Extraction of bioactive compounds from Rubus idaeus bioresidues: a full screening on phenolic composition and bioactive potential

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Abstract

Purpose: *Rubus idaeus* cultivation has boosted productivity due to its high nutritional value. In consequence, waste production increased. The discarded biomass, including leaves and aerial components, can be transformed into valuable functional ingredients for industrial applications, such as cosmetics. Studying their bioactivity potential is of high relevance.

Methods: According to the present idea, the research involved the extraction of phenolic compounds from raspberry plant branches and leaves by employing four different techniques, namely aqueous decoction, aqueous infusion, hydroethanolic maceration, and ultrasound-assisted extraction (UAE). Subsequently, these compounds were screened for their bioactive potential, including antioxidant, antibacterial, anti-inflammatory, and cytotoxic properties.

Results: The UAE-assisted extraction has yielded extracts with more phenolic compounds, while the infusion and maceration result in higher contents of flavonols. Among the identified phenolic compounds, hydrolysable tannins, particularly galloyl-*bis*-HHDP-glucose, were found to be the most dominant ones. Regarding antioxidant potential, the decoction extract was the strongest, while the infusion showed the greatest potential for inhibiting lipid peroxidation. The UAE extract was found to be highly effective as an antibacterial agent. Both infusion and UAE extracts demonstrated the highest anti-inflammatory potential.

Conclusion: The combination of these results highlights the sample's bioactive potential and the importance of exploiting bioresidues as unique, sustainable candidates for industrial applications.

Statement of Novelty

This study examines the bio-residues of the *Rubus idaeus* species, a two-meter-high deciduous bush. Raspberries, known for their antioxidant properties, are widely exported, consumed and used in various industrial sectors. A by-product of their cultivation, the leaves, and other aerial parts, which are commonly used in infusions for having properties similar to berries, are emerging as an abundant and valuable raw material to be exploited as a source of functional ingredients, becoming an important focus of study. Their extracts hold important bioactivity, indicating the potential to be used in key industries such as cosmetics. In this context, this work addresses the full characterisation of extracts obtained by different techniques ultrasound-assisted, maceration, infusion and decoction envisaging identifying the ones with higher potential focusing on antioxidant, antimicrobial, and anti-inflammatory applications.

Introduction

This work comprised a study on a species from the *Rubus* genus belonging to the Rosaceae family: *Rubus idaeus*, a two-meter-high deciduous bush. Its white flowers produce a red and spherical berry fruit that grows on its branches, composed of many connected drupelets, which are individual sections of the fruit, each one with its seed, surrounding a central core, commonly called raspberry [1]. In general, berry species are widely known, and their fruits are appreciated for their antioxidant properties. In 2015 the biggest raspberry exporters were Serbia, Poland, and Chile [2], and the largest consumer of fresh and frozen raspberries was the United States.

Due to their short shelf-life, most traded raspberries are sold in frozen blocks and treated as a sensitive commodity that is subsequently used in beverages, teas, artificial sweeteners, and fruited wines. Until now, the production and the harvested area have increased for *R. idaeus*, most significantly since 2003, reaching in 2018 almost 900 kilotonnes. Regarding its global distribution, Europe is responsible for 74.2% of net production [3]. One solution for bioresidues is the recovery and incorporation into new agri-food derived, cosmetic or pharmaceutical products. Due to their high content of phenolic acids, flavonoids, and fibres, these by-products could be successfully recovered for different industrial purposes.

While raspberries have already been studied in detail, their bio-residues have not been exploited so far, but some results point out the leaves as a potential source of phenolic compounds, around 800 mg/100 g, by fresh weight [4]. These compounds have been reported as excellent antioxidants that could be used in cosmetic formulations.

Regular consumption of vegetables and fruits with high loads of polyphenols can help protect from the damaging effects of UV radiation. This text highlights the health benefits of botanicals with photoprotective properties that reduce oxidative stress, inflammation and cancer risk. It is gaining attention from researchers in the cosmetic and pharmaceutical industries due to its cost-effectiveness [5]. Antioxidants can be a useful tool to reduce oxidative damage in skin applications [6]. Since the start of the 21st century, there has been a growing trend towards using natural extracts, especially those obtained from plants. Many of these plants have adapted to their environment by developing photo-adaptive mechanisms. These mechanisms include producing antioxidant and UV-absorbing compounds in response to intense radiation. [5]. Cosmetic formulations with natural benefits are popular due to advances in plant constituent knowledge, human molecular biology, and cell physiology.

