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Article

Nutraceutic potential of two *Allium* species and their distinctive organosulphur compounds: a multiassay evaluation

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Abstract: This study aimed to evaluate the biological activities of two *Allium* species (Garlic and onion) as well as diallyl disulphide (DADS) and dipropyl disulphide (DPDS) as their representative bioactive compounds in a multi-assay experimental design. The genotoxic, antigenotoxic and lifespan effects of garlic, onion, DADS and DPDS were checked in *Drosophila melanogaster* and their cytotoxic, pro-apoptotic and DNA-clastogenic activities were analysed using HL60 tumoral cells. All compounds were non-genotoxic and antigenotoxic against H_2O_2-induced DNA damage with a positive dose-response effect and different inhibition percentages (the highest value: 95% for DADS) at all tested concentrations. Daily intake of *Allium* vegetables, DADS or DPDS had no positive effects on flies' lifespan and healthspan. Garlic and DADS exerted the highest cytotoxic effects in a positive dose-dependent manner. Garlic and DADS exerted a DNA-internucleosomal fragmentation as an index of induced proapoptotic activity on HL60 cells. *Allium* vegetables and DADS were able to induce clastogenic strand breaks in the DNA of HL60 cells. This study showed the genomic safety of the assayed substances and their protective genetic effects against the hydrogen peroxide genotoxine. Long-term treatments during the whole life of *Drosophila* genetic model were beneficial only at low-median concentrations. The chemopreventive activity of garlic could be associated to its distinctive organosulphur DADS. We suggest that supplementary studies are needed to clarify the cell death pathway against garlic and DADS.

Keywords: Garlic, onion, antigenotoxicity, longevity, cytotoxicity, comet assay

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1. Introduction

Mediterranean diet is one of the best nutritional patterns for humans due to its demonstrated beneficial effects on health. This diet, based on the high consumption of fruit, vegetables, wine and olive oil, and fish as the main animal protein contribution, is a prototype of a healthy and well balanced food intake [1]. Today, most of the studies asserting these well-being effects agree to point the increased antioxidant and phenolic contents as the cause of its properties [2]. Diet-derived antioxidants are implicated in maintaining a balanced homeostasis and scavenging of reactive oxygen species (ROS) as a major part of a highly efficient defensive biological network which neutralizes the oxidative stress and complements the endogenous defense enzymes [3].

Garlic (*Allium sativum*) and onion (*Allium cepa*) are two native vegetables from Asia, widely used in different gastronomic cultures and traditional medicines for centuries [4]. According to the Food

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Administration Organization (FAO), these vegetables are two of the most important crops worldwide with a production of 24,255.303 and 85,795.195 tons of garlic and onion respectively in 2015, showing a trending towards an increased consumption in the recent years due to the expansion of the Mediterranean and Asian cuisine. These vegetables have been linked to preventive effects against several diseases such as cancer, obesity, diabetes type-2, coronary heart disease and hypertension, among others [5-8]. These pleiotropic effects were associated to the high content of thiosulfinates, a group of volatile organosulfur compounds originated from the decomposition of the allicin, which are also responsible for their typical pungent aroma and taste [9-12]. However, both vegetables showed a high variability with respect to the thiosulfinate profiles among strains being diallyl sulfide (DAS), diallyl disulfide (DADS) and diallyl trisulfide (DATS) normally higher in garlics and dipropyl sulfide (DPS) and dipropyl disulfide (DPDS) higher in onions [13, 14].

Garlic oils and extracts were associated to several health-benefit activities, such as a protective capacity against DNA damage induced by oxidative stress, increased hydrogen peroxide (H_2O_2) scavenging activity and ability to reduce the bioactivity of carcinogens and tumor cells proliferation [15-18]. These capacities were directly linked to DADS, one of their major and most garlic distinctive constituents, which was widely studied and characterized as non-genotoxic, antigenotoxic, inhibitor of cell proliferation and pro-apoptotic in different cancer cell lines like leukaemia, colon, prostate, lung, bladder and skin [19-26].

On the other hand, onions are more versatile vegetables that can also be consumed as fresh and processed products. In both presentations, they also showed a high oxy-radical scavenging capacity [27] as well as an antigenotoxic effect [28]. In addition, garlic ethanolic extracts and oils showed antimutagenic activity [29] and also decreased the viability and increased the apoptosis in several cancer cell lines like HL60, MDA-MB-231, A549 and B16F10 [30-33]. In this case, their pro-healthy properties were widely related to DPDS, one of its most representative organosulfur compounds. This molecule was previously associated to a strong anticarcinogenic activity [34] and a protective effect against DNA strand break and oxidative damage [35, 36]. Nevertheless, this compound had not antitumor effects in mice [37], did not decrease tumor cell growth and did not induce DNA-internucleosomal fragments on cancer cell lines by acting alone [26, 37-39].

Hereby, we performed a qualitative and quantitative evaluation of the health-beneficial properties of garlic, onion and their representative organosulfur compounds (DADS and DPDS) in a multi-assay experimental design using *in vivo* and *in vitro* models. We assessed on their genotoxic, antigenotoxic and lifespan effects in *Drosophila melanogaster* flies, a widely used experimental model closely related to humans and additionally, we evaluated their proapoptotic capacities against cancer processes through the determination of their cytotoxicity and clastogenic DNA activity against an *in vitro* human cancer model (HL60 cell line).

2. Materials and Methods

2.1. Allium vegetables and single compounds

Two *Allium* species and two of its most distinctive organosulfur compounds were assayed. Garlic (*Allium sativum*, purple variety) and onion (*Allium cepa*, Victoria variety) were purchased in a local market. Thiosulfinates, DADS from garlic and DPDS from onions, were purchased from Sigma (Cat numbers 317691 and 43550 respectively).

