applied sciences

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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 analyzed through the Folin–Ciocalteu procedure, while LC coupled to a single mass spectrometer (MS) Orbitrap using an electrospray interface (ESI) was performed to determine the phenolic profiles. 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 for *R. nigrum* 'Hara katarlik', showed higher activity than *R. rubrum* (GI_{50} at 72 h: 99 μg/mL). Interestingly, the extract from *Ribes* 'Erkeeni', which exhibited high bioactivity, contains all detected phenolic compounds, unlike *R. nigrum* 'Koksa', which lacks only populnin. Therefore, the high bioactivity found for such extracts could be due to a synergy of 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

Citation: Lyashenko, S.; López-Ruiz, R.; García-Cervantes, A.M.; Rodríguez-García, I.; Yunusova, S.; Guil-Guerrero, J.L. Phenolic Profiles and Antitumor Activity against Colorectal Cancer Cells of Seeds from Selected *Ribes* Taxa. *Appl. Sci.* **2024**, *14*, 2428. https://doi.org/10.3390/app14062428

Academic Editor: Maria Filomena Botelho

Received: 19 February 2024
Revised: 6 March 2024
Accepted: 11 March 2024
Published: 13 March 2024

1. Introduction

The genus *Ribes* belongs to the family Grossulariaceae, and it includes more than 150 diploid species, distributed in the temperate latitudes of South America and the Northern Hemisphere [1]. Nowadays, about 12 species of *Ribes* are cultivated to harvest their fruits, almost all of them being 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.) [2]. Black currants are fast-expanding crops that generate substantial income, as they are among the most valued by consumers after strawberries [2,3].

Ribes fruits, which are consumed both fresh and dehydrated, achieve high demand in international markets due to their good nutritional qualities and appropriate sensorial attributes, in addition to being considered foods of high functional value [4]. Furthermore, *Ribes* seed oil has immunomodulation and anti-inflammatory effects, i.e., the use of blackcurrant seed oil in preventing illnesses, like hypertension, psoriasis, and atopic dermatitis, has been reported [5,6].

Moreover, the fruit industry generates an increased number of fruit by-products each year, increasing the tonnage amount of seeds, and the seeds of some *Ribes* taxa contain valuable amounts of γ-linolenic acid (GLA, 18:3*n*-6), which can account for more than 20% of total fatty acids (FAs) [7,8]. Furthermore, blackcurrant seed oil, rich in GLA, is also appreciated for its phenolic composition [9], which constitutes a large fraction of the unsaponifiable material (the lipid fraction that is not formed by fatty compounds) of most vegetable oils [7].

The biochemical composition of European currants has been previously described, especially 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* taxa and cultivars are extremely limited. Considering the benefits of the unsaponifiable components in seed oil and the limited information on various *Ribes* taxa, this study aimed to unravel the phenolic profiles and the in vitro antiproliferative activity against colorectal cancer cells of the phenolic-containing seed extracts from selected *Ribes* taxa. All studied species/cultivars have been previously typified as potential GLA producers [7].

2. Materials and Methods

2.1. Reagents and Chemicals

All the chemicals used, including the solvents, were of analytical grade. Water was purified using a Milli-Q system (Millipore, Burlington, MA, USA). Aluminum chloride (99% purity), doxorubicin (98.0–102%, D1515), and sodium carbonate (99.5% purity) were obtained from Sigma-Aldrich Co. (St Louis, MO, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (98% purity), acetic acid (≥99.8%), amphotericin (suitable for cell culture), caffeic acid (≥98.0%), dimethylsulfoxide (DMSO, ≥99.7%, suitable for cell culture), F-C reagent, L-glutamine (suitable for cell culture), methanol (LC grade), hydrochloric acid (ACS reagent, 37%), penicillin-streptomycin (suitable for cell culture), petroleum ether (puriss. p.a., ACS reagent), and sodium pyruvate (suitable for cell culture, ≥99%) were purchased from Merck (Madrid, Spain).

