Microwave-assisted extraction of phenolic compounds with antioxidant and anti-proliferative activities from supercritical CO_2 pre-extracted mango peel as valorization strategy

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Abbreviations

EC_{50}: Half maximal effective concentration, EY: Extraction yield, IC_{50}: Half maximal inhibitory concentration, MAE: Microwave-assisted extraction. MP: Mango peel. MWP: Microwave power. SFE: Supercritical fluid extraction. SFE-MP: SFE pre-treated mango peel. UHPLC-Q-TOF-MS/MS: Ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry

Abstract

This work includes the second/sequential stage of a green-based valorization strategy of mango peel. An exhausted biomass from a pilot-scale CO_2 supercritical extraction process was reused for obtaining phenolic-rich extracts with high antioxidant and anti-proliferative activity, employing microwave-assisted extraction. The effects of microwave power (400-800 W), liquid-to-solid ratio (10-50 mL/g) and extraction time (60-120 s) on process yield, phenolic content, and antioxidant capacity were investigated using a Box-Behnken design. A solution consisting of 60% aqueous ethanol was used as extraction solvent. The results showed that microwave power and liquid-to-solid ratio were the most influential factors on the responses variables. The highest total phenolic content (52.08 mg gallic acid eq. /g d.w.) and antioxidant activities (2.75 mmol trolox eq./g extract, and of 6.47 μg/mL expressed in DPPH, EC_{50}) were obtained at 800 W, 50 g/mL, and 90 s. Mango peel extract recovered at optimal conditions provided high anti-proliferative activity against HT-29 colon cancer cells line, after 24 h treatment (IC_{50}=22.98 μg/mL). Gallic acid derivatives, such as galloyl-esters, xanthones like mangiferin, flavonoids, including quercetin and quercetin glycosides were tentatively identified by ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry. Most probably, the compounds responsible for the outstanding anti-proliferative activity.

Keywords: Fruit processing by-products; green extraction processes; HT-29 colon cancer cells; *Mangifera indica* L.; Box-Behnken experimental design.

1. Introduction

The global production of the main tropical fruits attained 93.6 million tons in 2017, from which mango (*Mangifera indica* L.) represented 52% (FAO, 2019). Brazil, Perú and Colombia are the major producers of mango in South America. Colombia produced near to 265.000 tons of mango in 2017, and its per capita consumption increased on average 2.5% between 2014 and 2017, reaching 5.9 kg/person/year (AGRONET, 2019). Manufacturing industries produces mainly pulp, juice/nectars and jam/puree, as a tentative to valorize the agro-food chain of mango (Wall-Medrano et al., 2020). Mango processing generates large amounts of by-products, such as kernel and peel (Jahurul et al., 2015). Mango peel (MP) represents about 15–20% of the fresh fruit, and has been described as potential source of food biomolecules, including dietary fiber, carotenoids, and phenolic compouds (Banerjee et al., 2018; Blancas-Benitez et al., 2015; Jahurul et al., 2015; Masibo & He, 2009; Puligundla et al., 2014; Sánchez-Camargo et al., 2019; Serna-Cock et al., 2016). Several authors have studied the phenolic profile of MP from different varieties, such as Attaulfo, Keitt, Sensacion, Osteen, Haden and Tommy Atkins (Coelho et al., 2019; López-Cobo et al., 2017; Ruales et al., 2018; Souza et al., 2019; Wall-Medrano et al., 2020). The most abundant phenolic compounds reported from MP are gallic acid, chlorogenic acid, epicatechin gallate, epigallocatechin gallate, kaempferol and its related conjugates, quercetin and quercetin derivatives, rutin, mangiferin, and procyanidins (Bai et al., 2018; Coelho et al., 2019; Dorta et al., 2014; Lauricella et al., 2019; López-Cobo et al., 2017; Luo et al., 2014; Velderrain-Rodríguez et al., 2018). Some of these compounds have been recognized by their antioxidant capacity (Bai et al., 2018; Kim et al., 2010; Rojas et al., 2020), and anti-tumoral effects in some cancer cell lines (Bai et al., 2018; Ediriweera et al., 2017; Kim et al., 2010; Noratto et al., 2010; Taing et al., 2015). Gallic acid-rich MP extracts exhibited antiproliferative activity (IC_{50} = 46 µg/mL) against LS180 colon cancer cells, mediated by an antioxidant mechanism (Velderrain-Rodríguez et al., 2018). Likewise, hydro-alcoholic extracts of MP, containing mainly methylgallate, methyl-

digallate ester and gallic acid, affected the cell viability associated to γH2AX-mediated apoptosis and inhibited the colony formation trend of diferent tumor cell lines: HT-29 (90 µg/mL), Caco-2 (30 µg/mL), and HCT116 (30 µg/mL) (Lauricella et al., 2019). On the other hand, maceration (Pal & Jadeja, 2020; Palmeira et al., 2012; Rojas et al., 2015, 2020; Ruiz-Montañez et al., 2014; Souza et al., 2019) and Soxhlet extraction (Castro-Vargas et al., 2019; Ruiz-Montañez et al., 2014; Souza et al., 2019; Tunchaiyaphum et al., 2013) have been extensively applied as conventional methods for obtaning the aforementioned phenolic compounds from MP. However, those processes are associated with high solvent consumtion, high temperatures, and long extraction times. When the conventional processes are not efficient to provide high process yield and selectivity towards the target compounds, a process intensification may improve the performance by combining with non-conventional extraction technologies (Perino & Chemat, 2019). Recently, microwave and ultrasonic irradiations, or even pulse electric fields have been used, before or during the extraction process, to enhance the recovery of phenolic compounds from several food by-products, as intensification strategy (Al Khawli et al., 2019; Chemat et al., 2017; Grillo et al., 2019; Perino & Chemat, 2019). In the case of MP, ultrasound assisted extraction (UAE) (Ruiz-Montañez et al., 2014; Safdar et al., 2017a, 2017b; Souza et al., 2019), high hydrostatic pressure extraction (HHPE) (Ruiz-Montañez et al., 2014), microwave assisted extraction (MAE) (Dorta et al., 2013, 2014; Pal & Jadeja, 2020; Rojas et al., 2020; Ruiz-Montañez et al., 2014), and subcritical water extraction (Souza et al., 2019) have been succesfuly applied. As pointed by Chemat et al., (2020), these extraction technologies are considered as sustainable techniques, since they can complete processes in shorter times with high reproducibilty and simplified manipulation, resulting in a higher quality of the final products. In addition, regards the enviromental impacts, those processes require only a fraction of the energy demanded by the conventional extraction methods. Concerning the use of solvents (an important aspect of green chemistry principles), ethyl acetate, ethanol, water, and most frequently, mixtures of the last two have

been succesfully used to obtain phenolic compounds. However, other type of compounds in MP such as lipids, carotenoids, and pectin are co-extracted, reducing the selectivity of the extraction process. For this reason, the need to develop a sequential green extraction for obtaining different fractions enriched in bioactive compounds has gained attention in the last years (Gallego, Bueno, et al., 2019; Perino & Chemat, 2019). Diverse strategies can be developed for attaining different bioproducts from the same initial agroindustrial biomass, using a sequential approach of green technologies (Cherubini, 2010; Perino & Chemat, 2019). A biorefinery process for obtaining polyphenols and pectin from MP using conventional extraction techniques, was recently described (Arora et al., 2018). However, the extraction of phenolic compounds from MP employing sequential emerging extraction technologies have been scarcely explored. Recently, the use of UAE for a sequential extraction of phenolics and pectin from MP was evaluated by Guandalini et al., (2019). Initially, phenolics were extracted from MP using ultrasound and subsequently, pectin was extracted by UAE from the residue obtained in the first extraction process. We have proposed a similar approach, which includs a first valorization step, consisting in the production of carotenoids-enriched extracts from dried MP var. Sugar, using supercritical fluid extraction (Sánchez-Camargo et al., 2019). The optimal extract obtained was used as food additive and it efficiently protected sunflower oil against lipid oxidation. In this work, we present the second stage of that valorization strategy, in which MAE has been applied to the exhausted biomass resulting from the first SFE stage, for obtaining phenolic compounds with antioxidant and anti-proliferative activities, that still remains in such biomass. The influence of microwave power (MWP), liquid-to-solid (L/S) ratio and extraction time on the processing yield, total phenolic content and extract quality was investigated by means of response surface methodology (RSM). The anti-proliferative activity against HT-29 colon cancer cells and the phytochemical profile of the extract obtained under optimal conditions were determined.

2. Materials and methods

145 *2.1. Sample preparation*

146 MP (var. Sugar) was supplied by a local fruit processing industry (Fast Fruit Ltda., Bogotá,
147 Colombia). The sample was air-dried (50 °C for 24 h), milled and chemically characterized (proximal
148 composition) as described recently (Sánchez-Camargo et al., 2019). The previously lab optimized SFE
149 conditions (25.0 MPa, 60 °C and ethanol 15% w/w) (Sánchez-Camargo et al., 2019) were used for
150 obtaining a representative sample of SFE pre-treated MP, hereinafter called SFE-MP. For this
151 purpose, a pilot scale SFE instrument (Thar Technologies, model SF2000, Pittsburg, PA) equipped
152 with a 0.5 L extraction cell and two 0.5 L separators with independent pressure and temperature
153 controls was employed. The SFE scaling-up procedure was followed by keeping constant the
154 geometric factors L/D (L = Height; D = diameter), at small and large scale, as described by Fernández-
155 Ponce et al. (2016). In this way, the $Q \times D/F$ ratio, where Q is the mass flow of CO_2 and F is the mass
156 of MP charged into the extraction cell, were used as scaling criteria. Four assays were carried out to
157 obtain approximately 900 g of SFE-MP extracted sample, for the subsequent MAE process.Table S1
158 (Supplementry material) shows the comparison of the conditions used in both pilot and laboratory
159 stages and the selected scaling criteria.

160 *2.2. Reagents*

161 Carbon dioxide 99.5% (w/w) (Carburos métalicos, Barcelona, Spain), ethanol absolute (Merck,
162 Colombia) and distilled water were employed as solvents. 2,2-Diphenyl-1-picrylhydrazylhydrate
163 (DPPH, 99%), gallic acid (> 98%), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox,
164 ≥ 97%), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS, ≥ 99%) were purchased from
165 Sigma-Aldrich. The Folin-Ciocalteu phenol reagent (2N) was provided by Merck (Darmstadt,
166 Germany). For the UHPLC-Q-TOF-MS/MS analyses, Acetronitrile (ACN) and water MS grade from
167 LabScan (Dublin, Ireland) were employed. For the inhibition of cell proliferation experiments, the dry

168 extracts were dissolved in DMSO (Sigma-Aldrich) at the appropriate concentrations and stored as
169 aliquots at -80°C until future use.