The ultimate goal of using a natural extract is to provide the basis for a better cosmetic product benefiting the consumer [6]. A study on cosmetic formulations with natural products reported that the preparation of a topical skin cream formulation containing both placebo and raspberry extract concluded that their stability was similar. This assay was preceded by a fruit extract selection, between two different cultivars of *Rubus idaeus*, and the one presenting the better antioxidant capacity was used in the assay [7]. In a recent report, researchers found that the wild red raspberry variety *R.*

idaeus has the highest concentration of anthocyanins and antioxidant compounds among other cultivars studied. Based on these findings, the authors decided to use the stem cells of *R. idaeus* to develop a new cosmetic active ingredient. Additionally, they extracted stem cells from the plant's leaves, which had potential cosmetic applications [8].

One of the most significant characteristics to monitor as a guarantee of consumer safety is the microbiological quality of cosmetic products; therefore, preservatives are mandatory. However, bacteria have developed resistance to some of the commonly applied preservatives due to their comparable mechanism of action to antibiotics. On the other hand, their usage is restricted because it can harm human health at certain levels. Researchers face new problems in developing new products with anti-microbiological capabilities while remaining non-toxic to consumers, increasing the need for new biocidal compounds with excellent toxicological compatibility [9].

Therefore, this work intended to evaluate the high added value potential of biowaste resulting from the production of raspberry, namely from the pruning process. The obtained extracts were characterised by their content of phenolic compounds and also studied according to the envisioned cosmetic future application through antioxidant, antimicrobial and anti-inflammatory.

Material and Methods

Plant material

The aerial sections of *Rubus idaeus* were courteously donated by the private enterprise Hortitool Consulting Lda based in Baião, Portugal, a city located at an elevation of 577 meters above sea level, in September 2019. After 5 days of air drying at ambient temperature (about 25 ºC), the raw material was ground to 20 mesh (0.8 mm) using a ZM200 Retsch-type grind. Following that, the material that was dried had been shielded from light and humidity until analysis.

Preparation of the R. idaeus extracts

Aqueous extracts

Infusion

In a boiling solution of 200 mL distilled water, 1 g of powdered sample was added and kept for 5 minutes to extract bioactive components.

Decoction

After adding 1g of powdered material to 200mL of water, the mixture was boiled for 5 minutes.

The samples were gravity filtered with a Whatman paper filter no. 4 for both aqueous extracts. After that, the aqueous extracts were frozen at -24°C, lyophilised (Freeze Dryer Scanvac, Coolsafe, 4x10-4mbar, Lillerod, Denmark) at -100°C, and stored in a dry environment.

Hydroethanolic extracts

Maceration: About 1 gram of milled material was added to 30 mL of 80:20 ethanol-water solution and stirred at room temperature for an hour.

Ultrasound-assisted Extraction (UAE): This extraction was carried out in a sonicator (Qsonica, Q500, ultrasonic processor sonicator, 20 KHz, Newtown, USA) following the procedure described by Heleno et al. (2016) with slight modifications to improve the extraction. In short, 1 g of powdered sample was mixed with 150 mL of an ethanol-water solution (80:20, *v/v*). To avoid scorching the samples, the extraction was carried out over 10 min, with 30-sec cycles and 10-sec pauses in between, at 375 W (75% of 500 W) using an ice bath.

The mixtures were gravity filtered before being evaporated at 40°C, 100 rpm, and 90 mbar under reduced pressure (Büchi R-114, rotary evaporator; Büchi B-480, water bath, and Büchi B-721, vacuum controlling system, Flawil, Switzerland). The aqueous residue was then lyophilised, stored in a dry chamber, and then frozen at -24°C.

The extracts were subjected to UV decontamination before each bioactivity evaluation.

Chemical characterisation in terms of phenolic compounds

The phenolic compounds in each extract were identified and quantified using the procedure described by Bessada et al. [10]. Each 10 mg of weighed sample was dissolved in 1 mL of ethanol-water solution (20:80 *v/v*), then filtered through a 0.22 µm disposable filter. The phenolic profile was determined using a Dionex Ultimate 3000, a high-performance liquid chromatographic system with a diode array detector and an electrospray ionisation mass spectrometry detector (HPLC-DAD-ESI/MS) (Dionex Ultimate 3000 UPLC and Linear Ion Trap LQT XL, Thermo Scientific, San Jose, CA, USA).

