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Article

Phenolic Profiles and Antitumor Activity against Colorectal Cancer Cells of Seeds from Selected *Ribes* taxa

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Abstract: Seeds from several *Ribes* taxa were surveyed for phenolic compounds and in vitro antiproliferative activity against HT-29 colorectal cancer cells. Total phenolic compounds were analysed through the Folin-Ciocalteu procedure, while phenolic profiles were analysed by LC coupled to a single mass spectrometer Orbitrap using an electrospray interface (ESI). Antitumor effects were established using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Total phenolics ranged from 11.4 in *R. alpinum* to 94.8 mg of caffeic acid equivalents (CAE)/g in *R. nigrum* 'Koksa'. Concerning phenolic compounds, four were hydroxylated benzoic acids, four cinnamic acid derivatives, eight flavonoids, and nine flavonoid glycosides. The growth inhibition against HT-29 cancer cells was exercised much better by *R. nigrum* 'Koksa' and *Ribes* 'Erkeeni' (GI_{50} 37 and 42 μg/ml). All *Ribes* extracts, except *R. nigrum* 'Hara katarlik', showed higher activity than that of *R. rubrum* (GI_{50} at 72 h: 99 μg/ml). Interestingly, the highly bioactive extract from *Ribes* 'Erkeeni' contains all detected phenolics, while *R. nigrum* 'Koksa' lacks only populnin. Therefore, the high bioactivity found for such extracts could be due to a synergy due to all detected compounds. This work constitutes a comprehensive action for expanding knowledge on the phenolic profiles and antitumor activity of GLA-rich *Ribes* seeds.

Keywords: *Ribes*; blackcurrant cultivars; phenolic compounds; LC-MS; HT-29; MTT assay

1. Introduction

The genus *Ribes* (fam. Grossulariaceae) includes more than 150 diploid species, distributed in the temperate regions of the Northern Hemisphere and South America (Brennan, 1996). At present, 10-12 species of *Ribes* are propagated for fruit production, the majority of which are black (*Ribes nigrum* L.), red, and white currant (*R. rubrum* L., synonyms *R. vulgare* Jancz. and *R. sativum* Syme) and gooseberry (e.g., European gooseberry: *R. uva-crispa* L., synonym *R. grossularia* L., and American hairystem gooseberry: *Ribes hirtellum* Michx.) (Brennan, 2009). Black currants are considered as promising crops with high economic value, especially since they are ranked second in consumer preferences after strawberries (Brennan, 2009; Jiménez-Aspee et al., 2015).

Fresh edible *Ribes* fruits are promising crops with high economic value, not only due to their desired taste and nutritional value but also for their well-known health-promoting properties (Mikulic-Petkovsek et al., 2015). Furthermore, *Ribes* seed oil has immunomodulation and anti-inflammatory effects, i.e., the use of blackcurrant seed oil in preventing illness like hypertension, psoriasis, and atopic dermatitis has been reported (Jurgoński et al., 2015; Michalak & Kiełtyka-Dadasiewicz, 2018).

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Moreover, the fruit industry generates year by year increased amounts of fruits by-products, in which occurs tonnage amounts of seeds, while the seeds of some species/cultivars constitute a valuable source of γ-linolenic acid (GLA, 18:3*n*-6), which can account for more than 20% of total fatty acids (FA) (e.g., Lyashenko et al., 2019; Golovenko et al., 2021). Furthermore, in addition to its high content in GLA, blackcurrant seed oil has also been reported to be a good source of phenolic compounds (Bakowska-Barczak et al., 2009), which constitute a large fraction of the unsaponifiable material of most vegetable oils (Fabrikov et al., 2019).

The chemical profiling of European currants has been extensively described, mainly for *R. nigrum* (black currants) and *R. rubrum* (red currants), while data on the composition of phenolic compounds and biological activity of the remaining species of *Ribes* genus and cultivars is extremely limited. Taking into account the benefits of the unsaponifiable material contained in the seed oil and the lack of information about several *Ribes* taxa, the aim of present study was to determine the phenolic profiles and the in vitro antitumor activity against colorectal cancer cells of the phenolic-containing seeds extracts from selected *Ribes* taxa. All studied species/cultivars have been previously typified as potential GLA producers (Lyashenko et al., 2019).

2. Results and Discussion

2.1. Total Phenolics and Oil Content

Table 1 shows the amounts of oil content, the total phenolic content (TPC) in mg of caffeic acid equivalents (CAE)/g seeds and mg of CAE/g oil, as well as data on GLA content in seeds (% of total FA) previously reported by our Research Team (Lyashenko et al., 2019). Notably, seed samples vary in their total oil content, with values ranging from 12.7 (*R. alpinum* 1B) to 25.6 g/100 g of seeds (*R. hudsonianum*). A significant variability was observed in TPC amounts among different samples and sections. In sect. Berisia, *R. alpinum* 1A have a lower oil content compared to 1B, but show higher TPC, while *R. pulchellum* stands out with the highest content of oil and TPC. As for sect. Coreosma, *R. hudsonianum* has the highest oil content and TPC in oil, and *R. nigrum* 'Koksa' has the highest TPC in seeds. Concerning sect. Ribes, *R. rubrum* has the lowest oil and TPC content in seeds. Regarding GLA values, *Ribes* 'Myuryucheene' shows the highest percentages of total FA, but unfortunately neither its oil content nor TPC are notable. Conversely, *R. nigrum* 'Koksa' has 17.0% GLA of total FA, and given its high TPC (15.5 mg CAE/g oil), this cultivar constitutes a promising source of GLA-rich oil containing good amounts of phenolics, thus, hosting healthy properties related to such compounds.

Table 1. Data on collection, oil, and total phenolics content of *Ribes* samples.