170 *2.3. Conventional extraction of soluble phenols in SFE-MP.*

171 As a benchmark method, a conventional organic-aqueous extraction was performed in tripicate, to
172 determine the total soluble phenols in SFE-MP, as described by Blancas-Benitez et al. (2015). Briefly,
173 0.5 g of sample was mixed with 20 mL of the acidified methanol solution (50:50 v/v, 0.8% HCl 2N)
174 and stirred for 1 h at room temperature. After centrifuging the mixture (3500 rpm, 10 min, 25 °C), the
175 supernatant was separated and the residual biomass was submitted to another extraction with 20 mL
176 of the aqueous acetone solution (80:20 v/v), repeating the centrifugation and combining the
177 supernatants with those obtained previously. The whole extract was stored in the dark at -10°C, until
178 analysis. This methodology was considered as that allowing 100% recovery of soluble phenolic
179 compounds from SFE-MP, and it was used to assess the extraction efficiency of MAE.

180 *2.4. Microwave-assisted extraction optimization*

181 A Box-Behnken experimental desing (BBD) was proposed to optimize the MAE of phenolic
182 compunds from SFE-MP, as a second stage of green emerging process-based stategy. The BBD
183 consisted of 17 randomized runs with five replicates at centralpoint. The codified and real levels for
184 the experimental factors and the response variables are shown in **Table 1**. The effects of MWP (400,
185 600 and 800 W), L/S ratio (10, 30 and 50 mL per gram of SFE-MP), and extraction time (60, 90 and
186 120 s) were investigated on *i*) the extraction yield (EY), *ii*) total phenolic content (TPC), *iii*) phenolic
187 concentration in the extract (PCE), *iv*) Trolox equivalent antioxidant capacity (TEAC), *v*) EC_{50} (by
188 radical scavenning DPPH method), and *vi*) individual phenolic compounds content (galic acid,
189 quercetin and mangiferin). The experimental desing was carried out in a microwave extraction system
190 (Ethos X, Milestone, Monroe, CT, USA) operating an a frequency of 2.54 GHz, equipped with a 360°
191 rotating carousel that had the capacity to hold up to 12 microwave digestion Teflon vessels. The unit

192 was provided with a temperature sensor, which combines infrared and fiber optics technologies, a
193 power control, and a magnetic stirring. The extractions were developed in the closed teflon vessels
194 (50 mL), using a 60% v/v hydroetanolic solution as solvent, according to previous studies (Coelho et
195 al., 2019; Palmeira et al., 2012; Safdar et al., 2017b). The obtained extracts were centrifuged, filtered
196 and completed to 50 mL with the extraction solvent and kept refrigerated (-10 °C).

197 *2.4.1 Statistical analysis*

198 The data analysis was accomplished by the RSM using the software STATISTICA 12 (Stat Soft,
199 Inc., Tulsa, OK 74104, USA). The effects of the independent variables on the response variables in
200 MAE process were calculated using the pure error, considering a 95% confidence interval. The
201 suitability of the 2nd order polynomial models was evaluated by the correlation coefficient (R^2) and
202 the F-test from the analysis of variance (ANOVA, including the test of lack-of-fit). For the graphical
203 analyses, pareto charts for the standardized effects of independent variables on the response factors
204 were also ploted. A multiple response optimization was carried out by combining the experimental
205 factors, seeking maximizing the desirability function. Pearson's correlation coefficients were also
206 calculated to corroborate relationships between the individual phenolic compounds and antioxidant
207 activity values.

208 *2.5 Determination of extraction yield (EY)*

209 The EY was determined gravimetrically, after solvent removal by oven drying from a known
210 aliquot of each obtained extract. The yield assays were conducted in triplicate and the results
211 expressed in terms of grams of extract per 100 grams of SFE-MP.

212 *2.6 Estimation of total phenolic content (TPC)*

213 The quantification of TPC in both SFE-MP (extract obtained as described in section 2.3) and MAE
214 extracts were assessed according to the Folin-Ciocalteu method with some modifications (Singleton
215 et al., 1999). In brief, 10 µL of each extracts and 600 µL of water were mixed to 50 µL of undiluted

Folin–Ciocalteu reagent (2N). After 1 min, 150 µL of 20% (w/v) sodium carbonate and 190 µL of water were added. After 2 h of dark incubation at 25 °C, 300 µL of the mixture was transferred into a well of a 96-well microplate, and the absorbance was measured at 760 nm (Multiskan Sky Microplate spectrophotometer, Thermo Scientific®, USA). A gallic acid calibration curve (0.032–2.00 mg/mL) was performed following the same procedure. All experiments were performed in triplicate. The results were expressed as mg of gallic acid equivalents (GAE) per gram of sample (TPC), and mg of gallic acid equivalents per gram of extract (PCE).

2.7 Capacity antioxidant in vitro assays

2.7.1 Trolox equivalent antioxidant capacity (TEAC) analysis

The antioxidant capacity of the different MAE extracts was determined by the TEAC assay following the ABTS radical method as described elsewhere (Sánchez-Camargo et al., 2016) and based on the procedure of Re et al. (1999). Brielfly, $ABTS^{\bullet+}$ radical was produced by reacting 2.5 mL of 7 mM ABTS and 44 µL of 2.45 mM potassium persulfate solutions in the dark at room temperature during 16 h before its use. The aqueous $ABTS^{\bullet+}$ solution was diluted with 5 mM phosphate buffer (pH 7.4) until achieve an absorbance of 0.7 (±0.02) at 734 nm. Then, ten microliters of sample (5 different concentrations) were mixed with one mL of $ABTS^{\bullet+}$ solution in a 2-mL vial and 300 µL of the mixture were transferred into a 96-well microplate. After that, the absorbance was measured at 734 nm every 5 min during 45 min in a microplate spectrophotometer reader (Multiskan Sky Microplate spectrophotometer, Thermo Scientific®, USA). As reference, trolox standard was used and the results were expressed as TEAC values (mmol of Trolox equivalents (TE)/g extract). These values were obtained from five different concentrations of each tested extract in the assay (between 0.0625-1 mg/mL), giving a linear response between 20 and 80% blank absorbance. All analyses were performed in triplicate.

2.7.2 Half maximal effective concentration (EC_{50}) by DPPH radical scavenging assay

The concentrations of the extracts (expressed in μg/mL) responsible for a 50% decrease in the initial activity of the DPPH radical (EC_{50}) was determined following the procedure developed by Brand-Williams et al. (1995), and in detail specified by Sánchez-Camargo et al., (2016).The lower the EC_{50} value, the higher the antioxidant capacity. Experiments were done in triplicate.

2.8 Analysis of phenolic compounds by liquid chromatography-quadrupole time-of-flight mass spectrometry (LC-Q/TOF-MS/MS)

Liquid chromatography coupled to a high-resolution mass spectrometer was employed to analyze and quantify phenolic compounds in MAE extracts. These analyses were performed using an ultrahigh performance liquid chromatography (UHPLC) system 1290 from Agilent (Agilent Technologies, Santa Clara, CA, USA) coupled to a quadrupole-time-of-flight mass spectrometer (Q/TOF MS) Agilent 6540 that was equipped with an orthogonal ESI source (Agilent Jet Stream, AJS, Santa Clara, CA, USA), and controlled by a PC running the Mass Hunter Workstation software 4.0 (MH) from Agilent. Chromatographic separation of the extracts was achieved using a Zorbax Eclipse Plus column (100 mm × 2.1 mm, d.p. 1.8 μm) (Agilent Technologies, Santa Clara, CA) with a mobile phase composition of water (+0.01% formic acid, A) and acetonitrile (+0.01% formic acid, B). The gradient program was as follows: 0 min, 0% B; 7 min, 30% B; 9 min, 100% B; 13 min, 100% B; 14 min, 100% A. The flow rate was 0.5 mL/min with an injection volume of 5 μL.The analyses were performed in negative ion mode. The extracts were injected to a concentration of 50 μg/mL. The mass spectrometer was used in MS and MS/MS modes for the structural analysis of all compounds. MS parameters were the following: Capillary voltage, 4000 V; nebulizer pressure, 30 psi; drying gas flow rate, 10 L/min; gas temperature, 350°C; skimmer voltage, 45 V; and fragmentor voltage, 110 V. The QTOF-MS was set to acquire m/z ranging between 50 and 1300 (MS) amu and 50 and 1000 (MS/MS) amu at a scan rate of 5 spectra per s. For post-acquisition data processing, Agilent Mass Hunter Qualitative analysis software (B.07.00) was used. The accurate mass data, isotopic patterns, ion source fragmentation,

MS/MS fragmentation patterns, MS databases (i.e., MassBank, HMDB, Metlin, among others) and bibliographic search were used for tentative identification of phenolic compound present in the optimal MAE extract. Quantitative data for acid galic, mangiferin and quercetin were obtained by calibration curve constructed (with $R^2 > 0.99$) with the standard compounds in the range of 0.1–100 μg/mL. All analyses were carried out in triplicate.

2.9 Cell culture

HT-29 (human colon adenocarcinoma) cells were purchased from the American Type Culture Collection. Cell lines were cultured in RPMI 1640 medium, supplemented with Hepes 25 mM, L-glutamine 2.05 mM, 10% fetal bovine serum and 50 μg/mL gentamicin, and incubated at 37°C under 5% CO_2 in a humidified atmosphere. When the cell achieved 80-90% confluent, it was detached by trypsin-EDTA and sub-cultured into new sterile culture flasks for further propagation.

2.10 Antiproliferative activity assay

The antiproliferative activity of optimal MAE extract was evaluated by MTT assay. HT-29 cells in exponential growth phase (80-90% confluence) were trypsinized, counted and seeded in 96-well plates at a density of 1.0×10^4. The wells with seeded cells were incubated for 24 hours at 37°C to allow the cell adhesion. Cells were treated with the vehicle (DMSO 0.1% v/v) regarded as untreated controls or with different concentrations of extracts (6.25-100 μg/mL) and incubated at three different time points 24, 48, and 72 hours. After the incubation, the medium was removed and 100 μL of MTT solution (0.25 mg/mL RPMI 1640 medium) was added to each well and the plate was incubated for 4 h. After, medium was discarded and cells were washed with 200 μL of phosphate buffer saline (PBS). 100 μL of DMSO were added to each well to dissolve the formazan crystals. The absorbance was measured at 570 nm using a microplate reader (Tecan, Infinite® 200 PRO). Triton X-100 (1.0%) was used as a positive control. The cell viability was expressed as percentage of live cells relative to controls. The IC_{50} values (concentration of extract that causes 50% inhibition or cell death) were

288 determined based on the dose-dependent response curves of extract using SigmaPlot v12.5 software
289 (Systat Software Inc., Erkrath, Germany). Each experiment was performed as three independent test
290 with minimum three replicated.