To quantify the identified molecules, calibration curves were developed for each chemical using authentic standards (Extrasynthése S.A., Genay, France). The phenolic compounds contained in the extracts were identified using the referred standards and data from the literature. The Xcalibur®

data system was used to collect and process the data (Thermo Scientific, San Jose, CA, USA). The results were provided in milligrams per gramme of extract.

Bioactive properties

Antibacterial activity

Different bacterial strains isolated from hospitalised patients (Hospital Center of Trás-os-Montes e Alto Douro, Vila Real, Portugal) were tested to determine the antibacterial capacity of the developed extracts, namely five Gram-negative bacteria: *Escherichia coli* (isolated from urine), *Proteus mirabilis* (isolated from wound exudate), *Klebsiella pneumoniae* (isolated from urine), *Pseudomonas aeruginosa* (isolated from expectoration) and *Morganella morganii* (isolated from urine); and three Gram-positive bacteria: *Enterococcus faecalis* (isolated from urine), *Listeria monocytogenes* (isolated from cerebrospinal fluid), and methicillin-resistant *Staphylococcus aureus* (MRSA) (isolated from expectoration). As indicated in the work of Pires et al. [11], the experiment was carried out using the microdilution technique with the addition of de *p*-iodonitrotetrazolium chloride (INT) (Panreac Applichem-Barcelona, Spain) as a marker. Ampicillin (from 0.15 to 20 mg/mL), imipenem, and vancomycin (from 0.0078 to 1 mg/mL) were used as positive controls. Bacterial viability control was also created using the medium applied with each bacterial strain. The extracts were evaluated at various concentrations, and the minimum inhibitory/bactericidal concentrations (MIC/MBC) were calculated and represented as mg/mL.

Antioxidant activity

The study used two tests, Oxidative Haemolysis Inhibition (OxHLIA) and Thiobarbituric Acid Reactive Substances (TBARS), to measure the antioxidant activity. The OxHLIA test was conducted based on Lockowandt *et al.* [12], which monitored the extracts' ability to protect erythrocyte membranes against free radical-induced oxidation. The extracts were dissolved in phosphate-buffered saline (PBS) to create a stock solution of 1.0 mg/mL, which was then diluted to make various working concentrations. Trolox (7.81–250 g/mL) was utilized as a positive control. The findings were presented as IC50 values (µg/mL) for Δt of 60 and 120 minutes, indicating the extract concentration needed to preserve 50% of the erythrocyte population unharmed for 60 and 120 minutes, respectively.

The TBARS experiment was performed according to Gómez-Mejía et al. [13], in which the ability of the extracts to prevent lipidic peroxidation is measured by the decrease of TBARS formation via the creation of malondialdehyde (MDA)-TBA, which can be measured at 532 nm (Specord 200 spectrophotometer, Analytik Jena, Jena, Germany). The EC_{50} values were calculated using Trolox as a positive control and expressed as the concentration (mg/mL) providing 50% antioxidant activity.

Cytotoxicity

The extracts were tested against MCF-7, NCI-H460, AGS, and CaCo2 cell lines representing breast adenocarcinoma, non-small-cell lung cancer, gastric adenocarcinoma, and colorectal adenocarcinoma respectively. The non-tumour cell line consisted of porcine liver cells (PLP2), and African green monkey kidney cells (VERO), using the sulphorodamine B technique to detect the potential toxicity of the produced extracts in non-tumour cell lines [14]. Using a positive control, ellipticine, the extracts were evaluated in a 400 to 1.56 mg/mL concentration range. The GI_{50} values, which represent the concentration inhibiting 50% of cell growth, were measured in µg/mL.

Anti-inflammatory activity

Mice macrophage cell lines (RAW264.7) were adopted in this study, according to the technique described by Taofiq et al. [15]. Different concentrations of the extracts were evaluated, ranging from 400 to 1.56 µg/mL. This effect was established on the ability of the extract to suppress NO generation, resulting in an active concentration with anti-inflammatory properties. NO generation was measured in a microplate reader (Bio-Tek Instruments, ELX800) using dexamethasone as the positive control, and absorbance at 540 nm for a calibration curve.