Code	Samples	Sample location	Total oil content g/100 g seeds	TPC (mg CAE/g seeds)^{abc}	TPC (mg CAE/g oil)^{abc}
		Subgenus Ribes (Currants)			
		Sect. Berisia Spach (Alpine currants)			
1A	*R. alpinum*	Sukachev Institute of Forest of the Siberian Branch of the RAS, Krasnoyarsk, Russia	19.9±0.5^{b}	36.9±1.8^{d}	7.3±0.3^{e}
1B	*R. alpinum*	Sierra de Baza, Granada, Spain	12.7±0.4^{f}	33.4±0.9^{de}	4.2±0.1^{hi}
2	*R. pulchellum*	Sukachev Institute of Forest of the Siberian Branch of the RAS, Krasnoyarsk, Russia	23.0±1.0^{a}	34.2±1.2^{de}	7.9±0.2^{de}
		Sect. Coreosma (Spach) Jancz. (Black Currants)			
3	*R. dikuscha*	Botanical Garden of North-Eastern Federal University, Yakutsk, Russia	17.8±0.2^{c}	30.5±2.4^{e}	5.4±0.0^{g}
4	*R. hudsonianum*	Botanical Garden of North-Eastern Federal University, Yakutsk, Russia	25.6±0.8^{a}	46.1±3.2^{c}	11.8±0.1^{b}

 doi:10.20944/preprints202402.1139.v1

5A	*R. nigrum* 'Hara katarlik'	Botanical Garden of North-Eastern Federal University, Yakutsk, Russia	18.4±0.1[b]	53.4±2.5[b]	9.8±0.2[c]
5B	*R. nigrum* 'Koksa'	Botanical Garden of North-Eastern Federal University, Yakutsk, Russia	16.3±0.0[de]	94.8±3.4[a]	15.5±0.1[a]
6	*R.* 'Algo' Yakutskaya	Botanical Garden of North-Eastern Federal University, Yakutsk, Russia	17.0±0.3[cd]	48.9±2.8[bc]	8.3±0.2[d]
7	*Ribes* 'Erkeeni'	Botanical Garden of North-Eastern Federal University, Yakutsk, Russia	18.3±0.2[bc]	49.0±2.6[b]	9.0±0.2[cd]
8	*Ribes* 'Myuryucheene'	Botanical Garden of North-Eastern Federal University, Yakutsk, Russia	17.7±0.6[c]	34.4±1.9[de]	6.1±0.4[f]
		Sect. Ribes (Red Currants)			
9	*R. glabellum*	Botanical Garden of North-Eastern Federal University, Yakutsk, Russia	14.9±0.4[e]	30.8±2.0[e]	4.6±0.2[h]
10	*R. triste*	Dendropark "Alexandria" NAS of Ukraine, Belaja Tserkov, Ukraine	18.5±0.5[bc]	31.2±2.9[e]	5.8±0.3[fg]
11	*R. rubrum*	Botanical Garden of North-Eastern Federal University, Yakutsk, Russia	15.0±0.2[e]	25.8±3.1[f]	3.9±0.1[i]

[a]Data represent means ± standard deviation of samples analyzed in triplicate; [b]Differences in TPC amounts were tested according to one-way ANOVA followed by Duncan's test; [c] Within a column, means followed by different letter are significantly different at $P<0.05$.

In short, a proper selection of *Ribes* varieties can significantly influence the content of bioactive compounds in seeds, i.e., GLA and TPC, whose values in the species and cultivars focused here are among the highest reported for *Ribes* taxa. Previous analysis effected to the seeds of other *R. nigrum* cultivars revealed that 'Ben Tirran' and 'Ben Sarek' ones are good sources of GLA (15.2-16.7% of total FA), although these have very low TPC amounts quantified by the Folin Ciocalteu (F-C) method: 1.99 and 2.31 mg of Gallic Acid Equivalents (GAE)/g seed residue, respectively (Bakowska-Barczak et al., 2009. However, Van Hoed et al. (2011) indicated figures obtained by the F-C methodology in the range showed here for several *Ribes* cultivars (5.6-11.3 mg CAE/g oil). Other works focused on residues from the extraction of *Ribes* fruits cannot be compared with the results obtained here, since such works were focused on the residual cake from the extraction of the fruit, which in addition to the seeds contains several other tissues of the fruit (e.g., Kapasakalidis et al., 2006; Granato et al., 2022).

2.2. Phenolic Compounds Profiles

The phenolic compounds profiles obtained by the LC-MS system of the seeds of *Ribes* species/cultivars focused here are reported in Table 2. The identification was achieved by means of retention time (Rt) and *m*/*z* of both, the adducts and fragment ions. All compounds were properly identified, and the bases for the identification of each compound are described in Table 2.

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Table 2. Identification of phenolic compounds in the seeds of selected *Ribes* taxa using LC-MS.