291 3. Results and discussion

292 *3.1 Optimization of microwave assistant extraction variables*

293 Valorization strategies based on green technologies have been recently proposed for the sustainable
294 recovery of valuable compounds from natural sources (Herrero & Ibañez, 2018; Perino & Chemat,
295 2019). Some approaches have been based on green downstream process using solvents with an
296 increasing polarity, for obtaining different fractions with added-value (Gallego, Martínez, et al., 2019;
297 Gilbert-López et al., 2017). Thus, the integration of extraction processes is gaining more relevance.
298 The sequential extraction mechanism proposed herein is based on increasing the solvent polarity,
299 where high value compouds with low polarity can be selectively extracted, followed by the extraction
300 of most polar compounds. This fractionation strategy allows to enhance the funcionalities of the
301 obtained extracts, by the enrichment of bioactive compounds in such fractions. Besides, it not only
302 takes in advantage of the most valuable compouds of a natural matrix, but contributes to elucidate the
303 relationship between chemical composition and functional activities, since more purified extracts are
304 obtained. This approach can be applied not only for the valorization of MP samples, but other natural
305 matrices composed by families of compounds with different polarities.

306 The values for the response variables studied by MAE from the SFE-MP sample are shown in
307 **Table 1**. A comprenhensive ANOVA is provided on **Table S2** (Supplementary Material). The F-test
308 for the lack-of-fit showed that only the resulted models for *i*) TPC, *ii*) TEAC and *iii*) EC_{50} were
309 adequate (p-value > 0.05) to describe the observed data at the 95.0% confidence level. The coefficients
310 of determination (R^2) of such models were close to 1.0 (0.93-0.97), indicating their high ability to
311 explain and to predict the obtained outcomes. Therefore, the response surfaces (**Figure 2**) were

prepared only for those variables. Despite that, the results obtained from the response variables, extraction yield, PCE, gallic acid, mangiferin, and quercetin contents, are essential to infer about the conditions that affect the MAE process. For this reason, Pareto charts were generated and presented in **Figure 1** to support the analysis of those response variables.

3.1.1 Effect of extraction factors on extraction yield

High extraction yields (varying from 33.58 to 48.87%) were obtained under the conditions studied. As shown in **Figure 1A**, EY was mainly influenced by the linear possitive effect of L/S ratio. When this factor increased from 10 to 50 mL/g (compare assays 3 and 6, 9 and 13, and 12 and 15 in **Table 1**), the EY increased remarkably. This result indicates that enough solvent is needed to guarantee a chemical potential gradient that promotes the mass transfer for the matrix exhaustion. In a solid-liquid system, microwave heating generates temperature gradients between the matrix cells and the solvent phase. Thus, as the amount of solvent increases, more solid material is wetted and swelled, which causes and increase in its surface area and facilitates the migration of the solvent into the cells. Consequently, the internal cell pressure increases, which could lead to the breaking of the cellular structure, enhancing the mass transfer towards the solvent phase (Taqi et al., 2020).

The MWP showed a linear negative effect on the EY. Usually, high MWP increases the system temperature, which improves the solvent power due to the decreasing of its viscosity and surface tension, facilitating the solubilization of compouds and reducing the extraction time (Veggi et al., 2013). Nevertheless, according to **Table 1**, values above 600 W showed a decline in EY. At 800 W, the slurry in the extraction vessel achieved temperatures in the range of 90 to 135°C (depeding on the extraction time), which might degradate thermolabile compounds, reducing the EY (Veggi et al., 2013).

Regarding the extraction time, the interaction and quadratic terms had a contribuion effect on this response variable. However, the linear effect of this factor showed a negative and unsignificant effect

on the EY, which is favorable for the process (**Figure 1A**). This avoids long-time heating and a posible degradation of the phenolic compounds. Thus, the highest EY (48.87±0.24%) was found at the conditions of 600 W, 50 mL/g, and 120 s; however, assays employing 90 s provided similar results. In general, the EY values obtained via MAE were quite close to those attained with the conventional extraction (45.12±2.25%) (**Table 1**), which demostrates the high efectivity of MAE, in saving solvent and energy consumption.

3.1.2 Effect of extraction factors on total and individual phenolic content

Figure 2A represents the Pareto chart and response surface obtained for the TPC. Similar to EY, this variable was principally affected by the linear positive effect of the L/S ratio. Furthermore, the linear variation of the extraction time did not have influence on TPC either; however, the interactions with the other two factors (power and extraction time) were highly significant. The highest values of TPC were found at the highest L/S ratio (50 mL/g) and MWP above 600 W. The response surface and the coefficients of regression equation for TPC are presented in the **Figure 2A** and **Table S2**, respectively. On the other hand, the benchmark method (section 2.3) was able to extract 54.96±2.03 mg GAE/g d.w. (**Table 1**). Therefore, 92.34 and 91.41% of the phenolic compounds present in SFE-MP sample were recovered by MAE employing 600 W, 50 mL/g, and 120 s and 800 W, 50 mL/g and 90 s, respectively, which also indicates that high MWP requires less extraction time for similar recoveries. Comparable results were recently obtained by Pal & Jadeja (2020), who optimized a microwave-assisted deep eutectic solvent extraction (MADESE) of phenolic antioxidants from MP. In that work, L/S ratio also was the main influence factor on TPC. The highest TPC (55.28 mg GAE/g d.w.) was achieved at a L/S ratio of 59.82 mL/g, MWP of 436.45 W, and extraction time of 19.66 min, using an aqueous solution of lactic acid-based DES as solvent. Our results are up to 9.8 times faster, and even more when compared to other conventional extraction of phenolic compounds from MP, such as maceration using water (60 °C, 30 min, 5 mL/g; 25.01 mg GAE/g d.w) (Rojas et al.,

2015, 2020), subcritical water extraction (180 °C, 90 min, 40 mL/g; 50.25 mg GAE/g d.w.) and Soxhlet using ethanol (78.3 °C, 240 min, 25.13 mg GAE/g d.w.) (Tunchaiyaphum et al., 2013). In addition, it is worth mentioning that the TPC values obtained from our SFE-MP sample are quite higher in comparison to other by-products such as apple peel (9.95 mg GAE/g d.w.) (Kschonsek et al., 2018), avocado peel (12.52 mg GAE/g d.w.), pineapple peel (3.74 mg GAE/g d.w.), and papaya peel (3.15 mg GAE/g d.w.) (Morais et al., 2015), among others.

Regarding the phenolic concentration in the extract (PCE), the linear effect of MWP had the most significant influence, followed by L/S ratio (**Figure 1B**). As discussed before, this behavior is related to the mechanisms of the microwave heating and their effects on the solid matrix cells and solid-solvent contact, which increases the recovery of phenolic compounds, reaching interesting values of about 11.3% of the extract (Assay 15, **Table 1**). Similar to the two last dependent variables studied, irradiation time did not have a significant effect on the PCE. Analyzing these results, if one wishes to obtain extracts enriched in phenols, the MWP should be prioritized, while, if one wants to extract as many phenols as possible per gram of sample, the L/S ratio should prevail. On the other hand, the effect of the studied factors on the content of some prominent phenolic compounds (gallic acid, mangiferin, and quercetin) in the mango peel extracts was also evaluated. Likewise, the quantification of these compounds was carried out to investigate if they could be responsible for or be associated to the antioxidant activity studied in the extracts, as is discussed in the next section. Among the compounds evaluated, gallic acid was the most abundant (2.72-5.65 mg/g), followed by mangiferin (0.33-1.25 mg/g) and quercetin (0.31-0.57 mg/g) (**Table 1**). These response variables showed a different trend about the effects, in both linear and quadratic, as well as interaction effects. In the case of gallic acid (**Figure 1C**) and mangiferin (**Figure 1D**), the three assessed factors significantly influenced their concentration in the extracts. The linear contribution of MWP had the most relevant negative effect, since high increments in the extraction temperature, may cause degradation on these

384 compounds. On the contrary, an increase in the L/S ratio offered a relative positive effect, having a
385 maximum at 30 mL/g, for the recovery of those compounds. Meanwhile, although small, extraction
386 time presented also a significant positive effect. Pal & Jadeja (2020) reported similar values of
387 mangiferin (0.93 mg/g) in extracts from MP obtained by MADESE. Other study employing MAE for
388 isolating mangiferin from MP Ataulfo varieties was developed by Ruiz-Montañez et al., (2014). The
389 authors used ethanol–water (80:20 v/v) solution as extraction solvent, at a ratio of 1:10 (g sample: mL
390 solvent), and operating at 600 W for 1 min in 30 s irradiation cycles. Under those conditions, contents
391 of mangiferin around of 4 mg/g sample were found, being the lowest value achieved when compared
392 to other extraction methods assessed such as UAE (~13 mg/g sample), HHPE (~11 mg/g sample),
393 maceration (~5 mg/g sample) and Soxhlet (~ 9.5 mg/g sample). Nevertheless, in a preparative scale
394 using UAE (the best extraction method), a maximum value of 1.89 mg/g extract was achieved.

395 On the other hand, quercetin (**Figure 1E**) showed a dissimilar behavior in regard to gallic acid and
396 mangiferin. The most significant effect in the extraction of quercetin was the interaction between
397 MWP and L/S ratio (A×B, in Table S2) followed by the linear effect of L/S ratio. An increase in the
398 MWP improved the concentration of this compound in the extract, which could demonstrate its
399 thermal stability.

400 *3.1.3 Effect of extraction factors on in vitro antioxidant capacity*

401 The antioxidant capacity measured by trolox equivalent (TEAC) on MAE extracts was also greatly
402 influenced by MWP, as presented in **Figure 2B**. At low powers, the extraction time did not have any
403 impact and poor antioxidant capacities were achieved. However, as power and extraction time
404 simultaneously increased, ABTS radical scavenging capacities from the extract samples reached
405 remarkable values. Although the linear contribution of the L/S ratio did not have any influence on
406 TEAC, its quadratic and linear interaction with MWP had great positive effects. As also noted
407 previously by Dorta et al. (2013), high values of L/S ratio (50 g/mL) have a positive impact on the

antiradical activity, determined by TEAC and DPPH radical scavenging methods. In this sense, long extraction times, high MWP and L/S ratios seem to result in high antioxidant capacities. According to the Box-Behnken experimental design explored, the highest TEAC value found was 2.75 mmol TE/g (or 0.69 g TE/g) using 800 W, 50 mL/g, and 90 s as operating conditions. Interestingly, analogous values (2.64 ± 0.08 mmol TE/g) were found by the benchmark method.

