

Article

Chemical Composition and Biological Activity of *A. cepa* L. and *A. × cornutum* (Clementi ex Visiani 1842) Methanolic Extracts

Željana Fredotović¹, Matilda Šprung², Barbara Soldo², Ivica Ljubenkov², Irena Budić-Leto³, Tea Bilušić⁴, Vedrana Čikeš-Čulić⁵ and Jasna Puizina^{1, *}

¹ Department of Biology, University of Split, Faculty of Science, R. Boškovića 33, 21 000 Split, Croatia; zfredotov@pmfst.hr (Ž.F.)

² Department of Chemistry, University of Split, Faculty of Science, R. Boškovića 33, 21 000 Split, Croatia; msprung@pmfst.hr (M.Š.); barbara@pmfst.hr (B.S.); iljubenk@pmfst.hr (I.LJ.)

³ Institute for Adriatic Crops and Karst Reclamation, Put Duilova 11, 21 000 Split, Croatia; irena.budic-letto@krs.hr (I.B-L.)

⁴ Faculty of Chemistry and Technology, University of Split, R. Boškovića 35, 21 000 Split, Croatia; tea@ktf-split.hr (T.B.)

⁵ School of Medicine, University of Split, Šoltanska 2, 21 000 Split, Croatia; vedrana.cikes.culic@mefst.hr (V.Č-Č.)

* Correspondence: puizina@pmfst.hr; Tel.: +0038521619260

Abstract: Here we report a comparative study of the phytochemical profile and a biological activity of the two onion extracts, namely *A. cepa* L. and *A. × cornutum* (Clementi ex Visiani 1842). Identification of flavonoids and anthocyanins and their individual quantities were determined by high performance liquid chromatography (HPLC). The potency of both extracts to scavenge free radicals was determined by DPPH (2,2'-diphenyl-1-picrylhydrazyl) radical-scavenging activity and oxygen radical absorbance capacity (ORAC) methods. DNA protective role was further tested by single-cell gel electrophoresis (COMET) assay and by Fenton's reagent causing double strand breaks on pUC19 plasmid. In the presence of both extracts, a significant decrease in the DNA damage was observed, which indicates a protective role of *A. cepa* and *A. × cornutum* on the DNA strand breaks. Additionally, cytotoxicity was tested on glioblastoma and breast cancer cell lines. The results showed that both extracts had antiproliferative effect, but the most prominent decrease in cellular growth was observed with glioblastoma cells.

Keywords: onions; *A. × cornutum*; *A. cepa*; phenolic compounds; antioxidant activity; genotoxicity; antimutagenic activity; antiproliferative activity

1. Introduction

Oxidative stress is the disturbance between the production of reactive oxygen species (ROS) and antioxidant defenses. Overproduction of ROS can cause damage of important biomolecules such as DNA, proteins, lipids, and carbohydrates resulting in several diseases [1]. For this reason, living cells have developed antioxidant systems to control free radicals, lipid peroxidation and maintain oxidative-antioxidative balance [2]. Dietary antioxidants also play an important role in the suppression of oxidative stress that may cause initiation and progression of various diseases, including cancer. Therefore, the consumption of dietary antioxidants is considered to be an effective tool in preventing diseases that may be caused by oxidative stress [3]. The genus *Allium* covers more than 750 species distributed all over the northern hemisphere [4]. The members of this genus are known not only as flavored vegetables and spice, but also as medical plants that have been used in traditional medicine [5,6]. Among other *Allium* species, *Allium cepa* L. (common onion) is one of the oldest plants cultivated worldwide [7]. Many epidemiological studies confirmed that dietary consumption of onions is associated with reduced risk for developing many forms of cancer,

cardiovascular and neurodegenerative diseases [8-10]. Their beneficial effect on health is attributed to a high content of biologically active phytomolecules such as phenolic compounds, especially flavonoids and several organosulfur compounds [11]. The most abundant flavonoids found in onions are quercetins, namely quercetin-4'-monoglucoside and quercetin-3,4'-diglucoside which account for more than 85% of total flavonoid content [12,13]. Besides flavonoids, onions, especially the red varieties, are a rich source of anthocyanins. The most frequently reported anthocyanins in red onions are cyanidin derivatives, although minor amounts of peonidin, petunidin and delphinidin derivatives have also been identified [14]. *A. × cornutum* (Clementi ex Visiani 1842) is a triploid hybrid onion ($2n=3x=24$) originating from three parental species (*A. cepa*, *A. pskemense* B. Fedt. and *A. roylei* Stearn), traditionally cultivated in costal Croatia under the name 'Ljutika' (shallot) [15]. Due to its tasty bulbs and leaves it is widely used as a spice and similarly cultivated in other parts of the world such as South-East Asia and Europe. To our knowledge, the chemical composition and biological activity of *A. × cornutum* have not been studied so far. This motivated us to identify and quantify major phenolic compounds present in bulbs of *A. × cornutum* and *A. cepa* and to assess the biological activity (free radical scavenging potential, antimutagenic activity, antiproliferative activity on cancer cells, and antigenotoxic activity) of these widely used plants.

2. Results and Discussion

2.1. HPLC analysis of flavonols and anthocyanin and total phenolic content of the two onion species

Two major and three minor fine peaks were successfully resolved by HPLC analysis of flavonols in *A. × cornutum* and *A. cepa* methanolic extracts (Figure 1). The resulting chromatograms were qualitatively similar, which is in good agreement with other reported studies [16-20]. Here, two major peaks were identified as quercetin 3,4'-diglucoside (**1**) (t_R for *A. cepa* 33.26 min; t_R for *A. × cornutum* 33.30 min) and quercetin 4'-monoglucoside (**2**) (t_R for *A. cepa* 41.41 min; t_R for *A. × cornutum* 41.45 min). Three minor peaks were identified as myricetin (**3**) (t_R for *A. cepa* 43.34 min; t_R for *A. × cornutum* 43.37 min), quercetin aglycone (**4**) (t_R for *A. cepa* 48.13 min; t_R for *A. × cornutum* 48.17 min) and isorhamnetin (**5**) (t_R for *A. cepa* 52.94 min; t_R for *A. × cornutum* 52.97 min). Two main quercetin conjugates, (**1**) and (**2**), together account for about 80% of total flavonol content in both onions (Table 1).

