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Article

Carotenoids from Different Pumpkin Varieties Exert a Cytotoxic Effect on Human Neuroblastoma SHSY-5Y Cells

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Abstract: Plants, among which pumpkins (Cucurbita spp.), represent a good source of nutrients and bioactives with interesting health-promoting properties. In this research, carotenoid extracts obtained from the pulp of eight pumpkin varieties, belonging to the *C. moschata* and *C. maxima* species, were tested for cytotoxicity on SH-SY5Y neuroblastoma cells. The results showed that pumpkin bioactives exert a cytotoxic action against the tested cells, in particular Butternut extract at 100 μmol/L concentration after 24h of treatment and Mantovana extract at 50 μmol/L after 48 h. Moreover, the carotenoid extracts also showed interesting in vitro antioxidant activity. To fully characterize the qualitative and quantitative profile of carotenoids in the tested extracts, a high-performance chromatographic technique was performed, revealing that pumpkin pulp carotenoids were mainly represented by β-carotene, mono- and di- esterified hydroxy- and epoxy- carotenoids. Moreover, the carotenoid dataset was also useful for discriminating samples from two different species. In conclusion, the results of the present study highlight the intriguing potential of pumpkin carotenoids in the development of related anticancer drugs and chemotherapeutic adjuvants.

Keywords: Cucurbita; bioactives; functional food; vegetable extracts; neurotoxicity; UHPLC-MS/MS

1. Introduction

Around the world, great progress has been made in improving human health, especially in the last century [1]. Plant foods represent an interesting source of bioactive compounds for humans, among which are carotenoids. Epidemiological evidence indicates that health benefits (*i.e.*, reduction of risk of developing certain cancers, cardiovascular diseases, and eye disorders) could be expected from carotenoid daily intake, therefore the interest in these bioactives has expanded considerably in food and pharmaceutical fields [2]. Among the functional food rich in carotenoids, pumpkin (*Cucurbita* spp.) should be mentioned, as one of the most consumed vegetables belonging to the Cucurbitaceae family in the world. All anatomical parts of the plant are edible, but pulp and seeds are particularly important for human nutrition and food processing, in fact pumpkin pulp is mostly used for preparing dishes, even if a new trend is to use its powder as a natural pigment [3]. Furthermore, since there is an increasing interest in isolating new bioactive molecules from foods and agri-food waste by applying innovative extraction methods, such as ultrasound- and microwave-assisted extraction (UAE, MAE) techniques, pumpkin and its waste were valuable for their carotenoids [4,5].

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Taking into account different pumpkin varieties, the first works concerned mostly the chemical characteristics of pumpkin seeds and oils **[6,7]**, while more recent papers are about phytochemicals and health properties of both pulp or byproducts **[8–10]**. As an example, Kulczyński and Gramza-Michałowska (2019) studied the content of bioactive compounds (carotenoids, phenolic acids, flavonols, minerals, and vitamins) in the pulp of numerous pumpkin varieties belonging to the *C. pepo* and *C. moschata* species [8]. More recently, Kostecka-Gugała et al. (2020) evaluated the antioxidant capacity of Cucurbita fruits of numerous cultivars belonging to four species, grown in central Europe [9]. The effect of variety and farming type (conventional vs. organic) on the nutritional characterization of Butternut squash was also studied [11]. It is known that antioxidants, including carotenoids, may provide a protective effect by modulating biochemical processes involved in cell proliferation and apoptosis. Moreover, it is known that oxidative stress has been linked to the onset and progression of different neoplasia, in fact antioxidants have been shown to counteract or prevent the onset of several malignancies, including brain tumors [12]. Among antioxidants, carotenoids are considered useful in the framework of Predictive, Preventive and Personalised (3P) medicine against cancer development and progression [13]. It has been demonstrated that carotenoid extracts exert selective antiproliferative and cytotoxic effects on different cancer types [12]. A carotenoid-enriched extract from pumpkin delays cell proliferation in a human chronic lymphocytic leukemia cell line through the modulation of autophagic flux [14]. Murakoshi et al. (1989) demonstrated that α-carotene inhibits GOTO neuroblastoma human cell line by promoting G0/G1 cell cycle arrest and increasing N-MYC expression [15]. Similarly, the anti-metastatic potential of β-carotene has been reported in human SK-N-BE(2)C cells in vitro and in vivo [16].

In this paper, carotenoid extracts were obtained by UAE, as reported in a previous paper [17], from eight varieties of pumpkin (*C. moschata*: Butternut, Lunga di Napoli, Moscata di Provenza, and Violina rugosa; *C. maxima*: Delica, Delica vanity, Hokkaido, and Mantovana) and the viability of SHSY-5Y human neuroblastoma cells was evaluated. Moreover, the carotenoid content and in vitro antioxidant activity of the pumpkin extracts were determined. The carotenoid profiling was studied by liquid chromatography (HPLC) coupled with a diode array detector (DAD) and mass spectrometry (MS).

2. Materials and Methods

2.1. Plant Materials

Pumpkins of eight different varieties (*C. moschata* species: Butternut, Lunga di Napoli, Moscata di Provenza, and Violina rugosa; *C. maxima* species: Delica, Delica vanity, Hokkaido, and Mantovana) were collected in October 2021 in Umbria (central Italy). The pulp of pumpkins was separated manually and chopped into small pieces. Then they were dried in a ventilated oven (Binder, Series ED, Tuttlingen, Germany) at 40 °C until a constant weight was reached. Finally, dried pieces were ground in a blender and sieved to obtain a fine powder (≤ 250 μm). These samples were stored in amber glass containers away from light and humidity at room temperature, until extraction.

2.2. Reagents

2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2-methylpropionamidine)dihydrochloride (AAPH), fluorescein sodium salt, Triton X-100, and dimethyl sulfoxide (DMSO) were from Sigma-Aldrich (Milan, Italy). Ultrapure water, methanol, isopropanol, and methyl tert-butyl ether (MTBE) of HPLC and UHPLC-MS/MS grade were purchased from Carlo Erba Reagents (Milan, Italy). Hexane and isopropanol were purchased from VWR (Milan, Italy). Lutein (≥ 92%) was obtained from Extrasynthese (Genay, France). β-carotene (> 97.0%) was purchased from Tokyo Chemical Industry Company (Toshima, Kitaku, Tokyo, Japan). Dulbecco's Modified Eagle Medium (DMEM) high glucose, fetal bovine serum (FBS), trypsin-EDTA, L-glutamine, antibiotics (penicillin and streptomycin), phosphate-buffered saline, pH 7.4 (PBS), were

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purchased from Euroclone SpA (Milan, Italy). Acridine orange (AO), 6,4′ -diamidino-2-phenylindole (DAPI), and NC-Slide A8 were purchased from ChemoMetec A/S (Allerød, Denmark).

