



Evaluation of the Leaf Extracts of *Kalanchoe Pinnata* and *Kalanchoe Daigremontiana* Chemistry, Antioxidant and Anti-inflammatory Activity

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The aim of this study was to analyze the chemical components and evaluate the biological activity of the extracts from the leaves *Kalanchoe pinnata* and *Kalanchoe daigremontiana*, which are cultivated in the province of Chiriqui, Republic of Panama. Phytochemicals components, antioxidant and anti-inflammatory activities were studied. The composition of the obtained petroleum ether, ethanol and aqueous extracts was analyzed by phytochemical screening. The antioxidant activity of the extracts was studied using three *in vitro* model systems (DPPH radical scavenging assay, nitric oxide radical scavenging assay, and superoxide radical scavenging activity). The anti-inflammatory activity of these species was studied using an *in vivo* model (λ -carrageenan-induced paw edema in rats). Phytochemical analysis of the extracts showed the

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presence of alkaloids, steroids, triterpenes, flavonoids, phenolic compounds, saponins and glycosides. The greatest radical inhibitory effect was observed in the DPPH model where the ethanolic extracts of both species developed a concentration-dependent inhibitory effect, the *K. pinnata* extract reached a maximum inhibitory effect of $49.5 \pm 5.6\%$ ($2000 \mu\text{g /mL}$) and *K. daigremontiana* $34.7 \pm 3.1\%$ ($1000 \mu\text{g /mL}$). The aqueous extract and the petrol ether extract (100 mg/kg) of *K. daigremontiana* showed high anti-inflammatory effect compared to diclofenac (100 mg/kg) used as a positive control, while the rest of the extracts showed slight effects. Our results showed that the two evaluated species exhibited a similar chemical composition. However, *K. pinnata* extracts showed better antioxidant activity while those of *K. daigremontiana* had better anti-inflammatory activity.

Keywords: *Kalanchoe daigremontiana*; *Kalanchoe pinnata*; phytochemical constituents; antioxidant activity; analgesic activity, anti-inflammatory activity, Chiriqui province.

1. INTRODUCTION

Plants belonging to the genus *Kalanchoe*, which are native to South Africa and Madagascar, are widely cultivated around the world as ornamental plants. In recent years there has been an increased interest in their culture for their medicinal properties. This genus comprises approximately 125 species some of which are used in traditional medicine to treat conditions such as pain, inflammation [1], diabetes, wounds, insect bites, respiratory infections, cutaneous leishmaniasis [2] among others.

Kalanchoe pinnata (Lam.) (Synonym: *Bryophyllum pinnatum*, local name: Siempreviva) is a succulent plant which can grow up to 1.5 m in height [3] The lower leaves are simple whereas the upper ones are 3-7 foliate and long-petioled. Their upper leaves are fleshy dark green that are distinctively scalloped and trimmed in red [4].

Several medicinal activities have been reported for this species, such as anti-inflammatory properties [5], antihistamine action, wound healing [6] and analgesics activities [7]. This plant has also been reported as beneficial for improving the digestive system's health [8]. In the province of Chiriqui it is generally classified as a weed due to its easy reproduction and people use it to relieve discomfort related to pain, inflammation and to treat respiratory conditions such as asthma.

Previous phytochemical research of different extracts of *K. pinnata* has shown the presence of alkaloids, phenols, saponins, tannins, carotenoids, glycosides, sitosterol, anthocyanins, malic acid, quinines, tocopherol, lectines, coumarines and bufadienolides [9]. Some flavonoids have been isolated from aqueous

flower extract [10] and ethanolic dried leaves extracts [11].

Another plant used in traditional medicine belonging to the same genus is the *Kalanchoe daigremontiana* (synonym: *Bryophyllum daigremontianum* Raym.-Hamet & H. Perrier, local name: Mala Madre). This species can reach 1.5 m in height and can alter the biology of the soil in semi-arid areas due to its invasive characteristics [12].

