Universidade Estadual Paulista "Júlio de Mesquita Filho"

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Departamento de Alimentos e Nutrição

Tese de Doutorado

Efeito do suco de laranja sobre biomarcadores relacionados ao excesso de peso e ao câncer

Grace Kelly Zanotti Simões Dourado

Araraquara - SP

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Tese apresentada ao Programa de Pós Graduação em Alimentos e Nutrição como requisito para obtenção do título de Doutor em Alimentos e Nutrição e área de concentração em Ciências Nutricionais.

Grace Kelly Zanotti Simões Dourado

Orientador: Profa. Dra. Thais Borges César

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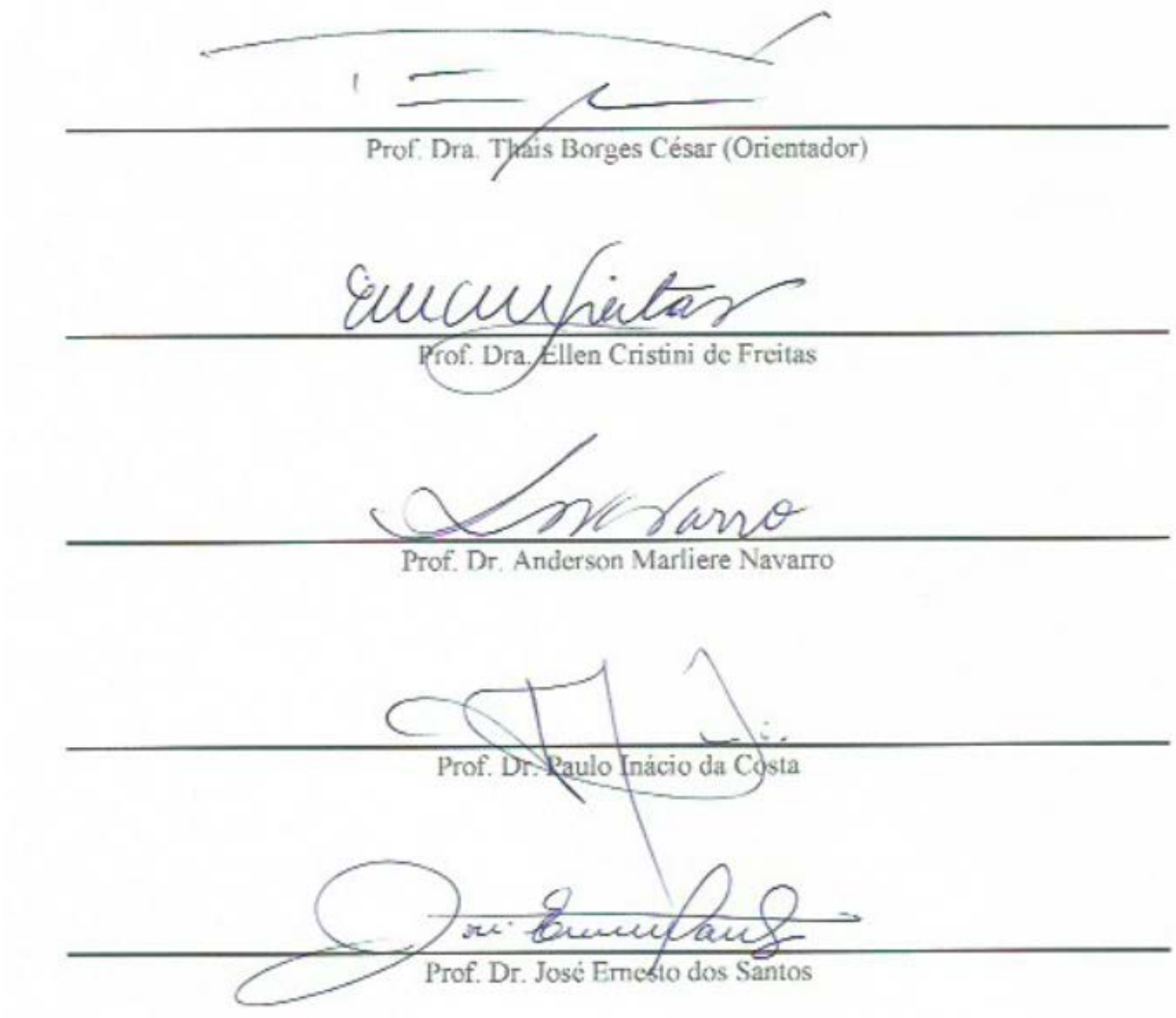

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EPÍGRAFE

“O sucesso nasce do querer, da determinação e persistência em se chegar a um objetivo. Mesmo não atingindo o alvo, quem busca e vence obstáculos, no mínimo fará coisas admiráveis”.

José de Alencar

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RESUMO

O objetivo desta pesquisa foi verificar o efeito do suco de laranja (SL) sobre dois tipos de condições, cuja as etiologias podem estar intimamente associadas a uma alimentação desequilibrada: o excesso de peso e o câncer. Baseado nessa abordagem, nós escolhemos avaliar os efeitos do SL em dois modelos experimentais diferentes: em humanos com excesso de peso (*in vivo*) e em cultura de células leucêmicas (*in vitro*). No experimento *in vivo* foram avaliados os níveis séricos de biomarcadores metabólicos, inflamatórios e oxidativos de indivíduos com peso normal e com sobrepeso após o consumo de oito semanas de suco de laranja blond (SLB). Os indivíduos foram divididos de acordo com o índice de massa corporal (IMC) em dois grupos: Peso normal e Sobrepeso. Em ambos os grupos, os indivíduos ingeriram 750 mL de SL diariamente. Foram avaliados a composição corporal (peso, estatura, IMC, gordura e circunferência da cintura), biomarcadores metabólicos (colesterol total, LDL-C, HDL-C, triglicérides, glicose, insulina, resistência à insulina e hemoglobina glicada), inflamatórios (proteína C reativa, IL-4, IL-10, IL-12, TNF-α e IFN-γ) e oxidativos (malondialdeído e DPPH$^{\bullet}$). Os resultados mostraram que após o consumo de SLB, as medidas da composição corporal não foram alteradas em nenhum dos grupos. Entretanto, houve redução nos níveis de lipídeos séricos, aumento da citocina IL-12, diminuição da proteína C reativa e aumento na capacidade antioxidante em indivíduos com peso normal e sobrepeso. No experimento *in vitro* foram examinados o crescimento, o ciclo e apoptose celular após a administração do suco de laranja blond (SLB), do suco de laranja vermelha (SLV) e da hesperidina (HSP) em uma nova linhagem celular de leucemia linfoblástica aguda de células T (Loucy). A distribuição do ciclo celular e a morte celular por apoptose foram determinadas utilizando citometria de fluxo. A secreção das citocinas IL-1α, IL1-β, IL-2, IL-4, IL-6, IL-10, IL-17A, IFNγ, TNFα, TGFβ e MIPα foram avaliadas por ELISA array. Os SLB e SLV não causaram alterações na proliferação celular, porém ambos reduziram a viabilidade celular em cerca de 25%. SLB induziu o bloqueio celular na fase G0/G1 e diminuiu o acúmulo de células na fase G2/M. SLV diminuiu a porcentagem de células na fração G0/G1, enquanto HSP não afetou a distribuição do ciclo celular. Ambos os tratamentos com SLA e SLV induziram a apoptose. Houve secreção espontânea das citocinas IL-6 e IL-10, que foram similarmente suprimidas pelos tratamentos com SLB, SLV e HSP. Portanto, nós concluímos que ambos os estudos evidenciaram o papel promissor do suco de laranja, na prevenção e/ou terapia de condições associadas com excesso de peso e também no câncer.

Palavras-chave: suco de laranja, flavonoides, sobrepeso, câncer, humano.

ABSTRACT

The objective of this study was to verify the effect of orange juice (OJ) on two types of conditions whose etiologies may be closely associated with an unbalanced diet: overweight and cancer. Based on this approach, we chose to evaluate the effects of OJ in two different experimental models: in overweight humans (*in vivo*) and in cultured leukemic cells (*in vitro*). In the *in vivo* experiment were evaluated serum levels of metabolic, inflammatory and oxidative biomarkers of normal weight and overweight after consuming eight weeks of blond orange juice (BOJ). The subjects were divided according to body mass index (BMI) in two groups: normal weight and overweight. In both groups, subjects ingested 750 ml of BOJ daily. Body composition (weight, height, BMI, fat and waist circumference), metabolic biomarkers (total cholesterol, LDL-C, HDL-C, triglycerides, glucose, insulin, insulin resistance and glycated hemoglobin), inflammatory (protein were evaluated C reactive protein, IL-4, IL-10, IL-12, TNF-α and IFN-γ) and oxidation (malondialdehyde and DPPH$^{\bullet}$). The results showed that after BOJ consumption, the measurements of body composition were not altered in either group. However, there was a reduction in serum lipid levels, increased cytokine IL-12, decreased C-reactive protein and increased of antioxidant capacity in normal and overweight. In the *in vitro* study, the growth, cycle and apoptosis cell were examined after administration of BOJ juice, red orange juice (ROJ), and hesperidin (HSP) into a new acute lymphoblastic leukemia cell line T cells (Loucy). The cell cycle distribution and apoptotic cell death were determined using flow cytometry. The secretion of IL-1α, IL1-β, IL-2, IL-4, IL-6, IL-10, IL-17A, IFNγ, TNFα, TGFβ, and MIPα were evaluated by ELISA array. BOJ and ROJ caused no alterations in cell proliferation, but both reduced cell viability by about 25%. BOJ induced cell cycle arrest in G0/G1 phase and decreased the accumulation of cells in phase G2/M. ROJ decreased the percentage of cells in fraction G0/G1, while HSP did not affect the cell cycle distribution. Both treatments BOJ and ROJ induced apoptosis. There was a spontaneous secretion of IL-6 and IL-10, which were similarly abrogated by treatment with BOJ, ROJ and HSP. Therefore, we conclude that both studies highlight the promising role of orange juice in the prevention and/or therapy of degenerative diseases, including those associated with excess weight and also in the cancer.

Keywords: orange juice, flavonoids, overweight, cancer, human.

SUMÁRIO

INTRODUÇÃO GERAL

O suco de laranja é um alimento singular devido ao seu elevado conteúdo de vitamina C e quantidades significativas de nutrientes essenciais, como açúcares simples, potássio e folato (COELHO et al., 2013). Além disso, o suco de laranja é fonte exclusiva das flavanonas dietéticas, hesperidina e naringina, uma subclasse de flavonoides, que contribuem para a ingestão total de compostos bioativos (RAMPERSAUD, 2007). Estudos recentes têm evidenciado que a ingestão de suco de laranja promove efeitos imunomoduladores, anti-inflamatórios, antioxidantes, e hipolipidêmicos (BENAVENTE-GARCIA e CASTILLO, 2008; SILALAHI, 2002). Por exemplo, o consumo de suco de laranja tem sido associado com a redução de peroxidação lipídica e aumento da capacidade antioxidante no sangue de indivíduos (JOHNSTON, 2003). Além disso, a ingestão de suco de laranja tem sido positivamente associada com adequação e qualidade da dieta, e também baixo risco no desenvolvimento de sobrepeso e obesidade em crianças e adultos (APTEKMANN e CESAR, 2013; O'NEIL et al., 2011; O'NEIL et al., 2012). O estresse oxidativo e inflamatório induzido por dietas ricas em carboidratos e lipídeos também foram prevenidos em indivíduos saudáveis após o consumo de suco de laranja (GHANIM et al., 2007 e 2010; DEOPURKAR et al., 2010). Estas ações promovidas pelo suco de laranja foram atribuídas não somente á vitamina C, mas á hesperidina e naringina e do provável efeito adicional e/ou sinérgico entre os flavonoides e os demais nutrientes presentes no suco de laranja (LIU et al., 2003 e 2004).

A incidência de doenças associadas à dieta está aumentando progressivamente devido à disponibilidade de refeições hipercalóricas e o estilo de vida sedentário. Obesidade, diabetes, aterosclerose e neurodegeneração são as principais condições relacionadas à dieta e que compartilham de um denominador patogênico de inflamação de baixo grau

(MAGRONE et al., 2013). Assim, o aumento na concentração de marcadores inflamatórios, tais como fator de necrose tumoral alfa (TNF-α), proteína C reativa, entre outros, tem sido encontrado em níveis elevados nos indivíduos com sobrepeso e obesidade (COELHO et al., 2013). Neste contexto, o consumo de suco de laranja poderia ser uma estratégia interessante para reduzir ou até mesmo neutralizar marcadores inflamatórios que frequentemente estão alterados em indivíduos com excesso de peso (GHANIM et al., 2007). Estudo recente mostrou que a administração de naringina atenuou os efeitos da síndrome metabólica (obesidade abdominal, dislipidemia, disfunção e acúmulo de gordura no fígado e resistência á insulina) induzida por dieta hiperlipídica, através de diferentes mecanismos de sinalização intracelulares e redução do estresse oxidativo (PU et al., 2012).

Além dos efeitos favoráveis relacionados ao excesso de peso, síndrome metabólica e dislipidemias, o suco de laranja também tem sido apontado com um agente quimiopreventivo, pois tem habilidade de inibir, retardar ou reverter alguns estágios no processo de carcinogênese (FANKE et al., 2013). Por exemplo, se o suco de laranja for consumido como uma parte das cinco porções de frutas recomendadas, poderá prevenir alguns tipos de câncer em adultos saudáveis (MCCALLAND FREI, 1999). Os benefícios do suco de laranja no câncer são atribuídos aos seus efeitos anti-inflamatórios, citoprotetores/pro-apoptóticos, moduladores de sinalização celular, antioxidante, anti-genotóxico e sobre enzimas metabólicas (FRANKE et al., 2013). As propriedades anti-proliferativas dos flavonoides do suco de laranja são possivelmente devido à regulação do ciclo celular, entre outras (GUTHRIE e CARROLL, 1998; SO et al., 1996). Estudo prévio mostrou que a administração do metabolito hesperitina, inibiu a proliferação celular e bloqueou o ciclo celular na fase G1 em células humanas de câncer de mama (CHOI, 2007).

