Blackberries and Mulberries from Covilhã as Health Promotors: Physical and Phytochemical Characterization and Biological Potential

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Mariana Sofia Morgado Martins

Dedication

To the woman who always tries to be better tomorrow than she was today
To the woman who didn't know she would be here
To the woman who doesn't know where she will go
To the woman who doesn't dare to give up!

To my mum,
who taught me how to be this woman.

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Obrigado, mãe, por ser uma inspiração constante e por ser a base sólida sobre a qual construí a minha vida.
Que eu um dia seja metade da mulher que tu és!

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Resumo

As amoras são pequenos frutos de verão, delicados e muito apreciados pelos consumidores devido à sua versatilidade de implementação na alimentação, apreciáveis características organoléticas e riqueza nutricional. De facto, esta pequena baga possui uma cor apelativa, sabor doce, elevado teor em água e baixo conteúdo de carbohidratos. Além das suas características nutricionais apreciáveis numa dieta equilibrada, a presença de compostos bioativos na sua constituição contribui para a promoção de efeitos metabólicos e fisiológicos importantes, sendo expectável algum impacto positivo na prevenção de algumas patologias, tais como cancro, doenças neurodegenerativas e cardiovasculares. Perante as evidências, o interesse do potencial biológico das amoras tem despertado a atenção das comunidades académicas, médicas, produtores e dos consumidores.

Tendo em consideração as potenciais propriedades promotoras da saúde das amoras e uma vez que a produção desta fruta também tem vindo a aumentar a nível mundial, em termos científicos é de extrema importância a caraterização físico-química e a avaliação do potencial biológico deste fruto vermelho. Assim sendo, neste trabalho procedeu-se à caracterização físico-química e à avaliação do potencial biológico de três espécies de amoras pretas portuguesas, mais concretamente (i) a amora cultivada (*Rubus fruticosus* L), (ii) a amora silvestre (*Rubus ulmifolius* Schott) e (iii) a amora de árvore (*Morus nigra* L). Tendo em conta os dados físico-químicos obtidos, o comprimento e a largura variaram entre 14,34-20,42 mm e 14,31-25,58 mm, respetivamente, tendo a espécie *R. fruticosus* apresentado o maior comprimento e a espécie *R. ulmifolius* a maior largura. O peso variou entre 2,16 a 7,75 g, tendo a espécie *R. fruticosus* apresentado o valor mais alto. Por outro lado, a firmeza variou de 5,5 N (*R. ulmifolius*) a 5,6 N (*R. fruticosus*), enquanto os teores de humidade e cinzas variaram entre 75,8% (*M. nigra*) e 79,1% (*R. fruticosus*) e, entre 97,7% (*R. ulmifolius*) e 99,5% (*R. fruticosus*), respetivamente. A espécie *R. fruticosus* foi a que exibiu a cor mais escura. Relativamente ao perfil fenólico obtido por HPLC-DAD-ESI/MS^n, foram identificados um total de 20 compostos, nomeadamente 8 antocianinas, 6 flavonóis, 1 ácido hidroxicinâmico, 3 ácidos hidroxibenzóicos e 2 taninos, que foram, posteriormente, quantificados por HPLC-DAD. Nas matrizes de amoras estudadas, a cianidina-3-*O*-glucósido (2) foi o composto maioritário, com valores que variaram entre 9202,2 *µg/g* na espécie *R. fruticosus* e 26845,2 µg/g na espécie *R. ulmifolius*. Por outro lado, a análise por ICP-MS e FAAS permitiu a identificação de um total de 27 minerais, incluindo 13 essenciais e 14 não-essenciais. O Ca foi o composto predominante em todas as amostras estudadas, enquanto o mineral Pb foi o menos abundante. Tendo em conta o aroma agradável das amoras, o seu conteúdo em compostos voláteis orgânicos foi também explorado por SPME/GC–MS. No total foram identificados 68 elementos, distribuídos por 7 classes, nomeadamente, 17 aldeídos, 13 álcoois, 3 cetonas, 20 ésteres, 2 furanos, 8 hidrocarbonetos e 5 ácidos. Como esperado, foram encontradas diferenças qualitativas e quantitativas dos parâmetros

avaliados entre as diferentes espécies. Comparando os valores obtidos, a *R. fruticosus* aparenta ser a espécie com as características mais apelativas, tanto para o consumidor como para os produtos e mercado.

Perante o valor bioativo e fitoquímico apresentado pelas amoras, nomeadamente o seu conteúdo fenólico, e sabendo que o mesmo está intimamente relacionado com o potencial biológico atribuído a este fruto, procedeu-se à obtenção de extratos concentrados nestes compostos. Os mesmos foram obtidos por extração em fase sólida. Numa primeira parte, avaliou-se a atividade antioxidante *in vitro* destes contra os radicais 1,1-difenil-2-picril-hidrazila (DPPH•), oxido nítrico (•NO) e superóxido ($O_2^{\bullet -}$). Após a análise dos resultados obtidos, constatou-se que a espécie *R. fruticosus* foi a mais ativa contra o DPPH•, enquanto a espécie *R. ulmifolius* foi a mais ativa contra o •NO (IC_{50}=34,29 ± 0,55 µg/mL e IC_{50}=59,49 ± 0,81 µg/mL, respetivamente). Por outro lado, no ensaio do $O_2^{\bullet -}$, foi o extrato rico em fenólicos proveniente da *M. nigra* aquele que apresentou os resultados mais notáveis, seguido do extrato da *R. fruticosus* (IC_{25}=14,26 ± 0,47 µg/mL e IC_{25}=14,70 ± 0,58 µg/mL, respetivamente). Adicionalmente, e de maneira a despistar possíveis efeitos sinérgicos, cada um dos três extratos foi ainda combinado com diferentes concentrações do controlo positivo, i.e., ácido ascórbico. De uma forma geral, os resultados obtidos foram muito promissores e incentivam a realização de outros ensaios complementares.

Posteriormente, avaliou-se a capacidade citotóxica dos extratos em células provenientes dos fibroblastos (NHDF) de forma a encontrar concentrações não tóxicas para células não-cancerígenas. De seguida, e usando as concentrações selecionadas tendo como base o estudo anterior, foram estudados os possíveis efeitos destas na inibição da proliferação de células de carcinoma Caco-2 do cólon humano. Entre as espécies estudadas, o extrato que apresentou um potencial anticancerígeno mais notável, após exposição por 24 horas, foi o extrato de *R. fruticosus*. Adicionalmente, e tendo em conta que o uso de moléculas anticancerígenas sintéticas intensifica o surgimento de vários efeitos secundários indesejáveis, foi decido estudar o efeito de um dos fármacos antitumorais sintéticos mais conhecidos, i.e., 5-fluorouracilo, de forma a perceber se a sua combinação com o extrato proveniente da espécie *R. fruticosus* apresenta potencial sinérgico ou antagónico. Uma vez mais, os resultados obtidos foram bastantes promissores.

De uma forma geral, os resultados obtidos neste estudo permitiram concluir que as amoras derivadas das três espécies de plantas estudadas possuem um grande potencial biológico e incentivam a sua incorporação em suplementos alimentares e em novas aplicações terapêuticas, farmacêuticas e nutracêuticas. Todavia, é indispensável a realização de mais estudos, principalmente estudos clínicos e em modelos animais, para demonstrar todo o potencial biológico destas frutas e determinar a dosagem segura a ingerir.

Palavras-chave

Amoras; Compostos fenólicos; Benefícios para a saúde; Potencial biológico; Propriedades antioxidantes; Potencial anticancerígeno.

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Resumo Alargado

Os frutos e vegetais são cada vez mais o foco de estudos científicos devido à sua relevância a nível de alimentação saudável, bem-estar, e importâncias médica e terapêutica. Este destaque está intimamente relacionado com os vários compostos bioativos presentes nos mesmos que, por sua vez, conferem-lhes notáveis propriedades antioxidantes, anti-inflamatórias, anticancerígenas e de antienvelhecimento, assim como proteção contra doenças cardiovasculares, neurodegenerativas e metabólicos (diabetes, hipertensão arterial, entre outras).

De entre os frutos, as amoras (*Rubus* e *Morus*) são um dos frutos de verão mais apreciados pelos consumidores, devido ao seu sabor doce e aroma agradável, à sua cor, elevada percentagem de água, e baixo teor de gorduras e calorias, assim como também devido à sua riqueza em vários compostos bioativos que já demonstraram capacidade para produzir notórios efeitos fisiológicos e metabólicos importantes na prevenção de algumas patologias, tais como doenças cardiovasculares, neurodegenerativas e cancro.

Tendo em conta o mencionado, neste projeto de investigação realizou-se a caracterização de 3 frutas vermelhas semelhantes e altamente apreciadas pelos consumidores, 2 espécies de arbusto (*R. fruticosus* e *R. ulmifolius*) e 1 espécie de árvore (*M. nigra*). Tendo em conta o elevado interesse e consequente aumento da sua produção, que se traduz no aumento do seu impacto económico, foi determinado estudar-se as características físico-químicas destas, nomeadamente o seu peso, firmeza, acidez, sólidos solúveis totais, entre outras características, e os seus valores nutricionais a fim de selecionar aquela com maior valor comercial e potencial biológico. Tendo em conta os dados físico-químicos, o comprimento e a largura variaram entre 14,34-20,42 mm e 14,31-25,58 mm, respetivamente, tendo a espécie *R. fruticosus* apresentado maior comprimento e a espécie *R. ulmifolius* maior largura. O peso variou entre 2,16 e 7,75 g, tendo a espécie *R. fruticosus* apresentado o valor mais alto. Por outro lado, a firmeza variou de 5,5 N (*R. ulmifolius*) a 5,6 N (*R. fruticosus*), enquanto os teores de humidade e cinzas variaram entre 75,8% (*M. nigra* L.) e 79,1%. *Fruticosus*) e, entre 97,7% (*R. ulmifolius*) e 99,5% (*R. fruticosus*), respetivamente. A espécie *R. fruticosus* foi a que exibiu a cor mais escura. O perfil fenólico foi identificado por HPLC-DAD-ESI/MS^n e quantificado por HPLC-DAD. No total, foram determinados 20 compostos fenólicos, incluindo 8 antocianinas e 12 compostos fenólicos não corados. A cianidina 3-*O*-glucósido foi a antocianina predominante e os derivados de quercetina foram os compostos fenólicos não corados mais abundantes. Por outro lado, através de ICP-MS e FAAS foram determinados um total de 27 minerais, incluindo 13 elementos essenciais e 14 não essenciais. O cálcio e o magnésio foram os mais abundantes em todas as espécies, enquanto o Pb foi o encontrado em menores quantidades. Um total de 68 voláteis foram identificados usando SPME/GC-MS, nomeadamente 17 aldeídos, 13 álcoois, 3 cetonas, 20 ésteres, 2 furanos, 8 hidrocarbonetos e 5 ácidos. Como esperado, foram

observadas diferenças qualitativas e quantitativas entre as espécies. Todas as famílias descritas influenciam o potencial nutricional, o aroma e o interesse dos consumidores.

A caracterização inicial das amoras permitiu concluir que as amoras da região da Covilhã apresentam uma boa composição nutricional e bioativos, o que pode ser uma mais-valia para o desenvolvimento de novos produtos dietéticos, nutracêuticos, farmacêuticos e outros. Além disso, foram encontradas diferenças qualitativas e quantitativas nos parâmetros avaliados entre as diferentes espécies. Comparando os valores obtidos, a *R. fruticosus* aparenta ser a espécie com as características mais apelativas, tanto para o consumidor como para os produtos e mercado.

De seguida, e usando os extratos concentrados em fenólicos das variedades, obtidos por extração em fase sólida, avaliou-se o potencial antioxidante destes, assim como o potencial proveniente da sua combinação com o controlo positivo usado, que foi o ácido ascórbico. O objetivo deste último ensaio foi despistar a existência de uma possível interação sinérgica, potenciando a atividade de ambos.

De facto, é do conhecimento geral que o metabolismo celular gera radicais livres de azoto e de oxigénio necessários para a sobrevivência e atividade das células, cujos níveis são maioritariamente controlados por enzimas antioxidantes intracelulares, de nomear a catalase, a glutationa peróxido e a superóxido dismutase. Contudo, a idade, o estilo de vida sedentário, os hábitos tabágicos e etílicos, entre outros, contribuem para um anormal aumento destas espécies que, consequentemente, podem reagir com proteínas, lípidos e DNA de células saudáveis, danificando-as, contribuindo deste modo para o aceleramento do envelhecimento e para o desenvolvimento de variadíssimas doenças, como o cancro, doenças neurodegenerativas e cardiovasculares, diabetes, entre outras. Portanto, para diminuir a formação excessiva de radicais livres e os seus efeitos deletérios no corpo humano, é essencial a adoção de uma dieta rica em compostos antioxidantes. De facto, é altamente recomendado a ingestão diária de 400 g de frutas e vegetais. Sendo assim, a atividade antioxidante dos extratos das amoras foi avaliada através de ensaios *in vitro* contra os radicais 1,1-difenil-2-picril-hidrazila (DPPH•), óxido nítrico (•NO) e superóxido ($O_2^{\bullet-}$). A espécie *R. fruticosus* revelou ser a mais ativa contra o DPPH• (IC_{50}=34,29 ± 0,55 µg/mL) enquanto a espécie *R. ulmifolius* foi a que revelou ter maior potencial em capturar o •NO (IC_{50}=59,49 ± 0,81 µg/mL). Por outro lado, no ensaio do $O_2^{\bullet-}$, as espécies *M. nigra* e a *R. fruticosus* foram as que revelaram melhores resultados (IC_{25}=14,26 ± 0,47 µg/mL e 14,70 ± 0,58 µg/mL, respetivamente). Relativamente à sua combinação com o controlo positivo, e entre as misturas realizadas, a combinação da espécie *M. nigra* com ácido ascórbico (25:75) foi a mistura que apresentou a maior atividade contra o DPPH•, revelando um IC_{50} de 3,14 ± 0,18 µg/mL, apresentando um potencial sinérgico. Por outro lado, a nível de atividade contra o •NO, a mistura *M. nigra* + ácido ascórbico (75:25) foi a mais promissora (IC_{50}=16,97 ± 0,88 µg/mL). Por outro lado, no ensaio de captação do $O_2^{\bullet-}$, a combinação da espécie *R. ulmifolius* +

ácido ascórbico (25:75) foi a que revelou ser a mais promissora, apesentando um valor de IC_{25} de 7,73 ± 0,33 µg/mL.

Os dados obtidos através deste estudo suportam as evidências já reportadas de que as amoras apresentam uma promissora capacidade antioxidante, sendo capazes de capturar radicais livres e, muito provavelmente, proteger as células dos danos causados por estes.

Finalmente, os efeitos de citotoxicidade dos extratos foram testados, primeiro em células normais de fibroblastos dérmicos humanos (NHDF) e, depois, em células de carcinoma Caco-2 do cólon humano. A primeira avaliação foi a fim de selecionar concentrações não tóxicas para células não-cancerígenas. Com as concentrações selecionadas, testou-se o efeito dos extratos nas células Caco-2. A realização deste ensaio permitiu verificar que, entre os 3 extratos estudados, o da espécie de cultivo *R. fruticosus* é aquele que possui um efeito anti-proliferativo mais notável. De seguida, combinou-se o extrato mais promissor, com o fármaco anti-tumoral 5-fluorouracilo (5-FU) a fim de perceber se a combinação de ambos potência os efeitos de inibição do crescimento das células Caco-2. Com este último ensaio realizado, foi possível perceber que, a combinação 50:50 entre a concentração mínima de 5-FU estudada (0,65 µg/mL) em conjunto com a concentração mais alta do extrato promissor testada (800 µg/mL) apresenta potencial sinérgico e altamente notável contra esta linha tumoral estudada. Estes resultados, são altamente promissores, incentivando a combinação de extratos de amoras com novas abordagens terapêuticas, farmacológicas e nutracêuticas, assim como a incorporação destes em suplementos alimentares.

Palavras-chave

Amoras; Compostos fenólicos; Benefícios para a saúde; Potencial biológico; Potencial antioxidante; Potencial anticancerígeno.

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Abstract

Fruits and vegetables are increasingly the focus of scientific studies due to their relevance in terms of healthy eating, well-being, and medical and therapeutic importance. This highlight is closely related to the various bioactive compounds present in them, which in turn, gives them notable antioxidant, anti-inflammatory, anti-cancer and anti-aging properties, as well as protection against cardiovascular, neurodegenerative and metabolic diseases (diabetes, hypertension, among others).

Among the fruits, blackberries (*Rubus* and *Morus*) are one of the summer fruits most appreciated by consumers, due to their sweet flavor and pleasant aroma, their colour, high percentage of water, and low fat and calorie content, as well as due to its richness in several bioactive compounds that have already demonstrated the ability to produce notable physiological and metabolic effects that are important in the prevention of some pathologies, such as cardiovascular, neurodegenerative diseases and cancer.

This research project carried out the characterization of 3 similar red fruits highly appreciated by consumers, which were 2 species of shrub blackberries (*R. fruticosus* and *R. ulmifolius*) and 1 species of tree mulberry (*M. nigra*). Taking into account the increase in their interest and in their production, which translates into an increase in their economic impact, it was decided to study their physicochemical characteristics, namely their weight, firmness, acidity, total soluble solids, among other characteristics, and their nutritional values in order to select the one with the greatest commercial value and biological potential. Considering the physicochemical data, the length and width varied between 14.34-20.42 mm and 14.31-25.58 mm, respectively, with the species *R. fruticosus* having the greatest length and the species *R. ulmifolius* the greatest width. The weight varied between 2.16 g and 7.75 g, with the species *R. fruticosus* presenting the highest value. On the other hand, firmness varied from 5.5 N (*R. ulmifolius*) to 5.6 N (*R. fruticosus*), while moisture and ash contents varied between 75.8% (*M. nigra*) and 79.1% *R. fruticosus*) and between 97.7% (*R. ulmifolius*) and 99.5% (*R. fruticosus*), respectively. The species *R. fruticosus* was the one that exhibited the darkest color.

The phenolic profile was identified by HPLC-DAD-ESI/MS^n and quantified by HPLC-DAD. In total, 20 phenolic compounds were determined, including 8 anthocyanins and 12 unstained phenolic compounds. On the other hand, through ICP-MS and FAAS, a total of 27 minerals were determined, including 13 essential and 14 non-essential elements. Ca and Mg were the most abundant in all species, while Pb was found in smaller quantities. A total of 68 volatiles were identified using SPME/GC-MS, namely 17 aldehydes, 13 alcohols, 3 ketones, 20 esters, 2 furans, 8 hydrocarbons and 5 acids. As expected, qualitative and quantitative differences were observed between species. All families described influence the nutritional potential, aroma and consumer interest.

The initial characterization of blackberries allowed us to conclude that blackberries from Covilhã region have a good nutritional and bioactive composition, which could be an added value for the development of new dietary, nutraceutical, pharmaceutical and other products. Furthermore, qualitative and quantitative differences were found in the parameters evaluated between the different species. Comparing the values obtained, R. *fruticosus* appears to be the species with the most appealing characteristics, both for the consumer and for the products and market.

Then, using the concentrated phenolic extracts of the varieties, obtained by solid phase extraction, their antioxidant potential was also evaluated, as well as the potential arising from their combination with the positive control used, which was ascorbic acid. The objective of this last test was to detect the existence of a possible synergistic interaction, enhancing the activity of both.

In fact, it is common knowledge that cellular metabolism generates nitrogen and oxygen free radicals necessary for the survival and activity of cells, whose levels are mostly controlled by intracellular antioxidant enzymes, namely catalase, glutathione peroxide and superoxide dismutase. However, age, a sedentary lifestyle, smoking and drinking habits, among others, contribute to an abnormal increase in these species, which can consequently react with proteins, lipids and DNA of healthy cells, damaging them, thus contributing for the acceleration of aging and the development of a wide range of diseases, such as cancer, neurodegenerative and cardiovascular diseases, diabetes, among others. Therefore, to reduce the excessive formation of free radicals and their harmful effects on the human body, it is essential to adopt a diet rich in antioxidant foods. In fact, a daily intake of 400 g of fruits and vegetables is highly recommended. Therefore, the antioxidant activity of blackberry extracts was evaluated through in-house tests against the radicals 1,1-diphenyl-2-picrylhydrazyl (DPPH•), nitric oxide (•NO) and superoxide ($O_2^{\bullet-}$). The species *R. fruticosus* proved to be the most active against DPPH• (IC_{50}=34.29 ± 0.55 μg/mL) while the species *R. ulmifolius* was the one that revealed the greatest potential in capturing •NO (IC_{50}=59.49 ± 0.81 μg /mL). On the other hand, in the $O2^{\bullet-}$ test, the species *M. nigra* and *R. fruticosus* showed the best results (IC_{25}=14.26 ± 0.47 μg/mL and 14.70 ± 0.58 μg/mL, respectively). Regarding its combination with the positive control, and among the mixtures made, the combination of the species *M. nigra* with ascorbic acid (25:75) was the mixture that showed the greatest activity against DPPH•, revealing an IC_{50} of 3.14 ± 0.18 μg/mL, presenting synergistic potential. On the other hand, in terms of activity against •NO, the mixture *M. nigra* + ascorbic acid (75:25) was the most promising (IC_{50}=16.97 ± 0.88 μg/mL). On the other hand, in the $O2^{\bullet-}$ uptake assay, the combination of the species *R. ulmifolius* + ascorbic acid (25:75) was the one that proved to be the most promising, presenting an IC_{25} value of 7.73 ± 0.33 μg/mL.

The data obtained through this study supports the evidence already reported that blackberries have a promising antioxidant capacity, being able to capture free radicals, and most likely, protect cells from the damage caused by them.

Finally, the cytotoxicity effects of the extracts were tested, first on normal human dermal fibroblast (NHDF) cells and then on human Caco-2 colon carcinoma cells. The first evaluation was to select non-toxic concentrations for non-cancerous cells. With the selected concentrations, the effect of the extracts on Caco-2 cells was tested. Carrying out this test made it possible to verify that, among the 3 extracts studied, that of the cultivated species *R. fruticosus* is the one that has the most notable anti-proliferative effect. Next, the most promising extract was combined with the anti-tumor drug 5-fluorouracil (5-FU) in order to understand whether the combination of both would enhance the effects of inhibiting the growth of Caco-2 cells. With this last test carried out, it was possible to see that the 50:50 combination between the minimum concentration of 5-FU studied (0.65 μg/mL) together with the highest concentration of the promising extract tested (800 μg/mL) presents potential synergistic and highly notable against this studied tumor line. These results may also be highly promising, encouraging the combination of blackberry and mulberry extracts with new therapeutic, pharmacological and nutraceutical approaches, as well as their incorporation into dietary supplements.

Keywords

Blackberries; Mulberries; Phenolic compounds; Health benefits; Biological potential; Antioxidant potential; Anticancer potential.

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List of Acronyms

5-FU	5-Fluorouracil
Ca	Calcium
Caco-2	Human colonrectal adenocarcinoma cells
Cu	Copper
DAD	Diode array detector
DMEM	Dulbecco´s Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DPPH•	1,1-Diphenyl-2-picrylhydrazyl radical
DW	Dry weight
EDI	Estimated Daily Intake
EWI	Estimate Weekly Intake
Fe	Iron
FW	Fresh weight
GC-MS	Gas chromatography-mass spectrometry
H_2O_2	Peroxide hydrogen
LOD	Limit of detection
HEPES	L-glutamine, 4-(2-652 hydroxyethyl)-1-piperazineethanesulfonic acid
HNO_3	Nitric acid
HPLC	High-performance liquid chromatography
HS-SPME	Headspace solid-phase microextraction
IC_{25}	25% Inhibitory concentration
IC_{50}	Half-maximal inhibitory concentration
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
IL	Interleukins
K	Potassium
MBCs	Minimum bactericidal concentrations
Mg	Magnesium
Mn	Manganese
MICs	Minimum inhibitory concentrations
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Na	Sodium
NBT	Nitro Blue Tetrazolium
NADH	*β*-Nicotinamide adenine dinucleotide
NHDF	Normal Human Dermal Fibroblast

$^{\bullet}NO$	Nitric oxide radical
$O_2^{\bullet-}$	Superoxide radical
P	Phosphorus
PBS	Phosphate-buffered saline
PMS	Phenazine methosulfate
PTWI	Provisional Tolerable Weekly Intake
ROS	Reactive oxygen species
SNP	Sodium nitroprusside dihydrate
TA	Titratable acidity
TNF	Tumour necrosis factor
TSS	Total soluble solids
WHO	World Health Organization

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List of Publication

Published manuscripts

1) **Mariana S. Martins**, Ana C. Gonçalves, Gilberto Alves, Luís R. Silva. Blackberries and mulberries: Berries with significant health-promoting properties (2023). International Journal of Molecular Sciences 24(15), 12024. https://doi.org/10.3390/ijms241512024

Conferences presentations

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I. Introduction

Fruit consumption is promoted globally, being considered an essential part of any diet because it helps people to ingest more vitamins, minerals, dietary fiber, and phytochemicals. Therefore, it should not be surprising that recent epidemiological and clinical studies have shown the importance of a fruit-rich diet for the prevention of many illnesses, including cardiovascular diseases, cancer, and metabolic disorders [1,2]. The World Health Organization (WHO) suggests that individuals have a minimum intake of 400 g of fruits (five servings) per day in order to prevent chronic diseases and other illnesses, as well as to prevent micronutrient deficiencies [1,3].

All fruits have gained in appeal and interest over the past few decades. Among them, red fruits from many families, such as *Rosaceae* (strawberry, raspberry, blackberry, and sweet cherry), *Ericaceae* (blueberry, cranberry), and *Moraceae* (mulberry) have received special attention, due to their high nutritive value, distinctive taste, flavor, and nutraceutical properties, as well as their health-promoting properties [4].

Vitamins A, C, and E, minerals [calcium (Ca), phosphorus (P), iron (Fe), magnesium (Mg), potassium (K), sodium (Na), manganese (Mn), and copper (Cu)], dietary fiber, and phenolics are just a few of the bioactive compounds and nutrients found in these fruits. Among them, this last subclass has undergone extensive research mainly due to its notable anti-inflammatory and antioxidant properties [5,6]. Indeed, phenolics are considered the main factors responsible for the health benefits attributed to these berries, and stand out due to their capacity to prevent cardiovascular diseases [7], reduce inflammation [8], improve neurological function and boost immune system [9], and offer resistance against oxidative stress (Figure 1) [10].

As well as those of other fruits and vegetables, the contents of red fruits also differ in terms of their nutritional value, consumer acceptability, and qualitative and quantitative composition depending on the species, cultivar, genotype, maturity stage, agricultural practices, environmental factors, soil conditions, and subsequent storage conditions [4,11].

Given these facts, as well as the increasing economic value of red fruits, the current review focuses on the nutritional and chemical composition, as well as the health benefits, of two blackberry species (*Rubus fruticosus* L.) and (*Rubus ulmifolius* Schott) and one mulberry species (*Morus nigra* L.).

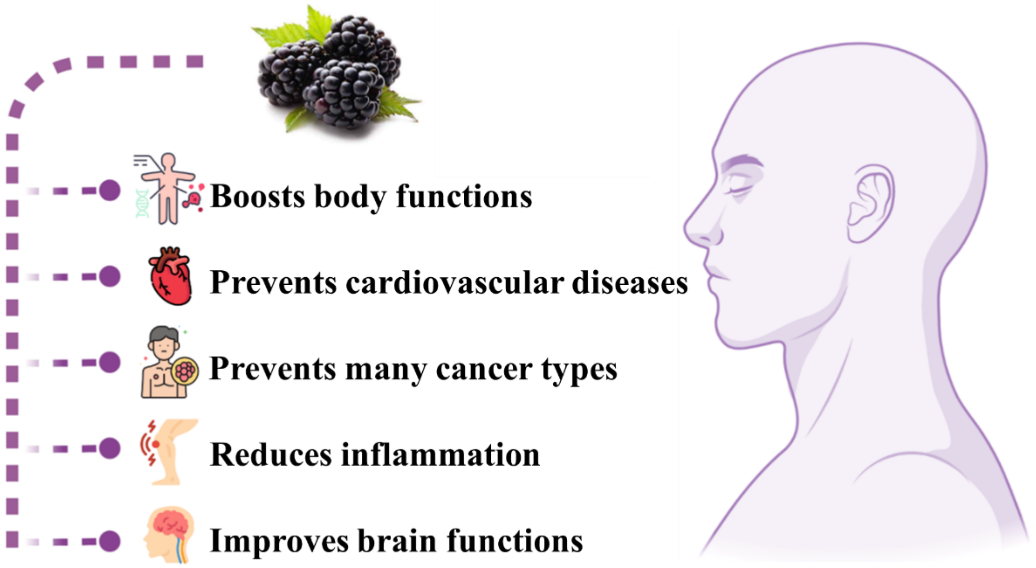


Figure 1. The main benefits linked to blackberries and mulberries consumption.

1. *Rubus fruticosus*, *Rubus ulmifolius* and *Morus nigra*

Focusing on *R. fruticosus* L. and *R. ulmifolius*, both are semi-prostrate erect, scrambling, and perennial deciduous prickly fruits whose shrubs grow up to 3 m at a rapid rate [12]. Their stems are up to 7 m long and are stretched out nearly upright with leaves [6]. Unlike *R. ulmifolius*, *Rubus fruticosus* is a cultivated shrub with no thorns. In addition, *R. ulmifolius* is widespread in forests, hedges, and deserted fields, and along water lines, walls, and fences, and its stems are thorny (Figure 2A,B) [13].

Figure 2. **(A)** *R. fruticosus,* (**B**) *R. ulmifolius* [14] *and* (**C**) *M. nigra* fruits [15].

The red fruits of this plant are blackberries. This species, a drupe-like aggregate fruit composed of numerous drupelets, belongs to the Rosaceae family, subfamily *Rosoideae* and genus *Rubus*, and has a morphology similar to that of raspberries. *Rubus* has over 740 species and 12 subgenera worldwide [6,7,16]. There are over 40 distinct species of blackberries worldwide today, although regions with mild winters and long temperate summers are better suited for their development [17,18]. It is believed that this plant originates from Armenia. On a worldwide basis, blackberries are becoming increasingly popular, and are mostly farmed in North America, Europe, Asia, South America, Central America, and Africa [14,17].

The main blackberry-growing regions in Europe are Serbia and Hungary, with Serbia accounting for 90% of processed and exported production [19]. Furthermore, the yield of wild blackberries is significant, accounting for 154,000 tons in 2005 [20]. The United States of America (USA) is the world's top producer of blackberries, with a production that, in 2017, reached a value of USD 31 million [21]. In 2020, Portugal exported around 29,848 tons of raspberries, strawberries, and mulberries [22]. In fact, the world production of these small fruits is growing due to the new trend towards biological products and the growing interest in their nutritional characteristics. They are rich in antioxidants and fiber, vitamins A, B, C, E, and K, Ca, Mg, and K, and are beneficial to promoting health status at many levels [23,24,25].

Blackberry fruits are commonly consumed fresh or frozen/processed, including when turned into jams, juices, syrups, and wines [24,25]. Phenolic concentrations is influenced by food processing technology used to produce a desirable product. The antioxidant potential significantly decreases as a consequence of jam manufacturing. The principal cause of these declines is the inclusion of glucose-fructose syrup. Total phenolic compounds, total flavonoids and monomeric anthocyanins, and total antioxidant capacity values were found to be lower (between 76–89%) after the addition of glucose-fructose syrup than those recorded in the frozen sample [26].

