

## Article

# Role of Zucchini and Its Distinctive Components in the Modulation of Degenerative Processes: Genotoxicity, Antigenotoxicity, Cytotoxicity and Apoptotic Effects

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**Abstract:** Zucchini (*Cucurbita pepo* subsp. *pepo*) is a seasonal vegetable with high nutritional and medical value. Many useful properties of this fruit are attributed to its bioactive compounds. Zucchini fruits (“Yellow” and “Light Green” varieties) and four distinctive components (lutein,  $\beta$ -carotene, zeaxanthin and dehydroascorbic acid) were selected. Firstly it was determined the lutein,  $\beta$ -carotene, zeaxanthin and dehydroascorbic acid content in these fruits and then, in order to measure the safety and suitability of their use different assays were carried out: (i) Genotoxicity and antigenotoxicity tests to determine the safety and DNA-protection against hydrogen peroxide; (ii) cytotoxicity and (iii) DNA fragmentation assay to evaluate the proapoptotic effect. Results showed that: (i) all the substances were non-genotoxic, (ii) all the substances were antigenotoxic except the highest concentration of lutein, (iii) “Yellow” Zucchini epicarp and mesocarp exhibited the highest cytotoxic activity ( $IC_{50} > 0.1$  mg/mL and 0.2 mg/mL, respectively) and iv) “Light Green” Zucchini skin and pulp induced internucleosomal DNA fragmentation. To sum up, Zucchini fruit could play a positive role in human health and nutrition due to this fruit and its components were safe, able to inhibit significantly the  $H_2O_2$ -induced damage and exhibit antiproliferative and proapoptotic properties toward HL60 tumour cells. The information generated from this research should take into account to select potential accessions for breeding programs purposes.

**Keywords:** cancer; carotenoid; chemoprevention; *Cucurbita*; cytotoxicity; DNA-protection; tumor cells

## 1. Introduction

It is currently accepted that diet can affect the overall process of carcinogenesis by different mechanisms: its constituents may contain cancer-causing substances as well as many cancer preventive agents [1]. Inappropriate dietetic habits are estimated to be the cause of more than one third of cancer deaths [2].

There are more than 20000 species of plants used in traditional medicines, alleged to be all potential reservoirs for new drugs [3] and are a potential source of chemical constituents with antitumor and cytotoxic activities [4]. Protective elements in a cancer prevention diet include selenium, folic acid, vitamin B-12, vitamin D, chlorophyll and antioxidants such as the carotenoids ( $\alpha$ -carotene,  $\beta$ -carotene, lycopene, lutein, cryptoxanthin) [5].

Summer squash (*Cucurbita pepo* subsp. *pepo*) is a seasonal vegetable that contains a number of beneficial micronutrients such as minerals, carotenoids, vitamin C, phenolic compounds, etc. [6-9]. It has been used in traditional folk medicine to treat colds and alleviate aches, due to its antioxidant/anti-radical, anti-carcinogenic, anti-inflammatory, antiviral, antimicrobial and analgesic activities [10-14].

Studies of genotoxicity/antigenotoxicity and cytotoxicity are rapid methods to assess the innocuousness and possible beneficial effects of single compounds or complex mixtures and foods [15-17]. The Somatic Mutation and Recombination Test (SMART) of *Drosophila melanogaster* is a suitable eukaryotic tool for genotoxicity and antigenotoxicity studies [18] and is a one-generation test based on the loss of heterozygosity of two suitable recessive markers (*mwh* and *flr*), due to different genotoxic events (*i.e.* mitotic recombination, mutation and chromosomal aberration). HL60 human leukaemia cells have been used in cytotoxicity assays in order to determine the tumoricide activity of some vegetable matrices [19].

There are a few studies where the cytotoxic activity of *C. pepo* has been checked. Shokrzadeh et al. [13] reported different cytotoxic activities of the *C. pepo* leaf extracts on normal and cancer cell lines. Wang et al. [14] observed a significant dose-dependent inhibitory effect against HeLa and HepG cell growth of the extract of *C. pepo* fruits. Menéndez et al. [10] also reported also a significant decrease of the growth of prostatic hyperplasia by extract of *C. pepo* seeds at the tested concentrations (400 and 200 mg/kg). However no studies have been carried out to demonstrate the presence or absence of *in vivo* activity of Zucchini fruit and about the mechanisms of action of their antioxidant compounds.

In the case of the genotoxic or antigenotoxic activities of antioxidant compounds such as the ascorbic acid (the reduced form of dehydroascorbic acid), most of the reported data [20-22] indicates that it is anti-mutagenic. Although antioxidant protection is assumed for ascorbic acid against oxidative damage, the details of this protection are not yet completely understood. In fact, under different conditions, ascorbic acid seems to have co-genotoxic activity instead of the normal antigenotoxic action [23].

Studies about carotenoids have also been performed in relation to decrease cancer risk. Thus, the chemopreventive action of  $\beta$ -carotene is effective mainly at the beginning of the carcinogenic process or in the initial stages of its promotion, inhibiting the formation of pre-neoplastic lesions in some *in vitro* and *in vivo* experimental models [24-26]. Lutein and zeaxanthin, naturally occurring carotenoids, have shown to reduce the risk of cataracts and age-related macular degeneration [27]. Zeaxanthin is related with the reduction of melanoma cells viability [28]. Lutein caused significant DNA damage in human retinal pigment epithelial cells [29].

The aim of this work was to give an added value to simple nutritional to the Zucchini fruit, along with some of its active components, carotenoids (lutein,  $\beta$ -carotene, and zeaxanthin) and Vitamin C, with respect to DNA integrity, and to establish the possible *in vivo* genotoxic and *in vitro* cytotoxic effects. In order to measure these properties, different assays were carried out: genotoxicity and antigenotoxicity (*in vivo* analysis using the *Drosophila melanogaster* model), and cytotoxicity and proapoptotic activity (*in vitro* assays using the promyelocytic leukaemia HL60 cell line). In this way, the beneficial health effects of the consumption of Zucchini could be explained and used for breeding program purposes.

## 2. Materials and Methods

### 2.1 Plant material

Two different varieties of *C. pepo* belonging to Zucchini morphotype were evaluated in this work: "Light Green" (elongated in shape with light green skin) and "Yellow" (elongated in shape with yellow skin). They were representatives of Zucchini commercial cultivars currently offered in the market. Seeds of these varieties were germinated on wet filter paper in Petri dishes at room temperature for 2-4 days in the dark, after which they were transplanted into rock-wool cubes (Grodan BV, 6040KD Roermond, NL) in a greenhouse. When plants had developed three to four leaves they were transferred to 1 m large rock-wool slabs at a density of two plants/slab. Plants were

grown in a greenhouse in the IFAPA Center in La Mojonera, Almería, Spain (36°47'19"N, 02°42'11"W; 142 m a.s.l.) from March to June 2011 following standard local cultural practices for both plant nutrition and insect pest and disease control. Six fruits of each variety were harvested at an immature stage, and they were processed preserving epicarp and mesocarp of each fruit separately, packaged in polypropylene plastic containers and stored at -80°C. Sample was lyophilized using freeze drier equipment (Telstar LyoQuest, Germany) at -55 °C under vacuum (133 x 10<sup>-3</sup> mBar) for 96 h per sample. Then, the samples were ground and frozen at -80°C for further extractions and biological analyses.

