Eur Food Res Technol (2017) 243:1343–1353
DOI 10.1007/s00217-017-2845-y

ORIGINAL PAPER

Valorization of kiwifruit production: leaves of the pruning branches of *Actinidia deliciosa* as a promising source of polyphenols

Joana Henriques[1] · Maria João Ribeiro[1] · Pedro L. Falé[1,2] · Rita Pacheco[1,3] · Lia Ascensão[1,4] · Maria Helena Florêncio[1,2] · M. L. M. Serralheiro[1,2]

Received: 13 October 2016 / Revised: 12 December 2016 / Accepted: 7 January 2017 / Published online: 4 February 2017

Abstract The present work concerns the novel application of a phenolic compound extraction methodology to leaves of *Actinidea deliciosa*. Recent studies have shown that crop residues could be raw material for recovery of natural bioactive compounds. Phenolic compounds from *Actinidea deliciosa* leaves were extracted with hot water, purified using reverse phase chromatography and mucilage precipitation with ethanol. The composition of the purified fraction was determined by HPLC-DAD and LC-MS^n. Quercitrin, rutin, proantocyanidin B and C, quinic acid, myricitrin, and triterpene acid-*O*-hexoside were found. These compounds were present in all the fractions. The antioxidant activity was determined as general radical scavenging capacity, lipid peroxidation prevention, and NO radical scavenging activity. Values of EC_{50} of 9.4 µg/mL, IC_{50} of 152.5 µg/mL, and IC_{50} of 81 µg/mL were determined, respectively. The best period of the year to obtain a high fraction of phenolic compounds (120 µg/mg of extract) from *A. deliciosa* leaves was December. The phenolic fraction obtained with hot water and ethanol precipitation is a promising good source of natural bioactive compounds and an easy method of taking advantage of the leaves from *A. deliciosa*. To the best of our knowledge, there are no previous works on the use of the residual leaves of this fruit tree. Several phenolic compounds with high antioxidant activity were extracted and identified in this plant for the first time.

Keywords *Actinidia deliciosa* · Crop residues · Phenolic compounds · Extraction and purification · Antioxidant activity · Radical scavenging activity

Introduction

In the last years, numerous studies have shown that the agricultural wastes generated in the farms can be sources of a wide range of bioactive compounds [1], but to date, no report on the residues from *Actinidea deliciosa* leaves is available. Plants contain, particularly in the leaves and fruits, different polyphenol compounds [2] that due to their antioxidant activities may find applications in several areas, such as food and functional food industries, cosmetics, and human and animal health as well [3]. In food industry, these compounds may contribute to the colour, flavour, odour, and oxidative stability of products [4]. European Union approved the use of rosemary extract (E392) as antioxidant [5], and the search for natural antioxidants still goes on [6]. In human and animal health, polyphenols may play an important role in the prevention and treatment of many diseases, such as atherosclerosis, inflammatory conditions, certain cancers, and aging related-neurological disorders [3]. Plants also contain mucilages, complex acidic,

Electronic supplementary material The online version of this article (doi:10.1007/s00217-017-2845-y) contains supplementary material, which is available to authorized users.

✉ M. L. M. Serralheiro
mlserralheiro@fc.ul.pt

[1] Centro de Química e Bioquímica, Faculdade de Ciências, Universidade de Lisboa, Edifício C8, Campo Grande, 1749-016 Lisboa, Portugal

[2] Departamento de Química e Bioquímica, Faculdade de Ciências, Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal

[3] Área Departamental de Engenharia Química, Instituto Superior de Engenharia de Lisboa, Av. Conselheiro Emídio Navarro, 1959-007 Lisboa, Portugal

[4] Centro de Estudos do Ambiente e do Mar, Faculdade de Ciências, Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal

or neutral polysaccharide polymers with high-molecular weight formed by sugars and uronic acid units [7]. These compounds also have a large wide of applications in food industry as a gelling non-toxic agents and in pharmaceutical and cosmetic industries especially due to their high water-binding capacity, antioxidant properties, and wounds healing [8].

Most often, the extraction processes to recover phenolic compounds from crop residues or fruit-industry wastes use organic solvents [9], microwaves [10, 11], ultrasounds [10], supercritical fluids [11], or two-phase systems (ATPS) [12]. Water has been used along the time to prepare infusions and decoctions from aromatic and medicinal plants, producing beverages that we know now, that have high amounts of bioactive phenolic compounds [13–16]. However, not only polyphenols were extracted with these aqueous systems, but also mucilages may be co-extracted [17]. In fact, the extraction procedures may have an important effect on the recovery of bioactive compounds and efforts must be made to optimize the processes.

The main goal of this study was to evaluate if the leaves of *Actinidia deliciosa* contained phenolic compounds with antioxidant activity and if these compounds could be recovered with an easy purification method.

Materials and methods

Chemicals

All chemicals were of analytical grade. 3,5-dinitrosalicylic acid (DNS), gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydrochloric acid were obtained from Sigma (Barcelona, Spain). Sodium hydroxide, Folin–Ciocalteu reagent, iron (III) chloride, potassium hexacyanoferrate (III), tannic acid and trifluoroacetic acid, quercitrin, methanol, ethanol, acetic acid, and formic acid were obtained from Merck (VWR, International, Darmstadt, Germany). The water (H_2O)—resistivity 18.2 MΩ cm, 25 °C was purified using a Milli-Q water system from Millipore (USA). The iron (II)-sulfate-7hydrate and trichloroacetic acid were bought from Riedel-de Haën.

Plant material

Leaves from *Actinidia deliciosa* (A. Chev) C.F. Liang et A.R. Ferguson (Actinidiaceae) were collected at the flowering time (April 2014) at the fructification/harvesting period (September to November 2015) and after the pruning branches (December 2015) from plants cultivated in the region of Beira Baixa, Portugal (39°46′ 47.783″N; 7°48′ 20.171″W; 244 m). The extraction was done in the 24 h after collection. Once in the laboratory, they were washed, dried with a filter paper, and immediately used for preparation of decoction.

Extract preparation

Aqueous plant extracts were prepared as decoctions, using 10 g of grounded fresh leaves in 100 mL of distilled water, boiling for 10 min. The decoctions were filtered through number 1 Whatman grade paper and lyophilized. The yield of extraction was between 101 and 123 mg of extract/g of plant.

Chromatographic analytical conditions (RP-HPLC-DAD)

The chromatographic analysis was carried out using reverse phase chromatography with a diode array detector (RP-HPLC-DAD), as described in [14].

Phenolic compounds isolation

Purification methods were applied to the lyophilized extract using reverse phase chromatography and ethanol precipitation of mucilage.

