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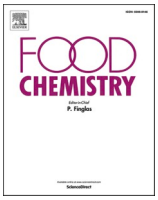

# Microwave-assisted extraction of raspberry pomace phenolic compounds, and their bioaccessibility and bioactivity

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ABSTRACT

Raspberry pomace (RBP) is rich in phenolic compounds. This study aims to optimize the extraction of phenolics from RBP and assess their bioaccessibility and bioactivity. The extraction process revealed that ethanol 50 % was the most effective solvent. Microwave-assisted hydroethanolic extraction (MAE) significantly outperformed conventional methods, with optimal conditions of 200 °C for 10 min yielding the highest concentrations of total phenolics (68 mg GAE/g RBP) and flavonoids (63 mg RE/g RBP). Eleven phenolic compounds were identified by HPLC-ESI-MS, with gallic acid and protocatechuic acid being the most prevalent. The gastrointestinal digestion revealed that although some phenolics suffered degradation (like ferulic acid and quercetin), phenolics are more bioaccessible and have relevant antioxidant activity. RBP extract exhibited anti-inflammatory activity by downregulating pro-inflammatory IL-1β and upregulating anti-inflammatory IL-10 cytokines in LPS-activated macrophages. These findings underscore the effectiveness of MAE in extracting bioactives from RBP, highlighting its potential in developing functional foods and nutraceuticals.

## 1. Introduction

Berries by-products from the food industry have received more attention in the last few years concerning their characterization and upcycling (Jaouhari et al., 2024; Nabih, 2023). Red raspberry (*Rubus idaeus* L.) is one of the most consumed and appreciated berries by consumers for their nutrients and aromatic taste (Chironi et al., 2017). It is distinguished by its color, pleasant aroma, acidity as well as high nutritional value and great content of antioxidant compounds. The latest FAO data show that global raspberry production reached 940,979 tons in 2023 (FAOSTAT, *2021*). Notably, Russia emerged as the leading producer, harvesting 197,700 tons, followed by Mexico and Serbia. Fresh raspberry remain the main market; however, their seasonal nature and susceptibility to spoilage necessitate industrial processing to obtain alternative products, like juices (Rocha et al., 2020). The process of raspberry transformation results in significant quantities of pomace (skin, seeds, and pulp) rich in valuable nutrients and bioactive non-nutrients, which hold potential for applications across various industrial sectors (Brodowska, 2017; de Ribeiro et al., 2019). Moreover, an efficient waste management and the ability to convert this waste into by-products provide a sustainable basis for realizing the transition from the linear to the circular economy (Jaouhari et al., 2023). In fact, the only beverage industry generates approximately 26 % of the total industrial food waste and the re-introduction of vegetal-based pomace in the food value chain represents an environmentally and economically friendly action (Baiano, 2014).

Most of the bioactive compounds present in the fresh raspberry remain in the solid bioresidues because they are originally located in the skins and seeds of the fruit (Struck et al., 2016). Phenolic molecules, especially non-anthocyanin flavonoids and phenolic acids, are among

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the most targeted bioactive compounds from the raspberry pomace (RBP) due to their strong bond with the polysaccharide matrix and high stability to degradation (Yao et al., 2021). Phenolic compounds are secondary metabolites that have important taxonomic significance in plants and fruits (Crozier et al., 2006). Deriving from the shikimic acid pathway, these phytochemicals are recognized by the presence of several phenolic hydroxyl groups, whose number and position in the molecular structure largely determine their antioxidant properties (Ferreira-Santos et al., 2020). Besides the antioxidant activity, different studies have reported that the characteristic polyphenols' structural configuration may have significant potential for other important biological activities, exploited in functional supplements and bioactive ingredients, including prebiotic, anti-inflammatory, and anti-diabetic, particularly for type 2 diabetes (Alves-Santos et al., 2020; Bahadoran et al., 2013; Yahfoufi et al., 2018).

Recently, the extraction of these valuable bioactive compounds has been undertaken using emerging technologies such as microwaves, ultrasounds, supercritical fluids, and electrotechnologies (ohmic heating, pulsed electric fields, *etc.*) which have gained interest since they could overcome the conventional extraction disadvantages (Ferreira-Santos et al., 2019, 2024; Talmaciu et al., 2015). Among these, microwave-assisted extraction (MAE) is a relatively modern technology that operates at higher temperatures and pressures, and, above all, requires a shorter extraction time than conventional solid-liquid extraction (Ameer et al., 2017; Ekezie et al., 2017; Rodríguez-Martínez et al., 2023). Microwave power and irradiation time enhance solvent penetration into the plant matrix and the subsequent cell's membrane rupture, facilitating the dissolution and extraction of the phenolic compounds, increasing the effectiveness of the process (Del-Castillo-Llamosas et al., 2023).

With this in mind, the first aim of the present study was to obtain an extract rich in phenolic compounds with higher antioxidant capacity from RBP using MAE technology, applying different extraction conditions (temperature and time). The selection of the best extracting solvent followed an initial conventional extraction process involving water and ethanol:water at different concentration. At selected conditions, in order to validate the nutraceutical activity of the RBP extract (RBPE), the simulated gastro-intestinal digestion was performed to evaluate the bioaccessible fraction of single phenolic compounds by HPLC-ESI-MS and its antioxidant potential. Additionally, the anti-inflammatory activity of the RPBE was evaluated in activated macrophages.

## 2. Materials and methods

### 2.1. Plant material and sample preparation

The pomace of raspberry (*Rubus idaeus* L.) (RBP) obtained from the juice pressing process was kindly supplied by the "Polo AGRIFOOD - MIAC Scpa" (Cuneo, Italy) in September 2023. The raw material, consisting of skin, seeds and pulp, was lyophilized in a laboratory freeze dryer (Lio 5P, Cinquepascal SrL, Trezzano sul Naviglio, Italy) and powdered using a ball mill (Rentsch GmbH, Haan, Germany).

### 2.2. Reagents and solvents

All chemical reagents and standards were obtained from Sigma Aldrich (St. Louis, MO, USA): Ferulic acid 99 %, *p*-Coumaric acid 98 %, Protocatechuic acid 99.9 %, Gallic acid 99 %, 4-Hydroxibenzoic acid 99 %, Vanillic acid 97 %, Rutin 99 %, Catechin 96 %, Epicatechin 98 %, Vanillin 99 %, Quercetin 98 %, 2,2′-azino-di(3-ethylbenzo-thia-zoline-6-sulfonic acid (ABTS), 2,2-Di(4-*tert*-octylphenyl)-1-picrylhydrazyl (DPPH), 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), aluminum chloride ($AlCl_3$), absolute ethanol anhydrous (99.9 %), Folin-Ciocâlteu reagent, bile extract, porcine pepsin, pancreatin, $(NH_4)_2CO_3$, $NaHCO_3$, $MgCl_2{\cdot}6H2O$, HCl (37 % (*w/v*)), NaCl, KCl, NaOH, $KH_2PO_4$, $CaCl_2{\cdot}2H_2O$. Other reagents were analytical grade, and ultra-pure water was used throughout the experiments.

