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Phytochemical Profiles and Anti-Glioma Activity of Bearberry *Arctostaphylos uva-ursi* (L.) Spreng. Leaf Extracts

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Abstract: The use of diversified raw materials and various extractant types is justified because the varied chemical composition of extracts obtained via extraction determines their biological activity. Therefore, the objective of this study was (i) to characterize the chemical profile of two types of bearberry extracts (70% ethanolic and water) and (ii) to investigate the biological activity of the analyzed extracts through an assessment of their possible proapoptotic effects on glioma cell lines. The HPLC-UV analysis of individual compounds was performed for the determination of the phytochemical profile of the bearberry extracts, and their total phenolic content (TPC) and total flavonoid content (TFC) were determined spectrophotometrically. The induction of apoptosis, autophagy, and necrosis in anaplastic astrocytoma MOGGCCM and human glioblastoma LN229 cell lines were investigated. The results indicated that the ethanolic (Et) and aqueous (Aq) extracts had different chemical profiles. The TPC in the Et was ca. 60% higher than in the Aq. Similarly, the TFC and methylarbutin (mARB) concentrations were significantly higher in the Et. On the other hand, the concentration of hydroquinone (HQ) was ca. 70% and that of corilagin (COR) was ca. 100% higher in the Aq. In turn, the presence of ursolic acid (UA) and oleanolic acid (OA) was confirmed solely in the Et. In contrast to Aq, Et demonstrated high proapoptotic activity. At the concentration of 2 µL/mL, the level of apoptosis varied between 14.7% and 26% in the case of the MOGGCCM cells and between 12.3% and 33.3% in the case of the LN229 cell line. The knowledge and information obtained in this study indicate a need for further research on the anticancer effect of the studied bearberry phytochemicals on the MOGGCCM and LN229 cell lines and for the elucidation of their molecular anticancer mechanisms.

Keywords: *Arctostaphylos uva-ursi*; chemical compounds; anaplastic astrocytoma MOGGCCM cell line; human glioblastoma LN229 cell line

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1. Introduction

Phytochemicals, mostly secondary metabolites obtained from different plant sources, are unique bioactive organic compounds involved in plant defense systems and plant

interactions with environmental factors [1,2]. In recent decades, medicinal plants and their molecules have gained considerable attention due to their potent biological activities [3–6]. Many of them are used as pharmaceuticals, nutraceuticals, food additives, and natural agents against pathogens [5]. Natural products provide a sustainable source with considerable efficacy to treat and overcome several disorders and fatal diseases [6]. Medicinal plants and their bioactive compounds also have potential as anticancer agents [4–8] and are one of the main sources of drugs [3,4,9]. Almost half of them are either natural compounds or directly derived therefrom [3,4]. Alternative treatments and therapies based on medicinal plants offer a wide range of benefits, especially for patients who are looking for options with fewer side effects compared to conventional medications [10].

Arctostaphylos uva-ursi (L.) Spreng., commonly known as bearberry, represents plants containing very interesting phytochemicals. For many years, the leaves of this plant have been used in traditional medicine [11]. The raw material is obtained through both wild harvesting and cultivation [12]. Due to the excessive pressure exerted by the collection of the raw material, many populations and habitats of bearberry have been destroyed; hence, the species is subject to strict protection and its collection is controlled in several countries [13,14]. This wild perennial shrub is widely distributed in the circumboreal area of Europe, North America, and Asia, and is a source of raw material for the pharmaceutical and herbal medicine sectors [12,15,16]. Its leaves contain several active compounds, including arbutin, hydroquinone, tannins, and flavonoids, which contribute to its therapeutic effects [16,17]. Arbutin, one of its active components, is metabolized into hydroquinone, which has antiseptic and antiadhesion properties in the urinary tract [18]. Recent research results also confirm a wider spectrum of action, e.g., antibacterial, antifungal, antiseptic, anti-inflammatory, and antioxidant properties of bearberry extracts [18–20].

Currently, bearberry extracts are being modified for the improvement of their biological properties and enhancement of the content of their secondary metabolites [21]. In turn, the use of chemically diverse eluents makes it possible to obtain extracts characterized by different chemical profiles. Therefore, it is interesting to use different types of plant extracts to determine the variability of their biological properties. In the current research, we assume that the use of different eluents will give chemically different extracts, consequently, with different anticancer activities.

A strong and reliable correlation was found between the antioxidant and anticancer activities of different plant extracts [22,23]. Therefore, it should be assumed that bearberry leaf extracts, which, in previous studies showed antioxidant activity due to the presence of arbutin, hydroquinone, methylarbutin, hyperoside, and flavonoids [19,24,25], will also be active against glioblastoma, which is one of the most perplexing cancers with one of the worst prognoses in modern medicine [26]. Therefore, the use of diversified raw materials and various extractant types is justified because the varied chemical composition of extracts determines their biological activity. This prompted us to further investigate the possible proapoptotic effects of bearberry extracts on III-grade and IV-grade glioma cell lines. Therefore, the objective of this study was (i) to characterize the chemical profile of two types of bearberry extracts and (ii) to investigate the biological activity of the analyzed extracts through the evaluation of their impact on the induction of apoptosis, autophagy, and necrosis in anaplastic astrocytoma MOGGCCM and human glioblastoma LN229 cell lines.

2. Materials and Methods

2.1. Reagents and Chemicals

Chromatography analytical standards [arbutin (≥98%), corilagin (≥96%), hydroquinone (≥99%), hyperoside (≥95%), methylarbutin (≥97%), oleanolic acid (≥97%), penta-O-galloyl-β-D-glucose (≥96%) picein (≥98%), and ursolic acid (≥95%)], eluent components (HPLC-grade formic acid and acetonitrile), reagents, and standards for the spectrophotometric analysis of total phenolic content [(Folin–Ciocalteu reagent and gallic acid (≥95%), respectively] and total flavonoid content [aluminum chloride (anhydrous) and quercetin

(≥95%)] were supplied by the Merck company (Darmstadt, Germany). All other chemicals were of analytical grade.