Solid-phase extraction (SPE)

The solid-phase extraction was developed, as described in [18], with a slight modification. The cartridge was conditioned with 1 mL methanol and 1 mL of 0.05 M HCl. Next, 5 mg/mL extract (*Actinidia deliciosa*/ April extract) in 0.05 M HCl solution was placed in a C_{18} cartridge (Merck, VWR International) and eluted. Subsequently, the analytes adsorbed on the cartridge were eluted with a methanol/1% acetic acid in water (70:30). The extraction resulted in two fractions: fraction I collected during the permeation of the sample and fraction II collected during the elution of the sample. These two fractions were dried under nitrogen and reconstituted with methanol and water (33:67). The fractions were then analysed by RP-HPLC-DAD, their total phenolic and tannins content were quantified, and the antioxidant activity determined.

RP-HPLC purification

For RP-HPLC purification, an extract solution of *A. deliciosa* (April extract), 10 mg/mL was prepared. The purification was carried out using the equipment and the analytical conditions previously described. Multiple injections were made, and several fractions were collected totalising 9 mg of injected sample. For each injection in the column, three fractions were collected at different retention times, fraction I from time of injection until 3 min retention time, fraction

II at retention time 3–17 min, and fraction III at retention time 17–30 min. The fractions collected were then injected again on the RP-HPLC-DAD. Total phenols and tannins were quantified and the antioxidant activity determined.

Mucilage separation

The separation of mucilage was carried out, as described in [19], with slight modifications. For the determination of mucilage, 10 mg of extract (for each extract of *A. deliciosa*) were initially weighed to which 1 mL of water was added to extract dissolution. Next, 4 mL of 96% ethanol was added. The solution was placed in the cold for 15 h and centrifuged for 30 min at 4 °C with 4500*g*. The pellet was removed and washed three times with Milli-Q water, and the supernatant was collected and pooled. Both were lyophilized and reconstituted with Milli-Q water to final concentrations of 1 mg/mL for analysis. The samples were then injected on RP-HPLC-DAD. Total phenols, tannins, reducing sugars, and mucilage were determined and the antioxidant activity evaluated.

Total phenol content quantification

Total phenolic compound content was determined spectrophotometrically, as described in [20] with some modification, using gallic acid as a standard. Briefly, 30 µL of the extract solution containing 1 mg of the extract/mL were diluted with 1.35 mL of each column eluent used. To this solution, 30 µL of Folin–Ciocalteu were added and the final solution was mixed. After 3 min, 90 µL of a 2% solution of sodium carbonate was added and the mixture was stirred for 2 h. The absorbance (A) was measured at 760 nm in triplicates. The concentration of the total phenolic compounds was determined as micrograms of gallic acid equivalents using the equation above as the mean of three replicates (mg of gallic acid equivalents per mg of dry extract).

Tannins quantification

The tannins content was determined spectrophotometrically, based on Van-Burden and Robinson method [21] with some modification using tannic acid as a standard. Briefly, 100 µL of the solution containing 1 mg of the dried extract or fraction/mL were diluted with 300 µL of water or water/methanol. To this solution, 300 µL of iron (III) chloride 0.1 M (in 0.1 M HCl) and 300 µL of potassium ferricyanide 0.008 M were added and solution was mixed. The assay was developed in microplates. The absorbance (A) was measured at 605 nm in triplicates. The concentration of tannins was determined as micrograms of tannic acid equivalents using the equation above as the mean of three replicates (mg of tannic acid equivalents per mg of dry extract).

Reducing sugars quantification

The content in reducing sugars was determined according to the DNS method [22].

Mucilage quantification

After obtaining the pellet from the ethanol precipitation process, the pellet was lyophilized and weighted.

Analysis by FTIR and observations by SEM of the fractions obtained from ethanol purification

FTIR analysis

After the precipitation of mucilage with ethanol, the analysis of the functional groups present in mucilage was carried out using spectroscopy in the infrared region, Fourier transform infrared spectroscopy (FTIR) (Nicolet™ FT-IR spectrometers from Thermo Electron Corporation) with DTGS TEC detector in the spectral range of 400–4000 cm^{-1}, and a resolution of 4 cm^{-1}. The dry powder (1 mg) was mixed with KBr and pressed into pellets under mechanical pressure.

SEM observations

The structure of the initial leaf decoction, the ethanol precipitated fraction (mucilage), and the supernatant was investigated by SEM. The power of the three lyophilized samples was scattered carefully over a double-side carbon tape attached to the aluminium stub surfaces. The stubs were then coated with a thin layer of gold and the metalized samples observed on a JEOL 5200LV scanning electron microscopy (JEOL Ltd, Tokyo, Japan) at an accelerating voltage of 20 kV.

LC-MS^n

The LC-MS^n analysis was carried out on a liquid chromatograph Surveyor Plus Modular LC system connected to a LCQ Duo ion trap mass spectrometer equipped with an electrospray ionisation (ESI) source, from Thermo Scientific (Bremen, Germany). The column and gradient used were the same as in the RP-HPLC–DAD analysis. The identification was carried out on the initial extract, without purification, by injecting 25 µL at a concentration of 10 mg/mL.

The mass spectrometer was operated in negative ion mode in the range *m*/*z* 50–2000, and the parameters were

adjusted to optimize the signal-to-noise ratios (*S*/*N*) for the ions of interest [14].

Antioxidant activity

DPPH test

Antioxidant activity was measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH) method, as described in [14].

Lipid peroxidation inhibition

Lipid peroxide formation was measured as described in [23] using the thiobarbituric acid reactive substance method (TBARS).

Determination of nitric oxide radical scavenging activity

The scavenging effect on nitric oxide was determinate, as described in [24] with slight modifications. Initially, 0.5 mL of sample was added in the test tubes to 0.9 mL sodium nitroprusside (20 mM) in phosphate buffered saline and the tubes were incubated at 23 °C for 2 h. The solution was diluted with 0.9 mL of Griess reagent. The absorbance of the chromophore was immediately read at 450 nm.

Statistical analysis of the results

The results were analysed using and variance analysis (ANOVA) in a Microsoft® Excel 2007 program.