N	Rt min	Mass[a] *m/z*	Adduct	Fragment[b]	Formula	Identification	Identification basis	Occurrence in samples[c]
1	3.88	153.01868	[M-H]$^-$	109.02970	$C_7H_6O_4$	3,4-Dihydroxybenzoic (protocatechuic) acid	Molecular ion [M–H]$^-$ *m/z* 153, and at *m/z* 109, produced after the neutral loss of CO_2 (44 Da)	1B,5B,6,7,8,9,10
2	5.12	139.03909	[M-H]$^-$	93.03460	$C_7H_6O_3$	Salicylic acid	Molecular ion [M–H]$^-$ *m/z* 137, which further yielded a fragment ion at *m/z* 93, due to the loss of a CO_2 group	5A,5B,6,7,10,11
3	8.72	179.03498	[M-H]$^-$	135.04810	$C_9H_8O_4$	Caffeic acid	Molecular ion [M–H]$^-$ *m/z* 179 and its characteristic product ion 135, due to the loss of the CO_2 group	1A,1B,2,5A,5B,6,7,10
4	13.92	167.03498	[M-H]$^-$	152.00996	$C_8H_8O_4$	Vanillic acid	Molecular ion [M–H]$^-$ *m/z* 167 and its characteristic product ion 152, due to the loss of CH_4	1A,1B,2,5B,6,7,8,10
5	16.68	163.04007	[M-H]$^-$	119.04881	$C_9H_8O_3$	*p*-coumaric acid	Molecular ion [M–H]$^-$ *m/z* 163 and its characteristic product ion 119, due to the loss of the CO_2 group	1A,1B,2,5A,5B,6,7,8,9,11
6	24.56	137.02442	[M-H]$^-$	93.03325	$C_7H_6O_3$	4-hydroxybenzoic acid	Molecular ion [M–H]$^-$ *m/z* 137 and its characteristic product ion 93, generated by the loss of the CO_2 group	1A,1B,2,3,4,5A,5B,6,7,8,9,10,11
7	26.41	223.06120	[M-H]$^-$	121.02821	$C_{11}H_{12}O_5$	Sinapic acid	Molecular ion [M–H]$^-$ *m/z* 223, and the loss of $2CH_3$ – CO_2 – CO (*m/z* 121) (Marcum 2016)	1A,1B,5B,6,7,8,9,11
8	28.01	447.09328	[M-H]$^-$	257.04496	$C_{21}H_{20}O_{11}$	Populnin (kaempferol-7-*O*-glucoside)	Molecular ion [M–H]$^-$ *m/z* 447 and *m/z* 257, corresponding to the fragment [M–H–CO]− . The ejection of CO is notably followed by B-ring rotation and bonding with the A-ring to form the fused ring structure of *m/z* 257 (March and Miao, 2004).	1A,1B,6,7,8
9	28.2	193.05063	[M-H]$^-$	134.03690	$C_{10}H_{10}O_4$	Ferulic acid	Molecular ion [M–H]$^-$ *m/z* 193, and *m/z* 134 corresponding to the loss of CO_2 and CH_3	1A,1B,2,4,5A,5B,6,7,8,9,10,11
10	28.62	303.04993	[M+H]$^+$	178.99749	$C_{15}H_{10}O_7$	Quercetin	Molecular ion [M–H]$^-$ *m/z* 303 and *m/z* 179, originated after cleavage of the B ring by a Retro Diels-Alder (RDA) mechanism (Dos Santos et al., 2018)	1A,1B,5B,6,7,8,9,11
11	28.81	463.08820	[M-H]$^-$	302.03696	$C_{21}H_{20}O_{12}$	Isoquercitrin (quercetin-3-*O*-glucoside)	Molecular ion [M–H]$^-$ *m/z* 463, and *m/z* 302, corresponding to the aglycone of quercetin following the loss of a hexose ([M – H – 162]$^-$	1B,2,4,5A,5B,6,7,8

 doi:10.20944/preprints202402.1139.v1

12	28.83	609.14611	[M-H]$^-$	301.03474	$C_{27}H_{30}O_{16}$	Rutin (quercetin 3-*O* rutinoside)	Molecular ion [M–H]$^-$ *m/z* 609, and fragment *m/z* 301 due to the loss of 308 Da (rutinose)	1A,1B,2,3,4,5A,5B,6,7,8,11
13	29.57	287.05501	[M+H]$^+$	153.01760	$C_{15}H_{10}O_6$	Kaempferol	Molecular ion [M–H]$^-$ *m/z* 287, and m/z 153 formed by RDA fragmentation wherein bonds 1 and 3 undergo scission leading to the formation of the A^+ ion (*m/z* 153) (Ma et al., 1997)	1A,1B,5B,6,7,8,9,11
14	29.75	447.09328	[M-H]$^-$	230.98517	$C_{21}H_{20}O_{11}$	Quercitrin (quercetin 3-*O*-rhamnoside)	Molecular ion [M–H]$^-$ *m/z* 447, and fragment *m/z* 231, corresponding to [quercetin-H-CO_2-CO]$^-$	5B,6,7,8,9,10,11
15	29.77	317.03029	[M-H]$^-$	151.00262	$C_{15}H_{10}O_8$	Myricetin	Molecular ion [M–H]$^-$ *m/z* 317, and typical MS/MS fragment at *m/z* 151, that corresponded to retrocyclization on the A–C ring ($^{1,2}A^-$) and the consecutive loss of CO ($^{1,2}A^-$-CO) (Chernosov et al., 2017)	1A,1B,2,3,4,5A,5B,6,7,8, 10
16	29.80	285.04046	[M-H]$^-$	121.02799	$C_{15}H_{10}O_6$	Fisetin	Molecular ion [M–H]$^-$ *m/z* 285, and *m/z* 121, that correspond to fragmentation of B ring ($^{1,2}B^-$), as described by Fabre et al. (2001)	5B,6,7,9
17	29.80	285.04046	[M-H]$^-$	175.03898	$C_{15}H_{10}O_6$	Luteolin	Molecular ion [M–H]$^-$ *m/z* 285, and *m/z* 175, corresponding to the loss of C_3O_2 – C_2H_2O (Śliwka-Kaszyńska et al., 2022)	1A,1B,5B,6,7,8,9
18	29.90	447.09328	[M-H]$^-$	285.03995	$C_{21}H_{20}O_{11}$	Juncein (luteolin-4′-*O*-glucoside)	Molecular ion [M–H]$^-$ *m/z* 447, and m/z 285 corresponding to luteolin aglycone, indicating the loss of a hexose	1A,1B,5B,6,7,8,11
19	29.92	447.09328	[M-H]$^-$	255.02924	$C_{15}H_{10}O_6$	Astragalin (kaempferol-3-*O*-glucoside)	Molecular ion [M–H]− *m/z* 447, and *m/z* 255, corresponding to the loss of the CH_2O from the aglycone (30 Da) (Dantas et al., 2021).	1A,1B,5B,6,7,10,11
20	29.95	593.15119	[M-H]$^-$	285.03973	$C_{27}H_{30}O_{15}$	Nicotiflorin (kaempferol-3-*O*-rutinoside)	Molecular ion [M–H]$^-$ *m/z* 593, and *m/z* 285 corresponding to a deprotonated kaempferol aglycone, and further loss of the rutinoside moiety	1A,1B,5B,6,7,8,10,11
21	30.06	287.05611	[M-H]$^-$	135.04382	$C_{15}H_{12}O_6$	Eriodictyol	Molecular ion [M–H]$^-$ *m/z* 287, and *m/z* 135 corresponding to fragmentation of the B ring ($^{1,3}B^-$), as described by Fabre et al. (2001)	5B,6,7
22	30.53	435.12967	[M-H]$^-$	273.07598	$C_{21}H_{24}O_{10}$	Phloridzin (phloretin-2′-*O*-glucoside)	Molecular ion [M–H]$^-$ *m/z* 435, and *m/z* 273 corresponding to phloretin (dihydronaringenin), after the losses of a hexosyl (glucose, 162 Da)	5B,6,7
23	30.78	269.04555	[M-H]$^-$	213.0545	$C_{15}H_{10}O_5$	Galangin	Molecular ion [M–H]$^-$ *m/z* 269, and *m/z* 213 corresponding to the loss of 2CO (56 Da)	1A,3,4,5A,5B,6,7,8,9,10,11