In addition to its versatility, blackberry fruit is particularly valuable to producers due to its low cost of production and cultivation [27]. Since the fruit's external appearance and internal quality are directly linked to the amount of primary and secondary metabolites present, fruit quality is crucial to both consumers and the food industry. It is also essential to remember that fruit with higher quality has a higher market value. Smaller fruits are firmer because they have the same number of cells as larger fruits, giving a higher density to the plant tissue. Fruit size is typically negatively correlated with firmness and berry phenolic content [18,19].

This fruit has the highest quality and flavor when it is fully ripe. From a business perspective, the color of the fruit and juice is crucial, because customers evaluate products based on their visual appearance. The color of blackberry fruits is influenced by a number of variables, including genotype, production conditions, fruit ripening stage, harvesting time, climate, soil, and storage conditions [28, 29]. In terms of climate, some environmental elements influence the fruit composition, which is defined by the presence of substances known as nutraceuticals, which offer health advantages and assist in the treatment of disorders [30].

Blackberries and their by-products have been used since ancient times in traditional medicine, but recently the knowledge concerning their health-promoting components has received a lot of attention, particularly due to their richness in different bioactive compounds, with the presence of vitamins, minerals, fiber, and phenolic compounds standing out [19,23,25,31]. Therefore, it is not surprising that consumers favor the nutritional and antioxidant qualities associated with these fruits [4]. These characteristics depend on the region, variety, and time of harvest [32,33]. Additionally, blackberry phenolic content can also be affected by soil composition, which, in turn, results in variations between cultivars produced in the same area [5,18].

The fruit of *M. nigra*, a member of the *Moraceae* family and the genus *Morus*, is frequently compared with that of *R. fruticosus* and *R. ulmifolius* (Figure 2A–C) [25, 26]. Although these three species have comparable appearances and chemical properties, and are consumed fresh, as well as processed to make jam, marmalade, syrup, a variety of soft beverages, and traditional items, *M. nigra* develops from trees that can reach a height of 10–13 m and exhibits higher potential for adaptation to diverse soil and environmental conditions [21, 22]. Their origin was India and China, but nowadays, they are commonly found in Asia, Europe, America, and Africa [21,24]. They have a wide range of varieties; however, the three most popular types are black mulberry (*M. nigra*), white mulberry (*M. alba*), and red mulberry (*M. rubra*) [33]. Among these, the black mulberry is an edible fruit that is 2–3 cm long, with a complex cluster of several tiny drupes, and is dark purple, almost black, when completely mature. In Xinjiang, a region of China, and Eastern Anatolia, a region of Turkey, black mulberry fruits are used as a traditional medicine for the prevention and treatment of hypertension, tonsillitis, sore throat, anemia, and iron deficiency [34]. According to recent studies, black mulberries have more flavonoids, anthocyanins, and antioxidant abilities than red or white mulberries [26,32]. Since this fruit has a high concentration of naturally occurring phenolic compounds, such as phenolic acids, flavonols, and anthocyanins, it shows a wide range of biochemical activities, including antioxidant, anti-hyperlipidemia, and anticancer properties [33,35,36].

1.1. Nutritional and Chemical Composition

Berries, including blackberries and mulberries have high nutritional content, including of fatty and organic acids, minerals (Mg, Fe, K, and Ca), vitamins (A, B, C, K, and E), proteins and amino acids, and carbohydrates (sugars, and fiber) [16,24,27,32]. In addition, and focusing on blackberries and mulberries, they are also a great source of bioactive compounds with pharmaceutical potential, including phenolics (e.g., anthocyanins, hydroxycinnamic acids, and flavonols) and volatiles [13,25,30,33,35,37]. As already mentioned, and like other fruits, the nutritional content and quality of blackberries and mulberries are influenced by their chemical composition [36]. Additionally, the physiological age of red fruits at harvest has a significant impact on post-harvest quality, having noticeable variations in colour, hardness, acidity, and TSS as both berries grow [24,30,36,38]. Several studies identified several characteristics of farmed and wild blackberry fruits [17,38]. In general, most quality assessments are based on the sugar/acid ratio level, calculated from total soluble solid (TSS; °Brix) and titratable acidity (TA). The TSS parameter indicates sugar content in fruits, while pH and TA represent total acids that contribute to sweetness and acidity, respectively, of fruits and related products [39,40].

Berry weight ranges from 1.2 g to 5.4 g for cultivated blackberries, such as *R. fruticosus*, whereas in wild blackberries, e.g., *R. ulmifolius*, it varies from 0.4 g to 1.2 g. This indicates that cultivated berries have a higher mean weight compared to wild genotypes. A similar trend is observed for length and width. However, TSS values are lower in cultivated fruits (8.6%–14.1%) than in wild genotypes (12.9%–22.3%). The mean TSS of wild genotypes is around 20%, whereas the mean pH of the wild genotypes is higher than that of the cultivated genotypes [38]. Concerning ash content, *R. ulmifolius* possesses around 0.58 g per 100 g fresh weight (fw). A higher degree of moisture was found for wild blackberries, with a value of 70 g per 100 g fw [13].

The weight of *M. nigra* ranges from 4.18 g to 5.55 g, while moisture content is around 78.03 fw and is the highest found on the *Morus* species [30,37,41]. Total ash content is around 0.50 g per 100 g dry weight (dw), whereas pH values range from 3.43 to 4.78 [37,42] and TA between 0.17% and 1.97% [43,44]. Recently, it was reported, in Chinese mulberries, TA values were between 5.82 and 48.49 mg citric acid per g fw [37]. The TSS content fluctuates between 6.20 to 19.43 °Brix [36,44].

1.1.1. Macronutrients

Macronutrients are chemicals that humans ingest in large quantities and are the primary body source of energy. The most well-known are carbohydrates, proteins, and organic and fatty acids. Among these, carbohydrates are considered the main source of energy used by human organisms. However, all of them are considered vital for preserving our health and life [43,45].

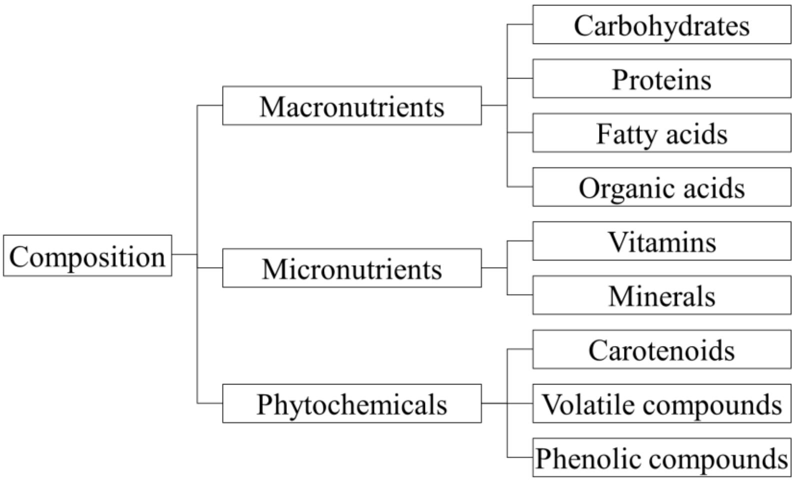


Figure 3. General composition of fruits.

1.1.1.1. Carbohydrates

Sugars are essential to a fruit's general taste character, nutritional value, and caloric density. They are the primary result of photosynthesis and are required for the development of plant cell walls, energy production, and the formation of a number of signaling molecules at cellular and tissue levels, participating in the formation of aroma compounds [41]. Since most customers prefer sweet fruits, a higher fructose concentration is preferred because fructose is typically sweeter than glucose and sucrose [24].

Fructose, glucose, sucrose, trehalose, and raffinose are found in *R. fruticosus* and *M. nigra*. Comparing both, *M. nigra* contains more total and reduced sugars, but lower levels of saccharose (Table 1) [25,41, 46,47].

The carbohydrates most found in blackberries and mulberries are glucose and fructose. Among these, fructose is the most abundant [47,48].

1.1.1.2. Proteins and Amino Acids

Proteins are chains of amino acids linked together by peptide linkages. Proteins are essential in the human organism. They can heal cells and structures, providing structural support, and contribute to pH and fluid equilibrium. They also enhance the immune system by transporting and storing nutrients and providing energy when needed [49]. Although fruits are not considered an excellent source of proteins, these berries present considerable amounts of proteins when compared to other fruits, with amounts around 1.39–2.4 proteins per 100 g for blackberries and about 1.44 g per 100 g for mulberries (Table 1) [47,48].

1.1.1.3. Fiber

Fiber is classified into (i) water-soluble fiber and (ii) insoluble fiber. Soluble fiber delays digestion and improves nutrient uptake. By restricting the enterohepatic circulation of cholesterol, soluble and insoluble fibers improve gut health and reduce the risk of cardiovascular diseases [49].

Dietary fiber is a non-caloric carbohydrate that human small intestines cannot process or ingest. Fruits contain dietary fiber, particularly soluble fiber, in quantities higher than 7%, and, therefore, they can reduce the risk of cardiovascular and coronary heart diseases. Thus, the primary nutritional reason for including fruits in a healthy diet is due to their fiber content, principally due to their gastrointestinal regulatory abilities, which contribute to human health maintenance. Additionally, fiber works together with vitamins, increasing the biological activities of foods [49].

Among berries, blackberries present the higher fiber content (approximately 5.3 g per 100 g) (Table 1). On the other hand, black mulberries only possess around 1.7 g per 100 g [47,48].

Table 1. Basic chemical composition, macronutrients, and mineral content of blackberry and mulberry (per 100 g of fw) [41,47,48].

Nutrient (Unit)	Basic chemical composition	
	Raw blackberry	**Raw black mulberry**
Water (g/100 g)	88.2	87.7
Energy (kcal/100 g)	43–125.25	43
Macronutrients		
Protein (g/100 g)	1.39–2.4	1.44
Total lipid (fat)	0.49–1.22	0.39
Fatty acids, total monounsaturated (g/100 g)	0.047	0.041
Fatty acids, total polyunsaturated (g/100 g)	0.28	0.207
Ash (g/100 g)	0.37–0.58	0.69
Carbohydrate, by difference (g/100 g)	9.61–26.2	9.8
Dietary fiber (g/100 g)	5.3	1.7
Total sugars (g/100 g)	4.78–16.3	10.14–21.32
Sucrose (g/100 g)	0.07–0.34	1.08–2.14
Glucose (g/100 g)	2.31–8.1	7.18–10.33
Fructose (g/100 g)	2.4–7.8	1.88–8.85
Maltose (g/100 g)	0.07	-
Galactose (g/100 g)	0.03	-
Micronutrients		
Minerals		
Calcium, Ca (mg/100 g)	12.5–29	39–502
Iron, Fe (mg/100 g)	0.62–3.4	1.85–77.6
Magnesium, Mg (mg/100 g)	20	18–386
Phosphorus, P (mg/100 g)	22	38–2520
Potassium, K (mg/100 g)	11.9–162	194–2234
Sodium, Na (mg/100 g)	1	5.9–302
Zinc, Zn (mg/100 g)	0.53	0.10–62
Cooper, Cu (mg/100 g)	0.165	0.06–0.10
Manganese, Mn (mg/100 g)	0.646	0.40–19
Selenium, Se (µg/100 g)	0.4	0.008–0.6

1.1.1.4. Fatty acids

Fatty acids are part of triglycerides and are the principal form in which fat occurs. Fatty acids can exist naturally, presenting different chain lengths and double bonds. They may be saturated, monounsaturated, or polyunsaturated. Fatty acids are required for the formation and reparation of cell structures, including cell walls. In addition, they are crucial to human well-being [49].

Blackberries have extremely little fatty acid content, with saturated fats making up about 0.014 g per 100 g, monounsaturated fats around 0.047 g per 100 g, and polyunsaturated fats approximately 0.28 g per 100 g of fruit (Table 1) [34 ,47,48]. Concerning *M. nigra*, they contain oleic acid (26.0%), palmitic acid (23.8%), and linoleic acid (23.1%) [34]. However, their percentages are widely variable. For example, Jiang and Nile [30] reported that the average linoleic acid concentration is 4.1 times higher than that of palmitic acid and 4.8 times higher than that of oleic acid of *M. Nigra* from Xinjiang, a province of China. These variations could be attributed to different cultivars, as well as the ecological circumstances under which the species are produced [30,34,37].

1.1.1.5. Organic acids

Organic acids are primary metabolites found in abundance in all plants, particularly in fruits and vegetables. The most well-known include citric, malic, and galacturonic acids. These compounds have a significant impact on the organoleptic properties of fruits and vegetables, particularly flavour, colour, and aroma [13,35]. When the fruit is immature, it has higher acid content, which decreases with the harvest. Organic acids are freely accessible and help to stabilize anthocyanins [50].

These primary metabolites can also inhibit the development of microorganisms in fruit juices, thereby improving product quality preservation [49]. The total quantity of organic acids found in several species of berries has been reported to range from 21.5 to 235 mmol/kg. The *R. fruticosus* species is the one that presents the highest content (45.1 mmol/kg) [51].

R. ulmifolius presents oxalic, quinic, malic, shikimic, ascorbic, and fumaric acids (Table 2), accounting for around 238 mg per 100 g fw. Quinic acid is the compound with the highest concentration (119 mg per 100 g fw), followed by oxalic (71 mg per 100 g fw), malic (29 mg per 100 g fw), shikimic (11.33 mg per 100 g fw), and ascorbic acids (6.66 mg per 100 g fw); fumaric acid is only detected in trace amounts [13]. On the other hand, the organic acids found in *R. fruticosus* are citric, oxalic, malic, ascorbic, and fumaric acids. Malic acid is predominant (5706.37 mg per 100 g dw), while ascorbic acid is the lowest (6.00 mg per 100 g dw). Other organic acids, namely, quinic, shikimic, tartaric, and succinic acids, have not been identified [52]. Relative to black mulberry fruits, these contain a variety of organic acids (Table 2). To date, citric, tartaric, malic, and succinic acids are the only organic acids detected in *M. alba*, *M. nigra*, and *M. rubra* species [35].

Table 2. Organic acids identified in *R.ulmifolius* and *R. fruticosus* blackberries, and *M. nigra* mulberry fruits (mg per 100 g) [13, 52, 53, 54].

Organic Acid	***R. ulmifolius***	***R. fruticosus***	***M. nigra***
Citric acid	-	125.54 dw	1084–7020 fw
Oxalic acid	71 fw	59.51 dw	450–1250 fw
Quinic acid	119 fw	-	-
Malic acid	29 fw	5706.37 dw	1323–13,650 fw
Succinic acid	-	-	342 fw
Shikimic acid	11.33 fw	-	1.36 fw
Tartaric acid	-	-	220–860 fw
Ascorbic acid	6.66 fw	6.00 dw	12.81–15.37 fw
Fumaric acid	tr	230.25 dw	-
Total	238 fw	6127.67 dw	2951 fw

tr: traces; -: no data; fw: fresh weight; dw: dry weight.

1.1.2. Micronutrients

Although micronutrients (e.g., vitamins and minerals) are consumed in small amounts, they are essential for health and vital functions [49]. They are essential elements that the organism requires to stay healthy (Figure 3). This requirement is determined by each person's unique needs, varying according to various metabolic circumstances throughout the life cycle (age, lifestyle, hormonal activity, exercise, etc.) [54]. All of the essential micronutrients cannot be synthesized within the body and are supplied by the diet. As a result, a diverse range of foods is important in our nutrition [48].

1.1.2.1. Minerals

A sufficient mineral intake is needed for good nutrition and food quality, and to avoid chronic nutrition-related illnesses. Certain elements, such as Ca, Fe, and zinc (Zn), are deficient in certain populations [48]. Fruit mineral composition is affected by growth circumstances, such as soil and geographical location, as well as species or varieties [41].

A total of ten minerals have been reported in raw blackberries, namely, Ca, Fe, Mg, P, K, Na, Zn, Cu, Mn, and selenium (Se) [47,48]. Black mulberry possesses all the minerals mentioned above (Table 1), with K, P and Ca found in higher concentrations [30,34,42,47,48]. In particular, Ca is necessary for the growth of bones and muscles, while Fe is required for the formation of hemoglobin, and to help oxygen and electron transfer [49,54].

1.1.2.2. Vitamins

Vitamins are complex organic essential compounds that are classified into two types: (i) fat-soluble and (ii) water-soluble [55]. They are required for the organism's functions and normal growth. Each vitamin serves a particular purpose in regular metabolism, development, vitality processes, and energy transformation. Furthermore, some of them are antioxidants. Fruits are the most significant source of vitamins in the human diet [49].

In particular, blackberries have higher levels of vitamins C and K. Vitamin C is a water-soluble vitamin that is present in higher amounts in fruits and vegetables, which contain up to 50% [53]. It is known that blackberries contain around 21 mg per 100 g of vitamin C, whereas black mulberries contain 17.41–28.33 mg per 100 g of fruit [30,33,41,43,56,57]. The amount of vitamin K in blackberries is approximately 19.79 mg per 100 g (Table 3) [47]. This vitamin can help the human body to fight against free radicals. Furthermore, blackberries contain approximately 1.17 mg of vitamin E per 100 g (Table 1) [47]. This vitamin can serve as a safeguard and protect the human body from free radicals, as well as strengthening the immune system and retarding skin aging. Finally, diets with higher amounts of vitamin C may reduce the risk of acquiring various types of malignancies, e.g., cardiovascular diseases and sicknesses caused by environmental factors [54,55].

Table 3. Vitamin content of raw blackberry and mulberry fruits [30,33,41,43,47,48,56,57].

Vitamins	Raw blackberry	Raw black mulberry
Vitamin C (mg/100 g)	21.0	19.3–36.4
Thiamin (mg/100 g)	0.02	0.029
Riboflavin (mg/100 g)	0.026	0.04–0.10
Niacin (mg/100 g)	0.646	0.62–1.60
Vitamin B-6 (mg/100 g)	0.03	0.05
Folate total (µg /100 g)	25.0	6.0
Folate, DFE (µg /100 g)	25.0	6.0
Folate, food (µg /100 g)	25.0	6.0
Choline, total (mg/100 g)	8.5	12.3
Vitamin K (phylloquinone) (µg /100 g)	19.8	7.8

1.1.2.3. Tocopherols

Vitamin E consists of the generic denomination of eight liposoluble compounds, alpha (*α*), beta (*β*), gamma (*γ*) and delta (*δ*)-tocopherols, each of which has specific biological activities. Among these, *α*-tocopherol is the compound with the highest antioxidant capacity [13]. The function of vitamin E as an antioxidant in the peroxidation of cell membranes occurs by supplying a hydrogen atom to the peroxide radical formed, acting as a scavenger of free radicals, hence protecting cell membranes from possible damage. Vitamin E is mainly found in products rich in fat, such as almonds, vegetable oils, and some fruits and vegetables. Blackberry exhibits very small amounts of tocopherols which can be explained by the low amounts of fat found in this fruit [58]. Blackberry fruit contains all of the tocopherols' isoforms, with *γ*-and *δ*-tocopherol being present at higher concentrations.

Tocopherols found in *R. ulmifolius* are described in Table 4. Isoforms, namely, α-, *β*-, *γ*-, and *δ*-tocopherol are present, representing quantities of 5.1–13.48 mg per 100 g fw [13]. *γ*-Tocopherol was highlighted as a major isoform present, with a concentration ranging from 1.34 to 3.73 mg per 100 g fw, followed by *δ*-tocopherol and *α*-tocopherol with similar contents (0.9–3.69 and 1.15–3.38 mg per 100 g fw, respectively) [13,58]. *β*-Tocopherol is detected at low concentrations (values of 0.020–0.24 mg per 100 g fw) [13]. In *R. fruticosus*, only α-tocopherol was found, in a concentration of 610 mg per 100 g. On the other hand, *M. nigra* shows nearly seven times more *α*-tocopherol than *R. fruticosus*. Additionally, in *M. nigra*, the four isoforms were found (Table 4), with the prevalence of α-tocopherol (4300 mg per 100 g), followed by γ-tocopherol (1250 mg per 100 g). δ-Tocopherol (550 mg per 100 g) and β-tocopherol (127 mg per 100 g) were less abundant [58,59].

In a general way, γ-tocopherol has been shown to be a highly effective molecule in postponing arterial thrombus development, lowering LDL oxidation and superoxide production, and avoiding lipid peroxidation. It has also been mentioned that regular consumption of food rich in this isoform reduces the risk of myocardial infarction and death from ischemic heart disease. Regarding antioxidant and protective effects of tocopherols, many studies focus primarily on α-tocopherol, which is the main form of vitamin E, in over-the-counter supplements [13,58].

Table 4. Tocopherols present in in *R. ulmifolius* and *R. fruticosus* blackberries, and *M. nigra* mulberry fruits [13,46,58].

Tocopherols	***R. ulmifolius*** **(mg per 100 g fw)**	***R. fruticosus*** **(mg per g extract)**	***M. nigra*** **(mg per g extract)**
α-tocopherol	1.15–3.38	6.1	43
β-tocopherol	0.02–0.24	nd	1.27
γ-tocopherol	1.34–3.73	nd	12.5
δ-tocopherol	0.9–3.69	nd	5.5
Total	5.1–13.48	6.1	62

nd: not detected; fw: fresh weight.

1.1.3. Phytochemicals

Phytochemicals are non-nutrient bioactive plant molecules found in fruits, vegetables, whole grains, and other plant foods [49,54]. Blackberries have a high amount of environmental variation due to their extensive geographic distribution, which influences their physical and chemical characteristics, and, hence, the profiles of bioactive substances, including anthocyanins, flavonoids, and carotenoids (Figure 4) [4,11,38]. *Rubus* berries are thought to be an abundant source of phytochemicals that play an important role in the prevention of modern chronic illnesses [19,35]. The physicochemical characteristics of mulberry cultivars are essential for economic and dietary benefits [19-21].

Phytochemicals are important antioxidants, having a positive impact on human health, particularly in the prevention of cardiovascular, inflammatory, and cancer diseases. Therefore, it is essential to identify and quantify the bioactive constituents of plant extracts because they are mainly responsible for the biological and pharmacological actions exhibited by foods [26,32,49].

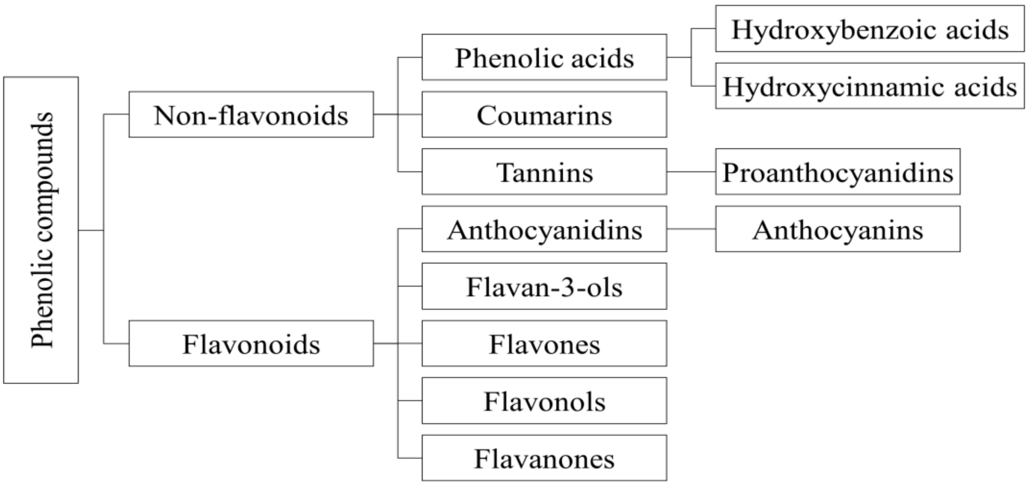


Figure 4. Phenolic compounds classification.

1.1.3.1. Carotenoids

Carotenoids are a class of fat-soluble natural pigments that have a variety of health benefits. These natural pigments metabolized by plants are responsible, along with anthocyanins, for the yellow, orange, and red colours in fruits and vegetables. The term carotenoid refers to a family of structurally similar pigments found primarily in plants [59]. Based on their functional groups, carotenoids are classified into two groups: (i) xanthophylls, which contain oxygen as a functional group (e.g., lutein and zeaxanthin), and (ii) carotenes, which contain only the parent hydrocarbon chain and no functional group, such as α-carotene, *β*-carotene, and lycopene [49].

Their content and types in plants are affected by several pre- and post-harvesting variables, genotype, ripening time, cultivation technique, climatic conditions, and processing methods [59]. Additionally, different parts of the same plant may also contain varying types and quantities of carotenoids. For example, the peel of fruits is typically higher in carotenoids than the pulp. Climate and growth circumstances can also have an impact on the quantity of carotenoids in plants. According to these findings, fruits exposed to higher temperatures and more sunlight may boost carotenoid production in order to defend the plant from photo-oxidation [49].

The daily ingestion of carotenoids is important to increase antioxidant activity, intercellular communication, gene regulation, and immune system activity. Indeed, carotenoid-rich diets have been linked to a lower incidence of many types of cancer, cardiovascular diseases, age-related macular degeneration, and cataract formation [48,49].

Unfortunately, when compared to other red fruits, such as blueberries and raspberries, the quantity of carotenoids in blackberries is small: 128 µg per 100 g of fruit (*β*-carotene) (Table 5) [13,47,61].

Table 5. Carotenoids present in *R. fruticosus* blackberry and *M. nigra* mulberry fruits [47].

Carotenoids	*R. fruticosus*	*M. nigra*
Carotene, beta (µg per 100 g)	128.0	9.0
Carotene, alfa (µg per 100 g)	0.0	12.0
Vitamin A, RAE (µg per 100 g)	11.0	1.0
Vitamin A, IU (µg per 100 g)	214.0	25.0
Lutein + zeaxanthin (µg per 100 g)	118.0	136.0

1.1.3.2. Volatile compounds

Flavour and aroma are two of the most essential aspects of fruits' excellence and acceptance. The aroma of some fruits has been linked to their concentration of volatile organic compounds. They derive from fatty acids, amino acids, carotenoids, and phenolics [49]. Additionally, the metabolism of fruits produces volatile compounds during the ripening, harvesting, post-harvesting, and storage. As a result, the volatile composition of blackberries is affected by the genotype, origin, technological treatment (freezing, drying, among others), ripening stage, harvest, and storage conditions [62,63,64,65]. Therefore, the analysis of volatile compounds is critical for understanding the components responsible for their flavour and aroma, as well as the best harvest period for higher quality and phytosanitary qualities [4,62].

Although several volatile compounds exist, regarding blackberry fruits, aldehydes, alcohols, ketones, esters, hydrocarbons, terpenoids, furanones, and sulfur compounds are the main contributors to their aroma [8,66]. Hence, terpenoids (75.38%) are the most abundant chemical category of volatile chemicals in *R. fruticosus*, whereas aldehydes (0.53%) are the least abundant [65].

On the other hand, *R. ulmifolius* possesses around 33 different volatile compounds: nine aliphatic alcohols, three branched alcohols, six aldehydes, two ketones, six terpenoid compounds (including *β*-myricene, D-limonene, *β*-linalool, L-*α*-terpineol, sulcatol, and sulcatone), four compounds containing a benzene-ring (including methoxyphenyl oxime, methyl salicylate, benzyl alcohol, and phenylethyl alcohol), and ethyl octanoate (an ester), 2-methylbutanoic acid (a carboxylic acid), and 2-ethylfuran (a cyclic ether). This species of blackberry contains high amounts of benzenoids, aldehydes, and alcohols [63,67].

Focusing on *M. nigra*, a previous study determined the presence of 67 volatiles: five acids, twenty-five alcohols, two aldehydes, twenty-six esters, five hydrocarbons, one ketone, and three phenols. The most prevalent chemicals in samples were aliphatic alcohols, which accounted for 47.5% of the total volatile component. The majority of the alcohol was ethanol (82.3%). Furthermore, ten aliphatic alcohols (ethanol, 1-propanol, 2-butanol, 2,3-butanediol, 2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol, benzyl alcohol, phenylethyl alcohol, and terpene-4-ol) were also found. Surprisingly, although aldehydes are abundant in many fruits, only two aldehydes (acetaldehyde and benzaldehyde), accounting for only 2.1% of the total volatile compounds, were detected. Relative to *M. alba*, esters are largely found, representing 36.3% of all volatile compounds found in this mulberry. In addition, isovaleric acid (94.4%) was revealed to be the most abundant fatty acid [64].

Finally, using solid-phase microextraction and gas chromatography-mass spectrometry, 45 volatile compounds have been reported in *R. fruticosus*. Terpenoids made up the vast majority (97.7%), with limonene being the most frequent compound. The discovered volatiles extracted with hexane were largely hydrocarbons, whereas those extracted with acetone were furans and pyrans. Hexane-extracted volatiles were also identified, with the majority of the compounds being the aliphatic ones, and just 13% were aromatic. The identified compounds accounted for 82% of the overall peak area in the acetone extract chromatogram. Altogether, the most essential volatile components responsible for the blackberry flavor are heptanol and *p*-cymen-8-ol [65].

Table 6. Volatile compounds identified in *R. fruticosus*, *R. ulmifolius*, and *M. nigra* fruits [67,68].

