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Hardy kiwifruit leaves (*Actinidia arguta*): An extraordinary source of value-added compounds for food industry

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ABSTRACT

The present study reports for the first time the identification and quantification of phenolic compounds, the antioxidant and antimicrobial activities as well as the *in vitro* radical scavenging activity and intestinal cell effects of *A. arguta* leaves extracts. Extractions were carried out under water, water:ethanol (50:50) and ethanol. The highest antioxidant activity were obtained in alcoholic extract (IC_{50} = 53.95 ± 3.09 μg/mL for DPPH; 6628.42 ± 382.49 μmol/mg dry weight basis for FRAP) while the phenolic profile confirmed by HPLC analysis revealed highest amounts of phenolic acids (hydroxycinnamic acid derivatives) and flavonoids (flavan-3-ol and flavonols derivatives). An excellent scavenging activity against reactive oxygen and nitrogen species were determined for all extracts as well as no adverse effects on Caco-2 and HT29-MTX cells in concentrations below 100 μg/mL and 1000 μg/mL, respectively. These results highlight the potentialities of hardy kiwi leaves valorization.

1. Introduction

In the last decades, plants and their derivatives have been a valuable source of natural products for application in different areas, including food, pharmaceutical and cosmetics (Braga, Rodrigues, & Oliveira, 2015; Costa et al., 2014; Rodrigues, Santos, et al., 2015). Likewise, more attention has been given to the sustainability concept, whereas sustainable production and consumption should embrace waste reduction and energy efficiency (Rodrigues et al., 2013). Nowadays, one of the greatest challenges for food researchers and manufacturers are the sustainable food production and the delivery of high quality food products. At the same time, an added functionality to prevent life-style related diseases is also advisable. For those reasons, a great number of research activities in the field of health related dietary aspects demonstrated the relation between the regular intake of phytochemicals (e.g. polyphenols, carotenoids and phytosterols), and the prevention of life-style related diseases (such as cancer, obesity, diabetes and cardiovascular complications or premature aging) (Ahmadi, Sadeghi, Modarresi, Abiri, & Mikaeli, 2010; Lim et al., 2016). In this context, characterizing the main bioactive compounds from natural sources can provide several benefits, not only at the biocompatibility level, but also as sustainability aspects and preservation of the environment.

The genus *Actinidia* (Actinidiaceae) is a deciduous woody, fruiting vine, which integrates different species and cultivars that exhibit a variety of physical characteristics and sensory attributes (Singletary, 2012). Kiwifruit is native of China, but it is now cultivated worldwide (~15 *Actinidia* species). Nevertheless, only three species have commercial importance: *A. deliciosa* (green kiwi), *A. chinensis* (gold kiwi) and *A. arguta* (hardy kiwifruit). Currently, commercial growth of hardy kiwifruit has spread to many countries including the United States, Italy, France, Greece, Brazil and Portugal (Nishiyama, 2007), wherein New Zealand and Chile are the most significant producers, having produced in 2013, 318 632 and 212 999 metric tons, respectively (O'Rourke, 2014). It is believed that the world production of kiwifruits tends to increase, considering the consumer's interest and the nutritional reports. According to several authors, hardy kiwifruit pulp contains high amounts of ascorbic acid (50–430 mg/100 g fresh weight) and relatively high contents of other nutraceuticals (Nishiyama, 2007; Nishiyama et al., 2004). Gallic acid is described as the major phenolic compound (~2 mg/g fresh weight) in the pulp (Lim et al., 2016), also

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presenting natural pigments (Antunes, Dandlen, Cavaco, & Miguel, 2010; Hunter, Skinner, Ferguson, & Stevenson, 2010). Furthermore, hardy kiwifruit possess 4- to 6-fold higher levels of myoinositol compared with the green or the yellow kiwi, being one of the richest dietary sources of this sugar alcohol, recognized as an important nutrient for human cells (Singletary, 2012). Nevertheless, until now no studies of *A. arguta* leaves have been published regarding its bioactivity and safeness. The aim of this study is to assess the health-promoting properties of *A. arguta* leaves on the basis of a comprehensive study of the phenolic profile, bioactivity and cell viability (through intestinal cells).

2. Material and methods

2.1. Chemicals and reagents

All chemicals and solvents used were of analytical grade, obtained from commercial sources and used as received or dried by standard procedures. 2,2-diphenyl-1-picryl-hydrazyl (DPPH) were obtained from Aldrich Chemical Co. (Steinheim, Germany). Folin–Ciocalteu reagent, gallic acid, catechin and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, USA). Dihydrorhodamine 123 (DRH), 4,5-diaminofluorescein (DAF-2), hydrogen peroxide 30%, sodium hypochlorite solution with 4% available chlorine, 3-(aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5), b-nicotinamide adenine dinucleotide (NADH), phenazine methosulphate (PMS), nitroblue tetrazolium chloride (NBT), α,α'-Azodiisobutyramidine dihydrochloride (AAPH), Tiron, Trolox, fluorescein sodium salt, lucigenin and quercetin were obtained from Sigma Aldrich (Steinheim, Germany). Butylated hydroxytoluene (BHT) and ascorbic acid were purchased from Merck (Darmstadt, Germany) while dimethylsulfoxide (DMSO) was from AppliChem (Darmstadt, Germany). Caco-2 clone type C2BBe1 and HT29-MTX cell lines were obtained from the American Type Culture Collection (ATCC, USA). Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), non-essential amino acids, penicillin and streptomycin, trypsin–EDTA and Hank's Balanced Salt Solution (HBSS) were purchased from Invitrogen Corporation (Life Technologies, S.A., Madrid, Spain).

2.2. Samples

Hardy kiwifruit leaves were collected during May 2016, in a production farm, located in Vila Nova de Famalicão, north of Portugal (GPS: 41.376830,-8.469748). After harvesting, the leaves were stored in sample bottles at −80 °C for subsequent lyophilization (Telstar, Cryodos, Spain). Thereafter they were ground in a mill (Grindomix GM200, Rech, Germany) to obtain a fine and homogeneous powder, which subsequently was stored in plastic tubes at 4 °C until the extracts preparation.