Além disso, flavonoides cítricos podem regular genes sensíveis ao fator de transcrição nuclear kappa B (NF-κB) e a cascata de sinalização de proteínas quinases

ativadas por mitógenos (MAPK). O NF-κB está envolvido em um mecanismo de controle da transcrição de respostas celulares a estímulos como o estresse oxidativo, inflamação e infecções bacterianas e virais, que são associados com câncer (MILLER et al., 2008). MAPK é um sistema enzimático que responde a estímulos mitogênicos extracelulares e regula várias atividades celulares, como a divisão e proliferação celular, diferenciação e apoptose (BRADHAM e MCCLAY, 2006; WU et al., 2008).

Assim, baseado em evidências recentes, nós hipotetizamos que o suco de laranja poderia estimular e/ou suprimir, positivamente, biomarcadores relacionados com o excesso de peso (indivíduos) e também com o câncer (leucemia linfoblástica aguda de células T). Portanto, o objetivo deste estudo foi investigar o efeito do consumo de suco de laranja em indivíduos com excesso de peso (*in vivo*) e o efeito da sua administração em uma nova linhagem celular de câncer – células Loucy (*in vitro*).

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OBJETIVOS

Investigar o efeito do suco de laranja em dois modelos experimentais nos quais biomarcadores séricos e celulares estão sendo afetados pela condição de sobrepeso (experimento *in vivo*) e câncer (experimento *in vitro*).

A) Experimento em humanos *in vivo*

Avaliar o efeito da ingestão do suco de laranja sobre biomarcadores metabólicos, inflamatórios e oxidativos em indivíduos com sobrepeso.

B) Experimento em cultura celular *in vitro*

Avaliar propriedades anti-proliferativas, pro-apoptóticas e imunomoduladoras do suco de laranja blond e vermelho e da hesperidina em uma linhagem de células leucêmicas (Loucy).

CAPÍTULO I: REVISÃO DA LITERATURA

1. Frutas cítricas e sucos de laranja: história, classificação e tipos

As frutas cítricas originaram no Sudeste Asiático, sendo que as laranjas doces podem ter se originado na Índia, a tangerina na China, e os outros tipos de frutas cítricas na Malásia. A primeira menção sobre frutas cítricas na Europa foi em 310 a.C por Teofrasto, o pai da botânica. Mais tarde, as frutas cítricas se espalharam por toda a Ásia, Norte da África e Europa através do comércio. Para as Américas, elas foram trazidas pelos exploradores como Cristóvão Colombo no final dos anos 1400 e início de 1500. Ponce de Leon introduziu as primeiras laranjeiras em St. Augustine, Flórida, EUA entre 1513 e 1565 (NEVES, 2009; ROUSEFF et al., 2008; WEBBER, 1967). As *grapefruit* foram estabelecidas pela primeira vez, na Flórida em 1823 por Don Philippe (um nobre espanhol), que plantou o primeiro bosque de *grapefruit* perto de Tampa, FL (ROUSEFF et al., 2008).

No Brasil, durante o período da colonização, a laranja foi introduzida e encontrou excelentes condições para vegetar e expandiu-se por todo o território nacional. A citricultura destacou-se em vários Estados até atingir a região do Vale do Paraíba em 1940, Limeira – SP, e posteriormente Araraquara em 1950 e Bebedouro ao final dessa década, e se estendeu ao norte e noroeste do Estado de São Paulo (NEVES, 2009). A identificação taxonômica das frutas cítricas é difícil porque existem muitos híbridos espontâneos e comerciais, entretanto elas podem ser classificadas nas seguintes categorias: laranjas doces, sanguíneas e sem acidez (C. sinensis), mandarins (Satsuma C. unshi), tangerinas (C. tangerina e reticulata) e clementinas (C. clementina), laranjas azedas/amargas (Sevilha, C. aurantium), limões (C. limon), limas (C. aurantifolia e latifolia), *grapefruit* (C. paradisi; C. grandis) e cidras (C. medica) (ORTIZ et al, 2002; TURNER e BURRI, 2013).

Dietas ricas em frutas e hortaliças têm sido fortemente associadas com inúmeros benefícios à saúde e menor risco no desenvolvimento de doenças, pois são importantes fontes de nutrientes, fibras e fitoquímicos (BOIENG et al., 2012). Neste cenário, as frutas cítricas têm sido muito valorizadas por conter nutrientes e compostos bioativos que apresentam atividades biológicas, tais como antioxidante, antimutagênica e imunomodulatórias (BENAVENTE-GARCIA e CASTILLO, 2008). Por causa da preferência pelo sabor de laranja, preço acessível e principalmente uma maior conscientização do consumidor sobre os efeitos positivos na saúde humana, as frutas cítricas e seus produtos são muito consumidos e difundidos nos países desenvolvidos e em desenvolvimento (LIU et al., 2012).

O suco de laranja é um dos sucos de frutas mais consumidas em todo o mundo e é reconhecido como uma bebida com elevada densidade nutricional, ou seja, fornece, simultaneamente, quantidades substanciais de vitaminas e minerais e valor calórico reduzido (RAMPERSAUD, 2007). Um recente estudo longitudinal comparou adultos consumidores e não consumidores de suco de laranja e mostrou que a ingestão habitual *per capita* de suco de laranja 100%, ou seja, sem adição de açúcares foi de 50 mL/dia. Também, o consumo regular de suco de laranja foi associado a uma melhor qualidade da dieta, contribuindo para atingir o requerimento médio de ingestão de nutrientes essenciais (principalmente vitamina C e folato) e flavonoides (O'NEIL et al., 2012).

Em relação à produção do suco de laranja, de cada cinco copos consumidos no mundo, três são produzidos nas fábricas brasileiras. O Brasil conta com 50% da produção mundial de suco de laranja, e exporta 98% do que produz (NEVES, 2009). O tipo de suco produzido é determinado pelo consumidor em mercados de poder aquisitivo elevado, que nos últimos anos passou a preferir o NFC (not frozen concentrate) ao FCOJ (frozen concentrated orange juice), por ser um produto de paladar mais agradável, com sabor mais

aproximado ao do suco espremido na hora e por apresentar aspecto mais saudável (NEVES, 2009).

Beaseado na coloração da polpa, as laranjas podem ser divididas em dois grandes grupos: laranjas claras (que neste trabalho nós iremos chamar de laranja blond) e as laranjas sanguíneas. As laranjas blond seriam caracterizadas pela cor laranja na polpa e no suco, devido à presença de carotenoides, que são pigmentos que podem variar entre o amarelo e o vermelho. As variações de cor da polpa de frutos observadas entre as variedades deste grupo seriam devidas as flutuações na quantidade dos diferentes carotenoides presentes. A este grupo pertencem quase que a totalidade das laranjas comerciais cultivadas no mundo, incluindo as variedades de mesa (laranjas Baías, Navel, Baianinhas e outras), variedades usadas para a extração de suco (Pêra, Valência, Natal, Hamlin e outras), laranjas sem acidez (Laranja Lima, Serra d´agua, por exemplo) (LATADO et al., 2009).

As laranjas sanguíneas são caracterizadas pela coloração vermelho-intenso da polpa e do suco, devido à presença do pigmento antocianina. As antocianinas são pigmentos pertencentes à família dos flavonoides e possuem uma série de funções na natureza, tais como a proteção de plantas contra o ataque de patógenos, proteção contra os danos causados pela radiação UV e a pigmentação de flores, frutos e sementes, com as finalidades de atração de insetos polinizadores e de dispersão de sementes. Como exemplos de variedades mais conhecidas e utilizadas de laranjas sanguíneas podemos citar: Tarroco, Moro, Sanguigno doppio, Sanguinella e Sanguinelo, entre outras que são mais cultivadas nas regiões do mediterrâneo e na Índia, por apresentarem maior aceitação comercial (LATADO et al., 2009).

No entanto, estudos prévios também atribuem á coloração vermelha, aos pigmentos carotenoides, como o licopeno, em vez de antocianinas. Laranjas doces de polpa vermelha (Laranja de Mombuca ou Falsa sanguínea) vêm sendo cultivadas desde 2005 no Brasil, e

como estas laranjas são uma nova variedade de frutas cítricas, os seus efeitos na promoção da saúde ainda não são foram explorados (LEE, 2001). O suco de laranja doce de polpa vermelha é diferente de outros sucos de laranjas sanguíneas porque contém os carotenoides licopeno, β-caroteno, xantina, entre outros (Alquézar et al, 2008 e 2009). Além disso, este tipo de suco contém quantidades semelhantes de nutrientes e flavonoides presentes no suco de laranja blond, porém, possui 2 vezes mais carotenoides totais, incluindo β-caroteno (5 vezes mais) e licopeno (MOURA, 2010). A presença de licopeno em laranja doce não é comum, mas é de especial interesse, devido aos estudos nutricionais e epidemiológicos recentes que têm investigado a importância do licopeno na prevenção de alguns tipos de cânceres (GANN et al., 1999; SALMAN et al., 2007; TEODORO et al., 2012).

Quadro 1. Composição de carotenoides do suco de laranja vermelha e clara/blond, integral e pasteurizado

Suco de Laranja (250 mL)	**Blond**	**Vermelho**
Carotenoides totais (μg)	1761	2800
β-caroteno (μg)	20	300
Criptoxantina (μg)	67	101
Luteína + Zeoxantina (μg)	67	92
Licopeno (μg)	0	430

Fonte: LATADO et al., 2009

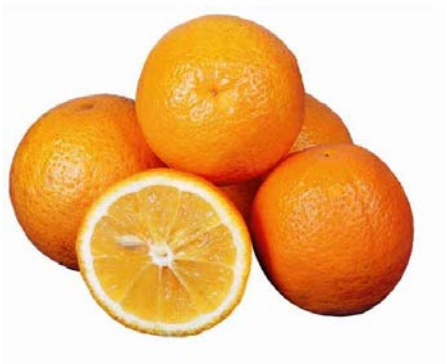

Figura 1. Fruto de laranja da variedade Valência (claras/blond)

Fonte: LATADO et al., 2009

Figura 2. Fruto de laranja da variedade sanguínea de Mombuca (vermelhas)

Fonte: LATADO et al., 2009

Figura 3. Sucos de laranja vermelha (sanguínea de Mombuca) e clara/blond (Pera)

Fonte: LATADO et al., 2009

2. Aspectos nutricionais do suco de laranja

Caracterizado pelo aroma distinto e delicioso sabor, o suco de laranja têm sido reconhecido como uma bebida importante e integrado na alimentação diária, contribuindo para o fornecimento de energia e nutrientes, e consequentemente, na promoção da saúde (LIU et al., 2012). O suco de laranja possui baixo teor de proteínas e lipídeos, e é rico carboidratos, como sacarose, glicose e frutose (2:1:1), os quais são responsáveis por conferir teor de doçura ao suco (TING e ATTAWAY, 1971). Dependendo da fruta, o conteúdo total de açúcar no suco pode variar de menos 1%, em alguns limões até 15% em algumas laranjas (RANGANNA et al., 1983). As composições nutricionais dos sucos de laranjas variam significativamente devido ao tamanho dos frutos, variedade, maturidade, armazenamento, condições de horticultura e clima flutuante sugerindo, que as análises de nutrientes fornecem informações apenas entre variedades de frutos similares (LIU et al., 2012). O quadro 1 mostra a composição nutricional contido em 1 copo de 250 mL de suco de laranja.

Sucos de laranja não são considerados uma importante fonte de proteínas, lipídeos e fibras. Portanto, a ingestão do suco de laranja como fonte desses nutrientes tem pouco impacto na nutrição humana (LIU et al., 2012). Por outro lado, os sucos de laranjas representam uma excelente fonte de vitamina C, ácido fólico, carotenoides e flavanonas, os quais serão descritos com mais detalhes abaixo.

Vitamina C (ácido ascórbico)

A vitamina C (ácido ascórbico) é um nutriente essencial que atua como um antioxidante e está envolvida no metabolismo do ferro, na biossíntese de carnitina, neurotransmissores, colágeno e ligamentos das fibras ósseas, e é um co-fator de vários processos enzimáticos e hormonais (DRI, 2003; MEISTER, 1994). Além disso, a vitamina C também está envolvida com o sistema imunológico, pois estimula a função das células brancas do sangue (WINTERGERST et al., 2006).

In vitro, o ácido ascórbico pode contribuir com 40% a 54% do potencial antioxidante de laranjas, tangerinas e *grapefruits* (SZETO et al., 2002). A maioria das pessoas pode alcançar 100% da RDA (Recommended Dietary Allowance) de vitamina C ao consumir quantidades moderadas de frutas ou sucos cítricos. As recomendações de ingestão mais recentes são 75 a 90 mg para adultos e valores ainda mais elevados para os indivíduos que são fumantes e mulheres durante a gravidez e lactação (USDA, 2011a e b).

Estudos em humanos também têm mostrado fortes evidências para as propriedades antioxidantes relacionadas à vitamina C em frutas cítricas (LIU et al., 2012). Por exemplo, Sánchez-Moreno et al (2003) mostrou que o consumo de 500 mL/dia de suco de laranja durante duas semanas (~ ácido ascórbico 250 mg/dia) aumentou as concentrações plasmáticas de vitamina C de 40% a 64% e diminuiu marcadores de oxidação (8-epi-PGF2) em adultos, com um efeito mais pronunciado em fumantes.

Em outro estudo, foi observada uma redução de 47% da peroxidação lipídica no plasma de adultos saudáveis após o consumo de ~ 236 mL de suco de laranja (~ 70 mg de vitamina C) todos os dias durante duas semanas (JOHNSTON et al., 2003). Esses dados mostram quão rápidos ocorre o aumento da vitamina C no plasma e o quanto a capacidade antioxidante pode ser alcançada através do consumo de quantidades razoáveis de suco de laranja (TURNER e BURRI, 2012). A laranja ou *grapefruit* de tamanhos médios contém aproximadamente 56 - 70 mg de ácido ascórbico e 225 mL de suco de laranja normalmente contém em média 125 mg de ácido ascórbico (WHITNEY et al., 2009).

A vitamina C do suco fresco é bastante estável durante curtos períodos de armazenamento e processamento, sendo que nenhuma perda grave de vitamina C foi observada após a temperatura de refrigeração durante um período de tempo razoavelmente longo (HORTON e DICKMAN, 1977). Mesmo em recipientes abertos, tais como vidro e latas, a perda de vitamina C é mínima quando os produtos são mantidos em baixa

temperatura. No entanto, a degradação da vitamina C pode ser mais pronunciada em contato com oxigênio atmosférico e elevadas temperaturas (SMOOT e NAGY, 1980).