## 2.2 Antioxidant compounds

The compounds used in this study were purchased from Fluka (lutein: Cat. Number 07168 and  $\beta$ -carotene: Cat. Number 22040), Extrasynthese (zeaxanthin: Cat. Number 0307S) and Sigma-Aldrich (ascorbic acid: Cat. Number 255564 and dehydroascorbic acid: Cat. Number 261556). The carotenoids were dissolved in ethanol prior to addition to the corresponding culture media *i.e.* in water for fly treatment, or in RPMI 1640 medium for HL60 cell culture at the time of the experiment. The final concentration of ethanol was 1% in the culture media.

## 2.3 Determination of the carotenoid content

All manipulations were performed in ice and under subdued artificial light conditions with headspaces of containers flushed with oxygen free nitrogen to help prevent carotenoid degradation. Individual carotenoid concentration was determined by reverse phase HPLC (High-Performance Liquid Chromatography) after saponification following the methods described by Martínez-Valdivieso et al. [9]. The carotenoids were extracted from the rehydrated sample with 5 mL ethanol containing 1 mg/mL butylated hydroxytoluene (BHT) using a Polytron homogenizer. Samples were saponified in order to hydrolyze esterified carotenoids that might complicate the chromatographic determinations [30]. One millilitre of a 40% w/v KOH methanolic solution was added to each tube, and the samples were saponified for 10 min at 85°C. The samples were cooled in an ice bath, and 2 mL of ice-cold water was added. The suspensions were extracted twice with 2 mL of hexane by vigorous vortexing followed by a 2000g centrifugation for 10 min at room temperature. The upper hexane layers were pooled and evaporated to dryness in a Savant SpeedVac apparatus and resuspended. Immediately before injection the carotenoids were dissolved in 800  $\mu$ L of an acetonitrile/methanol/dichloromethane (45:20:35 v/v/v) solution, filtered through a 0.22  $\mu$ m PTFE syringe filter (Millipore) directly to sample vials, and 10  $\mu$ L were injected into the chromatograph. The initial mobile phase consisted of acetonitrile/methanol (97:3, v/v/v) containing 0.05% (v/v) triethylamine. We used a linear gradient of dichloromethane from 0 to 10% in 20 min at the expense of acetonitrile, and then the dichloromethane was kept constant at 10% until the completion of the runs. The flow rate was 1.0 mL/min while the column temperature was 30°C. The analyses were carried out on a HPLC apparatus equipped with binary pump, in-line vacuum degasser, auto sampler injector, a Waters Symmetry C18 column (4.6 mm x 150 mm, 5  $\mu$ m) and a 996 diode array detector (Waters, Milford, MA) supported by the Empower chromatography manager computing system (Waters) was used to detect colored carotenoids at 450 nm. Compounds were identified by comparison of retention times, co-injection with known standards, and comparison of their UV-visible spectra with authentic standards. Quantification was carried out by external standardization. Full standard curves were constructed with five different concentrations for each carotenoid in triplicate. The curves passed through or were very near the origin, were linear and bracketed the concentrations expected in the samples. Results were expressed on a dry weight (DW) basis.

Once the content of the selected antioxidant compounds was evaluated in the epicarp and the mesocarp of *C. pepo* fruit, it performed the genotoxicity, cytotoxicity and apoptosis assays.

## 2.4 Extraction and analysis of vitamin C

The vitamin C analysis was carried out with freeze dried lyophilized samples stored at  $-80^{\circ}\text{C}$ . Five grams of samples were homogenized in 10 mL of MeOH/H<sub>2</sub>O (5:95) plus citric acid (21 g L<sup>-1</sup>) with EDTA (0.5 g L<sup>-1</sup>) and 4 mM NaF. Homogenates were then filtered through cheesecloth and C18 Sep-Pak cartridges (Waters, Milford, MA). Ascorbic acid (AA) and dehydroascorbic acid (DHA) contents were determined following the methods described by Zapata and Dufour [31]. HPLC analyses were performed after derivatization of DHA into the fluorophore 3-(1,2-dihydroxyethyl) furo[3,4-b]quinoxaline-1-one (DFQ), with 1,2-phenylenediamine dihydrochloride (OPDA). Samples of 20  $\mu\text{L}$  were analyzed by using a Merck-Hitachi (Tokyo, Japan). The analyses were carried out on a HPLC apparatus equipped with binary pump, in-line vacuum degasser, autosampler injector, a Waters and a 996 diode array detector (Waters, Milford, MA) supported by the Empower chromatography manager computing system (Waters). Separations of DFQ and AA were achieved on a Kromasil 100 C18 column (250 mm $\times$ 4 mm; 5  $\mu\text{m}$  particle size; Tecnokroma, Barcelona, Spain). The mobile phase was MeOH/H<sub>2</sub>O (5:95, v/v) containing 5mM cetrimide and 50 mM potassium dihydrogen phosphate at pH 4.5. The flow rate was 0.9 mLmin<sup>-1</sup>. The detector wavelength was initially set at 348 nm and after elution of DFQ, the wavelength was manually shifted to 261 nm for AA detection. Standard solutions, column conditioning and derivatization procedures have been previously described by Gil et al. [32].

## 2.5 Genotoxicity and antigenotoxicity tests

The principles and basic procedures for the *Drosophila* wing spot test have been described by Graf et al. [18], and in previous works of our group [15, 16]. Two strains of flies carrying wing genetic markers on the left arm of chromosome 3: *multiple wing hair* (*mwh*, 3-0.3) and *flare* (*flr*<sup>3</sup>, 3-38) are used. The transheterozygous larvae were obtained by crossing *mwh/mwh* males and *flr*<sup>3</sup>/TM3, *BdS* virgin females. Hybrid eggs derived from crossing optimally fertile flies were collected over an 8 h period. Larvae emerged  $72 \pm 4$  h later were cleaned from remaining feeding medium with distilled water, and subsequently transferred to treatment vials. These vials contained 0.85g of *Drosophila* Instant Medium (formula 4-24, Carolina Biological Supply, Burlington NC, USA) wetted with 4 ml of the epicarp and mesocarp of *C. pepo* and their antioxidant compounds solutions at physiological concentrations for *Drosophila melanogaster*: 0.25 and 8 mg/mL of epicarp and mesocarp of each variety, 0.039 and 0.615  $\mu\text{M}$  lutein, 0.0003 and 0.0689  $\mu\text{M}$   $\beta$ -carotene, 0.0001 and 0.105  $\mu\text{M}$  zeaxanthin, and 0.003 and 0.107 mM dehydroascorbic acid. Concurrent negative controls with the solvent alone (water) and positive controls with hydrogen peroxide (120 mM) were also run.

Antigenotoxicity tests were carried out by mixing the mutagen (hydrogen peroxide, 120 mM) with the compounds solution. After emergence, adult flies were collected from the treatment vials and stored in 70% ethanol. The wings of the flies were removed under a stereomicroscope using a pair of entomological tweezers, similar number of males and females-wings were mounted in Faure's solution on microscope slides and inspected, under 400 X magnification, for the presence of clones of cells. The mutant clones were classified into three types: (1) small single spots, containing one or two cells; (2) large single spots, containing three or more cells; and (3) twin spots, containing adjacent *mwh* and *flr*<sup>3</sup> cells [18]. The appearance of twin spots indicated the recombinogenic activity of the chemotherapeutic agent.

## 2.6 Data evaluation and statistical analysis

Differences between quality compounds present in epicarp and mesocarp from "Yellow" and "Light Green" zucchini were assessed by Student's t-test. Data normality was tested prior to analysis. SPSS Version 10.0 software was used to perform all statistical analyses (SPSS, 2000).

