melo et al., 2009). The samples (10 µL) were run at 0.6 mL/min for 50 min in an RP-18 column (5 µm, 250 × 4 mm, Lichro-CART®/Lichrospher®100; Merck®) kept at 35 °C. The mobile phase consisted of an aqueous solution of 0.1% formic acid (A) and acetonitrile (B). The gradient used was 0-20 min (0-20% B), 20-40 min (20-22% B), 40-43 min (22-30% B), 43-45 min (30-100% B), 45-50 min (100% B), and the absorbance was monitored at 254 and 365 nm. MS measurements were carried out using helium as the collision gas in the ion trap, and nitrogen as the sheath and auxiliary gas in the source. For ESI in the negative ion mode, the following parameters were used: capillary temperature 400 °C, sheath gas flow 35 L/min, spray voltage 5.5 Kv, and capillary voltage 24 V. An isolation width of 2 Da was used with a 30 ms activation time for the MS/MS experiments. The collision energy value was set at 30% of the instrument maximum. The mass range was analyzed from m/z 50 to 1000.

Antioxidant activity assays

The antioxidant activity of the *K. fedtschenkoi* extracts was measured by the 2,2-diphenyl-1-picrylhydrazyl (DPPH; Sigma-Aldrich, Missouri, EUA) free-radical method, as described by Nascimento et al. (2013). An antioxidant standardized extract of *Ginkgo biloba* (Tebonin®; Herbarium - Paraná, Brazil) was used as a positive control. The percentage of antioxidant activity and the concentration of extract sufficient to obtain 50% of the total antioxidant activity (EC_{50}) were calculated using Microsoft Excel software (Microsoft Corporation, Washington, USA) (Nascimento et al., 2013).

Results and discussion

Anatomical characterization of the leaves

K. fedtschenkoi has petiolate, decussate, glabrous leaves (Fig. 1). The leaf blade is symmetrical and obovate, with green to grayish color, and the margin shows a pinkish color that tends to be more intense under high sunlight. Dormant buds are present at the margins, being located between the leaf teeth.

The epidermis has cells with sinuous anticlinal walls (Fig. 2a and 2b). The leaves are amphistomatic with the stomata containing one to four subsidiary cells, depending on the degree of development (Fig. 2a and 2b). The mature stomata are anisocytic or heliocytic (Fig. 2a and 2b). Cross sections of the leaf blade reveal an unstratified epidermis covered by a smooth cuticle. The cells exhibit rectangular to rounded sections (Fig. 2c and 2f). Under the epidermis a layer of rounded cells is observed, suggesting the presence of a hypodermis (Fig. 2c).

The chlorenchyma cells are isodiametric in the subepidermal position and elongated in the innermost layers, having large vacuoles (Fig. 2c - 2f). Three collateral bundles depart from the leaf base towards the apex. From these main veins smaller caliber collateral bundles depart, which almost reach the leaf edges in the region of the teeth. The main bundle is protected by collenchymatic tissue that is restricted to

Fig. 1: *Kalanchoe fedtschenkoi* R. Hamet & H. Perrier. Bar 10 cm.

the abaxial side at the base and middle third of the leaf blade (Fig. 2d) and surrounds the bundle at the leaf apex.

The petiole has an unstratified epidermis, composed of lightly flat cells, coated by the cuticle (Fig. 3b and 3e). Similar to that observed in the leaf blade, a distinguished layer of cells is also observed below the epidermis, which might be classified as a hypodermis. The chlorenchyma cells exhibit isodiametric sections (Fig. 3b - 3e), and the vascular bundles are collateral. There is one large vascular bundle in the center of the organ, and smaller caliber ones facing the adaxial side (Fig. 3c and 3d). The smaller bundles vary in number, from four in the proximal region to fifteen in the distal region.

Idioblasts occur throughout the leaf, mainly in the subepidermal layers, but also surrounding the vascular bundles, in vascular tissues, and randomly dispersed in the parenchyma (Fig. 2c - 2f; Fig. 3). These idioblasts are pink in the fresh material (Fig. 4 and 5), due to the presence of anthocyanins. When stained with a mixture of astra blue and safranin, they show a red to dark brown color (Fig. 3d).

In the Crassulaceae family, leaves usually have an unstratified epidermis with cells transversely elongated in relation to the longitudinal axis (Metcalfe and Chalk, 1950), as observed here for *K. fedtschenkoi*. This was also observed in *K. daigremontiana*, *K. pumila* Baker, and *K. delagoensis* Ecklon & Zeyher (Balsamo and Uribe, 1988; Chernetskyy and Weryszko-Chmielewska, 2012; Casanova et al., 2020). Similar to our findings, the sinuous anticlinal walls on the epidermal cells were already described in *K. pinnata* (Moreira et al., 2012). Studies indicate that this sinuosity varies according to the plant environment, being less pronounced when the environment is more arid (Sharma and Dunn, 1968). As such, this anatomical feature might change according to the environmental conditions in which this plant grows.

The presence of amphiestomatic leaves is a common characteristic of the Crassulaceae family. These stomata are usually anisocytic, but helicocytic stomata may also occur (Metcalfe and Chalk, 1979). Anisocytic stomata have been also described for *K. pinnata*, *K. crenata* (Andrews) Haw., *K. pumilla*, and *K. delagoensis* (Chernetskyy and Weryszko-Chmielewska, 2012; Moreira et al., 2012; Casanova et al., 2020). Similarly, the occurrence of stomata at different stages of development is also a common trait of the genus and has already been noted in *K. pinnata*, *K. crenata*, *K. pumilla*, and *K. delagoensis* (Chernetskyy and Weryszko-Chmielewska, 2012; Moreira et al., 2012; Casanova et al., 2020), being related to the leaf development process of succulent plants. The formation of stomata in these plants begins during leaf expansion, allowing new stomata to appear among those already developed (Gibson, 1996).

The presence of a hypodermis in leaves of *Kalanchoe* species have been already described for *K. tubiflora* and *K. daigremontiana* (Balsamo and Uribe, 1988; Abdel-Raouf, 2012). However, as the correct classification of a hypodermis requires an ontogenetic analysis, which was not conducted in our study, we cannot definitely classify the subepidermal tissue observed here in the leaves of *K. fedtschenkoi* as a hypodermis, even with its similar characteristics. Indeed, the presence of a hypodermis in *K. fedtschenkoi* was not mentioned in any publication referring to this species.

Anatomical features common to CAM plants, such as succulent leaves and a mesophyll with isodiametric parenchyma cells exhibiting thin walls and large vacuoles, were also observed here in *K. fedtschenkoi*. These characteristics allow the storage of water and malic acid during carbon dioxide fixation at night, promoting the good metabolic performance of these species. A chlorophyll parenchyma differentiated in palisade and sponge is rare in succulent leaves (Gibson, 1996). Despite the lack of differentiation in a palisade parenchyma, the parenchymatic cells present in *K. fedtschenkoi* leaves are not isodiametric. These cells are isodiametric near to the epidermis but elongated in the innermost layers of the leaf limb. No record of this pattern has been mentioned in the literature, neither for plants of the genus *Kalanchoe*, nor for other Crassulacean species.

The vascular bundles of *K. fedtschenkoi* are collateral, as already described for other species of the genus (Chernetskyy and Weryszko-Chmielewska, 2012; Moreira et al., 2012; Casanova et al., 2020). The larger caliber bundles have already been shown to be surrounded by collenchymatous parenchyma in *K. pinnata* and *K. crenata* (Moreira et al., 2012), or by sclerenchymatous fibers in *K. pumila* (Chernetskyy and Weryszko-Chmielewska, 2012).

Moreover, in the leaves and petiole of *K. fedtschenkoi*, the vascular bundles are arranged in an arch when viewed in cross sections. Such a feature is mentioned by Metcalfe and Chalk (1950) for other members of the Crassulaceae family.

The presence of anthocyanic idioblasts (naturally colored pink) is common in the Crassulaceae family, as observed here in the leaves of *K. fedtschenkoi*, and they may occur isolated in some organs or in the whole plant (Metcalfe and Chalk, 1950). These anthocyanins are easily noticed in the margins of *K. fedtschenkoi* leaves. Idioblasts with the same characteristics and location have also been observed for *K. pinnata*, *K. crenata*, *K. daigremontiana*, and *K. pumila* (Balsamo and Uribe, 1988; Chernetskyy and Weryszko-Chmielewska, 2012; Moreira et al., 2012).

Anthocyanins are water-soluble pigments, especially found in the vacuole of plant cells. These compounds are the most important group of non-photosynthetic pigments and have colors that vary between red, pink, and purple. As the main pigment of fruits, flowers, and leaves, anthocyanins play an essential role in the attraction of pollinators and in seed dispersal in reproductive organs, besides having a high antioxidant capacity (Kong et al., 2003).