2.3. Extracts preparation

Powdered samples (1 g) were extracted with 50 mL of ethanol, water and ethanol-water (50:50 v/v) during 1 h, at 50 °C, on a heating plate (Mirak, Thermolyse, USA) under constant stirring (600 rpm) (Costa et al., 2014). Extracts were filtered through Whatman No. 1 filter paper, concentrated under vacuum (Vaccum Controller V-800, Büchi, Switzerland) at 37 °C and then lyophilized (Telstar, Cryodos, Spain). All extracts were stored at −20 °C for future analysis.

2.4. Determination of total phenolic content

Total phenolic content (TPC) was determined spectrophotometrically according to the Folin-Ciocalteu procedure described by Wootton-Beard *et al.* (Wootton-Beard, Moran, & Ryan, 2011). Briefly, 30 μL (1 mg/mL) of hardy kiwifruit leaves extracts (aqueous, hydroalcoholic and alcoholic) were mixed with 150 μL of Folin-Ciocalteu reagent (1:10, v/v) and 120 μL of 7.5% Na_2CO_3 solution, and allowed to stand for 15 min at 45 °C before the absorbance was determined at 765 nm using a Synergy HT GENS5 Microplate Reader (BioTek Instruments, Inc., Winooski, VT, EUA). Gallic acid was used as standard to obtain a calibration curve (linearity range = 5–100 ppm, R^2 = 0.9987). The TPC of the extracts was expressed as milligrams of gallic acid equivalents (GAE) per gram of plant material on dry weight basis (dw).

2.5. Determination of total flavonoid content

Total flavonoid content (TFC) was determined using a colorimetric assay previously validated (Rodrigues et al., 2013). Briefly, 30 μL of each extract was mixed with 75 μL of distilled water and 45 μL of 1% (w/v) $NaNO_2$ solution. After 5 min, 45 μL of 5% (w/v) $AlCl_3$ solution were added, and after 1 min, 60 μL of NaOH (1 M) and 45 μL of distilled water were also added. The absorbance was read at 510 nm using the Synergy HT Microplate Reader. Catechin was used as a reference to plot the standard curve (linearity range = 5–300 ppm, R^2 = 0.9999). TFC was expressed as mg of catechin equivalents (CAE) per g of plant material on dw.

2.6. In vitro antioxidant activity

2.6.1. DPPH free radical scavenging assay

The DPPH assay was used to evaluate the free radical scavenging activity of hardy kiwifruit leaves extracts. The reaction mixture was prepared directly on a 96 well plate between different sample concentrations (30 μL) and an ethanolic solution (270 μL) containing DPPH radicals (6×10^{-5} M) in each well. The reduction of the DPPH radical was determined at 517 nm at two-minute intervals, during 30 min (Barros, Baptista, & Ferreira, 2007). The radical scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the equation:% RSA = $[(A_{DPPH} - A_S)/A_{DPPH})] \times 100$, where A_S is the absorbance of the solution when the sample extract has been added at a particular level, and A_{DPPH} is the absorbance of the DPPH solution (Barros et al., 2007). The extract concentration providing 50% of radical scavenging activity (IC_{50}) was calculated from the graph of RSA percentage against the extract concentration.

2.6.2. Ferric reducing antioxidant power (FRAP) assay

The FRAP assay followed the protocol described by Benzie and Strain (1999) with minor modifications. An aliquot (35 μL) of each extract was added to 265 μL of the FRAP reagent (10 parts of 300 mM sodium acetate buffer at pH 3.6, 1 part of 10 mM TPTZ solution and 1 part of 20 mM $FeCl_3 \cdot 6H_2O$ solution) and the reaction mixture was incubated at 37 °C, during 30 min before reading at 595 nm. Solutions of known Fe(II) concentrations ($FeSO_4 \cdot 7H_2O$) were used to perform calibration curve (linearity range: 75–1000 μM, R^2 = 0.9992). Final results were expressed as ferrous sulphate equivalents per gram of plant material on dw.

2.7. Phenolic profile identification by HPLC-DAD

To study the phenolic profile of the different *A. arguta* leaves extracts, the dried residue obtained was dissolved in acetic acid solution (1%) at 5 mg/mL. The solutions were filtered with syringe filter (0.22 μm) prior to injection in a HPLC-DAD system (Jasco, Tokyo, Japan) equipped with AS-2057 automated injector, a PU-2089 pump and a MD-2018 multi-wavelength diode array detector (DAD). The chromatographic separation was achieved by gradient elution with 1% acetic acid solution (A) and methanol (B) accordingly to the following: 0 min, 90% A; 20 min, 70% A; 50 min, 25% A; 63 min, 0% A; 65 min, 90% A; 70 min, 90% A. The column, a Zorbax-SB-C18 (5 μm; 25 × 4.6 mm; Agilent Technologies, USA), was maintained at 20 °C during the analysis; a flow rate of 1 mL/min and an injection volume of

20 μL were used. The diode array detector recorded the spectra from 200 to 600 nm, being each run monitored at 280, 320 and 335 nm. The phenolic compounds identification was accomplished by comparison of the retention time and UV–Vis spectra with commercial standards, when available. Otherwise, peaks were tentatively identified comparing their UV–VIS spectra with available data in literature for the same type of samples (Cyboran, Oszmiański, & Kleszczyńska, 2014). The identified compounds were quantified by external calibration curves constructed based on their maximum UV signal. When the commercial standard was not available, the quantification was performed through the calibration curve of a compound from the same phenolic group. Specifically, all flavan-3-ol compounds were expressed as catechin equivalents and the hydroxycinnamic acids as caffeic acid equivalents (with exception of the caffeoyl-quinic compounds that were quantified as chlorogenic acid equivalents). The flavonols were either expressed as rutin equivalents or kaempferol equivalents, accordingly to their UV–Vispectra. The results were expressed in μg/mg dry extract, as mean ± standard deviation of two extracts.

2.8. Reactive nitrogen species and reactive oxygen species scavenging assays

The Reactive Nitrogen Species (RNS) and Reactive Oxygen Species (ROS) assays were carried out in a Synergy HT Microplate Reader, equipped with a thermostat for fluorescence, UV–Vis and chemiluminescence measurements. The hardy kiwifruit leaves extracts and the quercetin standard were dissolved in ethanol for ROS and RNS scavenging assays, except for superoxide radical ($O_2^{\cdot -}$) assay, dissolved in DMSO. Ascorbic acid standard was dissolved in Tris-HCl buffer (pH 7.4)/ethanol (1:1, v/v) in the hydrogen peroxide assay (H_2O_2). Quercetin and ascorbic acid standards were used as positive controls in the scavenging assays of nitric oxide ($^{\cdot}NO$) and H_2O_2, while quercetin, ascorbic acid and Tiron solutions were the positive controls in the $O_2^{\cdot -}$ scavenging assay. The IC_{50} values (the concentration required to obtain an inhibition capacity of 50%) were obtained from the curves of percentage of inhibition versus extract concentration, using the GraphPad Prism 7 software.