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24	30.87	433.11292	$[M+H]^+$	271.05908	$C_{21}H_{20}O_{10}$	Apigetrin (apigenin-7-*O*-glucoside)	Molecular ion $[M+H]^-$ *m*/*z* 433, and *m*/*z* 271 corresponding to the aglycon apiginin, by the loss of a glucose (162 Da)	5B,6,11
25	31.14	271.06120	$[M-H]^-$	119.04879	$C_{15}H_{12}O_5$	Naringenin	Molecular ion $[M–H]^-$ *m*/*z* 271, and *m*/*z* 119 that correspond to fragmentation of the B ring ($^{1,3}B^-$), as described by Fabre et al. (2001)	1A,1B,2,4,5A,5B,6,7,10

[a] mass error lower than 5 ppm; [b] mass error lower than 10 ppm; [c] Sample codes as in Table 1

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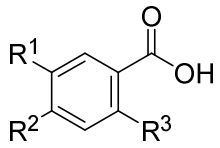
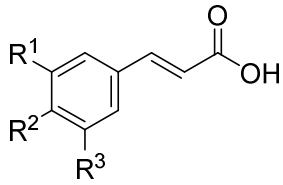
Among detected phenolics, four consisted of hydroxylated derivatives of benzoic acids (3,4-dihydroxybenzoic, salicylic, vanillic, and 4-hydroxybenzoic acid) (Figure 1A); four were cinnamic acid derivatives (caffeic, *p*-coumaric, chlorogenic, and ferulic acids) (Figure 1B); two flavone derivatives (luteolin and apigetrin) (Figure 1C); three flavanone derivatives (juncein, eriodictyol, and naringenin) (Figure 1D). The largest group was the flavonol derivatives, with eleven compounds (populnin, quercetin, isoquercitrin, rutin, kaempferol, quercitrin, myricetin, fisetin, astragalin, nicotiflorin, and galangin) (Figure 1E). Finally, a dihydrochalcone glucoside was also detected (phloridzin) (Figure 1F). Anthocyanins, usually found in blackcurrant and redcurrant pomaces, such as delphinidin-3-glucoside and cyanidin-3-glucoside (Mikulic-Petkovsek e al., 2015), were not found, which was due to the fact that the analyzed material only included seeds and no other fruit tissues where such compounds occur. However, some authors reported low quantities of anthocyanins (3–6 mg/100 g), such as delphinidin and cyaniding glycosides, in black currant seeds (e.g., Bakowska-Barczak et al., 2009), which can be due to an incomplete removal of pulp tissues from seeds.

A) Benzoic acid derivatives



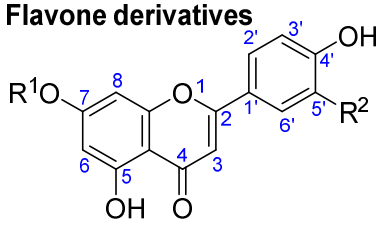
1: 3,4-Dihydroxybenzoic acid R^1=OH R^2=OH R^3=H
2: Salicylic acid R^1=H R^2=H R^3=OH
4: Vanillic acid R^1=OMe R^2=OH R^3=H
6: 4-Hydroxybenzoic acid R^1=H R^2=OH R^3=H

B) Cinnamic acid derivatives



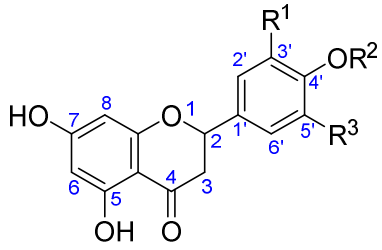
3: Caffeic acid R^1=OH R^2=OH R^3=H
5: *p*-Coumaric acid R^1=H R^2=OH R^3=H
7: Sinapic acid R^1=OMe R^2=OH R^3=OMe
9: Ferulic acid R^1=OMe R^2=OH R^3=H

C) Flavone derivatives



17: Luteolin R^1=H R^2=OH
24: Apigetrin R^1=Glc R^2=H

D) Flavanone derivatives



18: Jucein R^1=OH R^2=Glc R^3=H
21: Eriodictyol R^1=H R^2=H R^3=OH
25: Naringenin R^1=H R^2=H R^3=H

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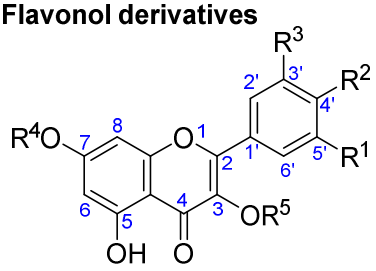
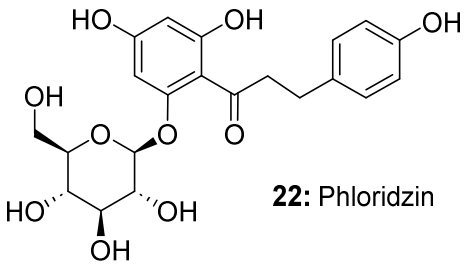
E) Flavonol derivatives



Glc: glucopyranoside
Rha: rhamnopyranosidr
Rut: rutinoside

8: Populnin	R^1=H	R^2=OH	R^3=OH	R^4=Glc	R^5=H
10: Quercetin	R^1=H	R^2=OH	R^3=OH	R^4=H	R^5=H
11: Isoquercitrin	R^1=OH	R^2=OH	R^3=H	R^4=H	R^5=Glc
12: Rutin	R^1=OH	R^2=OH	R^3=H	R^4=H	R^5=Rut
13: Kaempferol	R^1=H	R^2=OH	R^3=H	R^4=H	R^5=H
14: Quercitrin	R^1=H	R^2=OH	R^3=OH	R^4=H	R^5=Rha
15: Myricetin	R^1=OH	R^2=OH	R^3=OH	R^4=H	R^5=H
16: Fisetin	R^1=OH	R^2=OH	R^3=H	R^4=H	R^5=H
19: Astragalin	R^1=H	R^2=OH	R^3=H	R^4=H	R^5=Glc
20: Nicotiflorin	R^1=H	R^2=OH	R^3=H	R^4=H	R^5=Rut
23: Galangin	R^1=H	R^2=H	R^3=H	R^4=H	R^5=H

F) Dihidrochalcone derivative



22: Phloridzin

Figure 1. Structure of phenolic compounds detected in *Ribes* seeds.