2.2 Preparation of the samples

Garlics and onions were washed twice with distilled water, cut in slim slices, and freeze-dried at -80ºC. After that, both samples were lyophilized, pulverized with a mortar pestle, sieved and stored at 25 ºC in dark until use.

2.3 In vivo assays

2.3.1. SMART (Somatic Mutation and Recombination test)

Two *Drosophila melanogaster* strains carrying visible wing genetic markers were used in our experimental design: the flare (*flr*) strain *flr^3/ln (3LR) TM3, Bd^s* and the multiple wing-hair (*mwh*) strain *mwh/mwh*. The multiple wing hairs (*mwh, 3_0.3*) marker is a recessive viable mutation in homozygous flies, producing multiple-hairs trichomes in the fly adult body [40]. The flare (*flr^3, 3_38.3*) marker is a homozygous recessive lethal mutation which produces malformed individual wing hairs in somatic cells of larvae. The *flr^3* allele is retained in a balancer chromosome carrying multiple inversions and a homozygous lethal dominant visible marker expressed in the edge wing [41].

Genotoxicity was determined using the SMART test as described by Graf and Wurgler [42] including a negative control of pure water. The antigenotoxic activity was also determined using a modified SMART test following our standard protocols [43]. Briefly, optimally virgin *flr^3/ln* (3LR) TM3, *ri p^p sep bx^{34e} e^s Bd^S* (flare) females were crossed with *mwh/mwh* strain males, obtaining 72 h transheterozygous F1 larvae after an 8 h egg-laying on fresh yeast. Larvae were fed with *Drosophila* Instant Medium (Formula 4-24, Carolina Biological Supply, Burlington, NC) in 4 mL vials. Genotoxicity assays consisted of eight experimental groups by supplementing the base larvae food (0.85 g) with different concentrations of onion (0.625 and 5 mg/mL), garlic (0.625 and 5 mg/mL), DADS (4 mM and 34 mM) and DPDS (4 mM and 33 mM). The concentration ranges of single compounds were selected to mimic those described in the fresh *Allium sp*. [44]. Negative (distilled water) and positive (0.12 M H_2O_2) concurrent controls were included. Antigenotoxicity experimental design was similar to the genotoxicity assays by concurrently treating the larvae with the tested substances supplemented with H_2O_2 (0.12 M) as positive genotoxicant control. The emerged adults in each group were finally stored in 70 % ethanol until analysis.

Forty wings of heterozygous flies (mwh/flr^3) treated with each compound and concentration were removed and mounted on slides with Faure´s solution (Arabic gum 50 g, glycerol 20 mL, chloral hydrate 50 g, distilled water 50 mL). Both dorsal and ventral surfaced were screened under a bright light microscope at 400x magnification to detect small single spots (1-2 mwh or flr^3 cells), large single spots (3 or more cells) and twin spots (adjacent mwh and flr^3 cells). Single spots are produced by gene mutation, somatic recombination and deletion between the two markers. Twin spots are produced uniquely by recombination between the *flr^3* marker and the centromere.

In order to evaluate the possible genotoxic effect, the frequencies of total spots per wing of each series were statistically compared with the total spots of the negative control with the non-parametric U-test of Mann, Whitney and Wilcoxon [45]. Antigenotoxicity was determined as the inhibition percentage (IP) using the total spots per wing determined at each concentration with the following formula [46]:

$$IP= [(a - b)/a] \times 100$$

where ***a*** represents the frequency of total spots induced by the treatment with genotoxine alone, and ***b*** represents the frequency of total spots obtained with genotoxine plus substance tested in the different combined treatments.

2.3.2 Longevity assays

All the longevity experiments were performed following our standard procedures [47]. Transheterozygous larvae from a 12 h egg-laying with the same genetic background described above were used in the life and health-span trials. Healthspan is the healthy adult period of unimpaired life that precedes functional decline [48]. It is important to consider the quality of a prolonged life and for this reason healthspan is a new focus in aging research. Synchronized larvae of 72 ± 12 h were clustered in groups of 100 individuals in glass vials with 0.85 g of *Drosophila* Instant Medium in 4 mL of water solutions of the different experimental concentrations assayed (0.625, 1.25, 2.5 and 5 mg/mL for *Allium* vegetables; 4, 8, 16 and 33 mM for DPDS; 4, 8, 17 and 34 mM for DADS). The emerged flies were anesthetized under CO_2, separated into 10 single-sex groups, transferred to longevity vials and fed with the same treated medium during the whole experimental design. A concurrent treatment was also included using distilled water as negative control. The survivors were counted and the

medium was renewed twice a week until all individuals die. Survival curves were plotted as estimated by the Kaplan-Meier method and the statistical significance of curves were assessed using the Log-Rank (Mantel-Cox) method using the SPSS 15.0 statistics software (SPSS Inc. Headquarters, Chicago, IL, USA).

2.4 In vitro assays

2.4.1 Cell line cultures and cytotoxicity assay

In vitro assays were performed using the promyelocytic leukemia HL60 cell line. Cells were cultured at 2.5 x 10^5 cells/mL following our standard protocol [49] in complete RPMI 1640 medium (BioWhittaker, BE12-167F) containing 10% heat-inactivated foetal bovine serum (BioWhittaker, de14-801F), L-glutamine 200 mM (Sigma, G7513) and antibiotic-antimycotic solution (Sigma, A5955) at 37ºC in a humidified atmosphere of 5% CO_2. Two passes per week were performed and the experiments were carried with cells with no more than 20 passes. Cell viability was evaluated by Trypan blue exclusion assay. Cells (1 x 10^5 cells/mL) were seeded and incubated for 72 h in 96 well plates supplemented with 6 different concentrations *Allium species* (ranging from 0.002 mg/mL to 0.06 mg/mL) and 6 different concentrations of thiosulfinates (ranging from 0.012 mM to 0.4 mM). A concurrent negative control (base medium without supplementation) was also run. After incubation, Trypan blue was added to the cell suspension (1:1 ratio) and cells were counted in a Neubauer chamber under an inverted microscope at 100x magnification. Cell viability was expressed as percentage of survival with respect to control after 72 h period. IC_{50} values (concentration of tested compound causing 50% of cell growth inhibition) were estimated for each treatment. Viability curves were plotted as mean viability ± Standard deviation of three independent replicas in each substance and concentration.