Histochemical location and differentiation of phenolic substances in leaves using NP reagent

NP or Naturstoff's reagent (2-aminoethyl diphenylborate) is used in thin layer chromatography analyses (Wagner and Bladt, 2001) in

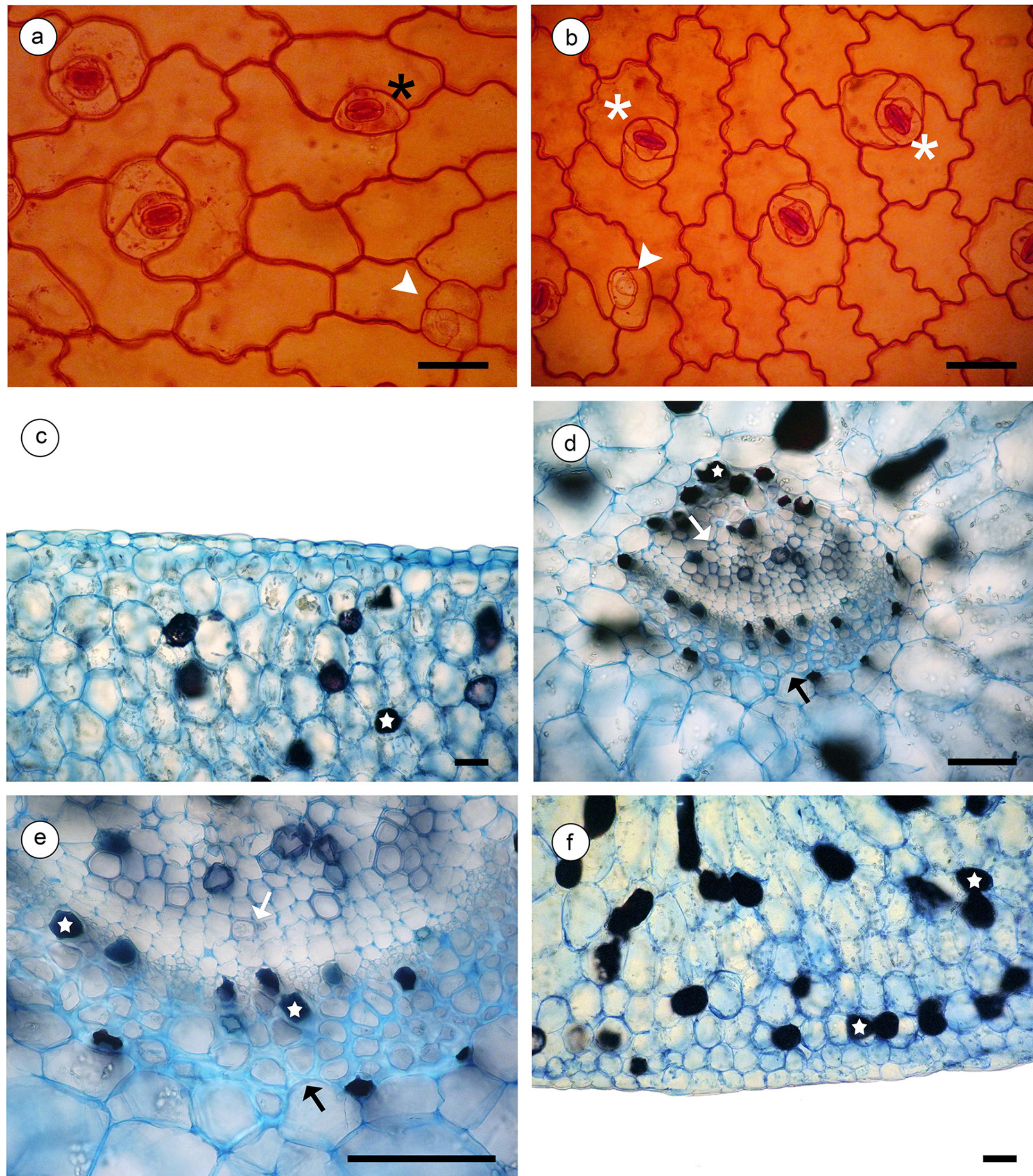


Fig. 2: Sections of the middle third of the *Kalanchoe fedtschenkoi* leaf blade. a - b – paradermal sections revealing cells with sinuous anticline walls: adaxial surface (a) and abaxial surface (b); black asterisks: anisocytic stomata; white asterisks: helicocytic stomata; arrowheads: developing stomata. c - f – cross sections: adaxial side (c); main vascular bundle with collenchymatic tissue (black arrow) (d); detail of the main vascular bundle (e); abaxial side (f); idioblasts with dark brown content (stars). Bars 100 µm.

order to evidence the presence of different classes of polyphenols in plant samples. It is also used to reveal these substances in plant tissue sections (CASANOVA et al., 2020). While flavonoids are revealed as orange, yellow, or green by this reagent; hydroxycinnamic and hydroxybenzoic acid derivatives are highlighted in blue and greenish-blue color (WAGNER and BLADT, 2001). Some compounds exhibit an intrinsic fluorescence (autofluorescence), which does not require the addition of NP in order to be observed. These include chlorophyll, which shows a red color (LANG et al., 1991) and lignin, which fluoresces in blue (DONALDSON and RADOTIC, 2013).

Untreated *K. fedtschenkoi* leaf material (control) revealed autofluorescence of the cell constituents when observed under a fluorescence microscope (UV radiation, 340-380 nm). Red chloroplasts (Figs. 4b, 4d, and 4f; Figs. 5b, 5d, and 5f), vessel elements in blue (Fig. 4d; Fig. 5d), and some blue scattered cells in the mesophyll and around the vascular bundle (Figs. 4b, 4d, and 4f; Figs. 5b, 5d, and 5f) can be observed. Some cells containing anthocyanins showed autofluorescence (Figs. 4a, 4b, 4e, and 4f; Figs. 5a and 5b), while others did not (Fig. 5a-d). These aspects were observed in the apex, middle third, base, and leaf margin, as well as in the petiole.

In the sections treated with NP (Fig. 6; Fig. 7), it was possible to notice the presence of different colors when observed under UV radiation (340-380 nm), indicating the presence of distinct phenolic substances. A yellow color, indicating the presence of flavonoids such as kaempferol and/or apigenin derivatives, was observed in the regular epidermis cells, subsidiary cells, and leaf mesophyll (Figs. 6b, 6d, and 6f; Figs. 7a, 7b, and 7f), in the apex, middle third, base, and leaf margin. In addition, an orange fluorescence was observed in the cells located below the epidermis, throughout the leaf blade, in the leaf apex, and in the stomata guard-cells (Fig. 7a and 7b), suggesting

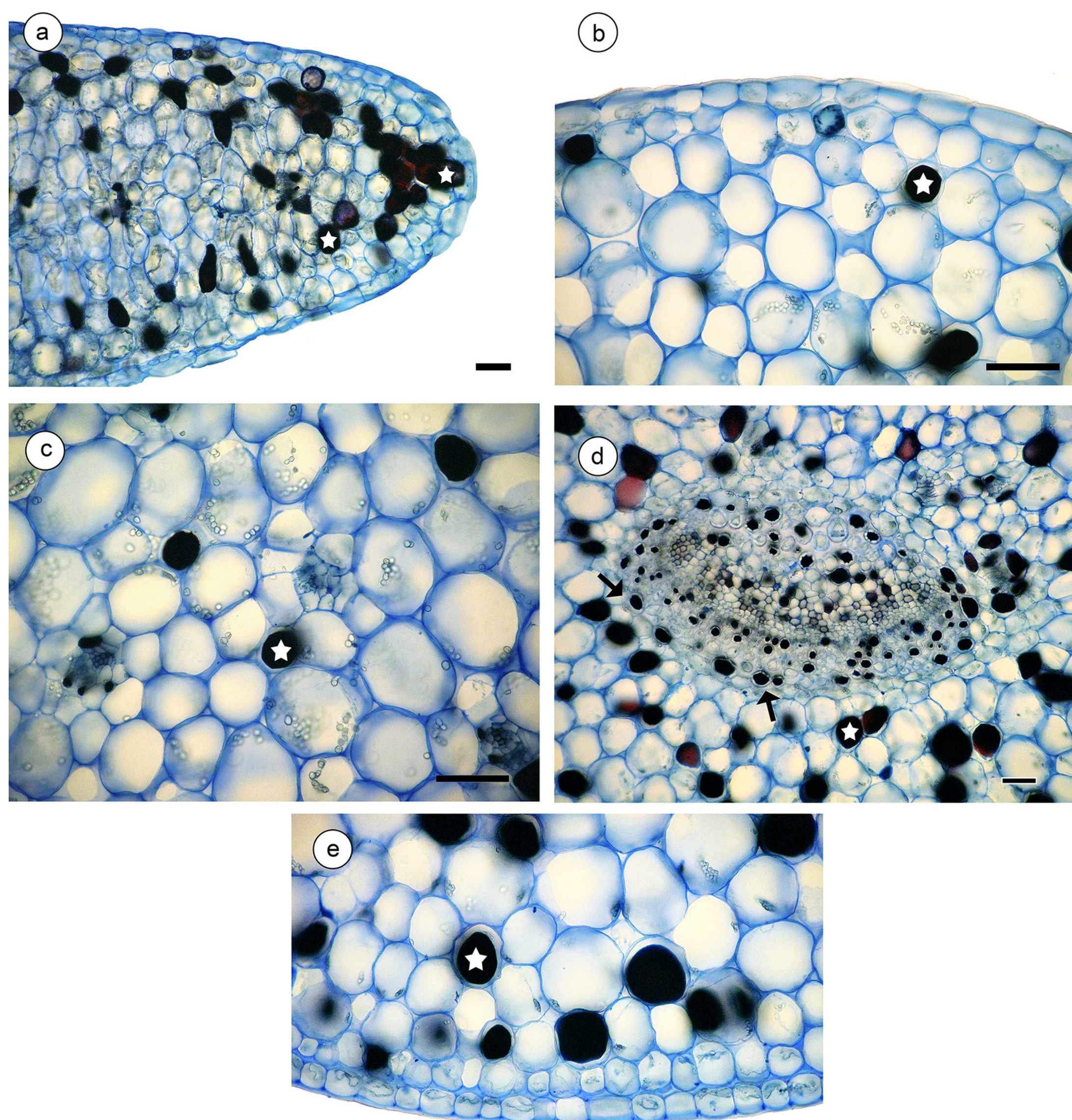


Fig. 3: Cross sections of *Kalanchoe fedtschenkoi* leaves. a - leaf blade margin at the middle third, b - e – petiole at the median region: adaxial surface (b); small caliber vascular bundles (c); main vascular bundle (d); abaxial side (e); parenchyma with thickened wall (black arrows); idioblasts with reddish brown content (stars). Bars 100 µm.

