Article

Phenolic Composition of *Dipteryx alata* Vogel Pulp + Peel and Its Antioxidant and Cytotoxic Properties

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Dipteryx alata Vogel is a native fruit from Brazil, which has been poorly investigated concerning its phenolic composition and the biological effects of its pulp + peel. Thus, in this study we evaluated the antioxidant activity and total phenolic content of the *D. alata* pulp + peel extracts obtained with different solvents, as well as determined the phenolic compounds by ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) analysis. In addition, cytotoxic effects of *D. alata* pulp + peel extract on non-tumor and cancer cell lines were investigated for the first time. The results showed that phenolic compounds can be efficiently extracted from pulp + peel of *D. alata* by organic solvent-water mixtures as an extraction system. The developed UHPLC-MS/MS method allowed the quantification of eighteen phenolic compounds in *D. alata* pulp + peel extract for the first time, which luteolin and *trans*-cinnamic acid were predominant. In addition, *D. alata* pulp + peel extract exhibited better cytotoxity against SiHa and C33A cervical cancer cell lines, while weak cytotoxicity was noticed against non-tumor HaCaT and L929 cell lines, pointing out its safety and providing preliminary evidence of its anticancer potential. Our findings indicate that *D. alata* pulp + peel can be explored as a natural source of phenolic compounds with promising antioxidant and cytotoxic properties.

Keywords: Cerrado fruits, baru, phenolic compounds extraction, UHPLC-MS/MS, MTT assay

Introduction

In the last decades, the health-promoting effects of fruits have attracted extensive attention from scientists worldwide. The health benefits of fruits are mainly ascribed to their antioxidant properties.[1] Human cells are continuously exposed to pro-oxidants species, so, in addition to the endogenous antioxidants, dietary antioxidants have a critical role in balancing oxidation-reduction in the body and in preventing oxidative stress related disorders, such as neurodegenerative and cardiovascular diseases, diabetes and cancer.[2,3]

Phenolic compounds have been shown to be the most bioactive among the natural antioxidant agents and they are mainly found in fruits and vegetables.[2] In this way, fruits and vegetables have been extensively studied for their phenolic composition, antioxidant activity and other biological properties.[4-7] However, some fruit species or fruit parts are still scientifically underexploited concerning their phenolic composition and potential biological action, such as *Dipteryx alata* Vogel pulp + peel.

Dipteryx alata Vogel is a native tree species to the Brazilian Cerrado belonging to the Fabaceae family. Its fruits are commonly known as "baru" in Brazil and they are characterized by a pulp covered by a thin brown peel (mesocarp) with a sweet taste, that envelope a single edible oleaginous seed, generally named almond.[8,9] The pulp along with the peel of *D. alata* fruit can be consumed *in natura*; while the almond contains a trypsin inhibitor, which must be inactivated by heat treatment before consumption.[10] *D. alata* fruits are also used for candies, cakes, sorbets, cereal bars, cookies and breads production.[8,9]

Studies have demonstrated the protective effect of the consumption of *D. alata* almonds on the oxidative status

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of rats supplemented orally with iron[11] and of rats fed with high-fat diets.[12] Moreover, clinical trials showed that diet supplementation with a *D. alata* almonds improved high-density lipoproteins and reduced abdominal adiposity[13] and increase the activity of glutathione peroxidase, an antioxidant enzyme, in overweight and obese women.[14] These almonds present great nutritional value, being considered a source of minerals, proteins and unsaturated fatty acids.[6,10] Also, the presence of phytosterols, terpenes, tocopherols and phenolic compounds have been reported in extracts from the of *D. alata* almonds.[6,10,15,16] However, pulp and peel of *D. alata* has been poorly studied about their biological activities and/or phenolic composition, with only two studies recently published. Santiago *et al.*[8] evaluated the proximate composition, antioxidant activity, and total phenolic content (TPC) from the extracts of peel, pulp, and raw and roasted almonds of *D. alata* fruit. Leite *et al.*[17] identified the chemical composition, including the phenolic compounds, of the pulp of *D. alata* fruit, and evaluated its antioxidant activity *in vitro* and *in vivo*, its toxic effects and ability to increase the life expectancy in the nematode *Caenorhabditis elegans*.

Given this background, the present study aimed to evaluate the antioxidant activity and TPC of the *D. alata* pulp + peel extracts obtained with different solvents, as well as to determine the phenolic compounds by ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) analysis. In addition, their cytotoxic effects were evaluated on human cervical and colon cancer cell lines and compared to non-tumor cell lines.