2.4.2 Internucleosomal DNA fragmentation assay

HL60 cells (1.5 x 10^6 cells/mL) were incubated with the same compounds and concentrations as in cytotoxicity assays for 5 h in 12-well plates. Thereafter cells were harvested, centrifuged at 2500 x g for 5 min. and washed with phosphate buffer saline (PBS). Total DNA was extracted using a commercial DNA-extraction kit (Blood Genomic DNA Extraction Mini Spin Kit, Canvax Biotech, Cordoba, Spain) according to the manufacturer instructions and subsequently treated with RNase overnight in order to eliminate false positive. DNA yielding was quantified in a Nanodrop™ (Thermo Scientific, Madrid, Spain). A total of 1500 ng of DNA per sample was electrophoresed in a 2% agarose gel, stained with ethidium bromide and run by 120m at 60V. Internucleosomal DNA fragmentation was determined by the presence of a ladder band patterns with 200 bp multiple fragments. 2.4.3. Evaluation of DNA breakage ability: comet assay DNA strand break ability of the compounds was determined by the alkaline comet assay as described Olive and Banáth [50] with minor modifications. HL60 cells (5x10^5 cells) were plated in 1.5 mL of culture medium supplemented with different concentrations of onion (0.004, 0.016 and 0.06 mg/mL), garlic (0.002, 0.004 and 0.008 mg/mL), DPDS (0.025, 0.1 and 0.4 mM) and DADS (0.01, 0.025 and 0.05 mM) and incubated for 5 h. After treatment, cells were washed and adjusted to 6.25 x 10^4 cells/ml in PBS. Then, cells (1.6 x 10^4) were suspended in 75 µL pre-warmed low melting point agarose (A4018, Sigma) and 50 µL of the suspension were rapidly spread on microscope slides and covered with coverslips. After gelling for 30 min at RT, the coverslips was gently removed and the slides were put in a tank filled with lysis solution (2.5M NaCl, 100mM Na-EDTA, 10mM Tris, 250mM NaOH, 10% DMSO and 1% Triton X-100; pH= 13) at 4ºC for 1 h. Next, slides were removed from the lysis solution and incubated in alkaline electrophoresis buffer (300mM NaOH and 1mM Na-EDTA, pH= 13) at 4ºC for 20-30 min. Electrophoresis was then carried out in a fresh-made electrophoresis buffer for 15 minutes at 20 V and 400 mA in dark conditions. After electrophoresis, slices were gently washed in cold fresh-made neutralization buffer (0.4M Tris-HCl buffer, pH 7.5) for 10 min and allowed to dry overnight at RT in dark conditions. Finally, gels were stained with 7 µL propidium iodide, covered with a coverslip and photographed at 400 X magnification in a Leica DM2500 epifluorescence microscope with a microscope. At least 50 cells were

assessed for each treatment. Data were analysed using the Open CometTM software [51]. The statistical ANOVA-Tukey test was applied [52] using the SPSS 15.0 statistics software (SPSS Inc. Headquarters, Chicago, IL, USA) in order to compare the results obtained for the different treatments and the negative control.

3. Results

3.1 SMART test

The results of genotoxicity and antigenotoxicity are shown in **Table 1**. All the assayed compounds were non-genotoxic in the flies at all tested concentrations. Both *Allium* vegetables showed no differences compared with water control in single and total spots. Validation of the experimental design was assessed by the results of positive control (H_2O_2; 0.37 total spots/wing) which agreed with our previous results [47, 53]. The antigenotoxic potency of *Allium* sp. vegetables, DPDS and DADS against H_2O_2 exhibited a clear positive dose-response effect although the lowest concentration of garlic was not statistically different respect to the positive control (**Figure 1**), showing the DADS the highest IP value (95%).

Figure.1. Mutagenicity inhibition percentages produced by onion, garlic, DPDS and DADS against H_2O_2–DNA induced damage (*Drosophila melanogaster* model). *: Statitiscal significance compared with positive control.

