U.PORTO

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Delphinidin-(3-*O*-glucoside and 3-*O*-rutinoside): Contents on *Ribes nigrum* infusions and synergistic effect with docetaxel on MDA-MB-231 cells

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Abstract

Delphinidin-(3-*O*-glucoside and 3-*O*-rutinoside): Contents on *Ribes nigrum* infusions and synergistic effect with docetaxel on MDA-MB-231 cells

Ribes nigrum L. berries stand out by their high content of anthocyanins, being a rare dietary source of delphinidin-3-*O*-rutinoside (D3R). Recent studies have shown that delphinidin-based anthocyanins play an important role in cancer prevention, namely in triple negative breast cancer (TNBC), therefore may be used in combination with chemotherapeutic drugs to increase their efficacy. Blackcurrant production has increased worldwide, but fresh supply is still limited due to their high perishability and seasonality, which highlights the need for new and more available blackcurrant-based products, such as infusions. Blackcurrant infusions have been pointed as a valuable source of anthocyanins; however, preparation conditions affect their composition.

In order to characterize freeze-dried blackcurrant berries and respective infusions, the anthocyanins composition, total phenolic content, and antioxidant activity were determined. Berries' anthocyanins, determined by UHPLC-DAD, ranged from 537 to 2381 mg/100 g DW, with D3R as the main anthocyanin (226 – 873 mg/100 g DW). Regarding blackcurrant infusions, a three-level factorial Box–Behnken design (BBD) was employed to optimize the preparation conditions (time: 3 - 7 min; temperature: 80 - 100 °C; ratio: 0.5 - 4.0 g/200 mL). Mass/volume ratio was the only variable with a significant impact on anthocyanins content. Regardless of the conditions used during infusion preparation, the extraction efficiency generally ranged from 70 to 120%. For a ratio of 4.0 g freeze-dried berries/200 mL, anthocyanins content ranged from 15.5 – 103 mg/200 mL.

Due to the promising effect of delphinidin-based anthocyanins on TNBC, the isolated and combined effect of delphinidin-3-*O*-glucoside (D3G) and D3R with docetaxel (DT) on MDA-MB-231 cell proliferation were evaluated based on Chou-Talalay method. D3G and D3R showed a dose-effect response on cell proliferation. D3G-D3R-DT combination resulted in a selective synergistic effect on MDA-MB-231 cell proliferation at all inhibition levels. This synergistic effect is of therapeutic importance, since D3G and D3R allowed the reduction of DT dosages while maintaining the efficacy of the treatment. Since blackcurrant infusions are simple food matrixes with high contents of D3G and D3R, they have the potential to be used as dietary supplements to improve DT efficacy in the treatment of TNBC, which deserves to be evaluated in further studies.

Keywords: Anthocyanins; Blackcurrants; Infusions; Triple negative breast cancer; Synergism.

Resumo

Delfinidina-(3-*O*-glucósido e 3-*O*-rutinósido): concentração em infusões de *Ribes nigrum* e efeito sinérgico com o docetaxel nas células MDA-MB-231

As bagas *Ribes nigrum* L. destacam-se pelo seu elevado teor em antocianinas, sendo a principal fonte alimentar de delfinidina-3-*O*-rutinósido (D3R). Estudos recentes têm demonstrado que as antocianinas à base da delfinidina desempenham um papel promissor na prevenção do cancro, nomeadamente no cancro da mama triplo negativo (TNBC), podendo ser utilizadas em combinação com quimioterápicos para aumentar a sua eficácia. A produção mundial de groselhas negras tem aumentado, mas a oferta de groselhas frescas ainda é limitada devido à sua elevada perecibilidade e sazonalidade, salientando a necessidade de desenvolver novos produtos à base de groselha negra, como é o caso das infusões. As infusões de groselha negra têm sido apontadas como uma fonte interessante de antocianinas, contudo, as condições de preparação afetam a sua composição.

A composição em antocianinas, o teor de fenólicos totais e a atividade antioxidante de diferentes amostras de groselhas negras liofilizadas e respetivas infusões foram determinadas. O teor de antocianinas das bagas foi determinado por HPLC-DAD e variou entre 537 e 2381 mg/100 g DW, sendo a D3R a principal antocianina (226 – 873 mg/100 g DW). Com o objetivo de otimizar as condições de extração (tempo: 3 - 7 min; temperatura: 80 - 100 °C; rácio: 0.5 – 4.0 g/200 mL) de antocianinas em infusões de groselha negra, foi aplicado um desenho fatorial do tipo Box-Behnken (BBD). O rácio massa/volume foi a única variável com impacto significativo no teor de antocianinas. Independentemente das condições utilizadas, a eficiência da extração variou entre 70 e 120%. Para um rácio de 4.0 g de baga liofilizada/200 mL, o teor de antocianinas variou entre 15.5 – 103 mg/200 mL.

Dado o efeito promissor das antocianinas à base da delfinidina no TNBC, o efeito isolado e combinado da Delfinidina-3-*O*-glucósido (D3G) e da D3R com o docetaxel (DT) na proliferação das células MDA-MB-231 foi avaliado com base no método de Chou-Talalay. Ambas as antocianinas apresentaram um efeito dose-resposta na proliferação celular. A combinação D3G-D3R-DT apresentou um efeito sinérgico seletivo na proliferação das células MDA-MB-231 para todas as concentrações. Este efeito sinérgico permite a redução das doses de DT, mantendo a eficácia do tratamento. Uma vez que as infusões de groselha negra são uma matriz simples com elevado teor de D3G e D3R, é de todo o interesse estudar no futuro o seu potencial como suplemento alimentar para melhorar a eficácia do DT no tratamento do TNBC.

Palavras-chave: Antocianinas; Groselhas negras; Infusões; Cancro da mama triplo negativo; Sinergismo.

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List of Abbreviations

ANOVA – Analysis of variance

ANT – Anthocyanin

BBD – Box–Behnken design

BSA - Bovine serum albumin

C3G – Cyanidin-3-*O*-glucoside

C3R – Cyanidin-3-*O*-rutinoside

CI – Combination index

DAD – Diode array detectors

D3G – Delphinidin-3-*O*-glucoside

D3R – Delphinidin-3-*O*-rutinoside

DF – Dilution Factor

DMEM/F12 – Dulbecco's modified Eagle's medium and Ham's F12 cell growth medium

DMEM-HG – Dulbecco´s modified Eagle´s high glucose medium

DMSO – Dimethyl sulfoxide

DT – Docetaxel

DPPH• – 2,2-diphenyl-1-picrylhydrazyl

DRI – Dose reduction index

DW – Dried weight

ECACC – European Collection of Authenticated Cell Cultures

EFSA – European Food Safety Authority

ER – Estrogen Receptor

ESI-MS – Electrospray ionization

F-C – Folin-Ciocalteau

FW – Fresh weight

GAE – Gallic acid equivalents

GLUT – Glucose transporter

HCl – Hydrochloric acid

hEGF – human epidermal growth factor

HOMA-IR – Homeostasis model assessment of insulin resistance

HPLC – High-performance liquid chromatography

HER2 – Human epidermal growth factor receptor 2

IARC – International Agency for Research on Cancer

IC – Inhibition concentration

IC_{50} (D_m) – Half maximal inhibitory concentration

LOD – Limit of detection

LOQ – Limit of quantification

LR – Linear range

MS – Mass-spectrometry

MTT – 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide

MUFA – Monounsaturated fatty acids

MW – Molecular weight

NE – Niacin equivalents

P.a. – *Pro analysis*

PBS - Phosphate-buffered saline

PR – Progesterone receptor

PTFE – Polytetrafluoroethylene

PUFA – Polyunsaturated fatty acids

RE – Retinol equivalents

RSD – Relative standard deviation

RSM – Response Surface Methodology

RT – Retention time

SEM – Standard error of the mean

SFA – Saturated fatty acids

SGLT1 – Sodium–glucose cotransporter 1

SRB – Sulforhodamine B

TE – Trolox equivalents

TNBC – Triple negative breast cancer

TPC – Total phenolic compounds

UHPLC – Ultra-high-performance liquid chromatography

1. Introduction

1.1. *Ribes nigrum* berries

The genus *Ribes* belongs to Grossulariaceae family and comprises black (*R. nigrum* L.), red, and white currants (*R. rubrum* L.) as well as gooseberries (European gooseberries: *R. uva-crispa*; American gooseberries: *R. hirtellum*) (1). In recent years, currants production has grown all over the world not only due to their organoleptic properties, but also for their nutritional and phytochemical composition and potential health benefits (2, 3). Blackcurrant (*Ribes nigrum* L.) is a woody, deciduous shrub, that produces small edible berries characterized by their black-purple colour, seed-containing pulp, and sweet-sour flavour. This shrub is native to colder countries of northern and central Europe and Asia, with Poland being the world´s leading producer of blackcurrant berries (Table 1), producing 105 000 of the 150 898 tonnes of blackcurrants cultivated worldwide, in 2020 (4-6). Although blackcurrants are economically important crops in Europe, the blackcurrant market is still underexplored in the United States of America (USA). At the beginning of the 20th century, the production, sale, and transport of blackcurrants was prohibited in the USA, as some cultivars acted as vectors of the fungus *Cronartium ribicola,* responsible for the white pine blister rust. The production of resistant blackcurrant cultivars, along with the development of new fungicides and growing consumer interest in these berries led some states to lift their bans in 2003. Currently, blackcurrants are already cultivated and sell in USA, however, their production is still very restricted compared to Europe (6-8).

Table 1: Global blackcurrant production in 2020 (6).

Blackcurrant producing countries	Production (tonnes), 2020
Denmark	1750
France	3994
Germany	4520
Latvia	4300
Lithuania	3740
Netherlands	1100
Oversea countries (Americas, Oceania, Asia)	14 630
Poland	105 000
Russian Federation	1450
United Kingdom	12 600
European countries	136 648
Non-European countries	14 250
World	150 898

1.1.1. Nutritional Composition

Blackcurrant berries are mostly cultivated for juice and beverage production due to their sweet-sour flavour, intense colour, and short shelf-life, but they can also be consumed in *natura* (3, 9). Regarding nutritional composition of these berries, they are mainly characterized by high moisture content (~79 %) and low energy (~57 kcal/100 g fresh weight, FW), fat (~0.4 g/100 g FW), protein (~1.3 g/100 g FW), and carbohydrate (~9.5 g/100 g FW) content (Table 2). Blackcurrants have higher content of sugars (~9.4 g/100 g FW), fibre (~5.7 mg/100 g FW) and organic acids (~3.9 g/100 g FW) than strawberries, blueberries, and blackberries where the content of these nutrients ranges from 4.5 to 6.4, 2.0 to 4.6, and 0.8 to 1.4 g/100 g FW, respectively (10).

Table 2: Proximate composition, energy value and fat and sugar profiles of blackcurrants.

		Mean ± SD	Range	References
Components (unit/100 g FW)				
Energy	kJ	237.7 ± 56.6	121.0 – 318.0	(11-21)
	kcal	56.7 ± 13.6	28.0 – 75.0	(11-21)
Moisture (g)		79.3 ± 2.7	72.1 – 87.7	(11-36)
Protein (g)		1.3 ± 0.5	0.7 – 3.1	(12-15, 18, 21, 22, 34)
Fat (g)		0.4 ± 0.4	0.1 – 1.3	(11-22, 34, 36)
SFA (g)		0.10 ± 0.07	0.03 – 0.24	(12-15, 17, 21, 22)
MUFA (g)		0.07 ± 0.06	0.00 – 0.20	(12-15, 17, 21, 22)
PUFA (g)		0.34 ± 0.21	0.10 – 0.73	(12-15, 17, 21, 22)
Available Carbohydrates (g)		9.5 ± 2.3	6.6 – 14.8	(11-17, 19, 20, 22)
Sugars (g)		9.4 ± 2.5	3.8 – 18.0	(1, 11-22, 28, 33, 36-42)
Fructose (g)		4.5 ± 2.0	1.1 – 8.5	(11-15, 22, 28, 36, 38, 40, 41, 43)
Glucose (g)		4.1 ± 1.8	1.4 – 8.3	(11-15, 22, 28, 36, 38, 40, 41, 43)
Sucrose (g)		0.9 ± 0.6	0.10 – 3.7	(11-15, 18, 20, 22, 28, 33, 36-41, 43)
Total dietary fibre (g)		5.7 ± 1.1	3.6 – 7.0	(12-19, 22)
Organic Acids (g)		3.9 ± 1.8	0.8 – 7.3	(16, 18, 22, 38, 40, 44, 45)
Ash (g)		1.1 ± 0.7	0.8 – 2.9	(12-15, 18, 21, 22, 34)

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

Concerning micronutrients, blackcurrants stand out by high vitamin C content (~186.9 mg/100 g FW), since they contain more than twice the value of major dietary sources of vitamin C, as sweet pepper (90.0 mg/100 g FW), kiwi (72.0 mg/100 g FW), boiled brussels sprouts (60.0 mg/100 g), lychee (58.3 mg/100 g FW), and orange (57 mg/100 g FW) (10, 46). For instance, the daily consumption of a single serving size (115 g) of

blackcurrants (12, 21) provides more than 100% of EFSA Dietary Reference Value for vitamin C (47). However, some studies have reported that blackcurrant processing into dehydrated and frozen products, juices, liqueurs, and jams contribute to vitamin C losses due to exposure to oxygen and high temperatures (48-50). On the other hand, vitamin C content of blackcurrants is also influenced by location, genotype, and environmental conditions (39, 51-53). According to Woznicki *et al.* (51), higher post-flowering temperatures (12 – 24 °C) resulted in lower sugar and vitamin C content and higher citric acid concentration in blackcurrant berries. These results were later confirmed by Allwood *et al.* (53) that found a similar trend in vitamin C accumulation. Therefore, cold summer conditions favour the production of sweeter berries with higher content of vitamin C (51).

Table 3: Vitamin and mineral composition of blackcurrants.

	Mean ± SD	Range	References
Vitamins (unit/100 g FW)			
Vitamin A (µg RE)	13.6 ± 6.8	3.0 – 27.0	(11-21)
Vitamin E (mg)	1.5 ± 0.6	0.8 – 2.2	(11-19, 21, 22)
Thiamin (mg)	0.04 ± 0.02	0.02 – 0.08	(11-19, 21, 22)
Riboflavin (mg)	0.05 ± 0.02	0.01 – 0.07	(11-19, 21, 22)
Niacin (mg NE)	0.41 ± 0.09	0.29 – 0.52	(11-14, 18)
Pantothenic acid (mg)	0.39 ± 0.03	0.32 – 0.40	(11-14, 18, 19, 21, 22)
Vitamin B6 (mg)	0.08 ± 0.03	0.06 – 0.17	(11-14, 16-19, 21, 22)
Biotin (µg)	2.3 ± 0.2	1.9 – 2.4	(11-14)
Folate (µg)	13.6 ± 11.1	7.7 – 40.0	(12-14, 18, 19, 21, 22)
Vitamin C (mg)	186.9 ± 78.0	68.0 – 541.0	(1, 11-22, 24, 28, 29, 31, 32, 36-40, 42, 43, 45, 50, 54-59)
Minerals (unit/100 g FW)			
Ca (mg)	47.0 ± 12.7	23.4 – 72.0	(11-22, 28, 33, 36, 60)
Cl (mg)	14.0 ± 1.4	12.0 – 15.0	(11, 12, 19)
Cu (µg)	123.6 ± 42.7	83.1 – 236.0	(11-14, 16-18, 21, 22, 36, 60)
Fe (µg)	1211.2 ± 321.9	503.1 – 1720.0	(11-22, 28, 33, 36, 60)
I (µg)	1.0 ± 0.4	0.2 – 1.5	(12-14, 22)
K (mg)	284.1 ± 53.7	171.7 – 370.0	(11-22, 28, 33, 36, 60)
Mg (mg)	30.0 ± 17.4	13.7 – 65.9	(11-22, 28, 33, 36, 60)
Mn (µg)	196.3 ± 68.3	100.0 – 310.0	(11-14, 21, 22, 28, 36, 60)
Na (mg)	3.9 ± 4.0	0.5 – 14.0	(11-16, 18-22, 28, 33, 60)
P (mg)	49.9 ± 9.3	34.0 – 68.0	(11-19, 21, 22, 60)
Se (µg)	1.8 ± 1.2	0.2 – 4.0	(12, 14, 16, 20, 22, 33)
Zn (µg)	235.8 ± 79.1	97.1 – 430.0	(11-14, 16-22, 28, 33, 36, 60)

RE: retinol equivalents; NE: niacin equivalents.

During juice production, approximately 25% of blackcurrants remain as pomace (61, 62). Blackcurrant pomace is composed by the skin, pulp and seeds of these berries and is commonly discarded as waste into soil or landfills. However, this by-product retains high contents of fibre, polyunsaturated fatty acids (PUFAs), vitamins, and polyphenols, therefore it can be used to formulate new food products or supplements, reducing food waste, and adding more value to blackcurrant juice industry (63-66). Currently, blackcurrant seeds are separated from blackcurrant pomace and are used to produce an oil rich in polyunsaturated fatty acids (PUFA; 73.34% – 79.3%), namely linoleic (43.27% - 47.5%), α-linolenic (14.1% – 18.1%), and ɣ-linolenic (10.9% – 16.7%) acids, with low n-6/n-3 ratio (2.7 – 3.6) and low atherogenic and thrombogenic indexes (0.39 and 0.18, respectively) (67, 68). After removing the seeds, blackcurrant pomace has been dehydrated, milled, and incorporated into cereal-based products to increase their fibre content (62, 63). Schmidt *et al.* (62) reported that the supplementation of savoury crackers with blackcurrant pomace not only increase their fibre content as their acidic and fruity flavour, with no differences in consumer acceptance.

1.1.2. Anthocyanin Composition

Blackcurrants are also known to have high contents of polyphenols (~721 mg gallic acid equivalents (GAE)/100 g FW), especially anthocyanins, flavonols, flavan-3-ols, and hydroxycinnamic acids (Table 4) (24, 25, 44). Polyphenols are secondary metabolites produced by plants, which play vital roles such as protection against multiple stresses, growth, and reproduction functions. Furthermore, polyphenols also contribute to sensory quality of berries, influencing their colour, bitterness, and astringency (69, 70).

Table 4: Total polyphenols content of blackcurrants (expressed as mg of gallic acid equivalents - GAE per 100 g fresh weight - FW).

Total Polyphenols (mg GAE/100 g FW)	**Mean ± SD**	**Range**	**References**
	721 ± 329	83 - 1500	(24-27, 29, 31, 37, 38, 40, 44, 45, 55, 71-77)

Anthocyanins are the major polyphenol found in blackcurrants (83 – 670 mg/100 g FW), being responsible for the intense black-purple colour of these berries (Table 5) (5, 35). The daily consumption of 115 g (one cup) of blackcurrant berries can provide more than 363 mg of anthocyanins (12, 21). Blackcurrant anthocyanin content and profile vary widely according to cultivars, geographical location, growing conditions, and ripening stage (35, 53, 71, 78). During ripening, total anthocyanin content increase in parallel with sugar

content, contributing to the sweeter flavour and darker colour of ripe blackcurrants. In general, the darker the berries, the higher the anthocyanin content (44, 78). Apart from maturation level, growth temperature also determines the concentration of anthocyanins in blackcurrants (31, 53). Allwood *et al*. (53) observed that blackcurrants cultivated at 24 °C accumulated less anthocyanins than the berries cultivated at 12 and 18 °C, resulting in berries with less colour and lower nutritional value. Thus, current breeding programmes have focused on the development of cultivars more resistant to extremely hot summers and richer in anthocyanins (7).

Table 5: Anthocyanin composition of blackcurrants.

	Mean ± SD	**Range**	**References**
Anthocyanins (mg/100 g FW)			
Delphinidin-3-*O*-rutinoside	156.0 ± 65.2	40.2 – 347.9	(30, 32, 35, 79-83)
Cyanidin-3-*O*-rutinoside	95.1 ± 47.7	15.6 – 244.0	(30, 32, 35, 40, 45, 79-84)
Delphinidin-3-*O*-glucoside	45.5 ± 34.4	7.6 – 237.0	(27, 30, 32, 35, 40, 45, 79-83)
Cyanidin-3-*O*-glucoside	16.9 ± 10.2	0.6 – 63.8	(25, 27, 30, 32, 35, 40, 45, 73, 75, 79-84)
Petunidin-3-*O*-rutinoside[a]	4.05 ± 1.29	1.84 – 5.67	(25)
Petunidin-3-*O*-(6"-*p*-coumaroyl-glucoside)[a]	3.74 ± 2.05	1.43 – 6.84	(25)
Delphinidin-3-*O*-(6"-*p*-coumaroyl-glucoside)	3.71 ± 2.39	0.82 – 9.17	(83)
Peonidin-3-*O*-rutinoside	2.67 ± 0.89	1.06 – 4.09	(83)
Petunidin-3-*O*-glucoside[a]	2.04 ± 1.56	0.13 – 3.97	(25)
Cyanidin-3-*O*-(6"-*p*-coumaroyl-glucoside)	1.44 ± 1.90	0.24 – 7.95	(83)
Malvidin-3-*O*-rutinoside	1.41 ± 0.66	0.24 – 2.59	(83)
Peonidin-3-*O*-glucoside[a]	1.28 ± 0.62	0.64 – 2.46	(25)
Pelargonidin-3-*O*-rutinoside[a]	0.86 ± 0.26	0.51 – 1.20	(25)
Total Anthocyanins	316.2 ± 168.1	83.0 – 670.0	(27, 30, 32, 35, 40, 79-81)

[a] Quantified as cyanidin-3-*O*-glucoside equivalents.

The average anthocyanin content of blackcurrants is approximately 316 mg/100 g FW, which is 2 and 10 times higher than the anthocyanin concentration of blackberry (149.9 mg/100 g FW) and red raspberry (33.3 mg/100 g FW), respectively, and similar to the anthocyanin content of black raspberry (381.4 mg/100 g FW) (85). In addition to their high anthocyanin content, blackcurrants stand out from other berries due to their unique anthocyanin profile. The major anthocyanins present in blackcurrants are delphnidin-3-*O*-rutinoside > cyanidin-3-*O*-rutinoside > delphinidin-3-*O*-glucoside > cyanidin-3-*O*-glucoside

accounting for ~97-98% of total anthocyanins. Delphinidin-based anthocyanins represent ~67% of total anthocyanins of blackcurrants, justifying the more bluish colour of these berries (84). Additionally, blackcurrants also contain minor anthocyanins, ranging from 0.9 to 4.1 mg/100 g FW. Among these minor anthocyanins, the most common are the rutinoside form of petunidin, peonidin, malvidin, and pelargonidin. Blackcurrant berries have been considered one of the most important food sources of delphinidin-3-*O*-rutinoside (40.2 – 347.9 mg/100 g FW) (Table 6). Beyond blackcurrant, this anthocyanin has only been found in jostaberry (*Ribes x nidigrolaria* Rud. Bauer & A. Bauer.) (84), a cross between blackcurrant and gooseberry, mulberry (*Morus alba* L.) (86), Ceylon gooseberry (*Dovyalis hebecarpa*) (87), *Clidemia rubra* berry (88), eggplant (*Solanum melongena* L.) (89), and tamarillo (*Solanum betaceum* Cav.) (90-92). Although Ceylon gooseberry peel presents high delphinidin-3-*O*-rutinoside levels, this fruit is commonly consumed after peel removal, which highlights the relevance of blackcurrants as a unique dietary source of this anthocyanin. Considering their singular profile of anthocyanins, blackcurrants and their by-products have been used as natural colourants and bioactive ingredients in the processing and development of multiple food products (5, 65).

Table 6: Content of delphinidin-3-*O*-rutinoside in fresh (FW) and dried weight (DW) in fruits and vegetables.

