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Phenolic profiling and *in vitro* bioactivities of three medicinal *Bryophyllum* plants

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ABSTRACT

Bryophyllum constitutes a subgenus within the genus *Kalanchoe* that contains several plant species used in traditional medicine worldwide for the treatment of several diseases. However, little is known about the phytoconstituents of *Bryophyllum* spp. and previous reports have pointed at their low *in Planta* concentrations of bioactive compounds. In this work, we take advantage of plant *in vitro* culture for the study of the phenolic compounds found in the aerial parts of *Bryophyllum* spp. and their associated bioactivities. Our results show that the induction of nutritional stress leads to an improved accumulation of phenolic compounds, mainly flavonols and anthocyanins, represented by myricetin and malvidin glycosides, respectively. This effect is mainly found for *B.* × *houghtonii*, whose hydroethanolic extracts promoted the highest antioxidant, cytotoxic and anti-inflammatory activities. In the case of cytotoxic activity, *Bryophyllum* extracts showed an enhanced activity against the MCF-7 human breast carcinoma cell line. Meanwhile, extracts from *B. daigremontianum* promoted a wide range of effectiveness against different bacterial and fungal strains. This study is committed to shed light about the phytochemical potential associated to this unexplored subgenus, with the aim of considering *Bryophyllum* spp. as a valuable source of bioactive compounds for their exploitation in food, cosmetic and pharmaceutical industries.

1. INTRODUCTION

The subgenus *Bryophyllum* (genus *Kalanchoe*, Crassulaceae) contains more than 25 species widely used for ornamental and medicinal purposes (Descoings, 2006). The medicinal properties attributed to *Bryophyllum* spp. have been reported by their use as part of plant-derived formulations in the traditional medicine across Africa, Asia and South America (Fürer et al., 2016; García-Pérez et al., 2019). Hence, *Bryophyllum* formulations have been used in the traditional medicine for the treatment of several ailments including infections, diabetes, rheumatism and inflammatory and neoplastic diseases across Africa, Asia and South America (Kolodziejczyk-Czepas and Stochmal, 2017; Quazi Majaz et al., 2011). Such medicinal properties are a consequence of the vast number of secondary metabolites found in several *Bryophyllum* species, that can be classified into different families, such as phenolic compounds, bufadienolides and isoprenoids, among others (Milad, 2014).

Nevertheless, the phytochemical studies of these species have focused on the identification and quantification of bufadienolides and their associated cytotoxic bioactivity (Fürer et al., 2016; Kolodziejczyk-Czepas and Stochmal, 2017; Stefanowicz-Hajduk et al., 2020a). In contrast, the analysis of phenolic compounds is scarce and only limited information is available about this family of phytoconstituents on *Bryophyllum* spp. (Bogucka-Kocka et al., 2016), which have been widely reported as antioxidant, anti-inflammatory, anti-microbial and cytotoxic agents (Oroian and Escriche, 2015).

Moreover, phytochemical studies conducted on *Bryophyllum* spp. have been performed using raw plant materials from conventional breeding. Consequently, an excessive amount of plant biomass was needed, as very low yields were achieved (de Paiva et al., 2008d; Mahata et al., 2012). As a solution, the establishment of *Bryophyllum* plant *in*

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vitro culture emerges as a powerful tool to consider their potential as a valuable source of bioactive compounds (García-Pérez et al., 2019a, 2020a, 2020b, 2020c). Plant tissue culture technology offers some advantages to accomplish this goal, such as the independence of geo-climatic conditions, the absence of potential pollutants (avoiding the use of pesticides and other chemicals), and the capability to get a stable production of true-to-type plant-derived products (Dias et al., 2016). Additionally, plant tissue culture enables the modulation of secondary metabolite biosynthesis, mediated by the induction of plant stress throughout a phenomenon known as elicitation (Moon et al., 2020). Elicitation has been implemented as an effective strategy for enhancing secondary metabolite activity, enabling their production at industrial level, as reported for a wide range of plant species (Narayani and Srivastava, 2017). Furthermore, the success of *Bryophyllum in vitro* culture is supported by the exceptional asexual reproduction developed along the leaf margins of mature plants, which involves the generation fully-developed epiphyllous plantlets (Garcês and Sinha, 2009; Garcês et al., 2014), as already demonstrated (García-Pérez et al., 2020d).

In the present work, the identification and quantification of phenolic compounds from three *in vitro*-cultured *Bryophyllum* species was performed, providing valuable information about the heterogeneity of these compounds. In addition, the induction of nutritional stress by reducing the concentration of macronutrients on the culture media as a potential strategy to enhance the production of phenolic compounds (García-Pérez et al., 2020b), was evaluated. Finally, different biological activities, including antioxidant, cytotoxic, anti-inflammatory and antimicrobial were assessed, in order to determine the health-enhancing properties associated to *Bryophyllum* cultured *in vitro*. The knowledge provided by this work will aid considering the unexploited potential of these medicinal plants from the *Bryophyllum* subgenus as valuable sources of bioactive compounds for industrial purposes.

2. MATERIALS AND METHODS

2.1. Plant material and culture conditions

Three different species belonging to *Bryophyllum* subgenus were used in this work, namely: *Bryophyllum daigremontianum* Raym. – Hamet et Perr. (syn. *Kalanchoe daigremontiana*), *Bryophyllum* × *houghtonii* D.B. Ward (*B. daigremontianum* × *tubiflorum*, syn. *Kalanchoe daigremontiana* × *tubiflora*) and *Bryophyllum tubiflorum* Harv. (syn. *Kalanchoe tubiflora*). One-year-old plants were harvested in a local greenhouse (Vigo, Spain) and epiphyllous plantlets were detached from mother plants. Then, plantlets were disinfected and subjected to *in vitro* culture conditions as previously reported (García-Pérez et al., 2019a). Briefly, after disinfection, plantlets were transferred in pairs to glass culture vessels containing 25 mL of previously autoclaved full-strength MS medium (Murashige and Skoog, 1962) or half-strength macronutrient MS medium (1/2MS; García-Pérez et al., 2020b). The 1/2MS formulation was already applied to *Bryophyllum in vitro* culture for the induction of nutritional stress, leading to the production of phenolic compounds (García-Pérez et al., 2020b). Cultures were then randomly placed in growth chambers under a photoperiod of 16 h light and 8 h dark at 25 ± 1 °C for 12 weeks. Plants were subcultured every 12 weeks by transferring newly-formed plantlets to fresh culture medium, as previously established (García-Pérez et al., 2020b). After each subculture, aerial parts from 12-week-old *in vitro*-grown plants were excised and immediately frozen at −20 °C until use. Aerial parts from three consecutive subcultures were merged prior sample extraction, as described in the next section.

2.2. Sample extraction

Once frozen, samples were lyophilized, powder homogenized and subjected to solid-liquid extraction, using 1.0 g of lyophilized materials and 30 mL of 80% (v/v) EtOH for 1 h at 150 rpm at room temperature. Samples were extracted twice and supernatants were combined to get the raw extract. Extracts were then filtered throughout Whatmann paper no. 4 and concentrated under reduced pressure in a rotary evaporator. The aqueous phase was removed by lyophilization and the solid dry extract was resuspended with 20% (v/v) EtOH at a final concentration of 5 mg mL^{-1}. Hydroethanolic extracts were filtered using 0.22 μm disposable LC filter disks, prior to their chromatographic analysis.

For the analysis of anthocyanins, the sample extraction procedure was performed under the same conditions but using acidified 80% (v/v) EtOH with 1% (v/v) trifluoroacetic acid as solvent.

2.3. Analysis of phenolic compounds

The analysis of phenolic compounds was performed by HPLC-DAD-ESI/MS^n (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA), as previously reported by Bessada et al. (2016) for the non-anthocyanin phenolic compounds and by Gonçalves et al. (2017) for the anthocyanin compounds. The detection of phenolic compounds was achieved using diode-array detector programmed with the preference wavelengths at 280, 330 and 370 nm for non-anthocyanin phenolic compounds, and 520 nm for anthocyanin compounds, also coupled to a Linear Ion Trap LTQ XL mass spectrometer (Thermo Finnigan, San Jose, CA, USA), working in negative (identification of non-anthocyanin phenolic compounds) and positive (identification of anthocyanin compounds) mode and was equipped with an ESI source.

Identification of phenolic compounds was achieved through their comparison to commercial standards, when available, in terms of their retention times and UV-Vis spectra. When standards were not available, tentative identification was offered, based on additional information from the literature. Quantitative analysis was developed using calibration curves obtained from the UV-Vis signal of available phenolic standards: naringenin ($y = 18433x + 78903$; $R^2 = 0.9998$), apigenin-6-*C*-glucoside ($y = 107025x + 61531$; $R^2 = 0.9989$), quercetin-3-*O*-rutinoside ($y = 13343x + 76751$; $R^2 = 0.9998$), myricetin ($y = 23287x - 581708$; $R^2 = 0.9988$), quercetin-3-*O*-glucoside ($y = 34843x - 160173$; $R^2 = 0.9998$) and peonidin-3-*O*-glucoside ($y = 151438x - (3\ 10^6)$; $R^2 = 0.9977$). Results were expressed as mg g^{-1} of dry extract.

2.4. Evaluation of in vitro biological activities

2.4.1. Antioxidant activity

In order to determine the antioxidant activity associated to *Bryophyllum* extracts, two different *in vitro* assays were conducted, including the determination of extracts capacity on the inhibition of thiobarbituric acid reactive substances (TBARS) and oxidative hemolysis (OxHLIA), based on previous works (Lockowandt et al., 2019). In both cases, oxidizable subtracts were used: porcine brain cells for TBARS assay and sheep erythrocytes for OxHLIA assay ($\Delta t = 60$ min). Trolox was selected as positive control for both assays and results were expressed as IC_{50} values, in μg mL^{-1}.