2.2. Plant Material and Extraction of Phytochemicals

In each of the 6 bearberry compact patches localized in pine forests in Eastern Poland, representative samples of plant material were taken in August 2020 for the phytochemical analyses. The plant material was placed in a refrigerator and transported to the laboratory. The leaves were dried at room temperature in the laboratory. For the preparation of aqueous extracts (Aq), powdered plant material (1 g) was brewed using 100 mL of distilled H_2O (100 °C) and incubated with continuous shaking in an orbital shaker at room temperature for 15 min. Afterward, the samples were subjected to ultrasound-assisted extraction at 40 °C for 45 min. For the preparation of ethanolic extracts (Et), powdered plant material (1 g) was combined with 100 mL of 70% ethanol in distilled water (v/v) and incubated at 40 °C for 60 min in an ultrasound water bath. To remove solid-state residues, the samples were centrifuged (9000 RPM, 15 min, 20 °C), and supernatants were vacuum filtered using qualitative cellulose filter discs.

2.3. Individual Compounds (HPLC Analysis)

The content of arbutin, corilagin, hydroquinone, hyperoside, methylarbutin, penta-O-galloyl-β-D-glucose, and picein was determined based on the previously described procedure [24] using a Varian ProStar HPLC separation system with UV detection (Varian Inc., Walnut Creek, CA, USA). In brief, the extracts were separated using a gradient mode, where eluent A was 0.1% HCOOH (formic acid) in H_2O (v/v) and eluent B was 0.1% HCOOH (formic acid) in ACN (acetonitrile) in the following mode: 0 min-4%B, 25 min-22%B, 40 min-25%B, 50 min-100%B, 55 min-100%B, 60 min-4%B, and 65 min-4%B. The temperature of separation was 25 °C, and the flow rate was set at 1 mL/min. The chromatograms for arbutin, corilagin, hydroquinone, methylarbutin, penta-O-galloyl-β-D-glucose, and picein were collected at 280 nm and at 350 nm for hyperoside.

Oleanolic acid and ursolic acid contents were determined using the same equipment, temperature of separation, flow rate, and eluents, but the following gradient mode was employed: 0 min-5%B, 20 min-80%B, 35 min-85%B, 40 min-100%B, 45 min-100%B, 50min-5%B, and 65 min-5%B. The chromatograms for oleanolic acid and ursolic acid were collected at 210 nm.

External calibration curves were applied for the quantification of the separated compounds, and the results were expressed as mg of individual compounds per g of plant material (dry weight).

2.4. Total Polyphenols and Flavonoids

The total phenolic content was determined based on the reaction with Folin–Ciocalteu reagent [27]. The method was adapted to microplate measurements according to a previously described procedure [24]. In brief, 10 µL of extract diluted 1:24 (v/v) with a corresponding solvent (water or 70% ethanol) was mixed with 40 µL of Folin–Ciocalteu reagent (diluted in distilled water 1:5; v/v). After 3 min, 100 µL of 10% sodium carbonate (w/v) and 150 µL of distilled water were added. The absorbance of the reaction mixture was measured at 765 nm after 30 min of incubation at room temperature. The results were expressed as mg GAE (gallic acid equivalents) per g of plant material (dry weight). The total flavonoid content was determined based on the reaction with Al^{3+} cations according to the procedure adapted to microplate measurements [28]. In brief, 150 µL of extract diluted 1:4 (v/v) with a corresponding solvent (water or 70% ethanol) was mixed with 150 µL of 3% aluminum chloride in 70% ethanol (w/v). The absorbance of the reaction mixture was measured at 430 nm after 30 min of incubation at room temperature. The results were expressed as QE (quercetin equivalents) per g of plant material (dry weight).

2.5. Cells and Culture Conditions

In order to perform the experiments, two human commercial cell lines—glioblastoma LN229 (ATCC) and anaplastic astrocytoma MOGGCCM (ECACC)—were used. The MOGGCCM cells were grown in a 3:1 mixture of Dulbecco's Modified Eagle Medium (DMEM) and Ham's nutrient mixture F-12 (Corning (Corning, NY, USA)) supplemented with 10% fetal bovine serum (FBS, Corning), while the LN229 cells were cultured in DMEM with 5% FBS. Both cell lines were supplemented with 100 µg/mL of penicillin and streptomycin and incubated at 37 °C in 95% air and 5% CO_2. The density of the seeded cells was 1×10^5.

2.6. Drug Treatment and Microscopic Detection of Apoptosis and Necrosis

The studied cells were incubated with 6 Et and 6 Aq at concentrations of 0.5, 1, 2, and 5 µL/mL for 24 h. Apoptosis, autophagy, and necrosis were identified using a fluorescence microscope (Nikon E–800, objective 40×/0.75, camera Nikon D-200, Tokyo, Japan) upon staining with Hoechst 33342 (Sigma, St. Louis, MI, USA) and propidium iodide (Sigma), as described in detail previously [29]. Each experiment was performed in triplicate.

2.7. Statistical Analysis

After testing the data for normality and homoscedasticity, adequate statistical tools (analysis of variance, post-hoc test, and correlation coefficient) were used the statistical analyses were carried out using the Statistica 6.0 software (Stat. Soft, Inc., Krakow, Poland). Cluster analysis and principal component analysis (PCA) were carried out using the statistical package (MVSP) program version 3.1 [30].

3. Results

3.1. Characteristics and Differentiation of the Chemical Composition of Bearberry Leaf Extracts

The analyzed extracts varied in terms of their chemical composition even though the plants came from pine forests, i.e., six sites/patches that were very similar to each other in terms of the vegetation species composition (Table 1). The cluster analysis of the chemical composition of the bearberry leaf extracts showed two separate groups (Figure 1). The ethanolic and aqueous extracts are clearly distinguished among the studied groups. The percent similarity of the two groups is ca. 86%; however, the similarity of the first cluster representing the ethanolic extracts (Et) is ca. 96.5% and that of the second cluster representing aqueous extracts (Aq) is ca. 95%.

Table 1. Content of phytochemicals in ethanolic (Et) and aqueous (Aq) bearberry leaf extracts. Mann–Whitney U test; values designated by different letters are significantly different ($p < 0.05$).