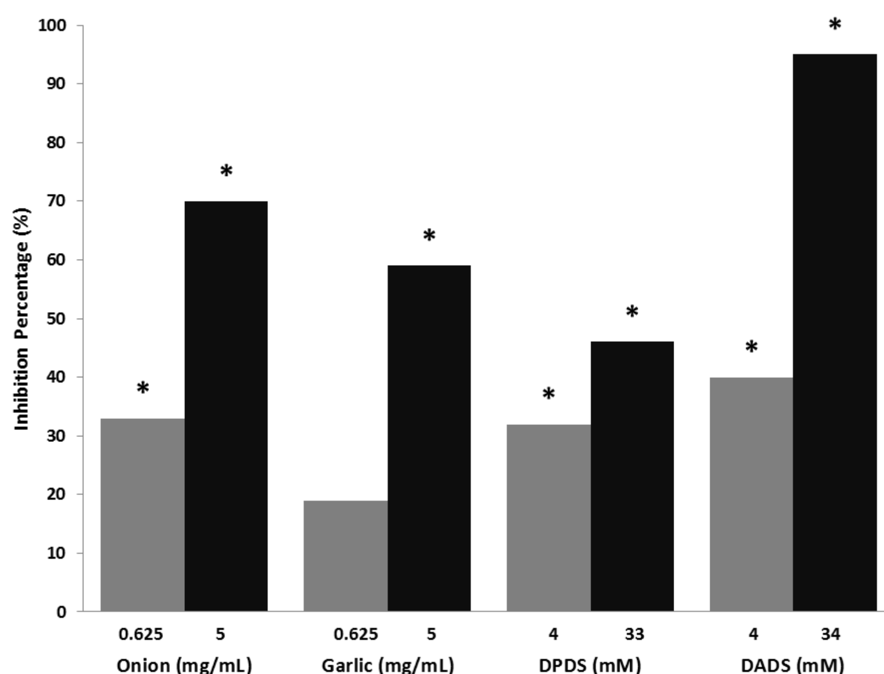

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Table 1. Genotoxicity and antigenotoxicity results obtained in the SMART test when flies were fed with different concentrations of onion, garlic and organosulfur DPDS and DADS in single and combined treatments.

		Clones per wings (number of spots) [1]				
Compounds	**N**	**Small spots (1-2 cells)**	**Large spots (>2 cells)**	**Twin spots**	**Total spots**	**Mann-Whitney Test [3]**
Controls						
H_2O	40	0.10(4)[2]	0	0	0.10(4)	
H_2O_2(0.12M)	40	0.30(12)	0.05(2)	0.02(1)	0.37(15)+	**
Onion (mg/mL)						
0.625	40	0.17(7)	0	0	0.17(7)i	Δ
5	38	0.08(3)	0.03(1)	0	0.10(4)i	Δ
0.625 + H_2O_2	40	0.20(8)	0	0.05(2)	0.25(10) λ	Ω
5 + H_2O_2	38	0.08(3)	0	0.03(1)	0.11(4) β	
Garlic (mg/mL)						
0.625	40	0.07(3)	0	0	0.07(3)i	Δ
5	40	0.05(2)	0	0	0.05(2)i	Δ
0.625 + H_2O_2	40	0.27(11)	0	0.02(1)	0.30(12) λ	Δ
5 + H_2O_2	40	0.15(6)	0	0	0.15(6) β	
DPDS (mM)						
4	40	0.22(9)	0.02(1)	0.02(1)	0.27(11)i	Δ
33	40	0.07(3)	0.07(3)	0	0.15(6)i	Δ
4 + H_2O_2	40	0.20(8)	0.05(2)	0	0.25(10) λ	Ω
33 + H_2O_2	40	0.17(7)	0.02(1)	0	0.20(8) λ	Ω
DADS (mM)						
4	40	0.15(6)	0	0	0.15(6)i	Δ
34	26	0.04(1)	0	0	0.04(1)i	Δ
4 + H_2O_2	40	0.20(8)	0.02(1)	0	0.22(9) λ	Ω
34 + H_2O_2	40	0.02(1)	0	0	0.02(1) β	

[1] Statistical diagnosis according to Frei and Würgler [45]. + (positive) and i (inconclusive) versus negative control; β (significantly different) and λ (inconclusive) versus positive control. m: multiplication factor. Kastenbaum-Bowman Test without Bonferroni correction, probability levels $\alpha = \beta = 0.05$. [2] Number of spots or clones in parentheses. [3] Inconclusive results were resolved using Mann-Whitney U-test. Delta marker (Δ) means no differences between the treatments and the concurrent control. Ohm marker (Ω) means differences between the treatments and the concurrent control.

3.2. Longevity assays

Flies' survival curves for all treatments are plotted in **Figure 2**. In general, all treatments induce lifespan maintenance. As shown in **Table 2**, only DPDS significantly decreased the lifespan at two supplementation levels (8 and 16 mM). DPDS and DADS significantly decreased the mean healthspan by 17% and 14% respectively only at highest concentrations.

It is noteworthy that there is an agreement between lifespan and healthspan significances of DPDS at 8 and 16 mM.

Figure 2. Effects of garlic, onion, DADS and DPDS supplementation on the lifespan of *Drosophila*

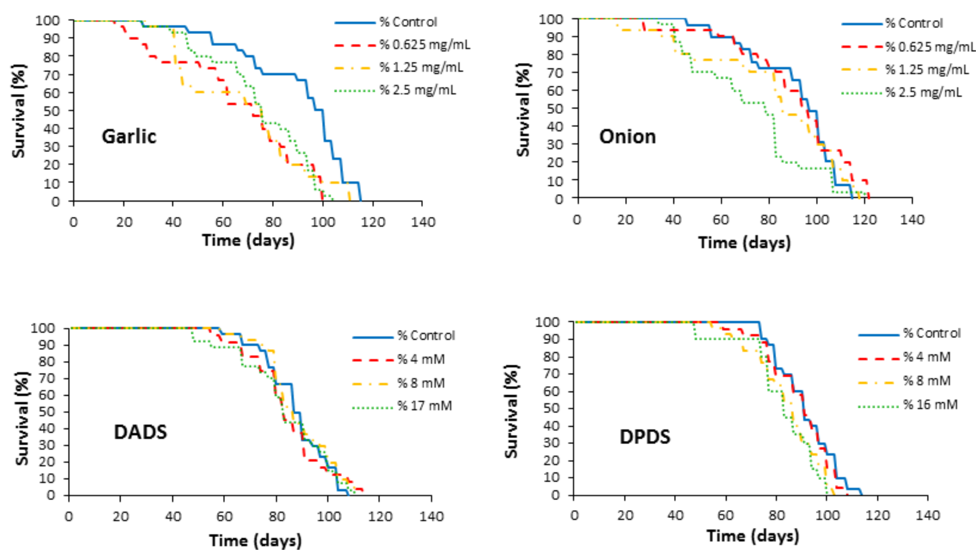

Table 2. Effects of the tested compounds at different concentrations on the *Drosophila melanogaster* mean lifespan and healthspan.