the presence of other classes of flavonoids (quercetin and/or luteolin derivatives). In the petiole (Fig. 7c - 7f), cells colored in yellow were observed in the epidermis and in one to three layers below it (Fig. 7f). In addition, cells fluorescing in orange were observed in the epidermis and in the first subepidermal layer (Fig. 7d). These results were observed in the proximal, median, and distal regions of the expanded petiole.

As described above, flavonoids were mainly observed in superficial layers or especially facing the adaxial side. Therefore, we could suppose that they might be related to the protection against high solar/UV radiation and oxidative stress. Indeed, flavonoids play an important role in many aspects of plant survival, development, and interactions with environmental factors such as UV protection (BASKAR et al., 2018).

UV-B radiation is known to be harmful, not only to animals but also to plants (JANSEN et al., 1998). It can damage the DNA, RNA, proteins, and cell membranes, and also destroy the photosynthetic apparatus, causing changes in plant growth, development, and morphology (JANSEN et al., 1998). The distribution pattern of phenolic substances in leaf tissues is closely related to their protective function against UV-B radiation and also to their high antioxidant activity. Flavonoids located in the cellular cytoplasm are believed to play a role in antioxidant defense, whereas those located in the cell walls and vacuoles most likely act to protect the plant against excess UV-B radiation (AGATI et al., 2020).

Phenolic substances, including flavonoids and other phenylpropanoids, have a high antioxidant potential. This property is attributed to their chemical structures; once oxidized, they are capable of generating radicals (or ions) stabilized by the delocalization of the electron over the adjacent *p* orbitals of their conjugated double bonds. Moreover, since phenolic compounds are weak acids, they are able to donate protons. Thus, these compounds can stabilize highly reactive molecules such as free radicals and remain stable due to the resonance effect. They can still act as reducing agents when complexed with

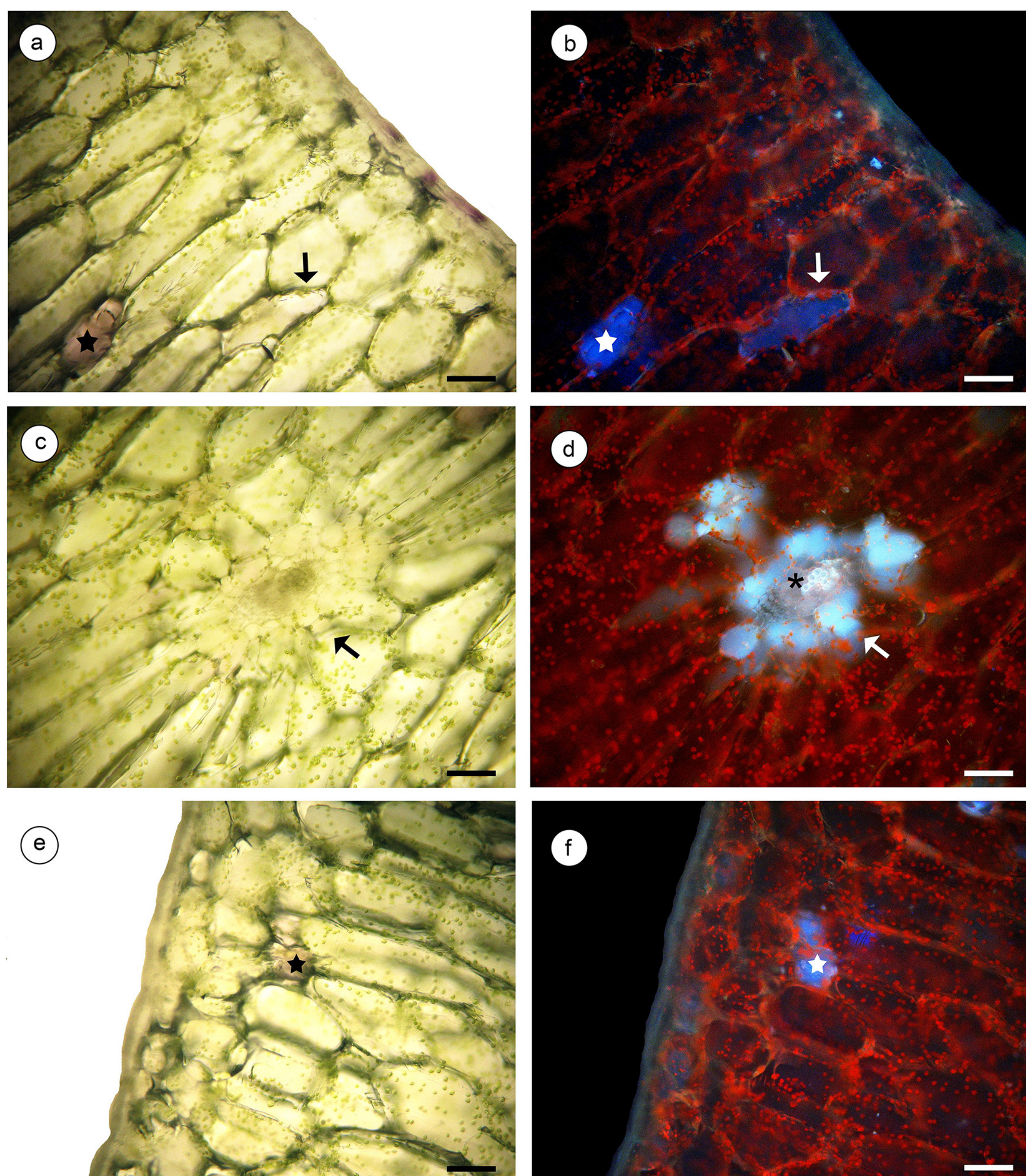


Fig. 4: Cross sections of *Kalanchoe fedtschenkoi* leaf blade at the middle third (control): adaxial side (a, b); main vascular bundle (c, d); abaxial side (e, f); visible light (a, c, e); UV light (b, d, f); anthocyanic idioblasts with pink color under white light and with blue fluorescence under UV (stars); blue fluorescent cells (arrows); vessel elements fluorescent in light blue (asterisk). Bars 100 µm.

metals such as Fe^{3+}, Mg^{2+}, and Al^{3+} (Vermerris and Nicholson, 2006).

Flavonoid composition and antioxidant activity of *Kalanchoe fedtschenkoi* aqueous leaf-extract

Aqueous leaf extracts of *K. fedtschenkoi* were analyzed by HPLC-DAD-ESI-MS/MS in the negative ion mode. The structures of the substances corresponding to the 9 peaks of the chromatogram (Fig. 8, Tab. 1) were inferred based on their UV spectra, MS/MS fragmentation pattern, the literature, and databases (Pereira et al., 2015; MoNA, 2020).

Quercetin di-*O*-glycosides were attributed to peaks at Rt 15.0 min, Rt 15.8 min, and Rt 17.8 min, while peaks at Rt 15.4 min, Rt 19.4 min, and 20.0 Rt min were putatively identified as kaempferol tri-*O*-glycosides. Flavonoid *O*-glycosides, such as those detected in the extracts of *K. fedtschenkoi*, present a typical MS/MS fragmentation pattern resulting from the cleavage of interglycosidic bonds, originating aglycone ions with characteristic m/z (kaempferol: m/z 285; quercetin: 301 and methylquercetin 315) (Cuyckens and Claeys, 2004).