	Et		Aq	
Phytochemicals	**Mean**	**SD**	**Mean**	**SD**
Total flavonoids (mg GAE g^{-1})	3.39 ^{a}	0.317	2.58 ^{b}	0.424
Total phenolics (mg QE g^{-1})	271.68 ^{a}	8.889	170.26 ^{b}	21.528
Arbutin (mg g^{-1})	88.79 ^{a}	13.757	87.08 ^{a}	17.764
Hydroquinone (mg g^{-1})	9.22 ^{b}	1.583	13.09 ^{a}	1.047
Ursolic acid (mg g^{-1})	9.32 ^{a}	0.727	0.00 ^{b}	0.000
Hyperoside (mg g^{-1})	5.16 ^{a}	1.087	4.43 ^{a}	1.175
Methylarbutin (mg g^{-1})	5.98 ^{a}	0.990	2.21 ^{b}	1.336
PGG (mg g^{-1})	3.39 ^{a}	0.317	2.58 ^{a}	0.424
Picein (mg g^{-1})	1.54 ^{a}	0.109	1.66 ^{a}	0.180
Oleanolic acid (mg g^{-1})	1.25 ^{a}	0.138	0.00 ^{b}	0.000
Corilagin (mg g^{-1})	0.85 ^{b}	0.379	1.77 ^{a}	0.079

The two groups of extracts had different chemical profiles (Table 1). In the case of the total phenolic content (TPC), the concentration of these metabolites in Et was ca. 60% higher than in Aq, which is in agreement with earlier studies [24]. Similarly, the total flavonoid content (TFC) and the concentration of methylarbutin (mARB) were significantly higher in Et. In turn, the concentration of hydroquinone (HQ) was ca. 70% and that of corilagin

(COR) was ca. 100% higher in Aq in relation to Et. The presence of ursolic acid (UA) and oleanolic acid (OA) was confirmed solely in Et, and the concentrations of arbutin (ARB), hyperoside (HYP), picein (PIC), and penta-O-galloyl-β-D-glucose (PGG) in both extract types were similar. The amount of the main component arbutin (ARB) depended on the ecological habitat conditions and the season [16]. Therefore, the noticeable variability in their concentration was probably determined by the habitat conditions. The abovementioned research results indicate that, in most cases, the bearberry leaves meet the requirements (>70 mg g^{-1}) specified in the European Pharmacopoeia [31]. The concentration of this component determined in Et and Aq is the minimum content given by Asensio [16] from populations growing in northern locations in Spain, i.e., in harsher conditions—relatively higher altitudes characterized by lower mean temperatures, higher annual precipitations, and lower global radiation levels.

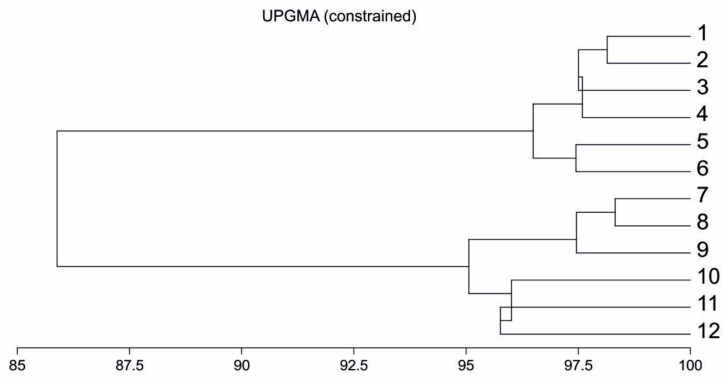

Figure 1. Cluster analysis based on the mean content of phytochemicals in bearberry leaf extracts. Ethanolic extracts: 1–6 and aqueous extracts: 7–12.

3.2. Antiglioma Activity of Bearberry Extracts

The results of the multi-way ANOVA confirmed the significant impact of all the studied parameters, namely, the site (SI), solvent (SO), and concentration (C), and their interactions on apoptosis (Ap) in the human anaplastic astrocytoma MOGGCCM cell line and the human glioblastoma LN229 cell line in both the Et and Aq variants (Table 2). A similar result was obtained in the necrosis (Nec) analysis. The analyzed extracts had no impact on the initiation of cell death in normal fibroblasts. Our observation showed that the studied Et and Aq extracts had no influence on autophagy initiation in all the MOGGCCM and LN229 cell lines; therefore, these data are not presented.

Table 2. Results of multi-way ANOVA. Effects of SI—site, SO—solvent, and C—concentration and their interactions on the levels of apoptosis (Ap) and necrosis (Nec) in MOGGCCM—human anaplastic astrocytoma cell line and LN229—human glioblastoma cell line.

Cell Line	MOGGCCM		LN229	
	Ap	**Nec**	**Ap**	**Nec**
SI	7.31	8.26	15.91	51.76
	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
SO	540.50	1069.24	799.29	2494.59
	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
C	884.55	2580.79	822.44	3622.77
	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
SI × SO	18.17	4.30	14.51	48.97
	$p < 0.001$	$p < 0.01$	$p < 0.001$	$p < 0.001$
SI × C	10.60	7.63	20.78	48.03
	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
SO × C	917.05	845.03	688.96	2602.87
	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
SI × SO × C	11.03	3.88	18.96	46.10
	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$

3.2.1. Apoptosis

The application of the increasing doses of Et and Aq prepared from bearberry leaves taken from the natural sites to the human anaplastic astrocytoma MOGGCCM cells caused an increase in the Ap level. At the concentration of 2 µL/mL, the level of Ap varied between 14.7% and 26% for Et and between 2% and 3% for Aq. The highest level of MOGGCCM cell death (over 20% for Et) was recorded using raw material obtained from sites 3 and 4. The increase in the concentration to 5 µL/mL significantly decreased the level of Ap for Aq. However, no apoptosis was induced by Et at this concentration. A statistically significantly higher level of Ap was noted after the addition of Et versus Aq to the MOGGCCM culture medium in the vast majority of the extract doses (Figure 2).