	Mean lifespan (days)	Mean lifespan difference (%)[a]	Health-span (75th percentile) (days)	Health-span difference (%)[a]
Onion (mg/mL)				
Control	92.24±3.58	0	76.00±12.63	0
0.625	95.77±3.45	4	83.00±5.04	9
1.25	92.83±3.36	1	83.00±5.08	9
2.5	81.92±4.98	-11	65.00±13.34	-11
Garlic (mg/mL)				
Control	81.25±4.57	0	51.14±4.31	0
0.625	79.51±3.30	-2	58.83±1.76	15
1.25	76.21±4.46	-6	47.29±3.73	-7
2.5	77.68±3.35	-4	53.57±3.48	5
DPDS (mM)				
Control	91.63±2.06	0	77.37±1.05	0
4	89.58±2.39	-2	73.00±2.80	-6
8	84.23±2.38*	-8	67.12±2.69**	-13
16	82.25±3.23*	-10	64.20±6.63*	-17
DADS (mM)				
Control	88.00±2.26	0	73.11±2.40	0
4	84.64±3.01	-4	68.00±3.34	-7
8	88.83±2.36	1	75.22±2.50	3
17	86.21±3.27	-2	62.87±3.96*	-14

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[a] Difference between treated flies and the concurrent negative control (water) in percentage. Positive results indicate that lifespan was increased and negative results indicate that lifespan was decreased. Statistical significance: * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$ (log-Mantel-Cox test)

3.3 Cytotoxicity and proapoptotic assays in leukaemia cells

The cytotoxic effects of *Allium* vegetables and their distinctive compounds (DADS and DPDS) on the survival of HL60 cells are shown in **Figure 3**. Garlic and DADS exerted a cytotoxic effect on cell growth in a positive dose-dependent manner after 72 h of incubation, with IC_{50} of 0.003 mg/mL and 0.06 mM respectively. On the contrary, the effect observed in DPDS was smaller, with a high IC_{50} of 0.25 mM and it was absent in onion treatments in which the cytotoxic effect resulted only in a growth inhibition of 30% at the higher tested concentrations.

Figure 3. Viability of HL60 cells treated during 72 hours with different concentrations of onion, garlic and their respective organosulfur compound, DPDS and DADS. Curves are plotted as mean percentages with respect to the control (three independent replicates). IC_{50}: Inhibition concentration 50.

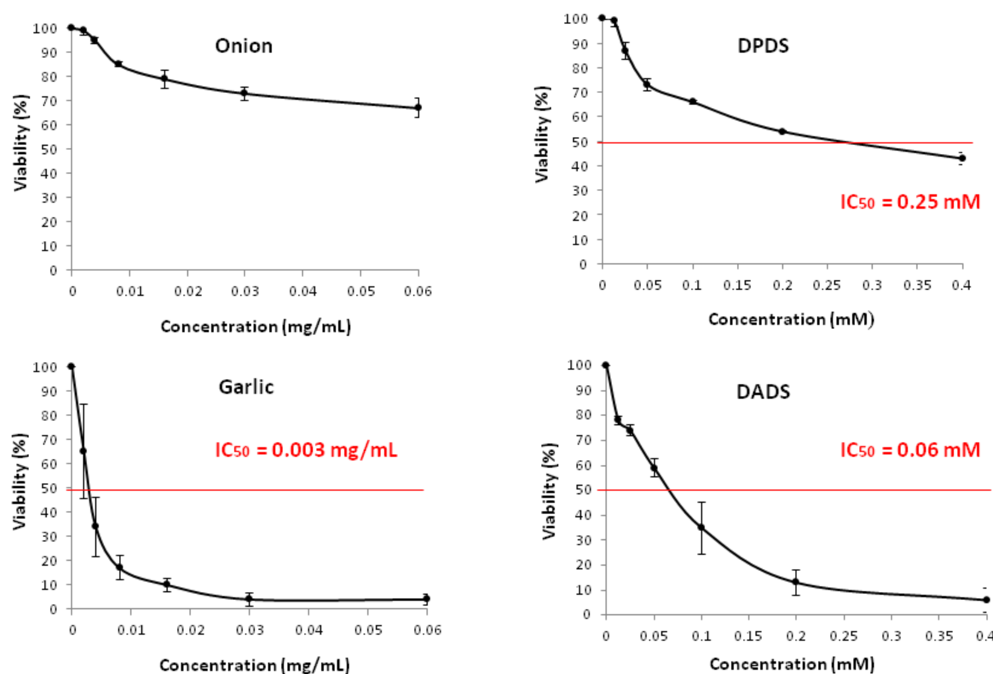

Figure 4 shows the results of proapoptotic effects of different concentrations of garlic, onion, DADS and DPDS in HL60 cells measured as internucleosomic programmed fragmentation [54]. DNA fragmentation was observed at high concentrations of garlic (0.03 and 0.06 mg/mL) and DADS (0.1, 0.2 and 0.4 mM). Nevertheless, any DNA internucleosomal fragments were induced neither by onion nor by DPDS at the assayed concentrations.

3.4 DNA single strand breaks

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Both vegetables induced a significant (p≤0.001) increase in the tail moment (TM) at all tested concentrations. On the contrary, only DADS -garlic most important organosulfur- was able to induce a significant (p≤0.01) increase of this parameter at 28 and 56 μM (**Figure 5**).