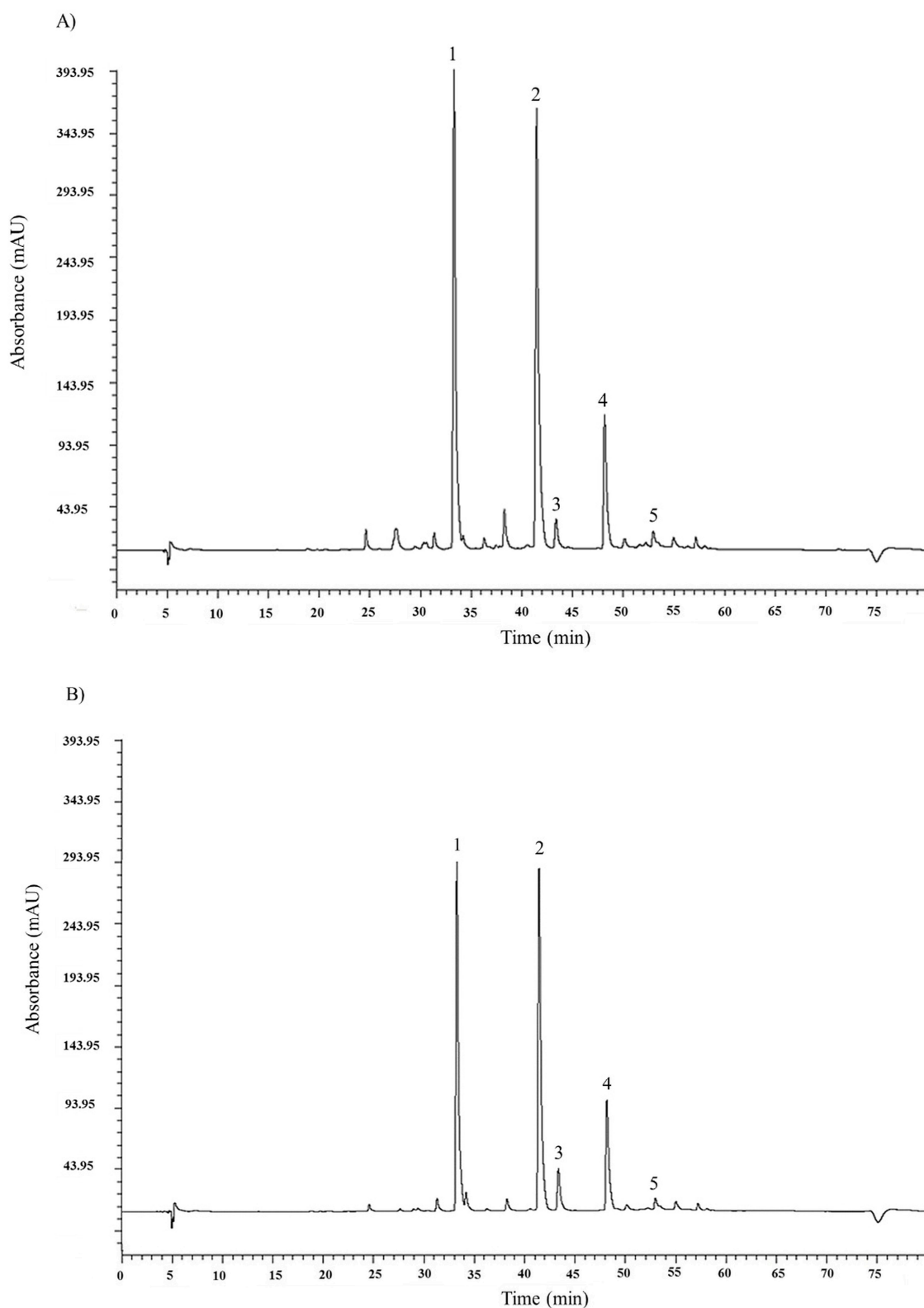


Figure 1. HPLC chromatograms of *A. x cornutum* (A) and *A. cepa* (B) metanolic extracts. Depicted are peaks: (1), quercetin 3,4'-diglucoside, (2) quercetin 4'-monoglucoside, (3) quercetin, (4) ishorhamnetin and (5) kaempferol.

Similar concentration of the two quercetin conjugates was previously observed in other studies [16–19], confirming that the two are indeed predominant flavonols in onions. However, in this work somewhat higher concentration of (1) and (2) was observed in *A. x cornutum* which is in agreement with higher total phenolic content (*A. x cornutum* TPC 6.63 ± 0.31 mg CA/g DW; *A. cepa* TPC 6.24 ± 0.23 CA/g DW) accounted for this onion species.

Table 1. HPLC quantification of flavonols and anthocyanins in *A. cepa* and *A. × cornutum* extracts.

	<i>A. × cornutum</i>	<i>A. cepa</i>
Flavonols*		
Quercetin 3,4'-diglucoside (1)	240.01 ± 0.39	171.34 ± 0.13
Quercetin 4'-monoglucoside (2)	159.86 ± 0.09	117.38 ± 0.17
Myricetin (3)	6.22 ± 0.09	8.02 ± 0.02
Quercetin aglycone (4)	24.13 ± 0.08	19.85 ± 0.03
Isorhamnetin (5)	7.43 ± 0.05	4.74 ± 0.01
Anthocyanins*		
Peonidin 3'-glucoside (6)	0.54 ± 0.00	0.19 ± 0.00
Petunidin 3'-glucoside acetate (7)	0.52 ± 0.00	0.13 ± 0.01
Delphinidin 3'-glucoside (9)	0.15 ± 0.00	nd
Malvidin 3'-glucoside (8)	0.01 ± 0.00	0.03 ± 0.00

* Concentrations in mg/100 g of dry weight

Our obtained TPC values show good correlation to those reported by Santas, Carbo, Gordon, and Almajano (2008) [21]. In their study, TPC values of the two methanolic extracts of Spanish onion varieties ranged from 5.15 to 6.33 mg GAE/g DW for white onion, and 2.48 to 2.58 mg GAE/g DW for Calçot de Valls onion. However, it should be noted that TPC values could vary depending on the location of the cultivar and on the solvent used during the extraction process [1,21–24].

Furthermore, quantification of individual anthocyanins was carried out on HPLC (Table 1) whereat three anthocyanins in *A. cepa* and four in *A. × cornutum* extract were identified. Three anthocyanins were the same for both onions and were further identified as peonidin glucoside (6), petunidin glucoside (7) and malvidin glucoside (8). Interestingly, the fourth anthocyanin, delphinidin glucoside (9), was found only in the *A. × cornutum* extract. The only study reporting the presence of the delphinidin glucoside in Tropea red onion is the one reported by Gennaro et al. (2002) [14]. Beside delphinidin glucosides, they also identified a petunidin derivatives and together they accounted for ~30% of total anthocyanins in onion bulbs.

2.2. Antioxidant activity

In order to determine the antioxidant potential of our extracts, two tests were performed, DPPH and ORAC assay. Both onion showed mutually similar DPPH (% DPPH inhibition for *A. cepa* 64.82 ± 5.31 and *A. × cornutum* 60.50 ± 3.84) and ORAC values (μmol TE/mL for *A. cepa* 17.62 ± 0.57 and *A. × cornutum* 19.38 ± 2.21). However, slightly higher antioxidant activity was observed for *A. × cornutum* in ORAC assay, which can be attributed to a bit higher total phenolic content. The high antioxidant scavenging activity of *Allium* species was reported in other studies and it was shown that it depends on the existence of both phenolic and organosulfur compounds [24–29].