Statistical Analysis

Experiments were conducted in triplicate, and findings were given as average ± standard deviation. The data was analysed using an analysis of variance (ANOVA), with a Tukey's test for homoscedastic samples and a Tamhane T2 for non-homoscedastic samples. When comparing two independent samples, the variance was analysed using a t-test. All analyses were performed at a threshold of significance of 0.05.

Results and Discussion

Phenolic compounds

Table 1 shows the chromatographic characteristics, tentative identification, and individual quantification of phenolic compounds in *R. idaeus* leaf extracts obtained by HPLC-DAD/ESI-MS. Sixteen phenolic compounds were tentatively identified: five phenolic acids (ellagic and chlorogenic acid derivatives), three flavonoids (one flavonol and two flavan-3-ols), and eight hydrolysable tannins.

Peak 1 ($[M-H]^-$ at *m/z* 633) and peaks **8**-**10** ($[M-H]^-$ at *m/z* 935) were assigned as hexahydroxydiphenoyl group, being identified as galloyl-HHDP-glucose and galloyl-bis-HHDP-glucose, respectively. The fragment at *m/z* 301 represented evidence of the presence of an (HHDP) group [ellagic–H]$^-$ in

the molecule [16]. Raspberry leaf extracts have revealed the presence of compounds that showed an UV spectrum coherent with galloyl and HHDP derivatives [17], therefore confirming these identifications. **Peaks 5** and **6** ($[M-H]^-$ at *m/z* 783) were assigned to pedunculagin isomers according to their pseudomolecular ion and fragmentation pattern as described by [18, 19]. Similarly, **peak 7** ($[M-H]^-$ at *m/z* 1251) was identified as punicalagin gallate [20].

Peak 2 ($[M-H]^-$ at *m/z* 355) was tentatively identified as dihydrocaffeoylquinic based on the parent ion, leading to the MS^2 base peak of *m/z* 191 corresponding to dihydrocaffeic acid mainly by the neutral loss of 164 u.

Peaks 11-14 were tentatively identified as ellagic acids derivatives. Thus, **peaks 11** and **14** ($[M-H]^-$ at *m/z* 433) were assigned as ellagic acid pentoside, producing a base fragment at *m/z* 301, corresponding to a deprotonated ellagic acid, revealing the loss of a pentose moiety (−132 u). Similarly, **peak 12** ($[M-H]^-$ at *m/z* 477) was tentatively identified as a methyl ellagic acid hexoside. **Peak 13** was positively identified as ellagic acid according to its retention time, mass spectra and UV–Vis characteristics by comparison with standards found in the market. According to the literature, *Rubus* leaves are rich in ellagic acid derivatives [21, 22], where ellagic acid may occur in its free form, but also be released through hydrolysis of ellagitannins [17, 23, 24].

Flavan-3-ols were also detected in raspberry leaves, revealing **peaks 3** ($[M-H]^-$ at *m/z* 577) and **4** ($[M-H]^-$ at *m/z* 865) a λ_{max} spectra around 276–281 nm, characteristic of this group of compounds, these compounds were tentatively assigned to a procyanidin dimer and procyanidin trimer, respectively. The only flavone (**peak 15**) detected was positively identified compared to a commercial standard.

Hydrolysable tannins, including peaks **1, 5-10,** and **16**, were the primary phenolic compounds in *R. idaeus* leaf extracts.Considering the different extraction methods, ultrasound-assisted extraction was more effective in extraction in terms of total phenolic compounds (109 ± 3 mg/g of extract), while for the flavonols and flavan-3-ols class, the most efficient extraction method was infusion and maceration.

Altogether, the extracted phenolic compounds are abundant, giving those extracts the characteristics sought for cosmetic use. Previous studies show that high polyphenols content extracts are useful for treating and preventing oxidative stress-induced premature skin aging. The advantageous characteristics of polyphenols, which are especially pertinent for topical administration, include antioxidant activity, protection against UV damage, inhibition of skin proteinases, anti-microbial activity, and anti-carcinogen action [25].

Ellagic acid may lead to improvement of hyperpigmentation and dark spots, melasma, increased UV damage protection, and seems to have antiwrinkle activity according to Sharifi-Rad et al. [26]. Chlorogenic acids skin-related benefits are described as antioxidant and anti-aging; photoprotective and antitumor; anti-inflammatory, and antibacterial [27]. Procyanidin possesses antioxidant, antitumor, anti-inflammatory, immunosuppressive, and antiallergy properties and protective properties against chronic diseases and metabolic disorders [28]. The identified flavone, luteolin-7-*O*-glucuronide, may be an ideal candidate to alleviate severe inflammatory responses and oxidative stress according to Cho et al. [29].

