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Role of Citrus Juices and Distinctive Components in the Modulation of Degenerative Processes: Genotoxicity, Antigenotoxicity, Cytotoxicity, and Longevity in *Drosophila*

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ROLE OF CITRUS JUICES AND DISTINCTIVE COMPONENTS IN THE MODULATION OF DEGENERATIVE PROCESSES: GENOTOXICITY, ANTIGENOTOXICITY, CYTOTOXICITY, AND LONGEVITY IN *DROSOPHILA*

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It is well established that breakfast beverages contain high quantities of *Citrus* juices. The purpose of the present study was to assess the nutraceutical value of orange and lemon juices as well as two of their active compounds: hesperidin and limonene. Indicator assays were performed at three levels to evaluate different biological health promoter activities: (i) determination of the safety and DNA-damage protecting ability against free radicals by using the somatic mutation and recombination test (SMART) in *Drosophila melanogaster*, (ii) study of the modulating role for life span in *Drosophila melanogaster*, and (iii) measurement of the cytotoxic activity against the human tumor cell line HL60. The highest concentrations assayed for lemon juice and limonene (50% v/v and 0.73 m*M*, respectively) showed genotoxic activity as evidenced from SMART. Orange and lemon juices as well as hesperidin and limonene exhibit antigenotoxic activity against hydrogen peroxide used as an oxidative genotoxin. Life-span experiments revealed that the lower concentrations of orange juice, hesperidin, and limonene exerted a positive influence on the life span of *Drosophila*. Finally all substances showed cytotoxic activity, with hesperidin being least active. Taking into account the safety, antigenotoxicity, longevity, and cytotoxicity data obtained in the different assays, orange juice may be a candidate as a nutraceutical food as it (1) is not genotoxic, (2) is able to protect DNA against free radicals, and (3) inhibits growth of tumor cells.

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Inappropriate dietetic habits are estimated to be the cause of more than one-third of cancer deaths. Many of these cancers might be avoided with an increased consumption of fruits and vegetables (Smith-Warner et al. 2006). Plant-based foods provide the organism with high content in antioxidants that might help to protect cells from the biological damage produced by free radicals that trigger cancer development (Shi et al, 1998; Reddy et al. 2003). More precisely, fruit consumption has been associated with reduced risk of cancer of the upper digestive tract, stomach, and urinary tract (La Vecchia and Bossetti, 2006).

Orange juice (OJ) and lemon juice (LJ) contain a number of beneficial micronutrients, including phenols, vitamin C, minerals, dietetic fiber, essential oils, and carotenoids, that help to prevent degenerative processes such as diabetes, cardiovascular diseases, or certain types of cancer (A. A. Franke et al. 2005; González-Molina et al. 2010). The major flavonoid in sweet oranges and lemon is hesperidin (Gattuso et al. 2007; Garg et al. 2001), which is hydrolyzed by gut microflora into aglycone to form hesperetin (Vallejo et al. 2010). Hesperidin is used in treatment for hair fragility due to its ability to reduce the permeability of the vascular endothelium. This phenol exhibits antioxidative activity via antiradical and anti-lipoperoxidation activities (Tripoli et al. 2007). Hesperidin also exerts anti-

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inflammatory activity because it inhibits the LOX, COX, and phospholipase A enzymes (Benavente-García et al. 1997) and modulates glucose, cholesterol, and fatty acid metabolism (Jung et al. 2004; 2006). Hesperidin prevents bone mass loss (Chiba et al. 2003) and prevents chemically induced breast cancer (So et al. 1996), bladder cancer (Yang et al. 1997), and colon cancer (Tanaka et al. 1997a; 1997b; Miyagi et al. 2000) in animals.

The distinctive flavor component in OJ and LJ is limonene. This monocyclic terpene is the major component in the citrus essential oils (Crowell, 1999; González-Molina et al. 2010), used as flavor in cosmetics, beverages, foods, and gums. Although mutagenicity assays showed negative results in *Salmonella* (National Toxicology Program, 1990) and rats (Turner et al. 2001), it is considered a nongenotoxic carcinogen (Tennant and Ashby, 1991). Lu et al. (2004) suggested that limonene may be of interest in chemoprevention because it inhibits tumor growth and metastasis via apoptosis.

Fresh homemade citrus juices are one of the most popular fruit beverages as a member of so-called healthy breakfasts. Therefore, it was of interest to evaluate the nutraceutical potency of a chronically consumed food through the entire life of subjects Several testing protocols need to be established for a food to be considered a health promoter: (i) safety with respect to genetic damage; (ii) potential protective role of DNA integrity; (iii) influence on life-span extension as a complex biological trait; and (iv) specific cytotoxic activity against transformed cells as chemopreventive agent.