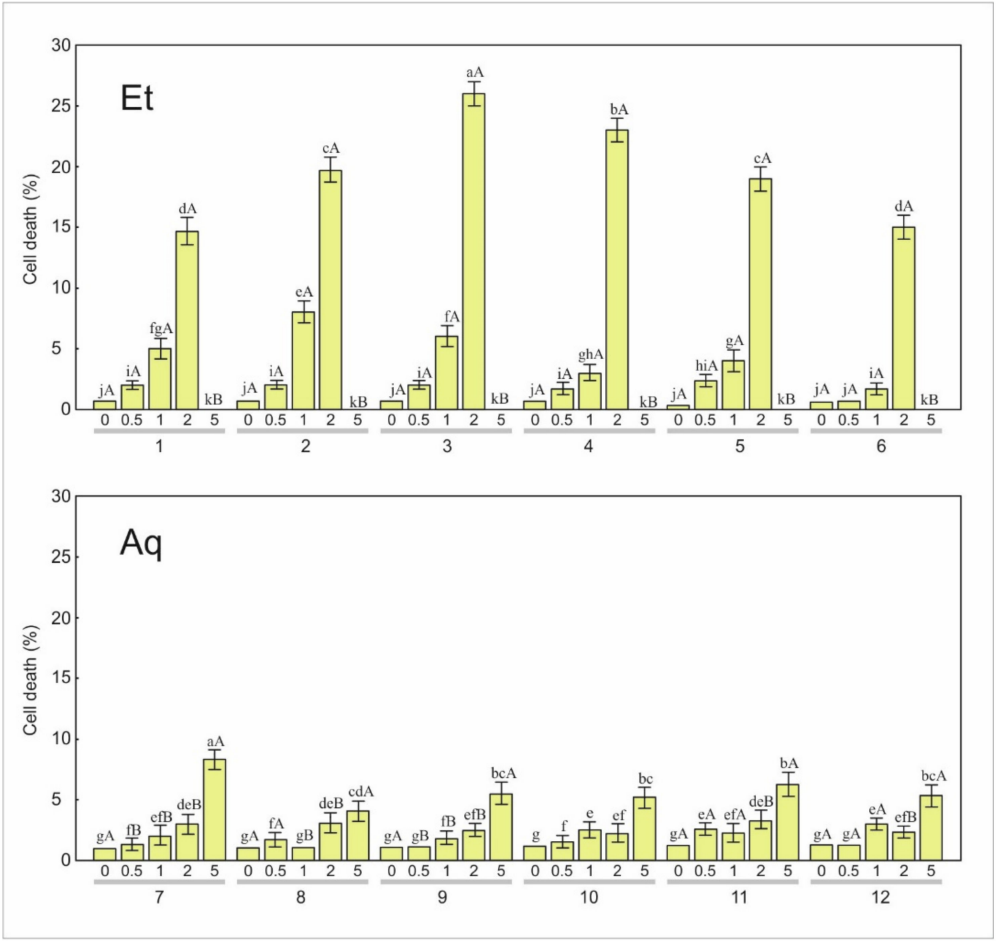

Figure 2. Level of apoptosis (%) observed in human anaplastic astrocytoma MOGGCCM cells treated with the ethanolic extracts (Et) and aqueous extracts (Aq) (concentrations: 0, 0.5, 1, 2, and 5 µL/mL) from the leaves of bearberry plants. Values designated by different small letters are significantly different ($p < 0.05$). A,B—statistically significant difference ($p < 0.05$) between the values of apoptosis induced by Et and Aq prepared from raw material taken from different sites.

Our experiments demonstrated that the application of the increasing doses of Et and Aq prepared from bearberry leaves taken from natural sites to the human glioblastoma LN229 cells caused an increase in the Ap level. As in the case of the MOGGCCM cell line, the highest Ap level was found at the concentration of 2 µL/mL; it varied between 12.3% and 33.3% for Et and between 2% and 3% for Aq. The highest level of LN229 cell death (over 20% for Et) was recorded using raw material obtained from sites 2, 3, 4, and 6. The increase in the concentration to 5 µL/mL significantly decreased the level of Ap for Et. A statistically significantly higher level of Ap was noted after the addition of Et to the LN229

culture medium in comparison with the Aq treatment in the vast majority of the extract doses (Figure 3).

