Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Bioactive compounds, phenolic profile, antioxidant capacity and effectiveness against lipid peroxidation of cell membranes of *Mauritia flexuosa* L. fruit extracts from three biomes in the Ecuadorian Amazon

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ARTICLE INFO

ABSTRACT

The bioactive compounds, total antioxidant capacity and protective effect against lipid oxidative damage of red blood cell (RBC) membranes of *Mauritia flexuosa* L. extracts from three altitude levels (low, middle and high zones) were analyzed. The identification of the main polyphenols in the fruit was performed using High Performance Liquid Chromatography/Electrospray Ionization Tandem Mass Spectrometry analysis, while High-Performance Liquid Chromatography - Diode Array Detector was applied for the vitamin C and carotenoid analysis. The ability of the extracts (methanolic, free polyphenols and bound polyphenols) to protect RBC membranes against oxidative destruction was assessed utilizing the thiobarbituric acid reactive substance assay (TBARS) assay. Among the 14 phenolic compounds identified, quercetin glycosides were the most predominant ones. The fruit from the middle altitude zone showed higher vitamin C and lutein contents than those from the low and high areas, whilst higher values of β-Carotene were obtained in *M. flexuosa* L. from the low zone. The contents of flavonoids and total polyphenols were lower in fruit from the low zone, whilst the fruit from the middle zone had the highest values. Similar results were observed in the results of the antioxidant capacity assays. However, a significant difference was not found in the protective effect of the extracts from the three altitude zones against lipid peroxidation in RBC membranes.

1. Introduction

Palm species constitute a substantial part of the world's tropical and commercially useful trees. They can be employed to fabricate a broad range of products. Most of those items are not industrially produced, however they do offer revenue and employment to many individuals in the Amazon region. *Mauritia flexuosa* L. is one of the most commonly sold types of palm fruit in local markets (Bernal et al., 2011; Holm et al., 2008).

The *M. flexuosa* L. palm is one of two species of the *Mauritia* genus belonging to the Arecaceae family, which is native to South America and is widely distributed (especially in the Amazon region) in Bolivia, Brazil, Colombia, Ecuador, the Guianas, Peru, Trinidad and Tobago, and Venezuela. In Ecuador, it is found mainly in the western part of the Amazon basin (Henderson et al., 2019). Its common name can vary according to the country or even the region. Examples include: "caranday-guazu" or "palma real" (Bolivia), "buriti" (Bolivia and Brazil), "miriti" (Brazil), "aguaje" (Colombia and Peru), "canangucho" (Colombia and Ecuador), "moriche" (Colombia and Venezuela), "morete" (Ecuador), and "ite palm" (Guyana).

The *M. flexuosa* L. fruit is highly nutritious. People consider it an important source of bioactive compounds. The carotenoid content for *M. flexuosa* L. pulp has been previously reported, wherein β-carotene is generally higher in this fruit than in carrot and spinach (dos Santos Freitas and Alcantara, 2018; Dos Santos et al., 2015; Freitas et al., 2018; Lescano et al., 2018). It also has a high content of lipids, proteins,

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carbohydrates, tannins, phenols, flavonoids, fiber, iron, copper and potassium (Koolen et al., 2013; Leão et al., 2017; Lescano et al., 2018; Nobre et al., 2018). Due to its high content of bioactive compounds that help prevent oxidative stress and chronic diseases, it can be considered a functional food (Cordeiro et al., 2015; Manhães et al., 2015; Pereira Freire et al., 2016). However, all these studies have been performed on fruit collected in Brazil, while studies on *M. flexuosa* L. from the Ecuadorian Amazonian region are scarce.

Cândido et al. (2015) reported that the bioactive compound contents in *M. flexuosa* L. extract may vary according to the difference in biome conditions (Cerrado and Amazon regions, Brazil). In addition, coinciding results were described by Tanilgan et al. (2007) and Ouni et al. (2011) in their studies on the characterization and quantification of the phenolic compounds of *Olea europea* L. in relation to different varieties and geographic origins. They found significant quantitative differences between the phenolic fractions, but did not find qualitative differences.

Within the aforementioned research projects into the bioactive compounds and antioxidant capacity of *M. flexuosa* L. pulp extract, the analyses focused on the soluble phenolic compounds, which were extracted directly by organic (or "free phenolic") solvents. In general, the solid residues of these extractions are not thought to be sources of bioactive compounds (Perez-Jimenez and Torres, 2011). Nevertheless, noteworthy amounts of phenolic compounds may remain in the residues, becoming up to 20.0% of the total phenols (Alu'datt et al., 2014). These are non-extractable in methanol or ethanol but may be released through hydrolysis pretreatment. They are also called insoluble, unextractable or bound phenolics, and are mainly proanthocyanidins, phenolic acids, and hydrolysable tannins, which may contribute to significant health benefits as free phenolics (Alu'datt et al., 2019; Perez-Jimenez and Torres, 2011).

As far as we are aware, there exists no information concerning either the chemical composition or the biological effect of *M. flexuosa* L. fruit extracts from the Ecuadorian Amazon, or regarding the possible influence that various environmental conditions might have, such as altitude, on these characteristics. Thus, the objective of this study was to compare the bioactive compounds, antioxidant capacity and protective effect against lipid oxidative destruction of *M. flexuosa* L. fruit extracts from different altitude gradients in the Ecuadorian Amazon.

2. Materials and methods

All chemicals and solvents were of analytical grade and were purchased from Sigma-Aldrich Co (St. Louis, MO, USA).

2.1. Collection of fruit and sample preparation

Ripe *M. flexuosa* L. fruit was collected between January and February 2018 from 3 zones of the Ecuadorian Amazon rainforest with different altitudes, specifically from the cantons of La Joya de los Sachas, Loreto and Tena, which will be referred to as low, middle and high zones, respectively (Table 1).

On two separate occasions, blemish-free and damage-free fruit were chosen, based on having similar degrees of ripeness. For each region, we chose three batches of samples (1 kg) at random on each occasion. Specialists at the Jardín Botánico de Quito (Ecuador) identified the specimens utilizing specimen reference vouchers found at the facility's herbarium. A pooled sample was prepared for each region, which consisted of the three batches randomly collected by region.

The fruit were washed with plenty of running water to remove all traces of soil or foreign material and subsequently with distilled water. Then, the parts of the fruit were manually separated (peel, mesocarp and seed) and the mesocarp corresponding to each sample was frozen for 20 min at -80 °C. Following that, the samples were lyophilized using BUCHI Lyovapor™ L-200 (Büchi Labortechnik AG, Flawil, Switzerland) and ground to a fine powder in a mill (Model: IKA A11BS001, IKA®-Werke GmbH & Co., Staufen, Germany).