The somatic mutation and recombination test (SMART) has been used to detect mutagenic and recombinagenic activity in the clone expansion of imaginal discs of *Drosophila melanogaster* larvae. This wing spot test was found to be a versatile and reliable system to test genotoxicity and antigenotoxicity of single compounds as well as complex mixtures due to the capabilities of treated larvae to bioactivate metabolites (Graf et al. 1994; Anter et al. 2010). The ability of LJ, OJ, and two of their major components (hesperidin and limonene) to inhibit the mutagenicity induced by a model oxidative genotoxin such as hydrogen peroxide (H_2O_2) was examined. H_2O_2 produces oxidative damage to DNA by generating adducts, such as 8-hydroxyguanine, that exert an important role in the mutagenesis process with an increase of induced transitions (Shi et al. 1998; Lim and Lim 2006). Hydrogen peroxide induces also a deregulation of methylation patterns of oncogenes (Cerda and Weitzman 1997) and inhibition of DNA repair enzymes (Hu et al. 1995).

The expected health-promoting properties of LJ, OJ, and their distinctive compounds might extend the longevity in *Drosophila melanogaster*. The life span of this insect is relatively short and adults seem to show many of the cell senescence features seen in mammals (Fleming et al. 1992). For this reason the fruit fly has been extensively used in studies of physiological, pathological, and other processes involved in life expectancy, as well as to understand the relationships between food metabolism and ageing (Li et al. 2010). Average life-span data of *Drosophila melanogaster* vary widely and are dependent on the rearing conditions (Trotta et al. 2006; Li et al. 2008; Mockett and Sohal, 2006).

Cytotoxicity bioassays in vitro are also needed in the assessment of the chemopreventive effects of a substance as a fast, not expensive, and informative first step for screening. The human cell line HL60 provides a reliable model to study the cytotoxic effect of chemopreventive substances and the mechanisms underlying this potential activity (Villatoro-Pulido et al. 2009). Once the cytotoxic activity of a nutraceutic had been assayed, a visible test of DNA fragmentation was carried out in order to investigate whether the mechanism undergoing the cytotoxicity was mediated via apoptosis.

METHODS

Fruits and Single Compounds

Juices from two *Citrus* species and two single compounds were selected. Oranges (*Citrus sinensis* var. Valencia Late) and lemons (*Citrus*

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limon var. Lunario) were obtained from a local market. Hesperidin and limonene were purchased from Sigma and Fluka (H5254 and 62118, respectively).

Preparation of the Samples

Fruits were washed with ethanol (70%) prior to extraction of the juice. Both OJ and LJ were prepared using a domestic manual squeezer. Fresh juices from 10 fruits were mixed, aliquoted, and stored at –80°C until utilization. In the case of cytotoxicity assays, juices were centrifuged for 1 min at 12,000 × g and the supernatant was stored at –80°C. Limonene was dissolved in ethanol.

Genotoxicity and Antigenotoxicity Assays (SMART)

Drosophila melanogaster strains. Two *Drosophila* strains were used, each with a hair marker in the third chromosome:

- *mwh*/*mwh*, carrying the recessive mutation *mwh* (*multiple wing hairs*) that produces multiple tricomas/cell (Yan et al. 2008).
- *flr*^{3}/*In (3LR) TM3, ri p*^{p} *sep bx*^{34e} *e*^{s} *Bd*^{S}, where the *flr*^{3} (*flare*) marker is a homozygous recessive lethal mutation that produces deformed tricomas but is viable in homozygous somatic cells once larvae start the development (Ren et al. 2007). Lindsley and Zimm (1992) provide more detailed information on the rest of the genetic *Drosophila* markers.

Drosophila were maintained at 25°C, 80% humidity, with a homemade meal (1 L water, 0.5 g NaCl, 100 g yeast, 25 g sucrose, 12 g agar-agar, 5 ml propionic acid, 3.5 ml of a 0.2% sulfate streptomycin solution) with three changes per week.

Treatments. The genotoxicity assays were carried out following the method described by Graf et al. (1984). Briefly, trans-heterozygous larvae for *mwh* and *flr*^{3} genes were obtained by crossing 200 optimally virgin females (4 d old) of *flr*^{3} strain with 100 males of *mwh* strain. Four days after fertilization, females were allowed to lay eggs in fresh yeast medium for 8 h in order to obtain synchronized larvae. After 72 h, larvae were washed with distilled water and groups of 100 individuals were placed in different treatment vials where a chronic treatment was followed until pupation. Treatment vials contained 0.85 g of *Drosophila* Instant Medium (Formula 4-24, Carolina Biological Supply, Burlington, NC) and 4 ml of different concentrations of the substance to be tested. Two concentrations of each citrus juice were assayed (0.75% v/v and 50% v/v) as well as hesperidin and limonene (0.0038 and 0.34 m*M*, 0.011 and 0.73 m*M*, respectively). Single compound concentrations correspond to the content level found in the fresh juices (Gattuso et al. 2007; Selli et al. 2004). The negative controls were prepared with medium and water and positive controls with medium and 0.15 *M* H_2O_2 (Sigma, H1009) as an oxidative genotoxicant (Anter et al. 2010).

The antigenotoxicity tests were performed following the method described by Graf et al. (1998), which consisted of combined treatments of genotoxin (0.15 *M* H_2O_2) and different concentrations of juices, hesperidin, or limonene. After emergence, adult flies were stored in 70% ethanol.

