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Comparative Study on Phytochemical Composition, Antioxidant, and Anti-HSV-2 Activities of *Sambucus nigra* L. and *Sambucus ebulus* L. Extracts

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Abstract: *Sambucus nigra* (SN) and *Sambucus ebulus* (SE) are widely used in folk medicine, primarily as antiviral agents for colds and influenza. In the current study, the antiviral activity of extracts of SN and SE fruits, flowers, and leaves were tested against herpes simplex virus type-2 (HSV-2). The HPLC analysis of the investigated extracts revealed the presence of phenolic acids, flavonoids, and anthocyanins. Rutin and chlorogenic acid were the main polyphenol constituents in flower and leaf extracts, whereas anthocyanins were predominant in fruit extracts. The flower extract of SN was characterized by the highest content of rutin and chlorogenic acid—14,232.1 mg/100 g dry weight (DW) and 7086.7 mg/100 g DW, respectively. SN fruit extract revealed the highest antioxidant activity measured using ORAC and HORAC methods—11,443.1 µmol TE/g and 8198.9 µmol GAE/g, respectively. To evaluate cytotoxicity, antiviral, and virucidal activities against HSV-2, the MTT assay and method of Reed and Muench were used. The least toxic extracts were PSNFrE and PSEFrE. The maximum tolerable concentration (MTC) of PSNFrE was 2000 µg/mL and the calculated CC_{50} value for that extract was 3570 µg/mL. The inhibitory activity against virus replication was established for three of the extracts—PSNFlE, PSNLE, and PSNFrE. PSEFrE showed neither activity against virus replication, nor virucidal activity. The data suggest a significant inactivation of more than 98% after 60 min of contact of HSV-2 virions with the PSNFrE applied in MTC. The current study provides evidence that *Sambucus nigra* reveals anti-HSV-2 activity; however, the most active parts of the species were fruits. Therefore, SN fruits and their extracts can be used as an attendant therapy for HSV-2 viral infections.

Keywords: black elder (*Sambucus nigra*); dwarf elder (*Sambucus ebulus*); herpes simplex type-2; antioxidant activity; anthocyanins; antiviral agents; antiherpetic activity; phytochemical composition

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1. Introduction

The herpes simplex virus (HSV) is a prevalent, human DNA virus belonging to the Herpesviridae family, which can remain dormant in the neurons of infected people for life. Periodically, it can become active again, leading to recurrent infections, shedding of the virus, and transmission to new hosts. HSV-2 typically affects the genital and mucosal areas. In some cases, infections can be serious, resulting in significant health problems and, in rare instances, even leading to conditions like viral encephalitis, meningitis, or blindness [1]. The virus is endemic worldwide and has no effective vaccines or prophylaxis that provide complete protection or immunity [2]. According to the World Health Organization, about

417 million people aged 15–49 have HSV-2 infections [3]. The available pharmaceuticals for treating viral infections are relatively restricted. The conventional approach to treating herpesvirus infections in humans involves antiviral chemotherapy. Presently, there are approximately 11 authorized drugs designed for this purpose [4]. The most commonly used one is the nucleoside analog acyclovir and its derivatives [4,5]. However, continuous therapy can result in the emergence of resistant strains [4,6]. This fact underscores the need for the creation of novel antiviral compounds. In this context, natural products have been reported as promising antiviral agents [7]. Plant antiviral therapy offers advantages over currently utilized chemotherapeutic agents since they tend to have fewer adverse effects. Additionally, their complex chemical composition often hinders the development of resistant strains [8]. Plants are widely known for their powerful antiviral effects. It has been demonstrated by some researchers that phytocompounds derived from various plants, such as polyphenols, have antiherpetic activities. Specifically, polyphenol-rich extracts have been shown to have anti-herpetic activities [9]. In recent studies, it has been found that caffeic acid and its derivatives, and kaempferol, can inhibit the activity of HSV [10]. Rutin, a flavonoid glycoside generally found in many plants, is highly effective against HSV-1, HSV-2, and other viruses, and is among the bioactive compounds produced by different plant species [11].

Sambucus nigra and *Sambucus ebulus*, commonly known as Black elder and Dwarf elder, respectively, belong to the Adoxaceae family (previously Caprifoliaceae). SN is widespread across Europe, west and central Asia, northern Africa, and North America [12]. Ethnobotanically, it is one of the most commonly used medicinal plants worldwide, and it is commonly used for both food and medicine [12,13]. SE is a perennial plant or bush and is mainly widespread across southern and central Europe, Southwest Asia, and northwest Africa [14]. The fruits and flowers of SN and SE are rich in bioactive phenolics including phenolic acids, flavonoids, and anthocyanins, which underlie their health benefits, supported by strong scientific evidence [14–16]. They act as antioxidants, reduce inflammation, modulate the immune system, demonstrate activity against cancer, and exhibit a broad spectrum of antiviral properties [14–23]. Extracts and products from SN fruits that exhibit antiviral activity are currently some of the most significant highlights of research [24]. SN fruit extracts and flower infusions have demonstrated effectiveness in alleviating symptoms of a variety of viral infections, independently of their mechanisms [12]. In vitro studies have indicated that Sambucol (extract prepared from fruits) significantly inhibits HSV-1, even against strains resistant to different conventional antiviral drugs [25]. In addition, in vitro experiments have demonstrated the effectiveness of Sambucol as an antiviral agent against HIV [26]. An infusion comprising flowers from *S. nigra*, aerial parts of *Hypericum perforatum*, and roots of *Saponaria officinalis* inhibited HSV-1 in vitro, and influenza A and B, both in vitro and in animal models [27–29]. For a long time, SE has been used in folk medicine to treat fever, different infections, or related inflammatory diseases. However, we found only one report about the antiherpetic activity SE fruits against HSV-1 [16].

Although there is strong evidence for the antiviral properties of SN, only a few studies specifically focus on the antiviral effects of SN against HSV-2. Moreover, it is not clear which anatomical part of the plant reveals the highest anti-HSV-2 activity. Therefore, the objective of this research was to investigate the polyphenol content and composition and antiviral activities, against HSV-2 of SN and SE fruit, flower, and leaf extracts. The antioxidant activity of the extracts was also investigated. To clarify the effect of polyphenol compounds in the observed effects, some extracts were additionally enriched in phenolic compounds through solid-phase extraction. The findings of our investigation may offer practical insights for formulating nutraceuticals with precise polyphenol composition and heightened biological benefits.