2.2. Extraction procedures

Methanolic extract (ME) was carried out according to the procedure described by Tulipani et al. (2008). A lyophilized sample (2 g) was added to a methanol:water solution (20 mL, 80:20, v/v). This was stirred at room temperature and protected from the light for 2 h. Next, solutions were centrifuged at 1000 rpm for 10 min at 10 °C. Subsequently, the supernatants were recovered and filtered using a 0.45 μm syringe filter (MILLEX GP). The extracts were stored in amber flasks at -20 °C until analysis.

Polyphenols (free and bound) were extracted as described by Zhong et al. (2018) with slight modifications. Briefly, for the extraction of free polyphenols (FP), 10 mL of hexane was added to 1 g of lyophilized samples and stirred for 10 min at room temperature. Next, the extracts were centrifuged at 2700 rpm, 4 °C for 5 min and the supernatants from each sample were collected (hexane layer). Next, hexane was evaporated under nitrogen flow, 20 mL of methanol/water (80:20, v/v) were added to the dry residue and the mixture was vortexed for 10 min. The samples were then centrifuged at 2700 rpm at 4 °C for 5 min, the supernatant was collected and the washing process repeated until the supernatants were clear. Finally, the supernatants were removed at 45 °C using a rotary evaporator. The dry residue was resuspended in 20 mL of methanol (80%) and stored at -20 °C until analysis.

Bound polyphenols (BP) were separated from the samples after free polyphenols were extracted. The residues obtained after the extraction of FP were mixed with NaOH (20 mL 2 M) for alkaline digestion under a flow of liquid nitrogen for 2 min. Then, they were kept for 60 min at room temperature with rotary stirring and the suspensions were acidified to pH 2 with concentrated HCl (6 mol/L). Hexane was then added as described above for free polyphenols. Subsequently, ethyl acetate (20 mL) was added to the residues and mixed using rotary stirring for 10 min at room temperature, followed by centrifugation under the conditions described above. The resulting organic layers were collected. This process was repeated five times. The supernatants were removed on a rotary evaporator at 45 °C. The dry residues were resuspended in methanol (20 mL, 80%) and stored at -20 °C until analysis.

Table 1. Origin and geographic data of *M. flexuosa* L. fruit collection locations in 2018, Ecuadorian Amazon region.

Geographic data	Low zone	Middle zone	High zone
Origin	Orellana	Orellana	Napo
Latitude (S)	00° 19′ 05.53″	00° 45′ 05.82″	00° 43′ 47.19″
Longitude (W)	76° 48′ 30.98″	77° 27′ 22.96″	77° 45′ 40.99″
Altitude (m.a.s.l)	273	639	1155
Average annual temperature (°C) (min – max)*	18.0–27.8	19.5–32.1	19.3–28.7
Precipitation (mm)*	373.4	273.7	288.7

* Source: *Instituto Nacional de Meteorología e Hidrología* (*INAMHI*, 2019).

2.3. High Performance Liquid Chromatography/Electrospray Ionization Tandem Mass Spectrometry (HPLC/ESI-MS/MS) analyses for phenolic composition

For the HPLC/ESI-MS/MS analysis for phenolic composition, a Vanquish HPLC system coupled with an LTQ-XL (Thermo Fisher Scientific) was used and controlled by Xcalibur software. An Accucore C18 column (2.1 μm, 4.6 × 150 mm) thermostated at 35 °C was used as a stationary phase. Formic acid (0.1 %) (A) and acetonitrile (B) were used as a mobile phase at 0.2 mL/min of flow rate and the elution gradient was isocratic 2% B for 4 min, 4–40% B over 36 min, 40–70% B over 10 min, 70–98% B over 5 min, 98-2% B over 5 min, and re-equilibration of the column to the initial solvent conditions. A double online detection was carried out in the DAD at 280, 360 and 520 nm, while the MS was operated in negative and positive mode and data were acquired in scan mode using a m/z range between 50 to m/z 2000. The MS detections were obtained a) through a full scan experiment, and b) dependent on MS2 data of the 5 most intense ions (from the full scan) with a normalized collision energy of 35. ESI conditions were set at a capillary temperature of 275 °C, a source voltage of 5 kV, and a capillary voltage of -35 V. The compounds were tentatively identified based on their mass spectra and data previously reported in the literature. . The data files from the complete scan and from the MS2 were previously converted to mzML files and were also processed through the GNP platform (http://gnps.ucsd.edu), thus confirming the identification of the compound with the data from the available libraries.

2.4. High Performance Liquid Chromatography - Diode Array Detector (HPLC-DAD) determination and quantification of vitamin C and carotenoid content

Both HPLC-DAD analyses of vitamin C and carotenoid content were realized by following the method previously described by Alarcón-Barrera et al. (2018) using an Agilent Technologies Series 1260 HPLC system (Santa Clara, California, United States) furnished with an Agilent 1260 Infinity Binary Pump and an Agilent 1200 Infinity Series Diode Array Detector (DAD).

For vitamin C content analysis, the lyophilized fruit powder (0.5 g) was added to 10 mL of a metaphosphoric acid and acetic acid (73/84, w/v) solution, left in sonication for 20 min, filtered through a syringe filter (0.45 μm) and immediately injected onto an HPLC system. An Eclipse Plus C18 (5 μm, 4.6 × 150 mm) chromatographic column was used for separation, while KH_2PO_4 (50 mM, pH 2.5) was used for elution at a 1 mL/min flow rate for 20 min at 245 nm. Ascorbic acid was used as an external standard (5–50 mg/L) and the vitamin C content was expressed as mg of vitamin C per 100 g of dry weight (mg Vit C/100 g DW).

For carotenoid content analysis, one gram of freeze-dried fruit powder was mixed with chloroform (20 mL) and left in continuous stirring at room temperature and protected from the light for 24 h. Next, the solution was filtered through a Minisart® syringe filters (0.45 μm, PBI International) and concentrated in a rotary evaporator. The dry residue was then saponified by the reflux method with 100 mL of 5% KOH in methanol for 4 h at 50 °C in the dark. The saponified material was combined with petroleum ether (100 mL) and distilled water (100 mL) and allowed to stand until the organic layer was formed. The organic layer was then collected and dried in a rotary evaporator and the resulting dried residue was resuspended in methanol-isopropanol (35:65, v/v), filtered through a Minisart® syringe filters (0.45 μm, PBI International) and analyzed on an HPLC system. Methanol-isopropanol (35:65, v/v) was used as a mobile phase for the elution in isocratic mode at a 1 mL/min flow rate for 20 min at 450 nm β-carotene (0.1–10 mg/mL) and lutein (0.1–50 mg/mL) calibration curves were used and the results were expressed as grams per 100 g of dry weight (DW) of fruit (g/100 g of DW).