### 2.3. Conventional and microwave-assisted extraction (MAE) conditions

Conventional extractions were performed in a 100 mL glass, weighing 1 g of freeze-dried RBP mixed with 20 mL of solvent (water, 30 % ethanol, 50 % ethanol, 70 % ethanol, and 90 % ethanol (*v/v*)). The ethanol concentrations were prepared by diluting absolute ethanol with distilled water (*w*/w) to achieve the specified percentages. The extractions were performed in a rotary incubator shaker at 40 °C for 2 h (in triplicate).

The MAE treatment was carried out using a Monowave single-mode microwave reactor (Anton Paar GmbH, Austria) operating with a maximum power of 850 W, equipped with a temperature sensor mechanism. RBP and solvent were introduced into a 30 mL Pyrex vial (Reaction Vial G30, Anton Paar), sealed with a cap and septum, at a liquid-to-solid-ratio (LSR) of 20:1 (20 mL of 50 % ethanol/g of RBP). The mixture was magnetically stirred at 600 rpm and heated up to the targeted temperature (100–200 °C) for a period ranging from 1 to 40 min. Finally, the mixtures were filtered with paper filters Whatman Grade 41 (20–25 μm) and the liquid extracts were stored at −20 °C until analysis. All experiments were performed at least in triplicate.

### 2.4. In vitro digestion of phenolic extract

The lyophilized phenolic extract obtained at optimum conditions from MAE was subjected to *in vitro* static simulated digestion, following the INFOGEST 2.0 protocol as described by Brodkorb et al. (2019). This experiment simulates the human digestion process (*i.e.*, oral, gastric, and intestinal phases). In order to have a detectable concentration of the bioactive compounds in each digestion phase, the dried RBPE was suspended in ultrapure water to an initial concentration of 60 mg/mL (volume of 5 mL). A blank test tube without samples (5 mL of water) but with all digestion fluids and enzymes was also subjected to analysis.

### 2.5. Total phenolic content and total flavonoid content assays

The total phenolic content (TPC) and total flavonoid content (TFC) were measured spectrophotometrically using the Folin-Ciocâlteu and aluminum chloride methods, respectively, as described by del Río et al. (2022). Regarding TPC, the results were expressed as mg of gallic acid equivalents/g of RBP (mg GAE/g RBP), while for TFC, the findings were expressed as mg of rutin equivalents/g of RBP (mg RE/g RBP).

### 2.6. Total monomeric anthocyanins content

Total monomeric anthocyanins content (TMAC) was measured using the spectrophotometric pH-differential method as described by Ferreira-Santos et al. (2022). TMAC was expressed as mg of cyanidin-3-*O*-glucoside equivalents (C3GE) per gram of dry extract (mg C3GE/g RBP).

### 2.7. Identification and quantification of phenolic compounds by HPLC-ESI-MS

RBPE phenolic compounds were identified and quantified in an Agilent 1260 series HPLC (Palo Alto, CA, USA) with AB SCIEX Triple Quad 3500 detector (Foster City, CA, USA), equipped with an electrospray source of ionization (ESI). For analysis, 5 μL of samples were injected in a Luna C18 column (150 mm × 2 mm; 3 μm particle diameter) from Phenomenex. For the separation, 0.1 % formic acid (solvent A) and acetonitrile with 0.1 % formic acid (solvent B) were used as eluents in a gradient (98 % of A from 0 to 4.0 min, 98–80 % of A from 4.0 to 7.0 min, 80–10 % of A from 7.0 to 14.0 min, 10 % of A from 14.0 to 15.0 min, 10–98 % of A from 15.0 to 17.0 min) at a flow of 0.3 mL/min. A positive/negative ionization source with turbo V™ (ion spray voltage

of 4500 V), with nitrogen as nebulizer and collision gas, were employed at a source temperature of 400 °C. Multiple reaction monitoring (MRM) was used to obtain the data using Analyst 1.6.2 software (AB Sciex, Foster City, CA). Phenolic compound standards were injected separately for quantification. Finally, the identification of each individual phenolic compound was determined by taking into account the retention time (RT), mass spectra (*m/z* of the ions), and the concentration was accessed using the calibration curve of each corresponding standards.

### 2.8. Bioaccessibility index (BI %)

To analyze the effect of the *in vitro* GID on phenolic content, the bioaccessibility index was calculated according to the following Eq. (1):

$$BI\% = \left(\frac{A}{B}\right) \text{x } 100 \quad (1)$$

where A is the phenolic content in the bio-accessible fraction (after *in vitro* GID), and B is the initial phenolic content before *in vitro* GID.

### 2.9. Antioxidant capacity assays

The determination of the antioxidant capacity of the liquid extracts was carried out following three methods described by Gullón et al. (2017): 2,2-Di(4-*tert*-octylphenyl)-1-picrylhydrazyl radical scavenging assay (DPPH), the 2,2-azino-bis-3-ethylbenzothiazoline-6- sulphonic acid radical cation decolorization assay (ABTS) and the ferric reducing antioxidant power (FRAP), using Trolox as standard. Each analysis was assessed in triplicates and results were expressed as mg of Trolox equivalents/mg of dried RBP (mg TE/mg RBP).

### 2.10. Anti-inflammatory activity

The macrophage cell line (J774A.1, ATCC TIB-67 ™) was cultured in DMEM high glucose, supplemented with 10 % FBS, 2 mM glutamine, 1 mM sodium pyruvate, and 25 mM HEPES buffer. The cultures were maintained in tissue culture flasks (Nagle Nunc, Int., Hereford, UK) with a humidified atmosphere containing 5 % CO2 at 37 °C (Binder CB150; Tuttlingen, Germany). After confluent growth, macrophage cells were washed with a fresh medium and recovered by scrapping. Viable cells were counted by Trypan blue exclusion in the hemocytometer and resuspended in DMEM to a final concentration of $1 \times 10^6$ cells/mL. A volume of 300 µL of the macrophage suspension was then cultured in 48-well tissue culture plates and incubated overnight with 1 µg/mL of lipopolysaccharides (LPS, Merk) to establish a cell-activation model. Following incubation, cells were washed with fresh medium and treated with 300 µg/mL of RBPE for 24 h. The extract concentration was selected based on preliminary results (data not shown). After treatment, the supernatants were collected and stored at −20 °C for cytokine quantification and the metabolic viability of the cells was confirmed using the MTT assay (American Type Culture Collection, 2011). Formazan crystals formed were dissolved in a DMSO:ethanol (1:1) solution, and absorbance was measured at 570 nm. Controls included cells incubated with the extracts' solvent (0.5 % DMSO), and with 1 µg/mL of LPS.