2.2. Plant Material

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

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

Code	Samples	Sample Location	Total Oil Content g/100 g Seeds [1,2,3]	TPC (mg CAE/g Seeds) [1,2,3]	TPC (mg CAE/g Oil) [1,2,3]	Botanical Garden Accession Number
		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}	45
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}	79
		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}	41
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}	47
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}	50

Table 1. *Cont.*

Code	Samples	Sample Location	Total Oil Content g/100 g Seeds [1,2,3]	TPC (mg CAE/g Seeds) [1,2,3]	TPC (mg CAE/g Oil) [1,2,3]	Botanical Garden Accession Number
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]	49
6	*Ribes* '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]	48
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]	52
8	*Ribes* 'Myuryucheene'	Botanical Garden of North-Eastern Federal University, Yakutsk, Russia Sect. Ribes (Red Currants)	17.7 ± 0.6 [c]	34.4 ± 1.9 [de]	6.1 ± 0.4 [f]	51
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]	46
10	*R. triste*	Botanical Garden of North-Eastern Federal University, Yakutsk, Russia	18.5 ± 0.5 [bc]	31.2 ± 2.9 [e]	5.8 ± 0.3 [fg]	41
11	*R. rubrum*	Dendropark "Alexandria" NAS of Ukraine, Belaja Tserkov, Ukraine	15.0 ± 0.2 [e]	25.8 ± 3.1 [f]	3.9 ± 0.1 [i]	- [4]

[1] Data represent means ± standard deviation of samples analyzed in triplicate; [2] differences in TPC amounts were tested according to one-way ANOVA followed by Duncan's test; [3] within a column, means followed by different superscript lower-case letters (a–i) are significantly different at $p < 0.05$; [4] included in the catalog of woody plants of the Oleksandria Dendrological Park of the National Academy of Sciences of Ukraine without numbering.

2.3. Seed Oil Extraction

The seeds from wild-collected fruits 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 gravimetrically determined by the Weibull and Stoldt method [10]. For this, seeds were heated with hydrochloric acid to denature proteins and release bound lipids. After digestion, the samples were filtered before oil determination by solvent extraction using petroleum ether.

2.4. Extraction of Phenolics from Ribes Seeds

Extraction and analysis of phenolic compounds from *Ribes* seeds were accomplished according to Lyashenko et al. [11], with some modifications. All powdered seed samples (~0.2 g) were extracted three times with 3 mL of methanol/water (60:40, v/v). After experimenting with different solvent ratios, the ratio between both solvents was selected because it yields the highest amount of phenolics. After centrifuging at 1000× g for 10 min, the supernatants were collected and combined, and the solvent was evaporated under vacuum at 60 °C 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 before chromatographic analysis. Determinations were affected in triplicate.

2.5. Determination of Total Phenol Content

TPC was determined using the F-C method as developed by Singleton et al. [12], with minor modifications. Briefly, 10 µL of phenolic seed extracts, prepared as described above, 0.79 mL of MiliQ water, and 50 µL of the F-C reagent were mixed, vortexed, and allowed to stand for 5 min at room temperature. Next, 150 µL of a 20% sodium carbonate solution was added and vortexed. 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 water as a blank. The results were expressed as mg of Caffeic Acid Equivalents (CAEs) per 100 g of sample using a standard curve of caffeic acid (ranging from 50 to 900 µg/mL). Such a standard was used to express the total phenolic content because it is widely distributed in the analyzed samples. Determinations were performed in triplicate.

2.6. Characterization of Phenolics by Liquid Chromatography–Mass Spectrometry

Chromatographic separation was performed on a Thermo Fisher Scientific Transcend 600 LC (Thermo Scientific Transcend™, Thermo Fisher Scientific, San Jose, CA, USA) using a Hypersil Gold column (250 × 4.6 mm, 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 MS 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 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; and 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-ion 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). The 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). An unknown analysis was carried out with Compound Discoverer™ version 2.1.