2.5. Total flavonoid and total polyphenol content

Total flavonoid content (TFC) was determined using the aluminum chloride method previously proposed by Zhishen et al. (1999). An aliquot of the sample, standard or blank (100 μL) was mixed with a 5 % $NaNO_2$ solution (30 μL) and distilled water (500 μL). The samples were left at room temperature protected from light for 6 min and then 60 μL of 10% aluminum chloride hexahydrate solution was added and the reaction mixture was allowed to react in the dark for 5 min. Subsequently, distilled water (10 μL) and NaOH (200 μL 1M) were added and the samples' absorbance was determined in a plate reader at 510 nm against a blank of distilled water. A catechin calibration curve (0.05–0.50 mM) was used and results were expressed in mg equivalent of catechin (mg Cateq/100 g DW).

Total polyphenol content (TPC) in the samples was determined using the Folin-Ciocalteu method (Singleton et al., 1999). An aliquot (100 μL) of the extract, standard or blank and Folin-Ciocalteu reagent (500 μL, 0.2 N 1:10, v/v) were mixed and incubated in the dark at room temperature for 5 min. A solution (Na_2CO_3 400 μL 0.7 M) was added and it was incubated again for 2 h at room temperature in the dark and then the absorbance was measured in a plate reader (Synergy HT, BioTek Instruments, Inc., Winooski, VT, USA) at 760 nm against a blank of distilled water. A gallic acid calibration curve (0.075–1.5 mM) was made and results were expressed in mg equivalent of gallic acid (mg GAE/100 g of DW).

2.6. Total antioxidant capacity assays

The total antioxidant capacity of the three extracts (ME, FP and BP) was determined using in parallel the DPPH assay (1,1 - Diphenyl - 2 - Picrylhydrazyl Free Radical) and the Ferric Reducing Antioxidant Power (FRAP) assay. In both analyses, a standard of Trolox (10–1000 μM) was used for the calibration curve and results were expressed in μmol Trolox equivalent per gram of dry weight (μmol TE/g DW).

The DPPH assay was carried out following the method previously described by Prymont-Przyminska et al. (2014). A DPPH· solution (80 μL 0.2 mM) in absolute methanol was mixed with the sample extract (10 μL) and ethanol (110 μL, 70%). Two blanks were prepared: blank 1 (DPPH reagent, 80 μL), absolute methanol (10 μL) and ethanol (110 μL, 70%), while in blank 2, the DPPH reagent was replaced with absolute methanol (80 μL). The mixture was then incubated at room temperature and protected the from light for 15 min and the absorbance was measured at 517 nm in a plate reader (Synergy HT, BioTek Instruments, Inc., Winooski, VT, USA) against the aforementioned blank samples.

For the FRAP assay, the method by Benzie and Strain (1996) was used. The sample extract, standard or blank (10 μL) and FRAP reagent (180 μL) [sodium acetate trihydrate buffer (300 mM), 2,4,6 - tri (2 - pyridyl) - 1,3,5 - triazine (TPTZ) (10 mM) and $FeCl_3$ solution (20 mM) in ratios of 10:1:1 respectively] were mixed and incubated at 37 °C for 30 min. Next, samples were read in a plate reader (Synergy HT, BioTek Instruments, Inc., Winooski, VT, USA) at 539 nm against the corresponding blank.

2.7. Evaluation of the ability of extracts to protect cell membranes against oxidative damage

2.7.1. Red blood cell (RBC) ghost membrane preparation

RBC ghost membrane preparation was performed as described by Alvarez-Suarez et al. (2012). The protocol was approved by the Universidad de Las Américas' Ethical Committee (Approval No: 2017–0701) in compliance with the principles of the Declaration of Helsinki, as revised in 2000. Healthy non-smoking adult volunteers were invited to participate in the study and, after obtaining signed informed consent, blood samples were obtained. Approximately 20 mL of blood were collected by antecubital venipuncture using a sodium citrate vacutainer (BD Vacutainer® CPTTM) after an overnight fast. Heparinized blood was

centrifugated at 1000 *g* for 10 min at 10 °C and after the removal of plasma and buffy coat, RBCs were obtained. RBCs were washed once with cool 0.9% NaCl solution, then three times with PBS (150 mM NaCl, 1.9 mM Na2HPO4 and 8.1 mM NaH2PO4, pH 7.4) at 4 °C. RBC hemolysis was induced with phosphate buffer (5 mM, pH 7.4, 1:20) for 30 min at 4 °C. The hemolysate was centrifuged (15000 *g* for 20 min at 8 °C), the supernatant discarded, and the cell pellet resuspended in phosphate buffer (2.5 mM, pH 8). In the last step, and to complete the hemolysis, the cell pellet were resuspended in phosphate buffer (1.25 mM, pH 8), a vortex for 5 min, and centrifuged at 15000 *g* for 20 min at 8 °C. At the end of this procedure, RBC ghost membranes appeared completely clear in the pellet. Finally, the ghost membranes were resuspended in PBS buffer/2.2 mM EDTA (1:5) and stored at -80 °C until analysis. Total proteins in RBC ghost membrane was estimated by means of the Lowry method (Lowry et al., 1951).

2.7.2. Thiobarbituric acid reactive substance assay (TBARS assay) for lipid peroxidation

Before the experiment, samples were centrifuged (1800 *g* for 20 min at 8 °C), the supernatant removed, and the packed ghost membranes were washed twice in 0.9% NaCl. Next, the ghost membranes were resuspended in 0.9% NaCl and incubated for 1 h at 37 °C with the different extracts (ME, FP and BP) at a final concentration of 20, 40, 80, 160, 320, 500 and 1000 μg/mL in darkness with gentle shaking. After incubation with the extracts, the membranes were washed with PBS to avoid the antioxidants coming into contact with the oxidant and were then subjected to an oxidative stress model using the reagent 2,2′-azo-bis(2-amidinopropane) dihydrochloride (AAPH) at 50 mM, stirring for 1 h at 37 °C.

Thiobarbituric acid reactive substances (TBARS) formation was determined using a modified TBARS assay reported by our group (Alvarez-Suarez et al., 2012). Briefly, 2 mL of thiobarbituric acid (0.375% w/v) – trichloroacetic acid (15% w/v) – HCl (0.2 M HCl) solution was added to the sample (0.6 mL) containing butylated hydroxytoluene (0.3 mM) to prevent the possible peroxidation of the lipids present in the samples during the assay. The reaction was started by heating the samples for 20 min at 95 °C, followed by cooling on ice. Then, the samples were centrifuged at 15000 g for 20 min at 8 °C and the

absorbance of the supernatant was determined using a plate reader (Synergy HT, BioTek Instruments, Inc., Winooski, VT, USA) at 535 nm. TEP (1,1,3,3-tetraethoxypropane) was used as standard and results were expressed as μg of extract/mL required to inhibit 50% (IC_{50}) of lipid peroxidation in membranes.