As the EC_{50} is concerned, MWP presented a significantly negative effect **(Figure 2C)**, where its increment produced high antioxidant capacities. It is worth clarifying that low EC_{50} values provide higher antioxidant capacity, since lower extract concentration is necessary to reduce the DPPH radical concentration by 50%. Extraction time factor also presented a significantly negative but minor effect, while L/S ratio did not present any influence. However, quadratic interactions of those last factors were highly significant, thus decreasing the antioxidant capacity. In this context, MWP above 600 W and irradiation times greater than 90 s, reached outstanding EC_{50} values between to 6.1-7.1 μg/mL.

As a way to correlate the presence of gallic acid, mangiferin and quercetin as the possible responsible compounds for the high antioxidant capacity of the extracts, their concentrations were plotted against TEAC and DPPH radical scavenging values. As shown in **Figure 2S (A-F),** the coefficients of regression R^2 were between 0.014-0.26, and the Pearson´s correlation coefficient's (r) were not direct (away from 1 or -1) as summarized in **Table 3**. These results suggest that the antioxidant capacity could not be attributed to a single component, but to the synergistic effect of all the compounds present. This behavior is in agreement with the results obtained by Berardini et al. (2005), who established that the antioxidant capacity of MP extracts was higher than that of standard mangiferin and quercetin-3-O-β-glucoside.

3.1.4 Selection of the optimal conditions for MAE process

According to the previous analyses, a multiple-response optimization was carried out. Then, a desirability function, combining TPC, TEAC and DPPH responses, was calculated. Optimal

conditions found were 800 W, 47 mL/g, and 98 s at 0.97 desirability value. The predicted responses values were 51.4 mg GAE/g d.w., 2.75 mmol TE/g, and 6.14 μg/mL, which are quite similar to the experimental outcomes obtained from assay number 15 of the experimental design (**Table 1**). The desirability value, very close to 1.0, indicates high maximization degree for multi-response optimization. Despite the optimum conditions were at the experimental region limit, the proximity between predictive and experimental data confirmed that the selected RSM model may be applied for MAE extracts with maximum TPC and antioxidant activity.

3.2 Anti-proliferative assays of the optimal MAE extract

Several studies focusing on the anti-proliferative activity of mango by-products extracts using cancer colon cells have been carried out (Ballesteros-Vivas et al., 2019; Castro-Vargas et al., 2019; Lauricella et al., 2019; Noratto et al., 2010; Velderrain-Rodríguez et al., 2018). HT-29 were selected as model, since it is considered as one of the most refractive colon cancer line (Ballesteros-Vivas et al., 2019). With this in mind, the anti-proliferative activity of MAE extract obtained under optimal conditions (assay number 15) was tested against HT-29. Cells were incubated with different concentrations of such extract (from 6.25 to 100 μg/mL) for 24, 48 and 72h. Cell proliferation was evaluated by the MTT assay, and the results are shown in **Figure 3**. As observed, the extract exerts a dose-dependent manner reduction on the cell proliferation after each treatment. It is worth noting that even at low concentrations of the extract (6.25 and 12.5 μg/mL), the cell viability decreased near to 45-50%. Similar trends about decreasing of cell proliferation were found at 24h (IC_{50}=22.98 μg/mL) and 48h (IC_{50}=38.37 μg/mL), however at 72h (IC_{50}=56.23 μg/mL), the highest concentration tested (100 μg/mL) caused a drastic drop in the cell viability. In order to determine the mechanisms that may explain the inhibitory activity of the optimal MAE extract on HT-29 cells, the percentage of growth (PG) of the extract was determined, and showed in **Figure 4**. Values above zero (Y axis) are indicative of cytostatic activity, since they represent the PG relative to the number of control cells since the start

456 of treatment. Conversely, negative PG values indicate cytotoxicity, which result in fewer cells
457 compared to the start of treatment. The results showed that concentrations of 6.25 and 12.5 μg/mL
458 produced PG values between 10 and 20, indicating a cytostatic effect. Regarding the concentration of
459 25 μg/mL, the extract exerts an intermediate cytostatic effect on cell proliferation. Finally, a clear
460 cytotoxic effect was evident when the cells were exposed to the highest extract concentration (100
461 μg/mL) for the longest treatment (72h). In a recent study, the antiproliferative activity of methanolic
462 extracts from sugar mango by-products (MP, seed coat and seed kernel) obtained using Soxhlet was
463 evaluated against a panel of human cancer cell lines that included MDA-MB-231 (breast
464 adenocarcinoma), PC-3 (prostate adenocarcinoma), A-549 (lung adenocarcinoma) and HT-29
465 (Castro-Vargas et al., 2019). MP extract did not affect the viability of cells at the evaluated
466 concentrations (1.25, 12.5 and 125 μg/mL). In contrast seed coat and seed kernel extracts showed a
467 decrease of HT-29 cell viability (~75%) at 125 μg/mL. The anti-proliferative potential of seed kernel
468 from sugar mango was further enhanced by Ballesteros-Vivas et al., (2019) after a two steps extraction
469 sequential process using pressurized liquid extraction technique. In the first step the nonpolar
470 compounds were removed while at the second step the polar fraction, enriched in phenolic compouds,
471 was recovery showing an important antiproliferative efect against HT-29 cells (IC_{50}=28.67 μg/mL at
472 72 h). That work and the present study demonstrate the great potential of the integrated processes to
473 obtain fractions with improved bioactivity from mango by-products as a contribution for their
474 valorization.

475 *3.3 UHPLC-Q-TOF-MS/MS profiling analysis*

476 A tentatively characterization of the optimal MAE-MP extract was carried out using UHPLC-Q-
477 TOF-MS/MS, to determine which compounds could be responsible for its outstanding anti-
478 proliferative activity. Based on accurate mass, MS/MS fragmentation patterns, MS databases and
479 previously reported data in literature, 18 compounds were identified (**Figure 5**) and listed in **Table 3**.

480 The optimal MAE extract is a phenolic-rich fraction with low complexity since it contains less than
481 20 compounds. This suggests that the valorization strategy not only allows taking advantage of
482 different natural matrices, but can also lead to a fractionation and purification process that could
483 contribute to a more detailed study of the relationship between chemical composition and biological
484 activity. Quinic acid, gallic acid and some of its glycosylated esters, mangiferin, ethyl gallate,
485 quercetin and some of its esters were the main compounds found. According to the chromatographic
486 profile, quinic acid, ethyl gallate and heptagaloylglucose are apparently the major compounds present
487 in this extract. Some works have previously described these compounds with anti-proliferative activity
488 against different human cancer cell lines. Recently, Bai et al. (2018) tested the antioxidant and anti-
489 proliferative activity of a hydroalcoholic extract (70% ethanol) from MP against A549 cell line of
490 liver cancer, and also tested standards of phenolic compounds that were identified in the extract
491 (vanillin, caffeic acid, oleanolic acid, chlorogenic acid, gallic acid, and procyanidin B2). The results
492 showed that gallic acid provided higher antioxidant activity compared to other phenolics. However,
493 oleanolic acid showed the highest anti-proliferative activity (IC_{50}=4.7 μM), being quite similar to 5-
494 fluorouracil (IC_{50}=3.8 μM), a compound used as a positive control. On the other hand, Velderrain-
495 Rodríguez et al. (2018) evaluated the antioxidant and anti-proliferative activity of various phenolic
496 compounds identified from MP extract (Ataulfo variety) against LS80 colon cancer cells. These
497 authors reported that gallic acid had higher antioxidant capacity than other compounds from the
498 extract, such as mangiferin, quercetin, or syringic acid. This fact may be related to the high anti-
499 proliferative activity (IC_{50}=46 μg/mL) of gallic acid against that cell line. Olivas-Aguirre et al. (2017)
500 reported that glycosylated esters of gallic acid such as penta-O-galloyl-glucoside inhibited the growth
501 of MDA-B-231 breast cancer cells (33 μg/mL), HepG2 liver cancer cells (8 μg/mL) and HL-60
502 leukemia (5 μg/mL), in a similar way as gallic acid (16, 6 and 2 μg/mL, respectively). Based on this
503 evidence, gallic acid has a high anti-proliferative activity and seems to be responsible for the anti-

504 proliferative capacity of the Ataulfo MP polyphenols. According to Lozano et al. (2006), the pro-
505 oxidant action of phenolic compounds, flavonoids, anthocyanins and carotenoids is typically
506 catalyzed within cells by transition metals such as Fe and Cu, under certain conditions of pH and O_2.
507 Eghbaliferiz & Iranshahi (2016) suggested that the antioxidant/pro-oxidant reactions of catechins are
508 responsible for their anti-proliferative effects on HT-29 cell lines, since these molecules are associated
509 with an efficient capacity for electron transfer. Therefore, phenolic compounds with low molecular
510 weight (e.g. gallic acid and quercetin) may exhibit pro-oxidant activity. Otherwise, bounded or
511 polymerized phenolic compounds (e.g. phenols and hydrolysable proanthocyanidins) have little or no
512 pro-oxidant properties. This pro-oxidant activity improves the production of ROS (reactive oxygen
513 species) at cytotoxic levels in this cell line (Eghbaliferiz & Iranshahi, 2016). In this context, it is
514 possible to infer that this type of compounds may be responsible for the anti-proliferative activity of
515 the optimal MAE sample. However, additional studies are necessary to assess whether synergistic or
516 antagonistic effects are taking place between the compounds present in that extract.

517 **4. Conclusions**

518 The results combined support the fact that MAE is suitable as second step of a green processes-
519 based approach for the recovery of extracts with high antioxidant and anti-proliferative activity from
520 MP. Despite that some works dealt with the recovery of phenolic compounds from MP by MAE, none
521 have used a pre-extraction process of this by-product using SFE, to improve the recovery of
522 carotenoids and phenolic compounds in a sequential green extraction process. This sequential process
523 provided more active extracts, which were fast recovered using reduced amounts of solvent in
524 comparison to conventional methods, while providing better selectivity towards the extraction of key
525 compounds. Using an exhausted biomass from SFE, the MAE process was successfully investigated
526 by RSM, where MWP and L/S ratio were the most influencing factors on the TPC and antioxidant
527 capacity. Bioactive extract obtained under optimal MAE conditions (after RSM optimization) showed

satisfactory EY, outstanding TPC and PCE, as well as a remarkable antioxidant capacity. The relevant anti-proliferative activity exhibited against human colon adenocarcinoma cell line HT-29 was supported by the profiling analysis of the phenolic compounds by UHPLC coupled to Q-TOF-MS/MS. Such analysis demonstrated the presence of phenolic acids, xantanoids, as well as a family of gallate derivatives with demonstrated in *vitro* bioactivity. As future trend, the use of green-based approach may be massively employed to produce bioactive extracts from promissory agri-food biomasses, with the aim of generating biorefineries with cleaner and more efficient processes, incorporating sustainability concepts, integration and intensification of processes.