Results and discussion

Quantification of total of phenols, tannins, mucilage, protein, and reducing sugars in *A. deliciosa* leaves during the flowering, fruitification/harvesting, and pruning branch months

Aqueous extracts of leaves from *A.deliciosa*, collected during different periods of the year, were analysed for their content in total phenols, tannins, reducing sugars, and mucilage (Fig. 1a). An increase from flowering to fruit harvesting periods (April to September–November) was observed in total phenol (with the greatest increase, 78.5%, between the months of November to December, followed by 61.1% during the months of September to October) and tannins content (the greatest increase, 71.9%, was also detected from November to December). Mucilage's seem to have a slight decrease, approx. 28%, from April to October. According to the literature, plant secondary metabolism is markedly influenced by ripeness at the time of harvest and also by environmental factors [2, 4, 25]. In fact, environmental factors that change with season of the year, such as sun exposure (light and temperature) and water, affect the plant's performance and have a major effect on its polyphenol content. The secondary metabolite profiles and their content can be related to conditions, such as season, time of day, tissue age, and temperature [26]. Increasing temperature has a negative effect on the content of quercetin derivatives [27]. The degree of ripeness considerably affects the concentrations and proportions of the various polyphenols [2, 4, 25]. The reducing sugars content increased till November, about 86% and decreased after this month. However, the amount of the mucilage in different leaf extracts was higher in April and September, and the ratios mucilage/total phenols were 19.6 and 3.2., respectively. Due to the higher ratio of phenolic compounds to mucilage in December, this should be chosen as the period of the year to collect the leaves, taking advantage of the higher phenolic content.

Influence of purification procedures, on the phenolic composition of *A. deliciosa* extract

Mucilages have been reported to occur in the leaves of numerous plant species [28]. Therefore, plant crude extracts usually contain large amounts of carbohydrates and/or lipidic material and the concentration of the phenolics in these extracts may be low [9]. To obtain a rich fraction in polyphenolic compounds, strategies, such as reverse phase chromatography, using either solid-phase extraction (SPE) or RP-HPLC and ethanol precipitation, were used [19]. The objective of these purification strategy was mainly to separate mucilages from phenolic compounds. The leaves extract from the pruning branches of *A. deliciosa* (April extract), which have the higher ratio mucilage's/phenolic compounds, was chosen to perform this study, as the objective was to devise a process to separate these two types of compounds.

The purification process was followed by measuring total phenol and tannin content per mg of dry matter and the values are shown in Fig. 1b. Two fractions were obtained using SPE, fraction I collected during the permeation of the sample and fraction II collected during the sample elution from the cartridge. In Fig. 1b, the results with fraction II are shown. The purification processes by SPE increased total phenols in 68.7% (24.6 and 41.5 µg/mg, before and after purification, respectively) and tannin content in 94.4% (14.4 and 28 µg/mg, before and after purification, respectively). On the other hand, the HPLC fraction collected increased total phenols in 45.5% (24.6 and 35.8 µg/mg, before and after purification, respectively) and tannins in 57.6% (14.4 and 22.7 µg/mg, before and after purification, respectively) (Fig. 1b). The fraction resulting from ethanol precipitation had an increase in total phenolics

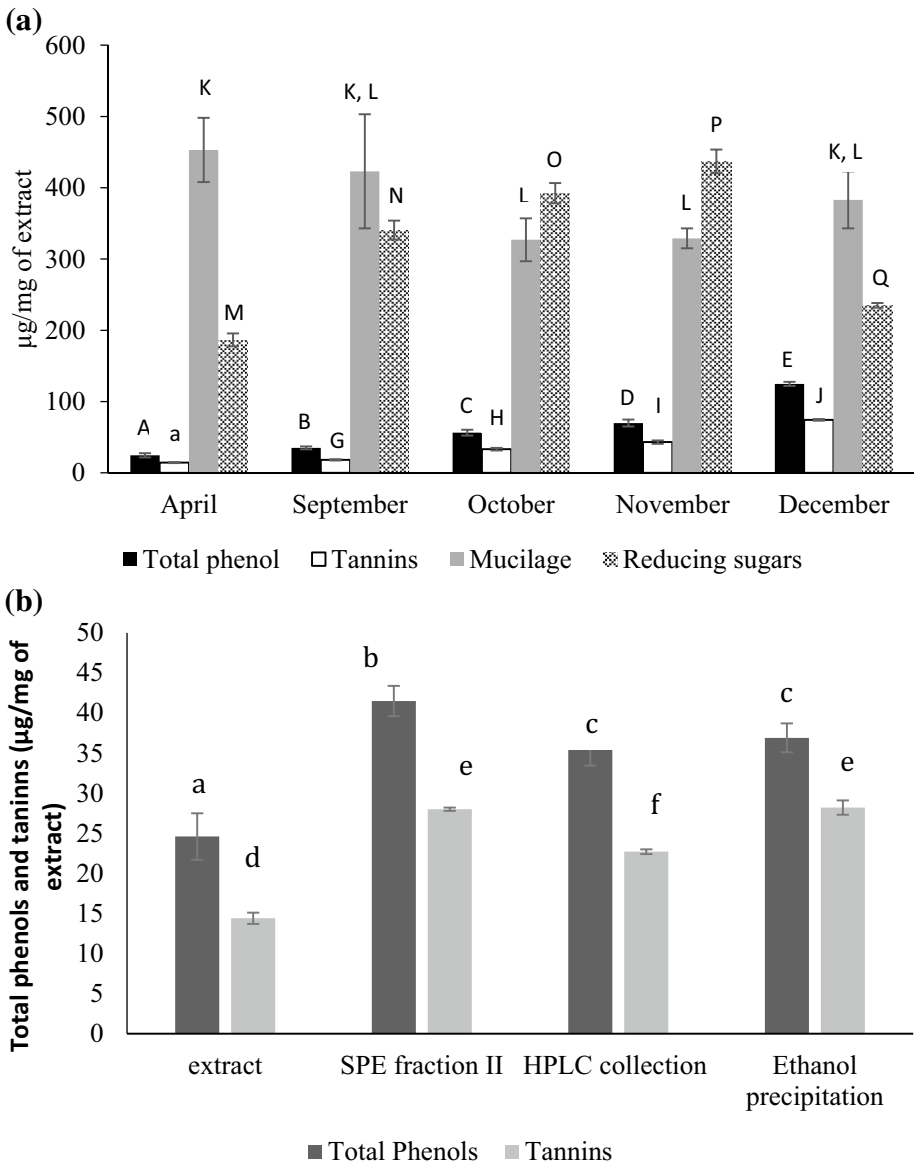

Fig. 1 Quantification of different components of the *A.deliciosa* leaf aqueous extracts: **a** total phenols, tannins, proteins, mucilage, and reducing sugars of the water extract from *A. deliciosa* leaves; **b** phenols and tannins content of the water extract from *A. deliciosa* leaves collected in April before and after the purification methods. Three independent measurements were made for each studied parameter and results are presented with means and standard deviations. *Letters* indicate differences significant at 95% confidence level

of 50% (24.6 and 36.9 µg/mg, before and after purification, respectively) and 74.3% for tannins (14.4 and 25.1 µg/mg, before and after purification, respectively). The precipitation method was applied to the extracts obtained during the year. Total phenols were also quantified in the mucilage extracts, and 6.2 ± 1.1 µg/mg was obtained. This value is 90% lower than the value obtained for the supernatant, meaning that the phenolic compounds stayed soluble and only 10% co-precipitated with the mucilage.