Figure 4. Internucleosomal DNA fragmentation. HL-60 cells were exposed to various concentrations of onion, garlic and their distinctive organosulfurs for 5h. DNA was extracted from cells and was subject to 2% agarose gel electrophoresis at 50 V for 90 min. M: DNA size marker.

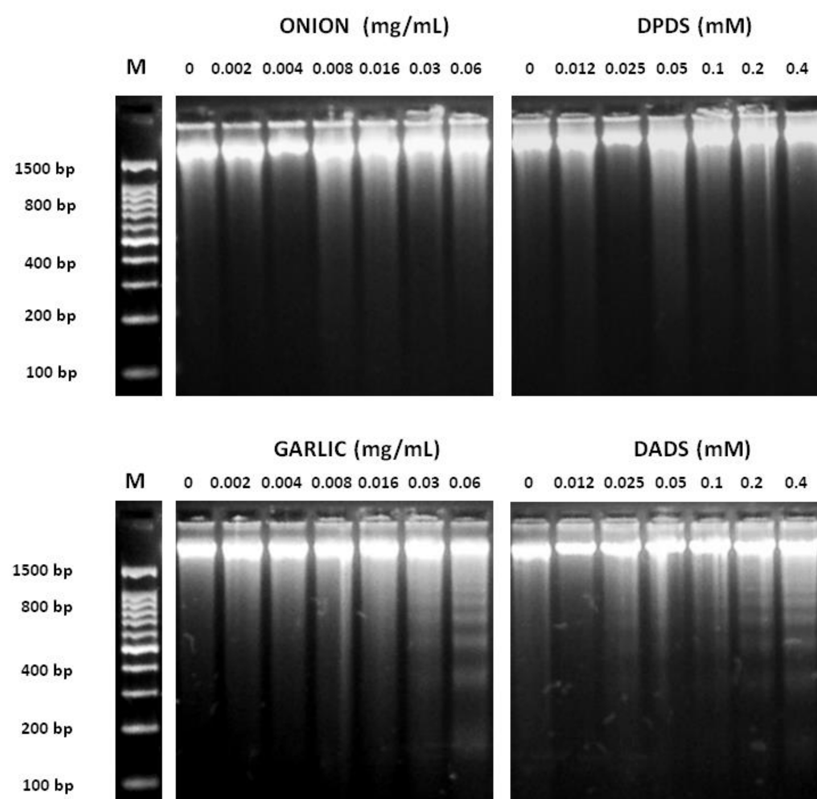
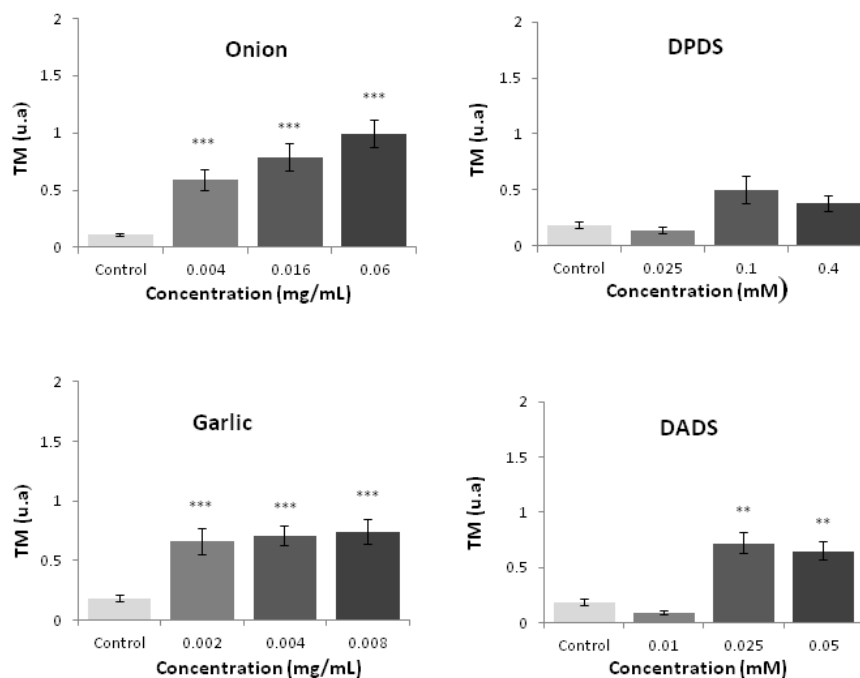


Figure 5. HL60 DNA integrity measured by the comet assay after 5 h-treatment with different concentrations of the tested compounds. Data are expressed as TM parameter [50]. Statitiscal significance compared with negative control: *** = p ≤ 0.000, ** = p ≤ 0.01 and * = p ≤ 0.05, for mean values of three independent replicates.



4. Discussion

4.1 In vivo assessment of the safety, protection and lifespan modulation.

Garlics and onions have traditionally been used as food sources around the world over centuries probably due to their demonstrated particular flavor but also due to the health benefits, such as the prevention of cardiovascular diseases, cancer and even aging [9]. Despite their popularity, the number of systematic, integrated and multifocal studies assessing the genotoxic, antigenotoxic and health-span effects are scarce, and even less for assessing their distinctive organosulfur compounds (DADS and DPDS).

Our *in vivo* DNA stability studies (genotoxicity, antigenotoxicity and longevity) were carried out using *D. melanogaster* flies. These organisms are widely used as a genetic animal model due to their homology with several mammal models in biological, physiological and neurological traits [55, 56]. It was demonstrated that more than 70 % of human disease-causing genes have a functional homologous in this fly model [57]. Additionally, this particular model was also largely used to evaluate the genotoxicity of different biological compounds and molecules due to its accuracy, robustness and reproducibility [58-60].