The frequencies of spots per fly of treated series were compared with its corresponding negative control series (water) to analyze SMART data. For maximum power, statistical analyses were done exclusively for the total number of spots recovered. The non-parametric *U* test of Mann, Whitney and Wilcoxon was used to avoid weak positive and inconclusive results on SMART and to achieve a well-

defined statistical diagnosis whether a given treatment should be regarded as genotoxic or not [33]. The inhibition percentages (IP) were calculated using the total spots per wing with the following formula [34]:

$$IP = [(Genotoxine\ alone - Genotoxine\ plus\ active\ compound) / Genotoxine\ alone] * 100$$

### 2.7 Cell culture and cytotoxicity assay

HL60 cells were cultured in suspension at 37 °C in RPMI 1640 medium (Biowhittaker, BE12-167F) supplemented with 10% fetal bovine serum (Biotewhittaker, DE14-801F), Glutamine (Sigma, G7513) and an antibiotic-antimycotic solution (Sigma, A5955) in a humidified atmosphere containing 5% CO<sub>2</sub>. Cultured HL60 cell (2×10<sup>5</sup> cells/mL) was treated for 72 h with epicarp, mesocarp and different compounds at the concentrations in which they are found in the fruit: epicarp (0.015-0.25 mg/mL) and mesocarp (0.015-0.5 mg/mL) from “Yellow” *C. pepo*, and epicarp and mesocarp (0.031-0.5 mg/mL) from “Light Green” *C. pepo*, lutein (0.0039-0.25 µM), β-carotene (0.023-0.466 µM), zeaxanthin (0.022-0.879 µM), dehydroascorbic acid (0.0341-0.681 mM). A mix of β-carotene, zeaxanthin and dehydroascorbic acid at the same concentrations above indicated was also prepared to check if the cytotoxic effects were in additive or synergic way.

The reactivity of trypan blue (Sigma, 93595), a vital dye, is based on the fact that the chromophore is negatively charged and does not interact with the cell unless the membrane is damaged. Therefore, all the cells that exclude the dye are viable. Non-viable cells stained purple-violet, whereas viable ones remained unstained. Trypan blue was added to cell cultures at a 1:1 ratio, and 20 µl of cell suspension was loaded into a Neubauer chamber. The cells were counted under an inverted microscope at 100 × magnification. After the incubation period, a growth curve was established and IC<sub>50</sub> values (concentration of test compound causing 50% inhibition of cell growth) were estimated. Curves are expressed as survival percentage with respect to controls growing at 72 h. All data represent the average of at least three independent experiments.

### 2.8 Assessment of proapoptosis DNA fragmentation

The HL60 tumoral cell line was used with the aim of analyse the apoptotic induction. HL60 cells (1.5×10<sup>6</sup> cells) were treated with the same concentrations of the different compounds for 5 h. A non-treated cells control was included for each experiment. Treated cells were collected, were centrifuged at 4000 rpm for 5 minutes and washed with PBS. The DNA was extracted using a commercial kit (Dominion mbl, MBL 243) and the resulting total DNA was treated with RNase in order to eliminate false positive for 30 minutes at 37°C. Samples of 1.5 µg of DNA were mixed with loading buffer and loaded onto a pre-solidified 2% agarose gel containing ethidium bromide. The agarose gels were run at 50 V for 90 minutes in TBE buffer and then observed and photographed under UV light.

## 3. Results

### 3.1 Quantitation of antioxidant compounds

The mean content values of the different active compounds in epicarp and mesocarp of the two summer squash varieties are listed in **Table 1**. The two varieties differed in the content of all the compounds analyzed. The “Yellow” Zucchini carotenoid content was significantly higher than “Light Green” Zucchini in both tissues, except zeaxanthin in mesocarp. It should be noted that the lutein epicarp content of “Yellow” Zucchini is seven times higher than “Light Green” Zucchini. Further in the latter variety, β-carotene was not detected in epicarp. Dehydroascorbic acid content of “Light Green” Zucchini was similar in both tissues, but mesocarp of “Yellow” Zucchini was tenfold higher than epicarp. In addition, dehydroascorbic acid mesocarp content of “Yellow” Zucchini was significantly higher than “Light Green” Zucchini.



**Table 1.** Epicarp and mesocarp mean content of utein,  $\beta$ -carotene, zeaxanthin and dehydroascorbic acid from two Zucchini varieties (“Yellow” and “Light green”) expressed in mg/kg dry weight.

	Yellow		Light	
<b>Epicarp</b>				
Lutein	1036.9	a	135	b
β-carotene	99.5	a	n.d	b
Zeaxanthin	18.6	a	1.7	b
DHA	369.3	b	592	a
<b>Mesocarp</b>				
Lutein	362.7	a	63.2	b
β-carotene	31.6	a	4.0	b
Zeaxanthin	1.9	a	1.7	a
DHA	3200	a	683.3	b

Means within the same row followed by the same letter are not significantly different using Student's test at  $P < 0.05$ .

### 3.2 Genotoxicity and antigenotoxicity analysis of *C. pepo* and their components

The results of chronic treatment of *D. melanogaster* transheterozygous larvae with Zucchini epicarp and mesocarp and their different antioxidant compounds, along or combined with one fixed concentration (120 mM) of hydrogen peroxide in the *Drosophila* wing spot assay (SMART) are presented in **Table 2** and **Table 3**. The positive control hydrogen peroxide ( $H_2O_2$ ) behaved as a genotoxin inducing oxidative stress and DNA damage, giving the expected result. It exhibited a total mutation rate (0.52 mutant clones/wing), which quadrupled the negative control (distilled water) rate (0.13). This ratio ensures the accuracy of the genotoxicity and antigenotoxicity concurrent assays [35].

All the compounds were non-genotoxic in the SMART at the tested concentrations (**Table 2**). There was no significant increase in the numbers of small single spots and total spots in the case of Zucchini epicarp and mesocarp and single compounds at the studied doses. Mutation rates were even lower than the negative water control. The only case with high but non-significant mutation rate was the highest concentration of lutein with 0.45 total spots/wing for 0.615  $\mu$ M. Zeaxanthin and dehydroascorbic acid induced a non-significant increase of mutation frequencies at the highest tested concentrations (0.15 total spots/wing at 0.105  $\mu$ M, and 0.18 at 0.107 mM, respectively) after larval feeding. Thus, in the wing spot test in vivo model, carotenoids and dehydroascorbic acid are not genotoxic.

Table 3 shows the results of the antigenotoxicity assays. The results obtained in the combined treatment of larvae with Zucchini epicarp and mesocarp (0.25 and 8 mg/mL) showed non-significant differences when compared to the negative control. Then the SMART test showed that Zucchini epicarp and mesocarp and bioactive compounds were able to detoxify the genotoxic activity of hydrogen peroxide although no dose effect was observed in general (Table 3). The mutation frequency in  $\beta$ -carotene, zeaxanthin and dehydroascorbic acid was even smaller than that obtained with the negative control. Only the highest concentration of lutein showed significant differences with the negative control (0.47 for 0.615  $\mu$ M).