Food	Tissues	Delphinidin-3-*O*-rutinoside		References
		mg/100 g FW	mg/100 g DW	
Ceylon gooseberry (*Dovyalis hebecarpa*)	Peel	324.08 - 403.00[a]	-	(87)
	Pulp	35.39 - 48.17[a]	-	
Clidemia rubra berry	Pulp + skin	23.74 ± 1.18	-	(88)
Eggplant *(Solanum melongena L.)*	Peel	21.51 - 116.22	-	(89)
	Pulp	7.24 - 19.46	-	
Jostaberry (*Ribes x nidigrolaria* Rud. Bauer & A. Bauer.)	Pulp + Skin	57.53 ± 3.49[b]	-	(84)
Tamarillo (*Solanum betaceum* Cav.)	Peel	-	0.43 - 49.11	(90)
	Pulp	-	29.17 - 273.36	
	Pulp	-	21.79 - 87.43[c]	(91)
	Pulp	-	32.70 ± 0.40[d]	(92)

[a] Quantified as cyanidin-3-*O*-rutinoside equivalents; [b] quantified as delphinidin equivalents; [c] quantified as delphinidin-3-*O*-glucoside equivalentes; [d] quantified as cyanidin-3-*O*-glucoside equivalents.

1.1.3. *Ribes nigrum* berry-based products

Blackcurrant production is a slow and expensive process as it takes at least three years to get a crop. The price of blackcurrants is also significantly influenced by weather conditions throughout the year. These shrubs require specific climatic conditions to produce quality fruits: during the dormancy period, blackcurrants require cold winters, while during flowering they need sunny springs. Therefore, mild winter and late spring frost result in blackcurrant yield losses and higher prices (1, 93, 94). Once harvested, ripe blackcurrants are quickly frozen, dehydrated or processed into juices, jams, jellies, liqueurs, infusions, and yogurts, to increase their self-life and market accessibility throughout the year. Consequently, the consumption of fresh blackcurrant is constrained due to its high cost and low availability in local supermarkets (9, 94). Although some losses of vitamins and polyphenols may occur during the processing and storage of blackcurrant juices and jams, these products retain high levels of vitamin C and phenolic compounds as well as elevated antioxidant activity (49, 77, 95). According to the literature, the content of total polyphenols in commercial blackcurrant jams ranged from 280 to 474 mg/100 g FW (77, 95). Regarding blackcurrant juices, Mattila *et al.* (49) reported a wide variation in vitamin C (2.6 – 46.8 mg/10 mL) and anthocyanins (1.7 – 23.2 mg/100 mL) content between 12 European commercial juices. The different vitamin C content of blackcurrant juices may result from the application of different processing techniques as well as from the fortification of some juices with this vitamin (49). On the other hand, the high polyphenol content of these blackcurrant-based products contributes to their astringency and bitterness. In this way, high amounts of sugar are added to these products to mask this undesired flavour and to increase consumer acceptance (7, 95).

In recent years, the health benefits associated with blackcurrants consumption, particularly in cardiovascular disease (96, 97), cancer (98, 99), diabetes mellitus type 2 (100), and neurodegenerative diseases (101), have led to the development of dietary supplements from blackcurrant leaves, buds, seeds, and berries. These supplements are concentrated sources of fibre, essential fatty acids and polyphenols extracted from blackcurrants that are available in the form of oils, powders, or capsules. However, as blackcurrant supplement market is recent, the supply of berry supplements in grocery stores, pharmacies and herbalists is still very restricted and their price is very high. Therefore, considering the role of blackcurrant anthocyanins on oxidative stress and inflammation is crucial to develop new anthocyanin-rich blackcurrant products that are both cheaper and more accessible to consumers (102-104).

Herbal infusions are beverages prepared by steeping dried aromatic parts of plants as roots, twigs, leaves, flowers, fruits, and seeds in hot or boiling water (105). Herbal

infusions are one of the most consumed beverages in the world, being a key element of Mediterranean diet (106, 107). In recent years, the interest in berry infusions has increased due to their fragrance, fruity flavour, colour, lower astringency, and high polyphenol content. In fact, berry infusions have been suggested as a valuable source of anthocyanins (108, 109). Savikin et *al.* (109) reported a total anthocyanin content of 8.63, 8.94 and 25.68 mg cyanidin-3-*O*-glucoside equivalents (C3G eq.)/100 mL in chokeberry, blackcurrant, and bilberry infusions, respectively. Hence, given the increasing availability of dried blackcurrant-based products in European market, berry infusions may represent a cheaper source of anthocyanins, namely delphindin-3-*O*-rutinoside. However, previous studies have shown that the chemical composition of teas is mainly affected by process conditions as the water volume, the amount of fruits or plants, the temperature, and the steeping time (106, 110, 111). Thus, infusion preparation conditions must be optimized to achieve the maximum extraction of polyphenols, especially anthocyanins, and ensure the consistency of these beverages over time.

1.2. Anthocyanins

1.2.1. Anthocyanin Chemistry

Anthocyanins are natural pigments widespread in the nature, responsible for the red-blue colour of multiple fruits, flowers, leaves, and roots. These secondary metabolites play diverse roles in plants: protection against biotic (pathogen attacks) and abiotic (extreme temperatures, drought, ultraviolet (UV) radiation, and lack of nutrients) stresses, and reproductive functions as pollination and seeds dispersal (112, 113). Anthocyanins are flavonoids that occur as the glycosylated form of anthocyanidins (aglycones). Chemically, anthocyanidins are hydroxylated and methoxylated derivates of phenyl-2-benzopyrylium or flavylium cation (Figure 1). Their basic structure consists of a C6-C3-C6 skeleton with two aromatic rings (A and B rings) linked by three carbon atoms that form an oxygenated heterocyclic ring (C ring) (114, 115). According to the literature, more than 23 anthocyanidins have been described, considering the different number and position of hydroxyl and methoxyl groups. Among these 23 anthocyanidins, only six of them (cyanidin, delphinidin, malvidin, pelargonidin, peonidin, petunidin) are ubiquitously distributed in plants (Table 7). Cyanidin (50%) is the most common anthocyanidin, followed by delphinidin (12%), pelargonidin (12%), peonidin (12%), malvidin (7%), and petunidin (7%) (114, 116). Cyanidin, delphinidin, and pelargonidin-based anthocyanins are mainly found in fruits, while malvidin, peonidin, and petunidin are predominant in flowers (113). The colour of anthocyanidins depends on the number of attached hydroxyl and methoxyl groups: higher number of hydroxyl groups translate to a more bluish colour, while higher number of

methoxyl groups result in a reddish colour (117). Anthocyanidins are quite unstable, therefore, they are mainly found in their glycoside form. The most common sugars attached to anthocyanidins are glucose, rhamnose, xylose, galactose, and arabinose in the form of mono-, di-, and tri-saccharides. Additionally, the sugar moiety may also be acylated by aromatic and aliphatic acids, increasing the stability of anthocyanins. Until today, more than 500 anthocyanins have been reported, depending on the basic anthocyanidin structure; the nature, number, and position of the sugar moiety attached to the anthocyanin; and the identity and position of acylating compound (112, 114). The most abundant anthocyanins in nature are mainly glycosylated in the 3-OH position (113, 117).

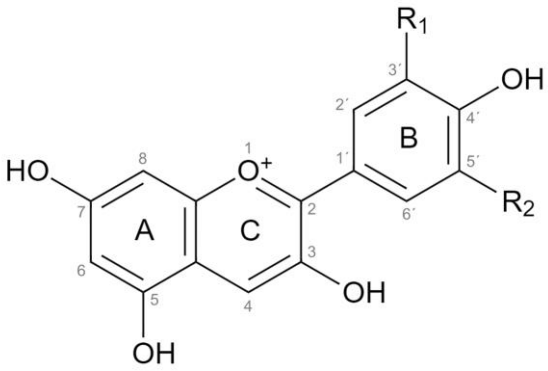


Figure 1: Basic structure of anthocyanidin pigments. R_1 and R_2 can be H, OH, or OCH_3. Created using ChemDraw®.

Table 7: Six most common anthocyanidins found in nature. Adapted from Prior and Wu (115).

Anthocyanidin	R_1	R_2
Cyanidin	OH	H
Delphinidin	OH	OH
Pelargonidin	H	H
Malvidin	OCH_3	OCH_3
Peonidin	OCH_3	H
Petunidin	OCH_3	OH

The stability and, consequently, the colour of anthocyanins is affected by several factors as temperature, light, pH, oxygen, chemical structure and concentration of anthocyanins, presence of other phenolic compounds, enzymes, proteins, carbohydrates, metal ions, and vitamin C (112, 113). Due to their ionic nature, anthocyanins undergo structural rearrangements depending on the pH of the solution. Therefore, four different structural forms of anthocyanins exist in equilibrium at specific pH conditions: flavylium

cation, quinoidal base, hemiketal base, and chalcone base (Figure 2) (112, 115). In acidic conditions (pH < 2), anthocyanins are more stable and exist predominantly in the form of the red flavylium. As the pH increases, the flavylium cation is converted into the hemiketal form, resulting in lower colour intensity. The hemiketal is then transformed into the pale yellow *cis*-chalcone form via tautomerization, which isomerizes to *trans*-chalcone. At low acidic, neutral, and basic conditions, flavylium cation can also be deprotonated, originating the purple/blue quinoidal form (118, 119).

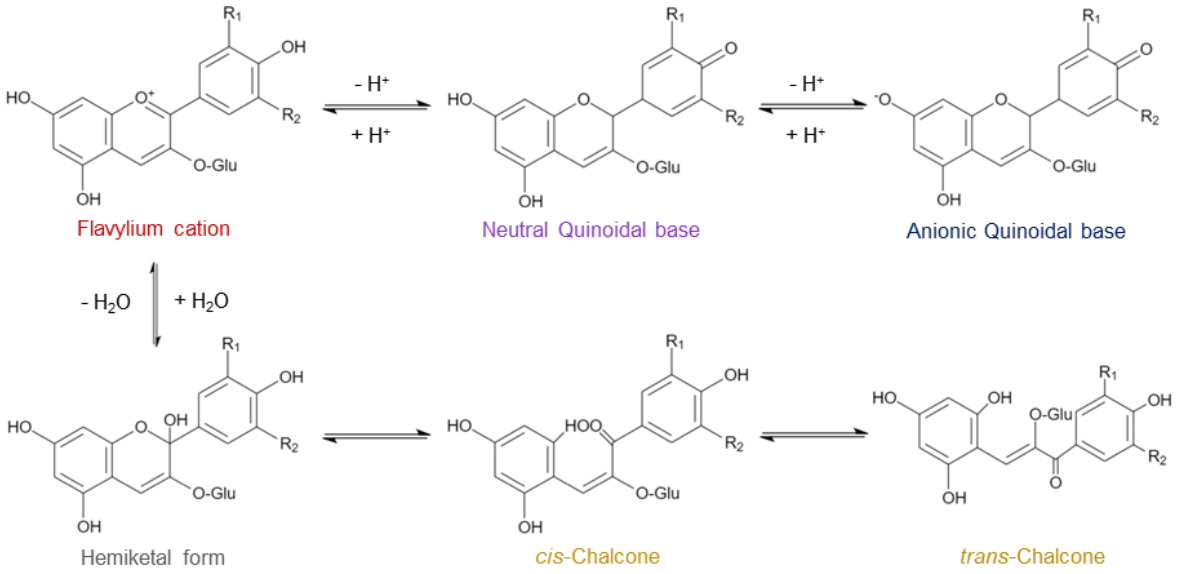


Figure 2: Anthocyanin equilibrium in aqueous solution [adapted from Oliveira *et al.* (117)] Created using ChemDraw®.

The colour of anthocyanins is also influenced by co-pigmentation effect. Co-pigmentation derives from molecular or complex associations between anthocyanins and other organic compounds, or metal ions present in the solution. The most common co-pigments are flavonoids, alkaloids, amino acids, organic acids, nucleotides, polysaccharides, and metal ions. Generally, co-pigments are colourless compounds that, when interacting with anthocyanins, produce a hyperchromic effect and a bathochromic shift in the absorption spectra, increasing the intensity and stability of anthocyanin colour. Therefore, co-pigmentation strategies have been used by food industry to stabilize the colour of food products (113, 116, 120).

1.2.2. Anthocyanins analysis - Extraction, separation, and quantification

There are several methods that can be used to extract anthocyanins from food samples. The most popular method is the solvent extraction (116, 121). Based on the

anthocyanin polarity, the commonly used solvents are ethanol, methanol, acetone, or water (122, 123). In food industry, ethanol is the organic solvent preferred due to the lower toxicity (116). In general, as anthocyanins are more stable in acidic conditions, formic, hydrochloric, or acetic acid are added to the solvent to increase yield extraction. However, the use of strong acids, as hydrochloric acid, should be avoided, since in strong acidic media, the glycosidic bond of anthocyanins might be hydrolysed (116, 123). Although solvent extraction method has numerous advantages (convenient operation, easy to implement, simple and inexpensive equipment), the need for faster, more efficient, and environmentally friendly methods has led to the development of new extraction techniques such as: ultrasound assisted extraction, microwave-assisted extraction, supercritical fluid extraction, high-pressure liquid extraction, pulsed electric fields, high voltage electrical discharge, and enzyme assisted extraction (122, 124). These extraction methods are non-selective for anthocyanins and, consequently, the obtained extracts contain many impurities as carbohydrates, proteins, and organic acids. Therefore, solid phase extraction, liquid-liquid extraction, and chromatographic methods are used to isolate anthocyanins of interest (122, 123).

Total monomeric anthocyanins can be quantified through the pH differential spectrophotometric method, which is based on the reversible structural rearrangements of anthocyanins at different pH (125). As previously referred, at pH 1, anthocyanins are predominantly in the red flavylium form, with a maximum absorption between 490 and 540 nm, while at pH 4.5 they are mainly in the colourless hemiketal form (117). Therefore, the absorbance of sample is recorded at pH 1 and 4.5 at the $\lambda_{vis\text{-}max}$ (ca 520 nm), as well as, at 700 nm (to correct for haze). Considering that polymerized and degraded anthocyanins and other pigments are resistant to color change at different pH, the difference in the absorbance at the $\lambda_{vis\text{-}max}$ will be proportional to the concentration of anthocyanins in the sample. Finally, the total anthocyanin content should be calculated based on the molecular weight and molar extinction coefficient of the major anthocyanin present in the sample (usually cyanidin-3-*O*-glucoside), using the following equations (125, 126):

$$A = (A_{\lambda max} - A_{700nm})_{\text{pH 1.0}} - (A_{\lambda max} - A_{700nm})_{\text{pH 4.5}}$$

$$\text{Anthocyanin (mg/L)} = \frac{A \times MW \times DF \times 10^3}{\varepsilon \times l}$$

where A = Absorbance; MW = Molecular weight (g/mol) of the major anthocyanin; DF = Dilution factor; ε = Molar extinction coefficient (L/mol/cm) of the major anthocyanin; l = pathlength in cm.

Although spectrophotometric methods are relatively inexpensive and simple, they are unable to discriminate and quantify the specific anthocyanins of different foods as well

as they over- or underestimate the content of anthocyanins due to the presence of similar compounds (117, 127). Therefore, high-performance, and ultra-high-performance liquid chromatography (HPLC and UHPLC, respectively) techniques coupled with diode array detectors (DAD) and/or mass-spectrometry (MS) detectors are currently the most widely used methods for the identification and quantification of anthocyanins (125, 127). In recent years, UHPLC techniques have been increasingly used since they provide higher sensitivity, shorter elution time and lower consumption of solvents (128). In general, HPLC and UHPLC methods for anthocyanins use reversed phase C18 columns, and a binary elution system composed by aqueous acidified solvents (acetic, phosphoric, or formic acid) and organic solvents as methanol or acetonitrile (127, 128). The use of acidic mobile phases ensure that anthocyanins are in the more stable flavylium form (128). Regarding detection methods, DAD is the most used and, generally, the selected wavelength varies between 510 and 540 nm. Although widely used, DAD has some disadvantages as the low availability of reference compounds and spectral similarities between anthocyanins. Therefore, this detection method has been replaced by MS detection, which is usually coupled to electrospray ionization (ESI-MS) (125, 127).

1.2.3. Dietary intake, bioavailability, and health-benefits of anthocyanins

The daily intake of anthocyanins ranges from several milligrams to hundreds of milligrams according to dietary habits, sex, age, level of education, physical activity level and body mass index (129-131). In Europe, the average daily intake of anthocyanins varies from 19.83 to 64.88 mg/day for men and 18.73 to 44.08 mg/day for women, while in the USA and Australia it is around 12.5 and 24.2 mg/day, respectively (Table 8) (129, 130, 132). Since anthocyanins are not nutrients, no dietary reference values have been established (131, 133). However, considering the health-benefits associated with anthocyanin consumption, China has suggested a daily anthocyanin intake of 50 mg/day (134). In Mediterranean countries (Italy, France, Spain), the daily intake of anthocyanins is higher mainly due to the greater consumption of fruits, vegetables, and red wine (129). In fact, the main dietary sources of anthocyanins are red-blue fruits, namely berries (blueberries, blackberries, raspberries), providing 38 – 70% of total anthocyanin daily intake (Table 9) (129, 130, 132). Apart from fresh fruits and vegetables, their derived products, obtained by thermal processing, are important dietary sources of anthocyanins, especially in winter, when fresh berries are not available. However, thermal processing leads to anthocyanin degradation, contributing to the reduction of anthocyanin content in these products (112, 113).

Table 8: Daily intake (mg/day) of total and single anthocyanins (expressed as the respective anthocyanidin) in Australia, European countries, and United States of America.

Countries (Reference)	Anthocyanins (mg/day)		Cyanidin (%)		Delphinidin (%)		Malvidin (%)		Pelargonidin (%)		Peonidin (%)		Petunidin (%)	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Australia (132)	23.62	24.72	39.8	37.5	12.0	13.0	31.6	31.4	0.5	0.5	4.6	4.7	4.2	4.4
Denmark (129)	28.0−31.6	26.2−27.0	32.2−41.6	37.8−47.2	7.8−10.6	6.9−9.5	35.1−42.0	32.5−37.1	4.1−5.8	5.0−5.9	4.7−5.3	4.2−4.8	5.0−5.8	4.3−4.9
France (129)	-	32.4−40.8	-	41.3−47.5	-	4.9−6.2	-	22.5−32.7	-	11.9−18.3	-	4.3−5.2	-	2.5−3.0
Germany (129)	29.8−33.7	36.0−40.8	41.3−50.0	45.2−50.5	7.5−7.8	8.4−9.2	21.9−32.8	22.1−24.0	9.5−14.4	12.4−14.8	3.7−4.5	3.6−3.9	2.6−4.6	2.6−3.2
Greece (129)	37.9	25.8	38.4	48.2	5.8	5.0	45.7	37.0	1.6	2.5	5.4	5.1	3.0	2.2
Italy (129)	44.4−64.9	27.6−44.1	39.2−44.0	46.3−63.9	6.1−7.5	4.9−5.8	36.1−40.7	22.3−31.6	2.2−5.4	2.5−10.0	4.6−5.6	3.7−5.1	3.6−4.4	1.7−2.6
Norway (129)	-	25.3−27.8	-	34.0−36.8	-	15.0−15.7	-	30.7−33.1	-	7.9−8.0	-	3.9−4.5	-	4.9−5.5
Spain (129)	36.5−47.5	18.7−26.3	26.3−45.9	46.3−63.9	5.7−7.5	3.4−8.8	37.3−51.2	21.9−31.6	2.5−6.5	2.5−10.0	4.5−6.3	3.7−5.1	4.1−6.1	1.7−2.6
Sweden (129)	20.2−21.2	20.1−22.3	31.6−34.2	32.3−38.4	19.8−25.5	17.5−25.9	22.2−29.3	19.8−30.9	6.3−7.9	6.2−8.6	4.3−4.6	3.7−4.6	6.8−7.4	6.1
The Netherlands (129)	19.8	23.3−24.6	46.6	48.9−53.2	12.8	10.7−14.3	22	16.6−19.1	11.3	10.1−14.4	3.7	3.0−3.8	3.5	2.9−3.0
UK (129)	21.8−27.8	24.1−30.8	35.9−52.5	34.5−44.4	8.3−8.7	5.8−7.3	18.0−29.1	27.4−31.9	9.9−15.1	8.8−11.5	3.0−13.0	9.2−13.2	2.7−3.9	2.9−3.1
USA (132)	12.53		44.7		20.7		15.4		3.3		6.9		9	

UK: United Kingdom; USA: United States of America.

Table 9: Main food sources of anthocyanins in Australia, European countries, and United States of America.

Main food sources of anthocyanins (% total anthocyanin daily intake)		
Australia (130)	**Europe** (129)	**United States of America** (132)
1. Blackberry (5.1 - 65.2%)	1. Wine (14.4 - 24.5%)	1. Blueberry (27.1%)
2. Eggplant (2.0 - 26.4%)	2. Grape (10.6 - 18.9%)	2. Grape (14.1%)
3. Cherry (2.6 - 23.9%)	3. Apples/Pears (10.0 - 14.2%)	3. Onion (7.7%)
4. [a]Raspberry (2.9 - 16.2%)	4. Berries (6.3 - 16.5%)	4. Raspberry (7.4%)
5. Grape (3.4 - 15.4%)	5. [c]Stone fruits (2.8 - 14.8%)	5. Grape juice (7.4%)
6. [b]Blueberry (3.8 - 12.7%)	6. [d]Fruit/vegetable juices (1.7 - 13.4%)	6. Red cabbage (6.5%)
7. Wine (3.8 - 10.3%)	7. [e]Carbonated/soft drinks (0.0 - 15.8%)	7. Apple (5.6%)
8. Cranberry (4.8 - 8.2%)	8. [f]Leafy vegetables (0.4 - 7.1%)	8. Wine (5.3%)
9. Red cabbage (2.4 - 8.8%)	9. [g]Cereal/Confectionery (1.0 - 6.5%)	9. Plum (5.1%)
10. Plum (2.4 - 5.4%)	10. Olives (0.8 - 4.5%)	10. Cherry (4.5%)
11. Radish (3.2 - 4.4%)	11. [h]Soups/Bouillons (0.0 - 5.9%)	11. Strawberry (3.3%)
12. Pear (1.3 - 1.5%)	12. [i]Root vegetables (1.2 - 2.8%)	12. Cranberry (1.4%)
13. Red apple (0.9 - 1.7%)	13. [j]Other fruits (1.1 - 2.5%)	13. Red Radish (1.1%)

[a] Include fresh and frozen raspberries; [b] include fresh and frozen blueberries; [c] stone fruits include plums, peaches, nectarines, apricots, mangoes and paraguayos; [d] Fruit/vegetable juices include blackcurrant juice, cranberry juice, redcurrant juice, cherry juice, peach juice, apricot juice, plum juice and beetroot juice; [e] Carbonated/soft drinks include blackcurrant syrups, syrups of fruits and berries, cherry coke, pommac and jaffa; [f] leafy vegetables include red leaf lettuce, red chicory, radicchio and trevise (red Treviso lettuce); [g] Cereal/Confectionery include fruit cakes, biscuits with jam and plum cake; [h] soups and bouillons include bilberry soup, berry soup and elderberry soup; [i] root vegetables include beetroot, red radish and black radish; [j] other fruits include sour cherries, persimmon, sharon fruit and pomegranate.

Cyanidin and malvidin are the most prevalent anthocyanidins in Australia (37.5 – 39.8% and 31.4 – 31.6%, respectively) and European countries (26.3 – 63.9% and 16.6 – 37.1%, respectively). This high contribution of malvidin to total anthocyanin intake could be explained by the significant consumption of red wine, which has high content of malvidin (129, 130). On the other hand, in United States cyanidin and delphinidin are the most consumed anthocyanidins, accounting for 44.7% and 20.7% of total anthocyanin daily intake. Furthermore, 73.1% of total anthocyanins are monoglucosides, whereas di- and

triglucosides represent 17.3% and 9.6% of total anthocyanin intake, respectively. Concerning anthocyanin acylation, 77% of total anthocyanins are non-acylated (132).