2.8.1. Superoxide radical scavenging assay

Scavenging capacities of hardy kiwifruit leaves extracts and positive controls against $O_2^{\cdot -}$ were measured using a colorimetric method, based on the reduction of NBT into a purple coloured diformazan by reaction with $O_2^{\cdot -}$. These ROS were generated through the non-enzymatic NADH/PMS/O_2 system, and the absorbance was read at 560 nm for 5 min at 37 °C (Gomes et al., 2007). Results were expressed as the inhibition, in IC_{50}, of the NBT reduction to diformazan.

2.8.2. Hydrogen peroxide scavenging assay

The H_2O_2 scavenging activity was measured by monitoring the $H_2O_2^-$ induced oxidation of lucigenin as previously described (Gomes et al., 2007). The endogenous antioxidant ascorbic acid was used as positive control. The results were expressed as the inhibition (in percentage) of the $H_2O_2^-$ induced oxidation of lucigenin (Boeing et al., 2017; Ribeiro et al., 2014).

2.8.3. Hypochlorous acid scavenging assay

The HOCl was measured by using a fluorescent methodology, described by Gomes *et al.* (Gomes, et al., 2007), based on the HOCl-induced oxidation of DHR to rhodamine. HOCl was prepared by adjusting the pH of a 1% (m/v) solution of NaOCl to 6.2 with addition of H_2SO_4 (10%). Quercetin and ascorbic acid were used as positive control. The results were expressed as the inhibition (in percentage) of HOCl-induced oxidation of DHR (Pistón et al., 2014; Ribeiro et al., 2014).

2.8.4. Peroxyl radical scavenging assay

The $ROO^{\cdot}$ scavenging capacity was measured by monitoring the effect of the tested extracts on the fluorescence decay resulting from ROO-induced oxidation of fluorescein (Boeing et al., 2017; Ou, Hampsch-Woodill, & Prior, 2001). ROO was generated by thermo decomposition of AAPH. Trolox was used as standard control and the results were expressed as ROO-induced oxidation of fluorescein calculated according to Gomes et al. (Gomes et al., 2007).

2.8.5. Nitric oxide scavenging assay

Scavenging capacity of hardy kiwifruit leaves extracts and positive controls against $^{\cdot}NO$ were carried out by monitoring the oxidation of the non-fluorescence DAF-2 to the fluorescent triazolofluorescein by the reaction with $^{\cdot}NO$, generated by the thermal decomposition of NOC-5 at 37 °C (Gomes et al., 2007). Results were expressed as the inhibition, in IC_{50}, of inhibition of $^{\cdot}NO$-induced oxidation of DAF-2.

2.8.6. Peroxynitrite scavenging assay

The $ONOO^-$ scavenging capacity was measured by monitoring the effect of the extracts and the standard (quercetin and ascorbic acid) on $ONOO^-$-induced oxidation of non-fluorescent DHR to fluorescent rhodamine 123, according to Gomes *et al.* (Gomes et al., 2007). The assays were performed in parallel in the absence and the presence of 25 mM $NaHCO_3$ to simulate the physiological CO_2 concentrations. The results were expressed as the inhibition, in percentage, of $ONOO^-$-induced oxidation of DHR.

2.9. Cell viability assay

Caco-2 clone type C2BBe1 (passage 75–79) and HT29-MTX (passage 30–32) cells have grown separately in tissue culture flasks (Orange Scientific, Belgium) in a complete medium, consisting of DMEM supplemented with 10% (v/v) inactivated FBS, 1% (v/v) l-glutamine, 1% (v/v) non-essential amino acids and 1% (v/v) antibiotic–antimitotic mixture (final concentration of 100 U/mL Penicillin and 100 U/mL Streptomycin) at 37 °C and 5% CO_2 in a water saturated atmosphere in an incubator (CellCulture® CO_2 Incubator, ESCO GB Ltd., UK).

The effect of hardy kiwi leaves extracts on cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTT) conversion assay. Caco-2 and HT29-MTX cells were seeded separately in 96-well microplates at 25×10^3 cells/well in supplemented DMEM and incubated for 24 h at 37 °C in 5% CO_2 environment. Extracts, negative control (DMEM), and positive control (1% (v/v) Titron X-100) were added in triplicate to the cell culture. The medium was then changed, and the cells treated with test samples for 24 h. Each treatment was tested in six individual wells. The supernatant was removed, and MTT solution added to each well and incubated for 3 h at 37 °C to allow the formation of formazan crystal. After that the medium was removed, and blue formazan eluted from cells using 150 μL of DMSO. The absorbance was measured at 590 nm with background subtraction at 630 nm.

2.10. Antimicrobial activity

Extracts were screened at different concentrations for the antimicrobial activity in different agar methods (embedding, surface scattering and agar diffusion). The positive results were submitted to the broth microdilution method in order to determine the minimum inhibitory concentration (MIC). Four microorganisms were included in the study (*Staphylococcus aureus* ATCC® 25923, *Escherichia coli* ATCC® 25922, *Pseudomonas aeruginosa* ATCC® 27853 and *Candida albicans* ATCC® 10231). For each experiment microorganisms were subcultured twice in Nutrient agar (Difco Laboratories, USA). For the evaluation of antimicrobial activity, cell suspensions (SC) were prepared in 1 mL of sterile 0.85% saline solution, with turbidity equivalent to 0.5 of the Mac Farland scale. Three methods were performed on agar to screen the antimicrobial activity. The presence of a growth inhibition halo greater than the halo containing only solvent was considered as a positive result.

2.10.1. Incorporation method

1 mL of cell suspension was added in the petri dish to 25 mL of MH agar. The mixture was homogenized by circular motions and placed for solidification at room temperature. After solidification, cavities of 6 mm diameter were made using sterile disposable tips and the bottom of the well was sealed with 10 µL of Mueller Hinton broth (MH; Difco Laboratories, USA). After solidification of the bottom sealing of the wells, 50 µL of the extract and the same volume of the solvent were added to another well. The plates were incubated during 24 h (48 h in the case of the yeast) at 37 °C.