Carcinogen molecules and mutagenic properties should be taken into account and carefully evaluated in every complex mixture to be proposed for food. For this reason, genotoxic screening assays are considered as the first mandatory step, being the *Drosophila* wing spot test one of the most reliable methodologies to be employed as an ideal assay to evaluate biological products aimed to be used in human and animal diets. To our knowledge, this is the first study to characterize the genotoxic effect of garlic, onion and their two major and distinctive organosulfur constitutive molecules (DADS and DPDS respectively) using *D. melanogaster* animal model. Previous studies determined the lack of mutagenicity of these vegetables in a *Salmonella typhimurium* and in yeast models [61, 62]. It has also been demonstrated that aqueous garlic extracts (5% v/v), fine garlic powder supplementation (7.5, 5 and 2.5 g/Kg body weight) and fresh garlic bulb extracts (3, 6 and 12 mg/culture) were safe *in vitro* (cell lines) and non-animal models [62-65].

Our results for onion supplementation in the *Drosophila* model demonstrated a lack of genotoxicity, validating previous reports obtained by Kulkarni et al. [66] in several *Salmonella* strains. In the same way, DPDS and DADS, the active principles of garlic and onion, were also non-genotoxic in our SMART trials. It is noteworthy that despite the fact that onions are widely employed in the

human diet, the number of genotoxicity studies carried out of DPDS are scarce [67, 68] being our study the first time to test the safety and protective effects of this compound using *in vivo* models. Nevertheless, previous reports assessing these particular molecules are controversial. For instance, Musk et al. demonstrated that DADS induced both chromosome aberrations and sister chromatid exchanges, characterized as genotoxic effects, at lower concentrations (below 0.07 mM) in a Chinese hamster ovary cell line [69]. However, this controversy could partially be explained due to methodological differences (*in vivo vs in vitro* models) and the concentrations tested. Controversial results are commonly found for a single molecule when it is tested in different assays and *in vivo* carcinogenic trials are needed.

One of the strategies for coping with the food and environmental genotoxic compounds is to identify effective antimutagens and anticarcinogens in order to increase man´s exposure to them as a way for decreasing the cancer incidence [70]. This is the second step in the search of real nutraceutic substances. In our case, antigenotoxicity assays were conducted using hydrogen peroxide as positive genotoxicant model since this compound is able to induce somatic mutation and mitotic recombination in *D. melanogaster* [53] affecting the DNA integrity and stability.

Similar results to ours were reported on the desmutagenic activity of onions. Ethanolic extracts showed a strong inhibitory effect against NDBA in prokaryotes [29] and Welsh onion juice suppressed the mutagenic activity of Benzo [a] Pyrene (BaP) and 4-Nitroquinoline 1-oxide (4QNO) and reduced the number of 2,4-Dimethoxybenzaldehyde (DMBA)-induced chromosome aberrations in rats [71] whilst onion supplementation protected *D. melanogaster* against urethane-induced DNA damage [28]. All those reports validate our findings since onion supplementation reduced the mutagenic effects of H_2O_2 as much as 65% in a dose dependent manner. In the same way, DPDS, showed desmutagenic properties when it was tested as an individual molecule although in a lower extent compared with the effect on onions. In this sense, DPDS strongly increased dimethyl nitrosamine (DMN) mutagenicity in *S. typhimurium* [34] and reduced NPYR/NDMA-induced oxidative DNA damage in HepG2 cells at 5 μM [35]. However, our study is the first one demonstrating that *Allium* vegetables have a protective role against H_2O_2 induced damage using the *D. melanogaster* model, which is a more adequate model widely used to extrapolate to mammals. This effect could be due to its well-known scavenging potential against free-radicals of their respective organosulfur compounds [15, 72, 73], since similar results were observed in the vegetables and simple molecule assessments.

The desmutagenic activity of garlic and different types of garlic extracts was previously described in several induced mutagenesis models. It was demonstrated that garlic and garlic water extracts protected against gamma-radiation and cyclophosphamide in mice [63, 65, 74]. In the same way, methanolic and ethanolic garlic extracts, even prepared by different processing methods (raw, grilled and pickled), showed inhibitory activities on H_2O_2-induced DNA damage in human leukocytes [75] and reduced the chromosomal aberrations induced by DMBA in mice bone marrow [76]. In the same way, raw garlic methanolic extracts reduced the urethane mutagenicity in standard and high bioactivated *D. melanogaster* crosses [28]. In our experimental design, garlic clearly behaves as an antigenotoxine, which could potentially be explained by the fact that concurrent experiments using DADS as simple molecule also inhibited the 95% of the H_2O_2 -induced DNA damage. This desmutagenic property of DADS was previously proposed in several reports using different mutagenic substances such as (+)-anti-7β,8α-dihydroxy-9α,10α-oxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE), styrene oxide (SO), 4-NQO, aflatoxin B1 (AFB1), N-nitrosodimethylamine (NDMA) and 1-Nitrosopyrrolidine (NPYR) [35, 77, 78].

Longevity assays are one of most simple and efficient methodological approaches to evaluate the aging and anti-aging effects of simple compounds and complex mixtures on higher organisms. *D. melanogaster* is considered a very useful genetic model on aging research since its similarities with human metabolic pathways controlling nutrient uptake, storage and metabolism [79, 80]. In addition, this model has a short lifespan compared with similar *in vivo* models, reducing the experimental periods.