2.4.2. Cytotoxic and hepatotoxic activity

Plant extracts at the concentration of 50-400 μg mL^{-1} (in water) were used for the determination of cytotoxic activity by the sulforhodamine B method (Guimarães et al., 2013) against different human tumor cell lines: NCI-H460 (non-small cell lung carcinoma), MCF-7 (breast adenocarcinoma), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma). The non-tumor PLP2 cell line (porcine liver primary cells) was used for the determination of hepatotoxicity. Elipticine was selected as the positive control, and the results were expressed as GI_{50} values, in μg mL^{-1}.

2.4.3. Anti-inflammatory activity

Plant extracts at the concentration of 0.125-0.4 mg mL^{-1} (in water) were tested for the determination of anti-inflammatory activity. Briefly, the stimulation of murine macrophages RAW 264.7 cell line (provided

by European Collection of Animal Cell Culture, ECACC, Salisbury, UK) by lipopolysaccharide (LPS), was measured by means of nitric oxide (NO) production by using the Griess Reagent System kit (Promega, WI, USA), as previously described (Souilem et al., 2017). Dexamethasone was used as positive control and the results were expressed as IC_{50} values, in μg mL^{-1}.

2.4.4. Antimicrobial activity

Plant extracts at the concentration of 0.1-20 mg mL^{-1} (in 30% ethanol) were tested for antibacterial and antifungal assays through the microdilution method (Heleno et al., 2013). All microorganisms were provided by the Institute for Biological Research "Sinisa Stanković" – National institute of Republic of Serbia, University of Belgrade, Serbia. For antibacterial analysis the next three Gram-positive strains: *Bacillus cereus* (food isolate), *Listeria monocytogenes* (NCTC7973) and *Staphylococcus aureus* (ATCC 6538); and the next Gram-negative strains: *Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (ATCC 35030) and *Salmonella* Typhimurium (ATCC13311) were used. For antifungal activity, the selected *Aspergillus* strains: *A. fumigatus* (ATCC 1022), *A. ochraceus* (ATCC 12066) and *A. niger* (ATCC 6275); and *Penicillium* strains: *P. ochrochloron* (ATCC 9112), *P. funiculosum* (ATCC 36839) and *P. verrucosum* var. *cyclopium* (food isolate) were used. Streptomycin and ampicillin were used as a positive control for antibacterial assays, whereas ketoconazole and bifonazole were selected as positive control for the antifungal assays, while 30% ethanol was used as a negative control. The results were presented as minimum inhibitory concentration (MIC, required for microbial growth inhibition), bactericidal (MBC) and fungicidal concentrations (MFC), expressed in mg mL^{-1}.

Table 1
Identification and quantification of phenolic compounds by HPLC-DAD-ESI/MS^n of hydroethanolic extracts from *Bryophyllum* spp. cultured *in vitro*.

Peak	Rt (min)	λ_{max} (nm)	[M-H]-/ $[M]^+$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Content (mg g^{-1} extract)					
						B. daigremontianum		*B. × houghtonii*		*B. tubiflorum*	
						MS	1/2MS	MS	1/2MS	MS	1/2MS
Non-anthocyanins phenolic compounds											
1	8.76	298	449	287(100)	Eriodictyol-*O*-hexoside[1]	tr	0.53 ± 0.02^{A}	tr	tr	nd	0.16 ± 0.01^{B}
2	9.62	327	593	473(100), 383 (11), 353(19)	Apigenin-*C*-dihexoside[2]	nd	nd	0.26 ± 0.01^{bB}	0.40 ± 0.01^{aB}	0.798 ± 0.002^{bA}	0.93 ± 0.04^{aA}
3	14.06	265/ sh309	725	563(40), 285 (100)	Kaempferol-*O*-deoxyhexoside-pentoside-*O*-hexoside[3]	tr	tr	tr	tr	nd	nd
4	15.12	352	479	317(100)	Myricetin-3-*O*-glucoside[4]	5.20 ± 0.01^{bA}	5.28 ± 0.02^{aA}	5.09 ± 0.01^{aB}	5.11 ± 0^{aB}	5.063 ± 0.002^{aB}	5.07 ± 0.01^{aC}
5	17.41	345	463	317(100)	Myricetin-*O*-deoxyhexoside[4]	5.11 ± 0.00^{bA}	5.22 ± 0.01^{aA}	5.042 ± 0.003^{bC}	5.08 ± 0.01^{aB}	5.08 ± 0.01^{aB}	5.06 ± 0.01^{aC}
6	18.07	350	595	317(100)	Myricetin-*O*-pentosyl deoxyhexoside[4]	5.29 ± 0.01^{bA}	5.62 ± 0.02^{aA}	5.06 ± 0.01^{bB}	5.12 ± 0.01^{aB}	5.064 ± 0.002^{aB}	5.07 ± 0.02^{aB}
7	18.60	348	595	317(100)	Myricetin-*O*-pentosyl deoxyhexoside[4]	5.18 ± 0.01^{bA}	5.28 ± 0.02^{aA}	5.04 ± 0.01^{aB}	5.07 ± 0.01^{aB}	nd	nd
8	19.08	345	595	317(100)	Myricetin-*O*-pentosyl deoxyhexoside[4]	5.113 ± 0.003^{bA}	5.19 ± 0.01^{aA}	5.030 ± 0.003^{aB}	5.05 ± 0.01^{aB}	nd	nd
9	20.17	334	549	505(25), 463 (20), 301 (100)	Quercetin-*O*-malonylhexoside[3]	tr	tr	nd	nd	nd	nd
10	20.17	334	463	301(100)	Quercetin-*O*-glucoside[5]	nd	1.008 ± 0.004^{B}	1.32 ± 0.01^{aA}	1.24 ± 0^{bA}	1.08 ± 0^{aB}	1.07 ± 0.02^{aB}
11	21.00	328	461	285(100)	Kaempferol-O-glucuronide[4]	nd	tr	tr	tr	tr	tr
12	22.32	345	579	301(100)	Quercetin-*O*-deoxyhexoside-*O*-pentoside[4]	tr	tr	tr	tr	tr	nd
13	23.33	269/ sh348	507	345(100)	Spinacetin-*O*-hexoside[4]	tr	tr	tr	tr	tr	tr
14	26.46	265/ sh342	563	285(100)	Kaempferol-*O*-deoxyhexoside-*O*-pentoside[4]	tr	0.43 ± 0.02	tr	tr	tr	nd
15	30.11	336	591	283(100), 269 (19)	Acacetin-*O*-rutinoside[4]	tr	tr	nd	tr	tr	tr
Anthocyanins											
16	14.92	523	655	493(100), 331 (35)	Malvidin-*O*-dihexoside[6]	4.18 ± 0.01^{bA}	4.588 ± 0.001^{aA}	4.102 ± 0^{bB}	4.27 ± 0.01^{aB}	nd	nd
17	24.89	525	493	331(100)	Malvidin-3-*O*-glucoside[6]	4.37 ± 0.02^{bA}	5.08 ± 0.02^{aB}	4.757 ± 0.001^{bA}	6.19 ± 0.05^{aA}	4.096 ± 0.002^{bA}	5.00 ± 0.01^{aB}
18	35.18	524	463	331(100)	Malvidin-*O*-pentoside[6]	nd	nd	4.025 ± 0.001^{b}	4.12 ± 0^{aA}	nd	4.002 ± 0.004^{B}
Σ Non-anthocyanin phenolic compounds						**25.89 ± 0.03^{bB}**	**28.54 ± 0.04^{aA}**	**26.85 ± 0.02^{bA}**	**27.07 ± 0.02^{aB}**	**17.09 ± 0.01^{bC}**	**17.34 ± 0.07^{aC}**
Σ Anthocyanin phenolic compounds						**8.55 ± 0.01^{bB}**	**9.66 ± 0.02^{aB}**	**12.884 ± 0.002^{bA}**	**14.58 ± 0.04^{aA}**	**4.096 ± 0.002^{bC}**	**9.00 ± 0.01^{aC}**
Σ Phenolic compounds						**34.44 ± 0.05^{bB}**	**38.20 ± 0.05^{aB}**	**39.73 ± 0.02^{bA}**	**41.64 ± 0.02^{aA}**	**21.18 ± 0.01^{bC}**	**26.34 ± 0.08^{aC}**

Statistical differences were assessed by one-way ANOVA, followed by Tukey's HSD *post hoc* test (α = 0.05): lower-case letters indicate significant differences among treatments within the same species, whereas upper-case letters indicate significant differences among species within the same treatment. [M-H]- was used for non-anthocyanins phenolic compounds and $[M]^+$ for anthocyanin compounds. Rt - retention time (min); λ_{max} - maximum absorption wavelengths and their eventual shifts (sh); [M-H]- and $[M]^+$ - mass spectral data; MS^2 - fragment ions generated and their abundance (in brackets); nd – not detected; tr – trace amounts. Standard compounds used for quantification: [1]Naringenin ($y = 18433x + 78903$, $R^2 = 0.9998$, LOD = 0.17 μg/mL; LOQ = 0.81 μg/mL); [2]Apigenin-6-*C*-glucoside ($y = 197337x + 30036$, $R^2 = 0.9997$, LOD = 0.19 μg/mL; LOQ = 0.63 μg/mL); [3]Quercetin-3-*O*-rutinoside ($y = 13343x + 76751$, $R^2 = 0.9998$, LOD = 0.14 μg/mL; LOQ = 0.45 μg/mL); [4]Myricetin ($y = 23287x - 581708$, $R^2 = 0.9988$, LOD = 0.23 μg/mL; LOQ = 0.78 μg/mL); [5]Quercetin-3-*O*-glucoside ($y = 34843x$ - 160173, $R^2 = 0.9998$, LOD = 0.21 μg/mL; LOQ = 0.71 μg/mL); [6]Peonidin-3-*O*-glucoside ($y = 151438x$ - 3E + 06, $R^2 = 0.9977$, LOD = 0.12 μg/mL; LOQ = 0.39 μg/mL).