**Table 2.** Genotoxicity (single treatment) of epicarp and mesocarp of two varieties of *C. pepo* and its active compounds (lutein,  $\beta$ -carotene, zeaxanthin and dehydroascorbic acid) in the *Drosophila* Wing Spot Test

Clones per wing (Number of spots) <sup>(1)</sup>						
Compound	N	Small Spots (1-2 cells) m = 2	Large Spots (>2 cells) m = 5	Twin Spots m = 5	Total spots m = 2	Mann- Whitney Test <sup>(3)</sup>
Negative Control (H <sub>2</sub> O)	40	0.08 (3) <sup>(2)</sup>	0.00 (0)	0.05 (2)	0.13 (5)	
EPICARP						
Yellow (mg/mL)						
0.25	40	0.15 (6)	0.00 (0)	0.03 (1)	0.18 (7)	n.s.
8	40	0.00 (0)	0.00 (0)	0.03 (1)	0.03 (1)	n.s.
Light Green (mg/mL)						
0.25	40	0.10 (4)	0.03 (1)	0.00 (0)	0.13 (5)	n.s.
8	40	0.05 (2)	0.00 (0)	0.00 (0)	0.05 (2)	n.s.
MESOCARP						
Yellow (mg/mL)						
0.25	40	0.08 (3)	0.03 (1)	0.00 (0)	0.10 (4)	n.s.
8	40	0.05 (2)	0.00 (0)	0.00 (0)	0.05 (2)	n.s.
Light Green (mg/mL)						
0.25	40	0.03 (1)	0.03 (1)	0.00 (0)	0.05 (2)	n.s.
8	40	0.03 (1)	0.03 (1)	0.00 (0)	0.05 (2)	n.s.
SINGLE COMPOUNDS						
Lutein ( $\mu$ M)						
0.039	40	0.15 (6)	0.08 (3)	0.00 (0)	0.23 (9)	n.s.
0.615	40	0.33 (13)	0.08 (3)	0.05 (2)	0.45 (18)	n.s.
$\beta$ -carotene ( $\mu$ M)						
0.0003	36	0.08 (3)	0.00 (0)	0.00 (0)	0.08 (3)	n.s.
0.0689	40	0.03 (1)	0.03 (1)	0.00 (0)	0.05 (2)	n.s.
Zeaxanthin ( $\mu$ M)						
0.0001	40	0.05 (2)	0.00 (0)	0.00 (0)	0.05 (2)	n.s.
0.105	40	0.10 (4)	0.03 (1)	0.03 (1)	0.15 (6)	n.s.
DHA (mM)						
0.003	40	0.10 (4)	0.03 (1)	0.00 (0)	0.13 (5)	n.s.
0.107	40	0.15 (6)	0.03 (1)	0.00 (0)	0.18 (7)	n.s.

(1) Statistical diagnosis by Frei and Würgler [36]. N: number of wings.

(2) Number of clones or spots per wing.

(3) The inconclusive results were resolved using the non-parametric U-Test Mann Whitney and Wilconson. n.s. (non-significative)

**Table 3.** Antigenotoxicity (combined treatment) of epicarp and mesocarp of two varieties of *C. pepo* and its active compounds (lutein,  $\beta$ -carotene, zeaxanthin and dehydroascorbic acid) in the *Drosophila* Wing Spot Test

Clones per wing (Number of spots) <sup>(1)</sup>						
Compound	N	Small Spots (1-2 cells) m = 2	Large Spots (>2 cells) m = 5	Twin Spots m = 5	Total spots m = 2	Mann- Whitney Test <sup>(3)</sup>
Negative Control (H <sub>2</sub> O)	40	0.08 (3) <sup>(2)</sup>	0.00 (0)	0.05 (2)	0.13 (5)	
Positive Control (H <sub>2</sub> O <sub>2</sub> 120 mM)	40	0.38 (15)	0.13 (5)	0.03 (1)	0.52 (21)	*
EPICARP						
Yellow (mg/mL)						
0.25	16	0.13 (5)	0.00 (0)	0.00 (0)	0.13 (5)	n.s.
8	40	0.15 (6)	0.03 (1)	0.00 (0)	0.18 (7)	n.s.
Light Green (mg/mL)						
0.25	40	0.13 (5)	0.05 (2)	0.03 (1)	0.20 (8)	n.s.
8	40	0.05 (2)	0.03 (1)	0.03 (1)	0.10 (4)	n.s.
MESOCARP						
Yellow (mg/mL)						
0.25	18	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)	n.s.
8	40	0.38 (15)	0.00 (0)	0.03 (1)	0.40 (16)	n.s.
Light Green (mg/mL)						
0.25	40	0.05 (2)	0.03 (1)	0.00 (0)	0.08 (3)	n.s.
8	40	0.05 (2)	0.00 (0)	0.00 (0)	0.05 (2)	n.s.
SINGLE COMPOUNDS						
Lutein ( $\mu$ M)						
0.039	32	0.06 (2)	0.06 (2)	0.00 (0)	0.13 (4)	n.s.
0.615	32	0.25 (8)	0.16 (5)	0.06 (2)	0.47 (15)	*
$\beta$ -carotene ( $\mu$ M)						
0.0003	12	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)	n.s.
0.0689	38	0.08 (3)	0.03 (1)	0.00 (0)	0.11 (4)	n.s.
Zeaxanthin ( $\mu$ M)						
0.0001	40	0.03 (1)	0.00 (0)	0.03 (1)	0.05 (2)	n.s.
0.105	30	0.07 (2)	0.00 (0)	0.00 (0)	0.07 (2)	n.s.
DHA (mM)						
0.003	40	0.08 (3)	0.00 (0)	0.00 (0)	0.08 (3)	n.s.
0.107	40	0.10 (4)	0.03 (1)	0.00 (0)	0.13 (5)	n.s.

(1) Statistical diagnosis by Frei and Würigler [36]. N: number of wings.

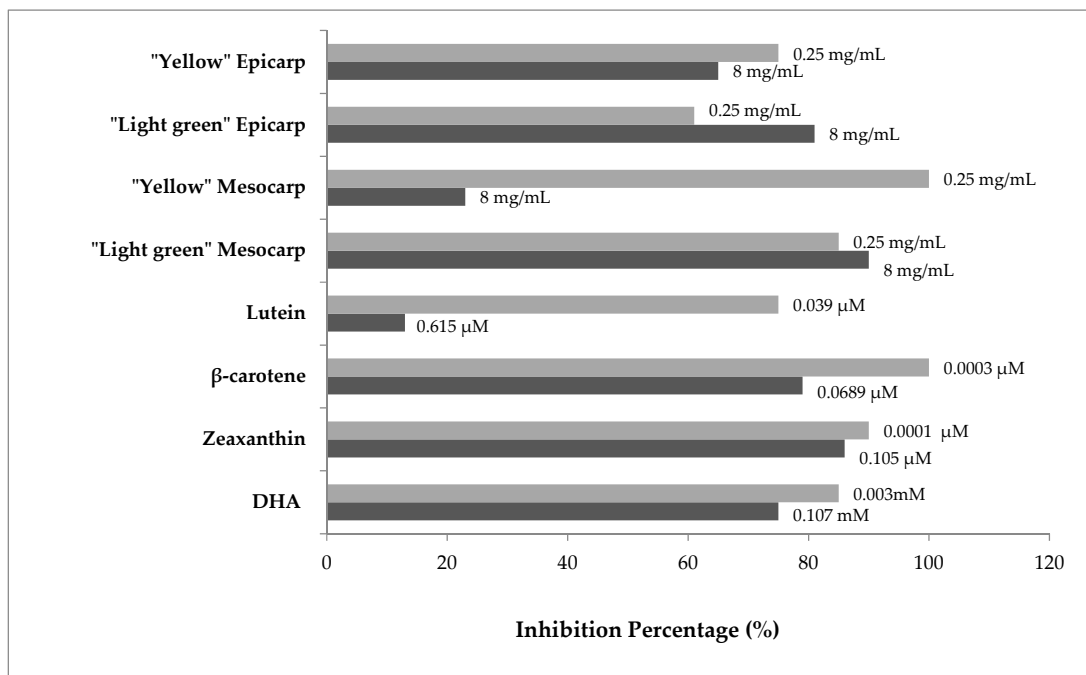
(2) Number of clones or spots per wing.