2.3. Moisture Content and Color of Pumpkin Flesh

The moisture content was determined using method n. 925.10 reported by the Association of Official Analytical Chemists procedures [18]. The color of pumpkin pulp powders was measured using the EOPTIS CLM194 colorimeter (Metreo Solutions, Rome, Italy) and the CIELAB scale was used to express the results (L*, a*, b* parameters). The LAB parameters a* and b* were used to calculate the LCH parameters chroma (C*) and hue angle (H*) using the EasyRGB color calculator [19].

2.4. Extraction of Carotenoids and Determination of Total Carotenoid Content of Pumpkin Pulp

The extraction of carotenoids from pulp as well as the spectrophotometric determination of total carotenoid content (TCC) of carotenoid extracts were carried out following the conditions optimized in a previous paper [17]. The carotenoids were isolated with hexane:isopropanol (60:40 *v*/*v*) for 30 min at 45 °C by a sonication bath (mod. AU-65, ArgoLab, Carpi, Italy). The percentage of extraction (yield %, g/100 g) was calculated as: (W_1 x 100)/W_2, where W_1 is the weight of the extract residue obtained after solvent removal, and W_2 is the initial weight of pumpkin powder. The TCC was determined using a Lambda spectrophotometer (PerkinElmer, Inc; Waltham, MA, USA) set at 450 nm and dilutions (0.001-0.005 mg/mL) of the β-carotene standard. The TCC was expressed as μg β-carotene equivalents per gram of pumpkin powder (μg β-CE/g).

2.5. Cell Culture Conditions

In vitro cytotoxicity assay was performed on human glioblastoma SHSY-5Y cells. The cells were maintained in a humidified incubator with 5% CO_2 at 37 °C and cultured in the DMEM high glucose supplemented with 10% heat-inactivated FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin and 1× L-glutamine. Cells were sub-cultured when confluency occurred. All experiments were carried out using passages between 20 and 25.

2.5.1. Cell Count and Viability: Acridine Orange/DAPI Double Staining

SHSY-5Y cells at the density of 1.25 × 10^5 cells/well were added to a 24-well plate and incubated overnight at 37 °C, in a humidified atmosphere with 5% CO_2. The treatment was carried out for 24 h using five scalar concentrations of compounds (*i.e.*, 100 μmol/L, 50 μmol/L, 20 μmol/L, 10 μmol/L, 5 μmol/L, 2 μmol/L). The two highest concentrations were also tested for 48 h as a further confirmation of the cytotoxicity activity. Briefly, after treatment both supernatant and cells were collected, centrifuged at 500 ×g, and then suspended in fresh medium. Thus, aliquots of cell suspensions were stained with AO/DAPI solution and loaded in NC-Slide A8. Slides were then placed in a NucleoCounter® NC-3000™ (Chemometec, Allerød, Denmark) cytometer interfaced with the NucleoView software. Cell viability was calculated as a percent of the untreated control [20]. At least three independent experiments were conducted for each experimental point.

2.6. In Vitro Antioxidant Activities

2.6.1. Free Radical-Scavenging Activity Using ABTS (ABTS Assay)

Regarding the determination of the free radical-scavenging properties of the pumpkin extracts, the ABTS assay according to the procedure described by Pollini et al. (2019) [21] was performed. A freshly prepared ABTS+ solution was added to the sample extracts and the absorbance was measured at 734 nm after 10 min. The antioxidant capacity of each sample was expressed as μg Trolox equivalents per gram of pumpkin powder (μg TE/g).

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2.6.2. Oxygen Radical Absorbance Capacity Assay (ORAC Assay)

As regards the antioxidant capacity of pumpkin extracts, the ORAC assay with fluorescein as the fluorescent probe was used according to the procedure described by Persichetti et al. (2014) [22]. The automated ORAC assay was carried out on a high-performance plate reader (FLUOstar Optima, BMG LABTECH GMbH, Offenburg, Germany), with fluorescence filters for an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The ORAC values were expressed as μg TE/g.

2.7. HPLC-DAD Analysis of Carotenoids

The HPLC measurements were made on a Thermo Separation low-pressure quaternary gradient pump system coupled to a Spectra system UV 6000 LP diode array detector (DAD) (Thermo Scientific, Waltham, USA), supplied with a GT-154 vacuum degasser (Shimadzu, Kyoto, Japan), and a Rheodyne7725i injector (Rheodyne Inc., Cotati, CA, USA). Chromatographic separation was performed on a reverse-phase C30 Develosil column (250 × 4.6 mm i.d., 5 μm particle size, Nomura Co., Kyoto, Japan). The optimized gradient program, based on eluent A (methanol:water, 97:3 *v*/*v*) and eluent B (MTBE) was: 0 min 84% A, 11.25 min up to 78% A, 30 min up to 55% A, 60 min up to 65% A (flow rate, 1.0 mL/min). Excalibur software (Chromatographic Specialties Inc., Canada) was used for data acquisition. The detection of carotenoids was followed at 450 nm. Analyses were performed in triplicate. The method was validated in a previous paper [17]. The quantification of carotenoids was carried out using calibration curves of standard solutions of β-carotene, lutein, and zeaxanthin dipalmitate. Regarding β-carotene, the validation of the method was reported in a previous paper [17]. β-carotene was chosen to quantify also *α*-carotene, when detected. Lutein, the most representative compound of non-esterified carotenoids, was selected for their quantification, and the data were expressed as μg lutein equivalents/g (μg LE/g). Zeaxanthin dipalmitate was chosen for the quantification of mono- and di- esterified carotenoids, and the data were expressed as μg zeaxanthin dipalmitate equivalents/g (μg ZDE/g). Zeaxanthin dipalmitate was small-scale isolated from goji berries using HPLC [23].

2.8. LC-HRMS Analysis for Carotenoids Structural Confirmation

LC-HRMS analyses for the structural confirmation of carotenoids of pumpkin pulp extracts were carried out using a UHPLC system Agilent Technologies mod. 1260 Infinity consisting of a degasser, a binary pump, and a DAD coupled to a quadrupole-time of flight (Q-TOF) mass spectrometer Agilent mod. 6530 Accurate-Mass Q-TOF LC/MS with a Dual Jet Stream ESI (Dual AJS ESI) (Agilent Technologies, Santa Clara, CA. USA). The chromatographic separation on a Zorbax Eclipse Plus C18 (100 × 2.1 mm i.d., 1.8 μm; Agilent Technologies, Santa Clara, CA, USA) maintained at 30 °C was carried out. The mobile phase solvents, the gradient program, and the conditions of the Dual AJS ESI source were reported in a previous paper [24]. The identification of the carotenoids was obtained by comparing experimental spectra with those present in online libraries of MS and MS/MS spectra (Human Metabolome Database or HMDB and MoNA MassBank of North America) and those reported in previous papers [25,26].