Sensitivity and Specificity of the LC-MS Technique

In LC-Orbitrap MS, sensitivity is often excellent due to the high-resolution capabilities of the Orbitrap mass analyzer and the sensitivity of modern LC systems. The instrument was able to detect analytes at parts-per-trillion (ppt) range.

Specificity refers to the ability of the LC-Orbitrap MS system to differentiate between analytes of interest and other compounds present in the sample matrix. The high resolution and mass accuracy of the Orbitrap mass analyzer contribute to excellent specificity by enabling precise determination of the mass-to-charge ratios (m/z) of analytes (up to 5 decimal points). Additionally, LC separation prior to MS analysis helps to resolve complex mixtures, further enhancing specificity.

2.7. 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 were used to check antiproliferative activities. Cultures were supplied by the Technical Instrumentation Service of the University of Granada (Granada, Spain). First, they were 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, Newton, NC, USA). Cell culture and cell assay, that is, the MTT test, were accomplished as previously described [13].

In the MTT assay, cells were divided into 96-well microtiter plates, adjusted to 1×10^4 cells/well, and cultivated in a medium at 37 °C and 5% CO_2 prior to adding the different extracts dissolved in the medium. The phenolic-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 im-

munosorbent assay (ELISA) plate reader (Thermo Electron Corporation, Sant Cugat del Valles, Barcelona, Spain). The formazan crystals produced were solubilized using 100 µL of DMSO. Cells without phenolic extracts were considered negative controls, which were used for all concentrations and tested extracts. Cell survival in exposed cultures relative to unexposed cultures was calculated, and the number of viable cells was calculated using the following equation:

Percentage of viable cells (%) = (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 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 [14].

2.8. Statistical Analysis

Data on seeds from botanical gardens correspond to the analyses effected by seeds received in a single shipment, which were analyzed three times each. Seeds from the wild were collected from three different species populations, each of which was analyzed in triplicate. The statistical significance was calculated using Student's *t*-tests and one-way analysis of variance (ANOVA) using Statgraphics Centurion XVI.I (Warrenton, VA, USA) and expressed as the average ± SD. Differences among mean values were tested by Duncan's test at $p < 0.05$ and for antiproliferative activity at $p < 0.05$ and $p < 0.01$.

3. Results and Discussion

3.1. Total Phenolics and Oil Content

Table 1 shows the amount of oil content, the total phenolic content (TPC) in mg of caffeic acid equivalents (CAE)/g seeds and mg of CAE/g oil, and data on GLA content in seeds (% of total FA) previously reported by our Research Team [7]. 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 has a lower oil content compared to 1B but shows higher TPC, while *R. pulchellum* stands out with the highest content of oil and TPC. 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 is 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, it hosts healthy properties related to such compounds.

In short, a proper selection of *Ribes* varieties can significantly influence the number 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 on the seeds of other *R. nigrum* cultivars revealed that 'Ben Tirran' and 'Ben Sarek' 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 Equivalent (GAE)/g seed residue, respectively [9]. However, Van Hoed et al. [15] indicated figures obtained by the F-C methodology in the range shown here for several *Ribes* cultivars (5.6–11.3 mg CAE/g oil). Other works that focused on residues from the extraction of *Ribes* fruits cannot be compared with the results obtained here since such works focused on the residual cake from the extraction of the fruit, which, in addition to the seeds, contains several other tissues of the fruit [16,17].

3.2. Phenolic Compound Profiles

The phenolic compound 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 the retention time (Rt) of analytes and m/z of molecular adduct and fragment ions. All compounds were properly identified, and the bases for the identification of each compound are described in Table 2.

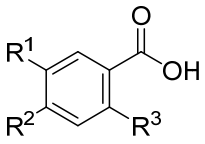
Among detected phenolics, four consisted of hydroxylated derivatives of benzoic acids (3,4-dihydroxybenzoic, salicylic, vanillic, and 4-hydroxybenzoic acid) (Figure 1A); four were cinnamic acid derivatives (caffeic, *p*-coumaric, chlorogenic, and ferulic acids) (Figure 1B); two were flavone derivatives (luteolin and apigetrin) (Figure 1C); and three were 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 [4], 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., [9]), which can be due to an incomplete removal of pulp tissues from seeds.