(3) The inconclusive and positive results were resolved using the non-parametric U-Test Mann Whitney and Wilconson. n.s.:non-significative; \*: significative ( $p \leq 0.05$ )



The inhibition percentage (IP) of genotoxicity by epicarp and mesocarp of “Yellow” Zucchini was 75% and 100% at 0.25 mg/mL and 65% and 23% at 8 mg/mL, respectively. In the case of the epicarp and mesocarp of “Light Green” Zucchini a dose-dependent IP was observed being the highest detoxifying activity by 90 % at 8 mg/mL of epicarp. The fruit antioxidant compounds would act as antimutagens against hydrogen peroxide in SMART. The lowest concentration of “Yellow” Zucchini mesocarp (0.25 mg/mL) showed the highest IP (100 %). The bioactive Zucchini selected compounds exhibited an IP that ranged from 13% to 100% (on average 75%) (Figure 1).

It should be noted that the SMART test is suitable to detect the anti/pro-mutagenic effects of some chemicals [2, 15, 16, 23, 37]. The results reported here are of interest when investigating the different ways in which summer squash and their antioxidant compounds can interfere *in vivo* with the mechanisms of genotoxic agents.



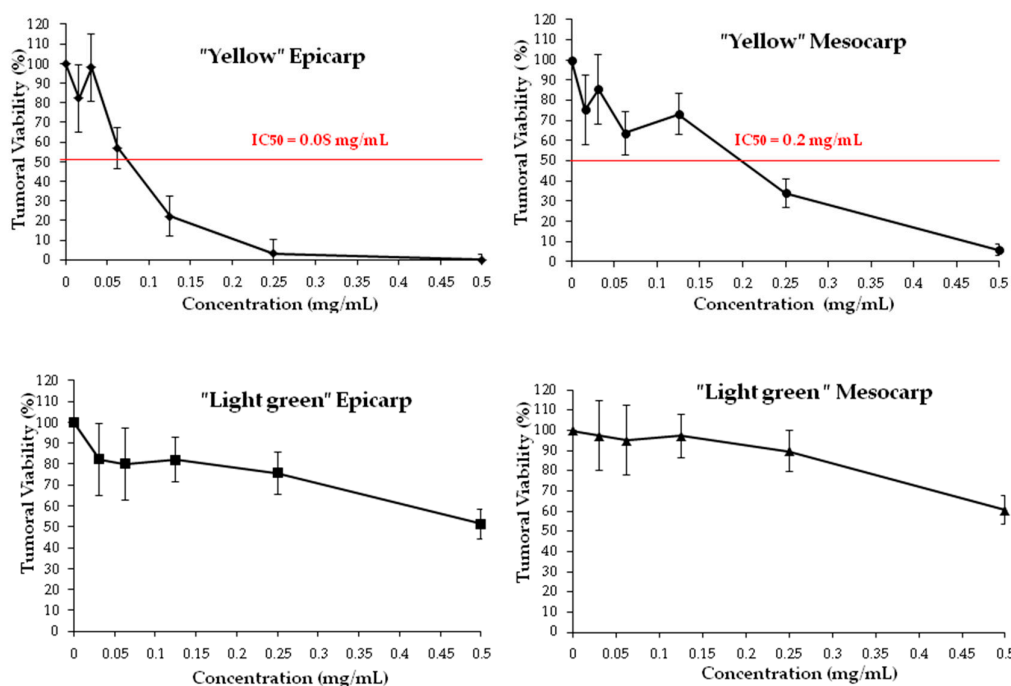
**Figure 1.** Inhibition percentages (IP) of Zucchini epicarp and mesocarp and its bioactive compounds against H<sub>2</sub>O<sub>2</sub>-induced damage in the *Drosophila* wing spot test.

### 3.3 Effects on tumoral growth of HL60 cells and apoptosis

Cultured mammalian cells provide an important tool for evaluating the cytotoxicity of compounds with potential therapeutic activity [38]. Trypan blue assessment was used to check the cytotoxicity of epicarp and mesocarp of two summer squash varieties and the main antioxidant compounds of the fruit.

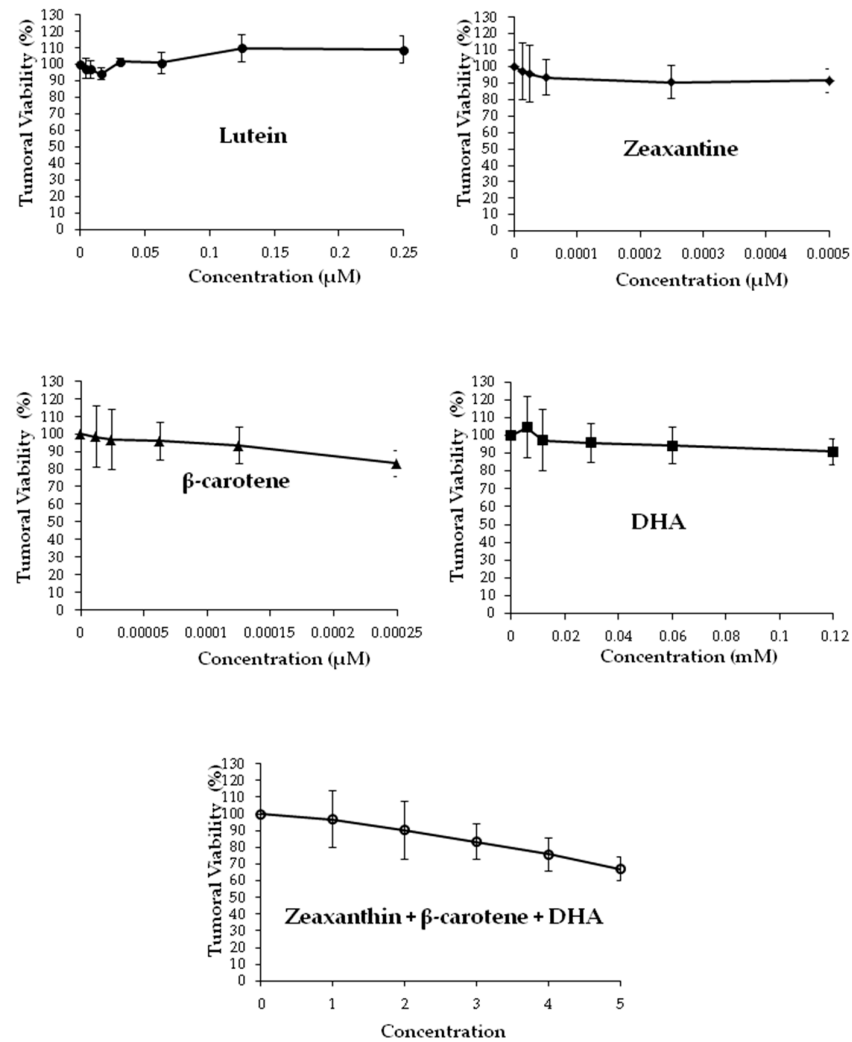
A wide range of concentrations was used for every compound. Figures 2 and 3 show the relative tumour growth inhibition activity of the substances assayed. The results are expressed as survival percentages with respect to the controls (% tumoral viability). The shapes of the curves were different for each case. Epicarp and mesocarp of “Yellow” Zucchini showed the most negative slope and the highest tumoricidal effect in the trypan blue exclusion assay ( $IC_{50}$  = 0.08 mg/mL and 0.2 mg/mL, respectively). Epicarp and mesocarp of “Light Green” Zucchini also showed a negative slope, but not reaching  $IC_{50}$ . The rest of compounds assayed did not reach  $IC_{50}$  at the tested concentrations, although slight cytotoxic activities were observed in some of them (zeaxanthin, dehydroascorbic acid, β-carotene and the mix of the compounds) (Figure 3). We did not observe any cytotoxic effect of lutein at the chosen concentrations. Taking these observations together, it appears that the cytotoxic action of Zucchini epicarp and mesocarp cannot be related to the

antioxidant effect of a single antioxidant compound, but rather to the total interactions between different compounds of such a complex mixture.

