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Article

Physico-chemical characterization and biological activities of black and white garlic: *in vivo* and *in vitro* assays

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Abstract: White and three types of black garlic (13, 32 and 45 days of fermentation, named 0C1, 1C2 and 2C1 respectively) were selected in order to check possible differences in their nutraceutic potential. For this purpose, garlic were physico-chemically characterised, and both *in vivo* and *in vitro* assays were carried out. Black garlic showed higher polyphenol content and antioxidant capacity than white garlic. The biological studies have shown that only white garlic was not safe showing toxicity effect. Furthermore, none garlic exert protective effects against H_2O_2, except the 0C1 black garlic. Moreover, garlic was non-genotoxic with the exception of the highest concentration of white garlic. On the other hand, 0C1 was the most antigenotoxic substance. The *in vivo* longevity assays yielded significant extension of lifespan results in some concentrations of white and 0C1and 1C2 black garlic. The *in vitro* experiments showed that all studied garlic induced a decrease in leukaemia cells growth. However, none type of garlic was able to induce proapoptotic internucleosomal DNA fragmentation. Taking into account the physicochemical and biological data, black garlic could be considered as a potential functional food and used in preventive treatment of age-related diseases. In addition, our findings could be relevant for the black garlic processing agrifood companies as the economical and timing costs are significantly reduced to 13 days aging.

Keywords: Black garlic; *Drosophila melanogaster*; Physico-chemical profile; Polyphenol content; HL-60 cell line

1. Introduction

Garlic (*Allium sativum*) is probably one of the oldest medicinal plants known, used from ancient time to cure different disease conditions in humans. Garlic started taking part in the daily diet since Egyptians age.[1] Several scientific research and clinical trials have been conducted during the last decade to determine the effects of garlic consumption. Garlic's principal medicinal uses focused on prevention and treatment of cardiovascular disease by lowering blood pressure and cholesterol, and, more recently, both as an antimicrobial and preventive agent for cancer. [2]·[3]

The physiological effects of garlic are mainly due to the presence of volatile sulfur compounds like thiosulfates which give it the characteristic pungent aroma. [4] Several recent studies have shown that these organosulfur compounds have anti-cancer, anti-cardiovascular, anti-neurological, and anti-liver disease effects, as well as effects for prevention of allergy and arthritis. [5-8] This group of compounds, originated from the allicin decomposition, was associated to the *Allium sp.* pungent aroma and taste and to an antioxidant activity. [5,9] However, garlic should be consumed in appropriate amount because cytotoxicity was reported at high doses. [10]

Black garlic products have emerged as one of the fastest-growing health-oriented food product in world markets with the growing awareness of the health benefits of garlic. [11] Thermal processes are commonly used in food manufacturing. One of the important objectives of thermal processes is to raise the sensory quality of foods, their palatability and to extend the range of colours, tastes, aromas and textures in food. [12] In addition, heating processes lead to the formation of biological compounds that are not originally present in food.[13] However, influences of thermal processes on the concentration of single flavonoids and phenolic acids in garlic are unknown.

Black garlic is produced through natural fermentation by aging whole ordinary garlic under controlled high temperature (70 °C) and humidity condition (90 %) for several days without any artificial treatments and additives.[14] During the aging production, the cloves of normal garlic change its colour from white to brown and finally became black, caused by the Maillard Reaction. The properties beneficial to health of black garlic have been described in many research works. [10,15-18] Compared with fresh garlic, the black one contains sevenfold polyphenol content, [19] which indicated the increase in the antioxidant activity. The amino acid, the carbohydrate and the S-allyl-L-cysteine contents are increased in 2.5-fold, 28.7 - 47.0 % and 8 times respectively. [20,21] Black garlic exhibited a wide range of biological activities, such as antioxidant, [10] anticancer ,[17] hypoglycaemic,[18] hypolipidemic,[22] antiinflammatory, [15] hepatoprotective [23] and immunostimulatory. [16] Furthermore, black garlic shows stronger antioxidant activity *in vivo* [24] and higher free radical scavenging properties *in vitro* compared with fresh garlic.[10] Black garlic has soft, sour and fruit-like sweetness, comestible just by peeling without any unpleasant smell in it. [15,25] These organoleptic characteristics are due to the conversion of unstable and odorous compounds of raw garlic to stable and odourless compounds such as S-allyl-L-cysteine (SAC) or decomposed to organosulfur compounds such as diallyl sulphide (DAS), diallyl disulphide (DADS), diallyl trisulphide (DATS), dithiins and ajoene. [5,7] During heat-treatment, unstable compounds in raw garlic are transformed into stable soluble compounds with a high antioxidant power. [7,26] Previous studies on black garlic reported that this increase in its antioxidant capacity could be due to the increase in polyphenols and S-allyl-cysteine, a compound derived from alliin. [27]

Drosophila melanogaster is an excellent model organism to the study of aging, due to its relatively short life expectancy; a large number of individual can be in controlled laboratory conditions and because adults show many aspects of the cellular senescence observed in mammals. [28] Thus flies have been used frequently to study physiological and pathological processes that affect life expectancy, and can help to understand the relationship between nutrient metabolism and the mechanisms of aging. [29]

The HL-60 cell line belongs to the undifferentiated immortal lines, as they are tumour cells. It is widely investigated as a model for inducible cell differentiation. [30] This phenomenon might affect the cell ability to proliferate and therefore their immortality with the appearance of apoptosis. [31] Compounds capable to induce differentiation and apoptosis are candidates to act as chemopreventive agents or cancer chemotherapeutic.[32]

The aim of the present study is to perform a qualitative and quantitative evaluation of the health-beneficial properties of white and three types of black garlic, using a multi-assay experimental design at the individual, cellular and DNA levels. We assessed on their genotoxic, antigenotoxic and lifespan effects in an *in vivo* animal model (*Drosophila melanogaster*) and their proapoptotic capacities against cancer processes: cytotoxicity and clastogenic DNA activity using an *in vitro* human cancer model (HL-60 cell line).

2. Materials and Methods

2.1. Preparation of Samples

White and black garlic were selected for this study. White garlic was purchased in a local market. Black garlic was manufactured at 60 °C and 90 % RH. Samples at 0 (White), 13 (0C1), 32 (1C2) and 45 (2C1) days were taken during the manufacturing process. After peeling bulbs, samples were crushed and divided into three sub-samples. Before to carry out the biological assays, garlic were lyophilised and dissolved in distilled water in order to obtain the different concentrations tested. The lyophilised extracts were stored, until use, at room temperature in a dark and dried atmosphere.

The concentrations of garlic for the bioassays were established taking into account the average daily food intake of *D. melanogaster* (1 mg/day) and the average body weight of *D. melanogaster* individuals (1 mg). [33] The concentration range for all tested substances was calculated in order to make it comparable to fit them into the recommended garlic daily intake for humans. Although there is no standard intake for garlic, the 1988 German Kommission E monograph proposed that daily intake of approximately 1 - 2 garlic cloves (about 4 g) of intact garlic may have benefits on health. [34] Unfortunately, this recommendation is not substantiated by any scientific reference.