Flavonoids corresponding to peaks at Rt 17.9 min, Rt 21.3 min, and Rt 32.0 min were previously isolated from leaves of the species (Castricini, 2004; Costa et al., 2008) and were fully identified in the present study by comparison of their retention time and MS spectra with those of the isolated substances. Their structures are shown in Fig. 9: kaempferol 3-*O*-glucopyranoside 7-*O*-rhamnopyranoside (Rt 17.9 min; **1**), kaempferol 3-*O*-β-D-xylopyranosyl (1→2)-*O*-α-L-rhamnopyranoside 7-*O*-α-L-rhamnopyranoside or sagittatin A (Rt 21.3 min; major flavonoid; **2**), and kaempferol 3-*O*-β-D-xylopyranosyl (1→2)-*O*-α-L-rhamnopyranoside (Rt 32.0 min; **3**). The complete structural identification of flavonoids **1** and **3** was previously reported by our group (De Melo et al., 2009; Ürményi

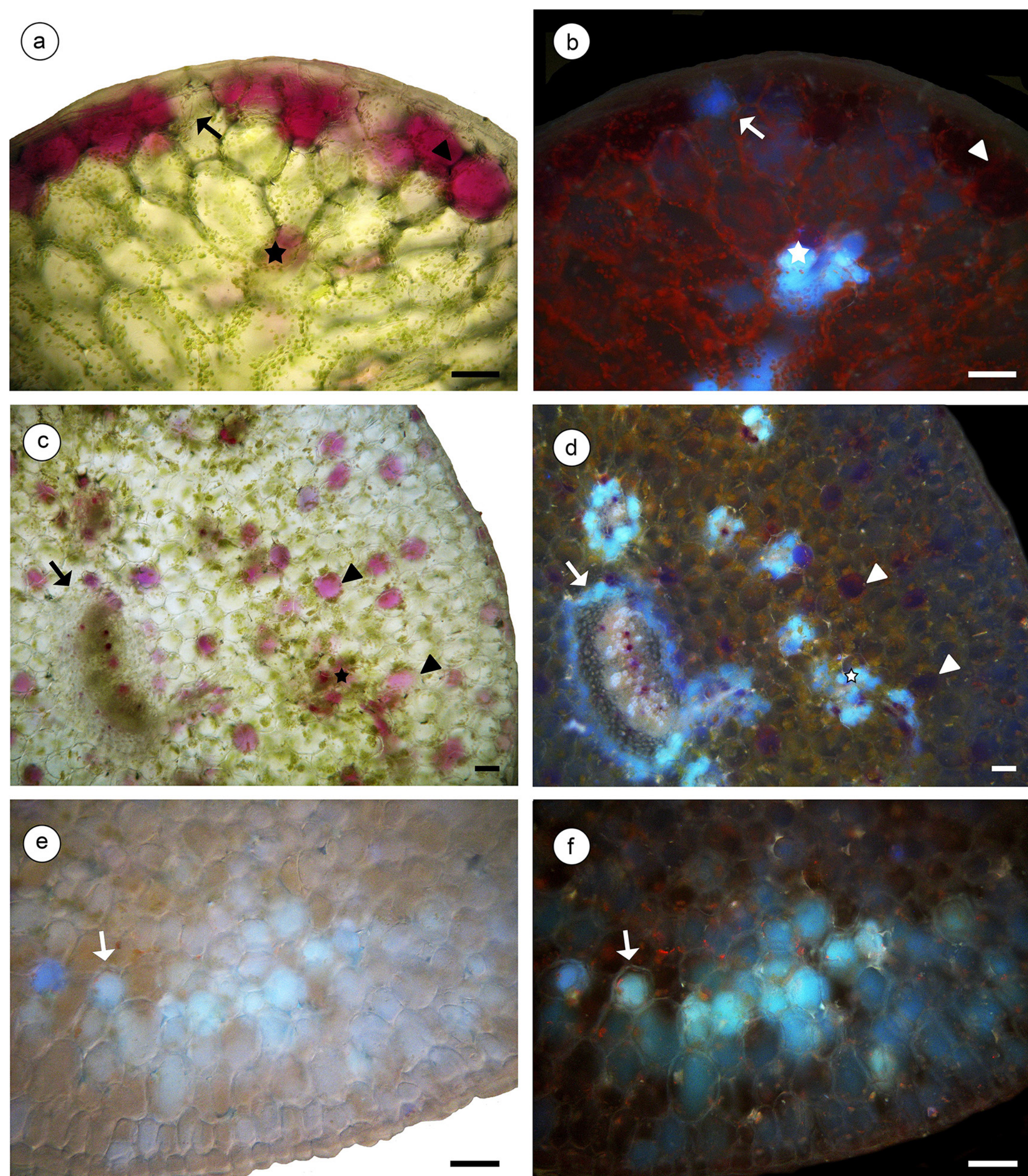


Fig. 5: Cross sections of *Kalanchoe fedtschenkoi* leaves. a - b – margin of the leaf blade at the middle third (control): visible light (a); UV light (b). c - f – petiole in median region: adaxial side and main vascular bundle (c, d); abaxial side (e, f); visible light (c); UV light (d, f); overlap of visible light and UV light (e); anthocyanic idioblasts with pink color under white light and with blue fluorescence under UV (stars); anthocyanic idioblasts with pink color under white light and without fluorescence under UV (arrow heads); blue fluorescent cells (arrows). Bars 100 µm.

et al., 2016). The spectroscopic data of flavonoid 2, identified as sagittatin A, are shown in Supplementary file. Structural elucidation of this flavonoid was based on analysis of ^{1}H NMR, ^{13}C NMR, HMQC and HMBC data obtained in CD_3OD (300 MHz spectrometer; Tab. S1 and Fig. S1-S9). The kaempferol aglycone was deduced from the ^{1}H NMR pattern in the aromatic region (Fig. S2 and S3): two doublet signals at δ 6.92 and 7.73 assigned to H-3', -5' and H-2', -6', respectively; and two doublet signals at δ 6.38 (H-6) and 6.64 (H-8). The presence of two rhamnopyranosyl units (H-1'', 5.42 ppm, singlet; H-1'''' 5.56 ppm, singlet) and a xylopyranosyl moiety (H-1''', 4.32 ppm; doublet) was deduced from ^{1}H NMR, ^{13}C NMR (Fig. S4) and HMQC data (Fig. S5 and S6). The spin systems of the carbohydrates as well as their attachment position was assigned based on ^{13}C NMR, HMQC and HMBC data (Fig. S7-S10). ^{1}H-^{13}C long-range correlations (HMBC) were observed between H-1'' (δ 5.42) and C-3 (δ 136.6) and between H-1'''' (δ 5.56) and C-7 (δ 163.0), which allowed to stablish the link of the two rhamnopyranosyl units to the 3- and 7-positions at the aglycone, respectively. Moreover, the long-range correlation between H-1''' (δ 4.32) and C-2'' (δ 82.3) allowed us to deduce the link of the xylopyranosyl moiety to the 2''-position of the 3-*O*-rhamnopyranosyl unit. The comparison of the ^{1}H and ^{13}C NMR data of the flavonoid with those of sagittatin A, previously isolated from *Epimedium sagittatum* (Oshima et al., 1989) and from *Kalanchoe streptantha* (Costa et al., 1996) afforded further confirmation of the structure.

It is noteworthy that most flavonoids occur as glycosides in nature. The glycosylation increases the solubility and facilitates their transportation in aqueous environments (such as cells), besides protecting the hydroxyl groups from autooxidation. In particular, the main compounds here detected in leaf aqueous extracts of *K. fedtschenkoi* were flavonol glycosides (quercetin and kaempferol derivatives). Indeed, flavonoids are known to be the most important chemical class