Ácido fólico (folato)

As frutas e sucos cítricos também são fontes naturais de ácido fólico, entretanto o suco de laranja se destaca por conter altas concentrações de folato em relação a outros sucos de frutas comumente consumidos (USDA, 2011a e b). O ácido fólico atua como uma coenzima essencial e está envolvido em várias funções biológicas importantes, tais como a síntese, reparação e metilação de DNA; divisão celular e crescimento; e no metabolismo da homocisteína (KAMEN 1997; FENECH et al., 1998). Recomendações dietéticas atuais definem 400 μg de ácido fólico/dia de acordo com RDA para adultos (USDA, 2011a e b). Embora alguns alimentos sejam ricos em ácido fólico, como vegetais de folhas verdes (espargos, espinafres, e nabo), gema de ovo e leguminosas (feijão e ervilha), o seu nível na dieta geralmente é baixo (USDA, 2011a e b). A exigência de enriquecer grãos processados pela adição de ácido fólico em alimentos como pães, cereais, farinhas e farinha de milho colaborou para a ingestão de ácido fólico na dieta (CRANDALL et al., 1998; MALINOW et al., 1998).

Quadro 2. Composição nutricional do suco de laranja blond integral e pasteurizado

Nutrientes	250 mL
Energia (kcal)	120
Água (g)	222
Proteínas (g)	1,5
Gorduras totais (g)	0,4
Carboidratos (g)	29
Açúcares totais (g)	21
Fibra dietética (g)	0,5
Cálcio (mg)	20
Ferro (mg)	1,1
Magnésio (mg)	27
Fósforo (mg)	1,1
Potássio (mg)	436
Sódio (μg)	0,2
Zinco (mg)	0,2
Vitamina A (μg)	22
Vitamina C (mg)	86
Folato (μg)	47
β-caroteno (μg)	20
Hesperitina (μg)	13,8
Naringenina (μg)	3,9

Fonte: USDA, 2014

Carotenoides

Os carotenoides são responsáveis pela coloração de um grande número de alimentos, entre eles, a cenoura, o suco de laranja, o tomate, o salmão e a gema de ovo. Foi reconhecido durante muitos anos o papel destes compostos, especialmente, os carotenos alfa e beta, e a beta-criptoxantina como excelentes fontes precursoras de vitamina A (MELÉNDEZ-MARTÍNEZ et al., 2004). No entanto, estudos recentes têm destacado as propriedades dos

carotenoides e a sua eficiência na prevenção de algumas doenças, incluindo propriedades antioxidantes, estímulo do sistema imune e comunicação celular (BURRI et al., 2011; MATSUMOTO et al., 2007; STAHL et al., 1997), proteção da região macular da retina humana e de formação de catarata, (SNODDERLY, 1995), e diminuição no risco de câncer (GALLICCHIO et al., 2008; NISHINO et al., 2009). Assim, o interesse nestes compostos tem aumentado muito do ponto de vista nutricional (MELÉNDEZ-MARTÍNEZ et al., 2004). Mais de 600 carotenoides foram identificados e cerca de 50 estão presentes na dieta humana (BRITTON, 1995).

Os carotenoides mais abundantes na dieta humana são a luteína, zeaxantina, licopeno, e os carotenoides pró-vitamina A, α-e β-caroteno e β-criptoxantina, os quais são encontrados em frutas e vegetais (BURRI et al., 2011). Sucos de laranjas podem fornecer uma quantidade substancial de β-criptoxantina, que é altamente biodisponível (BURRI et al., 2011). De acordo com dados do NHANES (2009-2010), a maioria das pessoas nos EUA não está consumindo quantidade suficiente de vitamina A em alimentos, e apenas uma pequena proporção de vitamina A é consumido como carotenoides (~ 20% - 35% da RDA de vitamina A) (USDA, 2014). Por outro lado, o consumo elevado de vitamina A a partir de suplementos têm sido associados com efeitos negativos para a saúde, incluindo diarreia, náuseas, vômitos, dores de cabeça, alterações ósseas e osteoporose, danos no fígado, perda de cabelo, e, possivelmente, defeitos de nascimento (PENNISTON e TANUMIHARDJO et al., 2006).

Flavonoides cítricos

Polifenóis são metabolitos secundários das plantas caracterizados por uma estrutura básica de um anel aromático com um ou mais grupos hidroxilas (Figura 1). De acordo com suas estruturas químicas, polifenóis podem ser divididos em quatro principais classes: ácidos

fenólicos, flavonoides, estilbenos, e lignanas (MANACH et al., 2004). Os principais polifenóis encontrados na dieta humana são os ácidos fenólicos e os flavonoides, os quais representam aproximadamente 2/3 e 1/3 do total da ingestão diária de polifenóis, respectivamente (PEREZ-JIMENEZ et al., 2011; CHANET et al., 2012).

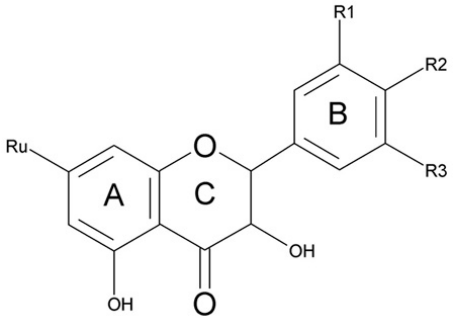

Figura 4. Estrutura básica de um flavonoide

Fonte: ROSS e KASUM, 2002

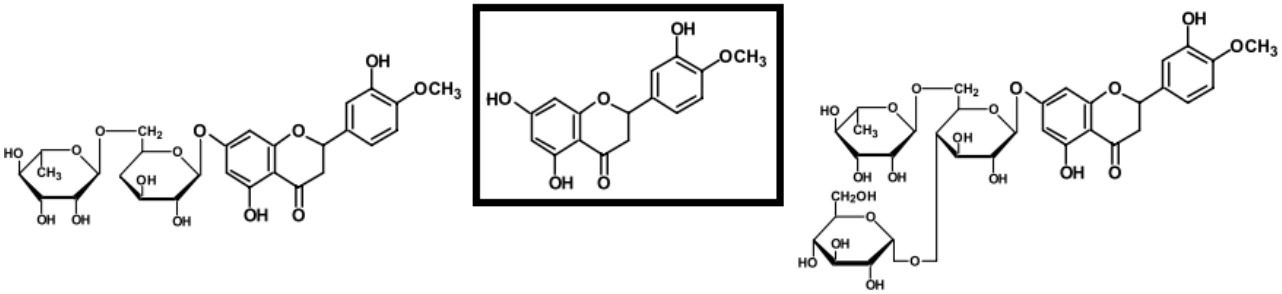

Figura 5. Estrutura química da hesperidina (á esquerda) hesperitina (ao centro) e da hesperidina glicosilada (á direita)

Fonte: http://examine.com/supplements/Hesperidin/

Flavonoides dietéticos são divididos em seis subclasses de acordo com suas características estruturais em: flavonas, flavonols, flavanonas, catequinas, antocianidinas se

isoflavonas. Uma das classes mais comuns de flavonoides encontrados exclusivamente em frutas cítricas, e em menor grau, em tomates e algumas ervas aromáticas são as flavanonas, hesperidina e naringina (ROSS e KASUM 2002; LIU et al., 2012). Nas frutas cítricas, as flavanonas são responsáveis por aproximadamente 95% dos flavonoides totais, sendo que as principais agliconas são a naringenina em *grapefruit*, hesperitina em laranja e tangerina, e eriodictiol em limão. Além disso, as flavanonas são geralmente glicosiladas por um dissacarídeo que confere um sabor amargo, como naringina e hesperidina em laranjas (BERHOW, 1998; PETERSON et al., 2006a e b).

O teor de flavanonas em frutas cítricas varia de acordo com a parte do fruto. A figura 1 mostra as partes sólidas da fruta, em particular o albedo (a parte esponjosa branca) e as membranas que separam os segmentos, os quais são os mais ricos em flavanonas comparado ás vesículas de suco (celulose), o que explica o maior teor de flavanonas no fruto do que no suco. Além disso, as flavanonas no albedo e segmentos são descartados (TOMAS-BARBERAN e CLIFFORD, 2000). Na fração comestível das laranjas, o conteúdo glicosídeo (hesperidina) varia de 35 a 147 mg/100 g (PETERSON et al., 2006a e b; NOGATA et al., 2006). Após compilação de dados sobre composição de alimentos, Tomas-Barberan e Clifford (2000) estimaram que teor médio das flavanonas em suco de laranja (hesperidina) estavam na faixa de 14 a 77 mg/100 mL. Em outro estudo, o conteúdo de glicosídeos de naringenina em várias marcas de sucos de *grapefruit* variaram entre 17 e 76 mg/100 mL (ROSS et al., 2000). Devido à falta de um banco de dados com informações sobre polifenóis em longo prazo, a ingestão de flavanonas não tem sido estimada em muitos estudos epidemiológicos.

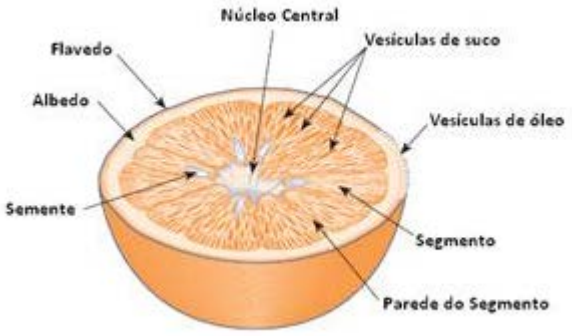

Figura 6. Estruturas da parte interna da laranja

Fonte: http://www.citrusbr.com.br/exportadores-citricos/laranja-suco/por-dentro-da-laranja-192885-1.asp

Contudo, a ingestão diária de flavanonas em adultos de diferentes países foi estimada entre 2,7 a 78 mg de aglicona (ERDMAN et al., 2007; PEREZ-JIMENEZ et al., 2011), sendo que as laranjas (frutas inteiras e sucos) parece ser o principal contribuinte para a ingestão das flavanonas (ZAMORA-ROS et al., 2010). No entanto, a biodisponibilidade da maioria das

flavonoides, após administração oral é bastante lento e irregular devido à sua natureza pouco solúvel em solução aquosa. Por exemplo, a glicosil hesperidina (G-hesperidina) um derivado estruturalmente modificado de hesperidina parece ser dez mil vezes mais solúvel em água do que a hesperidina. Entretanto, ambos as formas apresentaram *in vitro* e *in vivo* o mesmo perfil metabólico após a absorção (YAMADA et al., 2006). Um grande corpo de evidências, incluindo estudos do nosso grupo de pesquisa tem mostrado que as flavanonas são compostos biologicamente ativos provavelmente por contribuir para a prevenção de doenças cardiovasculares e câncer (de OLIVEIRA et al., 2012; DOURADO et al., 2014; MANTHEY et al, 2001; MORAND et al, 2011).

3. Digestão, absorção e metabolismo das flavanonas do suco de laranja

Tem sido descrito que antes da absorção, as formas glicosiladas das flavanonas são hidrolisadas pelas enzimas glicosidases da microflora colônica (30%). As agliconas livres liberadas são então captadas e conjugadas pelas enzimas de fase II no intestino e no fígado. Como resultado, as flavanonas circulam no plasma principalmente na forma de metabolitos sulfatados e glicuronídeos (URPI-SARDA et al., 2012). As flavanonas alcançam o cólon para serem absorvidas, assim sua concentração no plasma ocorre até 6 h após a ingestão (MANACH et al., 2004). Depois do consumo do suco de laranja, os metabolitos identificados no plasma foram hesperetina-7-glucoronideo, hesperetin-3′-glucoronideo, hesperetina-3′-sulfato, naringenina-4′-glucoronideo, e naringenina-7-glucuronídeo (BRETT et al., 2009; VALLEJO et al., 2010).

O principal pico de concentração no plasma das flavanonas variam entre 0.1 a 1 μM em uma ingestão que varia de 150 g de fruta até 500 mL de suco de laranja. Também, a concentração plasmática de 0,47 e 0,6 μM foram medidas depois da ingestão de 230 g da laranja (fruta) ou 550 mL de suco de laranja (ambos também fornecem 115 mg de agliconas), respectivamente. A similaridade entre as concentrações no plasma das flavanonas, observadas após administração da frutas cítricas inteiras ou sucos, sugerem que a matriz do alimento não afeta significativamente a biodisponibilidade destes flavonoides (VALLEJO et al., 2010, MANACH et al., 2004). A excreção urinária das flavanonas ocorre principalmente durante as 24 h após a ingestão com um pico entre 6 à 12h. Após o consumo de uma laranja (como fruta ou suco), a relativa excreção urinária de hesperitina varia entre 2 e 6% (VALLEJO et al., 2010, MANACH et al., 2004). A excreção de naringenina após o consumo de suco de laranja foi entre 1 até 18% da sua ingestão (MULLEN et al., 2004; ERLUND et al., 2001).

4. Evidências sobre o consumo de suco de laranja e flavonoides cítricos na prevenção de desordens metabólicas associadas ao excesso de peso

A obesidade pode ser definida como o aumento da ingestão de energia em relação ao gasto, resultando na deposição de gordura e ganho de peso. O acúmulo e armazenamento de lipídeos no tecido adiposo podem ser promovidos pela ingestão de dieta hipercalórica rica em carboidratos e gorduras, associada a um estilo de vida sedentário (TITTA et al., 2010). De acordo com as diretrizes da Organização Mundial da Saúde (OMS), o excesso de peso em adultos é definido como um índice de massa corporal (IMC: entre 25,0-29,9 kg/m^2), e a obesidade (IMC $\geq$ 30,0 kg/m^2) (WHO, 2002). O excesso de gordura corporal aumenta o risco para o desenvolvimento de várias doenças, incluindo diabetes, hiperlipidemia e hipertensão, que podem favorecer o desenvolvimento de aterosclerose e síndrome metabólica (GUH et al., 2009; ALAM et al., 2014). Assim, as organizações de saúde recomendam limitar o consumo de alimentos com elevado teor de açúcares e gorduras e aumentar o consumo de alimentos com baixo valor energético, especialmente frutas e hortaliças com o objetivo de prevenir o ganho de peso (WHO et al., 2003).