To our knowledge, this is the first assessment on the effect of onions, DADS and DPDS on the *D. melanogaster* lifespan and one of the few available assessing this effect in garlics [81]. These results

support the hypothesis that individual organosulfur compounds can reduce longevity in some extent. These compounds could primarily be responsible for the apparent reduced viability observed in some cohort groups of flies. A similarity between the complete food and their distinctive compounds in the lifespan behavior is observed, although in the case of vegetables the lifespan is not significantly reduced when comparing to the concurrent negative controls. Being onion and garlic complex mixtures of many individual molecules, the final outcome of such a complex trait longevity appears to be an additive combination of positive and negative synergic effects of the molecular components of vegetables, many of them phenolic and organosulfur which are not included in the present study. Previous reports showed beneficial effects of garlic extracts on animal lifespan, including *D. melanogaster* and *C. elegans* [18]. Those differences could be due to the different tested samples being raw garlic in our study and garlic extracts in previous reports. In this sense, Prowse et al. demonstrated the insecticidal activity of garlic juices across several life stages of flies at a wide range of concentrations (0.25-5 %) in two dipteran pests (*Delia radicum* and *Musca domestica*) [82]. These results agree with the fact that similar but not significant effects on lifespan were caused by garlic, onion, DADS and DPDS in our *D. melanogaster* experiments. It is noticeable that high doses were used for medicinal purposes in human acute treatments [83]. Thus, high dosages of garlic would not be advisable to be used in long term chronic treatments due to the adverse effects that could be associated, although nutraceuticals or dietary supplements include the bioactive compounds at higher doses than those used here.

4.2 In vitro assessment of the cytotoxic and clastogenic activities

Our results showed that only garlic and DADS have a strong cytotoxic effect and induce a clear DNA pro-apoptotic internucleosomal fragmentation against HL60 cells. Previous reports demonstrated that garlic and DADS exerted a chemopreventive effect through different pathways: (i) by increasing apoptosis and *Bcl-2* expression and decreasing p53 protein and *Bax* expression in lung cancer cells (NCI-H1299)[84]; (ii) by increasing intracellular ROS in A549 cells [19]; (iii) by inhibiting cell proliferation in CaCo-2 and HT-29 cells repressing histone deacetylase activity and histone hyperacetylation and increasing the p21(waf1/cip1) expression [85] and (iv) by inducing apoptosis through activation of caspase-3 expression in HL60 cells [86]. In addition, Yang et al. observed that DADS supplementation (0.5, 10 and 25 μM) had a pro-apoptotic effect in COLO 205 cell line through the induction of reactive oxygen species and caspase cascade [20]. On the contrary, the cytotoxic effect exerted by the onion and DPDS is relatively weak and their molecular mechanism is less explained. As example, Sundaram and Milner [87] demonstrated that DPDS (100 μM) was an inefficient molecule to inhibit the cell growth and to induce programmed cell death in tumour cells (HCT-15). However, Wu et al. suggested that onion oil induces cell cycle arrest and apoptosis through ROS production in A549 cells [32]. It was also been proposed that the carcinogenic inhibition mechanism of DADS is mediated through a modulation of the P450 cytochrome–dependent monooxygenases and/or the acceleration of carcinogen detoxification through phase II-enzymes upregulation [88, 89]. In our case, the chemopreventive properties of raw onion samples and DADS were weak despite the type of sample employed.

DNA internucleosomal fragmentation was defined as one of the hallmark of cellular apoptosis, although it cannot be considered as a single criterion to assess the apoptotic cell death [90]. In order to determine the ability of our tested substances to induce DNA breaks in HL60 cells, we employed a single cell gel electrophoresis (comet) assay, which it is widely used to detect the apoptotic capability of mixtures and single compounds to induce DNA damage [91, 92]. Nowadays, this procedure is being widely employed to evaluate the DNA stability in normal and carcinogenic cell lines against different substances, due to its robustness and reliability [93]. In this methodology we employed the tail moment (TM) index, which is an accurate parameter to quantify the DNA migration and thus, the DNA fragmentation status [50]. With this parameter we differentiated apoptosis-induced from necrosis-induced DNA damage as follows: a TM>30 is considered as an indicator of apoptosis and a TM between 5 and 30 is considered as a necrotic process [94].

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In this study we determined for the first time the DNA-damage exerted by garlic, onion, DADS and DPDS trough the alkaline "comet assay" in HL60 leukemic cells in order to assess their potential anticarcinogenic effect. Our results showed that onion and garlic induced DNA damage in HL60 by necrosis (short tails, TM<2) being in concordance with our cytotoxic and DNA-fragmentation results. Similar results were observed in DADS and DPDS but in a lower extent, suggesting the total absence of proapoptotic activity in the entire compound tested at the different assayed concentrations.

Our results with DPDS disagree with those obtained by Arranz, Haza [35], who showed that DPDS could act in a positive dose-dependent manner since the higher concentrations tested (>5 μM) caused DNA damage in HepG2 cells (data not shown) by comet assay. Arranz et al. assaying higher concentrations of DADS (>5 μM), showed DNA damage in HepG2 cells in the alkaline comet assay [35]. However, controversial results were also reported by Belloir et al. suggesting that DADS was not genotoxic at concentrations between 5-100 μM in the same *in vitro* model [95].

5. Conclusions

To sum up, our experimental results provide the evidence that (i) garlic, onion, DADS and DPDS are safe substances, exerting an antigenotoxic effect against oxidative mutagens in a dose dependent manner. (ii) The decrease of lifespan induced in the *Drosophila* animal model by DPDS at the highest concentrations could be a signal that the long term consumption of complex mixtures is safe only at low. (iii) Garlic exerted a clear chemopreventive effect, being its distinctive organosulphur DADS the most likely cause of such as activities. (iv). The slight cytotoxic effect of onion is probably mediated by non-apoptotic mechanism. Overall, this study could be a baseline for further supplementary studies to clarify the cell death pathway induced by garlic and DADS.

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