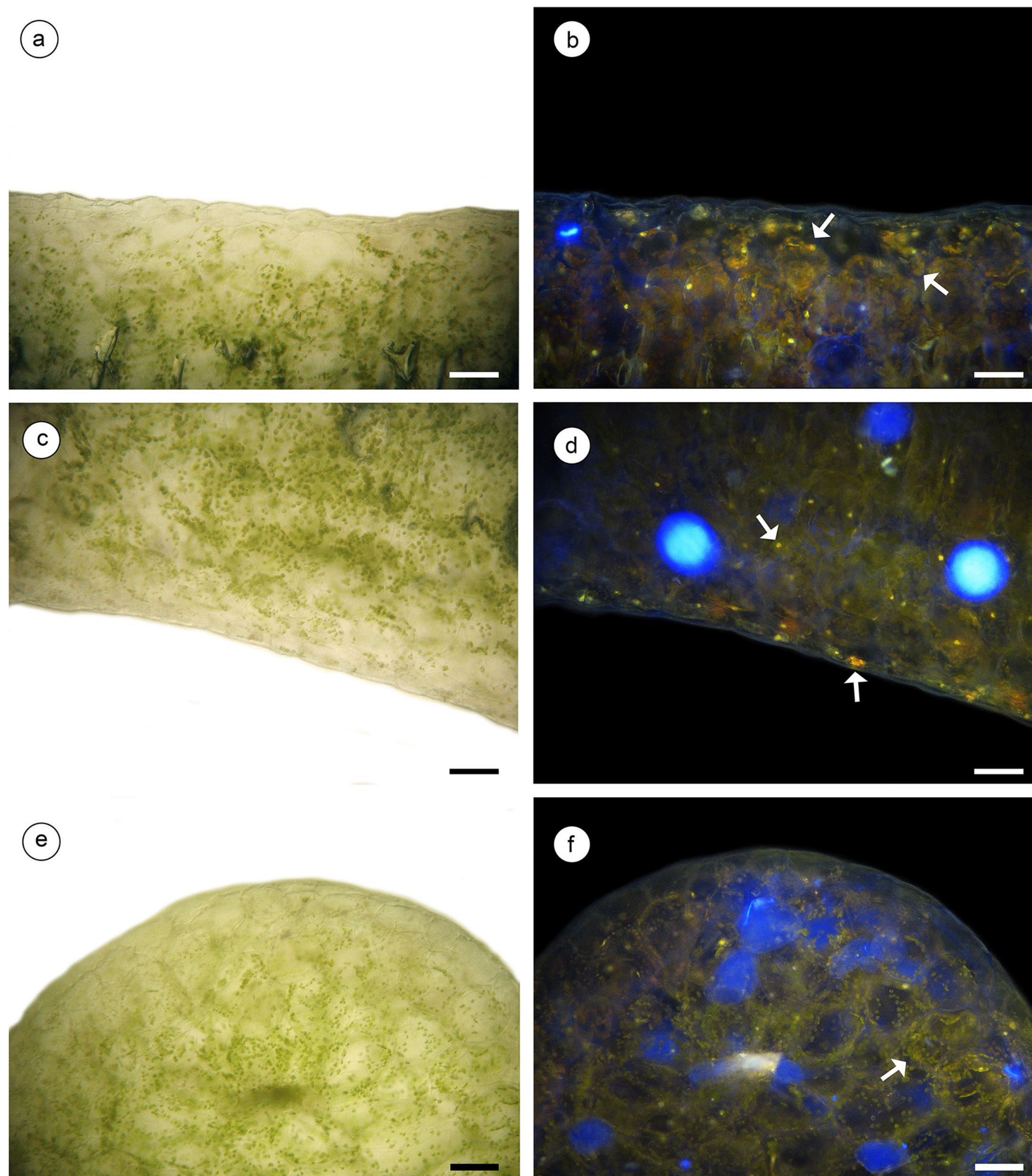


Fig. 6: Cross sections of *Kalanchoe fedtschenkoi* leaf blade at the middle third (NP treatment): adaxial side (a, b); abaxial side (c, d); leaf margin (e, f); visible light (a, c, e); UV light (b, d, f); yellow flavonoids (arrows). Bars 100 µm.

in *Kalanchoe* species, mostly represented by flavonols (COSTA et al., 2008). Therefore, considering the richness of glycosylated flavonoids in plants of the genus and their water-soluble nature, water, commonly used in homemade preparations, is expected to be an excellent solvent for the obtention of these nutraceutical molecules.

In addition, it is important to highlight that the extraction process used in this study do not reflect the native phenolic profile of the plant but corresponds to the chemical constitution of an aqueous preparation such as those commonly used for *K. fedtschenkoi* consumption. In fact, we cannot exclude the possible enzymatic transformation of some phenolic metabolites present in the native plant material since we did not apply an enzyme deactivation process. However, the use of fresh leaf material and their aqueous extraction is very common in studies focusing on nutraceutical and pharmaceutical applications (TCHABO et al., 2018).

The major flavonoid, a kaempferol triglycoside (sagittatin A), was previously reported in *K. streptantha* leaves (COSTA et al., 1996). Sagittatin A was originally isolated from *Epimedium sagittatum* (OSHIMA et al., 1989), a well-known Chinese medicinal plant from the Berberidaceae family. Later, this flavonoid was also found in other plant species (YONG and NTIE-KANG, 2015), including the leaves of *Cinnamomum osmophloeum*, which have the potential application in food supplements (SENTHIL KUMAR et al., 2019). As far as we know, there are no studies on the therapeutic properties of this flavonoid.

Kaempferol 3-*O*-β-D-xylopyranosyl (1→2)-*O*-α-L-rhamnopyranoside was also reported in *K. daigremontiana* and *K. pinnata* (FÜRER et al., 2013; ÜRMÉNYI et al., 2016). The latter is also a local food in Brazil (KINUPP and LORENZI, 2014). This flavonoid showed *in vitro* activity against herpes virus (HSV-1 and HSV-2) (ÜRMÉNYI et al., 2016). In addition, the minor kaempferol di-*O*-glycoside identified as kaempferol 3-*O*-glucopyranoside 7-*O*-rhamnopyranoside has already been reported in the leaves of *K. pinnata* (TATSIMO et al., 2012). This

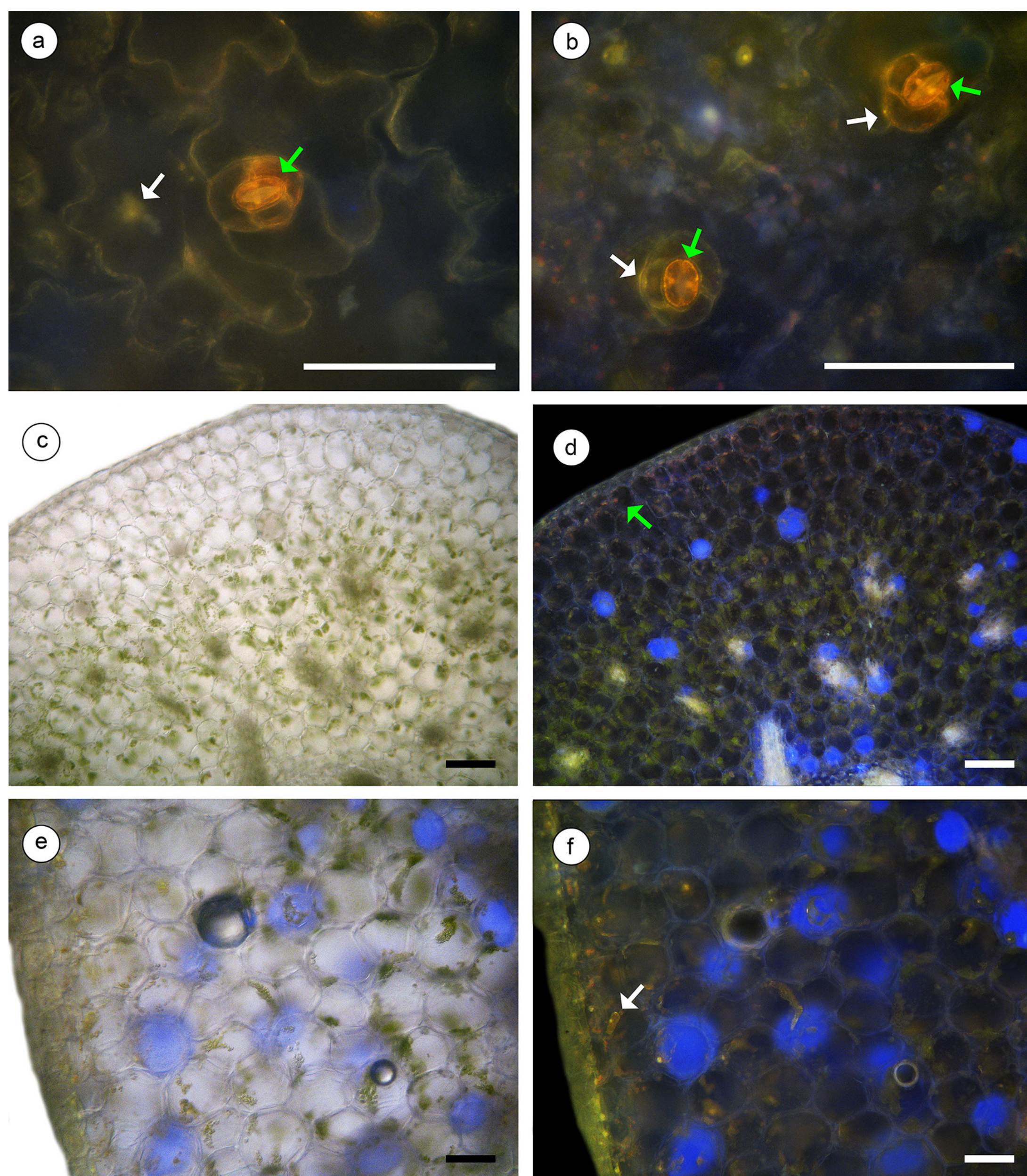


Fig. 7: Paradermal sections of *Kalanchoe fedtschenkoi* leaf blade at the middle third (NP treatment): adaxial surface (a, b). Cross sections of petiole at the median region (NP treatment): adaxial side (c, d); abaxial side (e, f); visible light (c); UV light (d, f); overlap of visible light and UV light (e); yellow flavonoids (white arrows); orange flavonoids (green arrows). Bars 100 µm.

flavonoid has anti-inflammatory (De Melo et al., 2009), antioxidant, and antimicrobial activity (Tatsimo et al., 2012). Other authors have demonstrated this flavonoid has inhibitory activity towards acetylcholinesterase and β-amyloid aggregation (both related to Alzheimer's disease) (Guo et al., 2016), and neuroactive behavior (Loscalzo et al., 2009), as well as inhibiting lipid accumulation in liver cells (An et al., 2019). Additionally, it also showed cytotoxicity against human tumor cell lines HL60 and AZ521 (Zhang et al., 2018).

Kaempferol glycosides were predominant in *K. fedtschenkoi* leaf extracts, along with minor quercetin derivatives. Flavonols are known to be the most common flavonoids in the *Kalanchoe* species, most of them with patuletin, quercetin, and kaempferol aglycones, while flavones are less frequent (Costa et al., 2008). These substances are frequently related to the biological activities of the *Kalanchoe* species (Costa et al., 2008; Ferreira et al., 2014; Ürményi et al., 2016). As no apigenin or luteolin derivatives (flavones) were identified in the analyzed extracts, the substances revealed in yellow and orange in leaf tissues stained with NP probably correspond to kaempferol and quercetin derivatives, respectively.