5. Acknowledgements

A.P.S-C. thanks to Ministerio de Ciencia Tecnología e Innovación (Colombia) for her postdoctoral fellowship (784-2017). A.P.S-C and S.R.S.F thank to CAPES-PRINT, Project number 88887.310560/2018-00 for the financial support.

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Figure captions

Figure 1. Standardized Pareto charts for the response variables A) extraction yield, B) phenolic content in extract (PCE), C) gallic acid, D) mangiferin, and E) quercetin contents.

Figure 2. Standardized Pareto charts for the significant response variables studied in the experimental design, and their corresponding response surfaces.

Figure 3. HT-29 colon cancer cell viability upon treatment for 24 h (▲), 48 h (■) and 72h (●) with different concentrations of optimal MAE extract. Error bars are given as 95% confidence interval.

Figure 4. Percentage of growth (PG) of HT-29 colon cancer cells exposed to the different extracts concentrations: 100 µg/mL (--●--), 50 µg/mL (♦), 25 µg/mL (▲), 12.5 µg/mL (—●—),and 6.25 µg/mL (■) for 24, 48 and 72 h. Error bars are given as the mean standard error.

Figure 5. UHPLC-Q-TOF-MS/MS profile of the optimal MAE extract.

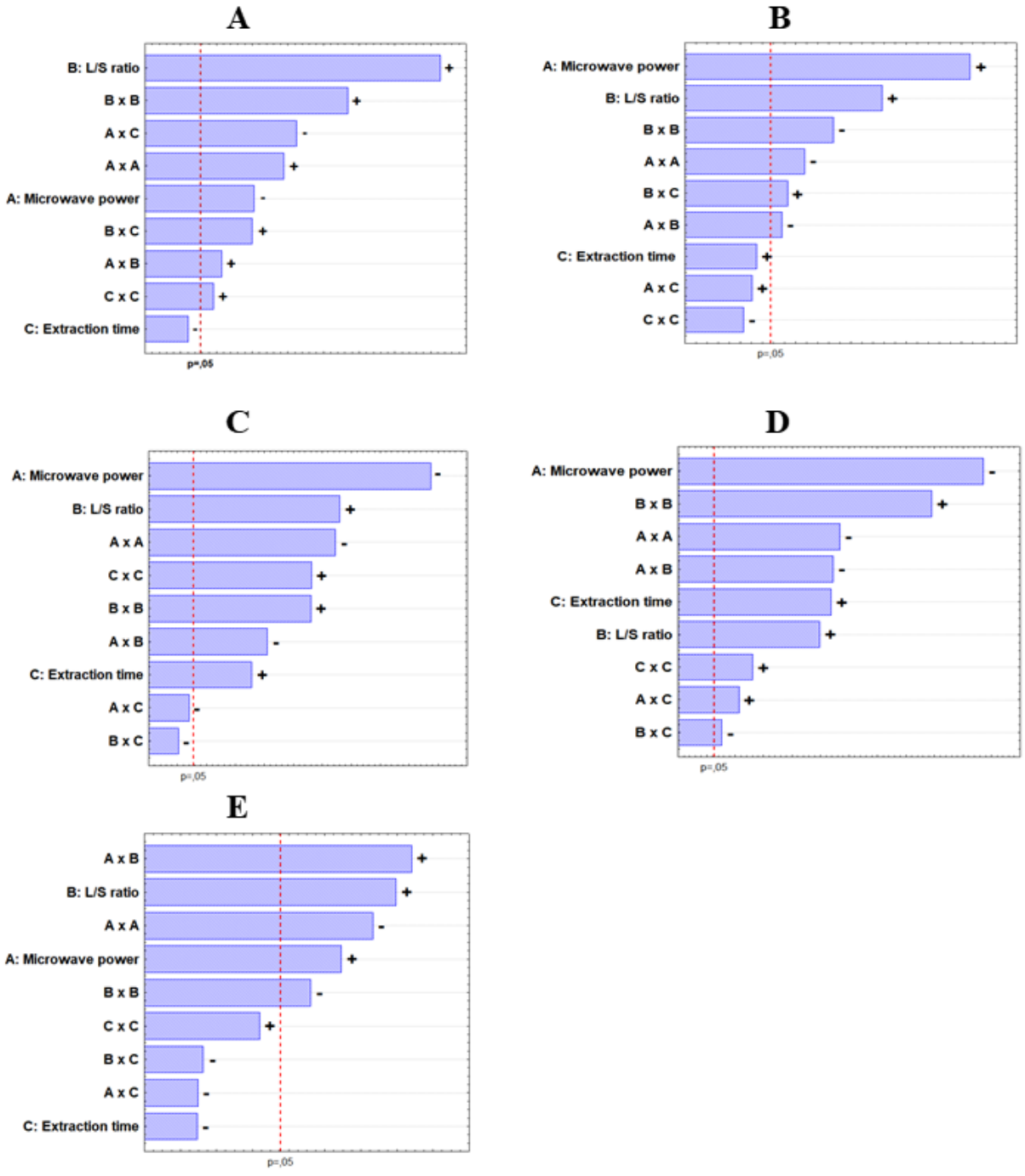
A
B: L/S ratio
B x B
A x C
A x A
A: Microwave power
B x C
A x B
C x C
C: Extraction time
p=,05
B
A: Microwave power
B: L/S ratio
B x B
A x A
B x C
A x B
C: Extraction time
A x C
C x C
p=,05
C
A: Microwave power
B: L/S ratio
A x A
C x C
B x B
A x B
C: Extraction time
A x C
B x C
p=,05
D
A: Microwave power
B x B
A x A
A x B
C: Extraction time
B: L/S ratio
C x C
A x C
B x C
p=,05
E
A x B
B: L/S ratio
A x A
A: Microwave power
B x B
C x C
B x C
A x C
C: Extraction time
p=,05

Figure 1.

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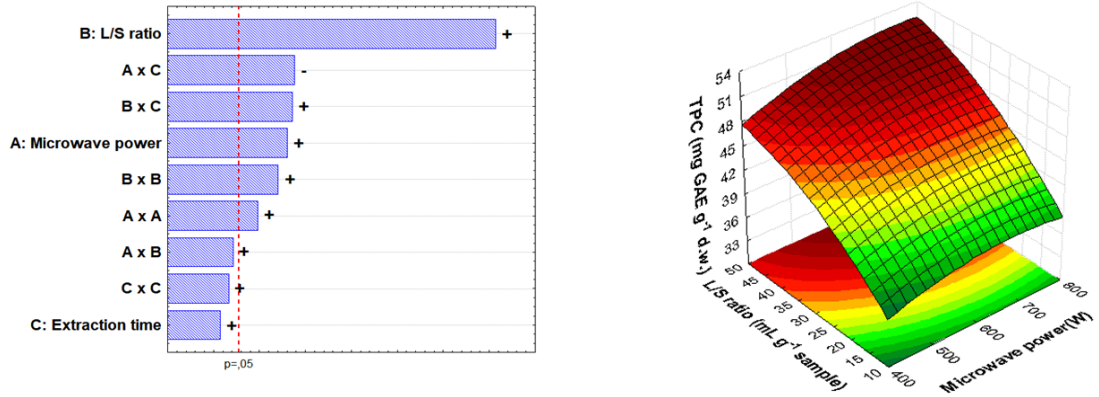
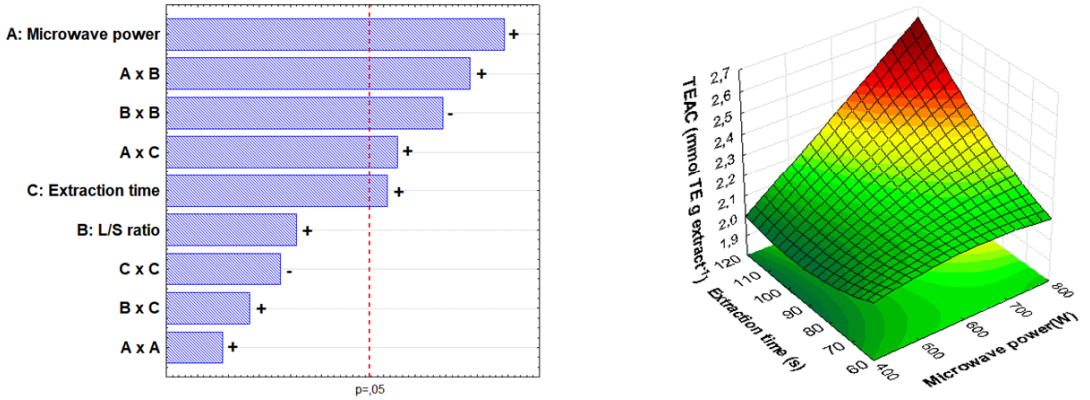
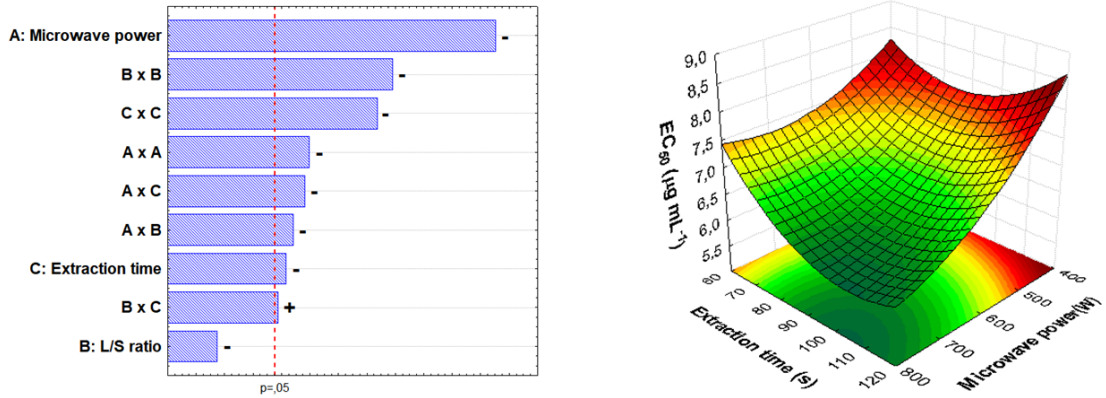
A. Total Phenolic content (mg GAE g^{-1} d.w.)