2.2. Measurement of soluble solid content, pH, a_w, and Browning intensity

Total soluble solid content (°Brix), pH, water activity (a_w), and browning intensity (*L* value) were determined in all samples during heat treatment. Determinations in triplicate were carried out. Garlic soluble solids (°Brix) were measured by an *Abbe Refractometer*. Garlic pH was measured with a pH meter *Crison Basic 20*. Garlic water activity (a_w) was measured with an *Aqualab Series 3/3TE* meter with a temperature stabiliser. Garlic browning intensity was determined by a *Konica Minolta CR-410 Croma Meter colorimeter* as *L* value (*L*=100, white; *L*=0, black).

2.3. Total polyphenol content and antioxidant capacity

A *Perkin Elmer Lambda 20 UV VIS spectrophotometer* was used to analyse raw and heated garlic, total polyphenol content and antioxidant capacity. A previous garlic extraction was prepared to analyse antioxidant properties. Briefly, samples were lyophilised and five extracts per sample were obtained. Garlic extract was prepared dissolving 0.3 g of the lyophilised sample in 10 mL of a mixture at 50 % v/v of ethanol and distilled water. Next, samples were stirred during one hour and then filtered using a Buchner funnel with *Whatman paper* into a vacuum flask connected to a vacuum pump filter. The filtered extract was levelled at 25 mL with a hydroalcoholic solution of 50 % v/v.

The polyphenol concentration of garlic samples was determined by *Folin-Ciocalteu* method. [35] To a volumetric 25 mL flask, 0.5 mL of extract, 10 mL of distilled water, 1 mL of *Folin-Ciocalteu* reagent and 3 mL of carbonate sodium 20 % w/v were added, and diluted to volume (25 mL) with distilled water. The mixture was heated at 50 °C during 5 minutes to accelerate the coloration reaction. Subsequently, it was cooled with water and the reading was performed in the *spectroPHOTO''meter* at 765 nm. The reading was compared to a calibration curve prepared with different gallic acid solutions: 75, 100, 200, 250, 300 ppm. Polyphenol content results were expressed considering the dilution of the sample (0.3 g in 25 mL) in grams of gallic acid equivalent per kilogram of lyophilised sample.

Raw and heated garlic antioxidant capacity was determined by *ABTS radical method.* [36] A mix of 2.557 mL of a solution of 7 mM *ABTS reagent* and 0.333 mL of a solution of 2.25 mM potassium persulfate in distilled water was made. The prepared solution was stored in darkness during 16 hours, enough time for the radical formation ($ABTS^{\cdot+}$). Then, 0.15 mL of the $ABTS^{\cdot+}$ solution was diluted in 15 mL of ethanol. The absorbance value at 734 nm was adjusted near 0.7 (A_0). Next, 0.980 mL of $ABTS^{\cdot+}$ solution and 0.02 mL of garlic extract sample were added. After stirring it, the absorbance was read at 734 nm after 7 minutes (A_1). The inhibition percentage was calculated by the following expression:

144 $$\% \text{ inhibition} = (A_0 - A_1)*100/ A_0$$

145 A calibration curve was built with the following Trolox concentrations: 0.1; 0.5; 1 and 1.5 mM.
146 Considering the sample dilution, results were expressed in mmolTrolox-equivalent per kilogram of
147 lyophilised sample.
148 The statistical analysis of the solid content, pH, a_w, browning intensity, polyphenol content,
149 antioxidant capacity and total polyphenol index for each type of garlic was evaluated with the SPSS
150 Statistics 17.0 software, using the one-way ANOVA method. The significance of the subsets was
151 determined using the Tukey HSD method (Homogeneous Subsets).

152 *2.4. In vivo assays*

153 2.4.1. Drosophila melanogaster strains

154 The following *Drosophila* strains, each carrying a hair marker on the third chromosome, were
155 used: (i) *mwh*/*mwh*, with the recessive mutation *multiple wing hairs* (*mwh*) that produces multiple
156 tricomas per cell instead of one, [37] and (ii) *flr*^{3}/*In (3LR) TM3, ri*p^p*sep bx*^{34e}*e*^{s}*Bd*^{S}, where the *flr*^{3}(*flare*)
157 marker is a homozygous recessive lethal mutation that produces deformed tricomas but is viable in
158 homozygous somatic cells once larvae start the development. [38] For detailed information of
159 mutations, see Lindsley and Zimm (2012). [39]

160 2.4.2. Toxicity and antitoxicity assays

161 In the toxicity assays five concentrations (4, 2, 1, 0.5 and 0.25 mg/mL) for each tested garlic, and
162 a negative (H_2O) and positive (0.12 M H_2O_2) controls were assayed. Toxicity index was expressed as
163 the percentage of the number of individuals born in each treatment with respect to the number of
164 individuals born in the negative control were analysed. The antitoxicity tests consisted of combined
165 treatments using the same concentrations as in toxicity assays, with the exception of the highest one
166 of 4 mg/mL, by adding the toxicant hydrogen peroxide at 0.12 M [40] and comparing the percentage
167 of emerging adults with the positive control.
168 Significant differences with respect to the concurrent control in toxicity assay were determined
169 using the Chi-square method described previously by Merinas-Amo et al. (2016) being a
170 concentrations resulted in toxic when Chi-square value is higher than 5.02.[41]

171 2.4.3. Genotoxicity and Antigenotoxicity assays

172 The genotoxicity assays were carried out following the method described by Graf et al. (1984).
173 [42] Briefly, trans-heterozygous larvae for *mwh* and *flr*^{3} genes were obtained by crossing four day-old
174 virgin *flr*^{3} females with *mwh* males in a 2:1 ratio. Four days after fertilisation, females were allowed to
175 lay eggs in fresh yeast medium (25 g of yeast and 4 mL of sterile distilled water) for 8 h in order to
176 obtain synchronised larvae. After 72 ± 4 h, larvae were collected, washed with distilled water to
177 remove the remaining medium, and transferred, in groups of 100 individuals, to the treatment tubes
178 where they were fed chronically with the different compounds. The treatment tubes contained 0.85 g
179 of *Drosophila* Instant Medium (Formula 4-24, Carolina Biological Supply, Burlington, NC) and 4 mL
180 of solutions with different concentrations of garlic (2 mg/mL and 0.25 mg/mL).
181 The antigenotoxicity trials were carried out following the method described by Graf et al.
182 (1998),[43] which consisted of combined treatments of genotoxin (0.12 M H_2O_2) and the same
183 concentrations used in genotoxicity assays of lyophilised garlic. For significance and evaluation of
184 the inhibition potency, negative (H_2O) and positive (0.12 M H_2O_2) controls were carried out. After
185 emergence, adult flies were stored in 70 % ethanol until the wings were removed, mounted on slides
186 using Faure's solution to the scrutiny of the mutations under photonic microscope at 400x
187 magnification.
188 Similar numbers of male and female wings for each treatment and concentration were mounted
189 and wing hair mutations were scored from a total of 24,400 monotricoma wild type cells per wing.
190 [44]

Wing hair spots were grouped into three different categories: *S*, a small single spot corresponding to one or two cells clones exhibiting the *mwh* phenotype that occur in the late stages of the mitotic division; *L*, a large single spot with three or more cells clones showing *mwh* or *flr*^{3} phenotypes and occur in the early stages of larval development; or *T*, a twin spot corresponding to two juxtapositioning clones, one showing the *mwh* phenotype and other the *flr*^{3} phenotype. Small and large spots are originated by somatic point mutation, chromosome aberration, and somatic recombination, while twin spots are produced exclusively by somatic recombination between the *flr*^{3} locus and the centromere.