Three taxa among the four with the highest TPC, i.e., *R. nigrum* 'Koksa', *Ribes* 'Algo' Yakutskaya, and *Ribes* 'Erkeeni' (with 94.8, 48.9, and 49.0 mg CAE/g seeds, respectively), also showed the highest variety of phenolic compounds. *Ribes* 'Algo' Yakutskaya contained all detected compounds, while in *R. nigrum* 'Koksa' and *Ribes* 'Erkeeni', 24 out of 25 different detected compounds were detected.

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 the taxonomic category. This is interpreted as meaning that, within the *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 that high insolation and temperature positively correlated with low amounts of the main phenolic compounds in all the cultivars studied [18]. However, the influence of the environment on the phenolic content of seeds remains unstudied.

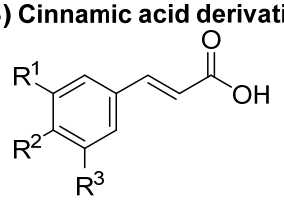
Some authors reported flavonols as the main phenolic group in black currant seeds. Among flavonols, quercetin-3-glucoside, myricetin-3-glucoside, and kaempferol-3-glucoside were the main compounds detected [9]. In this study, kaempferol-7-glucoside was detected instead of 3-glucoside, in addition to the aglycone of these compounds but not the glycosides of myricetin. In any case, most compounds found here were previously reported in *Ribes* species. For instance, Wójciak et al. [19], reported the black currant seed glucoside and rutinoside derivatives of quercetin and kaempferol, in addition to the aglycones of most compounds reported here. However, to the best of our knowledge, this paper is the first to report on some compounds occurring in *Ribes* taxa, such as fisetin, luteolin, eriodictyol, phloretin, galangin, and naringenin, as well as some of their glycosides.

(A) Benzoic acid derivatives



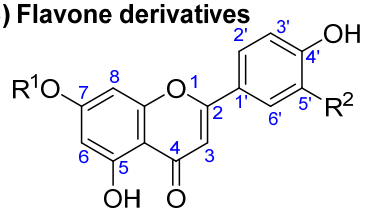
1: 3,4-Dihydroxybenzoic acid R^1=OH R^2=OH R^3=H
2: Salicylic acid R^1=H R^2=H R^3=OH
4: Vanillic acid R^1=OMe R^2=OH R^3=H
6: 4-Hydroxybenzoic acid R^1=H R^2=OH R^3=H

(B) Cinnamic acid derivatives



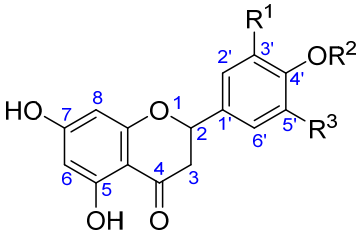
3: Caffeic acid R^1=OH R^2=OH R^3=H
5: *p*-Coumaric acid R^1=H R^2=OH R^3=H
7: Sinapic acid R^1=OMe R^2=OH R^3=OMe
9: Ferulic acid R^1=OMe R^2=OH R^3=H

(C) Flavone derivatives



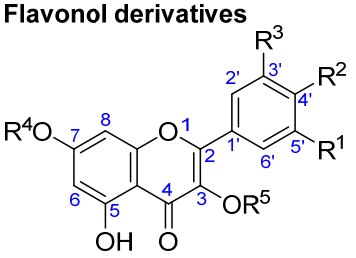
17: Luteolin R^1=H R^2=OH
24: Apigetrin R^1=Glc R^2=H