Experimental

Chemicals

Phenolic compounds standards (all with purity ≥ 95%) of caffeic acid, chlorogenic acid, gallic acid, hydroxybenzoic acid, *p*-coumaric acid, protocatechuic acid, sinapic acid, syringic acid, *trans*-cinnamic acid, (−)-epicatechin, (−)-epicatechin gallate, apigenin, luteolin, myricetin, naringenin, quercetin, *trans*-resveratrol, and rutin were acquired from Sigma-Aldrich (St. Louis, USA), whereas ellagic, ferulic and vanillic acids were acquired from Fluka (Buchs, Switzerland). Stock standard solutions were prepared by dissolving 10 mg of each phenolic compounds, with the exception of apigenin (5 mg), luteolin (4.4 mg), and rutin (3.6 mg), in 10 mL of methanol and stored at –18 °C. An intermediate solution containing all standard compounds was prepared in methanol, and dilutions from this solution were done at different levels for the construction of analytical curves. HPLC-grade ethanol, methanol and formic acid

were purchased from Sigma-Aldrich (St. Louis, USA). Ultrapure water was obtained from a Milli-Q ultrapure water purification system (Millipore, Burlington, USA).

Folin-Ciocalteu's phenol reagent, 2,2-azobis (2-methylpropanimidamide) dihydrochloride (AAPH), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) and 2,2-diphenyl-1-picrylhydrazyl (DPPH·) were obtained from Sigma-Aldrich (St. Louis, USA), whereas disodium fluorescein was obtained from Fluka (Buchs, Switzerland). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were obtained from Invitrogen (Carlsbad, USA) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) was supplied by Sigma-Aldrich (St. Louis, USA).

Samples

Fresh ripe *D. alata* fruits, approximately 3.0 kg, were collected from a farm located in Campo Grande, Mato Grosso do Sul, Brazil (20°26'34"S and 54°38'47"W), in September 2016. The ripening of the fruits was determined only visually by the color and texture. In the laboratory, the ripe fruits were washed and the pulp along with the peel were manually separated from the rest of the fruit (endocarp and almond), and then frozen and lyophilized. The dried pulp + peel was milled in a domestic grinder to obtain a homogeneous sample, vacuum-packed, and stored at −18 °C until analysis.

Preparation of extracts

D. alata pulp + peel powder (1.00 ± 0.01 g) was transferred to a 50 mL Falcon tube along with 10.0 mL of the extracting solvent, and the tube was immersed into the ultrasonic bath (Elmasonic P30H, Singen, Germany) with 320 W of potency and 37 kHz of frequency. The extraction was performed at 30 °C for 30 min, according to Boeing *et al.*[5] After extraction, the solution was centrifuged at 6535 × g for 10 min and the supernatant was filtered through 0.22 μm poly(tetrafluoroethene) (PTFE) syringe filters. Different solvents were used for extraction of phenolic compounds from *D. alata* pulp + peel. Solvents included methanol and ethanol, in the pure form (absolute) and also in mixtures with water to obtain a concentration of 80% (v/v), aqueous methanol and aqueous ethanol. Furthermore, 100% ultrapure water (H2O) was also used.

Antioxidant activity and total phenolic content

The antioxidant activity was evaluated by DPPH radical scavenging and oxygen radical absorbance

capacity (ORAC) assays. DPPH$^{\bullet}$ scavenging activity was measured using the method described by Ma *et al.*[18] Methanol solutions of Trolox (0-2000 µmol L^{-1}) were used to prepare the analytical curve ($y = -2.98 \times 10^{-4}x + 0.689$, coefficient of determination (r^2) = 0.995), and the results were expressed as micromoles of Trolox equivalents *per* gram of freeze-dried sample (µmol TE g^{-1}). The ORAC assay was performed according to Ou *et al.*[19] Trolox solutions (0-50 µmol L^{-1}) were used to prepare the analytical curve ($y = 0.261x + 2.07$, r^2 = 0.993), and the results were expressed as micromoles of Trolox equivalents *per* gram of freeze-dried sample (µmol TE g^{-1}).

The total phenolic content (TPC) of the extracts was determined according to Singleton and Rossi.[20] Methanolic solutions of gallic acid (0-180 mg L^{-1}) were used to prepare the analytical curve ($y = 0.0058x - 0.0179$, r^2 = 0.993), and the results were expressed as milligrams of gallic acid equivalents *per* gram of freeze-dried sample (mg GAE g^{-1}).

