RESEARCH ARTICLE

Chemopreventive Actions of Blond and Red-Fleshed Sweet Orange Juice on the *Loucy* Leukemia Cell Line

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Abstract

Background: Red-fleshed sweet orange juice (ROJ) comes from a new variety of citrus cultivated in Brazil that contains high levels of β-carotene and lycopene, and similar amounts of hesperidin (HSP) and nutrients, equivalently to blond orange juice (BOJ). Such bioactive compounds are associated with chemopreventive actions in several cancer cell lines. The purpose of this study was to examine the cytotoxicity, cell cycle, apoptosis, and cytokine secretion after BOJ, ROJ, and HSP treatment of a novel T acute lymphoblastic leukemia cell line, *Loucy*. Materials and Methods: *Loucy* cells were incubated for 24-h with BOJ, ROJ, and HSP, and the viability was measured using trypan blue. Cell cycling and apoptosis were assessed by propidium iodide (PI) and annexin V-FITC/PI flow cytometry, respectively. Secretion of cytokines IL-1α, IL1-β, IL-2, IL-4, IL-6, IL-10, IL-17A, IFNγ, TNFα, TGFβ, MIPα, and MIPβ was determined by ELISA array. Results: BOJ and ROJ treatments promoted *Loucy* cell cytotoxicity. Additionally, BOJ induced cell cycle arrest in the G0/G1 phase, and decreased the cell accumulation in the G2/M. ROJ decreased only the G0/G1 fraction, while HSP did not change the cell cycle. BOJ led to apoptosis in a different fashion of ROJ, while the first treatment induced apoptosis by increase of late apoptosis and primary necrotic fractions, the second increased early and late apoptosis, and primary necrotic fraction compared to positive controls. HSP had no effect on apoptosis. IL-6 and IL-10 were abrogated by all treatments. Conclusions: Taking together, these results suggest potential chemopreventive effects of BOJ and ROJ on *Loucy* cells.

Keywords: orange juice - hesperidin - cell cycle - apoptosis - leukemia - *Loucy* cells

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Introduction

Currently has been evidenced that dietary components may modify the risk of cancer by influencing multiple processes, including DNA repair, cell proliferation, differentiation, and apoptosis (Percival et al., 2008, Alshatwi et al., 2011; Akan et al., 2013; Tawil et al., 2015). New strategies for suppressing cancer cells with bioactive compounds from food have been investigated, for instance the suppression of the nuclear factor kappa B (NF-κB), a transcription factor linked to carcinogenesis (Barthi and Aggarwal, 2002). Chemoprevention is a promising and rational approach that uses synthetic, natural, or biological agents to reduce or block the occurrence of cancer (Franke et al., 2013; Steward and Brown, 2013). In line with this perspective, epidemiological studies have shown that the consumption in long-term of balanced diets, particularly those rich in vegetables and fruits, can promote chemopreventive activities (Steinmetz and Potter, 1996; Meiyanto et al., 2012). Indeed, the beneficial effects of these diets are attributable, at least in part, to polyphenols (Ullah and Khan, 2008; Singh et al., 2012).

Oranges and orange-derived products, such as orange juices, are an abundant source of citrus flavonoids, a subclass of polyphenols that represent an important dietary component. Inverse correlations have been reported between the consumption of orange juice and the incidence of cancer (So et al., 1996; Miyagi et al., 2000; Tanaka et al., 2012; Franke et al., 2013; Farooqi et al., 2015). The anticancer properties of orange juice have been focused on hesperidin and naringin, flavanones found almost exclusively in citrus (Guthrie et al., 1998; Ghorbani et al., 2012; Zeng et al., 2014). Hesperidin exhibited cancer chemopreventive actions, in part by arresting cell cycle progression (Choi, 2007), as well as by triggering cell death through a pro-apoptotic pathway (Nazari et al., 2011; Sambantham et al., 2013). A recent study revealed that hesperidin promoted the accumulation of protein 53 (p53) and down-regulated nuclear factor kappa B (NF-k), resulting in apoptosis through the recruitment of peroxisome proliferator-activated receptor-gamma (PPAR-γ) in NALM-6 cells, a B cell precursor leukemia cell line (Ghorbani et al., 2012).

Red-fleshed sweet oranges have been cultivated since 2005 in Brazil and, as a new variety of citrus, its health-promoting effects are still not fully studied (Merussi et

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al., 2013) Juice from red-fleshed sweet oranges (ROJ) is different from blood orange juice because, instead of anthocyanin, it has carotenoids as lycopene, β-carotene, xanthine and others, responsible for the intense reddish-orange color of the pulp (Rodrigo et al., 2015). Compared to the blond orange juice (BOJ), ROJ has 2-fold more total carotenoids, including β-carotene (5-fold more) and lycopene, which has been associated to antioxidant effects and specific properties against cancer malignancies (Salman et al., 2007; Alquezar et al., 2009; Tanaka et al., 2012; Teodoro et al., 2012).

T acute lymphoblastic leukemia (T-ALL) is a malignancy of thymo cells that affects 10-15% of children and 25% of adults (Roti and Stegmaier, 2014) and is associated with poor prognosis for both adults and children (Pui et al., 2008). Based on the current literature that has revealed the anticancer role bioactive compounds from orange, we hypothesized that the administration of BOJ and ROJ exerts chemopreventive effects on a new model of T acute lymphoblastic leukemia cell line, *Loucy* cells (Ben-Bassat et al., 1990). The aim of the present study was to exam the effects of orange juices, BOJ and ROJ, and their major flavanone, hesperidin, on cell viability, cell cycle distribution, apoptosis, and cytokine secretion of *Loucy* cells.

Materials and Methods

Cell line and chemicals

Loucy T-acute lymphoblastic leukemia cell line was obtained from American Type Culture Collection (ATCC® CRL-2629™) Manassas, VA, USA. The growth medium RPMI 1640 without L-glutamine and phenol red, HEPES buffer 1 M, L-glutamine, streptomycin, and penicillin were obtained from Mediatech Cellgro, Manassas, VA, USA. Fetal bovine serum (FBS) and phosphate-buffered saline (PBS) were obtained from Gibco, Life Technologies, Grand Island, NY, USA. Glucose solution 45%, triton X-100, hesperidin powder ≥ 80%, and trypan blue were obtained from Sigma-Aldrich, St. Louis, MO, USA. Sodium pyruvate, RNase A, DNase and protease-free were obtained from Thermo Scientific, Pittsburgh PA, USA. Dimethyl sulfoxide (DMSO) was obtained from Fisher Scientific, Houston, TX, USA. Membrane filters of 8 μm and 0.22 μm pore sizes were obtained from Millipore, Billerica, MA, USA. Propidium iodide (PI) was obtained from Santa Cruz Biotechnology, Dallas, Texas, USA. Annexin V-fluorescein isothiocynate/PI (FITC/PI) staining method and staurosporine were obtained from Abcam, Cambridge, MA, USA. Multi-Analyte ELISA array kits for cytokines were obtained from Quiagen, Valencia, CA, USA. Culture plates were obtained from Costar, Corning Incorporated, Corning, NY.