The concentrations of Tumor Necrosis Factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-10, and Transforming growth factor (TGF)-β1 in cell culture supernatants were measured using the corresponding Mouse Uncoated ELISA kit (Invitrogen), following the manufacturer's instructions.

### 2.11. Statistical analysis

All the statistical analyses and data visualizations were performed using the statistical software R 4.2.1 (Boston, USA) and GraphPad Prism 9.5 (San Diego, USA). Experiments were performed in triplicates, and the results were expressed as mean ± standard deviation (SD). Differences were estimated by analysis of variance (ANOVA) followed by Tukey's honest significant difference test and the statistical significance level was set to $p < 0.05$.

## 3. Results and discussion

### 3.1. Screening and selection of the best extraction solvent

The choice of the suitable solvent is highly related to the chemical characteristics and diffusion rate of the targeted bioactive compounds. To obtain extracts characterized by elevated levels of phenolic compounds and antioxidant capacity, this study tested environmentally friendly and food-grade solvents represented by water and ethanol at different concentrations (30, 50, 70, and 90 % (*v/v*)).

TPC, TFC, TMAC and antioxidant capacity (ABTS, DPPH, and FRAP) of the liquid extracts using the different extraction solvents are delineated in Table 1. As expected, hydroethanolic solutions were more efficient than water in recovering phenolic compounds. It is widely recognized that most phenolic compounds demonstrate a stronger affinity for organic solvents when combined with water, compared to extractions performed solely with water (Alara et al., 2021; Ameer et al., 2017).

Specifically, TPC varied from 15.26 to 29.52 mg GAE/g of RBP, obtaining the higher content employing EtOH 50 % as well as EtOH 70 % (28.54 mg GAE/g RBP) and 90 % (24.42 mg GAE/g RBP), which did not show any significant difference among them.

These values were in accordance with those from Jara-Palacios et al. (2019) who employed methanol, known as a non-green solvent, to obtain a maximum value of 20.15 mg GAE/g of RBP. Moreover, our finding was also similar to the whole raspberry (33 mg GAE/g RBP) obtained by Marino et al. (2024). Similarly, TFC was higher in EtOH 50 % (32.99 mg RE/g), 70 % (32.85 mg RE/g RBP) and 90 % (33.78 mg RE/g RBP), reporting no significant differences. Once again, it has been demonstrated that the extraction efficiency decreases with increasing water concentrations, reaching values of 23.10 mg RE/g RBP with EtOH 30 % and 18.18 mg RE/g RBP with just water.

TMAC of RBP was measured using the pH-differential absorbance method. Anthocyanins are polar compounds and water-soluble pigments that are negatively affected by temperature and light exposure (Câmara

**Table 1**
Phenolic content and antioxidant capacity (DPPH, ABTS and FRAP assays) in raspberry pomace (RBP) extracted with different solvents using conventional extraction method.

| Solvent | TPC (mg GAE/g RBP) | TFC (mg RE/g RBP) | TMAC (mg C3GE/g RBP) | Antioxidant capacity | | |
|---|---|---|---|---|---|---|
| | | | | DPPH (mg TE/g RBP) | ABTS (mg TE/g RBP) | FRAP (mg TE/g RBP) |
| Water | 15.26 ± 1.34$^{c}$ | 18.18 ± 0.24$^{b}$ | 0.312 ± 0.046$^{b}$ | 77.75 ± 1.52$^{d}$ | 31.39 ± 3.18$^{c}$ | 47.59 ± 4.11$^{c}$ |
| Ethanol 30 % | 18.11 ± 1.12$^{c}$ | 23.10 ± 0.81$^{b}$ | 0.320 ± 0.006$^{b}$ | 135.48 ± 6.69$^{c}$ | 41.18 ± 0.92$^{b}$ | 67.25 ± 2.45$^{b}$ |
| Ethanol 50 % | 29.52 ± 1.90$^{a}$ | 32.99 ± 2.19$^{a}$ | 0.269 ± 0.004$^{b}$ | 211.92 ± 4.56$^{a}$ | 62.05 ± 1.41$^{a}$ | 130.58 ± 0.87$^{a}$ |
| Ethanol 70 % | 28.54 ± 0.23$^{ab}$ | 32.85 ± 1.10$^{a}$ | 0.462 ± 0.031$^{a}$ | 180.67 ± 6.57$^{b}$ | 57.82 ± 2.24$^{a}$ | 132.43 ± 0.20$^{a}$ |
| Ethanol 90 % | 24.42 ± 0.17$^{b}$ | 33.78 ± 0.90$^{a}$ | 0.549 ± 0.021$^{a}$ | 150.25 ± 2.22$^{c}$ | 62.91 ± 1.21$^{a}$ | 63.84 ± 2.87$^{b}$ |

Values are expressed as mean ± standard deviation of three experiments. Data are reported on a dry weight basis. Different letters in the same column represent statistically different results ($p \leq 0.05$).
TPC: total phenolic content; TFC: total flavonoid content; TMAC: total monomeric anthocyanin content; GAE: gallic acid equivalent; RE: rutin equivalent; C3GE: cyanidin-3-*O*-glucoside equivalent; TE: Trolox equivalent.

et al., 2022). The use of polar solvents like ethanol makes the extraction of these molecules efficient. Indeed, analyzing the results obtained it is possible to observe that ethanol 70 % and 90 % hold the highest anthocyanins concentration (0.462 and 0.549 mg C3GE/g RBP, respectively), while ethanol 50 %, 30 %, and water are characterized by the lowest levels (0.269, 0.320 and 0.312 mg C3GE/g RBP, respectively).

The antioxidant capacity of RBP was determined by three methods: ABTS, DPPH and FRAP (Table 1).