As for the distribution of compounds among the various taxa, the highest diversity of compounds was detected in *Ribes* 'Algo' Yakutskaya and *R. nigrum* 'Koksa', while the compounds that were identified in all *Ribes* samples were ferulic and 4-hydroxybenzoic acids. Some compounds were restricted to few taxa; for instance, eriodictyol and phloridzin occurred only in *R. nigrum* 'Koksa', *Ribes* 'Algo' Yakutskaya, and *Ribes* 'Myuryucheene'. It has not been possible to establish a correlation between the presence of phenolic compounds and taxonomic category. This is interpreted as meaning that, within *Ribes* genus, environmental factors (temperature, soil, light, fertilizer, etc.) are more decisive in terms of the occurrence of phenolic compounds than any genetic proximity. This has been investigated in berries, and it has been reported that TPC was higher in fruits cultured in the north than in the south, and also that high radiation and temperature were associated with low contents of the major phenolic compounds in all the cultivars studied (Yang et al., 2013). However, the influence of the environment on the phenolic content of seeds remains unstudied.

Some authors reported flavonols as the main phenolic group in black currant seeds. Among flavonols, quercetin-3-glucoside, myricetin-3-glucoside, and kaempferol-3-glucoside were the major compounds detected (Bakowska-Barczak et al., 2009). In this study, kaempferol-7-glucoside was detected instead the 3-glusoside, in addition to the aglycone of these compounds, but not the glycosides of myricetin. Any case, most compounds found here were previously reported from *Ribes* species. For instance, Wójciak et al. (2022), reported for black currant seeds glucosides and rutinosides derivatives of quercetin and kaempferol, besides the aglycones of most compounds reported here. However, to the best of our knowledge, this work constitutes the first report for some compounds detectedt in *Ribes* taxa: e.g., fisetin, luteolin, eriodictyol, phloretin, galangin, and naringenin, as well as some of their glycosides.

The great variety of flavonoids found in the focused *Ribes* seeds has deep significance for health. Such compounds exhibit high activity against several diseases including cancer, without showing significant toxicity towards normal cells. Flavonoids can enhance drug sensitivity and suppress proliferation, metastasis, and angiogenesis of cancer cells by modulating several oncogenic or oncosuppressor microRNAs (miRNAs, miRs) (Tuli et al., 2023). For instance, quercetin is active against lung, breast, and prostate cancer cells, and luteolin is active against glioblastoma and colon

 doi:10.20944/preprints202402.1139.v1

cancers (Tuli et al., 2023). The same is true for flavonoid glycosides; e.g., populnin (kaempferol-7-*O*-glucoside) has potent anti-Herpes simplex virus activity, and significant anti-HIV-1 reverse transcriptase activity, which leads to consider it as an anti-HIV potential drug for the early treatment of HIV infection (Behbahani et al. 2014).

Previous reports indicated that berries cultivated in cold climates accumulate significantly higher levels of phenolic compounds than those grown in milder climates. In this way, flavonoids are accumulated in response to abiotic stresses such as low temperature, which increased the abundance of enzymes involved in flavonoid biosynthesis and the expression of flavonoid biosynthesis genes in various plant species (Schulz al., 2016). Consistently, the great diversity of phenolic compounds found in this work could be interpreted considering that plants were cultivated in very difficult climatic conditions of Siberia (Yakutia and Krasnoyarsk krai). These are the coldest regions of Russia, characterized by a protracted cold season and exceptionally low winter temperatures (-40 °C). Over time, the evolutionary processes in these challenging conditions have shaped a unique gene pool in plants, endowing them with complex resistance, such as frost resistance, high levels of biologically active compounds and key nutritional components. In fact, *Ribes* cultivars from Yakutia are characterized by higher levels of polyunsaturated FA (PUFA) than other *Ribes* species, particularly GLA (Lyashenko et al., 2019).

2.3. Antiproliferative Activity of the Water:Methanol Seed Extracts on HT-29 Cancer Cells

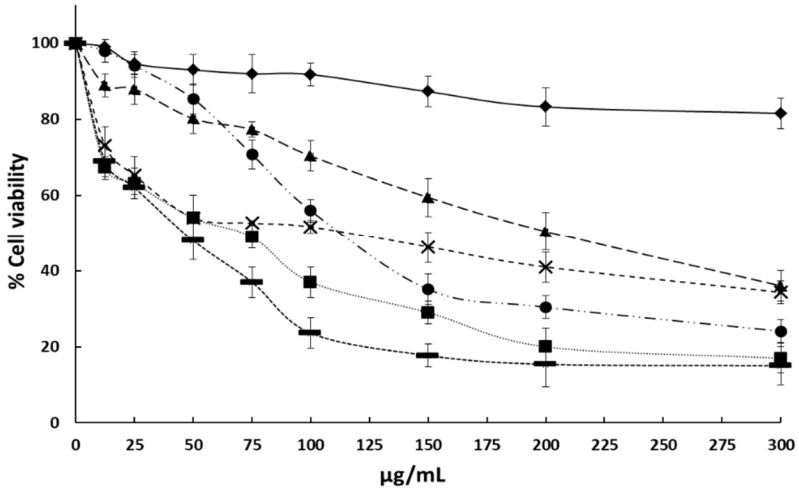
To determine the in vitro anticancer activity, we selected blackcurrant cultivars from Yakutia, since their seed oils showed high content of GLA (Lyashenko et al., 2019). A sample of red currant (*R. rubrum*) was also checked with comparative purposes since it is a widely cultured and commercialized berry. Previously, the antitumor activity of phenolic extracts from fruits and leaves of *Ribes* species has been studied. For instance, the apoptotic effects and mechanisms of blackcurrant extracts in MKN-45 (human gastric adenocarcinoma) and TE-1 (human esophageal cancer) cells were assessed. It was demonstrated that such extracts induced caspase-dependent apoptosis through downregulation of Bcl-2, a mitochondrial pathway involving activation of p38 (mitogen-activated protein kinases) and JNK (c-Jun N-terminal kinase), and inactivation of Akt (a central kinase that controls diverse processes including cell survival and apoptosis). Thus, such extract has been proposed as a potential candidate for cancer therapy (Liu and Li, 2016). However, the antitumor activity of phenolic-rich seed extracts of any *Ribes* species remains unchecked.