Cell culture

Loucy cells were grown in a complete RPMI 1640 medium containing a 10 mM HEPES buffer, 0.3 g of L-glutamine supplemented with 10% of fetal bovine serum (FBS), 100 μg/ml of streptomycin, 100 U/ml of penicillin, 1mM sodium pyruvate, and 25 mM of glucose solution 45%. The cells were incubated in a 5% CO_2 humidified incubator at 37°C and seeded at a density of 5 x10^5, unless otherwise indicated.

Orange juices and hesperidin preparation

100% BOJ and ROJ commercially pasteurized, obtained from orange varieties Pera and Sanguinea de Mombuca were respectively provided by Citrosuco, Matao, SP, Brazil. The characteristics of BOJ and ROJ were previously measured in our Department and described respectively, as follows: Brix° 11.5 and 9.6 (g sucrose/100g); titratable acidity 1.01 and 0.50 (% citric acid); ascorbic acid 26.9 and 28.2 (mg/100 ml); total polyphenols 1.66 and 1.80 (mg/100 ml gallic acid); total flavonoids 31.6 and 36.9 (mg rutin/ml); total carotenoids 2.00 and 3.99 (mg β-carotene/ml), and hesperidin 155 and 135 (mg/l). Before the cell culture experiment, each type of orange juice (100 ml) was lyophilized and stored at 4°C. The analytical methods of oranges juice are described below. For the assays, the juices were reconstituted in complete RPMI 1640, to obtain the final concentration of hesperidin of 10 μM for each juice in culture. Content of β-carotene and lycopene for the concentration of 10 μM of hesperidin per well were 0.00042 μM for BOJ and 0.00182 μM for ROJ. The concentration of hesperidin was chosen due to possibility of dilution of juices in culture i.e., concentrations above of 10 μM of hesperidin impaired the regular growing of *Loucy* cells (data not shown). Because hesperidin is a main bioactive compound present in both juices, we prepared a solution with purified hesperidin diluted in DMSO at 0.1% and complete RPMI 1640 media at 10 μM, in order to compare the isolated compound with hesperidin present in orange juices.

Total soluble solids and titratable acidity of OJs

Total soluble solids content (Brix) and titratable acidity (expressed in % citric acid) of samples was performed according to AOAC International (AOAC, 1998).

Ascorbic acid of OJs

Ascorbic acid content was determined by titration, which is a method

based on the reduction of 2,6-dichlorophenol-indophenol by ascorbic acid, and the results expressed in mg ascorbic acid/100 ml of juice (AOAC, 1998).

Total phenolics of OJs

Total phenolics content were determined by the Folin-Ciocalteu method using gallic acid as standard (Singleton and Rossi, 1965). Absorbance was read at 760 nm in a Beckman Spectrophotometer DU-640. The results were expressed in mg of gallic acid/100 ml of juice.

Total flavonoids of OJs

Extraction of flavonoids was perfomed using cold acetone (Yu and Dahlgren, 2000). Total flavonoid content was determined by colorimetric method (Zhishen et al., 1999), with absorbance at 510 nm. Rutin was used as a standard to obtain a calibration curve. Results were expressed in equivalent mg of rutin/ml.

Total carotenoids of OJs

Carotenoid pigments extract was partition to petroleum ether. The total carotenoid content was determined by measure of the absorbance at 450nm in a UV/V spectrophotometer using the absorption coefficient of β -carotene (A1% 1cm = 2592) (Rodriguez-Amaya, 1999). The total carotenoids content was expressed as mg β-carotene/ml. Carotenoid pigments were analyzed by RP-HPLC using ternary gradient of elution and Symmetry C18 column (4.6 mm x 150 mm ID., 3.5μm) from Waters. Chromatographic system was equipped with a photodiode array detector (DAD) and the mobile phase consisted of acetonitrile: methanol: ethyl acetate containing 0:05% triethylamine at a flow rate of 0.6 ml/min. A gradient was applied from 99:1:0 to 64:1:35 in 30 min and 99:1:0 in 60 min. The injection volume was 20μL. Detection was performed at 350-550 nm. Carotenoids individually isolated in samples were identified by comparing its retention time in HPLC, and by the spectra characteristics of diode array with patterns, and also with literature values.

Flavanones hesperidin and narirutin of OJs

Extraction of flavanones was according to Bocco et al. (1998). It was mixed 3 ml of juice plus 5 ml of methanol, followed by heating at 55°C for 15 min, and centrifugation at 3150 rpm for 15 min. Supernatant was collected and the procedure was repeated twice. Methanol extracts were mixed and evaporated in a water bath at 50°C under nitrogen flow, resulting in a final volume of 10 ml with methanol. The column used in the separation of flavanones was C18 Shimadzu Shim-pack CLC-ODS (M) (4.6 x 250 mm, 5 mm), and a mixture acetonitrile: water:acetic acid (21:75:4) (v/v/v) was used as mobile phase with a flow rate of 1.0 ml/min with photodiode array detector (DAD) Waters 996. Running time was 30 min and the detection was read at 280 nm. Flavanones identification were performed by comparison of retention times and spectra obtained by a photodiode array detector (DAD) of the samples and their respective standards (hesperidin and narirutin) between 200 and 450nm. The results were expressed as mg/l.

Orange juices and hesperidin in cell culture

Loucy cells were cultured at 5 x10^5 cells/ml in 24 well plates and incubated for 24-h with the followings treatments: (a) BOJ, *Loucy* cells cultured in RPMI 1640 plus blond orange juice (at 10 μM of hesperidin); (b) ROJ, *Loucy* cells cultured in RPMI 1640 plus red-fleshed sweet orange juice (at 10 μM of hesperidin); (c) HSP, *Loucy* cells cultured in RPMI 1640 plus hesperidin solution (at 10 μM); (d) Control, *Loucy* cells cultured only in complete RPMI 1640. Prior to culture we tested whether the administration DMSO 0.1% on cell culture (without hesperidin) could change the cell growth and the results showed no modification in *Loucy* cells viability, which was statistically similar to control (data not shown). The acidity of pure juice was 3.7 and 4.0 respectively for BOJ and ROJ, while on culture medium the pH values for both, BOJ and ROJ were approximately 7.2. All assays were performed in triplicates.