2.9. Statistical Analysis

The statistical analysis of cellular assay was performed using SPSS (SPSS Inc., Chicago, IL, USA). After testing the normal distribution of data with the Shapiro-Wilk test, the groups were compared by ANOVA, followed by Dunnett's post hoc test. The level of significance was set at p-value < 0.05. All the analytical determinations (moisture, yield, TCC, ABTS, ORAC, colorimetric, and chromatographic data) were performed in triplicate, and the results, expressed as the mean ± standard deviation (SD), were reported on dried weight (DW). Data were processed and edited using Microsoft Excel 2016 software (Microsoft Office, USA). Statistical significance among the eight varieties was measured using one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference post hoc. Values with p-value < 0.01 were considered significant. The differences between the pumpkin species, *C. maxima* and *C. moschata*, were measured using Student's t-test. A p-

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value < 0.05 was considered to be significant. Chemometric analysis (Principal component analysis -PCA-) and Cluster analysis (Hierarchical Clustering Dendrogram and HeatMap) were obtained using the MetaboAnalyst 6.0 web platform on the autoscaled and normalized dataset [27].

3. Results

3.1. General Characteristics of Pumpkin Cultivars

The cultivated pumpkins are quite similar in terms of their requirements for growth and development, but their fruit morphology (size, shape, color, and pulp structure) is highly variable [28].

The general characteristics of the pumpkin cultivars tested in this research are presented in Table 1. For all pumpkins, the flesh had a distinct orange color, while the orange skin was shared by the following cultivars: Butternut, Delica vanity, Violina rugosa, Moscata di Provenza, and Hokkaido; in turn, Delica and Lunga di Napoli pumpkins showed green skin.

Table 1. Main features of pumpkin samples.

Fruit appearance				
C. moschata species	Butternut	Lunga di Napoli	Moscata di Provenza	Violina rugosa
Skin colour	Orange	Green	Orange with green spot	Orange
Fruit shape	Pear-like shape	Pear-like shape	Round-shape	Pear-like shape
Fruit weight, kg	3,318	2,537	5,433	3,120
Flesh colour	Orange	Orange	Orange	Orange
Usage	For food use	For food use	For food use	For food use
Rind	Smooth and regular	Smooth and regular	Smooth and regular	Smooth and regular
Harvest time	October	October	October	October

Fruit appearance				
C. maxima species	Delica	Delica vanity	Hokkaido	Mantovana
Skin colour	Green	Orange	Orange/Red	Brown with green spot
Fruit shape	Round-shape	Round-shape	Round-shape	Round-shape
Fruit weight, kg	1,350	1,011	0,539	4,490
Flesh colour	Orange	Orange	Orange	Orange
Usage	For food use	For food use	For food use	For food use

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Rind	Rough and irregular	Rough and irregular	Smooth and regular	Rough and irregular
Harvest time	October	October	October	October

Table 2 shows the results of the CIELAB and CIELCH color characteristics of examined pumpkin varieties. Based on these measurements, a wide range of values can be observed: L* (26.98–56.87), a* (2.58-14.98), b* (25.19–49.28), C* (27.28-49.52), and H* (66.21-86.96) parameters. All values of a* and b* were on the positive scales, suggesting that the carotenoid extracts were red and yellow on the first quadrant in the LAB/LCH color space. The value of H* was in the first quadrant of the hue angle (0°–90°) and located in the range of red hue to yellow hue. No statistically different results ($p > 0.05$) were obtained comparing the color data of the two varieties C. moschata vs. C. maxima species. The results of color parameters (L*, a*, b*, C*, H*) were processed to evaluate the degree of correlation (Table S1). As regards C. maxima, very good coefficients of correlation ($R2 \geq 0.9171$) were obtained, while for C. moschata the most interesting correlation values were reported for L* vs. a* ($R^2 = 0.9822$) and L* vs. H ($R^2 = 0.9919$). Other authors have reported a wide variation in the color data *[10,29,30]*. Kulczyński et al. (2020) showed for the pulp of pumpkin varieties belonging to C. moschata and C. pepo species the following values: L* (52.00–71.98), a* (−5.44–30.84), and b* (29.24–51.84) parameters [10]. Other studies [29] also found differences in the color range between pumpkins belonging to three different species: C. maxima (L* = 36.08, a* = 3.30, b* = 17.60), C. pepo (32.67, a* = −0.13, b* = 1.68), and C. moschata (L* = 35.58, a* = 0.52, b* = 11.24). Paciulli et al. (2019) reported a deep colorimetric characterization of Delica and Butternut pumpkin species as raw samples and after high-pressure treatments, showing interesting color changes [30]. Also, Norfezah et al. (2011) reported a change in pulp color parameters after pumpkin flour production by cabinet drying (from 67.94 to 75.84 for L*; from 12.29 to 5.77 for a*; from 42.75 to 37.76 for b*) for mature Crown pumpkin (C. maxima) [31]. C* and H* values ranged respectively from 31.9 to 72.2, and from 77.5 to 99.0 for pumpkin cultivars belonging to C. moschata species [32]. Pumpkin pulp moisture was also determined and values between 81.65 and 96.86% for C. moschata species and between 83.34 and 89.61% for C. maxima species were obtained. Statistically different results were obtained from the comparison between moisture values of the two species ($p < 0.05$), but also considering different varieties belonging to the same species ($p < 0.01$). Other authors have also reported similar values. Norfezah et al. (2011) reported a moisture value of 84.34% for C. maxima species [31], while Karanja et al. (2014) showed a range from 75.08% to 91.16% for thirteen groups of pumpkins cultivated in Kenya [33].

Table 2. Color characteristics (L*, a*, b*, C*, H*) of pumpkin powder.

Cultivar	L*	a*	b*	C*	H*
C. moschata species					
Butternut	51.10 ± 0.89^{a,d}	7.28 ± 0.61^{a,d}	44.73 ± 0.58^{a,e}	45.32 ± 0.71^{a,e}	80.76 ± 0.78^{a}
Lunga di Napoli	55.58 ± 0.93^{a,e}	3.97 ± 0.12^{b}	40.15 ± 1.09^{a}	40.35 ± 0.87^{b}	84.35 ± 0.20^{a,c,d}
Moscata di Provenza	55.17 ± 1.20^{a,e}	4.87 ± 0.93^{a,b}	40.98 ± 0.29^{a}	41.27 ± 0.75^{a,b}	83.22 ± 1.19^{a,c,d}
Violina rugosa	34.52 ± 0.85^{b}	14.98 ± 0.31^{c}	33.98 ± 0.41^{b}	37.13 ± 0.33^{b}	66.21 ± 1.15^{b}
C. maxima species					
Delica	52.71 ± 1.08^{a,e}	4.87 ± 0.21^{a,b}	49.28 ± 0.89^{c,e}	49.52 ± 0.97^{c,e}	84.36 ± 1.51^{c,d}
Delica vanity	26.98 ± 1.08^{c}	10.47 ± 0.42^{d}	25.19 ± 1.13^{d}	27.28 ± 1.22^{d}	67.43 ± 1.94^{b}
Hokkaido	45.98 ± 1.95^{d}	5.18 ± 0.18^{a,b}	41.58 ± 2.43^{a}	41.90 ± 1.97^{a,b}	82.90 ± 1.08^{c}
Mantovana	56.87 ± 2.47^{e}	2.58 ± 0.05^{b}	48.62 ± 1.77^{e}	48.69 ± 1.15^{e}	86.96 ± 2.03^{d}