This species is traditionally used to treat burns and to reduce inflammation. In turn, anti-inflammatory, antidiabetic, antioxidant [13] antimicrobial and cytotoxic activities have been reported. Previous phytochemical studies with *K. daigremontiana* revealed the presence of organic acids, alkaloids, tannins and steroids. Bufadienolides have also been isolated from roots of this plant [14]. In the province of Chiriqui, *K. daigremontiana* has no ethnobotanical use and it is very common to see it adorning gardens and houses.

Due to its easy reproduction, adaptation and the numerous properties reported elsewhere, we decided to carry out this research as part of a study about medicinal plants from the province of Chiriqui, Panama.

2. MATERIAL AND METHODS

2.1 Plant Material

The plant material was collected around the province of Chiriqui, Republic of Panama, in the districts of Boquete, Tierras Altas, David, Bugaba and Alanje, between March and November 2018. A voucher of each species was deposited at the Universidad Autónoma de Chiriquí Herbarium, with registration code (C-017-012-2018). The

plant samples were taxonomically identified by the MSc. Rafael Rincón as *Kalanchoe pinnata* (Lam.) Pers. var. *pinnata* and *Kalanchoe daigremontiana* Raym. Hamet & H. Perrier, both belonging to the Crassulaceae family. In this work, the name sp.1 was used to refer to *K. pinnata* and sp.2 to *K. daigremontiana*.

2.2 Preparation of Extracts

The fresh plant material (leaves) of both species was cleaned, cut and dried in an oven at a temperature of 50 °C for 3 days and then pulverized to obtain a fine powder that was used in the production of the extracts. Twenty grams of dry plant material was weighed in a flask, and 200 mL of distilled water were added (<AqE> aqueous extract), and stirred for 24 h. This same procedure was performed using other solvents separately, such as ethanol (<EE> ethanolic extract) and petrol ether (<PEE> petrol ether extract). Each extract was subjected to a phytochemical screening following the methodology described by Sanabria [15].

2.2.1 Evaluation of photochemistry

The effect of light on the morphology and phytochemistry of both was studied following the methodology established by Leal-Costa et al [16]. with modifications. Seedlings of both species were used and exposed to two treatments of light: white lamp (WL) General Electric 13 W and blue light lamp (BL) General Electric 13 W. The treatment was performed for 30 days, with continuous daily photoperiods of 16

hours and room temperature of 25 ± 1 °C. Plant stem growth was recorded weekly and phytochemical screening was performed after a 30 days period.

The plants were obtained from an internal garden of the Chemistry School, where the specimens were grown under natural conditions. The leaf borders were cut into sections of 2 cm², washed and rinsed with sterilized water. Explants were transferred to a black soil substrate.

2.3 Evaluation of antioxidant activity

2.3.1 Determination of DPPH inhibition percentage

The DPPH free radical scavenging activity was estimated as described in the literature [17]. All extracts were dissolved in DMSO at different concentrations (31.3 – 2000 µg/mL). Quercetin was used as positive control. The reaction mixtures contained 100 µl of sample solution and 100 µl DPPH methanol solution (0.3 mM), and the absorbance was measured at 515 nm after incubation at 37 °C for 30 min. The DPPH radical scavenging effect was calculated using the following formula:

$$\% \text{ of free radical scavenging activity} = \left[\frac{A_0 - A_1}{A_0} \right] \times 100 \quad (\text{Eq.1})$$

Comment: A0 is the absorbance of the DPPH instead of the sample, and A1 is the absorbance of the sample.



Fig. 1. *Kalanchoe pinnata* (A); *Kalanchoe daigremontiana* (B)

2.3.2 Nitric oxide radical scavenging activity

Nitric oxide (NO) scavenging activity was estimated by assay based on Griess Illosvoy reaction [17]. About 50 μ L of the test sample (31.3 – 250 μ g/mL in DMSO) was mixed with 50 μ L of sodium nitroprusside (10 mM), and 50 μ L of Griess reagent prepared in saline phosphate buffer (pH 7.4). The microplates were incubated for 150 min at room temperature and the absorbance was recorded at 546 nm. Curcumin was used as a positive standard. The percentage of NO \cdot radical scavenging was calculated according to Eq. 1.