Mutations scoring. Forty trans-heterozygous marker wings (*mwh flr*$^+$/*mwh*$^+$ *flr*^{3}) of each control and concentration were mounted on slides using Faure's solution and scored under a photonic microscope at 400× magnification. Similar numbers of male and female wings were mounted and wing hair mutations were scored from a total of 24,400 monotricoma wild-type cells per wing (Alonso-Moraga and Graf 1989). In the balancer-heterozygous genotypes (*mwh*/TM3, *Bd*^{S}), *mwh* spot phenotypes are produced predominantly by somatic point mutation and chromosome aberrations, since mitotic recombination between the balancer chromosome and its structurally normal homologue is a lethal event. To quantify the recombinagenic potency of the positive control, the frequency of *mwh* clones on the marker trans-hetorozygous wings (*mwh* single spots plus twin spots) was compared with the frequency of *mwh* spots

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on the balancer trans-heterozygous wings. The difference in *mwh* clone frequency is a direct measure of the proportion of recombination (Frei et al. 1992). In the case of genotoxic results for single treatments, balancer wings (*mwh*/*Bd*^{s}) were also mounted in order to quantify the somatic recombinogenic activity (R) of the substance (Zordan et al. 1991) using the following formula:

$$R = (1 - \textit{mwh} \text{ spots on the balancer wings/}\ \textit{mwh} \text{ spots on the marker wings}) \times 100$$

Data evaluation and statistical analysis. Wing hair spots were grouped into three different categories: S, a small single spot corresponding to one or two cells exhibiting the *mwh* phenotype; L, a large single spot with three or more cells showing *mwh* or *flr*^{3} phenotypes; 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 spots was also determined.

A multiple-decision procedure was applied to determine whether a result is positive, inconclusive, or negative (Frei and Würgler, 1988, 1995). The frequencies of each type of mutant clone per wing were compared to the concurrent negative control and the significance was taken at the 5% level. All inconclusive and positive results were analyzed with the nonparametric *U*-test of Mann, Whitney, and Wilcoxon ($\alpha = \beta = .05$, one sided). In combined treatments the inhibition of mutagenic events for juices and single compounds was calculated for total spots as proposed by Abraham (1994) by means of the following formula:

$$\text{Inhibition} = (\text{genotoxin alone} - \text{sample plus genotoxin}) \times 100/\text{genotoxin alone}$$

Lifespan Assays

Drosophila melanogaster strains. Animals who undergo the longevity experiments exhibited the same genotype as in genotoxicity assays in order to compare genotoxicity and longevity results. The F_1 progeny from *mwh* and *flr*^{3} parental strains produced by an egg lying of 24 h in yeast was used. Longevity experiments were carried out at 25°C following the procedure of Chavous et al. (2001). Briefly, synchronized trans-heterozygous 72-h larvae were washed and separated into groups of 100 individuals in vials with a mixture of Instant Medium and 4 ml of different concentrations of the 4 substances selected. Emerging adults were collected, anesthetized under CO_2, and placed in 1-ml longevity vials in groups of 10 individuals. Three replicates were used during the complete live extension for each control and concentration established. The survivals were counted and media renewed twice a week.

Statistical analysis of life span. The Kaplan–Meier estimates of the survival function for each control and concentration are plotted as survival curves. The statistical analyses and significance of the curves were assessed by SPSS 15.0 statistics software (SPSS, Inc., Chicago) using the log-rank (Mantel–Cox) method.

Cytotoxicity Assays

Cell culture. The promyelocytic leukemia cell line HL60 was used to assess the cytotoxic effects of juices and phenols. Cells were cultured in RPMI 1640 medium (Biowhittaker, BE12-167F), supplemented with 10% heat-inactivated bovine serum (Biowhittaker, DE14-801F), 200 m*M* L-glutamine (Sigma, G7513), and an antibiotic–antimycotic solution with 10,000 U penicillin, 10 mg streptomycin, and 25 µg amphotericin B per milliliter (Sigma, A5955). Cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The cultures were plated at a density of 25×10^4cells/ml and transferred every 2 d.

Assessment of cell viability. HL60 cells were placed in 12-well culture plates

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(1×10^5cells/ml) and treated for 72 h with different concentrations of OJ, LJ, hesperidin, and limonene. The cell viability was assessed utilizing the trypan blue exclusion method. Trypan blue (Fluka, 93595) was added to the cell culture with a volume ratio of 1:1. The number of living cells was counted using a hemocytometer under an inverted microscope (Motic, AE30/31) at 100× magnification. Each experiment was repeated in triplicate, growth curves were established, and IC_{50} values were estimated. Curves are plotted as percent survival with respect to control growth at 72 h.

Analysis of DNA fragmentation. In order to detect DNA fragmentation in cells entering apoptosis, HL60 cells (1.5×10^6/ml) were treated with different concentrations of the substances for 5 h. Treated cells were collected, centrifuged at 2500 × g for 5 min. and washed with PBS. DNA was extracted using a commercial DNA extraction kit (Dominion mbl, 243) and was treated with RNase before loading. A final quantity of 1500 ng DNA was subjected to a 2% agarose gel electrophoresis (50 V for 2 h) and stained with ethidium bromide.