Data are reported as mean value ± SD of three independent measurements (n = 3) and are expressed on dry weight. L*, color lightness; a*, color in the range from green (negative) to red (positive); b*, color from blue (negative) to yellow (positive); C*, Chroma; H*, Hue angle. Different letters in each column indicate significant differences with $p < 0.01$

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3.2. Cell Count and Viability: Acridine Orange/DAPI Double Staining

To evaluate the cytotoxicity of pumpkin carotenoids on neuroblastoma cells, an optimal extraction procedure must be followed, providing both an efficient extraction, and also limiting the chemical decomposition and biological activity modification of bioactive compounds [34]. Based on a previous paper, an unconventional extraction technique (UAE), and the hexane:isopropanol (60:40 v/v) mixture were used to perform the isolation of carotenoids from pumpkin pulp, belonging to two species/eight varieties [17]. Neuroblastoma is a solid tumor often diagnosed in childhood. It represents 8-10% of all pediatric tumors and has unfavorable outcomes due to drug resistance and high metastatic potential [35]. To date, most of the available therapies remain unsuccessful against neuroblastoma and the discovery of new treatment approaches is required for this detrimental disease [16]. In this study, we evaluated the viability of SH-SY5Y human neuroblastoma cells after exposure to six increasing concentrations of carotenoid pumpkin extracts. In addition, SH-SY5Y cells were challenged with the same concentration of β-carotene used as positive control. Total/dead cell double-staining technique, using AO and DAPI fluorochromes, was employed. Results were expressed as percentage variation of cell viability concerning the untreated control. After 24 h of treatment (Figures 1A and 2A), only the highest concentration of Butternut (i.e., 100 μmol/L) caused a significant decrease in viability percentage compared to the untreated control ($p = 0.043$). On the contrary, after 48 h of treatment (Figures 1B and 2B), all extracts promoted cell death at 100 μmol/L concentration (Butternut, $p = 0.016$; Delica, $p = 0.008$; Delica Vanity, $p = 0.024$; Hokkaido, $p = 0.001$; Lunga di Napoli, $p = 0.001$; Mantovana, $P = 0.035$; Moscata di Provenza, $p = 0.037$; Violina rugosa, $p = 0.001$; β-carotene, $p < 0.0001$), whereas both Mantovana and β-carotene showed cytotoxicity at 50 μmol/L ($p = 0.038$ and $p < 0.0001$, respectively). Regarding the results reported in Figures 1A and 2A, it should be pointed out that at 2-100 μmol/L no significant differences ($p > 0.05$) were observed.

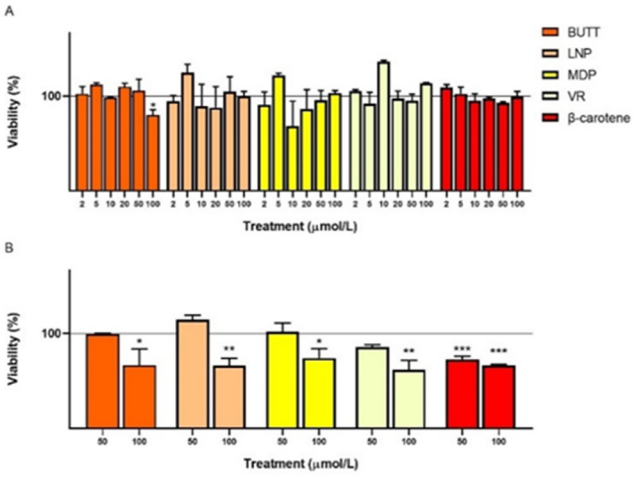

Figure 1. Effects of scalar concentrations of pumpkin (*C. moschata* species) pulp extracts on SH-SY5Y viability. Experimental groups comprised cells treated for 24 (A) and 48 h (B). The results of each experimental set are expressed as percent of negative control (taken as unit, 100%), and summarized as the mean ± standard error of the mean of at least three independent experiments. Statistical analysis: one-way ANOVA followed by Dunnett's post hoc analysis. * ($p < 0.05$), ** ($p < 0.01$), or *** ($p < 0.0001$). BUTT, Butternut; LNP, Lunga di Napoli; MDP, Moscata di Provenza; VR, Violina rugosa.

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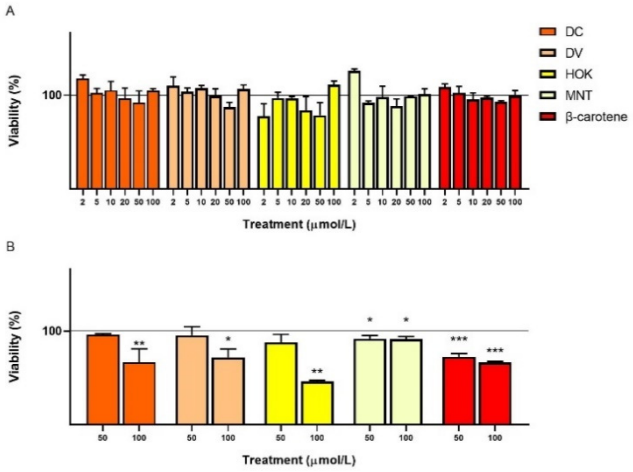

Figure 2. Effects of scalar concentrations of pumpkin (*C. maxima* species) pulp extracts on SH-SY5Y viability. Experimental groups comprised cells treated for 24 (A) and 48 h (B). The results of each experimental set are expressed as percent of negative control (taken as unit, 100%), and summarized as the mean ± standard error of the mean of at least three independent experiments. Statistical analysis: one-way ANOVA followed by Dunnett's post hoc analysis. * ($p < 0.05$), ** ($p < 0.01$), or *** ($p < 0.0001$). DC, Delica; DV, Delica vanity; HP, Hokkaido; MNT, Mantovana.

Our findings are in line with a previous paper in which β-carotene at 60 μmol/L (i.e., 112 μmol/L) was reported to induce apoptosis in SH-SY5Y cells through increasing intracellular reactive oxygen species (ROS) production [36]. Indeed, in certain doses, almost all antioxidants can behave as prooxidants, triggering cell death pathways mediated by increased ROS production [12]. The current study indicates that pulp pumpkin extracts exert cytotoxic action against SH-SY5Y neuroblastoma cells, laying an attractive basis for further research. Several investigations will be required to determine the mechanism underlying the extract's anti-cancer activity, which is most likely owing to the synergistic effect of several compounds in the phytocomplex.