Among fruit and vegetable consumers, anthocyanins are major dietary components, however, these flavonoids have been considered the ones with the lowest bioavailability (112). In contrast to other flavonoid glycosides, anthocyanins undergo structural rearrangements according to pH, influencing their absorption in the gastrointestinal tract as well as their identification and quantification. Therefore, recent studies have suggested that anthocyanin bioavailability has been underestimated (135, 136). Beyond pH, anthocyanin bioavailability is also highly dependent on their chemical structure (number of hydroxyl and methoxyl groups, number, type, and position of sugar moiety and acylating compounds, degree of polymerization and conjugation with other compounds), as well as on food matrix and cooking conditions (112, 131).

Anthocyanins have been found in plasma 15 minutes after the consumption of anthocyanin-rich foods, as blackberry purees and table and Port red wine, suggesting that anthocyanins are rapidly absorbed by the stomach in their intact form (137, 138). In acidic gastric conditions, anthocyanins are mainly found in their stable form, the flavylium cation, increasing their transport efficiency in the stomach (139, 140). It has been suggested that bilitranslocase and glucose transporters are involved in gastric absorption of anthocyanins (135, 139). According to Oliveira *et al*. (139), the presence of glucose reduced the transport efficiency of anthocyanins through MKN-28 cells. Furthermore, the gastric uptake of anthocyanins also decreased when gastric cells were exposed to cytochalasin B, a glucose transporter inhibitor, confirming the involvement of glucose transporters in gastric absorption of anthocyanins. On the other hand, the pre-treatment of cells with anthocyanins resulted in an increase in anthocyanin transport, probably by inducing the expression of glucose transporters. The competition of anthocyanins and glucose for the same transporters may decrease glucose absorption and, therefore, improve glycaemic profile (112, 139).

The fraction of anthocyanins that is not absorbed in the stomach, reaches the small intestine, where anthocyanins are absorbed as glycosides or aglycones by SGLT1 (sodium–glucose cotransporter 1) and GLUT2 (glucose transporter 2) transporters or by passive diffusion, respectively (135, 141). After absorption, anthocyanins are extensively metabolized by phase II enzymes in the liver and kidneys (125, 135). However, the occurrence of anthocyanin conjugates in plasma 15 minutes after blackberry purees consumption suggests that anthocyanins are also metabolized in the stomach by conjugative enzymes before post-absorption metabolization in the liver (137). In addition to stomach and small intestine, colon also plays a significant role in the absorption of

anthocyanins (112). Unabsorbed anthocyanins reach the colon, being hydrolysed into aglycones and metabolized into phenolic acids by microbiota and, consequently, absorbed by the colonic mucosa (125, 135). If, on the one hand, microbiota influences anthocyanin biotransformation and uptake, on the other hand, anthocyanin consumption can modify the profile and functionality of gut microbiota (142). Therefore, anthocyanins health-benefits derived from two different effects: direct effects of absorbed native anthocyanins and respective metabolites in target organs and indirect effects by non-absorbed anthocyanins and metabolites that modulate intestinal microbiota (135).

The consumption of anthocyanin-rich fruits and vegetables or anthocyanin supplements has been associated with lower risk of non-communicable diseases, namely cardiovascular disease, diabetes mellitus type 2 and cancer (143-145). According to Xu *et al.*, the administration of anthocyanin supplements or anthocyanin-rich berries was associated with a lower incidence and total cardiovascular mortality due to the improvement of blood lipid profiles and the reduction of inflammatory markers (143). Furthermore, Fallah *et al.* reported that dietary anthocyanin intake was associated with reduced levels of fasting and postprandial blood glucose, hemoglobin A1c and homeostasis model assessment of insulin resistance (HOMA-IR) (144). Regarding anticancer effects of anthocyanins, recent studies have demonstrated that they are based on different biological activities: antioxidant and anti-inflammatory effects; inhibition of mutagenesis, cell proliferation, cell invasion, and metastasis; induction of cell differentiation, cell cycle arrest, apoptosis, and increased sensitivity to chemotherapy (145). Among the different types of cancer, anthocyanins have demonstrated high anti-proliferative potential on breast cancer cells, namely on triple negative breast cancer cells (146-149).

1.2.4. Anthocyanins and Triple Negative Breast Cancer

Breast cancer is the type of cancer with the highest incidence and mortality rate in women (150). The International Agency for Research on Cancer (IARC) estimates that by 2040 the number of new breast cancer cases per year will increase by more than 40%, from 2.26 million new cases/year in 2020 to 3.19 million in 2040 (151). Despite the improvement of screening and diagnostic tools and the development of more effective treatments, the low five-year survival rate for triple negative breast cancer remains (152). Triple negative breast cancer (TNBC) is a subtype of breast cancer characterized by the lack of expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). TNBC accounts for 15-20% of total breast cancer tumours, being more prevalent in premenopausal women (under 40 years) of African-American or Hispanic descent or in women who carry a BRCA1 mutation (152, 153). Due to the lack of target

receptors, endocrine therapy and HER2 target therapy are ineffective, contributing to the high metastatic capacity, high rate of distant recurrence and poor prognosis (154). Therefore, systemic chemotherapy is the mainstay of treatment for TNBC (155). Despite the poor survival outcome of TNBC, epidemiological studies have suggested that this type of breast cancer is more sensitivity to diet (156-159). Link *et al.* (156) and Romanos-Nanclares *et al.* (157) reported that the adherence to a plant-based diet reduced the risk of ER-negative breast cancer, with no effect on ER-positive and PR-positive breast cancer. Moreover, the consumption of a Mediterranean-diet has been associated with a lower risk of breast cancer, especially ER-negative tumours (159). Fung *et al.* (158) also found an inverse association between the consumption of berries and peaches and the risk of ER-negative, but not ER-positive breast cancer among postmenopausal women. These studies suggest a potential protective role of diet on triple negative breast cancer. *In vitro* studies have also demonstrated an inhibitory effect of anthocyanin-rich foods on the viability of TNBC cells. In two *in vitro* studies, it was found that both blueberry extract and dark sweet cherry extract enriched in anthocyanins inhibited MDA-MB-231 (a TNBC cell model) cell proliferation with no effect on healthy breast cells (MCF-10A cell line) growth (160, 161). Among anthocyanins, delphinidin-based anthocyanins have been suggested as the most effective against TNBC cell proliferation. Viegas et al. (149) reported that delphinidin and delphinidin-3-*O*-glucoside (D3G) at 200 µM inhibited MDA-MB-231 cell proliferation, while cyanidin had no effect. Furthermore, a recent study found that, among the four anthocyanins tested (D3G, cyanidin-3-*O*-glucoside (C3G), malvidin-3-*O*-glucoside and luteolinidin), D3G was the only one with a dose-effect response on MDA-MB-231 cell growth (162).

The most common chemotherapy drugs used in treatment of TNBC are anthracyclines, taxanes, and platinum agents. Taxanes include paclitaxel and docetaxel (163). Docetaxel is a mitotic inhibitor that bind specifically to β-subunit of microtubules, stabilizing microtubules and preventing their depolymerization. As a result, cell division stops at G2/M phase of the cell cycle, resulting in cell death by apoptosis (164, 165). According to Viegas *et al.*, D3G and C3G led to the formation of tubulin complexes and the increase of tubulin units in MDA-MB-231 cells, suggesting a similar mechanism of action between anthocyanins and docetaxel (149). Although its large use and efficacy in the treatment of TNBC, some TNBC cell lines, namely MDA-MB-231, have developed resistance to docetaxel (164, 166, 167). Thus, combined therapies have been used to reduce the dose of pharmacological drugs and the associated adverse effects as well as to overcome chemotherapy resistance (164, 165). Considering the anti-proliferative effect and the similar mechanism of action of delphinidin-based anthocyanins and docetaxel, the combination of these compounds may represent a new strategy for the treatment of TNBC.

2. Objectives

Based on the potential health benefits of the highly perishable blackcurrant fruits, the two main objectives of this dissertation were: i) to optimize the preparation conditions of blackcurrant infusions that maximize anthocyanin extraction; and ii) to evaluate the combined effect of docetaxel with the two main delphinidin-based anthocyanins found in blackcurrant (delphinidin 3-*O*-glucoside and 3-*O*-rutinoside) on triple negative breast cancer cells. To achieve these main objectives, the following specific objectives were established:

- To validate a UHPLC-DAD method for anthocyanin analysis on blackcurrant-based products;
- To apply a Box–Behnken design to select the optimum conditions (temperature, time, and mass/volume ratio) that maximize anthocyanin content of blackcurrant infusions;
- To characterize and quantify the anthocyanin composition, phenolic compounds content and antioxidant activity of different freeze-dried blackcurrant berries and their infusions;
- To assess the isolated effect of docetaxel on MDA-MB-231 cell proliferation and its combined effect with delphinidin-3-*O*-rutinoside and delphinidin-3-*O*-glucoside;
- To evaluate the effect of docetaxel and anthocyanins on healthy breast cells (MCF-12A cell line).

3. Materials and Methods

3.1. Chemicals and Reagents

For high performance liquid chromatography (HPLC) methanol and acetonitrile of HPLC grade were obtained from Honeywell, Riedel-de-Haën (Seetze, Germany), while formic acid (purity = 99-100%) was purchased from AnalaR NORMAPUR, VWR (Radnor, PA, USA) and hydrochloric acid (HCl) from Merck (Darmstadt, Germany). Ultrapure water was obtained by using a Milli-Q water purification system (Millipore, Bedford, MA, USA). The standards delphinidin-3-*O*-glucoside (D3G), delphinidin-3-*O*-rutinoside (D3R), cyanidin-3-*O*-glucoside (C3G), and cyanidin-3-*O*-rutinoside (C3R) were purchased from Extrasynthese (Lyon, France). Stock standard solutions (500 µg/mL) were prepared with methanol containing 2% (v/v) hydrochloric acid and stored at -20 °C.

Gallic acid (≥98% purity), sodium carbonate, 2,2-diphenyl-1-picrylhydrazyl (DPPH•) solution, and trolox (97% purity) were acquired from Sigma-Aldrich Corp. (St. Louis, MO, USA). Folin-Ciocalteau (F-C) reagent was obtained from Merck (Darmstadt, Germany), while acetone (p.a.) and methanol (p.a.) were bought from Honeywell, Riedel-de-Haën (Seetze, Germany).

Dulbecco´s modified Eagle´s high glucose medium (DMEM-HG), fetal bovine serum (FBS; EU Approved, South American), penicillin-streptomycin solution (10,000 Units/mL – 10,000 µg/mL), and trypsin-EDTA (0.25%) were obtained from Gibco (Gibco/Life Technologies, Paisley, UK). Dulbecco's modified Eagle's medium and Ham's F12 cell growth medium (DMEM/F12), horse serum, human epidermal growth factor (hEGF), cholera toxin from *Vibrio cholerae*, bovine insulin (10 mg/mL insulin in 25 mM HEPES, pH 8.2), hydrocortisone, paraformaldehyde, Hoechst 33258, bovine serum albumin (BSA), Triton X-100, dimethyl sulfoxide (DMSO), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), phosphate-buffered saline (PBS) and docetaxel (DT) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Rabbit anti-α-tubulin primary antibody was obtained from Abcam (Cambridge, UK) and goat anti-rabbit Alexa Fluor® 594 secondary antibody was obtained from Invitrogen (Thermofisher, MA, USA). For cellular assays, anthocyanin stock solutions (40 mM) were prepared in 10% DMSO and stored at -20 °C. Docetaxel solution at 1000 nM concentration was solubilized in DMSO and diluted in PBS. During experiments, DMSO concentration in growth medium never exceed 0.1% (v/v) and was always considered as a control.

3.2. Samples

Four freeze-dried blackcurrant samples were purchased from online stores (Table 10). The obtained blackcurrant samples were produced in different countries: product A was produced in New Zealand, product B and C were produced in Lithuania, and product D was produced in Chile. Two blackcurrant samples (products A and B) were sold whole and the other two (products C and D) were sold in powder form. Therefore, whole blackcurrants were ground with a knife mill (Grindomix GM 200, Retsch, Hann, Germany) before analysis and infusion preparation. All the samples were stored protected from the light, at -80 °C, until infusions were prepared and analysed.

Table 10: Characterization of blackcurrant samples according to the label.

	Product A	**Product B**	**Product C**	**Product D**
Ingredients	Freeze-dried blackcurrant	Freeze-dried blackcurrant	Freeze-dried blackcurrant powder	Freeze-dried blackcurrant powder
Country of origin	New Zealand	Lithuania	Lithuania	Chile
Nutritional value per 100 g				
Energy (kJ)	1450	1200	1200	-
Energy (kcal)	347	290	290	-
Fat (g)	2.0	1.0	1.0	-
Of which saturated fat (g)	0.5	0.1	0.1	-
Carbohydrates (g)	65.5	33.0	33.0	-
Of which sugars (g)	46.8	33.0	33.0	-
Fibre (g)	23.0	37.0	37.0	-
Protein (g)	4.8	7.0	7.0	-
Suggested Use	1 tablespoon (4 grams) per day	-	-	2 teaspoons per day

3.3. Analysis of anthocyanins

3.3.1. Anthocyanin extraction from freeze-dried blackcurrant berries

Anthocyanin extraction was performed according to the method previously described by Peixoto *et al.* (140) with slight modifications (Figure 3). One hundred milligrams of freeze-dried blackcurrant powders were weighted in 15 mL centrifuge tube before the addition of 2 mL of methanol acidified with formic acid (9:1, v/v). The mixture was homogenized in vortex for 1 min and sonicated for 10 min prior to centrifugation at 3000 × g for 10 min at 20 °C. The supernatants were collected into 15 mL tube, and all the steps of the procedure were repeated until the purple colour of the samples disappeared. The

combined extract was centrifuged at 3000 × g for 10 min and 100 µL of supernatant were dissolved in 900 µL of a solution of formic acid/water (1:9, v/v). Diluted extracts were filtered through a 0.45 µm PTFE membrane and analysed by UHPLC-DAD. Triplicate independent extractions of blackcurrant powders were performed.

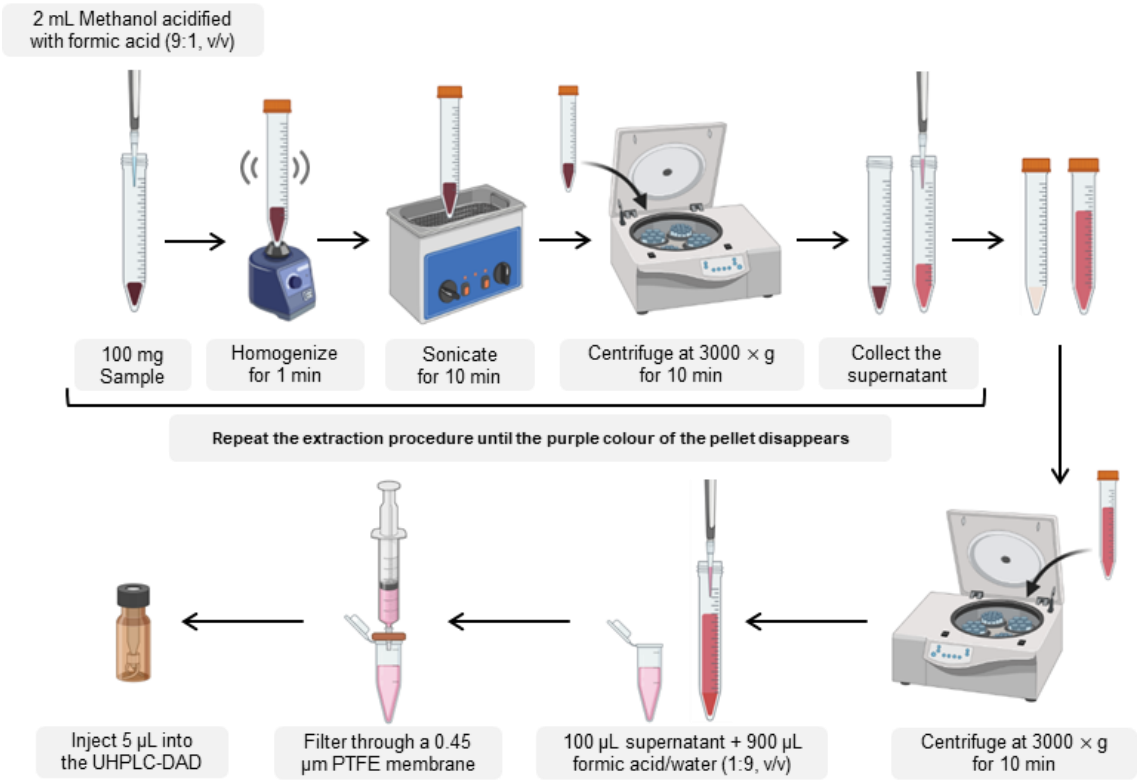


Figure 3: Representative scheme of anthocyanin extraction from freeze-dried blackcurrant berries. Created with BioRender.com.

3.3.2. Experimental Design

Infusion preparation conditions, as temperature, time, and fruit mass/water volume ratio, influence the extraction of phenolic compounds (106, 110, 168). Therefore, a three-level factorial Box–Behnken design (BBD) of Response Surface Methodology (RSM) was used to optimize infusion conditions that can affect anthocyanin extraction. Table 11 presents the BBD with three independent variables (X_1: temperature (°C); X_2: time (min); X_3: mass/volume ratio (g/200 mL) at three levels, coded -1, 0, +1 for low, medium, and high level. The low (-1) and high (+1) level of each experimental variable was defined according to the preparation instructions described on the label of different commercially available herbal/fruit tea bags.

Table 11: Experimental levels of independent variables in Box–Behnken design.

Variables	Levels		
	Low (-1)	**Medium (0)**	**High (+1)**
(X_1) Temperature (°C)	80.0	90.0	100.0
(X_2) Time (min)	2.0	4.5	7.0
(X_3) Ratio (g/200 mL)	0.5	2.25	4.0

The BBD resulted in 17 combinations, including 5 replicates of the center point (X_1: 90 °C; X_2: 4.5 min; X_3: 2.25 g/200 mL) to estimate the pure error sum of squares (Table 12). All the experiments were undertaken in random order to reduce the effect of uncontrolled variables. The anthocyanin content of infusions (expressed as milligram per 200 mL of infusion – brew volume recommended for commercial tea bags, considered a portion) was used as response. A full quadratic model was used to fit the data. Adequacy and significance of the quadratic models were evaluated by analysis of variance (ANOVA) through Fisher's F-test. The analysis of the experimental design was executed using the Design-Expert® software Trial Version 10 (Stat-Ease Inc., Minneapolis, MN, USA).

Table 12: Box–Behnken design to evaluate the effect of infusion conditions on the anthocyanin content of different blackcurrant infusions.

Standard order	Independent variables			**Response** (Anthocyanin content; mg/200 mL)			
	X_1	X_2	X_3	Product A	Product B	Product C	Product D
1	80.0	2.0	2.25	58.61	30.43	28.81	8.45
2	100.0	2.0	2.25	51.98	24.81	29.59	11.18
3	80.0	7.0	2.25	53.33	26.70	25.19	8.88
4	100.0	7.0	2.25	58.54	25.17	30.32	9.91
5	80.0	4.5	0.5	13.41	7.10	6.35	2.57
6	100.0	4.5	0.5	13.88	7.69	5.79	2.58
7	80.0	4.5	4.0	84.50	59.33	41.92	15.54
8	100.0	4.5	4.0	86.27	44.65	34.39	17.86
9	90.0	2.0	0.5	13.79	7.74	7.03	2.52
10	90.0	7.0	0.5	14.32	5.65	7.21	2.42
11	90.0	2.0	4.0	68.59	56.95	36.87	17.49
12	90.0	7.0	4.0	103.09	55.53	42.48	17.00
13	90.0	4.5	2.25	42.47	31.73	27.33	10.26
14	90.0	4.5	2.25	63.47	19.38	26.82	7.21
15	90.0	4.5	2.25	65.13	29.62	25.01	10.38
16	90.0	4.5	2.25	62.41	31.09	25.92	8.15
17	90.0	4.5	2.25	61.09	22.40	26.58	9.06

X_1: temperature (°C); X_2: time (min); X_3: mass/volume ratio (g/200 mL)

3.3.3. Preparation of blackcurrant infusions

Blackcurrant infusions were prepared following the brew conditions resulting from the experimental design. Briefly, 200 mL of distilled water were heated in a Thermomix (Vorwerk, Germany) to 80, 90 or 100 °C and added to blackcurrant powder (0.5, 2.25 or 4.0 g). Then, blackcurrant infusions were homogenized with a glass rod, covered, and incubated for 2, 4.5 or 7 minutes. After the incubation time, infusions were centrifuged at 3000 × g for 5 minutes and decanted to a 50 mL tube. For chromatographic analysis, 100 µL of the supernatant were dissolved in 900 µL of a solution of formic acid/water (1:9, v/v) and filtered through a 0.45 µm PTFE membrane (Figure 4). Both infusion aliquots were stored protected from the light, at -20 °C, until analysis.

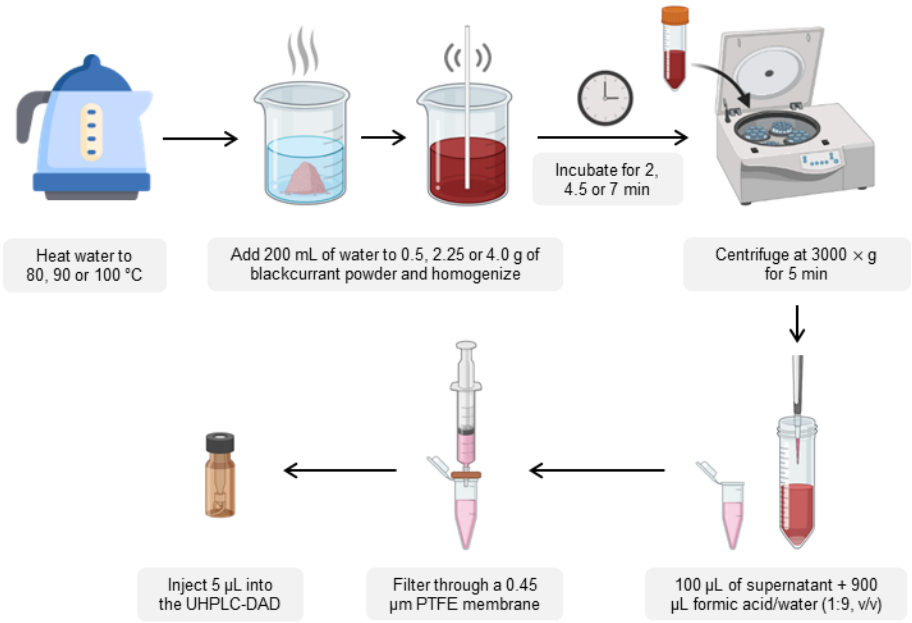


Figure 4: Representative scheme of blackcurrant infusion procedure and anthocyanin analysis. Created with BioRender.com.

3.3.4. UHPLC-DAD conditions

Separation and quantification of anthocyanins were performed by UHPLC-DAD. The chromatographic analysis was carried out in a Dionex UltiMate™ 3000 UHPLC system (ThermoFisher Scientific, MA, USA) equipped with a Phenomenex Gemini reversed phase C18 column (15 cm length, 3.0 mm internal diameter, 5 µm particle size, 110 Å pore size; Phenomenex inc., Torance, CA, USA) thermostated at 35 °C with the aid of a SecurityGuard ULTRA C18 guard column and a KrudKatcher ULTRA HPLC In-Line Filter (Phenomenex

inc., Torance, CA, USA). The Chromeleon™ Chromatography Data System (CDS) Software (ThermoFisher Scientific, MA, USA) was used for data processing.

Chromatographic separation conditions (Table 13) were selected according to the Application Note 281 of Dionex (ThermoFisher Scientific, MA, USA) with slight modifications (169). In this way, anthocyanins were separated using a mobile phase consisting of water and formic acid (9:1 v/v) as solvent A, and water/methanol/acetonitrile/formic acid (4.5:2.25:2.25:1 v/v) as solvent B with a flow rate of 0.500 mL/min. The gradient program is represented in Table 14. Run time was 22 min and the injection volume was 5 µL. After separation, anthocyanins were detected by a Dionex UltiMate™ 3000 Diode Array Detector at 520 nm and were identified by comparing the retention time and spectra of unknown peaks with standards. The concentrations of anthocyanins were determined using corresponding standard calibration curve based on the peak area for the selected wavelength. Anthocyanin concentrations of blackcurrant berries and infusions were expressed as milligrams per 100 g DW and per 200 mL, respectively.