2.10.2. Surface spreading method

Cell suspensions were seeded by surface scattering with a sterile swab on MH. Then wells were formed and the extract and solvent were added following the procedure previously described (see Section 2.10.1).

2.10.3. Agar diffusion method

Filter paper discs with 20 µL of extract and solvent were placed on the previously seeded MH agar surface, using a sterile swab with cell suspensions.

2.11. Statistical analysis

All experimental analyses were performed in triplicate and means and standard deviations were calculated. Statistical analysis of the data was carried out using IBM SPSS Statistics Version 24.0 (SPSS, Inc. Chicago, IL). One-way ANOVA was used to investigate the differences between different extracts for all assays. Post hoc comparisons of the means were performed according to Tukey's HSD test. In all cases, $p < 0.05$ was accepted as denoting significance. The IC_{50} values of ROS and RNS scavenging activity were calculated from the curves of percentage of inhibition versus antioxidant concentration, using the GraphPad Prism 5 software.

3. Results and discussion

3.1. Antioxidant activity evaluation

The total phenolic and flavonoid contents of *A. arguta* leaves extracts, as well as the IC_{50} values and the antioxidant activity (based on the FRAP assay) were summarized in Table 1.

To the best of our knowledge, few studies were carried out on the evaluation of *A. arguta* leaves (Ravipati et al., 2012). Nevertheless, the extraction conditions such as solvents and temperatures should be taken into account. For example, Ravipati *et al.* evaluated the antioxidant activity of some Chinese medicinal plants, including hardy kiwi leaves, and reported values considerably lower than the ones presented in this study (respectively, 4.71 mg GAE/g and 15.26 mg GAE/g for the aqueous and alcoholic extracts) (Ravipati et al., 2012). However, the authors used different extraction conditions, namely for the aqueous (121 °C for 1 h) and the alcoholic extract (70 °C for 6 h), that can be responsible for the hydrolyzation of the phenolic compounds present in the extracts (Oliveira et al., 2017). In another study, Latocha *et al.* reported the TPC of *A. arguta* fruits from different varieties ranging from 79.0 to 128.5 mg GAE/100 g (Latocha, Krupa, Wołosiak, Worobiej, & Wilczak, 2010). According to the same authors, the hardy kiwi peel was up to 12.6 and 3.0 times richer, respectively, in phenolics and ascorbate than the flesh fruit, being considered a significant source of bioactive compounds (Latocha, Łata, & Stasiak, 2015).

Flavonoids are considered the largest group of plant polyphenols, accounting for more than half of the eight thousand phenolic compounds identified in nature (Balasundram, Sundram, & Samman, 2006). According to Table 1, the aqueous extract presented the lowest result (136.72 mg CAE/g dw), followed by the hydroalcoholic (173.80 mg CAE/g dw) and the alcoholic one (318.11 mg CAE/g dw). These results suggest that the solvent used exerted a significant effect on the extraction efficiency, being ethanol the best one, clearly due to the solubility characteristics of this group of compounds (Rodrigues, Palmeira-de-Oliveira et al., 2015).

Different methodologies are available to evaluate the antioxidant activity of foodstuffs, being advisable the use of at least two, considering that each one has advantages and limitations. In the present study, the DPPH scavenging activity and the ferric-reducing antioxidant assays were used to evaluate the antioxidant activity. Regarding IC_{50} obtained on the DPPH assay, the best result was achieved with the alcoholic extract (53.95 µg/mL). For the aqueous extract it was not possible to evaluate the IC_{50}, even with the highest concentration tested (10520 µg/mL). Regarding the evaluation of the antioxidant capacity of extracts based on the FRAP assay, the obtained results are in line with the DPPH results, showing also the highest antioxidant activity in the alcoholic extract (6628.42 µmol of ferrous sulphate/g dw). This value is two times higher than the ones determined for the aqueous and hydroalcoholic extracts.

According to Rodrigues *et al.*, polyphenols are the main responsible for the antioxidant activity of plant extracts due to their ability to act as reducing agents or hydrogen-atom donors (Rodrigues et al., 2013). A regression analysis performed between the FRAP results (used to evaluate the antioxidant activity) and the TPC, achieved a positive correlation ($r^2 = 0.790$; $P < 0.01$). When the correlation study was performed between the IC_{50} values and the TPC (only considering the two samples for which the IC_{50} were achieved), the Pearson correlation was extremely negative ($r^2 = -0.997$; $P < 0.01$). Regarding the TPC and TFC the correlation determined was extremely positive ($r^2 = 0.918$; $P < 0.01$). These results are in accordance with other authors that also determined high correlations between TPC and antioxidant activity (Malheiro et al., 2012; Malheiro, Sousa, Casal, Bento, & Pereira, 2011; Velioglu, Mazza, Gao, & Oomah, 1998).

3.2. Phenolic profile of A. arguta extracts

The phenolic profile of a plant is directly influenced by intrinsic and extrinsic factors, like the plant development stage or the agronomic and environmental conditions during the growth (Santos, Oliveira, Ibáñez, & Herrero, 2014). The interaction of these factors will lead plants metabolism to produce different bioactive compounds, such as different

Table 1
Total polyphenol content (TPC), total flavonoid content (TFC), radical scavenging activity (RSA) IC_{50} values and antioxidant activities based on their abilities to reduce the ferric iron (Fe^{3+}) to the ferrous iron (Fe^{2+}) in the *A. arguta* leaves extracts. Values are expressed as means ± standard deviation ($n = 6$). GAE, gallic acid equivalents. CAE, catechin equivalents. Different letters (a, b, c) in the same row indicate significant differences between extracts ($P < 0.05$).

	Aqueous	Hydroalcoholic	Alcoholic
TPC (mg GAE/g dw)	189.39 ± 5.62^{b}	140.72 ± 10.22^{c}	440.71 ± 53.15^{a}
TFC (mg CAE/g dw)	136.72 ± 2.02^{c}	173.80 ± 2.26^{b}	318.11 ± 6.84^{a}
IC_{50} (µg/mL)	*	1097.28 ± 78.72^{a}	53.95 ± 3.09^{b}
FRAP (µmol of ferrous sulphate/g dw)	3398.78 ± 349.46^{c}	3076.35 ± 324.69^{b}	6628.42 ± 382.49^{a}

* IC_{50} was not determined up to the highest tested concentration (10520 µg/mL).