2.4.5. Statistical analysis

All determinations and assays were carried out in triplicate, and results were expressed as the mean ± standard deviation (SD). Statistical analysis was performed by using the STATISTICA v.12 software (StatSoft Inc., 2014, OK, USA). Firstly, to meet the requirements for the performance of analysis of variance (ANOVA) the normality of residuals and homoscedasticity were assessed by conducting the Saphiro-Wilk's and Levene's tests, respectively. Then, one-way ANOVA was carried out to evaluate statistical differences between treatments, followed by Tukey's HSD *post hoc* test. Statistical differences with respect to positive controls were evaluated by Student's *t*-test The significance level was adjusted at $\alpha = 0.05$ in all cases.

3. RESULTS AND DISCUSSION

3.1. Identification and quantification of phenolic compounds

The results from the analysis performed by HPLC-DAD-ESI/MS^n on the hydroethanolic extracts of *Bryophyllum* cultured *in vitro* are presented in Table 1. A total of 18 flavonoids, grouped as non-anthocyanin and anthocyanin phenolic compounds, were tentatively identified by this chromatographic method: 1 flavanone (peak **1**), 2 flavones (peaks **2** and **15**), 12 flavonols (peaks **3** to **14**), and 3 anthocyanins (peaks **16** to **18**).

The main non-anthocyanin phenolic compounds reported on the three *Bryophyllum* species were flavonol glycosides (peaks **3** to **14**), mainly represented by myricetin glycosides (peaks **4** to **8**). Peaks **4** to **8** exhibit a DAD and an ion fragmentation pattern coherent with myricetin glycoside derivatives. All five compounds revealed a major MS^2 ion at *m/z* 317, characteristic of an ionized myricetin. For peaks **4** and **5**, the loss of an hexosyl (−162 u) and deoxyhexosyl (−146 u) moieties, was observed, being assigned as myricetin-3-*O*-glucoside (in comparison with commercial standard) and myricetin-*O*-deoxyhexoside, respectively. Peaks **6** to **8**revealed the same pseudo-molecular ion $[M–H]^-$ at *m/z* 595 and presented only one MS^2 fragment, at *m/z* 317 (myricetin aglycone; pentosyl residue (−132 u) and a deoxyhexosyl moiety (−146 u)), being tentatively identified as myricetin-*O*-pentosyl-deoxyhexoside derivatives (Ghouti et al., 2018; Ziani et al., 2018).

Peaks **3**, **11**, and **14** presented a DAD spectrum and a MS^2 fragmentation pattern coherent with kaempferol glycoside derivatives. Peak **3**($[M–H]^-$ at *m/z* 725) was tentatively identified as kaempferol-*O*-deoxyhexoside-pentoside-*O*-hexoside; the fragmentation pattern in MS^2 gave two ions, a minor one at *m/z* 563, suggesting the loss of a hexosyl unit ($[(M–H) -162]^-$), and major at *m/z* 285, corresponding to kaempferol aglycone after the loss of both a pentosyl and a deoxyhexoside residue $[(M–H)−132 − 146]^-$. Similarly, peak **14** ([M-H]- at *m/z* 563) was tentatively identified as kaempferol-*O*-deoxyhexoside-*O*-pentoside. Peak **11**($[M–H]^-$ at *m/z* 461) revealed one major ion fragment at *m/z*285, resulting from the loss of a glucuronyl acid residue ($[M–H–176]^-$) and therefore was tentatively identified as kaempferol-*O*-glucuronide (Barros et al., 2011; Geng et al., 2016).

Moreover, peaks **9**, **10** and **12** showed a DAD and MS^2 fragment at *m/z* 301, characteristic of quercetin glycoside derivatives. Peak **9** ($[M–H]^-$at *m/z* 549) exhibited daughter peaks at *m/z* 505 and 463, which correspond to the loss of a malonyl moiety $[(M–H)−44 + 42]^-$, followed by the base peak at *m/z* 301 corresponding to quercetin aglycone, after the hexosyl moiety loss (−162 u). This compound was tentatively assigned as quercetin-*O*-malonylhexoside. Peak **10** was positively identified as quercetin-3-*O*-glucoside ($[M–H]^-$ at *m/z* 463), in comparison with the commercial standard. Peak **12** was tentatively identified as quercetin-*O*-deoxyhexosyl-*O*-pentoside, revealing the loss of a pentosyl and a deoxyhexosyl residue $[(M–H) −132 − 146]^-$ (Ghouti et al., 2018; Ziani et al., 2018).

Finally, with respect to flavonol glycosides, peak **13** ([M-H]- at *m/z* 507) was tentatively assigned to spinacetin-*O*-hexoside taking into account its fragmentation pattern (Boukhris et al., 2016). All these compounds (peaks **3** to **14**) belong to the flavonol subclass, which constitutes one of the most abundant flavonoid subclasses previously described for the *Bryophyllum* subgenus (Fürer et al., 2013; Stefanowicz-Hajduk et al., 2020b, 2020a; Tatsimo et al., 2012).

Besides flavonol glycosides, other flavonoid subclasses were also described in the *Bryophyllum* extracts, including flavones, such as apigenin (peak **2**) and acacetin (peak **15**) glycosides, and one flavanone (peak **1**; Table 1). Peak **2** was tentatively identified as apigenin-*C*-dihexoside, with a pseudo-molecular ion at *m/z* 593, and a base peak at *m/z* 473 $[(M–H)−120]^-$, a typical fragmentation of di-C-glycosyl flavones (Tahir et al., 2012). The MS^n fragments at *m/z* 353 $[(M–H) − (120 + 120)]^-$ and at *m/z* 383 $[(M–H) − (90 + 120)]^-$ indicated the presence of apigenin (mw 270) as aglycone and two hexosyl moieties (El Sayed et al., 2016; Geng et al., 2016). Peak **15**($[M–H]^-$ at *m/z* 591) was tentatively identified as acacetin-*O*-rutinoside. The MS^2 analysis gave two ions, a major one at *m/z* 283, characteristic of an ionized acacetin, which suggests the loss of a rutinosyl group ($[(M–H)−146 + 162]^-$); and a minor one at *m/z* 269, suggesting the loss of a methyl group $[(M–H)−14]^-$, characteristic of the apigenin structure (Lin and Harnly, 2010; Parejo et al., 2004). Peak **1**($[M–H]^-$ at *m/z* 449) presented a typical MS^2 fragment at *m/z* 287, indicating a deprotonated molecule [eriodictyol–H] and the loss of one hexosyl moiety $[(M–H)–162]^-$, respectively, and was tentatively identified as eriodictyol-*O*-hexoside (Pereira et al., 2013; Vallverdú-Queralt et al., 2011). Other authors reported acacetin glycosides in the leaves of *B. pinnatum* (Fürer et al., 2013) together with other flavones (Muzitano et al., 2006; Nascimento et al., 2015), as already detected in *B. tubiflorum* (Huang et al., 2013a) and *B. daigremontianum* (Stefanowicz-Hajduk et al., 2020a).

Concerning anthocyanins, only malvidin glycosides were identified in the leaf extracts of *in vitro*-cultured *Bryophyllum* spp. (peaks **16** to **18**; Table 1). Peaks **16** to **18** were tentatively identified as glycosidic forms of anthocyanidins found in plants; the MS^2 fragment at *m/z* 331 is the typical base peak of malvidin derivatives. Peak **16**($[M–H]^-$ at *m/z* 655) presented a consecutive loss of two hexosyl moieties $[(M–H)−162 − 162]^-$ and was tentatively identified as malvidin-*O*-dihexoside. Peak **17** ($[M–H]^-$ at *m/z* 493) exhibited a base peak at *m/z* 331, corresponding to the loss of an hexosyl moiety (−162 u), being assigned as malvidin-3-*O*-glucoside. Whereas, peak **18** ($[M–H]^-$ at *m/z* 463) revealed the loss of a pentosyl residue $[(M–H)−132]^-$, being identified as malvidin-*O*-pentoside (Flamini, 2013). These compounds are associated with flower pigmentation of other species belonging to the *Kalanchoe* genus, together with other anthocyanins, such as peonidin and delphinidin (Nielsen et al., 2005). However, they have also been produced in root cultures (Góraj-Koniarska et al., 2015). The presence of anthocyanins in the leaves of *Bryophyllum* spp. might be responsible for the dark, pigmented strands located in the abaxial leaf surface of these plants, as suggested by other authors (Casanova et al., 2020). This hypothesis is reinforced by the presence of idioblasts found in the leaf structure of *B. daigremontianum* and *B. tubiflorum*, considered cell reservoirs for phenolic compounds (Casanova et al., 2020; Chernetskyy et al., 2018).

Regarding the quantification of phenolic compounds, culture media composition and species were two significant factors affecting the accumulation of the different compounds (Table 1). As a general rule, the macronutrient reduction on 1/2MS caused a significant positive effect ($p < 0.05$) on the accumulation of phenolic compounds, including both non-anthocyanins and anthocyanins in all species (Table 1), due to a potential induction of phenolic compound biosynthesis caused by nutritional stress.

Myricetin glycosides were the non-anthocyanin flavonoids found in highest concentrations, with a maximum value of 5.62 mg g^{-1} in *B. daigremontianum* cultured on 1/2MS, corresponding to the compound myricetin-*O*-pentosyl deoxyhexoside (peak **6**; Table 1). These results are in line with the previously reported contents for *Bryophyllum* spp. cultured *in vitro*, in which macronutrient reduction led to an increased accumulation of phenolic compounds, being *B. daigremontianum* the species showing the highest accumulation of flavonoids (García-Pérez

et al., 2020b). Such nutrient reduction can be seen as a form of abiotic stress, considered as nutritional stress, which results in the induction of secondary metabolite biosynthesis (García-Pérez et al., 2020b). As it was shown for low nitrogen, potassium, and phosphate concentrations (Blasco et al., 2018; Cramer et al., 2011), the phenylpropanoid biosynthetic pathway was enhanced via the modulation of key biosynthetic genes, such as phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS) (Sharma et al., 2019). In the case of *in vitro*-cultured *Bryophyllum* spp., ammonium was predicted to promote the accumulation of phenolic compounds, as a result of the application of artificial intelligence tools (García-Pérez et al., 2020b), being also a critical factor on plant growth (García-Pérez et al., 2020d). This stress-induced overproduction of secondary metabolites is called elicitation, and was previously reported for *Bryophyllum* spp. (García-Pérez et al., 2019b).