B. TEAC (mmol TE g^{-1} extract)



C. EC_{50} (µg^{-1} mL)



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785 **Figure 2.**

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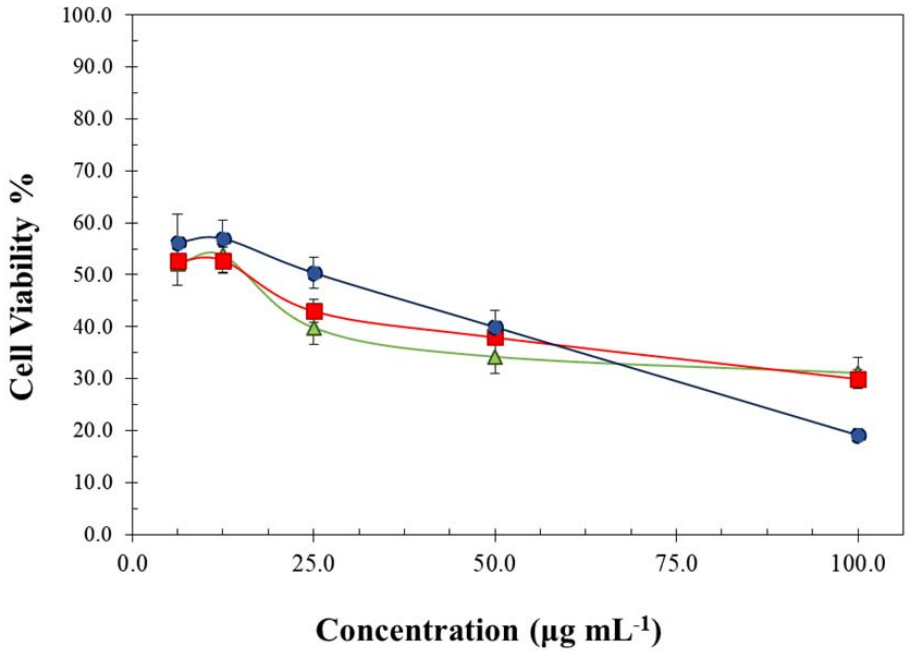
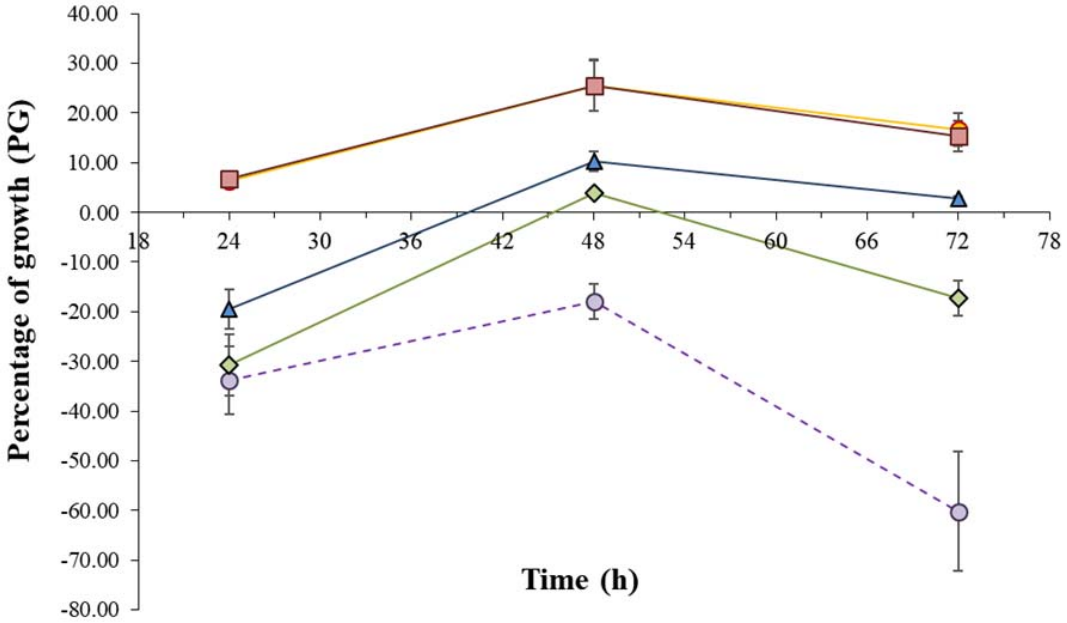

Cell Viability %
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0.0
25.0
50.0
75.0
100.0
Concentration (µg mL-1)

Figure 3.


Percentage of growth (PG)
40.00
30.00
20.00
10.00
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-10.00
-20.00
-30.00
-40.00
-50.00
-60.00
-70.00
-80.00
18
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30
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42
48
54
60
66
72
78
Time (h)

Figure 4.

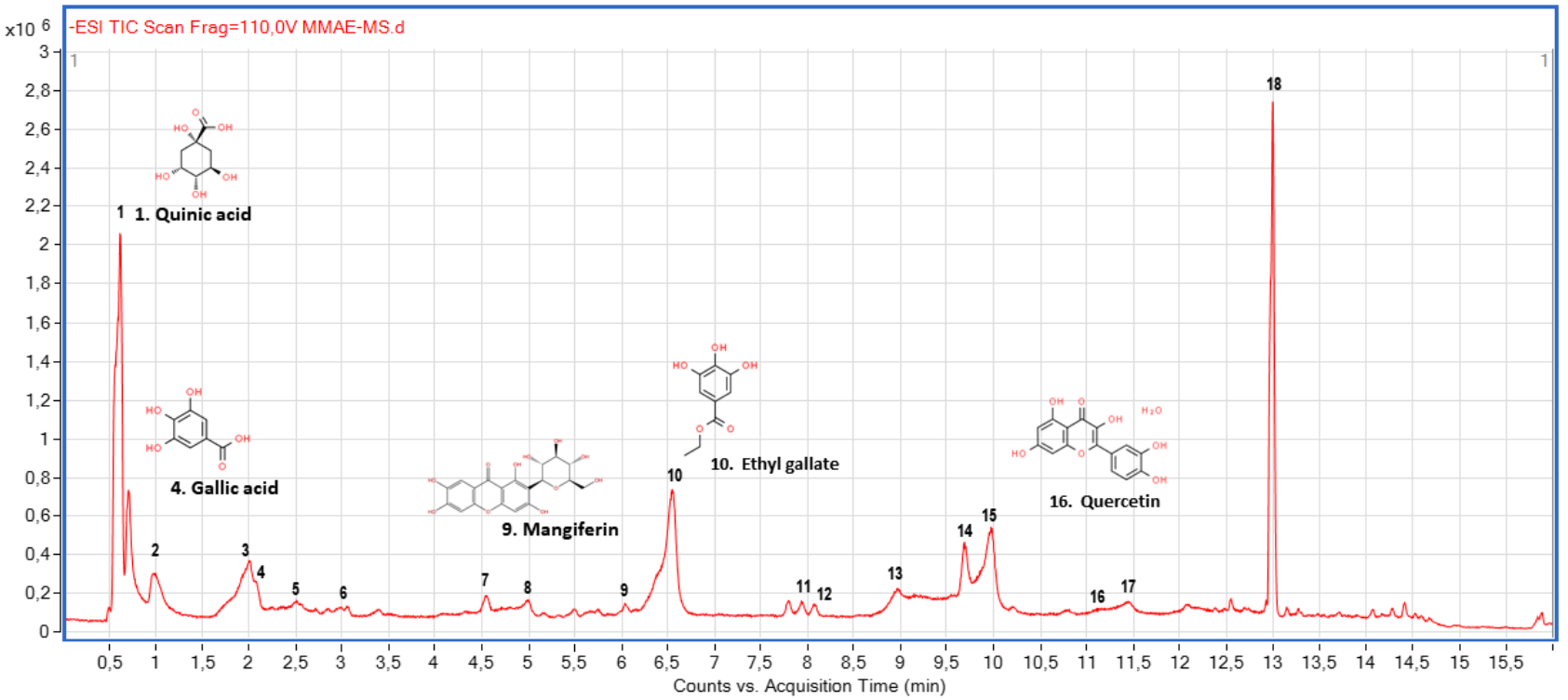
-ESI TIC Scan Frag=110,0V MMAE-MS.d
x10 6
1. Quinic acid
4. Gallic acid
9. Mangiferin
10. Ethyl gallate
16. Quercetin
Counts vs. Acquisition Time (min)

Figure 5.

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830 **Table 1**. Experimental conditions and results obtained for the optimization of MAE stage and benchmark method of SFE-MP.