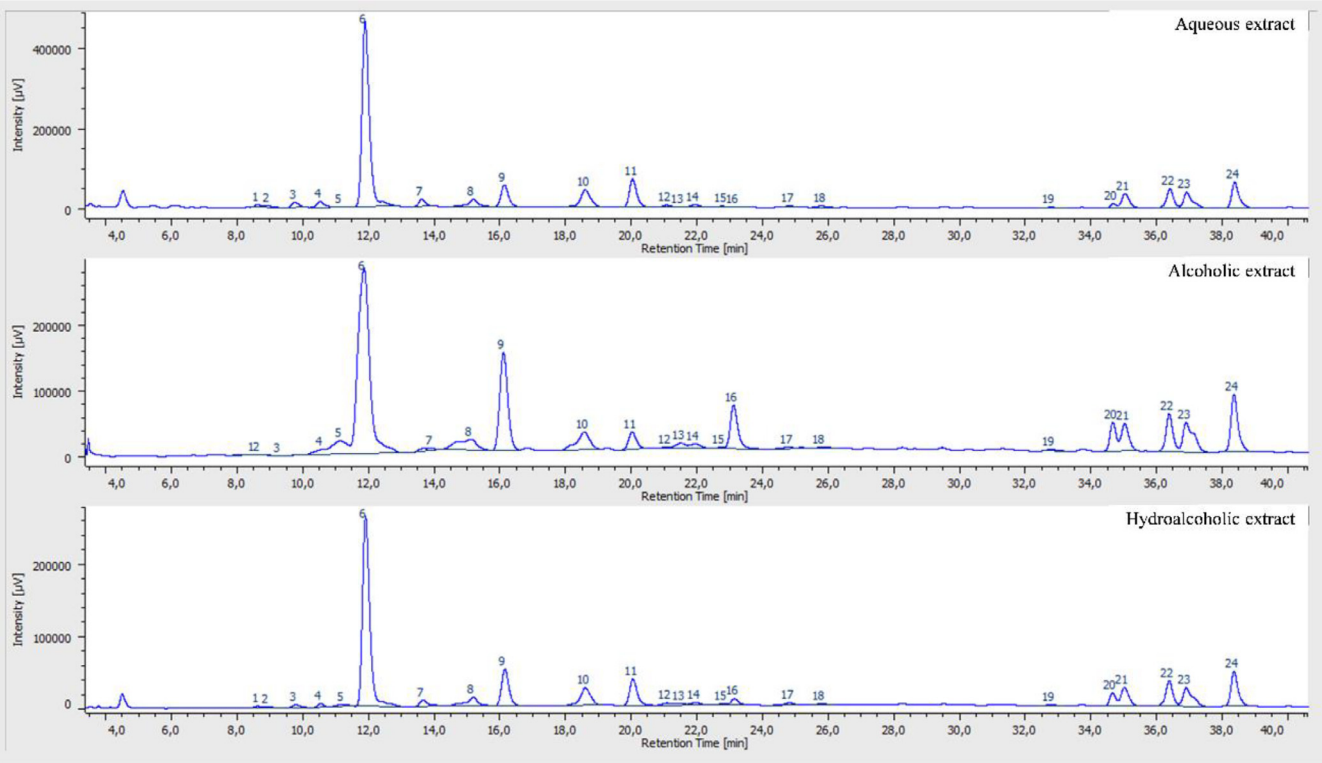

Fig. 1. Chromatogram obtained by HPLC–DAD of the aqueous, hydroalcoholic and alcoholic extracts from *A. arguta* leaves. Chromatographic conditions: see text. Processed at 280 nm. Peaks: see Table 2.

types of polyphenols. This group of substances will also respond differently to the extraction methods applied, due to their different solubility in several solvents (Das, Adsare, Das, Kulth, & Ganesan, 2017). The phenolic profile obtained for each extract studied is presented in Fig. 1 and Table 2, and the results of validation parameters of the HPLC method are shown in Table 3.

A total of 24 compounds were investigated in the extract's phenolic profile. Most of the compounds were detected in the three extracts, with exception of some minor compounds (peaks 1, 2 and 3) that were not present in the alcoholic extract. The UV–Vis spectra and retention time of each compound was first compared with the available standards, which allowed for the identification of peak 10 (Table 2) as being chlorogenic acid in all extracts. The subsequent attempt to identify the other compounds, presented in Table 2, was based on what was already described for this matrix and on their UV–Vis spectra maxima. The remaining compounds were classified in two main phenolic groups: the phenolic acids (in particular as derivatives of hydroxycinnamic acid) and flavonoids (derivatives of flavan-3-ol and flavonols). Flavan-3-ols (peaks 2, 5, 7, 12, 13, 14, 16) were classified by their spectrum absorbance maximum at 280 nm (Santos et al., 2014) in the three extracts. Most of these compounds were also identified as being catechin derivatives, being their presence already described in the phenolic composition of *A. arguta* leaves (Cyboran et al., 2014). Other flavon-3-ols were detected (peaks 4, 8, 9, 15, 17, 18 and 19 in the alcoholic extract, and peaks 8, 17, 18 and 19 in the hydroalcoholic extract), whose spectra and the available bibliographic information did not allow a secure tentative identification. These peaks also showed a different spectrum among extracts, being classified as hydroxycinnamic acids in the aqueous extract. The compounds derived from hydroxycinnamic acids had a UV–Vis spectrum with a maximum of absorbance around 310–330 nm (Santos et al., 2014). Within this phenolic group, it was possible to identify the already mentioned chlorogenic acid and the neochlorogenic acid (peak 6), both with the same spectra, but with different retention times. This identification was also based in the information about *A. arguta* phenolic profile made by Cyboran *et al.* (Cyboran et al., 2014). Other hydroxycinnamic acids with similar spectra (peaks 1, 3, 11) were tentatively identified as caffeic acid derivatives, as no further information was available to allow a complete identification. No tentative identification was possible in the hydroxycinnamic acids that were only present in one of the three extracts (peaks 4, 8, 17, 18, and 19). The third group of phenolic compounds, the flavonols, is characterized by having a spectra with a maximum of absorbance between 348 and 356 nm (band I) and a second one between 256 and 264 (band II) in their spectra (Carazzone, Mascherpa, Gazzani, & Papetti, 2013; Cyboran et al., 2014). In the *A. arguta* leaves, 5 peaks were classified as flavonols, being identified as glycosylated quercetin (peak 20–22) and glycosylated kaempferol (peak 23–24) derivatives.