UHPLC-MS/MS analysis

Chromatographic analysis was performed employing an Acquity UPLC H-Class system coupled to a Xevo TQD triple-quadrupole mass spectrometer equipped with a Z spray™ source (Waters, Milford, USA). The chromatographic separation was achieved using an Acquity UPLC® BEH C18 column (50 mm × 2.1 mm, internal diameter (i.d.), 1.7 µm) at 30 °C and flow rate of 0.15 mL min^{-1}. The mobile phase was composed of solvent A (ultrapure water acidified with 0.1% formic acid) and solvent B (methanol). Gradient elution was used and the organic solvent percentage was changed linearly as follows: 0 min, 10%; 4 min, 70%; 8 min, 100%; 11 min, 50%; 12.5 min, 10%; 15 min, 10%. The injection volume was 1.5 µL.

The electrospray ionization (ESI) source was operated in negative mode under the following conditions: capillary voltage, 3.0 kV; extractor voltage, 3.0 V; source temperature, 130 °C; desolvation gas temperature, 550 °C; desolvation gas (N$_2$) flow, 700 L h^{-1}; cone gas (N$_2$) flow, 50 L h^{-1}; collision induced dissociation gas pressure (Ar), 3.00×10^{-3} mbar. The mass spectrometer was operated in MS/MS mode using selected reaction monitoring (SRM). Precursor and product ions, associated with instrumental parameters optimized, and the retention times for the target analytes are presented in Table S1 (Supplementary Information (SI) section) and a representative chromatogram obtained from the standard mixture is shown in Figure S1 (SI section). Data were acquired and processed by MassLynx software version 4.1 (Waters, Milford, USA).[21]

Quantification of phenolic compounds in *D. alata* pulp + peel was achieved by the standard addition method, since blank samples were not available, and the results were expressed as milligram *per* kilogram of freeze-dried sample (mg kg^{-1}). Limits of detection and quantification (LOD and LOQ, respectively) were estimated for each analyte considering the concentration that produced a signal-to-noise ratio equal or higher than 3 and 10 times the noise of the baseline, respectively, in a chromatogram of a non-fortified sample, after estimating the endogenous amount.

Cell culture and cytotoxicity assay

Six cell lines were used throughout this study: murine fibroblast (L929), human immortalized keratinocytes (HaCaT), human cervical cancer (C33A, SiHa, HeLa) and human colon cancer (Caco-2). The HeLa (HPV18-positive), SiHa (HPV16-positive), C33A (HPV-negative) and HaCaT cell lines were provided by Dr Luiza L. Villa (ICESP, School of Medicine, University of São Paulo/Brazil) and Dr Silvya S. Maria-Engler (Faculty of Pharmaceutical Sciences, University of São Paulo/Brazil). L929 (ATCC® CCL1™) and Caco-2 (ATCC® HTB-37) cell lines were supplied by the American Type Culture Collection (Manassas, USA). The cell lines were cultured with DMEM (pH 7.4) supplemented with 10% (v/v) FBS and maintained in an incubator at 37 °C in a humidified atmosphere with 5% CO$_2$. The cell culture passage number is from the 4-6th passage for the cytotoxicity assay.

In vitro cytotoxicity of *D. alata* hydroethanolic extract was determined by the MTT assay and the conditions employed in this assay was described by Boeing *et al.*[5] Results were expressed as the 50% cytotoxic concentration (CC$_{50}$), defined as the extract's concentration that reduce 50% of cell viability, which were calculated by regression analysis.

Statistical analysis

Results were expressed as mean ± standard deviation (SD). Data were submitted to analysis of variance (ANOVA) using one-way with comparison of means by Tukey's test from Statistica 7.0 software (StatSoft, Tulsa, USA).[22] Differences were considered significant at $p < 0.05$. All experiments were performed in triplicate (n = 3).

Results and Discussion

Total phenolic content and antioxidant activity

Ultrasound-assisted extraction (UAE) is a rapid, easy to use, inexpensive, safe, and eco-friendly extraction