The major phenolic compound present, galloyl-*bis*-HHDP-glucose, is described in the literature as exerting anti-inflammatory effects [30, 31]. Pedunculagin isomers, other hydrolyzable tannins, are described as antioxidants and anticancer [32].

Insert Table 1

Bioactive potential

Antibacterial activity

The microdilution method was used to study the antibacterial properties against pathogenic bacteria. All extracts inhibited most of the tested bacterial strains, with UAE extracts being the most effective. UAE samples inhibited the Gram-negative bacteria *E. coli*, *K. pneumoniae*, *M. morganii*, and *P. mirabilis* with a 5 mg/mL MIC. In a report by Khalifa et al. [33] when studying raspberry aqueous extracts to inhibit *E. coli* and *P. aeruginosa*, concentrations of 25 mg/mL and 50 mg/mL were used. Also, whilst in that work, a MIC of 25 mg/mL against *L. monocytogenes* was found, in the present work, 5 mg/mL was determined.

Regarding the Gram-positive bacteria, it was achieved for raspberry UAE extract a MIC of 2.5 mg/mL against *E. faecalis* and the methicillin-resistant *S. aureus*. In contrast, previous works have achieved a MIC of 50 mg/mL for this last strain with aqueous raspberry extracts [33].

In another report, Krauze-Baranowska et al. [34] studied the antimicrobial activity of raspberry shoot aqueous extracts reporting a MIC of 60 mg/mL against *K. pneumoniae* and 120 mg/mL against *E. faecalis*, whilst in this work, a MIC of 5 mg/mL and 2.5 mg/mL respectively, were determined. That report presented better results using the MRSA, where a MIC of less than 1 mg/mL was achieved compared with the present work's best result of 2.5 mg/mL using the UAE extract. In another study, Bauza-Kaszewska et al., [35] found that all three *R. idaeus* preparations tested (seedless, seeds, and pomace) had no or low antibacterial activity against the tested microorganisms, one of which being *L. monocytogenes*, which was also studied in the current study in which the infusion extract presented a MIC of 5 mg/mL.

Regarding Minimum Bactericidal Concentration (MBC) results, none of the studied extracts presented bactericide effects. Khalifa et al. [33] determined bactericide results against the Gram-negative strain *P. aeruginosa* and the Gram-positive *L. monocytogenes*, finding a 100 mg/mL result

for both.

Krauze-Baranowska et al. [34], obtained a MBC of 120 and 60 mg/mL alongside *E. faecalis* and *K. pneumoniae*, respectively. Again, the present work obtained a better result against MRSA, with an MBC of 1 mg/mL. Thus, the tested extracts could be used as bioactive compounds capable of inhibiting the bacterial growth of some pathogenic strains.

Antimicrobial activity is an important bioactivity in the development of a cosmetic, as it is a parameter that will contribute to increasing the shelf life of the final product. The antimicrobial activity of extracts from raspberry residues can be explained by the presence of dihydrocaffeoylquinic acid [27].

Insert Table 2

Antioxidant activity

The study evaluated the antioxidant activity of raspberry extracts from its aerial parts, using two methods - OxHLIA and TBARS **(Table 3)**. Among the extracts, the one obtained through decoction showed the highest antioxidant activity after 60 minutes, followed by macerations. However, there were no significant differences found between the antioxidant activities of the extracts obtained through infusion and UAE. At 120 minutes, the only statistical difference found was between infusion and UAE. At 60 minutes, none of the samples showed better activity than the positive control, Trolox, although decoction had a similar response, while at 120 minutes, both the decocted and macerated samples showed better activity than the pure compound (Trolox). Comparing the two analysis times, for all extraction types, the best antioxidant activity was found after 60 minutes, with a statistically significant decrease at 120 minutes. Considering the TBARS assay, the best extract was the one of infusion, showing a statistically lower EC_{50} than the maceration. In contrast, the decoction and UAE did not show significant differences. The differences in the antioxidant activity between the two assays are mostly related to the antioxidant mechanisms of each assay.