2.8. Statistical analysis

The SPSS statistical software package for Windows V 22.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. All analyses were performed in triplicate and results are shown as mean ± standard deviation (SD). Statistical significance was determined using a one-way analysis of variance (ANOVA) and Tukey's Test at $p < 0.05$. In addition, Pearson's test was used to determine the degree of correlation between chemical composition and antioxidant capacity.

3. Results and discussion

3.1. Phenolic composition of M. flexuosa L. Fruit

The phenolic profile of *M. flexuosa* L. fruit was determined in the methanolic extract (ME) by HPLC-DAD-ESI/MS^n based on the deprotonated ions in negative (flavonoids) and positive (anthocyanins) mode and the fragments released in MS/MS experiments. The characterization was carried out in the methanolic extract in order to have an overview of the main compounds present in it, which in many cases would also be present (either free or conjugated) in the extracts studied. Figure 1 shows a representative HPLC chromatogram of the polyphenol profile in the studied samples, while Table 2 shows the mass data and the tentative identity of the peaks.

A total of 14 peaks were cautiously identified, corresponding to flavanols (9), flavanone (1), flavone (1), hydroxycinnamoyl derivatives (1) and anthocyanins (2). Chlorogenic acids, namely caffeoylquinic and dicaffeoylquinic acids, were the main phenolic acid derivatives identified, similar to those previously reported in *M. flexuosa* fruit (Koolen et al., 2013), whereas the majority of the flavonoid identified corresponded to quercetin glycosides. A similar polyphenolic profile was observed in the fruit from the three altitudinal zones; however, it should
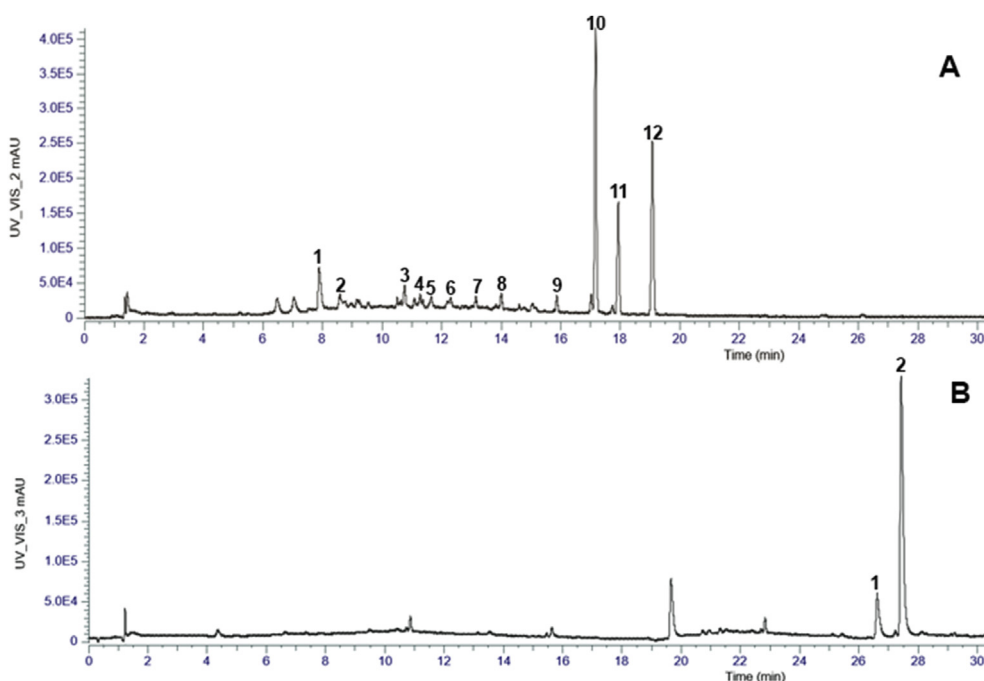
Figure 1. HPLC chromatograms of: (A) flavonoids (recorded at 360 nm), and (B) anthocyanins (recorded at 520 nm) in the hydroalcoholic extract of *M. flexuosa* L. fruit.

Table 2. HPLC-ESI-MS^n data and tentative compounds identification in *M. flexuosa* L. fruit.

Peaks	Rt (min)	$[M-H]^-$	Main MS^2 products (m/z)	Tentative identification	Molecular formula
Flavonoids					
1	7.88	625	301	Quercetin-dihexoside	$C_{27}H_{30}O_{17}$
2	8.56	493	317	Myricetin glucuronide	$C_{21}H_{18}O_{14}$
3	10.73	507	331, 193, 165	Methylmyricetin-*O*-glucuronide	$C_{22}H_{20}O_{14}$
4	11.26	609	301	Quercetin-*O*-rutinoside	$C_{27}H_{30}O_{16}$
5	11.38	463	301	Quercetin-*O*- glucoside	$C_{21}H_{18}O_{13}$
6	12.22	477	301	Quercetin-3-*O*-glucuronide	$C_{21}H_{18}O_{13}$
7	13.14	447	285	Kaempferol-3-*O*-glucoside	$C_{21}H_{20}O_{11}$
8	14.01	461	315	Kaempferol-3-*O*-glucuronide	$C_{21}H_{18}O_{12}$
9	15.86	433	271, 163, 155 151	Naringenin hexoside	$C_{21}H_{22}O_{10}$
10	17.19	431	285, 255	Luteolin-*O*-deoxyhexoside	$C_{21}H_{20}O_{11}$
11	17.95	271	227, 177, 151	Naringenin	$C_{15}H_{12}O_5$
12	19.39	301	272, 256, 179 151	Quercetin	$C_{15}H_{10}O_7$
Peaks	**Rt (min)**	$\mathbf{[M-H]^+}$	**Main MS^2 products (m/z)**	**Tentative identification**	**Molecular formula**
Anthocyanins					
1	26.76	595	449, 287	Cyanidin-3-rutinoside	$C_{27}H_{33}ClO_{15}$
2	27.53	449	287	Cyanidin-3-glucoside	$C_{21}H_{23}ClO_{11}$

be taken into account that some (minority) compounds could have escaped due to the methodological approach used, which should be studied more deeply for a more detailed chemical characterization.