Cell viability assay

To investigate a possible inhibitory effect on cell viability, we evaluated the number of *Loucy* cells before and after BOJ, ROJ, and HSP incubation. Following 24-hof incubation with the treatments, 20 μl of each cell suspension sample was taken out and added in 20 μl of trypan blue. Tubes were gently mixed and 20 μl of cell suspension was loaded into Cellometer® counting chambers. Cellometer® counting chambers was placed into the Cellometer® T4, Nexcelom, and the viability were determined using Cellometer® software. *Loucy* cells viability was expressed in percentage.

Cell cycle flow cytometer assay

After 24-h of BOJ, ROJ, and HSP incubation, a volume equivalent to 1x10^6 of *Loucy* cells suspension from each sample was collected, centrifuged, and the cell pellets were re-suspended in 1 ml of cold PBS. Next, the cells were fixed in 1 ml of ice-cold 70% ethanol. Prior to analysis, the cells were stained with PI (10 μg/ml) solution containing RNase A, DNase and protease-free (100 μg/ml), and Triton X-100 (0.1% v/v) diluted in PBS (Pozarowski and Darzynkiewicz, 2004). The distribution of G_0/G_1, S and G_2/M phases of the cell cycle was analyzed in Accuri C6 flow cytometer and software (BD Biosciences Immunocytometry Systems, San Jose, CA) and the results were expressed in percentage.

Annexin V-FITC/PI apoptosis assay

Apoptosis was induced in a 1x10^6 cell/ml suspension by the addition of 1 mg/ml staurosporine (Stp) for 2-h in a 37°C, 5% CO_2 incubator. The followins treatments were performed for assay apoptosis: Assay controls: (a) PI + Stp: *Loucy* cells culture in RPMI 1640 + propidium iodide + staurosporine; (b) Annexin V: *Loucy* cells culture in RPMI 1640 + annexin V; (c) Annexin V + Stp: *Loucy* cells culture in RPMI 1640 + annexin V + staurosporine. Treatment controls: (a) Annexin V + PI + Stp (+): *Loucy* cells culture in RPMI 1640 + annexin V + propidium iodide + staurosporine (positive control of the treatments); (b) Annexin V + PI (-): *Loucy* cells culture in RPMI 1640 + annexin V + propidium iodide (negative control of the treatments); (c) Untreated cells: *Loucy* cells culture only in RPMI 1640. Treatments: (a) BOJ: *Loucy* cells culture in RPMI1640 + blond orange juice (at 10 uM of hesperidin); (b) ROJ: *Loucy* cells culture in RPMI 1640 + red-fleshed orange juice (at 10 uM of hesperidin); (c) HSP: *Loucy* cells culture in RPMI 1640 + hesperidin (at 10 uM) solubilized in DMSO (0.1%); (d) DMSO: *Loucy* cells culture in RPMI 1640 + DMSO solution (0.1%). After 2-h of incubation with staurosporine for perform assay controls and treatments controls, and 24-h with the compounds of the treatments (BOJ, ROJ, HSP, DMSO), *Loucy* cells were washed twice with cold PBS, re-suspended in 500 μl of annexin V binding buffer and incubated with 5 μl of annexin V-FITC and 5 μl of PI in the dark at room temperature for 5 min. Posteriorly, *Loucy* cells were analyzed by Accuri C6 flow cytometer and software (BD Biosciences Immunocytometry Systems, San Jose, CA). The fluorescence intensity of FITC/PI were differentiated in viable (annexin V-/PI-), early apoptotic (annexin V+/PI-), late apoptotic (annexin V+/PI+), and

primary necrotic cells (annexin V-/PI+) in triplicate and the results were expressed in percentages.

Cytokines assay

After 24-h of incubation with treatments, cell culture supernatants were collected and stored at -80ºC. The levels of IL-1α, IL1-β, IL-2, IL-4, IL-6, IL-10, IL-17A, IFNγ, TNFα, TGFβ, MIPα and MIPβ cytokines secreted by *Loucy* cells were measured in undiluted supernatant using Multi-Analyte ELISA array with kit standards and controls in accordance with the manufacturer's instructions. Final absorbance was measured at 450 nm on a SPECTRAmax 340PC plate reader and data analyzed using SOFTmax® Pro 5.2 (Molecular Devices, Sunnyvale, CA). To obtain the corrected absorbance values, the initial absorbance of each cytokine was subtracted by the absorbance of the negative control from the kit. The results were expressed in percentages as the difference of control cells (untreated) *vs* all treated cells.

Statistical analysis

All values were expressed as means ± standard deviation (SD). Comparisons were performed using a One Way ANOVA, among the treatments for cell cycle (treatments *vs* control) and apoptosis (treatments *vs* positive control), followed by post-hoc Tukey test.

Cell viability was compared using a T test between each treated *vs* control. Statistical analysis data were performed using Sigma Stat software version 3.11 (Sigma Stat for Windows, Systat Software Inc., San Jose, USA) and the statistical significance was set at P≤0.05

Results

Cell viability

The effects of BOJ, ROJ, and HSP on *Loucy* cells viability were analyzed using trypan blue exclusion assay, which live cells possess intact cell membranes that exclude the dye, while the dead cells remained stained. We observed the cytotoxic effect after the BOJ and ROJ treatments as result of decreases by 23% and 27% in cell viability, respectively, while the HSP treatment was statistically similar to control (Figure 1).

Cell cycle

The cell cycle analysis has evaluated a possible cell cycle redistribution or arrest by BOJ, ROJ, and HSP treatments. The cell cycle distribution of untreated *Loucy* cells (control) was 46% in the G_0/G_1 phase, 28.8% in the S phase, and 26.8% in the G_2/M phase (Table 1). Compared to the control, BOJ treatment induced the cell cycle arrest by increase of 6.9-fold in the G_0/G_1 phase. In addition, this treatment left the S phase unaltered, and decreased the 9.2-fold in the G_2/M phase. ROJ treatment decreased 2.9-fold the G_0/G_1 phase, while the S and G_2/M phases remained unaltered in relation to the control. HSP treatment did not affect the *Loucy* cell cycle distribution (Table 1).