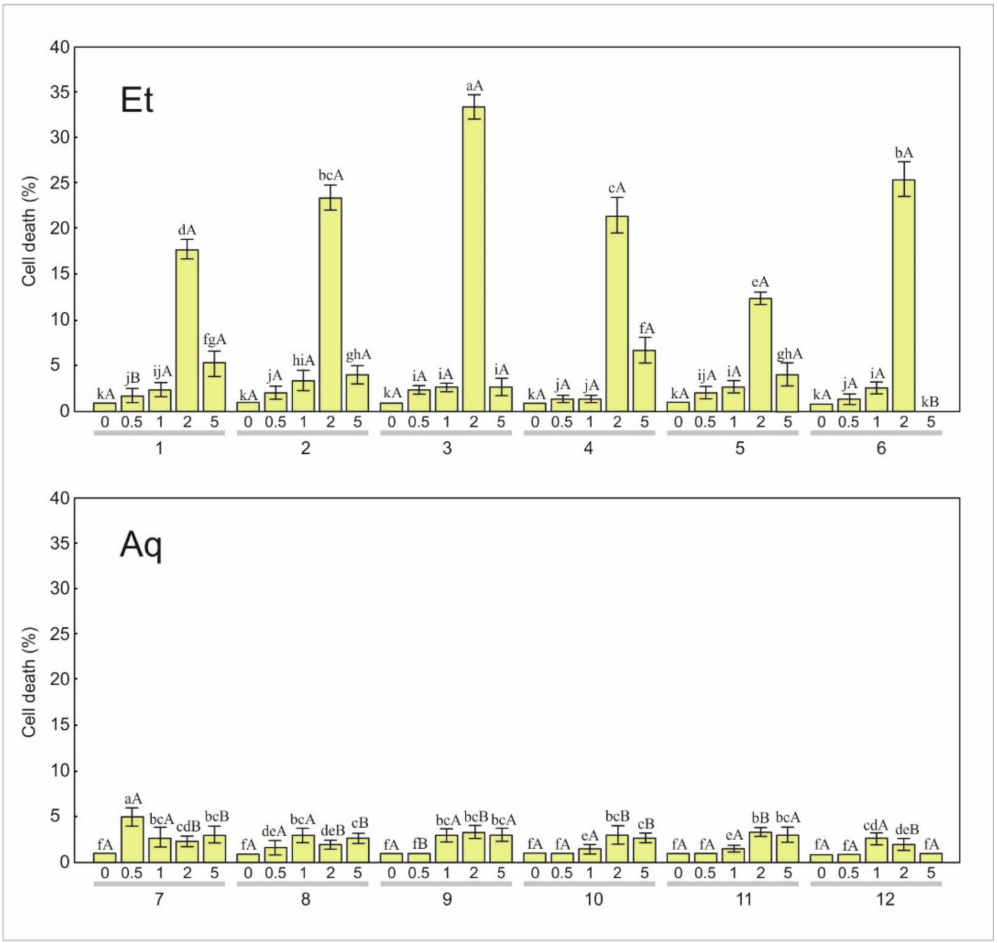

Figure 3. Level of apoptosis (%) observed in human glioblastoma LN229 cells treated with the ethanolic extracts (Et) and aqueous extracts (Aq) (concentrations: 0, 0.5, 1, 2, and 5 µL/mL) from the leaves of bearberry plants. Values designated by different small letters are significantly different ($p < 0.05$). A,B—statistically significant difference ($p < 0.05$) between the values of apoptosis induced by Et and Aq prepared from raw material taken from different sites.

3.2.2. Necrosis

We observed a general increase in the level of necrosis in the human anaplastic astrocytoma MOGGCCM cells, successively, with the concentration growth (Table 3). At the concentration of 2 µL/mL, the level of Ap observed in the Et variant was very promising (Figure 2) and, at the same time, a low level of Nec was found—2.7–8% depending on the different sites (Table 3). A similar level of Nec was found at the same concentration after the application of Aq. The increase in the concentration to 5 µL/mL caused a rapid increase in the Nec level, as in the case of Ap and Aq.

We observed an increase in Nec in the human glioblastoma LN229 cells, successively, with the concentration growth. At the concentration of 2 µL/mL, the level of LN229 apoptosis observed in the Et treatment was in the range of 12.3–33.3% (Figure 3) and, at the same time, the level of necrosis was in the range of 2–3%, depending on the different sites (Table 4). A similar level of Nec was found at the same concentration after the application of Aq. The increase in the concentration to 5 µL/mL caused a rapid increase in the Nec level, as in the case of Ap and Aq. A higher Nec level in the MOGGCCM and LN229 lines was found in the treatment with Et.

Table 3. Level of necrosis (%) observed in human anaplastic astrocytoma MOGGCCM cells treated with the ethanolic extracts (samples 1–6) and aqueous extracts (samples 7–12) (C—control, concentrations: 0, 0.5, 1, 2, and 5 µL/mL) from the leaves of bearberry plants. Values designated by different letters are significantly different ($p < 0.05$).

Concentration	C		0.5		1		2		5	
Samples	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	0.3 ^{g}	0.6	0.3 ^{g}	0.6	3.3 ^{ef}	1.5	4.0 ^{e}	1.0	91.7 ^{b}	11.8
2	0.0 ^{h}	0.0	0.7 ^{g}	0.6	3.7 ^{ef}	0.6	4.7 ^{e}	1.5	100.0 ^{a}	0.0
3	0.0 ^{h}	0.0	0.0 ^{h}	0.0	1.3 ^{g}	0.6	2.7 ^{f}	0.6	91.7 ^{b}	6.4
4	0.0 ^{h}	0.0	0.3 ^{g}	0.6	2.3 ^{f}	1.5	5.3 ^{e}	1.5	66.0 ^{c}	9.5
5	0.3 ^{g}	0.6	0.7 ^{g}	0.6	2.0 ^{f}	1.0	8.0 ^{d}	1.0	92.7 ^{b}	11.8
6	0.0 ^{h}	0.0	0.3 ^{g}	0.6	2.7 ^{f}	1.5	7.0 ^{de}	1.0	96.3 ^{b}	6.4
7	0.0 ^{g}	0.0	0.3 ^{f}	0.6	1.0 ^{f}	0.0	1.0 ^{f}	1.0	24.0 ^{c}	3.0
8	0.0 ^{g}	0.0	0.3 ^{f}	0.6	0.7 ^{f}	0.6	0.0 ^{g}	0.0	26.3 ^{bc}	1.2
9	0.3 ^{g}	0.6	1.0 ^{f}	0.0	1.0 ^{f}	0.0	1.3 ^{f}	0.6	27.0 ^{a}	3.6
10	0.0 ^{g}	0.0	0.0 ^{g}	0.0	0.0 ^{g}	0.0	0.3 ^{f}	0.6	20.3 ^{d}	1.5
11	0.0 ^{g}	0.0	0.3 ^{f}	0.6	0.7 ^{f}	0.6	1.0 ^{f}	1.0	18.3 ^{e}	0.6
12	0.3 ^{f}	0.6	0.0 ^{g}	0.0	0.7 ^{f}	0.6	0.0 ^{g}	0.0	30.0 ^{a}	4.4

Table 4. Level of necrosis (%) observed in human glioblastoma LN229 cells treated with the ethanolic extracts (samples 1–6) and aqueous extracts (samples 7–12); (C—control, concentrations: 0, 0.5, 1, 2, and 5 µL/mL) from the leaves of bearberry plants. Values designated by different letters are significantly different ($p < 0.05$).