2. Materials and Methods

2.1. Chemicals

All chemicals used in the present study were of analytical and pharmaceutical grade. Folin–Ciocalteu's phenol reagent was purchased from Merck (Darmstadt, Germany). Gallic acid, 2,2-azobis-(2-amidinopropane) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), fluorescein disodium salt, chlorogenic acid, neochlorogenic acid, benzoic acid, *p*-coumaric acid, caffeic acid, ferulic acid, rosmarinic acid, cinnamic acid, vanilic acid, quercetin, quercetin-3-glucoside, quercetin-3-rutinoside, myrecetin, kaempherol, and apigenin were delivered from Sigma-Aldrich (Steinheim, Germany). Cyanidin-3-glucoside, cyanidin-3-sambubioside, cyanidin-3,5-diglucoside, cyanidin-3-galactoside, cyanidin-3-rutinoside, cyanidin-3-arabinoside, pelargonidin-3-glucoside, cyanidin, delphinidin, pelargonidin, malvidin, peonidin were purchased from Extrasynthese S.A. (Genay Cedex, France). All other reagents and solvents used purchased from local distributors.

2.2. Plant Materials

Sambucus nigra L. and *Sambucus ebulus* L. flowers and leaves were collected from natural populations in the territory of Rodopi mountain (Ravnogor village) Bulgaria, located at 1319 m altitude (41°57′4.28″ N 24°22′4.37″ E) in June 2019. The plant SN was identified by the authors according to the *Sambucus nigra* monograph in the *European Pharmacopoeia* [30]. The plant SE was authenticated by the authors according to *Flora of the Republic of Bulgaria* [31]. Flowers and leaves were allowed to dry at room temperature, whereas fruits from both species were collected in the stage of full maturity (in the period 1–15 September), put in polyethylene bags, frozen, and stored at −18 °C until analyzed.

2.3. Preparation of Dry Extracts

The dried flowers and leaves of SN and SE were ground in a laboratory grinder to obtain a homogeneous powder. An amount of 20 g of the powders was accurately weighed and extracted using 400 mL 60% (*v*/*v*) ethanol for 1 h and shaking on a thermostatic water bath (NUVE ST 402, Turkey), at a temperature of 60 °C. After that, the obtained extract was filtered and concentrated using a vacuum evaporator while the ethanol was evaporated. Frozen SN and SE fruits (200 g) were defrosted at room temperature and homogenized using a laboratory blender. Then, 100 g of the mash was transferred to a brown glass bottle and incubated with 300 mL 60% (*v*/*v*) ethanol in a thermostatic shaker water bath (NUVE, Akyurt/Ankara, Turkey) for one hour at a temperature of 50 °C. After that, the fruit mash was filtered through cheesecloth and the liquid phase was centrifuged for 30 min at 6000 g (MPW-260R, MPW Med. Instruments, Warszawa, Poland). Obtained extracts were filtered and concentrated using a vacuum evaporator while the ethanol was evaporated. Half the obtained amount of ethanol-free, liquid flower and leaf extracts, and the entire amount received from fruits were subjected to purification through solid-phase extraction, according to the procedure described by Denev et al. [32]. Purified extracts were frozen, lyophilized, and stored at −18 °C until analyzed.

2.4. Total Polyphenol Content (TPC) Analysis

The total polyphenols content was determined according to the method of Singleton and Rossi with Folin–Ciocalteu's reagent [33] and results are expressed as milligrams of gallic acid equivalents (GAE) per 100 g dry weight (DW).

2.5. High-Performance Liquid Chromatography (HPLC) Analysis of Phenolic Compounds

HPLC analysis of phenolic components was performed according to Denev et al. [34] on an HPLC system (Agilent 1220, Agilent Technology, Santa Clara, CA, USA), using a binary pump and UV-Vis detector (Agilent Technology, USA). Separation was performed on an Agilent TC-C18 column (5 µm, 4.6 mm × 250 mm) at 25 °C and a wavelength of 280 nm was used. The following mobile phases were used: 0.5% acetic acid (A) and 100%

acetonitrile (B) at a flow rate of 0.8 mL/min. The gradient elution started with 14% B, between 6 min and 30 min, linearly increased to 25% B, and then to 50% B at 40 min. Results are expressed as mg/100 g DW of the extract.

2.6. High-Performance Liquid Chromatography (HPLC) Analysis of Anthocyanins and Anthocyanidins

Anthocyanins were determined on an HPLC system (Agilent 1220, Agilent Technology, Palo Alto, CA, USA), with a binary pump and UV-Vis detector (Agilent Technology, USA). A wavelength of 520 nm was used. Anthocyanins were separated using an Agilent TC-C18 column (5 µm, 4.6 mm × 250 mm) at 25 °C. The following mobile phases were used: 5% formic acid (A) and 100% methanol (B) at a flow rate of 1.0 mL/min. The gradient condition started with 15% B and linearly increased to 30% B at 20 min. Results are expressed as mg/100 g DW of the extract.

For the determination of anthocyanidin content and composition (cyanidin, delphinidin, pelargonidin, and malvidin), extracts were subjected to an acid hydrolysis. Briefly, 2 mg of extract was added to 10 mL 3N HCl in MeOH and hydrolyzed at 100 °C for 3 h. The solution was then filtered through a 0.45 µm filter (Supor-450 Membrane, Waters) and injected into an Agilent 1020 chromatographic system equipped with a UV-VIS detector and a Zorbax C18 column, wavelength λ = 520 nm, eluent MeOH: 5% HCOOH (35:65), 0.5 mL/min at 25 °C. Results are expressed as mg/100 g DW of the extract.

2.7. Antioxidant Assays

Oxygen Radical Absorbance Capacity (ORAC) was measured according to the method of Ou, Hampsch-Woodill, and Prior [35], with some modifications described by Denev et al. [32], whereas Hydroxyl Radical Averting Capacity (HORAC) was measured according to Ou, Hampsch-Woodill, and Prior [36]. ORAC and HORAC analyses were carried out on FLUOstarOPTIMA plate reader (BMG Labtech, Ortenberg, Germany) with an excitation wavelength of 485 nm and emission wavelength of 520 nm, and the results are expressed in µM Trolox equivalents (µM TE) and µM gallic acid equivalents (µM GAE) per gram of dry extract, respectively.