Table 13: Selected chromatographic conditions for the analysis of anthocyanins.

	Chromatographic conditions
Run Time	22 min
Flow	0.500 mL/min
Mobile Phase	**A:** 90% H_2O/10% Formic acid **B:** 45% H_2O/22.5% Methanol/22.5% Acetonitrile/10% Formic Acid
DAD selected λ	520 nm
T sampler	4 °C
T oven	35 °C
Sample volume	5 µL

Table 14: Mobile phase gradient program used to separate anthocyanins.

Mobile phase gradient program		
Time (min)	**% Eluent A**	**% Eluent B**
0	85	15
15	75	25
17	75	25
19	85	15
22	85	15

3.3.5. Analytical performance

The analytical performance of the proposed method was assessed through the study of the linearity, limit of detection, limit of quantification, and precision. In order to study linearity, and to obtain regression equations and correlation coefficients (r^2), calibration curves were constructed using eleven concentration levels of standard mix solutions with D3G, D3R, C3G, C3R in a linear range of 0.05 to 50 µg/mL. The study of linearity also included the determination of standard deviation of slope ($S_{(b)}$), intercept ($S_{(a)}$), and y-residuals ($S_{(y/x)}$) according to Miller and Miller (170). The limit of detection (LOD), defined as the lowest amount of analyte which can be detected, and the limit of quantification (LOQ), defined as the lowest amount of analyte which can be quantified were determined based on the calibration curve parameters:

$$LOD = \frac{3 \times S_{(y/x)}}{b} \qquad LOQ = \frac{10 \times S_{(y/x)}}{b}$$

where $S_{(y/x)}$ is the standard deviation of y-residuals and b is the slope of the regression equation.

The precision of the chromatographic method was evaluated by repeatability and intermediate precision which expresses the precision under the same conditions during the same day and the within-laboratory variations on different days, respectively, according with ICH guidelines. Therefore, four different concentration levels (2.5, 10, 25 and 50 µg/mL) of each anthocyanin, within the linear range, were assessed in triplicate (repeatability) in three different days (intermediate precision) and expressed as the relative standard deviation (RSD %).

3.4. Determination of total phenolic compounds and antioxidant activity

3.4.1. Extraction of phenolic compounds from blackcurrant berries

Extraction of total phenolic compounds of freeze-dried blackcurrant berries were preformed according to the method described by Pérez-Jiménez *et al.* (171) with some modifications (Figure 5). Briefly, 4 mL of methanol 50% (v/v) were added to 100 mg of freeze-dried blackcurrant powder, homogenized in vortex for 1 min and placed in constant agitation for 1 hour. Methanolic extract was centrifuged at 5000 × g for 5 min at 4 °C and 2 mL of supernatant were collected into a 15 mL tube. Then, the resulting sample was extracted one more time with 4 mL of acetone 70% (v/v). After centrifugation (at 5000 × g for 5 min at 4 °C), 2 mL of the supernatant were added to the 2 mL of methanolic extract.

Combined extracts were stored protected from the light, at -20 °C, until analysis. Triplicate independent extractions were performed.

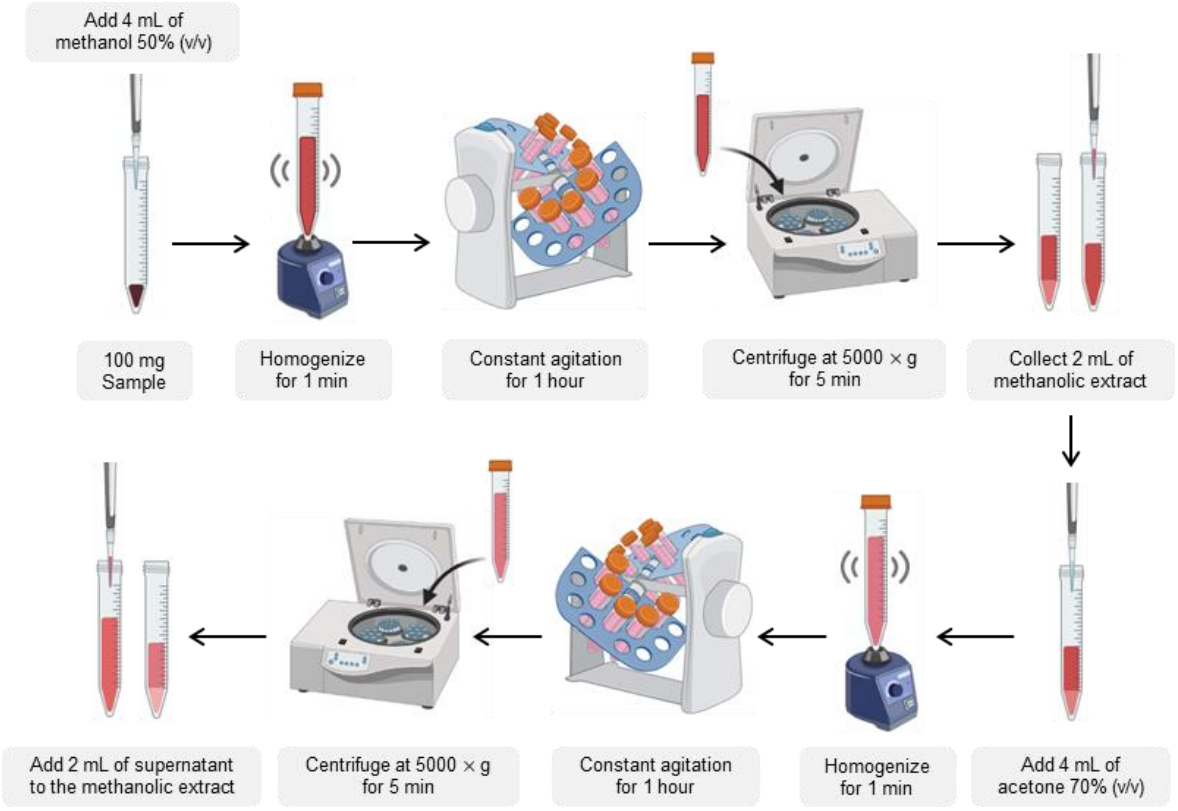


Figure 5: Representative scheme of the extraction of phenolic compounds from freeze-dried blackcurrant berries. Created with BioRender.com.

3.4.2. Quantification of total phenolic compounds

Total phenolic compounds were determined through the Folin–Ciocalteu F–C) method. This assay is based on the transfer of electrons of phenolic compounds to phosphomolybdic/phosphotungstic acid reagent in alkaline conditions. The reduction of the phosphomolybdate-phosphotungstate complex results in blue complexes that can be detected spectrophotometrically at 765 nm (172, 173).

Determination of phenolic compounds of blackcurrant berries and infusions were carried out according to the conditions described by Magalhães *et al.* (172) with minor modifications. Firstly, blackcurrant extracts and blackcurrant infusions were diluted 1:5 (v/v) and 1:2 (v/v) with water, respectively. In a 96-well plate, 75 µL of blackcurrant extracts were successively diluted (1:10, 1:20, 1:40, 1:80, 1:160) with 75 µL of a solution equivalent to the extracts (methanol/acetone/H_2O; 2.5/3.5/94, %v/v), while 75 µL of blackcurrant infusions were diluted (1:4, 1:8, 1:16, 1:32, 1:64) with 75 µL of water, as shown in figure 6. Then, 25 µL of F-C reagent (4:10, v/v), were added to all wells, except for those intended for the

evaluation of the intrinsic absorption. To assess the intrinsic absorption of the samples, 25 µL of water were added instead of 25 µL F-C reagent. After the addition of F-C reagent, the plate was shaken in the microplate reader for 1 min and incubated for 10 min in the dark. Subsequently, 100 µL of sodium carbonate (6%, v/v) were added to every well. After 1 hour of reaction, the absorbance was measured, at 765 nm, using a SPECTROstar Nano Microplate Reader (BMG Labtech, Ortenberg, Germany). Gallic acid (550 µM – 15.63 µM) was used as standard. The content of phenolic compounds in blackcurrant berries and infusions was expressed in milligrams of gallic acid equivalents per 100 g DW (mg GAE/100 g DW) and per 200 mL (mg GAE/200 mL), respectively.

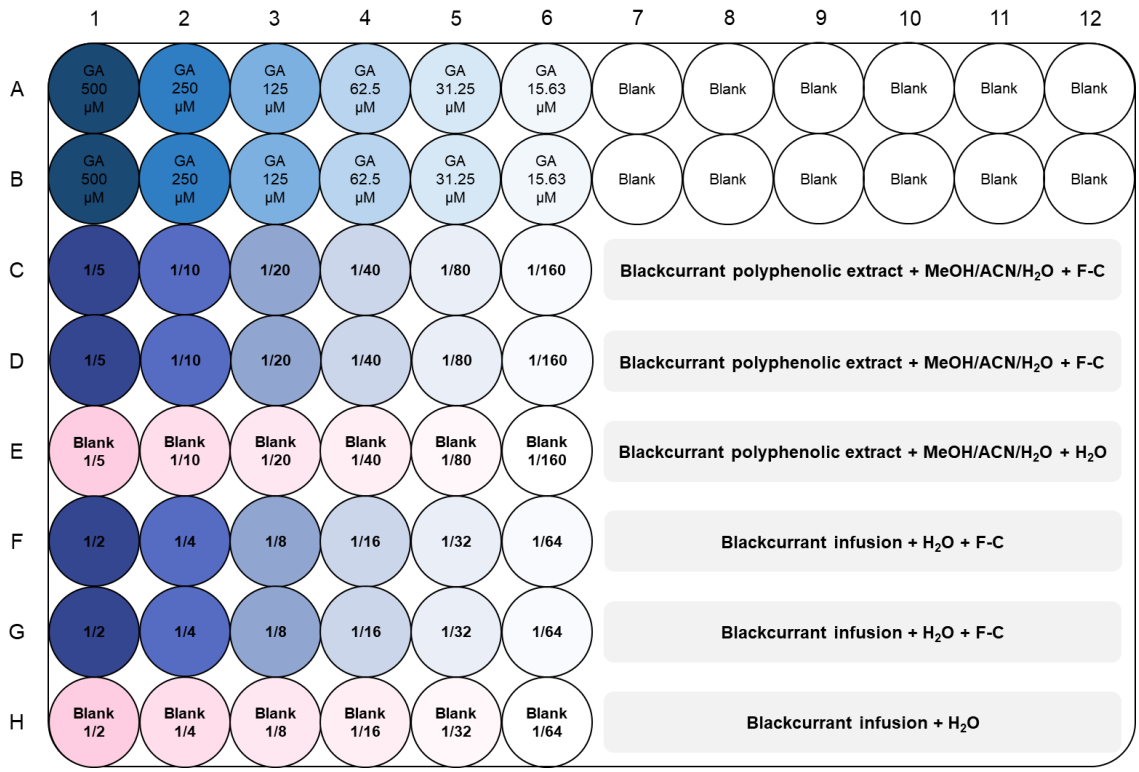


Figure 6: Representative scheme of the microplate layout for the Folin–Ciocalteu assay.

3.4.3. Determination of antiradical activity by DPPH• assay

Antiradical activity of blackcurrant berries and infusions were determined through the DPPH• assay. The purple free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•) can be scavenged by antioxidant species, resulting in its yellow-pale reduced form (DPPH-H). Therefore, antioxidant capacity of compounds can be assessed spectrophotometrically, at 517 nm, based on the discolouration that occurs during the scavenging of DPPH• by antioxidant species (174, 175).

The DPPH• assay was performed according to the modified methods described by Magalhães *et al.* (176) and Viegas *et al.* (177). In the day of the assay, DPPH• solution (1 mM) was prepared with absolute methanol and diluted until an absorbance of 0.800 ± 0.020 was obtained. The extracts of blackcurrant berries (prepared in 3.4.1.) were diluted 1:5 with methanol 50% (v/v), while blackcurrant infusion extracts were diluted 1:2 with absolute methanol. 250 µL of blackcurrant berries and infusions were successively diluted (1:10, 1:20, 1:40, 1:80, 1:160 and 1:4, 1:8, 1:16, 1:32, 1:64, respectively) with 250 µL of methanol 50% (v/v) in 1.5 mL microcentrifuge tubes. Then, 250 µL of DPPH• solution were added to all tubes. To assess the intrinsic absorption of the samples, 250 µL of absolute methanol were added to the tubes containing 250 µL of sample + 250 µL of methanol 50% (v/v). DPPH• controls were prepared by adding 250 µL of DPPH• solution to 250 µL of methanol 50% (v/v). One hour after the addition of DPPH• solution, 200 µL of the reaction solutions were transferred to each well of the microplate, as shown in figure 7. Finally, the absorbance was read at 517 nm, using a SPECTROstar Nano Microplate Reader (BMG Labtech, Ortenberg, Germany). Trolox (550 µM – 15.63 µM) was used as standard. The antioxidant activity of blackcurrant berries and infusions was expressed in milligrams of trolox equivalents per 100 g DW (mg TE/100 g DW) and per 200 mL (mg TE/200 mL), respectively.

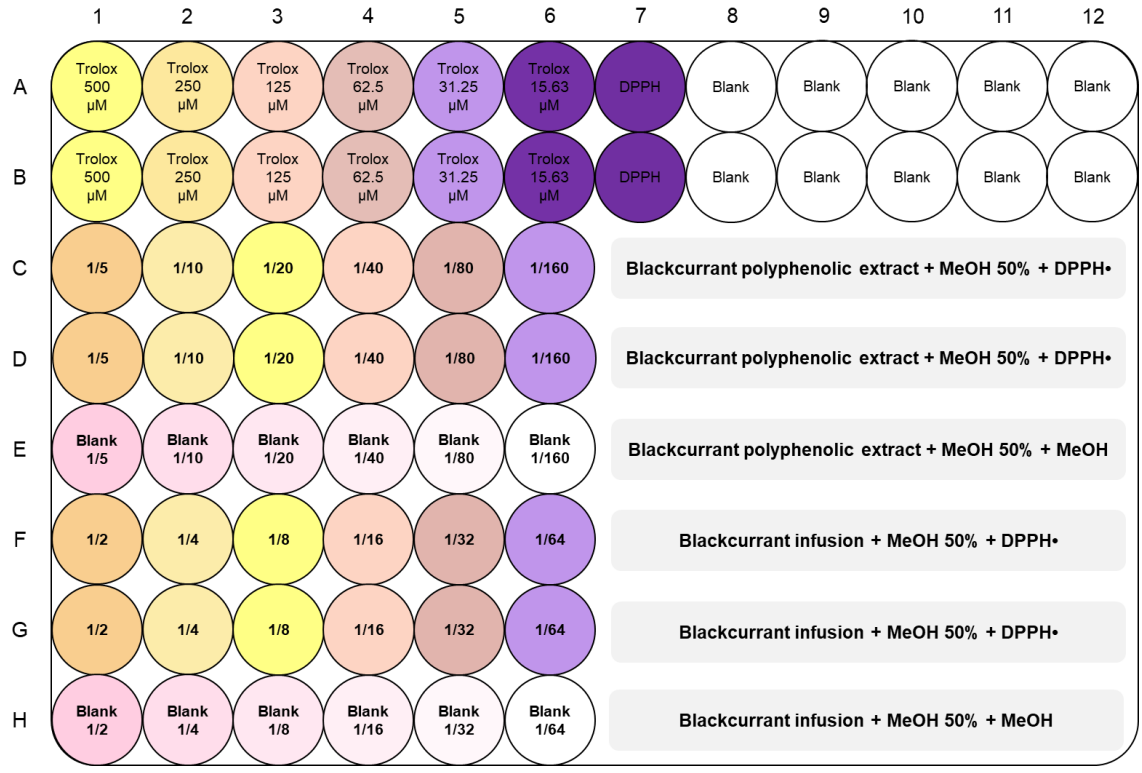


Figure 7: Representative scheme of the microplate layout for the DPPH• assay.

3.5. Statistical analysis

Samples were analysed in triplicate. Data were tested for normal distribution of residuals with Shapiro–Wilk's test. The results were analysed statistically by one-way analysis of variance (ANOVA), followed by Tukey post-hoc test for mean comparison, when the normal distribution of residuals and the homogeneity of variances was confirmed. Welch´s correction followed by Tamhane's T2 post hoc test was applied when homogeneity of variances was not verified. A p value < 0.05 was considered to denote statistically significant differences. Statistical analyses were performed using GraphPad Prism version 8.0.1 for Windows (GraphPad Software, La Jolla California USA).

3.6. Determination of combined effect of docetaxel and delphinidin-3-*O*-glucoside and 3-*O*-rutinoside on cell proliferation

3.6.1. Cell culture

MDA-MB-231 (human Caucasian triple negative (ER−, PR−, Her2−), claudin-low breast carcinoma) and MCF-12A (non-neoplastic breast cells) cell lines were purchased from European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK). MDA-MB-231 cells were cultured in DMEM-HG supplemented with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin. MCF-12A cells were grown in DMEM/F12 medium supplemented with 20 ng/mL hEGF, 100 ng/mL cholera toxin, 0.01 mg/mL bovine insulin, 500 ng/mL hydrocortisone and 5% (v/v) horse serum. Both cell lines were cultured in monolayers in sterile environment at 37 °C and 5% CO_2 and were subcultured at 80% confluence. Under these conditions, MDA-MB-231 and MCF-12A cells presented a population doubling time of 24.9 ± 1.4 h and 19.9 ± 4.8 h, respectively. Mycoplasma contamination of cell cultures was routinely screened according to the protocol described by Van Kuppeveld *et al.* (178). Cell viability assays were carried out during the logarithmic phase of cell growth.

3.6.2. Isolated and combined effects

In order to assess the isolated effect of D3G, D3R and DT on cell proliferation, MDA-MB-231 cells were exposed to six different concentrations of each isolated compound: 10, 50, 125, 250, 325 and 400 μM for anthocyanins and 0.01, 0.1, 1, 10, 100 and 200 nM for docetaxel. Stock solutions of anthocyanins (40 mM) were dissolved (1:100) in growth medium, filtered through a 0.22 μm nylon filter and, subsequently, diluted in growth medium (from 400 to 10 μM). Docetaxel working solutions were also prepared in complete medium. The concentrations of tested compounds were selected based on previous works, namely Viegas *et al.* for anthocyanins and Carvalho *et al.* for docetaxel. The results obtained from

the exposure of TNBC cells to anthocyanins and docetaxel were used to fit a dose-response curve for each isolated compound and to calculate the half maximal inhibitory concentration (IC_{50}) of D3G, D3R and DT in MDA-MB-231 cells. To assess the combined effect of DT with each anthocyanin separately and with both anthocyanins simultaneously, MDA-MB-231 cells were exposed to five concentration levels based on the constant combination ratio design proposed by Chou-Talalay method (179). According to Chou and Talalay (179), the combination ratio should be kept at an equipotency ratio (i.e. $(IC_{50})_1/(IC_{50})_2$ ratio and $(IC_{50})_1/(IC_{50})_2/(IC_{50})_3$ for 2- and 3-drug combination, respectively) to assure that each compound contributes similarly to the combination. The isolated and combined effect of anthocyanins and docetaxel on the proliferation of non-neoplastic cells was assessed by exposing MCF-12A cells to the IC_{50} of each compound in MDA-MB-231 cells.

3.6.3. MTT cell viability assay

The isolated and combined effect of DT, D3G and D3R on the proliferation of MDA-MB-231 and MCF-12A cells was assessed by the MTT assay, according to the method described by Soares *et al* (180). Briefly, 200 µL of cell suspension (9.0×10^{-3} cells/mL) were seeded in 96-well plates and incubated for 24 h at 37 °C to allow cell adhesion. Thereafter, the growth medium was replaced by 200 µL of complete medium containing DT, D3G and D3R isolated or in combination and incubated for another 72 h. At the end of incubation period, cells were exposed to 100 µL of MTT solution (0.5 mg/mL) for 2 h. The blue formazan crystals, resulting from the mitochondrial reduction of MTT by viable cells, were dissolved with 100 µL of DMSO and the absorbance was evaluated at 570 nm using a SPECTROstar Nano Microplate Reader (BMG Labtech, Ortenberg, Germany). The cells treated with DMSO 0.1% (v/v) were used as controls. The anti-proliferative effect of tested compounds was expressed as a percentage of control.

3.6.4. Data analysis

All the experiments were performed at least in two independent experiments with 6 replicates per condition. Data were expressed as mean ± standard error of the mean (SEM). Dose-effect curves of docetaxel and anthocyanins isolated or in combination were performed using GraphPad Prism version 8.0.1 for Windows (GraphPad Software, La Jolla, CA, USA). CompuSyn software version 1.0 (ComboSyn Inc., Paramus, NJ, USA) was used to determine the IC_{50} of each compound and to assess the combined effect of anthocyanins and docetaxel based on the calculation of the combination index (CI). Compusyn software calculates the IC_{50} (D_m) using the median-effect principle of the mass-action law, considering the shape of dose-response curve:

$$\log(f_a/f_u) = m\log(D) - m\log(D_m)$$

where D is the dose of a compound, f_a is the fraction affected by D, f_u is the fraction unaffected ($f_u = 1 – f_a$), m is the shape of the dose-response curve (where m = 1, m > 1 and m < 1 indicate hyperbolic, sigmoidal, and flat sigmoidal curves, respectively), and D_m is the dose that inhibits 50% of cell proliferation (181).

To assess the combined effect of docetaxel and anthocyanins on TNBC cell proliferation, CI was calculated based on the following formula:

$$CI = \sum_{j=1}^{n} \frac{(D)_j}{(D_x)_j}$$

where $(D_x)_j$ are the doses of each compound alone that inhibit x% of cell proliferation, and D_j are the doses of each compound that in combination inhibits x% of cell proliferation. CI is used to quantify the synergism and antagonism for two or more compounds. Therefore, a CI around 1 (0.9 < CI < 1.1) indicates an additive effect of docetaxel and anthocyanins on cell proliferation. On the other hand, if CI < 0.9 or CI > 1.1 means that docetaxel and anthocyanins have synergistic or antagonistic effects, respectively (181). CI values were used to construct CI-plot and isobologram graphs. For those combinations with synergistic effects, dose reduction indexes (DRI) were calculated. DRI indicates how many folds the dose (or concentration) of each compound in a synergistic combination may be reduced to achieve the same anti-proliferative effect compared with the doses of each compound isolated. Thus, DRI of each compound can be determined based on the inversion of each term of CI equation:

$$DRI = \frac{(D_x)_j}{(D)_j}$$

where $(D_x)_j$ is the dose of each compound alone that inhibit x% of cell proliferation, and D_j is the dose of each compound that in combination inhibits x% of cell proliferation (179).

3.6.5. Immunocytochemistry

MDA-MB-231 cells were seeded in 384-well black microplates ($1x10^4$ cells/mL) and allowed to attach for 24 h. The cells were treated with the IC_{50} of DT isolated and in combination with D3G and D3R for 24 h. After incubation period, cells were fixed with paraformaldehyde solution (pH 7.0) for 10 min at room temperature and washed twice with PBS (100 mM NaH_2PO_4, 50 mM NaCl). Prior immunostaining, cells were permeabilized with 0.1% Triton X-100, 0.2 M glycine, 10% FBS, 1% BSA in PBS for 1 h. Next, cells were washed twice with PBS and α-tubulin was stained with rabbit anti-α-tubulin (1:200 dilution,

for 12 h at 4 °C) primary antibody. After washing cells with PBS, goat anti-rabbit Alexa Fluor® 594 secondary antibody solution (1:500 dilution) was added and incubated for 1 h at room temperature in dark. Cell nuclei were stained with Hoechst 33258 solution (5 mg/mL) for 5 min in dark. Finally, cells were washed twice with PBS for 5 min and cell micrographs were immediately captured with 20x objective using The Lionheart™ FX Automated Live Cell Imager (BioTek, Bad Friedrichshall, Germany). Image analysis was performed using Gen5 Image Prime 3.02 (Biotek, Bad Friedrichshall, Germany). To quantify α-tubulin per cell, automated threshold-based masks corresponding to the microtubule fluorescence staining were generated. Mean fluorescence intensity was quantified per cell. Results were expressed as percentage of tubulin-positive cells (percentage of cells with fluorescence intensity > mean fluorescence intensity + 2SD). Data were analysed statistically by one-way ANOVA followed by Dunnett's multiple comparisons for analysis of means, using GraphPad Prism version 8.0.1 for Windows (GraphPad Software, La Jolla, CA, USA). A p-value < 0.05 was considered statistically significant.

