UNIVERSIDADE ESTADUAL DE CAMPINAS

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In vitro evaluation of the anti-lipogenic and anti-inflammatory potential of biotransformed extract from citrus residues

Avaliação *in vitro* do potencial anti-lipogênico e antiinflamatório de extrato biotransformado de resíduos de cítricos

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Orientadora: Profª. Drª. Juliana Alves Macedo

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RESUMO

A obesidade é caracterizada pelo excesso de tecido adiposo com aumento na infiltração de macrófagos, e secreção de citocinas pró-inflamatórias, levando a um estado inflamatório subclínico crônico. Diversos produtos têm sido avaliados na tentativa de prevenir e tratar a obesidade e suas complicações. Dentre os compostos estudados, destaca-se a importância dos fenólicos. As frutas cítricas são importante fonte de flavonoides, com destaque a casca, que apresenta teor mais elevado que a polpa. Assim, este estudo objetivou avaliar o potencial anti-lipogênico e anti-inflamatório de extratos de flavonoides de cítricos. Os extratos foram obtidos a partir do resíduo industrial de frutas cítricas, modificados por bioprocesso fermentativo (extrato "Biotransformado"), e dois extratos controles, o primeiro sem processamento ("In Natura"), e o segundo com o processamento térmico equivalente ao sofrido pelo extrato fermentado (extrato "Autoclavado"). Realizaram-se estudos *in vitro* para determinar atividade antioxidante, ação na lipogênese e lipólise em células 3T3-L1, e o efeito anti-inflamatório em células RAW264.7 e 3T3-L1. O extrato do resíduo de cítricos obtido após a biotransformação apresentou-se como boa fonte de hesperitina e naringenina, flavanonas encontradas em baixa quantidade na natureza. O extrato "Biotransformado" apresentou menor quantidade de flavanonas totais, no entanto, a capacidade antioxidante foi semelhante de acordo com DPPH e ORAC, indicando um novo perfil fenólico com maior potencial bioativo que o original. Os extratos apresentaram baixa citotoxicidade nas concentrações entre 0,01-1,00 mg/mL. As amostras não apresentaram muita influência nos processos de diferenciação dos adipócitos a partir de fibroblastos, porém a adição de extrato no meio de maturação causou uma diminuição dose dependente na acumulação de lipídeos das células, com reduções entre 22% e 48% para os extratos "Biotransformado" e "In Natura". O extrato "Biotransformado" foi o único que apresentou efeito na liberação de glicerol livre no sobrenadante da cultura (2,39 ± 0,17 - 5,24 ± 0,29 mg/mL de glicerol), indicando seu papel na lipólise. A adição dos extratos em macrófagos (RAW264.7) causou menor secreção de indicadores inflamatórios, como TNF-α e NO, com melhores resultados para o extrato "Biotransformado". A adição dos extratos também causou menor expressão proteica do fator de transcrição NFκB. Em co-cultura de RAW264.7 e 3T3-L1, o tratamento com 1,0 mg/mL de extratos "Biotrasformado" e "In Natura" reduziu a secreção de TNF-α (30,7% e 14,9%) e IL-6 (43,4% e 42,7%) em relação ao Controle sem tratamento. Ainda, o extrato "Biotransformado" a uma concentração de 1,0 mg/ml promoveu maior aumento de adiponectina em relação ao "In Natura" (66,0% e 35,3%, respectivamente). Quando a co-cultura recebeu estímulo com LPS, a adição do extrato reduziu a concentração de IL-6 e

TNF-α e causou maior aumento na concentração de adiponectina. A biotransformação dos compostos fenólicos do extrato de resíduo de cítricos foi capaz de modificar o perfil de flavanonas, aumentando as agliconas. Ainda, o extrato demonstrou potencial para uso na indução da lipólise e atividade anti-inflamatória em cultura de macrófagos e em co-cultura de macrófagos/adipócitos. Assim, podemos concluir que o bioprocessamento pode contribuir para o desenvolvimento de um produto com potencial uso no tratamento da obesidade e da inflamação associada.

Palavras-chave: obesidade, inflamação, cítricos, cultura de células.