RESULTS

Genotoxicity and Antigenotoxicity Testing of Citrus Juices and Components

The SMART assay was used to assess the health-promoting properties of citrus species and its distinctive compounds. Table 1 shows the results for genotoxicity testing of the four substances assayed using SMART. All substances were nonmutagenic at the lowest concentration used. This lower concentration was chosen taking into account the daily food intake for a *Drosophila* larva and giving a similar juice intake to a human consumption of 250 ml/d. Nevertheless, LJ and limonene were mutagenic (0.325 spots/wing) in the SMART at the highest concentration (50% v/v and 0.73 m*M*, respectively). In order to evaluate the recombinogenic potency of mutagenic concentrations, the spots per wing scored in balancer-heterozygous wings were examined where *mwh* clones reflect only somatic point mutations and chromosome aberrations, since somatic recombination is a lethal event. Values of recombinogenicity with respect to the total induced clones were 77 and 62.5% for LJ and limonene, respectively.

Table 2 shows the results for antigenotoxicity assays performed in the combined treatments where larvae were fed chronically with the genotoxicant H_2O_2 (0.15 *M*) and different concentrations of OJ, LJ, or components. Hydrogen peroxide is a well-known mutagen in *D. melanogaster* and has been used to induce microsatellite instability in mismatch repair mutants (López et al. 2002). Hydrogen peroxide induced a mutation rate of 0.45 spots/wing. This result is in agreement with other data using the same genetic background (Anter et al. 2010; Villatoro-Pulido et al. 2009). The antigenotoxic potency of four substances studied against H_2O_2 showed no clear-cut dose-response effect. Average values for the inhibition percent genotoxicity of H_2O_2 were 16.5, 41.6, 47, and 64% for LJ, hesperidin, OJ, and limonene, respectively.

Longevity Assays

Figure 1 shows the survival curves obtained by the Kaplan–Meier method for *Drosophila melanogaster* under chronic treatment with different concentrations of LJ, OJ, hesperidin, and limonene and controls. The entire life-span curves were analyzed statistically by the log-rank (Mantel–Cox) method (data not shown). For controls, average and maximum of entire life span values were 99.2 and 123 d, respectively. Log-rank (Mantel–Cox) analyses for complete life span showed no significant differences between treatment curves and control for OJ. In the case of LJ, curves for higher concentrations (3, 12.5, and 50% v/v) were statistically different from the water control, and the lower concentration (0.75% v/v) ones produced a decrease of life span. Hesperidin curves at 0.15 and 0.06 m*M* were also statistically lower than water control and the lowest concentration (0.0038 m*M*) curves. Finally, 0.0111 and 0.18 m*M* limonene supplementation significantly

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TABLE 1. Genotoxicity of Lemon and Orange Juices, Hesperidin, and Limonene in the *Drosophila* Wing Spot Test

Compounds	Number of wings	Small spots (1–2 cells) $m = 2$			Large spots (more than 2 cells), $m = 5$			Twin spots, $m = 5$			Total spots, $m = 2$			c
		No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	
Negative control (H_2O)	40	6	0.15		0	0		0	0		6	0.15		
Lemon juice (% v/v)														
0.75	40	11	0.275	i(ns)	0	0	–	0	0	–	11	0.275	i(ns)	1.146
50.00	40	12	0.3	i(ns)	0	0	–	1	0.02	i(ns)	13	0.325	i(*)	1.354
50.00 *Serrate*	40	3	0.075		0	0		0	0		3	0.075		
Orange juice (%v/v)														
0.75	40	9	0.225	i(ns)	0	0	–	0	0	–	9	0.225	i(ns)	0.937
50.00	40	6	0.15	i(ns)	1	0.02	i(ns)	0	0	–	7	0.175	i(ns)	0.729
Hesperidin (m*M*)														
0.0038	40	11	0.275	i(ns)	0	0	–	0	0	–	11	0.275	i(ns)	1.146
0.2400	40	6	0.15	i(ns)	0	0	–	0	0	–	6	0.15	i(ns)	0.625
Limonene (m*M*)														
0.011	40	11	0.275	i(ns)	0	0	–	0	0	–	11	0.275	i(ns)	1.146
0.73	40	12	0.3	i(ns)	1	0.02	i(ns)	0	0	–	13	0.325	i(*)	1.354
0.73 *Serrate*	40	5	0.125		0	0		0	0		5	0.125		

Note. No., number of spots; Fr., frequency; D., statistical diagnosis according to Frei and Würgler (1988); m, multiplication factor; c, frequency of clone formation per 10^5 cells; +, positive; Serrate, balancer-heterozygous Beaded Serrate genotype wings; −, negative; ns, nonsignificant ($p > .05$), Asterisk indicates significant ($p < 0.05$). The inconclusive and positive data were evaluated by the nonparametric *U*-test of Mann, Whitney, and Wilcoxon according to Frei and Würgler (1995).