2.8. Viruses and Cells

HSV-1, strain Vic, and HSV-2, strain BA, were supplied by the National Center of Infectious and Parasitic Diseases (NCIPD), Bulgaria. For virus production, monolayers of MadinDarby Bovine Kidney (MDBK) cells in 75 cm^2 tissue culture flasks were infected with HSV at a multiplicity of infection of 0.01. After 48 h at 37 °C, the infected cells were harvested with three freeze-and-thaw cycles and the cellular debris were removed through low-speed centrifugation. The cell line MDBK was provided by the National Bank for Industrial microorganisms and Cell Cultures. The cells were cultured in Dulbecco's modified eagle medium (DMEM; GibcoÒ by life technologies) supplemented with 10% fetal bovine serum (FBS) and 1:1000 penicillin/streptomycin (P/S), at 37 °C and 5% CO_2 atmosphere. The cells were passed on and harvested on a regular basis according to a constant scheme.

2.9. Cytotoxicity Assay

The cytotoxicity was determined through a comparative microscopic examination of the cell morphology in treated and untreated cultures. The maximum concentration, which did not alter the morphology of the cells, was recognized as maximal tolerated concentration (MTC) [37]. In a second experiment, the cell viability was determined by the ability of the cells to cleave the tetrazolium salt 3-(4,5dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, Burlington, MA, USA) through the mitochondrial enzyme succinate dehydrogenase, which gives a formazan blue product, following the procedure described earlier [38]. The intensity of the absorbance of the released triazolium crystals from cells treated with serial dilutions of the analyzed extracts (250, 500, 750, 1000, 1250,

1500, 1750, 2000 and 2500 mg/mL) was measured on the Elisa reader and, consequently, a standard curve of the cell viability was produced. The 50% cytotoxicity concentration (CC_{50}) was calculated based on the curve.

2.10. Effect on the Extracellular Virus (Virucidal Effect)

Equal volumes of viral stock containing $10^{5.5}$ $CCID_{50}/_{mL}$ and media with MTC of the appropriate extract were mixed and incubated at different times (5, 10, 15, 30, 60, 120, 250, and 360 min) at 37 °C. The samples were frozen and thawed. Infectious virus titers were calculated at the 48th hour of culturing through the method of Reed and Muench [39]. The virucidal effect was determined by the reduction in the infectious virus titer of each sample as compared to the relevant viral control equal volumes of viral stock and medium incubated, as described above.

2.11. Statistical Analysis

The results are obtained by two independent experiments performed in duplicates and triplicates. The statistical analysis of data was performed using MS Excel 2016 software and the results are expressed as mean ± SD. In the case of chemical analysis, one-way analysis of variance (ANOVA) and Student's *t*-test were used to evaluate the differences in the mean between groups. In the case of antioxidant activity determination, significant differences between groups were assessed through one-way analysis of variance with multiple comparisons (ANOVA), followed by Tukey's post hoc test. *P* values less than * 0.05, ** 0.01, and *** 0.001 were considered to be significant.

3. Results

3.1. Polyphenol Content and Composition

3.1.1. Phenolic Acids and Flavonoids Content and Composition

The content and composition of the major polyphenols present in the investigated *S. nigra* and *S. ebulus* extracts are given in Table 1. Different extracts significantly differ in the content of total polyphenol content, which varied in a broad range from 9803.3 mg GAE/100 g DW (SEFlE) to more than a six-fold higher value of 59,596.6 mg GAE/100 g DW (PSNFrE). The total polyphenol content of purified dry extracts PSNFlE and PSNLE was 42,647.1 ± 1331.8 mg GAE/100 g DW and 30,120.5 ± 1748.8 mg GAE/100 g DW, respectively. In flower, leaf and fruit extracts, chlorogenic acid was the predominant representative. The chlorogenic acid content varied in the range of 2068.7 ± 88.3–7086.7 ± 312.8 mg/100 g DW. The highest amount of this acid was found in PSNFlE. Chlorogenic acid, followed by neochlorogenic and benzoic acids, was detected in all investigated extracts. The results also show that the flowers are the richest in chlorogenic acid, followed by fruits and leaves. The amount of chlorogenic acid was 1.5 times higher in PSNFlE (7086.7 mg/100 g DW) compared to SNFlE (4673.3 mg/100 g DW).

Table 1. Content and composition of the major phenolic acids and flavonoids (mg/100 g DW) of dry *S. nigra* L. and *S. ebulus* L. flower, leaf, and fruit extracts.