The total number of clones was also annotated and a multiple-decision procedure was applied to determine whether a result is positive, inconclusive or negative. [45,46] The frequency of each type of mutant clone/wing was compared with the concurrent negative control and the significance was taken at the 5 % level. Inconclusive and positive results were further analysed with the nonparametric *U*-test of Mann Whitney and Wilcoxon ($\alpha = \beta = 0.05$). The inhibition percentages (IP) for the combined treatments are calculated from total spots per wing with the following formula [47]:

$$IP= [(\text{single genotoxin - combined treatment})/ \text{single genotoxin}] \times 100$$

2.4.4. Lifespan assays

In order to compare genotoxicity and longevity results, flies who undergo the lifespan trials exhibited the same genotype as in genotoxicity assays. Hence, the F1 progeny from *mwh* and *flr*^{3} parental strains produced by a 24 h egg-laying in yeast medium was used in longevity experiments. All experiments were carried out at 25 °C according to the procedure described by Tasset-Cuevas et al. (2013). [48] Briefly, synchronised 72 ± 12 hours old trans-heterozygous larvae were washed, collected and transferred in groups of 100 individuals into test vials containing 0.85 g of *Drosophila* Instant Medium and 4 mL of the different concentrations of the compounds to be assayed.

Sets of 25 emerged individuals of the same sex were selected and placed into sterile vials containing 0.21 g of *Drosophila* Instant Medium and 1 mL of the different concentrations of solution of the compounds to be tested (4 mg/mL - 0.25 mg/mL).Two replicates were followed during the complete life extension for each control and concentrations established. Alive animals were counted and media renewed twice a week.

The statistical treatment of survival data for each control and concentration was assessed with the SPSS Statistics 17.0 software (SPSS, Ink., Chicago), using the Kaplan-Meier method. The significance of the curves was determined using the Log-Rank method (Mantel-Cox).

2.5. In vitro assays

2.5.1. HL-60 cell line culture conditions

Cells were grown in RPMI-1640 medium (Sigma, R5886) supplemented with 50 mL heat-inactivated foetal bovine serum (Linus, S01805), L-glutamine at 200 mM (Sigma, G7513) and antibiotic-antimycotic solution with 10.000 units of penicillin, 10 mg of streptomycin and 25 µg amphotericin B per mL (Sigma, A5955). Cells were incubated at 37 °C in a humidified atmosphere of 5 % CO_2 (Shel Lab, Cornelius, OR, USA). [49] The cultures were plated at 2.5 x 10^4 cells/mL density in 10mL culture bottles and passed every 2 days.

2.5.2. Cytotoxicity assay

HL-60 cells were placed in 96 well culture plates (2 x 10^4 cells/mL) and treated for 72 h with the lyophilised white and black garlic at different concentrations (4 mg/mL, 2 mg/mL, 1 mg/mL, 05 mg/mL, 0.25 mg/mL, 0.12 mg/mL, 0.06 mg/mL, 0.03 mg/mL and 0.015 mg/mL for white garlic and 4 mg/mL, 2 mg/mL, 1 mg/mL, 05 mg/mL and 0.25 mg/mL for black garlic). This wide range of tested concentrations is intended to estimate the cytotoxicity inhibitory concentration 50 (IC_{50}).

Cell viability was determined by the trypan blue dye (Sigma, T8154) exclusion test. Trypan blue was added to the cell cultures at a 1:1 volume ratio and 20 µl of cell suspension was loaded into a

Neubauer chamber. The cells were counted with an inverted microscope at 100x magnification (AE30/31, Motic). Curves were plotted as survival percentage with respect to the control growing at 72 h. At least three independent repetitions of the assays were carried out to calculate means for statistical analysis.

In order to obtain the tumour growth inhibition curves, the mean of three independent assays of the alive-treated cells for each compound and concentration was used. The standard errors of the three replicas were calculated and the curve given by the Excel program was added. Finally, an estimation of inhibitory concentration 50 (CI_{50}) was calculated.

2.5.3. Determination of DNA fragmentation

DNA fragmentation is a hallmark of apoptosis and has been regarded as a critical process in apoptosis (Nagata, 2000). Briefly, HL-60 cells (1 x 10^6 cells/mL) were treated with different concentrations of lyophilised garlic (4 mg/mL, 2 mg/mL, 1 mg/mL, 0.5 mg/mL and 0.25 mg/m, respectively) for 5 h. Treated cells were collected and centrifuged at 3,000 rpm for 5 min, and DNA was extracted with lysis, precipitation and wash steps according to Merinas-Amo et al. (2016). [41] The total extracted DNA was quantified in a spectrophotometer (Nanodrop® ND-1000) and 1200 ng of DNA were loaded into a 2 % agarose gel electrophoresis, stained with ethidium bromide and visualised under UV light.

3. Results and Discussion

3.1. Soluble solids content, pH, water activity, and Browning intensity

A weight reduction was observed during the different garlic procedure being the 0C1 black garlic the sample with the nearest weight with respect to the white garlic. According to similar studies in garlic, the changes in the garlic weight during the processing are mainly caused by a reduction of the water quantity in the black garlic. [20] The most important organosulfur in black garlic are considered to be the water-soluble S-allyl-L-cysteine (SAC).[50] Hence, after aging of garlic, SAC increased in the processed black and its precursor garlicγ-Glutamyl-S-allyl-L-cysteine decreased. The creation of black garlic in this manner is not a microbe-associated fermentation, but a Maillard and Browning reaction, because the processing temperature of garlic does not allow bacterial growth to elicit fermentation.[20]

Soluble solids content (ºBrix), pH, water activity (a_w) and browning intensity (L) are shown in Table 1. During heat-treatment, soluble solid content increased in garlic whereas its pH, a_w and browning intensity decreased. Similar Tukey tests were showed in °Brix readings between white and 0C1 black garlic (40.47), meanwhile significant soluble solids content differences were observed in the 1C2 and 2C1 black garlic (43.17 and 45.67, respectively). The sugar content (°Brix) of black garlic increased. This result is in agreement with the data of Choi et al. (2008), which show that sugar content (e.g., glucose, fructose, sucrose, and maltose) increased in black garlic compared to fresh and steamed garlic. [51] Furthermore, this increased sugar content of black garlic might be related to its sweet taste. [20] pH decreased significantly during the manufacturing process. White garlic pH was the highest one with a value of 5.94, whereas black garlic pH decreased rapidly fewer than 3.69 reaching 3.49 value in the 45 days of aging process. This result is in agreement with the report of Shin et al. (2008), which showed that the pH of black garlic decreased from 6.40 to 5.29 after 6 days of aging. [52] The same observation has recently been described. [53,54] A heating temperature over 60 °C and a decrease in pH below 4.2 are two important factors for preventing the possibility of anaerobic bacteria proliferation. Water activity (a_w) decreased in a lesser extent than other parameters because black garlic was manufactured maintaining high relative humidity. According to Kaanane and Labuza (1989) and Labuza and Saltmarch (1981), the rate of the browning reaction is known to reach a maximum at a_w values in the range of 0.5 - 0.7. [55,56] However, white and black garlic a_w showed significant differences (Table 1). The 90 % RH and time required for producing black garlic in the present study might have created a situation in which the a_w of the heated garlic sample reached a state of equilibrium with the RH in the chamber in which the black garlic was

produced. This a_w condition is thought to facilitate the browning reaction in heated garlic samples. As Table 1 shows, browning intensity (*L*) in white and black garlic was significantly different with more than 28 units of difference between them, even though the 1C2 and 2C1 black garlic showed similar luminescence (17.85 and 17.58, respectively). It was observed that at higher temperatures garlic browning intensity happened earlier. Studies have shown that when the temperature increased, the browning product formation also increased; however, the initial induction period decreased. [57,58] Garlic colour finally changed to dark brown and black, mainly due to the formation of numerous compounds resulting from the reaction of non-enzymatic browning or Maillard.