Assay	Microwave power (MWP) (W)	Liquid-to-solid ratio (mL/g)	Extraction Time (s)	Extraction yield (EY) (g/100 g SFE-MP d.w.)	Total Phenolic Content (TPC) (mg GAE/g d.w.)	Phenolic concentration in the extract (PCE) (mg GAE/g extract)	Individual phenolic compounds concentration (mg/g extract)			TEAC (mmol TE/g extract)	EC_{50} (μg/mL)
							Galic acid	Mangiferin	Quercetin		
1	800 (+1)	30 (0)	60 (-1)	47.36 ± 0.15	48.46 ± 0.65	102.34 ± 1.37	2.72 ± 0.08	0.37 ± 0.02	0.33 ± 0.02	2.08 ± 0.03	7.51 ± 0.06
2	600 (0)	10 (-1)	60 (-1)	40.44 ± 0.11	41.14 ± 1.04	101.73 ± 2.56	2.49 ± 0.05	0.33 ± 0.01	0.31 ± 0.01	2.27 ± 0.01	8.68 ± 0.04
3	600 (0)	50 (+1)	120 (+1)	48.87 ± 0.24	52.62 ± 2.22	107.67 ± 4.53	3.40 ± 0.10	0.57 ± 0.02	0.31 ± 0.02	2.65 ± 0.12	7.82 ± 0.07
4	400 (-1)	30 (0)	120 (+1)	46.99 ± 0.28	46.71 ± 0.44	99.40 ± 0.93	5.64 ± 0.13	1.25 ± 0.03	0.34 ± 0.01	2.02 ± 0.06	8.56 ± 0.11
5*	600 (0)	30 (0)	90 (0)	47.22 ± 0.34	46.07 ± 0.59	97.57 ± 1.24	4.09 ± 0.05	0.75 ± 0.02	0.33 ± 0.01	2.27 ± 0.06	6.52 ± 0.02
6	600 (0)	10 (-1)	120 (+1)	37.36 ± 0.26	34.89 ± 0.84	93.38 ± 2.24	2.82 ± 0.09	0.50 ± 0.03	0.32 ± 0.01	2.37 ± 0.09	7.58 ± 0.10
7*	600 (0)	30 (0)	90 (0)	47.24 ± 0.13	46.63 ± 1.08	98.71 ± 2.30	4.09 ± 0.08	0.75 ± 0.01	0.28 ± 0.01	2.20 ± 0.05	6.40 ± 0.09
8*	600 (0)	30 (0)	90 (0)	47.84 ± 0.29	47.62 ± 1.99	99.55 ± 4.16	4.00 ± 0.00	0.76 ± 0.00	0.29 ± 0.00	2.20 ± 0.05	6.77 ± 0.22
9	400 (-1)	10 (-1)	90 (0)	40.00 ± 0.33	36.07 ± 1.12	90.19 ± 2.81	3.41 ± 0.11	0.50 ± 0.02	0.30 ± 0.02	2.34 ± 0.10	8.07 ± 0.10
10	800 (+1)	30 (0)	120 (+1)	41.12 ± 0.10	45.05 ± 1.59	109.58 ± 3.86	3.18 ± 0.08	0.69 ± 0.02	0.31 ± 0.01	2.63 ± 0.08	6.07 ± 0.14
11*	600 (0)	30 (0)	90 (0)	47.51 ± 0.34	47.20 ± 2.65	99.36 ± 5.59	4.00 ± 0.02	0.76 ± 0.02	0.30 ± 0.01	2.14 ± 0.07	6.43 ± 0.06
12	800 (+1)	10 (-1)	90 (0)	33.58 ± 0.19	37.93 ± 0.91	112.96 ± 2.71	3.52 ± 0.19	0.56 ± 0.06	0.32 ± 0.01	2.30 ± 0.06	7.07 ± 0.04
13	400 (-1)	50 (+1)	90 (0)	47.19 ± 0.20	47.71 ± 0.61	101.11 ± 1.30	5.50 ± 0.14	1.03 ± 0.04	0.33 ± 0.01	1.94 ± 0.05	9.03 ± 0.19
14	600 (0)	50 (+1)	60 (-1)	45.25 ± 0.28	48.74 ± 1.75	107.73 ± 3.86	3.08 ± 0.01	0.46 ± 0.03	0.32 ± 0.01	2.38 ± 0.14	7.62 ± 0.26
15	800 (+1)	50 (+1)	90 (0)	44.66 ± 0.27	52.08 ± 1.46	116.63 ± 3.28	4.12 ± 0.06	0.53 ± 0.04	0.57 ± 0.01	2.75 ± 0.06	6.47 ± 0.11
16	400 (-1)	30 (0)	60 (-1)	42.29 ± 0.23	39.71 ± 0.44	94.26 ± 0.52	4.99 ± 0.36	1.08 ± 0.03	0.34 ± 0.01	2.10 ± 0.10	8.26 ± 0.09
17*	600 (0)	30 (0)	90 (0)	47.95 ± 0.35	46.76 ± 2.62	97.52 ± 5.46	4.02 ± 0.04	0.76 ± 0.02	0.32 ± 0.01	2.01 ± 0.02	6.14 ± 0.06
Benchmark method				45.12 ± 0.38	56.98 ± 2.03	126.29 ± 4.49	ND	ND	ND	2.64 ± 0.08	5.76 ± 0.08

831 *Central points assays. SFE-MP: Supercritical CO_2 pre-treated mango peel; GAE: Galic acid equivalent; TEAC: Trolox equivalent antoxidant capacity. EC_{50}: Half maximal effective concentration; ND: no
832 determined

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Table 2. Tentatively identified compounds from optimal MAE extract by LC-Q-TOF-MS/MS analysis.

Peak	t_R (min)	Molecular ion $[M–H]^-$ (m/z)		Formula	Tentatively Identified Compound	Error (Δ ppm)	MS/MS Fragment ions (m/z)
		Measured	Theoretical				
1	0.619	191.0565	191.0561	$C_7H_{12}O_6$	Quinic acid	2.1	93; 85
2	0.998	331.0669	331.0671	$C_{13}H_{16}O_{10}$	Galloyl glucose isomer I	-0.6	169; 125
3	2.001	331.0674	331.0671	$C_{13}H_{16}O_{10}$	Galloyl glucose isomer II	0.9	169; 125
4	2.078	169.0139	169.0142	$C_7H_6O_5$	Gallic acid*	-1.8	125; 79
5	2.511	331.0678	331.0671	$C_{13}H_{16}O_{10}$	Galloyl glucose isomer III	2.1	169; 125
6	3.068	331.0683	331.0671	$C_{13}H_{16}O_{10}$	Galloyl glucose isomer IV	3.6	169; 125
7	4.624	645.1285	645.1309	$C_{26}H_{30}O_{19}$	Digalloyl diglucoside	-3.7	483; 321; 169
8	4.997	321.0260	321.0252	$C_{14}H_{10}O_9$	Digallic acid	2.5	169; 125
9	6.047	421.0779	421.0776	$C_{19}H_{18}O_{11}$	Mangiferin*	0.7	331; 301; 271
10	6.543	197.0462	197.0455	$C_9H_{10}O_5$	Ethyl gallate	3.6	169
11	7.933	463.0901	463.0882	$C_{21}H_{20}O_{12}$	Quercetin glucoside isomer I	4.1	301
12	8.073	463.0881	463.0882	$C_{21}H_{20}O_{12}$	Quercetin glucoside isomer II	-0.2	301
13	8.973	349.0576	349.0565	$C_{16}H_{14}O_9$	Galloyl ethylgallate isomer I	3.2	197; 169
14	9.642	349.0582	349.0565	$C_{16}H_{14}O_9$	Galloyl ethylgallate isomer II	4.9	197; 169
15	9.979	349.0581	349.0565	$C_{16}H_{14}O_9$	Galloyl ethylgallate isomer III	4.6	197; 169
16	11.118	301.0368	301.0354	$C_{15}H_{10}O_7$	Quercetin*	4.7	191; 127
17	11.432	501.0694	501.0675	$C_{23}H_{18}O_{13}$	Ethyl trigallate	3.8	349; 212; 197
18	12.955	1243.1321	1243.1330	$C_{55}H_{40}O_{34}$	Heptagalloylglucose	-0.7	545; 621; 939

* Identification confirmed by commercial standard.

840 **Table 3**. Pearson's correlation coefficients (r) between phenolic compounds (gallic acid, mangiferin and quercetin) quantified in MAE extracts and the
841 antioxidant capacity (TEAC and EC_{50})

Assay	Phenolic compounds		
	Gallic acid	**Mangiferin**	**Quercetin**
TEAC	- 0.47	- 0.51	0.45
EC_{50}	0.21	0.17	-0.12

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857 **SUPPLEMENTARY MATERIAL**

858 **Microwave-assisted extraction of phenolic compounds with antioxidant and anti-proliferative**
859 **activities from supercritical CO_2 pre-extracted mango peel as valorization strategy**

860 Andrea del Pilar Sánchez-Camargo, Diego Ballesteros-Vivas, Luis Miguel Buelvas-Puello, Hugo A.
861 Martinez-Correa, Fabián Parada-Alfonso, Alejandro Cifuentes, Sandra R.S. Ferreira, Luis-Felipe
862 Gutiérrez

863

864 **Table S1**. SFE process parameters at laboratory and pilot scales for the first valorization step of
865 mango peel

Parameter	10 mL cell	500 mL Cell
	Lab Scale	Pilot Scale
Temperature (°C)	60	60
Pressure (MPa)	25	25
Ethanol (% w/w)	15	15
Mango peel mass, F (g)	5	222
CO_2 mass flow, Q_{CO2} (g/min)	6.7	90
Ethanol volumetric flow (mL/min)	1.5	20
Internal Diameter, D(cm)	1.3	4.3
Height, L (cm)	5.5	18.8
Extraction time (min)	180	180
Effective cell volume (mL)	7.3	270
Bulk density of mango peel (g/mL)	0.89	0.89
L/D	4.23	4.37
Q_{CO2}×D/F (cm/min)	1.74	1.74

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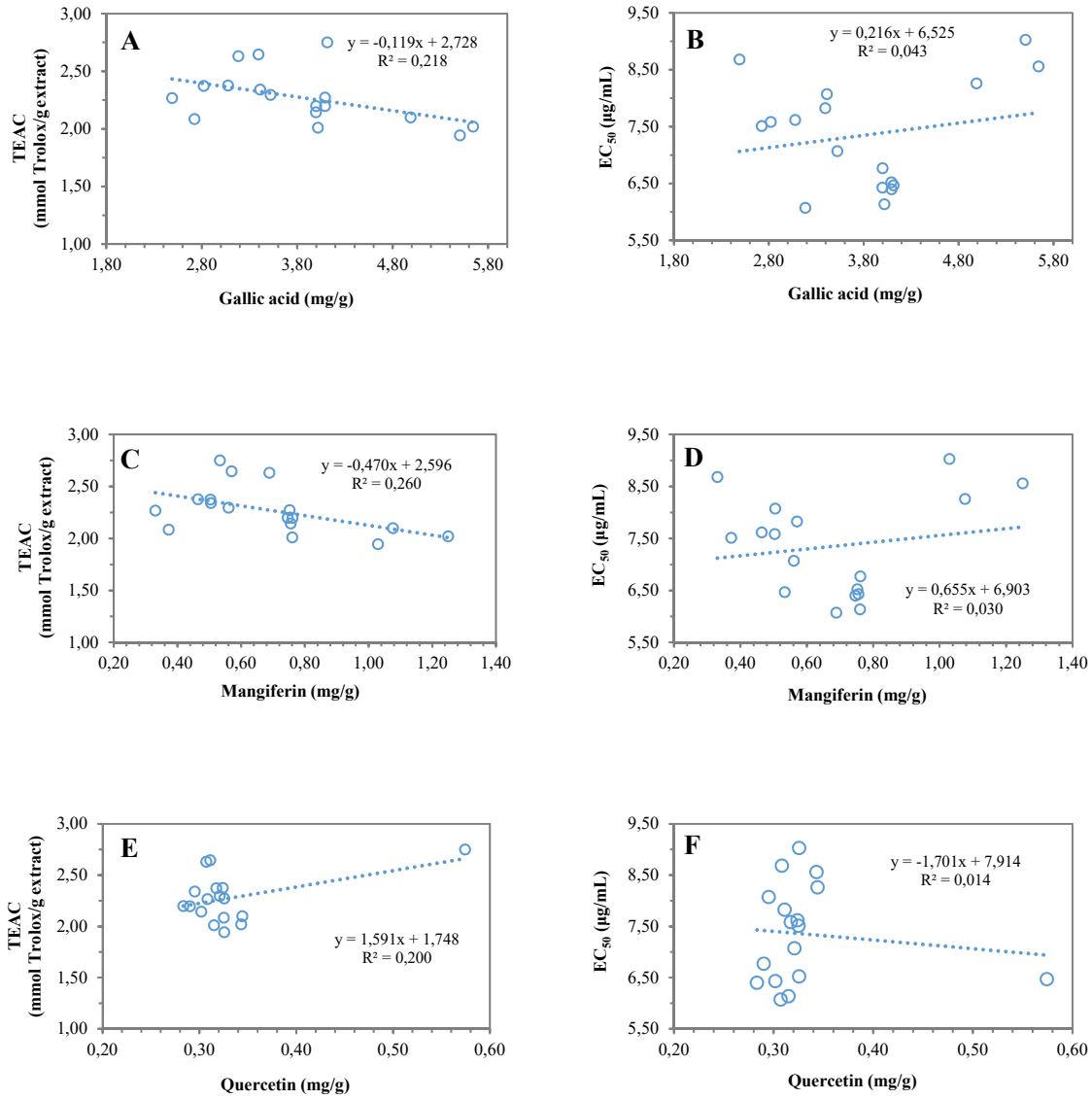
867 **Table S2**. ANOVA for response surface modeling showing linear, quadratic and interaction relations
868 of each variable and coefficient for model prediction.