3.3. TCC and Antioxidant Activity of Pumpkin Pulp

The tested pumpkin extracts were characterized for their carotenoid content and antioxidant properties. Table 3 shows the values of the yield of carotenoid extraction, TCC, and in vitro antioxidant activity (ABTS and ORAC assays) of pulp extracts. The percentage of extraction (yield, %), determined by the gravimetric method, was calculated using the equation reported in a previous paper [24]. It is possible to observe a wide range of values even if the same pumpkin species were considered (i.e., from 1.03% of Delica vanity to 4.15% of Delica). In addition, no statistical differences ($p > 0.05$) were found between C. moschata and C. maxima species, even if the same extraction method and conditions (solvent, temperature, time, solid/liquid ratio) were used. After that, a spectrophotometric characterization was carried out to determine the TCC and antioxidant properties of the extracts. The TCC results of the considered eight varieties change over the range 161.08 μg/g of Butternut to 443.89 μg/g of Violina rugosa, both varieties belonging to C. moschata species, while for C. maxima the values ranged from 241.32 to 379.36 μg/g (Delica vanity and Delica, respectively). To make an interesting comparison with literature, it must be taken into consideration that TCC values, as well as biological properties, could change from pumpkin variety to variety, or in the same variety, they could change based on the extraction method and conditions and many other variables [9,17].

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Table 3. Values of extraction yield, TCC and in vitro antioxidant activity (ABTS and ORAC assays) of pulp extracts.

Cultivar	Yield (%)	TCC (μg β-CE/g)	ABTS (μg TE/g)	ORAC (μg TE/g)
C. moschata species				
Butternut	1.63 ± 0.20^{a,b}	161.08 ± 7.80^{a}	280.91 ± 27.45^{a}	1352.34 ± 10.34^{a}
Lunga di Napoli	1.29 ± 0.06^{a,d}	303.27 ± 2.08^{b}	343.13 ± 18.82^{a,b}	1802.54 ± 76.54^{a,b}
Moscata di Provenza	2.00 ± 0.25^{b,e}	365.73 ± 9.49^{c}	417.62 ± 53.94^{b,c,e}	1500.30 ± 53.04^{a,d}
Violina rugosa	1.42 ± 0.15^{a,d}	443.89 ± 7.58^{d}	525.39 ± 32.24^{c,f}	2560.11 ± 324.24^{b}
C. maxima species				
Delica	4.15 ± 0.00^{c}	379.36 ± 44.08^{c}	1192.11 ± 48.44^{d}	3996.18 ± 72.58^{c}
Delica vanity	1.03 ± 0.01^{d}	241.32 ± 21.55^{e}	313.23 ± 16.22^{a,e}	1267.86 ± 166.22^{a}
Hokkaido	1.58 ± 0.06^{a,b}	310.77 ± 23.82^{b}	548.41 ± 68.45^{f}	2341.24 ± 68.45^{b,d}
Mantovana	2.20 ± 0.28^{e}	247.05 ± 13.62^{e}	500.90 ± 11.69^{c,f}	2615.96 ± 52.10^{b}

Data are reported as mean value ± SD of three independent measurements (n = 3) and are expressed on dry weight. TCC, total carotenoid content; ABTS, 2,20-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; ORAC, oxygen radical absorbance capacity; TE, Trolox equivalents; β-CE, β-carotene equivalents. Different letters in each column indicate significant differences with $P < 0.01$.

The TCC values reported in this paper are similar to those found by other authors, for example de Carvalho et al. (2012) [37]. They found a range of TCC from 234.21 to 404.98 μg/g in two landrace pumpkins (C. moschata) cultivated in Brazil. Higher or lower values have been found compared to other papers. For example, Armesto et al. (2020) reported TCC levels of 34.54-39.53 μg/g for Butternut (C. moschata) pumpkin from Spain, extracted by UAE with acetone [11]. Hussain et al. (2022) reported a TCC value of 35.2 mg/100 g for flesh powder from C. maxima of Pakistan, extracted with 80% methanol, in an orbital shaker for 120 h at room temperature [38]. Biesiada et al. (2011) reported that the highest content of carotenoids was recorded in a cultivar belonging to C. maxima from Poland, Amazonka (18.40 mg/100 g fresh weight), while the lowest (0.57 mg/100 g fresh weight) for C. pepo, Pyza cultivar [39]. Azizah et al. (2009) evaluated twenty-two cultivars of C. moschata reporting a TCC ranging from 7.02 to 138.56 μg/g, using ethanol for the extraction by a flask placed in a water bath at 25 °C for 1h [40].

As regards antioxidant activity results, the ABTS values ranged from 280.91 μg TE/g of Butternut to 1192.11 μg TE/g of Delica, while ORAC values ranged from 1267.86 μg TE/g of Delica vanity to 3996.18 μg TE/g of Delica. Statistically different results were obtained from the comparison between ABTS values of C. moschata and C. maxima species, as well as between ORAC values ($p < 0.05$), but also taking into consideration different varieties belonging to the same species ($p < 0.01$). The results of spectrophotometric analyses (TCC, ABTS, ORAC) were processed to evaluate their degree of correlation (Table S2), and a good positive linear relationship between all parameters ($R^2 \geq 0.5666$) was found.

For literature comparison, it is worth noting that some papers reported ABTS and ORAC values of extracts obtained with polar solvents (methanol, water), where the antioxidant activity was probably linked to polyphenols, but not to carotenoids. As an example, Kulczyński et al. (2020) reported for Butternut lower ABTS values both taking into consideration aqueous-methanol extract (126.92 mg TE/100 g), and aqueous extract (138.36 mg TE/100 g) and found the lowest ABTS values for Futsu and Table Queen pumpkin cultivars [10]. As regards ORAC values, the same authors found the lowest and the highest values for cultivars belonging to the C. pepo (i.e., Table Queen 28.12 μmol TE/g; Delicata 108.3 μmol TE/g). Only a few papers reported the values of antioxidant assays carried out on carotenoid extracts. As an example, Pinna et al. (2022) reported values of 958.88 and 2832.76 μg TE/g for ABTS and ORAC respectively for extracts of C. moschata obtained with hexane:isopropanol (60:40 v/v) [17].

3.4. Carotenoid Composition of Pulp and Multivariate Statistical Analysis

Established that carotenoid fraction isolated from pumpkin powder showed interesting biological activity, the in-depth profiling of bioactives of extracts was the next key step. The

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qualitative and quantitative analysis of carotenoids by an HPLC-DAD procedure was performed, taking advantage of a method validated in previous works [17,24]. A UHPLC-MS/MS technique was also carried out for the structural confirmation of the analytes.