As reported in a previous work involving the study of leaf extracts from seven different raspberry cultivars concerning their antioxidant activity, they showed values of 88.1 mmol of Trolox equivalents for the most active sample [21]. Vara et al. [36] studied red raspberry hydroethanolic extracts, which presented an IC_{50} of 298 mg/mL for OxHLIA and an EC_{50} of 122 mg/mL for TBARS assay. Thus, *R. idaeus* aerial parts presented better antioxidant activity than its fruits.

UV light and pollutants may also produce oxidative stress, which can deplete the natural antioxidant reserve. This can lead to loss of cellular integrity, increased matrix metalloproteinases, wrinkle formation, metastases, and peroxidation of fatty acids. Natural polyphenols have shielding effects against radical oxygen species, making them promising for antiaging applications. Antioxidant activity manifested by raspberry waste extracts can be explained by the presence of dihydrocaffeoylquinic acid, luteolin-7-*O*-glucuronide, pedunculagin isomers, and mostly due to procyanidin.

Insert Table 3

Cytotoxicity

Insert Table 4

The UAE and infusion extracts showed the lowest IC_{50} values in the anti-inflammatory assay, indicating stronger activity. Still, the ones obtained by infusion did not show statistically significant differences towards the maceration sample. Finally, the decoction was the least effective extraction type in terms of anti-inflammatory activity. The UAE was also the extract with higher antitumor activity against all tested cell lines, showing statistically significant lower IC_{50} values than the extracts obtained by the other techniques. This shows that UAE preserves polyphenols with higher biological properties towards cell lines. Still, none of the samples showed an activity close to the positive controls. In terms of cytotoxicity to normal cell lines, none of the extraction techniques showed extracts with toxicity, representing more than 400 cells per plate.

Proof of the cytotoxic safety of extracts from raspberry bioresidues allows taking advantage of their excellent antitumor and anti-inflammatory properties. The antitumor behavior can be mainly attributed to dihydrocaffeoylquinic acid and procyanidin isomers and, in a more residual way, to pedunculagin. Inflammation is a tissue's defence mechanism against pathogen invasions, cell injury, and irritation, and acts as a means of removing injured and necrotic cells. It can be acute or chronic, and chronic inflammation is thought to be a major factor in the emergence of chronic illnesses. The studied extracts have been shown to have relevant anti-inflammatory properties. They may have been evidenced by the major presence of the compound galloyl-*bis*-HHDP-glucose, as well as dihydro caffeoylquinic acid, procyanidin isomers, and luteolin-7-*O*-glucuromide, also present in the extracts.

Conclusions

The exponential evolution of waste biomass derived from berry crops, in which raspberry is included, is a new study focus since producers are increasingly interested in its valorisation in high-added-value products, under the European Commission's recommendations to achieve the zero-waste goal. According to the data obtained from the various types of extractions, all the employed methods enabled the obtention of the target molecules, namely phenolic compounds, which exhibited bioactive potential. It should be noted that the tested extraction techniques may be used to achieve significant quantities of phenolics; for example, in the UAE, achieving the greatest yields for some of the key compounds was possible. Maceration

was the most effective technique for extracting phenolic acids and flavonols. In terms of bioactivity, all of the extracts were active, with the UAE extract being the most active. The antioxidant capabilities of the extracts were also shown to be favourably linked with the level of hydrolysable tannins. These findings highlight the sample's bioactive potential and the necessity of exploiting bioresidues as distinct options for industrial applications by harnessing their biological properties. In this sense, the leaves and other aerial components are good examples of waste biomass that can be exploited for several applications, namely cosmetic formulations.

Declarations

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Conflict of interest The authors declare they have no conflict of interest.

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Tables

Table 1. Raspberry aerial parts different extracts retention time (Rt), maximum absorption wavelengths (λmax), mass spectral data, and phenolic component quantification (mg/g extract).