(D) Flavanone derivatives



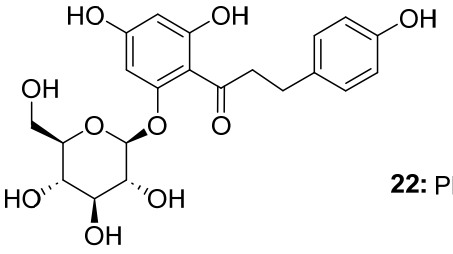
18: Jucein R^1=OH R^2=Glc R^3=H
21: Eriodictyol R^1=H R^2=H R^3=OH
25: Naringenin R^1=H R^2=H R^3=H

(E) Flavonol derivatives



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 (numbering as in Table 2); Glc: glucopyranoside; Rha: rhamnopyranoside; Rut: rutinoside.

The great variety of flavonoids found in the studied *Ribes* seeds has deep significance for health. Such compounds exhibit high activity against several illnesses, including cancer, but lack noticeable toxicity to normal cells. Particularly, flavonoids can boost drug sensitivity while suppressing the proliferation, metastasis, and angiogenesis of cancer cells by regulating several oncogenic or oncosuppressor microRNAs (miRNAs, miRs) [20]. For instance, quercetin is active against lung, breast, and prostate cancer cells, and luteolin is active against glioblastoma and colon cancers [20]. 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 considering it as an anti-HIV potential drug for the initial therapy of HIV infection [21].

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 increases the abundance of enzymes implicated in flavonoid biosynthesis and the expression of genes regulating this in several plant species [22]. Consistently, the great diversity of phenolic compounds found in this work could be interpreted considering that plants were cultivated in the 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 amounts of bioactive 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 [7].

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 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) [23]	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 $[M\text{-}H\text{-}CO]^-$. 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 [24]	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 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 [25]	1A,1B, 5B, 6, 7, 8, 9, 11

Table 2. *Cont.*

N	Rt Min	Mass [a] *m/z*	Adduct	Fragment [b]	Formula	Identification	Identification Basis	Occurrence in Samples [c]
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 ($[M-H\text{-}162]^-$	1B, 2, 4, 5A, 5B, 6, 7, 8
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) [26]	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 $[\text{quercetin-H-}CO_2\text{-CO}]^-$	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 a typical MS/MS fragment at m/z 151, which corresponded to retrocyclization on the A-C ring ($^{1,2}A^-$) and the consecutive loss of CO ($^{1,2}A^-$-CO) [27]	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, which correspond to fragmentation of the B ring ($^{1,2}B^-$) [28]	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 [29]	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 CH_2O from the aglycone (30 Da) [30]	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 ($^{1,3}B^-$) [28]	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 loss of 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
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 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, which correspond to the fragmentation of the B ring ($^{1,3}B^-$) [28]	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.

3.3. Antiproliferative Activity of the Water/Methanol Seed Extracts on HT-29 Cancer Cells

To determine the in vitro antiproliferative activity, we selected blackcurrant cultivars from Yakutia since their seed oils showed a high content of GLA [7]. A sample of red currant (*R. rubrum*) was also checked for 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 reported that such extracts induced caspase-dependent apoptosis through the downregulation of Bcl-2, a mitochondrial pathway involving the activation of p38 (mitogen-activated protein kinases) and JNK (c-Jun N-terminal kinase) and the inactivation of Akt (a central kinase that controls diverse processes, including cell survival and apoptosis). Thus, such an extract has been

proposed as a potential candidate for cancer therapy [31]. However, the antitumor activity of phenolic-rich seed extracts of any *Ribes* species remains unchecked.