Quercetin has also been previously reported in this fruit, as well as another group of flavonoids that includes naringenin and myricetin. However, in our study, glycosylated forms of these flavonoids were predominantly identified, in comparison with the previous studies that only reported aglycone forms (Koolen et al., 2013). Quercetin is one of the most studied flavonoids due to its abundant presence in nature and the human diet, as well as its marked biological activity, among which its antioxidant capacity stands out (Anand David et al., 2016). Its aglycone form of flavonoid glycosides, has been associated with beneficial effects against several diseases, which include chronic non-communicable diseases (Anand David et al., 2016). In addition to other biological effects that have also been reported, such as anti-inflammatory, anti-ulcer, anti-allergy, anti-viral gastroprotective, antihypertensive, immunomodulatory and anti-infective effects (Anand David et al., 2016). However, the main problem that this form encounters is its low bioavailability (Kaşıkcı and Bağdatlıoğlu, 2016). In contrast, we demonstrated that quercetin glucoside's bioavailability was higher than that of quercetin aglycone (Kaşıkcı and Bağdatlıoğlu, 2016). This could be due to various facts, for instance: (i) quercetin glucoside is more water-soluble than quercetin aglycone, or (ii) the sodium-dependent glucose transporter 1 (SLGT1) favors the absorption of quercetin glycosides, while this is not the case for quercetin aglycone (Guo and Bruno, 2015). This is an important aspect considering that the main form of this flavonoid found in the *M. flexuosa* fruit studied here are glycosides.

Another important flavonoid identified was kaempferol glycosides. Previous studies have reported the capacity of kaempferol in the prevention and reduction the risk of chronic diseases, especially cancer. Kaempferol has been related with an increase in antioxidant defense against free radicals and its capacity to modulate different intracellular signal transduction pathways linked to apoptosis, angiogenesis, inflammation, and metastasis (Chen and Chen, 2013). Similarly to quercetin, glucoside conjugates in kaempferol appear to increase its bioavailability in humans. Once absorbed, kaempferol is rapidly is transformed by hepatic metabolism and released into the bloodstream as methyl, glucuronide and sulfate metabolites, which has a direct influence on the biological effects that have been attributed to it.

Cyanidin derivatives were also identified, which correspond to the anthocyanins identified in the fruit. Anthocyanins are another important group of bioactive compounds to which important biological properties have also been attributed, such as antioxidant (Rio et al., 2013), anti-tumor (Amatori et al., 2016), and anti-inflammatory activity (Gasparrini et al., 2017). Therefore, this chemical composition could partly justify the biological effects previously reported for this fruit, such as its antioxidant capacity (Ramful et al., 2011).

3.2. Vitamin C and carotene content in M. flexuosa L. Fruit

Fruit is a relevant source of bioactive compounds. The latter play an important role in disease prevention and degenerative processes, such as cancer, cardiovascular diseases and inflammation (de Almeida Siqueira et al., 2013; Habibi and Ramezanian, 2017). Currently, the search for novel and exotic sources of bioactive compounds has focused attention on Amazonian regions because of their variability in native species and the use of those species in traditional medicine by the regions' indigenous peoples. *M. flexuosa* L. fruit have been used and consumed since ancient times by native Amazonian populations, especially the *Achuar* people. The fruit pulp is highly nutritious and contains protein, fat, vitamins and carbohydrates. It is eaten fresh or used to make beverages, such as *moriche carato,* and even to extract oil or make a type of sweet called *moriche* nougat.

It has been reported that climatic and regional conditions can directly influence the composition of the fruit, hence the importance of studying its composition and relating it to the regions where it is produced (Pérez-Balladares et al., 2019). Within this context, here we studied the chemical composition and antioxidant properties of *M. flexuosa* L. fruit produced at different altitude levels in the Amazon region of Ecuador. Table 3 shows the vitamin C, β-Carotene and lutein content determined in the *M. flexuosa* L. mesocarp collected in the three Ecuadorian Amazon zones studied.

The vitamin C content for *M. flexuosa* L. for the three zones was significantly different ($p < 0.05$): fruit in the middle and high zones had greater concentrations than those in the low zone. This difference in vitamin C content in *M. flexuosa* L. pulp is also evident if we compare two Brazilian regions in studies carried out by Schiassi et al. (2018) (7.42 ± 0.19 mg Vit C/100 g DW) in the Cerrado region, and Sandri et al. (2017) (49.67 ± 0.92 mg Vit C/100 g DW) in the city of Diamantino, Mato

Table 3. Vitamin C, β-carotene and lutein content determined in *M. flexuosa* L. mesocarp originating in three zones of the Ecuadorian Amazon rainforest.

Altitude zone	Vitamin C (mg Vit C/100 g DW)	β-carotene (mg β-Carotene/100 g DW)	Lutein (mg Lutein/100 g DW)
Low	3.28 ± 0.78[c]	19.5 ± 0.13[a]	5.74 ± 0.06[c]
Middle	33.4 ± 0.48[a]	3.29 ± 0.02[b]	22.6 ± 0.55[a]
High	31.8 ± 0.12[b]	3.08 ± 0.02[c]	16.4 ± 0.01[b]

Means followed by different lowercase letters in the same column are significantly different according to Tukey's test (p < 0.05).

Grosso. The Vit C content of our samples in the low zone is similar to fruit such as: *Pyrus communis* L (2.8 mg Vit C/100 g), *Eriobotrya japonica* Lindl (3.2 mg Vit C/100 g) and *Eugenia malaccensis* L (3.8 mg Vit C/100 g), while the middle and high zone results are close to *Citrus limoniaI Osbeck* (32.8 mg Vit C/100 g), *Citrus aurantifolia* (34.5 mg Vit C/100 g) and *Arábic comosus* (L.) Merril (34.6 mg Vit C/100 g). Vitamin C is recognized for being a promoter of biochemical and physiological processes, formation of collagen, immune cell maturation and iron absorption. In addition, it decreases cardiovascular risk due to the protection of the vascular tissue and the improvement of lipid metabolism and arterial pressure (Pereira Freire et al., 2016).

β-Carotene content was higher for fruit in the low zone than in the middle and high zones. However, the β-Carotene values determined in different research projects about *M. flexuosa* L. pulp vary quantitatively, for instance: 4.65 (Schiassi et al., 2018), 9.01 (Sandri et al., 2017) and 34.1 mg β-Carotene/100 g DW (Lima et al., 2009). This difference can be due to the changes produced during pulp processing and freezing, as well as soil properties, climate, harvest season and genetic variability in cases where the pulps are from native fruit (Schiassi et al., 2018).

As regards lutein content, the values varied greatly between the zones studied, being higher in the middle and high zones. These values are within the range (1.88–22.4 mg Lutein/100 g DW) determined by Guevara et al. (2019) concerning the 19 main types of fruit consumed in the Western coastal region of Ecuador. Those authors presumed that the differences within the chemical composition can be influenced by factors including altitude, incidence of ultraviolet light and temperatures changes. In Ecuador, the sun's rays fall almost perpendicularly onto the Earth's surface. These conditions that the plants are exposed to could be associated with the high concentrations of carotenoids. The high amount of lutein present in *M. flexuosa* L. is consistent with the results proposed by Priyadarshani and Jansz (2014) that yellow-orange colored fruit and vegetables are a good source of carotenoids.