2.3. DNA nicking assay

As it was shown that both onions have relatively high amount of phenolic compounds, we further wanted to investigate their potential in protecting DNA from ROS. In that sense, pUC19 plasmid exposed to Fenton's reagent served as a biological probe.

Hydroxyl radicals generated by the Fenton reaction cause oxidatively induced DNA strand breaks to yield its open circular or relaxed form [1]. Figure 2. show the DNA protecting effect of *A. cepa* and *A. × cornutum* where it can be seen that lower extract concentrations show poor DNA protection and an increase in open circular and linear forms of DNA. On the contrary, higher concentrations show significant free radical scavenging activity and the ability to maintain a supercoiled form of DNA. Our results clearly show that preservation of intact circular plasmid is concentration dependent (5–100 μg/ml).

Figure 2. Protective effect of onion extracts on supercoiled pUC19 DNA. Lane 1: pUC19 plasmid; Lane 2: pUC19 incubated with Fenton's reagent; Lane 3: pUC19, Fenton's reagent and catalase (5 U); Lane 4-9: pUC19, Fenton's reagent and 5, 10, 15, 20, 50 and 100 µg/mL of *A. × cornutum* extract; Lane 10-14: pUC19, Fenton's reagent and 5, 10, 20, 50 and 100 µg/mL of *A. cepa* extract.

Since both extracts have a relative high amount of phenolic compounds (quercetin and its derivatives) which act as antioxidants, we presume they can be responsible for such a good scavenging activity. Several other previous studies have in turn also reported that high phenolic content prevents DNA damage caused by hydroxyl radicals [1,30].

The antioxidant activity of phenolic compounds is associated with the total number and position of hydroxyl functional groups that are able to reduce highly oxidizing free radicals such as superoxide, peroxy, alkoxy and hydroxyl radicals [31]. Other than ROS reduction by hydrogen donation, antioxidant action can include suppression of the ROS formation by chelating metal ions, scavenging ROS and inhibition of oxidases [6]. Since phenolic compounds act as antioxidants, it is justified to conclude that higher phenolic content leads to stronger antioxidant capacity. This conclusion agrees with our findings that TPC values are in correlation with DPPH-scavenging and ORAC values for both onions.

2.4. Comet assay

The comet assay was performed to determine a possible protective effect of both onions on DNA damage in human leukocytes. Incubation of leukocytes with DNA damage inducing reagent (H_2O_2) resulted in a significant increase of DNA damage comparing to the untreated control ($p < 0.001$) (Figure 3.).

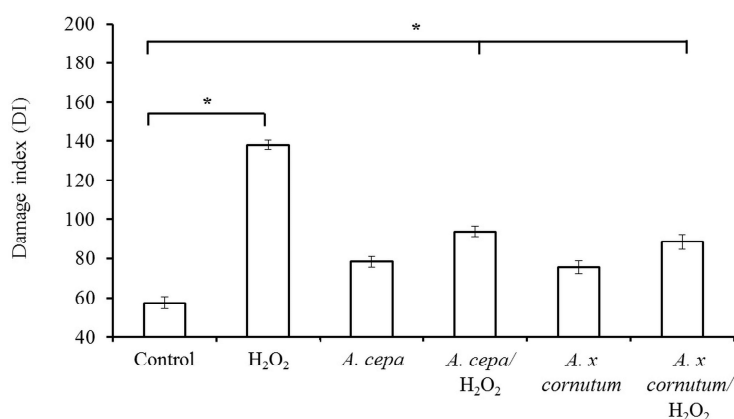


Figure 3. DNA damage index (DI) of treated human leukocytes: control, H_2O_2 , *A. cepa*, *A. cepa* with H_2O_2 , *A. × cornutum*, and *A. × cornutum* with H_2O_2 (* $p < 0.001$).

Cells treated with *A. × cornutum* or *A. cepa* extracts showed only moderate level of the DNA damage indicating that some of the phytochemicals present in onions can also provoke oxidative DNA damage. This is considered to be a structural property of phenolic compounds that can act as, both, antioxidants and prooxidants at the same time. In the presence of oxygen, some transition metals such as Cu and Fe can cause oxidation of flavonoids leading to the formation of ROS, which can damage DNA molecule [32]. When leukocytes were simultaneously incubated with onion

extracts and H_2O_2 , a significant decrease in DNA damage was observed ($p < 0.0001$ for *A. × cornutum* and *A. cepa*).

Hydrogen peroxide generates hydroxyl radicals (OH^\bullet) that cause DNA strand breaks and fragmentation. Thanks to the high levels of phenolic compounds, especially quercetin and its derivatives, *A. × cornutum* and *A. cepa* are able to catch and inactivate those radicals before they can induce DNA damage. Quercetin and its glycosides may work in a way that donate a hydrogen atom from its phenolic hydroxyl group in B-ring to remove hydroxyl radicals generated from hydrogen peroxide. Similar protective effect of quercetin on hydrogen peroxide induced DNA damage was reported before [33–35]. Our result demonstrate that both extracts are able to prevent DNA damage caused by oxidative DNA damaging agents such as H_2O_2 in human leukocytes.

2.5. Cell proliferation assay

To evaluate whether *A. × cornutum* and *A. cepa* methanolic extract have cytotoxic effect on breast and glioblastoma cancer cell lines, cells were treated with onion extracts for 4, 24, 48 and 72 hours and the amount of metabolically active cells was determined by MTT assay.

As shown in Figure 4, both extracts significantly reduced ($p < 0.001$) the number of viable cells, albeit *A. × cornutum* which showed slightly stronger inhibitory effect on all cancer cell lines. The most striking observation was the low viability of glioblastoma, which among other tested cancer cells, showed the highest susceptibility in the presence of both onion extracts. Our results indicate that both plants, *A. × cornutum* and *A. cepa*, are effective inhibitors of tumor cell proliferation which is in correlation with study of Boivin et al., (2009) [26], who have also shown strong antiproliferative effect of *Allium* vegetables on different cancer cell lines.