Regarding ABTS, the scavenging antioxidant capacity showed a similar trend to the phenolic content, showing the order of EtOH 90 % / EtOH 50 % / EtOH 70 % > EtOH 30 % > water, with no significant difference between EtOH 50 %, 70 % and 90 %. When water served as the sole extracting solvent, the antioxidant activity was 31.39 mg TE/g of RBP, attaining its maximal level of 62.91 mg TE/g of RBP when EtOH 90 % was employed. Likewise, DPPH analysis also shows the same trend where EtOH 50 % holds the maximum value (211.92 mg TE/g RBP) followed by EtOH 70 % (180.67 mg TE/g RBP), EtOH 90 % (150.25 mg TE/g RBP), EtOH 30 % (135.48 mg TE/g RBP), and water (77.75 mg TE/g RBP). Finally, testing the antioxidant capacity through an electron transfer mechanism of ferric reducing antioxidant power (FRAP) rather than the scavenging mechanism based on the previous assays, the trend remained practically unchanged. More specifically, the most notable levels were consistently achieved through the utilization of EtOH 50 % (148.25 mg TE/g RBP) and EtOH 70 % (132.43 mg TE/g RBP), followed by EtOH 90 % (63.84 mg TE/g RBP), EtOH 30 % (67.25 mg TE/g RBP) and water (47.59 mg TE/g RBP) which did not demonstrate significant differences.

Recapitulating, values measured by TPC, TFC, ABTS, DPPH, and FRAP were higher using EtOH 50 %. The only exception was TMAC, which higher concentrations were obtained using EtOH 70 % and 90 %. The use of EtOH 50 % for recovering value-added compounds from agri-food by-products such us grape, apple and olive pomace is described in literature (Gullón et al., 2018; del Razola-Díaz et al., 2022; Rodrigues et al., 2023).

According to these results, it is evident that phenolic compounds tend to dissolve more in ethanolic solution, especially at higher concentrations (up to 50 %), than in water. The trend observed indicates that as the concentration of ethanol increases, there is a subsequent increase in the extraction efficiency of total phenolic and flavonoid content, as well as antioxidant capacity measured through radical scavenging and ferric-reducing antioxidant power assays. The physicochemical characteristics of the solvent, in this case ethanol, can play a key role in the extraction efficiency due to its direct properties, especially its polarity and hydrophilic interaction, which improve the solubilization of the phenolic compounds.

It could be concluded that EtOH 50 % possessed the right compromise of extraction efficiency, resulting in a high-yield antioxidant-rich extract, and feasibility in economic terms given the use of water at concentrations higher to that of ethanol compared to EtOH 70 % and 90 %.

### 3.2. Characterization of extracts from MAE

Once ethanol 50 % (*v:v*) was chosen as the extraction solvent, the different extraction conditions were tested, such as temperature (100, 140, 180, and 200 °C) and time (1, 10, 20, 30, and 40 min), on the microwave extraction process.

Microwave irradiations enhance solvent penetration into the plant cell walls during the operation process, achieving the dissolution and extraction efficiency of the polyphenol compounds.

The extracts resulting from MAE were analyzed to study the content of phenolics, flavonoids, anthocyanins, and antioxidant capacity, and the results are exhibited in **Figs. 1** and **2**. Significant differences were observed for all the parameters studied during different extracting conditions (temperature and time).

In the case of TPC (Fig. 1A), extractions conducted at 200 °C for 10 and 20 min showed the greatest recovery of phenolics from RBP (68.10 and 66.43 mg GAE/g RBP, respectively). It should be noted that these results were approximately two times higher than the concentration obtained using EtOH 50 % *via* the traditional extraction method (29.52 mg GAE/g RBP). When moderating temperature and time to their minimum levels, the MAE efficiency decreases. Specifically, extractions performed at 100 °C for 1 and 10 min recorded the lowest values of total phenolic compounds (31.61 and 31.67 mg GAE/g RBP, respectively).

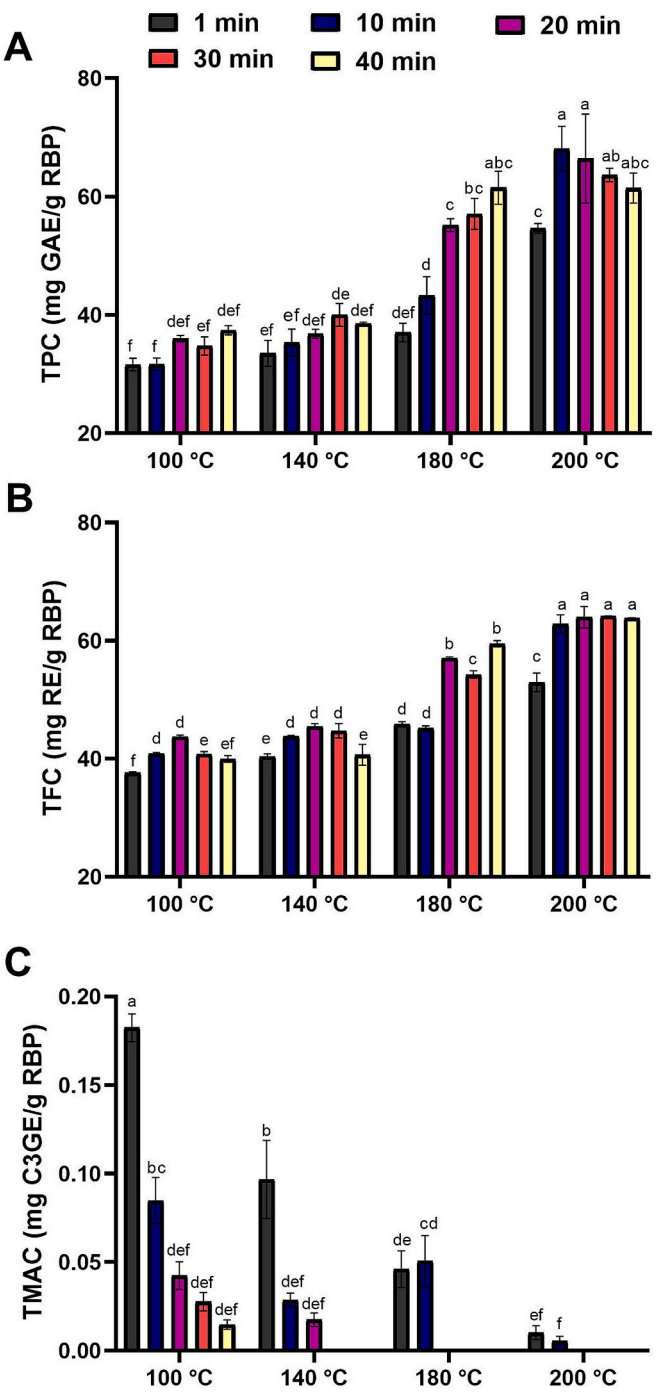


**Fig. 1.** Phenolic content of obtained raspberry pomace (RBP) extracts expressed as mean ± standard deviation. Data are reported on a dry weight basis. Bars with different letters are significantly different ($p \leq 0.05$). (**A**) total phenolic content (TPC), (**B**) total flavonoid content (TFC), and (**C**) total monomeric anthocyanin content (TMAC). GAE: gallic acid equivalent; RE: rutin equivalent; C3GE: cyanidin-3-*O*-glucoside equivalent.