3.3. Total flavonoid content (TFC) and total phenolic content (TPC) for the three types of extracts of M. flexuosa L. Pulp

Figures 2 and 3 present the TFC and TPC determined for the different types of extract of *M. flexuosa* L. pulp for the zones studied. Both colorimetric methods are used as routine methods to produce a general idea of the amount of different groups of compounds. However, it must be taken into account that they may lead to an overestimation of compounds, especially in the case of the Folin-Ciocalteu method, as it
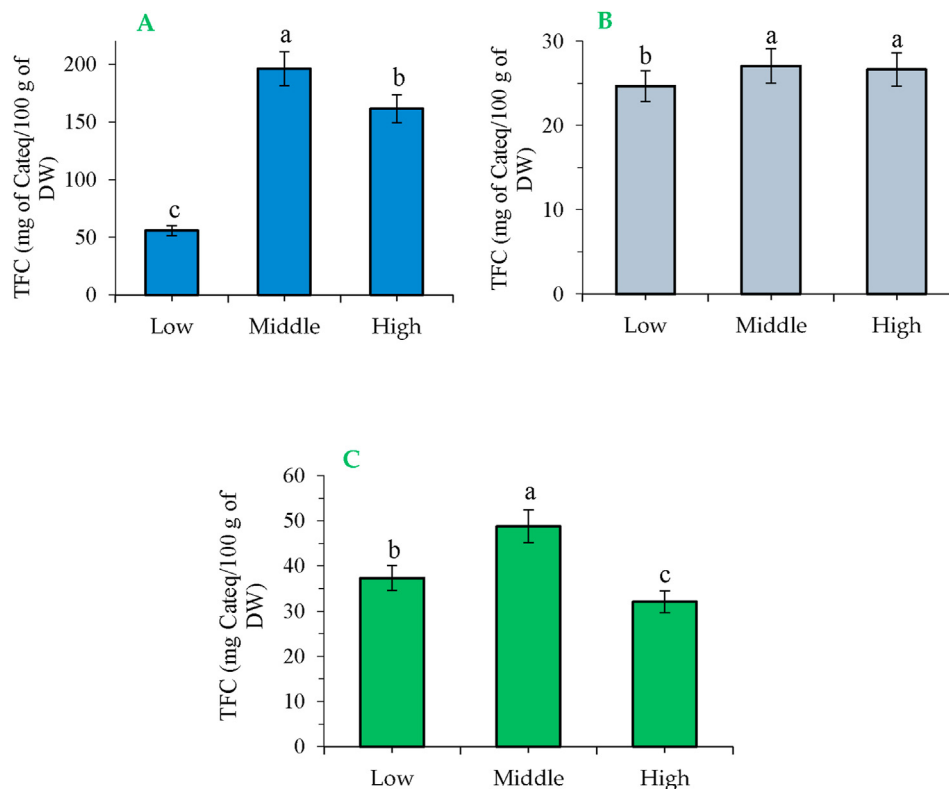
Figure 2. Total flavonoid content (TFC) for the three type of extracts: (A) methanolic extract, (B) free polyphenols and (C) bound polyphenols obtained for *M. flexuosa* L. pulp from low, middle and high zones. Means followed by different letters are significantly different according Tukey's test (*p* < 0.05).
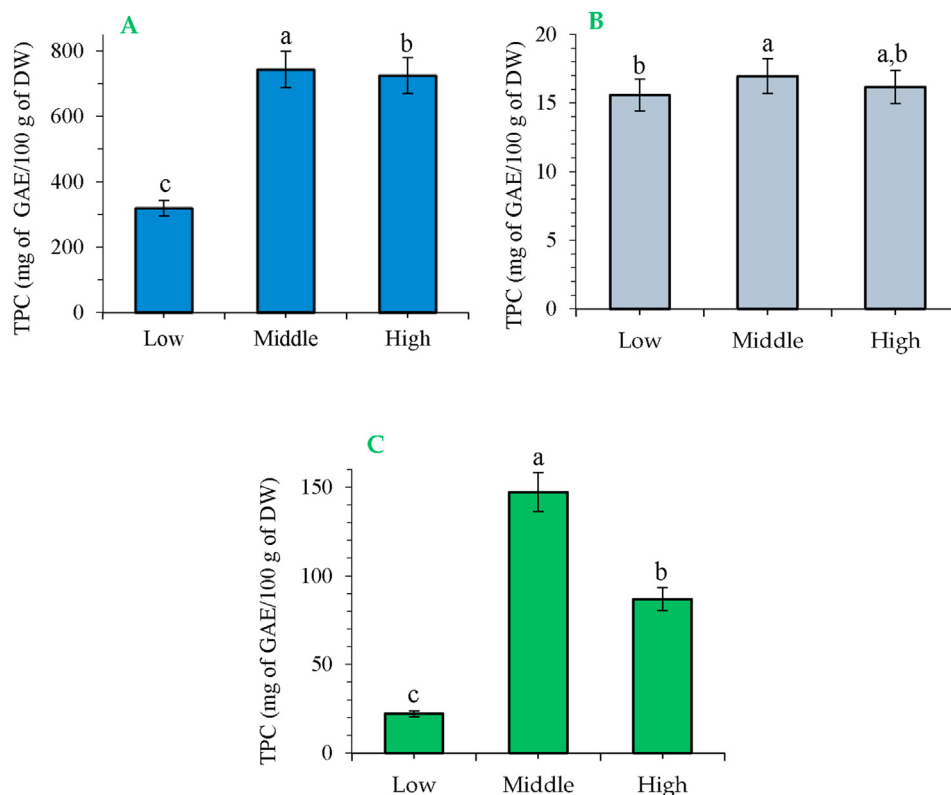
Figure 3. Total phenolic content (TPC) for the three types of extracts: (A) methanolic extract, (B) free polyphenols and (C) bound polyphenols obtained from *M. flexuosa* L. pulp from low, middle and high zones. Means followed by different letters are significantly different according to Tukey's test ($p < 0.05$).

actually determines reducing substances present in the extract. However, it is a universal method used by most authors, and the expression of the results as "gallic acid equivalents" (GAE) allows one to compare the results here obtained with those previously reported in Morete and other fruit.