A inflamação de baixo grau tem sido reconhecida como uma relação causal entre adiposidade e o risco de desordens metabólicas crônicas, tais como obesidade, síndrome metabólica e diabetes (LUMENG et al., 2011). O excesso de tecido adiposo no abdômen favorece o disparo de uma cascata de eventos que levam à ativação crônica do sistema imune inato, resultando no desenvolvimento da inflamação de baixo grau. Esta condição é caracterizada pelo excesso de produção de mediadores inflamatórios, incluindo proteína C-reativa (CRP), TNFα, e IL-1β, entre outros (BASTARD et al., 2006). Além disso, o estresse oxidativo é o fator comum subjacente a estas doenças, provocando uma resposta inflamatória precoce e liberação de moléculas pró-inflamatórias (CERIELLO e MOTZ, 2004). A elevação dos triglicérides intracelulares que causam o aumento da formação de superóxido e

estimula adipócitos ou pré adipócitos a produzirem citocinas inflamatórias, as quais induzem a formação de vários radicais oxidativos (BAKKER et al., 2000; COPPACK, 2001; FENSTER et al., 2002).

Tem sido sugerido que os flavonoides cítricos, naturalmente presente em laranjas e no suco, apresentam propriedades anti-inflamatórias e antioxidantes que auxiliam na prevenção e no tratamento das condições acima mencionadas (BENAVENTE-GARCIA e CASTILLO, 2008; GARCÍA-LAFUENTE et al., 2009; GHANIM et al., 2010). Estudo prévio mostrou que a hesperidina e naringina apresentaram atividade anti-inflamatória em modelo experimental, sendo observada a normalização na concentração elevada de TNF-α e da infiltração de células inflamatórias (JAIN e PARMAR, 2012). É bem conhecido que o fígado pode ser afetado pela secreção de mediadores pro-inflamatórios do tecido adiposo, por exemplo, a ativação crônica do NF-kB por citocinas tem sido diretamente relacionado ao desenvolvimento de resistência à insulina (CAI et al., 2005). Por outro lado, extratos de frutas cítricas possuem grandes quantidades de flavonoides e mostraram potente atividade sequestradora de radicais livres (GUIMARÃES et al., 2010). Naringina e hesperidina e suas formas agliconas, são potentes sequestradores de radicais livres e inibidores de peroxidação lipídica (CAVIA-SAIZ et al., 2010; SANCHEZ-MORENO et al., 2003).

Níveis elevados de lipídeos no sangue (hiperlipemia) é um sintoma característico em indivíduos obesos e/ou com distúrbios metabólicos. Tem sido mostrado que flavonoides cítricos são capazes de diminuir as concentrações de lipídios plasmáticos em adultos (APTEKMANN e CESAR, 2013; de OLIVEIRA et al., 2012). As suplementações com naringina e hesperidina reduziram lipídios plasmáticos em modelos experimentais de hiperlipidemia e obesidade (MOLLACE et al., 2011; PU et al., 2012). Também a administração de naringina reduziu as concentrações de lipídios plasmáticos que estavam elevados em ratos alimentados com dieta rica em gordura (PU et al., 2012), e melhorou os

níveis de colesterol, triglicérides e ácidos graxos livres circulantes no plasma em ratos obesos alimentados com dieta com alto teor de gordura e carboidratos (ALAM et al, 2013).

Ensaio clínico mostrou que a suplementação com naringina (400 mg/dia) reduziu as concentrações plasmáticas de colesterol e LDL-C, enquanto triglicérides e HDL-colesterol permaneceram inalterados em pacientes hipercolesterolêmicos (JUNG et al., 2004). Efeito semelhante também foi observado em um estudo conduzido por nosso grupo, no qual foi verificado que o consumo de suco de laranja em longo prazo (480 mL de suco de laranja/dia por 12 meses) reduziu as concentrações de colesterol total, LDL-Ce apo B e a razão LDL/HDL, em comparação com os indivíduos não consumidores de suco. Contudo, o percentual de gordura abdominal nos consumidores de suco de laranja não diferiu dos não consumidores (APTEKMANN e CESAR, 2013). De maneira similar, estudo prévio mostrou efeito positivo da ingestão de 750 mL de suco de laranja/dia nas concentrações de HDL-C (KUROWSKA et al., 2000).

A hiperglicemia e resistência à insulina são características comuns de síndrome metabólica. A resistência à insulina pode ser definida como a diminuição da resposta dos tecidos periféricos à ação da insulina. Estudo prévios mostraram que a IL-6 e as concentrações de TNF-α estavam aumentados em indivíduos com a resistência à insulina e diabetes tipo 2 (KADO et al., 1999; PICKUP et al., 2000). TNF-α e IL-6 podem causar a disfunção do receptor de insulina periférica e resistência à insulina resultando no aumento das concentrações de glicose no plasma (KROGH-MADSEN, 2006; DANDONA et al., 2004).

As dietas ricas em gorduras aumentam a secreção de citocinas inflamatórias, o risco de resistência à insulina e causam hiperglicemia (LEE et al., 2010; GHANIM et al., 2010). O efeito hipoglicemiante de naringina foi evidenciado (JUNG et al., 2004; ALI e KADER, 2004) pela suplementação do flavonoide (30 mg/kg de peso) acompanhado de vitamina C

(50 mg/kg de peso) levando a melhora na concentração de insulina e prevenção do estresse oxidativo em ratos (PUNITHAVATHI et al., 2008). Tem sido sugerido também que o efeito hipoglicemiante observado após a administração flavonoides cítricos está relacionado a ação que esse compostos exercem sobre atividade das enzimas reguladoras da glicemia (JUNG et al, 2004).

5. Evidências sobre o consumo de suco de laranja e flavonoides cítricos na prevenção/terapêutica do câncer

O câncer é uma doença hiperproliferativa que envolve transformação morfológica celular, desregulação da apoptose, proliferação celular descontrolada, invasão, angiogênese e metástase (HANAHAN e WEINBERG, 2000). A leucemia linfoblástica aguda de células T (LLA-T) é um tipo de câncer induzido pela transformação de células T progenitoras e ocorre principalmente em crianças e adolescentes. Embora o resultado do tratamento em pacientes com LLA-T tenha avançado nos últimos anos, os pacientes com recidiva da doença continuam a ter um prognóstico ruim (PUI et al., 2008).

Aproximadamente 15% e 25% dos casos diagnosticados de leucemia linfoblástica aguda (LLA) em crianças e adultos, respectivamente, são de células T e estão historicamente ligados a um mau prognóstico. LLA-T é definida por características clínicas e biológicas distintas e desfavoráveis, como uma alta contagem de células brancas do sangue, adenopatias volumosas e envolvimento do sistema nervoso central. No entanto, apesar dessas características, o prognóstico para pacientes com LLA-T melhorou significativamente, devido à aplicação de regimes de quimioterapia intensiva. Recentemente tem sido imortalizado uma linhagem permanente de células de LLA-T (Loucy). As células Loucy expressam típicas características morfológica, citoquímica e antigênica associada com a linhagem celular derivada de LLA-T (Vide detalhes em Anexo II).

Atualmente está bem estabelecido que os componentes da dieta podem modificar o risco de câncer por influenciar vários processos, incluindo a reparação do DNA, a proliferação celular, diferenciação, apoptose, e a angiogênese, entre outros (PERCIVAL et al., 2008). Novas estratégias para suprimir as células cancerígenas através do consumo regular de compostos bioativos naturais estão atraindo a atenção de pesquisadores, principalmente devido às habilidades destes compostos limitar a resistência de células cancerígenas a apoptose (PATIL et al., 2009).

Quimioprevenção é uma abordagem promissora que utiliza agentes naturais, biológicos ou sintéticos para reduzir ou retardar a ocorrência de câncer (STEWARD e BROWN, 2013). Estudos epidemiológicos têm mostrado que dietas, principalmente aquelas ricas em frutas e hortaliças apresentam atividades quimiopreventivas devido ao elevado conteúdo de flavonoides, principais compostos ativos em alimentos vegetais apontados como agentes quimiopreventivos (SURH et al, 2003).

O câncer pode ser controlado por muitas vias, incluindo supressão, bloqueio, e transformação. Agentes supressores previnem a formação de novos cânceres por meio de fatores pró-carcinogênicos; agentes bloqueadores impedem que fatores cancerígenos alcancem locais críticos de início da proliferação celular; e agentes de transformação atuam facilitando o metabolismo dos componentes cancerígenos em elementos menos tóxicos ou impedem as suas ações biológicas. Os flavonoides podem atuar em todas as três formas (MANTHEY et al., 2001).

Além disso, estudos recentes tem atribuído aos flavonoides da dieta, proteção do DNA contra danos oxidativos, inativação de substâncias cancerígenas, inibição da expressão de genes mutagênicos e enzimas responsáveis pela ativação de substâncias pró-carcinogênicas, e ativação dos sistemas responsáveis pela desintoxicação de xenobióticos (BRAVO et al., 1998). Muitos destes estudos têm mostrado uma relação entre a estrutural

química e as funções que os flavonoides irão desempenhar (KAWAII et al., 1999; MANTHEY e GUTHRIE et al., 2002).

Neste contexto, o consumo regular de frutas cítricas e, particularmente, do suco de laranja associado a uma dieta balanceada podem ser promissores. Os efeitos positivos de suco de laranja em relação as suas propriedades anticancerígenas têm sido focados nas flavanonas, hesperidina e naringina. A hesperidina parece exibir ações quimiopreventivas, em parte por bloquear a progressão do ciclo celular, bem como por desencadear a morte celular através de vias pró-apoptóticas (PAN et al., 2002; BENAVENTE-GARCIA et al., 2008; FRANKE et al., 2013).

Outras evidências também têm mostrado que fatores estruturais das moléculas de alguns flavonoides cítricos podem explicar os efeitos o antioxidantes e anti-proliferativos (MARTINEZ et al., 2003; RODRIGUEZ et al., 2002). Entre esses fatores incluem o estágio de oxidação da estrutura (flavanona, flavona, entre outros), substituintes (posição, número e natureza dos grupos nos anéis A e B da estrutura dos flavonoides), e a presença de glicosilação (MANTHEY et al., 2001; MANTHEY e GUTHRIE et al., 2002; BENAVENTE-GARCIA et al., 2005).

Se o suco de laranja for consumido como uma parte das cinco porções de frutas recomendadas, poderá prevenir alguns tipos de câncer em adultos saudáveis (MCCALL e FREI, 1999). As propriedades anti-proliferativas dos flavonoides do suco de laranja são possivelmente devido à regulação do ciclo celular, entre outros efeitos (SO et al., 1996; GUTHRIE e CARROLL, 1998). Estudo prévio mostrou que a administração do metabolito hesperitina, inibiu a proliferação celular e bloqueou o ciclo celular na fase G1 em células humanas de câncer de mama (CHOI, 2007).

Tem sido observado que flavonoides cítricos bloqueiam a progressão do ciclo celular, em ambas os limites das fases G1/S ou G2/M. No entanto, os mecanismos

moleculares envolvidos no bloqueio do ciclo celular permanecem desconhecidos. O ciclo celular eucariótico é regulado através da ativação sequencial e inativação de quinases dependentes de ciclina (CDKs) que conduzem a progressão do ciclo celular, através da fosforilação e desfosforilação de várias proteínas reguladoras (PAN et al., 2002). Progressão do G1 para a fase S em células de mamíferos é regulada pelo acúmulo de ciclinas D, E e A, que se ligam e ativam diferentes subunidades catalíticas Cdk. A transição da fase G1 cedo para meados é regulada pelo complexo D Cdk4-ciclina D e/ou Cdk6-ciclina. Transição a partir da fase G1 para a fase S é regulada pela ativação do complexo E Cdk2-ciclina. Transição de G2/M é regulada pela Cdk1-ciclina complexo A/B. Estudos recentes têm estabelecido uma relação estrutura-atividade para a inibição da Cdks por flavonoides. Flavonas cítricas, tais como tangeritina, induziram o bloqueio celular na fase G1 em células coloretais humanas (COLO 205) (PAN et al., 2002). Estes resultados sugerem que a detenção do ciclo celular por flavonoides ou tangeritina é um efeito que é dependente dos grupos estruturais destes agentes.

Além disso, flavonoides cítricos podem regular genes sensíveis ao fator de transcrição nuclear kappa B (NF-kappa B) e pela cascata de sinalização de proteínas quinase ativadas por mitógenos (MAPK). NF-kappa B está envolvido em um mecanismo de controle da transcrição de respostas celulares a estímulos como o estresse oxidativo, inflamação e infecções bacterianas e virais, que são associados com câncer (MILLER et al., 2008). MAPK é um sistema enzimático que responde a estímulos mitogênicos extracelulares e regula várias atividades celulares, como a divisão e proliferação celular, diferenciação e apoptose (BRADHAM e MCCLAY, 2006; WU et al., 2008). Estudo recente mostrou que que a hesperidina promoveu a acúmulo da proteína 53 (p53) e inibiu o fator de transcrição nuclear NF-kB, resultando em apoptose através do recrutamento de receptor ativado por proliferadores de peroxissona gama (PPAR-γ) em células NALM-6 (uma linhagem de

células leucêmicas) (GHORBANI et al., 2013).