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TABLE 2. Antigenotoxicity of Lemon and Orange Juices, Hesperidin, and Limonene in the *Drosophila* Wing Spot Test

Compounds	Number of wings	Small spots (1–2 cells), $m = 2$			Large spots (more than 2 cells), $m = 5$			Twin spots, $m = 5$			Total spots, $m = 2$			IP (%)
		No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	
Negative control (H_2O)	40	6	0.15		0	0		0	0		6	0.15		
Positive control (H_2O_2; 0.15 *M*)	40	16	0.4	+(*)	1	0.02	i(ns)	1	0.02	i(ns)	18	0.45	+(*)	
Lemon juice (% v/v)														
0.75	40	13	0.325	i(ns)	0	0	–	1	0.02	i(ns)	14	0.35	i(ns)	22.2
50.00	40	16	0.4	+(*)	0	0	–	0	0	–	16	0.4	+(*)	11.1
Orange juice (% v/v)														
0.75	40	10	0.25	i(ns)	0	0	–	0	0	–	10	0.25	i(ns)	44.4
50.00	40	4	0.1	i(ns)	5	0.125	+(*)	0	0	–	9	0.225	i(ns)	50.0
Hesperidin (m*M*)														
0.0038	40	7	0.175	i(ns)	1	0.02	i(ns)	0	0	–	8	0.2	i(ns)	55.5
0.2400	40	12	0.3	i(ns)	0	0	–	1	0.02	i(ns)	13	0.325	i(ns)	27.8
Limonene (m*M*)														
0.011	40	8	0.2	i(ns)	0	0	–	0	0	–	8	0.2	i(ns)	55.5
0.73	40	3	0.075	i(ns)	1	0.02	i(ns)	1	0.02	i(ns)	5	0.125	i(ns)	72.2

Note. No., number of spots; Fr., frequency; D., statistical diagnosis according to Frei and Würgler (1988); i, inconclusive data; *m*, multiplication factor; +, positive; −, negative; ns, nonsignificant ($p > .05$), Asterisk indicates significant ($p < 0.05$). The inconclusive and positive data were evaluated by the nonparametric *U*-test of Mann, Whitney, and Wilcoxon according to Frei and Würgler (1995). IP, inhibition percentage.

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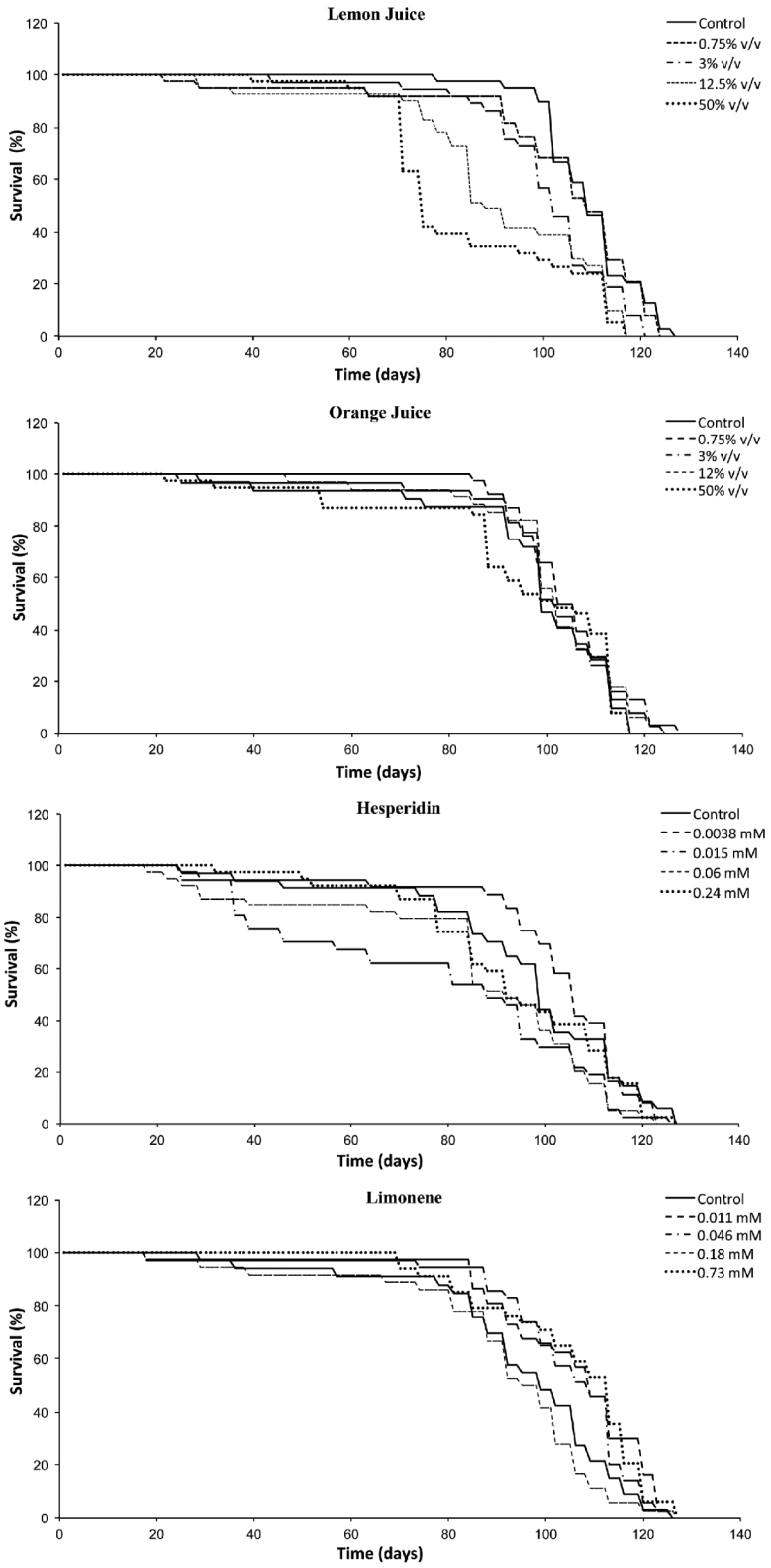

FIGURE 1. Survival curves of *Drosophila melanogaster* fed with different concentrations of lemon and orange juices, hesperidin, and limonene over time.

increased the average life span compared to control flies fed normal food.