Concentration	C		0.5		1		2		5	
Samples	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	0.0 ^{i}	0.0	1.0 ^{h}	0.0	1.0 ^{h}	0.0	2.3 ^{g}	0.6	53.7 ^{e}	2.5
2	0.0 ^{i}	0.0	0.3 ^{h}	0.6	1.0 ^{g}	1.0	2.0 ^{g}	0.0	80.7 ^{c}	7.4
3	0.0 ^{i}	0.0	1.0 ^{h}	1.0	1.3 ^{h}	0.6	3.0 ^{g}	1.0	95.3 ^{b}	4.0
4	0.0 ^{i}	0.0	0.3 ^{h}	0.6	0.0 ^{i}	0.0	2.7 ^{g}	0.6	49.7 ^{f}	4.5
5	0.0 ^{i}	0.0	0.7 ^{h}	0.6	1.3 ^{g}	1.5	2.3 ^{g}	0.6	59.3 ^{d}	6.8
6	0.0 ^{i}	0.0	1.0 ^{h}	0.0	1.3 ^{g}	1.5	2.7 ^{g}	0.6	100.0^{a}	0.0
7	0.0 ^{j}	0.0	1.7 ^{h}	0.6	1.0 ^{i}	0.0	2.7 ^{gh}	0.6	8.0 ^{b}	1.0
8	0.7 ^{hi}	1.2	2.3 ^{g}	0.6	1.0 ^{hi}	1.0	2.0 ^{gh}	1.0	7.7 ^{bc}	1.5
9	0.0 ^{j}	0.0	1.0 ^{hi}	1.0	1.3 ^{hi}	0.6	4.0 ^{e}	1.0	9.0 ^{a}	1.0
10	0.0 ^{j}	0.0	1.7 ^{hi}	0.6	0.0 ^{j}	0.0	3.7 ^{e}	1.2	6.7 ^{c}	1.5
11	0.0 ^{j}	0.0	0.3 ^{i}	0.6	1.3 ^{gh}	1.5	3.0 ^{fg}	1.0	6.0 ^{c}	2.0
12	0.0 ^{j}	0.0	0.7 ^{hi}	1.2	1.3 ^{gh}	1.5	2.3 ^{g}	0.6	6.0 ^{c}	1.0

3.3. Bearberry Secondary Metabolites vs. the Level of Apoptosis and Necrosis

The results of the principal component analysis performed on the basis of the chemical composition of bearberry leaf extracts and the levels of apoptosis and necrosis in the MOGGCM cell lines are presented in Figure 4. The eigenvalues of the first (9.65) and second axis (2.61) indicated the presence of two gradients. The first two axes explained 87.7% of the variability (68.9%—Axis 1 and 18.8%—Axis 2). The content of TPC, TFC, mARB, UA, and OA in the bearberry extracts and the levels of Ap and Nec in the MOGGCCM cell lines were positively correlated with Axis 1, whereas HQ and COR were negatively correlated. Therefore, both apoptosis and necrosis in the MOGGCCM cell line were determined by the abovementioned metabolites. In turn, the contents of ARB, HYP, and PIC were negatively correlated with Axis 2.

The results of the multidimensional analysis performed on the basis of the chemical composition of bearberry leaf extracts and the levels of apoptosis and necrosis in the LN229 cell line are presented in Figure 5. The eigenvalues of the first (9.03) and second axis (2.57) indicated the presence of two gradients. The first two axes explained 82.8% of the variability

(64.5%—Axis 1 and 18.4%—Axis 2). The content of TPC, TFC, mARB, UA, and OA in the bearberry extracts and the level of Ap in the LN229 cell line were positively correlated with Axis 1, whereas HQ, COR, and the Nec level were negatively correlated. Therefore, it can be concluded that TPC, TFC, mARB, UA, and OA were responsible for the level of Ap in the LN229 cell line, while HYP and PIC were responsible for Nec.

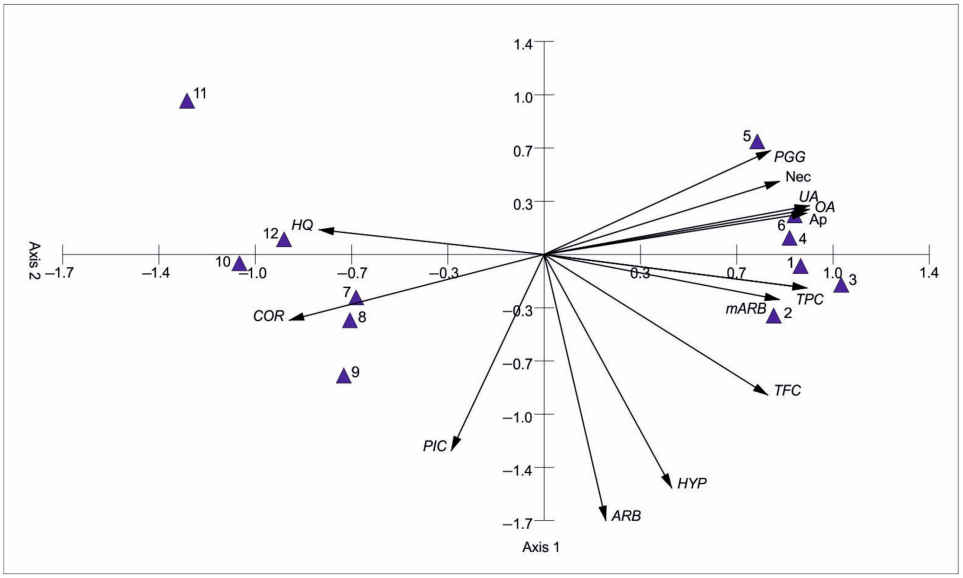

Figure 4. Principal component analysis based on the phytochemistry of bearberry leaf extracts and the level of apoptosis (Ap) and necrosis (Nec) in the MOGGCM cell line. TFC—total flavonoid content, TPC—total phenolic content, ARB—arbutin, HQ—hydroquinone, UA—ursolic acid, HYP—hyperoside, mARB—methylarbutin, PGG—penta-O-galloyl-β-D-glucose, PIC—picein, OA—oleanolic acid, and COR—corilagin.