ABSTRACT

Obesity is characterized by excess adipose tissue with increased macrophage infiltration and pro-inflammatory cytokines secretion, leading to a subclinical chronic inflammatory state. Many food products have been evaluated in an attempt to prevent and treat obesity and its complications. Among the compounds studied, phenolics are of great interest. Citrus fruits are an important source of flavonoids, especially citrus peel, which present higher content in relation to pulp. Thus, this study aimed to evaluate anti-lipogenic and anti-inflammatory potential of a citrus flavonoid extract. The extracts were obtained from industrial residue of citrus fruits, modified by fermentative bioprocess ("Biotransformed" extract) and two control extracts, the first one without any processing ("In Natura"), and the second with the equivalent thermal process suffered by fermented extract ("Autoclaved"). For this purpose, *in vitro* assays were performed to determine their antioxidant activity, lipogenesis and lipolysis activity in 3T3-L1 cell line, and anti-inflammatory effect on RAW264.7 and 3T3-L1.The citrus residue extract obtained after "Biotransformation" was a good source of hesperitin and naringenin, flavanones often found in low quantity in nature. Biotransformed residue presented smaller amount of total flavanones, however the antioxidant capacity of the extracts was similar according to DPPH and ORAC assays, indicating a new phenolic profile with greater bioactive potential than the original. The extracts showed low cytotoxicity in concentrations ranging from 0.01-1.00 mg / mL, in the cells of interest. Samples did not have much influence in the new adipocytes differentiation processes from fibroblasts, on the other hand, the addition of the extracts in maturation medium in adipocytes caused a dose-dependent decrease in lipid accumulation, reaching a diminution of 22% and 48% for Biotransformed and In Natura extracts. Biotransformed extract was the only that presented some effect on glycerol release (2.39 ± 0.17 - 5.24 ± 0.29 mg/mL of glycerol), indicating its role in lipolysis. Treatment of RAW 264.7 cell with extracts caused lower secretion of inflammatory indicators, as TNF-α and NO, with greater results for "Biotransformed" extract. There was also lower protein expression of NFκB with the treatment. In RAW264.7 and 3T3-L1 co-culture, treatment with 1.0mg/mL of "Biotrasformed" and "In Natura" extracts reduced secretion of TNF-α (30.7% and 14.9%) and IL-6 (43.4% and 42.7%) compared to Control without any treatment. Still, Biotransformed extract at a concentration of 1.0mg/mL promoted greater increase in adiponectin in relation to In Natura (66.0% and 35.3% respectively). When the co-culture received LPS stimulus, addition of the extract reduced IL-6 and TNF-α concentration and caused a greater increase in adiponectin. Biotransformation of phenolic compounds from citrus

extract residue was able to modify flavanones profile, increasing the aglicones. Still, the extract showed potential for use in inducing lipolysis and anti-inflammatory activity in macrophages culture and co-culture of macrophages with adipocytes. Thus, we can conclude that bioprocess can contribute to the development of a product with potential use in treatment of obesity and associated inflammation.

Keywords: obesity, inflammation, citrus, cell culture.

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ABBREVIATIONS AND ACRONYMS LIST

AAPH	2,2’-Azobis(2-amidinopropane) dihydrochloride
ACC	Acetyl-CoA carboxilase
ACC1	Acetyl-CoA carboxylase 1
Akt	serine/treonine kinase
AMPK	AMP-activated protein kinase
aP2	Activating protein 2
C/EBPα	CCAAT-enhancer-binding protein alpha
C/EBPβ	CCAAT-enhancer-binding protein beta
cAMP	Cyclic adenosine monophosphate
CCK	Cholecystokinin
CPT1	Carnitine palmitoyltransferase 1
CPT-1a	Carnitine palmitoyl transferase 1a
DEX	Dexamethasone
DMEM	Dulbecco's Modified Eagle Medium
DPPH	2,2-diphenyl-1-picrylhydrazyl
ERK1/2	Extracellular signal–regulated kinase 1/2
FAS	Fatty acid synthase
FBS	Fetal bovine serum
FL	Fluorescein
FW	Fresh weight
GAE	Gallic Acid Equivalent
GSK3β	Glycogen synthase kinase 3 beta
HPLC-MS	High performance liquid chromatography-Mass spectrometer
HSL	Hormone sensitive lipase
HSP70	Heat shock protein 70
IBMX	3-isobutyl-1-methylxanthine
IFN-γ	Interferon gamma
IKK	IκB kinase
IL-1	Interleukin-1
IL-6	Interleukin 6
iNOS	Inducible nitric oxide synthase
IκB	Inhibitor of κB

JNK	c-Jun N-terminal kinase
LKB1	Liver kinase B1
LPS	Lipopolysaccharides
LXR	Liver X receptor
MCP-1	Monocyte chemotatic protein-1
MDA	Malondialdehyde
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Myd88	Myeloid differentiation primary response gene 88
NFκB	Nuclear factor-κB
NO	Nitric oxide
ORAC	Oxygen Radical Absorbance Capacity
PKA	Protein kinase A
PMF	Polymethoxyflavone
PPARα	Peroxisome proliferator-activated receptor alpha
PPARγ	Peroxisome proliferator-activated receptor gamma
SCD1	Stearoyl-CoA desaturase 1
SDS	Sodium dodecyl sulfate
SRBP-1c	Binding protein sterol regulatory element 1
TLR-4	Toll-Like Receptor 4
TNF-α	Tumor necrosis factor-alpha
TRAF6	TNF receptor associated factor 6
Trolox®	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
TRP	Transient receptor potential
U.V.	Ultraviolet
UCP2	Uncoupling protein 2