Volatile compounds	Fruit species	Volatile compounds	Fruit species
Esters			
Methoxyphenyl oxime	*R. ulmifolius*	Methyl salicylate	*R. ulmifolius*
Ethyl octanoate	*R. ulmifolius*	Methyl acetate	*M. nigra*
Etyl acetate	*M. nigra*	Etyl propanoate	*M. nigra*
Ethyl 2-metylbutanoate	*M. nigra*	Propyl acetate	*M. nigra*
Ethyl 3-metylbutanoate	*M. nigra*	Etyl butanoate	*M. nigra*
Isopentyl acetate	*M. nigra*	Etyl pentanoate	*M. nigra*
Ethyl 2-hydroxyhexanoate	*M. nigra*	Ethyl lactate	*M. nigra*
Isoamyl lactate	*M. nigra*	Ethyl octanoate	*M. nigra*
Ethyl decanoate	*M. nigra*	Ethyl 9-decenoate	*M. nigra*
Diethyl succinate	*M. nigra*	Benzyl acetate	*M. nigra*
2-Phenylethyl acetate	*M. nigra*	Methyl salicytate	*M. nigra*
Ethyl dodecanoate	*M. nigra*	Diethyl pentanedioate	*M. nigra*
Ethyl-3phenylpropanoate	*M. nigra*	Ethyl phenoylethanoate	*M. nigra*
Ethyl tetradecanoate	*M. nigra*	Ethyl hexadecanoate	*M. nigra*
Metyl-hexanoate	*R. futicosus*	Ethyl-hexanoate	*R. fruticosus* *M nigra*
Ethyl benzoate	*R. futicosus*	Methyl salicylate	*R. futicosus*
Terpenes			
D-limonene	*R. ulmifolius*	β-Linalool	*R. ulmifolius*
L-α-terpineol	*R. ulmifolius*	β-Myricene	*R. ulmifolius*
Terpenoids			
α-Thujene	*R. futicosus*	β-Myrcene	*R. futicosus*
α-Pinene	*R. futicosus*	α-Phellandrene	*R. futicosus*
1-Octanol	*R. futicosus* *M. nigra*	Terpinolene	*R. futicosus*
Camphene	*R. futicosus*	Limonene	*R. futicosus*
o-Cimene	*R. futicosus*	α-Terpinene	*R. futicosus*
Linalool	*R. futicosus*	Linalool oxide	*R. futicosus*
trans-Limonene oxide	*R. futicosus*	Isoborneol	*R. futicosus*
Isopinocarveol	*R. futicosus*	Terpinen-4-ol	*R. futicosus*
(-)-Carvone	*R. futicosus*	*p*-Cymen-8-ol	*R. futicosus*
Geraniol	*R. futicosus*	α-Copaene	*R. futicosus*
Vitispirane	*R. futicosus*	α-Terpineol	*R. futicosus*
Theaspirane	*R. futicosus*	-	-
Aldehydes			
Pentanal	*R. ulmifolius*	Hexanal	*R. futicosus* *R. ulmifolius*
E-2-Pentenal	*R. ulmifoliu*	Nonanal	*R. futicosus* *R. ulmifolius*

E-2-Hexenal	*R. ulmifolius*	Z-2-Heptenal	*R. ulmifolius*
2-Hexenal	*R. futicosus*	Octanal	*R. futicosu*
Heptanal	*R. futicosus*	Decanal	*R. futicosus*
Nonenal	*R. futicosus*	*p*-Mentenal	*R. futicosus*
Acetaldehyde	*M. nigra*	Benzaldehyde	*R. futicosus* *M. nigra*
Alcohols			
2-Ethyl-1-pentanol	*R. ulmifolius*	Phenylthyl alcohol	*M. nigra*
1-Penten-3-ol	*R. ulmifolius*	1-Octen-3-ol	*R. ulmifolius*
Isoamyl alcohol	*R. ulmifolius*	Sulcatol	*R. ulmifolius*
2-Heptanol	*R. ulmifolius* *R. futicosus* *M. nigra*	(*s*)-3-Ethyl-4-methylpentanol	*R. ulmifolius*
Z-2-Penten-ol	*R. ulmifolius*	*Z*-5-Octen-1-ol	*R. ulmifolius*
1-Hexanol	*R. ulmifolius* *M. nigra*	Benzyl alcohol	*M. nigra* *R. ulmifolius*
1-Heptanol	*R. futicosus* *R. ulmifolius*	*E*-2-Hexen-1-ol	*R. ulmifolius*
Z-3-Hexen-1-ol	*R. ulmifolius*	2-Tetradecanol	*M. nigra*
2-Butanol	*M. nigra*	2-Pentadecanol	*M. nigra*
1-Propanol	*M. nigra*	2-Nonanol	*M. nigra*
3-Methyl-2-butanol	*M. nigra*	1-Octanol	*M. nigra* *R. fruticosus*
2-Metyl-1-butanol	*M. nigra*	4-Methyl-1-pentanol	*M. nigra*
3-Methyl-1-butanol	*M. nigra*	3-Methyl-1-pentanol	*M. nigra*
3-Methyl-3-buten-1-ol	*M. nigra*	Terpene-4-ol	*M. nigra*
1,3-Butanediol	*M. nigra*	2-Decanol	*M. nigra*
2-Undecanol	*M. nigra*	Ethanol	*M. nigra*
2-Methyl-1-propanol	*M. nigra*	2,3-Butanediol	*M. nigra*
2-Butyl-1-octanol	*M. nigra*	3-Ethyl-4-methyl-pentanol	*M. nigra*
Ketones			
Methyl ethyl ketone	*R. futicosus*	Damascenone	*R. futicosus*
2-Heptanone	*R. futicosus*	Verbenone	*R. futicosus*
3-Hydroxy-2-butanone	*M. nigra*	-	-
Hydrocarbons			
Pentadecane	*M. nigra*	Dodecane	*M. nigra*
Nonadecane	*M. nigra*	Tridecane	*M. nigra*
Heptane	*R. futicosus*	Tetradecane	*M. nigra*
Toluene	*R. futicosus*	-	-
Acids			
Hexanoic acid	*M. nigra*	Acetic acid	*M. nigra*
Octanoic acid	*M. nigra*	Butanoic acid	*M. nigra*
Isovaleric acid	*M. nigra*	-	-

Carbonyls			
1-Penten-3-one	*R. ulmifolius*	2-Heptanone	*R. ulmifolius*
Sulcatone	*R. ulmifolius*	2-Methyl butanoic acid	*R. ulmifolius*
Phenols			
2,4-Di-tert-butylphenol	*M. nigra*	2-Methoxyphenol	*M. nigra*
4-Methyl-2-methoxyphenol	*M. nigra*	-	-

-: not reported until date.

1.1.4. Phenolic compounds

Phenolic compounds can be classified into (i) non-flavonoids and (ii) flavonoids. Phenolic acids, coumarins, and tannins are examples of non-flavonoids. Flavonoids are further classified into five main subgroups: (i) anthocyanidins and their glycosides anthocyanins, (ii) flavan-3-ols, (iii) flavones, (iv) flavonols, and (v) flavanones (Figure 4). They are regarded as non-nutrient physiologically active molecules capable of functioning as free radical scavengers [49].

This subclass is composed of secondary metabolic products found in fruits, vegetables, leaves, nuts, seeds, flowers, and barks which are kept in cell structures of the fruit skin, pulp, and seeds of fruits [68]. They are essential for plant reproduction, development, and metabolism, as well as for defense against pathogenic viruses and infections [11,12]. In addition to their activities in plants, in our diet, phenolics may lower the risk of chronic illnesses, such as cancer, heart disease and diabetes [31,35,49,68]. As mentioned above, their content in berries may be influenced by genotype, geographic region, storage conditions, ripeness, and climate, among others [11,33,39,41,43]. According to a previous study, polyphenols steadily rise throughout the last phase of maturity in blackberry and mulberry fruits [34].

Table 7. Phenolic compounds reported in *R. fruticosus*, *R. ulmifolius*, and *M. nigra* fruits grown in Covilhã region (Portugal).

Phenolic Compounds	*R. fruticosus*	*R. ulmifolius*	*M. nigra*	Ref
Phenolic Acids				
Hydroxybenzoic acids				
p-Hydroxybenzoic acid	1.44 mg per 100 g fw	-	0.053–0.47 mg per 100 g dw	[63]
Gallic Acid	145.85 mg per 100 g fw	268.72 mg per 100 g fw	21.83–40.90 mg per 100 g fw	[41,67,69]
Syringic acid	-	40.84 µg per 100 g dw	-	[69]
Vanillic acid	14.72 mg per 100 g	-	0.014–0.10 mg per 100 g dw	[70,71]
Salicylic acid	-	296.62 µg per 100 g dw	0.007–0.12 mg per 100 g dw	[74]
Ellagic acid	30.01–33.81 mg per 100 g fw	-	1.36–6.32 mg per 100 g fw	[41,45,70]
Hydroxycinnamic acids				
Caffeic acid	-	75.52 µg per100 g dw	6.14–21.93 mg per 100 g fw	[41,45,71]
Ferulic acid	2.99–22.09 mg per 100 g fw	388.59 µg per 100 g dw	0.009–00.056 mg per 100 g dw	[70, 71,72]
Chlorogenic acid	-	-	43.76–97.59 mg per 100 g fw	[41,45]
p-Coumaric acid	0.40–2.08 mg per 100 g fw	39.65 µg per 100 g dw	-	[70, 71]
Sinapic acid	-	228.69 µg per 100 g dw	0.013–0.11 mg per 100 g dw	[36,72]
Flavonoids				
Flavonols				
Quercetin	20.62 mg per 100 g fw	5509.61 µg per 100 g dw	2.33–11.25 mg per 100 g fw	[41,67,72]
Rutin	4.16–6.45 mg per 100 g	-	32.06–133.60 mg per 100 g fw	[41,73,74]
Quercetin 3-*O*-galactoside	5.44 mg per 100 g fw	-	-	[74]
Quercetin 3-*O*-glucoside	18.18 mg per 100 g fw	36.46 mg per 100 g	-	[69,73]
Kaempferol	0.63 mg per 100 g	399.48 µg per 100 g dw	0.009–0.17 mg per 100 g dw	[71,73]
Flavan-3-ols				
(+)-Catechin	265.75–312.86 mg per 100 g fw	156.61 µg per 100 g dw	228–10.54 mg per 100 g fw	[41,70,73]
(+)-Epicatechin	-	250.82 µg per 100 g dw	0.004–0.054 mg per 100 g dw	[69,71,74]
(-)-Epicatechin	94.29 mg per 100 g fw	-	-	[73]
Flavone				
Myricetin	9.99 mg per 100 g fw	-	-	[36,70]
Luteolin	-	5.97 µg per 100 g dw	0.098–2.26 mg per 100 g dw	[33,74]
Flavanone				
Naringenin	-	28.34 µg per 100 g dw	-	[36,73]
Anthocyanins				

Cyanidin 3-*O*-glucoside	19.49–86.73 mg per 100 g fw	92.33 mg per 100 g	6.010 mg per g extract	[39,72,73]
Cyanidin *O*-hexoside	-	3.761 mg per g extract	-	[77]
Cyanidin 3,5-diglucoside	55,447.28 µg per 100 g	-	0.51–7.28 mg per 100 g dw	[36]
Cyanidin 3-*O*-rutinoside	330,616.73 µg per 100 g	-	1.00–9.21 mg per 100 g dw	[36,74]
Cyanidin *O*-rhamnoside-*O*-hexoside	-	-	2.43 mg per g extract	[77]
Cyanidin *O*-pentoside	-	1.265 mg per g extract	-	[77]
Cyanidin 3-*O*-xyloside	2.62 mg per g extract	12.1–47.1 mg per 100 g	-	[13,39]
Cyanidin 3-*O*-malonylglucoside	-	5.7–20.9 mg per 100 g	-	[39]
Cyanidin 3-*O*-dioxalylglucoside	1.198–2.04 mg per g extract	16.9–107.5 mg per 100 g	-	[13,39,60]
Delphinidin 3-*O*-glucoside	-	-	0.24–7.42 mg per 100 g dw	[39]
Pelargonidin 3-*O*-glucoside	102,936.30 µg per 100 g	-	0.012–0.068 mg per 100 g dw	[36,72]
Pelargonidin 3-*O*-rutinoside	4.23 mg per 100 g fw	-	-	[74]

-: not reported until date.

Phenolic acids are frequent and widespread bioactive molecules in nature. They are commonly found in bound forms, such as amides, esters, or glycosides, with the exception of caffeic and ferulic acids, which are mainly esterified with other molecules such as carbohydrates and organic acids [6].

There are two major groups of phenolic acids: hydroxybenzoic acid derivatives and hydroxycinnamic acid derivatives [49].

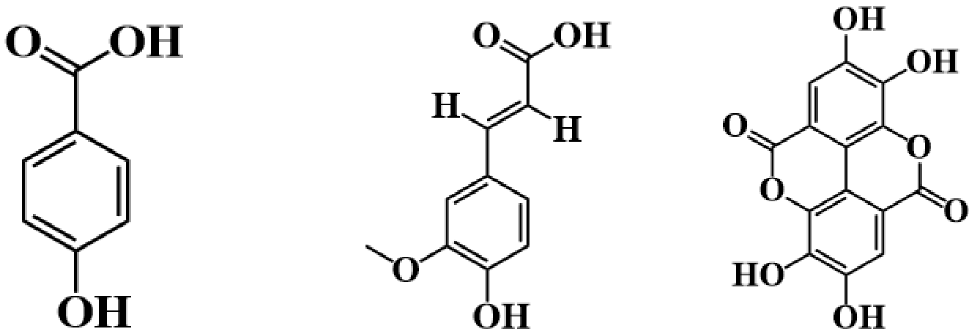
Hydroxycinnamic acids are composed of a nine-carbon structure (C6-C3) with a side-chain double bond (with *cis* or *trans* configuration). The most prevalent hydroxycinnamic acids are caffeic, *o*-coumaric, *p*-coumaric, *m*-coumaric, and ferulic acids [69,70]. In a general way, caffeic, ferulic, chlorogenic and *ρ*-coumaric acids were the main ones identified and quantified in both berries (Figure 5) [36,44,71,72]. Ferulic acid is predominately found in *R. ulmifolius* (388.59 µg per 100 g dw) [80]. Comparative to other red fruits, strawberries present higher amounts of *ρ*-coumaric acid (concentrations around 0.7-4.1 mg per 100 g fw, double that reported in *R. fruticosus*) [71].

Hydroxybenzoic acid is generated from cinnamic acid and is commonly found in food as esters with quinic acid or glucose. This subgroup of phenolic acids is produced from benzoic acid and has a typical common structure of C6-C1. *ρ*-Hydroxybenzoic, protocatechuic, vanillic, syringic, tannic, and gallic acids are the principal ones reported

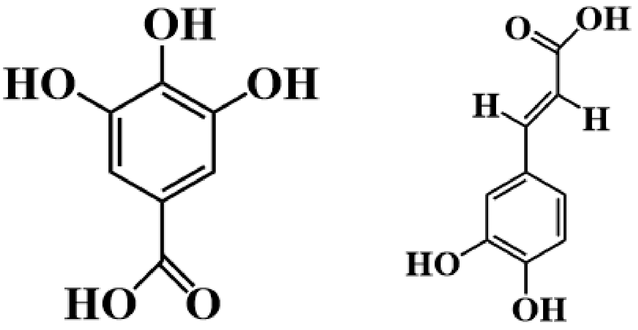
[49]. They form components of complex structures, such as lignins and hydrolysable tannins, and contribute to formation of cell walls and proteins [82]. In comparison to hydroxycinnamic acids, hydroxybenzoic acids are present in relatively modest concentrations in red fruits. Gallic acid is present in *M. nigra*, *R. fruticosus*, and *R. ulmifolius* in higher concentrations (21.83 to 40.90 mg per 100 g fw, 145.85 mg per 100 g fw, and 268.72 mg per 100 g fw, respectively) [44,66,75]. Comparing the three species, *R. ulmifolius* showed the largest level of this hydroxybenzoic acid. Relative to other red fruits, sweet cherries possess amounts fluctuating from 0.73 to 10.64 mg per 100 g of fw, and this concentration is much lower than that of blackberries and mulberries [84].

Concerning *M. nigra*, the major hydroxybenzoic acid present in this fruit is gallic acid (21.83 to 40.90 mg per 100 g fw), followed by ellagic acid (1.36 to 6.32 mg per 100 g fw) [44].

Although the precise role of phenolic acids is uncertain, it is known that they help with food intake, structural support, enzyme activity, protein synthesis, photosynthesis, and allelopathy. Phenolic acids are also the ancestor of bioactive compounds used in food, cosmetics, and pharmaceutical industries [74]. According to research, in individuals, this subclass of fruit compounds has the potential to improve brain function, protect against heart disease, and stop the growth of some cancers [69,70,77].



p-Hydroxybenzoic acid Ferulic acid Ellagic acid

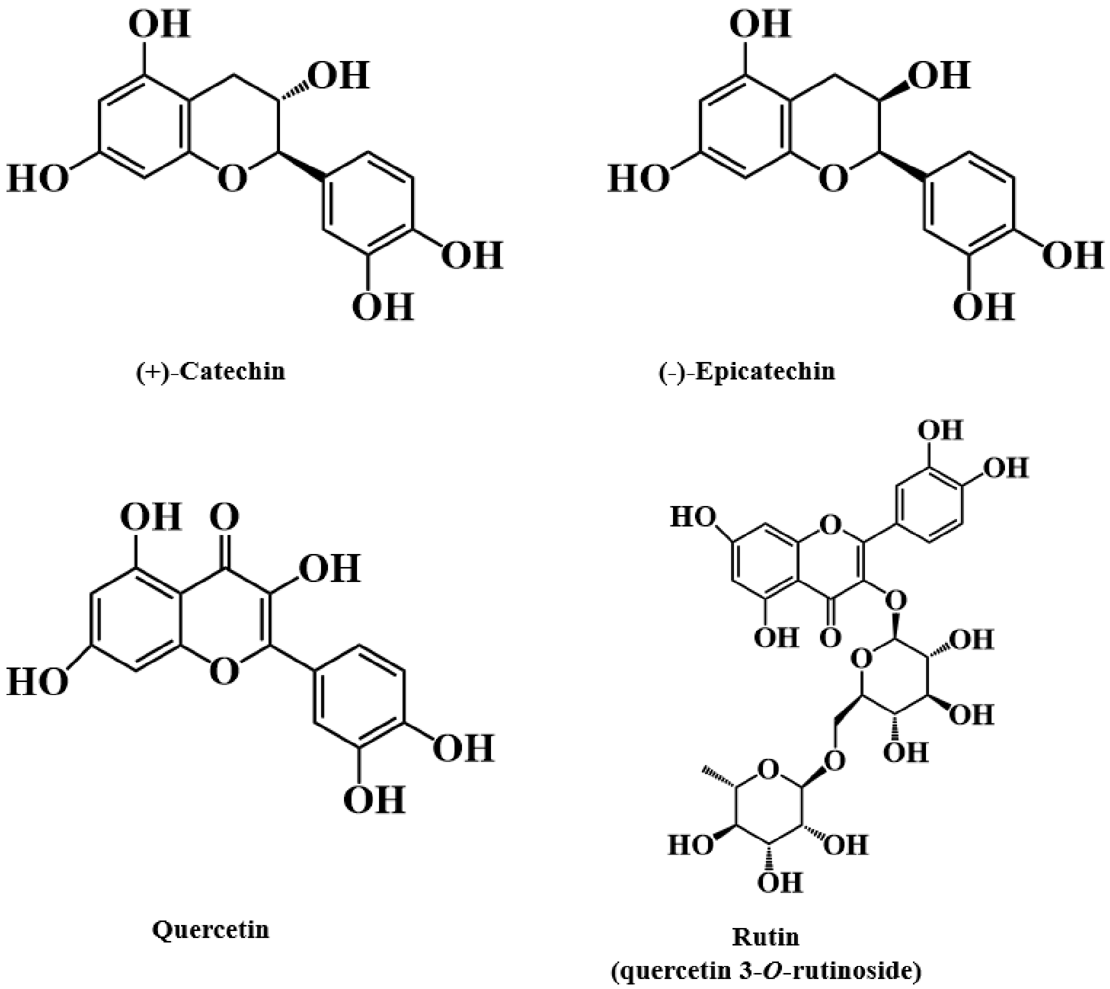


Gallic acid Caffeic acid

Figure 5. Principal phenolic acids found in *R. fruticosus*, *R. ulmifolius*, and *M.nigra*.

Flavonoids are a subgroup of phenolic compounds that fall into several groups, such as anthocyanidins, flavan-3-ols, flavones, flavonols, and flavanones (Figure 4) [49].

The total flavonoid content in *M. nigra* fruit is around 254.0 mg catechin equivalent per 100 g fw [86]. The predominant flavonoids reported in black mulberry fruits are rutin, quercetin, and (+)-catechin (Figure 6), with values varying from 32.06 to 133.60 mg per 100 g fw for rutin, followed by quercetin (2.33 to 11.25 mg per 100 g fw) and catechin (2.28 to 10.54 mg per 100 g fw) [29,41]. The total flavonoids of *R. fruticosus* fruit fluctuating from 30.4 to 82.2 mg catechin equivalent per 100 g of fw, with quercetin, rutin, (+)- catechin, (-)-epicatechin, and myricetin the most abundant [66,75,71,79]. In particular, the level of quercetin in blackberries (20.62 mg per 100 g) is significantly higher than that in black mulberries (2.33 to 11.25 mg per 100 g) [44,66].



(+)-Catechin

(-)-Epicatechin

Quercetin

Rutin
(quercetin 3-*O*-rutinoside)

Figure 6. Principal flavan-3-ols and flavonols present in *R. fruticosus*, *R. ulmifolius*, and *M. nigra*.

Other flavonoids found in both berries are anthocyanins. These are considered the primary factor responsible for the color of many fruits and vegetables. Anthocyanins can be found in the cell at locations known as anthocyanoplasts, which are vacuole sites [88], and are responsible for the red, purple, and black pigments of fruits and vegetables, as well as being recognized for their notable health benefits [49]. The colors produced by anthocyanins depend on pH, light, and temperature, appearing reddish in more acidic conditions and turning blue as the pH rises [89].

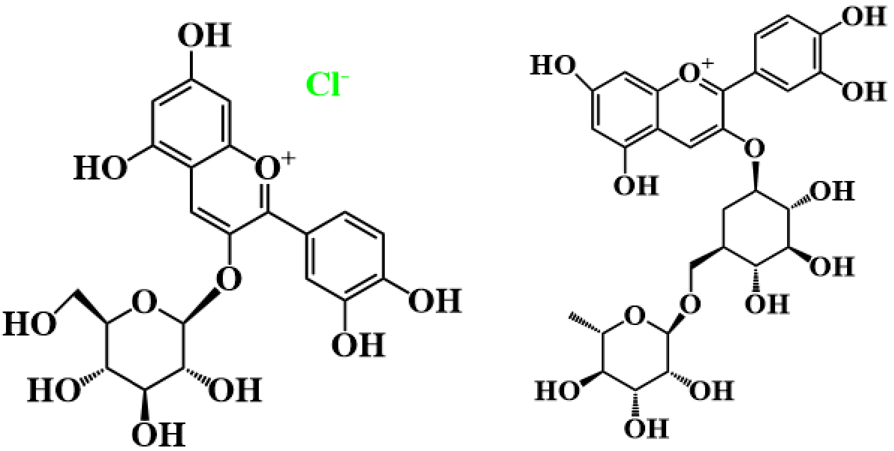
The blackberry is an excellent source of natural antioxidants. Indeed, the total anthocyanin content in *R. fruticosus* ranges from 70 to 180 mg per g fw [82,83], while in *R. ulmifolius*, the total anthocyanin content ranges from 5.87 to 35.55 mg per g fw [92].

Blackberry anthocyanins in *R. fruticosus* are mostly cyanidin derivatives (Figure 7). Cyanidin 3-*O*-glucoside is the most abundant anthocyanin found in blackberry at the ripened stage (92.3 to 335.6 mg per 100 g), followed by cyanidin 3-*O*-dioxalylglucoside

(16.9-107.5 mg per 100 g) [39]. Other anthocyanins found in blackberry fruit include cyanidin 3-*O*-xyloside, cyanidin 3-*O*-dioxaloylglucoside, cyanidin 3-*O*-(6-malonyl)-glucoside, pelargonidin 3-*O*-glucoside, malvidin 3-*O*-glucoside, cyanidin 3-*O*-arabinoside, cyanidin 3-*O*-xyloside, cyanidin 3-*O*-dioxalylglucoside, and cyanidin-3-*O*-glucoside acylated with malonic acid [13,36,39,85]. Anthocyanins such as cyanidin 3-*O*-glucoside (92.3-335.6 mg per 100 g fw) and cyanidin 3-*O*-dioxalylglucoside (16.9-107.5 mg per 100 g fw) were also detected in *R. ulmifolius* [76].

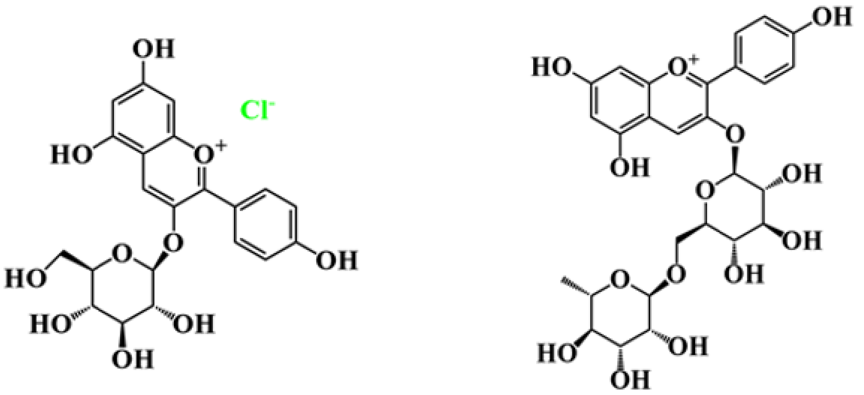
On the other hand, mulberries present anthocyanin levels ranging from 184.3 to 227.0 mg per 100 g of fruit [87]. Among anthocyanins, cyanidin 3-*O*-glucoside, cyanidin 3-*O*-rutinoside, pelargonidin 3-*O*-glucoside, and pelargonidin 3-*O*-rutinoside are abundant in *M. nigra* [47]. According to research, the anthocyanin contents of blackberries vary depending on variety, environmental conditions, cultivation site, degree of ripeness, and processing. Fruit maturation is reported to influence the total amount of anthocyanin in blackberries. The antioxidant capacity peaks in some species at early stages of development. However, from a practical perspective, berries should be harvested when fully ripe because the maturity stage has a significant impact on their flavour and taste [11,33,39,41,59].

Regarding anthocyanins' biological potential, it was reported that these phenolics have notable antioxidant abilities and capacity to induce enzyme activation, and hence inhibit possible DNA damage by carcinogens, reduce body inflammation, protect brain health, and enhance cognitive function [6]. According to existing data, the antioxidant potential of wild berries is higher than that of domesticated and genetically modified crops when comparing *R. fruticosus* and *R. ulmifolius*. In terms of anthocyanin content and antioxidant capacity, wild species are highly impressive. Anthocyanins are the main phenolic subclass found in *R. ulmifolius* fruits (23.8 mg per g extract), representing about 35% of the total phenolic compounds identified in them [13]. Evidence that anthocyanin levels found in these fruits are higher compared to other small fruits supports the enormous potential of blackberry and mulberry fruits as natural colour additives in the food, drug, nutraceuticals, and cosmetic industries, and their incorporation in pharmaceutics [82,83,85,87,88,89].



Cyanidin 3-*O*-glucoside

Rutin
(quercetin 3-*O*-rutinoside)



Pelargonidin 3–*O*–glucoside

Pelargonidin 3–*O*–rutinoside

Figure 7. Principal anthocyanins present in *R. fruticosus*, *R. ulmifolius*, and *M. nigra*.

1.2. Health benefits

Many studies have shown that the daily consumption of blackberries is an exceptionally essential source of health-promoting substances. Dietary improvements, particularly increased consumption of plant-based foods, may prevent more than 30% of all fatalities [78]. Blackberry fruit has been the subject of extensive research due to its high antioxidant content, which can normalize stress oxidative and inflammatory levels, as well as reduce cancer risk and cardiovascular complications, and has demonstrated biological activity against esophageal, colon, and oral cancers [24,91]. According to recent research, mulberries have positive biological properties that can help in the prevention of chronic diseases, such as cancer, neurotoxicity, obesity, diabetes, and memory loss [36,89,92].

The application in pharmaceutical sectors is critical for improving health naturally and without side effects. As far as we know, no negative effects of the administration of blackberries or mulberries have been observed, making it a feasible and potentially effective dietary strategy to improve disease prognosis [99].

1.2.1. Antidiabetic properties

Diabetes mellitus is a chronic endocrine condition in which the pancreas either stops producing insulin or produces inadequate insulin. Diabetes affects about 425 million people globally and is defined by a rise in blood glucose concentration (>7 mmol/L) [100]. It has been associated with the development of various significant problems at cardiovascular, neurological, and renal levels, leading to increased morbidity and mortality [98]. The International Diabetes Federation anticipated that, by 2030, there will be 552 million diabetics globally [101].

To establish glycemic control, diabetics use insulin and other therapy drugs, such as metformin, sodium-glucose cotransporter-2 inhibitors, and glucagon-like peptide 1 [102]. Before the development of insulin, medicinal plants were used to treat this condition. Because of their low cost, availability, and lack of negative effects, the use of natural plants was and still is an alternative for many people. Various plant genera and phytochemical constituent types with anti-diabetic properties have been used in this context [31,49,96,97]. Therefore, it is not surprising that formulations using anti-diabetic plant extracts or phytocompounds have been derived. Additionally, nowadays, systems such as "Herbal-based anti-diabetic drug delivery systems" are largely used to provide herbal medicines to treat diabetes [103].

Certain regions of the world employ black mulberry leaves, fruits, and barks as anti-diabetic medications, believing in their efficacy in lowering blood glucose levels [31,98-101]. In accordance with this, *M. nigra* has been shown to have a wide range of biological and pharmacological therapeutic benefits, including antidiabetic, anti-obesity, and anti-hyperlipidemic effects [103]. Hydroethanolic freeze-dried extracts of this fruit revealed potential for inhibiting pancreatic lipase, displaying a half maximal inhibitory concentration of 6.32 mg/mL [106].

Among both berries' constituents, quercetin has been demonstrated to have considerable antioxidant and anti-inflammatory characteristics and the ability to interfere with a variety of antidiabetic activities, including insulin secretion and sensitization, glucose level improvement, and inhibition of intestinal glucose absorption. By activating adenosine monophosphate and preventing lipid peroxidation, this phenolic molecule promotes glucose transporter 4, the principal facilitative mediator of glucose uptake in skeletal muscles, adipose tissues, and other peripheral tissues. Given that, it is not surprising that quercetin can be used to stabilize blood glucose and body weight [103, 104]. Furthermore, a single oral dosage of quercetin (400 mg) decreased α-glucosidase activity and reduced postprandial hyperglycemia in rats with type 2 diabetes [108].

Ferulic acid, another berry phenolic component, at 1000 mg per day for six weeks, showed the capacity to decrease total cholesterol, malonylaldehyde, tumour necrosis factor (TNF)-α, and triglycerides by 8.1, 24.5, 13.1, and 12.1%, respectively, and increase HDL cholesterol by 4.3% [109]. These findings suggested that ferulic acid can also help diabetic patients with hyperlipidemia. Ferulic acid was found to be generally safe, with LD_{50} values of 2445 mg /kg in male rats and 2113 mg /kg in female rats [110].

Additionally, diabetic male Wistar rats received injections of black mulberry fruit extracts at 150 and 300 mg/kg body weight for 4 weeks. After this time, microalbuminuria, albumin, glucose, insulin, creatinine, and creatine levels in the serum were measured. The study discovered that diabetic animals considerably improved in all of the measures tested. The activity of catalase activity was also improved. Furthermore, the histological examination of their kidney tissues revealed a significant reduction in degenerative anomalies and glomerular sclerosis. TNF-α, vascular cell adhesion molecule-1, and fibronectin mRNA expression were all downregulated in treated rats [105]. Therefore, the downregulation of TNF-α, VCAM-1, and fibronectin levels in diabetic rats avoids, or retards, the development of diabetic nephropathy. Altogether, these data support the evidence that mulberry fruit extract may be a potential agent in the treatment of diabetic nephropathy [111].

1.2.2. Antimicrobial properties

Plant-derived antimicrobial chemicals may limit the development of bacteria, fungi, viruses, and protozoa by different processes from those utilized by synthetic antimicrobials, and thus exhibit substantial therapeutic benefit in the treatment of resistant microbial strains. The antimicrobial activity of an agent is generally due its potential to chemically interfere with the manufacture or function of key components of bacteria and/or evade established antibacterial resistance mechanisms [49,108,109].

The majority of phytochemicals with therapeutic value found in fruits are secondary metabolites. Their antimicrobial activity varies depending on the structure, number, and position of substituent groups, the presence of glycosidic linkages, and the alkylation of hydroxyl groups [50,109]. As expected, blackberries' antimicrobial properties differ among cultivars and ambient and soil factors. Furthermore, it is important to note that it is not possible to associate the antimicrobial activity with a specific compound due to the capacity of phenolic compounds to act synergistically [31,88,91,110,111].

Recent research has revealed that blackberries and mulberries have notable antimicrobial properties. The antimicrobial activity of different *R. fruticosus* extracts was investigated against *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, *B. subtilis*, *B. mojavensis*, *Salmonella Hartford*, *Proteus vulgaris*, *Pseudomonas baetica*, *Micrococcus luteus*, and *Saccharomyces cerevisiae*. The inhibition zone diameter (mm) was measured, revealing that the ethanolic extracts are more competitive than the crude extracts, and show a notable antimicrobial potential against *Proteus vulgari* (20.53 mm). The lowest activity was observed against *S. Hartford* bacteria (9.54 mm). In this study, minimum inhibitory concentrations (MICs) and minimum bactericidal concentration (MBC) were not calculated [114].