Compounds	SNFlE	PSNFlE	SEFlE	PSEFlE	SNLE	PSNLE	SELE	PSELE	PSNFrE	PSEFrE
Phenolic acids, mg/100 g										
Chlorogenic acid	4673.3 ^{b} ± 217.6 ***	7086.7 ^{a} ± 312.8 ***	2307.5 ^{d} ± 74.8 **	3953.6 ^{c} ± 167.6 ***	2149.1 ^{d} ± 97.4 ***	4810.4 ^{b} ± 210.5 ***	2068.7 ^{d} ± 88.3 ***	3026.6 ^{c} ± 154.2 *	3614.9 ^{c} ± 190.1 *	3214.6 ^{c} ± 122.7 *
Neochlorogenic acid	998.5 ^{b} ± 53.8 **	1342.2 ^{a} ± 69.2 **	395.6 ^{d} ± 19.2 ***	550.4 ^{c} ± 21.8 ***	485.3 ^{cd} ± 19.4 ***	1082.8 ^{b} ± 52.7 *	583.5 ^{c} ± 19.7 ***	1300.1 ^{a} ± 60.0 **	417.7 ^{d} ± 16.3 ***	305.1 ^{e} ± 18.7 ***
Caffeic acid	131.6 ^{d} ± 6.2 *	234.5 ^{b} ± 14.3 *	n.d.	n.d.	507.9 ^{a} ± 20.1 ***	328.1 ^{b} ± 16.7 ***	80.3 ^{e} ± 3.2 ***	37.6 ^{f} ± 1.7 **	294.1 ^{bc} ± 5.9 ***	373.8 ^{b} ± 14.2 ***
p-Coumaricacid	51.1 ^{d} ± 3.1 ***	103.3 ^{c} ± 4.7 *	62.5 ^{d} ± 3.7 ***	111.7 ^{c} ± 4.6 ***	110.4 ^{c} ± 4.0 ***	366.7 ^{a} ± 17.5 ***	25.6 ^{e} ± 0.9 ***	46.7 ^{d} ± 0.8 *	151.2 ^{b} ± 6.9 ***	164.7 ^{b} ± 6.8 ***
Rosmarinic acid	212.4 ^{e} ± 5.8 **	480.2 ^{d} ± 17.9 **	915.1 ^{a} ± 38.9 ***	686.7 ^{b} ± 28.4 ***	274.2 ^{e} ± 13.6 **	734.1 ^{b} ± 21.7 ***	562.8 ^{c} ± 22.3 ***	978.5 ^{a} ± 61.3 ***	237.6 ^{e} ± 11.4 ***	588.3 ^{c} ± 24.0 ***
Cinnamic acid	96.9 ^{c} ± 3.8 ***	188.9 ^{b} ± 8.7 ***	133.9 ^{c} ± 3.7 *	254.1 ^{a} ± 12.1 ***	42.1 ^{d} ± 1.9 ***	101.9 ^{c} ± 4.9 ***	11.8 ^{e} ± 0.6 ***	16.8 ^{e} ± 0.7 ***	55.8 ^{d} ± 2.3 **	41.4 ^{d} ± 1.6 ***
Vanillic acid	176.1 ^{e} ± 10.7 *	296.0 ^{d} ±13.2 **	86.1 ^{f} ± 1.8 ***	180.2 ^{e} ± 5.4 **	471.0 ^{c} ± 19.4 *	2117.6 ^{a} ± 101.6 **	237.1 ^{d} ± 8.6 **	290.7 ^{d} ± 10.0 ***	678.5 ^{b} ± 24.7 ***	286.1 ^{d} ± 9.6 ***
Benzoic acid	560.2 ^{e} ± 33.6 *	733.8 ^{d} ± 19.7 *	312.5 ^{f} ± 19.5 *	547.4 ^{e} ± 25.2 *	1048.1 ^{c} ± 46.6 *	1685.5 ^{a} ± 76.5 ***	1237.7 ^{b} ± 49.3 *	1557.5 ^{a} ± 61.9 *	1038.2 ^{c} ± 92.6 *	816.4 ^{d} ± 69.1 *
Ferulic acid	128.7 ^{b} ± 3.5 ***	227.7 ^{a} ± 8.2 ***	50.8 ^{d} ± 3.0 ***	155.7 ^{b} ± 10.8 ***	79.7 ^{c} ± 2.6 **	167.8 ^{b} ± 5.5 **	27.1 ^{d} ± 0.3 ***	41.8 ^{d} ± 0.9 ***	136.2 ^{b} ± 4.5 ***	218.2 ^{a} ± 4.8 ***
Gallic acid	87.2 ^{f} ± 3.6	91.8 ^{f} ± 3.3 ***	481.5 ^{b} ± 17.2 ***	458.7 ^{b} ± 12.3 ***	206.0 ^{d} ± 9.3 **	322.1 ^{c} ± 6.8 ***	243.2 ^{d} ± 11.0 ***	147.1 ^{e} ± 5.4	644.6 ^{a} ± 26.5 ***	512.7 ^{b} ± 22.9 ***
Flavonoids, mg/100 g										
Quercetin	115.9 ^{d} ± 4.6 **	203.8 ^{b} ± 12.2 **	163.9 ^{c} ± 5.9 *	227.3 ^{b} ± 8.3 ***	177.3 ^{c} ± 7.3 *	395.1 ^{a} ± 15.7 ***	92.1 ^{d} ± 2.7 ***	142.8 ^{c} ± 5.6 ***	256.1 ^{b} ± 11.4 ***	295.0 ^{b} ± 14.5 ***
Quercetin-3-glucoside	1120.8 ^{d} ± 35.6 ***	2963.0 ^{a} ± 132.7 ***	1146.2 ^{d} ± 69.1 ***	2028.2 ^{b} ± 94.3 ***	349.5 ^{g} ± 7.7 ***	717.5 ^{e} ± 23.8 *	248.2 ^{g} ± 11.3 ***	489.2 ^{f} ± 23.4 ***	1513.3 ^{c} ± 68.8 *	1676.5 ^{c} ± 33.4 **
Quercetin 3-rutinoside	8414.2 ^{b} ± 250.7 ***	14,232.1 ^{a} ± 648.9 ***	2010.9 ^{e} ± 73.8 ***	4550.0 ^{c} ± 224.5 ***	4271.1 ^{c} ± 178.9 ***	7004.1 ^{b} ± 235.3 ***	768.9 ^{g} ± 47.1 ***	1092.7 ^{f} ± 43.4 ***	4623.0 ^{c} ± 283.7 ***	3465.6 ^{d} ± 144.8 ***
Myricetin	138.5 ^{c} ± 6.4 ***	294.4 ^{a} ± 14.8 ***	167.6 ^{b} ± 7.7	280.8 ^{a} ± 10.3 ***	119.6 ^{c} ± 3.0 ***	227.4 ^{b} ± 9.5 **	86.6 ^{d} ± 4.7 ***	178.2 ^{b} ± 10.9 **	276.8 ^{a} ± 16.9 ***	316.7 ^{a} ± 10.1 ***
Kaempferol	38.4 ^{d} ± 1.4 **	64.6 ^{c} ± 2.0 **	116.9 ^{b} ± 4.8 ***	210.4 ^{a} ± 8.6 ***	27.3 ^{d} ± 0.9 ***	92.2 ^{b} ± 2.4 ***	24.2 ^{d} ± 0.8 **	57.0 ^{c} ± 3.4 **	57.7 ^{c} ± 1.5 **	86.3 ^{b} ± 2.8 ***
Apigenin	125.3 ^{b} ± 3.7 ***	207.1 ^{a} ± 8.3 ***	95.8 ^{c} ± 4.8 ***	137.5 ^{b} ± 6.0 ***	32.6 ^{e} ± 1.1 ***	94.7 ^{c} ± 5.7 *	34.7 ^{e} ± 1.2 ***	47.3 ^{d} ± 2.0 ***	19.8 ^{f} ± 0.9 ***	53.0 ^{d} ± 2.0 ***
Total polyphenols, mg GAE/100g	17,944.8 ^{d} ± 515.3 ***	42,647.1 ^{b} ± 1331.8 ***	9803.0 ^{f} ± 443.9 ***	14,617.9 ^{e} ± 590.7 **	17,842.6 ^{d} ± 124.9 **	30,120.5 ^{c} ± 1748.8 ***	11,568.5 ^{f} ± 644.1 **	13,928.7 ^{e} ± 63.4 **	59,596.6 ^{a} ± 1796.3 ***	32,204.8 ^{c} ± 598.4 ***

Data represent mean ± SD; n.d.—not detected; SNFlE (SN flower extract); PSNFlE (purified SN flower extract); SEFlE (SE flower extract); PSEFlE (purified SE flower extract); SNLE (SN leaf extract); PSNLE (purified SN leaf extract); SELE (SE leaf extract); PSELE (purified SE leaf extract); PSNFrE (purified SN fruit extract); PSEFrE (purified SE fruit extract). Different letters in the same row indicate significant differences (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

3.1.2. Anthocyanin and Anthocyanidin Content and Composition

The results of the HPLC analyses clearly show that fruit extract from SN is richer in anthocyanins than SE extract (Table 2). Cyanidin glycosides were the predominant anthocyanins in both SN and SE extracts.