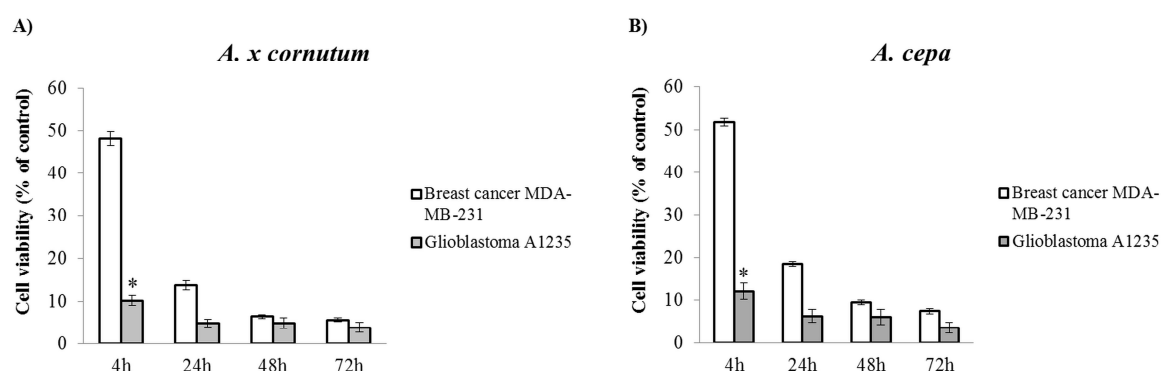


Figure 4. Cytotoxic effect of *A. × cornutum* and *A. cepa* methanolic extracts on two human cancer cell lines: breast and glioblastoma cancer cells. The antiproliferative effect of *A. × cornutum* (A) and *A. cepa* (B) after 4, 24, 48 and 72 h of exposure. The percentage of metabolically active cells is expressed in comparison to the untreated control. The data are presented as a mean value \pm SD of the three independent experiments. The statistically significant difference of glioblastoma cell line is represented as * $p < 0.001$ for *A. × cornutum* and *A. cepa*.

It's worth noting that *Allium* extracts used in their study, also displayed strong inhibitory effect against glioblastoma cancer cells. Most importantly, the authors showed that onion extract had no impact on the growth of normal fibroblast, suggesting their selective antiproliferative activity exclusively on tumor cells. Similar results were obtained by Yang, Meyers, van der Heide, and Liu (2004) [36] as they confirmed antiproliferative effect on human epithelial colorectal adenocarcinoma cells (Caco-2) and liver hepatocellular carcinoma cells (HepG2). The authors also observed that different range of antiproliferative activities depends on onion cultivar and the type of cancer cells used in the experimental process. Therefore, the authors hypothesized that the reason for these differences could be attributed to a different type and concentration of bioactive phytochemicals, which can target different molecules or different signaling pathways in distinct cancer cell lines. Millet, Lamy, Jonas, Stintzing, Mersch-Sundermann, and Merfort (2012) [37] tested antiproliferative activity of three onion extracts: fermented aqueous extract (FAE), aqueous (AE) and methanolic (ME). Only FAE extract showed significant toxicity on HepG2 cancer cell growth, while the other

two did not have any impact on cell proliferation. It should also be emphasized that the inhibition of cancer cell proliferation by the two tested extracts may not be exclusively due to their polyphenolic content, but might be also attributed to their other bioactive compounds, for example organosulfur compounds, typical for the majority of *Allium* species. These findings are in agreement with known anticancer properties of *Allium* species noticed in many epidemiological studies [9].

3. Materials and methods

3.1. Chemical reagents

LMP, NMP agarose, hydrogen peroxide (H₂O₂), acetate, ethanol, methanol, ferric chloride (FeCl₃), caffeic acid, phosphate buffer saline pH 7.4, dimethyl sulfoxide (DMSO), ascorbic acid, hydrochloric acid (HCl), sodium chloride (NaCl), sodium hydroxide (NaOH), Na-lauryl sarcosine, disodium salt ethylenediaminetetraacetic acid (Na₂EDTA), Tris-Cl, Triton X-100, Folin-Ciocalteu reagent, 2,2-diphenyl-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox), fluoroshield with DAPI, quercetin 4'-monoglucoside (spiraeoside), quercetin, isorhamnetin and myricetin were obtained from Sigma-Aldrich (St. Louis, MO, USA). pUC19 plasmid DNA was purchased from Invitrogen, Life Technologies, Carlsbad, CA, USA. Quercetin 3,4'-diglucoside was obtained from Polyphenols AS, Sandnes, Norway, and malvidin-3-O-glucoside chloride was obtained from Extrasynthese, Genay, France. All chemicals and reagents were AR or HPLC grade.

3.2. Plant material

Allium × cornutum was obtained from local gardens of Croatian coasts and islands and the *Allium cepa* plants were purchased at a local market.

3.3. Preparation of onion extracts

The phenolic compounds were extracted from the homogenized dry plant material (10 g) using 70% v/v methanol/water (100 ml). After 30 min of extraction with magnetic stirring at room temperature (ca 20°C), the extract was centrifuged at 3000 rpm for 15 min. All three supernatants were pooled and dried under a vacuum using rotary evaporator at 50°C. The dry residues were dissolved with 10% DMSO. For HPLC analysis, the solution was filtered through a 0.45 µm nylon filter disc prior to analysis.

3.4. Analysis of the phytochemicals

3.4.1 HPLC analysis of flavonols

High performance liquid chromatography (HPLC) measurement of flavonols were carried out on Perkin Elmer HPLC system (Waltham, Massachusetts, USA) consisting of a binary pump Series 200, an autosampler, Peltier column oven Series 200, UV/VIS detector Series 200 and UltraAqueous C18 column (250 × 4.6 mm, Resek). TotalChrom Workstation software was used to process the chromatographic data. After filtration through 0.45 µm syringe filter, extract was injected directly through a 20 µl fixed loop into a guard of C18 column. Each sample was injected three times in order to check reproducibility. A gradient consisting of a solvent A (0.2% H₃PO₄) and solvent B (MeOH/acetonitrile, 1:1 v/v) was applied at the flow rate of 0.8 ml/min as follows: 0-0.5 min 96% A and 4% B; 0.5-40 min 50% A and 50% B; 40-45 min 40% A and 60% B; 45-60 min 0% A and 100% B; 60-68 min 0% A and 100% B; 68-70 min 96% A and 4% B; 70-80 min 96% A and 4% B. Detection of the elution peaks was at 360 nm. Flavonoid compounds were identified on the basis of their retention times and quantified using external standard calibration curves. Standards for identification purposes were: quercetin, quercetin 4'-monoglucoside, quercetin 3,4'-diglucoside prepared in methanol. The resultant concentrations are expressed as mg/100 g of dry weight.

3.4.2. HPLC analysis of anthocyanins

HPLC analysis of anthocyanins was performed using a Varian HPLC system (Varian, Inc., CA, USA), consisting of a Star 9010 pump, a Rheodyne 7125 syringe loading sample injector, a 500-LC module for a column oven, a ProStar 330 Photodiode Array Detector, and a Star Chromatography

workstation, version 5. The separation was carried out using a Kinetex C18 core-shell column (150 x 4.6 mm), filled with 5 µm particle size, and furnished with the SecurityGuard ULTRA Cartridge UHPLC C18 for 4.6 mm ID column (Phenomenex, USA), both thermostated at 35°C. Two eluents were used: A was 0.3% HClO₄ and B was MeOH. The linear gradient was as follows: from 28% B to 51% B in 42 min, then to 69% in 3 min and to 80% B in 1 min 80% B for 3 min. The time of equilibration for the column to the initial gradient was 6 min, and the injection volume was 10 µL. The flow rate was 0.6 mL/min. Samples and the standards were filtered before analysis through a 0.45 µm pore size membrane syringe filters. Anthocyanins were identified according to retention times and UV-DAD spectra of each peak at 520 nm. Quantifications were performed using a standard curve of malvidin-3-O-glucoside chloride. The resultant concentrations are expressed as mg/100 g of dry weight.