Additionally, hydroethanolic extracts of *R. ulmifolius* proved to have bacteriostatic effects against three Gram-negative bacteria (*E. coli*, *Morganella morganii*, and *P. mirabilis*), four Gram-positive bacteria (MRSA-methicillin-resistant *S. aureus*, MSSA-methicillin susceptible *S. aureus*, *Listeria monocytogenes*, and *Enterococcus faecalis*), and one fungus (*Candida albicans*). The results obtained in this work revealed activity in some tested strains, with MIC values ranging between 5 and >20 mg/mL. To inhibit the growth of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, a concentration above 20 mg/mL was necessary. For the remaining Gram-negative strains, the most effective results were shown against *M. morganii* (MIC = 5 mg/mL) and *E. coli* (MIC = 5 mg/mL), followed by P. mirabilis (10 mg/mL) (Table 8) [13]. In another study, methanolic extracts of *R. ulmifolius* showed antimicrobial potential against two Gram-negative bacteria (*E. coli* and *Salmonella* typhimurium), three Gram-positive bacteria (*S. aureus*, *Enterococcus feacium*, *Streptococcus agalactiae*) and one fungus (*Candida albicans*). The most notable values were observed against *S. agalactiae* and *E. coli* bacteria (Table 9) [116].

The antimicrobial effects of *M. nigra* were also evaluated, especially in *S. aureus*, *P. aeruginosa*, and *E. coli*, where the ability of its extracts to inhibit the production of proinflammatory cytokines and interfere with *i*NOS and NF-*κ*B pathways was observed [116]. Considering the higher content of anthocyanins in this species, these effects could be attributed to these compounds. In fact, anthocyanins have potent antiviral and antibacterial properties, being already known for their antimicrobial potential against

K. pneumonia, *P. aeruginosa*, *S. aureus*, *E. coli*, H1N1, SARS CoV-2, and rabies and herpes simplex virus [49,50,112].

Additionally, the antibacterial efficacy of mulberry total flavonols was assessed against three bacteria (*E. coli*, *P. aeruginosa*, and *S. aureus*), revealing interesting MBC results against *S. aureus* and *E. coli* (Table 9) [117]. Another investigation demonstrated the potential of *M. nigra* ethanolic extracts to be used in acne-treatment beauty care products given their capacity to inhibit *S. epidermis* and *P. acnes* growth, revealing MIC values of 2.5% for both bacteria, and MBC scores of 2.5% and 5% against *S. epidermidis* and *P. acnes*, respectively [118].

Black mulberry juice also has antibacterial properties, with its ability against three Gram-negative strains (*E. coli*, *P. aeruginosa*, and *S.* Typhimurium) and five Gram-positive strains (*Bacillus spizizenii*, *B. subtilis*, *Corynebacterium diphtheriae*, *Enterococcus faecalis*, and *S. aureus*) being previously reported. The maximum zone of inhibition was against *P. aeruginosa* (19.87 mm), followed by *Bacillus spizizenii* (19.68 mm) and *B. subtilis* (18.46 mm). The minimum zone of inhibition was obtained against *E. coli* (9.98 mm). Among the Gram-positive species, *Bacillus* species exhibited the highest zones of inhibition while, regarding Gram-negative bacteria, *P. aeruginosa* had higher inhibition than *S.* Typhimurium and *E. coli* [119].

Table 8. Antimicrobial effect of *M. nigra* juice, *R. fruticosus* (crude and ethanolic extracts), and *R. ulmifolius* (methanolic and hydroethanolic extracts) [13,110,112-115].

Antimicrobial Activity								
Microorganisms	***M. nigra* juice (100 µL)**	***R. fruticosus***		***R. ulmifolius***				
		Crude Extract	**Ethanolic Extract**	**Methanolic Extract (15 µL)**			**Hydroethanolic Extract**	
	Mean Zone of Inhibition (mm)				**MIC**	**MBC**	**MIC**	**MBC**
Gram-negative bacteria								
Escherichia coli	9.98	9.37	16.70	28	4.03	8.92	5	>20
Klebsiella pneumoniae	-	-	-	-	-	-	>20	>20
Morganella morganii	-	-	-	-	-	-	5	>20
Porteus mirabilis	-	-	-	-	-	-	10	>20
Proteus vulgaris	-	12.75	20.53	-	-	-	-	-
Pseudomonas aeruginosa	19.87	-	-	-	-	-	>20	>20
Pseudomonas baetica	-	9.76	14.30	-	-	-	-	-
Salmonella Typhimurium	11.73	-	-	225	4.13	8.24	-	-
Salmonella Hartford	-	14.49	9.54	-	-	-	-	-
Gram-positive bacteria								
Enterococcus faecium	-	-	-	16	4.76	8.70	-	-
Enterococcus faecalis	16.03	-	-	-	-	-	5	>20
Listeria monocytogenes	-	-	-	-	-	-	5	>20
Bacillus spizizenii	19.68	-	-	-	-	-	-	-
Bacillus cereus	-	11.20	14.00	-	-	-	-	-
Bacillus subtilus	18.46	8.10	14.04	-	-	-	-	-
Bacillus mojavensis	-	9.79	15.43	-	-	-	-	-
Corynebacterium diphtheriae	15.57	-	-	-	-	-	-	-
Micrococcus luteus	-	10.64	15.00	-	-	-	-	-
Saccharomyces cerevisiae	-	-	11.52	-	-	-	-	-
Staphylococus aureus	17.37	7.28	15.64	39	3.22	7.17	-	-
Streptococcus agalactiae	-	-	-	50	2.29	4.38	-	-
MRSA	-	-	-	-	-	-	10	>20
MSSA	-	-	-	-	-	-	-	>20
Fungi								
Candida albicans	-	-	-	39	-	-	-	-

-: no data; MIC: minimal inhibitory concentration; MBC: minimum bactericidal concentration.

1.2.3. Antioxidant activity

Reactive species are products of normal cellular metabolism and play key roles in signal transduction pathways, growth regulation, gene expression, and immune responses. In the human body, various mechanisms are necessary to maintain redox homeostasis [49,116]. These mechanisms include non-enzymatic and enzymatic antioxidant defenses created in the body (endogenous), as well as those given by the food (exogenous). However, the overproduction and accumulation of free radicals can lead to oxidative damage [6]. This biological condition may be caused by a lack of antioxidant defense mechanisms, excessive reactive species production, and excessive activation of their systems, increasing aging and the pathology of many chronic diseases, such as cancer, cardiovascular disease, inflammation, diabetes, and Parkinson's and Alzheimer's disease [49,117]. Therefore, it is essential to reduce their levels. Flavonoids, stilbenes, and tannins are examples of exogenous antioxidants. For example, scavenging and detoxifying radical oxygen species and preventing their production, influencing the cell cycle, avoiding tumor suppression, and modulating signal transduction, apoptosis events, and metabolism, are all biologically relevant mechanisms attributed to phenolic compounds [11,49,58,118,119,120]. Their antioxidant diversity and concentration are greatly dependent on the species and cultivars. Pre-harvest practices, environmental conditions, harvest ripeness, postharvest storage, and processing operations are also key drivers of phytochemical profiles [11,17,40].

Blackberries are considered one of the richest sources of natural antioxidants due to their high content of phenolic compounds, such as anthocyanins, ellagitannins, flavonols, and flavanols [13,41,44,51,63]. In fact, they present an extraordinary capacity to scavenge chemically generated radicals, thus preventing a wide range of human disorders and maintaining a healthy balance between free radicals and antioxidant systems. In particular, blackberries have notable antioxidant abilities against superoxide radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\bullet}OH$), and singlet oxygen (O_2) [121,122].

The antioxidant capacity of blackberries was previously determined by in vitro assays, by the lipid peroxidation inhibition assays (TBARS), oxidative reactive oxygen and nitrogen species (ROS/RNS), hemolysis inhibition assay, the ORAC method, 2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid ($ABTS^{\bullet +}$), the ferric-reducing/antioxidant power (FRAP) method, 2,2-diphenylpicrylhydrazyl ($DPPH^{\bullet}$), and Trolox equivalent antioxidant capacity (TEAC) assay [23,36,39,46,52,56,86, 122,124].

In the TBARS experiment, *R. fruticosus* extract revealed a high antioxidant activity, displaying an IC_{50} value of 100 ug/mL, which is substantially lower than that obtained with the positive control, Trolox (139 ug/mL) [46]. Additionally, using FRAP assay, $ABTS^{\cdot+}$, and $DPPH^{\cdot}$, the obtained results were between 4.45-14.16 for FRAP, 2.28-8.89 for $ABTS^{\cdot+}$, and 2.63-9.35 mmol Trolox equivalents per·100 g fw for $DPPH^{\cdot}$ [76].

Furthermore, methanolic extracts of *M. nigra* at 76 μg showed the capacity to inhibit lipid oxidation by 28.7%, while ethanolic extracts exhibited lower inhibitory capacity (23.7–47.6%) [128]. The antioxidant abilities of its aqueous extracts were also evaluated, revealing lower abilities than the methanolic ones; at 100 μg/mL, the values obtained were 1.1% and 7.1% for aqueous and methanolic extracts, respectively, whereas at 300 μg/mL, the corresponding values were 7.1% and 21.6%, respectively [128]. However, when comparing wild blackberries (*R. ulmifolius*) with the cultivated ones (*R. fruticosus*), substantial differences were found, with the latter having higher antioxidant content [126].

To summarize, mulberries have lately gained a large amount of interest as prospective sources of functional foods due to a variety of biological benefits [107,125]. The obtained findings on the antioxidant activity of mulberry fruits support their incorporation in biological applications [100,107,123,126].

1.2.4. Anti-Inflammatory properties

Inflammation is the immune system's reaction to potentially damaging stimuli such as infection or injury. In the presence of stressors, immune cells release inflammatory substances, such as inflammatory cytokines, including TNF-α and interleukins (IL)-6 and 10, leading to increased nitric oxide (NO) levels and prostaglandins via the catalysis of cyclooxygenase-2 (COX-2) and NF-κB pathways [49,127,128]. Blackberry freeze-dried powders are capable of reducing mRNA expression of NF-*k*B and COX-2 in the liver [132].

A healthy lifestyle that includes physical activity, stopping smoking, and moderate alcohol intake, associated with a diet rich in fruits, vegetables, and whole grains, decreases the risk of developing chronic diseases. As expected, phenolic compounds, carotenoids, vitamins, and dietary fiber contribute to the anti-inflammatory and antioxidant effects of fruits and vegetables [48,49,129,130,131]. In particular, high quantities of dietary anthocyanins may be viewed as a feasible nutraceutical in the context of inflammatory disease. Among these, cyanidin 3-*O*-glucoside can reduce cytokine-induced inflammation in intestinal cells by inhibiting the production of NO, PGE2, and IL-8, and the expression of *i*NOS and COX-2 [50,130-132,135].

Focusing on blackberries and mulberries, anthocyanin-enriched fractions from fermented blueberry and blackberry beverages inhibited dipeptidyl peptidase-IV activity in LPS-stimulated murine macrophages. Computational docking demonstrated that this effect could be mainly attributed to delphinidin 3-*O*-arabinoside, which effectively inactivates dipeptidyl peptidase-IV by binding with a low interaction energy (−3228 kcal/mol). Additionally, anthocyanins and proanthocyanidins (100 μM cyanidin 3-*O*-glucoside and epicatechin equivalents, respectively) extracted from them reduced LPS-induced inflammatory response in mouse macrophages by stopping the NF-*κ*B pathway [136]. Another study that used RAW 264.7 macrophages stimulated with LPS demonstrated that blackberry anthocyanin extract (0–20 μg/mL)-treated macrophages presented lower levels of IL-1 and TNF-*α* [137]. Once again, this reduction is mainly associated with the ability of anthocyanins to interfere with NF-κB signaling [136], particularly of cyanidin 3-*O*-glucoside, which previously showed potential to decrease pro-inflammatory mediators NO, PGE2, COX-2, and IL-8 generated by cytokine-stimulated HT-29 cells [135]. In accordance with this, *R. fruticosus* also showed capacity to inhibit the secretion of pro-inflammatory IL-8 cytokines in two cellular models (HT-29 and T-84 cells) in a dose dependent-manner in both cell lines [97].

Ellagitannins are another significant polyphenol that has displayed anti-inflammatory properties. Previous research [138] examined their anti-inflammatory efficacy of TNF-α, IL-1B, IL-8, and NF-*κ*B on the AGS gastric cell line. Ellagitannins extracted from *R. fruticosus* suppressed TNF-*α*, showing an IC_{50} value of 0.67–1.73 mg/mL. At 2 mg/mL, ellagitannins inhibited TNF-*α* and NF-κB nuclear translocation by 57% and 67%, respectively. At lower doses, ellagitannins reduced IL-8 secretion, revealing an IC_{50} ranging between 0.7 and 4 mg/mL. Moreover, in a rat model of ethanol-induced stomach lesions, the protective effect of ellagitannins was also tested. Ellagitannins (20 mg/kg/day) were administered orally to rats for ten days, and ethanol was administered one hour before sacrifice. The mucosa of the stomach was separated and utilized to measure IL-8 release, NF-*κ*B nuclear translocation, TEAC, and superoxide dismutase and catalase activities. This investigation demonstrates that the treatment with these compounds can decrease NF-*κ*B nuclear translocation and suppress IL-8 production. The present work demonstrated that ellagitannins derived from *Rubus* berries definitively protect against the formation of gastric ulcers in rat animal models. In particular, ellagitannins can block the NF-*κ*B cascade either directly on the cell response to pro-inflammatory cytokines or act as antioxidant agents by inhibiting reactive species generated in several inflammatory conditions [139].

1.2.5. Neuroprotection

The human brain is responsible for a wide range of cognitive, motor and behavioral functions that require significant amounts of energy. Neurons are responsible for transmitting information to and from the brain. Neurodegenerative illnesses are distinguished by progressive brain cell death and neuronal loss, which impair motor or cognitive function. Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and spinocerebellar ataxia are examples of common neurodegenerative disorders. These disorders are a major public health concern, particularly among the elderly [121]. These disorders develop because the brain is more sensitive to oxidative stress than other organs due to the poor activity of antioxidant defense mechanisms [140].

Many epidemiological studies are being conducted to study the potential of phenolics to be used to promote neuronal health and prevent neural cells from being damaged through their antioxidant and anti-inflammatory properties, and thus delay Parkinson's and Alzheimer's diseases, ischemic diseases, and aging effects [129,137]. The inclusion of blackberries in the diet has been shown to reduce brain degeneration [9,138,139,140]. The neuroprotective capacity of this fruit mainly comes from its antioxidant capacity, promoted by the presence of phenolic compounds, such as anthocyanins, caffeic acid, and quercetin [49,141]. Indeed, these compounds can penetrate the hematoencephalic membrane and have neuroprotective effects on various cerebral structures in the brain, including the hippocampus and cortex [69]. Flavonoids may also have important impacts on mammalian cognitive function, perhaps halting the aging-related declines in memory and learning. These benefits are mostly sought for preventing brain damage, such as neurodegenerative diseases, and improving memory, learning, and cognitive functions [144]. Blackberries from the north of Portugal can lower intracellular reactive species levels, alter glutathione levels, and inhibit the emergence of caspases during treatment, hence reducing oxidative stress and preventing neurodegeneration [143]. Mulberry fruit extracts and cyanidin 3-*O*-glucoside have shown the ability to inhibit reactive species production and, consequently, neuronal injury [135].

Neuroblastoma cells exposed to H_2O_2 and treated with raw, digested, and dialyzed blackberry extracts at physiological concentrations revealed lower age-related neurodegeneration [9]. In addition, animal research found that an intermediate dosage of blackberry juice (5.83 mg/kg anthocyanins, 27.10 mg/kg polyphenols) enhanced mechanisms of behavioral coping with diazepam. The forced swim test supported these findings by demonstrating that blackberry juice, at moderate and high doses, improves the acute stress response [146]. These findings suggest that blackberry juice may have a therapeutic value in alleviating anxiety caused by stressful experiences. *M. nigra* revealed a notable protective effect against Alzheimer's disease, specifically by inhibiting amyloid-β-induced paralysis symptoms and suppressing over-sensitivity to exogenous serotonin by about 55.65% in transgenic Alzheimer's disease Caenorhabditis elegans models, which were treated with up to 1.00 mg/mL. These effects are due to the capacity of this fruit to activate the DAF-16/ SOD-3/ GST-4 pathway, improve antioxidant capacity, delay aging, and alleviate the symptoms caused by the amyloid-β protein [147]. These findings suggest that functional foods, such as mulberry, can be used to lower the risk of Alzheimer's disease.

1.2.6. Anticancer activity

Cancers are characterized by abnormal cell growth capable of invading other regions of the body, resulting in metastasis. A tumor is a complex multistage process that begins with the genesis of a cancer cell caused by DNA damage, followed by accumulation of mutations, progression to cell proliferation and tumor expansion, and, finally, progression to malignancy and metastasis. While new cancer incidence is expected to rise by 70% by 2034, approximately 35% of cancer deaths are attributed to behavioral and dietary risks, such as high body mass index, low fruit and vegetable intake, and lack of physical activity [148]. According to epidemiological and clinical research, a diet consisting of 400–800 g of various vegetables and fruits per day can prevent 20% or more of all cancer cases [2,48,129].

Phenolic berry content has shown the capacity to reduce inflammation, inhibit angiogenesis, protect against DNA damage, and influence apoptosis or proliferation rates in malignant cells. Indeed, they demonstrate the ability to interfere in all phases of cancer development, including initiation, promotion, progression, invasion, and metastasis [49,127,141,146]. Berry extracts also inhibited cancer-induced AP-1 and NF-κB, as well as decreasing the expression of the two proteins involved in tumor promotion and progression, i.e., vascular endothelial growth factor and COX2 [150]. These effects are intimately linked to the capacity of phenolics to alter the genomic stability at many phases in the cancer genesis process [133]. For example, anthocyanins have been shown to activate phase II enzymes, which may inactivate carcinogens triggered by phase I enzymes, and hence prevent DNA damage caused by the carcinogens [50,81,148].

Dietary bioactive compounds can also decrease telomerase activity by modifying histones or by inhibiting DNA methyltransferases. Telomerase activity has been detected in more than 80% of human malignancies, making the enzyme a promising target for anticancer treatment. According to research, the antiproliferative impact of blackberry fruits is mediated by their anti-telomerase activity [152]. Additionally, there have been no negative effects associated with the administration of blackberries, indicating that this fruit has the potential to be effective for a dietary plan to reduce cancer risk and assist cancer patients with illness prognosis [150].

Blackberries previously demonstrated significant chemo-preventative and antioxidant activities by inhibiting the growth, proliferation, and migration of the human A549 lung carcinoma cell line, and strong inhibitory effects on the cell growth of highly metastatic breast cancer HS578T cells, by inducing significant alterations in cell cycle regulators, causing G2/M arrests [153]. Blackberries and mulberries contain anthocyanin cyanidin-3-*O*-glucoside, which has promising qualities for usage in nutraceuticals, and has shown potential to limit cell proliferation, arresting the cell cycle in the G2/M phase, and inducing apoptosis in vitro [50,130,151]. In fact, in a recent investigation, rats were administered orally with purified cyanidin 3-*O*-glucoside (800 µmol/kg of body weight). After 30 min–2.0 h of delivery, this was detected in plasma, with a C_{max} value of 0.8 µM. This evidence represents added value regarding the incorporation of this anthocyanin in dietary supplements, aiding in the anticancer therapy of breast cancer [154].

M. nigra extracts have also been the subject of much research. A three-month enriched diet applied in $MUC2^{-/-}$ mice, with a model of spontaneous chronic intestinal inflammation and induced-intestinal tumors at three months, at 5% or 10%, resulted in a reduction in tumorigenesis and intestinal inflammation. Basically, mice aged 6 to 8 weeks that were supplemented with 5% or 10% *M. nigra* extracts for 10 days and there were observed improvements in their signs and symptoms caused by dextran sulfate sodium-induced acute colitis, preventing weight loss and bloody stools, and promoting positive changes in the histology of the colorectal lining [155].

1.2.7. Cardiovascular protection

Cardiovascular disorders affect the heart and blood vessels and are the major cause of death worldwide. People who have high blood pressure and cholesterol, as well as smokers, those who are sedentary or obese, and people who have a diet rich in salt, sugar, and fatty acids, are more susceptible to cardiovascular problems [156].

The current nutritional guidelines for the prevention of cardiovascular diseases include a Mediterranean-style diet rich in fruits, vegetables, and whole grains, as well as non-tropical vegetable oils, in order to reduce total cholesterol, oxidative stress, and inflammation [2,48, 60,129,154].

Blackberry phenolic compounds have demonstrated the capacity to diminish LDL oxidation, quench free radicals by hydrogen molecule donation, and interfere with liposome oxidation systems [155,156]. In particular, anthocyanins from M. nigra showed the capacity to protect human primary endothelial cells by decreasing the production of the cytokine-induced chemokine monocyte chemotactic protein 1, a protein directly linked to atherogenesis, and which is mainly responsible for attracting macrophages to sites of infection or inflammation [75]. Moreover, although not directly shown in blackberry flavonoids, several flavonoids also revealed the capacity to protect platelet function, which is crucial in the pathogenesis of these diseases. In fact, flavonoids can minimize platelet aggregation, reduce platelet generation of superoxide anions, and increase platelet NO production [160].

In epidemiological studies, diets high in plant-derived phenolic compounds have been shown to reduce the incidence of coronary heart disease. The chronic antioxidant and hypolipidemic characteristics of these compounds play critical roles in the prevention of lipoprotein oxidation and the formation of atherosclerotic lesions [2,142,156, 159].

II. Aims of study

The aim of the present study was to improve the knowledge about blackberries and mulberries, which are small, perishable, and seasonal red fruits, whose consumption is increasing worldwide, not only due to their organoleptic properties as well as their nutritional and phytochemical values.

The emergence of this consumer knowledge has been one of the driving reasons behind the development of food products that can satisfy basic dietary requirements as well as provide health benefits. Blackberry and mulberry fruits are well-known around the world for their mouth-watering taste that makes them suitable to consume either fresh or as an ingredient in value-added products and for culinary uses. Its popularity has grown because of consumer awareness and enthusiasm for healthier and lower-calorie foods. This has led to increased demand in the food processing industry.

To that objective, detailed goals for execution were developed, which were separated into two sections:

I. Physicochemical, colour and nutritional composition of three species of similar berry fruits, i.e., two blackberries (*R. fruticosus* and *R. ulmifolius*) and one mulberry (*M. nigra*)

- Study of their physicochemical characteristics, including size, weight, firmness, pH, moisture and ash, titratable acidity (TA), total soluble solids (TSS) and maturity index (TSS/TA ratio);
- Colour determination;
- Phenolic profile (coloured and non-coloured phenolics) by HPLC-DAD-ESI/MS^n and HPLC-DAD;
- Analysis of essential and non-essential minerals by Inductively Coupled Plasma Mass Spectrometry (ICP-MS); and
- Volatile composition through Headspace Solid-Phase Microextraction (HS-SPME).

II. Biological potential of blackberries and mulberries phenolic-rich extracts

- Phenolic profile analysis by HPLC-DAD-ESI/MS^n and HPLC-DAD;

- Evaluation of their antioxidant capacity against 1,1-diphenyl-2-picrylhydrazyl, nitric oxide and superoxide radicals ($\text{DPPH}^{\bullet}$, $^{\bullet}\text{NO}$ and $O_2^{\bullet -}$, respectively), alone and combined with positive control ascorbic acid to search possible synergic or antagonist effects;
- Evaluation of extracts' cytotoxicity effects in normal human dermal fibroblast (NHDF) cells, and human colonrectal adenocarcinoma (Caco-2) cells;
- The most promising phenolic-rich extract against Caco-2 cells' growth was also combined with positive control fluorouracil (5-FU) in order to investigate possible synergic or antagonist effects.

III. Materials and Methods

1. Standards and reagents

All chemicals used were of analytical grade. 1,1-Diphenyl-2-picrylhydrazyl (DPPH•), *β*-nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), nitrotetrazolium blue chloride (NBT), phosphate-buffered saline (PBS), trypan blue and were purchased from Sigma-Aldrich (St. Louis, MO, USA). N-(1-Naphthyl)ethylenediamine dihydrochloride, sulfanilamide, 4-nitrophenyl-alpha-D-glucopyranoside and sodium nitroprusside dihydrate (SNP) were obtained from Alfa Aesar (Karlsruhe, Germany). Trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA) solution, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), sodium nitroprusside dihydrate (SNP) were obtained from Alfa Aesar (Karlsruhe, Germany). High-glucose Dulbecco´s Modified Eagle Medium (DMEM), low-glucose DMEM, RPMI 1640 medium fetal bovine serum (FBS), L-glutamine, 4-(2-652 hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sodium pyruvate and antibiotics (penicillin, streptomycin and amphotericin B) were obtained from (Sigma Aldrich, Inc.). Normal human dermal fibroblasts (NHDF) and Human colorectal adenocarcinoma (Caco-2) cell lines were from American Type Culture Collection (ATCC, Manassas, VA, USA). Methanol and acetonitrile were from Fisher Chemical. Water was deionized using a Milli-Q water purification system (Millipore Ibérica, S.A.U., Madrid).

2. *Rubus fruticosus* and *R. ulmifolius* blackberries and *M.nigra* mulberry samples

Around 1 kg of *R. fruticosus*, *R. ulmifolius* and *M. nigra* (Figure 8A, B and C, respectively) samples, were collected at Covilhã region, between July and August 2022. Cultivated blackberry (*R. fruticosus*) and mulberry (*M. nigra*) were collected in July, and blackberry from brambles (*R. ulmifolius*) was collected at the end of August. All fruits were collected at commercial stage. The fruit collection was carried out manually carefully, with gloves, placed immediately in plastic bags, and transported to Health Science Research Centre facilities, at low temperatures, as soon as possible. The physicochemical characteristics and colour were immediately analysed. To explore the phenolic profile, essential and non-essential elements, volatile organic compounds and biological potential, *R. fruticosus* and *R. ulmifolius* blackberries and *M. nigra* mulberry were frozen with liquid nitrogen and maintained at -80 °C, to be further lyophilized

(SCANVAC CoolSafe^{tm}, Frilabo, Portugal) and powdered (mean particle size lower than 910 µm).

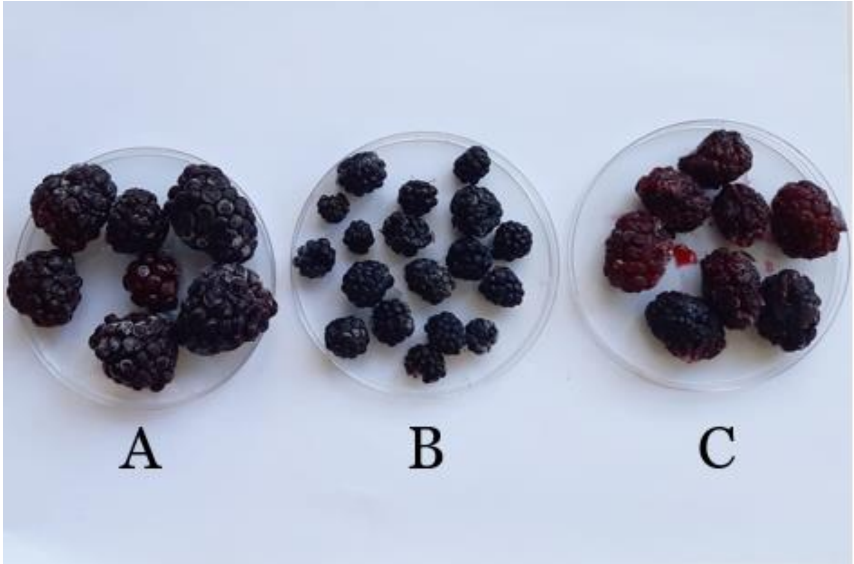


Figure 8. (A) *R. fruticosus*, **(B)** *R. ulmifolius* and *(C) M. nigra*.

2.1. Physicochemical characteristics analysis

2.1.1. Weight and size measurements

For each batch, twenty-five fruits from each species were selected randomly. Their weights were evaluated using a RADWAG Wagi Elektroniczne, Radom, Poland digital weighing scale, and fruit length and width were measured using a manual slide caliper ruler (Powerfix Profi Electronic Digital Calliper Modell-Nr. /Model No.: Z22855, Owing GmbH & Co. Kg, Stiftsbergstraβe 1 D-74167, Neckarsulm, Germany). Finally, results were reported in grams (g) and millimeters (mm) as mean of 25 fruits for weight and size, respectively.

2.1.2. Firmness

The firmness of blackberries and mulberries was measured using a texture analyzer (TA-XT plus, Stable Micro Systems, Surrey, UK) connected to a computer, equipped with a 50-N load cell. A 10 mm conical probe was used to penetrate the fruit while force required was measured in Newtons (N). The testing parameters used were penetration depth of 5 mm and a crosshead speed of 1 mm/sec. Results are mean of 25 measurements in different berries and are expressed in Newtons (N).

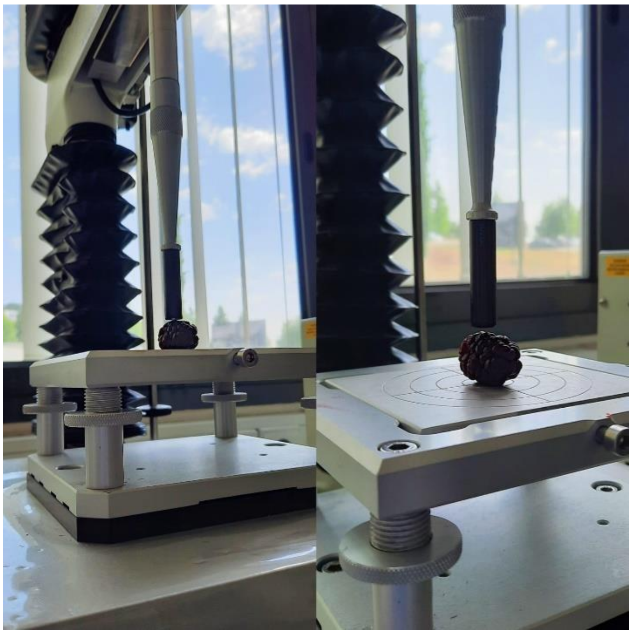


Figure 9. Firmness test on blackberries and mulberries.

2.1.3. Moisture and ash

Moisture and ash contents were performed according to the Association of Official Analytical Chemists methods [162]. A total of 5 g of each fresh sample was weighed in porcelain crucibles. Blackberries and mulberry samples were dried in an oven at 105 °C for 3 h to assess their moisture content. The samples were then transferred to a desiccator to cool and weigh. Dried samples were incinerated overnight at 550 °C in the furnace to determine the ash content (ashes were cooled in a desiccator and weighed). Moisture and ash determinations were done in triplicate.