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CAPÍTULO 2: Manuscrito 1

Orange juice consumption improves metabolic and inflammatory biomarkers in overweight subjects

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Abbreviations

BMI; Body mass index

hsCRP; High sensitive C-reactive protein

DPPH$^{\bullet}$; 2,2-dipheny-l-pikryl-hydrazyl

HOMA-IR; Homeostatic model assessment – insulin resistance

IFN-γ; Interferon gamma

NHANES; National Health and Nutrition Examination Survey

OJ; Orange juice

TBARS; Thiobarbituric acid reactive substances

TNF-α; Tumoral necrosis factor alpha

Abstract

Abdominal adiposity has been linked to metabolic abnormalities, including dyslipidemia, oxidative stress, and low grade inflammation. In this study we tested the hypothesis that 100% orange juice (OJ) consumption improves metabolic, oxidative and inflammatory biomarkers in overweight subjects with increased waist circunference. The subjects were divided into two groups: normal-weight and overweight in accordance with their body mass index and consumed 750 mL of orange juice daily. Body composition (weight, height, % fat mass, and waist circumference), metabolic (total cholesterol, LDL-C, HDL-C, triglycerides, glucose, insulin, HOMA-IR, glycated hemoglobin), oxidative (malondialdehyde and $DPPH^{\bullet}$), and inflammatory (CRP, IL-4, IL-10, IL-12, TNF-α, IFN-γ) biomarkers were evaluated before and after eight weeks of OJ consumption. The major findings of this study were: (1) OJ consumption did not alter body composition in either group; (2) improvement of the lipid profile, evidenced by a reduction in total cholesterol and LDL cholesterol; (3) a likely stimulation of the immune response due to the increase of IL-12 levels; (4) anti-inflammatory effects as a result of a marked reduction in CRP levels; and (5) antioxidant action by the enhancement of total antioxidant capacity and the reduction of lipid peroxidation-derived of malondialdehyde in both normal-weight and overweight individuals. In conclusion, OJ consumption has a positive effect on important biomarkers of health status in normal-weight and overweight subjects, thereby supporting evidence that OJ shows functional properties and could be consumed as part of a healthy diet to prevent metabolic and chronic diseases.

Keywords: Orange juice, Overweight, Inflammation biomarkers, Cytokines, Oxidative stress, Clinical Trial

1. Introduction

Easy access to hypercaloric food items, the ingestion of high-fat and high-carbohydrates meals, as well as ever more sedentary lifestyles have contributed to the progressive increase of overweight and obesity in Western societies [1]. Excess adipose tissue in the abdomen favors the induction of a cascade of events that lead to the chronic activation of the innate immune system, resulting in the development of low-grade inflammation. This condition is characterized by overproduction of proinflammatory mediators, such as circulating levels of C-reactive protein (CRP), TNF-α, and IL-1β, as well as insulin resistance and dyslipidemia, all of which are risk factors for cardiovascular diseases, metabolic syndrome, and diabetes [2]. Furthermore, oxidative stress is the common factor underlying these diseases, triggering an early inflammatory response and release of adhesion molecules and cytokines [3]. In contrast, the intake of various bioactive and antioxidant compounds commonly found in fruits and vegetables may counterbalance these events [4, 5].

Citrus fruits consumed as orange juice (OJ) constitute a significant source of the flavanones hesperidin and naringin in the diet [4, 6]. Several health benefits have been attributed to hesperidin, including anti-inflammatory, antioxidant, immunomodulatory, hypolipidemic, and anticancer effects [7]. However, evidence suggests that the effects of bioactive compounds are less pronounced when ingested as dietary supplements compared to the consumption of whole fruits and juices. This is a result of synergistic interactions that occur among phytochemicals and nutrients, which can target multiple signal transduction pathways in cells [8, 9]. A previous study showed that oxidative and inflammatory stress induced by high-fat and high-carbohydrate meals was prevented by the consumption of OJ in healthy subjects [10]. In addition, the consumption of 100% OJ was associated with better diet quality due to the increased intake of nutrients such as vitamin C, folate, and potassium,

reduced risk of obesity and metabolic syndrome, as well as the lowering of blood lipids [11] and lipid peroxidation [12, 13].

Due to paucity of clinical studies that evaluated the effect of OJ consumption on biomarkers commonly altered as a consequence of excess of weight and abdominal fat accumulation, the objective of this study was to test the hypothesis that the regular consumption of OJ improves metabolic, oxidative, and inflammatory biomarkers in overweight subjects. Further, the study aimed to verify whether OJ consumption could contributed to gain of weight, decrease the levels of serum lipids, CRP, proinflammatory cytokines, such as TNF-α, increase the antioxidant capacity, an amelioration in the quality of diet, and boost the immune response thought the interesting changes on cytokines levels.

2. Material and Methods

2.1. Orange juice

100% orange juice, commertially pasteurized, obtained from the variety of *Pera Rio,* was kindly provided by Citrosuco, Matao, Brazil. The characteristics of the OJ, assessed in this study, were measured in our Department in accordance with [14] and are describes following: Brix (11.5 g de sucrose/100 g), titratable acidity (1,01 %), ascorbic acid (26.9 mg/100 mL), total polyphenols (1.66 mg/100 mL gallic acid), total flavonoids (31.6 mg Rutin /mL), total carotenoids (2.00 mg β-carotene/mL), narirutin (15 mg/L), and hesperidin (103.3 mg/L).

2.2. Subjects and study design

A dietary intervention clinical trial, cross sectional was design to evaluate two groups of individuals in accordance with BMI, normal-weight (18.5-24.9 kg/m^2) and overweight (≥ 25 kg/m^2) [15]. Fifty-five healthy individuals, aged 23–59 years, of both genders were assessed for eligibility by individual interviews at Sao Paulo State University, Araraquara,

Brazil. Of this sample, 25 normal-weight and 25 overweight subjects were enrolled to the study due to their availability and willing to follow the research protocol, while 5 subjects were excluded. Exclusion criteria included the presence of cardiovascular, kidney, and thyroid disease, diabetes, pregnancy or lactation, drug treatment for hypertension or hyperlipidemia, or vitamin/mineral supplement. At the end of the study, 21 normal-weight and 25 overweight participants (n = 46) concluded the study, 4 dropped out during the course of the experiment because of professional or personal reasons, and therefore they were excluded from the data analysis.

In each group, the participants consumed 750 mL/day (3 cups) of OJ divided into at least two times a day during eight weeks. The period of intervention was based on previous studies that reported changes on serum biomarkers after OJ [16,17]. The subjects were also advised to continue their habitual diets during the study. The bottles of frozen OJ were delivered weekly to each participant and stored at 4^{o}C before consumption. Body composition measures (body weight, height, fat mass, and waist circumference), metabolic (glucose, insulin, homeostatic model assessment – insulin resistance (HOMA-IR), total cholesterol, HDL-C, LDL-C, triglycerides, glycated hemoglobin), oxidative (malondialdehyde and DPPH$^{\bullet}$ radical), inflammatory (C-reactive protein, TNF-α, IFN-γ, IL-12, IL-4, IL-10) biomarkers were analyzed in the serum of individuals before and after eight week OJ dietary intervention.

2.3. Body composition

Standardized weight, height, and waist circumference measurements were taken before and after the eight week period [15]. The percentage of fat mass was determined using bioelectrical impedance analysis (BIA, Biodynamics 310, Seattle, USA).

2.4. Ethics

The study was approved by Faculty of Pharmaceutical Sciences Review Board, Sao Paulo State University, for human experimentation (protocol n° 22/2009). All procedures of the study were performed in accordance with Good Clinical Practices and the World Medical Association Declaration of Helsinki. All individuals received suitable information regarding the goals of the study and the potential adverse effects and benefits in consume 750 mL of OJ. After comprehensive information, written informed consent was requested from all individuals. It was made clear that at any time the subjects could leave the study.

2.5. Blood samples

Blood samples were drawn from each participant via the cubital vein after 12 hours of fasting at baseline and at the end of the study period. The freshly drawn blood was centrifuged and the serum was stored at -80 ^{o}C.

2.6. Biomarkers

Total cholesterol, HDL-C, triglycerides, and glucose were determined by enzymatic colorimetric assay, whereas glycated hemoglobin was analyzed by immunoturbidimetric assay (Labtest Diagnóstica S/A, Brazil). Low density cholesterol (LDL-C) was indirectly estimated [18]. Insulin was measured by a chemiluminescent assay (Roche Diagnostics, USA). Insulin sensitivity was stimated by HOMA-IR using the equation: [fasting insulin (μU/l) × fasting glucose (mmol/l)] / 22.5, and the cut-off was associated to BMI [19]. Cytokines TNF-α, IFN-γ, IL-12, IL-4, and IL-10 were determined by ELISA assay (BD Pharmingen, USA). HsCRP was performed using the neflometry assay (Dade Behring Inc., USA). Lipid peroxidation was assessed by TBARS assay measuring the

amount of malondialdehyde, a secondary product of lipid peroxidation in serum [20]. Total antioxidant capacity in serum was determined by $DPPH^{\bullet}$ radical assay [21].

2.7. Nutritional assessment

A 24-hours recall was applied for dietary assessment. The participants were advised not to modify their food habits during the study. Energy, macronutrient, and micronutrient intakes were determined by the software Nut Win, version 1.5 (Federal University of Sao Paulo, Sao Paulo, Brazil).

2.8. Statistical analysis

The minimum number of individuals for each group was estimated, adopting 5% of significance level and 80% of desired power based on a previous clinical trials performed in our Lab [16,17]. The analysis indicated that at least 22 individuals in each group (n = 44) would significantly detect a difference in that parameter analyzed. All values were expressed as means ± standard deviation (SD), except inflammatory biomarkers, which were expressed as means ± standard error of the mean (SEM). Repeated measures two-way ANOVA followed by post-hoc Tukey test with statistical significance set at $P \leq 0.05$ were applied to compare the periods of OJ comsumption (before and after) and groups of individuals (normal-weight and overweight) by Sigma Stat version 3.11, Systat Software Inc., USA.

3. Results and Discussion

In the present study we tested the hypothesis that the regular consumption of OJ could improve metabolic, inflammatory, and oxidative biomarkers in overweight subjects with increased abdominal obesity. The main results found were: (1) Regular OJ consumption did not alter body composition in either group; (2) improvement of the lipid profile,

evidenced by a reduction in total cholesterol and LDL-C; (3) the likely stimulation of the immune defense due to the increase of IL-12; (4) anti-inflammatory effects as a result of a marked reduction in CRP levels; and (5) antioxidant action by the enhancement of total antioxidant capacity and the reduction of lipid peroxidation.

Glucose, insulin, and glycated hemoglobin levels were in accordance with reference values [22] in all subjects, but none of normal weight and three overweight subjects shown insulin resistance evaluated by HOMA-IR (Table 1) [19]. The blood serum lipid panel of normal-weight participants was in accordance with standard values, but overweight subjects initiated the study with total cholesterol and LDL-C at borderline high levels [23] (Table 1). Also, overweight subjects had higher levels of triglycerides than subjects with normal-weight; however, both groups were below the standard threshold values [23] (Table 1). Compared to normal-weight, overweight subjects have had higher dietary consumption of energy (38%), carbohydrates (25%), protein (66%), lipids (36%), cholesterol (83%) and saturated fatty acids (41%) (Table 2), and increased body weight (43%) and waist circumference (27%) (Table 3). Furthermore, also were increased the levels of total cholesterol (17%), LDL-C (33%), tryglicerides (32%), as well as TNF-α (2-fold) (Table 1).

The accumulation of adipose tissue *per se* is recognized as the main contributor to insulin resistance, due to over-secretion of pro-inflammatory adipokines, among them, the TNF-α which can alter insulin-mediated processes, such as glucose homeostasis and lipid metabolism [24]. Thus, the elevated concentration of TNF-α in overweight individuals can be associated with increased waist circunference, which predispose to the development of metabolic disorders, such as metabolic syndrome and type 2 diabetes (Table 1). Furthermore, higher ingestion of energy combined with an unbalanced diet in the long-term may contribute to the progression of others stages of obesity, as well as the emergence of diet-related disease [1].

Regarding nutritional assessment (Table 2), it was observed that the total energy diet was not altered during the eight-week intervention with OJ, showing that this addition of energy and sugar (340 kcal/day and 63 g/day of sugars) was balanced by a decrease in other dietary items. For instance, it was verified an slightly decreased on dietary lipids consumption in normal-weight and a significant decreased of lipids in overweight subjects diet, thereby balancing the total energy intake. Both groups experienced an increase in the intake of vitamin C after OJ consumption; with a 4.6 fold increase among normal-weight subjects and 2-fold increase among those in the overweight group. Folate intake increased as well, by two fold in each group (Table 2). These result were expected since OJ is a nutrient-dense beverage, and its consumption provides substantial amounts of vitamins and minerals with relatively few calories, thereby improving diet quality [25].

There were no differences in body weight, BMI, body fat mass, and waist circumference after consumption of OJ in normal-weight and overweight individuals (Table 3). These data showed that body composition in either groups were not affect by the addition of 340 kcal/d provided by OJ, because of the reduction and change of the macronutrients composition in the diet. The association between the consumption of fruit juices and body weight has recently discussed in the literature [11,26]. One study that analyzed data from NHANES (2003-2006) showed that consumers of 100% OJ had lower BMI, total cholesterol, and LDL-C levels, as well as a decreased risk for obesity and metabolic syndrome [11]. In addition, previous data from our group have shown that even in the long-term comsumption (≥ 12 months) of 2 cups/d of OJ, did not modify body composition, consequently opposing the idea that plain fruit juice contributes to weight gain [27].

There was no change in levels of glucose, insulin, HOMA-IR values, and glycated hemoglobin among normal-weight subjects after OJ treatment (Table 1). Concerning the lipid panel, there was a significant reduction in total cholesterol (-8%) and LDL-C (-11%),

whereas HDL-C, in both women and men, and triglyceride levels remained unchanged. In overweight subjects, there was no change in glucose, glycated hemoglobin, HOMA-IR values, and insulin levels after OJ intake. In addition, total cholesterol and LDL-C levels were significantly reduced by 7% and 8%, respectively. HDL-C and triglyceride levels were not altered (Table 1). Similarly, in a recent study, we showed that regular consumers of OJ had lower levels of total cholesterol (-11%), LDL-C (-18%), apolipoprotein B (-12%) and LDL/HDL ratio (12%) in comparison to non-consumers [27]. Thus, the present study highlights the lipid-lowering properties of OJ, as shown by the decreases in total cholesterol and LDL-C, suggesting that participants of both weight status were equally benefited by OJ intake. This effect has been attributed to the flavanones, hesperidin and naringin, that modulate expression and activity of key mediators related to the control of hepatic lipid homeostasis [6,28].