Although the three *A. arguta* leaves extracts showed mostly the same compounds in their phenolic profile, the total yield achieved was significantly ($p < 0.05$) different (Table 2). The alcoholic extract had more than the double of the total quantity found in the other two extracts. This extract also showed the highest yield of flavan-3-ols and flavonols. In the alcoholic and hydroalcoholic extracts, the flavan-3-ols represented about 62% and 45% of the total phenolic content, respectively. However, the hydroalcoholic extract had a higher amount (%) of hydroxycinnamic acids than the alcoholic extract in their composition (29% versus 19%, respectively). In the aqueous extract, the hydroxycinnamic acids prevailed, with 52% of the total phenolic content, showing also the highest quantity of these compounds among the three samples (60.19 ± 0.68 µg/mg dw). In this extract, the neochlorogenic acid (peak 6) was the main compound, representing 37% of the total phenolic content. The same occurred in the hydroalcoholic extract, where it represented 21% of the total phenolic content detected. In the alcoholic extract, the main compound was from the flavan-3-ols group (peak 9, Table 2), representing 28% of the phenolic content. These results are in agreement with the expected solubility of each type of compounds in the different solvent used, being the phenolic acids more

Table 2
Retention time (Rt), UV–Vis spectrum maxima, phenolic group, tentative identification and concentration (mean ± standard deviation) of phenolic compounds found on the three extracts (n.d. not detected; * identified by comparison with standard; Sh, spectral shoulder; different letters in the same line mean significant differences ($p < 0.05$) between extracts).

Peak	RT	Aqueous	Hydroalcoholic	Alcoholic	Phenolic group	Tentative dentification	Aqueous	Hydroalcoholic	Alcoholic
	min	λ_{max} (nm)					(μg/mg dry extract)		
1	8.6	324, sh298	324, sh298	n.d.	Hydroxycinnamic acid	Caffeic acid derivative	0.83 ± 0.02^a	0.37 ± 0.02^b	
2	8.9	284, 312	280	n.d.	Flavan-3-ol	Catechin derivative	2.09 ± 0.67	0.88 ± 0.01	
3	9.8	328, sh298	324, sh298	n.d.	Hydroxycinnamic acid	Caffeic acid derivative	0.74 ± 0.04^a	0.50 ± 0.03^b	
4	10.5	312, sh290	324, sh298		Hydroxycinnamic acid		0.45 ± 0.03	0.40 ± 0.02	
				280	Flavan-3-ol				4.12 ± 0.04
5	11.2	280	280	280	Flavan-3-ol	Catechin derivative	1.30 ± 0.02^b	1.97 ± 0.09^b	17.46 ± 0.40^a
6	11.9	324, sh298	324, sh298	324, sh298	Hydroxycinnamic acid	Neochlorogenic acid	43.14 ± 0.76^a	23.18 ± 0.93^c	34.55 ± 0.23^b
7	13.6	280	280	280	Flavan-3-ol	Catechin derivative	7.13 ± 0.82^a	4.00 ± 0.18^b	6.20 ± 0.00^a
8	15.1	288, 304			Hydroxycinnamic acid		0.57 ± 0.03	–	
			280, 314	280	Flavan-3-ol			8.24 ± 0.12^b	18.44 ± 0.49^a
9	16.1	312, sh288			Hydroxycinnamic acid		1.96 ± 0.02		
			280, 312	280, 312	Flavan-3-ol			22.14 ± 1.02^b	65.72 ± 1.01^a
10	18.6	324, sh298	324, sh298	324, sh298	Hydroxycinnamic acid	Chlorogenic acid*	4.94 ± 0.29^a	2.94 ± 0.14^b	3.32 ± 0.02^b
11	20	324, sh298	324, sh298	324, sh298	Hydroxycinnamic acid	Caffeic acid derivative	7.05 ± 0.14^a	3.62 ± 0.19^b	2.64 ± 0.10^c
12	21	280	280	280	Flavan-3-ol	Catechin derivative	2.48 ± 0.02^a	1.46 ± 0.10^b	0.41 ± 0.07^c
13	21.5	280	280	280	Flavan-3-ol	Catechin derivative	0.95 ± 0.08^b	1.06 ± 0.01^b	4.01 ± 0.31^a
14	21.9	280	280	280	Flavan-3-ol	Catechin derivative	3.55 ± 0.02^a	1.91 ± 0.12^b	3.46 ± 0.29^a
15	22.8	276, 348	276, 348		Flavonol		0.06 ± 0.01^a	0.52 ± 0.02^a	
				280	Flavan-3-ol				0.49 ± 0.03
16	23.1	280	280	280	Flavan-3-ol	Catechin derivative	1.31 ± 0.07c	3.18 ± 0.13^b	27.26 ± 0.01^a
17	24.8	284, 312			Hydroxycinnamic acid		0.23 ± 0.02		
			280, 312	280, 312	Flavan-3-ol			1.14 ± 0.10^b	1.51 ± 0.07^a
18	25.8	284, 328			Hydroxycinnamic acid		0.18 ± 0.00		
			280, 328	280	Flavan-3-ol			0.97 ± 0.05	1.10 ± 0.09
19	32.8	284, 316			Hydroxycinnamic acid		0.10 ± 0.00		
			280, 316	280, 316	Flavan-3-ol			0.78 ± 0.04^b	1.70 ± 0.16^a
20	34.6	256, sh268, 356	256, sh268, 356	256, sh268, 356	Flavonol	Glycosilated Quercitin derivative	2.42 ± 0.17^c	4.01 ± 0.12^b	9.22 ± 0.07^a
21	35	256, sh268, 356	256, sh268, 356	256, sh268, 356	Flavonol	Glycosilated Quercitin derivative	10.63 ± 0.11^a	7.62 ± 0.32^b	10.65 ± 0.42^a
22	36.4	256, sh268, 356	256, sh268, 356	256, sh268, 356	Flavonol	Glycosilated Quercitin derivative	13.15 ± 0.35^a	9.44 ± 0.31^b	13.29 ± 0.08^a
23	36.9	264, 348	264, 348	264, 348	Flavonol	Glycosilated Kaempferol derivative	4.37 ± 0.10^b	3.20 ± 0.10^c	5.72 ± 0.08^a
24	38.3	264, 348	264, 348	264, 348	Flavonol	Glycosilated Kaempferol derivative	6.51 ± 0.25^a	4.53 ± 0.34^b	7.48 ± 0.21^a
					Hydroxycinnamic acid		60.19 ± 0.68^a	31.01 ± 1.34^c	44.63 ± 0.07^b
					Flavan-3-ols		18.81 ± 1.45^c	48.26 ± 1.95^b	147.77 ± 1.03^a
					Flavonols		37.12 ± 0.78^b	28.8 ± 1.19^c	46.36 ± 0.28^a
					Total		**116.13 ± 2.9^b**	**108.07 ± 4.49^b**	**238.76 ± 1.38^a**