Annexin V-FITC/PI apoptosis

To investigate whether the cytotoxic effect promoted by BOJ and ROJ on *Loucy* cells was related to apoptotic cell death, the annexin V-FITC/PI double staining assay was applied. Our results correspond to the average of triplicates and are shown in the Figure 2/Table 2. There was a decrease in the number of cells only by the use of annexin V + PI (negative control), and annexin V + PI + staurosporine (positive control). These differences were significant and reduced the number of cells by 1.1 for negative control, and 1.3 for positive control compared

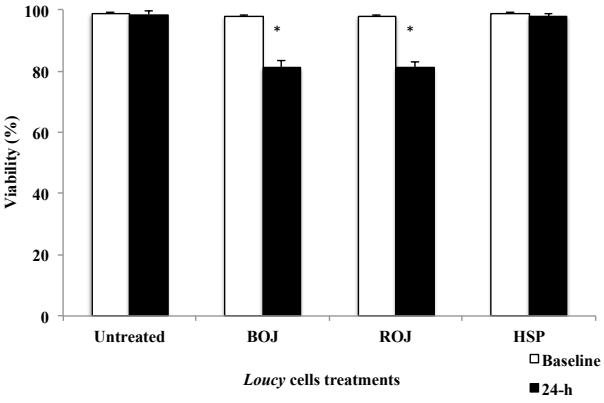

Figure 1. *Loucy* cell viability assay after 24-h of incubation with blond orange juice (BOJ), red-fleshed sweet orange juice (ROJ), and hesperidin (HSP) treatments. Untreated: cells culture only in RPMI 1640; BOJ: cells culture in RPMI 1640 plus blond orange juice; ROJ: cells culture in RPMI 1640 plus red-fleshed sweet orange juice; HSP: cells culture in RPMI 1640 plus hesperidin solubilized in dymethyl sulfoxide (0.1%). Trypan blue exclusion method was applied. ANOVA One Way followed by post-hoc Tukey test P≤0.05.*Statistical difference between baseline time *vs* 24-h of incubation with each treatment

Table 1. *Loucy* cell cycle distribution (G0/G1, S, and G2/M phases) after 24-h of incubation with blond orange juice (BOJ), red-fleshed sweet orange juice (ROJ), and hesperidin (HSP)

	Loucy cells treatments (24-h incubation)			
Cell cycle phases (%)	Untreated	BOJ	ROJ	HSP
G0/G1	46.0 ± 0.8[b]	52.9 ± 0.1[a]	43.1 ± 1.0[c]	43.9 ± 1.1[bc]
S	28.2 ± 0.2[a]	28.6 ± 0.8[a]	27.7 ± 0.7[a]	27.9 ± 0.2[a]
G2/M	26.8 ± 0.7[a]	17.6 ± 0.9[b]	26.1 ± 1.6[a]	27.7 ± 1.1[a]

*Untreated: cells cultured only in RPMI 1640; BOJ (blond orange juice): cells cultured in RPMI 1640 + blond orange juice (at 10 μM hesperidin); ROJ (red-fleshed sweet orange juice): cells cultured in RPMI 1640 + red-fleshed sweet orange juice (at 10 μM hesperidin); HSP: *Loucy* cells culture in RPMI 1640 + hesperidin (at 10 μM) solubilized in DMSO (0.1%). Results are expressed as mean ± SD. Values with the same letter in a row are not significantly different, while the different letters are statisticaly significant. Anova One Way by line followed by post-hoc Tukey test P ≤ 0.05.

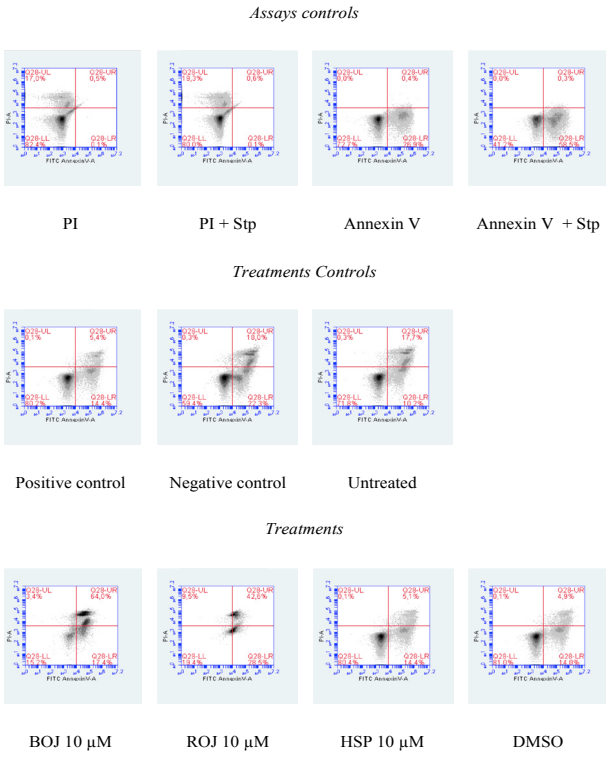

Figure 2. Effect of blond orange juice (BOJ), red-fleshed sweet orange juice (ROJ), and hesperidin (HSP) on induction-apoptosis and necrosis in *Loucy* cells. Double staining with annexin V-FITC/propidium iodide solution are distinguished among the percentage of cells live, early apoptotic, late apoptotic, and primary necrotic. Assay controls: (a) PI: cells cultured in RPMI 1640 + propidium iodide (PI); (b) PI + Stp: cells culture in RPMI 1640 + propidium iodide (PI) + staurosporine (Stp) (1mg/ml); (c) Annexin V: cells culture in RPMI 1640 + annexin V; (d) Annexin V +Stp: cells culture in RPMI 1640 + annexin V + staurosporine (Stp) (1mg/ml); Treatment controls: (a) Positive control (Annexin V + PI + Stp): cells culture in RPMI 1640 + annexin V + propidium iodide (PI) + staurosporine (Stp) (1mg/l); (b) Negative control (Annexin V + PI): cells culture in RPMI 1640 + annexin V + propidium iodide (PI); (c) Untreated cells: cells culture only in RPMI 1640. Treatments: (a) BOJ: cells culture in RPMI1640 + blond orange juice (at 10 µM hesperidin); (b) ROJ: cells culture in RPMI 1640 + red-fleshed orange juice (at 10 µM hesperidin); (c) HSP: cells culture in RPMI 1640 + hesperidin (10 µM) solubilized in DMSO (0.1%); (d) DMSO: cells culture in RPMI 1640 + DMSO solution (0.1%). Anova One Way followed by post-hoc Tukey test $P \leq 0.05$ comparing treatments vs positive control by each cell fractions (live-LL; early apoptotic-LR, late apoptotic-UR, and primary necrotic-UL)

to untreated cells. Staurosporine administration (positive control) was effective in inducing apoptosis in *Loucy* cells, because there was a decrease in viable cells by 1.2-fold, an increase of 2.2-fold in early apoptotic cells, while no changes were observed on late apoptotic or primary necrotic cells compared to negative control. After 24-h of incubation with BOJ, we observed a decrease by 3.8-fold in viable cells and 1.4-fold in early apoptotic cells. Additionally, there were an increase by 3.6-fold in late apoptotic cells, and 17-fold primary necrotic cells