In Vitro Human Leukemia Cytotoxicity Assay

A wide range of concentrations was used for every substance (0.625–2.5%, 0.75–20%, 0.37–25 m*M*, and 0.035–2.34 m*M*, for LJ, OJ, hesperidin, and limonene, respectively). Figure 2 shows the relative tumor growth inhibition for the substances assayed. Lemon juice presented an IC_{50} (1.4%) lower than for OJ (4.4%). The concentration-response curves were different for the two juices, with LJ exhibiting a wide plateau for the lower concentrations. Hesperidin and limonene exerted cytotoxic effects on HL60 cells, although the IC_{50} for limonene (0.2 m*M*) was lower than for hesperidin (14 m*M*).

Figure 3 shows the electrophoresis of the genomic integrity in HL60 cells treated for 5 h with different concentrations of the substances. DNA nucleosomal fragmentation was observed in median to highest concentrations of LJ (0.8, 1.2, 1.4, 1.6, 1.8, and 2% v/v) and at the three highest concentrations of limonene (0.6, 1.2, and 2.35 m*M*). This characteristic laddering of apoptotic activity was not observed with OJ or hesperidin.

DISCUSSION AND CONCLUSIONS

The results in the wing spot test for OJ yielded nonsignificant values at the assayed concentrations compared to control. Mutagenicity of OJ was previously found only in the Ames *Salmonella* test using the TA97a and TA98 strains with and without metabolic activation (Franke et al. 2004). In contrast, when the Swiss Webster mouse eukaryotic model was used to carry out the comet assay in peripheral white blood cells, OJ was nongenotoxic (S. I. R. Franke et al. 2005). As *Drosophila* is a eukaryotic model, our results in the wing spot test are in agreement with those of the comet assay in mouse. Lemon juice was tested in the wing spot test of *Drosophila* and data demonstrated a genotoxic inducing recombinogenic activity at the higher concentration (50% v/v). Our findings provide the first result available with respect to the genetic safety of LJ. Hesperidin was nongenotoxic in the SMART assay of

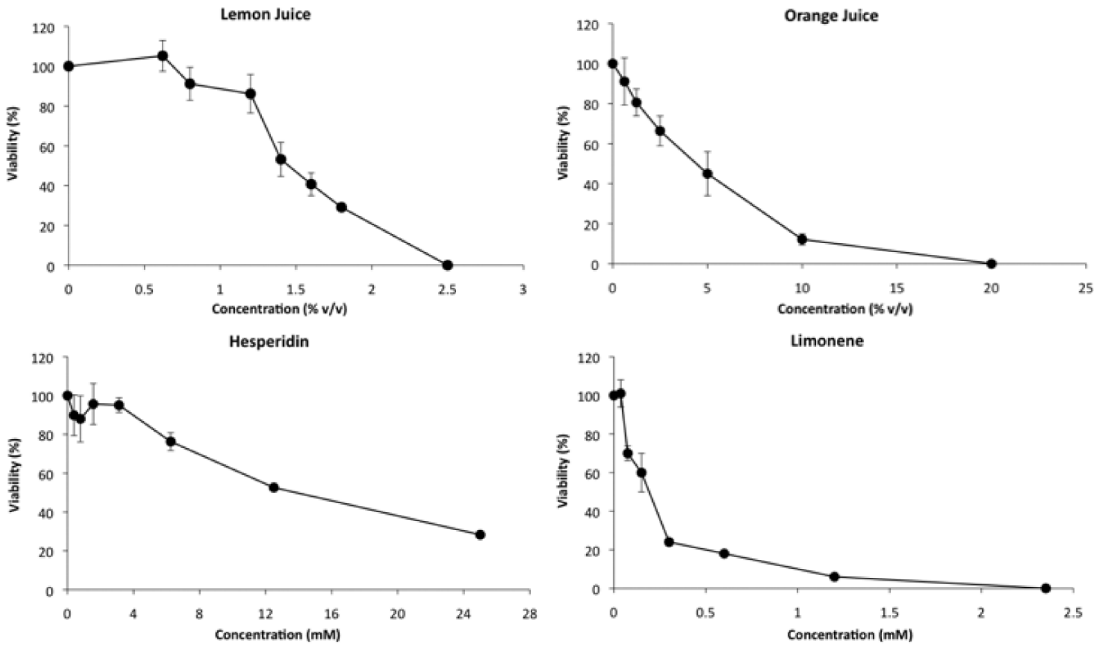

FIGURE 2. Cytotoxicity of lemon and orange juices, hesperidin, and limonene on HL60 cells.