The quantitation of carotenoids identified in all the investigated extracts was carried out based on calibration curves built up using standard solutions of lutein and zeaxanthin dipalmitate with concentration values in the range specified in Table S3. The calibration curve of β-carotene and the relative values of linearity and accuracy were reported in a previous paper [17]. The β-carotene standard was used for the quantification of non-esterified cyclic carotenes (β-carotene and α-carotene), other non-esterified carotenoids (i.e., violaxanthin, antheraxanthin, neoxanthin, lutein, zeaxanthin) were quantified using the regression equation of lutein (expressed as μg LE/g DW), while esterified (mono- and di-) carotenoids (among which violaxanthin and antheraxanthin myristate, lutein and antheraxanthin palmitate, and also violaxanthin di-myristate, antheraxanthin di-laurate and others) were quantified using the regression equation of zeaxanthin dipalmitate (expressed as μg ZDE/g DW). The regression model showed good linearity for both lutein and zeaxanthin dipalmitate (R^2 > 0.996 and 0.994, respectively), revealing useful for predictive purposes. Furthermore, the established RP-HPLC method was validated at a research level in terms of accuracy, precision, limit of detection (LOD), and limit of quantification (LOQ) (Table S4). This internal validation, performed at a basic level to demonstrate the suitability of the in-house developed method, provided useful results thus ensuring that the process is satisfactory and consistent within the scope of the present study. In Table S5 the UV-Vis and mass spectral data used for the identification of the carotenoids are shown.

Figure 3A,B shows the data of carotenoid composition, while Figure 4A,B the content of grouped carotenoids. The most abundant xanthophylls found in pumpkin pulp were monoesterified for C. maxima and diesterified for C. moschata. A study of correlation was carried out between all parameters (spectrophotometric and chromatographic data), and it can be noted that C. maxima data showed always high values of R2 which were between 0.5411 and 0.9907 (Table S2). The cultivars with the highest β-carotene content were Delica, Violina rugosa, and Moscata di Provenza (134.52-140.38 μg/g), while α-carotene was only detected in pumpkin belonging to C. moschata species (10.29-153.86 μg/g). Generally, it should be noted that carotenoid content varied in a wide range *[8,38,39,41]*, also considering the dependence of this data from cultivar, variety, harvesting time, storage, and processing. Hussain et al. (2022) reported a β-carotene content of 6.18 mg/100 g for flesh pumpkin (C. maxima) [38]. de Carvalho et al. (2012) found that all E-β-carotene was the most abundant isomer, varying from 141.95 to 244.22 μg/g, compared to α-carotene (67.06-72.99 μg/g), while 9- and 13-Z-β-carotene isomers were found in low concentrations [37]. Kreck et al. (2006) reported that pumpkin varieties belonging to C. maxima differ significantly in terms of carotenoids, in fact the concentration of β-carotene ranged from 17 mg/kg to 263 mg/kg [42]. Murkovic et al. (2002) reported a content of α-carotene from 0.03 mg/100 g for C. pepo (Carneval di Venezia variety) up to 7.5 mg/100 g for C. maxima (Flat White Boer variety) and a content of β-carotene from 0.06 to 7.4 mg/100 g [43]. Dhenge et al. (2022) reported the content of carotenoids after high-pressure processing (α-carotene 29.2-78.3 μg/g; β-carotene 10.5-20.4 μg/g) [44]. Recently, Grassino et al. (2023) reviewed the carotenoid content and profiles of pumpkin products and by-products. They reported β-carotene values of 5.70 and 17.04 μg/g for the pulp of C. moschata and C. maxima respectively, while the content of α-carotene was reported only for pumpkin seed oil, puree, juice, and extrudates [41].

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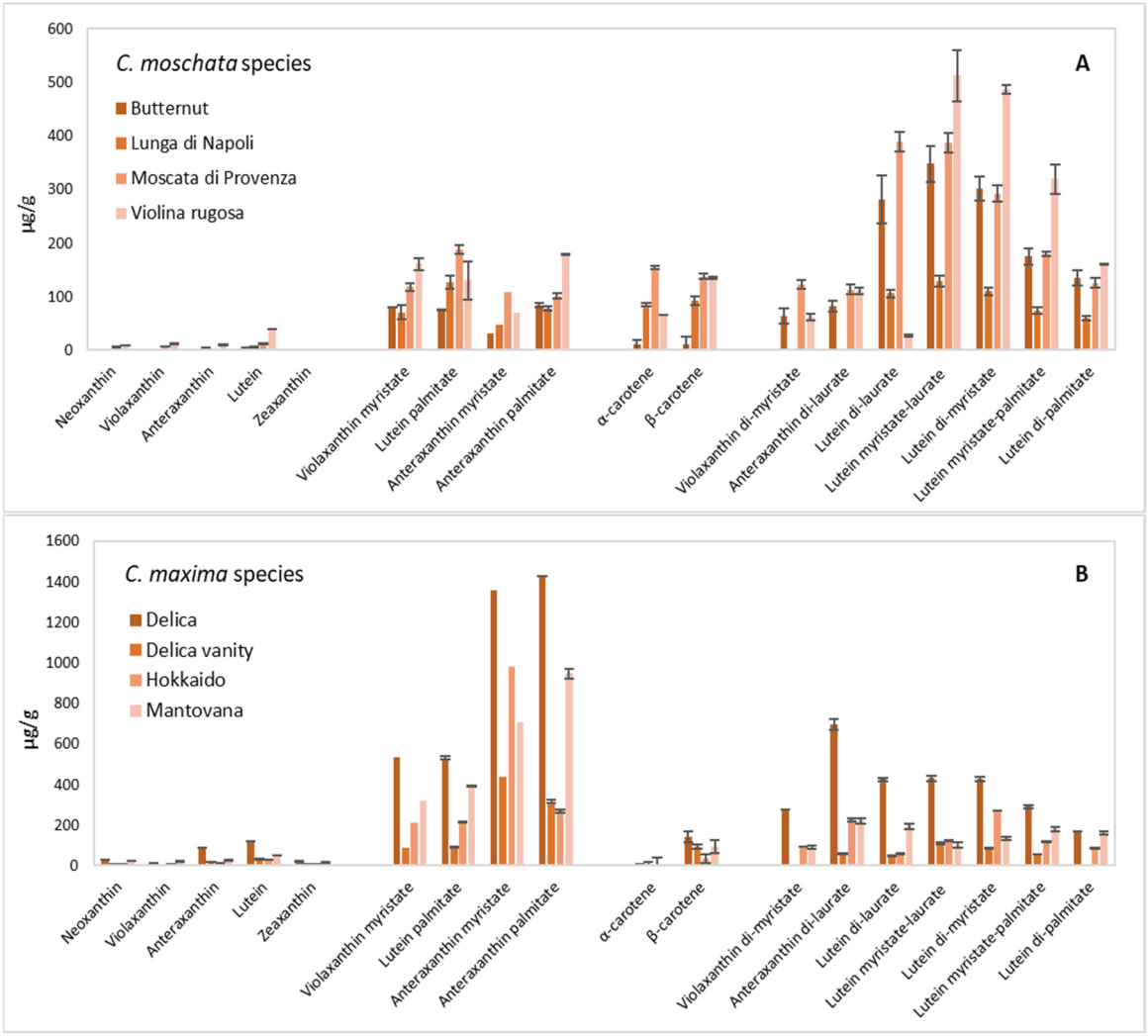

Figure 3. Content of pulp carotenoids (µg/g) of the pumpkin varieties belonging to C. moschata (A) and C. maxima (B) species.