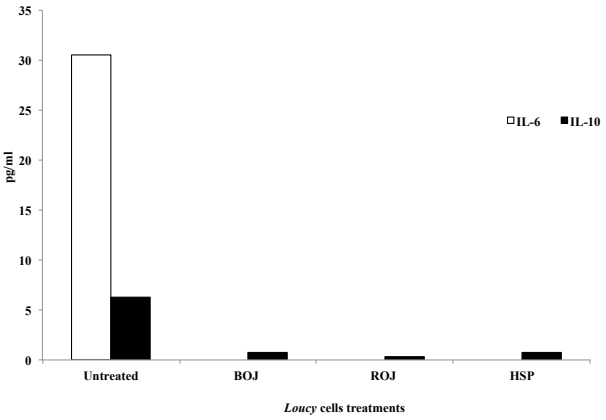

Figure 3. Cytokines Secretion from *Loucy* Cells Supernatant After 24-h of Incubation with Blond Orange Juice (BOJ), Red-Fleshed Orange Juice (ROJ), and Hesperidin (HSP). Untreated: cells culture only in RPMI 1640; BOJ: cells culture in RPMI 1640 plus blond orange juice; ROJ: cells culture in RPMI 1640 plus red-fleshed sweet orange juice; HSP: cells culture in RPMI 1640 plus hesperidin solubilized in dymethyl sulfoxide (0.1%). The results are expressed in percentages as the difference of Untreated vs each treatment. To obtain the corrected absorbance values, the initial absorbance of each cytokine was subtracted by the absorbance of the own negative control of the kit

compared to positive control. After ROJ treatment, there was a decrease by 3-fold in viable cells, a significant increase of early apoptotic cells, an increase of 2.4-fold of late apoptotic cells, and 33-fold of primary necrotic cells in relation to positive control. Regarding HSP treatment, there was an increase by 1.3-fold in viable cells, while was observed a decrease in early apoptotic (1.6-fold), late apoptotic (3.4-fold), and primary necrotic cells (3-fold) compared to positive control (Figure 2/Table 2).

Cytokines

Of the analyzed cytokines (IL-1α, IL1-β, IL-2, IL-4, IL-6, IL-10, IL-17A, IFNγ, TNFα, TGFβ, MIPα and MIPβ) only IL-6 and IL-10 were secreted by *Loucy* untreated cells (Figure 3). Compared to control, both BOJ and HSP treatments suppressed the secretion of IL-10 by 8.5 fold, whereas the ROJ treatment reduced similarly the secretion of this cytokine by 17 fold. The IL-6 levels were abrogated by all treatments in relation to control cells, however in undetectable levels by the assay (Figure 3).

Discussion

In this study, we investigated the effects of two different types of orange juice, BOJ and ROJ, and their most common flavonoid, hesperidin, on cytotoxicity, cell cycle, apoptosis, and cytokine secretion pattern of the *Loucy* leukemia cell line. The main findings of this study were: (1) BOJ and ROJ treatment induced a cytotoxic effect on *Loucy* cells, which were evidenced by a reduction of viable cells; (2) BOJ, but not ROJ, promoted the cell cycle arrest in G_0/G_1; (3) BOJ induced late apoptosis, while ROJ induced early and late apoptosis; (4) BOJ, ROJ, and HSP treatments suppressed the cytokines IL-6 and IL-10. Therefore, the results of this study revealed a potential

Table 2. Effect of blond orange juice (BOJ), red-fleshed sweet orange juice (ROJ), and hesperidin (HSP) on induction-apoptosis and necrosis in *Loucy* cells

	Loucy cells fractions			
	Live (%)	Early Apoptotic (%)	Late Apoptotic (%)	Primary necrotic (%)
Assay controls				
PI	81.9 ± 1.0^{f}	0.1 ± 0.0^{a}	0.5 ± 0.0^{a}	17.5 ± 1.1^{d}
PI + Stp	80.2 ± 1.5^{f}	0.1 ± 0.0^{a}	0.6 ± 0.0^{a}	19.1 ± 1.5^{d}
Annexin V	72.7 ± 0.1^{e}	27.0 ± 0.1^{e}	0.3 ± 0.1^{a}	0.0 ± 0.0^{a}
Annexin V + Stp	42.6 ± 2.0^{c}	56.5 ± 1.8^{f}	0.2 ± 0.0^{a}	0.7 ± 0.0^{a}
Treatments controls				
Annexin V + PI + Stp (+)	60.0 ± 1.6^{d}	22.3 ± 0.6^{d}	17.4 ± 1.1^{c}	0.3 ± 0.0^{a}
Annexin V + PI (-)	71.9 ± 0.5^{e}	10.1 ± 0.2^{b}	17.7 ± 0.4^{c}	0.3 ± 0.0^{a}
Untreated	79.3 ± 1.5^{f}	15.0 ± 1.2^{c}	5.6 ± 0.4^{b}	0.1 ± 0.0^{a}
Treatments				
BOJ 10 μM	15.8 ± 1.5^{a}	16.3 ± 1.5^{c}	62.7± 2.3^{e}	5.2± 2.4^{b}
ROJ 10 μM	19.5 ± 0.5^{b}	28.1 ± 1.0^{e}	42.6 ± 0.4^{d}	9.8± 0.9^{c}
HSP 10 μM	80.7 ± 1.4^{f}	14.1 ± 1.2^{c}	5.1 ± 0.3^{b}	0.1 ± 0.0^{a}
DMSO	80.8 ± 0.8^{f}	14.1 ± 0.4^{c}	5.0 ± 0.4^{b}	0.1 ± 0.0^{a}

*Double staining with annexin V-FITC/propidium iodide solution are distinguished among the percentage of *Loucy* cells live, early apoptotic, late apoptotic, and primary necrotic. Assay controls: (a) PI: cells cultured in RPMI 1640 + propidium iodide (PI); (b) PI + Stp: cells culture in RPMI 1640 + propidium iodide (PI) + staurosporine (Stp) (1mg/ml); (c) Annexin V: cells culture in RPMI 1640 + annexin V; (d) Annexin V +Stp: cells culture in RPMI 1640 + annexin V + staurosporine (Stp) (1mg/ml); Treatment controls: (a) Positive control (Annexin V + PI + Stp): cells culture in RPMI 1640 + annexin V + propidium iodide (PI) + staurosporine (Stp) (1mg/l); (b) Negative control (Annexin V + PI): cells culture in RPMI 1640 + annexin V + propidium iodide (PI); (c) Untreated cells: cells culture only in RPMI 1640. Treatments: (a) BOJ: cells culture in RPMI1640 + blond orange juice (at 10 μM hesperidin); (b) ROJ: cells culture in RPMI 1640 + red-fleshed orange juice (at 10 μM hesperidin); (c) HSP: cells culture in RPMI 1640 + hesperidin (at 10 μM) solubilized in DMSO (0.1%); (d) DMSO: cells culture in RPMI 1640 + DMSO solution (0.1%). Anova One Way followed by post-hoc Tukey test $P \leq 0.05$ comparing treatments vs positive control by each cell fractions (live, early apoptotic, late apoptotic, and primary necrotic). Values with the same letter in a column are not significantly different, while the different letters are statistically significant.