4. Results and Discussion

4.1. Analytical performance

The analytical performance of the chromatographic method for the identification and quantification of anthocyanins from different blackcurrant samples was evaluated. Figure 8 displays the chromatographic separation of D3G, D3R, C3G and D3R standards (10.0 µg/mL) in the selected chromatographic conditions.

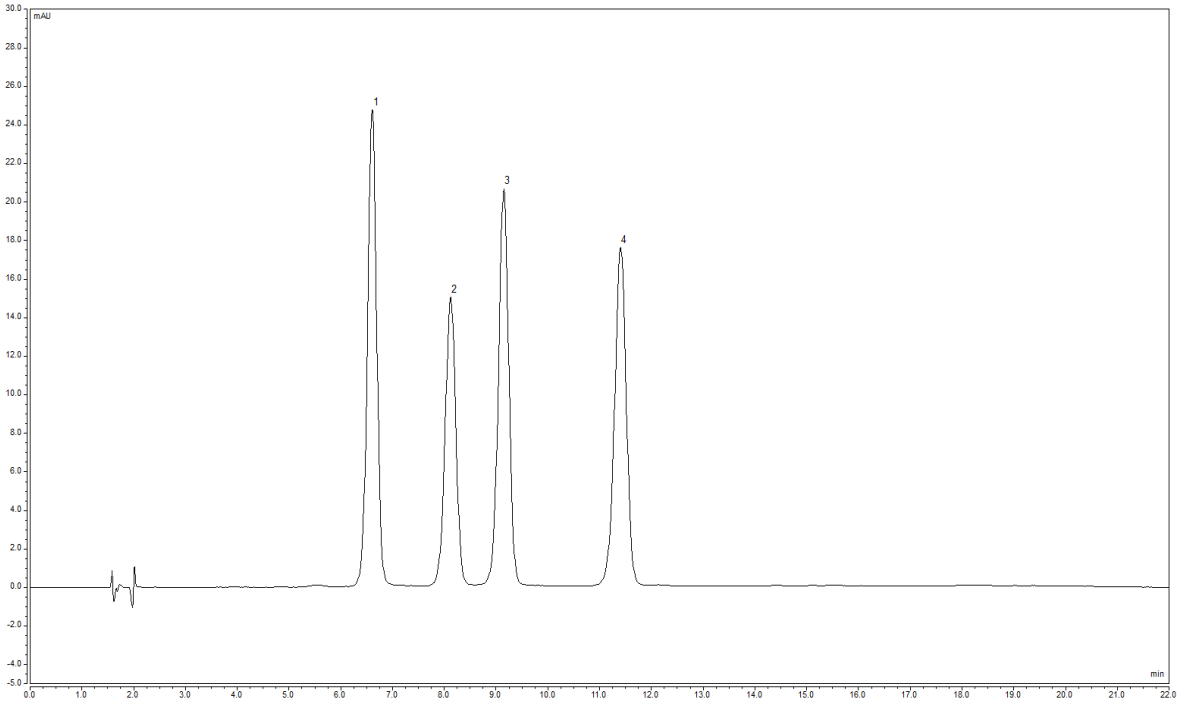


Figure 8: Typical chromatogram of a standard mix of anthocyanins with a concentration of 10.0 µg/mL each.

1 – Delphinidin-3-*O*-glucoside; **2** – Delphindin-3-*O*-rutinoside; **3** – Cyanidin-3-*O*-glucoside; **4** – Cyanidin-3-*O*-rutinoside.

Linearity, evaluated by the correlation coefficient (r^2), was observed using calibration standards in the range between 0.05 – 50.0 µg/mL for the major anthocyanins found in blackcurrants (D3G, D3R, C3G, C3R). Table 15 summarizes the calibration parameters calculated according to Miller and Miller (170). The correlation coefficient (r^2) was 1.000 for the four anthocyanins, which indicates an excellent linear response. LOD and LOQ were 0.04 and 0.14 µg/mL for D3R and C3R, respectively, and 0.05 and 0.15 µg/mL for D3G and C3G. Higher LOD and LOQ values were described for D3G (1.56 and 6.25 µg/mL, respectively) and C3G (0.78 and 3.13 µg/mL, respectively) using similar chromatographic conditions (169).

Table 15: Calibration parameters for anthocyanins identification and quantification by UHPLC-DAD.

ANT	RT (min)	LR (µg/mL)	b	a	r^2	$S_{(b)}$	$S_{(a)}$	$S_{(y/x)}$	LOD (µg/mL)	LOQ (µg/mL)
D3G	6.9	0.05 - 50.0	0.5280	0.0010	1.000	0.0002	0.003	0.0079	0.05	0.15
D3R	8.5	0.05 - 50.0	0.3699	0.0028	1.000	0.0001	0.002	0.0053	0.04	0.14
C3G	9.5	0.05 - 50.0	0.4956	0.0031	1.000	0.0001	0.003	0.0074	0.05	0.15
C3R	11.8	0.05 - 50.0	0.4780	0.0025	1.000	0.0001	0.002	0.0069	0.04	0.14

ANT: anthocyanin; D3G: delphinidin-3-*O*-glucoside; D3R: delphinidin-3-*O*-rutinoside; C3G: cyanidin-3-*O*-glucoside; C3R: cyanidin-3-*O*-rutinoside; RT: retention time; LR: linear range; b: slope; a: intercept; r^2: correlation coefficient; $S_{(b)}$ and $S_{(a)}$: standard deviations of the slope and intercept; $S_{(y/x)}$: standard deviation of y residuals of regression line; LOD: limit of detection; LOQ: limit of quantification.

The precision of the method was evaluated through repeatability (intra-day precision) and intermediate precision (inter-day precision). Four different concentration levels (2.5, 10, 25 and 50 µg/mL) of each anthocyanin were assessed in triplicate in three different days and expressed as the relative standard deviation (RSD). Results from repeatability and intermediate precision are presented in Table 16. The relative standard deviations ranged from 0.16 and 0.42% for repeatability and 0.49 and 3.00% for intermediate precision. These values were far below the upper limit (± 15%) set by the regulatory authorities (182, 183).

Table 16: Repeatability and intermediate precision of the chromatographic method at four levels of concentration (2.5, 10, 25 and 50 µg/mL)

	Repeatability (n = 3) RSD (%)				**Intermediate Precision (n = 9)** RSD (%)			
Concentration level (µg/mL)	2.5	10	25	50	2.5	10	25	50
D3G	0.21	0.35	0.32	0.19	0.49	1.46	2.09	2.86
D3R	0.29	0.3	0.25	0.17	0.70	1.5	2.13	3.00
C3G	0.42	0.40	0.30	0.19	0.93	1.46	1.99	2.96
C3R	0.16	0.29	0.26	0.19	0.89	1.55	2.22	2.91

D3G: delphinidin-3-*O*-glucoside; D3R: delphinidin-3-*O*-rutinoside; C3G: cyanidin-3-*O*-glucoside; C3R: cyanidin-3-*O*-rutinoside; RSD: relative standard deviation.

4.2. Freeze-dried blackcurrant berries

4.2.1. Identification and quantification of anthocyanins from freeze-dried blackcurrant berries

The anthocyanin profiles of blackcurrant samples are presented in Figure 9. Four major anthocyanins were identified in the analysed samples (delphinidin-3-*O*-glucoside; delphinidin-3-*O*-rutinoside; cyanidin-3-*O*-glucoside, and cyanidin-3-*O*-rutinoside) by comparing the retention time and spectra of unknown peaks with standards. In addition to delphinidin and cyanidin-based anthocyanins, additional peaks were detected in HPLC chromatograms, suggesting that blackcurrant samples have other anthocyanins. According to Dariusz *et al.* (83) and Bakowska-Barczak *et al.* (25), 3-*O*-glucoside and 3-*O*-rutinoside forms of delphinidin and cyanidin represent 93 – 99% of total anthocyanins, while the remaining 1 – 7% correspond to the 3-O-glucoside and 3-O-rutinoside forms of petunidin and peonidin.

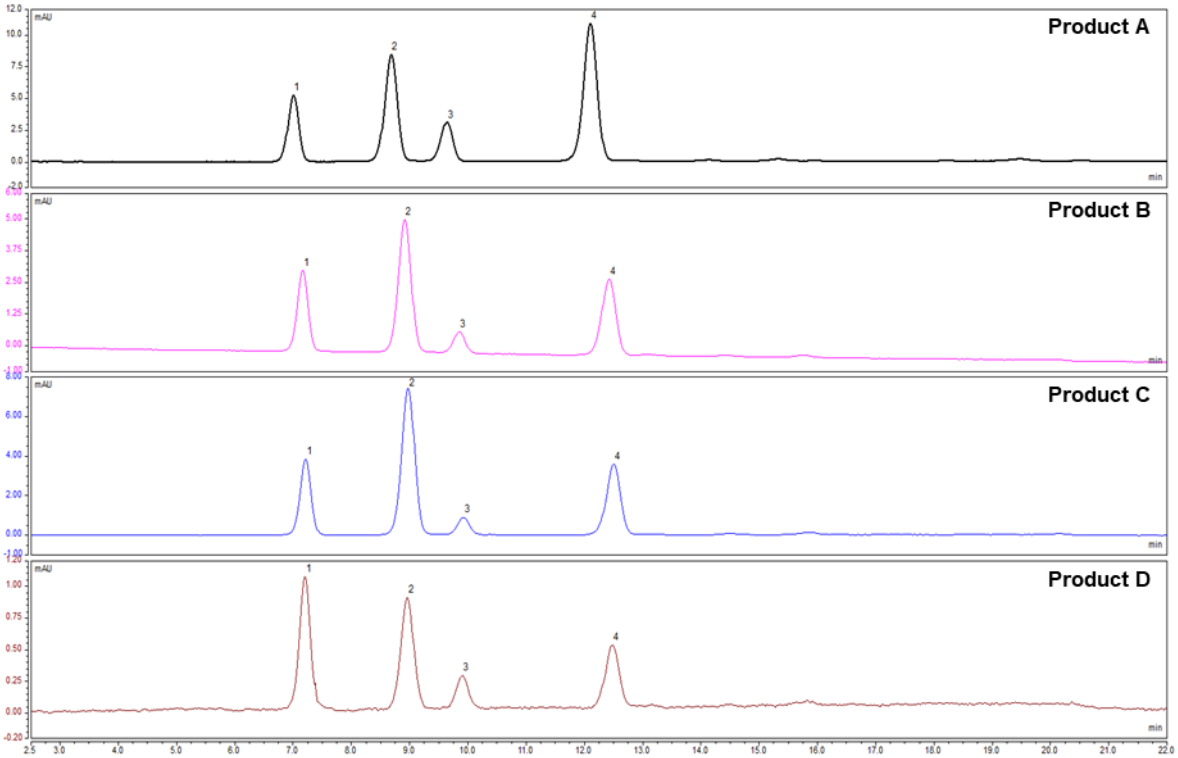


Figure 9: Chromatograms of the anthocyanins found in the 4 freeze-dried blackcurrant samples under study.

1 – Delphinidin-3-*O*-glucoside; **2** – Delphindin-3-*O*-rutinoside; **3** – Cyanidin-3-*O*-glucoside; **4** – Cyanidin-3-*O*-rutinoside.

The anthocyanin composition of *R. nigrum* berries is present in Table 17. The content of anthocyanins in blackcurrant samples ranged from 537.1 to 2380.6 mg/100 g DW. New Zealand blackcurrant berries (product A; total anthocyanins: ~2380.6 mg/100 g DW) had approximately 2 and 4 times more anthocyanins than Lithuanian (products B and C; total anthocyanins: ~1381.3 and 1105.8 mg/100 g DW) and Chilean (product D, total anthocyanins: 537.1 mg/100 g DW) samples. Product D presented significantly lower anthocyanin content ($p < 0.05$) than other blackcurrant berries.

Table 17: Content of anthocyanins in different freeze-dried blackcurrant samples.

	Product A	**Product B**	**Product C**	**Product D**	***p*-value**
D3G (mg/100 g DW)	327.4 ± 3.2^{a}	239.2 ± 10.0^{b}	206.7 ± 0.5^{c}	159.4 ± 3.5^{d}	<0.001
D3R (mg/100 g DW)	872.9 ± 10.6^{a}	764.3 ± 31.4^{b}	563.1 ± 6.0^{c}	225.6 ± 2.1^{d}	<0.001
C3G (mg/100 g DW)	228.8 ± 3.8^{a}	65.4 ± 2.8^{b}	65.1 ± 0.7^{b}	45.9 ± 0.0^{c}	<0.001
C3R (mg/100 g DW)	951.4 ± 9.5^{a}	312.4 ± 11.6^{b}	270.8 ± 0.5^{c}	106.1 ± 1.5^{d}	<0.001
Total ANT (mg/100 g DW)	2380.6 ± 27.2^{a}	1381.3 ± 55.8^{b}	1105.8 ± 7.7^{c}	537.1 ± 7.2^{d}	<0.001

Data expressed as mean ± standard deviation (n = 3). Different letters for each product in a row indicate statistically significant differences ($p < 0.05$) between blackcurrant samples. Results were analysed by one-way ANOVA, followed by Tukey post-hoc test for mean comparison. D3G: delphinidin-3-*O*-glucoside; D3R: delphinidin-3-*O*-rutinoside; C3G: cyanidin-3-*O*-glucoside; C3R: cyanidin-3-*O*-rutinoside; ANT: anthocyanin.

The obtained results are in line with the anthocyanin values reported by Mattila *et al.* (35) for different European blackcurrant varieties (1260 to 2878 mg/100 g DW). In this work, a two-fold difference was found between the varieties with the highest and the lowest anthocyanin content (35). Other authors have also reported higher anthocyanin contents in blackcurrants grown in New Zealand. Birgit *et al.* (184) concluded that anthocyanin content of blackcurrant juices from New Zealand cultivars were 1.5 times higher than that of non-New Zealand cultivars, which corroborates our findings. The different anthocyanin contents found among blackcurrant products may have result not only from genetic variation but also from different growing, harvesting, processing, and storage conditions. Rubinskiene *et al.* (78) found that blackcurrant anthocyanin content increased 3-7 times during ripening process. On the other hand, considering that anthocyanins are unstable to temperature, O_2, and light, some losses may have occurred during blackcurrant lyophilization and storage (25, 34, 113). In fact, compared to the values described in the literature, product D had

significant lower anthocyanin content, suggesting that some losses may occurred during fruit processing. Moreover, the brownish colour of these blackcurrant powder is another possible indicator of anthocyanin degradation into brown polymers (113, 185). Although products B and C were from the same country and the same brand, they presented significantly different anthocyanin levels (1381.3 and 1105.8 mg/100 g DW, respectively). This may be fundamentally due to two factors: the form of blackcurrant berries (whole or powdered) and the packaging material of each product. Product C was sold as a powder and packaged in a transparent bag, increasing exposure to light and O_2 and, consequently, contributing to the rapid degradation of anthocyanins.

The analysed blackcurrant products also had different anthocyanin profiles. In product A, delphinidin-based anthocyanins accounted for ~50% of total anthocyanins, while in products B, C and D, D3G and D3R accounted for more than 70%. D3R was the main anthocyanin found in products B, C, and D, ranging between 225.6 and 764.3 mg/100 g DW, which corresponds to approximately 42 and 55% of total anthocyanins content. In contrast, C3R accounted for ~40% of total anthocyanins of product A, being the major anthocyanin of these berries. In general, delphinidin- and cyanidin- 3-*O*-rutinoside corresponded to 62-78% of all anthocyanins. Similar anthocyanin profiles were found for different European blackcurrant varieties, where D3R was pointed as the main anthocyanin of these berries (35).

4.2.2. Total phenolic compounds and antioxidant activity of freeze-dried blackcurrant samples

Total phenolic compounds (TPC) of freeze-dried blackcurrants were quantified through the Folin–Ciocalteu assay and the results are shown in Figure 10. The sample with highest TPC content was product A (7399.9 mg GAE/100 g DW), followed by products B (5569.3 mg/100 g DW) > C (5362.2 mg GAE/100 g DW) > D (2150.1 mg GAE/100 g DW). Although product B contains significantly higher anthocyanin content than product C, both products have similar concentrations of phenolic compounds, suggesting that product C is richer in other polyphenols. As expected, product A presented significantly higher content of phenolic compounds ($p < 0.05$), while product D was the sample with the lowest values ($p < 0.05$). The results found for products A, B and C are higher than the ones found in the literature. The values reported by Vagiri *et al.* (71) and Orsavová *et al.* (24) ranged from 1920 to 3180 mg GAE/100 g DW and 1801 to 3077 mg GAE/100 DW, respectively .

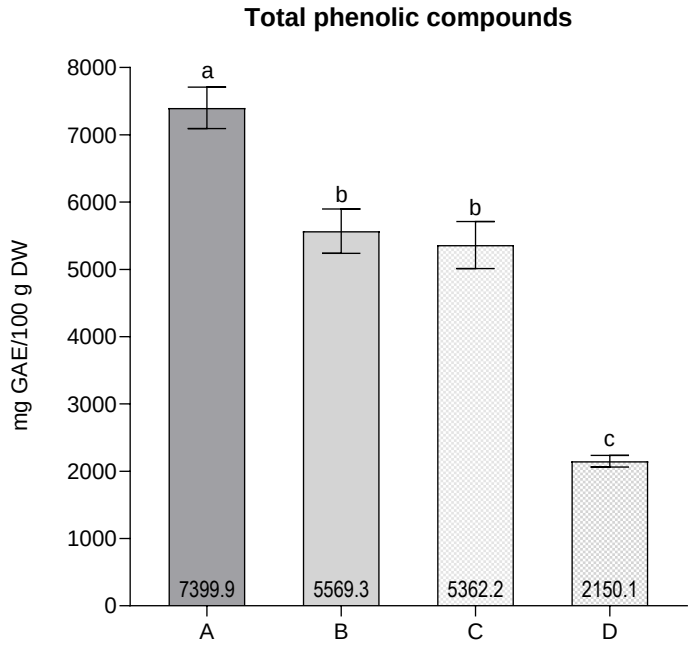


Figure 10: Content of total phenolic compounds in freeze-dried blackcurrant samples.

Results are expressed as mg of gallic acid equivalents (GAE) per 100 g of dried weight (DW). Different letters in each bar indicate statistically significant differences ($p < 0.05$) between blackcurrant samples. Data were analysed by one-way Welch ANOVA, followed by Tamhane's T2 test since homogeneity of variances was not confirmed. Values are expressed as mean ± standard deviation (n = 3).

Regarding the antioxidant activity of blackcurrant berries, the results from the DPPH• assay are shown in Figure 11. The results of antioxidant activity followed the same trend than the phenolic compounds, with product A (10193.3 mg TE/100 g DW) presenting the highest antioxidant activity, followed by products B (7023.8 mg TE/100 g DW) > C (4671.6 mg TE/100 g DW) > D (2426.0 mg TE/100 g DW). In contrast to the results obtained by the Folin–Ciocalteu assay, product B presented significantly higher antioxidant activity than product C ($p < 0.05$). Regarding products A and D, the antioxidant activity was in line with total phenolic contents. Therefore, the higher content of phenolic compounds in product A was translated into higher antioxidant activity. DPPH• inhibition values found for products A, B and C were higher than those described in the literature that ranged from 1553 to 1820 mg TE/100 g DW (24).

To understand the association of phenolic compounds with antioxidant activity, Pearson correlation analysis was carried out. A significant correlation ($p < 0.05$), with a Pearson r-value of 0.918, was found between phenolic content and DPPH• inhibition, confirming the strong contribution of phenolic compounds to the antioxidant activity of this fruit.

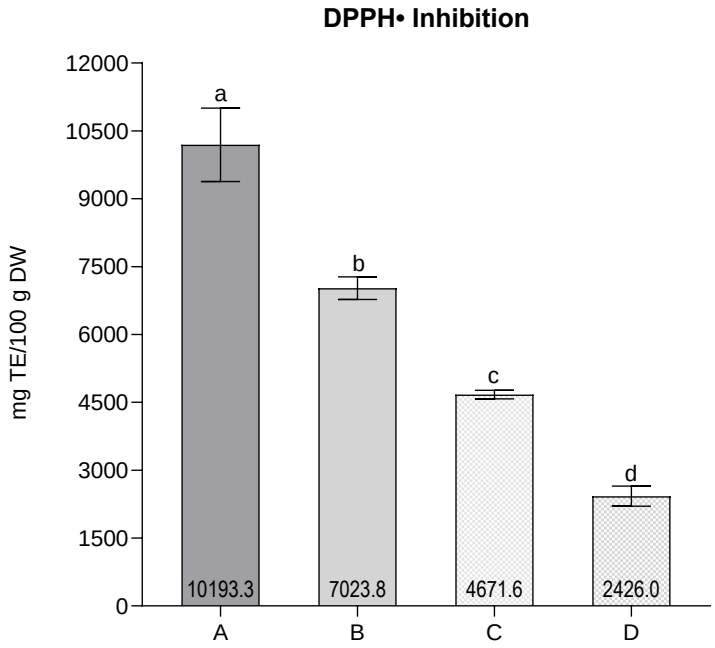


Figure 11: Antioxidant activity of freeze-dried blackcurrant samples assessed through the DPPH• assay.

Results are expressed as mg of trolox equivalents (TE) per 100 g of dried weight (DW). Different letters in each bar indicate statistically significant differences ($p < 0.05$) between blackcurrant samples. Data were analysed by one-way Welch ANOVA, followed by Tamhane's T2 test since homogeneity of variances was not confirmed. Values are expressed as mean ± standard deviation (n = 3).

4.3. Blackcurrant infusions

4.3.1. Optimization of blackcurrant infusion conditions

A three-level factorial Box–Behnken design, consisting of 17 experimental runs with five replicates of the centre point, was employed to optimize blackcurrant infusion conditions (X_1: temperature (°C); X_2: time (min); X_3: mass/volume ratio (g/200 mL). Total anthocyanins content as function of X_1, X_2 and X_3 was used as a response for the BBD. Compared to the original 3^3 design which generates 27 experiments, BBD is more economical, efficient, and environmentally friendly, as it requires less experimental runs and, consequently lower volumes of solvents and shorter analysis time. Regression analysis was performed to fit the response function, and final model was obtained for anthocyanin content. The obtained models presented a good fitness, with R^2 predicted and R^2 adjusted values ranging from 0.7563 to 0.9592 and 0.9439 to 0.9691, respectively. Adequate Precision, measured as the signal to noise ratio, ranged from 17.026 to 21.491, suggesting the adequacy of the generated models. The significance of the models was evaluated by ANOVA through

Fisher's F-test. As shown in Table S2, F values were 39.99, 30.89, 46.31 and 56.77 for infusions A, B, C, and D, respectively, which indicates that the obtained models are significant with only 0.01% of possibility that a large model F-value could occur due to noise. The independent variable X_3 and the quadratic term X_3^2 were the terms with a *p*-value lower than 0.05, indicating that the mass/volume ratio was the only term with a significant effect on total anthocyanins content. The lack of fit value was 0.8564, 0.9052 and 0.9247 for infusions A, B and D, which means that the models are valid for the present study.