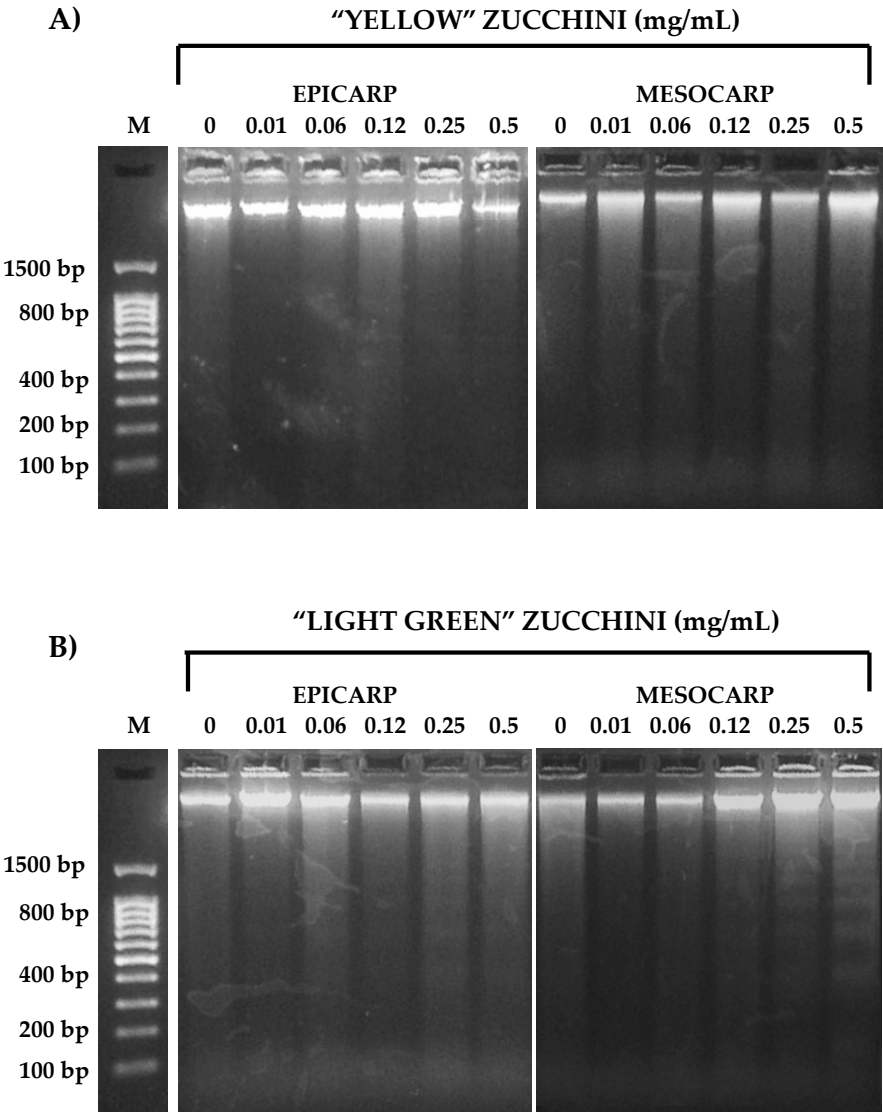


**Figure 2.** Effects of Zucchini epicarp and mesocarp on viability of HL60 tumoral cells. Cell viability was assessed after 72 hours by trypan blue exclusion test assay. The data are expressed as percentages of control (mean  $\pm$  SD from three independent experiments).

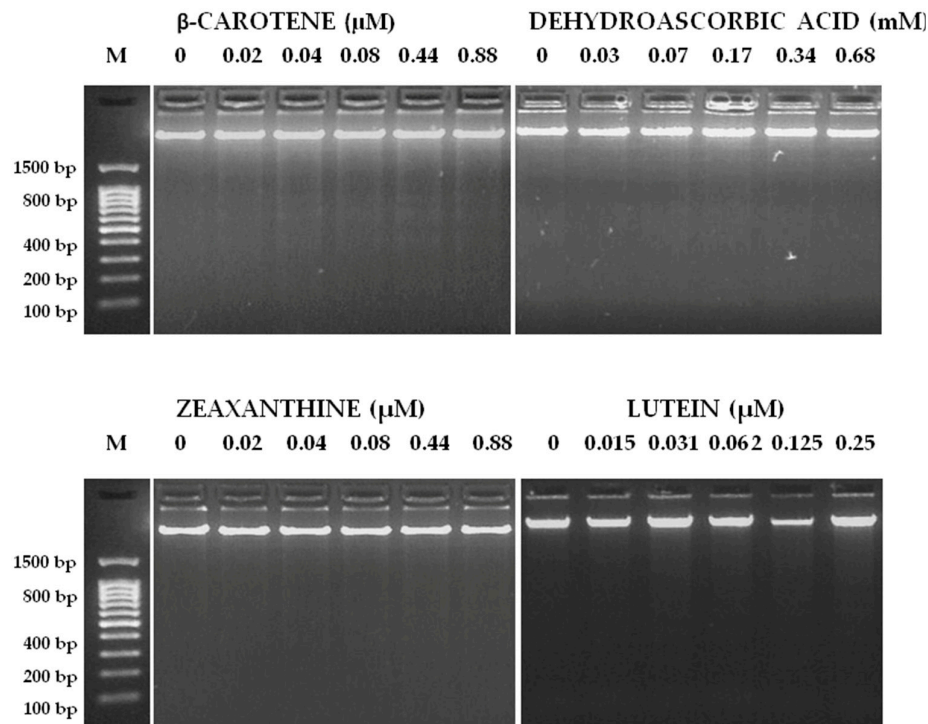
Once cytotoxicity assays of Zucchini epicarp and mesocarp and some of its antioxidant compounds were performed, a visible assay of DNA fragmentation was carried out in order to investigate whether the mechanism undergoing the cytotoxicity was mediated via apoptosis. The degradation of genomic DNA into oligonucleosomal fragments is a hallmark of apoptosis. This DNA fragmentation endpoint was analyzed by conventional agarose gel electrophoresis. The HL60 cell line was treated for 5 h with the same concentrations as in the trypan blue cytotoxicity assay. As shown in **Figure 4**, the highest concentrations of "Light Green" Zucchini mesocarp and, in a lesser extent, epicarp showed an internucleosomal fragmentation ladder pattern of DNA for HL60 treated cells. This did not occur in the control and some lower concentrations. A dose-dependent effect is suggested. "Yellow" Zucchini,  $\beta$ -carotene, lutein, zeaxanthin and DHA did not show any internucleosomal fragmentation ladder pattern (**Figures 4 and 5**).



**Figure 3.** Effects of lutein, zeaxanthin,  $\beta$ -carotene, dehydroascorbic acid (DHA) and a mix of compounds (zeaxanthin,  $\beta$ -carotene and DHA) on viability of HL60 cells. Cell viability was assessed after 72 hours by trypan blue exclusion test assay. The data is expressed as percentages of control (mean  $\pm$  SD from three independent experiments).



**Figure 4.** Nucleosomal DNA fragmentation. HL-60 cells were exposed to various concentrations of Zucchini epicarp and mesocarp for 5h. DNA was extracted from cells and was subject to 2% agarose gel electrophoresis at 50 V for 90 min. A) "Yellow" Zucchini and B) "Light Green Zucchini. M: DNA size marker.