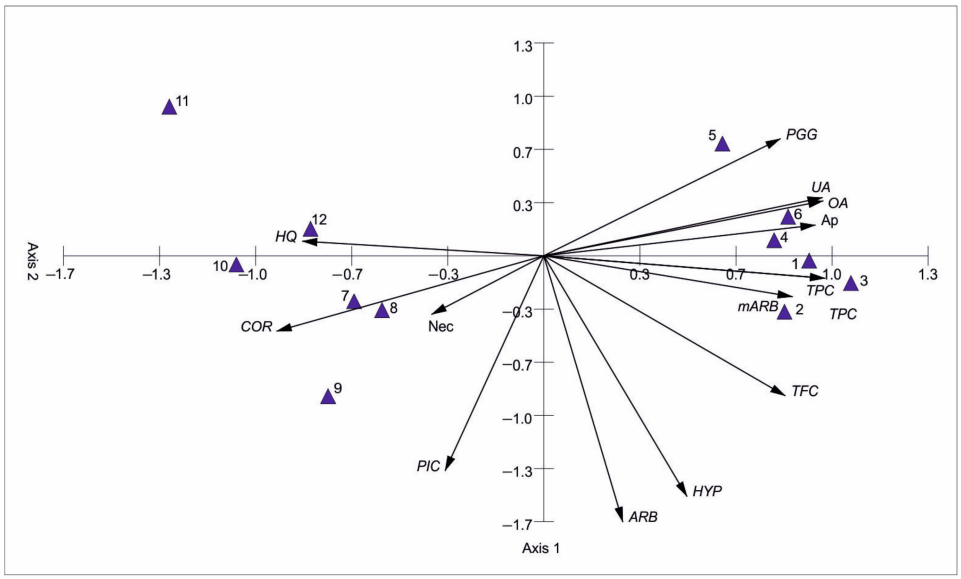

Figure 5. Principal component analysis based on the phytochemistry of bearberry leaf extracts and the level of apoptosis (Ap) and necrosis (Nec) in the LN229 cell line. TFC—total flavonoid content, TPC—total phenolic content, ARB—arbutin, HQ—hydroquinone, UA—ursolic acid, HYP—hyperoside, mARB—methylarbutin, PGG—penta-O-galloyl-β-D-glucose, PIC—picein, OA—oleanolic acid, and COR—corilagin.

The PCA analysis and inference were confirmed by the correlation between the secondary metabolites contained in the bearberry extracts and the Ap and Nec levels (Table 5).

The positive impact of TFC, TPC, UA, OA, and PGG on the level of Ap in all the glioma cells was clearly visible. On the other hand, a negative relationship was found in the case of HQ and COR. The lack of relationships between arbutin and the level of Ap in the MOGGCCM and LN229 cell lines was very interesting since ARB is the primary bioactive compound in this plant and bearberry is regarded as the main natural source of ARB to be used for phytotherapy purposes [32,33].

Table 5. Relationships between the analyzed secondary metabolites of bearberry extracts and the types of cell death (the level of apoptosis and necrosis) in the glioma cell lines irrespective of the solvent used. * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$.

Phytochemicals	Apoptosis		Necrosis	
	MOGGCCM	LN229	MOGGCCM	LN229
Total flavonoids	0.787 **	0.835 ***	0.642 *	−0.078
Total phenolics	0.815 **	0.670 *	0.582 *	−0.473
Arbutin	0.240	0.123	−0.130	−0.242
Hydroquinone	−0.829 ***	−0.775 **	−0.656 *	0.285
Ursolic acid	0.810 **	0.780 **	0.888 ***	−0.587
Hyperoside	0.441	0.604 *	0.298	0.128
Methylarbutin	0.734 **	0.800 **	0.758 **	−0.285
PGG	0.702 *	0.663 *	0.807 **	−0.337
Picein	−0.448	−0.505	−0.319	0.174
Oleanolic acid	0.825 ***	0.843 ***	0.788 **	−0.186
Corilagin	−0.826 ***	−0.684 *	−0.653 *	0.320

4. Discussion

In the present study, the site, solvent, and concentration and their interactions influenced the induction of apoptosis in the examined cell lines. The results revealed variability in the chemical composition of bearberry leaves originating from pine forest habitats (Table 1). Despite the raw material sampling in vegetation patches with a similar floristic composition, large differences were found in the content of all the analyzed components of bearberry leaves and, in consequence, the biological activity of the extracts. Generally, there are scarce investigations of the chemical profiles of bearberry populations occurring in natural habitats. In Europe, very detailed characteristics, such as the chemical composition and biological activity, have recently been presented from Spain [16,34], where the plant material was collected from typical bearberry habitats located in mountain plant communities. Factors such as the date of collection, altitude, elevation, UV radiation, and location of plants have been shown to influence chemical composition and differentiation [16,34]. Our earlier study conducted in natural habitats demonstrated higher TFC and HYP concentrations in bearberry leaves in heathland populations than in pine forest populations; on the other hand, higher COR and mARB content was observed in raw material taken from pine forests [24]. Therefore, many environmental factors can determine secondary metabolite production. It is often observed that stress and disturbances in harsh habitat conditions affect the response of plants through the accumulation of secondary metabolites [35]. Pine forests represent acidic and poor habitats, and the determination of the impact of any of the environmental factors on variability in the chemical composition of bearberry leaves requires comprehensive research of biotic and abiotic factors.

Flavonoids are powerful antioxidants that scavenge free radicals and reactive oxygen species (ROS), thus reducing oxidative stress linked to DNA damage, mutations, and the initiation of carcinogenesis [36]. This antioxidant property is crucial for their anticancer effects [37]. Bearberry exhibits antioxidant activities thanks to its content of flavonoids and phenolic acids [24,25], and our results additionally demonstrated the role of these compounds in proapoptotic activity (Figures 2 and 3). The large differences in the induction of apoptosis in the studied cells treated with the two tested extracts may be related to the presence of UA and OA in Et only (Figures 2 and 3). These penta-

cyclic triterpenoid isomers exhibit neuroprotective effects in multiple brain disorders [38]. Literature reports have repeatedly demonstrated their antidiabetic, antioxidant [39,40], anti-inflammatory [40,41], antimicrobial [42], antifungal [43], anticancer, and apoptotic [44,45] activities and their potential to be used in the prevention and therapy of cancer [46]. UA and OA are present in many medicinal plant species [47]. These metabolites often co-occur in *Ocimum sanctum* [48], *Ligustrum lucidum* [49], *Achyranthes aspera* [50], and some species of the Lamiaceae family [47]. As evidenced above, they may also play a role as proapoptotic molecules with high biological activity, which was confirmed in our study by the very high correlation coefficient between UA and OA and the level of apoptosis of all the studied cell lines (Figures 4 and 5 and Table 5). In further research on anti-glioma activity, these metabolites used individually and together should be taken into account.