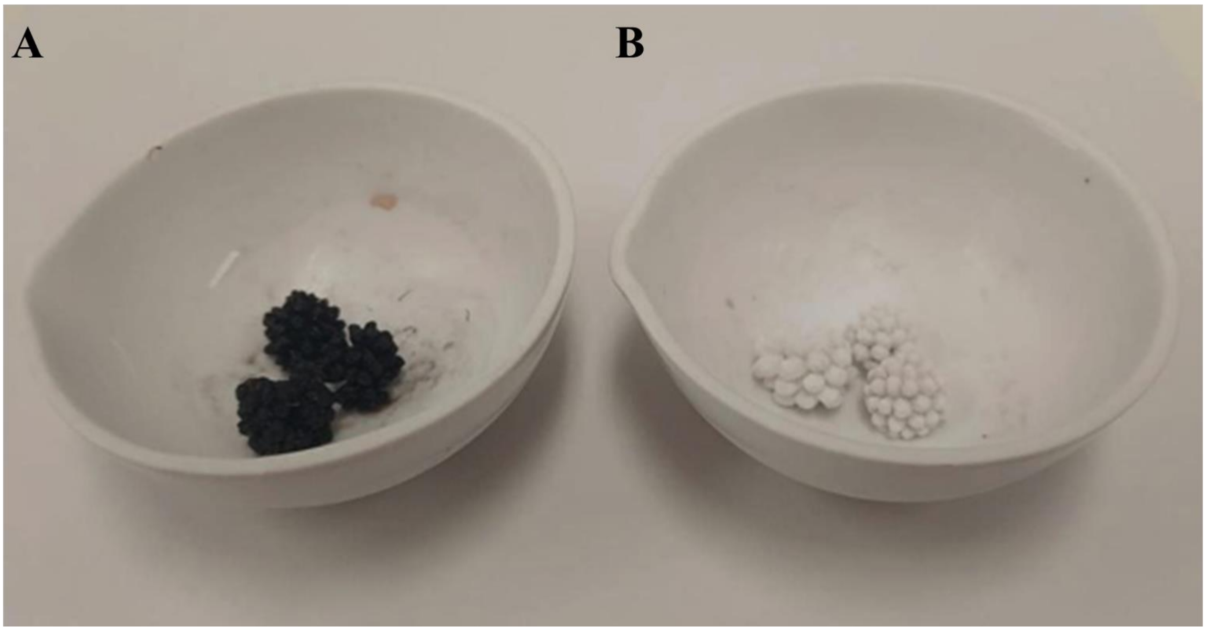


Figure 10. Moisture (A) and ash (B) of *R. ulmifolius*.

2.1.4. Total soluble solids (TSS), pH and titratable acidity (TA)

Fruit juice was extracted from 25 blackberries and mulberries with a juicer (Food Multi processor Hoffen food expert, PANK-H104 model (Lisboa Portugal). Total soluble solids (TSS) were measured with a refractometer (Labolan, Navarra, Spain). Before, the instrument was calibrated with distilled water, and measurements were made in triplicate for each independent homogenate. Results were expressed as ºBrix. To assess pH and TA, 5 g of fruit juice was mixed with 50 mL of deionized water and determined in triplicate for each independent homogenate. After, pH was measured using a benchtop pH meter (827 pH lab, Metrohm, Herisau, Switzerland). Titratable acidity (TA) was estimated by juice titration with 0.1 N NaOH up to a pH value of 8.1. Third step, results were expressed as malic acid content (g malic acid 100^{-1} fw). Finally, maturation index was calculated by making a ratio between TSS and TA.

2.1.5. Color measurements

Color measurements (520 nm) were assessed by tristimulus reflectance colorimetry in a colorimeter (CR-410 Konica Minolta, Tokyo, Japan) with an 8 mm diameter measuring area. CIE L*, a* and b* values were calculated using illuminant D65 and a 10º observer, according to CIEL*a*b* 76 Convention [40]. First, the analysis was performed by reflection, using a pulsed xenon arc lamp inside a mixing chamber. Second, colorimeter has been calibrated using a white tile. Skin color was evaluated in samples of 25 individual random blackberries from each species, by reading in two distinct points of the fruits. Results were represented as mean ± standard deviation of two measurements taken at equidistant points in an equatorial region of the respective fruit. Third, data obtained were used to calculate saturation mean values, chroma (*C*) and hue angle (h), using two formulas: [(a*) 2 + (b*) 2] 1/2 and arctan (b*/a*), respectively. The hue angle expresses color nuance and values are defined as follows: red-purple: 0º; yellow: 90º; bluish-green: 180º; blue: 270º. For ending the process, C* is a measure of chromaticity which defines purity or saturation of the color, data were processed using a processor by CR-410.

Figure 11. Color measurements.

2.2. Extraction of phenolic compounds

Phenolic compounds were extracted according to a method already described [163] to be possible to explore the antioxidant potential, aliquots of 1 g of powder sample were weighed and extracted with 20 mL of ethanol (70:30) for 2 hours under stirring and protected from light to avoid oxidations. Then, each extract of each species was centrifuged for 10 min at 2900 x g. The obtained supernatant was then evaporated to dryness under reduced pressure at 30 °C. The resultant extract was dissolved with 50 mL of deionised water and placed into the column. The solid-phase extraction cartridge was preconditioned with 20 mL of ethyl acetate, 20 mL of ethanol and 20 mL of 0.01 mol/L HCl. Phenolic-rich extracts were obtained by elution with 40 mL of ethanol containing 0.1% HCl, again diluted in deionised water, lyophilized and stored with silica and protected from light until analysis. The yield of extraction was 22.44 ± 0.11% for *R. fruticosus*, 47.4 ± 0.25% for *R. ulmifolius* and 28.15 ± 0.10 % for *M. nigra*.

On the other hand, and to analyze phenolics present in fresh fruits, samples were only lyophilized, obtaining extractions yields of 22.44 ± 0.11% for *R. fruticosus*, 47.4 ± 0.25% for *R. ulmifolius* and 28.15 ± 0.10 for *M. nigra*.

2.3. Phenolics identification and quantification

2.3.1. Identification of phenolic compounds via HPLC-DAD-ESI/MS^n

The phenolic compounds identification was performed in an Agilent HPLC 1100 series model equipped with a photodiode array detector (model G1315B), a mass detector in series (Agilent Technologies, Waldbronn, Germany), a binary pump (model G1312A), a degasser (model G1322A) and an autosampler (model G1313A), based on a method described by [164]. Injections (20 µL) of each species were performed in triplicate. Mass detector was an ion trap spectrometer (model G2445A) equipped with an electrospray ionization interface and was controlled by LC/MS software (Esquire Control Ver. 6.1. Build No. 534.1, Bruker Daltoniks GmbH, Bremen, Germany). A Nucleosil® 100-5 C18 column (25.0 cm x 0.46 cm; 5 µm particle size waters; Macherey-Nagel, Düren, Germany) was used, and mobile phase was composed of two solvents: eluent A consisted of water/formic acid (99:1, v/v), and eluent B of acetonitrile. The solvent system started with 8% of B, and reached 15% of B at 25 min, 22% at 55 min, and 40% at 60 min, with a wash-out period of 5 min and returned to initial conditions afterwards. Mass spectra were acquired with a scan range from m/z 100 to 1200 and MS parameters were set as follows: capillary temperature was 350 °C, capillary voltage was set at 4 kV, nebulizer pressure was 65.0 psi and nitrogen flow rate was 11 L/min. Flow rate was 0.8 mL/min during the whole run and all gradients were linear. Collision-induced fragmentation experiments were performed in ion trap using helium as collision gas, with voltage ramping cycles from 0.3 to 2 V. For anthocyanins, mass spectrometry data were acquired in positive ionization mode while for non-colored phenolics, acquisition was done in a negative ionization mode. MS^n was carried out in an automatic mode on more abundant fragment ion in $MS^{(n-1)}$ HPLC system was controlled by ChemStation for LC 3D Systems software Rev. B.01.03-SR2 (Agilent Technologies Spain S.L., Madrid, Spain). Phenolic compounds were tentatively identified based on their elution order, retention times, and ultraviolet-visible and mass spectra features as compared to authentic standards analyzed under the same conditions and data available in literature [164,165,166].

2.3.2. Quantification of phenolic compounds by HPLC-DAD analysis

Twenty microlitres of each sample were analyzed on a Shimadzu LC-2010A HT Liquid Chromatography system (Shimadzu Corporation, Kyoto, Japan) using a Nucleosil® 300 C18 column (250 x 4.6 mm; 5 µm particle size waters; MZ-Analysentechnik GmbH, Mainz, Germany). Detection was achieved with a Shimadzu SPD-M20A diode-array detector using LabSolutions software (Shimadzu Corporation, Kyoto, Japan), according to Gonçalves and co-workers [167].

2.3.2.1. Determination of anthocyanins

The mobile phase consisted of water/formic acid/acetonitrile (87:10:3, v/v/v; eluent A) and water/formic acid/acetonitrile (40:10:50, v/v/v; eluent B) using a gradient program as follows: from 10% to 25% B (10 min), from 25% to 31% B (5 min), from 31% to 40% (5 min), from 40% to 50% B (10 min), from 50% to 100% B (10 min), from 100% to 10% B (5 min). Total run time was 50 min. The flow rate was 0.8 mL/min. Peak purity was checked by the software contrast facilities. The compounds in each sample were identified by comparing their retention times and ultraviolet-visible spectra in the 200-600 nm range with the library of spectra previously compiled by the authors and by external standards at 520 nm [163]. Cyanidin 3-*O*-glucoside (1), cyanidin 3-*O*-glucoside (2), pelargonidin glucoside and pelargonidin deoxyhexoside-hexoside were quantified as petunidin3-*O*-rutinoside ($y = 26536x + 10906$; $R^2 = 0.999$), while cyanidin arabinose/xyloside, cyanidin-malonyl-glucoside and cyanidin-dioxalyl-glucoside were quantified as cyanidin 3-*O*-rutinoside ($y = 39242x + 18052$; $R^2 = 0.999$). The total anthocyanins (∑) were the result of the sum of each determined compound belonging to anthocyanins.

2.3.2.2. Determination of non-coloured phenolic compounds

The mobile phase used was composed of 2% (v/v) acetic acid in water (eluent A) and 0.5% (v/v) acetic acid in water and acetonitrile (50:50, v/v, eluent B). The solvent system starts with 10% of eluent B and installs a gradient to obtain 24% B at 20 min, 30% B at 40 min, 55% B at 60 min, 70% B 65 min, 80% B at 70 min, 100% B at 75 min, and maintain 100% B isocratic during 5 min (80 min). The solvent flow rate was 1,0 mL/min. Spectral data from all peaks were accumulated in the range of 200-400 nm and chromatograms were recorded at 280, 320 and 350 nm. Peak purity was checked by the software contrast facilities. Phenolic compounds quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. The quantification of phenolic compounds was achieved by the absorbance recorded in the chromatograms relative to external standard at 350 nm for flavonols, 320 nm for hydroxycinnamic acids and 280 nm for tannins and hydroxybenzoic acids [163]. Ellagitannins (Pedunculagin I and II), galloyl-hexahydroxydiphenoyl-glucoside and ellagic acid pentoside were quantified as 4-hydroxybenzoic acid ($y = 24800x - 4376{,}7$; $R^2 = 0.999$), whereas 5-*p*-Coumaroyl quinic acid as *m*-coumaric acid ($y = 17721x + 121678$; $R^2 = 0.999$). On the other hand, quercetin 3-*O*-glucuronide, quercetin 3-*O* glucoside derivative and quercetin 3-pentoside were quantified as quercetin 3-*O*-glucoside (y = 4393,5x - 5758,9; R^2 = 0.999). The total non-coloured phenolics (∑) were the result of the sum of each determined compound belonging to non-coloured phenolics.

3. Minerals Identification

3.1. Samples mineralization

A Milestone (Sorisole, Italy) MLS 1200 Mega high-performance microwave digestion unit equipped with an HPR-1000/10 S rotor was used for the acid mineralization of the samples. Approximately 250 mg of powdered *R. fruticosus* and *R. ulmifolius* blackberries and *M. nigra* mulberries were directly weighted into the microwave polytetrafluoroethylene (PTFE) vessels. Then, 3 mL of 69% (w/w) nitric acid (HNO_3) and 1 mL of 30% (v/v) peroxide hydrogen (H_2O_2) were added to each vessel. The mixture was submitted to the following microwave heating program: 250 W for 1 min, 0 W for 2 min, 250 W for 5 min, 400 W for 5 min, and 600 W for 5 min [168]. After cooling, the vessel content was transferred to 25 mL volumetric flasks and the volume was made up with ultrapure water. For analytical quality control, the certified reference material BCR-679 (white cabbage, supplied by EC Institute for Reference Materials and Measurements, Geel, Belgium) was used, and processed as the samples.

3.2. Samples solutions analysis

Samples solutions analysis was performed according to [168]. Ca, K, Na and Mg were determined by FAAS, using a PerkinElmer (Waltham, USA) Analyst 200 instrument. External calibration was carried out with Ca (0.1 to 2.5 mg/L), K (0.4 to 2 mg/L), Na (0.2 to 1 mg/L) and Mg (0.025 to 0.25 mg/L) standard solutions prepared with 0.1% m/v of La, used as a "releasing agent" to overcome potential chemical interferences in the atomization process at the air/acetylene flame. The remaining elements were determined by ICP-MS, using an iCAPTM Q instrument (Thermo Fisher Scientific, Bremen, Germany), equipped with a Meinhard® TQ+ high sensitivity nebulizer, a baffled cyclonic spray chamber (Peltier-cooled), a standard quartz torch and a two-cone design (nickel sample and skimmer cones). High purity argon (Gasin II, Leça da Palmeira, Portugal) was used as the nebulizer and plasma gas. The ICP-MS instrument operational parameters were: 1150 W (RF-power), 14 L/min (plasma gas flow), 0.8 L/min (auxiliary gas flow) and 0.95 L/min (nebulizer flow rate). The following elemental isotopes (m/z) ratios ^{7}Li, ^{9}Be, ^{27}Al, ^{51}V, ^{52}Cr, ^{55}Mn, ^{57}Fe, ^{59}Co, ^{60}Ni, ^{65}Cu, ^{66}Zn, ^{75}As, ^{82}Se, ^{85}Rb, ^{88}Sr, ^{95}Mo, ^{107}Ag, ^{111}Cd, ^{118}Sn, ^{121}Sb, ^{137}Ba and ^{205}Tl were monitored for analytical determinations. The elemental isotopes ^{45}Sc, ^{89}Y, ^{115}In, ^{159}Tb and ^{209}Bi were used as internal standards. The instrument was tuned for maximum signal sensitivity, stability, low oxides and doubly charged ion formation using the Tune B iCAP Q solution (Thermo Fisher Scientific; 1 µg/L of Ba, Bi, Ce, Co, In, Li and U in 2% HNO_3 plus 0.5% HCl). The limits of detection (LOD) and quantification (LOQ) were calculated as the concentration corresponding to 3.3 and 10 times the standard deviation of 10 replicate integrations of the calibration blank (HNO_3 2%), respectively. All samples were analysed in triplicate. Results were expressed as µg/g on dw basis.

3.3. Estimated daily intake of essential elements

The estimated daily intake (EDI) of essential elements resulting from blackberries and mulberries consumption was calculated by multiplying the determined mean content of each mineral (C_{element}: µg/kg dw basis) by the average per capita daily recommended consumption of these berries ($DC_{\text{blackberries and mulberries}}$) (I):

$$\text{(I)}\ EDI = C_{\text{elements}} \, x DC_{\text{blackberries and mulberries}}$$

The EDI value is expressed as mg/day, and the DC recommend was assumed to be 144 g/ person, according to Food and Agriculture Organization (FAO) data for the European Union [169]. The obtained EDI value was then compared with the corresponding Average Requirement (AR) or Adequate Intake (AI), as suggested by European Food Safety Authority (EFSA) [169]. Cobalt (Co) and Cs elements were not considered because, there is no clear and reproducible indicator based on the intake of Co and Cs levels, by humans, sufficient to establish its AR/AI.

3.4. Estimated weekly intake of non-essential elements

The Estimate Weekly Intake (EWI) value of non-essential elements resulting from blackberries and mulberries ingestion was calculated based on the determined elemental content (C_{element}: µ/g dw basis), the average per capita weekly recommended consumption of blackberries and mulberries ($WC_{\text{blackberries and mulberries}}$) and the standard human body weight (bw): according to the formula II:

$$\text{(II)}\ EWI = \frac{C_{\text{elements}} \, x WC_{\text{blackberries and mulberries}}}{bw}$$

The EWI is expressed as mg/week/bw. WC was assumed to be 42.21 g/person [169] and bw as 70 kg. The obtained EWI value was then compared with the corresponding

Provisional Tolerable Weekly Intake (PTWI) published by Joint FAO/WHO Expert Committee on Food Additives (JECFA) [170].

4. Volatile compounds analysis

4.1. Headspace solid-phase microextraction (HS-SPME)

The analysis of the volatile compounds (aldehydes, alcohols, ketones, esters, furans, hydrocarbons and acids) through HS-SPME with a 50/30 µm divinylbenzene/carboxen/polydimethylsiloxane (DVB/ CAR/PDMS) (Supelco Bellefonte, PA, USA) was performed according to publications [171] with slight modifications. Briefly, to analyse possible differences regarding volatile compounds between fresh and lyophilized samples, approximately 0.1 g of lyophilized *R. fruticosus* and *R. ulmifolius* blackberries and *M. nigra* mulberries fruits, or 1 g of fresh *R. fruticosus* and *R. ulmifolius* blackberries and *M. nigra* mulberry fruits, were stirred (500 rpm) with 5 mL of water and 1.5 g of sodium chloride (NaCl) at 45 °C, for 5 min. The fiber was then exposed to the headspace for 10 min, under continuous agitation (250 rpm) at 45 °C. Afterward, the analytes were thermally desorbed on the injector of an EVOQ 436 GC system (Bruker Daltonics, Fremont, USA) coupled to a SCION SQ mass detector and a Bruker Daltonics MS workstation software (version 8.2) coupled to a Combi-PAL autosampler.

4.2. Gas chromatography-mass spectrometry (GC–MS) analysis

The conditions used were like those described for GC-MS volatile compounds analysis [171], with some modifications. The injector port was heated to 220 °C and injections were performed in splitless mode. The chromatographic separation was carried out using a capillary column Rxi-5Sil MS (30 m × 0.25 mm × 0.25 µm) from RESTEK. High purity He C-60 (Gasin, Portugal) was used as the carrier gas at a constant flow rate of 1.0 mL min^{-1}. The oven temperature was held for 1 min at 40 °C, then increased at a rate of 5 °C/min until reaching 250 °C (held for 5 min) and followed by an increase of 5 °C/ min to 300 °C. The injection was made in splitless mode and the injector was at 250 °C (held for 20 min). The MS detector was operated in electron impact (EI) mode and data acquisition was performed in full scan mode with a mass range between 40 and 500 m/z at a scan rate of 6 scans/s. The analysis was performed in full scan mode. Compounds were identified according to their retention indices relative to C8-C20 n-alkanes and mass spectra, which were compared with those of the NIST 14MS Library Database (Match and R. Match greater than 80%), pure standards analyzed under the same conditions, NIST Chemistry WebBook, bibliography Viant and co-workers [166,167, 171]. Each sample was analysed three times.

5. Biological potential of *R. fruticosus* and *R. ulmifolius* blackberry and *M. nigra* mulberry phenolic-rich extracts

5.1. Antioxidant activity

As already mentioned, the analyses of the antioxidant capacity of these berries were done using the phenolic-rich extracts obtained by SPE to scavenging DPPH•, •NO and $O_2^{\bullet -}$, assessed spectrophotometrically using a Microplate Spectrophotometer Reader (Bio-Rad Laboratories, Hercules, CA, USA). In the three assays, ascorbic acid was used as positive control and each assay was done using seven concentrations for each extract and done, at least, in triplicate. The obtained results were expressed as 25% inhibitory concentration (IC_{25}) or half-maximal inhibitory concentration (IC_{50}) values (μg/mL).

5.1.1. 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH•) assay

The ability of blackberry and mulberry phenolic-rich extracts to act as a free radical scavenger against DPPH• was prepared in a 96-well plate (concentrations ranging between 3.3 and 211.11 μg/mL). The reaction mixtures in the sample wells consisted of 25 μL of extract (redissolved in methanol) and 200 μL of 150 mM methanolic DPPH•. Control was composed of replacing the sample with methanol, while blank was composed of methanol, or diluted extract, and methanol. After the addition of DPPH•, the plate was incubated for 30 min at room temperature, and the absorbance was measured at 515 nm. Similar conditions were also applied to study the potential effect of phenolic-rich fractions combined with ascorbic acid at different conditions (25:75, 75:25 and 50:50).

5.1.2. Nitric oxide radical (•NO) assay

Antiradical activity was determined following a previously described method [173]. This activity was measured spectrophotometrically in 96-well plate reader at 560 nm, using different sample extract concentrations (10.4-666.7 μg/mL). The reaction mixture in each well consisted of 100 μL of extract dissolved in buffer (KH_2PO_4 100 mM, pH 7.4) and 100 μL of SNP (20 mM). Blanks and controls contained phosphate buffer and SNP. The plates were incubated at room temperature for 60 min, under light. 100 μL of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine in 2% H_3PO_4) was then added (blanks received 100 μL H_3PO_4), and 60 min later, the observance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthylethylenediamine was determined. Similar conditions were also applied to study the potential effect of phenolic-rich fractions combined with ascorbic acid at different conditions (25:75, 75:25 and 50:50).

5.1.3. Superoxide radical ($O_2^{\bullet -}$) assay

The effect of the phenolic-rich extracts against $O_2^{\bullet -}$-induced reduction of nitro-tetrazolium blue chloride was monitored at 562 nm, using different extract concentrations (5.2-333.3 µg/mL). Superoxide radicals ($O_2^{\bullet -}$) were generated by the NADH/phenazine methosulfate system as previously reported [171]. Each well contained 50 µL each diluted extract dissolved in potassium phosphate buffer (19 mM, pH 7.4), 50 µL NADH, 150 µL NBT, and 50 µL PMS. The control was composed of phosphate buffer instead of diluted extract. After the addition of PMS, each plate was immediately collocated in the spectrophotometer and absorbance readings were collected every 10 seg over 2 min. Similar conditions were also applied to study the potential effect of phenolic-rich fractions combined with ascorbic acid at different conditions (25:75, 75:25 and 50:50).

5.2. Cell culture assays

5.2.1. Cell culture conditions and treatments

Normal human dermal fibroblasts (NHDF) and Caco-2 cells (Figure 12A and B, respectively) were cultured in 75 cm^2 culture flasks and incubated at 37 °C in a humidified atmosphere of 5% CO_2. NHDF cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, and 1% penicillin/streptomycin, while Caco-2 cells were cultured as a monolayer in DMEM supplemented with 20% FBS, 1% penicillin/streptomycin In order to evaluate the antiproliferative effects, 200 µL of NHDF and Caco-2 cells were seeded at density of 1.0×10^4 cells and 2.5×10^4 cells per mL, respectively, and after one day of incubation, both cell lines were treated with 200 µL of six different concentrations of *R. fruticosus* and *R. ulmifolius* blackberries and *M. nigra* mulberry extracts (50 to 800 µg/mL) for other 24 hours. After this time, the medium was completely removed, and the viability of cells was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Untreated cells were used as control and 5-fluorouracil (5-FU; 0.65, 6.50 and 65 µg/mL) was used as positive control. In addition, the most promising phenolic-rich extract was mixed with 5-FU to see possible synergic effects on Caco-2 cells. A total of six independent experiments per extract (or positive control), at least, were performed. For the several assays, NHDF cells were used between passages 14 to 19, and for Caco-2 cells, from 39 to 48.

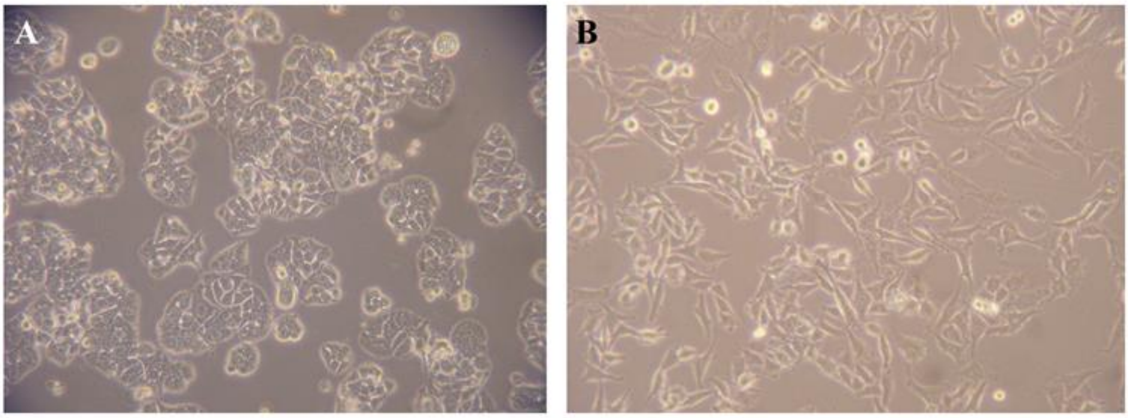


Figure 12. Image of (A) NHDF and (B) Caco-2. 400x magnification.

5.2.1. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The metabolic activity of cells was evaluated via their capacity to reduce the yellow MTT (0.5 mg/mL in the appropriate serum-free medium) into a blue formazan product after 4 hours of incubation at 37 °C. Therefore, after 24 hours of cells' exposition to each phenolic-rich extract, NHDF cells the medium of each well was removed, and twice washed with 200 μL of PBS. Then, for 4 hours, MTT was added. After this time, the MTT-containing serum-free medium was removed and the formazan crystals were dissolved using DMSO [175]. The absorbance was measured at 570 nm using a microplate reader Bio-Rad Xmark spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA). The values of cell proliferation were expressed as percentages based on the relative absorbance measured in the treated wells versus control wells [176].

6. Statistical analysis

All data were recorded as mean ± standard deviation of, at least, triplicate determinations. Mean values were compared using one-way analysis of variance (one-way ANOVA) and the means were classified by Tukey's test at a 95% level of significance (or two-way ANOVA and the Bonferroni test when applicable). Differences were considered significant for $P < 0.05$. To determine the contribution of the total phenolic compounds on the antioxidant activity showed by *R. fruticosus*, *R. ulmifolius* blackberries and *M. nigra* mulberry, Pearson's correlation coefficients were calculated.

All analyses were performed using Graph Pad Prism Version 5.01 (GraphPad Software, Inc., San Diego, CA).

IV. Results and Discussion

As a mentioned before, two samples of blackberries (*R. fruticosus* and *R. ulmifolius*) and one sample of mulberry fruits (*M. nigra*) from Covilhã region (Portugal) were studied. The fruits were characterized regarding their physicochemical characteristics (size, weight, color, firmness, pH, TA, TSS and TSS/TA). In addition, their phenolic profile (coloured and non-coloured phenolic compounds) by HPLC-DAD-ESI/MS^n and HPLC-DAD, essential and non-essential elements by ICP-MS and volatile organic compounds by HS-SPME were also explored.

In addition, the antioxidant capacity against1,1-diphenyl-2-picrylhydrazyl, nitric oxide and superoxide radicals (DPPH•, •NO and O_2•-, respectively) of *R. fruticosus* and *R. ulmifolius* blackberries and *M. nigra* mulberry phenolic-rich extracts were also assessed, as well as their normal human dermal fibroblasts (NHDF) and human colon Caco-2 carcinoma cells.

I. Physychochemical composition, color and nutritional characteristics

1.1. Physicochemical parameters

Both blackberries and mulberries are preferably consumed fresh, they can also be processed into juices, jams, purees, concentrates, and desserts. Size, weight and firmness, and color are the main contributors that influence consumers' and producers' choices when they are still fresh. The quantities of primary (e.g., sugars, amino acids, and organic acids) and secondary (e.g., phenolics and volatile organic compounds) metabolites are closely related to these factors [29,171].

A particular fruit variety's consumer attraction and acceptance can be attributed to its advantageous health-promoting properties, high nutritional content, and distinctive taste and aroma. As far as we know, it is largely accepted that blackberries and mulberries present considerable amounts of vitamins, minerals, and other beneficial substances like phenolics. These fruits are at their best and tastiest when fully matured. Based only on coloration, it is difficult to choose the optimal picking time. In actuality, this is the time when the fruits' rich dark color disappears and they are simple to harvest [29,38].

As a result, the physicochemical characteristics (size, weight, firmness, pH, TA, TSS and TSS/TA) and color (Table 9) of blackberries and mulberries were analysed in light of the health benefits associated with their consumption as well as their significance on the global market. Some statistical differences were discovered ($p < 0.05$).

R. fruticosus was therefore the largest species, followed by *M. nigra* in terms of size (Table 9). Regarding their weight, they range from 2.16 ±0.12 (*R. ulmifolius*) to 7.75 ±0.28 g (*R. fruticosus*). Lower weight was found in *M. nigra* from the East Anatolia Region of Turkey (4.37 g) compared to earlier research, providing another evidence that physicochemical characteristics also depend on origin and climate. Firmness, which is a key quality parameter for fruits and vegetables and is frequently used to evaluate their quality for selective harvesting and to determine post-harvest softening, influences consumers' preferences in addition to size and weight. Additionally, cultivar, maturity stage and time of harvest (early and late cultivars) have an impact on this feature [178]. Firmness values are shown in Table 8 and varied significantly from 5.51 N (*R. ulmifolius*) to 5.62 N (*M. nigra*). As is to be expected, firmness decays as consequence of weight loss and dehydration. According to published research, Romanian *R. fruticosus* cultivars have a firmness range of 5.23 to 8.56 N [179], which is comparable to the results of our study. Sweet cherries display values between 7.28 and 16.99 N, making them significantly firmer and more robust than other red fruits [40].

Table 9. Physicochemical parameters and colour of *R. fruticosus*, *R. ulmifolius* and *M. nigra* grown in Covilhã region, Portugal.

Berries		*Rubus fruticosus*	*Rubus ulmifolius*	*Morus nigra*
Harvest date		13 July, 2022	September 4, 2022	July 17, 2022
Size (mm)	Lenght	20.42 ± 0.27	14.34 ± 0.21^{a}	18.38 ± 0.24^{ab}
	Width	24.78 ± 0.35	14.31 ± 0.22^{a}	25.58 ± 0.35^{b}
Weight (g)		7.75 ± 0.28	2.16 ± 0.12^{a}	6.26 ± 0.23^{ab}
Firmness (N)		5.58 ± 0.06	5.51 ± 0.07	5.62 ± 0.074^{b}
Moisture (%)		79.34 ± 1.05	79.12 ± 0.75	75.75 ± 1.03
Ash (%)		99.48 ± 0.11	99.37 ± 0.14	97.74 ± 0.31
pH		3.39 ± 0.13	5.59 ± 0.26^{a}	3.93 ± 0.08^{ab}
TA (g malic acid)		1.65 ± 0.11	0.35 ± 0.12^{a}	1.93 ± 0.10^{ab}
TSS (°Brix)		11.05 ± 0.28	12.98 ± 0.29^{a}	18.72 ± 0.33^{ab}
Maturity Index (TSS/TA)		6.74 ± 0.27	34.58 ± 1.12^{ab}	9.57 ± 0.16
Colour	L*	47.02 ± 0.05	61.90 ± 0.06^{a}	50.16 ± 0.05^{ab}
	a*	1.81 ± 0.12	0.09 ± 0.13^{a}	2.72 ± 0.17^{ab}
	b*	1.63 ± 0.05	3.04 ± 0.04^{a}	1.59 ± 0.05^{b}
	Chroma	2.46 ± 0.10	3.04 ± 0.05	3.26 ± 0.145^{a}
	Hue	43.32 ± 0.08	88.22 ± 0.02^{a}	35.87 ± 0.11^{ab}
Yield %		22.44 ± 0.11	47.4 ± 0.25	28.15 ± 0.10

Values are expressed as mean ±standard deviation of three independent assays. Significant differences between cultivars according to the Tukey's test ($p < 0.05$) are indicated by: ^{a} vs *R. fruticosus*; ^{b} vs *R. ulmifolius*.