Table 1. Physicochemical characterization of four types of garlic according to the days of aging process. (1) Different letters mean significantly different values in one-way ANOVA method using the post hoc Tukey test.

Type of garlic	White	0C1 Black	1C2 Black	2C1 Black
Aging process (days)	0	13	32	45
Weight of 10 garlic cloves (g)	49.69 c(1)	45.83 bc	37.57 b	19.67 a
Soluble solid content (ºBrix)	40.47 a	40.47 a	43.17 b	45.67 c
pH	5.94 d	3.69 c	3.60 b	3.49 a
Water activity (a_w)	0.97 c	0.93 a	0.93 a	0.93 a
Browning intensity (*L*)	47.16 c	18.73 b	17.85 a	17.58 a
Polyphenol content (g/kg in Gallic)	4.30 a	10.94 b	14.67 c	16.17 d

3.2. Total polyphenol content (g/kg in Gallic) and antioxidant capacity (% inhibition)

Total polyphenol (g/kg in Gallic) content and antioxidant capacity (inhibition percentage) are shown in Table 1. During heat-treatment, unstable compounds in raw garlic are transformed into stable soluble compounds with a high antioxidant power. [7,26] Previous studies on black garlic reported that this increase in the antioxidant capacity could be due to the increase in polyphenols and S-allyl-cysteine, the compound derived from alliin. [27] The antioxidant power of polyphenols has been demonstrated so it seems logical to state that an increase in polyphenol content in black garlic is responsible for the antioxidant properties in this product. [59]

All studied garlic showed significant differences for the total polyphenol content and the antioxidant capacity among them. Both characteristic increased significantly with the heat-treatment. The highest concentration of polyphenol content was obtained in 2C1 black garlic, although all black garlic increased between 6-fold to 12-fold relating with the heat-treatment (Table 1). Previous black garlic studies carried out at 70, 72, 75 and 78 °C with whole bulbs have described an increase in polyphenol content of about 2 to 3-fold, compared to raw garlic. [14,54] Our results on the increase of polyphenol content after heating agree in part with those obtained by other authors who found an increase of 3-fold content [14].

To clarify the antioxidant properties of black garlic during aging, we focused on the analysis of total polyphenol content. At the end of the heating process, an increase of antioxidant capacity was observed in garlic. All the black garlic samples showed an increase rank of 5.7-fold and 7.8-fold, with respect to white garlic (Table 1). Several studies described that aged black garlic exerts stronger antioxidant activity than garlic *in vitro* and *in vivo* assays. [27,60] The total polyphenol contents of black garlic were not only significantly higher than those of raw garlic, but also increased significantly at the 13th day of aging. Similar results were obtained by Sasaki et al. (2007) exhibiting an antioxidant potency increased in aged black garlic extracts reaching 25-fold compared with fresh garlic. [20] According to Xu and Chang (2008) heat treatment of the phenolic compounds increased the free fraction of phenolic acids, whereas it decreased the ester, glycoside, and ester-bound fractions, leading to an increase in free phenol forms.[61] Gorinstein et al. (2006) showed that the garlic processing conditions lead to changes in the contents of its bioactive compounds, such as polyphenols, flavonoids, and anthocyanins, and that this is connected to the type and duration of treatment. [62] From the results of total polyphenols and antioxidant capacity it is possible to state

that the optimum aging period of black garlic to maximise antioxidant content may be the 13st day of aging.

3.3. Toxicity/Antitoxicity

Toxicity of the four types of tested garlic has been assessed in the *Drosophila in vivo* model. Figure 1A shows the relative percentage of emerging adults after treating larvae with different concentrations of these substances, showing significant values of toxicity for the fourth highest tested concentration of white garlic. Yun et al. (2014) reviewed experimental reports related with the toxicity and safety of garlic extracts not founding adverse effects in animal or human models. [63] The safety of garlic extracts has been well established by studying general, chronic, acute and subacute toxicity tests, teratogenicity tests and toxicity test conducted by the U.S. Food and Drug Administration, and clinical studies. [64-69]

The antitoxicity assays revealed a differential behaviour of the substances. Figure 1B shows that hydrogen peroxide is significantly toxic at 0.12 M with an average survival rate of 63.4 % with respect to the water control. 0C1 black garlic is the only preventive substance against H_2O_2 at the three highest assayed concentrations. On the other hand, white and 1C2 black garlic significantly did not exhibit protective effects against the genotoxin at 0.25 - 0.5 and 0.5 - 1 mg/mL, respectively. Only 2C1 black garlic did not show significant protective effects against the oxidative toxicant with respect to their concurrent positive control H_2O_2. Lei et al. (2014) studied the effects that black 10-15 days-aged extract garlic had in *Drosophila melanogaster*. The results from this study demonstrated black garlic extracts possessed strong antioxidant capacity *in vitro* and *in vivo*.[70]