In several chronic pathological conditions, including overweight and obesity, the local inflammatory process in the adipose tissue stimulates the excessive secretion or lower levels of cytokines in circulation [29]. Changes in the circulating levels of these proteins are linked to several disease states, making them valuable functional biomarkers [30]. Due to exploratory approach concerning the impact of OJ on cytokine secretion associatet with excess of weight, we chose a panel of anti-inflammatory (IL-4, IL-10) and pro-inflammatory cytokines (TNF-α, IFN-γ, IL-12). The levels of IL-12 was increased by approximately 2.5 fold in both normal-weight and overweight groups. However, IFN-γ, TNF-α, IL-4, and IL-10 cytokine levels did not change with OJ intake (Table 1). IL-12 is required for the development of T-helper (Th1) and is essential for the host's defense against bacteria, viruses, and complex parasites [31]. Although IL-12 induces production of IFN-γ and subsequent activation of phagocytic cells [31] the levels of IFN-γ were not altered in this study. Thus, it seems that OJ is able to affect specifically the secretion of IL-12, thereby

boosting partially the immune response, as previously observed in ex vivo macrophages from mice treated with hesperidin and orange juice [32] .

CRP levels among normal-weight subjects were reduced by 52%, while among overweight individuals the CRP reduction was more pronounced, by 66% (Table 1). High levels of CRP in blood serum (> 0.3 mg/dl) have been associated with systemic inflammation, risk of coronary events, and more recently with low grade inflammation induced by visceral adiposity [33, 34]. Participants of this experiment had baseline CRP between 0.1 – 0.3 mg/dl, which is associated with a moderate risk of cardiovascular diseases [34]. Thus, after OJ treatment, the overweight subjects decreased CRP levels to a lower risk category (< 0.1 mg/dl). Although CRP levels among normal-weight subjects dropped significantly as a result of dietary intervention with OJ, this decrease was not enough to shift the subjects to a lower cardiovascular disease risk category. Taking together, these results highlight the anti-inflammatory role of OJ and support previous evidences that OJ acted as a functional food due its ability to modulate CRP [35].

Oxidative stress is often assessed on the blood serum as TBARS that measure the amount of malondialdehyde, a secondary product of lipid peroxidation [35]. In this study, we have shown that the levels of malondialdehyde in normal-weight and overweight subjects were within reference values (1.86-3.94 μM of malondialdehyde) for both groups of individuals [20]. Furtheremore, after OJ intervention, the lipid peroxidation was significantly reduced in both normal-weight and overweight subjects by 35% and 55%, respectively. In addition, the total antioxidant capacity has confirmed the potent antioxidant action of OJ (>100%) in both groups of individuals. (Table 1). Althought, there was a significant reduction of malondialdehyde levels after OJ consumption in normal-weight subjects (-20%), as showed previously [12], this effect was more pronounced among overweight subjects (-55%). It has been established that excessive abdominal adiposity raises the

oxidative radicals that stimulates adipocytes to produce inflammatory cytokines [36], suggesting that overweight subjects were more susceptible to the antioxidant effects of OJ.

In conclusion, we confirmed the hypothesis that OJ has the ability to improve metabolic, inflammatory, and oxidative biomarkers in both groups of individuals studied. In addition, OJ enhances the levels of IL-12 cytokines linked with the host's defense not only in overweight subjects, but also in those normal-weight. Taken together, these results suggest that the regular consumption of OJ could be an strategy to prevent diseases-related to diet, since that its comsumption provides a unique dietary source of nutrients and bioactive compounds, which are associated with the reduction of the development of metabolic and chronic diseases.

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Table 1. Serum biomarkers of normal-weight and overweight subjects after eight week of dietary intervention with 750 mL of orange of juice

Subjects		Normal-weight (*n* = 21)		Overweight (*n* = 25)	
Orange Juice period		Before	After	Before	After
Metabolic biomarkers					
Glucose (mg/dL)		78 ± 7a	77 ± 7a	79 ± 11a	80 ± 11a
Glycated hemoglobin (%)		5.30 ± 0.31a	5.30 ± 0.30a	5.32 ± 0.54a	5.42 ± 0.39a
Insulin (μU/mL)		6.05 ± 2.86a	5.28 ± 2.83a	6.99 ± 2.66a	7.34 ± 2.89a
HOMA-IR		1.17 ± 0.60a	1.01 ± 0.58a	1.40 ± 0.71a	1.47 ± 0.74a
Triglycerides (mg/dL)		89 ± 29a	84 ± 29a	118 ± 37b	124 ± 36b
Total cholesterol (mg/dL)		173 ± 20a	159 ± 27b	203 ± 40c	188 ± 38d
LDL (mg/mL)		104 ± 21a	93 ± 22b	138 ± 37c	126 ± 37d
HDL (mg/dL)	Women	62 ± 15a	57 ± 12a	57 ± 18a	56 ± 17a
	Men	55 ± 12a	48 ± 13a	43 ± 9a	40 ± 7a
*Inflammatory biomarkers**					
CRP (mg/dL)		0.25 ± 0.04a	0.12 ± 0.03b	0.18 ± 0.02a	0.06 ± 0.01b
IFN-γ (pg/mL)		16.5 ± 1.8a	18.0 ± 3.4a	16.9 ± 1.8a	23.7 ± 4.6a
TNF-α (pg/mL)		21.5 ± 4.4a	19.1 ± 4.0a	44.2 ± 6.5b	40.7 ± 7.0b
IL-12 (pg/mL)		7.8 ± 1.3a	20.7 ± 4.0b	7.4 ± 1.0a	17.6 ± 3.6b
IL-4 (pg/mL)		23.2 ± 3.0a	19.5 ± 3.0a	23.5 ± 4.0a	29.7 ± 5.8a
IL-10 (pg/mL)		12.5 ± 3.3a	11.6 ± 1.6a	12.3 ± 2.7a	16.4 ± 3.1a
Oxidative biomarkers					
Malondialdehyde (μM)		2.40 ± 1.93a	1.55 ± 1.20b	2.20 ± 1.35a	1.00 ± 0.73b
DPPH• (%)		8.80 ± 6.80a	26.0 ± 8.64b	10.4 ± 7.65a	26.5 ± 8.07b

Normal-weight: BMI = 18.5-24.9 kg/m2; Overweight: BMI ≥ 25 kg/m2. Most of results are expressed as mean ± SD.

*Results are expressed as mean ± SEM. Means with the same letter in a column are not significantly different, while the different letters are statisticaly sifnificant. Repeated measures two-way ANOVA followed by post-hoc Tukey test $P \leq 0.05$

Table 2. Energy and nutrient intakes of normal-weight and overweight subjects before and after eight week of dietary intervention with 750 mL of orange of juice

Subjects	Normal-weight (*n* = 21)		Overweight (*n* = 25)	
Orange juice period	Before OJ	After OJ	Before OJ	After OJ
Energy (kcal)	1840 ± 284a	1886 ± 440a	2535 ± 334b	2607 ± 334b
Carbohydrates (g)	234 ± 52a	265 ± 56b	292 ± 79c	335 ± 86d
Protein (g)	77 ± 28a	78 ± 22a	128 ± 22b	121 ± 29b
Lipids (g)	69 ± 15a	63 ± 20a	94 ± 20b	82 ± 16c
Cholesterol (mg)	189 ± 61a	209 ± 67a	346 ± 191b	253 ± 108a
Saturated fatty acid (mg)	17 ± 6a	17 ± 6a	24 ± 8b	20 ± 8ab
Vitamin C (mg)	94 ± 58a	437 ± 40b	187 ± 107c	451 ± 75b
Folate (μg)	183 ± 97a	369 ± 70b	211 ± 89a	432 ± 115d
Calcium (mg)	691 ± 280a	635 ± 259a	637 ± 243a	634 ± 284a

Normal-weight: BMI = 18.5-24.9 kg/m2; Overweight: BMI ≥ 25 kg/m2 (WHO, 2000). Results are expressed as mean ± SD. Means with the same letter in a column are not significantly different, while the different letters are statisticaly sifnificant. Repeated measures two-way ANOVA followed by post-hoc Tukey test $P \leq 0.05$.

Table 3. Body composition of normal-weight and overweight subjects after eight week of dietary intervention with 750 mL of orange of juice

Subjects	Normal-weight (*n* = 21)		Overweight (*n* = 25)	
Orange juice period	Before	After	Before	After
Age	32.1 ± 8.6a		38.6 ± 10.5a	
Body mass (kg)	62.1 ± 9.4a	61.8 ± 9.4a	88.6 ± 11.4b	77.6 ± 3.7b
BMI (kg/m^2)	22.4 ± 1.7a	22.3 ± 1.7a	28.9 ± 3.0b	28.9 ± 2.9b
Body fat mass (%)	28.6 ± 6.5a	29.3 ± 4.6a	29.2 ± 6.0a	30.4 ± 5.3a
Waist circumference (cm)	77.1 ± 7.8a	76.6 ± 7.1a	98.1 ± 10.6b	97.4 ± 10.1b

Normal-weight: BMI = 18.5-24.9 kg/m^2; Overweight: BMI ≥ 25 kg/m^2 (WHO, 2000). Results are expressed as mean ± SD. Means with the same letter in a column are not significantly different, while the different letters are statisticaly sifnificant. Repeated measures two-way ANOVA followed by post-hoc Tukey test $P \leq 0.05$.

CAPÍTULO III: Manuscrito 2

Chemopreventive actions of blond and red-fleshed sweet orange juice in *Loucy* leukemia cell line

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ABSTRACT

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 as blond orange juice (BOJ). Such bioactive compounds are associated to chemopreventive actions in several cancer lines. We examined the cell growth, cell cycle, and apoptosis after administration of BOJ, ROJ, and HSP on a new T acute lymphoblastic leukemia cell line. Cells were incubated for 24-h with BOJ, ROJ, and HSP, and cell proliferation and viability were measure by trypan blue. Cell cycle and apoptosis were assessed by propidium iodide (PI) and annexin V-FITC/PI by flow cytometry, respectively. Secretion of IL-1α, IL1-β, IL-2, IL-4, IL-6, IL-10, IL-17A, IFNγ, TNFα, TGFβ, MIPα, and MIPβ were determined by ELISA array. Neither BOJ nor ROJ caused changes in cell proliferation, but both reduced cell viability. BOJ induced cell cycle arrest in the G0/G1 phase and decreased cell accumulation in the G2/M phase. ROJ decreased only the G0/G1 fraction, while HSP no changed the cell cycle distribution. IL-6 and IL-10 were abrogated by all treatments. In conclusion, these results suggest potential chemopreventive effects of BOJ and ROJ on *Loucy* cells.

KEYWORDS: orange juice, hesperidin, cell cycle, apoptosis, leukemia, experimental.

INTRODUCTION

Currently has been evidenced that dietary components may modify the risk of cancer by influencing multiple processes, including DNA repair, cell proliferation, differentiation, apoptosis, and angiogenesis (1). 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 (2). Chemoprevention is a promising and rational approach that uses synthetic, natural, or biological agents to reduce or block the occurrence of cancer (3, 4). 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 (5). Indeed, the beneficial effects of these diets are attributable, at least in part, to polyphenols (6).

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 (4, 7, 8, 9). The anticancer properties of orange juice have been focused on hesperidin and naringin, flavanones found almost exclusively in citrus (10, 11, 12). Hesperidin exhibited cancer chemopreventive actions, in part by arresting cell cycle progression (13), as well as by triggering cell death through a proapoptotic pathway (14). 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 (11).

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 (15). 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 (16). Compared to the blond orange juice (BOJ), ROJ has 2-fold more total carotenoids, including β-carotene (5-fold more) and lycopene (17), which has been associated to antioxidant effects and specific properties against cancer malignancies (18, 19, 20).

T acute lymphoblastic leukemia (T-ALL) is a malignancy of thymo cells that affects 10-15% of children and 25% of adults (21) and is associated with poor prognosis for both adults and children (22). 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) (23). The aim of the present study was exam the effects of orange juices, ROJ and BOJ, and their major flavanone, hesperidin, on cell proliferation, apoptosis, cell cycle distribution, and the pattern of cytokine secretion of Loucy cells culture.

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

BOJ and ROJ were obtained from orange varieties *Pera* and *Sanguinea de Mombuca*, respectively, provided by Citrosuco, Matao, SP, Brazil. The characteristics of both juices were previously measured (17). The data are described as follow for BOJ and ROJ, respectively: brix (g de sucrose/100 g) 11.5 and 9.6; titratable acidity (%) 1,01 and 0.50; ascorbic acid (mg/100 ml) 26.9 and 28.2; total polyphenols (mg/100 ml gallic acid) 1.66 and 1.80; total flavonoids (mg Rutin /ml) 31.6 and 36.9; total carotenoids (mg β-carotene/ml) 2.00 and 3.99, and 155 mg/L and 135 mg/L of hesperidin (17). Before the cell culture experiment, each type of orange juice (100 ml) was lyophilized and stored at 4^{o}C. For the assays, the juices were reconstituted in complete RPMI 1640, to obtain the hesperidin

concentration of 10 μM for both BOJ and ROJ. This concentration was applied for all assays based in previous studies (11, 13), and the juices were centrifuged, filtered, and added to the cell culture plates, which were incubated for 24-h. To assess the cell growth, the cell suspension was evaluated in an automatic cell counter, as described below. 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, which was applied to compare with both orange juices on cell culture.

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: BOJ, reconstituted blond orange juice plus Loucy cells cultured in RPMI 1640; ROJ, reconstituted red-fleshed sweet orange juice plus Loucy cells cultured in RPMI 1640; HSP, HSP solution plus Loucy cells cultured in RPMI 1640; and Control, Loucy cells cultured only in complete RPMI 1640. Prior to culture, we also tested, whether the administration DMSO 0.1% on cell culture (without hesperidin) could change the cell growth and the results showed any modification in Loucy cell viability, which was statistically similar to control (data not shown). To neutralize the acidity of orange juice on culture medium, the pH values for both BOJ and ROJ and hesperidin solution were set for 7.2. All assays were performed in triplicates.

Cell proliferation and viability assay

To investigate a possible inhibitory effect on cell proliferation, we evaluated the ratio between the number of Loucy cells before and after BOJ, ROJ, and HSP incubation. Following 24-hof incubation with treatments, 20μl of each cell suspension sample was taken out and added in 20μl 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 cell concentration and viability were determined using Cellometer® software. Loucy cell proliferation and viability were expressed in cells/ml, and in percentage, respectively.