The fractions obtained after each purification step were analysed by RP-HPLC (Fig. 2). The initial extract showed the existence of nine main compounds (Fig. 2a) that were followed throughout the purification steps and procedures. The extracts prepared with the leaves collected from the other months showed an identical chromatographic profile, differing solely in the amount of each component. The two fractions obtained through SPE purification, fraction I and II, were analysed by HPLC-DAD, Fig. 2a, b, respectively. In fraction I (Fig. 2b1), no phenolic compounds were detected, meaning that the compounds stayed bonded to the column, while in fraction II (Fig. 2b2), all compounds could be detected, eluting from the column. The purification through RP-Chromatography, using HPLC-DAD, exhibited a similar profile as SPE, as can be seen comparing Fig. 2c with Fig. 2b2. The analysis of the mucilage fraction obtained by ethanol precipitation process (Fig. 2d1) indicated that no phenolic compounds were present in significant amount, and all phenolic compounds were found in the supernatant fraction (Fig. 2d2). This chromatographic analysis demonstrated that the ethanol precipitation process proved to be an efficient method.

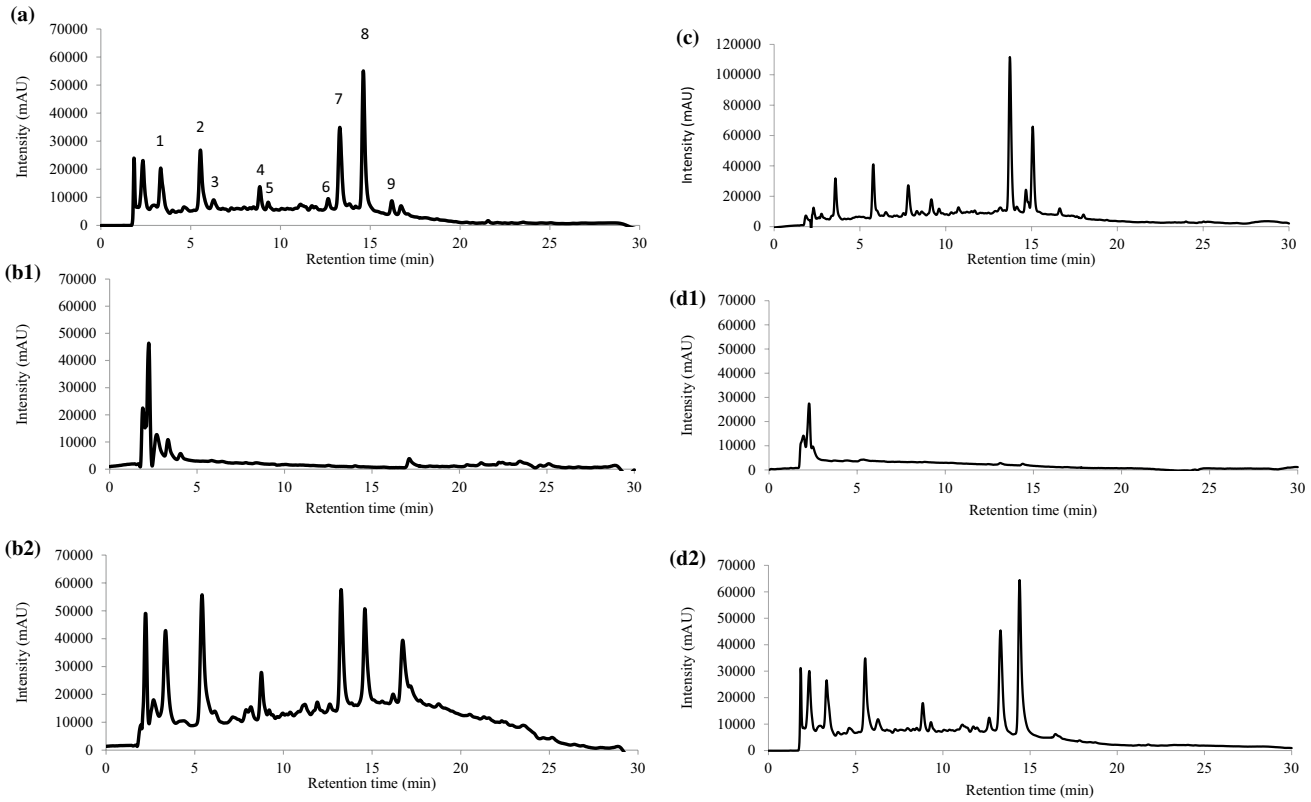

Fig. 2 RP-HPLC of extract from *A. deliciosa* leaves (April) and of the purified fractions: **a** initial water extract; **b** fractions collected from SPE purification: **b1** fraction I, collected during purification, **b2** fraction II, collected during elution; **c** fraction II from HPLC purification (eluted during 3–17 min); and **d** fraction II after mucilage precipitation with ethanol: **d1** solid precipitated phase and **d2** supernatant

After analysis of the chromatograms resulting from the purification methods, it is possible to verify that most of the compounds present in the initial extract were present at the end of each purification process. Analysing the area of each peak in the chromatograms, the effect of purification process on the amount of each component to be determined (Table 1). After SPE purification, the relative proportion of compounds 1, 2, 4 doubled relatively to the initial extract, and compounds 5 and 7 also increased (Fig. 2b2). In the case of HPLC-DAD preparative chromatography, peak 7 increased to three times its initial concentration (Fig. 3c). In the ethanol precipitation process, all the compounds were slightly more concentrated relatively to the non-purified extract.

When comparing the ethanol precipitation with the chromatographic processes on what concerns the yield of total phenols and mucilage, as well as the phenolic composition, the results were very similar. However, chromatographic processes are more expensive, time consuming, and only small volumes of sample can be handled comparatively to precipitation with ethanol [9, 29].