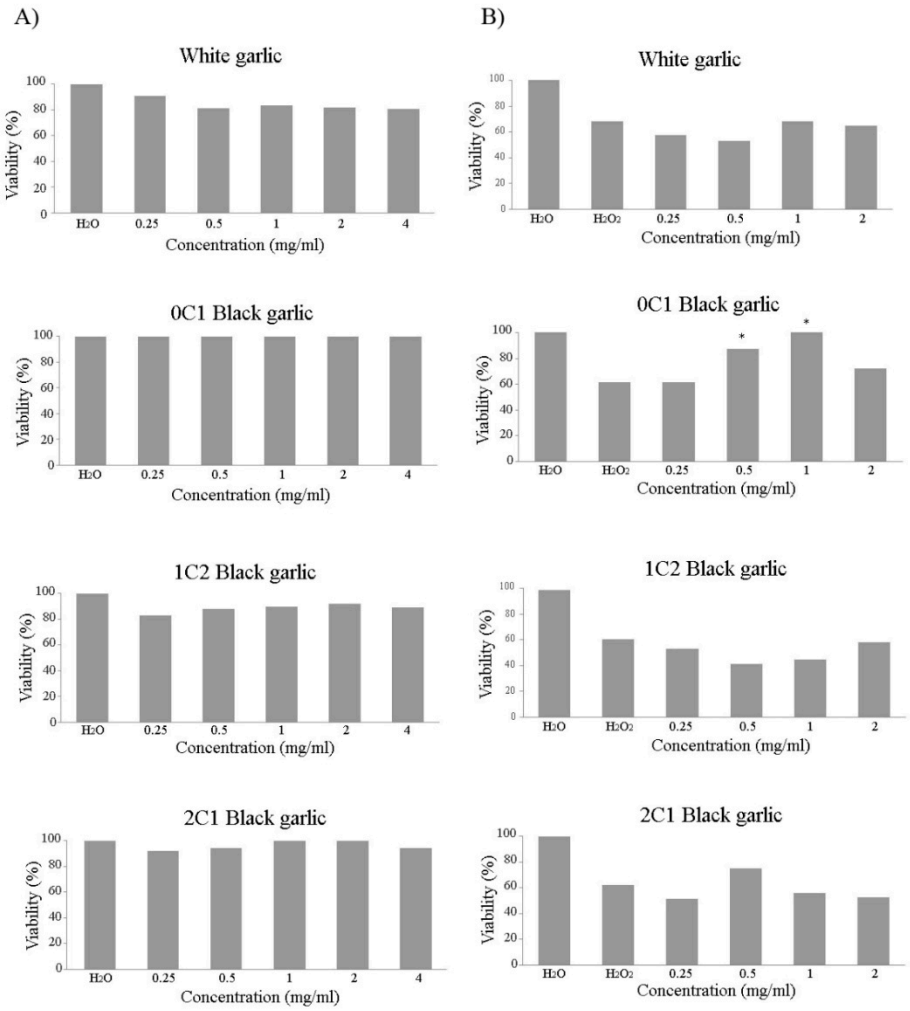

Figure 1. Toxicity (A) and antitoxicity (B) levels of white (0 days aging), 0C1 black (13 days aging), 1C2 black (32 days aging) and 2C1 black (45 days aging) garlic studied in *Drosophila melanogaster*. A) Percentage of viability of *Drosophila* treated with different concentrations of the assayed garlic. B) Viability of *Drosophila* tested with different concentrations of the tested garlic combined with the genotoxicant hydrogen peroxide at 0.12 M. * : significant ($p > 0.05$), with respect to their concurrent controls.

3.4. Genotoxicity/Antigenotoxicity

To assess the genotoxicity/antigenotoxicity of the studied compounds we used the SMART Test in *Drosophila melanogaster*.[43] Increasing concentrations of tested compounds, a negative control which corresponds to water used as a solvent, and a positive control (H_2O_2) for periodically validation of the assay were analysed concurrently. Furthermore, antigenotoxicity experiments were carried out using combined treatments consisting of repeating every concentration tested and by adding the same concentration of hydrogen peroxide, which we have demonstrated that is a potent mutagen in the SMART system. [71]

Table 2. Genotoxicity and antigenotoxicity of white (0 days aging), 0C1 Black (13 days aging), 1C2 Black (32 days aging) and 2C1 Black (45 days aging) garlic in the *Drosophila* wing spot test. (1) Statistical diagnosis according to Frei & Wurgler (1988, 1995). +: positive ($p < 0.05$), -: negative. m: multiplication factor. Levels of significance $\alpha = \beta = 0.05$, tail test without Bonferroni correction. Inconclusive results were resolved by U-test of Mann Whitney and Wilconxon. (2) The inhibition percentages for the combined treatments were calculated from total spots per wing according to S. K. Abraham (1994).

Compound	Clones per wing (nº spots) (1)					
	Number of wings	Small single clones (1-2 cells) m = 2	Large simple clones (more than 2 cells) m = 5	Twin clones m = 5	Total clones m = 2	Inhibition percentaje (%) (2)
H_2O	41	0.146 (6)	0.049 (2)	0	0.195 (8)	
H_2O_2	40	0.350 (14)	0.075 (3)	0	0.425 (17) +	
SIMPLE TREATMENT						
White garlic (mg/mL)						
0.25	40	0.225 (9)	0.025 (1)	0.025 (1)	0.275 (11) -	
2	40	0.375 (15)	0.050 (2)	0.000	0.425 (17) +	
0C1 Black garlic (mg/mL)						
0.25	40	0.175 (7)	0.025 (1)	0.000	0.200 (8) -	
2	41	0.122 (5)	0.000	0.000	0.122 (5) -	
1C2 Black garlic (mg/mL)						
0.25	40	0.200 (8)	0.025 (1)	0.025 (1)	0.250 (10) -	
2	40	0.175 (7)	0.025 (1)	0.000	0.200 (8) -	
2C1 Black garlic (mg/mL)						
0.25	40	0.175 (7)	0.05 (2)	0.000	0.225 (9) -	
2	40	0.250 (10)	0.000	0.000	0.250 (10) -	
COMBINED TREATMENT WITH H_2O_2 (0.12 M)						
White garlic (mg/mL)						
0.25	34	0.235 (8)	0.088 (3)	0.000	0.323 (11) -	24
2	34	0.265 (10)	0.206 (7)	0.000	0.500 (17) +	-17
0C1 Black garlic (mg/mL)						
0.25	30	0.5 (15)	0.033 (1)	0.000	0.533 (16) +	-25.4
2	30	0.233 (7)	0.033 (1)	0.000	0.266 (8) -	37.4
1C2 Black garlic (mg/mL)						
0.25	26	0.307 (8)	0.038 (1)	0.000	0.346 (9) -	18.6
2	38	0.368 (14)	0.053 (2)	0.000	0.421 (16) -	0.17
2C1 Black garlic (mg/mL)						
0.25	28	0.357 (10)	0.036 (1)	0.000	0.393 (11) -	7.5

2	28	0.357 (10)	0.250 (7)	0.000	0.607 (17) +	-42.8

Table 2 shows the results of genotoxicity assays in the SMART test for white and the three studied black garlics. Negative control showed a frequency of mutations per wing of 0.195, which fall into the historical range for the wing spot test. [41,72] The final concentration of H_2O_2 used (0.12 M) has been demonstrated to exert a potent genotoxic effect capable to induce somatic mutations and mitotic recombination in *D. melanogaster*.[73] The average frequency of total mutations per wing obtained in the treatment with H_2O_2 was 0.425. For each concentration and compound single small, single large, twin and total clones were analysed in the wings of chronically treated animals. The results showed that all white and black garlic showed a non genotoxic activity, except the white one which increased significantly the frequency of mutations in 0.425 at the highest concentration tested. Similar results were obtained by Abraham and Kesavan (1984) and Shukla and Taneja (2002), who demonstrated that aqueous garlic extracts (5% v/v) and fine garlic powder (7.5, 5 and 2.5 g/Kg body weight) supplementation did not induced chromosomal aberrations nor DNA damage in mouse bone marrow cells. [74,75] Same results were obtained by Sowjanya et al. (2009) at 3, 6 and 12 mg/culture in human lymphocytes [76] and by Chughtai et al. (1998) (extracts of fresh garlic bulbs) in yeast.[77]

Vegetables contain many polyphenols and oligoelements with antimutagenic activity.[78] The 0C1 black garlic was the only able to inhibit the genotoxic activity of hydrogen peroxide in a dose dependent manner (Table 2). The highest concentration tested for 0C1 black garlic in the combined treatments reached to eliminate part of the genotoxic effect of H_2O_2, showing a decreasing of the total mutation frequency in 0.266 spots/wing and inhibiting around 37 % of genotoxicity induced by H_2O_2 (without control correction). The rest of compound tested did not show significant protective results against the DNA damage at the highest concentration and a slight inhibition percentage of mutations induced by the genotoxine was observed (24 % for white, 18.6 % for 1C2 black and 7.5 % for 2C1 black garlic).