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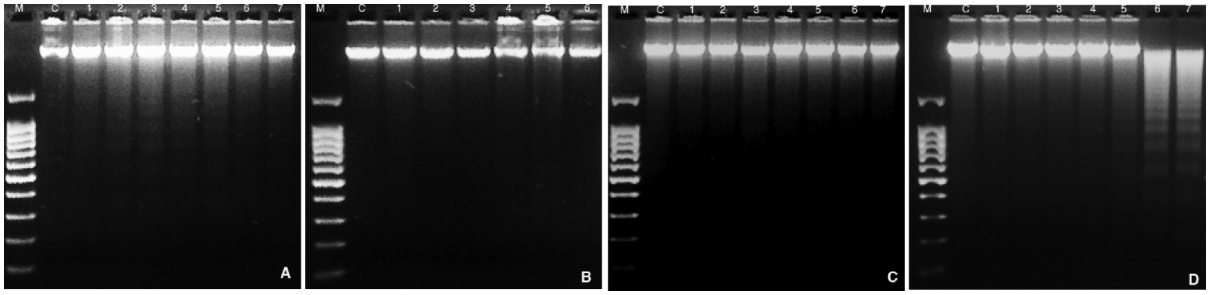

FIGURE 3. DNA fragmentation induced in HL60 cells by lemon and orange juices (A, B), hesperidin (C), and limonene (D). HL60 human leukemia cells were exposed for 5 h to different concentrations of tested compounds. DNA was extracted from cells and subsequently subject to 2% agarose gel electrophoresis at 50 V for 90 min. Lemon juice (A): marker (lane M); control (lane C); 0.62 % v/v (lane 1); 0.8 % v/v (lane 2); 1.2 % v/v (lane 3); 1.4 % v/v (lane 4); 1.6% v/v (lane 5); 1.8 % v/v (lane 6); 2 % v/v (lane 7). Orange juice (B): marker (lane M); control (lane C); 0.62 % v/v (lane 1); 1.25 % v/v (lane 2); 2.5 % v/v (lane 3); 5 % v/v (lane 4); 10 % v/v (lane 5); 20 % v/v (lane 6). Hesperidin (C): marker (lane M); control (lane C); 0.39 m*M* (lane 1); 0.78 m*M* (lane 2); 1.52 m*M* (lane 3); 3.12 m*M* (lane 4); 6.25 m*M* (lane 5); 12.5 m*M* (lane 6); 25 m*M* (lane 7). Limonene (D): marker (lane M); control (lane C); 0.037 m*M* (lane 1); 0.075 m*M* (lane 2); 0.15 m*M* (lane 3); 0.3 m*M* (lane 4); 0.6 m*M* (lane 5); 1.2 m*M* (lane 6); 2.35 m*M* (lane 7).

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Drosophila melanogaster, and our results agree with the lack of genotoxicity detected in the *Salmonella* TA98 assay with or without metabolic activation by Van der Merwe et al. (2006). Limonene was not mutagenic in the Ames system using four strains of *Salmonella typhimurium* (TA98, TA100, TA1535, and TA1537) (National Toxicology Program 1990). Nevertheless, our results indicated genotoxic activity for limonene at the higher assayed concentration in the *Drosophila* wing spot test. Our data showed that limonene may produce oxidative stress and ROS generation acting as a pro-oxidant at the highest concentration. This finding agrees with the results in eukaryotic cells that suggest that limonene may act as a pro-oxidant agent dependent upon concentration (Bakkali et al. 2008). Genotoxicity results of the citrus juices and components assayed suggested that limonene content of LJ might be responsible for the recombinagenic activity observed at the highest concentration of LJ. The differential content of limonene in LJ and OJ (86 and 17 mg/L) noted by Maccanore et al. (1998) might reflect an association between genotoxicity induced by LJ and limonene at the higher concentration.

Our antigenotoxicity data for OJ obtained in *Drosophila* against H_2O_2 (47% average percent inhibition) are in agreement with those obtained by S. I. R. Franke et al. (2005), who demonstrated that OJ inhibited DNA damage produced by alkylating agents in the mouse comet assay. Higashimoto et al. (1998) found a 36% mutagenicity-reducing activity of LJ against nitrite-treated 1-methyl-1,2,3,4-tetrahydro-carboline-3-carboxylic acid (MTCCA) using the TA100 strain of *Salmonella typhimurium*. Our results for LJ are also in agreement with the AMES test showing an average percent inhibition of 16.5%. The different antigenotoxic potencies of the OJ and LJ may be related to differential content of antioxidants. It is known that the antioxidant potency of citrus is due to ascorbic acid and phenolic content (Gardner et al. 2000) and that OJ contains higher β-carotene equivalents, ascorbic acid, and total phenolics than LJ (Xu et al. 2008).

The inhibitory capacity of hesperidin against the genotoxic effects of H_2O_2 in the imaginal discs of *Drosophila* was higher at the lowest concentration (55.5%). Kalpana et al. (2009) found hesperidin produced radioprotection by effectively decreasing MN frequency, dicentric aberrations, and comet attributes, and correlated this activity with an ability for ROS scavenging. The higher content of hesperidin in OJ in comparison to LJ, 58 and 20 mg/100 ml, respectively (Cano et al. 2008; Gattuso et al. 2007), may account for the antigenotoxic ability of OJ. Limonene inhibited the genotoxicity of H_2O_2, behaving as a reductor agent that would protect cells from H_2O_2-induced

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oxidative stress (Roberto et al. 2009; La Rotta-Hernández, 2007).