2.3.3 Superoxide radical scavenging activity

Measurement of superoxide scavenging activity of terpene compounds was based on the method described by Hsia-Yin and Cheng-Chun [18]. Superoxide radicals were generated in a PMS-NADH system by oxidation of NADH and assayed through reduction of NBT. The superoxide radicals were generated with 50 μ L of different concentrations of the sample or quercetin (31.3 – 250 μ g/ml), 50 μ L ml of NBT (150 μ M) solution, 50 μ L of NADH (936 μ M) solution. The reaction started by adding 50 μ L of PMS solution (120 μ M) to the mixture, followed by incubation at 25°C for 5 minutes, and the absorbance at 560 nm was then measured by an automated microplate reader (GloMax $^{\text{®}}$ -Multi+Detection System with Instinct $^{\text{™}}$ Software). Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage of superoxide radical scavenging was calculated according to Eq. 1.

2.4 Evaluation of Anti-inflammatory Activity

This analysis was conducted by the Laboratorio de Ensayos Biológicos (LEBi) of the Universidad de Costa Rica and it was directed in accordance with national laws and guidelines: Animal Welfare Law N $^{\circ}$ 7451 and MICITT Accordance 26668.

Rat paw edema was induced by the injection of 0.1 mL λ -carrageenan at 1% into the subplantar region of the right hinge paw according to the method described by Donoso et. al. [19]. Thirty female Wistar rats (150-200 g body weight) were randomly divided into six groups (n=5). The test groups were treated intraperitoneally with saline solution (0.1 ml/kg; negative control), diclofenac

(100 mg/kg; positive control), AqE sp1 (100 mg/kg), Aq sp2 (100 mg/kg), PEE sp1 (100 mg/kg) or PEE sp2 (100 mg/kg). The paw volume was measured in triplicate at 0, 1, 3, 5, and 7 hours of λ -carrageenan treatment using a plethysmometer (Ugo Basile $^{\text{®}}$ model 7140). The anti-inflammatory equipment used is shown in Fig. 2.



Fig. 2. Plethysmometer equipment used for rat paw edema model

2.5 Statistical Analysis

All experiments were conducted in triplicate. Values are expressed as mean \pm standard error of the mean (SEM). The significance level by the anti-inflammatory assay was set at $p = 0.05$. Results of antioxidant activity consider a minimum of 3 independent experiments. Statistical analysis was performed using one-way ANOVA and significant difference between the treatments was accepted at the level of $P < 0.05$. Analyses were performed using Graph Pad prism 5 software.

3. RESULTS AND DISCUSSION

3.1 Phytochemical compounds and photochemical analysis

We carried out a phytochemical study of both species in order to corroborate the presence of constituents that have been identified by other authors. As shown in Table 1, there is a broad

diversity of families of chemical compounds in the leaves of these plants. These results are consistent with those reported by Akinnibosun and Edionwe [20]; Puertas et al. [12] in their research. Literature shows that flavonoids represent the most frequently found class of secondary metabolites, being the main component of these plants [21,22] a fact that was obtained in our phytochemical screening. Another important aspect to highlight is that the composition of secondary metabolites is remarkably similar in both species. Based on this result, we decided to evaluate the effect of treatment with different types of light on the phytochemical composition of both species, since irradiation is the main environmental factor affecting morphological and physiological processes in plants.

The growth of the species was recorded for thirty (30) days under normal conditions and with exposure to white or blue light. Fig.1. shows that light treatment accelerated the growth of both species of *Kalanchoe*.

After thirty days of observation, the natural growth of species 1 is equivalent to 1.5 times its initial length. However, under the influence of blue light this species developed twice, while white light induced a growth of 1.8 times with respect to the basal determination (week zero). Although at first glance it appears that species 1 shows greater development than species 2 (Fig. 1), the reality is that the starting length differs between the two species. Hence, by the end of the fifth week of observation, *K. daigremontiana* had doubled in size under normal conditions, and had tripled in length under the influence of both white and blue lights. Post-treatment phytochemical screening does not differ from that shown in Table 1.