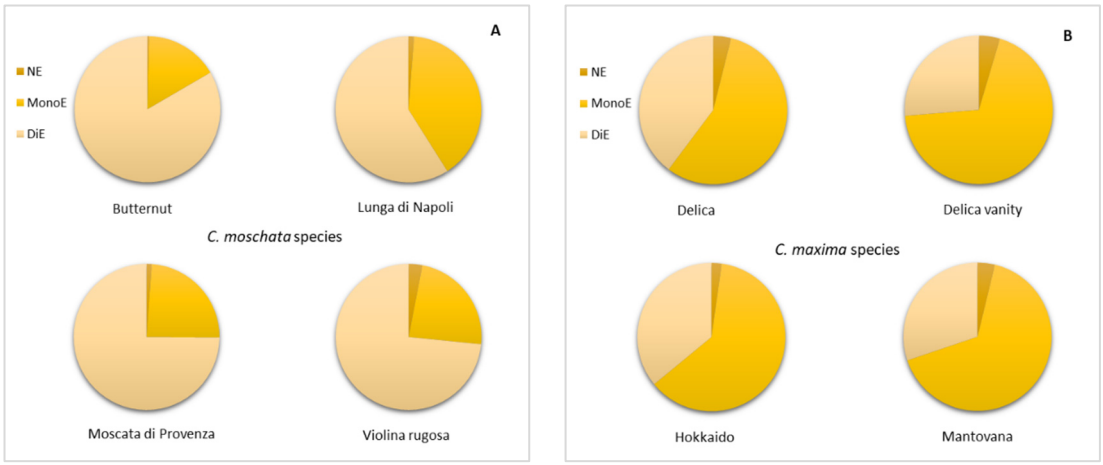

Figure 4. Content of pulp carotenoids (µg/g), grouped as non-esterified (NE), monoesterified (MonoE) and diesterified (DiE) of the pumpkin varieties belonging to C. moschata (A) and C. maxima (B) species.

In this research, in addition to cyclic carotenes (α- and β-carotene), other non-esterified carotenoids were detected, among which epoxycarotenoids (neoxanthin, violaxanthin, and antheraxanthin) and hydroxycarotenoids (zeaxanthin and lutein). Delica and Mantovana varieties,

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belonging to C. maxima species, showed the highest contents (266.195 and 135.23 μg LE/g, respectively), while the Butternut and Lunga di Napoli varieties of C. moschata species showed the lowest content (6.55 and 10.22 μg LE/g, respectively) of this class of non-esterified compounds. However, there was a notable difference among the single compound content (neoxanthin, violaxanthin, antheraxanthin, lutein, and zeaxanthin). As regards lutein content, in this work the values ranged from 5.24 μg LE/g of Butternut up to 119.26 μg LE/g of Delica. Generally, it can be observed that this last variety was the one richest in carotenoid content, in fact it showed the highest content of β-carotene, as well as non-esterified and esterified carotenoids. Kulczyński and Gramza-Michałowska (2019) reported lutein content ranging from 87.20 μg/g for Porcelain Doll to 388.79 for Melonowa Żółta variety, with a value of 130.23 μg/g for Hokkaido. Lutein content from 0.6 to 17.3 μg/g fresh weight was reported by Itle and Kabelka (2009) [32].

Among hydroxycarotenoids, zeaxanthin was quantified by some authors with a wide range of contents (Kurz et al. (2008); Kulczyński and Gramza-Michałowska, 2019; Hussain et al., 2022). Kurz et al. (2008) reported values of 0.57 μg/g for Halloween pumpkin to 22.45 μg/g for Hokkaido variety, while Kulczyński and Gramza-Michałowska (2019) from 19.57 μg/g for Buttercup to 192.53 μg/g of Melonowa Żółta variety [8]. In the research of Murkovic et al. (2002), lutein and zeaxanthin were not separated by routine HPLC method, so the authors reported that the content of lutein (+ zeaxanthin) changed from 0.8 to 17 mg/100 g for C. maxima species, and from 0.08 to 1.1 mg/100 g for C. moschata species [43]. In this deep characterization of carotenoid fraction of pumpkin, it was found that many carotenoids were linked to saturated and long-chain fatty acids, i.e., lauric (C12:0), myristic (C14:0) and palmitic (C16:0) acids.

Among esterified carotenoids, monoesterified carotenoids (i.e., violaxanthin and antheraxanthin myristate, lutein and antheraxanthin palmitate) and diesterified carotenoids (i.e., violaxanthin or lutein di-myristate, antheraxanthin di-laurate, lutein di-palmitate, lutein myristate-laurate, and zeaxanthin myristate-palmitate) were identified and quantified. The presence of fatty esters of xanthophylls especially of violaxanthin (monomyristate) and lutein (monomyristate and monopalmitate) was published for the first time in 1988 by the group of Khachik [45], but they did not quantify these compounds since no reference materials were available. Recently, Ouyang et al. (2022) studied the stability of carotenoids and carotenoid esters in pumpkin (C. maxima) slices during hot air drying. They identified and quantified six esterified carotenoids, besides free carotenoids. They reported essentially esterified forms of lutein with contents of 20.3 μg/g DW for lutein-stearate-palmitate up to 93.1 μg/g DW for lutein-palmitate-laurate, in addition to 37.8 μg/g DW of violaxanthin-dipalmitate [46].

The esterification of carotenoids has been correlated with greater stability and potentially higher or equivalent bioavailability compared to free carotenoids; in fact, it has been reported that xanthophyll esters probably need to be hydrolyzed during digestion before absorption and that the in vivo absorption of carotenoids is better if they are esterified rather than non-esterified *[47,48]*. In conclusion of this section, it must be emphasized that the presence of high content of carotenoids in pumpkin pulp plays an essential role in maintaining a healthy status, due to the broad spectrum of health-promoting effects of these bioactives, having neuroprotective, ophthalmological, antimicrobial, cardioprotective, antiplasmodial, and skin effects. As an example, for age-related eye disease (macular degeneration and cataract), it is sufficient to mention that β-carotene is a precursor of 11-cis retinal, a chromophore of rhodopsin found in rods, receptors enabling vision under low-light conditions, while lutein and zeaxanthin are the main antioxidants of the retina. They can absorb UV radiation and blue light as well as scavenge free radicals and reactive oxygen species. Prevention and treatment of age-related eye diseases include carotenoid supplementation *[49,50]*.

In this paper, to evaluate the possibility that carotenoids could represent valid biomarkers for pumpkin species differentiation (C. moschata vs. C. maxima), principal component analysis (PCA) was applied and the dataset was represented by the carotenoid content obtained by HPLC-DAD analysis.