Kaempferol glycosides, in particular, have been highlighted as important bioactive molecules, showing several pharmacological effects, including anti-inflammatory, analgesic, antimicrobial, anticancer, cardio and neuroprotective, antidiabetic, and anti-osteoporotic (Kim et al., 2018). Indeed, these glycosides are the most common form of dietary ingestion of kaempferol compounds. Despite their high polarity, which could hamper the absorption of these substances, there is evidence showing they can be absorbed without hydrolysis (Dabeek and Marra, 2019).

In this study, the aqueous leaf extracts of *K. fedtschenkoi* were also evaluated for their *in vitro* antioxidant activity. Their mean EC_{50} was

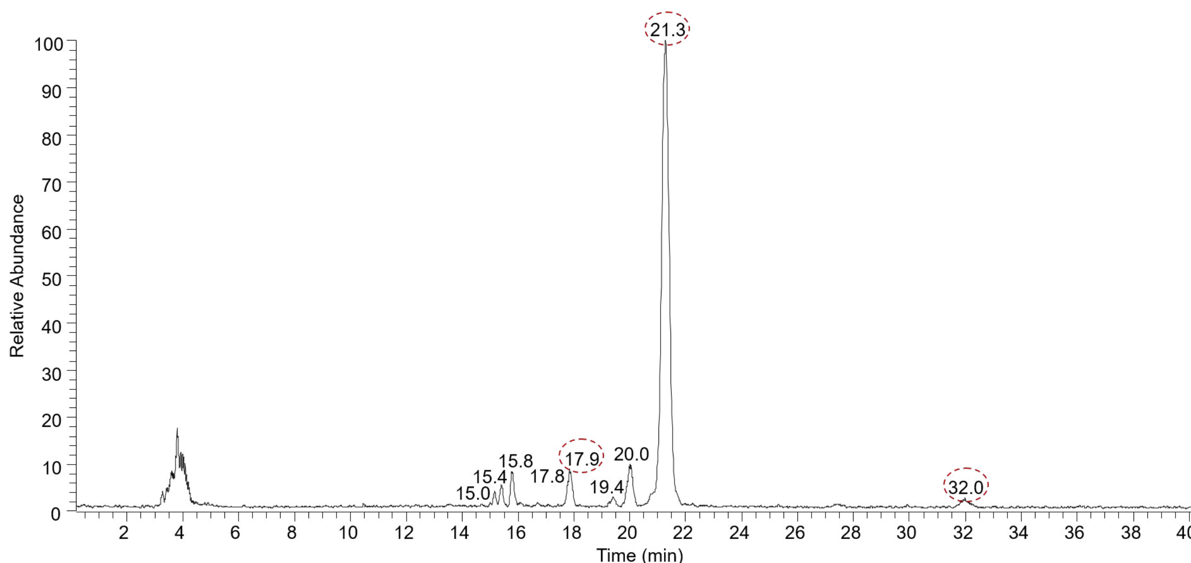
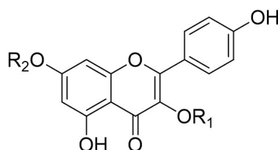


Fig. 8: Base peak chromatogram (HPLC-DAD-ESI-MS/MS) of a representative *Kalanchoe fedtschenkoi* aqueous leaf-extract (2 mg/mL) in the negative ion mode. Marked peaks correspond to substances identified based on comparison with pure compounds: kaempferol 3-*O*-glucopyranoside 7-*O*-rhamnopyranoside (Rt 17.9 min), kaempferol 3-*O*-β-D-xylopyranosyl (1→2)-*O*-α-L-rhamnopyranoside 7-*O*-α-L-rhamnopyranoside (Rt 21.3 min; major flavonoid), and kaempferol 3-*O*-β-D-xylopyranosyl (1→2)-*O*-α-L-rhamnopyranoside (Rt 32.0 min).

Tab. 1: Chromatographic and spectrometric properties of flavonoids detected in *Kalanchoe fedtschenkoi* aqueous leaf-extract by HPLC-DAD-ESI-MS/MS in the negative ion mode.

Rt (min)	λmax (nm)	$[M–H]^-$	Fragment ions	Proposed structure
15.0	270, 350	625	463, 301, 300	Quercetin di-*O*-hexoside
15.4	266, 341	725	563, 431, 284	Kaempferol-*O*-hexoside-*O*-deoxyhexoside-*O*-pentoside
15.8	266, 349	609	463, 301	Quercetin-*O*-hexoside-*O*-deoxyhexoside
17.8	270, 350	623	477, 461, 315	Methylquercetin-*O*-hexoside-*O*-deoxyhexoside
17.9	268, 344	593	447, 431, 285	Kaempferol 3-*O*-glucopyranoside 7-*O*-rhamnopyranoside*
19.4	266, 344	739	593, 285	Kaempferol *O*-hexoside-di-*O*-deoxyhexoside
20.0	266, 344	739	593, 431, 285	Kaempferol *O*-hexoside-di-*O*-deoxyhexoside
21.3	265, 341	709	563, 431, 285, 284	Kaempferol 3-*O*-β-D-xylopyranosyl (1→2)-*O*-α-L-rhamnopyranoside 7-*O*-α-L-rhamnopyranoside* (Saggitatin A)
32.0	264, 344	563	431, 413, 285	Kaempferol 3-*O*-β-D-xylopyranosyl (1→2)-*O*-α-L-rhamnopyranoside*

* Substances identified based on comparison of their retention time and spectra with those of the respective isolated compounds.



1. R_1 = glucose; R_2 = rhamnose
2. R_1 = xylose (1→2)-*O*-rhamnose; R_2 = rhamnose
3. R_1 = xylose (1→2)-*O*-rhamnose; R_2 = H

Fig. 9: Flavonoids identified in *Kalanchoe fedtschenkoi* aqueous leaf-extracts based on comparison with pure compounds: kaempferol 3-*O*-glucopyranoside 7-*O*-rhamnopyranoside (**1**), kaempferol 3-*O*-β-D-xylopyranosyl (1→2)-*O*-α-L-rhamnopyranoside 7-*O*-α-L-rhamnopyranoside (**2**; sagittatin A), and kaempferol 3-*O*-β-D-xylopyranosyl (1→2)-*O*-α-L-rhamnopyranoside (**3**).

8.32 ± 1.43 µg/mL, which was very similar to that of the positive control (8.94 ± 0.9 µg/mL), a standardized *Ginkgo biloba* extract (Tebonin®), well known for its high antioxidant activity. Our findings indicate that *K. fedtschenkoi* has great antioxidant potential, which should be confirmed in further *in vitro* and *in vivo* studies.

The overproduction of reactive oxygen species (ROS), which are known to cause oxidative stress and tissue damage, is involved in the physiopathological mechanisms of several inflammatory and neurodegenerative diseases (SOGA et al., 2012). Thus, natural antioxidant substances, especially flavonoids, are believed to play an important role in their prevention and treatment (YAO et al., 2004; AHN-JARVIS et al., 2019). Some studies have evidenced an inverse relationship between dietary intake of flavonoids and cardiovascular diseases, diabetes, mental illness, and the risk of some types of cancer (AHN-JARVIS et al., 2019). Indeed, the food concept is changing worldwide. Nowadays, there is an increased focus on the use of foods not only as nutritional sources, but also as functional and nutraceutical ingredients, aiming to provide health and better well-being (JANABI et al., 2020). Therefore, the search for food sources of bioactive molecules and powerful antioxidant agents, which include non-commercial crops, is progressing. The higher consumption of plant-based foods rich in these molecules might attenuate the incidence of ROS-related cardiovascular and degenerative diseases, consequently increasing the human lifespan (JANABI et al., 2020).

Due to its CAM characteristics, *K. fedtschenkoi* is a widespread species, even in harsh environments such as those of some Brazilian biomes. In addition, similar to other *Kalanchoe* species, *K. fedtschenkoi* is easily cultivated by vegetative propagation (Jaiswal and Sawhney, 2006; Kinupp and Lorenzi, 2014). Considering these features, the species might represent a rich and accessible source of nutraceutical compounds, supporting its use as a food by the population. It is important to point out that further analysis focusing on the toxicity of this plant material should be performed, despite a low toxicity being expected, considering previous reports on water preparations of other *Kalanchoe* species (Nascimento et al., 2018).

Conclusion

We report for the first time the complete anatomical description of *Kalanchoe fedtschenkoi* leaves and reveal the location of flavonoids in the leaf tissues, helping to better characterize the species through anatomical and histochemical analyses. In addition, we report the phenolic profile and the antioxidant activity of *K. fedtschenkoi* leaf extracts. Our findings contribute to the knowledge of the potential nutraceutical compounds of this non-conventional food plant and evidence the possible health benefits of this edible species, encouraging future investigations on its possible toxicity, and its therapeutic and nutritional properties.

Acknowledgments

This work was supported by the Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) APQ1- 210.573/2016. R. K. Y. thanks CNPq for PIBIC fellowship (Proc. 154048/2014-3, 155601/2013-0). We are grateful to Fernanda G. Ürményi and Giany O. Melo for samples of the flavonoids kaempferol 3-*O*-β-D-xylopyranosyl (1→2)-*O*-α-L-rhamnopyranoside and kaempferol 3-*O*-glucopyranoside 7-*O*-rhamnopyranoside, respectively.