Overall, a similar trend can be observed for the TFC and TPC results for the same type of extracts (ME, FP and BP). The content of bioactive compounds was highest for ME, while for the FP extract, the lowest values were obtained (ME > BP > FP). In both TFC and TPC, the bound phenolic compounds were higher than their free phenolic counterparts among the same zones. This interesting group of compounds (BP) has an important nutritional value upon ingestion. Their potential use as a dietary source of bioactive compounds is generally ignored, since most studies only report the TPC of ME from foods of vegetable origin. Nonetheless, the bioavailability of some of these compounds can be increased, since some of them can be hydrolyzed by intestinal enzymes within the small intestine and therefore become bioaccessible. As a result of the action of the microbiota, others can be released from the food matrix when they reach the colon and can be transformed into small phenolics and other metabolites that can be bioabsorbed (Perez-Jimenez and Torres, 2011).

Regarding the influence of altitude on the bioative compound contents, significant differences ($p < 0.05$) were observed between the values, being lower in the extracts from the low zone. However, the values in the middle and high zones were numerically close in most cases. The TFC and TPC determined in the FP extract from the middle and high zones did not show a significant difference ($p < 0.05$).

As altitude increases, the incidence of solar radiation gradually increases. This condition exposes the plants to a higher level of ultraviolet radiation (UV), where for every kilometer of altitude, UV radiation increases by 10% (Organization, 2020). When plants receive high level of UV radiation, a large number of cellular elements are interfered, in

particular cellular macromolecules (DNA and proteins) and as a consequence, oxygen radicals are induced (Xie et al., 2019). This condition, combined with the temperature changes that occur at higher zones (low at night and warm during the day), further accentuates oxidative stress. It is then that plants, as a protection mechanism, begin to produce a group of secondary metabolites with antioxidant capacity, such as polyphenols (Pandey and Rizvi, 2009) and Vitamin C (Pehlivan, 2017). This accumulation of antioxidant compounds in response to these environmental stimuli favors the antioxidant response of the plant to stress-generating factors and at the same time increases the quality of the fruit in terms of its antioxidant content and health benefits (Chen et al., 2018).

In addition, the TFC range in the ME for the three zones was from 55.8 ± 0.2 to 196.0 ± 0.6 mg Cateq/100 g DW. In previous papers, authors have reported the following values for the same species: 52.86 ± 10.71, Amazon (Pará) (Cândido et al., 2015), and 63.679 ± 9.51 mg/100g, Campo Grande (Mato Grosso do Sul) (Lescano et al., 2018), both in Brazil. These results are similar to those we determined for the extracts from the low zone and are lower than those obtained for the middle and high zones in our study. According to the classification of total flavonoid levels in pulp extracts proposed by Ramful et al. (2011), the fruit collected is categorized as medium (between 40 and 60 mg/100g) for the low zone, and as high (>60 mg/100g) for the other two zones.

The *M. flexuosa* L. pulp TPC values determined for the middle and high zones (743.2 ± 0.5 and 725.0 ± 0.5 mg GAE/100 g DW) are higher than what was reported by Cândido et al. (2015) for the same species in the Brazilian biomes of the Amazon and Cerrado (362.9 and 435.1 mg GAE/100 g DW) at 127 and 780 m.a.s.l., respectively. Nonetheless, the TPC quantified from our fruit collected in the low zone was lower (319.2 ± 07 mg GAE/100 g DW at 273 m.a.s.l.) than in the Brazilian zones. Tulipani et al. (2011), studied what effect various environmental conditions had on the TPC and TFC of four types of strawberry, and they confirmed a dependent response (TPC and TFC values) to environmental

Table 4. Total antioxidant capacity (μmol TE/g of DW) of the methanolic, free polyphenols and bound polyphenols extracts of *M. flexuosa* L. pulp.

Antioxidant activity	Extract type	Altitude zone		
		Low	Middle	High
DPPH assay	ME	14.76 ± 0.09^b	18.03 ± 0.07^a	17.98 ± 0.05^a
	FP	0.12 ± 0.04^c	1.28 ± 0.07^b	1.63 ± 0.07^a
	BP	0.74 ± 0.07^c	6.05 ± 0.09^a	4.75 ± 0.07^b
FRAP assay	ME	11.38 ± 0.10^c	15.4 ± 0.03^b	15.68 ± 0.06^a
	FP	0.49 ± 0.09^b	0.53 ± 0.05^b	0.80 ± 0.03^a
	BP	0.70 ± 0.06^c	6.24 ± 0.03^a	2.83 ± 0.06^b

Results are expressed as Means ± standard deviation. Means followed by different lowercase letters in the same row are significantly different according to Tukey's test ($p < 0.05$). ME: Methanolic extract; FP: Free polyphenols; BP: Bound polyphenols.

stress conditions, such as pluviometric index conditions and a higher sunlight incidence.

3.4. Total antioxidant capacity and protective effect against lipid peroxidation of M. Flexuosa L. Pulp extracts

The antioxidant capacity of *M. flexuosa* L. pulps extracts, are shown in Table 4. Both assays showed a similar trend for the three extracts. The highest results were obtained in extracts from fruit collected in the middle zone. However, the results for the middle and high zones were numerically close. Most of the values presented significant differences at $p < 0.05$. ME surpassed the antioxidant capacity of the FP and BP extracts. This behavior is similar to what was observed in contents of total phenols and flavonoids (Figures 2 and 3). It may be due to the fact that antioxidant capacity is associated with the differences in chemical constituents in each extract (Cândido et al., 2015; Pereira Freire et al., 2016), mainly in terms of phenols, flavonoids and anthocyanins, and that there is a direct correlation between the polyphenol content and the antioxidant capacity of the extracts, which agree with what was reported by Forero-Doria et al. (2016).

As regards DPPH radical scavenging capacity results for the ME, the low zone was similar to the values reported by De Souza Schmidt Goncalves et al. (2010) for *M. flexuosa* L (~15 μmol TE/g dw) and *Scheelea phalerata* (~14 μmol TE/g DW) originating in the Cerrado and Amazon biomes in Brazil, respectively. Our values for the middle and high zones are greater than these but lower than those for *Astrocaryum aculeatum* species (~132 μmol TE/g DW), also belonging to the Arecaceae family.

The FRAP assay show the reducing capacity of antioxidants present in the sample that react with ferric tripyridyltriazine complex and produces a colored ferrous. The FRAP results in the ME of the three Ecuadorian Amazon zones studied are lower than those reported by Cândido et al. (2015) in *M. flexuosa* L. from the Amazon and Cerrado biomes (~27 and ~38 μmol TE/g DW), Brazil. However, these values are higher than other types of fruit consumed in Ecuador, such as: *Cereus megalanthus* K. Schum. ex Vaupel (9.04 μmol TE/g DW), *Cereus ocamponis* Salm-Dyck (known as red pitahaya, 10.53 μmol TE/g DW) and *Borojoa patinoi* Cuatrec (3.66 μmol TE/g DW) (Guevara et al., 2019). In a study on flavonoid interaction and its effect on antioxidant capacity, Hidalgo et al. (2010) concluded that there are synergistic and antagonistic interactions between flavonoids that may in part explain the differences in the antioxidant effect of fruit extracts.