citotoxic, pro-apoptotic and immunomodulatory effect by cytokines after administration of both orange juices, which were more pronounced than the purified hesperidin on *Loucy* cell line. In the last years, new evidences have emerged about the anticancer role of citrus flavonoids, especially hesperidin and naringin due to their cytotoxic and pro-apoptotic effects in several cancer cell lines (Nazari, et al., 2011; Ghorbani et al., 2012; Zeng et al., 2014). ROJ differs from other blood oranges because is rich in lycopene, β-carotene, xanthine and others, but absent in anthocyanin (Lee, 2001; Xu et al., 2006), that in turn have been associated with anti-cancer actions in some types of cancer cells (Wang et al., 2012; Zhang et al., 2015). With this approach, the present study it has been the first one to investigate the potential chemopreventive of ROJ on T-ALL cell line, *Loucy*.

Both orange juices reduced the growth of *Loucy* cells induced by cytotoxicity, but none inhibition was observed on cell growth after HSP treatment. HSP was previously solubilized in DMSO assuring its incorporation by the cells (Yamada et al., 2006; Nazari et al., 2011), but even though HSP treatment has none effect. Recent study showed that hesperidin at 10-100 μM had a minimal effect on NALM-6 cells after 24-h, but after 48-h of treatment the decrease on the cell viability was marked (Ghorbani et al., 2012). On the contrary, 24-h of incubation for either BOJ or ROJ, at 10 μM of HSP, was enough in terms of concentration and time to decrease the number of *Loucy* leukemic cells. We suggested that results with both juices were mainly because of interaction between nutrients and other nonnutritive compounds, as the flavanones and carotenoids of OJs.

It was previously documented that orange juice contains compounds that may act alone or synergistically, i.e. the biological response to exposure to several chemicals could be greater than the sum of the effects of these individual agents (Nazari et al., 2011). For instance, ascorbic acid possesses proactive role against some types of cancer, including leukemia, and also boost host defense by immunomodulatory effects (Park et al., 1980; Head, 1998; Subramani et al., 2014), while lycopene protect against many types of cancer, as lung, prostate and colon, by modulating cell cycle progression and proliferation (Teodoro et al., 2012). Thus, since these compounds are present in ROJ, we suggest they could provide additional anti-cancer effect.

The cell cycle plays an important role in cell fate, including replication, death and cell function (Vermeulen et al., 2003; Lay et al., 2014). We examined if the cytotoxic effect promoted by orange juice was related to alterations in the *Loucy* cell cycle distribution. BOJ treatment induced cell cycle arrest in the G_0/G_1, while also decreasing the accumulation of cells in G_2/M phase. ROJ treatment promoted a slight decrease in the G_0/G1 phase, showing absence of cell cycle arrest after this treatment. Previous studies have reported that hesperidin/hesperitin were able to promote cell cycle arrest in G_0/G_1 phase, associated with modulation of the expression or activation of cell-cycle regulatory proteins (Choi et al., 2007; Jin et al., 2008; Morikawa et al., 2008). However, in this experiment HSP treatment resulted in none change to the cell cycle. Anti-proliferative action of citrus bergamot juice in neuroblastoma cells (SH-SY5Y) was attributed to the cell cycle arrest in the G1 phase through a mechanism involving a reduction of cyclin D1. This protein is associated with cell cycle progression

through the G1 phase and is responsible for controlling cell cycle checkpoints. It was observed any cytotoxic activity or apoptosis by citrus bergamot juice (Delle et al., 2013). Indeed, we were expecting the *Loucy* cell cycle arrest would be more pronounced with ROJ due to its appreciable amounts of β-carotene and lycopene, beyond of its content of hesperidin and vitamin C (Rodrigo et al., 2015). Instead, only BOJ induced the cell cycle arrest in the G_0/G_1phase, and the reason for this should continue to be deciphered in later studies.

Recent studies have used the process of programmed cell death as a therapeutic strategy to treat cancer (Patil et al., 2009; Ishii et al., 2010). To determine whether cytotoxicity of BOJ, ROJ, and HSP on *Loucy* cells was related to apoptotic cell death, the percentages of apoptotic cells were measured. ROJ was the treatment that more induced the early apoptotic cells, and also primary necrotic cells. BOJ treatment, in turn, also decreased the percentage of viable cells, leading to 1.4-fold early apoptosis cells than staurosporine. However, its pro-apoptotic effect was more pronounced in late apoptotic, and in primary necrotic cells. These results are very intriguing because shows that juices acted completely different on apoptosis process. Suggesting on more time, that de orange juice composition appear to determine the mechanisms that could be trigger in a programmed cell death. Previous study tested the polymethoxyflavones (PMF), tangeretin and nobiletin, on the growth of human T lymphoblastoid leukemia cells (MOLT-4) (Ishii et al., 2010). Both compounds have induced cytostatic effects (G_1/S accumulation), instead cytotoxicity, suggesting a selective interaction with mediators of cell cycle events. It appears that PMFs decrease the late apoptosis with necrosis especially tangeretin (Ishii et al., 2010), similar to our BOJ result.

Others studies revealed that 10 to 100 μM hesperidin upregulates p53 expression by recruiting PPARγ and down-regulates nuclear NF-kB in human NALM-6 pre-B cell lines (Ghorbani et al., 2012). Protein p53 is a tumor suppressor that induces either cell cycle arrest or apoptosis, whereas PPARγ induces apoptosis by inhibiting the activation of NF-ϰB (44), which is associated with survival pathways in most of cancer cells, including hematopoietic malignancies (Sun et al., 2008; Nazari et al., 2011). Taken together, BOJ treatment seems have potential to interfere either, on the cell cycle arrest and apoptosis (late apoptosis and primary necrotic fraction), while ROJ induced the cytotoxicity majoritarily by apoptosis (early and late apoptosis and primary necrotic cells fraction). Although, it was not observed the pro-apoptotic effect with isolated HSP on *Loucy* cells.