Ash and moisture are also crucial parameters in food quality. Ash is considered the total amount of minerals present in food, refers to the inorganic residues that remain after water and organic matter have been removed. Both moisture and ash contents (concentration and type of minerals) affect a number of quality characteristics, including appearance, taste, texture and stability [180].

The three berries under study had moisture levels of 75.75 ± 1.03 % for *M. nigra*, 79.12 ± 0.75 % for *R. ulmifolius* and 79.34 ± 1.05 for *R. fruticosus*. On the other hand, ash contents were very similar between the species, with *M. nigra* having a value of 97.74 ± 0.31 %, *R. ulmifolius* having a value of 99.37 ± 0.14 %, and *R. fruticosus* having a value of 99.48 ± 0.11 % (Table 9). *M. nigra* has similar moisture values previously reported in the literature, however, ashes can range from 3.5 to 6%, a complete different value compared to evaluated in our study [30,175].

The pH values of *R. fruticosus* and *R. ulmifolius* blackberries, and *M. nigra* mulberry fruits were also measured, because this characteristic has a significant impact on the quality of fruits, particularly berries. The obtained pH data for *R. ulmifolius*, *M. nigra* and *R. fruticosus* were 5.59 ± 0.26, 3.93 ± 0.08 and 3.39± 0.13, respectively (Table 9), which is slightly higher than other previously published results (values around 2.89) [181]. However, our results are in agreement with the literature in that wild blackberries have a higher pH and may taste sweeter than those cultivated blackberries [179]. In addition, a study from Italy reported pH values for *R. ulmifolius* from different regions of Calabria, from 3.30 to 5.12 [182], while a study from Romania found pH values for *R. fruticosus* between 3.01 and 4.07 [179]. Cultivar genotype, climate, type of soil, agronomic practices and conditions, post-harvest conditions and processing methods can all contribute to this variation.

Moreover, total soluble solids (TSS) and TA are significant factors in foods' quality, contributing to an intense flavor, which in turn, influences consumers' preference and acceptability. In a general way, the preference tends to increase with high TSS and TA levels. As far as we know, both parameters are strongly controlled by the genotype [178]. In addition, sugar levels and organic acids rise when fruit ripens, being lower in the early phases of fruit development, but higher as the fruit matures. In the current investigation, the obtained TA values were 0.35 ± 0.12, 1.65 ± 0.11 and 1.93 ± 0.10 for *R. ulmifolius*, *R. fruticosus* and *M. nigra*, respectively. On the other hand, the obtained TSS scores were 11.05 ± 0.28 °Brix for *R. fruticosus*, 12.98 ± 0.29 °Brix for *R. ulmifolius* and 18.72 ± 0.33 °Brix for *M. nigra* (Table 9). The most common sugars in these berries, according to the literature, are glucose and fructose [177] (Table 1), being both considered the main responsible for sweetness perception. On the other hand, the main organic acid detected was malic acid, being this responsible for sourness perception [51].

Maturity index (TSS/TA ratio), is positively correlated to pH, given that, as fruit maturity increases, acidic content decreases, but pH and sugar content increase. This ratio is related to the perception of sweetness and sourness, being crucial in determining whether consumers' approval. The TSS/TA ratio obtained in the present study is represented in Table 9, ranging from 6.74 ± 0.27 in *R. fruticosus* to 34.58 ± 1.12 in *R. ulmifolius*. Turkish-cultivated blackberry cultivars, in comparison to those evaluated in this work, showed higher TSS (8.98%–20.2%) and TA (1.0%–3.1%) [25], than that studied in this work. In addition, the TA ranges from 0.7% to 1.0% for wild blackberries located in Turkey [25]. Once more, genotype, climate, origin, and other factors are directly related to the observed disparities.

1.2. Colour measurements

Berries typically undergo a direct colour change during ripening from green to coloured (red, orange, blue, purple, depending on the type of berry). Different pigments, standing out the accumulation of anthocyanins and carotenoids in the fruit skin cells, dictate the colour type. As expected, fruit genotype, environmental factors, and maturity stage all have an impact on the colour of the fruit. [178]. The flesh of the fruit can also be evaluated regarding its colour, however, the first measure is largely important for consumers' choice in the marketplace. In addition, and as far as we know, the amount of coloured pigments like anthocyanins present in fruits has also had an impact on the interest in the flesh colour of fruits, which has only recently increased.

Therefore, especially in light of the aforementioned, it is accurate to state that colour directly affects fruit appearance and influences consumers' choice, since people are mainly attracted to fruits with shiny skins with intense and homogeneous colour.

Blackberries coloration is depends on various factors, such as genotype, production conditions, fruit ripening stage, harvesting time, climate and soil, as well as storage conditions [29]. The results obtained for CIEL*a*b* values, which stand for brightness, redness and yellowness, respectively, are displayed in Table 9.

In a general way, the L* values for *R. fruticosus*, *R ulmifolius* and *M. nigra* were 47.02 ± 0.05, 61.90 ± 0.06 and 50.16 ± 0.05, respectively. When compared to the literature, a previous study revealed lower L* values for *R. fruticosus*, than that obtained in the present study (varying from 19.8 to 23.9) [29], which revealed that our *R. fruticosus* samples are darker, almost black, than those studied in the literature. On the other hand, a* values were 1.81 ± 0.12 for *R. fruticosus*, 0.09 ± 0.13 for *R. ulmifolius* and 2.72 ± 0.17 for *M. nigra*, while b* levels were 1.63 ± 0.05 for *R. fruticosus*, *R. ulmifolius* 3.04 ± 0.04 and 1.59 ± 0.05 for *M. nigra*.

In addition, shiny fruit appearance is a result of the *C* parameter contributes, whereas *h* is related to how most people perceive the colour in question. Therefore, higher values of *C* are associated with a fruits' colour that is more intense and vibrant and so more attractive to the consumer [29]. In addition, lower quantities of *C* are associated with more intense, saturated and dark colours. In the present study, the obtained *C* values were 2.46 ± 0.10 for *R. fruticosus*, 3.04 ± 0.05 for *R. ulmifolius*, and 3.26 ± 0.15 for *M. nigra*, whereas *h* parameter scores were 43.32 ± 0.08 for *R. fruticosus*, 88.22 ± 0.02 for *R. ulmifolius* and 35.87 ± 0.11 for *M. nigra*. Comparing the obtained data with literature, higher values of *C* and *h* (22.7-25.5 for *C* and 346.90 to 349.66 for *h*) were reported for *R. fruticosus* from Slovenia [29]. These differences are once more closely linked to cultivar genotype, and origin, climate, agricultural conditions and processing techniques used.

Given the obtained data, it is possible to infer that colour attributes are closely linked to amounts of phenolic compounds, particularly anthocyanins, which are considered the most responsible for fruits' color.

Overall, based on the results, it is feasible to conclude that the quality of berries is a complex issue that may be explained by their organoleptic properties and nutritional values. If for many years, organoleptic qualities were the primary drivers of market choice for berries, nowadays, special attention has been given to their nutritional values and consequently, health-promoting properties. As a result, contemporary breeding programs are now focused on the identification of novel cultivars that combine plant resistance with yield, fruit sensory and nutritional quality development. To maximize the influence of genetic resources (including wild germplasm) on the content of specific bioactive chemicals in berries, proper integration of genetic resources, genetic improvement, and new breeding techniques are required [178]. However, the optimal agronomic performance of a new genotype can only be obtained by combining environmental circumstances and cultivation procedures that allow for the exploitation of higher agronomic performance together with the best fruit organoleptic and nutritional quality. Therefore, exploring the organoleptic characteristics is an added value, helping in the selection of the most valuable and desirable blackberries and mulberries.

In addition, it is expectable that berry cultivation in the future will be based on the use of new cultivation models created in conjunction with an interdisciplinary approach able to identify new high-performing cultivars for specific environmental and cultivation conditions, resulting in stable high-quality fruits with legally notable health effects.

2. Phenolic compounds characterization

Phenolic compounds are secondary metabolites found in many fruits and plants. They are mostly represented by (i) non-flavonoid compounds, namely phenolic acids; and (ii) flavonoids, including anthocyanins, flavan-3-ols, flavonols, among others. As was already indicated, the presence of these compounds have been associated to a number of physiological and health benefits, including antioxidant, anti-inflammatory, cardioprotective, and anti-microbial actions [68]. Their composition is directly related to the health benefits that fruits, notably red fruits, demonstrate.

A total of 20 different phenolic compounds were tentatively identified based on the interpretation of their fragmentation patterns obtained from mass spectra and by comparison with other published data (Table 10). Particularly, there were found 8 anthocyanins, 6 flavonols, 1 hydroxycinnamic acid, 3 hydroxybenzoic acids and 2 tannins (Table 10). The identified phenolics were measured using HPLC-DAD (Table 10). Anthocyanins, which make up around 40% of all phenolic compounds, are the most prevalent among them and are thought to be primarily responsible for the vibrant red and purple colours exhibited by these berries.

Table 10. Retention time (Rt), wavelengths of maximum absorption in the ultraviolet-visible region (λmax), mass spectral data and identification of anthocyanins and non-coloured phenolic compounds found in *R. fruticosus* and *R. ulmifolius* blackberries and *M. nigra* mulberry fruits grown in Covilhã region, Portugal, and respective phenolic-rich extracts.

Peak	**Phenolic compounds**	**HPLC-DAD-ESI-MSn characteristics**			
		Rt(min)	**ʎ max**	**Molecular ion[M+H] (m/z)**	**Fragments MSn (m/z)**
	Anthocyanins				
1	Cyanidin 3-*O*-glucoside (1)	7.5	518	449	287
2	Cyanidin 3-*O*-glucoside (2)	9.4	518	449	287
3	Cyanidin 3-*O*-rutinoside	10.2	518	595	449,287
4	Pelargonidin glucoside	11.5	-	433	287
5	Pelargonidin deoxyhexoside-hexoside	11.7	-	579	433,271
6	Cyanidin arabinose/xyloside	13.2	518	419	287
7	Cyanidin-malonyl-glucoside	14.5	518	535	287
8	Cyanidin-dioxalyl-glucoside	15.8	516	593	287
		Rt(min)	**ʎ max**	**Molecular ion[M-H] (m/z)**	**Fragments MS^n (m/z)**
	Non-coloured compounds				
9	Ellagitannin (Pedunculagin I)	4.1	376	783	481,301
10	Elagitannin (Pedunculagin II)	5.2	374	783	481,301
11	3-Caffeoyl-quinic acid (Neochlorogenic acid)	7.3	329	353	191,179
12	5-*ρ*-Coumaroyl quinic acid	11.2	320	337	191
13	Ellagic acid pentoside	14.9	364	433	301
14	Galloyl-Hexahydroxydiphenoyl-glucoside	16.8	360	633	301,275
15	Quercetin 3-*O*-rutinoside	17.3	364	609	301
16	Quercetin 3-*O*-glucuronide	18.5	354	477	301

17	Quercetin 3-*O*-glucoside derivative	20.7	354	603, 463	301
18	Quercetin acetyl-glucoside	20.8	354	505	301
19	Kaempferol 3-rutinoside	20.9	346	593	285
20	Quercetin 3-pentoside	21.3	356	433	301

Table 11. Quantification of non-coloured phenolic compounds and anthocyanins (µg/g of dried fruit) identified in *R. fruticosus* and *R. ulmifolius* blackberries and *M. nigra* mulberry fruits grown in Covilhã region, Portugal, and respective phenolic-rich extracts.

Peaks	**Phenolic compounds**	*R. fruticosus*		*R. ulmifolius*		*M. nigra*	
		Lyophilized fruit	**Phenolic-rich fraction**	**Lyophilized fruit**	**Phenolic-rich fraction**	**Lyophilized fruit**	**Phenolic-rich fraction**
	Anthocyanins						
1	Cyanidin 3-*O*-glucoside (1)	nq	946.79 ± 27.48	nq	2224.40 ± 81.70^{b}	nq	185.31 ± 11.87^{bd}
2	Cyanidin 3-*O*-glucoside (2)	9202.17 ± 104.51	24053.14 ± 34.88^{a}	26845.29 ± 50.89^{a}	39847.30 ± 73.40^{abc}	16380.79 ± 59.81^{abcd}	13252.31 ± 60.59a^{bcde}
3	Cyanidin 3-*O*-rutinoside	nq	nq	nd	nd	4966.82 ± 36.32	735.13 113.50^{e}
4	Pelargonidin glucoside	nd	nd	4568.79 ± 4.523	nq	437.10 ± 64.59^{c}	119.41 ± 32.40^{ce}
5	Pelargonidin deoxyhexoside-hexoside	nd	nd	nd	nd	nq	nd
6	Cyanidin arabinose/xyloside	nq	264.65 ± 10.43	nq	329.95 ± 16.23	nq	14554.56 ± 262.51^{bd}
7	Cyanidin-malonyl-glucoside	nq	nq	nq	13766.39 ± 161.81	nd	nd
8	Cyanidin-dioxalyl - glucoside	nq	11919.49 ± 117.57	nq	579.96 ± 24.63	nd	nd
	Σ	9202.17	37184.06	31414.08	56748.00	21784.68	28846.72
	Non-coloured phenolic compounds						
9	Ellagitannin (Pedunculagin I)	nq	1151.40 ± 41.95	nd	nd	nd	nd
10	Ellagitannin (Pedunculagin II)	636.47 ± 4.05	9964.30 ± 107.04^{b}	nd	nd	nd	nd
11	3-Caffeoyl-quinic acid (Neochlorogenic acid)	nd	nd	nq	nq	nd	nd
12	5-ρ-Coumaroyl quinic acid	nd	nd	nd	nd	nd	nq

13	Ellagic acid Pentoside	nq	9348.74 ± 109.27	nq	6975.72 ± 547.83[b]	nd	nd
14	Galloyl-Hexahydroxydiphenoyl-glucoside	nq	11382.10 ± 56.26	nq	nq	nd	nd
15	Quercetin 3-*O*-rutinoside	nq	nq	nd	nd	nq	nq
16	Quercetin 3-*O*-glucuronide	nq	42995.9 ± 539.94	nd	nd	nd	nd
17	Quercetin 3-*O*-glucoside derivative	nq	nq	nq	nq	3969 ± 55.64	18518.37 ± 370.71[e]
18	Quercetin acetyl-glucoside	nd	nd	nd	nd	nq	nq
19	Kaempferol 3-rutinoside	nd	nd	nd	nd	nd	nq
20	Quercetin 3-pentoside	nq	4229.38 ± 123.21	nd	nd	nd	nd
	Σ	636.47	79071.82	nd	6975.72	3969.00	18518.37

Values are expressed as mean ± standard deviation of three assays. ∑, sum of the determined anthocyanins; nd; not detectable; nq, not quantified. Significant differences between different extracts were obtained by the Tukey's test ($p < 0.05$) and indicated by: [a]: *R. fruticosus* Lyophilized fruit; [b] *R. fruticosus* rich-fraction phenolic; [c]: *R. ulmifolius* lyophilized fruit; [d]: *R. ulmifolius* rich-fraction phenolic; [e]: *M. nigra* lyophilized fruit.

2.1. Anthocyanins

Anthocyanins are the great responsible for the colour exhibited by fruits and vegetables. Significant correlations were found between their content and the biological potential attributed to natural products were also discovered. Therefore, studying their levels is essential.

In this work, the HPLC-DAD-ESI/MS^n analysis allowed the identification of six anthocyanins in *R. fruticosus*: **(1)** cyanidin 3-*O*-glucoside, **(2)** cyanidin 3-*O*-glucoside, **(3)** cyanidin 3-*O*-rutinoside, **(4)** cyanidin arabinose/xyloside, **(5)** cyanidin-malonyl-glucoside and **(6)** cyanidin-dioxalyl-glucoside. The anthocyanins **(1)** cyanidin glucoside, **(2)** cyanidin 3-*O*-glucoside, **(3)** cyanidin arabinose/xyloside, **(4)** pelargonidin glucoside, **(5)** cyanidin arabinose/xyloside, **(6)** cyanidin-malonyl-glucoside and **(7)** dioxalyl-glucoside were identified in *R. ulmifolius*. Six anthocyanins were found, namely, **(1)** cyanidin 3-glucoside, **(2)** cyanidin 3-*O*-glucoside, **(3)** cyanidin 3-*O*-rutinoside, **(4)** pelargonidin glucoside, **(5)** pelargonidin deoxyhexoside-hexoside and **(6)** cyanidin arabinose/xyloside were discovered in *M. nigra*. The detection of these compounds based on their *m/z* and fragment ions are in accordance with other studies [40,46,183–185].

Focusing on their quantification, their total levels in lyophilized samples varied between 9202.17 and 31414.08 dw in *R. fruticosus* and *R. ulmifolius*. On the other hand, in phenolic-rich extracts, the highest content was found in *R. ulmifolius* (56748.00 dw), and the lowest in *M. nigra* (28846.72 dw).

The predominant one was (2) cyanidin 3-*O*-glucoside in the three species. Among them, *R. ulmifolius* was the richer one in this compound in both lyophilized fruit and corresponding phenolic-rich extract (26845.29 ± 50.89 and 39847.30 ± 73.40, respectively).

The outcomes are consistent with previous research, with cyanidin-3-*O*-glucoside being the main anthocyanin present in this type of berries; cyanidin-3-xyloside, cyanidin-3-malonylglucoside cyanidin-3-dioxalylglucoside and cyanidin-3-sambubioside were also detected [25,80,186]. In particularly, other studies reported levels of cyanidin 3-*O*-glucoside for *R. fruticosus* ranging from 19.49 and 86.73 mg per 100 g fw, from 92.3 to 335.6 mg per 100 g for *R. fruticosus* and around 6.01 mg per g extract for *M. nigra*, whereas cyanidin 3-*O*-rutinoside in *R. fruticosus* were around 330,616.73 µg per 100 g, and fluctuating from 1.00 to 9.21 mg per 100 g dw in *M. nigra* [122]. Cyanidin 3,5-diglucoside and pelargonidin 3-*O*-rutinoside were both found in *R. fruticosus* at quantities of 55,447.28 g and 4.23 mg per 100 g, respectively, whereas cyanidin 3-*O*-dioxalylglucoside ranged in concentration from 16.90 to 107.50 mg per 100 g [36].

Cyanidin 3-*O*-dioxalylglucoside, on the other hand, was identified in *Rubus* species at concentrations from 1.20 to 2.04 mg per g extract for *R. fruticosus* and between 16.90 and 107.50 mg per 100 g for *R. ulmifolius* [76]. This unstained compound was not quantified in *M. nigra*, which agrees with the literature. Finally, pelargonidin 3-*O*-glucoside was identified in *R. fruticosus* (102,936.30 µg per 100 g) and *M. nigra* (0.012–0.068 mg per 100 g dw) [36,122]. In the present work, this compound was only quantified in the lyophilized fruit of *R. ulmifolius* (4568.79 ± 4.523 µg/g) and in the lyophilized fruit and phenolic-rich fraction of *M. nigra* (437.10 ± 64.59 µg/g dw and 119.41 ± 32.40 µg/g dw, respectively).

Comparing with other red fruits, peach are also richer in cyanidin 3-*O*-glucoside (1.6-135.2 µg/g dw), while sweet cherries have higher levels of cyanidin 3-*O*-rutinoside (10.41-164.60 µg/g dw) [167,187].

The majority of anthocyanins can be found in red fruits including grapes, strawberries, blueberries, and other red fruits and vegetables. Due to the large number of free hydroxyl groups surrounding the ring B, they are recognized as the main antioxidant molecules in human diet [188]. As far as we know, in blackberries and mulberries, they account for 90% of the antioxidant capacity [189]. They also have antibacterial, anti-inflammatory [83], and neuroprotective properties [142], as well as cellular signaling activity, cardiovascular [190], cancer prevention [152], and anti-diabetic, being also able to manage weight [114]. These effects are attributable to anthocyanins' ability to easily scavenge reactive species, chelate metals, and form direct bonds with proteins and active receptors on peroxisome proliferator, modifying its activity and influencing substrate metabolism and inflammation. All of these capabilities help to improve pathologic dangers such as cancer, diabetes, and cardiovascular diseases.

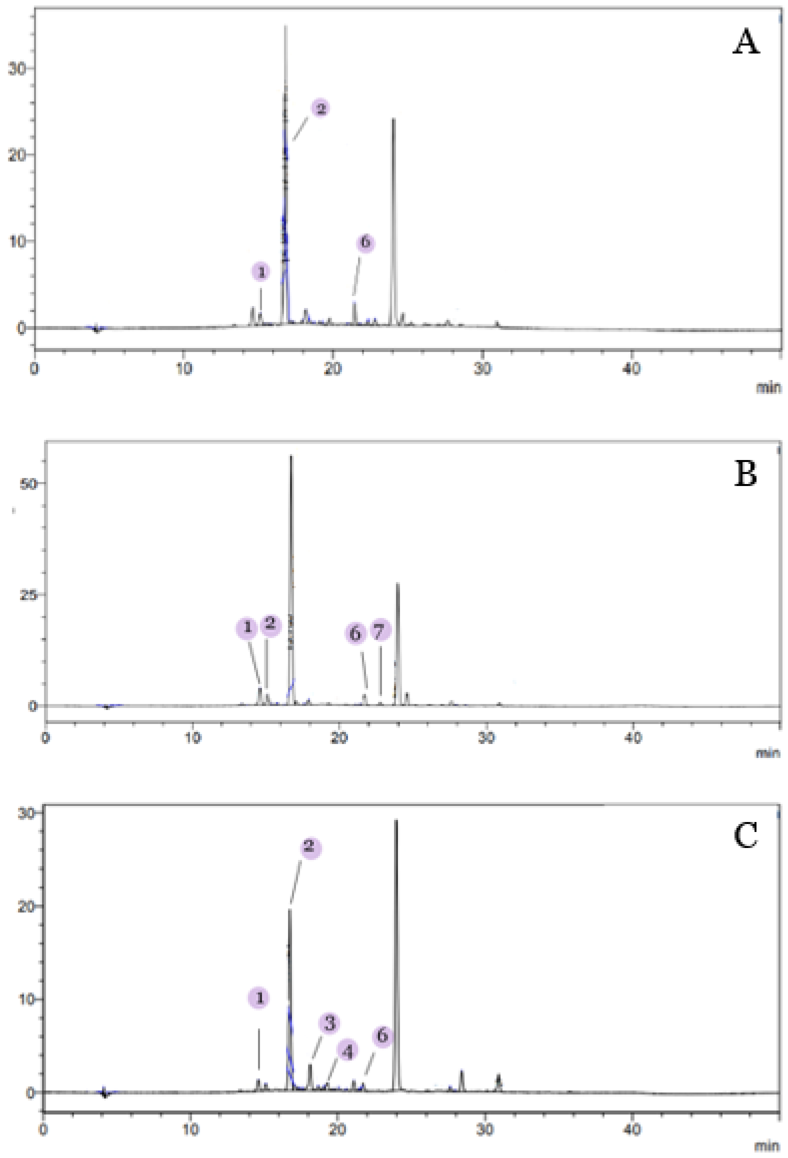


Figure 13. Anthocyanins found by HPLC-DAD at 520 nm in **(A)** *R. fruticosus* phenolic-rich extract, **(B)** *R. ulmifolius* phenolic-rich extract and **(C)** *M. nigra* phenolic-rich extract. **(1)** cyanidin 3-*O*-glucoside, **(2)** cyanidin 3-*O*-glucoside, **(3)** cyanidin 3-*O*-rutinoside, **(4)** cyanidin arabinose/xyloside and **(6)** cyanidin-dioxalyl-glucoside.

2.2. Non-coloured phenolic compounds

In terms of non-coloured phenolic compounds, the analysis of blackberries *R. fruticosus* and *R. ulmifolius* and mulberry *M. nigra* by HPLC-DAD-ESI/MS^n allowed the identification of 6 flavonols, 1 hydroxycinnamic acid, 3 hydroxybenzoic acids and 2 tannins. Their detection focused on their *m/z* and fragment ions is in accordance to previous peaks [46,183–185].

Regarding lyophilized samples, *M. nigra* (3969.00 µg/g dw) had the highest levels of non-coloured phenolics, whilst *R. ulmifolius* had the lowest levels in this subclass. The

R. fruticosus extract, on the other hand, had the highest concentration of non-coloured phenolic compounds (79071.82 g/g dw), whilst the *M. nigra* extract had the lowest concentration (18518.37 g/g dw).

As expected, the lyophilized extracts exhibit lower phenolic levels than their corresponding phenolic-rich extracts, once, by SPE, other constituents such as sugars, fatty acids, fibers were eliminated in order to obtain an extract concentrated in this phytochemical subclass.

In the current investigation, tannins were discovered in traces in both *R. fruticosus* and *R. ulmifolius* blackberries and *M. nigra* mulberry fruits and phenolic-rich extracts.

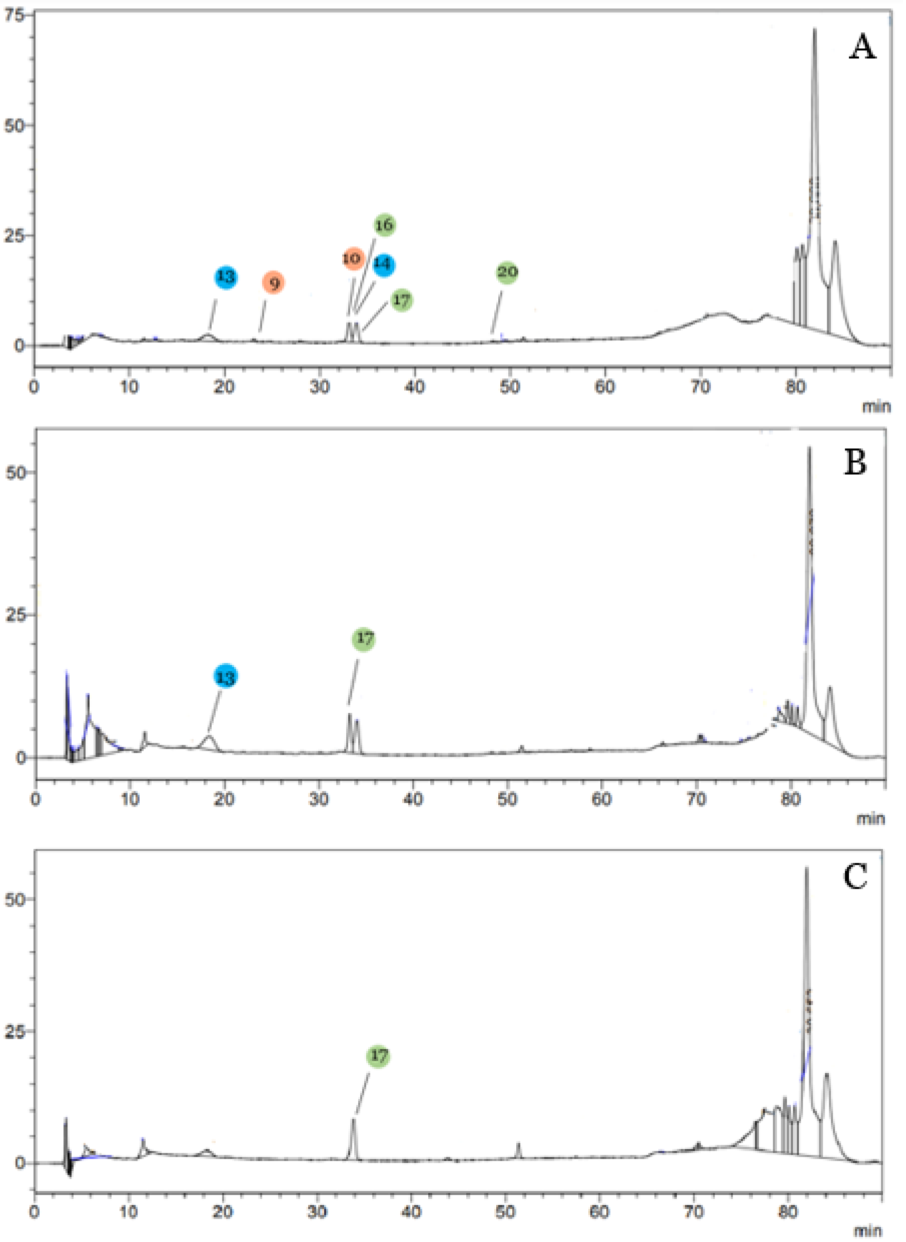


Figure 14. Non-coloured phenolic compounds obtained by HPLC-DAD at 350 nm found in **(A)** *R. fruticosus* phenolic-rich extract, **(B)** R. *ulmifolius* phenolic-rich extract and **(C)** *M. nigra* phenolic-rich extract. **(9)** ellagitannin (pedunculagin I), **(10)** ellagitannin (Pedunculagin II), **(13)** ellagic acid pentoside **(14)** galloyl-hexahydroxydiphenoyl-glucoside, **(16)** quercetin 3-*O*-glucuronide and **(20)** quercetin 3-pentoside.

Six compounds, including **(9)** ellagitannin (Pedunculagin I), **(10)** ellagitannin (Pedunculagin II), **(13)** ellagic acid pentoside **(14)** galloyl-hexahydroxydiphenoyl-glucoside, **(16)** quercetin 3-*O*-glucuronide and **(20)** quercetin 3-pentoside were found in lyophilized fruits and phenolic-rich extracts from *R. fruticosus*. *R. ulmifolius* samples and phenolic-rich extracts, on the other hand, presented **(13)** ellagic acid pentoside and **(14)** galloyl-hexahydroxydiphenoyl-glucoside. In addition, **(15)** quercetin 3-*O*-rutinoside and **(18)** quercetin 3-*O*-pentoside flavonols were detected in *M. nigra* lyophilized fruits and phenolic-rich extract (Table 11). Comparing all species investigated., *R. fruticosus* is the species where more non-coloured compounds were detected.

Focusing on the most obtained data in *Rubus* species, it is important to highlighting the presence of (13) ellagic acid pentoside and (14) galloyl-hexahydroxydiphenoyl-glucoside in *R. fruticosus* phenolic-rich extract (9348.74 ± 109.27 and 11382.10 ± 56.26 dw, respectively) and quercetin 3-*O*-glucuronide (42995.9 ± 539.94 dw). In *M. nigra* species, quercetin 3-*O*-glucoside derivative was the main non-coloured phenolic-rich fraction 18518.37 ± 370.71 µg/g dw in its phenolic-rich extract, whereas the lyophilized fraction, showed lower values of this quercetin derivative (only 3969 ± 55.64 µg/g dw).

According to the literature, the presence of hydroxycinnamic acids, flavonols and tannins in blackberries and mulberries increases their health benefits. The only hydroxycinnamics identified in the current study were neochlorogenic acid and 5-*ρ*-coumaroyl quinic acid. However, it has already been reported [36] that there are additional caffeic, ferulic acid and *ρ*-coumaric acid derivatives present. This variation can be associated with different genotypes of cultivars, soil, treatments and processing. The ability of tannins, on the other hand, to inhibit the growth of many fungi, yeasts, bacteria, and viruses makes them notable phenolics [191].

In contrast with other small fruits, sweet cherries present other compounds non identified in the present work, namely naringenin hexoside 1 and 2 [40]. In addition, *M. nigra* samples contained more contents of quercetin 3-*O*-glucoside than peach (values between 0.8 0.2 µg/g dw for cultivar *Royal Lu* and 12.6 0.1 µg/g dw *Royal Magister*) [187].