Table 2. Anthocyanin and anthocyanidin content and composition (mg/100 g DW) of dry *S. nigra* L. and *S. ebulus* L. fruit extracts.

Compounds	PSNFrE	PSEFrE
Anthocyanins, mg/100 g		
Cyanidin-3-glucoside	24,341.1 ^{a} ± 1017.4 **	30.7 ^{b} ± 1.1
Cyanidin-3-sambubioside	21,051.4 ^{a} ± 951.5 **	44.2 ^{b} ± 2.3
Cyanidin-3,5-diglucoside	4951.2 ^{a} ± 197.3 ***	32.3 ^{b} ± 1.6
Cyanidin-3-galactoside	108.7 ^{b} ± 4.4 ***	1830.1 ^{a}± 66.3
Cyanidin-3-rutinoside	40.5 ^{a} ± 1.4 **	10.4 ^{b} ± 0.5
Cyanidin-3-arabinoside	n.d.	2.8 ± 0.1
Pelargonidin-3-glucoside	64.2 ± 2.0	n.d.
Anthocyanidins, mg/100 g		
Cyanidin	36,500.6 ^{a} ± 1868.8 **	2063.7 ^{b} ± 96.9
Delphinidin	203.4 ± 9.1	n.d.
Pelargonidin	112.1 ± 4.8	n.d.
Malvidin	242.3 ± 12.4	n.d.
Peonidin	n.d.	n.d.

Data represent mean ± SD; n.d.—not detected; PSNFrE (purified SN fruit extract); PSEFrE (purified SE fruit extract). Different letters in the same row indicate significant differences (** $p < 0.01$; *** $p < 0.001$).

3.2. Antioxidant Activity

We used two complementary antioxidant activity assays that examine different aspects of the antioxidant activity of polyphenols (ORAC and HORAC) to define the antioxidant activity of SN and SE extracts (Figure 1).

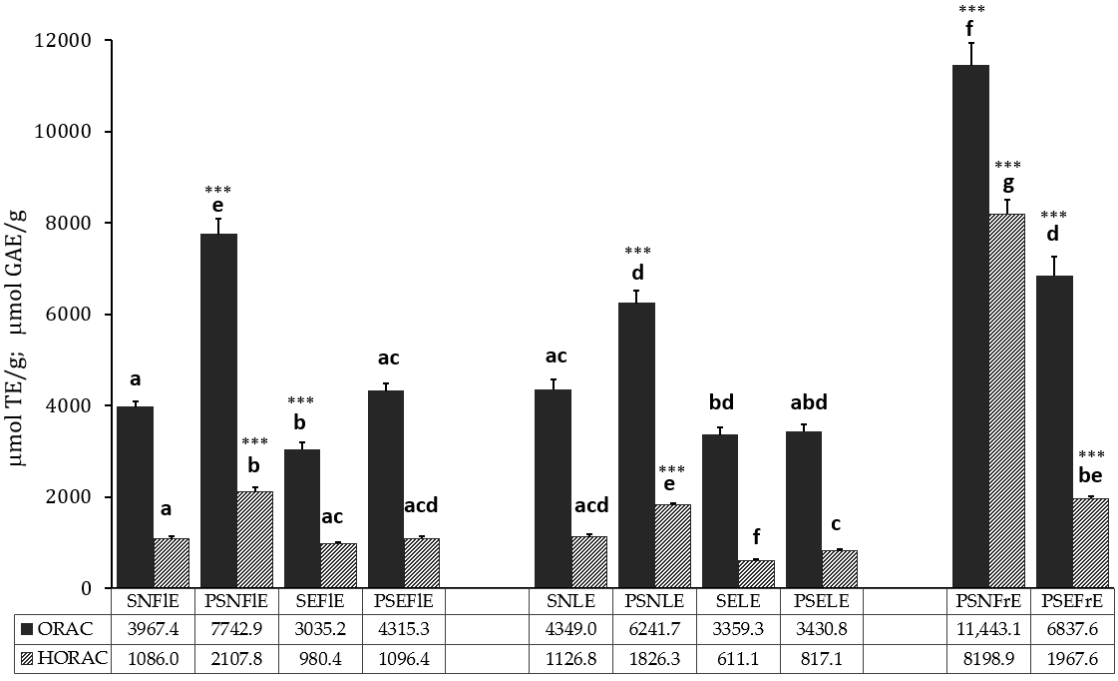

Figure 1. Antioxidant activity of SN and SE flower, leaf, and fruit extracts measured through ORAC and HORAC methods. Data represent mean ± SD; values with different letters are significantly different (*** $p < 0.001$).

3.3. Antiviral Activity

3.3.1. Determination of the Cytotoxic Effect of the Extracts on the MDBK Cell Line

Only three of the tested extracts showed inhibitory effects against HSV-2, as well as virucidal activity. Hence, we proceeded with screening for the antiviral activity of SN and SE extracts against human herpesvirus type 2 (HSV-2). To evaluate the bioactive potential of investigated extracts, we first started with the determination of their cytotoxicity in the MDBK cell line. The viability of the MDBK cell line upon exposure to the extracts was determined. Table 3 presents the results for the cytotoxic effect of the investigated extracts.

Table 3. Cytotoxicity of extracts from SN and SE in MDBK cell line.

Extract	MTC	CC_{50}
SNFlE	2000 µg/mL	3398 µg/mL
PSNFlE	1000 µg/mL	1700 µg/mL
SEFlE	1500 µg/mL	3410 µg/mL
PSEFlE	2000 µg/mL	3331 µg/mL
SNLE	2000 µg/mL	3300 µg/mL
PSNLE	2000 µg/mL	2700 µg/mL
SELE	1500 µg/mL	3310 µg/mL
PSELE	1500 µg/mL	3320 µg/mL
PSNFrE	2000 µg/mL	3570 µg/mL
PSEFrE	2500 µg/mL	3420 µg/mL

MTC (maximal tolerated concentration); CC_{50} (cytotoxicity concentrations).