Figure 12 shows the combined effect of the variables X_1 (temperature) and X_2 (time), while the variable X_3 (ratio) was fixed at central level (2.25 g/200 mL). Figure 13 shows the effect of mass/volume ratio (variable X_3) on total anthocyanin content of blackcurrant infusion, while the variables temperature and time were fixed at central levels (90 °C and 4.5 min, respectively). The contour plots for the combined effect of time and temperature showed that these two variables had no significant impact on total anthocyanin content of blackcurrant infusion. However, one-factor plots (Figure 13) shows that mass/volume ratio had a significant positive effect on total anthocyanin content of blackcurrant infusions. Therefore, to achieve the maximum content of anthocyanin in blackcurrant infusions, in the range of studied conditions (selected according with label recommendations of commercial fruit/herbal infusions and teas), a proportion of 4.0 g of blackcurrant powder per 200 mL of water should be used.

Although temperature and infusion time had no significant effect ($p > 0.05$) on anthocyanins content, the obtained models suggest a possible negative impact of these variables in some blackcurrant infusions. Increased temperatures and infusion times are required to induce the diffusion of anthocyanins from berry matrix to water. However, some studies have suggested that the use of extreme temperatures and longer infusion times may induce polyphenols degradation (106, 168). In contrast to our results, Salmerón-Ruiz *et al.* (168) found that both temperature and time had a significant effect on anthocyanin content of *H. sabdariffa* L. infusion. Nevertheless, in the mentioned study higher infusion times were tested (1–30 min), justifying the discrepancies found compared to our study (168). In fact, infusion times used by Salmerón-Ruiz *et al.* (168) were not representative of the recommended steeping times (2 – 10 min) for the most popular herbal infusions. Currently, consumers are looking for more convenient infusion options which require shorter steeping times (105). Rocha *et al.* (105) reported that Portuguese consumers take less than one minute to steep infusions. Therefore, blackcurrant infusions may represent a more convenient infusion for consumers since the extraction of their phenolic compounds, especially anthocyanins, and, consequently, their sensory properties are not compromised by shorter infusion times.

Figure 12: Contour plots for the combined effect of temperate (X_1) and time (X_2) on total anthocyanin contents of blackcurrant infusions.

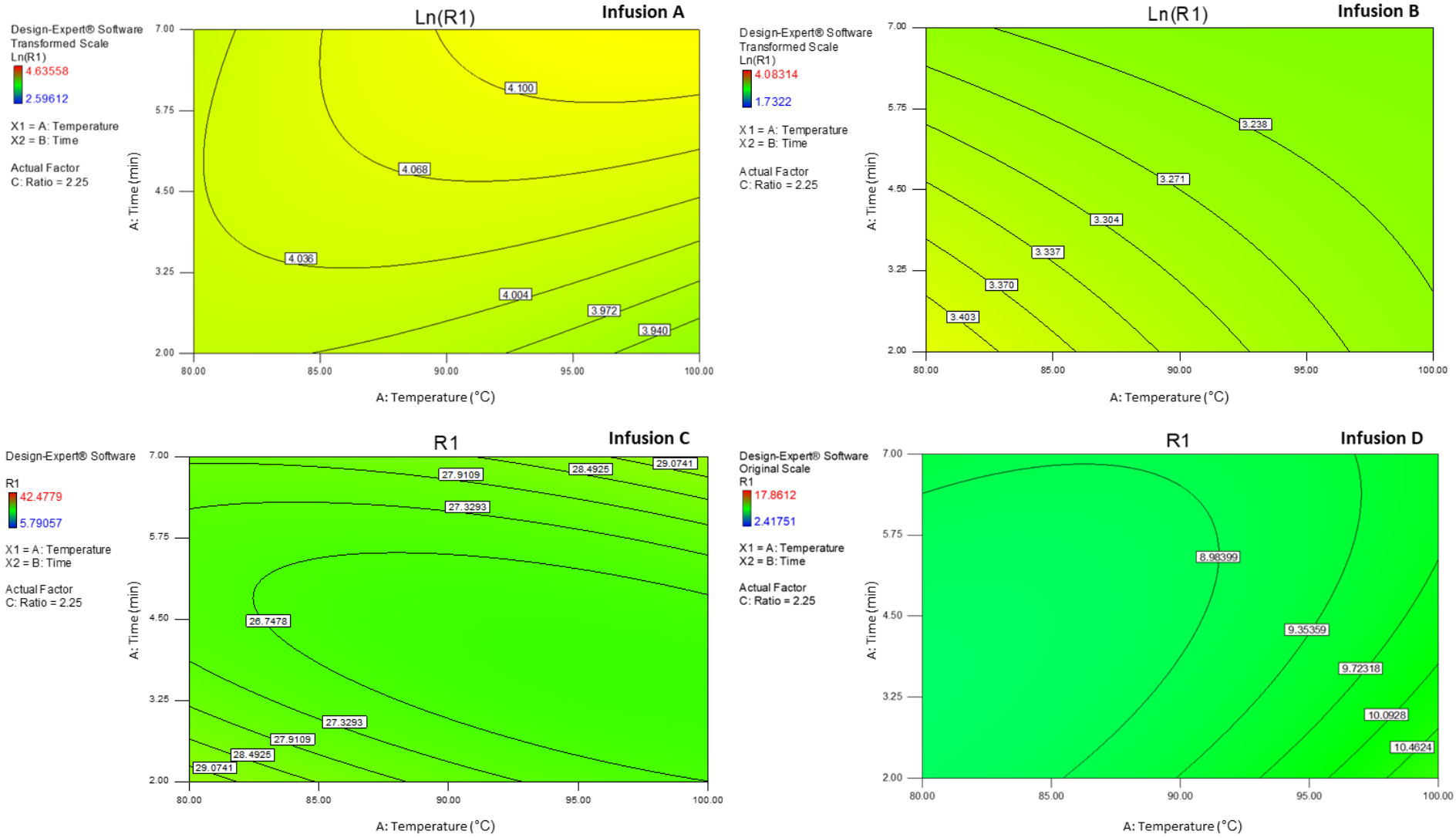
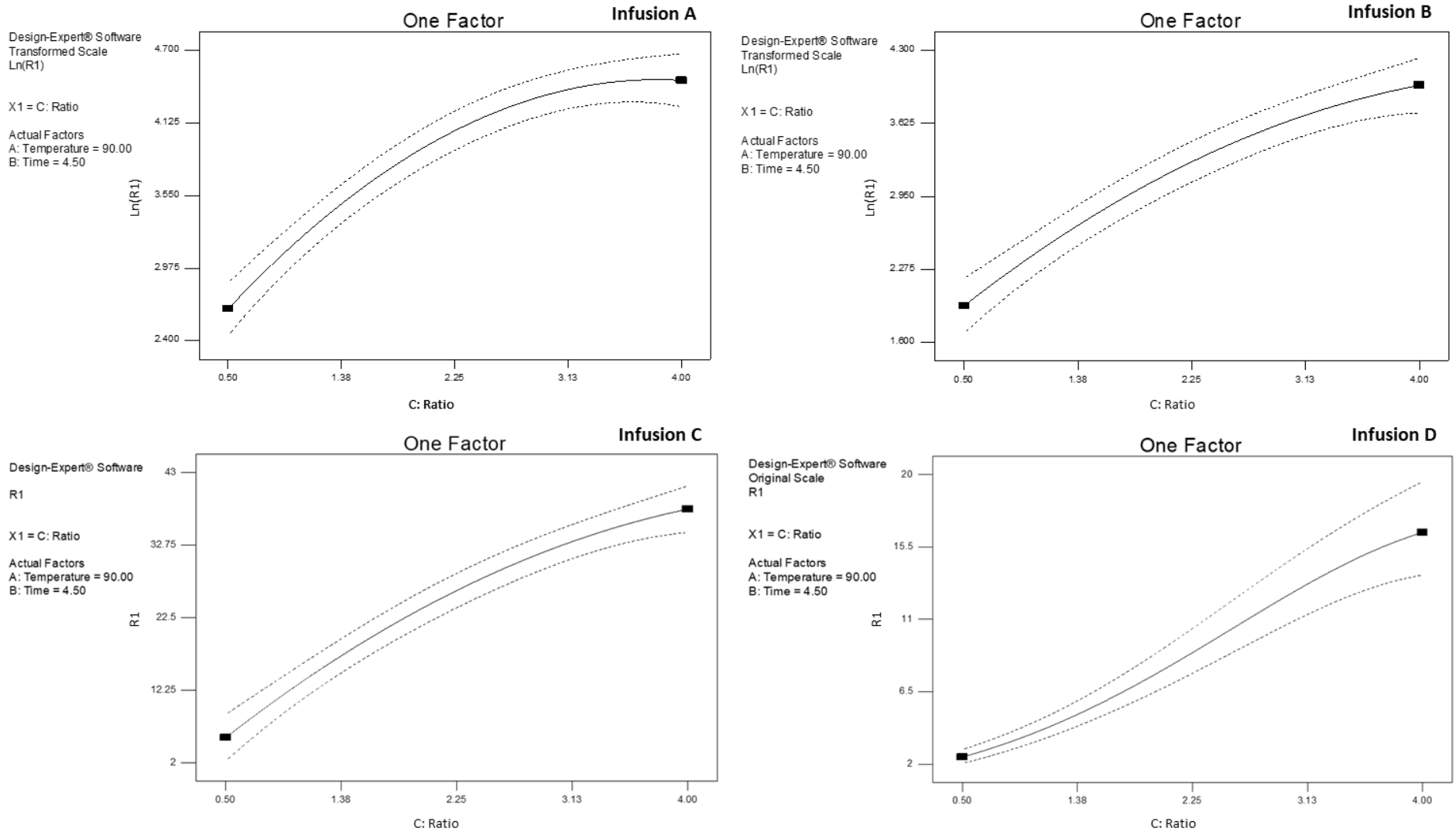


Figure 13: Effect of mass/volume ratio on total anthocyanin content of blackcurrant infusions.



4.3.2. Content of anthocyanins, total phenolic compounds, and antioxidant activity of blackcurrant infusions

Blackcurrant infusions were prepared according to the conditions defined in Box–Behnken design. Considering that mass/volume ratio was the only variable with a significant effect on total anthocyanin contents, results were grouped per ratio level: 0.5, 2.5 and 4.0 g/200 mL.

The anthocyanin content of blackcurrant infusions is presented in Table 18. According to the blackcurrant products used to prepare infusions, total anthocyanin content was considerably different. As expected, blackcurrant infusions prepared from New Zealand blackcurrants (A) presented higher content of anthocyanins (13.41 – 14.32 mg/200 mL, 42.47 – 65.13 mg/200 mL, and 68.59 – 103.09 mg/200 mL for a ratio of 0.5, 2.25 and 4.0 mg/200 mL, respectively) than the other infusions. Regarding anthocyanin profiles of blackcurrant infusions, they were similar to those found for each blackcurrant sample. In infusions B, C and D the main anthocyanin was D3R, while in infusion A was C3R. Although infusion B had lower concentration of total anthocyanins, it presented equivalent D3R contents to infusion A. At the highest ratio level (4 g/200 mL), anthocyanin content ranged from 69 - 103, 45 – 59, 34 – 42 and 16 – 18 mg/200 mL for infusion A, B, C, and D, respectively. Therefore, drinking one cup (200 mL) of blackcurrant infusions prepared with 4.0 g of products A and B provides at least 50.0 mg of anthocyanins (daily dose recommended by China), which is equivalent to consuming 14 - 21 g of fresh blackcurrants (134).

The obtained anthocyanin concentration values are relatively higher than the values described in literature for other berry-based infusions. Savikin *et al.* (109) studied the phenolic composition of dried chokeberry, bilberry and blackcurrant infusions and reported lower anthocyanin content in blackcurrant infusions (~8.94 mg/100 mL) prepared with 2 g of dried berries in 100 mL of boiled water. In contrast, our results showed that the anthocyanin content of infusions A, B and C prepared in similar conditions (4 g of berries per 200 mL, i.e., 2 g of berries per 100 mL) ranged from 17.20 mg to 51.55 mg/100 mL. Only anthocyanin contents of infusion D (7.7 – 8.93 mg/100 mL) were equivalent to those reported by Savikin *et al.* (109). These results can be explained by the lower anthocyanin content of dried blackcurrant samples studied by Savikin *et al.* (109) (< 400 mg/100 g DW). Bratu *et al.* (186) described similar anthocyanin contents (9.07 – 11.94 mg C3G equivalents/100 mL infusion) in three commercial berry fruit teas which according to label contained between 46 and 60% of berry fruits.

Table 18: Anthocyanin content (mg/200 mL) of blackcurrant infusions prepared following the conditions resulting from the Box–Behnken design.

Infusion	Ratio (g/200 mL)	D3G (mg/200 mL)	D3R (mg/200 mL)	C3G (mg/200 mL)	C3R (mg/200 mL)	Total ANT (mg/200 mL)	Amount of fresh blackcurrant with equivalent anthocyanin content (g)[#]
A	0.5	1.73 - 1.94	5.08 - 5.36	1.19 - 1.33	5.41 - 5.69	13.41 - 14.32	2.7 - 2.9
	2.25	5.60 - 8.66	15.96 - 24.50	3.75 - 5.81	17.16 - 26.16	42.47 - 65.13	8.6 - 13.2
	4	9.07 - 13.60	25.83 - 38.88	6.03 - 9.08	27.66 - 41.53	68.59 - 103.09	13.9 - 20.9
B	0.5	0.94 - 1.31	3.12 - 4.33	0.29 - 0.37	1.32 - 1.73	5.65 - 7.74	2.0 - 2.7
	2.25	3.30 - 5.36	10.78 - 17.68	0.88 - 1.48	4.42 - 7.22	19.38 - 31.73	6.8 - 11.1
	4	7.59 - 10.05	24.88 - 33.03	2.09 - 2.76	10.09 - 13.50	44.65 - 59.33	15.6 - 20.8
C	0.5	1.05 - 1.31	2.94 - 3.67	0.37 - 0.43	1.44 - 1.81	5.79 - 7.21	2.5 - 3.1
	2.25	4.53 - 5.45	12.77 - 15.23	1.45 - 1.85	6.26 - 7.86	25.01 - 30.32	10.9 - 13.2
	4	6.25 - 7.68	17.45 - 21-64	2.03 - 2.50	8.65 - 10.66	34.39 - 42.48	15.0 - 18.6
D	0.5	0.67 - 0.73	1.09 - 1.15	0.18 - 0.21	0.48 - 0.52	2.42 - 2.58	2.2 - 2.3
	2.25	1.97 - 3.22	3.25 - 4.92	0.54 - 0.87	1.45 - 2.17	7.21 - 11.18	6.5 - 10.1
	4	4.02 - 4.91	7.26 - 8.05	1.10 - 1.36	3.16 - 3.55	15.54 - 17.86	14.0 - 16.1

Blackcurrant infusions were prepared at three different ratios (0.5; 2.25 or 4 g/200 mL), at 80, 90 or 100 °C for 2, 4.5 or 7 minutes. [#]The amount of fresh blackcurrant berries with an anthocyanin content equivalent to the infusions was estimated based on the anthocyanin content per 100 g of dried blackcurrants, assuming 79.3% of moisture, with no anthocyanin losses during processing and storage. D3G: delphinidin-3-*O*-glucoside; D3R: delphinidin-3-*O*-rutinoside; C3G: cyanidin-3-*O*-glucoside; C3R: cyanidin-3-*O*-rutinoside; ANT: anthocyanin.

Table 19 presents the anthocyanin extraction efficiency (%) according to the mass/volume ratio used in the preparation of blackcurrant infusions. In general, the extraction efficiency ranged from 70 to 120% in all ratios studied, which is in line with the limit values established by ICH guidelines, demonstrating the potential of the selected conditions to extract anthocyanins from freeze-dried blackcurrants and the stability of these polyphenols during the preparation of the infusions. Altogether, these results highlight the potential of blackcurrant infusion as an important source of anthocyanins, namely D3R.

Table 19: Anthocyanin extraction efficiency (%) according to the mass/volume ratio used during the preparation of infusions.

Infusion	Ratio (mg/200 mL)	% D3G extracted	% D3R extracted	% C3G extracted	% C3R extracted	% Total ANT extracted
A	0.5	101.8 - 117.3	112.2 - 121.8	100.7 - 115.5	109.7 - 118.7	108.7 - 119.3
	2.25	76.0 - 117.6	81.2 - 124.7	72.9 - 113.0	80.1 - 122.2	79.3 - 121.6
	4	69.4 - 104.0	74.1 - 111.4	66.0 - 99.3	72.8 - 109.2	72.2 - 108.3
B	0.5	78.0 - 108.3	81.2 - 111.6	87.2 - 111.3	83.9 - 110.4	81.6 - 110.7
	2.25	61.2 - 99.5	62.6 - 102.7	59.7 - 100.2	62.9 - 102.5	62.3 - 102.0
	4	79.4 - 105.2	81.5 - 108.2	80.0 - 105.5	80.8 - 108.2	80.9 - 107.6
C	0.5	104.3 - 128.7	100.7 - 125.3	103.9 - 128.5	113.5 - 129.6	105.7 - 131.9
	2.25	97.4 - 117.1	100.8 - 120.1	98.8 - 126.4	103.5 - 129.0	100.6 - 121.8
	4	75.3 - 93.0	77.2 - 96.1	77.8 - 95.9	79.6 - 98.5	77.5 - 96.1
D	0.5	84.0 - 90.5	96.3 - 101.4	80.0 - 92.1	88.7 - 97.5	90.0 - 95.4
	2.25	54.8 - 89.6	64.0 - 96.8	52.7 - 84.3	60.7 - 91.0	59.7 - 92.4
	4	63.1 - 77.0	80.6 - 89.2	59.8 - 74.0	74.5 - 83.5	72.4 - 83.1

Blackcurrant infusions were prepared at three different ratios (0.5; 2.25 or 4 g/200 mL), at 80, 90 or 100 °C for 2, 4.5 or 7 minutes. D3G: delphinidin-3-*O*-glucoside; D3R: delphinidin-3-*O*-rutinoside; C3G: cyanidin-3-*O*-glucoside; C3R: cyanidin-3-*O*-rutinoside; ANT: anthocyanin.

The contents of total phenolic compounds and antioxidant activity of blackcurrant infusions are presented in Table 20. Total phenolic compounds and antioxidant activity followed the same trend observed for total anthocyanins content. Infusion A had the highest phenolic content and antioxidant activity ranging from 22.4 – 185.2 mg GAE/200 mL and 40.9 – 312.6 mg TE/200 mL, respectively, followed by infusions B > C > D. At the highest ratio level (4 g/200 mL), phenolic contents and antioxidant activity of blackcurrant infusions ranged from 34.8 to 185.2 mg GAE/200 mL and 63.7 to 312.6 mg TE/200 mL, respectively. Total phenolic results are in line with those described in the literature (109, 187). The level of phenolic compounds in the blackcurrant infusions studied by Savikin *et al.* (109) was

~60.07 mg GAE/100 mL, which is equivalent to the values found in our samples (54.35 – 64.85 and 59.25 – 63.95 mg GAE/100 mL for infusions B and C at ratio 4 g/200 mL, respectively). Regarding antioxidant activity, the content of commercial infusions was ~120.4 mg TE/100 mL, which is comparable to the scavenging capacity of blackcurrant infusions (117.5 – 137.9 and 126.0 – 134.8 mg TE/100 mL for infusions B and C at ratio 4 g/200 mL, respectively) (187). As observed in freeze-dried berries, significant correlation ($p < 0.05$), with a Pearson r-value of 0.974, was found between phenolic content and antioxidant activity of blackcurrant infusions.

Table 20: Content of total phenolic compounds and antioxidant activity of blackcurrant infusions prepared following the conditions resulting from the Box–Behnken design.

Infusion	Ratio (g/200 ml)	Total phenolic compounds (mg GAE/200 mL)	DPPH• inhibition (mg TE/200 mL)
A	0.5	22.4 - 24.9	40.9 - 50.9
	2.25	79.8 - 100.6	175.2 - 215.8
	4	169.6 - 185.2	269.8 - 312.6
B	0.5	16.4 - 17.9	30.8 - 38.0
	2.25	58.4 - 73.5	115.8 - 139.6
	4	108.7 - 129.7	234.9 - 275.8
C	0.5	13.2 - 15.4	26.0 - 34.7
	2.25	63.2 - 72.6	112.4 - 135.0
	4	118.5 - 127.9	252.0 - 269.5
D	0.5	5.3 - 6.2	9.9 - 12.7
	2.25	18.7 - 25.2	38.9 - 49.6
	4	34.8 - 41.5	63.7 - 85.1

Blackcurrant infusions were prepared at three different ratios (0.5; 2.25 or 4 g/200 mL), at 80, 90 or 100 °C for 2, 4.5 or 7 minutes. The content of total phenolic compounds was estimated by the Folin–Ciocalteu assay and expressed as mg of gallic acid equivalents (GAE) per 200 mL of infusion. The antioxidant activity of blackcurrant infusions was assessed through DPPH• assay and expressed as mg of Trolox equivalents (TE) per 200 mL of infusion.

4.4. Combined effect of delphinidin-3-*O*-glucoside and 3-*O*-rutinoside with docetaxel on MDA-MB-231 cell proliferation

Docetaxel is one of the most common and effective chemotherapeutics used in TNBC treatment, however, some TNBC cells, namely MDA-MB-231, have developed resistance to this drug. (164, 166, 167). Thus, new combined therapies have been developed to improve docetaxel efficacy, overcome chemotherapy resistance, and reduce

its associated adverse effects (164, 165). Given the higher anti-proliferative potential of delphinidin-based anthocyanins on TNBC cells, the cytotoxicity of D3G and D3R, two main anthocyanins found in blackcurrants, was assessed isolated and in combination with docetaxel on MDA-MB-231 cells.

4.4.1. Isolated effect of the main delphinidin-based anthocyanins found in blackcurrants and docetaxel on MDA-MB-231 cell proliferation

The isolated effect of D3G, D3R and DT on MDA-MB-231 cell proliferation after 72 h of incubation was assessed by the MTT assay, which evaluates mitochondrial activity of viable cells. Figure 14 presents the individual effects of anthocyanins and docetaxel on cell proliferation. Both anthocyanins induced a dose-effect response on MDA-MB-231 cell proliferation. However, TNBC cells were more sensitive to D3G than to D3R, resulting in different dose-response curves and, consequently, different IC_{50} values for each anthocyanin. The cytotoxic effect observed for D3G and D3R agreed with the results described in the literature. Viegas *et al.* (149) reported that delphinidin and D3G, at 200 µM, inhibited ~50% and ~70% of MDA-MB-231 cell proliferation, respectively. Although different works have focused on the study of the inhibitory effect of anthocyanins on the proliferation of TNBC cells, this is the first study reporting anti-proliferative effects of D3R. Regarding DT, no effects were observed at lowest doses (0.01 – 0.1 nM), while at higher concentration levels cell proliferation dramatically decreased.

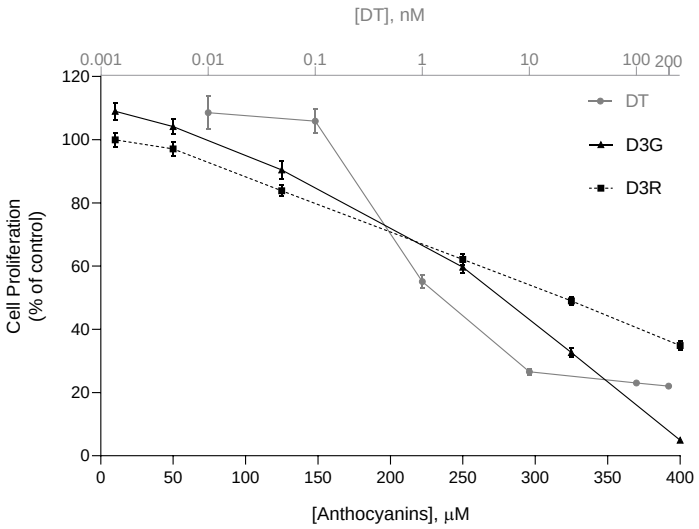


Figure 14: Isolated effect of docetaxel (DT; 0.01 – 200 nM), delphinidin 3-O-glucoside (D3G; 10 – 400 µM) and delphinidin-3-O-rutinoside (D3R; 10 – 400 µM) on MDA-MB-231 cell proliferation after 72 h exposure. The results are expressed as a percentage of control mean ± SEM.