Based on 2D scores plot (Figure 5A) and biplot (Figure S2), the samples were grouped both considering the varieties and the species. In particular, the samples belonging to C. maxima species

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(on the right) were separated from those of C. moschata (on the left). To explain the variance, the first two principal components (PC1 and PC2) were extracted, which together accounted for 74.9% of the variance (PC1: 53.8%; PC2: 21.1%). Figure S3 shows the PCA overview with the pairwise score plot for the top 5 PC, while Figure S4 shows the PCA scree plot, indicating the variance explained by individual PC and the accumulated variance. The HeatMap (Figure 5B) shows that 17 carotenoids were identified as interesting biomarkers. In particular, Violina rugosa showed lutein di-myristate, lutein myristate-laurate, and lutein myristate-palmitate as overexpressed carotenoids, while lutein palmitate, violaxantin-myristate, violaxanthin, and neoxanthin were overexpressed in Mantovana variety. Antheraxanthin myristate and lutein di-laurate were mainly represented in Hokkaido and Butternut, respectively. Moreover, hierarchical clustering dendrogram (Figure S5), the branching diagram representing the relationships of similarity among groups, highlighted the clear separation of pumpkin varieties belonging to the two different species (C. moschata vs. C. maxima).

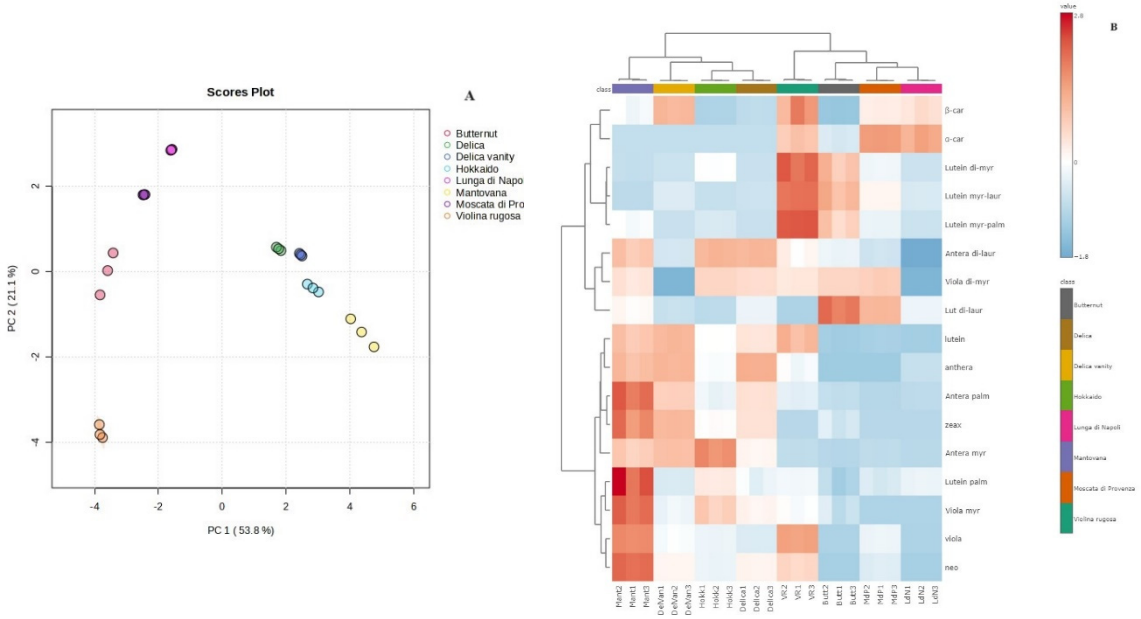

Figure 5. Chemometric analysis and hierarchical clustering of carotenoids of eight pumpkin varieties. Principal Component Analysis 2D scores plot (A) and HeatMap (B).

4. Conclusions

In this work, carotenoid extracts from pulp pumpkin of different varieties were assayed for their cytotoxic effect on human neuroblastoma SHSY-5Y and then deeply characterized for antioxidant properties and chemical profile. Interestingly, as far as the biological study is concerned, at all the tested concentrations after 48 h the pulp pumpkin extracts exert cytotoxic action against SH-SY5Y neuroblastoma cells, laying an attractive basis for further research. The data collected in this research could be useful for a deeper investigation of still scarcely explored biochemical processes modulated by carotenoids regarding cytotoxic effects on human neuroblastoma. Moreover, the results obtained valorize pumpkin as an excellent dietary source of antioxidants. In addition to the spectrophotometric measurement of TCC and antioxidant activity, the profiling of carotenoids was deepened by a newly developed HPLC-MS method, revealing at least 18 carotenoid compounds. The results of the quantitative analysis showed a high content of β-carotene for Delica and Violina rugosa pumpkins, while α-carotene is a typical carotenoid of pumpkins belonging to *C. moschata* species. Among *C. maxima* species, the Delica variety was the most abundant in carotenoids, while among *C. moschata* species the Violina rugosa variety was. In conclusion, the edible part of pumpkin is a significant natural source of bioactive substances against brain cancer, and it has intriguing potential in the development of related anticancer medications and/or as chemotherapeutic adjuvants.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Figure S1: A typical HPLC-DAD chromatographic profile of pumpkin pulp. A, Hokkaido variety (*C. maxima* species); B, Moscata di Provenza variety (*C. moschata* species). 1, neoxanthin; 2, violaxanthin; 3, antheraxanthin; 4, lutein; 5, zeaxanthin; 6, violaxanthin myristate; 7, lutein palmitate; 8, antheraxanthin myristate; 9, antheraxanthin palmitate; 10, α-carotene; 11, β-carotene; 12 violaxanthin di-myristate; 13, antheraxanthin di-laurate; 14, lutein di-laurate; 15, lutein laurate myristate; 16, lutein di-myristate; 17, lutein palmitate myristate; 18, lutein di-palmitate; Figure S2: PCA overview. Display pairwise score plot for top 5 PC; Figure S3: PCA scree plot. The green line on top shows the accumulated variance explained; the blue line underneath shows the variance explained by individual PC. Figure S4: Biplot for principal components (PC1 and PC2); Figure S4: Hierarchical Clustering Dendrogram. Distance Measure: Euclidean; Clustering Algorithm: Ward; Table S1: Correlation between color parameters (L*, a*, b*, C*, H*) of two different species (*C. moschata* and *C. maxima*); Table S2: Correlation between spectrophotometric parameters (TCC, ABTS, ORAC) of two different species (*C. moschata* and *C. maxima*); Table S3: Regression equation, R^2, linearity range, LOD, and LOQ of lutein and zeaxanthin dipalmitate, analyzed by HPLC-DAD; Table S4: Method validation for lutein and zeaxanthin dipalmitate: evaluation of precision (RSD %) and accuracy (recovery %) in the short- and long-term period (intra-day and inter-day precision and accuracy values); Table S5: HPLC retention times, ultraviolet (UV)/visible light (Vis) spectra, and MS spectral data of carotenoids from pumpkin pulp.

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