Table 1 Relative intensities for each compound using LC for the different purification methods

Compound	Purification methods		
	SPE[a]	HPLC collection[a]	Ethanol precipitation[a]
1	++	+	+
2	++	+	+
3	−	−	+
4	++	+	+
5	+	+	+
6	−	+	+
7	+	+++	+
8	−	+	+
9	−	−	−

[a]+++ tree times more concentrated, ++ two times more concentrated, + more concentrated, −less concentrated relatively to initial decoction of *Actinidia deliciosa*

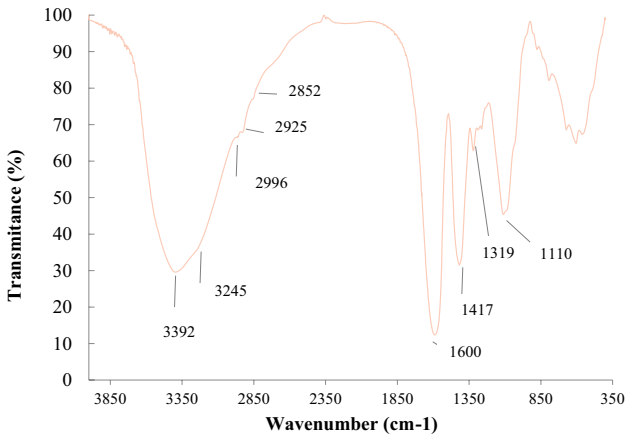

Fig. 3 FTIR spectrum of *Actinidia deliciosa* leaves mucilage from April

Analysis of mucilage by FTIR

To have some insight into the chemical functional groups present in the mucilage, FTIR analysis was carried out in the ethanol precipitate (Fig. 3). FTIR spectroscopy has been extensively applied to characterize polymer's molecular structure and composition [30]. The peaks obtained in the IR spectra are a result of different bond vibrations from molecules present and its interactions [31]. The FTIR spectrum of *A. deliciosa* leaf mucilage exhibits typical bands and peaks characteristic of mucilage, and these were assigned to functional groups according to the bibliography (Table 2). FTIR analysis showed that the mucilage contains major functional and structural groups, such as polymeric hydroxyl groups, alkanes, and carboxylate groups [30], ensure the presence of polymeric chain in mucilage [31]. OH and the carbonyl groups found at 3392 cm^{-1} seen in the mucilage FTIR spectrum cannot be assigned to phenolic compounds, as these were not detected in the chromatograms of the HPLC-DAD analysis (Fig. 2d1). Hence, these bands obtained in the FTIR analysis can be assigned polysaccharides present in plant mucilage fractions [32].

Analysis of the fractions from ethanol purification by SEM

To prove that the ethanol purified fraction was different from the starting material the initial water extract, SEM observations were carried out on the initial decoction, the ethanol precipitated fraction (mucilage), and the supernatant (Fig. 4). In fact, the observations shown that the microstructure of the three samples investigated was different. The particles of the lyophilized leaves decoction exhibit irregular profiles and sizes (Fig. 4a) and are constituted by groups of cubic or parallelepiped-shaped crystals that seem to be embedding in an amorphous matrix (Fig. 4b, c). The lyophilized mucilage particles, also with different shapes and dimensions (Fig. 4d), have a granulose structure (Fig. 4e, f). In very high magnification, aggregates of spherical granules with a uniform dimension are clearly seen (Fig. 4g). The particles of the lyophilized supernatant are more or less of spherical shape, mostly with a smooth surface (Fig. 4h). In higher magnifications, some wrinkled regions are visible

Table 2 FTIR Spectral data of *Actinidia deliciosa* leaves mucilage

Wavenumber (cm^{-1}) In this work	Absorption range (cm^{-1})	Type of bond	References
3392	3447.91–3354.35	CO and OH groups	Shingh and Bothara [30] and Shende and Marathe [31]
3245	3245	Intra- and inter-molecular hydroxyl groups that make up the gross structure of polymeric carbohydrates	Shende and Marathe [31]
2996	2400–3200	N─H bond (ammonium ions)	Ahad et al. [33]
2925	2925	C─H bond (alkyl, methyl)	Ahad et al. [33]
2852	2885.63–2811.37	Stretching modes of the alkyl C–H stretch, carboxylic acid C=O and O–H stretch and methylene ($–CH_2–$) C–H stretch	Shingh and Bothara [30] and Faccio et al. [32]
1600	1618–1430	Typical of carboxylate groups of the galacturonic acid residues	Shingh and Bothara [30]
1417	1420	Symmetrical deformation of the C–H and C–OH groups was associated with reducing sugars	Faccio et al. [32]
1319	1740–1258	Typical of acetyl group	Shingh and Bothara [30]
1110	1200–800	Finger print region for carbohydrates	Shingh and Bothara [30]
	1249–1018	C–O stretching vibration	Shende and Marathe [31]