Similarly, in the specific case of anthocyanins, their concentrations significantly increased ($p < 0.05$) by nutritional stress in all species (peaks **16-18**; Table 1). Although the highest values were found on *B.* × *houghtonii* cultured on 1/2MS, showing a concentration of 6.19 mg g^{-1} for malvidin-*O*-hexoside (peak **17**; Table 1), the most significant effect of nutrient stress was observed on *B. tubiflorum*, with a 54.4% increase (from 4.09 to 9.00 mg g^{-1} for anthocyanin phenolic compounds, Table 1). These findings reinforce the hypothesis that macronutrients present a species-dependent narrow range of efficacy, which should be optimized in order to meet specific requirements for every genotype (Castro et al., 2018; García-Pérez et al., 2020d).

The highest values for total phenolics were presented by *B.* × *houghtonii*, 41.64 mg g^{-1} under nutritional stress (1/2MS; Table 1). However, the strongest effect of nutritional reduction was observed for *B. tubiflorum*, showing a 19.6% increase (Table 1). This species-dependent behavior for phenolic accumulation was previously reported for *Bryophyllum* cultured *in vitro* (García-Pérez et al., 2020a, 2020b), suggesting that even genetically close species show different requirements for several physiological features, as it was also noted for other physiological features, such as *in vitro* growth and organogenesis (García-Pérez et al., 2020d, 2020e).

3.2. In vitro biological activities

3.2.1. Antioxidant activity

Table 2 shows the results for the *in vitro* antioxidant activity of *Bryophyllum* extracts through the two methods employed (TBARS and OxHLIA). The results from TBARS assay were given as IC_{50}, which indicates the extract concentration required to prevent by 50% the malondialdehyde formation throughout the H^+ donation to lipid radicals. In parallel, the results for OxHLIA assay were also given as IC_{50}, which indicates the extract concentration needed to protect the 50% of erythrocyte population. Hemolysis was induced by the free-radical initiator 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) and antioxidant activity was measured at $\Delta t = 60$ min, in order to allow the performance of all compounds present in the extracts, which can show different action ranges along time (Lockowandt et al., 2019). In both cases, lower IC_{50} values correspond to higher antioxidant activity of plant extracts.

As it can be seen in Table 2, the highest antioxidant activity was reported to extracts from plants cultured under nutritional stress (cultured on 1/2MS medium) in all species for both TBARS and OxHLIA assays, except for *B. tubiflorum* in OxHLIA. These findings correlate to the observed results for phenolic composition of *Bryophyllum* extracts, which suggest that phenolic compounds, such as myricetin and malvidin glycosides, are responsible for this antioxidant activity, as previously reported for both compounds (Barzegar, 2016; Huang et al., 2016). Specifically, the mechanism of action of these compounds has been associated to free-radical scavenging activity, due to their ability to donate H^+ and/or e- with the aim of forming more stable radicals (Heim et al., 2002).

According to the results for TBARS, the IC_{50} values for *B.* × *houghtonii* cultured on 1/2MS (27.7 μg mL^{-1}) were only 5.2-fold higher than the positive control, 5.4 μg mL^{-1}, represented by the artificial antioxidant Trolox, whereas the results for *B. daigremontianum* and *B. tubiflorum* were significantly higher, 45 μg mL^{-1} and 77 μg mL^{-1}, respectively (Table 2). It is noteworthy that Trolox is a single, artificial molecule with enhanced antioxidant activity, whereas plant extracts contain a heterogenous composition of phytoconstituents that may interact either positively or negatively, leading to different rates of antioxidant activity (Tsao and Deng, 2004).

Similarly, in the case of OxHLIA, the same patterns were observed, being the extracts from *B.* × *houghtonii* cultured on 1/2MS the ones showing the lowest IC_{50} values, 79 μg mL^{-1}. It supposes an increase of 28.8% and 30.7% in the antioxidant activity, with respect to their counterparts for *B. daigremontianum* and *B. tubiflorum*, respectively (Table 2). Again, the positive control reached significantly higher values (19 μg mL^{-1}).

The results for both antioxidant assays are in accordance to the previous knowledge provided by *in vitro*-cultured *Bryophyllum* spp. A

Table 2
Antioxidant, cytotoxic, hepatotoxic and anti-inflammatory activity of *Bryophyllum* extracts.

	B. daigremontianum		*B.* × *houghtonii*		*B. tubiflorum*		Positive controls
	MS	1/2MS	MS	1/2MS	MS	1/2MS	
Antioxidant activity (IC_{50}, μg mL^{-1})[1]							**Trolox**
TBARS	72 ± 2^{bA}	45 ± 1^{aB}	82 ± 5^{bB}	27.7 ± 0.3^{aA}	83 ± 2^{bB}	77 ± 1^{aC}	5.4 ± 0.3*
OxHLIA $\Delta t = 60$ min	124 ± 3^{bB}	111 ± 1^{aB}	180 ± 4^{bC}	79 ± 2^{aA}	110 ± 4^{aA}	114 ± 4^{aB}	19 ± 1*
Cytotoxic activity (GI_{50}, μg mL^{-1})[1]							**Ellipticine**
NCI-H460 (lung cancer)	15.0 ± 0.6^{aA}	24.9 ± 1.2^{bC}	15.4 ± 0.8^{bA}	13.8 ± 1.1^{aA}	20.2 ± 1.2^{bB}	16.8 ± 1.5^{bB}	0.9 ± 0.1*
MCF-7 (breast carcinoma)	11.6 ± 0.7^{aA}	19.7 ± 1.9^{bC}	11.1 ± 0.3^{bA}	9.6 ± 1.2^{aA}	14.9 ± 0.3^{bB}	13.4 ± 1.0^{aB}	1.21 ± 0.03*
HeLa (cervical carcinoma)	15.2 ± 0.6^{aA}	29.9 ± 1.6^{bB}	15.2 ± 0.6^{bA}	14.0 ± 0.2^{aA}	17.1 ± 0.3^{aB}	16.0 ± 1.3^{aA}	1.0 ± 0.1*
HepG2 (hepatocellular carcinoma)	16.5 ± 1.1^{aB}	34.5 ± 4.1^{bB}	13.1 ± 0.6^{aA}	13.6 ± 1.0^{aA}	15.30 ± 0.04^{bB}	13.9 ± 0.5^{aA}	1.1 ± 0.1*
Hepatotoxic activity (GI_{50}, μg mL^{-1})[1]							**Ellipticine**
PLP2 (liver primary culture)	118.9 ± 3.1^{aB}	146.2 ± 11.1^{bB}	112.6 ± 2.9^{bB}	101.4 ± 4.3^{aA}	89.2 ± 6.9^{aA}	99.29 ± 5.03^{bA}	3.2 ± 0.7*
Anti-inflammatory activity (IC_{50}, μg mL^{-1})[1]							**Dexamethasone**
RAW 264.7	26.5 ± 0.6^{aA}	40.3 ± 2.9^{bC}	26.3 ± 1.1^{aA}	25.2 ± 1.7^{aA}	31.3 ± 1.9^{aB}	29.7 ± 0.9^{aB}	16.0 ± 1.0*

Statistical differences were assessed by one-way ANOVA, followed by Tukey's HSD *post hoc* test ($\alpha = 0.05$): lower-case letters indicate significant differences among treatments within the same species, whereas upper-case letters indicate significant differences among species within the same treatment. Asterisks (*) indicate statistical differences between positive controls and every treatment according to Student's t-test ($\alpha = 0.05$).

[1] Note: lower IC_{50} and GI_{50} values correspond to higher antioxidant activity.

reduction in the macronutrient concentration of the culture media caused a significant 2.5-fold increase in the antioxidant activity, determined by the 2,2-diphenyl-picrylhidrazyl (DPPH) method, which was more evident in the case of *B.* × *houghtonii* (García-Pérez et al., 2020a, 2020b, 2019a). In fact, it was recently demonstrated that extracts from *in vitro*-cultured *B.* × *houghtonii* effectively prevent lipid oxidation on food models, such as omega-3 enriched fish oil-in-water emulsions (García-Pérez et al., 2020a).

A number of phytochemical reports have highlighted the antioxidant potential of different *Bryophyllum* species grown *ex vitro* and found higher IC_{50} values than the species employed in this work for TBARS assay, i.e.: 134.56 μg mL^{-1} for *B. pinnatum* (Mohan et al., 2012). This suggests that the establishment of plant *in vitro* culture emerges as an efficient strategy to enhance the antioxidant potential of *Bryophyllum* spp., as demonstrated for *B. daigremontianum*, *B.* × *houghtonii* and *B. tubiflorum*.

3.2.2. Cytotoxic and hepatotoxic activity

The results obtained for the cytotoxic activity of hydroethanolic *Bryophyllum* extracts against four different human cancer cell lines are shown in Table 2. The values for cytotoxic activity were expressed as GI_{50}, which indicates the extract concentration (in μg mL^{-1}) required to inhibit cell growth by 50%. In all cell lines tested, the extracts belonging to *B.* × *houghtonii* cultured on 1/2MS showed the highest cytotoxic activity, although no significant differences ($p > 0.05$) were found for HeLa and HepG2 between *B.* × *houghtonii* and *B. tubiflorum* (Table 2). In contrast, the extracts from *B. daigremontianum* showed the highest activity for MS-grown plants (Table 2). This differential observation may indicate that, besides phenolic compounds, there are other families of secondary metabolites that contribute to the development of cytotoxic activity in this species, i.e.: bufadienolides. Bufadienolides are C-24 steroidal compounds, widely distributed among *Bryophyllum* subgenus, that show a potent cytotoxic activity against a series of human cancer cell lines (García-Pérez et al., 2019, 2020c). However, the flavonoids present in *Bryophyllum* extracts, such as myricetin and malvidin, also present an associated cytotoxic activity, establishing a close relationship between plant phenolic content (Table 1) and cytotoxicity (Table 2), in agreement with previous results (Hyun and Chung, 2004; Semwal et al., 2016).