Peak	Rt (min)	λ_{max} (nm)	[M-H] *m/z*	MS^2 (*m/z*)	Identification	Decoction	Infusion	Maceration	UAE
1	4.55	286	633	481(13), 463(12), 301(100)	Galloyl-HHDP-glucose[1]	6.00±0.20^{a}	4.17±0.02^{b}	3.50±0.03^{c}	4.85±0.05^{b}
2	5.26	298sh319	355	209(32), 191(100)	Dihydrocaffeoylquinic acid[2]	8.50±0.40^{b}	10.10±0.30^{a}	10.70±0.50^{a}	9.60±0.40^{a}
3	6.69	281	577	451(41), 425(62), 407(100), 289(73), 287(17)	Procyanidin dimer[3]	8.80±0.10^{c}	8.69±0.03^{c}	10.40±0.20^{b}	13.00±1.00^{a}
4	7.51	281	865	739(15),577(33), 575(20), 425(21), 407 (100), 289(45), 287(85)	Procyanidin trimer[3]	6.07±0.03^{c}	11.05±0.03^{a}	10.10±0.10^{b}	10.00±1.00^{b}
5	8.18	269	783	633(52), 301(100)	Pedunculagin isomer 1 (bis-HHDP-glucose) [1]	3.21±0.04^{b}	3.89±0.03^{b}	3.79±0.03^{b}	4.74±0.05^{a}
6	10.24	275	783	481(11), 301(100)	Pedunculagin isomer 2 (bis-HHDP-glucose) [1]	5.60±0.20^{a}	4.59±0.02^{b}	2.98±0.01^{c}	4.37±0.05^{b}
7	12.23	280sh378	1251	1083(4), 781(13), 601(4), 301(13)	Punicalagin gallate[1]	8.50±0.20 ^{a}	6.70±0.10^{b}	4.08±0.02^{d}	5.77±0.05^{c}
8	13.36	276	935	633(15),301(18)	Galloyl-bis-HHDP-glucose isomer 1 [1]	12.60±0.30^{c}	16.80±0.20^{b}	21.90±0.20^{b}	33.30±0.50^{a}
9	14.56	281	935	633(15),301(18)	Galloyl-bis-HHDP-glucose isomer 2[1]	1.46±0.00^{a}	1.44±0.00^{a}	1.39±0.00^{a}	0.17±0.01^{b}
10	15.01	289	935	633(15),301(18)	Galloyl-bis-HHDP-glucose isomer 3[1]	1.58±0.03^{a}	1.70±0.02^{a}	1.70±0.02^{a}	0.66±0.04^{b}
11	16.85	248sh362	433	301(100)	Ellagic acid pentoside[1]	2.43±0.03^{a}	2.51±0.03^{a}	2.23±0.02^{a}	2.24±0.03^{a}
12	17.80	254sh362	477	301(100)	Methyl ellagic acid hexoside[1]	10.50±0.30^{a}	8.40±0.10^{b}	10.00±0.30^{a}	8.90±0.20^{b}
13	19.30	253sh360	301	256(6), 185(15)	Ellagic acid[1]	3.24±0.03^{a}	3.12±0.02^{a}	2.45±0.03^{b}	2.38±0.03^{b}
14	20.13	248sh355	433	301(100)	Ellagic acid pentoside isomer[1]	2.73±0.02^{b}	2.45±0.04^{c}	3.01±0.03^{a}	2.63±0.03^{b}
15	21.06	258sh357	461	285(100)	Luteolin-7-*O*-glucuronide[4]	5.40±0.10^{b}	5.69±0.05^{a}	5.70±0.10^{a}	4.70±0.10^{c}
16	22.92	350sh380	1083	781(12), 601(25), 301(93)	Punicalagin[1]	1.74±0.02^{a}	1.66±0.03^{a}	1.91±0.02^{a}	1.05±0.03^{b}
					TPA	32.73±0.03^{b}	32.00±1.00^{b}	34.17±0.03^{a}	30.00±1.00^{c}
					TF3O	14.90±0.10^{c}	19.70±0.10^{b}	20.50±0.20^{b}	23.00±1.00^{a}
					TOF	5.40±0.10^{a}	5.69±0.05^{a}	5.70±0.10^{a}	4.70±0.10^{b}
					THT	40.80±0.50^{b}	41.00±0.50^{b}	41.30±0.40^{b}	55.00±1.00^{a}
					TPC	88.00±1.00^{d}	93.00±1.00^{c}	96.00±1.00^{b}	109.00±3.00^{a}

TPA-Total phenolic acids, TF3O – Total flavan-3-ol, TOF-Total flavonols, THT-Total hydrolysable tannins, TPC-Total phenolic compounds; UAE-Ultrasound Assisted Extraction; calibration curves used: 1- ellagic acid (y = 26719x – 317255, R^2 = 0.9986; LOD = 0.41 µg/mL and LOQ = 1.22 µg/mL), 2- chlorogenic (y = 168823x - 161172; R^2 = 0.9999; LOD = 0.20 µg/mL; LOQ = 0.68 µg/mL), 3- catechin (y = 84950x – 23200, R^2 = 0.9999; LOD 0.17

µg/mL; LOQ 0.68 µg/mL), 4- apigenin-7-*O*-glucoside (y = 10683x - 45794; R^2 = 0.999; LOD = 0.10 µg/mL; LOQ = 0.53 µg/mL). nd- not detected. Different letters in the same row show significant difference between means of the same compounds in different extraction methods.