In general, garlic has significant antioxidant activity and protective effects against oxidative DNA damage regardless of processing method.[79] Our antitoxicity and antigenotoxicity results showed that 0C1 black garlic (13 days aged) is able to protect genomic damages against this genotoxin in a doses-dependent manner. This effect could probably be due to the antioxidative and free-radicals scavenging capacity of their respective organosulfur compounds, which agree with previous reports. [26,80,81] Besides the antioxidant activity, our results about the strongest antioxidant activity that black garlic showed compared with fresh garlic are in agreement with previous *in vivo* and *in vitro* garlic assays. [10,82]

3.5. Longevity assays

The entire lifespan curves obtained by the Kaplan-Meier method for each substance and concentration are shown in Figure 2. *Drosophila* had a lifespan expansion of 60 days for the control treatment. White and 1C2 black garlic increased significantly the *Drosophila's* lifespan at the lowest and the two moderated concentrations tested (0.25, 1 and 2 mg/mL) with an expansion of 10.1, 11.1 and 18.5 days for white garlic and 9.4, 10.1 and 9.8 days for black garlic, respectively, with respect to the concurrent control (Table 3). Furthermore, all concentrations assayed of 0C1 black garlic, except the highest one, induced a lifespan expansion in *D. melanogaster* compared to the control in a value over 10 days in each one with respect to the control. On the other hand, 2C1 black garlic did not have activity on the lifespan of *Drosophila melanogaster* at any tested concentration. No previous *in vivo* studies on longevity properties of black garlic as a food have been reported. However, several authors have reported beneficial effects on animal lifespan using garlic extracts in: *D. melanogaster* at 37.5 and 75 mg/mL; *C. elegans* at 50 ppm and senescence accelerated mice (SAMP8) at 2 % w/w.[70,83,84]

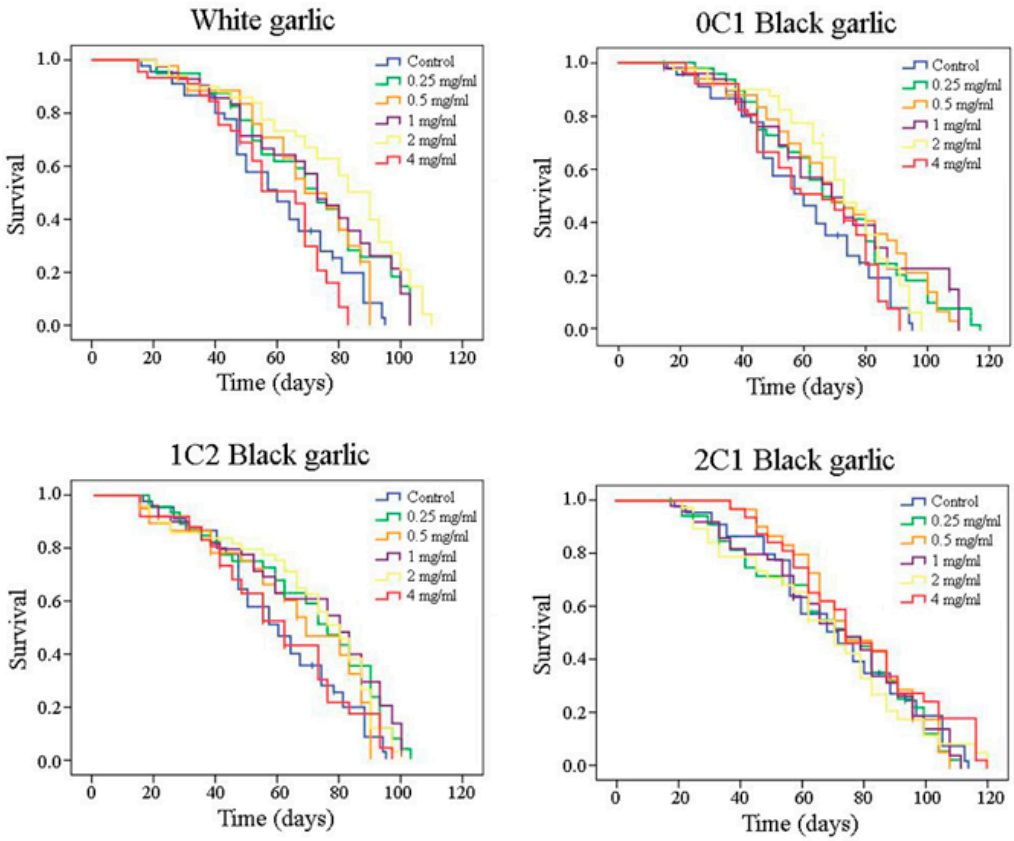

Figure 2. Survival curves of *Drosophila melanogaster* fed with different concentrations of white (0 days aging), 0C1 black (13 days aging), 1C2 black (32 days aging) and 2C1 black (45 days aging) garlic over time.

We suggest that our differences with these results could be due to the different type of sample presentation. We have used entire garlic material and all data available elsewhere on lifespan trials come from extracts but not crude material. In this sense, Prowse et al. (2006) demonstrated that garlic juice had insecticidal activity across life stages of flies at a wide range of concentrations (0.25-5 %) in two dipteran pests (*Delia radicum* and *Musca domestica*).[85] Lei et al. (2014) studied the effects of black 10-15 days-aged garlic extracts on lifespan in *Drosophila* through observation on half dead time, mean and maximum lifespan of organisms. The results suggested a significant longevity extension in *Drosophila* treated with black garlic extracts in a dose-dependent manner. [70]

3.6. Healthspan assays

In order to know the quality of life of the *Drosophila* treated in the longevity assays, we studied the 25 % of individual survival at the top of the lifespan curves obtained in the previous test for each substance and concentration tested. This part of the lifespan is considered as the healthspan of a curve, characterised by low and more or less constant age-specific mortality rate values. [86] The results are shown in Figure 3.

Only white and 0C1 black garlic induced a significant increase of healthspan in *Drosophila melanogaster* compared to the control in an average of 8 and 11.5 days, respectively. On the contrary, 1C2 and 2C1 black garlic induced a significant reduction of healthspan in *Drosophila* at moderate concentration with a value of 7.3 and 9 days, respectively with respect to the control (Table 3). No previous studies about the properties that white and black garlic have on the quality of life have been reported.