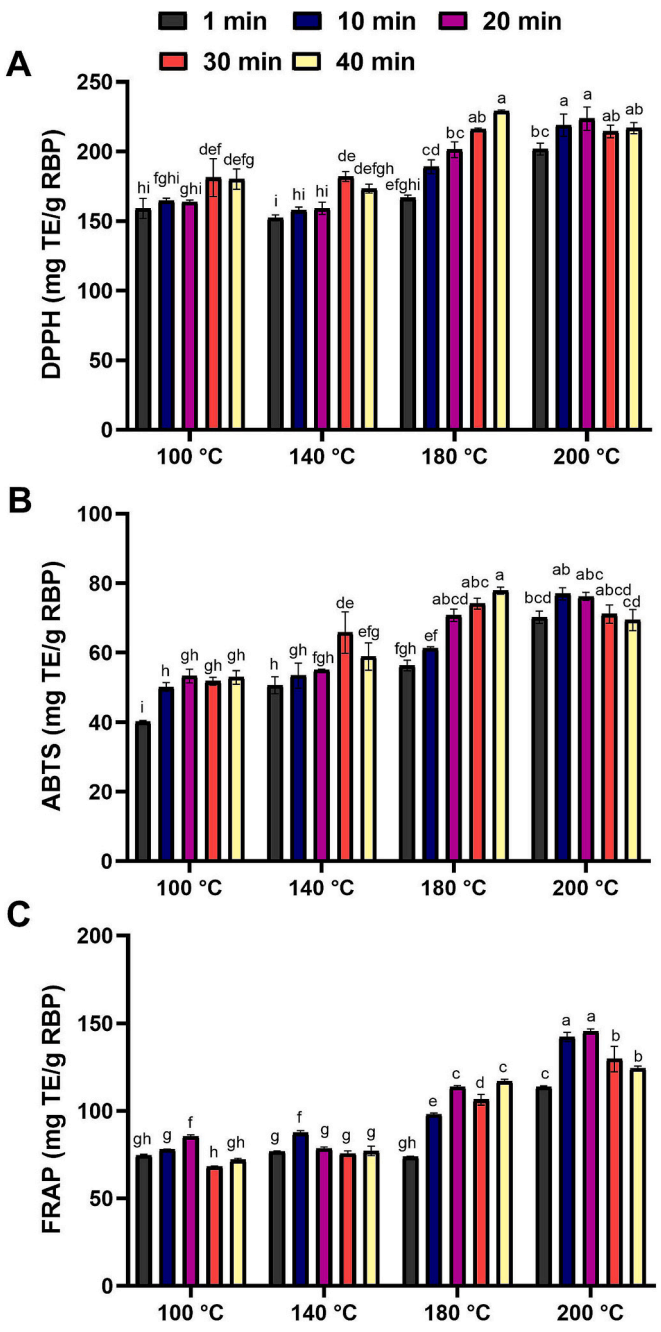


**Fig. 2.** Antioxidant capacity (DPPH, ABTS, and FRAP assays) in raspberry pomace (RBP) extracts expressed as mean ± standard deviation. Data are reported on a dry weight basis. Bars with different letters are significantly different ($p \leq 0.05$). (**A**) DPPH, (**B**) ABTS, and (**C**) FRAP assay. TE: Trolox equivalent.

Baltacıoğlu et al. (2021) reported that TPC in tomato samples increased increasing the microwave temperature.

In the current study, it has been investigated the impact of the MAE on the flavonoid molecular class. Based on the results shown in Fig. 1B, total flavonoids ranged from 37.59 to 64.12 mg RE/g RBP. TFC exhibited a marked increase employing the highest selected temperature for at least 10 min, recording values equal to 62.80 mg RE/g RBP (10 min), 62.71 mg RE/g RBP (20 min), 64.12 mg RE/g RBP (30 min) and 63.82 mg RE/g RBP (40 min) with no significant difference among them. The results presented herein were higher than the 33.78–18.18 mg RE/g RBP obtained through the conventional extraction method, which means approximately an increase of 90 and 250 %, respectively.

It can be noticed that the results of the antioxidant capacity of extracts measured by DPPH, ABTS and FRAP assays were in accordance with each other.

The results presented in Fig. 1C show a significant decline in anthocyanins content (TMAC results) of RBP extracts as the processing microwave temperature increases from 100 °C to 200 °C. The highest TMAC is observed at 100 °C and with a lower extraction time, while higher temperatures and longer extraction times significantly affect anthocyanin stability (thermal sensitivity of anthocyanins), emphasizing the need for optimized processing conditions.

Regarding the DPPH assay (Fig. 2A), the RBP extracts were affected significantly by temperature and time. The highest antioxidant activity was recorded at elevated temperature in particular at 200 °C for 10 min (218.99 mg TE/g RBP) and 20 min (223.56 mg TE/g RBP), and at 180 °C for 40 min (229.09 mg TE/g RBP), while the lowest was recorded at 140 °C for 1 min (152.31 mg TE/g RBP) of treatment.

In the ABTS assay (Fig. 2B), the measurements spanned from 40.08 mg TE/g RBP (100 °C for 1 min) to 77.84 mg TE/g RBP (180 °C for 40 min). Higher value was also registered at 200 °C after 10 min of MW extraction (76.92 mg TE/g RBP) which represents the maximum peak at this temperature.

In addition, for the antioxidant capacity measured by FRAP (Fig. 2C), values from 68.04 mg TE/g RBP at 100 °C for 30 min to 145.39 mg TE/g RBP at 200 °C for 20 min were obtained.

In summary, after the statistical analysis and following a reasoning on the operational timing, the condition of 200 °C for 10 min was selected as optimum to obtain the highest values of antioxidant capacity, total phenolics and flavonoids. The only exception was TMAC, which highest values were obtained at 100 °C for 1 min due to its rapid thermal degradation. Elez Garofulić et al. (2023) concluded that 60 °C with a shorter irradiation time less than 4 min is the optimal condition for MAE of chokeberry pomace's anthocyanins. Moreover, according to Chen et al. (2020) red raspberry anthocyanins degrade rapidly at 37 °C during storage producing small molecular constituents responsible for the color loss in juice and model systems. Despite this, the principal emphasis of this study is to obtain an extract rich in phenolic compounds resistant to heat treatment, storage and human digestion.