Regarding cancer cell lines, MCF-7 (breast carcinoma) was the most sensitive to *Bryophyllum* extracts, showing the lowest GI_{50} values for all species, mostly *B.* × *houghtonii* (9.6 μg mL^{-1}; Table 2). The rest of cell lines tested, including NCI-H460 (lung cancer), HeLa (cervix carcinoma) and HepG2 (hepatocellular carcinoma) presented a similar sensitivity against *B.* × *houghtonii* cultured on 1/2MS, with GI_{50} values ranging 13.6 – 14.0 μg mL^{-1}. These results show the same patterns as reported in recent reports conducted on *B. daigremontianum* cultured *ex vitro*, in which the cytotoxicity against MCF-7 (43.6 μg mL^{-1}) was significantly higher than against HeLa cell line (100 μg mL^{-1}) (Stefanowicz-Hajduk et al., 2020a). Several authors demonstrated that kaempferol and myricetin, found on *Bryophyllum* spp. hydroethanolic extracts, showed a potent cytotoxic effect against MCF-7 cell line, thus suggesting that both molecules may be the major responsible for such effects observed for this cell line (Jiao and Zhang, 2016; Srinivas Raghavan et al., 2015). Specifically, myricetin has been shown to exert a 7-fold higher cytotoxic activity against MCF-7 than the HeLa cell line (Semwal et al., 2016). In addition, the cytotoxic activity of *Bryophyllum* spp. is well-known for a series of different species, including *B. daigremontianum*, as described earlier, *B.* × *houghtonii* (Supratman et al., 2001) and *B. tubiflorum* (Hsieh et al., 2016). Nevertheless, to the best of authors' knowledge, this is the first work applying *in vitro* culture methodology as a solution to improve the phytochemical potential of *Bryophyllum* spp., in terms of cytotoxic activity.

Hepatotoxicity was assessed by the use of one non-tumor PLP2 porcine liver cell culture and results are shown in Table 2. Ellipticine was selected as the positive control, as it occurred for cytotoxic activity: despite its efficacy as cytotoxic agent (0.9 – 1.2 μg mL^{-1}) (Stiborová et al., 2011), ellipticine also caused the highest toxicity against porcine liver cell line PLP2 (3.2 μg mL^{-1}; Table 2). In this sense, *Bryophyllum* extracts showed a markedly lower hepatotoxicity, being MS-grown *B. tubiflorum* the species whose the extracts caused the highest hepatotoxicity, 89.2 μg mL^{-1}, with 27.9-fold higher GI_{50} values than ellipticine (Table 2). This lower hepatotoxicity associated to *Bryophyllum* extracts may be explained in the basis of their hepatoprotective properties, as demonstrated in both *in vitro* and *in vivo* assays (Yadav and Dixit, 2003).

3.2.3. Anti-inflammatory activity

The results obtained for the anti-inflammatory activity of *Bryophyllum* extracts are displayed in Table 2. Anti-inflammatory activity was assessed by IC_{50} values (μg mL^{-1}), which represent the extract concentration required to inhibit by 50% the NO production by LPS-induced macrophages. Our results show that *B.* × *houghtonii* presented the highest anti-inflammatory activity regardless of the composition of culture media, ranging 25.2 – 26.3 μg mL^{-1} together with MS-cultured *B. daigremontianum* extracts, whereas *B. tubiflorum* extracts promoted a significantly lower activity (Table 2). However, in all cases, dexamethasone, used as positive control, showed a more potent anti-inflammatory activity than *Bryophyllum* extracts. These patterns in the results are likely related to those from cytotoxic activity (Table 2). This observation may suggest a crosstalk between inflammation and cancer proliferation signaling pathways, in which macrophages play a relevant role, as demonstrated in many epidemiological studies (Elinav et al., 2013; García-Pérez et al., 2019). Therefore, the present work provides enough evidence showing that *Bryophyllum* phytochemical potential is developed by a wide range of bioactivities. Both myricetin and malvidin, as the most relevant phenolic compounds identified in *Bryophyllum* spp., were found to exert a potent effect on the LPS-induced NO biosynthesis by macrophages, via the inhibition of nuclear factor-kappa B (NF-κB) (Bognar et al., 2013; Hämäläinen et al., 2007). Previous phytochemical studies have pointed at flavonoids from *B. tubiflorum* as anti-inflammatory agents, showing different rates of activity depending on the compounds, whose IC_{50} values range between 4.5 – 19.6 μg mL^{-1} (Huang et al., 2013b). Furthermore, the ethanolic extracts from *B. pinnatum* maintained their anti-inflammatory activity when transferred to *in vivo* murine models (Tanko, 2012), thus suggesting a promising approach for their application from *in vitro* to *in vivo* conditions.

3.2.4. Antimicrobial activity

Table 3 includes the results for antimicrobial activity, for both antibacterial (expressed as MIC and MBC) and antifungal activities (expressed as MIC and MFC). Concerning the antibacterial activities, differential results were obtained depending on the genotype employed. *Bacillus cereus* was the strain showing the highest sensitivity against *Bryophyllum* extracts (MIC = 0.9 mg mL^{-1} for 1/2MS-cultured *B. daigremontianum* extracts), together with other Gram-positive strains, such as *Listeria monocytogenes* and *Staphylococcus aureus* in a lesser extent (MIC = 1.8 and 3.6 mg mL^{-1}, respectively). On the other hand, *Escherichia coli* was the Gram-negative strain showing the highest sensitivity against *Bryophyllum* extracts (MIC = 1.8 mg mL^{-1} for 1/2MS-cultured *B. daigremontianum* extracts), whereas *Enterobacter cloacae* and *Salmonella* Typhimurium showed an increased resistance (MIC = 7.2 mg mL^{-1}), both providing identical results (Table 3). The MBC values were in accordance with the results shown for MIC (Table 3).

In the case of antifungal activities, differential results were also obtained depending on the genotype employed. Our results reflected a differential efficiency towards fungal strains: *Aspergillus ochraceus* showed the greatest sensitivity against all the extracts, with MIC values ranging between 0.5 mg mL^{-1}, for *B. daigremontianum* and *B. tubiflorum*, and 0.8 mg mL^{-1}, for *B.* × *houghtonii* (Table 3). In addition, *B. daigremontianum* and *B.* × *houghtonii* also showed an enhanced antifungal activity against *Aspergillus fumigatus* and *Penicillium funiculosum*

Table 3
Antimicrobial activity of *Bryophyllum* extracts. Streptomycin and ampicillin were used as control for bacterial growth; ketoconazole and bifonazole for fungus growth. The results were given as minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC; all in mg mL^{-1}).

	B. daigremontianum				*B.* × *houghtonii*				*B. tubiflorum*				Positive controls			
	MS		1/2MS		MS		1/2MS		MS		1/2MS					
Antimicrobial activity													**Streptomycin**		**Ampicillin**	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
B. cereus	1.6	3.2	0.9	1.8	1.7	3.4	1.6	3.1	1.8	3.6	6.6	13.2	0.1	0.2	0.3	0.4
S. aureus	6.4	12.8	3.6	7.2	13.6	>13.6	12.4	>12.4	3.6	7.2	6.6	13.2	0.04	0.1	0.3	0.5
L. monocytogenes	6.4	12.8	1.8	3.6	6.8	13.6	6.2	12.4	3.6	7.2	6.6	13.2	0.2	0.3	0.4	0.5
E. coli	3.2	6.4	1.8	3.6	6.8	13.6	6.2	12.4	3.6	7.2	6.6	13.2	0.2	0.3	0.4	0.5
E. cloacae	3.2	6.4	7.2	>7.2	5.1	6.8	6.2	12.4	3.6	7.2	6.6	13.2	0.2	0.3	0.3	0.5
S. Typhimurium	3.2	6.4	7.2	>7.2	5.1	6.8	6.2	12.4	3.6	7.2	6.6	13.2	0.2	0.3	0.8	1.2
Antifungal activity													**Ketoconazole**		**Bifonazole**	
	MIC	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**
A. fumigatus	0.8	1.6	0.5	0.9	0.9	1.7	0.8	1.6	0.9	1.8	1.7	1.3	0.3	0.5	0.2	0.2
A. ochraceus	0.8	1.6	0.5	0.9	0.9	1.7	0.8	1.6	0.5	0.9	0.8	1.7	0.2	0.5	0.1	0.2
A. niger	1.2	1.6	0.9	1.8	1.7	3.4	2.3	3.1	1.8	3.6	1.7	3.3	0.2	0.5	0.2	0.2
P. funiculosum	0.8	1.6	0.9	1.8	1.7	3.4	0.8	1.6	0.9	1.8	1.7	3.3	0.2	0.5	0.2	0.3
P. ochrochloron	1.6	3.2	0.9	1.8	1.7	3.4	2.3	3.1	1.8	3.6	3.3	6.6	2.5	3.5	0.2	0.3
P. verrucosum	0.8	1.6	0.9	1.8	1.7	3.4	2.3	3.1	0.9	1.8	1.7	3.3	0.2	0.3	0.1	0.2

(MIC = 0.8 mg mL^{-1} in all cases). The MFC values are in accordance with the results shown for MIC (Table 3). In all cases, positive controls showed greater antimicrobial activity than *Bryophyllum* extracts, probably due to the existence of heterogenous compounds with complex interactions that modulate such bioactivity, as stated above.