Cell cycle flow cytometer assay

After 24-h of BOJ, ROJ, and HSP incubation, a volume equivalent to $1x10^6$ of Loucy cell suspension from each sample was collected, centrifuged, and the cell pellets were resuspended 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 (24). 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 for 2-h in a 37°C, 5% CO_2 incubator, which was determined as a positive control for the assay. After 2-h of incubation with staurosporine and 24-h with BOJ, ROJ, HSP, and control, Loucy cells were washed twice with cold PBS. After, the cells were resuspended in 500 µl of annexin V binding buffer and incubated with 5µl of annexin V-FITC and 5 µl of PI in the dark at room temperature for 5 minutes. Posteriorly. Loucy cells were immediately analyzed by Accuri C6 flow cytometer and software (BD Biosciences Immunocytometry Systems, San Jose, CA). The fluorescence intensity of FITC / PI differentiated 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 –80^{o}C. 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 the 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 BOJ, ROJ, HSP and control, for cell cycle and apoptosis, followed by post-hoc Tukey test. Cell proliferation was compared using a T test between 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 $P \leq 0.05$

RESULTS

Cell proliferation and viability

The effects of BOJ, ROJ, and HSP on the proliferation and viability of Loucy cells were analyzed using trypan blue exclusion assay, in that live cells possess intact cell membranes that exclude dye, while the dead cells remained stained. There were no significant differences in cell proliferation after 24-h among BOJ, ROJ, and HSP treatments (Figure 1A). However, we observed a 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 1B).

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, evidenced through an increase of 15% in the G_0/G_1 phase. In addition, this treatment left the S phase unaltered, and decreased the G_2/M phase by 34%. ROJ treatment decrease the G_0/G_1 phase by 6%, while the S and G_2/M phases remained unaltered in relation to the control. The 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, as the average of triplicates and exemplified in the Figure 2, showed that in the negative control, 79.3% of cells were viable, 15.0% were in early apoptosis, and 5.6 % were in late apoptosis. In the positive control (staurosporine), 60.0% of cells were viable, 22.3% were in early apoptosis, and 17.4% were in late apoptosis (Figure 2).

Compared to negative control, the staurosporine administration (positive control) was effective in inducing apoptosis in Loucy cells, because there was a decrease in viable cells by 0.8-fold, 1.5-fold increase in early apoptotic cells, and 3.1-fold increases in late apoptotic cells (Figure 2). After 24-h of incubation with BOJ, we observed that there was a decrease by 5-fold in viable cells, and increase by 11-fold in late apoptotic cells, compared to the negative control, while the percentage of early apoptotic cells was statistically similar to negative control (Figure 2). Following ROJ treatment, there was a decrease by 4-fold in viable cells compared to the negative control. In addition, there were an increase by 1.9-fold in early apoptotic cells, and an increase by 7.6-fold in late apoptotic cells. HSP treatment was statistically similar to the negative control (Figure 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 (control) (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 proliferation, 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 an anti-proliferative, pro-apoptotic potential and immunomodulatory effect by cytokines through administration of both orange juices, which were more pronounced than the purified hesperidin on Loucy cell line.

In the last years, new evidences has emerged about the anticancer role of citrus flavonoids, especially hesperidin and naringin, which have shown cytotoxic and pro-apoptotic effects in several cancer lines (11, 12, 14). This study was the first one to investigate the potential chemopreventive of ROJ on T-ALL cell line. ROJ differs from other blood oranges because is rich in lycopene, β-carotene, xanthine and others, but absent in anthocyanin (25, 26). 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 (13, 27). Recent study showed that hesperidin, at 10-100 μM for 24-h, had a minimal effect on NALM-6 cells, but cell viability was more pronounced after 48-h of treatment (11). Thus, 24-h of incubation for either BOJ or ROJ at 10 μM of HSP was enough to decrease the number of Loucy leukemic cells, then this incubation time was not enough for HSP treatment.

It was previously documented orange juice contains compounds with anti-cancer activity, and they may act alone or synergistically leading to the antiproliferative effect. For instance, ascorbic acid possesses proactive role against some types of cancer, including leukemia and immunomodulatory effects enhancing host defense (28, 29, 30, 31). Lycopene for its turn protect against many types of cancer, as lung, prostate and colon, by modulating cell cycle progression and proliferation (32).

The cell cycle plays an important role in cell fate, including replication, death and cell function (33). We examined if the cytotoxic effect promoted by orange juice was related

to alterations in the Loucy cell cycle distribution. Treatment with BOJ significantly induced the cell cycle arrest in the G_0/G_1, while also decreasing the accumulation of cells in the G_2/M phase. Treatment with ROJ promoted a slight decrease of 6% in the G_0/G_1 phase, showing the absence of cell cycle arrest with this treatment. Previous studies have reported that hesperidin was able to promote cell cycle arrest in the G0/G1 phase, associated with modulation of the expression or activation of cell-cycle regulatory proteins (13, 32, 34). However, in this experiment HSP treatment resulted in none change to the cell cycle distribution.

The antiproliferative 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 (35). Indeed, we were expecting that the Loucy cell cycle arrest would be more pronounced with ROJ due to its appreciable amounts of β-carotene and lycopene (17), beyond of its content of hesperidin and vitamin C, which have anti-cancer properties (11, 30). Instead, only BOJ induced the cell cycle arrest in the G0/G1phase, suggesting that the synergic and/or additive interactions between nutrients and bioactive compounds of fruit juices should be taken into account. Nevertheless, the findings of this study could be tested further in different doses and incubation times (36).

Apoptosis is a type of programmed cell death that plays an essential role in organism development and tissue homeostasis. Recent studies have used the process of programmed cell death as a therapeutic strategy to treat cancer (37, 38). To determine whether cytotoxicity of BOJ, ROJ, and HSP on Loucy cells was related to apoptotic cell death, percentages of apoptotic cells were measured by annexin V-FITC/PI staining. Our results indicated that proportions of apoptotic cells increased after BOJ treatment, with 63% of cells

in late apoptosis, and early apoptosis was similar to negative control. ROJ treatment showed 28% of cells in early apoptosis, whereas 42.6% were in late apoptosis. Previous study tested the polymethoxyflavones (PMF), tangeretin and nobiletin, on the growth of human T lymphoblastoid leukemia cells (MOLT-4). Both compounds have induced cytostatic effects (G1/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 (39).

Although it was not observed the pro-apoptotic effect with HSP on Loucy cells, others revealed that 10 μM hesperidin or more upregulates p53 expression by recruiting PPARγ and down-regulates nuclear NF-kB in human NALM-6 pre-B cell lines (11). Protein p53, a tumor suppressor, induces either cell cycle arrest or apoptosis, whereas PPARγ induces apoptosis by inhibiting the activation of NF-κB (40). In its turn, NF-κB is associated with survival pathways in most of cancer cells, including hematopoietic malignancies (14, 41). Taken together, our results showed that cytotoxicity promoted by BOJ seem be mediated by both cell cycle arrest and apoptosis, while the cytotoxicity induced by ROJ was mediated only by apoptosis.

Chronic inflammation is closely linked with development of cancer and has been related with cytokines that regulate and mediate inflammation (42). Alterations in the concentration of these mediators can promote growth, attenuating apoptosis and facilitating the invasion and metastasis (41). Furthermore, proinflammatory cytokines and other inflammatory markers, as cyclooxygenase (COX-2) and, inducible nitric oxide synthase (iNOS), have become targets for cancer chemoprevention (43, 44, 45). 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, suggesting some limitation of current methodology. On the

other hand, we verified expressive secretion of cytokines IL-6 and IL-10 by untreated cells. 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 and ROJ treatment by 17-fold, showing that the type of juice affects cytokine secretion differently.

Higher concentrations of IL-6 have been implicated in a different type of cancers; including leukemia are associated with poor clinical outcomes (45). Thus, such evidences suggest that inhibition or suppression of IL-6 may be a potential therapeutic strategy for cancer in which IL-6 is overproduced (46). In the similar fashion, IL-10 is an immunosuppressive and is expressed by malignant cells, resulting in increased circulating concentrations in most human cancers. There is a frequent association of elevated concentrations of IL-10 with negative prognosis links in the later stages of cancer (47). 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, these interpretations should be confirmed for 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, make them candidates for further studies to understand the effects of their bioactive components, 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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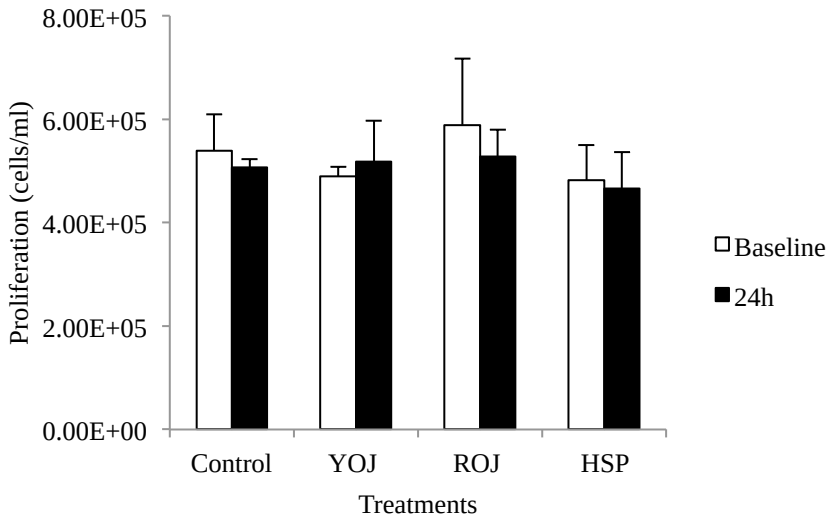

Figure 1A. Loucy cell proliferation after 24-h of incubation with blond orange juice (BOJ), red-fleshed sweet orange juice (ROJ), and hesperidin (HSP) treatments. Control: cells culture in RPMI 1640; BOJ: cells culture in RPMI 1640 + blond orange juice; ROJ: cells culture in RPMI 1640 + red-fleshed sweet orange juice, HSP: cells culture in RPMI 1640 + hesperidin solubilized in dymethyl sulfoxide (0.1%).
Anova One Way followed by post-hoc Tukey test $P \leq 0.05$ between baseline time *vs* 24-h of incubation with each treatment. Trypan blue exclusion method was applied.

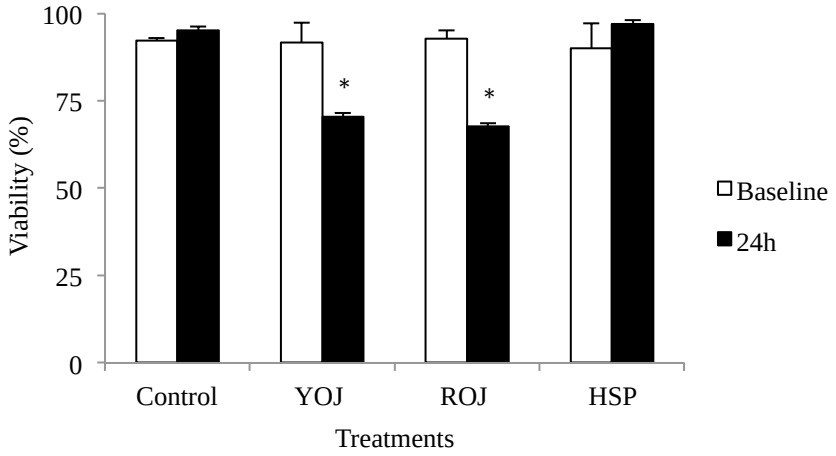

Figure 1B. Loucy cell viability assay after 24-h of incubation with blond orange juice (BOJ), red-fleshed sweet orange juice (ROJ), and hesperidin (HSP) treatments. Control: cells culture in RPMI 1640; BOJ: cells culture in RPMI1640 plus blond orange juice; ROJ: cells culture in RPMI 1640 plus red-fleshed sweet orange juice, HSP: cells culture in RPMI 1640 plus hesperidin solubilized in dymethyl sulfoxide (0.1%).
Anova One Way followed by post-hoc Tukey test $P \leq 0.05$.* Statistical difference between baseline time *vs* 24-h of incubation with each treatment. Trypan blue exclusion method was applied.

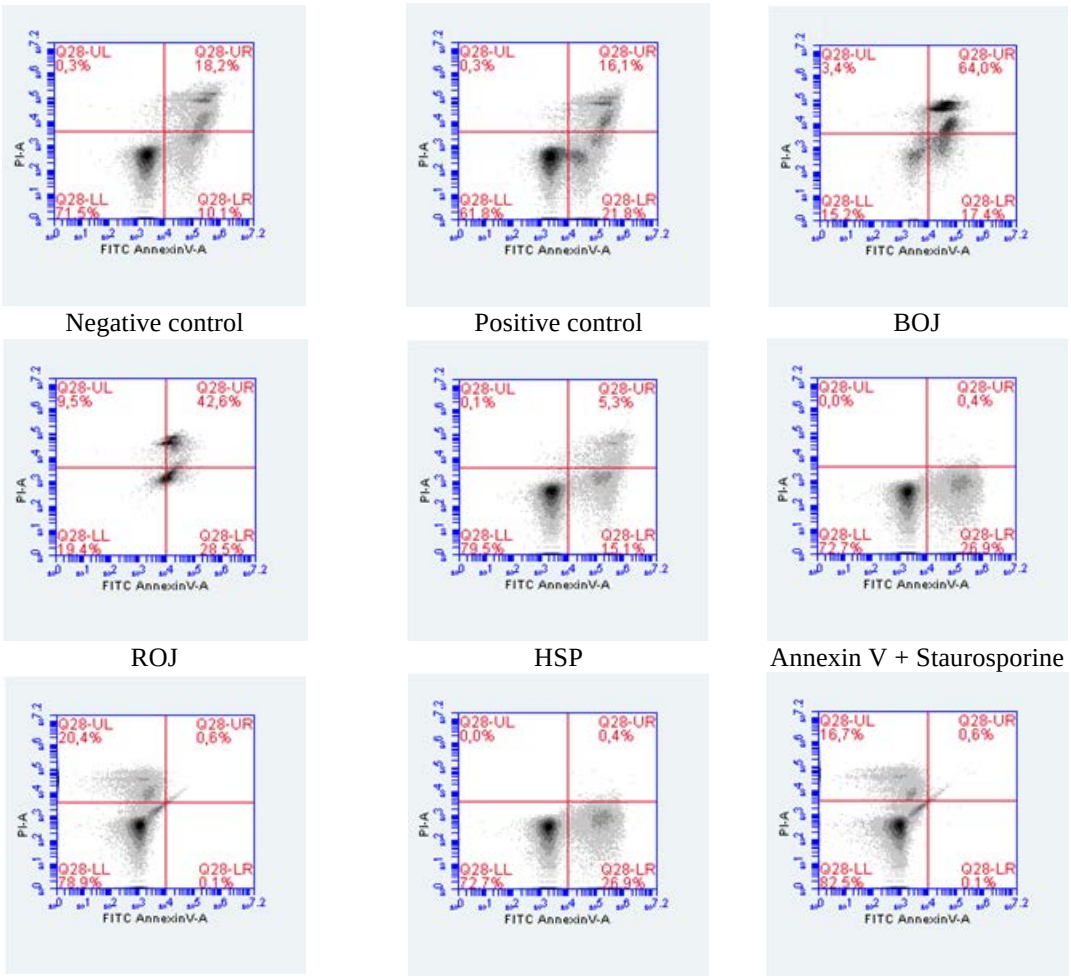

Figure 2. Effect of blond orange juice (BOJ), red-fleshed sweet orange juice (ROJ), hesperidin (HSP) treatments on induction-apoptosis and necrosis after 24-h of incubation with annexin V-FITC/propidium iodide flow cytometric assay. Double staining with annexin V-FITC/propidium iodide solution can distinguish among the percentage of live cells (LL), early apoptotic cells (LR) and late apoptotic cells (UR). Negative control: Loucy cells culture in RPMI + annexin V + propidium iodide; Positive controls: Loucy cells culture in RPMI + annexin V + propidium iodide + inducer-apoptosis staurosporine (1mg/l); BOJ: Loucy cells culture in RPMI1640 + blond orange juice; ROJ: Loucy cells culture in RPMI 1640 + red-fleshed orange juice, HSP: Loucy cells culture in RPMI 1640 + hesperidin solubilized in DMSO (0.1%). Anova One Way followed by post-hoc Tukey test $P \leq 0.05$ to compare the treatments *vs* the percentage of live, early apoptotic, or late apoptotic cells.