Figure 2 shows the results of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (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 ferulic acid and doxorubicin (positive control) is depicted in Figure 3. The concentration range shown in Figures 2 and 3 (0–300 μg/mL) was selected because it allows visualizing an asymptotic approximation to a certain formazan absorption level corresponding to maximum decreasing of cell viability. The Selectivity Index (SI) for 72 h exposed cells to seed extracts is shown in parentheses over the columns. After 48 and 72 h of treatment, the MTT test revealed time- and concentration-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 of culture, cell growth inhibition was exerted much better by the seed extracts of *R. nigrum* 'Koksa' and *Ribes* 'Erkeeni', which show a GI_{50} value of 37 and 42 μg/mL. According to the threshold proposed by Suffness and Pezzuto [32], crude extracts showing a $GI_{50} \leq 100$ μg/mL can be considered to be cytotoxic and selected for further studies, whereas the most promising ones are those with a GI_{50} lower than 30 μg/mL. Thus, all seed extracts whose GI_{50} is detailed in Figure 3 merit further research for their fractionation until pure active compounds are isolated. After that, the mechanisms of action of such compounds against various cancer cell lines would be established according to proper experimentation.

All assayed *Ribes* extracts, except *R. nigrum* 'Hara katarlik', showed activity higher than the *R. rubrum* extract (GI_{50} at 72 h of cell exposure to the 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, it needs to be considered that the polysaccharide-rich fraction in *R. nigrum* has been characterized as highly bioactive [33], 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 [11,34]. Finally, the selectivity index (SI) of HT-29 versus normal cells CCD-18 was calculated (see Material and Methods, Section 2). An extract with an SI value greater than two is considered as high selectivity against cancer cells, whereas one with an SI value less than two demonstrates general toxicity to normal cells [14]. Such value for any research on herbal drugs and/or isolated compounds is critical for determining whether further research can be continued [35]. The 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. However, the data exposed here should be complemented with further experiments before considering the extracts checked in this work as anticancer drug candidates. This is because although the measurement of drug/extract dose–response in cultured cells is the cornerstone of the preclinical assessment of anticancer drugs, the transition from in vitro antitumor activity to clinical relevance requires cautious interpretation and further validation.