Figure 2 shows the results of the MTT assay. The concentration-response plots for HT-29 cells after exposure to seed extracts after 48 and 72 h of treatment are drawn in Figures 2A and 2B, respectively, while GI_{50} for the previous assays and for ferulic acid and doxorubicin (positive control) is depicted in Figure 3. The Selectivity Index (SI) for 72-h exposed cells to seeds extracts is shown in parentheses over columns. After 48 and 72 h of treatment, the MTT assay revealed concentration- and time-dependent inhibitory effects on HT-29 cells for all assayed extracts. Cell viability at 48 h at the maximum concentration tested (300 μg/mL) and for the different species, was ~20% lower than that obtained at 72 h. After 72 h culture, cells growth inhibition was exercised much better by *R. nigrum* 'Koksa' and *Ribes* 'Erkeeni', which shows a GI_{50} value of 37 and 42 μg/ml. All assayed *Ribes* extracts, except *R. nigrum* 'Hara katarlik', showed activity higher than *R. rubrum* extract (GI_{50} at 72 h of cell exposure to extract of 99 μg/ml). The extracts of *R. nigrum* 'Hara katarlik' showed an undetermined GI_{50} value higher than 300 μg/mL. Interestingly, the highly bioactive extract from *Ribes* 'Erkeeni' contains all detected phenolics, while *R. nigrum* L. 'Koksa' lacks only populnin. Therefore, the high bioactivity found for such extracts could be due to a synergy between all the compounds detected. However, need to be considered that the polysaccharide-rich fraction from *R. nigrum* has been characterized as highly bioactive (Yang et al., 2020), and that the water:methanol extracts obtained from the seeds assayed in this work can include some amounts of polysaccharides, thus, a synergy between phenolic compounds and seed polysaccharides could be also responsible for the noted antitumor effects against HT-29 cells, especially considering that these cells are not too sensitive to phenolic compounds (Lyashenko et al., 2021; Chelh et al., 2022). Finally, the selectivity index (SI) of HT- 29 versus normal cells CCD-18 was calculated (see Material and Methods section, 3.7). An extract

 doi:10.20944/preprints202402.1139.v1

with SI value greater than 2 is considered as high selectivity against cancer cells, whereas one with SI value less than 2 demonstrates general toxicity to normal cells (Vichitsakul et al., 2023). Such value for any research on herbal drug and/or isolated compound is critical for determining whether further research can be continued (Peña-Morán et al., 2016). SI at 72 h ranged from 17 (*Ribes* 'Myuryucheene') to 32 (*Ribes* 'Erkeeni'), from which it can be concluded that the seed extracts of *Ribes* have a high selectivity against HT-29 human colorectal cancer cells. This means that the extracts evaluated here constitute promising candidates for obtaining active compounds against colorectal cancer.

(**a**)



(**b**)

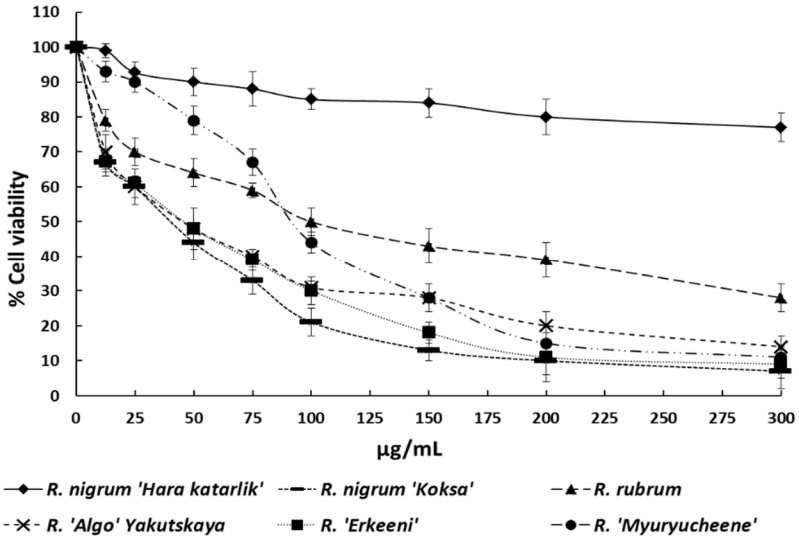


Figure 2. MTT assay. A: Concentration-response plot for HT-29 cells after exposure to seed extracts for 48 (A) and 72 h (B). Data represent the mean of three complete independent experiments ± SD (error bars).