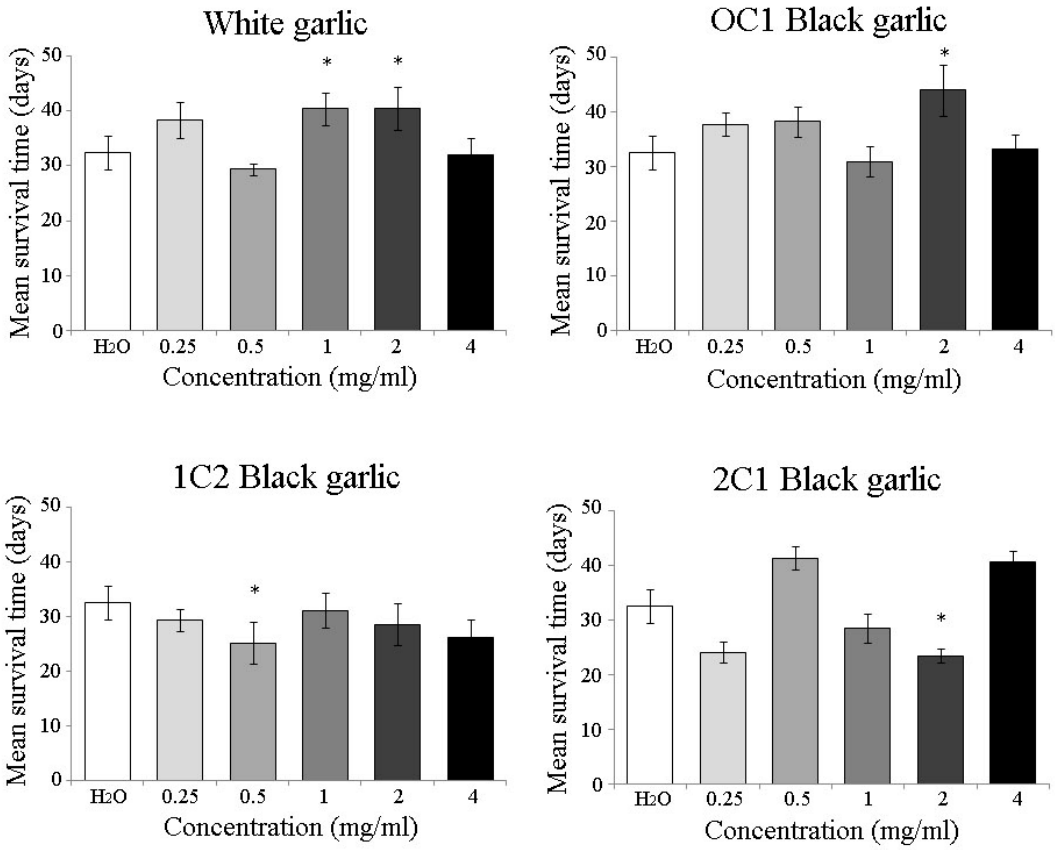

Figure 3. Healthspan effect on *Drosophila melanogaster* fed with different concentrations of white (0 days aging), 0C1 black (13 days aging), 1C2 black (32 days aging) and 2C1 black (45 days aging) garlic. *: significant ($p < 0.05$).

3.7. Cytotoxicity

All the substances assayed showed cytotoxic activity against HL-60 tumour cells (Figure 4). White and black garlic showed a dose dependent response, with an increase of the cytotoxicity level according to increased concentration of garlic. White garlic showed the main cytotoxicity effect against the tumour cell being the inhibitory concentration 50 (IC_{50}) under 0.03 mg/mL.

Cytotoxicity curve of 0C1 black garlic showed an increase of dose-dependent with a IC_{50} value of 1 mg/mL. In relation to 1C2 and 2C1 black garlic, no inhibition was observed at the lowest concentration tested, but contrarily strong tendency to increase the cell growth is observed with a IC_{50} value of 0.7 and 0.9 mg/mL, respectively. Moreover, a completely cells growth inhibition was observed in 1C2 and 2C1 black garlic at 2 mg/mL.

A number of studies have demonstrated the chemopreventive activity of garlic by using different garlic preparations including fresh garlic extract, aged garlic, garlic oil and a number of organosulfur compounds derived from garlic. [87,88] The chemopreventive activity has been attributed to the presence of organosulfur compounds in garlic. Therefore, the consumption of garlic may provide some kind of protection against tumour cells proliferation. [89] Studies on the preventive effects of black garlic extracts also shown an induction of inhibition in *in vitro* and *in vivo* gastric cancer cell growth, chemopreventive effects in rats colon tumour and increase the anti-tumour activity in mouse model treated. [18,20,90].

Table 3. Mean and significances of lifespan and healthspan curves for the different garlic treatments assayed in *Drosophila*. Results were calculated by the Kaplan-Meier method and significance of the curves determined by the Log-Rank method (Mantel-Cox). ns: non-significant ($p > 0.05$),*: significant ($p < 0.05$), **: significant ($p < 0.01$), ***: significant ($p < 0.001$). WG: white garlic (0 days aging); 0C1: Black garlic (13 days aging); 1C2: Black garlic (32 days aging); 2C1: Black garlic (45 days aging).

	Treatment (mg/mL)	Lifespan mean (days)		Healthspan mean (days)	
Negative Control	0	60.309		32.455	
WG					
	0.25	70.468	*	38.400	ns
	0.5	68.718	ns	29.400	ns
	1	71.429	**	40.385	*
	2	78.888	***	40.444	*
	4	58.214	ns	31.909	ns
0C1					
	0.25	70.146	*	37.667	ns
	0.5	71.716	**	38.183	ns
	1	70.732	*	30.848	ns
	2	71.800	*	43.900	*
	4	62.600	ns	33.100	ns
1C2					
	0.25	69.727	*	29.364	ns
	0.5	65.191	ns	25.145	*
	1	70.490	**	31.096	ns
	2	70.179	*	28.565	ns
	4	60.255	ns	26.200	ns
2C1					
	0.25	59.058	ns	24.071	ns
	0.5	64.754	ns	41.292	ns
	1	59.815	ns	28.500	ns
	2	57.304	ns	23.460	**
	4	66.375	ns	40.500	ns

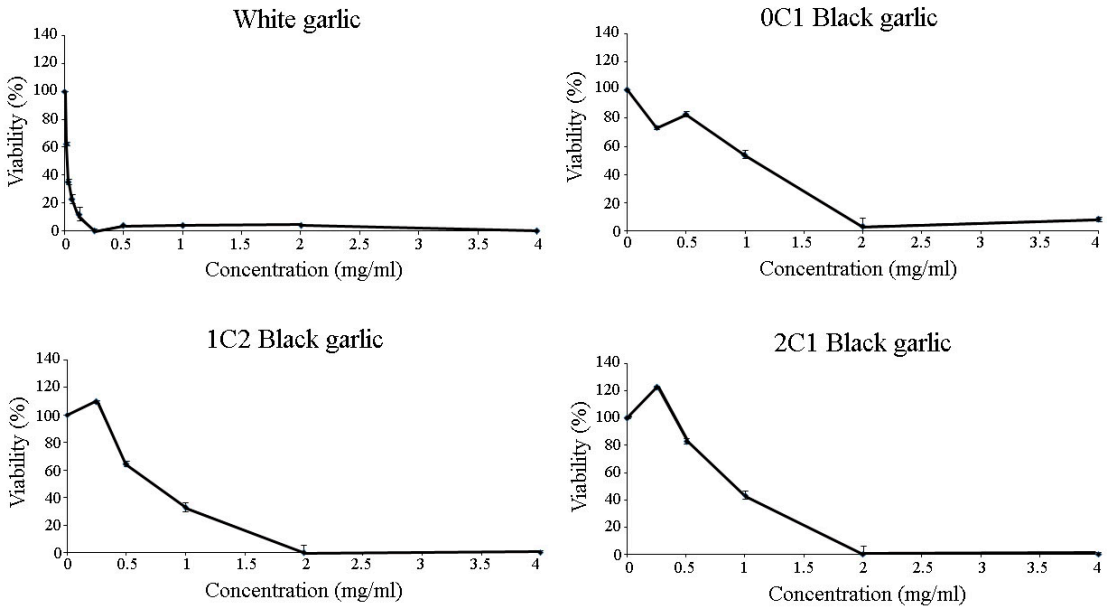

Figure 4. Viability of HL-60 cells treated with different concentrations of white (0 days aging), 0C1 black (13 days aging), 1C2 black (32 days aging) and 2C1 black (45 days aging) garlic for 72 hours.