The data suggest that the extracts slightly altered the cell morphology, as the MTC value of the extracts ranged from 1000 to 2500 µg/mL (Table 3). The results obtained are presented graphically on Figure 2. As shown, extracts were applied at concentrations ranging between 500 µg/mL and 4.5 mg/mL, and both MTC and CC_{50} were simultaneously determined.

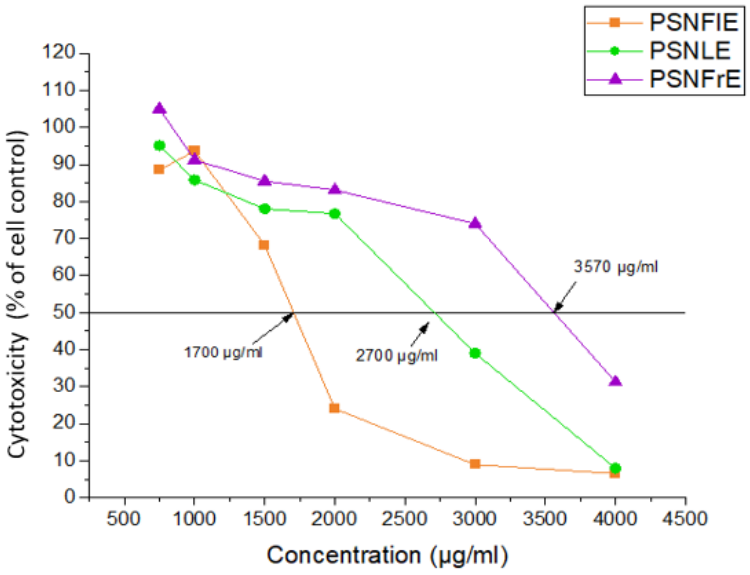

Figure 2. Cytotoxicity of purified SN flower, leaf, and fruit extracts against MDBK cell line.

The low toxicity obtained through the MTC assay was further confirmed through the MTT assay. The results for MTC and CC_{50} were measured at 48 and 72 h after the addition of the extracts using a colorimetric MTT test [38]. The calculated CC_{50} values of the extracts ranged from 1.70 mg/mL for PSNFlE to 3570 µg/mL for PSNFrE resp.

From the results obtained, we concluded that the least toxic were PSNFrE and PSEFrE. The highest cytotoxicity was shown by PSNFlE (CC_{50}—1700 mg/mL).

3.3.2. Virucidal Activity

When the extracts were applied to MTC against viral replication (intracellular stage), negligible inhibitory activities for PSNFlE, PSNLE, and PSNFrE were found. PSEFrE did not show activity against viral replication, nor virucidal activity against extracellular virion activity. PSNFlE, PSNLE, and PSNFrE at the same concentrations showed a strong inhibitory effect against the extracellular form of the virus (virucidal activity) (Figure 3).

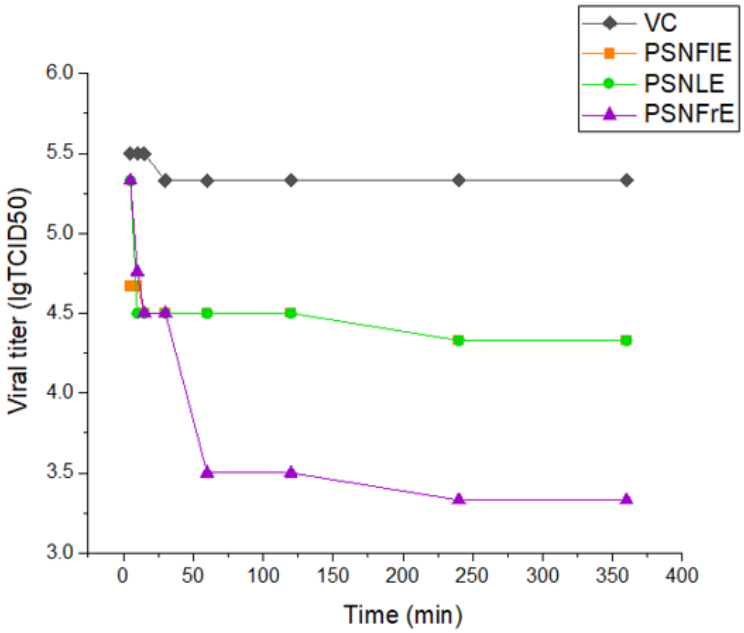

Figure 3. Virucidal activity (activity on extracellular HSV-2) of purified SN flower, leaf, and fruit extracts.

The data suggest a significant inactivation of more than 85% after 5 min of contact with HSV-2 virions with the flower extract applied in maximal nontoxic concentration. As seen in Figure 3, the other extracts tested (PSNLE, PSNFrE) inactivated the virions by 90%, 10 min from the beginning of the effect. The effect was permanently maintained during the incubation as the viral inactivation reached a value of 90% (Δlog = 1) for flower and leaf extracts. The highest inactivation was achieved through the application of the purified fruit extract of *S. nigra* L. After 60 min from the start of incubation, inactivation was 98%. For more than 4 h after administration of the PSNFrE, the inactivation of the extracellular virus was almost complete—99% (Δlog 1).

4. Discussion

Polyphenols, including flavonoids and anthocyanins, are secondary metabolites produced primarily in plants. They are highly studied compounds due to their important health benefits as they exhibit activities such as antioxidant, anti-cancer, anti-inflammatory, anti-aging, and antiviral [40–42]. Black and dwarf elder and their bioactive components, polyphenols, and anthocyanins are known for their powerful effects on oxidative stress [14,19,21]. As it is evident from the results, the studied extracts (from fruits, flowers, and leaves), and especially those purified through solid-phase extraction, are very a rich source of phenolic compounds. All extracts contain several classes of phenolic compounds: hydroxycinnamic acids (chlorogenic, neochlorogenic, caffeic, p-coumaric, rosmarinic, and cinnamic acid); hydroxybenzoic acids (benzoic, gallic, vanillic, and ferulic acid); flavonols (quercetin, quercetin-3-glucoside, quercetin-3-rutinoside, myricetin, kaempferol), the flavon apigenin, and anthocyanins. Different extracts significantly differ in total polyphenol content, which varied in a broad range from 9803.3 mg GAE/100 g DW (SEFlE) to more than a six-fold higher value of 59,596.6 mg GAE/100 g DW (PSNFrE). The total polyphenol

content of purified dry extracts PSNFlE and PSNLE was 42,647.1 ± 1331.8 mg GAE/100 g DW and 30,120.5 ± 1748.8 mg GAE/100 g DW, respectively. In flower and leaf extracts, chlorogenic acid was the predominant representative, whereas benzoic acid was the major phenolic acid only in the fruit extracts. The chlorogenic acid content varied in the range of 2068.7 ± 88.3–7086.7 ± 312.8 mg/100 g DW. The highest amount of this acid was found in PSNFlE. Chlorogenic acid, followed by neochlorogenic and benzoic acids, were detected in all investigated extracts. The results also show that the flowers are richest in chlorogenic acid, followed by fruits and leaves. The amount of chlorogenic acid was 1.5 times higher in PSNFlE (7086.7 mg/100 g DW) compared to SNFlE (4673.3 mg/100 g DW).