technique which is broadly used for the extraction of phenolic compounds from plant materials.[23] Cavitation is the main mechanism of action of the UAE; this favors solvent penetration and promotes the disruption of cell walls of the matrix, resulting in the mass transfer of the intracellular content into extraction solution.[24] In this study, UAE was used for extraction of phenolic compounds from *D. alata* pulp + peel and five extraction solvents, ultrapure water, absolute methanol, absolute ethanol, aqueous methanol (80%, v/v) and aqueous ethanol (80%, v/v), were compared in terms of TPC and antioxidant activity (DPPH⁺ and ORAC assays), as illustrated in Figures 1a and 1b, respectively.
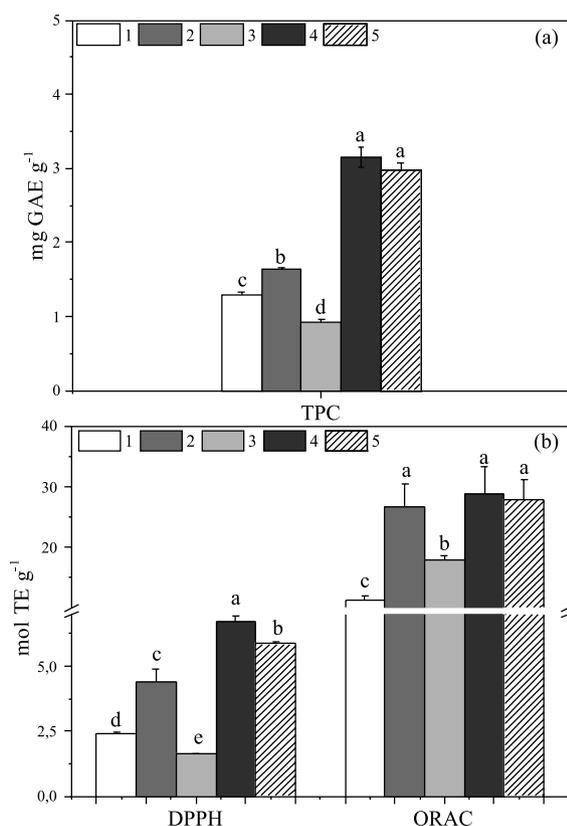

Figure 1. Comparison of total phenolic content (TPC) (a) and antioxidant activity measured by DPPH⁺ and ORAC assays (b) of extracts of *D. alata* pulp + peel obtained with different extraction solvents. 1: ultrapure water; 2: absolute methanol; 3: absolute ethanol; 4: aqueous methanol (80%, v/v); 5: aqueous ethanol (80%, v/v). Different letters between solvents represent results with statistical difference, according to the Tukey's test ($p < 0.05$).

The results showed that the TPC and antioxidant activity varied according to each solvent used in the extraction step. Overall, ultrapure water and absolute ethanol were the least favorable solvents for the extraction of the phenolic compounds from *D. alata* pulp + peel. These results are possibly associated to the differences of polarity and viscosity of the solvents used. The efficiency of ultrapure water as extraction solvent is reduced

because phenolic compounds are often more soluble in organic solvents that are less polar than water.[25] On the other hand, a decrease in solvent viscosity is related to better diffusion through the pores of the samples and to an increase in the propagation of ultrasonic waves in the solvent, improving the yield of the extraction.[24] Ethanol is the solvent with the highest viscosity followed by water and methanol, while the increasing polarity is displayed by water > methanol > ethanol. Aqueous methanol (80%, v/v) and aqueous ethanol (80%, v/v) showed the highest values of TPC (3.15 ± 0.14 and 2.98 ± 0.01 mg GAE g⁻¹, respectively) and antioxidant activity by ORAC assay (28.7 ± 4.5 and 27.9 ± 3.2 µmol TE g⁻¹, respectively). No significant difference was observed between these extraction solvents. For DPPH⁺ assay, aqueous methanol (80%, v/v) showed the highest antioxidant activity with a value of 6.70 ± 0.23 µmol TE g⁻¹ followed by aqueous ethanol (80%, v/v) with a value of 5.86 ± 0.10 µmol TE g⁻¹. The polarity of the medium increases with the addition of the water to organic solvents, which facilitates the extraction of compounds that are soluble in water and/or organic solvents.[25] Moreover, aqueous mixtures with organic solvents contribute to the disrupt cell walls of the matrix and dissolve the compounds at the same time.[26]

It is important to note that the TPC values obtained for the hydroalcoholic extracts of *D. alata* pulp + peel were higher than the values found for the methanolic extracts of raw and roasted *D. alata* almond (values ranged between 111.3 and 240.4 mg GAE 100 g⁻¹ dry weight) in the study of Lemos *et al.*[10] and aqueous extract of *D. alata* pulp (262.089 ± 0.60 mg GAE 100 g⁻¹).[17] Moreover, TPC values obtained for hydroalcoholic extracts of *D. alata* pulp along with peel were similar than values reported for the aqueous ethanol (50%, v/v) extracts of only pulp of *D. alata* (292 mg GAE 100 g⁻¹ of fresh weight) but lower compared to the other parts of the fruit (row and roasted almond and peel).[8] The antioxidant activity by DPPH⁺ assay was higher than that found for methanolic extracts of raw and roasted *D. alata* almond (22.8 and 13.9 µmol TE 100 g⁻¹ dry weight, respectively)[10] and aqueous extract of roasted *D. alata* almond (0.8 µmol TE g⁻¹ dry weight).[11] Regarding ORAC assay, antioxidant activity of hydroalcholic extracts of *D. alata* pulp + peel was lower than methanolic and hydrolyzed methanolic extracts of roasted *D. alata* almond (values ranged between 88.71 and 110.44 mg TE g⁻¹) reported by Oliveira-Alves *et al.*[6]