Compusyn software was used to estimate the half maximal inhibitory concentration (IC_{50}) of each compound. Table 21 presents the parameters (r, m, Dm) of the dose-effect response curves of individual anthocyanins and docetaxel. For DT, the IC_{25}, IC_{50} and IC_{75} were 2.3, 10.7 and 50.6 nM, respectively. Carvalho *et al*. reported a similar IC_{50} value (10.0 nM) for DT on MDA-MB-231 cells, using SRB (sulforhodamine B) proliferation assay. Regarding the isolated cytotoxic effect of both anthocyanins, D3G was the anthocyanin with highest anti-proliferative effect, presenting an IC_{50} of 253.3 µM. MDA-MB-231 cells were slightly less sensitive to D3R, therefore, this anthocyanin had higher IC_{50} value (341.3 µM).

Table 21: Isolated and combined effect parameters calculated based on Chou-Talalay method for combination assays by Compusyn software (179).

Isolated								
	r	**m**	**IC_{25}**		**IC_{50} (D_m)**		**IC_{75}**	
D3G (µM)	0.91	2.66	167.5		253.3		382.9	
D3R (µM)	0.84	1.68	177.8		341.3		655.3	
DT (nM)	0.85	0.71	2.3		10.7		50.6	
Combination								
	r	**m**	**IC_{25}**	**CI value**	**IC_{50} (D_m)**	**CI value**	**IC_{75}**	**CI value**
D3G+DT (µM/nM)	0.97	0.78	20.9+0.9	0.52 +++	85.9+3.7	0.68 +++	3523.0+15.1	1.22 – –
D3R+DT (µM/nM)	0.96	1.88	83.8+2.6	1.63 – – –	150.6+4.7	0.88 +	270.5+8.5	0.58 +++
D3G+D3R+DT (µM/µM/nM)	0.98	1.34	12.8+17.8+0.6	0.42 +++	29.0+40.3+1.3	0.35 +++	65.7+91.3+2.9	0.37 +++

D3G: delphinidin-3-*O*-glucoside; D3R: delphinidin-3-*O*-rutinoside; DT: docetaxel; CI: combination index. Symbol description of synergism: Synergism (+++); Slight synergism (+); Moderate antagonism (– –); Antagonism (– – –) (179). r values represent linear correlation coefficients; and m indicates the shape of the dose-effect curve (m = 1, hyperbolic; m > 1 sigmoidal; m < 1 flat sigmoidal).

4.4.2. Combined effect of docetaxel and anthocyanins on MDA-MB-231 cell proliferation

Considering that both D3G and D3R showed a dose-effect response, combined assays were performed using both anthocyanins separately and together, to infer their combined effects with DT on cell proliferation. Therefore, MDA-MB-231 cells were exposed to five concentration levels, based on the constant combination ratio design proposed by Chou-Talalay method (179). The combination ratios were calculated based on the equipotent IC_{50} values obtained from the individual exposure experiments, to assure that each compound contributed similarly to the combination. The final concentrations of D3G,

D3R and DT used for the combination assays are shown in Table 22 and ranged from 2 to 300 µM, 3 to 442 µM, and 0.09 to 12.84 nM, respectively. The concentration ratios were kept equal to 23:1, 32:1 and 23:32:1 for all D3G-DT, D3R-DT and D3G-D3R-DT combinations.

Table 22: Concentrations of each compound in mixtures and respective concentration ratios used for the combination assays on MDA-MB-231 cells.

Concentrations used for combination ratios									
D3G (µM)	DT (nM)	$IC50_{D3G}/IC50_{DT}$	D3R (µM)	DT (nM)	$IC50_{D3R}/IC50_{DT}$	D3G (µM)	D3R (µM)	DT (nM)	$IC50_{D3G}$/ $IC50_{D3R}/IC50_{DT}$
300	12.84	23:1	680	21.40	32:1	325	442	13.9	23:32:1
250	10.70	23:1	340	10.70	32:1	250	340	10.7	23:32:1
50	2.14	23:1	68	2.14	32:1	50	68	2.14	23:32:1
10	0.43	23:1	14	0.43	32:1	10	14	0.43	23:32:1
2	0.09	23:1	3	0.09	32:1	2	3	0.09	23:32:1

D3G: delphinidin-3-*O*-glucoside; D3R: delphinidin-3-*O*-rutinoside; DT: docetaxel.

The effect of D3G-DT, D3R-DT and D3G-D3R-DT combinations on MDA-MB-231 cell proliferation is presented in Figure 15. All three combinations presented a dose-effect response on TNBC cell line, decreasing cell proliferation at higher concentration ratios. D3G-D3R-DT combination was the most effective, achieving the same effect at lower doses than D3G-DR and D3R-DT combinations. However, at the highest concentration level, both D3R-DT and D3G-D3R-DT combinations inhibited 96% of cell proliferation. Although D3G-DT was more effective at lower concentrations, this combination only inhibited 69% of cell viability at highest concentration level.

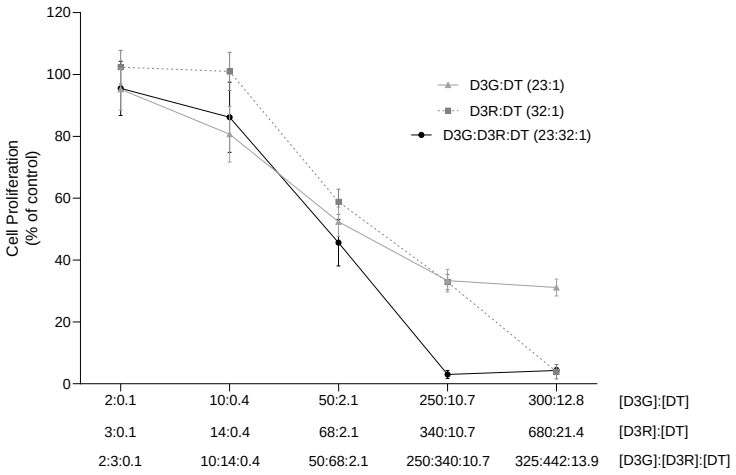


Figure 15: Combined effect of docetaxel (DT), delphinidin 3-*O*-glucoside (D3G) and delphinidin-3-*O*-rutinoside (D3R) on MDA-MB-231 cell proliferation after 72 h exposure. The results are expressed as a percentage of control mean ± SEM.

CI-plots (effect-oriented graphs) and isobolograms (dose-oriented graphs) were designed for each combination, based on CI values, and are shown in Figure 16. Combination indexes quantify the combined effect of docetaxel with both anthocyanins: synergistic if CI < 0.9, additive if 0.9 < CI < 1.1 and antagonistic If CI > 1.1.

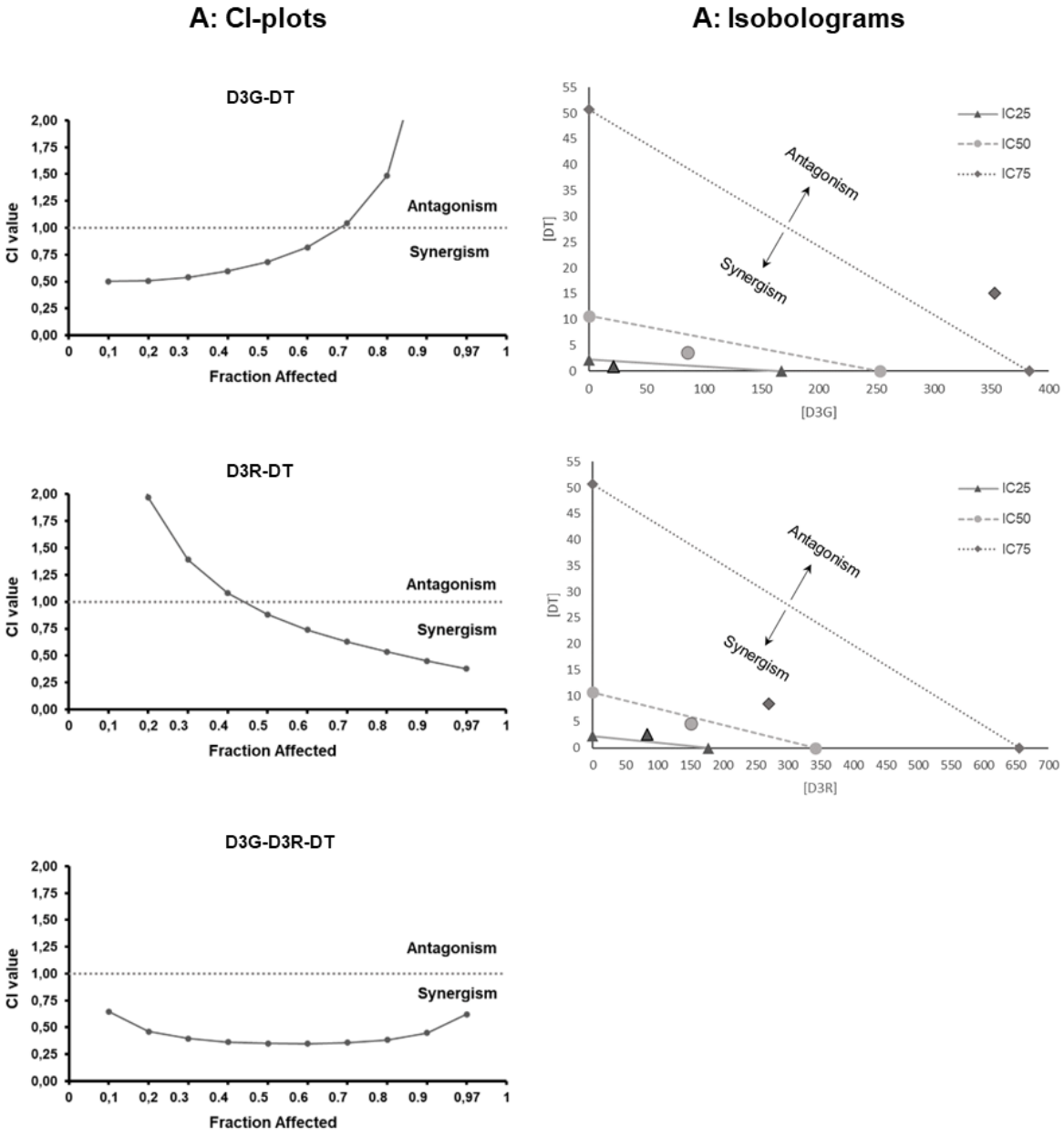


Figure 16: D3G-DT, D3R-DT, and D3G-D3R-DT combination plots after 72 h exposure on MDA-MB-231 cells: (A) combination index plot (CI-Plot). CI < 1, CI = 1, and CI > 1 indicate synergistic, additive, and antagonistic effects, respectively; (B) Isobologram illustrating the combined toxicity at IC_{25}, IC_{50} and IC_{75}. All these results were calculated using the Compusyn software.

D3G: delphinidin-3-*O*-glucoside; D3R: delphinidin-3-*O*-rutinoside; DT: docetaxel.

D3G-DT combination showed a synergistic effect (CI < 1) when fraction affected was lower than 70%, while for higher inhibition levels, this combination resulted in an antagonistic effect (CI > 1). In contrast, D3R-DT combination showed the opposite effect: at lower inhibition level (< 50%), D3R-DT had an antagonistic effect, but at higher inhibition levels, the response was reversed, and this combination presented a synergistic effect. On the other hand, the simultaneous combination of docetaxel with both anthocyanins (D3G-D3R-DT) resulted in a synergistic effect (CI < 1) regardless of the percentage of fraction affected. Liu *et al.* (188) demonstrated that the combination of C3G with trastuzumab potentiated the cytotoxic effect of this drug on different HER2-positive breast cancer cell lines (CI < 1). In the present study, the combined effect of docetaxel with C3G was not assessed since previous studies have shown that C3G do not induce a dose-effect response on MDA-MB-231 cell proliferation (149, 162). It was also previously reported that docetaxel combined with procyanidin B2 and catechin resulted in a synergistic effect on MDA-MB-231 cells, confirming the potential of these polyphenols as co-adjuvants of TNBC chemotherapy (189). To the best of our knowledge, this is the first study reporting the combined effect of DT with D3G and D3R on MDA-MB-231 cells.

Figure 15B presents the isobolograms of D3G-DT and D3R-DT combinations at three inhibition concentrations: IC_{25}, IC_{50} and IC_{75}. Isobologram is a graphical representation of equipotent combinations of different doses of two drugs. Considering that isobologram is a two-dimension graph, it is not for three-drug combinations. Therefore, no isobologram was constructed for D3G-D3R-DT combination. In isobologram graphs, the X-axis represents the doses of D3G/D3R, and Y-axis represents the doses of DT. For each inhibition concentration (IC) the diagonal line connects the IC of isolated D3G/D3R and DT, while the dot represents the IC of the combination (D3G-DT and D3R-DT). The position of dots in the isobologram indicates the type of interaction between D3G/D3R and DT: if the dot is positioned below the line, the combination is synergistic, while if the dot is positioned above the line, the combination is antagonistic. The distance of the dot to the line indicates the strength of the combined effect.

Through the analysis of D3G-DT isobologram, it can be inferred that this combination had synergistic effects at IC_{25} (CI = 0.52) and IC_{50} (CI = 0.68), whereas at IC_{75} the observed effect is moderately antagonistic (CI = 1.22). On the other hand, CI values of D3R-DT combination were 1.63 0.88 and 0.58 for IC_{25}, IC_{50} and IC_{75}, suggesting an antagonistic, a slightly synergistic and a synergistic effect, respectively. In the case of D3G-D3R-DT combination, CI values were always below 0.7, suggesting that this combination is synergic at all inhibition levels.

Dose reduction indexes were calculated for those combinations with synergistic effects and the results are shown in Table 23. DRI indicates how many folds the concentration of each compound in a synergistic combination may be reduced to achieve the same response compared to the concentrations of each individual compound. Thus, greater DRI values indicate a greater dose reduction for a given inhibitory effect.

Table 23: Dose reduction index values (DRI) at inhibition concentrations of 25, 50 and 75% for D3G-DT, D3R-DT and D3G-D3R-DT combination in MDA-MB-231 cells.

	DRI		
	IC_{25}	IC_{50}	IC_{75}
D3G	8.02	2.95	-
DT	2.53	2.91	-
D3R	-	2.27	2.42
DT	-	2.27	5.99
D3G	13.11	8.74	5.83
D3R	10.00	8.47	7.17
DT	4.07	8.50	17.73

D3G: delphinidin-3-*O*-glucoside; D3R: delphinidin-3-*O*-rutinoside; DT: docetaxel.

DRI values for DT ranged from 2.53 to 2.92, 2.27 to 5.99 and 4.07 to 17.73 for DG3-DT, D3R-DT and D3G-D3R-DT combinations. At IC_{50}, D3G-DT, D3R-DT and D3G-D3R-DT combinations require 2.9, 2.3 and 8.5-fold less DT to achieve the same 50% inhibition of this isolated compound. D3G-D3R-DT combination is able to reduce 75% of cell proliferation with a 17.7-fold lower DT concentration. For example, the estimated IC_{75} for docetaxel isolated is 50.6 nM, while for D3G-D3R-DT combination is 2.9 nM.

4.4.3. Combined effect of docetaxel and anthocyanins on microtubule dynamics

A previous study (149) showed that the anti-proliferative effect of anthocyanins on MDA-MB-231 cells was associated with their effects on microtubule dynamics. In the presence of delphinidin-based anthocyanins (200 μM), microtubules became disordered, tangled, and formed complexes, suggesting that anthocyanins may inhibit cell proliferation through similar mechanisms to those reported for taxanes In order to evaluate if the synergistic effect of D3G-D3R-DT combination occurred due to impaired microtubule dynamics, MDA-MB-231 cells were immunostained with species specific antibody for α-

tubulin, after 24 h exposure to D3G-D3R-DT at IC_{50} level, and α-tubulin was quantified. Table 24 presents α-tubulin quantification results expressed as percentage of tubulin-positive cells. As expected, 81.2 % of MDA-MB-231 cells treated with DT (10.7 nM) were tubulin-positive, confirming the ability of DT to inhibit microtubule depolymerization and, consequently, induce cell cycle arrest. When cells were treated with D3G-D3R-DT combination, the percentage of tubulin-positive cells (37.8 ± 5.4%) was approximately half the percentage observed for DT. However, the concentration of DT in the combination was 8.2-fold lower than the concentration tested for docetaxel isolated, suggesting that D3G and D3R potentiated DT anti-proliferative effect by compromising microtubule depolymerization. Nevertheless, other mechanisms could also be responsible for the synergistic effect of D3G-D3R-DT combination, therefore further studies are needed to fully understand the combined effect of docetaxel and anthocyanins on MDA-MB-231 cells.

Table 24: Percentage of tubulin-positive cells after 24 h exposure to docetaxel (DT) and D3G-D3R-DT combination at IC_{50} level.

Treatment	Concentration			% tubulin-positive cells	*p*-value
	D3G (µm)	D3R (µm)	DT (nM)		
Control	-	-	-	18.7 ± 2.4	-
DT	-	-	10.7	81.2 ± 3.4	<0.0001
D3G+D3R+DT	29.0	40.2	1.3	37.8 ± 5.4	0.0005

MDA-MB-231 cells were treated with DT and D3G-D3R-DT at IC_{50} level for 24 h and immunostained with species specific antibody for α-tubulin. Mean fluorescence intensity was quantified per cell and results were expressed as percentage of tubulin-positive cells (percentage of cells with fluorescence intensity > mean fluorescence intensity + 2SD). Results are expressed as mean ± SEM. One-way ANOVA followed by Dunnett's multiple comparisons test against controls was performed. A *p*-value < 0.05 was considered statistically significant. D3G: delphinidin-3-*O*-glucoside; D3R: delphinidin-3-*O*-rutinoside; DT: docetaxel.

4.4.4. Impact of docetaxel and anthocyanins on healthy breast cells proliferation

To evaluate the selective cytotoxicity of docetaxel-anthocyanin combinations for TNBC cells, MCF-12A non-tumour cells were exposed for 72 h to IC_{50} values estimated for MDA-MB-231 cells. Cytotoxic effect of DT on healthy breast cells was inhibited when combined with both anthocyanins (Figure 17). At 10.7 nM, DT inhibited 60% of MCF-12A cell proliferation, while D3G (253.3 µM) D3R (341.3 µM) had no effect on cell proliferation (94.4 ± 2.7 and 83.8 ± 2.7% of cell growth, respectively). The use of lower DT concentrations

in D3G-DT, D3R-DT and D3G-D3R-DT combinations had no effects on MCF-12A cell growth (98.3 ± 2.6; 89.5 ± 2.9 and 99.2 ± 2.2% of cell growth, respectively), suggesting that these combinations have selective cytotoxicity for TNBC cells. This selective effect was also described by Ozbay and Nahta (146), where the exposure to delphinidin at 165 µM for 6 days inhibited ~50% of MDA-MB-231 cell proliferation with no effects on the healthy cell line (MCF-10A). Viegas *et al.* (149) also reported that D3G at 200 µM reduced ~50% of MDA-MB-231 cell growth, with considerably lower inhibition of MCF-12A cell proliferation (69.2 ± 2.3%).

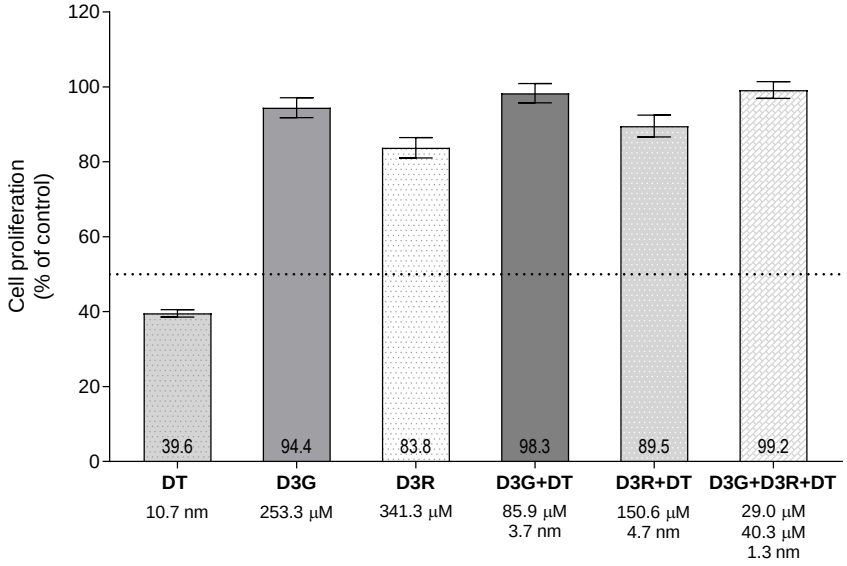


Figure 17: Cell proliferation of non-cancerous MCF-12A cell line upon 72 h exposure to docetaxel (DT), delphinidin-3-*O*-glucoside (D3G) and delphinidin-3-*O*-rutinoside (D3R), isolated or in combination, at IC_{50} values estimated for MDA-MB-231 cell line (the results are expressed as a percentage of control mean ± SEM). The dashed line indicates the expected effect of the tested doses on MDA-MB-231 cell proliferation.

Since no specific targeted therapy is available for TNBC, chemotherapy remains the mainstay of treatment. This systemic therapy is not specific for cancer cells, presenting multiple adverse effects. Therefore, new effective treatments are needed that are capable to inhibit cancer cell proliferation with minimal toxicity to healthy cells. According to our results, D3G-D3R-DT combination could represent a potential therapeutic strategy for the treatment of TNBC, as it allows the reduction of docetaxel doses, maintaining therapeutic efficacy and minimizing its adverse effects. In this study, intact compounds were exposed directly to cells, without considering the bioavailability of anthocyanins. As previously referred, the bioactivity of anthocyanins depends on their digestion, absorption, metabolism, and biological activity of the resulting metabolites. Therefore, future studies, should evaluate

the isolated and combined effect of D3G and D3R with DT on MDA-MB-231 cell growth after performing *in vitro* digestion and gastric and intestinal transport. Furthermore, since blackcurrant berries and infusions present D3G and D3R in similar proportions to those found in combinations assays, the combined effect of these digested products with docetaxel should be assessed to evaluate their potential adjuvant role in the treatment of TNBC.

Regarding the bioavailability of the studied anthocyanins, some works have reported higher urinary excretion levels (absolute bioavailability) for D3R than for D3G, suggesting that the rutinoside form of delphinidin is more bioavailable than the glucoside (190, 191). The higher bioavailability of D3R has been associated with its lower susceptibility to enzymatic deglycosylation and higher stability towards pH changes. Moreover, since human metabolism lacks rhamnoside enzymes, D3R deglycosilation mainly occurs in colon due to the presence of bacterial rhamnosides (192). Some studies have also reported that food matrix affects the bioaccessibility and bioavailability of blackcurrant anthocyanins (139, 191, 193). Nielsen *et al.* (191) studied the effect of the concomitant ingestion of a carbohydrate-rich meal (rice cake) and blackcurrant juice on anthocyanins bioavailability. The additional ingestion of a rice cake resulted in delayed gastric emptying and, consequently, slower absorption of blackcurrant anthocyanins, with no effects on total absorption and excretion (191). In contrast, the absorption and excretion of D3G and D3R in rats fed blackcurrant powder were impaired by the simultaneous intake of oatmeal (193). In a study using MKN-28 cell model, it was observed that the intake of glucose reduced gastric uptake of anthocyanins probably due to competition for the same transporters (GLUT1 e GLUT3) (139). Altogether these results highlight that complex food matrix with high contents of sugar and fibre may reduce anthocyanins bioavailability, suggesting that simpler food matrixes, such as berry infusions, should lead to higher and rapid absorption of D3G and D3R.