Table 2. Antibacterial ability of raspberry aerial parts extracts is reported in mg/mL.

	UAE		Maceration		Decoction		Infusion		Ampicillin		Imipenem		Vancomycin	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Gram-negative bacteria														
Escherichia coli	5	>20	5	>20	5	>20	5	>20	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
Klebsiella pneumoniae	5	>20	5	>20	5	>20	10	>20	10	20	<0.0078	<0.0078	n.t.	n.t.
Morganella morganii	5	>20	5	>20	5	>20	10	>20	20	>20	<0.0078	<0.0078	n.t.	n.t.
Proteus mirabilis	5	>20	10	>20	10	>20	20	>20	<015	<0.15	<0.0078	<0.0078	n.t.	n.t.
Pseudomonas aeruginosa	20	>20	20	>20	20	>20	20	>20	>20	>20	0.5	1	n.t.	n.t.
Gram-positive bacteria														
Enterococcus faecalis	2.5	>20	2.5	>20	5	>20	5	>20	<0.15	<0.15	n.t.	n.t.	<0.0078	<0.0078
Listeria monocytogenes	10	>20	20	>20	20	>20	5	>20	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
MRSA	2.5	>20	2.5	>20	2.5	>20	2.5	>20	<0.15	<0.15	n.t.	n.t.	0.25	0.5

n.t.- not tested; MIC- minimum inhibitory concentration in mg/mL; MBC- minimum bactericidal concentration in mg/mL; MRSA- methicillin resistant *Staphylococcus aureus*

Table 3. Antioxidant activity of raspberry aerial parts.

	OxHLIA (IC_{50} values, µg/mL)		TBARS (EC_{50} values, µg/mL)
	Δt = 60 min	Δt = 120 min	
Decoction	21.9 ± 0.3a,*	30.4 ± 0.4a	8.0 ± 0.1 c
Maceration	24.1 ± 0.4b,*	34.8 ± 0.5b	4.0 ± 0.2b
Infusion	26 ± 1c,*	49 ± 1c	3.00 ± 0.02a
UAE	28 ± 1c,*	57 ± 1d	8.0 ± 0.3c
Trolox	21.8 ± 0.2	43.5 ± 0.3	5.8 ± 0.6

Different letters in each column translate statistically significant differences ($p < 0.05$) between extraction techniques. Asterisks in the first column mean a significant difference between each of the analysed times of the OxHLIA assay. Both analyses were performed with a significance of 0.05. UAE-Ultrasound Assisted Extraction.

Table 4. Anti-inflammatory activity (IC_{50} µg/mL) and cytotoxicity (GI_{50} µg/mL) of raspberry aerial parts extracts.

	Decoction	Infusion	Maceration	UAE	Positive control
Anti-inflammatory activity					**Dexamethasone**
RAW 264.7	62 ± 7c	32 ± 3a,b	38 ± 5b	27 ± 3a	6.3 ± 0.4
Cytotoxicity in tumour cells					**Ellipticine**
MCF-7 (breast carcinoma)	257 ± 16c	220 ± 1b	290 ± 1d	183 ± 6a	1.02 ± 0.02
NCI-H460 (non-small cell lung carcinoma)	132± 4b	108 ± 7a	161 ± 9c	93 ± 8a	1.01 ± 0.01
AGS (gastric adenocarcinoma)	207 ± 6b	228 ± 6c	261 ± 6d	161± 10a	1.23 ± 0.03
CaCo2 (colorectal adenocarcinoma)	294 ± 8d	245 ± 13b	269 ± 8c	228 ± 13a	1.21 ± 0.02
Cytotoxicity in non-tumour cells					
VERO	>400	>400	>400	>400	1.41 ± 0.06
PLP2	>400	>400	>400	>400	1.4 ± 0.1

Different letters in each line mean statistically significant differences with a significance of 0.05. UAE-Ultrasound Assisted Extraction.

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