Myricetin, as the most prevalent non-anthocyanin flavonoid on *Bryophyllum* spp. aerial parts, has been shown to exert a potent antimicrobial activity. Specifically, this flavonol was found to exhibit a potent inhibition of both DNA and RNA polymerases in *E. coli* (Ong and Khoo, 1997) and showed a moderate growth inhibition against *S. aureus* and digestive-related bacteria (Xu and Lee, 2001), being our results in line with the previous literature. Malvidin, as the most representative anthocyanin in *Bryophyllum* spp., has also been associated to antimicrobial activity, including both antibacterial and antifungal activities (Cisowska et al., 2011). Thus, it is suggested that the phenolic constituents of *Bryophyllum* extracts are responsible for the inhibition of both bacterial and fungal growth. Previous works already reported that *Bryophyllum* extracts have a significant antimicrobial activity, as demonstrated by *B. daigremontianum*, showing a moderate activity against *B. cereus*, *S. aureus*, *E. coli* and *A. niger* (Anisimov et al., 2009; Nahar et al., 2008). The efficiency of *Bryophyllum* against enterobacteria shows a general pattern, showing an enhanced antibacterial activity, as demonstrated for *B. mortagei* and *B. fedtschenkoi* (Richwagen et al., 2019). In parallel, other authors have outlined the efficiency of *Bryophyllum* extracts against another relevant bacteria, such as *Pseudomonas aeruginosa*, as found for *B. daigremontianum* (Nahar et al., 2008) and *B. pinnatum* (Okwu and Nnamdi, 2011).

4. CONCLUSIONS

In this work, the establishment of plant *in vitro* culture was performed as an effective strategy to enhance the phytochemical potential of three medicinal plant species belonging to the subgenus *Bryophyllum*. The identification and quantification of phenolic compounds showed that these species are a valuable source of flavonols and anthocyanins. Concerning the genotype, the species *B. daigremontianum* showed the highest rates of myricetin glycosides, whereas *B.* × *houghtonii* presented the highest rates of malvidin glycosides. In both cases, the use of culture media formulation with a reduced macronutrient concentration, as an approach to induce nutritional stress, led to the accumulation of such compounds through a phenomenon called elicitation. To provide insight into the phytochemical potential of *in vitro*-cultured *Bryophyllum* spp., different biological activities were analyzed. According to both antioxidant activity assays employed in this work, the extracts from *B.* × *houghtonii* cultured under nutritional stress, showed the highest rates of antioxidant activity. The same extracts showed the highest cytotoxic activity, with an enhanced effectiveness towards MCF-7 breast carcinoma cell line. Moreover, the anti-inflammatory activity presented the same patterns as observed for antioxidant and cytotoxic activity, in which extracts from *B.* × *houghtonii* under nutritional stress exhibited the highest activity. The results displayed in this work suggest that the highest rates of antioxidant, cytotoxic and anti-inflammatory activities attributed to *B.* × *houghtonii* cultured under nutritional stress are caused by the accumulation of anthocyanins, specifically malvidin glycosides. In this way, thanks to their associated antioxidant, cytotoxic and anti-inflammatory bioactivities, these anthocyanin-rich extracts, are considered as promising candidates to promote an integrative effectiveness against the cancer-related phenomena, as it is the case of oxidative stress, inflammation and malignant cell proliferation, closely related during the initiation of carcinogenic process. Finally, concerning antimicrobial activity, the extracts obtained from *B. daigremontianum* cultured under nutritional stress showed the highest activity against both Gram-positive bacteria, such as *B. cereus* and *L. monocytogenes*, and Gram-negative bacteria, including *E. coli*, together with some fungal *Aspergillus* and *Penicillium* species. Overall, this study shed light about the phytochemical potential of an unexplored plant subgenus, by providing evidence for considering *in vitro*-cultured *Bryophyllum* spp. as a valuable source of bioactive compounds to be applied in different bio-based industrial areas.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Anisimov, M.M., Gerasimenko, N.I., Chaikina, E.L., Serebryakov, Y.M., 2009. Biological activity of metabolites of the herb *Kalanchoe daigremontiana* (Hamet de la Bathie) Jacobs et Perr. Biol. Bull. 36, 568–574. https://doi.org/10.1134/S1062359009060041.

Barros, L., Dueñas, M., Ferreira, I.C.F.R., Maria Carvalho, A., Santos-Buelga, C., 2011. Use of HPLC-DAD-ESI/MS to profile phenolic compounds in edible wild greens from Portugal. Food Chem. 127, 169–173. https://doi.org/10.1016/j.foodchem.2011.01.009.

Barzegar, A., 2016. Antioxidant activity of polyphenolic myricetin in vitro cell- free and cell-based systems. Mol. Biol. Res. Commun. 5, 87–95. https://doi.org/10.22099/mbrc.2016.3651.

Bessada, S.M.F., Barreira, J.C.M., Barros, L., Ferreira, I.C.F.R., Oliveira, M.B.P.P., 2016. Phenolic profile and antioxidant activity of *Coleostephus myconis* (L.) Rchb.f.: An underexploited and highly disseminated species. Ind. Crops Prod. 89, 45–51. https://doi.org/10.1016/j.indcrop.2016.04.065.

Blasco, B., Navarro-León, E., Ruiz, J.M., 2018. Oxidative stress in relation with micronutrient deficiency or toxicity. In: Hossain, M.A., Kamiya, T., Burritt, D., Tran, L.-S.P., Fujiwara, T. (Eds.), Plant Micronutrient Use Efficiency: Molecular and Genomic Perspectives in Crop Plants. Academic Press, London, UK, pp. 181–194. https://doi.org/10.1016/B978-0-12-812104-7.00011-3.

Bognar, E., Sarszegi, Z., Szabo, A., Debreceni, B., Kalman, N., Tucsek, Z., Sumegi, B., Gallyas, F., 2013. Antioxidant and anti-inflammatory effects in RAW264.7 macrophages of malvidin, a major red wine polyphenol. PLoS One 8. https://doi.org/10.1371/journal.pone.0065355.

Bogucka-Kocka, A., Zidorn, C., Kasprzycka, M., Szymczak, G., Szewczyk, K., 2016. Phenolic acid content, antioxidant and cytotoxic activities of four *Kalanchoë* species. Saudi J. Biol. Sci. 25, 622–630. https://doi.org/10.1016/j.sjbs.2016.01.037.

Boukhris, M.A., Destandau, É., El Hakmaoui, A., El Rhaffari, L., Elfakir, C., 2016. A dereplication strategy for the identification of new phenolic compounds from *Anvillea radiata* (Coss. & Durieu). Comptes Rendus Chim. 19, 1124–1132. https://doi.org/10.1016/j.crci.2016.05.019.

Casanova, J.M., dos Santos Nascimento, L.B., Casanova, L.M., Leal-Costa, M.V., Costa, S. S., Tavares, E.S., 2020. Differential distribution of flavonoids and phenolic acids in leaves of *Kalanchoe delagoensis* Ecklon & Zeyher (Crassulaceae). Microsc. Microanal. 1–8. https://doi.org/10.1017/s1431927620024344.

Castro, P.H., Lilay, G.H., Assunção, A.G.L., 2018. Regulation of micronutrient homeostasis and deficiency response in plants. In: Hossain, M.A., Kamiya, T., Burritt, D., Tran, L.-S.P., Fujiwara, T. (Eds.), Plant Micronutrient Use Efficiency: Molecular and Genomic Perspectives in Crop Plants. Academic Press, London, UK, pp. 1–15. https://doi.org/10.1016/B978-0-12-812104-7.00002-2.

Chernetskyy, M., Woźniak, A., Skalska-Kamińska, A., Żuraw, B., Blicharska, E., Rejdak, R., Donica, H., Weryszko-Chmielewska, E., 2018. Structure of leaves and phenolic acids in *Kalanchoë daigremontiana* Raym.-Hamet & H. Perrier. Acta Sci. Pol. Hortorum Cultus 17, 137–155. https://doi.org/10.24326/asphc.2018.4.13.

Cisowska, A., Wojnicz, D., Hendrich, A.B., 2011. Anthocyanins as antimicrobial agents of natural plant origin. Nat. Prod. Commun. 6, 149–156. https://doi.org/10.1177/1934578x1100600136.

Cramer, G.R., Urano, K., Delrot, S., Pezzotti, M., Shinozaki, K., 2011. Effects of abiotic stress on plants: A systems biology perspective. BMC Plant Biol. 11, 163. https://doi.org/10.1186/1471-2229-11-163.

de Paiva, L.S., Hayashi, E.A., De Melo, G.O., Costa, S.S., Koatz, V.L.G., Nobrega, A., 2008d. Inhibition of B cell development by kalanchosine dimalate. Int. Immunopharmacol. 8, 828–835. https://doi.org/10.1016/j.intimp.2008.01.028.

Descoings, B., 2006. Le genre *Kalanchoe* structure et définition par Bernard Descoings. Le J. Bot. 33, 3–28.

Dias, M.I., Sousa, M.J., Alves, R.C., Ferreira, I.C.F.R., 2016. Exploring plant tissue culture to improve the production of phenolic compounds: A review. Ind. Crops Prod. 82, 9–22. https://doi.org/10.1016/j.indcrop.2015.12.016.

El Sayed, A.M., Ezzat, S.M., El Naggar, M.M., El Hawary, S.S., 2016. In vivo diabetic wound healing effect and HPLC–DAD–ESI–MS/MS profiling of the methanol extracts of eight *Aloe* species. Rev. Bras. Farmacogn. 26, 352–362. https://doi.org/10.1016/j.bjp.2016.01.009.

Elinav, E., Nowarski, R., Thaiss, C.A., Hu, B., Jin, C., Flavell, R.A., 2013. Inflammation-induced cancer: Crosstalk between tumours, immune cells and microorganisms. Nat. Rev. Cancer 13, 759–771. https://doi.org/10.1038/nrc3611.

Flamini, R., 2013. Recent applications of mass spectrometry in the study of grape and wine polyphenols. ISRN Spectrosc. 2013, 1–45. https://doi.org/10.1155/2013/813563.