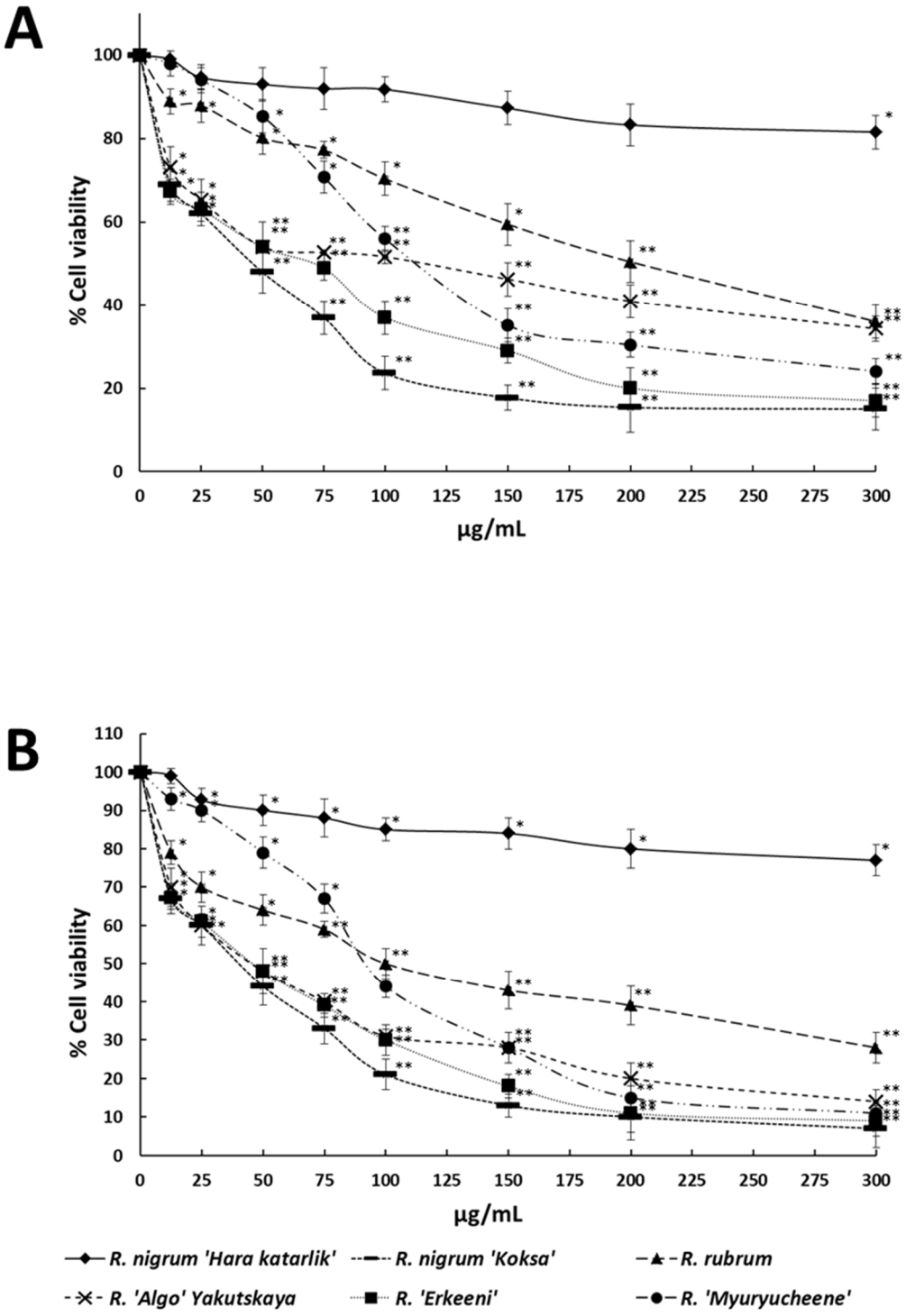


Figure 2. MTT assay. A: concentration–response plot for HT-29 cells after exposure to seed extracts for 48 (**A**) and 72 h (**B**). Data represent the mean of three complete independent experiments ± SD (error bars) with statistical significance equal to * $p < 0.05$; ** $p < 0.01$.