3. Essential and non-essential elements identification

Essential elements are required for cells, tissues, and organs growth as well as for the maintenance of essential body functions. According to Table 12, the main essential mineral elements present in higher amounts in this type of berries were Ca and Mg, followed by Mn > Fe > Zn > K > Cu. When comparing the obtained results, *R. ulmifolius* had a concentration of Ca four times higher compared to the others. Ca is essential for the development of bones and muscles as well as for the regulation of normal heart rhythms and nerve functions [53]. However, Mg is crucial to managing diabetes since it helps to maintain normal blood pressure and blood sugar levels [47].

Table 12. Content of essential and non-essential elements (µg/g dw) in the analysed *R. fruticosus* and *R. ulmifolius* blackberries, and *M. nigra* mulberry fruits grown in Covilhã region, Portugal.

Fruit cultivar Elements	*Rubus fruticosus*	*Rubus ulmifolius*	*Morus nigra*
Essential elements			
Mg	1616.30 ± 138.53	1802 ± 57.14	836.66 ± 85.28^{ab}
Ca	1739.69 ± 139	4372.49 ± 375.70^{a}	1992.56 ± 88.16^{b}
Mn	136.42 ± 7.10	28.32 ± 1.00^{a}	6.36 ± 0.30^{ab}
Fe	29.76 ± 2.60	32.77 ± 2.80	71.38 ± 49.87
Co	0.06 ± 0.005	0.05 ± 0.004	0.08 ± 0.09
Cu	4.18 ± 0.15	5.89 ± 0.27^{a}	4.08 ± 0.30^{b}
Zn	15.09 ± 0.93	11.43 ± 1.15^{a}	7.16 ± 0.43^{ab}
Se	<LOD	<LOD	<LOD
Mo	0.27 ± 0.03	0.06 ± 0.004^{a}	0.18 ± 0.003^{ab}
Cs	0.06 ± 0.006	0.02 ± 0.002^{a}	0.14 ± 0.006^{ab}
Hg	<LOD	<LOD	<LOD
Na	0.03 ± 0.03	0.010 ± 0.002	0.02 ± 0.003
K	12.9 ± 0.014	10.6 ± 0.010^{b}	20.6 ± 0.02^{ab}
Non-essential elements			
Li	0.21 ± 0.02	0.09 ± 0.03	0.19 ± 0.16
Be	0.01 ± 0.0	0.02 ± 0.0^{a}	<LOD^{ab}
Al	26.97 ± 2.07	28.70 ± 5.45	32.56 ± 4.68
V	<LOD	<LOD	<LOD
Cr	0.07 ± 0.007	0.06 ± 0.018	0.17 ± 0.038^{ab}
Ni	0.69 ± 0.11	0.39 ± 0.22	0.37 ± 0.25
As	<LOD	<LOD	<LOD
Rb	7.31 ± 0.58	10.03 ± 0.59^{a}	25.77 ± 1.59^{ab}
Sr	6.92 ± 0.79	20.10 ± 2.71^{a}	4.81 ± 0.24^{b}
Cd	0.03 ± 0.006	<LOD	<LOD
Sb	<LOD	<LOD	<LOD
Ba	5.47 ± 0.47	12.97 ± 0.40^{a}	4.06 ± 0.15^{ab}

TI	<LOD	<LOD	<LOD
Pb	0.01 ± 0.002	0.02 ± 0.003[a]	0.02 ± 0.002[ab]

Values are expressed as mean ± standard deviation of three independent assays. <LOD: lower than limit of detection. Significant differences between cultivars according to the Tukey's test ($p < 0.05$) are indicated by: [a] vs *R. fruticosus* ; [b] vs *R. ulmifolius*.

Sodium (Na), Cs, Mo and Co were found in traces in the studied species. Selenium (Se) and Hg were unidentified Se, a substance required for immune system activity, was, nevertheless, discovered in blackberries and mulberries, according the literature [32,41]. The presence of K was another distinction. In fact, several research have linked higher levels of K in mulberry fruits; nevertheless, in the current study, this critical element was found in modest quantities (approximately 10.6-20.6 g/g dw) in the species that were studied. These differences were to be expected, since the nutritional value of berries is largely determined by genotype, and less by soil, climate, origin and agricultural and processing treatments. These substances are essential for maintaining the pH and water balance of physiological fluids, the metabolism of carbohydrates and lipids, protein synthesis, control of muscular contraction, and nerve impulses [192].

Although there is no information regarding the daily consumption of these berries per capita, as already mentioned, USDA recommends the ingestion of 144 g per day. Therefore, the analysis of the estimated EDI (Table 13) reveals that both blackberries and mulberries will contribute about 46.58% (male) and 59.51% (female) for the AI of Mn, and 9.84% (both male and female) for the AI of Cu. On the other hand, these berries will not significantly contribute to the daily dietary intake of K and Na, accounting for <0.005% of the AI of K and Na.

Table 13. Estimated daily intake (EDI) of essential elements considering the average per capita recommended dose of this type of berries in Portugal (144 g/person/day) expressed as percentage of Recommended Dietary Allowance (RDA)/ Adequate Intake (As.)

	EDI (expressed as % of the RDA/Al)	
	Male	**Female**
Ca	5,074272	5,074272
Mg	6,34279752	8,32492174
K	0,00587456	0,00587456
Na	0,0000250	0,0000250
Mo	7,09565217	7,09565217
Zn	1,91696443	1,91696443
Cu	9,84347826	9,84347826
Mn	46,5754253	59,5130435
Fe	10,479913	4,65773913

When compared to our small red fruits, sweet cherries are richer in K, with values ranging from 11774 to 19524 µg/g dw, but they have lower amounts of Ca (mean value of 207 µg/g dw) than these berries [61].

In all species, Al was the non-essential element that was most abundant, followed by Li > Rb > Sr > Ba. Small levels of Lithium (Li), Be, Cr, Ni, Cd, and Pb were found, whereas V, As, Sb, and TI were either completely absent or present in amounts below the limits of detection. This situation is in line with that described in the literature and it is not alarming [47]. Indeed, analyzing the PTWI values, it is possible to see that the consumption of 144 g of both berries will not contribute to the dietary intake of Cd and Pb (Table 14). Regarding Al and Ni, the values are not harmful since their concentrations in these berries are below their dietary reference intake (<2 and <1 mg per day, respectively) [193,194].

Table 14. Provisional Tolerable Weekly Intake (PTWI) of non-essential and toxic elements considering the average recommended dose of both of this type of berries per capita (144 g/person/day).

	PTWI (expressed as % of the established PTWI)
Pb	0,96
Cd	6,171428571
Ni	139,2
Al	21,1752

4. Volatiles organic compounds identification

Flavor and fragrance are essential factors in influencing customer acceptance of fresh and processed foods. According to scientific research, the profile of volatile organic molecules in fruits and vegetables has a direct impact on the production of odor and flavor sensations as well as market acceptance [84].

In this study, 68 compounds were identified and quantified, including 17 aldehydes, 13 alcohols, 3 ketones, 20 esters, 2 furan, 8 hydrocarbons and 5 acids (Table 15). Once more, the cultivar, climate, production methods, maturation stage, and pre- and post-harvest handling conditions all have a major role in their levels [178]. As would be expectable, there were some discrepancies between the investigated species as well as between fresh and lyophilized samples. Since lyophilization concentrates the other compounds contained in the samples by removing all water, it is expected that the greatest values would be seen in these samples.

Regarding fresh samples, *M. nigra* had the most diversity of compounds, whilst *R. fruticosus* had the lowest concentration of volatile organic compounds. Aldehydes were therefore the most detected in *Rubus* fruits (were identified a total of 17 compounds); but alcohols were also found in the highest concentrations. Hexanal was the volatile compound found that was discovered in higher amounts in *M. nigra*. This compound is associated to fatty, green and grassy sensorial descriptors.

In particular, 1-hexanol (alcohol) and ethyl hexanoate (ester), which are commonly associated with floral, grape and apple, pear, pineapple, grape and strawberry sensations, were the volatile compounds found in larger amounts in *R. fruticosus* fresh samples. On the other hand, 1-hexanol, 1-octanol and 2-heptanone were the predominant volatile compounds in fresh samples of *R. ulmifolius*.

Focusing on the lyophilized samples, *R. fruticosus* had greater variation than the other lyophilized species, and that none of them presented acids, in contrast to the fresh samples. Another difference observed was the great levels of furan in all lyophilized species. Other families of volatile compounds, including terpenes, terpenoids, carbonyls and phenols, have been identified in these berries, including 2-heptanol in the three species and hexanal on both species of *Rubus* [67,68]. Despite this, our study provided pertinent data, detecting some volatiles that had not previously been reported, including aldehydes, alcohols, furans and some acids, including L-lactic acid, which was discovered for the first time in *M. nigra,* as well as undecanoid acid, among others.

Compared with other red fruits, on sweet cherries, benzaldehyde, hexanal, nonanal, benzyl alcohol, (*E*)-2-hexen-1-ol, 1-hexanol, (*Z*)-2-hexen-1-ol, 2-ethyl-1-hexanol, linalool, α-terpineol and α-ionone were the major ones. Qualitative and quantitative differences were observed among the cultivars, which influenced nutritional potential and aroma. Cherries cultivars from Fundão *4-84*, *Burlat* and *Celeste* might be considered some of the most interesting cultivars, since present high diversity in volatiles. The observed differences in the volatile composition can be used as differentiation markers to determine species' and possibly a locations' origin.

Table 15. Volatile composition in fresh *R. fruticosus* (FRF), *R. ulmifolius* (FRUS) and *M. nigra* (FMN) fruits grown in Covilhã region, Portugal.

Compounds	**Sensorial description**	**ID(Fit/Rfit)^a**	**RI^b**	**A^c/1000 (S.D.)**		
				FRF	**FRUS**	**FMN**
Aldehydes						
Acetaldehyde ^{L1}	Apple	92.2/95.3	2.144	nd	102.84 ± 0.10	84.78 ± 2.84
2-methyl-propanal ^{L1}	Floral, fresh	84.1/86.0	2.572	nd	5.8 ± 0.60	nd
3-Methyl-butanal ^{L1}	Fruity, nut, cocoa	91.0/91.0	3.385	9.95 ± 1.67	73.71 ± 3.10	12.51 ± 0.87
2-Methyl-butanal ^{L1}	Almond, cocoa, coffee	89.8/90.7	3.470	9.78 ± 0.14	34.71 ± 4.32	116.94 ± 5.81
Pentanal ^{L1}	Almond, malt, pugent	82.2/93.0	3.905	21.70 ± 7.57	11.99 ± 1.94	84.30 ± 6.41
Hexanal ^{L1}	Fatty, green, grassy	93.8/95.7	5.911	4456.77 ± 350.77	1534.67 ± 256.08	3234.65 ± 164.70
Furfural ^{L1}	Sweet, woody, almond	92.9/92.9	6.721	nd	nd	nd
(*E*)-2-Hexenal ^{L1}	Fresh, fruity, green	88.6/93.7	7.326	3711.38 ± 361.79	96.82 ± 3.73	258.55 ± 16.10
(*Z*)-2-Heptenal ^{L1}	Almond	88.9/91.9	10.329	nd	nd	48.88 ± 0.53
Benzaldehyde ^{L1}	Almond, cherry	91.9/93.5	10.498	nd	18.29 ± 0.78	nd
Octanal ^{L1}	Citrus, fatty, fruity, green, lemon, honey	91.5/95	11.731	nd	nd	118.02 ± 13.11
Benzaneacetaldehyde ^{L1}	Hyacinty, lilac	90.9/95.7	12.997	nd	43.80 ± 6.42	nd
(*E*)-2-Octenal ^{L1}	Fatty, green	87.9/89.7	13.425	nd	39.73 ± 13.71	176.05 ± 2.69
Nonanal ^{L1}	Apple, citrus, fruity, grape, green, orange, rose	91.5/91.7	14.826	nd	106.30 ± 4.78	693.72 ± 73.52
(*E*)-2-Nonenal ^{L1}	Green, citrus, melon	88.7/90.5	16.495	nd	25.34 ± 0.15	564.90 ± 94.46
Decanal ^{L1}	Sweet, Orange, floral	82.6/86.6	17.825	nd	nd	21.83 ± 0.03
4-Propyl benzaldehyde ^{L2}	Violet clove, lily	93.0/93.6	19.856	nd	nd	nd
Total aldehydes				8209.58	2094.00	5415.13
Alcohols						
1-penten-3-ol ^{L1}	Butter, mild, green	83.4/88.7	3.733	nd	nd	79.04 ± 7.73

1-Pentanol ^{L1}	Balsamic, sweet	87.3/87.3	4.583	nd	197.86 ± 13.07	nd
4-methyl-1-pentanol ^{L1}	Nutty	89.1/91.4	6.89	nd	43.60 ± 3.84	nd
1-Hexanol ^{L1}	Floral, grape	93.2/93.3	7.731	6947.17 ± 635.58	5004.09 ± 237.73	929.85 ± 54
2-Heptanol ^{L1}	Fruity, green	91.2/91.7	8.650	3867.88 ± 281.54	1737.31 ± 188.45	nd
1-Heptanol ^{L1}	Woody, fatty, pungent	91.8/92.0	10.727	110.73 ± 5.11	347.53 ± 20.12	10.51 ± 1.10
1-Octen-3-ol ^{L1}	Fruity, Woody, green, mushroom, nut	66.7/82.1	11.018	nd	nd	nd
1-Octanol ^{L1}	Floral, bitter almond	79.2/88.4	13.817	1026.71 ± 29.86	5203.93 ± 204.88	82.42 ± 1.76
(*E*,*Z*)-3,6-Nonadien-1-ol ^{L2}	Melon, tea, honey berry	80.9/83.7	14.737	nd	nd	119.57 ± 12.35
Phenylethyl alcohol ^{L1}	Floral, sweet, rose	93.9/94.9	15.133	nd	163.23 ± 10.79	nd
2,6-Nonadien-1-ol ^{L1}	Melon, strawberry, green apple	77.4/85.1	16.622	nd	nd	8.87 ± 0.50
1-Nonanol ^{L1}	Fat, floral, green	87.5/91.9	16.840	nd	201.39 ± 16.20	nd
2-Undecanol ^{L1}	Fruity	73/91.1	20.530	nd	6.53 ± 0.83	nd
Total alcohols				11952.49	12905.47	1230.26
Ketones						
2-Pentanone ^{L1}	Fruity, pungent	77.7/82.4	3.827	nd	nd	29.86 ± 3.95
2-Heptanone ^{L1}	Fruity, green, nut, spice	90.0/90.9	8.287	nd	5253.69 ± 16.50	167.39 ± 6.05
2-Nonanone ^{L1}	Fruity, green	85.7/88.1	14.393	nd	63.57 ± 9.55	140.17 ± 14.03
Total ketones				nd	10634.52	337.42
Esters						
Vinyl Acetate ^{L2}	Sweet, fruity, pleasant	81.7/95.1	2.727	nd	nd	nd
Methyl acetate ^{L2}	Green	78/89.4	2.413	nd	nd	71.65 ± 4.77
Ethyl acetate ^{L1}	Fruity, pineapple, pleasant	91.7/92.3	3.030	507.15 ± 19.20	nd	2382.15 ± 177.46
Ethyl propanoate ^{L1}	Sweet, fruity, grape	90.1/90.7	4.132	95.97 ± 14.51	nd	7.98 ± 1.30
Methyl butanoate ^{L2}	Pineapple, apple, strawberry	84.9/88.1	4.375	nd	nd	35.48 ± 2.99
Ethyl 2-Butenoate ^{L1}	Fruity, pineapple	81.9/86.2	7.061	nd	nd	174.64 ± 11.24
2-Methyl ethyl butanoate ^{L2}	Fruity, strawberry, blueberries, apples	93.8/94.7	7.145	nd	nd	nd

Methyl hexanoate ^{L2}	Sweet, fresh	90.3/90.5	9.278	nd	113.18 ± 16.73	nd
4-Methyl pentanoate ^{L2}	Acidic, chessy	79/89.6	10.206	nd	13.26 ± 0.43	nd
Ethyl hexanoate ^{L1}	Apple, pear, pineapple, grape, strawberry	90.8/91.9	11.553	1305.10 ± 187.09	nd	nd
Ethyl 2-hexenoate ^{L1}	Fruity, green, sweet	79.9/82.8	12.961	nd	nd	64.48 ± 5.78
2-Propenyl hexanoate ^{L1}	Pineapple, apple, peach, rum	78.1/82.1	14.092	nd	12.58 ± 0.20	nd
Methyl benzoate ^{L2}	Cherry	88/92.5	14.570	nd	nd	1436.98 ± 83.64
Methyl octanoate ^{L2}	Orange, winey, fruity	88.2/89.5	15.372	nd	41.59 ± 3.65	nd
Ethyl benzoate ^{L1}	Sweet, fruity	82/87.3	16.831	nd	nd	430.60 ± 45.98
(*E*)- Methyl cinnamate ^{L1}	Fruity, strawberry	88.3/93.6	22.780	nd	nd	77.76 ± 1.63
Ethyl caproate ^{L1}	Chessy, fatty	87.7/89.4	22.957	nd	nd	74.01 ± 11.01
Methyl benzoate ^{L1}	Herb, prune, violet	81.1/91.5	14.552	nd	nd	1162.71 ± 119.41
E-2-Hexenyl benzoate ^{L2}	Green, pear, floral, fruity	66.9/67.6	14.585	nd	6.83 ± 0.26	nd
Ethyl octanoate ^{L1}	Fruity, banana	89.6/92.2	17.511	302.36 ± 10.47	17.50 ± 1.55	nd
Total esters				2210.58	204.94	5918.44
Furans						
Dimethyl disulfide ^{L1}	Tomato, garlic, onion	96.2/96.8	4.716	nd	nd	nd
2-Pentyl-furan ^{L1}	Green, rum, chocolate	86.9/89.7	11.306	nd	nd	1854.49 ± 85.11
Total furans				nd	nd	1854.49
Hydrocarbons						
Styrene ^{L1}	Floral, cocoa, cinnamon	87.3/91.2	8.395	nd	nd	169.79 ± 13.97
Decane ^{L1}	Strawberry	93.3/93.8	11.644	nd	nd	nd
D-Limonene ^{L1}	Citrus, sweet, balsamic	92.8/92.8	12.610	2500.00 ± 348.07	nd	nd
Undecane ^{L1}	Gasoline-like	89.4/89.6	13.351	nd	nd	nd
Dodecane ^{L1}	Citrus, fatty	93.3/93.3	17.671	nd	nd	nd
Cyclodecane ^{L2}	N.A.	84/85.1	19.680	nd	124.90 ± 1.04	nd
Nonadecane ^{L1}	Watermelons, papaya	87.2/90.3	22.185	nd	nd	nd

Tetradecane [L1]	Gasoline like	87.3/94.4	23.124	nd	nd	nd
Total hydrocarbons				2500	124.90	169.79
Acids						
L-Lactic acid [L1]	Sweet	83.8/84.4	2.223	nd	nd	67.79 ± 10.32
Hexanoic acid [L1]	Acidic, chessy	80.3/85.2	11.302	nd	42.34 ± 4.04	nd
Octanoic acid [L1]	Acid, fat, sweat	69.2/75.1	17.440	nd	1.90 ± 0.26	nd
Oleic acid [L1]	odorless	73.4/78.8	19.449	12.05 ± 0.90	nd	12.61 ± 0.66
Undecanoid acid [L1]	waxy creamy cheesy fatty coconut	75.6/87.2	22.079	5.19 ± 0.14	nd	6.99 ± 0.60
Total acids				12.05	44.24	87.39

[a]Identification method tentatively identified by NIST14 Library Database (fit/retrofit values, %); [b] Kovats retention index; [c]Area expressed as arbitrary units; S.D. = standard deviation of three assays; nd: not detected; [L1]Identified metabolites (confirmed using a chemical reference standard); [L2]putatively annotated compounds (NIST14 database) [174].

Table 16. Volatile composition in lyophilized, *R. fruticosus* (LRF) and *R. ulmifolius* (LRUS) blackberries and *M. nigra* (LMN) mulberry fruits grown in Covilhã region, Portugal.

Compounds	**Sensorial description**	**ID(Fit/Rfit)[a]**	**RI[b]**	**A[c]/1000 (S.D.)**		
				LRF	**LRUS**	**LMN**
Aldehydes						
Acetaldehyde [L1]	Green apples,	92.2/95.3	2.144	81.54 ± 4.51	nd	nd
2-methyl-propanal [L1]	Floral, fresh	84.1/86.0	2.572	117.26 ± 7.13	154.90 c 19.42	nd
3-Methyl-butanal [L1]	Fruity, nut, cocoa	91.0/91.0	3.385	1848.23 ± 28.16	2103.45 ± 223.15	nd
2-Methyl-butanal [L1]	Almond, cocoa, coffee	89.8/90.7	3.470	1438.05 ± 143.97	nd	nd
Pentanal [L1]	Almond, malt, pugent	82.2/93.0	3.905	1408.41 ± 194.61	854.43 ± 44.45	1001.13 ± 156.39
Hexanal [L1]	Fatty, green, grassy	93.8/95.7	5.911	10091.45 ± 499.54	10121.62 ± 172	5215.07 ± 560.94
Furfural [L1]	Sweet, woody, almond	92.9/92.9	6.721	947.35 ± 16.90	nd	177.95 ± 23.49
(*E*)-2-Hexenal [L1]	Fresh, fruity, green	88.6/93.7	7.326	769.14 ± 119	3292.89 ± 633.41	nd
(*Z*)-2-Heptenal [L1]	Almond	88.9/91.9	10.329	nd	nd	352.06 ± 30.95
Benzaldehyde [L1]	Almond, cherry	91.9/93.5	10.498	nd	nd	nd
Octanal [L1]	Citrus, fatty, fruity, green, lemon, honey	91.5/95	11.731	nd	nd	211.37 ± 5
Benzaneacetaldehyde [L1]	Hyacinty, lilac	90.9/95.7	12.997	nd	nd	59.48 ± 8.46
(*E*)-2-Octenal [L1]	Fatty, green	87.9/89.7	13.425	nd	nd	nd
Nonanal [L1]	Apple, citrus, fruity, grape, green, orange, rose	91.5/91.7	14.826	4138.94 ± 362.93	6606.17 ± 1116.83	5623.71 ± 731.25
(*E*)-2-Nonenal [L1]	Green, citrus, melon	88.7/90.5	16.495	nd	nd	351.73 ± 3.82
Decanal [L1]	Sweet, Orange, floral	82.6/86.6	17.825	165.18 ± 18.59	nd	nd
4-propyl-Benzaldehyde [L2]	Violet, clove, lily	93.0/93.6	19.856	3486.73 ± 237.79	nd	662.13 ± 37.38
Total aldehydes				24492.28	23133.46	13654.63
Alcohols						
1-penten-3-ol [L1]	Butter, mild, green	83.4/88.7	3.733	nd	nd	nd
1-Pentanol [L1]	Balsamic, sweet	87.3/87.3	4.583	nd	nd	nd

4-methyl-1-pentanol ^{L1}	Nutty	89.1/91.4	6.89	nd	nd	nd
1-Hexanol ^{L1}	Floral, grape	93.2/93.3	7.731	nd	39201.92 ± 5127.02	nd
2-Heptanol ^{L1}	Fruity, green	91.2/91.7	8.650	2358.41 ± 232.78	16533.66 ± 1500.39	nd
1-Heptanol ^{L1}	Woody, fatty, pungent	91.8/92.0	10.727	nd	nd	126.87 ± 13.79
1-Octen-3-ol ^{L1}	Fruity, Woody, green, mushroom, nut	66.7/82.1	11.018	nd	nd	662.97 ± 49.43
1-Octanol ^{L1}	Floral, bitter almond	79.2/88.4	13.817	nd	21555.20 ± 989.02	nd
(*E*,*Z*)-3,6-Nonadien-1-ol ^{L2}	Melon, tea, honey berry	80.9/83.7	14.737	nd	nd	nd
Phenylethyl alcohol ^{L1}	Floral, sweet, rose	93.9/94.9	15.133	nd	nd	nd
2,6-Nonadien-1-ol ^{L1}	Melon, strawberry, green apple	77.4/85.1	16.622	nd	nd	nd
1-Nonanol ^{L1}	Fat, floral, green	87.5/91.9	16.840	nd	nd	nd
2-Undecanol ^{L1}	Fruity	73/91.1	20.530	nd	nd	nd
Total alcohols				2358.41	77290.78	789.84
Ketones						
2-Pentanone ^{L1}	Fruity, pungent	77.7/82.4	3.827	nd	nd	nd
2-Heptanone ^{L1}	Fruity, green, nut, spice	90.0/90.9	8.287	47.55 ± 1.65	3043.24 ± 203.86	48.78 ± 18.72
2-Nonanone ^{L1}	Fruity, green	85.7/88.1	14.393	nd	nd	47.90 ± 17.75
Total ketones				47.55	3043.24	96.68
Esters						
Vinyl Acetate ^{L2}	Sweet, fruity, pleasant	81.7/95.1	2.727	65.17 ± 5.30	nd	nd
Methyl acetate ^{L2}	Green	78/89.4	2.413	nd	98.73 ± 2.98	nd
Ethyl acetate ^{L1}	Fruity, pineapple, pleasant	91.7/92.3	3.030	1072.57 ± 104.59	204.11 ± 27.09	44.09 ± 4.83
Ethyl ester propanoic acid ^{L1}	Sweet, fruity, grape	90.1/90.7	4.132	86.79 ± 4.92	nd	nd
Methyl ester butyric acid ^{L2}	Pineapple, apple, strawberry	84.9/88.1	4.375	nd	nd	nd
Ethyl ester 2-Butenoic acid ^{L1}	Fruity, pineapple	81.9/86.2	7.061	nd	nd	nd
2-methyl ethyl ester butanoic acid ^{L1}	Fruity, strawberry, blueberries, apples	93.8/94.7	7.145	1856637.17 ± 1676991.15	nd	503.67 ± 26.74

Methyl ester Hexanoic acid ^{L2}	Sweet, fresh	90.3/90.5	9.278	nd	nd	105.36 ± 1.34
4-methyl-Pentanoic acid ^{L2}	Acidic, chessy	79/89.6	10.206	nd	nd	nd
Ethyl ester hexanoic acid ^{L1}	Apple, pear, pineapple, grape, strawberry	90.8/91.9	11.553	784.51 ± 65.58	180.31 ± 27.58	113.93 ± 15.85
Ethyl 2-hexenoate ^{L1}	Fruity, green, sweet	79.9/82.8	12.961	nd	nd	nd
2-propenyl eter hexanoic acid ^{L1}	Pineapple, apple, peach, rum	78.1/82.1	14.092	nd	nd	nd
Methyl benzoate ^{L1}	Herb, prune, violet	81.1/91.5	14.552	nd	nd	nd
Methyl ester benzoic acid ^{L2}	Cherry	88/92.5	14.570	nd	nd	292.73 ± 12.52
E-2-Hexenyl benzoate ^{L2}	Green, pear, floral, fruity	66.9/67.6	14.585	nd	nd	nd
Methyl ester octanoic acid ^{L2}	Orange, winey, fruity	88.2/89.5	15.372	nd	nd	nd
Ethyl ester benzoic acid ^{L1}	Sweet, fruity	82/87.3	16.831	nd	Nd	nd
Ethyl ester Octanoic acid ^{L1}	N.A.	89.6/92.2	17.511	351.73 ± 3.82	nd	nd
(*E*)-methyl ester Cinamic acid ^{L1}	Fruity, strawberry	88.3/93.6	22.780	nd	nd	nd
Ethyl ester capric acid ^{L1}	Chessy, fatty	87.7/89.4	22.957	nd	nd	nd
Total esters				1858911.15	483.15	1059.78
Furans						
Dimethyl disulfide ^{L1}	Tomato, garlic, onion	96.2/96.8	4.716	66943.95 ± 7180.78	71526.37 ± 464.11	28390.23 ± 1321.01
2-Pentyl-furan ^{L1}	Green, rum, chocolate	86.9/89.7	11.306	nd	nd	nd
Total furans				66943.95	71526.37	28390.23
Hydrocarbons						
Styrene ^{L1}	Floral, cocoa, cinnamon	87.3/91.2	8.395	nd	nd	nd
Decane ^{L1}	Strawberry	93.3/93.8	11.644	1886.14 ± 207.13	20751.31 ± 1470.21	4241.37 ± 578.55
D-Limonene ^{L1}	Citrus, sweet, balsamic	92.8/92.8	12.610	1547.79 ± 71.34	nd	nd
Undecane ^{L1}	Gasoline-like	89.4/89.6	13.351	4257.52 ± 511.87	24464.74 ± 1001.24	14006.95 ± 1708.50
Dodecane ^{L1}	Citrus, fatty	93.3/93.3	17.671	8745.13 ± 911.10	19196.60 ± 634.77	9676.62 ± 1507.04
Cyclodecane ^{L2}	N.A.	84/85.1	19.680	nd	nd	nd

Nonadecane ^{L1}	Watermelons, papaya	87.2/90.3	22.185	272.52 ± 40.49	353.45 ± 9.13	238.16 ± 5.96
Tetradecane ^{L1}	Gasoline like	87.3/94.4	23.124	195.22 ± 30.04	573.20 ± 11.41	447.48 ± 2.94
Total hydrocarbons				12647.19	65339.30	28610.58
Acids						
L-Lactic acid ^{L1}	Sweet	83.8/84.4	2.223	nd	nd	nd
Hexanoic acid ^{L1}	Acidic, chessy	80.3/85.2	11.302	nd	nd	nd
Octanoic acid ^{L1}	Acid, fat, sweat	69.2/75.1	17.440	nd	nd	nd
Oleic acid ^{L1}	oderless	73.4/78.8	19.449	nd	nd	nd
Undecanoid acid ^{L1}	Waxy, creamy, cheesy fatty, coconut	75.6/87.2	22.079	nd	nd	nd
Total acids				nd	nd	nd

[a] Identification method tentatively identified by NIST14 Library Database (fit/retrofit values, %); [b] Kovats retention index; [c] Area expressed as arbitrary units; S.D. = standard deviation of three assays.; nd: not detected; ^{L1}: Identified metabolites (confirmed using a chemical reference standard); ^{L2}: putatively annotated compounds (NIST14 database).

II. Biological potential of *R. fruticosus*, *R. ulmifolius* and *M. nigra* extracts

According to numerous studies, the phenolic content of these perishable fruits directly contributes to their noteworthy health advantages. Their antioxidant, anti-inflammatory and brain boost abilities had been researched in the past, these activities were strong correlated with the high concentration of phenolic compounds, particularly the presence of anthocyanins [195].

Considering these facts, the current study examined the capacity of *R. fruticosus* and *R. ulmifolius* blackberries, and *M. nigra* mulberry phenolic-rich extracts to scavenge DPPH$^{\bullet}$, $^{\bullet}$NO and $O_2^{\bullet -}$, as well as to interfere with growth of normal human dermal fibroblast (NHDF) cells and human colon Caco-2 carcinoma cells.

2.1. Antioxidant activity

The role of free radicals and reactive species in human metabolism is undeniable, particularly in the regulation of gene expression and cell growth, immune responses, and signal transduction pathways, their overproduction and subsequent accumulation cause DNA, lipid, and protein damage, necrosis, and exacerbated inflammatory responses, which in turn, promoting the onset of several diseases, including cancer, and cardiovascular, and neurological pathologies [175]. Phenolics have previously shown great potential in lowering oxidative stress levels with little to no negative side effects, in contrast to synthetic antioxidants, which are regarded to be dangerous and have unfavorable side effects.