UHPLC-MS/MS analysis of phenolic compounds

Firstly, the effect of the solvent on the extraction efficiency of the target phenolic compounds from

pulp + peel of *D. alata* fruits was evaluated. Figure 2 presents the relation of the phenolic compounds extracted with the respective amounts (in means of peak area) for each solvent evaluated. UHPLC-MS/MS analysis showed that the phenolic compounds extracted from *D. alata* pulp + peel with all evaluated extraction solvents presented the same profile, with exception of ultrapure water that did not extract apigenin; however, the amounts of the individual compounds extracted varied according to the solvent employed (Figure 2). The trend of TPC and antioxidant activity analysis was comparable with that obtained by UHPLC-MS/MS analysis, where ultrapure water and absolute ethanol were the solvents that showed the lowest extraction capacity of the target phenolic compounds, followed by absolute methanol. In general, as can be observed in Figure 2, higher amounts of the phenolic compounds were obtained when aqueous ethanol (80%, v/v) and aqueous methanol (80%, v/v) were used as extraction solvents. The better efficiency of aqueous methanol or ethanol for the extraction of phenolic compounds from plant-derived samples was also reported by other authors.[7,25] In this context, the use of ethanol is more recommended, especially for food matrices, due to the fact that it is non-toxic, inexpensive and not harmful to human health and to the environment.[27] Therefore, aqueous ethanol (80%, v/v) was selected as the extraction solvent for the quantification of phenolic compounds of *D. alata* pulp + peel.

Table 1 shows the linear range of analytical curves used for the quantification of the phenolic compounds and the correlation coefficients (r), LOD and LOQ values and the concentration of phenolic compounds quantified in *D. alata* pulp + peel. The linearity (r > 0.99) for authentic reference standards, in a concentration range expected for the phenolic compounds in samples (7.5-2667 µg kg^{-1}), was satisfactory. This shows that the UHPLC-MS/MS method allows the proper quantification of the target compounds in the hydroethanolic extract of *D. alata* pulp + peel. Also, the LOD and LOQ were satisfactory for quantifying the target compounds at the concentration levels found in the sample. The LOD ranged from 0.03 to 100 µg kg^{-1}, whereas LOQ ranged from 0.1 to 330 µg kg^{-1}.

Of the twenty-one phenolic compounds analyzed in this study, eighteen of them were found and quantified in the *D. alata* pulp + peel, evidencing the wide range of phenolic compounds in this underexploited part of the fruit. To the best of our knowledge, the quantification of phenolic compounds in *D. alata* pulp + peel was not reported hitherto in the literature. Regarding the identification of phenolic compounds, a single work was recently published, in which some phenolic compounds were putatively identified in the
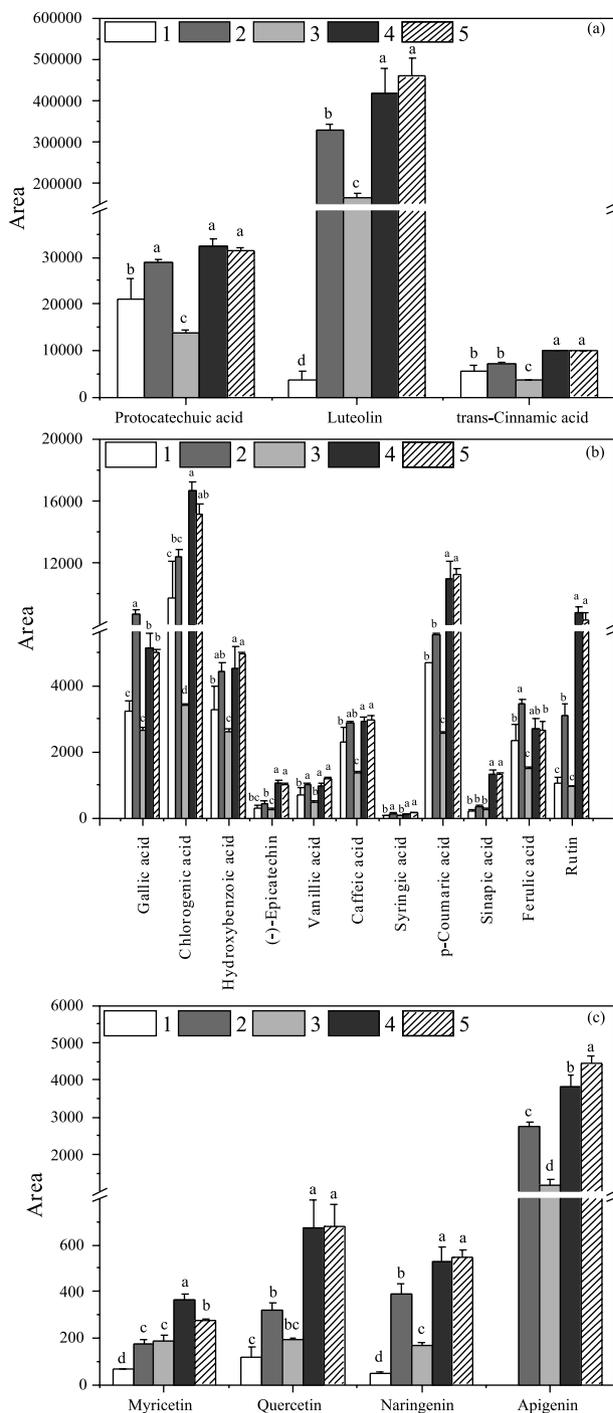