5. Conclusions

In the present study, freeze-dried blackcurrant anthocyanins were determined by UHPLC-DAD. The observed content and profile of anthocyanins were in line with the results reported in the literature. Delphinidin-3-*O*-rutinoside (D3R) was the main anthocyanin found in blackcurrant berries, followed by cyanidin-3-*O*-rutinoside (C3R) > delphinidin-3-*O*-glucoside (D3G) > cyanidin-3-*O*-glucoside (C3G) for all products, except for New Zealand blackcurrants, where C3R and D3R were the most abundant anthocyanins. Compared to the other blackcurrant berries (537 - 951 mg/100 g DW), New Zealand blackcurrants presented significantly higher content of total anthocyanins (2380.6 ± 27.2 mg/100 g DW), corroborating the results reported in previous studies. Beyond anthocyanin content, phenolic compounds and antioxidant activity were evaluated, ranging from 2150 to 7400 mg GAE/100 g DW and 2426 to 10193 mg TE/100 g DW. A significant correlation was found between phenolic compound content and antioxidant activity ($r = 0.918$), confirming the contribution of phenolic compounds to the antioxidant activity of these berries.

Despite their bioactive potential, the commercial availability of fresh blackcurrants is still limited due to their high perishability, which highlights the need for new and cheaper blackcurrant-based products, such as infusions. Infusions can be prepared in multiple ways, affecting their anthocyanin composition. Therefore, preparation conditions were optimized through a three-level factorial Box–Behnken design to maximize anthocyanins extraction. The obtained models for each infusion showed that the mass/volume ratio was the only variable with significant impact on total anthocyanin content of blackcurrant infusions. In general, anthocyanins extraction efficiency ranged from 70 to 120%, suggesting that the conditions used in the present study evidenced great potential to extract anthocyanins from blackcurrant berries. As expected, the anthocyanin profile of blackcurrant infusions was similar to those observed for freeze-dried samples, with D3R being the most abundant anthocyanin. Regarding the anthocyanin content of blackcurrant infusions, values ranged from 2.42 to 103.1 mg/200 mL. This wide variation in anthocyanin contents and profiles, makes it imperative to apply strict quality control to ensure the consistency of these plant-derived products, especially if used for therapeutical proposes.

Delphinidin-based anthocyanins have been suggested as the most effective anthocyanins against TNBC cell proliferation. D3G and D3R, abundant delphinidin-based anthocyanins in blackcurrants, demonstrated a dose-effect response on cell proliferation, therefore docetaxel (DT) was combined with each anthocyanin both separately and simultaneously. D3G-DT and D3R-DT combination presented a synergistic effect, however D3G-DT showed signs of antagonism at higher levels of toxicity, while D3R-DT presented the same behaviour at lower levels of toxicity. Notably, D3G-D3R-DT combination resulted

in a synergistic effect (CI < 0.7) on MDA-MB-231 cell proliferation at all inhibition levels. None of the combinations presented cytotoxicity in non-cancerous cells (MCF-12A). The synergistic effect of D3G-D3R-DT is of therapeutic importance in chemotherapy regimens, since D3G and D3R allowed the reduction of DT dosages while maintaining the efficacy of the treatment, which should reduce the adverse effects associated with docetaxel.

Since blackcurrant infusions are simple food matrixes with high contents of D3G and D3R, they have the potential to be used as dietary supplements to improve DT efficacy in the treatment of TNBC. However, further studies are needed, which should: i) determine the bioaccessibility and bioavailability of D3G and D3R of blackcurrant infusions; ii) evaluate the effect of blackcurrant infusions on MDA-MB-231 cell proliferation after *in vitro* digestion and transport assay; and iii) assess the combined effect of digested blackcurrant infusions and docetaxel on TNBC cell proliferation. Moreover, given that 25% of blackcurrant berries used in juice production remain as pomace, and that anthocyanins are prone to be extracted with green and food-grade solvents, these by-products should also be exploited as a source of new dietary supplements of D3G and D3R.

6. List of Publications and Communications

This dissertation work resulted in the following oral communications:

- Ribeiro, M.; Viegas, O.; Ferreira, I.M.P.L.V.O. *Impact of different preparation conditions on anthocyanins content of blackcurrant infusions*. I Jornadas do Mestrado em Controlo da Qualidade, Faculdade de Farmácia da Universidade do Porto, July 7th, 2022.
- Ribeiro, M., Faria, M., Viegas, O., Vojtek, M., Diniz, C., Ferreira, I.M.P.L.V.O. *Promising synergistic effect of blackcurrant anthocyanins and docetaxel on triple negative breast cancer cells.* IJUP'22 – Encontro de Investigação Jovem da Universidade do Porto, May 4-6th, 2022. (ISBN 978-989-746-329-7).
- Ribeiro, M., Viegas, O., Faria, M., Vojtek, M., Gonçalves-Monteiro, S., Diniz, C., Ferreira, I.M.P.L.V.O. Combined effect of docetaxel and delphinidin-3-*O*-glucoside on triple negative breast cancer cells. XIV Congresso de Nutrição e Alimentação da Associação Portuguesa de Nutrição, September 9-10th, 2020 (online). https://dx.doi.org/10.21011/apn.2020.2214

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8. Supplementary materials

Table S1a: Box–Behnken design to evaluate the effect of infusion conditions on the anthocyanin content of different blackcurrant infusion A.

Standard order	Independent variables			Anthocyanin content (mg/200 mL)				
	X_1	X_2	X_3	D3G	D3R	C3G	C3R	**Total**
1	80.0	2.0	2.25	7.73	22.10	5.18	23.60	58.61
2	100.0	2.0	2.25	6.89	19.52	4.63	20.94	51.98
3	80.0	7.0	2.25	7.00	20.14	4.73	21.46	53.33
4	100.0	7.0	2.25	7.76	22.06	5.18	23.54	58.54
5	80.0	4.5	0.5	1.73	5.08	1.19	5.41	13.41
6	100.0	4.5	0.5	1.84	5.21	1.26	5.56	13.88
7	80.0	4.5	4.0	11.09	31.89	7.45	34.06	84.50
8	100.0	4.5	4.0	11.45	32.46	7.62	34.73	86.27
9	90.0	2.0	0.5	1.84	5.19	1.23	5.53	13.79
10	90.0	7.0	0.5	1.94	5.36	1.33	5.69	14.32
11	90.0	2.0	4.0	9.07	25.83	6.03	27.66	68.59
12	90.0	7.0	4.0	13.60	38.88	9.08	41.53	103.09
13	90.0	4.5	2.25	5.60	15.96	3.75	17.16	42.47
14	90.0	4.5	2.25	8.46	23.90	5.65	25.46	63.47
15	90.0	4.5	2.25	8.66	24.50	5.81	26.16	65.13
16	90.0	4.5	2.25	8.32	23.54	5.57	24.97	62.41
17	90.0	4.5	2.25	8.14	22.97	5.46	24.53	61.09

X_1: temperature (°C); X_2: time (min); X_3: mass/volume ratio (g/200 mL); D3G: delphinidin-3-*O*-glucoside; D3R: delphinidin-3-*O*-rutinoside; C3G: cyanidin-3-*O*-glucoside; C3R: cyanidin-3-*O*-rutinoside.

Table S1b: Box–Behnken design to evaluate the effect of infusion conditions on the anthocyanin content of different blackcurrant infusion B.

Standard order	Independent variables			Anthocyanin content (mg/200 mL)				
	X_1	X_2	X_3	D3G	D3R	C3G	C3R	**Total**
1	80.0	2.0	2.25	5.13	17.00	1.41	6.89	30.43
2	100.0	2.0	2.25	4.22	13.79	1.17	5.63	24.81
3	80.0	7.0	2.25	4.39	14.99	1.20	6.11	26.70
4	100.0	7.0	2.25	4.27	14.02	1.16	5.72	25.17
5	80.0	4.5	0.5	1.22	3.94	0.34	1.59	7.10
6	100.0	4.5	0.5	1.30	4.29	0.36	1.73	7.69
7	80.0	4.5	4.0	10.05	33.03	2.76	13.50	59.33
8	100.0	4.5	4.0	7.59	24.88	2.09	10.09	44.65
9	90.0	2.0	0.5	1.31	4.33	0.37	1.73	7.74
10	90.0	7.0	0.5	0.94	3.12	0.29	1.32	5.65
11	90.0	2.0	4.0	9.68	31.76	2.66	12.85	56.95
12	90.0	7.0	4.0	9.41	30.97	2.61	12.53	55.53
13	90.0	4.5	2.25	5.36	17.68	1.48	7.22	31.73
14	90.0	4.5	2.25	3.30	10.78	0.88	4.42	19.38
15	90.0	4.5	2.25	5.05	16.48	1.38	6.70	29.62
16	90.0	4.5	2.25	5.25	17.35	1.45	7.04	31.09
17	90.0	4.5	2.25	3.79	12.51	1.02	5.07	22.40

X_1: temperature (°C); X_2: time (min); X_3: mass/volume ratio (g/200 mL); D3G: delphinidin-3-*O*-glucoside; D3R: delphinidin-3-*O*-rutinoside; C3G: cyanidin-3-*O*-glucoside; C3R: cyanidin-3-*O*-rutinoside.

Table S1c: Box–Behnken design to evaluate the effect of infusion conditions on the anthocyanin content of different blackcurrant infusion C.

Standard order	Independent variables			Anthocyanin content (mg/200 mL)				
	X_1	X_2	X_3	D3G	D3R	C3G	C3R	**Total**
1	80.0	2.0	2.25	5.24	14.69	1.70	7.18	28.81
2	100.0	2.0	2.25	5.45	14.98	1.76	7.41	29.59
3	80.0	7.0	2.25	4.55	12.88	1.45	6.31	25.19
4	100.0	7.0	2.25	5.38	15.23	1.85	7.86	30.32
5	80.0	4.5	0.5	1.17	3.22	0.38	1.58	6.35
6	100.0	4.5	0.5	1.05	2.94	0.37	1.44	5.79
7	80.0	4.5	4.0	7.49	21.31	2.50	10.61	41.92
8	100.0	4.5	4.0	6.25	17.45	2.03	8.65	34.39
9	90.0	2.0	0.5	1.31	3.54	0.41	1.77	7.03
10	90.0	7.0	0.5	1.30	3.67	0.43	1.81	7.21
11	90.0	2.0	4.0	6.67	18.77	2.21	9.22	36.87
12	90.0	7.0	4.0	7.68	21.64	2.49	10.66	42.48
13	90.0	4.5	2.25	4.92	13.89	1.63	6.88	27.33
14	90.0	4.5	2.25	4.91	13.66	1.60	6.65	26.82
15	90.0	4.5	2.25	4.53	12.77	1.46	6.26	25.01
16	90.0	4.5	2.25	4.71	13.19	1.51	6.50	25.92
17	90.0	4.5	2.25	4.84	13.56	1.57	6.61	26.58

X_1: temperature (°C); X_2: time (min); X_3: mass/volume ratio (g/200 mL); D3G: delphinidin-3-*O*-glucoside; D3R: delphinidin-3-*O*-rutinoside; C3G: cyanidin-3-*O*-glucoside; C3R: cyanidin-3-*O*-rutinoside.

Table S1d: Box–Behnken design to evaluate the effect of infusion conditions on the anthocyanin content of different blackcurrant infusion D.

Standard order	Independent variables			Anthocyanin content (mg/200 mL)				
	X_1	X_2	X_3	D3G	D3R	C3G	C3R	**Total**
1	80.0	2.0	2.25	2.33	3.81	0.66	1.65	8.45
2	100.0	2.0	2.25	3.22	4.92	0.87	2.17	11.18
3	80.0	7.0	2.25	2.40	4.07	0.64	1.77	8.88
4	100.0	7.0	2.25	2.72	4.43	0.77	1.99	9.91
5	80.0	4.5	0.5	0.70	1.15	0.21	0.52	2.57
6	100.0	4.5	0.5	0.73	1.13	0.21	0.50	2.58
7	80.0	4.5	4.0	4.02	7.26	1.10	3.16	15.54
8	100.0	4.5	4.0	4.91	8.05	1.36	3.55	17.86
9	90.0	2.0	0.5	0.72	1.12	0.20	0.48	2.52
10	90.0	7.0	0.5	0.67	1.09	0.18	0.48	2.42
11	90.0	2.0	4.0	4.78	7.94	1.33	3.44	17.49
12	90.0	7.0	4.0	4.52	7.77	1.28	3.43	17.00
13	90.0	4.5	2.25	2.81	4.61	0.83	2.01	10.26
14	90.0	4.5	2.25	1.97	3.25	0.54	1.45	7.21
15	90.0	4.5	2.25	2.86	4.66	0.81	2.06	10.38
16	90.0	4.5	2.25	2.26	3.67	0.63	1.60	8.15
17	90.0	4.5	2.25	2.44	4.10	0.68	1.83	9.06

X_1: temperature (°C); X_2: time (min); X_3: mass/volume ratio (g/200 mL); D3G: delphinidin-3-*O*-glucoside; D3R: delphinidin-3-*O*-rutinoside; C3G: cyanidin-3-*O*-glucoside; C3R: cyanidin-3-*O*-rutinoside.

Table S2a: Analysis of variance (ANOVA) for response surface quadratic model for total anthocyanin content of infusion A.

Infusion A	**Sum of Squares**	**df**	**Mean Square**	***F*-value**	***p*-value**	
Model	7.742337	9	0.86026	39.98832	< 0.0001	s.
X_1	9.64×10^{-5}	1	9.64×10^{-5}	0.004482	0.9485	n.s.
X_2	0.027580	1	0.02758	1.282018	0.2948	n.s.
X_3	6.563948	1	6.563948	305.1186	< 0.0001	s.
$X_1 X_2$	0.011367	1	0.011367	0.528364	0.4909	n.s.
$X_1 X_3$	4.34×10^{-5}	1	4.34×10^{-5}	0.002019	0.9654	n.s.
$X_2 X_3$	0.034133	1	0.034133	1.586622	0.2482	n.s.
X_1^2	0.003133	1	0.003133	0.14564	0.7141	n.s.
X_2^2	0.001706	1	0.001706	0.079282	0.7864	n.s.
X_3^2	1.082367	1	1.082367	50.31276	0.0002	s.
Residual	0.150589	7	0.021513	-	-	-
Lack of fit	0.023968	3	0.007989	0.252389	0.8564	n.s.
Pure Error	0.126621	4	0.031655	-	-	-
Cor Total	7.892926	16	-	-	-	-

df: degrees of freedom; s.: significant; n.s.: not significant; X_1: temperature (°C); X_2: time (min); X_3: mass/volume ratio (g/200 mL).

Table S2b: Analysis of variance (ANOVA) for response surface quadratic model for total anthocyanin content of infusion B.

Infusion B	**Sum of Squares**	**df**	**Mean Square**	***F*-value**	***p*-value**	
Model	8.854895	9	0.983877	30.89177	< 0.0001	s.
X_1	0.027275	1	0.027275	0.856378	0.3855	n.s.
X_2	0.025987	1	0.025987	0.815937	0.3964	n.s.
X_3	8.329051	1	8.329051	261.5155	< 0.0001	s.
X_1 X_2	0.005292	1	0.005292	0.166171	0.6957	n.s.
X_1 X_3	0.033254	1	0.033254	1.044106	0.3409	n.s.
X_2 X_3	0.020909	1	0.020909	0.65651	0.4445	n.s.
X_1^2	0.000601	1	0.000601	0.018881	0.8946	n.s.
X_2^2	5.59×10^{-6}	1	5.59×10^{-6}	0.000175	0.9898	n.s.
X_3^2	0.412015	1	0.412015	12.93644	0.0088	s.
Residual	0.222944	7	0.031849	-	-	-
Lack of fit	0.026433	3	0.008811	0.179348	0.9052	n.s.
Pure Error	0.196511	4	0.049128	-	-	-
Cor Total	9.077839	16	-	-	-	-

df: degrees of freedom; s.: significant; n.s.: not significant; X_1: temperature (°C); X_2: time (min); X_3: mass/volume ratio (g/200 mL).

Table S2c: Analysis of variance (ANOVA) for response surface quadratic model for total anthocyanin content of infusion C.

Infusion C	Sum of Squares	df	Mean Square	*F*-value	*p*-value	
Model	2215.338	9	246.1486	46.31333	< 0.0001	s.
X_1	0.586129	1	0.586129	0.110281	0.7495	n.s.
X_2	1,054145	1	1.054145	0.198339	0.6695	n.s.
X_3	2089.015	1	2089.015	393.0521	< 0.0001	s.
X_1 X_2	4.729501	1	4.729501	0.889865	0.3769	n.s.
X_1 X_3	12.13448	1	12.13448	2.283125	0.1745	n.s.
X_2 X_3	7.392497	1	7.392497	1.390912	0.2768	n.s.
X_1^2	0.784556	1	0.784556	0.147616	0.7122	n.s.
X_2^2	12.39302	1	12.39302	2.331769	0.1706	n.s.
X_3^2	90.98450	1	90.98450	17.11891	0.0044	s.
Residual	37.20398	7	5.314855	-	-	-
Lack of fit	33.98884	3	11.32961	14.09530	0.0136	s.
Pure Error	3.215145	4	0.803786	-		-
Cor Total	2252.542	16	-	-		-

df: degrees of freedom; s.: significant; n.s.: not significant; X_1: temperature (°C); X_2: time (min); X_3: mass/volume ratio (g/200 mL).

Table Sd: Analysis of variance (ANOVA) for response surface quadratic model for total anthocyanin content of infusion D.

Infusion D	**Sum of Squares**	**df**	**Mean Square**	***F*-value**	***p*-value**	
Model	7.816205	9	0.868467	56.7723	< 0.0001	s.
X_1	0.035441	1	0.035441	2.316786	0.1718	n.s.
X_2	0.002377	1	0.002377	0.155382	0.7052	n.s.
X_3	7.262368	1	7.262368	474.746	< 0.0001	s.
$X_1 X_2$	0.007207	1	0.007207	0.471140	0.5145	n.s.
$X_1 X_3$	0.004667	1	0.004667	0.305100	0.5979	n.s.
$X_2 X_3$	3.41×10^{-5}	1	3.41×10^{-5}	0.002232	0.9636	n.s.
X_1^2	0.005441	1	0.005441	0.355674	0.5697	n.s.
X_2^2	0.004089	1	0.004089	0.267330	0.6211	n.s.
X_3^2	0.501949	1	0.501949	32.81275	0.0007	s.
Residual	0.107082	7	0.015297	-	-	-
Lack of fit	0.010809	3	0.003603	0.149707	0.9247	n.s.
Pure Error	0.096272	4	0.024068	-	-	-
Cor Total	7.923287	16	-	-	-	-

df: degrees of freedom; s.: significant; n.s.: not significant; X_1: temperature (°C); X_2: time (min); X_3: mass/volume ratio (g/200 mL).

Table S3a: Content of total phenolic compounds and antioxidant activity of infusion A prepared following the conditions resulting from the Box–Behnken design.

Standard order	Independent variables			Total Phenolic compounds	DPPH• inhibition
	X_1	X_2	X_3	(mg GAE/200 mL)	(mg TE/200 mL)
1	80.0	2.0	2.25	93.89	214.10
2	100.0	2.0	2.25	80.13	175.20
3	80.0	7.0	2.25	83.58	192.18
4	100.0	7.0	2.25	89.86	215.79
5	80.0	4.5	0.5	22.23	40.85
6	100.0	4.5	0.5	24.74	50.85
7	80.0	4.5	4.0	169.58	312.59
8	100.0	4.5	4.0	181.09	299.00
9	90.0	2.0	0.5	23.41	42.79
10	90.0	7.0	0.5	24.86	44.66
11	90.0	2.0	4.0	172.97	269.84
12	90.0	7.0	4.0	185.18	296.54
13	90.0	4.5	2.25	85.52	184.47
14	90.0	4.5	2.25	94.01	209.16
15	90.0	4.5	2.25	87.00	200.37
16	90.0	4.5	2.25	79.82	212.17
17	90.0	4.5	2.25	100.63	187.12

X_1: temperature (°C); X_2: time (min); X_3: mass/volume ratio (g/200 mL); GAE: gallic acid equivalents; TE: trolox equivalents.

Table S3b: Content of total phenolic compounds and antioxidant activity of infusion B prepared following the conditions resulting from the Box–Behnken design.

Standard order	Independent variables			Total Phenolic compounds	DPPH• inhibition
	X_1	X_2	X_3	(mg GAE/200 mL)	(mg TE/200 mL)
1	80.0	2.0	2.25	59.63	124.13
2	100.0	2.0	2.25	68.48	132.47
3	80.0	7.0	2.25	58.39	115.79
4	100.0	7.0	2.25	73.46	139.57
5	80.0	4.5	0.5	16.35	33.19
6	100.0	4.5	0.5	17.29	37.99
7	80.0	4.5	4.0	118.60	234.89
8	100.0	4.5	4.0	129.57	247.07
9	90.0	2.0	0.5	17.89	30.81
10	90.0	7.0	0.5	16.77	31.78
11	90.0	2.0	4.0	129.65	253.35
12	90.0	7.0	4.0	108.69	275.77
13	90.0	4.5	2.25	65.16	128.25
14	90.0	4.5	2.25	71.11	130.30
15	90.0	4.5	2.25	70.33	121.80
16	90.0	4.5	2.25	67.79	139.35
17	90.0	4.5	2.25	61.11	130.46

X_1: temperature (°C); X_2: time (min); X_3: mass/volume ratio (g/200 mL); GAE: gallic acid equivalents; TE: trolox equivalents.

Table S3c: Content of total phenolic compounds and antioxidant activity of infusion C prepared following the conditions resulting from the Box–Behnken design.

Standard order	Independent variables			Total Phenolic compounds	DPPH• inhibition
	X_1	X_2	X_3	(mg GAE/200 mL)	(mg TE/200 mL)
1	80.0	2.0	2.25	72.57	130.03
2	100.0	2.0	2.25	69.92	128.22
3	80.0	7.0	2.25	63.16	133.36
4	100.0	7.0	2.25	67.13	131.76
5	80.0	4.5	0.5	15.36	25.98
6	100.0	4.5	0.5	15.00	28.58
7	80.0	4.5	4.0	124.64	255.22
8	100.0	4.5	4.0	125.94	254.62
9	90.0	2.0	0.5	15.30	27.09
10	90.0	7.0	0.5	13.20	34.70
11	90.0	2.0	4.0	127.88	269.47
12	90.0	7.0	4.0	118.48	251.96
13	90.0	4.5	2.25	67.20	120.19
14	90.0	4.5	2.25	69.66	123.25
15	90.0	4.5	2.25	71.06	116.58
16	90.0	4.5	2.25	64.98	125.60
17	90.0	4.5	2.25	68.14	135.01

X_1: temperature (°C); X_2: time (min); X_3: mass/volume ratio (g/200 mL); GAE: gallic acid equivalents; TE: trolox equivalents.

Table S3d: Content of total phenolic compounds and antioxidant activity of infusion D prepared following the conditions resulting from the Box–Behnken design.

Standard order	Independent variables			Total Phenolic compounds	DPPH• inhibition
	X_1	X_2	X_3	(mg GAE/200 mL)	(mg TE/200 mL)
1	80.0	2.0	2.25	18.86	38.85
2	100.0	2.0	2.25	25.21	49.57
3	80.0	7.0	2.25	18.67	40.95
4	100.0	7.0	2.25	22.01	43.26
5	80.0	4.5	0.5	5.34	9.89
6	100.0	4.5	0.5	6.07	12.65
7	80.0	4.5	4.0	34.80	63.71
8	100.0	4.5	4.0	41.45	85.09
9	90.0	2.0	0.5	6.18	11.73
10	90.0	7.0	0.5	6.02	11.08
11	90.0	2.0	4.0	37.40	73.92
12	90.0	7.0	4.0	39.88	70.50
13	90.0	4.5	2.25	20.68	40.57
14	90.0	4.5	2.25	20.03	42.94
15	90.0	4.5	2.25	21.48	41.64
16	90.0	4.5	2.25	20.94	40.26
17	90.0	4.5	2.25	19.94	40.54

X_1: temperature (°C); X_2: time (min); X_3: mass/volume ratio (g/200 mL); GAE: gallic acid equivalents; TE: trolox equivalents.