3.4.3. Determination of total phenolic content

The total phenolic content (TPC) of *A. × cornutum* and *A. cepa* methanolic extracts was determined using Folin-Ciocalteu method described by Singleton and Rossi (1965) [38]. Briefly, determination of TPC in tested samples was carried out using 10 mL of previously diluted Folin-Ciocalteu reagent (1:20 v/v) and 50 µL of onion extract. After 3 min of incubation, 1 mL of saturated sodium carbonate was added after which the reaction mixture was incubated for another 60 min in the dark and the absorbance was measured at 725 nm using the Perkin Elmer UV/VIS Lambda Bio 40 spectrophotometer. Caffeic acid (CA) served as the standard and results were expressed as mg of caffeic acid equivalents (CA) per g of dry weight. All measurements were carried out in triplicate and the results are expressed as mean values ± SD.

3.5. Antioxidant activity

3.5.1. Measurement of the DPPH (2,2'-diphenyl-1-picrylhydrazyl) radical-scavenging activity

Antioxidant capacity of extracts was evaluated using the DPPH method previously described by Kulišić, Dragović-Uzelac, and Miloš, (2006) [39]. Briefly, the method is based on the reduction of alcoholic DPPH[•] solution in the presence of a hydrogen donating antioxidant. An aliquot (50 µL) of the onion extract was mixed with methanolic solution of DPPH (1 mL, 0.1 mM) and the initial absorbance at 517 nm was measured immediately using EtOH as a blank. After an hour of incubation, the absorbance was measured again and the percentage of DPPH inhibition was calculated according to the formula by Yen and Duh (1994) [40]:

$$\% \text{ inhibition} = [(A_c(0) - A_A(t)) / A_c(0)] \times 100,$$

where $A_c(0)$ is the absorbance of the control at $t=0$ min, and $A_A(t)$ is the absorbance of the antioxidant at $t=1$ h. All measurements were done in triplicate.

3.5.2. Oxygen Radical Absorbance Capacity (ORAC) assay

The assay was performed in Perkin-Elmer LS55 spectrofluorimeter, using 96-well white polystyrene microtiter plates (Corning Costar). Each reaction contained 190 µL of fluorescein (160 µM), 60 µL AAPH (150 mM) and 30 µL of plant extracts or reference standard Trolox (6.25–50 µM). All experimental solutions and samples were prepared in phosphate buffer (0.075 mM, pH 7.0). The measurements were performed in triplicates at 37°C using the excitation wavelength of 485 nm and the fluorescence decay was monitored at 530 nm during the period of 60 min. The obtained fluorescence decay curves were analyzed by FL WinLab software and the resultant area under the curve (AUC) of each standard or sample was acquired after the blank AUC subtraction (Supplementary Fig. 1.). The standard curve was generated by plotting AUC of standards with corresponding nmol of trolox. The ORAC values of onion extracts were expressed as µmol of trolox equivalents (TE) per mL of tested sample. The results were obtained from three independent experiments.

3.6. Evaluation of biological activity

3.6.1. Blood sampling and treatment of human leukocytes

Blood samples were obtained from a healthy female donor (age 28, non-smoker, first author of this paper and protocol was approved by the Ethics Committee of School of Medicine, University of Zagreb). Venous blood was collected into heparinized vacutainer tubes (Becton Dickeson, Plymouth, UK) under sterile conditions. Leukocytes were isolated by gradient centrifugation with Histopaque-1077 (Sigma, St. Luis, USA), 400 x g for 30 min and washed twice with PBS. Cell viability was determined with trypan blue exclusion assay. Leukocytes (2×10^6 cells/mL) were then incubated in RPMI-1640 medium (Gibco, Invitrogen, USA) supplemented with *A. × cornutum* and *A. cepa* methanolic extract (100 µg/mL) for 30 min at 37°C in a humidified atmosphere with 5% CO₂. Cells growing in a medium supplemented with 1% DMSO were used as the negative control and those incubated with 200 µM of H₂O₂ (for 5 min, 4°C) served as the positive control. After incubation, cells were centrifuged at 300 x g for 5 min at 4°C and washed twice with PBS. Supernatant was discarded and pellet was placed on ice and resuspended in LMP agarose. All treatments were performed in duplicates.

3.6.2. Comet assay (single-cell gel electrophoresis)

The alkaline comet assay was carried out according to the procedure of Singh, McCoy, Tice, and Schneider (1988) [41]. Conventional microscope slides were precoated by dipping in a solution of 1% normal melting point (NMP) agarose (Sigma-Aldrich, MO, USA) and dried overnight. After treatment, cells were centrifuged (300 x g, 5 min, 4°C) and the supernatant was removed. The pellet was resuspended in RPMI medium and the cells were counted. Approximately 1×10^6 cells/mL were mixed with 100 µL 0.5% low melting point agarose (LMP) and placed on a precoated slide. After solidification of the agarose, the slides were immersed in fresh ice-cold lysis solution (2.5 M NaCl, 0.1 M Na₂EDTA, 10 mM Tris-Cl, 10% DMSO, 1% sodium lauryl sarcosine, 1% Triton X-100, pH 10) overnight at 4°C. Alkaline denaturation was performed in an electrophoresis buffer solution (1 mM Na₂EDTA and 300 mM NaOH, pH ≥ 13) for 20 min. Electrophoresis was carried out in a chilled electrophoresis buffer for 20 min (25 V, 300 mA, 4°C). The slides were then washed three times for 5 min in 0.4 M Tris-Cl, pH 7, treated with ethanol for another 5 min and dried. For comet analysis, slides were stained with DAPI (5 µg/mL). A total of 100 comets (50 cells from each of the two replicated slides) were scored visually in five classes according to tail size and intensity (from undamaged 0; to maximally damaged 4) as depicted in Figure 5. DNA damage index was determined according to the equation:

$$DI = 1n_1 + 2n_2 + 3n_3 + 4n_4,$$

where DI is damage index in arbitrary unit (AU), n_1 - n_4 are the number of comets with damage levels 1, 2, 3 and 4. DI values can indicate various situations, from all undamaged cells (class 0; 0 AU) to highly damaged cells (class 4; 400 AU).