**Figure 5.** Nucleosomal DNA fragmentation. HL-60 cells were exposed to various concentrations of different carotenoids ( $\beta$ -carotene, zeaxanthin and lutein) and dehydroascorbic acid for 5h. DNA was extracted from cells and was subject to 2% agarose gel electrophoresis at 50 V for 90 min.

#### 4. Discussion

Common prevention strategies include avoiding exposure to known cancer-causing agents, enhancement of host-defense mechanisms against cancer, lifestyle modifications and chemoprevention [39]. Chemoprevention is essential for the reversion, inhibition and prevention of cancer, and, optimally, requires the use of non-toxic agents that inhibit molecular steps during the carcinogenic pathway [40]. Prevention by dietary phytochemicals is an important approach in cancer management [41].

The first step of the present work was to characterise the different active compounds in Zucchini epicarp and mesocarp matrices. As the results show, both varieties differ greatly in bioactive compound contents in both tissues. The antioxidant contents exhibited by the samples were included in the variation interval found in the literature for *C. pepo*. Lutein and  $\beta$ -carotene contents in epicarp and mesocarp fall within the range reported in previous studies [9], but our lutein mesocarp content in “Yellow” Zucchini (362.7 mg/kg dry weight) was higher than reported in mesocarp by Tadmor et al. [42] (143.2 mg/kg dry weight, assuming 92% of moisture); El-Qudah [43] analyzed the lutein,  $\beta$ -carotene and zeaxanthin contents in Zucchini fruit and their results were lower than ours for the three carotenoids (23.4, 1.46 and 0.41 mg/kg dry weight, respectively). In relation to Vitamin C, in our study the loss of ascorbic acid was offset by the simultaneous increase of dehydroascorbic acid (a biologically active vitamin). As reported in previous studies, ascorbic acid (vitamin C) is a very unstable compound and it easily transforms into its oxidized form, dehydroascorbic acid [44]. Ascorbic acid can be easily degraded, depending on many variables such as pH, temperature, light, and presence of enzymes, oxygen, and metallic catalyzers [45]. Another important factors influencing Vitamin C retention in fresh-cut vegetables are the cutting mechanism and drying methods used. Cutting the product may promote the loss of vitamin C as a result of the increase of the surface area in contact with oxidizing agents such as oxygen that favor the action of ascorbate peroxidase (APX), the main enzyme responsible for degradation of vitamin C [46]. In relation to drying methods,

although freeze-drying is considered one of the main techniques to preserve phytochemicals, a negative effect on ascorbic acid, and other compounds such as total phenolics and carotenoids have been previously reported in different foodstuff [47-49].

The SMART test has been shown to be a good predictor of safe/harmful substances due to the ability of *Drosophila* larvae to activate many procarcinogens and the possibility for individually treating thousands of somatic cells [50]. The main objective of this assay was to evaluate the safety of Zucchini epicarp and mesocarp for the first time, as well as their major active components (lutein,  $\beta$ -carotene, zeaxanthin and dehydroascorbic acid) with respect to the lack of induction of genetic damage. The wing spot test for Zucchini tissues and their components resulted non-significant at the assayed concentrations when compared to the water control.

Antigenotoxic agents generally exhibit desirable chemotherapeutic effects that could be efficient in the strategy of cancer control [51]. Hydrogen peroxide was used as a positive control in SMART because it is reported that it generates DNA damage through oxygen-radical mechanisms, gene mutation, chromosomal aberration and DNA single-strand breaks [52]. Nevertheless, it is not known whether the interactions that can occur between oxidative elements ( $H_2O_2$ ) and other bioactive compounds (lutein,  $\beta$ -carotene, zeaxanthin and DHA) act mutually. The concentrations of the selected compounds were shown to be antigenotoxic in the SMART test of *Drosophila melanogaster*. The strongest inhibition ability was detected with the lowest concentration of "Yellow" Zucchini mesocarp (0.25 mg/mL) and the lowest concentration of  $\beta$ -carotene (0.0003  $\mu$ M) against the genotoxic effects of  $H_2O_2$  in the imaginal discs of *Drosophila* (100% for both).

The cytotoxicity assessment is an *in vitro* bioassay needed in the evaluation of the chemopreventive effects of a substance as a fast, non-expensive and informative first step of screening. The human cell line HL60 provides a reliable model to study the cytotoxic effect of chemopreventive substances and the mechanisms underlying this potential activity [37].

Some examples of similar results in relation to the cytotoxicity of *C. pepo* in the *in vitro* cytogenetic assay with different cell lines have been reported. Wang et al. [14] observed a significant dose-dependent inhibitory effect against HeLa and HepG cell growth using ethanolic extracts of *C. pepo* fruits. Menéndez et al. [10] remarked a significant decrease of the prostatic growth using a lipophilic extract of *C. pepo* seeds at doses of 400 and 200 mg/kg. Shokrzadeh et al. [13] used hydro-alcoholic extracts of *C. pepo* leaves on normal [Chinese hamster ovarian cells (CHO) and rat fibroblast] and cancer (HepG2 and CT26) cell lines with the following rank of inhibition ability: CHO < fibroblast < CT26 < HepG2 (being the lowest and the highest  $IC_{50}$  for HepG2: 132.6  $\mu$ g/mL, and for fibroblast: 293.2  $\mu$ g/mL) cell lines. Other studies revealed that aerial parts of other *Cucurbita* species such as *C. maxima* also possess significant anticancer activity in Ehrlich ascites carcinoma model in mice which may be due to its cytotoxicity and antioxidant properties [4].

Anti-proliferative properties and induction of apoptosis in tumor cells are suitable when the health-protecting effect of antioxidants is assessed [51]. Apoptosis plays critical roles in the development and maintenance of homeostasis, in multicellular organisms. During apoptosis, intracellular contents are not released and potentially harmful inflammatory responses are prevented. Apoptotic cell death is characterized by a DNA fragmentation into 180-200 pb nucleosomal units [16]. The detection of DNA fragmentation in HL60 exposed to different concentrations of epicarp and mesocarp of "Light Green" Zucchini variety indicated that the cells exposed to these concentrations suffer cell death via apoptosis and therefore their activity could be catalogued like an antiproliferative action. The compounds that promote apoptosis should become an important addition to the arsenal of target-specific drugs in the fight against cancer. The results of our study have shown fragmentation of DNA upon treatment of HL60 cells with epicarp and mesocarp of "Light Green" Zucchini, indicating the involvement of apoptosis.

In the present study, we demonstrated that a complex mixture such as Zucchini epicarp and mesocarp, inhibited proliferation in the human promyelocytic HL60 cell line. In addition, our results provide a basic knowledge about phytochemicals interactions with biological systems that may be useful for the design of functional foods. Sharoni et al. [53] hypothesized that a single micronutrient



cannot replace the power of the concerted action of multiple agents derived from a diet rich in fruits and vegetables.