Chronic inflammation is closely linked with development of cancer and has been related with cytokines that regulate the inflammation (Rakoff-Nahoum, 2006). Altered concentrations on levels of these mediators can promote growth, attenuating apoptosis and facilitating the invasion and metastasis (Dranoff, 2004). Furthermore, proinflammatory cytokines and other inflammatory markers, as cyclooxygenase (COX-2) and, inducible nitric oxide synthase (iNOS), have become targets for cancer chemoprevention (Ohshima et al., 2005; Fukumura et al., 2006; Yasui and Tanaka, 2009). Based on these evidences, we studied whether the secretion pattern of cytokines by *Loucy* cells could be shifting after the treatment with both juices and HSP. In our experiments, *Loucy* cells have secreted only two cytokines spontaneously by untreated cells (IL-6 and IL-10), meaning potential limitation of current methodology. IL-6 was completely abrogated by BOJ, ROJ and HSP treatment. BOJ and HSP treatment decrease the concentration of IL-10 by 8.5-fold, while ROJ decreases by 17-fold, suggesting the type of juice affects cytokine secretion differently.

High concentration of IL-6 have been implicated in different types of cancers, including leukemia, and are associated with poor clinical outcomes (Tanaka et al., 2012). Thus, seems the inhibition or suppression of IL-6 could be a potential therapeutic strategy for some types of cancer, when IL-6 is being overproduced (Lippitz, 2013). Similarly, IL-10 is an immunosuppressive cytokine, and is expressed by malignant cells, resulting in increased its concentrations circulating in most human cancers. There is a frequent association of elevated concentrations of IL-10 with negative prognosis in the later stages of cancer (Lippitz, 2013). Therefore, based on the founded results it seem that the treatments with BOJ and ROJ, and even HSP, would be interesting for cancer management, considering the suppressor effect on IL-6 and IL-10. However, such interpretations should be investigated in further studies, and the precise action mechanism of these compounds remains to be elucidated.

In conclusion, our findings show that both BOJ and ROJ exert differential effects in cell growth inhibition and cytokines secretion by *Loucy* cells. The ability of these juices to induce cell cycle arrest in the G_0/G_1phase, apoptosis and suppress two important cytokines (IL-6 and IL-10) involved with negative prognosis of cancer, can make them candidates in order to understand the effects of their bioactive compounds, synergic interactions, and mechanisms of inducing cell death. Furthermore, this study represents a basis for future investigations on the potential use of BOJ and ROJ in the field of nutrioncology.

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References

Akan Z, Garip AI (2013). Antioxidants may protect cancer cells from apoptosis signals and enhance cell viability. *Asian Pac J Cancer Prev*, **14**, 4611-14.

Alquezar B, Zacarias L, Rodrigo MJ (2009). Molecular and functional characterization of a novel chromoplast-specific lycopene beta-cyclase from Citrus and its relation to

lycopene accumulation. *J Exp Bot*, **60**, 1783-87.
Alshatwi AA, Shafi G, Hasan TN, et al (2011). Apoptosis-mediated inhibition of human breast cancer cell proliferation by lemon citrus extract. *Asian Pac J Cancer Prev*, **12**, 1555-59.
AOAC International (1995). Official methods of analysis of AOAC International (vol. II). Gaithersburg: AOAC International.
Ben-Bassat H, Shlomai Z, Kohn G, et al (1990). Establishment of a human T-acute lymphoblastic leukemia cell line with a (16;20) chromosome translocation. *Cancer Genet Cytogenet*, **49**, 241-48.
Bharti AC, Aggarwal, BB (2002). Chemopreventive agents induce suppression of nuclear factor-κB leading to chemosensitization. *Ann N Y Acad Sci*, **973**, 392-95.
Bocco A, Cuvelier ME, Richard H, et al (1998). Antioxidant activity and phenolic composition of citrus peel and seed extracts. *J Agric Food Chem*, **46**, 2123-29.
Choi EJ (2007). Hesperetin induced G1-phase cell cycle arrest in human breast cancer MCF-7 cells: involvement of CDK4 and p21. *Nutr Cancer*, **59**, 115-19.
Delle Monache S, Sanita P, Trapasso E, et al (2013). Mechanisms underlying the anti-tumoral effects of citrus bergamia juice. *PLoS One*, **8**, 61484.
Dranoff G (2004). Cytokines in cancer pathogenesis and cancer therapy. *Nat Rev Cancer*, **4**, 11-22.
Farooqi AA, Wang Z, Hasnain S, et al (2015). Citrus fruits and their bioactive ingredients: leading four horsemen from front. *Asian Pac J Cancer Prev*, **16**, 2575-80.
Franke SI, Guecheva TN, Henriques JA, et al (2013). Orange juice and cancer chemoprevention. *Nutr Cancer*, **65**, 943-53.
Fukumura D, Kashiwagi S, Jain RK (2006). The role of nitric oxide in tumour progression. *Nat Rev Cancer*, **6**, 521-34.
Ghorbani A, Nazari M, Jeddi-Tehrani M, et al (2012). The citrus flavonoid hesperidin induces p53 and inhibits NF-κB activation in order to trigger apoptosis in NALM-6 cells: involvement of PPARγ-dependent mechanism. *Eur J Nutr*, **51**, 39-46.
Guthrie N, Carroll KK (1998). Inhibition of mammary cancer by citrus flavonoids. *Adv Exp Med Biol*, **439**, 227-36.
Head KA (1998). Ascorbic acid in the prevention and treatment of cancer. *Altern Med Rev*, **3**, 174-86.
Ishii K, Tanaka S, Kagami K, et al (2010). Effects of naturally occurring polymethyoxyflavonoids on cell growth, p-glycoprotein function, cell cycle, and apoptosis of daunorubicin-resistant T lymphoblastoid leukemia cells. *Cancer Invest*, **28**, 220-29.
Jin YR, Han XH, Zhang YH, et al (2008). Hesperetin, a bioflavonoid, inhibits rat aortic vascular smooth muscle cells proliferation by arresting cell cycle. *J Cell Biochem*, **104**, 1-14.
Lay MM, Karsani SA, Malek SN (2014). 1-(2,6-dihydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl) ethanone-induced cell cycle arrest in G_0/G_1 in HT-29 cells human colon adenocarcinoma cells. *Int J Mol Sci*, **15**, 468-83.
Lee HS (2001). Characterization of carotenoids in juice of red navel orange (Cara Cara). *J Agric Food Chem*, **49**, 2563-68.
Lippitz BE (2013). Cytokine patterns in patients with cancer: a systematic review. *Lancet Oncol*, **14**, 218-28.
Meiyanto E, Hermawan A, Anindyajati (2012). Natural products for cancer-targeted therapy: citrus flavonoids as potent chemopreventive agents. *Asian Pac J Cancer Prev*, **13**, 427-36.
Merussi GD, Latado RR, Rossi EA, et al (2013). Book of abstracts and proceedings of the 7th international congress on pigments in food. Carotenoids in red fleshed sweet oranges, pp 63.
Miyagi Y, Om AS, Chee KM, et al (2000). Inhibition of azoxymethane-induced colon cancer by orange juice. *Nutr Cancer*, **36**, 224-29.
Morikawa K, Nonaka M, Mochizuki H, et al (2008). Naringenin and hesperetin induce growth arrest, apoptosis, and cytoplasmic fat deposit in human preadipocytes. *J Agric Food Chem*, **56**, 11030-37.
Nazari M, Ghorbani A, Hekmat-Doost A, et al (2011). Inactivation of nuclear factor-κB by citrus flavanone hesperidin contributes to apoptosis and chemo-sensitizing effect in Ramos cells. *Eur J Pharmacol*, **650**, 526-33.
Ohshima H, Tazawa H, Sylla BS, et al (2005). Prevention of human cancer by modulation of chronic inflammatory processes. *Mutat Res*, **591**, 110-22.
Park CH, Amare M, Savin MA, et al (1980). Growth suppression of human leukemic cells *in vitro* by L-ascorbic acid. *Cancer Res*, **40**, 1062-65.
Patil JR, Chidambara Murthy KN, Jayaprakasha GK, et al (2009). Bioactive compounds from Mexican lime (Citrus aurantifolia) juice induce apoptosis in human pancreatic cells. *J Agric Food Chem*, **57**, 10933-42.
Percival SS, Bukowski JF, Milner J (2008). Bioactive food components that enhance gamma delta T cell function may play a role in cancer prevention. *J Nutr*, **138**, 1-4.
Pozarowski P, Darzynkiewicz Z (2004). Analysis of cell cycle by flow cytometry. *Methods Mol Biol*, **281**, 301-11.
Pui CH, Robison LL, Look AT (2008). Acute lymphoblastic leukaemia. *Lancet*, **371**, 1030-43.
Rakoff-Nahoum, S (2006). Why cancer and inflammation? *Yale J Biol Med*, **79**, 123-30.
Rodrigo MJ, Cilla A, Barbera R, et al (2015). Carotenoid bioaccessibility in pulp and fresh juice from carotenoid-rich sweet oranges and mandarins. *Food Funct*, May 21.
Rodriguez-Amaya, DB (1999). A guide for carotenoid analysis in foods. washington, dc: international life sciences institute press.
Roti G, Stegmaier K (2014). New approaches to target T-ALL. *Front Oncol*, **8**, 170.
Salman H, Bergman M, Djaldetti M, et al (2007). Lycopene affects proliferation and apoptosis of four malignant cell lines. *Biomed Pharmacother*, **61**, 366-69.
Sambantham S, Radha M, Paramasivam A, et al (2013). Molecular mechanism underlying hesperetin-induced apoptosis by in silico analysis and in prostate cancer PC-3 cells. *Asian Pac J Cancer Prev*, **14**, 4347-52.
Singh M, Singh P, Shukla Y (2012). New strategies in cancer chemoprevention by phytochemicals. *Front Biosci*, **4**, 426-52.
Singleton VL, Rossi JA (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am J Enol Vitic*, **16**, 144-58.
So FV, Guthrie N, Chambers AF, et al (1996). Inhibition of human breast cancer cell proliferation and delay of mammary tumorigenesis by flavonoids and citrus juices. *Nutr Cancer*, **26**,167-81.
Steinmetz KA, Potter JD (1996). Vegetables, fruit, and cancer prevention: a review. *J Am Diet Assoc*, **96**, 1027-39.
Steward WP, Brown K (2013). Cancer chemoprevention: a rapidly evolving field. *Br J Cancer*, **109**, 1-7.
Subramani T, Yeap SK, Ho WY, et al (2014). Vitamin C suppresses cell death in MCF-7 human breast cancer cells induced by tamoxifen. *J Cell Mol Med*, **18**, 305-13.
Sun SC, Ley SC (2008). New insights into NF-kappa B regulation and function. *Trends Immunol*, **29**, 469-78.
Tanaka T, Narazaki M, Kishimoto T (2012). Therapeutic targeting of the interleukin-6 receptor. *Annu Rev Pharmacol Toxicol*, **52**, 199-219.