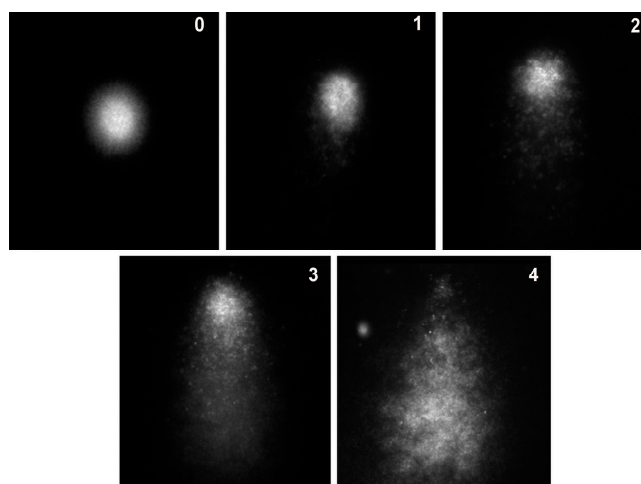


Figure 5. Grading of the DNA damage in human leukocytes: (0) no damage, (1) low level of DNA damage, (2) medium level of DNA damage, (3) high level of DNA damage, (4) maximum DNA damage.

3.6.3. DNA nicking assay

DNA nicking assay was performed using supercoiled pUC19 plasmid DNA by the method of Prakash et al. (2007). A reaction mixture containing different concentrations of plant extracts (10-100 µg/mL) and pUC19 plasmid DNA (0.5 µg) was incubated for 10 min at room temperature, followed by the addition of Fenton's reagent (30 mM H₂O₂, 50 µM ascorbic acid and 80 µM of FeCl₃). The reaction mixture was then incubated for 30 min at 37 °C and the DNA was analyzed on a 1% agarose gel.

3.6.4. Cell culture

Cells were purchased from ATCC (LGC Standards). Cancer cell lines (breast cancer cell line MDA-MB-231 and human glioblastoma cell line-A1235) were cultured in a humidified atmosphere with 5% CO₂ at 37°C, in a Dulbecco's modified Eagle's medium (DMEM Euroclone, Milano, Italy) containing 4.5 g/L glucose, 10% fetal bovine serum(FBS) and 1% antibiotics (Penicillin Streptomycin, EuroClone, Milano, Italy).

3.6.5. Cell proliferation assay

Cells were resuspended in a diluted solution of trypan blue and counted by a binocular inverted microscope, MOTIC AE30, using Neubauer chambers. The cell number was calculated according to the formula: number of counted cells × 10⁴/mL. The cells were then plated in 96-well plates at a density of 11,000 cells/well and incubated overnight. The cells were treated with *A. × cornutum* and *A. cepa* methanolic extract at a concentration of 100 µg/ml in a complete medium (in triplicate) for 4, 24, 48 and 72 h. Then, the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay was performed in such a manner that after the treatment with onion extracts, the cells were incubated with 0.5 g MTT/L at 37°C for 2 hours. After that, the medium was removed and dimethylsulphoxide (10% DMSO) was added and incubated for another 10 min at 37°C while shaking. The degree of formazan formation, an indicator of living and metabolically active cells, was measured photometrically at 570 nm. The data was calculated in relation to the untreated control (100%) from three independent measurements.

3.7. Statistical analysis

Results are expressed as mean values with depicted standard deviation. Microsoft Excel Student t-test was used to analyze data and to discriminate statistically significant results.

4. Conclusion

This was the first known thoroughly comprehensive study of *A. × cornutum* phytochemical composition and biological activity. HPLC analysis revealed two major quercetin conjugates (quercetin 3,4'-diglucoside and quercetin 4'-monoglucoside) as the most abundant flavonols in *A. × cornutum* and *A. cepa* extracts. Additionally, we successfully identified three anthocyanins in both onions, while the fourth anthocyanin, delphinidin glucoside, was observed only in *A. × cornutum*. Overall, *A. × cornutum* showed slightly higher concentrations of all identified phenolic compounds that might be a cause of a higher bioactivity profile accounted for this onion.

Our results clearly show that both onions have strong protective effects on DNA molecule as we have proven with several in vitro experiments. Both onions have also shown strong antiproliferative activity on tested human cancer cell lines. Susceptibility of glioblastoma cells to both extracts was higher than that of a breast cancer cell lines. Antiproliferative activity of the tested methanol extracts could be mediated by induction of apoptosis, alterations of the cell cycle or some other mechanism, and further research is needed to clarify the exact mechanism(s) of their anti-proliferative activity in vitro. Therefore, it can be concluded that *A. × cornutum* and *A. cepa* methanolic extracts have comparable composition and concentrations of phenolic compounds, antioxidant, antigenotoxic and antiproliferative effects. This research demonstrated that *A. × cornutum* and common onion might be considered as an important source of natural antioxidants that might have a beneficial protective effect on human health.

Supplementary Materials: The following are available online at www.mdpi.com/link, Figure S1: ORAC

Acknowledgments: This work was supported by the Croatian Ministry of Science, Education and Sports as part of multiannual financing intended for institutions.

Author Contributions: J.P. and Ž.F. conceived and designed the experiments; Ž.F., M.Š., B.S., I.L.J., I.B.-L., T.B. and V.Č.-Č. performed the experiments; Ž.F. and M.Š. analysed the data; J.P., Ž.F. and M.Š. wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest. The founding sponsors had no role in the collection, analyses, or interpretation of data, and in the writing of the manuscript.