Previous studies on *Sambucus nigra* reported TPC values in the range of 364 and 387 mg GAE/100 g and 510 and 582 mg GAE/100 g, as well as 371–432 mg GAE/100 g fresh mass for different SN varieties and genotypes [43,44]. Mikulic-Petkovsek et al. reported a TPC of 515 mg GAE/100 g FW for wild elderberry [45], while Duymuş et al.'s TPC values range from 1986 to 2333 mg GAE/100 g DW [46]. The total phenolic content data in our study are not comparable to these data, as our extracts were polyphenol-enriched. It is known that SN and SE fruits are relevant sources of phenolic acids and some studies indicate that chlorogenic and neochlorogenic acids are the main phenolic acids in SN and SE species [14,23,47–49]. Przybylska-Balcerek et al. reported that chlorogenic, sinapic, cinnamic, and ferulic acids are the major phenolic acids [24]. However, differences in the amount of phenolic acids could be expected due to various factors, including variety, environmental conditions (light, temperature, cultivation methods, etc.), processing method, and storage conditions [50,51]. Interestingly, some authors [52] reported the presence of salicylic, sinapic, protocatechuic, and gentisic acids, which were not found in our extracts. Data on the amount and composition of phenolic acids in SE are scarcer. According to the literature, the SE flowers contain caffeic acid and its derivatives, including chlorogenic and *p*-coumaric acids [14]. Chlorogenic acid, which was the major phenolic component of the extracts, is an important dietary polyphenol with numerous therapeutic effects, such as antioxidant, antibacterial, hepatoprotective, cardioprotective, anti-inflammatory, antipyretic, neuroprotective, anti-obesity, and antimicrobial activities [53]. It has antiviral effects against several viruses, including HIV [54,55], adenovirus [56], hepatitis B virus [56], and HSV [57], and also inhibits inflammation caused by viral infections [57].

The presence of various phenolic components such as flavonol derivatives in *Sambucus* species has been previously reported. A number of studies have investigated the flavonoid content and composition of SN and have demonstrated the presence of flavones, flavonols, and flavonol glycosides. The main phenolic components in black elderberry flowers are flavonols, namely quercetin-3-rutinoside (rutin), quercetin-3-glucoside (isoquercetin), kaempferol-3-glucoside, kaempferol-3-rutinoside, and isorhamnetin-3-rutinoside [58]. In our study, significant differences in the content of the investigated flavonoids among the studied extracts were observed. Quercetin and its glycosides (flavonols) were present in all extracts. PSNFlE was distinctive with the highest content of quercetin-3-rutinoside (14,232.1 ± 648.9 mg/100 g DW), followed by PSNLE (7004.1 ± 235.3 mg/100 g DW) and PSNFrE (4623.0 ± 283.7 mg/100 g DW). The content of isoquercetin was also higher (2963.0 ± 132.7 mg/100 g DW, 2028.2 ± 94.3 mg/100 g DW, 1513.3 ± 68.8 mg/100 g DW—determined for PSNFlE, PSEFlE, and PSNFrE, respectively). In addition, rutin and isoquercetin, the other flavonoids, appeared to be minor constituents. Despite the differences in the flavonoid content and composition, all extracts were distinctive with a very high content of rutin, which was the predominant flavonol in all extracts. The highest amount of rutin (14,232.1 mg/100 g DW) found in PSNFlE significantly exceeds the available results in the literature from 11.6 mg/100 g DW to 42.3 mg/100 g DW [59], and 14.93 mg/100 g DW [60], which is due the purification using solid-phase extraction. Previous studies indicate that SN flowers are the richest part of the plant in flavonoids [61], which is in agreement with our results. Christensen et al. reported that quercetin-3-rutinoside, kaempferol-3-rutinoside, and isorhamnetin-3-rutinoside are the major flavonoids composing over 90% of the total flavonoid content in black elderberry flowers [60].

Determining plant anthocyanin content is of great importance from both a nutritional and pharmacological point of view. We detected cyanidin-3-glucoside, cyanidin-3-sambubioside, cyanidin-3,5-diglucoside, cyanidin-3-galactoside, cyanidin-3-O-rutinoside, cyanidin-3-arabinoside, and pelargonidin-3-glucoside. The PSNFrE contained 24,341.1 mg/100 g DW cyanidin-3-glucoside and 21,051.4 mg/100 g DW cyanidin-3-sambubioside. The remaining anthocyanin representatives were in smaller quantities. Pelargonidin-3-glucoside was only found in SN fruit extracts, and cyanidin-3-arabinoside was only found in SE extracts. In order to reveal whether glycosides of other anthocyanidins were present, we hydrolyzed the investigated extracts and revealed that besides cyanididin, SN extract also contains small amounts of delphinidin, pelargonidin, and malvidin. After hydrolysis, SE extract revealed a slightly higher content of cyanidin, which indicates that it contains additional cyanidin glycosides. However, aglycones unlike cyanidin were not detected in SE extract. It is known that berry fruits are particularly rich in anthocyanins, whose content, however, depends mostly on the plant species and climate characteristics [62]. According to the literature data, the predominant anthocyanin in *S. nigra* fruit is cyanidin-3-glucoside (204.6–481.4 mg CGE/100 g FW), followed by cyanidin-3-sambubioside (122.2–269.1 mg CGE/100 g FW) [43]. Other cyanidin glycosides reported include cyanidin-3-sambubioside-5-glucoside, cyanidin-3,5-diglucoside, cyanidin-3-rutinoside, pelargonidin-3-glucoside, and pelargonidin-3-sambubioside [15,21]. In agreement with these results, in our study, cyanidin glycosides were found to be the predominant anthocyanins in both SN and SE extracts. However, SN fruit extract was a very rich source of anthocyanins.