Carotenoids have been studied widely to demonstrate if these colorful compounds can decrease the cancer risk [54]. Dias et al. [55] obtained similar results in the SMART test for  $\beta$ -carotene using higher concentrations than ours (1, 2 and 4 mg/mL):  $\beta$ -carotene was not genotoxic and was able to detoxify up to 95 % of the mutation induced by doxorubicin. Zhang and Omaye [56] demonstrated that the antioxidant and pro-oxidant effects of  $\beta$ -carotene are dependent of both, the level of oxygen and the  $\beta$ -carotene concentration. For most biological tissues, when the oxygen level is low,  $\beta$ -carotene, as other carotenoids, becomes important as an antioxidant [57]. The chemopreventive action of  $\beta$ -carotene is effective mainly in the beginning of the carcinogenic process or in the initial stages of its promotion, inhibiting the formation of pre-neoplastic lesions in experimental models *in vitro* and *in vivo* [24] [25] [26]. The ability of  $\beta$ -carotene to inhibit cell growth has been established in several tumor cells including melanoma [58], prostate [59], colon [60], lung, breast and oral mucosa cancer [61]. In our study  $\beta$ -carotene did not show cytotoxicity (0.5  $\mu$ M) which agrees with Sacha et al. [62] who used  $\beta$ -carotene in the concentrations available *in vivo* (10  $\mu$ M) and did not affect leukemic cell lines (HL60). Higher doses (ID<sub>50</sub> = 27  $\mu$ M) of  $\beta$ -carotene used in HL60 cells by Palozza et al. [63] modulated cell cycle progression and induced apoptosis in a dose-dependent manner; nevertheless the delay in cell growth by  $\beta$ -carotene was highly coincident with the increased intracellular ROS production and oxidized glutathione content induced by the  $\beta$ -carotene. On the other hand, Sacha et al. [62] also informed that  $\beta$ -carotene stimulated apoptosis in HL60 by modulating the expression of the regulatory genes at 10  $\mu$ M concentration. This pigment at 10  $\mu$ M and 50  $\mu$ M also stimulated apoptosis in B16F cells (melanoma) [64] and in the MCF-7 cell line [65], respectively. Although epidemiologic studies have demonstrated that a high intake of vegetables containing  $\beta$ -carotene decreases the risk of cancer, different studies have revealed that  $\beta$ -carotene supplementation to smokers resulted in a high incidence of lung cancer, it could alternatively behave as an antioxidant or as a prooxidant molecule, depending on its redox potential and on the cellular environment [63]. Studies tend to agree that the overall intake of carotenoids is more protective than a high intake of a single carotenoid [54] as  $\beta$ -carotene may be a marker for the intake of fruits and vegetables, but it does not have a powerful protective effect in isolated pharmacological doses. So, a variety of fruits and vegetables is still a better anti-cancer strategy than just using a single vegetable with high content in a specific carotenoid.

With respect to the rest of the carotenoids (lutein and zeaxanthine), The cytotoxic effects of zeaxanthin on two human uveal melanoma cell lines (SP6.5 and C918) were studied and compared to effects on normal ocular cells (uveal melanocytes, retinal pigment epithelial cells, and scleral fibroblasts); zeaxanthin reduced the cell viability of melanoma cells in a dose-dependent manner (10, 30, and 100  $\mu$ M), with IC<sub>50</sub> at 40.8 and 28.7  $\mu$ M in SP6.5 and C918 cell lines, respectively. Zeaxanthin did not affect the viability of normal ocular cells even at the highest levels tested (300  $\mu$ M), suggesting that zeaxanthin has a selectively cytotoxic effect on melanoma cells; in addition, zeaxanthin (30  $\mu$ M) induced apoptosis in melanoma cells [28].

Previous work on lutein indicated that it is able to induce significant DNA damage in a dose- and time-dependent manner in human retinal pigment epithelial cells (ARPE-19) at a concentration ranging from 10 to 50  $\mu$ M [29]. Lutein has an antiproliferative activity on HeLa, HCT116 and Hep2 cancer cell lines [66] [67] [68]. On the other hand, similar negative effects to ours were obtained by Fernández-Bedmar and Alonso-Moraga [15] when HL60 cells were treated with this carotenoid. Roberts et al. [69] demonstrated that when the human lens epithelial cells (HLE B-3) were pretreated with lutein (20  $\mu$ M added 2 h prior to irradiation with UVA), the singlet oxygen quencher-lutein significantly protected against fullerol photodamage, phototoxic damage decreased by half.

Other studies have been conducted with rats where the toxicity of zeaxanthin and lutein present in different commercial preparations have been evaluated, and no toxicologically relevant findings were noted in any study. Moreover in these studies, the results of zeaxanthin mutagenicity testing in the *Salmonella typhimurium* model did not reveal any genotoxicity [70] [27]

[71], and neither in *Escherichia coli* [71]. In the same way, lutein was not genotoxic in *Drosophila melanogaster* genetic model [15].

Low blood levels of ascorbic acid are detrimental to health and vitamin C is correlated with overall good health and cancer prevention [5]. Dehydroascorbic acid did not show cytotoxicity in our studies at 0.7 mM, but Park et al. [72] informed that L-ascorbic acid (the reduced form of dehydroascorbic acid) showed cytotoxicity and induces apoptosis of malignant cells (HL60, NB4 and NB4-R1) *in vitro*, when they used higher concentrations than ours (0.25-1 mM). Moreover, dehydroascorbic acid (0.5-1 mM) was tested for its protective effect against phototoxic damage induced by fullerol, resulting in some activity against metabolic damage [69].

The results obtained in genotoxicity and antigenotoxicity assays of Zucchini epicarp and mesocarp and its different bioactive compounds (lutein,  $\beta$ -carotene, zeaxanthin and dehydroascorbic acid) indicated that Zucchini is not genotoxic and safe. "Light Green" and "Yellow" Zucchini showed antimutagenic capacity, but the capacity of the "Light Green" variety was higher on average. This difference in the antigenotoxic activities could be due to the different content of bioactive molecules. With the current data, authors suggest that lutein could be the responsible molecule for the different antimutagenic activity of two varieties, as although lutein is not genotoxic, it is at the limit of significance. Moreover lutein was antigenotoxic at the lowest concentration, but at highest concentration enhances the genotoxicity of hydrogen peroxide. With respect to the cytotoxicity results in the present study, "Light Green" Zucchini showed a lower activity than "Yellow" Zucchini. The differences in the carotenoid's content could partially explain the different cytotoxic activities, as can be observed in Figure 3, where the inhibition of the tumoral growth of the mix of the three compounds is equivalent to the addition of the inhibitions of the separate individual compounds (zeaxanthin,  $\beta$ -carotene and dehydroascorbic acid).

## 5. Conclusions

*C. pepo* is an important crop and source of human food around the world. Our results confirmed the safety, antigenotoxicity and chemopreventive potential of Zucchini and some of its compounds using the SMART test *in vivo* model and the cytotoxicity HL60 cells *in vitro* model. Antigenotoxicity assays indicated that all the concentrations showed protective antigenotoxic activity with different inhibition percentages (ranging from 11 to 100 % inhibition) in combined treatments with hydrogen peroxide as a genotoxinant, exception made of the highest concentration of lutein. The leukaemic cell line HL60 provides an important model for studying the mechanisms and relationships between cytotoxicity, apoptosis and antitumor efficacy of different substances and compounds. Epicarp and mesocarp of "Yellow" Zucchini exhibited the highest cytotoxic activity ( $IC_{50} > 0.08$  mg/mL and 0.2 mg/mL, respectively); the rest of the bioactive compounds assayed did not reach  $IC_{50}$  at the tested concentrations.

The result of the present investigation is quite encouraging and it explores the potential anticancer activity of Zucchini, being probably due to its direct cytotoxic effect which is further enhanced by its antioxidant properties. The Zucchini fruit showed promising results, and could play a beneficial role in human nutrition and general health. We conclude that *C. pepo* and their components were safe, able to inhibit significantly the  $H_2O_2$ -induced damage and exhibit antiproliferative and pro-apoptotic properties toward HL60 tumour cells. The information generated from *in vitro* and *in vivo* studies about the Zucchini phytochemical profile that contributes to improve the consumer's health is essential in order to select potential accessions for breeding programs purposes.

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**Conflicts of Interest:** "The authors declare no conflict of interest."

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