### *3.3. Effect of in vitro digestion on bioaccessibility of phenolic compounds and antioxidant capacity of MAE extract*

It is important to understand how the digestion processes affect the bioaccessibility and bioactivity of phenolic compounds after consumption. The efficacy of functional ingredients regarding their therapeutic or physiological activities is strictly related to their bioaccessibility. Bioaccessibility is defined as the amount of bioactive compounds released from the food matrix (or drugs) during oral administration, which then become accessible for absorption in the small intestine or are subject to colonic fermentation by gut microbiota (Fernández-García et al., 2009). The bioaccessibility of phenolics is highly variable, as it is influenced by factors such as enzymes, pH and bile salts in the stomach and intestinal environments, which can lead to the degradation or transformation of these compounds. It has been reported that highly-polymerized phenolics (flavonoids and tannins) or those bound to nutrients, like proteins or sugars, could be hydrolyzed and biotransformed into low molecular weight compounds (Ferreira-Santos et al., 2024).

Polyphenols bioaccessibility after gastric and intestinal phase (TPC, TFC and phenolic profile by HPLC-ESI-MS) and their antioxidant activity are shown in Table 2. Considering the TPC measured by Folin-Ciocalteu assay, the oral and gastric digestion dramatically decreased the amounts of phenolics (79.86 and 77.21 mg GAE/g RBPE, respectively) compared to their initial concentration (95.76 GAE/g RBPE). However, the intestinal digestion led to an increase of TPC to 108.39 mg GAE/g RBPE which means a bioaccessibility of 113 %. This observation is in line with several findings which reported that the intestine plays a central role in the release of phenolic compounds. For instance, Yang et al. (2018)

**Table 2**
Influence of *in vitro* gastrointestinal digestion on phenolic composition, antioxidant capacity and bioaccessibility index (BI) of raspberry pomace extract (RBPE).

| Components | Non-digested | Oral | Gastric | Intestinal | BI (%) |
|---|---|---|---|---|---|
| TPC (mg GAE/g RBPE) | 95.76 ± 0.28$^{b}$ | 79.86 ± 1.22$^{c}$ | 77.21 ± 1.32$^{c}$ | 108.39 ± 4.22$^{a}$ | 113 |
| TFC (mg RE/g RBPE) | 67.06 ± 2.98$^{a}$ | 41.82 ± 0.46$^{c}$ | 38.39 ± 1.03$^{c}$ | 58.50 ± 2.14$^{b}$ | 87 |
| Individual phenolic compounds (μg/g RBPE) | | | | | |
| Ferulic acid | 0.12 ± 0.02$^{a}$ | 0.06 ± 0.01$^{b}$ | nd | nd | 0 |
| *p*-Coumaric acid | 5.57 ± 0.05$^{b}$ | 4.97 ± 0.26$^{b}$ | 14.01 ± 0.89$^{a}$ | 17.35 ± 1.67$^{a}$ | 311 |
| Protocatechuic acid | 99.25 ± 5.06$^{b}$ | 62.65 ± 2.96$^{c}$ | 50.74 ± 1.71$^{c}$ | 147.90 ± 9.39$^{a}$ | 149 |
| Gallic acid | 1955 ± 83$^{b}$ | 2193 ± 73$^{b}$ | 2484 ± 31$^{b}$ | 3826 ± 313$^{b}$ | 196 |
| 4-Hydroxibenzoic acid | 17.12 ± 0.89$^{c}$ | 28.18 ± 2.06$^{b}$ | 27.19 ± 0.93$^{b}$ | 58.17 ± 2.74$^{a}$ | 340 |
| Vanillic acid | 3.79 ± 0.24$^{b}$ | 2.76 ± 0.18$^{c}$ | 1.67 ± 0.08$^{d}$ | 6.50 ± 0.32$^{a}$ | 172 |
| Rutin | 1.94 ± 0.11$^{c}$ | 2.82 ± 0.27$^{bc}$ | 3.36 ± 0.39$^{b}$ | 6.11 ± 0.38$^{a}$ | 315 |
| Catechin | 11.63 ± 0.08$^{b}$ | 14.26 ± 0.35$^{b}$ | 1.51 ± 0.22$^{c}$ | 27.49 ± 2.14$^{a}$ | 236 |
| Epicatechin | 2.47 ± 0.07$^{b}$ | 3.87 ± 0.29$^{b}$ | 0.32 ± 0.01$^{c}$ | 5.52 ± 0.82$^{a}$ | 223 |
| Vanillin | 20.50 ± 0.19$^{c}$ | 37.0 ± 1.48$^{b}$ | 19.85 ± 2.69$^{c}$ | 70.74 ± 7.52$^{a}$ | 345 |
| Quercetin | 0.27 ± 0.00$^{a}$ | 0.06 ± 0.01$^{b}$ | nd | nd | 0 |
| Antioxidant capacity | | | | | |
| DPPH (mg TE/g RBPE) | 235.22 ± 1.27$^{a}$ | 216.48 ± 4.18$^{b}$ | 143.61 ± 4.49$^{d}$ | 180.80 ± 1.89$^{c}$ | – |
| FRAP (mg TE/g RBPE) | 186.34 ± 4.68$^{a}$ | 188.09 ± 5.39$^{a}$ | 82.65 ± 5.39$^{b}$ | 63.50 ± 5.45$^{c}$ | – |
| ABTS (mg TE/g RBPE) | 87.80 ± 0.48$^{a}$ | 78.53 ± 0.87$^{b}$ | 36.86 ± 2.01$^{d}$ | 43.88 ± 1.60$^{c}$ | – |

Values of phenolic content and antioxidant capacity are expressed as mean ± standard deviation. Data are reported on a dry weight basis. Different letters in the same raw represent statistically different results ($p \leq 0.05$).
TPC: total phenolic content; TFC: total flavonoid content; GAE: gallic acid equivalent; RE: rutin equivalent; TE: Trolox equivalent; nd: not detected.

demonstrated that *in vitro* digestion involving bile salts resulted in a fivefold increase in the release of phenolics compared to the counterpart without bile. This enhanced liberation of phenolics can be attributed to the emulsifying properties of bile salts, which facilitate the breakdown of non-covalent complexes formed between these bioactive molecules and the gastric enzymes, especially pepsin, thereby improving their solubility and accessibility. Moreover, flavonoid molecules suffer an intense degradation in the intestine into small metabolites, usually represented by phenolic acids, increasing the amount of antioxidant molecules (Tarko et al., 2013).

As expected, the TFC quantified by the aluminum chloride method showed a slight decrease after the intestinal digestion reporting a bioaccessibility index of 87 %. Similarly, Feng et al. (2023) reported a bioaccessibility of total flavonoid in lotus leaf after simulated gastric and intestinal digestions of 86 %.