The plants belonging to the genus *Kalanchoe* are rich in flavonoids [22] in their leaves and compounds that play an important role as protectors of plants against radiation [23]. In our study we only verified the qualitative changes of radiation on the plants. Other authors have reached similar conclusions and add that radiation exposure, in addition to qualitative changes, also modified the profile of phenolic compounds of the plant [24].

3.2 Antioxidant Activity Analysis

In the DPPH radical uptake model, we observed that quercetin, used as a positive control, reaches its highest radical inhibitory activity at the concentration of 500 $\mu\text{g}/\text{mL}$ (80.5 %). The

ethanolic extracts of both species 1 and 2 developed a concentration-dependent inhibitory effect, reaching a maximum inhibitory effect of $49.5 \pm 5.6\%$ for EE sp.1 (2000 $\mu\text{g}/\text{mL}$) and $34.7 \pm 3.1\%$ for EE sp.2 (1000 $\mu\text{g}/\text{mL}$). For the rest of the extracts no inhibitory activity was observed against DPPH radical.

The antioxidant properties of several species of *Kalanchoe* have been reported in numerous studies [25], [5] especially of aqueous and alcoholic extracts. However, few studies of organic extracts in other solvents have been reported so it is difficult to compare results. In the present work, the aqueous extracts for the *Kalanchoe* species presented solubility problems so they were not evaluated in the antioxidant activity assays.

The nitric oxide (NO) scavenging activity was studied of the ethanolic and petrol ether extracts for both species of *Kalanchoe*, using curcumin as standard. The results are presented in Fig. 3 and it can be observed that the maximum inhibitory effect against NO generated by the positive control was 40.4 % at the concentration of 250 $\mu\text{g}/\text{mL}$. Although the extracts do not appear to be effective in neutralizing the presence of the nitric oxide radical, it is interesting that at a concentration of 31.3 $\mu\text{g}/\text{mL}$, EE sp.1 was more effective than the standard ($23.2 \pm 1.5\%$ and $11.6 \pm 9.7\%$, respectively).

In the present study, curcumin, the positive control, developed a capacity of $68.4 \pm 2.6\%$ to inhibit the superoxide anion, so it can be concluded that none of the extracts showed antioxidant activity against this radical (Fig. 4.).

3.3 Anti-inflammatory Activity Analysis

The results of the aqueous and petrol ether extracts of both species showed different behaviors in regard to the inflammation triggered by the λ -Carrageenan, used as an agent propeller of the paw edema. Both the aqueous extract (0.782 mL, t: 7 hr.) and the petrol ether extract (0.766 mL, t: 3 hr.) of specie 2 showed a higher % inhibition of inflammation (AqE: -105.69 ± 0.40) and (PEE: -79.95 ± 0.37), which was comparable during the first 5 and 7 hours of treatment respectively, with the effect of diclofenac, used as a positive control drug. On the other hand, the AqE sp.1 did not show any anti-inflammatory activity while PEE sp1 had only activity at 5 hour during the assay. The results obtained in this set of experiments are summarized on Fig. 5.

Table 1. Phytochemical screening of different extracts from *Kalanchoe*

Extracts	<i>K. pinnata</i>			<i>K. daigremontiana</i>		
	AqE	EE	PEE	AqE	EE	PEE
Alkaloids	++	+++	++	+	+	+
Reducing sugars	-	-	-	-	-	-
Steroids/ Triterpenes	-	++	+++	-	+	++
Flavonoids	++	+++	-	+	+	-
Cardiotonic Glycosides	-	+++	++	-	++	+
Gum and Mucilages	++	-	-	+	-	-
Saponins	++	+++	-	+	++	-
Tannins and phenolic compounds	++	+++	-	+	++	-

Key: (+++) intense, (++) moderate, (+) mild, (-) absent.