In studies on molecular mechanisms of elimination of human glioma cells by programmed cell death, much attention is paid to the role of drug interactions. Experiments conducted in the last few years revealed that sorafenib and the natural flavonoid quercetin [51], sorafenib and temozolomide [29], sorafenib and tipifarnib [52], formononetin and temozolomide [53], or the single-targeted flavonoid derivative LY294002, in combination with sorafenib [54], were more effective in programmed cell death induction than in single application. To recapitulate, the combination of two drugs with different mechanisms of action is the best strategy that may lead to the elimination of cancer cells by programmed cell death initiation. Our results indicated that the bearberry extract concentration at the level of 2 µL/mL was very efficient in apoptosis induction in the glioma cells, with no significant necrotic effect (Figures 2 and 3 and Tables 3 and 4). The correlation between TPC, UA, OA, and the apoptosis level indicates an influence of these chemicals on programmed cell death in both MOGGCCM and LN229 (Table 5). It can be assumed that the obtained level of apoptosis is the result of the interaction between the metabolites contained in the extracts, which was confirmed by the multidimensional analyses (Figures 4 and 5). Earlier studies of the activity of bearberry extracts confirmed the impact of TPC and HQ on the DPPH level. Therefore, it cannot be excluded that such an interaction of metabolites should be expected in the case of apoptosis induction, taking into account the fact that extracts often have antioxidant and anti-cancer properties [22,23].

In studies on the role of a Bcl-2 and Beclin-1 complex in switching between apoptosis and autophagy in human glioma cells [55], the authors showed the impact of LY294002 (10 µL) alone, sorafenib (1 µL) alone, and LY294002 × sorafenib combination on the level of different types of cell death in glioma cells, including the MOGGCCM cell line. Apoptosis was the dominant type of death when sorafenib was used alone (ca. 5%) and combined with LY294002 (almost 20%). In the present study, a very promising level of apoptosis was obtained in the case of MOGGCCM (26%) and LN229 (33.3%) at a 2 µL/mL concentration of the extract, with a very low level of necrosis (Figures 2 and 3 and Tables 3 and 4).

In recent years, medical plants have attracted the interest of researchers as a source of metabolites with the potential to be used to fight cancer, including glioma. Selective induction of apoptosis in glioma tumor cells by a *Gynostemma pentaphyllum* extract was reported by Schild et al. [56]. Apoptosis induction in glioma cells by extracts of *Leonurus sibiricus* was presented by Sitarek et al. [57]. Radan et al. [58] showed that the majority of human glioblastoma A1235 cancer cells died via apoptosis induced by *Centaurea ragusina* aqueous extracts. The antiproliferative activity of *Centaurea castriferrei* extracts was tested in the human glioblastoma LN229 cell line as well [59]. Regardless of the extractant used, similar cytotoxicity of the methanol–water extracts and water extracts on this cell line and most cancer cell lines was observed. However, the methanol–water extracts containing significantly higher amounts of phenolic compounds and flavonoids exhibited stronger antioxidant properties in comparison to water extracts. Our study has shown that bearberry leaves are another source of compounds with proapoptotic activity against gliomas and that the Et extract has much higher anticancer activity than Aq (Figures 2 and 3). Additionally, ethanolic extracts contain UA and OA (Table 1), which are characterized by anticancer activity against different types of cancer [38,60,61]. Attention should also be paid to the

chemical profile of Aq characterized by higher contents of HQ and COR in comparison with Et but, above all, to the negative correlation between HQ-Ap and COR-Ap in the two studied cell lines.

It should be noted that the anti-glioma activity of the extracts, in addition to the main compounds identified in this study, may also have been influenced by other unidentified phytochemicals. The chromatograms of the ethanolic and aqueous extracts presented in Figure S1 indicated the presence of peaks corresponding with some unidentified compounds. Recent studies applying mass spectrometry systems for the identification of compounds reported a strong contribution of, e.g., various galloyl derivatives in the bearberry phytochemical profile [62,63]; however, only two main representatives of this group were identified and quantified in this study, i.e., COR and PGG, due to the unavailability of commercial standards facilitating the identification of other compounds. In addition, despite the considerable similarity in the chromatographic profiles of the studied extracts (Figure S1), some additional peaks corresponding with unidentified substances that may contribute to the activity of ethanolic extracts were also observed (especially at the retention time of 28.9 min).

Furthermore, it is known that, in complex mixtures of herbal preparations, despite their strict chemical composition, synergistic and antagonistic interactions among co-existing compounds may affect the final biological effects [64]. Therefore, further investigations should focus on the assessment of such interactive effects in model systems and the determination of their possible mechanisms.

5. Conclusions

In the present study, the site, solvent, and concentration and their interactions influenced the induction of apoptosis in the human anaplastic astrocytoma MOGGCCM cell line and the human glioblastoma LN229 cell line. The variability in the chemical composition of bearberry leaves originating from pine forest habitats demonstrated in the present study was probably determined by environmental factors. Despite the raw material sampling in vegetation patches with a similar floristic composition, large variability was found in the content of all the analyzed phytochemicals of bearberry leaves and, in consequence, the anticancer activity of its extracts. In our study, the impact of different *Arctostaphylos uva-ursi* extracts on programmed cell death induction in glioma cells was evaluated for the first time. The extracts had no influence on autophagy initiation in the MOGGCCM and LN229 cell lines. In contrast to the aqueous extracts, the ethanolic bearberry extracts exhibited high proapoptotic activity. At the extract concentration of 2 µL/mL, the level of apoptosis varied between 14.7% and 26% in the case of the MOGGCCM cells and between 12.3% and 33.3% in the case of the LN229 cell line. The knowledge and information obtained in this study indicate a need for further research on the anticancer effect of the studied bearberry phytochemicals on the MOGGCCM and LN229 cell lines and for the elucidation of their molecular anticancer mechanisms.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app14083418/s1, Figure S1: Exemplary HPLC chromatograms of bearberry leaf extracts. A—chromatogram for phenolic compounds at 280 nm; B—chromatogram for triterpenoids at 210 nm; Et—ethanolic extract, and Aq—aqueous extract.

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