References

- Prakash, D.; Singh, B.N.; Upadhyay, G. Antioxidant and free radical scavenging activities of phenols from onion (*Allium cepa*). *Food Chem.* **2007**, *102*, 1389–1393. [http://dx.doi.org/10.1016/j.foodchem.2006.06.063] [http://www.sciencedirect.com/science/article/pii/S0308814606006005]
- Razavi-Azarkhavi, K.; Behravan, J.; Mosaffa, F.; Sehatbakhsh, S.; Shirani, K.; Karimi, G. Protective effects of aqueous and ethanol extracts of rosemary on H₂O₂-induced oxidative DNA damage in human lymphocytes by comet assay. *J. Complement. Integr. Med.* **2014**, *11*, 27–33. [http://dx.doi.org/10.1515/jcim-2013-0063] [https://www.ncbi.nlm.nih.gov/pubmed/24497228]
- Özyürek, M.; Bener, M.; Güçlü, K.; Apak, R. Antioxidant/antiradical properties of microwave-assisted extracts of three wild edible mushrooms. *Food Chem.* **2014**, *157*, 323–331. [http://dx.doi.org/10.1016/j.foodchem.2014.02.053] [http://www.sciencedirect.com/science/article/pii/S0308814614002283]
- Puizina, J.; Javornik, B.; Bohanec, B.; Schweizer, D.; Maluszynska, J.; Papeš, D.; Schweizer, D. Random amplified polymorphic DNA analysis, genome size, and genomic *in situ* hybridization of triploid viviparous onions. *Genome*, **1999**, *42*, 1208–1216. [http://dx.doi.org/10.1139/g99-023] [https://www.ncbi.nlm.nih.gov/pubmed/10659789]
- Corzo-Martínez, M.; Corzo, N.; Villamiel, M. Biological properties of onions and garlic. *Trends Food Sci Tech.* **2007**, *18*, 609–625. [http://dx.doi.org/10.1016/j.tifs.2007.07.011] [http://www.sciencedirect.com/science/article/pii/S0924224407002282]
- Lanzotti, V. The analysis of onion and garlic. *J. Chromatogr. A* **2006**, *1112*, 3–22. [http://dx.doi.org/10.1016/j.chroma.2005.12.016] [http://www.sciencedirect.com/science/article/pii/S0021967305024064]
- Gurushizde, M.; Mashayekhi, S.; Blattner, F.R.; Friesen, N.; Fritsch, R.M. Phylogenetic relationship of wild and cultivated species of *Allium* section *Cepa* inferred by nuclear rDNA ITS sequence analysis. *Plant Syst. Evol.* **2007**, *269*, 259–269. [http://dx.doi.org/10.1007/s00606-007-0596-0] [http://link.springer.com/article/10.1007/s00606-007-0596-0]
- Kendler, B.S. Garlic (*Allium sativum*) and onion (*Allium cepa*): a review of their relationship to cardiovascular disease. *Prev. Med.* **1987**, *16*, 670–685. [http://dx.doi.org/10.1016/0091-7435(87)90050-8] [https://www.ncbi.nlm.nih.gov/pubmed/3317392]
- Nicastro, H.L.; Ross, S.A.; Milner, J.A. Garlic and onions: Their cancer prevention properties. *Cancer Prev. Res.* **2015**, *8*, 181–189. [http://dx.doi.org/10.1158/1940-6207.CAPR-14-0172] [https://www.ncbi.nlm.nih.gov/pubmed/25586902]
- Yang, E.-J.; Kim, G.-S.; Kim, J.A.; Song, K.-S. Protective effects of onion-derived quercetin on glutamate-mediated hippocampal neuronal cell death. *Pharmacogn. Mag.* **2013**, *9*, 302–308. [10.4103/0973-1296.117824] [https://www.ncbi.nlm.nih.gov/pubmed/24124281]
- Goldman, I.L.; Kopelberg, M.; Debaene, J.E.; Schwartz, B.S. Antiplatelet activity in onion (*Allium cepa*) is sulfur dependent. *Throm. Haemost.* **1996**, *76*, 450–452. [https://www.ncbi.nlm.nih.gov/pubmed/8883285]
- Bonaccorsi, P.; Caristi, C.; Gargiulli, C.; Leuzzi, U. Flavonol glucosides in *Allium* species: A comparative study by means of HPLC-DAD-ESI-MS-MS. *Food Chem.* **2008**, *107*, 1668–1673. [http://dx.doi.org/10.1016/j.foodchem.2007.09.053]
- Griffiths, G.; Trueman, L.; Crowther, T.; Thomas, B.; Smith, B. Onions- a global benefit to health. *Phytother. Res.* **2002**, *16*, 603–615. [http://dx.doi.org/10.1002/ptr.1222] [https://www.ncbi.nlm.nih.gov/pubmed/12410539]

14. Gennaro, L.; Leonardi, C.; Esposito, F.; Salucci, M.; Maiani, G.; Quaglia, G.; Fogliano, V. Flavonoid and carbohydrate contents in Tropea red onions: effects of homelike peeling and storage. *J. Agric. Food Chem.* **2002**, *50*, 1904-1910. [http://dx.doi.org/10.1021/jf011102r] [https://www.ncbi.nlm.nih.gov/pubmed/11902931]
15. Fredotović, Ž.; Šamanić, I.; Schneeweiss-Weiss, H.; Kamenjarin, J.; Jang, T.-S.; Puizina, J. Triparental origin of triploid onion, *Allium x cornutum* (Clementi ex Visiani, 1842), as evidenced by molecular, phylogenetic and cytogenetic analysis. *BMC Plant Biol.* **2014**, *14*, 14-24. [http://dx.doi.org/10.1186/1471-2229-14-24]
16. Caridi, D.; Trenerry, V.C.; Rochfort, S.; Duong, S.; Laughler, D.; Jones, R. Profiling and quantifying quercetin glucosides in onion (*Allium cepa* L.) varieties using capillary zone electrophoresis and high performance liquid chromatography. *Food Chem.* **2007**, *105*, 691-699. [http://dx.doi.org/10.1016/j.foodchem.2006.12.063] [http://www.sciencedirect.com/science/article/pii/S0308814607000441]
17. Lombard, K.A.; Geoffriau, E.; Peffley, E. Flavonoid quantification in onion by spectrophotometric and high performance liquid chromatography analysis. *HortScience* **2002**, *37*, 682-685.
18. Pérez-Gregorio, R.M.; García-Falcón, M.S.; Sinal-Gándara, J.; Rodrigues, A.S.; Almeida, D.P.F. Identification and quantification of flavonoids in traditional cultivars of red and white onions at harvest. *J. Food Comp. Anal.* **2010**, *23*, 592-598. [http://dx.doi.org/10.1016/j.jfca.2009.08.013] [http://www.sciencedirect.com/science/article/pii/S0889157509002634]
19. Price, K.R.; Rhodes, M.J.C. Analysis of the major flavonol glycosides present in four varieties of onion (*Allium cepa*) and changes in composition resulting from autolysis. *J. Sci. Food Agr.* **1997**, *74*, 331-335.
20. Rhodes, M.; Price, K. Analytic problems in the study of flavonoid compounds in onion. *Food Chem.* **1996**, *57*, 113-117. [10.1016/0308-8146(96)00147-1] [http://www.sciencedirect.com/science/article/pii/S0308814696001471]
21. Santas, J.; Carbo, R.; Gordon, M.H.; Almajano, M.P. Comparison of the antioxidant activity of two Spanish onion varieties. *Food Chem.* **2008**, *107*, 1210-1216. [http://dx.doi.org/10.1016/j.foodchem.2007.09.056] [http://www.sciencedirect.com/science/article/pii/S0308814607009739]
22. Nile, S.H.; Park, S. Total phenolics, antioxidant and xanthine oxidase inhibitory activity of three colored onions (*Allium cepa* L.). *Front. Life Sci.* **2014**, *7*, 224-228. [http://dx.doi.org/10.1080/21553769.2014.901926] [http://www.tandfonline.com/doi/abs/10.1080/21553769.2014.901926]
23. Sharma, K.; Assefa, A.D.; Kim, S.; Young Ko, E.; Lee, E.T.; Park, S.W. Evaluation of total phenolics, flavonoids and antioxidant activity of 18 Korean onion cultivars: a comparative study. *J. Sci. Food Agr.* **2014**, *94*, 1521-1529. [http://dx.doi.org/10.1002/jsfa.6450] [https://www.ncbi.nlm.nih.gov/pubmed/24136245]
24. Zill, H.; Fabiano-Tixier, A.-S.; Elmaataoui, M.; Dangles, O.; Chemat, F. A remarkable influence of microwave extraction: Enhancement of antioxidant activity of extracted onion varieties. *Food Chem.* **2011**, *127*, 1472-1480. [http://dx.doi.org/10.1016/j.foodchem.2011.01.112] [http://www.sciencedirect.com/science/article/pii/S0308814611002196]
25. Benekeblia, N. Free-radical scavenging capacity and antioxidant properties of some selected onions (*Allium cepa* L.) and garlic (*Allium sativum* L.) extracts. *Braz Arch Biol Technol.* **2007**, *48*, 753-759. [http://dx.doi.org/10.1590/S1516-89132005000600011] [http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1516-89132005000600011]
26. Boivin, D.; Lamy, S.; Lord-Dufour, S.; Jackson, J.; Beaulieu, E.; Côté, M.; Moghrabi, A.; Barrete, S.; Gingras, D.; Béliveau, R. Antiproliferative and antioxidant activities of common vegetables: A comparative study. *Food Chem.* **2009**, *112*, 374-380. [http://dx.doi.org/10.1016/j.foodchem.2008.05.084] [http://www.sciencedirect.com/science/article/pii/S0308814608006419]
27. Miller, H.E.; Rogelhof, F.; Marguart, L.; Prakash, A.; Kanter, M. Antioxidant content of whole grain breakfast cereals, fruits and vegetables. *J. Am. Coll. Nutr.* **2008**, *19*, 1-8. [http://dx.doi.org/10.1080/07315724.2000.10718966] [https://www.ncbi.nlm.nih.gov/pubmed/10875603]
28. Nuutila, A.M.; Puupponen-Pimiä, R.; Oksman-Caldentey, K.-M. Comparison of antioxidant activities of onion and garlic extracts by inhibition of lipid peroxidation and radical scavenging activity. *Food Chem.* **2003**, *81*, 485-493. [http://dx.doi.org/10.1016/S0308-8146(02)00476-4] [http://www.sciencedirect.com/science/article/pii/S0308814602004764]
29. Yin, M.; Cheng, W. Antioxidant activity of several *Allium* members. *J. Agric. Food Chem.* **1998**, *46*, 4097-4101. [10.1021/jf980344x] [http://pubs.acs.org/doi/abs/10.1021/jf980344x]