Figure 2. Effect of the solvent on the extraction of the phenolic compounds from *D. alata* pulp + peel. Major (a), intermediates (b) and minority (c) phenolic compounds found in the *D. alata* pulp + peel. 1: ultrapure water; 2: absolute methanol; 3: absolute ethanol; 4: aqueous methanol (80%, v/v); 5: aqueous ethanol (80%, v/v). Different letters between solvents represent results with statistical difference, according to the Tukey's test (*p* < 0.05).

pulp of *D. alata*.[17] In the present study, among the quantified phenolic compounds, luteolin and *trans*-cinnamic acid were the most abundant with concentrations of 153 ± 1 and 129 ± 4 mg kg^{-1}, respectively, followed by protocatechuic acid (24.0 ± 0.7 mg kg^{-1}). The other phenolic compounds

Table 1. Analytical performance of the developed method and concentration of phenolic compounds from *D. alata* pulp + peel

Phenolic compound	Linear range / (µg kg⁻¹)	Linear regression		LOD / (µg kg⁻¹)	LOQ / (µg kg⁻¹)	Concentration[a] / (mg kg⁻¹)
		$y = ax + b$	r			
Gallic acid	150-1200	$y = 5544.3x + 3810.4$	0.9990	11	37	6.69 ± 0.06
Protocatechuic acid	62.5-500	$y = 7757.3x + 1540.1$	0.9963	10	33	24.0 ± 0.7
Hydroxybenzoic acid	100-600	$y = 9735x + 2939.7$	0.9991	25	82	3.00 ± 0.02
Chlorogenic acid	400-2000	$y = 10502x + 9194.9$	0.9975	0.13	0.42	8.8 ± 0.1
Vanillic acid	500-2000	$y = 909.87x + 675.55$	0.9959	84	277	7.7 ± 0.1
(–)-Epicatechin	200-800	$y = 2591.5x + 825.71$	0.9958	13	43	3.2 ± 0.1
Caffeic acid	75-600	$y = 9111.9x + 2107.7$	0.9957	7	23	2.3 ± 0.2
Syringic acid	150-600	$y = 512.46x + 121.08$	0.9927	21	69	2.2 ± 0.2
(–)-Epicatechin gallate	40-240	$y = 7395.3x + 2.24$	0.9957	2.5	8.2	nd
p-Coumaric acid	150-1200	$y = 18846x + 7633.9$	0.9988	2.4	7.9	4.09 ± 0.05
Sinapic acid	200-800	$y = 2353.4x + 797.18$	0.9929	34	113	3.0 ± 0.1
Ferulic acid	250-2000	$y = 4742.2x + 2457.1$	0.9951	0.3	1	5.0 ± 0.2
Rutin	100-800	$y = 22399x + 5588.8$	0.9988	0.15	0.5	2.5 ± 0.1
trans-Resveratrol	60-360	$y = 2397.9x - 6.93$	0.9940	5	17	nd
Ellagic acid	100-600	$y = 1756.9x + 17.15$	0.9959	10	33	nd
Myricetin	10-80	$y = 8748.3x + 218.31$	0.9900	0.2	0.7	0.25 ± 0.03
Quercetin	12.5-100	$y = 15540x + 614.46$	0.9906	0.2	0.7	0.40 ± 0.02
Luteolin	292-1750	$y = 25178x + 32313$	0.9960	0.03	0.1	153 ± 1
Naringenin	7.5-60	$y = 18603x + 444.25$	0.9956	0.45	1.5	0.247 ± 0.003
trans-Cinnamic acid	667-2667	$y = 569.32x + 621.38$	0.9960	100	330	129 ± 4
Apigenin	40-320	$y = 35665x + 3035$	0.9990	0.58	1.9	0.87 ± 0.01