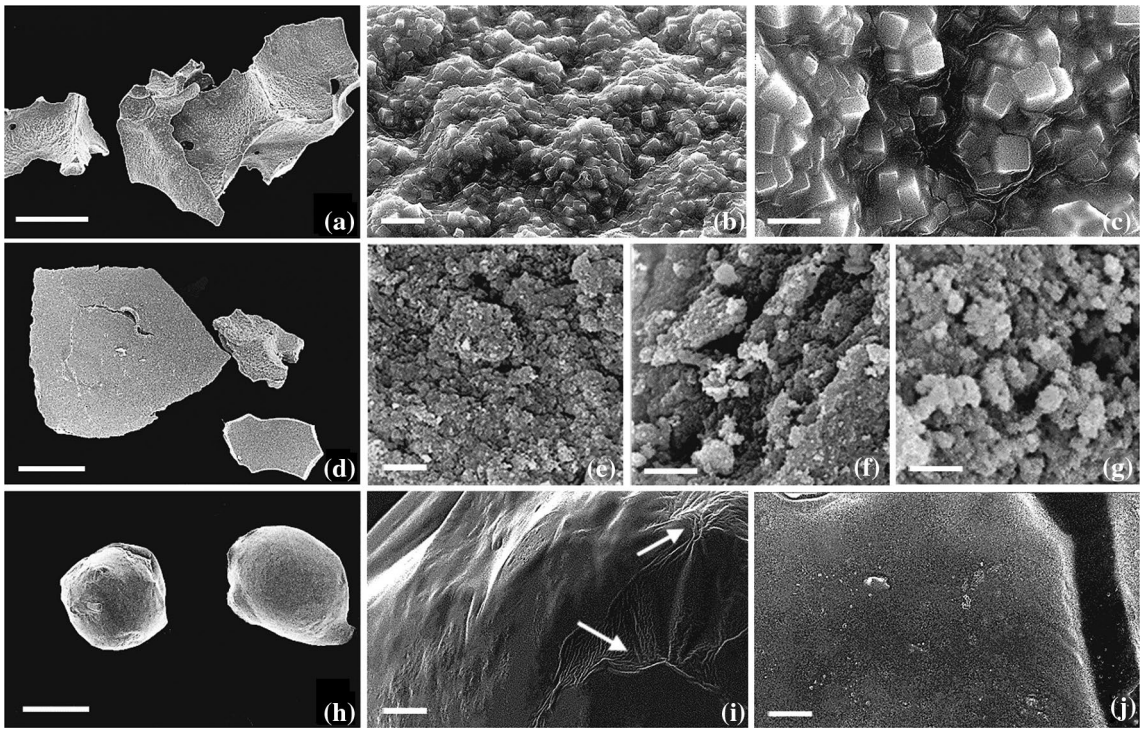

Fig. 4 Scanning electron micrographs of the lyophilized particles from April *Actinidia deliciosa* leaves: **a–c** initial extract; **d–g** ethanol precipitated fraction (mucilage); and **h–j** supernatant. **a, d** Particles displaying irregular profiles and sizes. **b, c** Groups of cubic or parallelepiped-shaped crystals embedding in an amorphous matrix of the initial extract. **e–g** Granulose microstructure is well seen, especially in high magnifications (**e**). **h** Lyophilized supernatant particles look like spherules. **i** Wrinkled regions are visible (*arrows*) on the particles smooth surfaces. **j** Amorphous nature of the particles is apparent. *Scale bars* 500 µm (**a, d, h**), 10 µm (**b, e, i**), 5 µm (**c, f**), and 1 µm (**j**)

(Fig. 4i, arrows), but the access to the inner structure is difficult even in the fragmentation zones (Fig. 4j). However, the amorphous nature of the particles is apparent, such as in the work of Singh and Bothara [30].

Identification of main compounds by HPLC-DAD and LC-MS^n

The identification of the nine compounds present in the initial and purified fractions was accomplished through LC-MS/MS and shown in Table 3. The structures of these compounds were proposed based on UV spectrum, as well

Table 3 Identification of the components of the *Actinidea deliciosa* decoction by mass spectrometry

HPLC Peak	Deprotonated molecule $[M–H]^-$	MS/MS fragment ions (Rel.Ab. %)	Identification
1	191	173 (100), 111 (12), 155 (4), 85 (2)	Quinic acid
2	577	407 (100), 425 (58), 451 (46), 289 (38), 559 (10), 245 (4)	Proanthocyanidin B
3	865	695 (100), 577 (46), 451 (36), 407 (34), 713 (30), 739 (29), 587 (28), 543 (26), 575 (22), 425 (18), 289 (6), 287 (4), 245 (2)	Proantocyanidin C
4	771	609 (100), 301 (9), 463 (2)	Quercetin 3-*O*-rutinoside-7-*O*-glucoside
5	609	447 (100), 463 (56), 301 (36)	Quercetin 3-*O*-rhamnoside-7-*O*-glucoside
6	463	316 (100), 317 (22), 179 (4), 271 (2), 151 (1)	Myricitrin
7	609	301 (100), 271 (10), 179 (6), 255 (5)	Rutin
8	447	301 (100), 151 (3), 179 (2), 271 (2), 255 (1)	Quercitrin
9	711[a]	665 (18), 503 (100)	Triterpene acid-*O*-hexoside

[a]The ion corresponds to $[M + HCOO]^-$

as on the fragmentation pattern obtained by ESI-MS-MS experiments. The loss of 162 Daltons is indicative of hexose (glucose or galactose, the most common sugars found in flavonoids), the loss of 146 Daltons was indicative of rhamnose, the loss of 133 Daltons was indicative of pentose (xylose or arabinose, the most common pentoses found in natural products), and the loss of 308 Daltons indicative of compounds having the disaccharide structure rutinose or neohesperidose linked thorough an-*O*-glycosidic bond [40]. The HPLC-DAD analysis of the initial and all the purified fractions by HPLC-DAD revealed two major peaks, (7) and (8); these two compounds were identified as quercitrin and rutin, respectively, Table 3. The detailed process of identification is described in Supplementary Material.

Antioxidant activity

The antioxidant activity was analysed using the DPPH scavenging activity, the prevention of lipid peroxidation, and the inhibition capacity of NO radical formation. In all the cases, there was a dose-activity relationship that allowed the determination of the amount of the extract that showed 50% of activity with a correlation higher than 0.940.

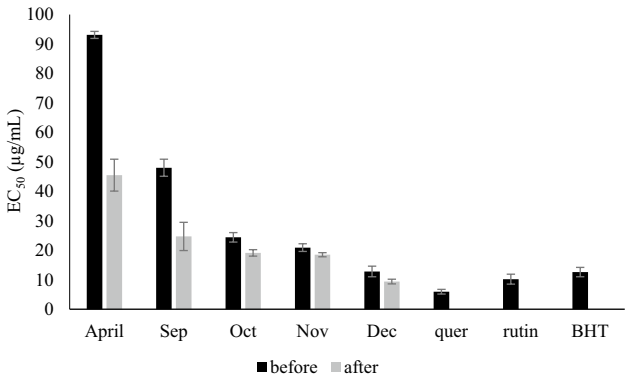

Fig. 5 EC_{50} (µg/mL) values for antioxidant activity evaluated before and after ethanol precipitation and for the standard of rutin, quercitrin and BHT

DPPH scavenging activity

The antioxidant activity was measured using DPPH free-radical test. The capacity of the antioxidants to scavenge free radicals is a measure of their antioxidant activity, reflecting the capacity to capture radicals before these can damage any cell constituent, such as proteins, DNA, or lipids [3].

The effect of the purification process with ethanol precipitation on the antioxidant activity was measured in all the extracts before and after mucilage removal, Fig. 5. The antioxidant activity of the extracts obtained during the different months indicated that the activity increases during the autumn and after precipitating the mucilage (values different at $P=0.05$). The extract from December after precipitation with ethanol has a stronger effect than the commercial antioxidant 2,6-di-tert-butyl-4-hydroxytoluene (BHT), Table 4. Considerable improvement with ethanol precipitation can be observed, with an increase of 51.1% in EC_{50} value, while with SPE and HPLC purification, 25 and 23.5% were obtained (data not shown), respectively. These results can be justified by a higher concentration of phenolic compounds by ethanol precipitation. Comparatively with other extracts used in traditional medicine, the December extract and ethanol purification fraction of *A. deliciosa* showed lower EC_{50} value, which are an indicative of greater antioxidant power [14].