A more detailed analysis of the phenolic composition in RPBE demonstrated the impact of *in vitro* digestion on these antioxidant compounds across the various digestive phases. As detailed in Table 2, the HCPL-ESI-MS analysis revealed initially eleven phenolic compounds in RBPE belonging phenolic acids (ferulic, *p*-coumaric, protocatechuic, gallic, 4-hydroxibenzoic and vanillic acid) and flavonoids (rutin, catechin, epicatechin, vanillin and quercetin), reaching a total concentration of 2.12 mg/g RBPE. Gallic acid was identified as the predominant phenolic compound in RBPE, with a concentration of 1955 μg/g RBPE, accounting for 90 % of the total phenolic content. In contrast, the literature reports a higher prevalence of anthocyanins and ellagitannins in fresh raspberries. The elevated concentration of gallic acid in RBPE is strictly related with the thermal degradation of these complex molecules during MAE. Ellagitannins are hydrolysable tannins consisting of a saccharide core, mainly glucose, esterified with hexahydroxydiphenic acid and gallic acid, which account approximately for 60 % of the total phenolic content. As explained by Sójka et al. (2019) the thermal degradation of ellagitannins in raspberry, particularly trimeric lambertianin C and dimeric sanguiin H-6, gives as main end products gallic and ellagic acids. Additionally, acid gallic is generated through the degradation pattern of anthocyanins, specifically cyanidin-3-*O*-glucoside (Hao et al., 2021). Significant alterations in the composition of phenolic compounds in RBPE were observed during the simulated *in vitro* gastrointestinal digestion. Quercetin and ferulic acid were not detected at the end of digestion. Recently, Kashyap et al. (2022) reported the degradation of these compounds after GID of meghalayan cherry (*Prunus nepalensis*) pomace extracts. Simultaneously, the bioaccesibility of *p*-coumaric acid, protocatechuic acid, gallic acid, 4-hydroxibenzoic acid, vanillic acid, rutin, catechin, epicatechin and vanillin increased by the end of the digestion process. This suggests that high-molecular-weight compounds, such as tannins or their derivatives, were biotransformed into simpler molecules through the action of digestive enzymes and pH variations. The depolymerization is essential, as previously explained in the case of gallic acid, leading to smaller monomeric and dimeric species with potentially higher bioaccessibility. This phenomenon was also documented in studies on grape and blackcurrant pomace, where especially phenolic acids exhibited an increase and high bioaccessibility post-digestion (dos Santos Lima et al., 2024; Ferreira-Santos et al., 2024).

Scientific evidence indicates that the phenolic compounds are the primary contributor to antioxidant capacity (Jacobo-Velázquez & Cisneros-Zevallos, 2009). In this work, we investigated the effect of *in vitro* digestion on two accepted mechanisms of antioxidant activity: electron donation (FRAP assay) and radical scavenging activity (DPPH and ABTS assays). As shown in Table 2, the undigested RBPE demonstrated antioxidant values of 235.22, 186.34 and 87.80 mg TE/g RBPE for DPPH, FRAP and ABTS, respectively. However, in contrast to the phenolic content, our results suggest that the antioxidant capacity increased only during the gastric phase and then declined significantly during the intestinal phase, indicating the low bioactivity of the smaller molecules formed. The latter is explained by the fact that the antioxidant properties are strictly influenced differently by each phenolic compound (Di Majo et al., 2008). After GID, the radical scavenging capacity, measured by DPPH and ABTS, decreased to 180.80 and 43.88 mg TE/g RBPE, respectively, as well as the reducing antioxidant power to 63.50 by FRAP assay. Similarly, Cruz et al. (2024) demonstrated that digestion affected the bioactivity of barbados gooseberry fruit, decreasing its antioxidant properties measured by FRAP, CUPRAC and ORAC assays.

### 3.4. Regulation of cytokine release by macrophages incubated with RBPE

The inflammatory response is a complex, tightly regulated immune process activated by tissue damage or infection. Pathogen-associated and damage-associated molecular patterns (PAMPs and DAMPs) bind to receptors in immune cells, triggering a signaling cascade that initiates inflammation. Normally, this response is temporary, but in chronic diseases, prolonged inflammation can contribute to cancer development. The inflammasome is a cytosolic protein complex that, upon assembly, activates caspase-1 to convert pro-IL-1β into IL-1β, promoting acute inflammation. This process is initiated by the binding of molecules to Toll-like receptors (TLRs), which produce pro-IL-1β, and inflammasome assembly then activates IL-1β release to promote acute inflammation (Lamkanfi & Dixit, 2012).

To assess the anti-inflammatory potential of plant infusions, the production of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) and two cytokines that can act as pro- or anti-inflammatory biomarkers (IL-10

and TGF-β1) were analyzed in macrophages. Pro- and anti-inflammatory cytokines promote and inhibit inflammation, respectively (L. Chen et al., 2017).

Phenolic compounds are key bioactive components in raspberry extracts (as reported in previous results), known to reduce interleukins involved in inflammation signaling (Jean-Gilles et al., 2012; Lopez-Corona et al., 2022). Phenolic compounds are known for their anti-inflammatory properties, with anthocyanins, flavonoids (quercetin, catechin, rutin, etc), and some phenolic acids like gallic acid, specifically reducing pro-inflammatory cytokines. The anti-inflammatory activity of phenolic compounds may be due to their ability to balance oxidative stress in cells and suppress the signaling of pro-inflammatory transduction (Dobani et al., 2021).

In this study, the RBPE, rich in phenolic compounds, especially flavonoids and phenolic acid (Table 2), was evaluated for its anti-inflammatory effect by measuring cytokine release from non-activated and activated macrophages *in vitro* (Fig. 3).

The macrophage treatment with RBPE increased IL-10 production, confirming its anti-inflammatory potential (Fig. 3D). However, its greatest impact was observed in LPS-activated macrophages, where RBPE reduced the pro-inflammatory cytokine IL-1β (Fig. 3B) and further enhanced IL-10 (Fig. 3D), effectively shifting the cytokine profile towards an anti-inflammatory response. These results suggest that RBPE suppresses inflammasome activation induced by LPS, as previously