Table 3
Phenolic standards identification and retention time, linearity, limits of detection (LOD), limits of quantification (LOQ) and repeatability obtained with the HPLC–DAD method (Sh, spectral shoulder).

Name	Detection		LOD	LOQ	Linearity		Repeatability (RSD%)			
	RT	UV–Vis maxima			Range tested	R^2	INTRA-DAY (n = 5)		INTER-DAY (n = 15)	
	(min)	(nm)	μg/mL	μg/mL	μg/mL		RT	Area	RT	Area
Catechin	15.9	280	2.4	7.3	0.9–270.0	0.999	0.36	1.36	0.47	1.24
Chlorogenic acid	18.4	296 sh; 324	5.2	16.0	2.3–145.0	0.999	0.35	1.21	0.49	1.93
Caffeic acid	21.6	296 sh; 324	5.7	17.3	2.3–150.0	0.999	0.29	1.68	0.40	2.77
Rutin	34.8	256; 268 sh; 356	4.1	12.5	1.6–105.0	0.999	0.08	1.54	0.18	3.06
Kaempferol	45.6	264;320 sh 364	3.0	9.1	0.6–40.0	0.996	0.06	1.76	0.11	2.87

easily extracted in the more polar solvent (water), and the flavan-3-ols compounds more soluble in higher concentrations of alcoholic solutions (Butsat & Siriamornpun, 2016; Do et al., 2014; Lou, Lin, Hsu, Chiu, & Ho, 2014).

3.3. In vitro scavenging capacity of A. arguta leaves extracts against ROS and RNS

ROS and RNS present key roles on the physiological processes in the organism, particularly in cell signaling and homeostasis. Inside ROS, $O_2^{\cdot -}$ is classified as an important precursor of different powerful oxidants, such as hydroxyl radicals, while H_2O_2 is one of the less reactive species among ROS (Pistón et al., 2014). Nevertheless, as $O_2^{\cdot -}$, H_2O_2 could generate other species with more potent and toxic effects, such as $HO^{\cdot}$ and HOCl. Regarding RNS, nitric oxide is the primary source of these species in biological systems, rapidly reacting with free radicals, such as superoxide (O_2^{-}), to form peroxynitrite ($ONOO^-$). This species is chemically unstable under physiological conditions,

Table 4
Superoxide anion radical ($O_2\cdot^-$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), peroxyl radical (ROO·), nitric oxide (·NO) and peroxynitrite ($ONOO^-$) scavenging capacities of *A. arguta* leaves extracts. Values are expressed as mean ± standard deviation ($n = 3$). Different letters (a, b) in the same column indicate significant differences between mean values ($p < 0.05$).

Extracts	Reactive Oxygen Species				Reactive Nitrogen Species		
	IC_{50} (μg/ml)						
						$ONOO^-$	
	O_2^-	H_2O_2	HClO	ROO·	·NO	Absence of $NaHCO_3$	Presence of $NaHCO_3$
Aqueous	24.44 ± 3.97[c]	451.70 ± 72.58[a]	82.33 ± 8.06[b]	NA	1.51 ± 0.26[a]	38.74 ± 5.33[a]	29.96 ± 0.04[a]
Hydroalcoholic	27.80 ± 2.92[b]	465.50 ± 36.69[a]	137.50 ± 12.63[a]	NA	1.75 ± 0.27[a]	5.48 ± 0.20[b]	5.16 ± 0.19[c]
Alcoholic	36.46 ± 1.00[b]	192.70 ± 24.01[b]	97.99 ± 9.32[b]	NA	0.83 ± 0.07[b]	4.66 ± 0.13[b]	8.58 ± 0.23[b]
Positive Controls							
Ascorbic acid	41.83 ± 4.23[b]	157.70 ± 23.30[b]	1.64 ± 0.11[c]	0.53 ± 0.06	–	0.62 ± 0.02[b]	1.01 ± 0.08[d]
Tiron	59.92 ± 1.59[a]	–	–	–	–	–	–
Quercetin	–	–	0.14 ± 0.02[c]	4.09 ± 0.52	0.028 ± 0.003[c]	2.02 ± 0.20[b]	3.87 ± 0.55[c]

IC_{50} = *in vitro* concentration required to decrease in 50% the reactivity of the studied reactive species in the tested media (mean ± standard error of the mean; $n = 3$); [NA] No activity was determined up to the highest tested concentration (1 mg/mL).

having the potential to initiate lipid peroxidation reactions by abstracting a hydrogen atom from a polyunsaturated fatty acid and the formation of nitrated lipids (Patel et al., 1999). Table 4 summarizes the *in vitro* scavenging capacity of *A. arguta* leaves extracts against ROS and RNS.

Noteworthy, all *A. arguta* leaves extracts showed scavenging capacity against almost all tested ROS (with exception of $ROO^{\cdot}$) and RNS. The alcoholic extract presented the most promising results probably due to their high phenolic content (238.76 ± 1.38 μg/mg dry extract determined by HPLC/DAD).

In what concerns to ROS, the alcoholic extract showed the highest scavenging efficiency against H_2O_2 and HOCl (with IC_{50} values of 192.70 ± 24.01 and 97.99 ± 9.32, respectively), while for the O_2^- the best result was achieved by the aqueous extract (IC_{50} = 24.44 ± 3.97). The ORAC assay allows the evaluation of *A. arguta* leaves extract capacity to scavenge ROO. Nevertheless, at the highest concentration tested (1 mg/mL) none of the extracts presented activity, probably due to the mechanism behind this particular assay.