Inhibition of lipid peroxidation by TBARS method

Lipid peroxidation is a well-established mechanism of cellular injury in both animals and plants and is used as an indicator of oxidative stress in cells and tissues. The formation of lipoperoxides is a result of a chemical process, in which unsaturated fatty acids are damaged by oxygen, forming free radicals [34]. Lipoperoxides are unstable and decompose to form a wide range of compounds, including reactive carbonyl compounds, mainly aldehydes [malondialdehyde (MDA), 4-hydroxy-2-nonenal (4-HNE)] that damage cells by binding the free amino groups of amino acids of proteins [35]. The lipid peroxidation was monitored by the thiobarbituric acid assay (TBA), one of the

Table 4 Antioxidant activity determined as free-radical (DPPH) scavenging, prevention of lipid peroxidation (TBARs), and NO radical formation by December aqueous extract of *A.deliciosa* leaves

	DPPH EC_{50} values (µg/mL)	TBARs IC_{50} values (µg/mL)	NO radical IC_{50} values (µg/mL)
Before purification	12.8 ± 1.8	330.8 ± 19.4	449 ± 24.3
After purification	9.4 ± 0.8	152.5 ± 31.6	81 ± 8.1
BHT	12.6 ± 1.6	48.6 ± 3.3	–
Ascorbic acid	–	–	45.5 ± 5.2
Rutin	12.2 ± 1.7	–	322.8 ± 36.7
Quercitrin	5.9 ± 0.8	–	149.5 ± 24.6

most common methods for the effect [36]. The December extract of *Actinidia deliciosa* was analysed for the lipid peroxidation inhibition capacity, before and after discarding the mucilage from the extract. IC_{50} values of 330.8 ± 19.4 and 152.5 ± 31.6 µg/mL were obtained, respectively. Comparatively to the standard BHT with IC_{50} value of 48.6 ± 3.3 µg/mL (Table 4), the activity is approximately three times lower; however, discarding the mucilage the activity increased (lower IC_{50} value). These values are similar to those detected also with plant extracts [37].

Determination of nitric oxide radical scavenging activity

Nitric oxide is a free radical that may function in the cell as a signalling molecule with several biological effects, such as neurotransmission, vasodilatation, and several cardiovascular functions [38], and therefore, it may be appropriate to find antioxidants with the ability of scavenging these radicals.

A test was developed using sodium nitroprusside thatis known to decompose in aqueous solution at physiological pH (7.2) to generate nitric oxide radicals that can be quantified using Griess reagent [24]. Scavengers of nitric oxide may compete with oxygen, reducing the production of peroxinitrite ions [24]. The IC_{50} value of *A. deliciosa* December extract is shown in Table 4. It can be seen the IC_{50} values for the supernatant after precipitation with ethanol increased 81% relatively to the initial one. It is not as effective as ascorbic acid, but the purified extract is better than pure rutin and quercitrin. Comparatively with others extracts, supernatant of *A. deliciosa* December extract showed lower IC_{50} value, which is an indicative of greater ability to scavenging the NO radicals [39].

Conclusions

This work presents innovative results concerning the extraction of polyphenolic compounds from *A. deliciosa* leaves. Although the extraction method itself is not new, this is the first time that it was applied to *A. deliciosa* leaves and proved to yield highly antioxidant compounds from this crop residue. Leaves from *A. deliciosa* after pruning and kiwifruit harvesting can be used as an easy and inexpensive accessible source of natural antioxidants. These crop residues present high amount of phenolic compounds, especially at the end of winter (December), in the post harvesting period. The water extraction process here reported and followed by ethanol purification processes revealed to be an efficient methodology. The phenolics obtained showed to have high antioxidant capacity, and, therefore, can also be useful for preventing damage of several food products, taking advantage of the wastes from an agricultural process. Our data showed that the leaves of *Actinidia deliciosa* could be successful used for the extraction of plant polyphenols, an important class of natural products with a wide range of bioactive functions. The mucilages also obtained as a by-product can be further analysed and food applications for human or animal health may also be developed. This work is also proof of concept that the methodology here described can be used to recover phenolic compounds with important biological activities from crop residues, and may pave the way to future applications to other crops.

Acknowledgements We acknowledge Fundação para a Ciência e Tecnologia (FCT) for financial support to Centro de Química e Bioquímica (PEst-OE/QUI/UI0612/2013; UID/MULTI/00612/2013) and to CESAM (UID/AMB/50017), through FCT/MEC National funds, and the co-funding by the FEDER, within the PT2020 Partnership Agreement and Compete 2020. Authors are also grateful to Prof. Maria Helena Mendonça (DQB, FCUL) for having collected the *A. deliciosa* leaves for this study.

Compliance with ethical standards

This article does not contain any studies with human or animal subjects.

Conflict of interest None.

References

1. Santana-Méridas O, González-Coloma A, Vioque RS (2012) Agriculture residues as source of bioactive. Phytochem Rev 11:447–466
2. Manach C, Scalbert A, Morand C, Rémésy C, Jiménez L (2004) Polyphenols: food sources and bioavailability. Am J Clin Nutr 79:727–747
3. Lobo V, Patil A, Phatak A, Chandra N (2010) Free radicals, antioxidants and functional foods: impact on human health. Pharmacogn Rev 4:118–126
4. Pandey KB, Rizvi SI (2009) Plant polyphenols as dietary antioxidant in human health and disease. Oxid Med Cell Longev 2:270–278
5. EU: Extension of use of extracts of rosemary (E 392) in fat-based spreads. https://www.efsa.europa.eu/en/efsajournal/pub/4090. Accessed 10 Oct 2016
6. Shebis Y, Iluz D, Kinel-Tahan Y, Dubinsky Z, Yaron Yehoshua Y (2013) Natural antioxidants: function and sources. Food Nutr Sci 4:643–649
7. Misra B, Kameshwari S (2016) Extraction and sugar composition of mucilage in *urginea indica*/*drimia indica* (roxb) kunth hyacinthaceae. Int. J Pharm Pharm Sci 8:335–338
8. Choudhary PD, Pawar HA (2014) Recently Investigated Natural Gums and Mucilages as Pharmaceutical Excipients: An Overview. J Pharm 2014 204849:9. doi:10.1155/2014/204849
9. Dai J, Mumper RJ (2010) Plant Phenolics: extraction, analysis and their antioxidant and anticancer properties. Molecules 15:7313–7352
10. Nayak B, Dahmnoune F, Moussi K, Remini H, Dairi S, Aoun O, Khodir M (2015) Comparison of microwave, ultrasound and accelerated-assisted solvent extraction for recovery of polyphenols from *Citrus sinensis* peels. Food Chem 187:507–516
11. Mustapa AN, Martin Á, Mato RB, Cocero MJ (2015) Extraction of phytocompounds from the medicinal plant *Clinacanthus*