 doi:10.20944/preprints202402.1139.v1

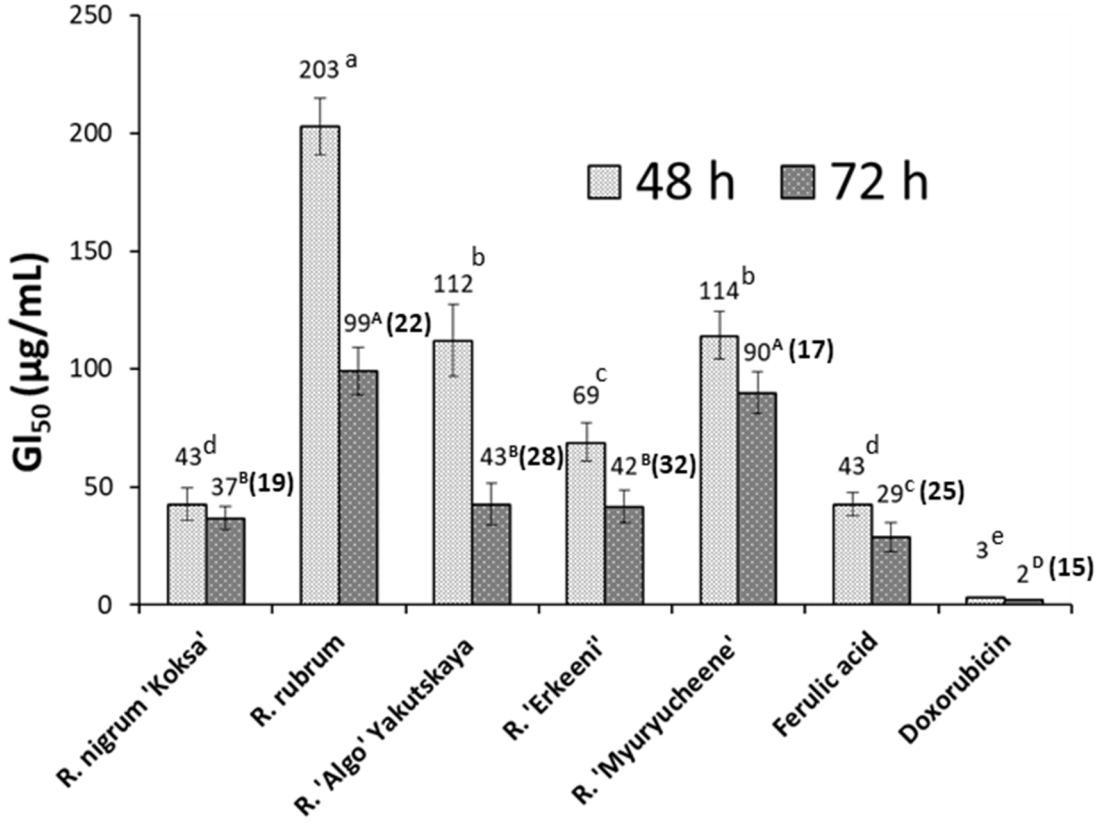


Figure 3. MTT assay. GI_{50} after HT-29 cells exposure for 48 and 72 h to seed extracts, as well as to ferulic acid and doxorucicin (positive control). The GI_{50} value is detailed over columns, and the Selectivity Index (SI) for 72-h exposed cells to seeds extracts is shown in parentheses. Data represent the mean of three complete independent experiments ± SD (error bars). In a bar, means followed by different lower case letters (for 48 h treatment) and capital letters (for 72 h) are significantly different at $p<0.05$.

Conclusions

As shown, the seeds of some unexplored taxa belonging to *Ribes* genus constitute potentially raw sources of healthy phenolic compounds-rich seed oils, in addition to their already known GLA-rich FA profiles. Among the different taxa analyzed here highlights *R. nigrum* cultivars, due to the diversity of compounds they show, especially *Ribes* 'Algo' Yakutskaya and *R. nigrum* 'Koksa'. The compounds that were identified in all *Ribes* samples were ferulic and 4-hydroxybenzoic acids, while some compounds were restricted to few taxa. It was not possible to establish a correlation between the presence of phenolic compounds and taxonomic rank and, probably, environmental factors were more decisive for compound occurrence than any genetic proximity. It highlights the antiproliferative activity of some seed extracts against HT-29 cells. Cell growth inhibition was strongly effected by *R. nigrum* 'Koksa' and *Ribes* 'Erkeeni', which shows very low GI_{50} values and contains most identified phenolics. Future research on this subject should be carried out to elucidate the composition of other components of the unsaponifiable of these oils, such as sterols and tocols, while it is recommended to obtain *Ribes* oils exclusively by cold pressing, so that they can be enriched in the healthy components contained in the unsaponifiable fraction. Other actions should be carried out to deepen the knowledge of the quantification of the detected phenolics, and their individual action against various cancer cell lines.

3. Materials and Methods

3.1. Reagents and Chemicals

 doi:10.20944/preprints202402.1139.v1

Unless otherwise indicated, all chemicals and solvents were purchased from Merck (Madrid, Spain). Aluminum chloride and sodium carbonate were obtained from Sigma-Aldrich Co. (St Louis, MO, USA). Water was purified using a Milli-Q system (Millipore, Burlington, MA, USA). All the chemicals used, including the solvents, were of analytical grade.

3.2. Plant Material

Data on analyzed *Ribes* seeds are detailed in Table 3. Seeds were donated by several botanical gardens. *R. alpinum* (1B) seeds were collected three well-differentiated subpopulations from their natural habitats in Sierra de Baza (Granada, Spain). Upon receipt, after cleaning, adequate amounts of each sample (2 g) were used for moisture analysis. This was carried out in a forced air oven at 103 ºC for 8 h, and all results in tables and figures are expressed on dry weight (dw) basis. Moisture content ranged from 7.1 (*R. rubrum*) to 8.3 g/100 g (*R. Erkeeni*). The remaining seeds were labeled and placed in plastic containers at -18 ºC until lab analysis. Immediately before starting each experiment, seeds were dried and grounded into fine powder using mortar and pestle.

3.3. Seed oil Extraction

Wild-collected seeds were separated from the pulp, exposed to air to dry at room temperature, and ground into powder. The powders from all seeds were analyzed without delay after crushing and the oil content was determined by the Weibull and Stoldt method (AOAC, 2000).

3.4. Extraction of Phenolics from Ribes Seeds

Extraction and analysis of phenolic compounds from *Ribes* seeds were carried out according to Lyashenko et al. (2021) with some modifications. Powdered seeds (~0.2 g) were extracted three times with 3 mL of methanol:water, (60:40, v/v). After centrifuging at 1,000×g for 10 min, the supernatants were collected, combined and the solvent was evaporated under vacuum at 60 ºC to dryness. The residue was dissolved in 1 mL of methanol:water (60:40, v/v), and filtered through a 0.22 μm membrane filter prior to the chromatographic analysis.