[a]Data given as the mean concentration ± standard deviation (n = 3); r: correlation coefficient; LOD: limit of detection; LOQ: limit of quantification; nd: not detect.

were found in intermediate concentrations (in the range between 2.2 and 8.8 mg kg⁻¹); while the concentration found for myricetin, quercetin, naringenin and apigenin was less than 1 mg kg⁻¹. Luteolin is one of the most important bioactive flavonoids. It possesses potent biological activities, including antioxidant, anti-inflammatory, neuroprotective, anti-tumor, cardioprotective and antidiabetic activity.[28] Also, *trans*-cinnamic acid has attracted a great deal of interest over the years because of its potential benefits to human health, including antioxidant, antifungal, anti-tumor, anti-malaria and anti-inflammatory activity.[29] The concentration of luteolin found in *D. alata* pulp + peel is higher in comparison to other fruits largely consumed in Brazil, such as *Euterpe precatoria* (21.61 µg g⁻¹ dry weight), *Malpighia emarginata* (3.16 µg g⁻¹ dry weight), *Myrciaria dubia* (3.16 µg g⁻¹ dry weight), and *Anacardium occidentale* (3.67 µg g⁻¹ dry weight).[4] Regarding *trans*-cinnamic acid, it was found in *D. alata* pulp at higher concentrations than in the fruits of wild plants, such as *Olea europaea* L. (32.43 mg kg⁻¹ dry weight), *Ziziphus jujuba* Mill. (5.77 mg kg⁻¹ dry weight), *Ficus carica* L. (11.70 mg kg⁻¹ dry weight)[30] and also at higher levels than described for the extracts of five tropical

fruits grown in Mexico, namely *Annona diversifolia* (2.64 mg 100 g⁻¹ dry weight), *Annona reticulata* (3.54 mg 100 g⁻¹ dry weight), *Diospyros digyna* (2.07 mg 100 g⁻¹ dry weight), *Manilkara sapota* L. (2.22 mg 100 g⁻¹ dry weight) and *Melicoccus bijugatus* (7.27 mg 100 g⁻¹ dry weight).[31]

In addition, studies describing the determination of phenolic compounds from different parts of *D. alata* fruits have been also reported in literature.[10,16,17,32] Among the phenolic compounds determined in *D. alata* pulp + peel extracts, several of them have already been reported for almond, bark or pulp of *D. alata* fruits. Lemos *et al.*[10] identified eight phenolic compounds in raw and roasted *D. alata* almonds, with and without peels; six of which are the same as those found in *D. alata* pulp + peel extracts in our study: gallic acid, (–)-epicatechin, ferulic acid, *p*-coumaric acid, caffeic acid and hydroxybenzoic acid. In addition, quercetin, rutin, chlorogenic acid and *trans*-cinnamic acid have already been determined in raw and roasted *D. alata* almonds by Campidelli *et al.*[16] Gallic acid was predominant in all the samples analyzed by Lemos *et al.*[10] and Campidelli *et al.*,[16] with concentrations varying between 66.7 and 224.0 mg 100 g⁻¹ and 45.83 and

48.90 mg 100 g^{-1} fresh weigh, respectively. Protocatechuic and vanillic acids were isolated from barks of *D. alata* tree[32] and luteolin was also identified from *D. alata* pulp by Leite *et al.*[17] To the best of our knowledge, syringic acid, sinapic acid, myricetin, naringenin and apigenin had not yet been determined in *D. alata* fruits.

Assessment of cytotoxicity using MTT assay

Cancer initiation and progression have been associated to oxidative stress by inducing deoxyribonucleic acid (DNA) damage and increasing DNA mutations, cell proliferation and genome instability, thereby compounds with antioxidant properties, such as phenolic compounds, could intervene with carcinogenesis.[33] Considering the wide range of phenolic compounds found in *D. alata* pulp + peel, its hydroethanolic extract was evaluated in relation to its cytotoxic effect in non-tumor and cancer cells.