The antiproliferative activity of OJ was tested in various K562 (human chronic myelogenous leukemia), HL60 (human leukemia), and MCF-7 (human breast adenocarcinoma) cell lines, showing that a concentration of 10% v/v was able to inhibit 73% HL60 cell growth (Camarda et al. 2007), corresponding to 85% in our experiments. The cytotoxicity of LJ against HL60 cells found in the present investigation was also reported for Caco-2 and HpG_2 cell lines (Lim and Lim 2006; Sun et al. 2002). The cytotoxicity of hesperidin was determined in different cell lines (MDA-MB-435 ER-, MCF-7 ER+, DU-145, HT-29, DMS-114, SK-MEL5) by Manthey and Guthrie (2002), who showed no marked antiproliferative activity due to the glysosylation of the molecule moiety. Nevertheless, many in vivo studies concluded that hesperidin presents anticancer activity in lung, oral, colon, and bladder carcinogenesis (Kamaraj et al. 2009; Tanaka et al. 1997a; 1997b; Yang et al. 1997); these results are in concordance with the in vitro data in the present study performed with HL60 cell line. With respect to the cytotoxicity of limonene, Tatman and Mo (2002) obtained an inhibitory concentration similar to that in the present study (0.18 and 0.20 m*M*, respectively). In vivo assays for limonene are contradictory, as it seems to inhibit the appearance of liver and gastric tumors in mice (Parija and Das 2003; Lu et al. 2004) but Turner et al. (2001) found that limonene induced kidney and bladder tumors in male rats.

Cleavage of chromosomal DNA into oligonucleosomal size fragments is a biochemical hallmark of apoptosis. The results of our study showed fragmentation of DNA upon treatment of HL60 cells with LJ and limonene, indicating the involvement of apoptosis. A concentration-dependent relationship in the treatment with LJ at lower concentrations was observed. At the two highest doses, it seems that cells are undergoing necrotic processes, which might explain the absence of DNA fragmentation. Limonene induced a slight DNA fragmentation at 0.6 m*M*. This effect was more apparent at highest concentrations (1.2 and 2.35 m*M*) resulting in a concentration-dependent response. These observations may be related to an initiation of the apoptotic process in the tumoral HL60 cells. Limonene seems to act like a pro-apoptotic agent with promising antitumoral properties. Rabi and Bishayee (2009) demonstrated the apoptotic effect of limonene in DU-145 prostate cancer cells but not in normal epithelial prostate PZ-HPV- 7 cells. In human colon cancer cells (SW-480) a DNA fragmentation and induction of caspase-3 by lime volatile oils were noted, which may be due to the involvement of apoptosis mechanism (Patil et al. 2009).

The survival curves for control and the rest of substances at $\geq$75% of living flies were compared. The health span significance was as follows: OJ lower concentration treatments of 0.75 and 3.25% v/v compared to nonenriched diet water control significantly increased health span. Every LJ treatment decreased health span when compared to control, as the low pH of lemon juice (2.3) negatively and differentially affected *Drosophila* adult survival (Mai et al. 2010). A lower concentration of hesperidin (0.0038 m*M*) significantly increased the health span, and the two lowest concentrations of limonene (0.011 and 0.046 m*M*) also significantly improved health span. Taking into account that the maximum average life spans for $\leq$75% survivals are 91, 98, 95, 92, 95, and 92 d for control and the already-mentioned corresponding OJ, LJ, hesperidin, and limonene significant concentrations, a general increase trend was observed in both mean and maximum life span. This implies there was an increase of the health span portion of the life span. Taken together, this study indicated the effects of OJ and LJ on *Drosophila melanogaster* longevity are a result of a combination of antioxidative and prooxidative activities. Given that the fruit fly is an important model for studies on human nutrition and pharmacology, the results of this study suggest that moderate consumption of OJ and its active and predominant components (hesperidin and limonene) may have the potential to strengthen the antioxidant defense system and consequently extend

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the life span and increase the health span. However, considering the fact that citrus juices may also exhibit pro-oxidant activities toward mitochondria, life-span extension may vary depending upon genetic and environmental factors (Arking 2005).

The results obtained in the present study showed different aspects of the activity of LJ, OJ as well as hesperidin and limonene. Genotoxicity data demonstrated mutagenic activity for LJ and limonene at the highest concentrations. Antigenotoxicity assays indicated that all the genetic safe concentrations are antigenotoxic, showing different percent inhibition. All substances exerted cytotoxic activity, although only LJ and limonene produced DNA fragmentation as an apoptotic mechanism. Finally, as a biological multivariate trait, life-span studies suggested that the lower concentrations of OJ, hesperidine, and limonene increased the health-span part of the life-span curves. Orange juice as a complex mixture and hesperidin and limonene as single compound may be proposed as substances to be studied more extensively as potential nutraceuticals.

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