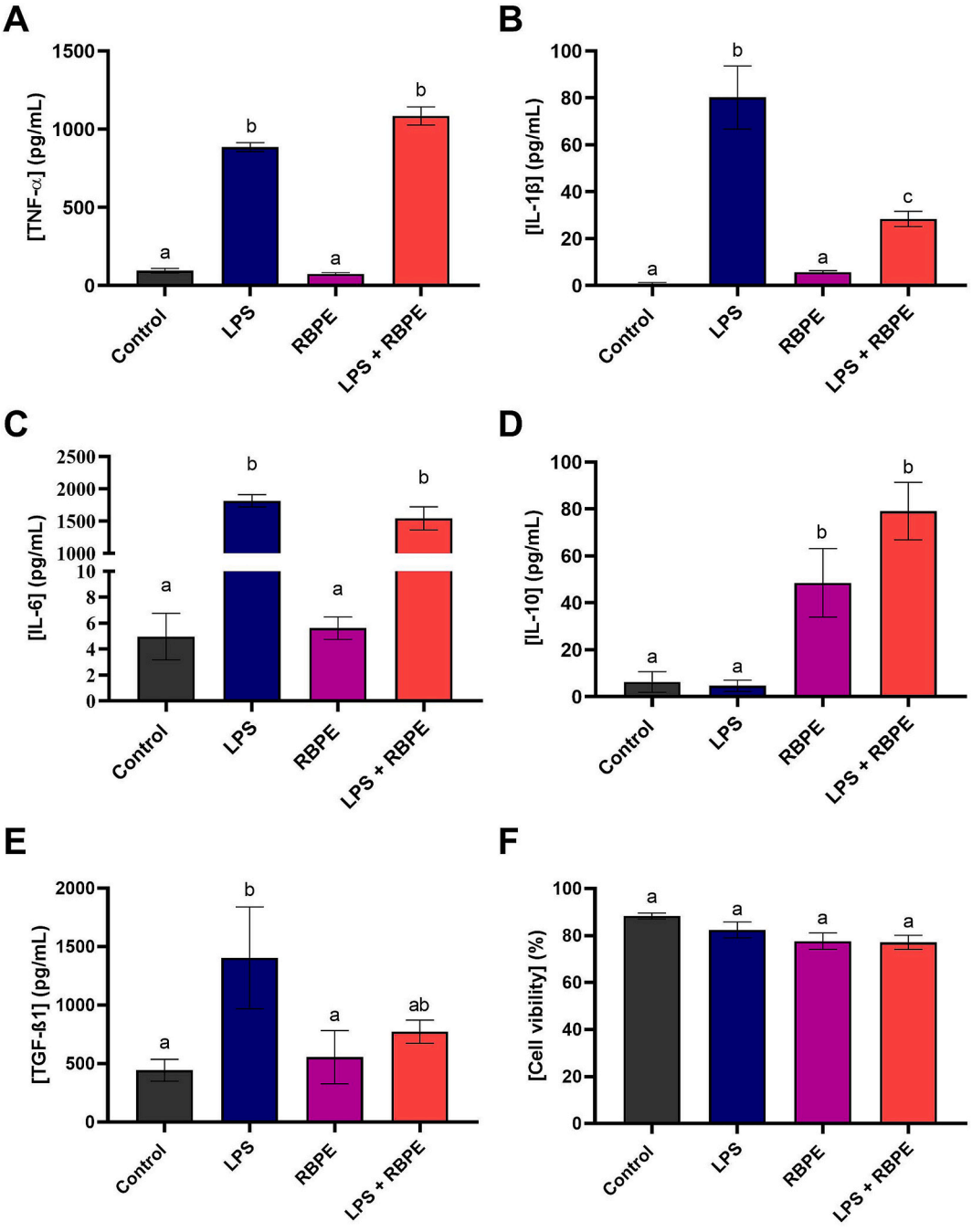


**Fig. 3.** Measurement of pro- and anti-inflammatory cytokine production in J774 macrophages treated with raspberry pomace extracts (RBPE, 300 μg/mL) for 24 h. The experimental conditions included: untreated cells (control), cells treated with RBPE alone, and cells pre-treated with LPS for 8 h before RBPE exposure (LPS groups). (**A**) tumor necrosis factor-α (TNF-α), (**B**) interleukin-1β (IL-1β), (**C**) interleukin-6 (IL-6), (**D**) Interleukin-10 (IL-10), and (**E**) transforming Growth Factor-Beta 1 (TGF-β1). J774 macrophage viability after treatments are presented in graph (**F**). Bars with different letters are significantly different ($p < 0.05$).

reported in Caco-2 cells infected with pathogenic *E. coli* O157 (Xue et al., 2019). Bibi et al. reported that raspberry intake reduces colorectal inflammation and carcinogenic risk in rats with dextran sulfate sodium-induced colitis by increasing the release of the anti-inflammatory cytokine IL-10 and observed attenuated pro-inflammatory markers and mediators (Bibi et al., 2018). In another study, Yahfoufi and colleagues showed high efficiency of raspberry extracts in suppressing nitric oxide synthesis and reduced the levels of IL-1β and IL-6 in LPS/IFN-γ-activated RAW 264.7 macrophages (Yahfoufi et al., 2018). Moreover, no significant difference was observed in cell viability with LPS and RPME treatments (Fig. 3F), noting more than 75 % viability for all treatments.

## 4. Conclusion

This study underscores the potential of RBP using green solvents and innovative extracting methods. Employing MAE under optimized conditions (200 °C for 10 min with 50 % ethanol), the research demonstrates a significant enhancement in the recovery of phenolics and flavonoids compared to conventional extraction methods.

A critical dimension of the study is its investigation into the bioaccessibility and bioactivity of the phenolic extract. The *in vitro* digestion experiments reveal that while certain phenolic compounds degrade during digestion, others, particularly phenolic acids, become more bioaccessible during the intestinal phase. This phenomenon highlights the pivotal role of digestive biotransformation in enhancing the physiological availability of bioactives.

The study also delves into the anti-inflammatory properties of the selected RBPE, demonstrating their ability to modulate inflammatory responses in activated macrophages. By suppressing pro-inflammatory cytokine IL-1β and promoting the release of anti-inflammatory IL-10, the extracts exhibit significant potential for therapeutic applications in managing inflammation-related disorders.

This research advocates for the integration of MAE into scalable industrial processes and calls for further studies to validate the *in vivo* efficacy of these bioactive-rich extracts. By addressing both environmental sustainability and human health, this work sets the stage for the development of innovative, eco-friendly solutions in food science and nutraceutical development.

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## CRediT authorship contribution statement

**Yassine Jaouhari:** Writing – original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. **Matteo Bordiga:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Fabiano Travaglia:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Jean-Daniel Coisson:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Augusto Costa-Barbosa:** Writing – review & editing, Investigation, Data curation. **Paula Sampaio:** Writing – review & editing, Methodology, Investigation, Data curation. **Cláudia Botelho:** Writing – review & editing, Data curation. **Beatriz Gullón:** Writing – review & editing, Resources, Project administration, Funding acquisition, Conceptualization. **Pedro Ferreira-Santos:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Data availability

Data will be made available on request.

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