Fürer, K., Raith, M., Brenneisen, R., Mennet, M., Simões-Wüst, A.P., Von Mandach, U., Hamburger, M., Potterat, O., 2013. Two new flavonol glycosides and a metabolite profile of *Bryophyllum pinnatum*, a phytotherapeutic used in obstetrics and gynaecology. Planta Med. 79, 1565–1571. https://doi.org/10.1055/s-0033-1350808.

Fürer, K., Simões-Wüst, A.P., Von Mandach, U., Hamburger, M., Potterat, O., 2016. *Bryophyllum pinnatum* and related species used in anthroposophic medicine: Constituents, pharmacological activities, and clinical efficacy. Planta Med. 82, 930–941. https://doi.org/10.1055/s-0042-106727.

Garcês, H., Sinha, N., 2009. The "Mother of Thousands" (*Kalanchoë daigremontiana*): A plant model for asexual reproduction and CAM studies. Cold Spring Harb. Protoc. 4, 1–9. https://doi.org/10.1101/pdb.emo133.

Garcês, H.M.P., Koenig, D., Townsley, B.T., Kim, M., Sinha, N.R., 2014. Truncation of LEAFY COTYLEDON1 protein is required for asexual reproduction in *Kalanchoë daigremontiana*. Plant Physiol. 165, 196–206. https://doi.org/10.1104/pp.114.237222.

García-Pérez, P., Barreal, M.E., Rojo-De Dios, L., Cameselle-Teijeiro, J.F., Gallego, P.P., 2019. Bioactive natural products from the genus *Kalanchoe* as cancer chemopreventive agents: A review. In: Ur-Rahman, A. (Ed.), Studies in Natural Products Chemistry, 61. Elsevier, Amsterdam, pp. 49–84.

García-Pérez, P., Losada-Barreiro, S., Bravo-Díaz, C., Gallego, P.P., 2020a. Exploring the use of *Bryophyllum* as natural source of bioactive compounds with antioxidant activity to prevent lipid oxidation of fish oil-in-water emulsions. Plants 9, 1012. https://doi.org/10.3390/plants9081012.

García-Pérez, P., Losada-Barreiro, S., Gallego, P.P., Bravo-Díaz, C., 2019a. Adsorption of gallic acid, propyl gallate and polyphenols from *Bryophyllum* extracts on activated carbon. Sci. Rep. 9, 14830. https://doi.org/10.1038/s41598-019-51322-6.

García-Pérez, P., Losada-Barreiro, S., Gallego, P.P., Bravo-Díaz, C., 2019b. Cyclodextrin-elicited *Bryophyllum* suspension cultured cells: Enhancement of the production of bioactive compounds. Int. J. Mol. Sci. 20, 5180. https://doi.org/10.3390/ijms20205180.

García-Pérez, P., Lozano-Milo, E., Landín, M., Gallego, P.P., 2020b. Combining medicinal plant in vitro culture with machine learning technologies for maximizing the production of phenolic compounds. Antioxidants 9, 210. https://doi.org/10.3390/antiox9030210.

García-Pérez, P., Lozano-Milo, E., Landín, M., Gallego, P.P., 2020c. From ethnomedicine to plant biotechnology and machine learning: the valorization of medicinal plant *Bryophyllum* sp. Pharmaceuticals 13, 444. https://doi.org/10.3390/ph13120444.

García-Pérez, P., Lozano-Milo, E., Landin, M., Gallego, P.P., 2020d. Machine Learning unmasked nutritional imbalances on the medicinal plant *Bryophyllum* sp. cultured in vitro. Front. Plant Sci. 11, 576177 https://doi.org/10.3389/fpls.2020.576177.

García-Pérez, P., Lozano-Milo, E., Landin, M., Gallego, P.P., 2020e. Machine Learning technology reveals the concealed interactions of phytohormones on medicinal plant in vitro organogenesis. Biomolecules 10, 746. https://doi.org/10.3390/biom10050746.

Geng, P., Sun, J., Zhang, M., Li, X., Harnly, J.M., Chen, P., 2016. Comprehensive characterization of C-glycosyl flavones in wheat (*Triticum aestivum* L.) germ using UPLC-PDA-ESI/HRMSn and mass defect filtering. J. Mass Spectrom. 51, 914–930. https://doi.org/10.1002/jms.3803.

Ghouti, D., Rached, W., Abdallah, M., Pires, T.C.S.P., Calhelha, R.C., Alves, M.J., Abderrahmane, L.H., Barros, L., Ferreira, I.C.F.R., 2018. Phenolic profile and in vitro bioactive potential of Saharan *Juniperus phoenicea* L. and *Cotula cinerea* (Del) growing in Algeria. Food Funct. 9, 4664–4672. https://doi.org/10.1039/c8fo01392f.

Gonçalves, G.A., Soares, A.A., Correa, R.C.G., Barros, L., Haminiuk, C.W.I., Peralta, R.M., Ferreira, I.C.F.R., Bracht, A., 2017. Merlot grape pomace hydroalcoholic extract improves the oxidative and inflammatory states of rats with adjuvant-induced arthritis. Journal of Functional Foods 33, 408–418.

Góraj-Koniarska, J., Stochmal, A., Oleszek, W., Mołdoch, J., Saniewski, M., 2015. Elicitation of anthocyanin production in roots of *Kalanchoe blossfeldiana* by methyl jasmonate. Acta Biol. Cracoviensia Ser. Bot. 57, 141–148. https://doi.org/10.1515/abcsb-2015-0007.

Guimarães, R., Barros, L., Dueñas, M., Calhelha, R.C., Carvalho, A.M., Santos-Buelga, C., Queiroz, M.J.R.P., Ferreira, I.C.F.R., 2013. Nutrients, phytochemicals and bioactivity of wild Roman chamomile: A comparison between the herb and its preparations. Food Chem. 136, 718–725. https://doi.org/10.1016/j.foodchem.2012.08.025.

Hämäläinen, M., Nieminen, R., Vuorela, P., Heinonen, M., Moilanen, E., 2007. Anti-inflammatory effects of flavonoids: Genistein, kaempferol, quercetin, and daidzein inhibit STAT-1 and NF-κB activations, whereas flavone, isorhamnetin, naringenin, and pelargonidin inhibit only NF-κB activation along with their inhibitory effect on i. Mediators Inflamm. 2007 https://doi.org/10.1155/2007/45673.

Heim, K.E., Tagliaferro, A.R., Bobilya, D.J., 2002. Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. J. Nutr. Biochem. 13, 572–584. https://doi.org/10.1016/S0955-2863(02)00208-5.

Heleno, S.A., Ferreira, I.C.F.R., Esteves, A.P., Ćirić, A., Glamočlija, J., Martins, A., Soković, M., Queiroz, M.J.R.P., 2013. Antimicrobial and demelanizing activity of *Ganoderma lucidum* extract, p-hydroxybenzoic and cinnamic acids and their synthetic acetylated glucuronide methyl esters. Food Chem. Toxicol. 58, 95–100. https://doi.org/10.1016/j.fct.2013.04.025.

Hsieh, Y.-J., Huang, H.-S., Leu, Y.-L., Peng, K.-C., Chang, C.-J., Chang, M.-Y., 2016. Anticancer activity of *Kalanchoe tubiflora* extract against human lung cancer cells in vitro and in vivo. Environ. Toxicol. 31, 1663–1673.

Huang, H.C., Huang, G.J., Liaw, C.C., Yang, C.S., Yang, C.P., Kuo, C.L., Tseng, Y.H., Wang, S.Y., Chang, W. Te, Kuo, Y.H., 2013a. A new megastigmane from *Kalanchoe tubiflora* (Harvey) Hamet. Phytochem. Lett. 6, 379–382. https://doi.org/10.1016/j.phytol.2013.04.002.

Huang, H.C., Lin, M.K., Yang, H.L., Hseu, Y.C., Liaw, C.C., Tseng, Y.H., Tsuzuki, M., Kuo, Y.H., 2013b. Cardenolides and bufadienolide glycosides from *Kalanchoe tubiflora* and evaluation of cytotoxicity. Planta Med. 79, 1362–1369. https://doi.org/10.1055/s-0033-1350646.

Huang, W., Zhu, Y., Li, C., Sui, Z., Min, W., 2016. Effect of blueberry anthocyanins malvidin and glycosides on the antioxidant properties in endothelial cells. Oxid. Med. Cell. Longev. 2016 https://doi.org/10.1155/2016/1591803.

Hyun, J.W., Chung, H.S., 2004. Cyanidin and malvidin from *Oryza sativa*cv. Heugjinjubyeo mediate cytotoxicity against human monocytic leukemia cells by arrest of G2/M phase and induction of apoptosis. J. Agric. Food Chem. 52, 2213–2217. https://doi.org/10.1021/jf030370h.

Jiao, D., Zhang, X.D., 2016. Myricetin suppresses p21-activated kinase 1 in human breast cancer MCF-7 cells through downstream signaling of the β-catenin pathway. Oncol. Rep. 36, 342–348. https://doi.org/10.3892/or.2016.4777.

Kolodziejczyk-Czepas, J., Stochmal, A., 2017. Bufadienolides of *Kalanchoe* species: an overview of chemical structure, biological activity and prospects for pharmacological use. Phytochem. Rev. 16, 1155–1171. https://doi.org/10.1007/s11101-017-9525-1.

Lin, L.Z., Harnly, J.M., 2010. Identification of the phenolic components of chrysanthemum flower (*Chrysanthemum morifolium* Ramat). Food Chem. 120, 319–326. https://doi.org/10.1016/j.foodchem.2009.09.083.

Lockowandt, L., Pinela, J., Roriz, C.L., Pereira, C., Abreu, R.M.V., Calhelha, R.C., Alves, M.J., Barros, L., Bredol, M., Ferreira, I.C.F.R., 2019. Chemical features and bioactivities of cornflower (*Centaurea cyanus* L.) capitula: The blue flowers and the unexplored non-edible part. Ind. Crops Prod. 128, 496–503. https://doi.org/10.1016/j.indcrop.2018.11.059.