Response variable	Source	DF	SS	MS	F-value	p-value		CE
Extraction yield (g/100 g sample d.b.)	Model	16	310.70					
	A: Microwave power	1	11.90	11.90	105.26	0.0005		
	B: L/S Ratio	1	149.43	149.43	1321.51	< 0.0001		
	C: Extraction time	1	0.13	0.13	1.13	0.3475		
	A × B	1	3.77	3.77	33.35	0.0045		
	B × C	1	11.23	11.23	99.35	0.0006		
	A × C	1	29.87	29.87	264.13	0.0001		
	A × A	1	23.61	23.61	208.81	0.0001		
	B × B	1	61.71	61.71	545.75	< 0.0001		
	C × C	1	2.34	2.34	20.69	0.0104		
	Lack of fit	3	9.30	3.10	27.42	0.0040*		
	Pure error	4	0.45	0.11				
	$R^2 = 0.967$							
	Adjusted $R^2 = 0.928$							
Total phenolic content (TPC) (mg GAE/g d.w.)	Model	16	437.76				β_0	1.23
	A: Microwave power	1	22.21	22.21	63.93	0.0013	β_1	8.42×10^{-2}
	B: L/S Ratio	1	326.59	326.59	939.85	< 0.0001	β_2	0.15
	C: Extraction time	1	0.18	0.18	0.52	0.5118	β_3	0.24
	A × B	1	1.58	1.58	4.54	0.1002	$\beta_{1.2}$	1.57×10^{-4}
	B × C	1	25.64	25.64	73.79	0.0010	$\beta_{2.3}$	4.22×10^{-3}
	A × C	1	27.05	27.05	77.85	0.0009	$\beta_{1.3}$	-4.33×10^{-4}
	A × A	1	8.08	8.08	23.25	0.0085	$\beta_{1.1}$	-3.46×10^{-5}
	B × B	1	17.20	17.20	49.51	0.0022	$\beta_{2.2}$	-5.05×10^{-3}
	C × C	1	1.00	1.00	2.89	0.1643	$\beta_{3.3}$	-5.43×10^{-5}
	Lack of fit	3	4.58	1.53	4.39	0.0934		
	Pure error	4	1.39	0.35				
	$R^2 = 0.986$							
	Adjusted $R^2 = 0.969$							
Phenolic content in extracts (PCE) (mg GAE/g extract)	Model	16	800.58					
	A: Microwave power	1	399.70	399.70	431.08	< 0.0001		
	B: L/S Ratio	1	152.03	152.03	163.96	0.0002		
	C: Extraction time	1	1.97	1.97	2.13	0.2185		
	A × B	1	13.13	13.13	14.16	0.0197		
	B × C	1	17.18	17.18	18.53	0.0126		
	A × C	1	1.10	1.10	1.18	0.3380		
	A × A	1	31.25	31.25	33.70	0.0044		
	B × B	1	66.07	66.07	71.26	0.0011		
	C × C	1	0.07	0.07	0.08	0.7941		
	Lack of fit	3	108.29	36.10	38.93	0.0020*		
	Pure error	4	3.71	0.93				
	$R^2 = 0.860$							
	Adjusted $R^2 = 0.680$							
TEAC (mmol TE/g extract)	Model	16	0.86				β_0	4.63
	A: Microwave power	1	0.23	0.23	24.34	0.0079	β_1	-2.51×10^{-3}
	B: L/S Ratio	1	2.42×10^{-2}	2.42×10^{-2}	2.56	0.1850	β_2	-6.34×10^{-2}

	C: Extraction time	1	8.85×10^{-2}	8.85×10^{-2}	9.34	0.0378	β_3	-2.69×10^{-2}
	A × B	1	0.18	0.18	19.17	0.0119	$\beta_{1.2}$	5.33×10^{-5}
	B × C	1	6.67×10^{-3}	6.67×10^{-3}	0.70	0.4484	$\beta_{2.3}$	6.81×10^{-5}
	A × C	1	0.10	0.10	10.34	0.0324	$\beta_{1.3}$	2.61×10^{-5}
	A × A	1	1.58×10^{-3}	1.58×10^{-3}	0.17	0.7037	$\beta_{1.1}$	-4.85×10^{-7}
	B × B	1	0.15	0.15	15.62	0.0168	$\beta_{2.2}$	4.69×10^{-4}
	C × C	1	1.69×10^{-2}	1.69×10^{-2}	1.79	0.2523	$\beta_{3.3}$	7.04×10^{-5}
	Lack of fit	3	1.75×10^{-2}	5.83×10^{-3}	0.62	0.6402		
	Pure error	4	3.79×10^{-2}	9.47×10^{-3}				
	$R^2 = 0.935$							
	Adjusted $R^2 = 0.852$							
EC_{50} (µg/mL)	Model	16	14.51				β_0	17.73
	A: Microwave power	1	5.77	5.77	110.94	0.0005	β_1	-8.05×10^{-3}
	B: L/S Ratio	1	0.03	0.03	0.53	0.5053	β_2	-0.11
	C: Extraction time	1	0.52	0.52	9.91	0.0346	β_3	-0.12
	A × B	1	0.61	0.61	11.63	0.0270	$\beta_{1.2}$	-9.73×10^{-5}
	B × C	1	0.43	0.43	8.20	0.0458	$\beta_{2.3}$	5.44×10^{-4}
	A × C	1	0.61	0.61	11.63	0.0270	$\beta_{1.3}$	-7.24×10^{5}
	A × A	1	0.82	0.82	15.73	0.0166	$\beta_{1.1}$	1.10×10^{-5}
	B × B	1	2.48	2.48	47.63	0.0023	$\beta_{2.2}$	1.92×10^{-3}
	C × C	1	2.11	2.11	40.54	0.0031	$\beta_{3.3}$	7.87×10^{-4}
	Lack of fit	3	0.19	0.06	1.24	0.4051		
	Pure error	4	0.21	0.05				
	$R^2 = 0.972$							
	Adjusted $R^2 = 0.937$							
Gallic acid (mg/g extract)	Model	16	15.37					
	A: Microwave power	1	1.67	1.67	709.96	< 0.0001		
	B: L/S Ratio	1	1.85	1.85	785.44	< 0.0001		
	C: Extraction time	1	2.11	2.11	895.43	< 0.0001		
	A × B	1	0.56	0.56	238.88	0.0001		
	B × C	1	3.6×10^{-5}	3.6×10^{-5}	0.02	0.9053		
	A × C	1	1.15	1.15	490.49	< 0.0001		
	A × A	1	3.69	3.69	1569.45	< 0.0001		
	B × B	1	2.96	2.96	1255.82	< 0.0001		
	C × C	1	0.28	0.28	116.84	0.0004		
	Lack of fit	3	1.43	0.48	202.23	0.0001*		
	Pure error	4	0.01	0.002				
	$R^2 = 0.906$							
	Adjusted $R^2 = 0.786$							
Mangiferin (mg/g extract)	Model	16	1.00					
	A: Microwave power	1	0.36	0.36	9442.12	< 0.0001		
	B: L/S Ratio	1	6.09×10^{-2}	6.09×10^{-2}	1581.11	< 0.0001		
	C: Extraction time	1	7.37×10^{-2}	7.37×10^{-2}	1915.03	< 0.0001		
	A × B	1	7.61×10^{-2}	7.61×10^{-2}	1976.83	< 0.0001		
	B × C	1	1.11×10^{-3}	1.11×10^{-3}	28.94	0.0058		
	A × C	1	5.08×10^{-3}	5.08×10^{-3}	131.98	0.0003		
	A × A	1	8.36×10^{-2}	8.36	2170.43	< 0.0001		
	B × B	1	0.24	0.24	6234.20	< 0.0001		
	C × C	1	1.01×10^{-2}	1.01×10^{-2}	263.39	0.0001		

	Lack of fit	3	0.10	3.39×10^{-2}	880.61	< 0.0001*
	Pure error	4	1.54×10^{-4}	3.85×10^{-5}		
	$R^2 = 0.899$					
	Adjusted $R^2 = 0.768$					
Quercetin (mg/g extract)	Model	16	6.78×10^{-2}			
	A: Microwave power	1	6.02×10^{-3}	6.02×10^{-3}	19.90	0.0111
	B: L/S Ratio	1	1.08×10^{-2}	1.08×10^{-2}	35.57	0.0040
	C: Extraction time	1	6.60×10^{-5}	6.60×10^{-5}	0.22	0.6646
	A × B	1	1.24×10^{-2}	1.24×10^{-2}	41.09	0.0030
	B × C	1	1.20×10^{-4}	1.20×10^{-4}	0.40	0.5630
	A × C	1	7.24×10^{-5}	7.24×10^{-5}	0.24	0.6502
	A × A	1	8.63×10^{-3}	8.63×10^{-3}	28.54	0.0059
	B × B	1	3.94×10^{-3}	3.94×10^{-3}	13.03	0.0226
	C × C	1	1.45×10^{-3}	1.45×10^{-3}	4.80	0.0936
	Lack of fit	3	2.30×10^{-2}	7.67×10^{-3}	25.37	0.0046*
	Pure error	4	1.21×10^{-3}	3.02×10^{-4}		
	$R^2 = 0.642$					
	Adjusted $R^2 = 0.183$					

DF-degree of freedom; CE- coefficients of regression equation (Uncoded units); SS-sum of squares; MS-mean squares
*Not significant.



872 **Figure S1.** Correlation between gallic acid (A and B), mangiferin (C and D), and quercetin content (E and F)
873 and the antioxidant activity by TEAC and DPPH radical scavenging assays, respectively.

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