Regarding RNS, once again the alcoholic extract exhibited the highest ability to scavenge ·NO and $ONOO^-$, namely IC_{50} of 0.83 ± 0.07 and 4.66 ± 0.13 μg/mL, respectively. Nevertheless, the hydroalcoholic extract was the best scavenger of $ONOO^-$ in the presence of $NaHCO_3$ (IC_{50} = 5.16 ± 0.19 μg/mL).

As well detailed in Table 2 these scavenging activities could be due to the presence of catechin derivatives whose scavenge potential has been well detailed by different authors (Iacopini, Baldi, Storchi, & Sebastiani, 2008; Nakagawa & Yokozawa, 2002). Thus, the chemical composition of *A. arguta* leaves extracts are probably the first responsible for the observed ROS and RNS scavenging activity. Nonetheless, it should be highlighted that the concomitant presence of the different compound in the extracts could lead to an increase of the potential scavenge activity observed.

3.4. Effects of extracts towards Caco-2 and HT29-MTX cells

A. arguta leaves extracts were added to Caco-2 and HT29-MTX cells to analyze their effects on cell viability (Fig. 2). Caco-2 and HT29-MTX were selected as intestinal cell models to evaluate the potential effects on the small intestine, where the majority of digestion and absorption of bioactive compounds occurs. All experiments are carried out up to 1000 μg/mL of extract.

According to the obtained results, the aqueous extract did not decrease the Caco-2 cell viability in all tested concentrations. Nevertheless, after exposure to the hydroalcoholic and alcoholic extracts at a concentration of 1000 μg/mL the cell viability of Caco-2 decrease to 58% and 60%, respectively. Between these two extracts there was no significant difference. In what concerns to HT29-MTX, none of the extracts lead to a decrease on cell viability at concentrations up to 1000 μg/mL. These data allowed selecting the optimal range of non-cytotoxic concentrations of each extract (up to 1000 μg/mL for the aqueous and 100 μg/mL for the hydroalcoholic and alcoholic) used in further experiments.

3.5. Antimicrobial assay

The antimicrobial activity was evaluated using different methodologies as mentioned above. The incorporation and surface scattering methods were used as screening tests while the diffusion method allowed the MIC determination. The microorganisms were selected to cover Gram-positive (*S. aureus*) and Gram-negative bacteria (*E. coli* and *P. aeruginosa*) as well as yeasts (*C. albicans*). Two different concentrations were tested: 20 mg/mL and 50 mg/mL.

A. arguta leaves hydroalcoholic and alcoholic extracts presented antimicrobial activity against *S. aureus* at the highest concentration tested, namely through the incorporation and surface scattering methodologies, which is justified based on the larger volume of extract used when compared to the diffusion method. Nevertheless, none of the extracts had activity against *E. coli*, *P. aeruginosa* and *C. albicans* at tested concentrations. Regarding the MIC value, the hydroalcoholic and alcoholic extracts presented a result of 3.12 mg/mL and 1.56 mg/mL, respectively. Since the MIC values were lower than 8 mg/mL, a potential antimicrobial effect could be highlighted (Fabry, Okemo, & Ansorg, 1998). Taking into account the phenolic composition of *A. arguta* leaves extracts, this activity is probably related to the presence of phenolic acids and flavonoid compounds.

4. Conclusion

Traditionally plant leaves are used for infusions and decoctions, but a careful previous evaluation of their phytochemicals bioactivity, bioavailability and even toxicity is of huge importance. In this work, the leaves of *A. arguta* were evaluated regarding antioxidant and antimicrobial activity, as well as phytochemical composition, radical scavenging activity and intestinal cell viability effects. The alcoholic extract displayed the highest antioxidant activity as well as TPC and TFC levels, being even superior to the results reported for hardy kiwi. In what concerns to scavenging capacity against reactive species, the alcoholic extract was again the most effective. The phenolic composition revealed that the alcoholic extract had more than the double of the total quantity found in the other two extracts and also showed the highest

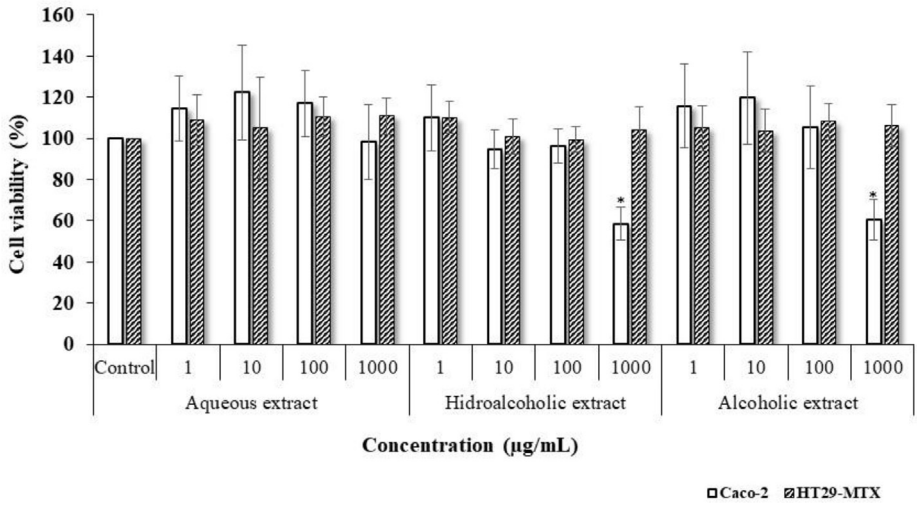

Fig. 2. Effect of *A. arguta* leaves extracts exposure on the viability of Caco-2 and HT29-MTX cells at different concentrations, as measured by the MTT assay. Values are expressed as means ± SD ($n = 6$). * $p < 0.05$ vs. control.

yield of flavan-3-ols and flavonols. Regarding cell viability, this extract of *A. arguta* do not present possible adverse effects on Caco-2 and HT29-MTX cells in concentrations below 100 µg/mL and 1000 µg/mL, respectively. It was also possible to verify the antimicrobial activity of the hydroalcoholic and alcoholic extracts in the concentration of 50 mg/mL. Further investigations are needed to explore the *in vivo* effects of *A. arguta* when prepared as infusions or decoctions.

Conflict of interest

The authors have declared no conflicts of interest.

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