30. Singh, B.N.; Singh, B.R.; Singh, R.L.; Prakash, D.; Singh D.P.; Sharma, B.K.; Upadhyay, G.; Singh, H.B. Polyphenolics from various extracts/fractions of red onion (*Allium cepa*) peel with potent antioxidant and antimutagenic activities. *Food Chem. Toxicol.* **2009**, *47*, 1161-1167. [http://dx.doi.org/10.1016/j.fct.2009.02.004] [https://www.ncbi.nlm.nih.gov/pubmed/19425188]
31. Kumar, S.; Pandey, A.K. Chemistry and biological activities of flavonoids: an overview. *Scientific World J.* **2013**, Article ID 162750, 16 pages. [http://dx.doi.org/10.1155/2013/162750] [https://www.hindawi.com/journals/tswj/2013/162750/]
32. Sakihama, Y.; Cohen, M.F.; Grace, S.C.; Yamasaki, H. Plant phenolic antioxidant and prooxidant activities: phenolics-induced oxidative damage mediated by metals in plants. *Toxicology*, **2002**, *177*, 67-80. [http://dx.doi.org/10.1016/S0300-483X(02)00196-8] [https://www.ncbi.nlm.nih.gov/pubmed/12126796]
33. Duthie, S.J.; Collins, A.R.; Duthie, G.G.; Dobson, V.L. Quercetin and myricetin protect against hydrogen peroxide- induced DNA damage(strand breaks and oxidized pyrimidines) in human lymphocytes. *Mutat. Res.* **1997**, *393*, 223-231. [http://dx.doi.org/10.1016/S1383-5718(97)00107-1] [https://www.ncbi.nlm.nih.gov/pubmed/9393615]
34. Noroozi, M.; Angerson, W.J.; Lean, M.E. Effects of flavonoids and vitamin C on oxidative DNA damage to human lymphocytes. *Am. J. Clin. Nutr.* **1998**, *67*, 1210-1218. [https://www.ncbi.nlm.nih.gov/pubmed/9625095]
35. Johnson, M.K.; Loo, G. Effects of epigallocatechin gallate and quercetin on oxidative damage to cellular DNA. *Mutat. Res.* **2000**, *459*, 211-218. [http://dx.doi.org/10.1016/S0921-8777(99)00074-9] [https://www.ncbi.nlm.nih.gov/pubmed/10812333]
36. Yang, J.; Meyers, K.J.; van der Heide, J.; Liu, R.H. Varietal differences in phenolic content and antioxidant and antiproliferative activities of onions. *J. Agric. Food Chem.* **2004**, *52*, 6787-6793. [10.1021/jf0307144] [https://www.ncbi.nlm.nih.gov/pubmed/15506817]
37. Millet, A.; Lamy, E.; Jonas, D.; Stintzing, F.; Mersch-Sundermann, V.; Merfort, I. Fermentation enhances the biological activity of *Allium cepa* bulb extracts. *J. Agric. Food Chem.* **2012**, *60*, 2148-2156. [http://dx.doi.org/10.1021/jf2041643]
38. Singleton, V.L.; Rossi, J.A. Colorimetry of total phenolics with phosphomolybdic phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1965**, *12*, 99-101.
39. Kulišić, T.; Dragović-Uzelac, V.; Miloš, M. Antioxidant activity of aqueous tea infusions prepared from oregano, thyme and wild thyme. *Food Technol. Biotechnol.* **2006**, *44*, 485-492.
40. Yen, G.C.; Duh, P.D. Scavenging effect of methanolic extracts of peanut hulls on free-radical and active oxygen species. *J. Agric. Food Chem.* **1994**, *43*(2), 629-632. [doi:10.1021/jf00039a005] [https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2723265/]
41. Singh, N.P.; McCoy, M.T.; Tice, R.R.; Schneider, E.L. A simple technique for quantification of low levels of DNA damage in individual cells. *Exp. Cell. Res.* **1988**, *175*, 184-191. [doi:10.1016/0014-4827(88)90265-0] [https://www.ncbi.nlm.nih.gov/pubmed/3345800]

Sample Availability: Samples are available from the authors.



© 2017 by the authors; licensee *Preprints*, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons by Attribution (CC-BY) license (<http://creativecommons.org/licenses/by/4.0/>).