nutans Lindau by microwave-assisted extraction and supercritical carbon dioxide extraction. Ind Crops Prod 74:83–94
12. Xavier L, Freire MS, Vidal-Tato I, González-Álvarez J (2015) Application of aqueous two phase systems based on polyethylene glycol and sodium citrate for the recovery of phenolic compounds from *Eucalyptus* wood. Maderas Cienc Tecnol 17:345–354
13. Falé PL, Amaral F, Madeira PJA, Silva MS, Florêncio MH, Frazão FN, Serralheiro ML (2012) Acetylcholinesterase inhibition, antioxidant activity and toxicity of *Peumus boldus* water extracts on HeLa and Caco-2 cell lines. Food Chem Toxicol 50:2656–2662
14. Falé PL, Ferreira C, Rodrigues AM, Cleto P, Amorim PJ, Florêncio MH, Frazão FN, Serralheiro ML (2013) Antioxidant and anti-Acetylcholinesterase activity of commercially available medicinal infusions after *in vitro* gastrointestinal digestion. J Med Plants Res 7:1370–1378
15. Li S, Li SK, Gan RY, Song F-L, Kuang L, Li H-B (2013) Antioxidant capacities and total phenolic contents of infusions from223 medicinal plants. Ind Crops Prod 51:289–298
16. Fattahi S, Zabihi E, Abedian A, Pourbagher R, Ardekani AM, Mostafazadeh A, Akhavan-Niaki H (2014) Total Phenolic and Flavonoid Contents of Aqueous Extract of Stinging Nettle and In Vitro Antiproliferative Effect on Hela and BT-474 Cell Lines. Int J Mol Cell Med 3:102–107
17. Wollgast J, Anklam E (2000) Polyphenols in chocolate: is there a contribution to human health? Food Res Int 33:449–459
18. Irakli MN, Samanidou VF, Biliaderis CG, Papadoyannis IN (2012) Simultaneous determination of phenolic acids and flavonoids in rice using solid-phase extraction and RP-HPLC with photodiode array detection. J Sep Sci 35:1603–1611
19. Ghanem ME, Classen B, Quetin-Leclerq J, Mahy G, Ruan CJ, Qin P, Pérez-Alfocea F, Lutts S (2010) Mucilage and polysaccharides in the halophyte plant species *Kosteletzkya virginica*: localization and composition in relation to salt stress. J Plant Physiol 167:382–392
20. Oktay M, Gülçin I, İrfan Küfrevioğlu Ö (2003) Determination of *in vitro* antioxidant activity of fennel (*Foeniculum vulgare*) seed extracts. Food Sci Technol 36:263–271
21. Van Buren JP, Robinson WB (1981) Formation of complexes between protein and tannic acid. J Agric Food Chem 17:772–777
22. Gusakov AV, Kondratyeva EG, Sinitsyn AP (2011) Comparison of two methods for assaying reducing sugars in the determination of carbohydrase activities. Int J Anal Chem 2011:1–4
23. Tokur B, Kormaz K (2007) The Effects of an iron-catalyzed oxidation system on lipids and proteins of dark muscle fish. Food Chem 104:754–760
24. Sakat SS, Juvekar AR, Gambhire MN (2010) *In vitro* antioxidant and anti-inflammatory activity of methanol extract of *Oxalis corniculata* Linn. Int J Pharm Pharm Sci 2:146–155
25. Babou L, Hadidi L, Grosso C, Zaidi F, Valentão P, Andrade PB (2016) Study of phenolic composition and antioxidant activity of myrtle leaves and fruits as a function of maturation. Eur Food Res Technol 242:1447–1457
26. Pavarini D, Pavarani S, Niehues M, Lopes N (2012) Exogenous influences on plant secondary metabolite levels. Anim Feed Sci Technol 176:5–16
27. Liu P, Kallio H, Yang B (2011) Phenolic compounds in hawthorn (*Crataegus grayana*) fruits and leaves and changes during fruit ripening. J Agric Food Chem 59:11141–11149
28. Clifford SC, Arndt SK, Popp M, Jones HG (2001) Mucilages and polysaccharides in *Ziziphus* species (Rhamnaceae): localization, composition and physiological roles during drought-stress. J Exp Bot 53:131–138
29. Magalhães PJ, Vieira JS, Gonçalves LM, Pacheco JP, Guido LF, Barros AA (2010) Isolation of phenolic compounds from hop extracts using polyvinylpolypyrrolidone: characterization by high-performance liquid chromatography-diode array detection-electrospray tandem mass spectrometry. J Chromatogr A 1217:3258–3268
30. Singh S, Bothara SB (2014) Physico-chemical and structural characterization of mucilage isolated from seeds of *Diospyros melonoxylon* Roxb. Braz. J Pharm Sci 50:713–725
31. Shende MA, Marathe RP (2015) Extraction of mucilages and its comparative mucoadhesive studies from *Hibiscus* plant species. World. J Pharm Sci 4:900–924
32. Faccio C, Machado RAF, De Souza LM, Zoldan SR, Quadri MG (2015) Characterization of the mucilage extracted from jaracatiá (*Carica quercifolia* (A. St. Hil.) Hieron). Carbohydr Polym 131:370–376
33. Ahad HA, Yesupadam P, Ramyasree P, Padmaja BS, Sravanthi M, Prakash PG (2011) Isolation and physicochemical cartcerization of *Hybiscus rosa sinesis* leaves mucilage. Int J Curr Res 3:210–212
34. Vaithiyanathan V, Mirunalini S (2015) Assessment of antioxidant potential and acute toxicity studies of whole plant extract of *Pergularia daemia* (Forsk). Toxicol Int 22:54–60
35. Ayala A, Muñoz MF, Argüelles S (2014) Lipid Peroxidation: Production, Metabolism, and Signaling Mechanisms of Malondialdehyde and 4-Hydroxy-2-Nonenal. Oxid Med Cell Longev 2014(360438):31
36. Garcia YJ, Rodriguez-Malaver AJ, Nancy Penaloza N (2005) Lipid peroxidation measurement by thiobarbituric acid assay in rat cerebellar slices. J Neurosci Methods 144:127–135
37. Kusirisin W, Jaikang C, Chaiyasut C, Narongchai P (2009) Effect of polyphenolic compounds from Solanum torvum on plasma lipid peroxidation, superoxide anion and cytochrome P450 2E1 in human liver microsomes. Med Chem 5:583–588
38. Forstermann U, Sessa WC (2001) Nitric oxide synthases: regulation and function. Eur Heart J 33:829–837
39. Boora F, Chirisa E, Mukanganyama S (2014) Evaluation of nitrite radical scavenging properties of selected zimbabwean plant extracts and their phytoconstituents. J Food Process 2014:1–7
40. Brito A, Raminez JE, Areche C, Sepúlveda B, Simirgiotis MJ (2014) HPLC-UV-MS Profiles of phenolic compounds and antioxidant activity of fruits from three *Citrus* species consumed in northern Chile. Molecules 19:17400–17421