AqE: Aqueous extract of dry leaves; EE: Ethanolic extract of dry leaves; PEE: Petroleum ether extract of dry leaves

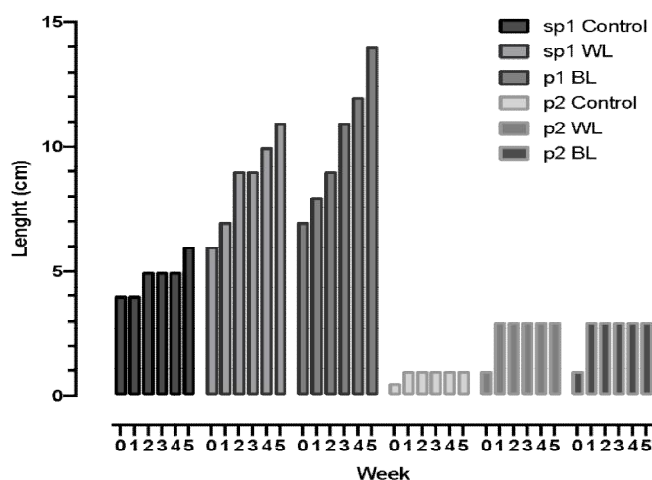


Fig. 3. Growth of *Kalanchoe* species cultured under normal conditions (control), white (WL) or blue light (BL)

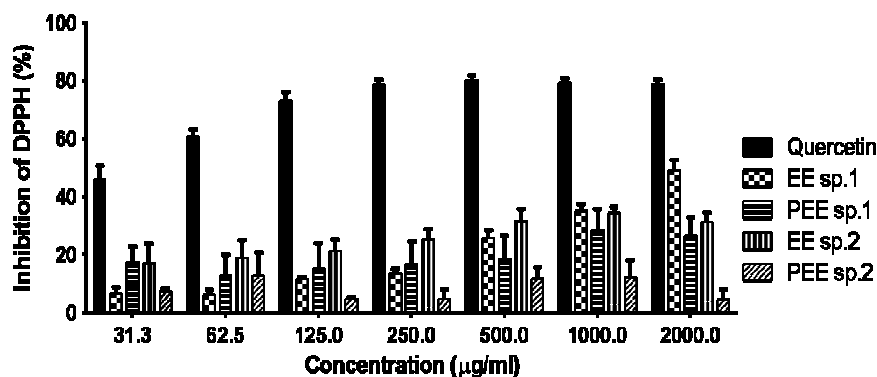


Fig. 4. Effect of different extracts of *Kalanchoe* evaluated on DPPH radical

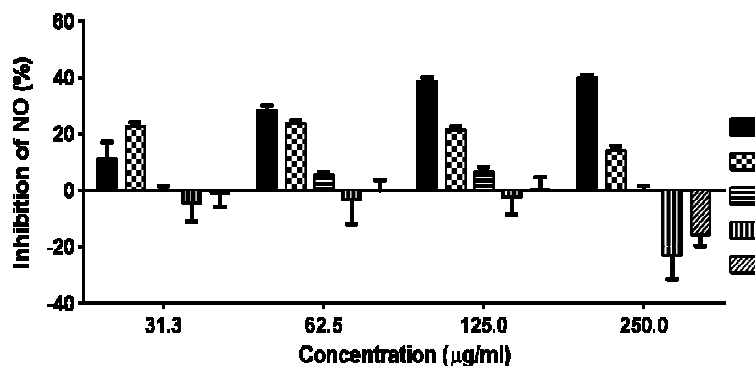


Fig. 5. Effect of different extracts of Kalanchoe evaluated on nitric oxide radical (NO)

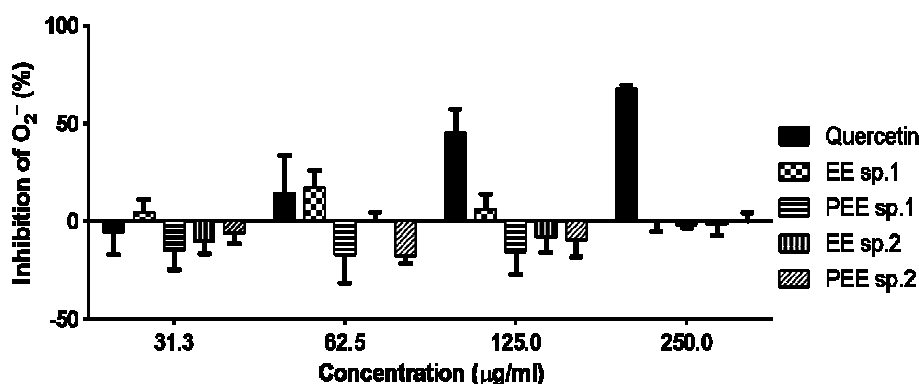


Fig. 6. Effect of different extracts of Kalanchoe evaluated on superoxide anion (O₂⁻)