3.5. Determination of Total Phenol Content

Total phenolics content (TPC) was measured using the F-C assay as developed by Singleton et al. (1999) with minor modifications. Briefly, 10 μL of phenolic seed extracts, prepared as above described, 0.79 mL of MiliQ water and 50 μL of the F-C reagent were mixed, vortex and allowed to stand for 5 min at room temperature. Next, 150 μL of a 20% sodium carbonate solution were added and vortex. A control sample was also prepared. After incubation at room temperature for 2 h in darkness, the absorbance of the mixture was read at 765 nm on a UV-VIS spectrophotometer using either water as blanks. The results were expressed as mg of Caffeic Acid Equivalents (CAE) per 100 g of sample using a standard curve of caffeic acid (ranging from 50 to 900 μg/mL). Determinations were done in triplicate.

3.6. Characterization of Phenolics by Liquid Chromatography-Mass Spectrometry

The chromatographic separation was performed on a Thermo Fisher Scientific Transcend 600 LC (Thermo Scientific Transcend™, Thermo Fisher Scientific, San Jose, CA, USA) by using a Hypersil Gold column (250 x 4.6mm, 5 μm). A flow rate of 0.65 mL/min was set. The compounds were separated with gradient elution using aqueous acetic acid (acetic acid: H_2O, 1:99, v/v) (A) and methanol (B) as eluents at ambient temperature. The step gradient was as follows: 0–20 min 80% of A; then, it was linearly decreased to 25% in 10 min and remained constant during 10 min. Later, it was increased to 80% in 10 min and remained constant during 5 min. The total running time was 55 min. The column temperature was 25 °C, and the injection was 10 μL.

The LC system is coupled to a single mass spectrometer Orbitrap Thermo Fisher Scientific (Exactive™, Thermo Fisher Scientific, Bremen, Germany) using an electrospray interface (ESI) (HESI-II, Thermo Fisher Scientific, San Jose, CA, USA) in positive and negative ion mode. ESI parameters

 doi:10.20944/preprints202402.1139.v1

were as follows: spray voltage, 4 kV; sheath gas (N_2>95%), 35 (adimensional); auxiliary gas (N_2>95%), 10 (adimensional); skimmer voltage, 18 V; capillary voltage, 35 V; tube lens voltage, 95 V; heater temperature, 305 °C; capillary temperature, 300 °C. The mass spectra were acquired employing two alternating acquisition functions: (1) full MS, ESI+, without fragmentation (higher collisional dissociation (HCD) collision cell was switched off), mass resolving power = 25,000 FWHM; scan time = 0.25 s; (2) all-ions fragmentation (AIF), ESI+, with fragmentation (HCD on, collision energy 30 eV), mass resolving power = 10,000 FWHM; scan time = 0.10 s, (3) full MS, ESI- using the aforementioned settings and (4) AIF, ESI- using the settings explained for (2). Mass range in the full scan experiments was set at *m*/*z* 50–1000. LC chromatograms were acquired using the external calibration mode and they were processed using Xcalibur™ version 3.0, with Qualbrowser and Trace Finder 4.0 (Thermo Fisher Scientific, Les Ulis, France). Unknown analysis was carried out with Compound Discoverer™ version 2.1.

3.7. Cell assays on Cancer and Normal Cell Lines

The anticancer activity was determined for seed extracts from *Ribes* cultivars, and *R. rubrum* extract, a widely used commercial *Ribes* species, was used for comparison. The HT-29 colon cancer cells line and the CCD-18 colonic human myofibroblasts cells line was used to check antiproliferative activities. Cultures were supplied by the Technical Instrumentation Service of University of Granada (Granada, Spain). First, it was checked for the absence of *Mycoplasma* and bacteria. Then, cells were grown at 37 °C and 5% CO_2 humidified atmosphere in medium RPMI-1640 supplemented with 5% fetal bovine serum, 2 mM L-Glutamine, 1 mM sodium pyruvate, 0.125 mg/mL amphotericin and 100 mg/mL penicillin-streptomycin.

All cultures were plated in 25 cm^2 plastic tissue culture flasks (Sarstedt, USA). All culture media and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cell culture and cell assay, that is, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test were accomplished as previous described (Ramos-Bueno et al., 2016).

In the MTT assay, cells were divided into 96-well microtiter plates, adjusted of 1×10^4 cell/well and cultivated in medium at 37°C, 5% CO_2 prior to adding the different extracts dissolved in medium. The phenolics-containing extracts were supplied to cells dissolved in a mixture of methanol:water, (60:40, v/v), and then in the culture medium at designed concentrations (0–300 μg/mL). After 48 and 72 h of cell exposure, 5 mg/mL of an MTT solution was added to the culture medium to determine the viability of cells. The absorbance was recorded at 570 nm on an enzyme-linked immunosorbent assay (ELISA) plate reader (Thermo Electron Corporation, Sant Cugat del Valles, Barcelona, Spain). The formazan crystals produced were solubilized using 100 μL dimethylsulfoxide (DMSO). Cells without phenolic extracts were considered as negative controls.

Cell survival in exposed cultures relative to unexposed cultures (negative controls) was calculated, and the number of viable cells was calculated using the following equation: Percentage of viable cell (%) = (Absorbance of treated cells/Absorbance of untreated cells) × 100%.

The concentrations causing 50% cell growth inhibition (GI_{50}) were calculated from the growth curves. Doxorubicin (98.0–102%, D1515), from Sigma-Aldrich (Madrid, Spain) was used as a positive control, while DMSO and methanol were used as the negative (vehicle) controls. Phenolic extracts and controls were evaluated in three independent assays. Values presented are mean ± standard error of the mean. The selectivity index (SI) of each compound was calculated as GI_{50} of the extract against the CCD-18 normal cell line/GI_{50} of the same extract against the HT-29 cancer cell line (Vichitsakul et al., 2023).

3.8. Statistical Analysis

Data on seeds from botanical gardens correspond to the analyses effected to seeds received in a single shipment, which were analyzed three times in triplicate each. Seeds from the wild were collected from three different species populations and each of which was analyzed in triplicate. All data in tables were analyzed using one-way ANOVA (Statgraphics Centurion XVI.I, Warrenton, VA,

 doi:10.20944/preprints202402.1139.v1

USA) and expressed as the average ± SD. Differences among mean values were tested by Duncan's test at $P<0.05$.

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Data availability statement: The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

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Conflicts of Interest: The sponsors had no role in the design, execution, interpretation, or writing of the study.

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