Mahata, S., Maru, S., Shukla, S., Pandey, A., Mugesh, G., Das, B.C., Bharti, A.C., 2012. Anticancer property of *Bryophyllum pinnata* (Lam.) Oken. leaf on human cervical cancer cells. BMC Complement. Altern. Med. 12, 15. https://doi.org/10.1186/1472-6882-12-15.

Milad, R., 2014. Genus Kalanchoe (Crassulaceae): a review of its ethnomedicinal, botanical, chemical and pharmacological properties. European J. Med. Plants 4, 86–104. https://doi.org/10.9734/ejmp/2014/5901.

Mohan, S.C., Balamurugan, V., Elayaraja, R., Prabakaran, A.S., 2012. Antioxidant and phytochemical potential of medicinal plant *Kalanchoe pinnata*. Int J Pharm Sci Res 3, 881–885.

Moon, K.B., Park, J.S., Park, Y. Il, Song, I.J., Lee, H.J., Cho, H.S., Jeon, J.H., Kim, H.S., 2020. Development of systems for the production of plant-derived biopharmaceuticals. Plants 9, 1–21. https://doi.org/10.3390/plants9010030.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. 15, 473–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x.

Muzitano, M.F., Tinoco, L.W., Guette, C., Kaiser, C.R., Rossi-Bergmann, B., Costa, S.S., 2006. The antileishmanial activity assessment of unusual flavonoids from *Kalanchoe pinnata*. Phytochemistry 67, 2071–2077. https://doi.org/10.1016/j.phytochem.2006.06.027.

Nahar, K., Khan, M.G.U., Rahman, M.S., Begum, B., Rashid, M.A., 2008. Antimicrobial and cytotoxic activities of *Bryophyllum daigremontianum*. Dhaka Univ. J. Pharm. Sci. 7, 99–101.

Narayani, M., Srivastava, S., 2017. Elicitation: a stimulation of stress in in vitro plant cell/tissue cultures for enhancement of secondary metabolite production. Phytochem. Rev. 16, 1227–1252. https://doi.org/10.1007/s11101-017-9534-0.

Nascimento, L.B.D.S., Leal-Costa, M.V., Menezes, E.A., Lopes, V.R., Muzitano, M.F., Costa, S.S., Tavares, E.S., 2015. Ultraviolet-B radiation effects on phenolic profile and flavonoid content of *Kalanchoe pinnata*. J. Photochem. Photobiol. B Biol. 148, 73–81. https://doi.org/10.1016/j.jphotobiol.2015.03.011.

Nielsen, A.H., Olsen, C.E., Møller, B.L., 2005. Flavonoids in flowers of 16 *Kalanchoë blossfeldiana* varieties. Phytochemistry 66, 2829–2835. https://doi.org/10.1016/j.phytochem.2005.09.041.

Okwu, D.E., Nnamdi, F.U., 2011. Two novel flavonoids from *Bryophyllum pinnatum* and their antimicrobial activity. J. Chem. Pharm. Res. 3, 1–10.

Ong, K.C., Khoo, H.E., 1997. Biological effects of myricetin. Gen. Pharmacol. 29, 121–126. https://doi.org/10.1016/S0306-3623(96)00421-1.

Oroian, M., Escriche, I., 2015. Antioxidants: Characterization, natural sources, extraction and analysis. Food Res. Int. 74, 10–36. https://doi.org/10.1016/J.FOODRES.2015.04.018.

Parejo, I., Jauregui, O., Sánchez-Rabaneda, F., Viladomat, F., Bastida, J., Codina, C., 2004. Separation and characterization of phenolic compounds in fennel (*Foeniculum vulgare*) using liquid chromatography-negative electrospray ionization tandem mass spectrometry. J. Agric. Food Chem. 52, 3679–3687. https://doi.org/10.1021/jf030813h.

Pereira, O.R., Peres, A.M., Silva, A.M.S., Domingues, M.R.M., Cardoso, S.M., 2013. Simultaneous characterization and quantification of phenolic compounds in *Thymus* x *citriodorus* using a validated HPLC-UV and ESI-MS combined method. Food Res. Int. 54, 1773–1780. https://doi.org/10.1016/j.foodres.2013.09.016.

Quazi Majaz, A., Tatiya, A.U., Khurshid, M., Nazim, S., Siraj, S., 2011. The miracle plant (*Kalanchoe pinnata*): a phytochemical and pharmacological review. Int. J. Res. Ayurveda Pharm. 2, 1478–1482.

Richwagen, N., Lyles, J.T., Dale, B.L.F., Quave, C.L., 2019. Antibacterial activity of *Kalanchoe mortagei* and *K. fedtschenkoi* against ESKAPE pathogens. Front. Pharmacol. 10, 1–13. https://doi.org/10.3389/fphar.2019.00067.

Semwal, D.K., Semwal, R.B., Combrinck, S., Viljoen, A., 2016. Myricetin: A dietary molecule with diverse biological activities. Nutrients 8, 1–31. https://doi.org/10.3390/nu8020090.

Sharma, A., Shahzad, B., Rehman, A., Bhardwaj, R., Landi, M., Zheng, B., 2019. Response of phenylpropanoid pathway and the role of polyphenols in plants under abiotic stress. Molecules 24, 1–22. https://doi.org/10.3390/molecules24132452.

Souilem, F., Fernandes, Â., Calhelha, R.C., Barreira, J.C.M., Barros, L., Skhiri, F., Martins, A., Ferreira, I.C.F.R., 2017. Wild mushrooms and their mycelia as sources of bioactive compounds: Antioxidant, anti-inflammatory and cytotoxic properties. Food Chem. 230, 40–48. https://doi.org/10.1016/j.foodchem.2017.03.026.

Srinivas Raghavan, B., Kondath, S., Anantanarayanan, R., Rajaram, R., 2015. Kaempferol mediated synthesis of gold nanoparticles and their cytotoxic effects on MCF-7 cancer cell line. Process Biochem. 50, 1966–1976. https://doi.org/10.1016/j.procbio.2015.08.003.

Stefanowicz-Hajduk, J., Asztemborska, M., Krauze-Baranowska, M., Godlewska, S., Gucwa, M., Moniuszko-Szajwaj, B., Stochmal, A., Ochocka, J.R., 2020a. Identification of flavonoids and bufadienolides and cytotoxic effects of *Kalanchoe daigremontiana* extracts on human cancer cell lines. Planta Med. 86, 239–246. https://doi.org/10.1055/a-1099-9786.

Stefanowicz-Hajduk, J., Hering, A., Gucwa, M., Hałasa, R., Soluch, A., Kowalczyk, M., Stochmal, A., Ochocka, R., 2020b. Biological activities of leaf extracts from selected *Kalanchoe* species and their relationship with bufadienolides content. Pharm. Biol. 58, 732–740. https://doi.org/10.1080/13880209.2020.1795208.

Stiborová, M., Poljaková, J., Martínková, E., Bořek-Dohalská, L., Eckschlager, T., Kizek, R., Frei, E., 2011. Ellipticine cytotoxicity to cancer cell lines-a comparative study. Interdiscip. Toxicol. 4, 98–105. https://doi.org/10.2478/v10102-011-0017-7.

Supratman, U., Fujita, T., Akiyama, K., Hayashi, H., 2001. Insecticidal compounds from *Kalanchoe daigremontiana* x *tubiflora*. Phytochemistry 58, 311–314. https://doi.org/10.1016/S0031-9422(01)00199-6.

Tahir, N.I., Shaari, K., Abas, F., Parveez, G.K.A., Ishak, Z., Ramli, U.S., 2012. Characterization of apigenin and luteolin derivatives from oil palm (*Elaeis guineensis* Jacq.) leaf using LC–ESI-MS/MS. J. Agric. Food Chem. 60, 11201–11210.

Tanko, Y., 2012. Antinociceptive and anti-inflammatory activities of ethanol extract of *Bryophyllum pinnatum* laboratory animals. IOSR J. Dent. Med. Sci. 3, 46–49. https://doi.org/10.9790/0853-0314649.

Tatsimo, S.J.N., Tamokou, J.D.D., Havyarimana, L., Csupor, D., Forgo, P., Hohmann, J., Kuiate, J.R., Tane, P., 2012. Antimicrobial and antioxidant activity of kaempferol rhamnoside derivatives from *Bryophyllum pinnatum*. BMC Res. Notes 5, 1–6. https://doi.org/10.1186/1756-0500-5-158.

Tsao, R., Deng, Z., 2004. Separation procedures for naturally occurring antioxidant phytochemicals. J. Chromatogr. B 812, 85–99. https://doi.org/10.1016/J.JCHROMB.2004.09.028.

Vallverdú-Queralt, A., Jáuregui, O., Di Lecce, G., Andrés-Lacueva, C., Lamuela-Raventós, R.M., 2011. Screening of the polyphenol content of tomato-based products through accurate-mass spectrometry (HPLC-ESI-QTOF). Food Chem. 129, 877–883. https://doi.org/10.1016/j.foodchem.2011.05.038.

Xu, H.X., Lee, S.F., 2001. Activity of plant flavonoids against antibiotic-resistant bacteria. Phytother. Res. 15, 39–43. https://doi.org/10.1002/1099-1573(200102)15:1<39::aid-ptr684>3.0.co;2-r.

Yadav, N.P., Dixit, V.K., 2003. Hepatoprotective activity of leaves of *Kalanchoe pinnata* Pers. J. Ethnopharmacol. 86, 197–202. https://doi.org/10.1016/S0378-8741(03)00074-6.

Ziani, B.E.C., Barros, L., Boumehira, A.Z., Bachari, K., Heleno, S.A., Alves, M.J., Ferreira, I.C.F.R., 2018. Profiling polyphenol composition by HPLC-DAD-ESI/MSn and the antibacterial activity of infusion preparations obtained from four medicinal plants. Food Funct. 9, 149–159. https://doi.org/10.1039/c7fo01315a.