Author contributions

Study design, conceptualization, and supervision: E.S.T. and S.S.C; Anatomical and histochemical analyses: E.S.T, J.M.C, and L.B.S.N; Chemical analyses: S.S.C., L.M.C., J.E.S., S.D.C and R.K.Y.; Writing – original draft preparation: J.M.C., L.M.C., and L.B.S.N.; Writing – review and editing: E.S.T., S.S.C, J.M.C., L.B.S.N., L.M.C., J.E.S., and R.K.Y. All authors have read and agreed to the published version of the manuscript.

Conflict of interest

No potential conflict of interest was reported by the authors.

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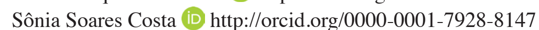
ORCID

Luana Beatriz dos Santos Nascimento https://orcid.org/0000-0002-1827-1672

Livia Marques Casanova http://orcid.org/0000-0002-0697-1575


Sônia Soares Costa http://orcid.org/0000-0001-7928-8147

Eliana Schwartz Tavares http://orcid.org/0000-0001-9668-2330

Address of the corresponding authors:
Eliana Schwartz Tavares: Laboratório de Anatomia Vegetal, Departamento de Botânica, Instituto de Biologia, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, 21 941-902, Rio de Janeiro, RJ, Brazil
E-mail: elianast@gmail.com
Sônia Soares Costa: Instituto de Pesquisas de Produtos Naturais, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, 21 941-902, Rio de Janeiro, RJ, Brazil
E-mail: sscostabh@gmail.com

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Supplementary material

Physical and spectroscopic data for Sagittatin A isolated from *Kalanchoe fedtschenkoi* leaves

$[\alpha]_D^{25}$ = -50 (c = 0.1; H_2O)

Melting point: 202-205 ºC

NMR data: Table S1 and Figures S1-S10.

NMR experimental conditions

The NMR spectra of sagittatin A were recorded on a Bruker VRX-300 (1H, 300 MHz; 13C, 75 MHz) spectrometer at IPPN, UFRJ. The sample (75 mg) was dissolved in 700 µL of deuterated methanol (CD3OD) and transferred to a 5 mm NMR tube. Spectra were recorded at room temperature (25 ºC).

Table S1: ^{1}H NMR data (300 MHz), ^{13}C (75 MHz) observed for sagittatin A (CD_3OD).

Aglycone	δC (ppm)	δH (ppm); m; *J* (Hz)
2	161.6	-
3	136.6	-
4	179.3	-
5	163.0	-
6	100.3	6.38; d; 1.9
7	163.0	-
8	95.1	6.64; d; 1.9
9	157.6	-
10	107.3	-
1’	121.8	-
2’/6’	131.6	7.73; d; 8.7
3’/5’	116.4	6.92; d; 8.7
4’	159.2	-
Carbohydrate 1	**3-*O*-α-L-rhamnopyranoside**	
1’’	102.7	5.42; *br* s
2’’	82.3	4.22; d; 2.0
3’’	71.7	3.85; dd; 9.4, 3.2
4’’	73.6	3.35; m
5’’	71.5	3.67; m
6’’	17.4	1.00; d; 6.1
Carbohydrate 2	**3-*O*-(1→2)-β-D-xylopyranosyl**	
1’’’	107.3	4.32; d; 7.5
2’’’	74.9	3.22; d; 7.5
3’’’	77.4	3.30; m
4’’’	70.9	3.60; m
5’’’	66.7	3.10, 3.67; m
Carbohydrate 3	**7-*O*-α-L-rhamnopyranoside**	
1’’’’	99.4	5.56; *br* s
2’’’’	71.3	4.04; *br* s
3’’’’	71.7	3.85; dd; 9.4, 3.2
4’’’’	73.3	3.51; m
5’’’’	71.5	3.67; m
6’’’’	17.8	1.26; 6.1

s: singlet; *br* s: broad singlet; d: doublet; m: multiplet.

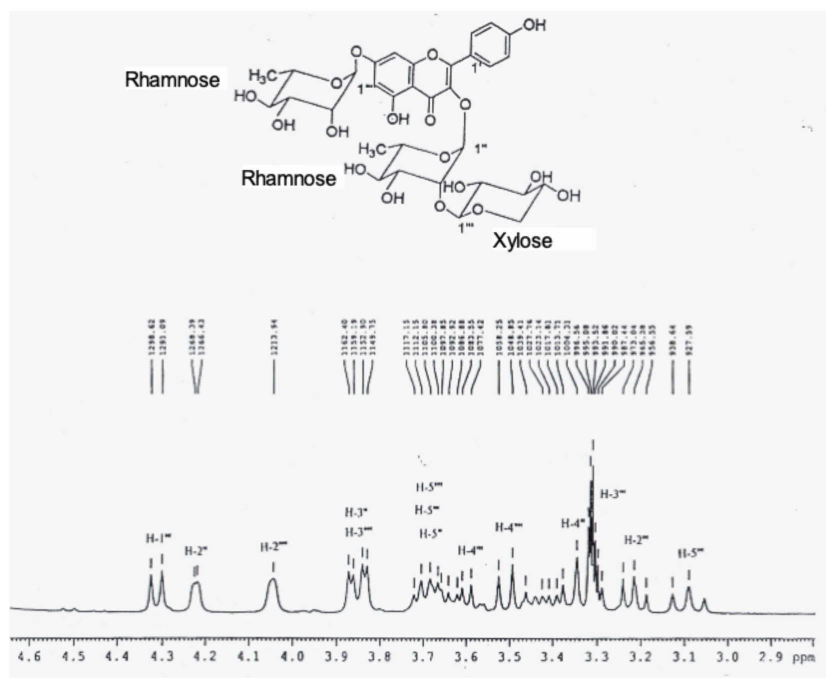

Figure S1: ^{1}H-NMR spectrum of sagittatin A (300 MHz; CD_3OD).
Magnification of 2.8 - 4.7 ppm region.

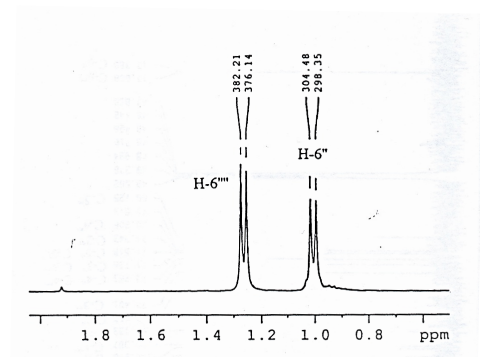

Figure S2: ^{1}H-NMR spectrum of sagittatin A (300 MHz; CD_3OD).
Magnification of 0.5 - 1.0 ppm region.

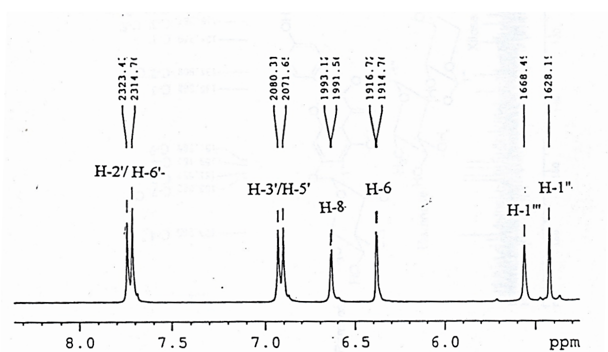

Figure S3: ^{1}H-NMR spectrum of sagittatin A (300 MHz; CD_3OD). Magnification of 5.5 - 8.5 ppm region.

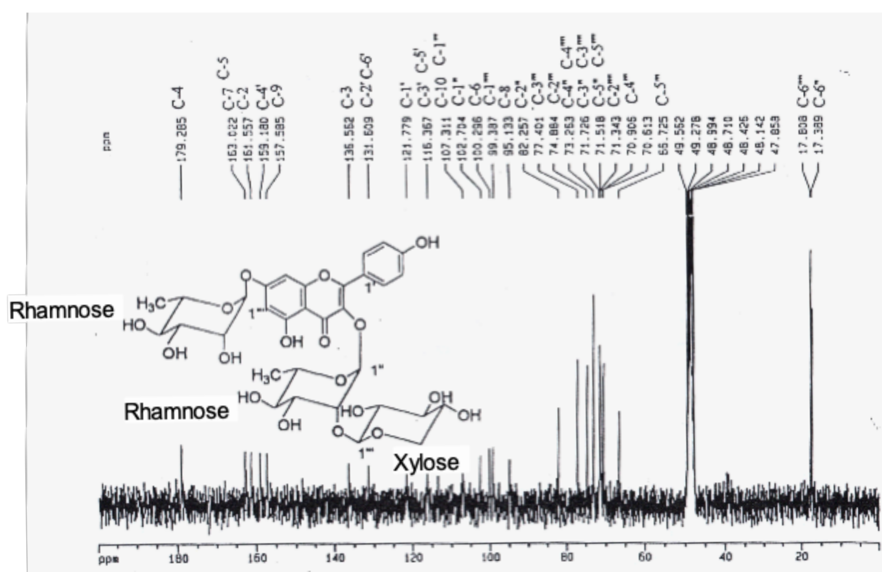

Figure S4: ^{13}C-NMR spectrum of sagittatin A (75 MHz; CD_3OD).