As far as we know, phenolics are effective in controlling oxidative stress and restoring redox homeostasis because of their ability to (i) neutralize and/or reduce free radicals and the formation of reactive species, (ii) chelate trace elements involved in the formation of these pro-oxidant species, (iii) modulate the activity of related enzymes in cell signalling cascades, and (iv) stimulate the endogenous defence system by stimulating the action of intracellular antioxidant enzymes (e.g., catalase, glutathione, and superoxide dismutase) [196].

As already mentioned, the antioxidant properties of the phenolic-rich extracts from blackberry and mulberry extracts were evaluated against DPPH$^{\bullet}$, $^{\bullet}$NO and $O_2^{\bullet -}$ species. The obtained values are represented in Table 17.

Table 17. Antioxidant capacity of *R. fruticosus* and *R. ulmifolius* blackberries and *M. nigra* mulberry phenolic-rich extracts grown in Covilhã region, Portugal. Values were expressed as **IC$_{25}$** or IC$_{50}$ values (μg/mL dw).

Assay	***Rubus fruticosus***	***Rubus ulmifolius***	***Morus nigra***
DPPH•	34.29 ± 0.55	62.55 ± 0.82^a	56.30 ± 0.96^a
•NO	202.98 ± 2.12	59.49 ± 0.81^a	65.01 ± 0.63^a
$O_2^{\bullet -}$	**14.70 ± 0.58**	**23.59 ± 0.73^a**	**14.26 ± 0.47^b**

Values are expressed as mean ± standard deviation of three assays concerning the antioxidant capacity against DPPH•, •NO and $O_2^{\bullet -}$. Significant differences between phenolic-rich extracts according to the Tukey's test ($p < 0.05$) are indicated by: ^{a} vs *R. fruticosus*; ^{b} vs *R. ulmifolius*.

DPPH is a synthetic radical, but its assay is routinely performed due to its stability and simplicity. This enables a general screening of antioxidant activity of several extracts and individual compounds. It is based on the transformations from violet to yellow that occurs when a tested substance or extract donates hydrogen to DPPH•, neutralizing it [168,169].

All tested extracts exhibited dose-dependent effects against DPPH• (Table 17 and Figure 15). *R. fruticosus* was the extract most effective in reducing this radical, with an IC$_{50}$ score of 34.29 ± 0.55 μg/mL dw, followed by *M. nigra* (IC$_{50}$ value of 56.30 ± 0.96 μg/mL dw). The least active species was *R. ulmifolius* (IC$_{50}$ = 62.55 ± 0.82 μg/mL dw). Nevertheless, all tested extracts displayed lower activity than ascorbic acid positive control (IC$_{50}$=5.53± 0.40 μg/mL).

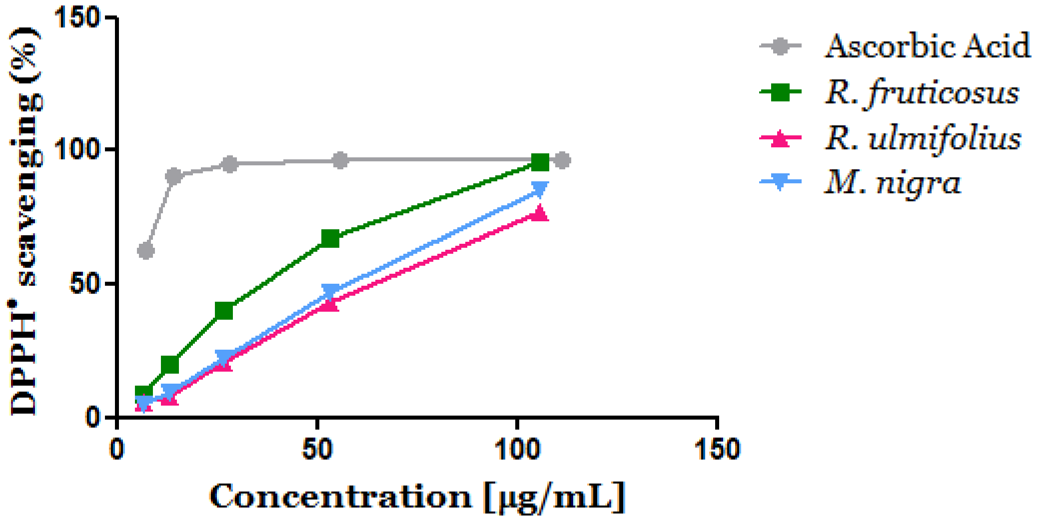


Figure 15. Antioxidant potential of *R. fruticosus* and *R. ulmifolius* blackberries, and *M.nigra* mulberry phenolic-rich extracts against DPPH•.

The studied extracts were more efficient than blueberry extracts when compared to other red fruits (IC_{50} values from 144.68 to 208.06 μg/mL= [199]. However, *R. fruticosus* demonstrated similar efficacy to phenolic-rich extracts of the cultivar *Sweetheart* sweet cherry (IC_{50}=43.03 ± 0.53 μg/mL dw) [167].

Nitric oxide (NO) is a chemical mediator produced by endothelial cells that is involved in several physiological effects to protect the organism against vascular, gastrointestinal, and nervous system vasodilation, as well as tumoral, microbial, and inflammatory processes [200]. However, excessive production has negative effects on proteins and mitochondria,, activating pro-inflammatory transcription factors that promote inflammation and promoting the development of neurodegenerative and chronic diseases like cancer diabetes, atherosclerosis, rheumatoid arthritis, and inflammatory bowel diseases [141].

Phenolic-rich extracts of blackberries from *R. fruticosus* and *R. ulmifolius* and mulberry *M. nigra* also shown the ability to capture this radical, in a dose-dependent manner (Table 17 and Figure 16). Comparing the studied extracts, *R. ulmifolius* had the highest activity (IC_{50}=59.49 ± 0.81 μg/mL dw), being nearly 1.7 times more potent than ascorbic acid positive control ($IC_{50=}$104.10 ± 0.96 μg/mL), followed by *M. nigra* (IC_{50}=65.01 ± 0.63 μg/mL dw) and *R. fruticosus* (IC_{50}=202.98 ± 2.12 μg/mL dw).

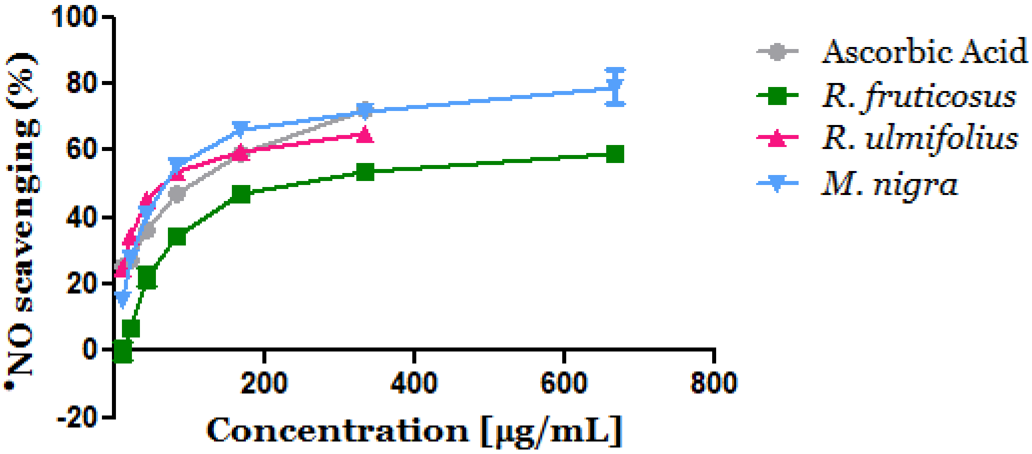


Figure 16. Antioxidant potential of *R. fruticosus* and *R. ulmifolius* blackberries and *M. nigra* mulberries phenolic-rich extracts against •NO.

Comparing with other red fruits, the capacity of *R. ulmifolius* and *M. nigra* extracts to quench •NO was stronger than *Sweetheart* sweet cherry phenolic-rich extract (IC_{50}= 358.64 ± 2.40 μg/mL dw) [199]. Saco sweet cherry extracts, on the other hand, demonstrated superior activity (IC_{50}=33.72 ± 0.89 μg/mL dw) [175]. In addition, *R.*

ulmifolius exhibited similar activity compared to *Legacy* and *Duke* blueberry phenolic-rich extracts (50.34 and 69.53 µg/mL of dw, respectively) [199].

The present study also assessed the ability of the phenolic-rich extracts from *R. fruticosus* and *R. ulmifolius* blackberries, and *M. nigra* mulberry to scavenge $O_2^{\bullet -}$ since NO can react with $O_2^{\bullet -}$, producing more toxic free radical species, such as hydrogen peroxide. This radical mainly results from purine metabolism and electron leakage from the respiratory chain [173]. As well as other radicals, $O_2^{\bullet -}$ also plays a significant role in signal transduction pathways, growth regulation, gene expression, and immunological responses [115]. However, this one is also active at greater levels in a variety of pathophysiologic events, including inflammation, oxygen toxicity, and phagocyte-mediated activity.

In the present study, the analyzed extracts were also effective in scavenging this radical, in a dose-dependent manner (Table 17 and Figure 17). *M. nigra* phenolic-rich extract was the most active (IC_{25}=14.26 ± 0.47 µg/mL) dw, followed by *R. fruticosus* (IC_{25}=14.70 ± 0.58 µg/mL dw). *R. ulmifolius* was the less efficient (IC_{25}=23.59 ± 0.73 µg/mL dw) This last one was the most active in comparison to the ascorbic acid positive control (IC_{25}=3.19 ± 0.30 µg/mL). Only the IC_{25} was possible to determine.

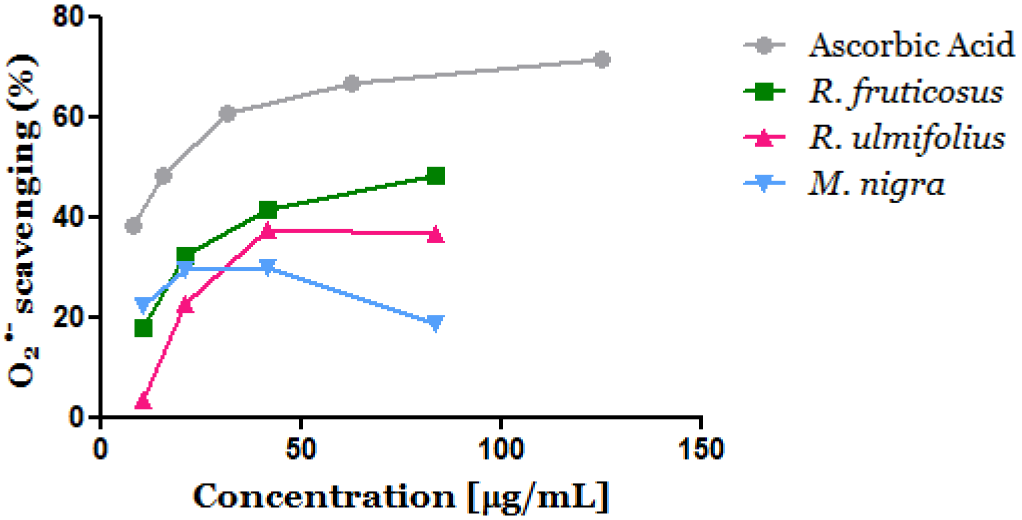


Figure 17. Scavenging activity of *R. fruticosus* and *R. ulmifolius* blackberries, and *M. nigra* mulberry phenolic-rich extracts against superoxide radicals ($O_2^{\bullet -}$).

Although, the obtained values are encouraging, phenolic-rich extracts from *Duke* and *Legacy* blueberries were more effective, showing better IC_{25} values around 1.00 µg/mL [199].

In a general way, fruits' antioxidant capacity is generally proportional to phenolic content. Accordingly, it was feasible to identify positive correlations ($r>0.30$; $P> 0.05$) between the DPPH$^\bullet$ values and the content of cyanidin derivatives quantified. However, despite the extracts' potential to trap NO$^\bullet$ negative correlations were discovered between the concentrations of cyanidin 3-*O*-glucosides (1) and (2) ($r<-0.20$), and cyanidin arabinose/xyloside ($r<-0.50$) and their capacity to quench this radical. In addition, strong positive correlations ($r>0.90$; $P>0.05$) were discovered between cyanidin derivatives and the assay to capture $O_2^{\bullet-}$.

In general, the presence of anthocyanins and non-colored compounds does not directly influence the capture of free radicals. In addition, in most cases, the highest content of phenolics, leadings to increase the antioxidant activity. Indeed, the chemical structure of phenolics, namely composed of catechol, pyrogallol and methoxy groups, confer to them an easy capacity to neutralize free radicals. Even so, the presence of other non-determined bioactive compounds able to interact in additive or synergistic ways with phenolics cannot be ignored.

2.1.1. Antioxidant mixtures

Despite potential synergistic effects, phenolic-rich extracts and ascorbic acid were combined to further the evidence gained (Table 18).

One of the most notable antioxidants and anti-inflammatory substances is ascorbic acid, also known as vitamin C, which is found in higher concentrations in citrus fruits, particularly oranges. This molecule is considered essential for the health of the human body owing to its role in a number of physiological processes, such as blood vessel strengthening and sealing, controlling leukocyte microbial absorption, lowering cholesterol levels, and speeding up the healing of wounds [201]. This molecule also controls the synthesis of collagen, slows down the aging process of the skin, and lowers blood pressure. Due to all its notable properties, it is largely incorporated in supplements and pharmaceutical formulations to promote a healthy status. In fact, clinical evidence has been reported that topical treatment of ascorbic acid alleviates the symptoms of skin aging and increases the production of collagen [202].

Table 18. Antioxidant capacity of *R. fruticosus* and *R. ulmifolius* blackberries and *M. nigra* mulberry phenolic-rich extracts grown in Covilhã region, Portugal combined with ascorbic acid positive control. Values were expressed as **(IC_{25})** or (IC_{50}) values (μg/mL).

Assay	Extract:Ascorbic acid	*Rubus fruticosus*	*Rubus ulmifolius*	*Morus nigra*
DPPH•	25:75	10.89 ± 0.14	4.18 ± 0.20[a]	3.14 ± 0.18[ab]
	50:50	9.42 ± 0.20	6.65 ± 0.21[a]	3.32 ± 0.12[ab]
	75:25	6.77 ± 0.15	4.04 ± 0.28[a]	6.68 ± 0.19[b]
•NO	25:75	295.77 ± 0.87	80.29 ± 0.19[a]	184.36 ± 1.41[ab]
	50:50	227.28 ± 1.65	28.27 ± 0.40[a]	176.25 ± 0.85[b]
	75:25	19.66 ± 0.28	31.70 ± 0.71[a]	16.97 ± 0.88[b]
$O_2^{\bullet -}$	**25:75**	**8.22 ± 0.41**	**7.73 ± 0.33**	**24.10 ± 0.63[ab]**
	50:50	**12.87 ± 0.23**	**8.19 ± 0.41**	**37.28 ± 1.01[ab]**
	75:25	**8.44 ± 0.20**	**13.11 ± 0.50**	**22.47 ± 0.98[ab]**

Values are expressed as mean ± standard deviation of three assays concerning the antioxidant capacity against 1,1-diphenyl-2-picrylhydrazyl, nitric oxide and superoxide radicals (DPPH•, •NO and $O_2^{\bullet -}$, respectively). [a] significant result ($p < 0.05$) is indicated as vs. *R. fruticosus*. [b] $p < 0.05$ is indicated as vs. *R. ulmifolius*.

When comparing the results, it is evident that ascorbic acid and phenolic-rich extracts typically interact synergistically to increase the antioxidant activities of the mixture. Focusing on *R. fruticosus* as an example, its phenolic-rich extract alone had an activity against DPPH• of IC_{50}= 34.29 ± 0.55 μg/mL, but when combined with ascorbic acid (50:50), it was almost four times more effective (IC_{50}= 9.42 ± 0.20 μg/mL), *R. ulmifolius* mixtures present a synergistic effect by using the same methodology as before, but now against •NO. In particular, the combination of extract with ascorbic acid (75:25) of *M. nigra* and *R. fruticosus,* results in lower values of IC_{50} when compared to the control ascorbic acid alone.

All the mixtures had an antagonistic effect on $O_2^{\bullet -}$, with IC_{25} values higher than those of ascorbic acid control (IC_{25}= 3.19 ± 0.30 μg/mL) and *R. ulmifolius* (IC_{25} = 7.73 ± 0.33 μg/mL). Taking into account the unintentional side effects of synthetic pharmaceutical products, the interest in natural products and nutraceuticals has increased the interest worldwide for incorporating them into pharmaceuticals or even replacing certain drugs with natural alternatives.

2.2. Cells culture

2.2.1. Normal human dermal fibroblasts (NHDF)

A preliminary study using NHDF cells was carried out in order to select non-toxic concentrations for non-tumorous cell line (Figure 18). Similar assays are routinely performed [73,197].

Analyzing the obtained data, it is possible to observe that the tested concentrations (50-800 μg/mL) do not exhibit any form of toxicity for normal cells.

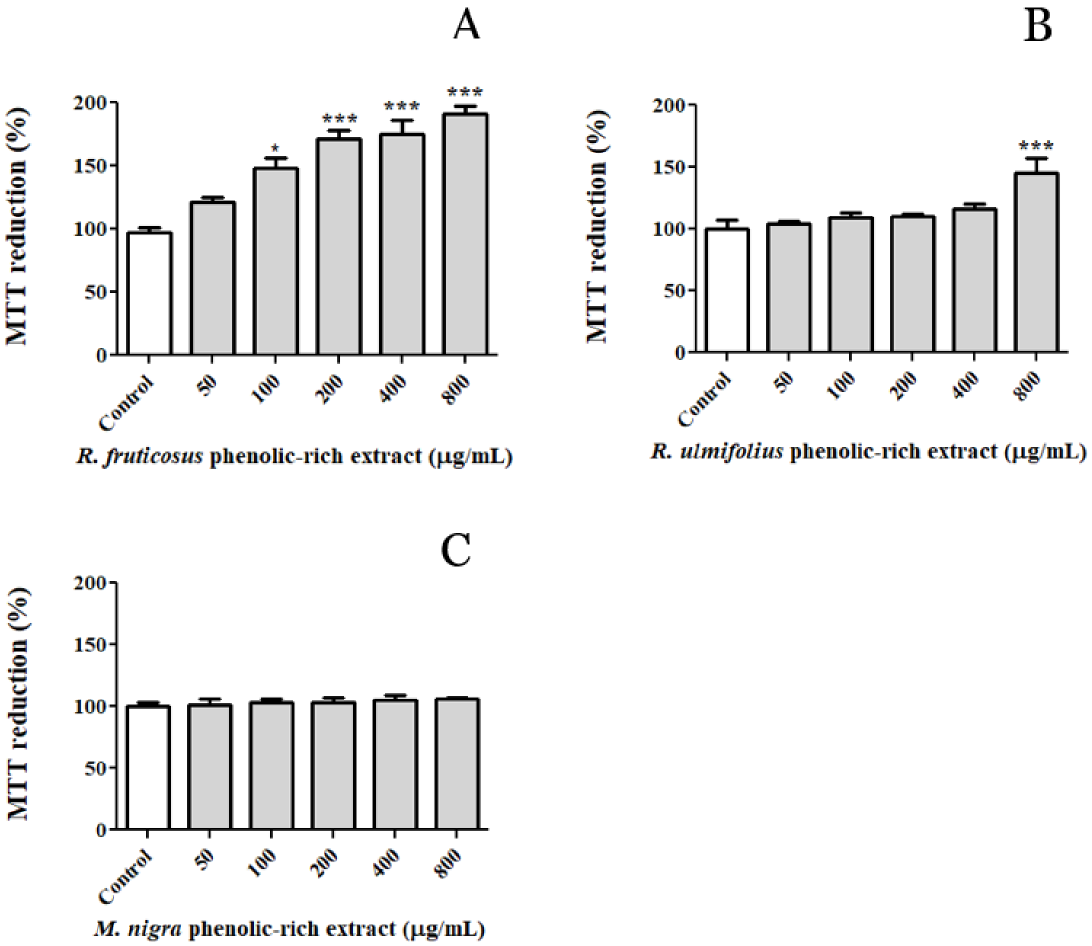


Figure 18. Effect of **(A)** *R. fruticosus* and **(B)** *R. ulmifolius* blackberries, and **(C)** *M. nigra* mulberry phenolic-rich extracts on NHDF viability after 24 hours of exposure, assessed by MTT reduction. Values show mean ± standard deviation of six independent assays, at least, performed in triplicate (* $p < 0.05$**, $p < 0.01$ and *** $p < 0.0001$ compared to the respective controls).

2.2.2. Human colonrectal adenocarcinoma (Caco-2) cells

Taking into account the non-cytotoxicity obtained in the previous studies, the effect of them on Caco-2 viability was further studied. This cell line was chosen, because it is a well-known model of the intestinal epithelium, because, after differentiation, it forms monolayers that mimic a number intestinal epithelial cell properties [204].

After 24 hours of exposure, it is feasible to compare the effects of phenolic-rich extracts to see how they affect Caco-2 cells' proliferation, and only *R. fruticosus* revealed the most notable activity at the highest dose examined (800 µg/mL). However, the anti-proliferative activity was less effective compared to that demonstrated by anti-tumoral drug 5-FU at 65.0 µg/mL.

Additionally, the phenolic-rich extract of *R. ulmifolius* showed a slight cytotoxic activity, whilst *M. nigra* did not exhibit any kind of anti-growth ability for these cancer cells in the assessed time exposure. However, it is anticipated that increasing the time of exposure of the extracts to cancer cells will result in an increase in the anticancer effects.

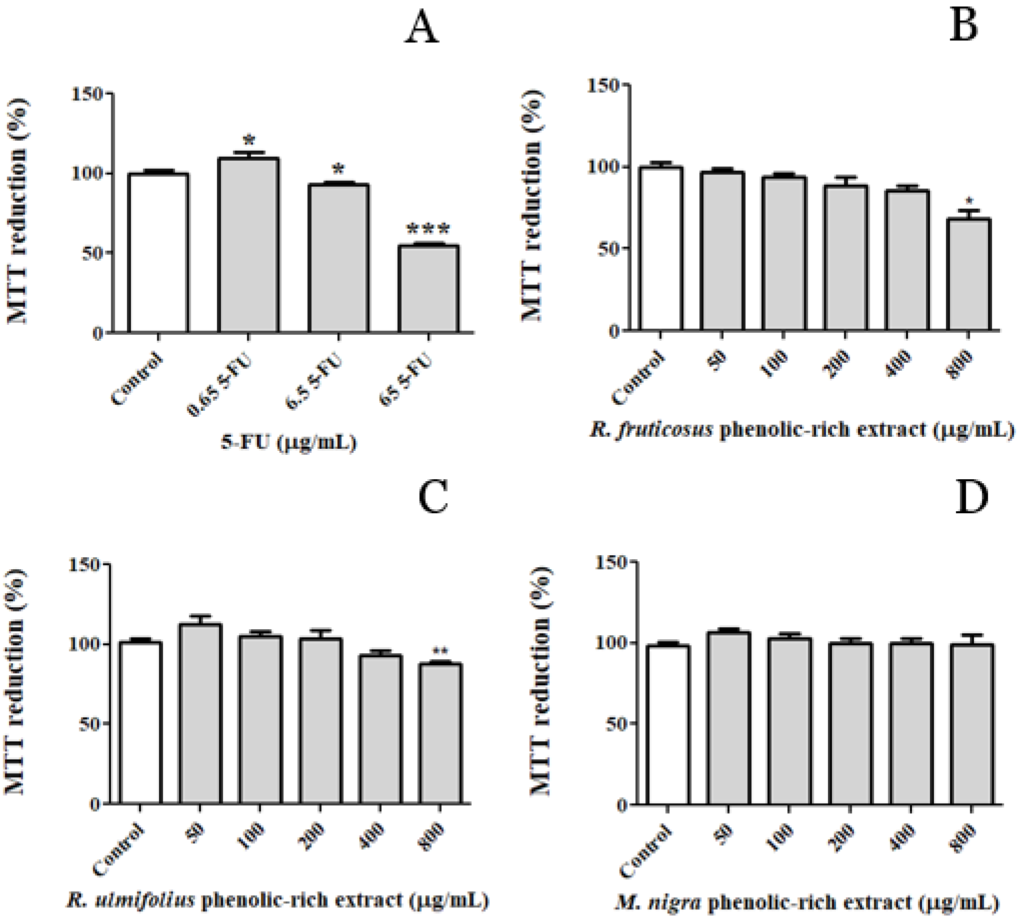


Figure 19. Effects of **(A) anti-tumoral drug** 5-FU, **(B)** *R. fruticosus* and **(C)** *R. ulmifolius* blackberries, *and* **(D)** *M. nigra* mulberry phenolic-rich extracts on Caco-2 cells viability after 24 hours of exposure, assessed by MTT reduction. Values show mean ± standard deviation of six independent assays, at least, performed in triplicate (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.0001$ compared to the respective controls).

Considering the obtained data of this study, it was possible to conclude that, of the three species, only *R. fruticosus* has ability to inhibit the proliferation of Caco-2 cells, probably owing to their high content in anthocyanins. Once more, the presence of phenolics, particularly anthocyanins, is associated with this ability. In fact, the coloured extract was the most effective in a study that compared the antiproliferative effects of sweet cherry extracts to prevent Caco-2 cells proliferation, giving an IC_{50} value of 667.84 ± 2.46 µg/mL [175].

2.2.2. Combination of *R. fruticosus* with 5-FU anti-cancer drug

5-Fluorouracil (5-FU) is a powerful anti-cancer drug frequently used to treat several malignancies, including colon and breast cancers. This drug is a heterocyclic aromatic organic compound with a structure similar to that of the pyrimidine molecules of DNA and RNA, being an analog of uracil with a fluorine atom at the C-5 position in place of hydrogen [205]. This chemotherapeutic drug has been used because it can inhibit the growth of cancer cells, but long-term use can have unfavorable side effects [206]. Knowing the current interest and incorporation of plant-based products in anti-cancer drugs [207] in the present study, the highest concentration of the most promising phenolic-rich extract, that from *R. fruticosus*, (800 µg/mL), was conjugated with the highest concentration of the anticancer drug 5-FU (0.65 µg/mL). These concentrations were chosen because 800 µg/mL demonstrated the most notable anti-proliferative effects and 0.65 µg/mL of 5-FU was the minimum concentration studied in order to see the antitumor potential of the extract when the drug is in low concentration compared to the nutraceutical.

Therefore, mixtures of *R. fruticosus* and 5-FU (75:25 and 50:50) were made and tested (Figure 20). The mixture of extract and 5-FU (25:75) was not evaluated because the major goal is to decrease the concentration of 5-FU synthetic drug to diminish its unwanted side effects and also its price.

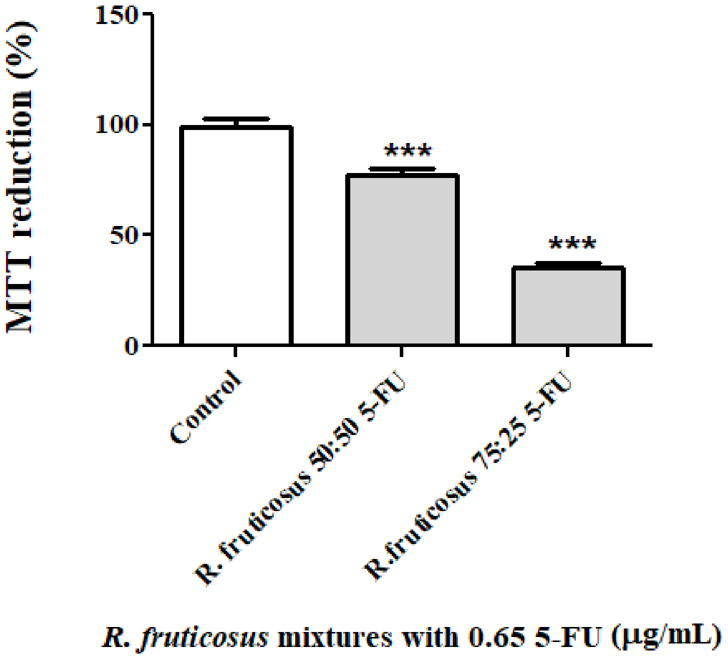


Figure 20. Effects of combined 50:50 and 75:25 of *R. fruticosus* and 5-FU anti-cancer drug on Caco-2 cells for 24 hours. After that time, cells' viability was assessed by MTT reduction. Values show mean ± standard deviation of six independent assays, at least, performed in triplicate (*** $p < 0.0001$ compared to the respective control).

The obtained data revealed that the combination of *R. fruticosus* with 5-FU exhibits a synergistic effect, improving the anticarcinogenic potential of both agents.

In a related investigation, the anti-cancer capacity of thiosulfinate-enriched *Allium sativum* extract combined with 5-FU chemotherapy against Caco-2 cells' growth was also evaluated. It was discovered that this combination is highly advantageous, boosting the potential of both agents [208].

This investigation is interesting given that natural products, including *R. fruticosus* berry, are natural agents, easily found in the environment and without harm to humans, unlike synthetic drugs used for treatment, which have unfavorable side effects and implications for the human body.

V. Conclusions

Currently, here is growing interest in using natural products for therapeutic purposes, especially those derived from plants and rich in antioxidant molecules, namely phenolic compounds. This occurs because they are inexpensive and believe they have fewer side effects and lower toxicity than synthetic pharmaceutical drugs.Considering these facts, the information gathered for this dissertation allows for the following conclusions:

- Focusing on the analysis of the physicochemical characteristics and colour, *R. fruticosus* was the species that presented the most desirable characteristics to consumers and markers, and as a result, had the highest economic value;

- The study of phenolic profile allowed the identification of several phenolic compounds, standing out the presence of cyanidin 3-*O*-glucoside; among the species studied, *R. ulmifolius* was the one with highest concentration of phenolics. As expected, qualitative and quantitative differences were observed among the species, which affects their nutritional potential;

- In addition, it was discovered that Portuguese blackberries and mulberries contain several minerals and volatile elements, which are frequently associated with to the health benefits of their consumption and aroma, respectively. Ca and Mg were the main elements found in all species, contributing in a significant way, to the daily mineral intake. On the other hand, aldehydes and alcohols are the main contributors to the pleasant aroma of these berries. Once more, and as expected, qualitative and quantitative differences were observed among the species, which influences their nutritional potential;

- Regarding the biological potential, particularly the antioxidant one, dose-dependent effects were observed. The phenolic-rich extract from *R. fruticosus* was the most active against the DPPH$^{\bullet}$, while *R. ulmifolius* was the most active against $^{\bullet}$NO. On the other hand, phenolic-rich extracts from both *M. nigra* and *R. fruticosus* exhibited similar and intriguing activity in the $O_2^{\bullet-}$. Concerning the anticancer properties of phenolic-rich extracts, *R. fruticosus* was the most promising, which inhibits Caco-2 cells' growth. Nonetheless, it is important to keep in mind that all species may exhibit cytotoxicity effects as exposure time increases. Additionally, the conjugation of *R. fruticosus* extract with 5-FU at ratios of 50:50 and 75:25 demonstrated a considerable increase in this activity, which may open the way to combining this drug already used against tumors with blackberries to enhance the anticancer effects.

Overall, the information acquired revealed that both blackberries and mulberries have strong antioxidant properties, which may be beneficial for treating oxidative-related diseases, as cardiovascular, inflammatory and tumor pathologies. This discovery is another argument in favor of include blackberries and mulberries in pharmaceutical and nutraceutical formulations, even though further research, particularly clinical trials, are required to fully explore the biological potential and safe dosage.

VI. References

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