To evaluate cytotoxic effect, different concentrations of the hydroethanolic extract of *D. alata* pulp + peel were applied on cervical (HeLa, SiHa and C33A) and colon (Caco-2) cancer cell lines and their viability was evaluated by MTT assay. HaCaT e L929 cell lines were used as non-tumor models for cytotoxic effect evaluation. The extract concentrations required to decrease 50% of the cellular viability (CC$_{50}$) are summarized in Figure 3. The *D. alata* pulp + peel extract inhibited the viability of both cancer and non-tumor cell lines in a concentration-dependent manner (data not shown). Of the tested cell lines, the SiHa and C33A cells exhibited the most sensitivity toward the *D. alata* pulp + peel extract, with the lowest CC$_{50}$ values (130 ± 19 and $142 \pm 44 \mu g$ mL^{-1}, respectively), followed by the HeLa cell line ($412 \pm 11 \mu g$ mL^{-1}). In contrast, *D. alata* pulp + peel extract showed hardly any cytotoxic effect against non-tumor HaCaT and L929 and Caco-2 cell lines, with CC$_{50}$ values higher than 700 μg mL^{-1}. Interestingly, *D. alata* pulp + peel extract demonstrated tumor-cell selective potential, being less cytotoxic in non-tumor cells than in cervical cancer cells. These findings suggest that the *D. alata* pulp + peel may be a potential agent to act against cervical cancer cells. Oliveira-Alves *et al.*[6] reported that *D. alata* almond also inhibiting cancer cell growth, in this case HT29 cell line was used as model for *in vitro* colon cancer studies.

The cytotoxicity of *D. alata* pulp + peel extract may be associated, at least partially, with the presence of phenolic compounds, such as luteolin and *trans*-cinnamic and protocatechuic acids, since it is well-known that this class of antioxidants can exert potent cytotoxic effect against different cancer cell lines.[3,34] Luteolin indicated cytotoxic effect against multiple cell lines, including breast

(MDA-MB-468 and MCF-7), colorectal (HCT-116 and LoVo) and prostate (PC3 and LNCaP) cancer cells.[35-38] Previous studies[34,39] have shown that protocatechuic acid has anticancer potential, possessing apoptotic activity on human breast (MCF-7), liver (HepG2), lung (A549), cervical (HeLa) and prostate (LNCaP) cancer cell lines. Furthermore, the phenolic compounds may interact synergistically among them inducing cytotoxic effects on cancer cells.
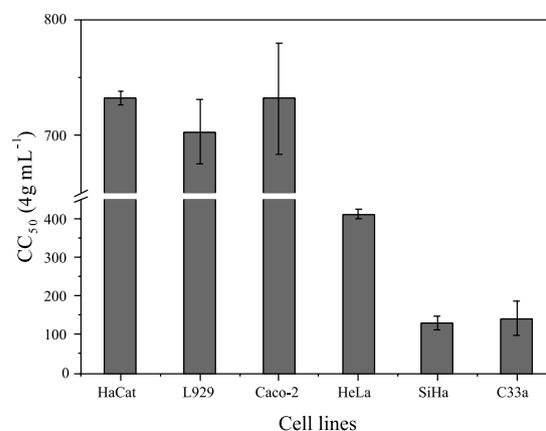

Figure 3. *In vitro* cytotoxicity of the hydroethanolic extract of *D. alata* pulp + peel against different non-tumor and cancer cell lines. CC$_{50}$: concentration of the extracts required to decrease 50% of the cellular viability.

Conclusions

This study provides information on the TPC, antioxidant activity by DPPH· and ORAC assays, phenolic composition and cytotoxic effects on non-tumor and cancer cell lines for *D. alata* pulp + peel extracts. The results showed that antioxidant compounds, especially phenolic compounds, can be efficiently extracted from pulp + peel of *D. alata* by using UAE and organic solvent-water mixtures as an extraction system. The developed UHPLC-MS/MS method allowed the proper analysis of twenty-one phenolic compounds from *D. alata* pulp + peel in just 15 min. Eighteen phenolic compounds were quantified for the first time in *D. alata* pulp + peel and the major compounds were luteolin (153 ± 1 mg kg^{-1}) and *trans*-cinnamic acid (129 ± 4 mg kg^{-1}). In addition, *in vitro* cytotoxicity, determined by the MTT assay, showed that the *D. alata* pulp + peel was potent against the cervical cancer cell lines (SiHa and C33A). Results of the present study show that the pulp + peel of *D. alata* possesses promising biological potential and a wide range of phenolic compounds. Therefore, just like the almond, the pulp + peel of *D. alata* can be considered a natural source of phenolic compounds which have potential in applications for human health, such as in functional foods and in the pharmaceutical industries.

Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br as PDF file.

Acknowledgments

Author Contributions

Érica O. Barizão was responsible for conceptualization, formal analysis, investigation, methodology, project administration and writing original draft; Joana S. Boeing for investigation and writing review and editing; Eliza M. Rotta for investigation and formal analysis; Hélito Volpato for investigation and formal analysis; Celso V. Nakamura for resources and writing review and editing; Liane Maldaner for conceptualization, supervision and writing review and editing; Jesuí V. Visentainer for funding acquisition and supervision.

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