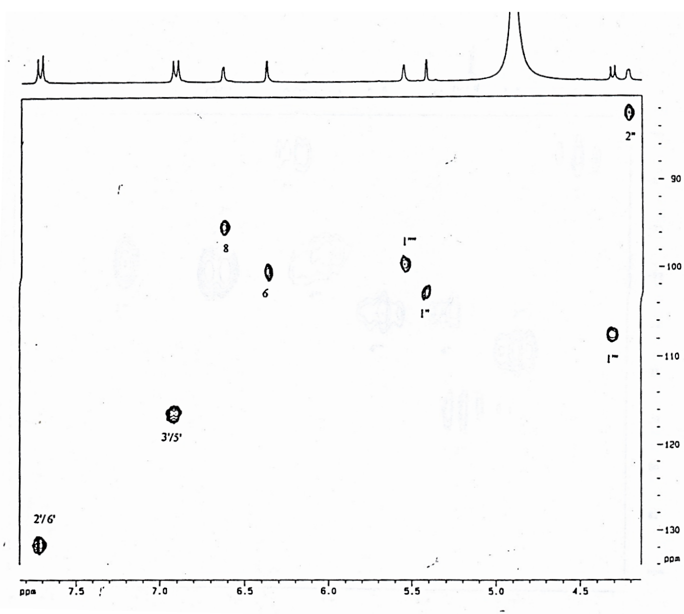

Figure S5: HMQC spectrum of sagittatin A (300 MHz; CD_3OD). Magnification of 4.0 - 8.0/80 - 140 ppm region.

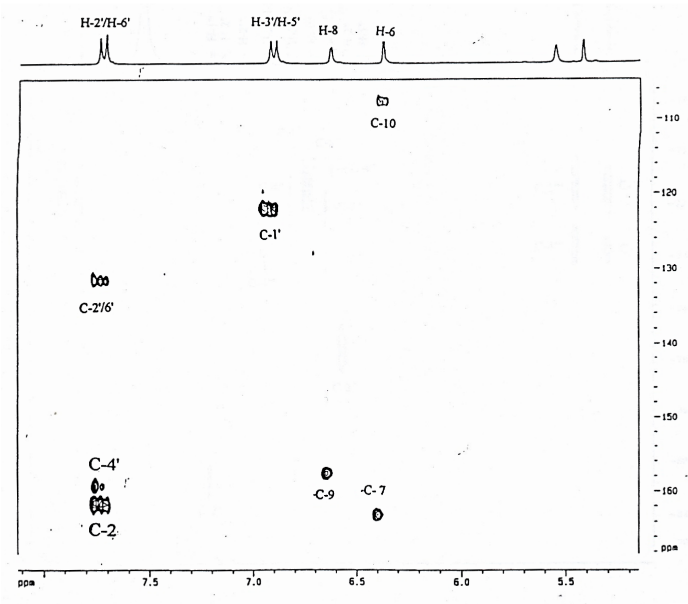

Figure S7: HMBC spectrum of sagittatin A (300 MHz; CD_3OD). Magnification of 5.0 - 8.0/100 -170 ppm region.

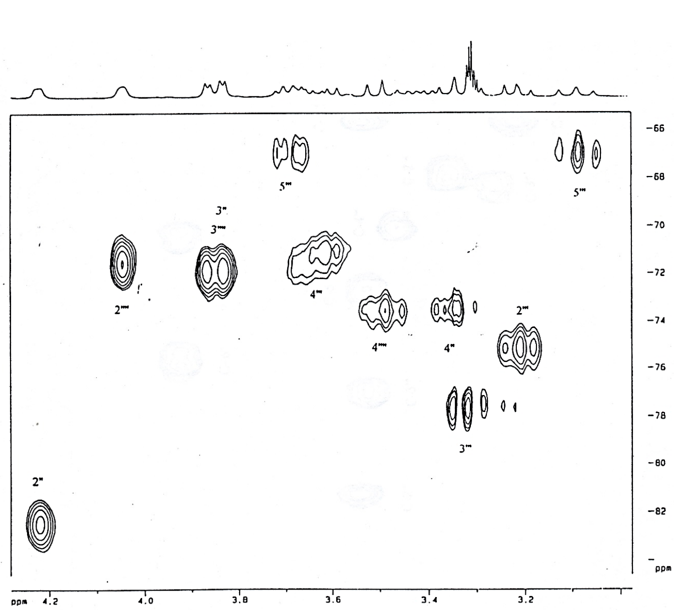

Figure S6: HMQC spectrum of sagittatin A (300 MHz; CD_3OD). Magnification of 3.0 - 4.3/65 - 83 ppm region.

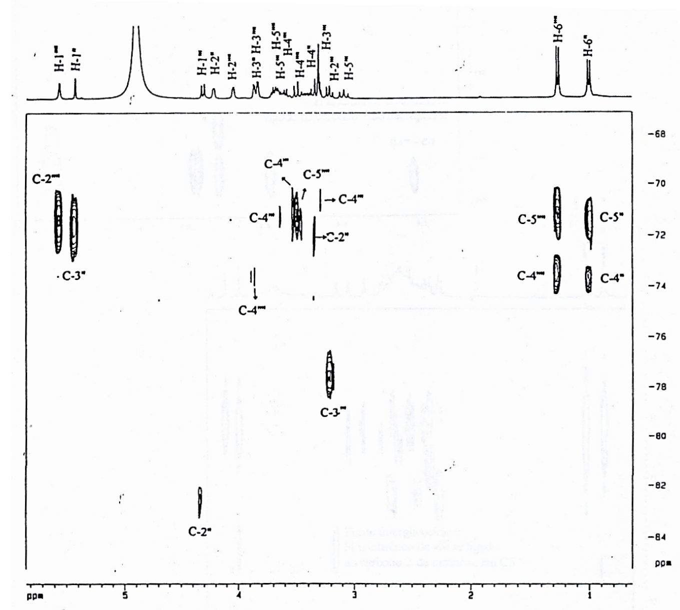

Figure S8: HMBC spectrum of sagittatin A (300 MHz; CD_3OD). Magnification of 0.5 - 6.0/67 - 85 ppm region.

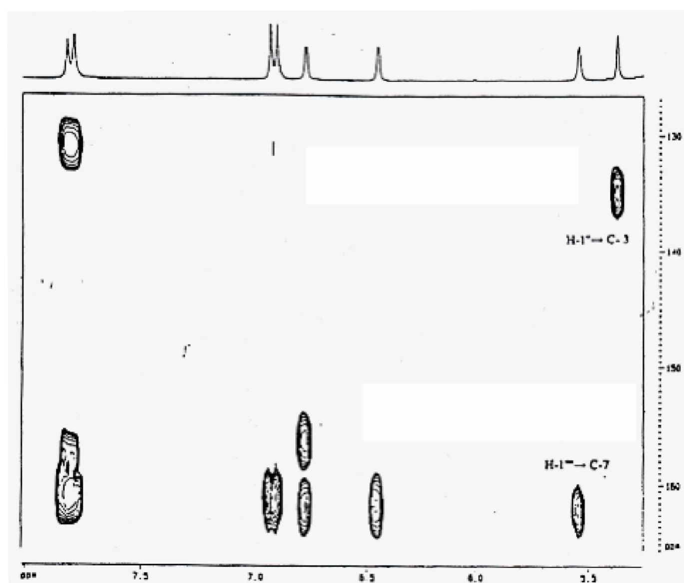

Figure S9: HMBC spectrum of sagittatin A (300 MHz; CD_3OD).
Magnification of 5.0 - 8.0/120 -170 ppm region.

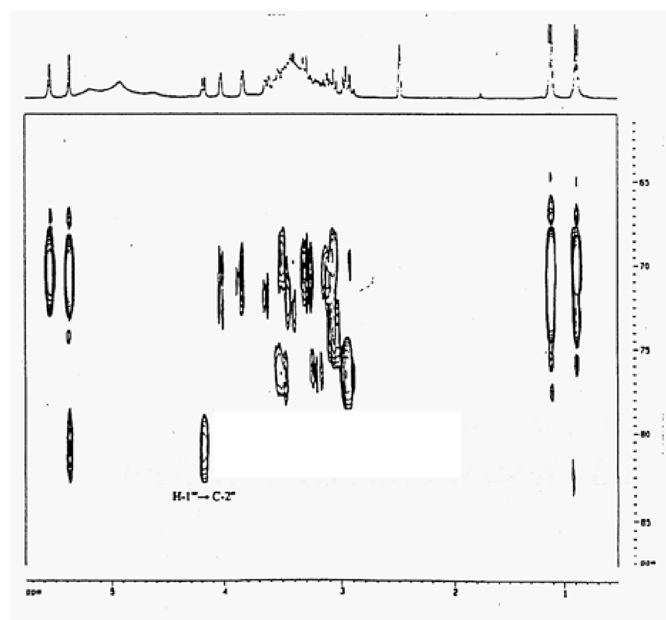

Figure S10: HMBC spectrum of sagittatin A (300 MHz; CD_3OD).
Magnification of 0.5 - 6.0/60 - 90 ppm region.