3.8. DNA internucleosomal fragmentation

Figure 5 shows the electrophoresis of the genomic DNA of HL-60 cells treated with different concentrations of white, 0C1, 1C2 and 2C1 black garlic.

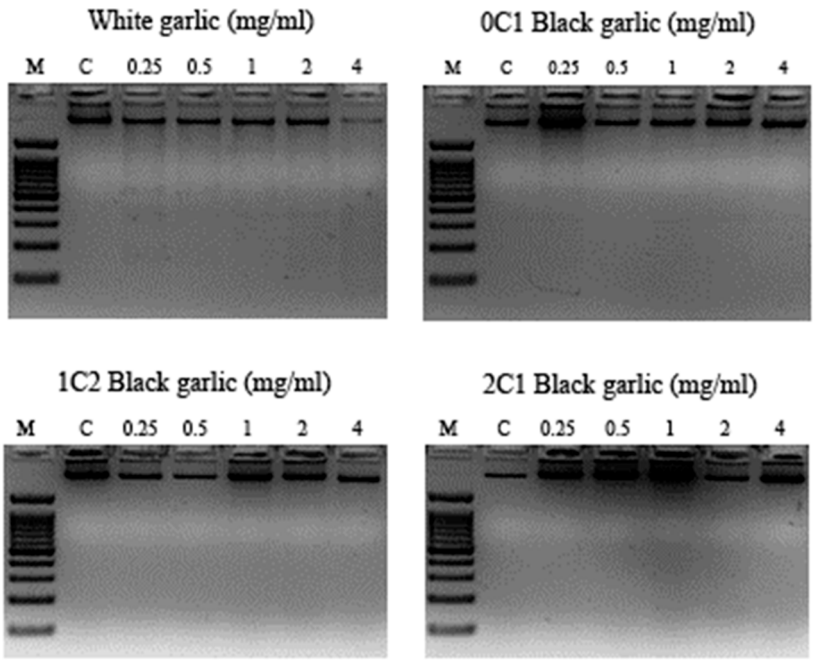

Figure 5. Internucleosomal DNA fragmentation in HL-60 cells treated for 5 hour with different concentrations of white (0 days aging), 0C1 black (13 days aging), 1C2 black (32 days aging) and 2C1 black (45 days aging) garlic. DNA fragmentation was detected following electrophoresis in agarose gels and staining with ethidium bromide. M: indicates DNA size marker; C: indicates control (lane 1); 0.25 mg/mL (lane 2); 0.50 mg/mL (lane 3); 1 mg/mL (lane 4); 2 mg/mL (lane 5) and 4 mg/mL (lane 6) of garlic sample.

DNA internucleosomal fragmentation is represented by a DNA laddering and it is associated to the activation of the apoptotic way in cancer cells being a hallmark of the genomic integrity. [91] None assayed concentration (4 mg/mL to 0.25 mg/mL) induced internucleosomal fragmentation in the different black garlic treatments, but a slight fragmentation is observed in the lowest assayed concentration of white garlic (0.25 mg/mL). Hence, the cytotoxic activity observed is only induced in a proapoptotic way in the white garlic.

Our results demonstrate that only white garlic have a strong cytotoxic effect and induce slight DNA pro-apoptotic internucleosomal fragmentation against HL-60 cells. These results agree with several reports demonstrating that garlic exerted a chemopreventive effect by increasing apoptosis in lung cancer cells (NCI-H1299). [92] On the other hand, our results do not agree with the results obtained by Wang et al. (2012), who detected a dose-dependent apoptosis in aged black garlic extract in *in vitro* studies. [18]

4. Conclusions

It is the first time that a relationship between the physico-chemical characterisation and the biological activities of white and black garlic is carried out. Systematic, integrate and multifocal studies assessing the toxicity, antitoxicity, genotoxicity, antigenotoxicity, longevity, cytotoxicity and pro-apoptotic properties of the different types of garlic were followed in order to propose black garlic as a nutraceutical or functional food. Mention should be made that we have used entire garlic material in all the *in vivo* and *in vitro* assays tested meanwhile most of available data in literature come from extracts but not crude material.

Analyzing the physico-chemical properties of the different studied garlic in this research, we must point out the improved qualities that the 13 days aged black garlic reaches with respect to the other processed black garlic and also the white one. The 0C1 black garlic (13 days aged) showed similar weight and °Brix like the raw garlic. Moreover, that black garlic improved the polyphenol

content and the inhibition percentage with respect to the white garlic and also with the other types of black garlic if we take into account the time of processing.

All types of garlic were safe without showing toxicity except the white one. Moreover, only 0C1 (13 days aged) black garlic showed a slight protection against the oxidative toxicant at three highest concentrations. With respect to the genotoxic potential, all raw and processed garlic were not genotoxic with the exception of the higher concentration of white garlic exhibits antigenotoxic effects when the imaginal discs are treated with the genotoxine hydrogen peroxide. The longevity assays in *Drosophila* yielded significant extension of lifespan results in some of the tested concentrations of white and 0C1 and 1C2 black garlic. Finally, the results achieved in the *in vitro* experiments for garlic cytotoxicity were hopeful. All studied garlic induced a decrease in leukaemia cells growth. However, none type of garlic was able to induce proapoptotic internucleosomal DNA fragmentation.

The present study of black garlic might be useful for understanding not only the antioxidant properties of this processed garlic, but also its optimum aging conditions for maximizing biological and antioxidant properties. Important information is added to the agrifood industry on the fermentation conditions data that improve the quality of garlic. Our data suggest that short-aged fermented black garlic has better properties than the longer-fermented ones and even more than white garlic. This latter could have industrial and economics consequences. Taking both the physicochemical and biological data, the 13 days aging black garlic has shown itself to be best nutraceutical. Our findings are relevant for the black garlic processing agrifood companies as the economical and timing incomes are significantly reduced to 13 days aging. The sector should know that black garlic obtained with less cost is healthier than the ones needing more time and processing costs.

Author Contributions: MATM, JPA and AMO performed all physicochemical analysis. ZFB, TMA, RF and MDRC carried out all genotoxicological and antigenotoxicological analysis. RMR and AAM designed this study and revised the manuscript. ZFB, TMA, RF and MDRC wrote this manuscript. TMA performed the longevity assays. ZFB, TMA and AAM performed all *in vitro* assays. All the listed authors have read and approved the submitted manuscript.

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