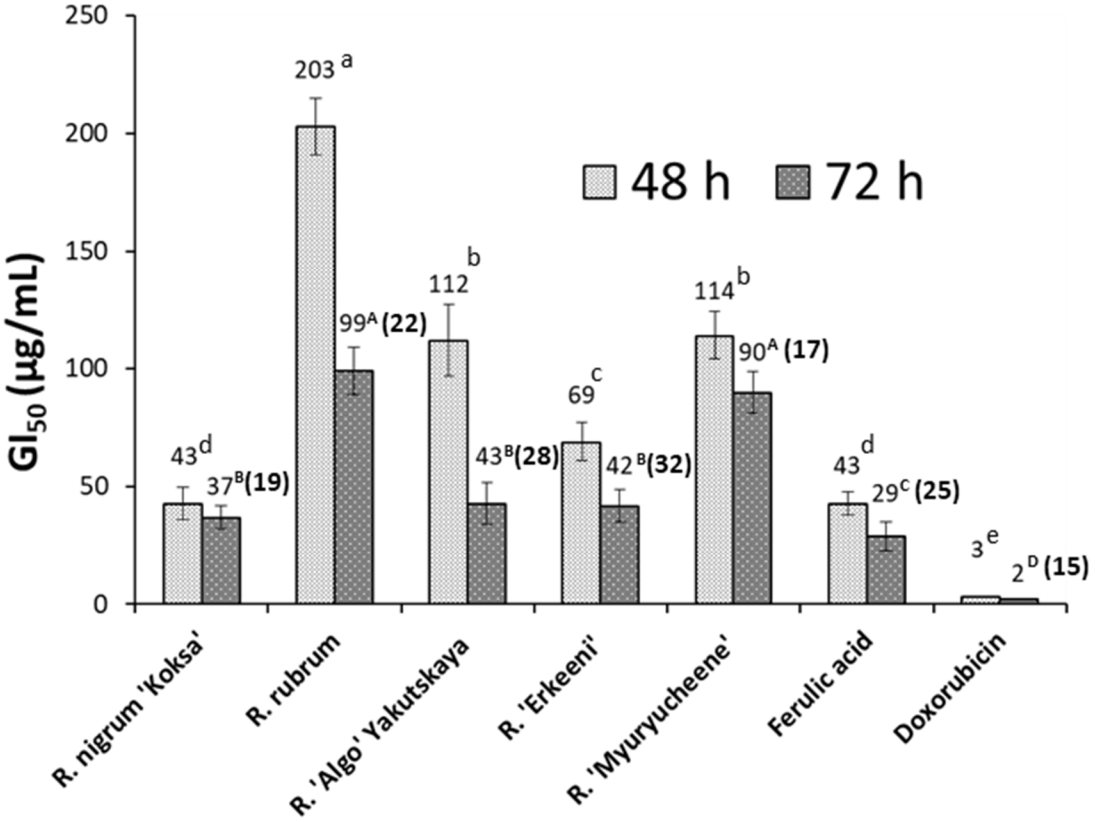


Figure 3. MTT assay. GI_{50} after HT-29 cell exposure for 48 and 72 h to seed extracts, as well as ferulic acid and doxorubicin (positive control). The GI_{50} value is detailed over the columns, and the SI for 72 h exposed cells to seed extracts is shown in parentheses. Data represent the mean of three 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 $p < 0.05$.

4. Conclusions

As shown, the seeds of some unexplored taxa belonging to the *Ribes* genus constitute potentially raw sources of healthy phenolic compound-rich seed oils, in addition to their already known GLA-rich FA profiles. Among the different taxa analyzed here, we highlight *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 a 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. This highlights the antiproliferative activity of some seed extracts against HT-29 cells. Cell growth inhibition was strongly affected by *R. nigrum* 'Koksa' and *Ribes* 'Erkeeni', which show very low GI_{50} values and contain 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, and 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 or combined actions against various cancer cell lines. Furthermore, research devoted to unraveling the molecular mechanisms underlying the antitumor activity displayed by each of the detected phenolics on colorectal cancer cells will be welcomed.

Author Contributions: Conceptualization, S.L., I.R.-G. and J.L.G.-G.; Methodology, S.L., R.L.-R. and J.L.G.-G.; Software, S.L., R.L.-R., I.R.-G. and J.L.G.-G.; Validation, S.L., R.L.-R., A.M.G.-C., I.R.-G., S.Y. and J.L.G.-G.; Formal Analysis, S.L., R.L.-R., A.M.G.-C., I.R.-G., S.Y. and J.L.G.-G.; Investigation, S.L., R.L.-R. and J.L.G.-G.; Resources, R.L.-R. and J.L.G.-G.; Data Curation, S.L., R.L.-R., A.M.G.-C., I.R.-G., S.Y. and J.L.G.-G.; Writing—Original Draft Preparation, S.L. and J.L.G.-G.; Writing—Review & Editing, S.L., I.R.-G. and J.L.G.-G.; Visualization, S.L., R.L.-R., A.M.G.-C., I.R.-G., S.Y. and J.L.G.-G.; Supervision, J.L.G.-G., Project Administration, R.L.-R. and J.L.G.-G.; Funding Acquisition, R.L.-R. and J.L.G.-G. All authors have read and agreed to the published version of the manuscript.

Funding: The authors acknowledge financial support from Junta de Andalucía (Project P20_00806), University of Almería (P_LANZ_2023/003), Campus de Excelencia Internacional Agroalimentario (ceiA3), and Centro de Investigación en Agrosistemas Intensivos Mediterráneos y biotecnología Agroalimentaria (CIAMBITAL), and the State Tasks 'FMRS-2022-0023-122031400260-7'.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the article, and further inquiries can be directed to the corresponding author.

Acknowledgments: The authors acknowledged the botanical gardens listed in Table 1 for providing plant material.

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

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