3.4.1. Protective effect against lipid peroxidation for TBARS of M. flexuosa L. Pulp extracts

The protective effect of lipid peroxidation was assessed by TBARS formation of the *M. flexuosa* L. extracts against AAPH-induced oxidative damage on red blood cell membranes. The results for the ME obtained from fruit pulp (*M. flexuosa* L.) from three zones studied in the Ecuadorian Amazon are shown in Figure 4.

The IC_{50} data (Figure 4) shows a slight increase from the low to high zones. However, a significant difference between the values was not
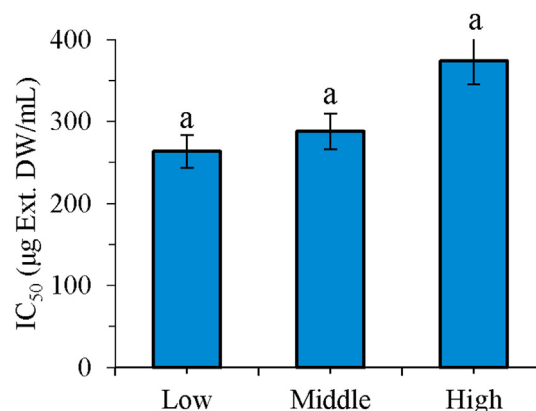
Figure 4. Protective effect against lipid peroxidation (TBARS) in human red blood cell membranes of the *M. flexuosa* L. methanolic extract from three zones of the Ecuadorian Amazon. Means followed by equal letters are not significantly different according to Tukey's test ($p < 0.05$).

found (Tukey's test, $p < 0.05$). The results of the extract from the low and middle zones are lower than those obtained by Nobre et al. (2018) for *M. flexuosa* L (297.1 μg/mL) collected in Crato, Ceará, Brazil. Meanwhile, the TBARS quantified for our fruit in the high zone are higher than those from Brazil. When comparing this result with those reported in other species, it was observed that the IC_{50} of *M. flexuosa* L. is higher than that found in *Olea europaea* (80.59 μg/mL) (Khaliq et al., 2015) and *Solanum guaraniticum* (54.23 μg/mL) (Zaidun et al., 2018).

The overproduction of free radicals has been associated with several pathologic consequences, such as the oxidation of cell structures, causing injury to cells, tissues, and organs. Therefore, the IC_{50} found in *M. flexuosa* L. suggests that it may be useful in counteracting pathologies associated with reactive oxygen species (da Silveira Agostini-Costa, 2018; Leão et al., 2017; Nobre et al., 2018). In assays that use lipids as an oxidizable substrate, the antioxidant's protective role differs according to its solubility, which determines its distribution in the system phase and its incorporation into the cellular membranes (Alvarez-Suarez et al., 2012). Additionally, the complex composition of plant extracts can cause synergistic or antagonistic interactions between the compounds, as previously mentioned, which can also modify their protective effect against peroxidation (Santos et al., 2015).

The linear relationship of bioactive compounds (phenols, flavonoids, vitamin C and β-carotene), the antioxidant capacity and the protective effect against lipid peroxidation was determined using an analysis of the Pearson's correlation (InfoStat). Table 5 shows the results obtained from the Pearson's correlation analysis.

A very good positive correlation (>0.80) was observed between the TPC *vs* TFC (0.974), β-carotene (0.862), FRAP (0.954) and DPPH assay (0.954), which is consistent with what was reported by Cândido et al. (2015), Pereira-Freire et al. (2018) and Abreu-Naranjo et al. (2018).

Table 5. Pearson's correlation coefficients of bioactive compounds, antioxidant activity and protective effect against lipid peroxidation.

	TPC	TFC	Vit-C	β -carotene	FRAP	DPPH	TBARS
TPC	1	-	-	-	-	-	-
TFC	0.974**	1	-	-	-	-	-
Vit-C	-0.899**	-0.805**	1	-	-	-	-
β -carotene	0.862*	0.717	-0.998**	1	-	-	-
FRAP	0.954**	0.898**	-0.938**	0.894*	1	-	-
DPPH	0.954**	0.872**	-0.964**	0.951**	0.968**	1	-
TBARS	0.393	0.281	-0.690*	0.781	0.485	0.514	1

*Significant correlations at $p < 0.05$ and **$p < 0.01$, respectively.

According to the methodology used, this result reinforces the fact that polyphenols and carotenoids may be the main components responsible for the antioxidant capacity of *M. flexuosa* L., as suggested by Càndido et al. (2015) in fruit collected in the Cerrado and Amazon biomes in Brazil.

In addition, a high correlation was found between the DPPH *vs* FRAP results (0.968), indicating that the results of antioxidant capacity of these assays may be comparable to each other. This is because the two assays use similar action mechanisms and are possibly linked to hydrophilic compounds (Maria do Socorro et al., 2010). However, the Pearson's correlation coefficient did not show a robust association between TBARS with the total antioxidant capacity, nor with the content of bioactive compounds (TPC, TFC, vit C and *β*-carotene). This behavior is consistent with the results reported by Pereira-Freire et al. (2018).

4. Conclusions

To the best of our knowledge, this paper is the first to report the distribution of free and bound phenolic compounds in *M. flexuosa* L. The results on bound and free phenolics extracts from *M. flexuosa* L. pulp confirm that this fruit has the potential to be used as a source of bioactive compounds and antioxidants for applications to functional food. Furthermore, our results highlight the importance of native fruit as sources of bioactive compounds and suggest that *M. flexuosa* L. represents an important natural source for preventing lipid peroxidation. The middle zone showed higher bioactive compound contents and total antioxidant capacity than the other two zones in the three types of extracts analyzed. In most cases, significant differences ($p < 0.05$) between the results of the extracts (ME, FP and BP) for the three zones were observed. However, the three zones did not present significant differences ($p < 0.05$) regarding the protective effect against lipid peroxidation (TBARS) in human red blood cell membranes of the *M. flexuosa* L. ME. A high correlation between the TPC *vs.* TFC, *β*-carotene, FRAP and DPPH was found, confirming that polyphenols and carotenoids may be the main components responsible for the total antioxidant capacity of *M. flexuosa* L.

Declarations

Author contribution statement

Reinier Abreu-Naranjo: Performed the experiments; Wrote the paper.

Jonathan G. Paredes-Moreta, Genoveva Granda-Albuja, Gabriel Iturralde: Performed the experiments.

Ana M. González-Paramás: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

José M. Alvarez-Suarez: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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