Tanaka T, Shnimizu M, Moriwaki H (2012). Cancer chemoprevention by carotenoids. *Molecules*, **17**, 3202-42.

Tanaka T, Tanaka T, Tanaka M, et al (2012). Cancer chemoprevention by citrus pulp and juices containing high amounts of β-cryptoxanthin and hesperidin. *J Biomed Biotechnol*, **2012**, 516981.

Tawil M, Bekdash A, Mroueh M, et al (2015). Wild carrot oil extract is selectively cytotoxic to human acute myeloid leukemia cells. *Asian Pac J Cancer Prev*, **16**, 761-7.

Teodoro AJ, Oliveira FL, Martins NB, et al (2012). Effect of lycopene on cell viability and cell cycle progression in human cancer cell lines. *Cancer Cell Int*, **12**, 36.

Ullah MF, Khan MW (2008). Food as medicine: potential therapeutic tendencies of plant derived polyphenolic compounds. *Asian Pac J Cancer Prev*, **9**, 187-95.

Vermeulen K, Van Bockstaele DR, Berneman ZN (2003). The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. *Cell Prolif*, **36**, 131-49.

Wang SK, Yang L, Wang TT, et al (2012). Inhibition of proliferation and induction of apoptosis by the combination of β-carotene and 1,25-dihydroxyvitamin D3 in human esophageal cancer EC9706 cells. *Asian Pac J Cancer Prev*, **13**, 6327-32.

Xu CJ, Fraser PD, Wang WJ, et al (2006). Differences in the carotenoid content of ordinary citrus and lycopene-accumulating mutants. *J Agric Food Chem*, **54**, 5474-81.

Yamada M, Tanabe F, Arai N, et al (2006). Bioavailability of glucosyl hesperidin in rats. *Biosci Biotechnol Biochem*, **70**, 1386-94.

Yasui Y, Tanaka, T: Protein expression analysis of inflammation-related colon carcinogenesis. *J Carcinog*, **8**, 10.

Yu Z, Dahlgren RA (2000). Evaluation of methods for measuring polyphenols in conifer foliage. *J Chem Ecol*, **26**, 2119-40.

Zeng L, Zhen Y, Chen Y, et al (2014). Naringin inhibits growth and induces apoptosis by a mechanism dependent on reduced activation of NF-κB/COX-2-caspase-1 pathway in HeLa cervical cancer cells. *Int J Oncol*, **45**, 1929-36.

Zhang YY, Lu L, Abliz G, et al (2015). Serum carotenoid, retinol and tocopherol concentrations and risk of cervical cancer among Chinese women. *Asian Pac J Cancer Prev*, **16**, 2981-6.

Zhishen J, Mengcheng T, Jianming W (1999). The determination of flavonoid content in mulberry and their scavenging effects on superoxide radicals. *Food Chem*, **64**, 555-9.