In order to assess the effect of phenolic compounds on the antioxidant and antiviral effects of the extracts, we used solid-phase extraction, which led to a significant increase in their polyphenol and anthocyanin contents [32]. During purification, impurities such as sugars, acids, proteins, and pectin are removed from the raw extract, thereby concentrating polyphenols, flavonoids, and anthocyanins. The purpose of purification was to remove primary metabolites and obtain polyphenol-enriched extracts. From the results on the phenolic content and composition of the extracts, it is evident that solid-phase extraction resulted in the production of extracts with a higher content of phenolic acids, flavonoids, and anthocyanins. This is likely a prerequisite for increased biological activity of the extracts, since a significant number of studies have demonstrated the effectiveness of polyphenols against different pathogens, including herpes simplex virus (HSV) [10,63].

To more fully characterize the obtained dry extracts and their expected high biological activity, we determined their antioxidant and anti HSV-2 activities. Since polyphenols have been described in the literature as potent antioxidants, we hypothesized that enriched polyphenol extracts would exhibit both higher antioxidant and antiviral activities against HSV-2. Given the differences in the polyphenol content and composition of the studied extracts, it could be expected that there would also be differences in their antioxidant and antiviral activities. As far as we know, no previous research has reported ORAC, HORAC, and anti-HSV-2 activities of polyphenol-enriched SN and SE fruit, flower, and leaf extracts. For this reason, we were unable to make comparisons of our results with literature data.

The highest ORAC antioxidant activity was measured for PSNFrE (11,443.1 ± 495.2 µmol TE/g) followed by PSNFlE (7742.9 ± 338.4 µmol TE/g) and PSEFrE (6837.6 ± 429.8 µmol TE/g). SEFlE (3035.2 ± 162.7 µmol TE/g) and SELE (611.1 ± 26.4 µmol GAE/g) revealed a lower activity measured using ORAC and HORAC, respectively. Due to the high amount of polyphenols, including anthocyanins, in PSNFrE, its ORAC value was extremely high. The HORAC value of PSNFrE was also high (8198.9 ± 315.4 mol GAE/g). The ORAC and HORAC values of PSNFlE were 7742.9 ± 338.4 mol TE/g and 2107.8 98.1 mol GAE/g, respectively. Similarly to TPC, ORAC and HORAC antioxidant activity values in purified extracts were significantly higher than those of the unpurified ones.

Given the scarcity of research on the antiviral effects against HSV-2 of studied plants, particularly of their polyphenol-enriched extracts, this study continued by investigating their activity against HSV-2. Our results confirmed the safety of *S. nigra* L. fruits, which have been recognized as a safe food additive by the US Food and Drug Administration

(FDA) and the European Medical Agency (EMA) [64]. A number of medicinal plants have shown antiviral activity against different virus types and polyphenols have shown an ability to inhibit viruses via different mechanisms [65]. In the available literature, there are data on the antiviral activity mainly for SN and SE fruits, while flowers and leaves are less studied. Fruit extracts have been studied mainly for their effects against influenza viruses—type A and type B, H5N1, and H1N1—HIV-1; coronavirus (CoV), causing infectious bronchitis in birds; and other human coronaviruses (HCoV 229E, HCoV OC43, HCoV HKU1, and HCoV NL63), which are responsible for about 20% of common colds and other respiratory diseases [12,66]. According to Vlachojannis et al., the results from several in vitro and in vivo human studies suggest that the aqueous elderberry extract Sambucol® (derived from *S. nigra* fruits) demonstrates potential in treating viral infections [67]. However, data for activity against herpes viruses of both species are scarce.

Given the fact that we studied complex extracts containing different phenolic compounds, it is a difficult task to seek a relation between the structure and activity of *Sambucus* metabolites. It is known that the antiviral effects of phenolic compounds could be due to different mechanisms. For example, chlorogenic acid exhibits activity against different viruses and a clear determination of the underlying mechanism of its antiviral action is difficult [65]. The antiviral properties of kaempferol and quercetin derived from elderberries have been documented for their impact on HSV-1 virus [68]. Among anthocyanins, cyanidin and its glycosidic forms have been studied in regard to their antiviral action against InfV A and B, HSV-1, and other viruses [2]. The hypothesized mechanisms for the antiviral effects of polyphenols include the inhibition of extracellular virions, prevention of their attachment to the cell membrane, and interference with intracellular viral replication. Caffeic acid and its derivatives do not demonstrate direct virucidal effects, and their antiherpetic activity is likely attributed to the interaction with cell membrane elements, which inhibits viral adsorption and penetration. Additionally, it may disrupt intracellular replication stages before the completion of viral DNA replication [69]. According to Jassim and Naji, anthocyanins derived from plants may possess significant antiviral properties, hence potentially serving as an effective treatment against viral infections [70].

It should be noted that in vitro studies of antiviral activity have their limitations, and the major one is likely related to the lack of a clinical picture and recovery after treatment. However, this is a further step in any research on the antiviral activity of untested substances. In conclusion, due to the low cytotoxicity and significant inhibition of extracellular virions, PSNFrE is promising for further studies as an anti-HSV-2 agent. The observed effect is likely attributable to the complex chemical composition of the extract, and particularly the significant content of anthocyanins. The results of our study showed that purification of the extracts resulted in differences in the amount of polyphenolic components, which was a prerequisite for increased biological activity. Purified SN fruit extracts can be used for the production of functional foods and dietary supplements with antioxidant and anti HSV-2 activities.

5. Conclusions

The current study indicates that SN and SE fruit, flower, and leaf extracts significantly differ both in the content and the composition of their phenolic compounds, which is a prerequisite for different antioxidant and antiviral activities. The highest antioxidant activity was exhibited by PSNFrE, which was also the richest source of polyphenols. The effect of the substances on viral replication was negligible. Our results indicate that three of the extracts (PSNFrE, PSNLE, and PSNFlE) demonstrate a pronounced effect on extracellular virions; however, PSNFrE revealed the highest activity against HSV-2. We consider the fact that the substances affect the extracellular form of a supercapsid virus to be extremely important. The antiviral properties of PSNFrE could likely be attributed to the high content of anthocyanins. The statement that *S. nigra* is the safest member of the genus *Sambucus* is reasonable and consistent with its ethnobotanical history of use. The

use of *S. nigra* fruits and extracts as anti-HSV-2 agents is promising and could lead to the development of novel immune-boosting and antiviral nutraceuticals and drugs.

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