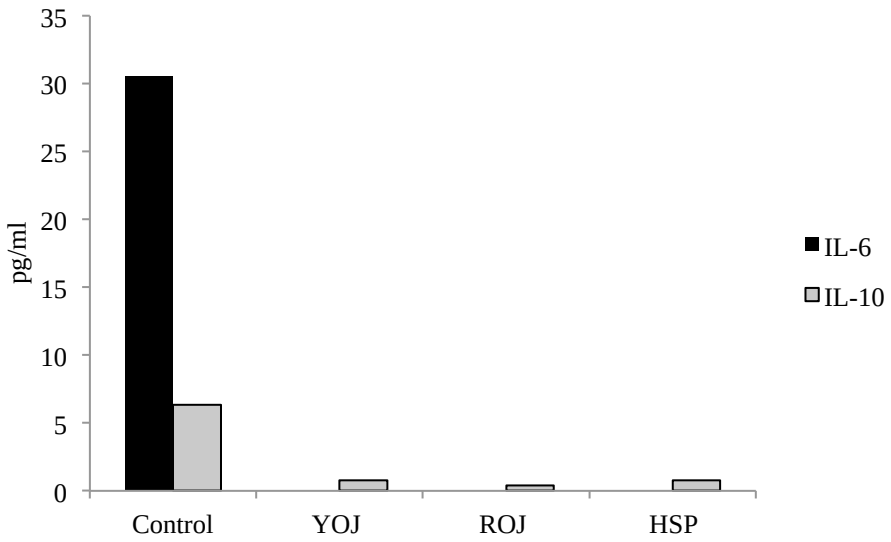

Figure 3. Cytokines secretion from Loucy cells supernatant after 24-h of incubation with BOJ: Loucy cells culture in RPMI1640 + blond orange juice; ROJ: Loucy cells culture in RPMI 1640 + red-fleshed orange juice, HSP: Loucy cells culture in RPMI 1640 + hesperidin solubilized in DMSO (0.1%), and Control: Loucy cells culture em RPMI 1640 by ELISA array assay. The results are expressed in percentages as the difference of Control *vs* each treatment. To obtain the corrected absorbance values, the initial absorbance of each cytokine was subtracted by the absorbance of the negative control of the kit.

Table 1. Loucy cell cycle distribution (G_0/G_1, S and G_2/M phases) after 24-h of incubation with blond orange juice (BOJ), red-fleshed sweet orange juice (ROJ), and hesperidin (HSP)

	Loucy cell treatments (24-h incubation)			
Cell cycle phases (%)	**Control**	**BOJ**	**ROJ**	**HSP**
G_0/G_1	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	28.6 ± 0.8	27.7 ± 0.7	27.9 ± 0.2
G_2/M	26.8 ± 0.7^{a}	17.6 ± 0.9^{b}	26.1 ± 1.6^{a}	27.7 ± 1.1^{a}

Control: Loucy cells culture in RPMI 1640; OJ: Loucy cells culture in RPMI 1640 + blond orange juice; ROJ: Loucy cells culture in RPMI 1640 + red-fleshed sweet orange juice, HSP: Loucy cells culture in RPMI 1640 + hesperidin solubilized in DMSO (0.1%). Results are expressed as mean ± SD. Letters means statistical significance among the treatments. Anova One Way by line followed by post-hoc Tukey test $P \leq 0.05$.

APÊNDICE I: Questionário de identificação dos voluntários

Você estaria disposto a ingerir 750 mL (3 copos) de suco de laranja diariamente, durante 2 meses, gratuitamente?
( ) Sim ( )Não

Você estaria disposto a participar de duas avaliações nutricionais, duas antropométricas e duas bioquímicas, sendo uma no início e a outra no término da pesquisa?
( ) Sim ( )Não

Se você respondeu "sim" para as duas perguntas, está convidado a participar de uma pesquisa científica com suco de laranja no qual serão avaliados: estado nutricional, perfil lipídico,glicêmico e insulínico, as variáveis inflamatórias e antioxidantes.

1. Dados pessoais

Nome:

E-mail:

Idade: Data de nascimento.: _____/_____/_____

Endereço:

Telefone res. ( ) Celular ( )

2. Dados Sócio-Econômicos

Escolaridade:

Profissão:

Numero de pessoas residentes: Adultos: _____Crianças (0 a 14 anos):____

Estado Civil: ( )Solteiro ( )Casado ( )Viúvo ( )Divorciado

3. Histórico Clínico e Avaliação do Risco Cardiovascular

Sexo: ( ) Masculino ( ) Feminino

Raça: ( )Branca ( ) Negra ( ) Parda ( ) Amarela

Faz uso de cigarros? ( )Sim ( )Não Quantidade/dia:

Faz uso de bebida alcoólica? Tipo:

Tempo de uso: Freqüência:

Faz uso de medicamentos?

Possui Hipertensão Arterial? ( )Sim ( )Não ( )Não sei PA: mm Hg

Faz atividade física?

( )Sim ( )Não

Qual? Quantas vezes/sem?

Você já fez exame de glicemia? ( )Sim ( )Não Qual foi o resultado?

Possui Diabetes? ( )Sim ( )Não ( )Não sei Qual tipo?

Você já fez exame de colesterol? ( )Sim ( )Não Qual foi o resultado?

Você já fez exame de triglicérides? ( )Sim ( )Não Qual foi o resultado?

Você tem doença coronariana? ( )Sim ( )Não ( )Não sei

Histórico Familiar de Diabetes? ( )Sim ( )Não Qual parentesco?

Histórico Familiar de HAS? ( )Sim ( )Não Qual parentesco?

Histórico Familiar de Hipercolesterolemia?

( )Sim ( )Não

Qual parentesco?

Histórico Familiar de doença coronariana?( )Sim ( )Não Qual parentesco?

Você se preocupa com a quantidade de gordura saturada, gordura trans, colesterol total e de gordura na sua dieta?

Dados Antropométricos:

Altura_____ Peso_____ IMC:_____

Classificação:_____

APÊNDICE II: Recordatório alimentar de 24 horas e antropometria

Nome:	No.		
Idade:	Peso:		
Data de nascimento: / /	Altura:		
Sexo:	C. Cintura:		
( ) Feminino ( ) Masculino	C. Quadril:		
Raça:	C. Punho:		
P.A:	C. Braço:		
BIA:	P. cutânea tricipital:		
1. Que tipo de exercício físico você pratica?	Com que frequência:		
	( ) regular	h/dia	Vezes semana
	( ) irregular		
	( ) raramente		
	( ) não pratica		

2. Qual é o seu padrão de refeição durante a semana?	
Café da Manhã Horário:	
Lanche da manhã Horário:	
Almoço Horário:	
Lanche da tarde Horário:	
Jantar Horário:	
Ceia Horário:	

APÊNDICE III: Termo de Consentimento Livre Esclarecido

Eu, ...
RG. . . . . , estado civil . . . . . ,
idade . . . . . , residente à Rua . . . . . .
. . . . . , bairro. . . . . .
. . . . . , cidade . . . . . , telefones de contato . . . .
. . . . . , declaro ter sido orientado e esclarecido sobre o protocolo de pesquisa a seguir:

Que a finalidade deste estudo será para verificar se o consumo de 750 mL de suco de laranja tomado diariamente reduz o colesterol, os triglicérides e o açúcar do sangue e melhora a capacidade antioxidante do corpo, prevenindo contra doenças do coração e o diabetes.

Que serei submetido à avaliação física e nutricional, em duas ocasiões, no início e final do estudo e responderei perguntas relativas à saúde pessoal e à dieta consumida por mim com relativa freqüência. Quando necessário poderei esclarecer minhas dúvidas em relação à pesquisa e receberei a orientação dietética adequada. Que deverei tomar 750 mL de suco de laranja diariamente durante dois meses.

Que terei de doar 50 mL de sangue em duas ocasiões (total de 100 mL), uma no início e outra no final do tratamento para exames bioquímicos e imunológicos. O local da coleta será o Laboratório de Análises Clínicas da Faculdade de Ciências Farmacêuticas da UNESP, situado na Rua Expedicionários do Brasil, 1621, Centro, Araraquara, SP.

Que a pesquisa terá a duração de dois meses e minha participação será voluntária e livre de qualquer ônus, inclusive receberei ressarcimento para deslocamento quando necessário para o encontro com os pesquisadores.

Que durante a pesquisa eu não estarei sob tratamento medicamentoso para controle do colesterol e de triglicérides, e se necessitar de medicamentos informarei imediatamente os pesquisadores sobre esta nova condição de saúde.

Que os riscos são mínimos ao participar desta pesquisa, apenas terei o desconforto das coletas de sangue, e que todos os materiais utilizados serão descartáveis.

Que concordo em retornar ao laboratório toda vez que for solicitado pelos pesquisadores, com ressarcimentos de despesas com transporte.

Que os procedimentos que estou sendo submetido não acarretarão qualquer dano físico ou financeiro e por isso não haverá necessidade de ser indenizado por parte da equipe ou instituição responsável por essa pesquisa (FCF/UNESP).

Que meu nome será mantido em sigilo, assegurando, assim, minha privacidade e se desejar, receberei informações sobre o resultado da pesquisa.

Que poderei desistir da pesquisa em qualquer momento, sem nenhum prejuízo ou penalização, mas que avisarei os pesquisadores se isto ocorrer.

Que a notificação de qualquer situação de anormal idade relacionada à pesquisa, eu deverei entrar em contato com a equipe científica pelo telefone (0XX16) 3301-6927.

Pelo presente esclarecimento, concordo em participar do estudo: “Avaliação do efeito do consumo habitual do suco de laranja vermelha sobre marcadores inflamatórios e antioxidantes, variáveis bioquímicas e nutricionais”, sob responsabilidade de Thais Borges César.

Assinatura do voluntário: _____

ANEXO I: Aprovação do comitê de ética em pesquisa

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unesp UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO" Câmpus de Araraquara

Protocolo CEP/FCF/CAr nº 22/2009
Interessado: PROFA. DRA. THAIS BORGES CESAR
Projeto: Avaliação do efeito do consumo habitual do suco de laranja vermelha sobre marcadores inflamatórios e antioxidantes, variáveis bioquímicas e nutricionais

Parecer nº 50/2009 – Comitê de Ética em Pesquisa

O projeto "Avaliação do efeito do consumo habitual do suco de laranja vermelha sobre marcadores inflamatórios e antioxidantes, variáveis bioquímicas e nutricionais", encontra-se adequado em conformidade com as orientações constantes da Resolução 196/96 do Conselho Nacional de Saúde/MS.

Por essa razão, o Comitê de Ética em Pesquisa desta Faculdade, considerou o referido projeto estruturado dentro de padrões éticos manifestando-se FAVORAVELMENTE à sua execução.

O relatório final do projeto de pesquisa deverá ser entregue em agosto de 2010, no qual deverá constar o Termo de Consentimento Livre Esclarecido dos sujeitos da pesquisa.

Araraquara, 09 de novembro de 2009.

Profª. Drª. AURELUCE DEMONTE
Coordenadora do CEP

ANEXO II: Informações sobre células Loucy

ATCC

Product Sheet

Loucy (ATCC® CRL-2629™)

Please read this FIRST

Storage Temp.
liquid nitrogen vapor phase

Biosafety Level
1

Intended Use

This product is intended for research use only. It is not intended for any animal or human therapeutic or diagnostic use.

Complete Growth Medium

The base medium for this cell line is ATCC-formulated RPMI-1640 Medium, Catalog No. 30-2001. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%.

Citation of Strain

If use of this culture results in a scientific publication, it should be cited in that manuscript in the following manner: Loucy (ATCC® CRL-2629™)

Description

Organism: *Homo sapiens*, human
Tissue: peripheral blood
Disease: T-cell acute lymphoblastic leukemia
Cell Type: T lymphocyte
Age: 38 years
Gender: female
Morphology: lymphoblast
Growth Properties: suspension
DNA Profile:
Amelogenin: X
CSF1PO: 11,12
D13S317: 11,13
D16S539: 11
D5S818: 12
D7S820: 9,10
THO1: 8,9.3
TPOX: 8,9
vWA: 16,17
Cytogenetic Analysis: 45,X,5q-,t(16;20)(p12;q13)