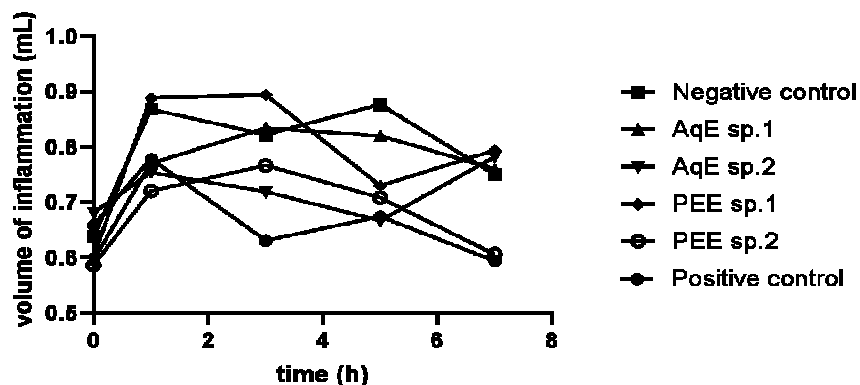


Fig. 7. Effect of different extracts from Kalanchoe species on rat paw edema induced by carrageenan

Data represents the mean SEM of 6 animals. AqE sp.1 ($p=0.003$), AqE sp. 2 ($p=1.000$), PEE sp. 1 ($p=0.001$), PEE sp. 2 ($p=0.341$) compared with positive control

This anti-inflammatory activity may be due to the presence of compounds of steroidal nature. These types of compounds possess anti-inflammatory activity and analgesic according to what is stated by Afzal et al. [26]. The present results of the *Kalanchoe* extracts are interesting,

since species 1 is used in traditional medicine as anti-inflammatory, whereas species 2 is generally used as an ornamental plant. In other countries sp2 is used in traditional medicine and its properties have been explored in several animal models [27].

Table 2. Maximal inhibitory effect and maximum effective concentration (Cmax) of different extracts of *Kalanchoe* evaluated on DPPH, NO and O₂⁻ radicals

Sample	DPPH		NO		O ₂ ⁻	
	Inhibition (%)	Cmax (µg/mL)	Inhibition (%)	Cmax (µg/mL)	Inhibition (%)	Cmax (µg/mL)
EE sp.1	49.5 ± 5.6	2000	24.3 ± 0.9	62.5	17.9 ± 2.6	62.5
PEE sp.1	28.9 ± 11.6	500	7.2 ± 2.0	125	Nv	-----
EE sp.2	34.7 ± 3.1	500	Nv	-----	Nv	-----
PEE sp.2	12.5 ± 9.6	500	0.8 ± 7.9	125	Nv	-----
Quercetin	80.5 ± 2.4	500	-----	-----	68.4 ± 2.6	250
Curcumin	-----	-----	40.4 ± 2.5	250	-----	-----

Nv: Negative values

4. CONCLUSION

It was possible to detect the presence of alkaloids, triterpenes, phenolic compounds, saponins, cardiotonic glycosides, steroids and flavonoids in the extracts of both species, with the flavonoids as most likely candidates for the biological activities of these plants. Based on the present results, both species displayed antioxidant and anti-inflammatory properties. These results will orient future studies of these plants in the search for alternative pharmacological uses that may expand the traditional applications already established in Panama. Clearly, the next step in this sense will be the identification of the active principles.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All animals procedure were within Law 7451 on Animal Welfare, the Decree 26668 of the Ministry of Science, Technology and Telecommunications (MICITT), the Guide of Laboratory Animals, the Institutional Committee for the Care and Use of Animals of the University of Costa Rica (CICUA) and the principles of Good Laboratory Practices (GLP) for the proper handling of laboratory animals.

DISCLAIMER

The products used for this research are commonly and predominantly used in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for

any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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