SUMARY

INTRODUÇÃO

A obesidade é uma doença caracterizada pelo excesso de tecido adiposo, e encontra-se associada a um estado inflamatório subclínico crônico, causado pelo aumento da secreção de adipocinas pró-inflamatórias (BALISTRERI; CARUSO; CANDORE, 2010). Em tecidos adiposos aumentados, além dos adipócitos, há aumento na infiltração de macrófagos no tecido, contribuindo para a maior secreção de citocinas com atividade pró-inflamatória (WEISBERG et al., 2003). Esse aumento na circulação de adipocinas e citocinas parece ser responsável pelo desenvolvimento das doenças crônicas não transmissíveis associadas a obesidade, causando resistência a insulina, aumento de pressão arterial, alteração dos lipídios séricos, aumento da resposta inflamatória, e formação de trombos (GRUNDY et al., 2004).

Além das diversas complicações associadas a obesidade, a elevada prevalência da doença tornaram-na um problema de saúde pública. Desta forma, diversas estratégias e produtos têm sido avaliados na tentativa de prevenir e tratar o excesso de gordura corporal e suas complicações. Dentre os compostos estudados, destaca-se a importância dos fenólicos presentes nos alimentos.

Uma fonte interessante de compostos fenólicos são as frutas cítricas. Dentre os cítricos de maior importância comercial, destaca-se a laranja, sendo o Brasil o maior produtor do mundo, atingindo uma produção de 16.850 mil toneladas em 2013/2014, segundo estimativa da Food and Agriculture Organization (FAO). No cenário nacional, o estado de São Paulo é o destaque com 70% do volume produzido, seguido da Bahia e Minas Gerais. Do total produzido, estima-se que 85% seja destinado a indústria de suco e o restante ao consumo *in natura* (CONAB, 2013). Na produção do suco, cerca de 50% do subproduto gerado é composto por casca e bagaço, utilizado comumente como componente de ração animal.

A principal classe de flavonoides de cítricos é a flavanona, possuindo também quantidades consideráveis de flavonas, flavonois e antocianinas (BENAVENTE-GARCÍA et al., 1997). Os flavonoides mais comuns em cítricos são hesperidina, naringina, narirutina, eriocitrina, nobiletina e tangeritina (SUN et al., 2013)

É importante ressaltar que a casca dos cítricos possuem maior conteúdo de polifenois e maior atividade antioxidante em relação a polpa, indicando que o subproduto de cítricos é uma fonte promissora de compostos bioativos (BARROS; FERREIRA; GENOVESE, 2012). Ainda, os efeitos positivos dos flavonoides de cítricos no tratamento da obesidade e suas

complicações são demonstrados em diversos estudos em cultura de células (KANG et al., 2012; KIM et al., 2012; YOSHIDA et al., 2010, 2013), ensaios biológicos (ALAM; KAUTER; BROWN, 2013; UM et al., 2013), e ensaios clínicos (DALLAS et al., 2008, 2013).

Nas fases iniciais do desenvolvimento de novos produtos, o uso de cultura de células apresenta vantagens devido a necessidade de pouca amostra inicial para os testes, e o menor custo. Além disso, nos estudos *in vitro* é possível definir as vias de ação que o novo produto pode atuar. Nos estudos *in vitro* associados a obesidade, a cultura de pré-adipócito 3T3-L1 é o modelo mais utilizado. Para mimetizar a inflamação presente no tecido adiposo durante a obesidade, é realizada a co-cultura de 3T3-L1 com RAW264.7, uma cultura de macrófago que secreta citocinas proinflamatórias (FREIWALD et al., 2013; YAMASHITA et al., 2007).

Nesse sentido, este trabalho avaliou o potencial biológico *in vitro* de um extrato rico em compostos fenólicos, obtido por processos biotecnológicos, a partir de resíduos de cítricos provenientes da indústria de extração de pectina. Destaca-se que o valor agregado deste resíduo é muito reduzido, sendo um produto derivado de dois processos industriais subsequentes: o primeiro, a extração de suco de laranja; e o segundo a extração de pectina.

Além da utilização de resíduos industriais de baixo valor agregado como fonte de compostos fenólicos, uma importante inovação proposta pelo trabalho foi a biotransformação dos polifenois dos resíduos de cítricos, pela ação do microrganismo *Paecilomyces variotii* em processo de fermentação sólida, gerando um produto com perfil de composição de polifenois diferente dos obtidos por simples extração. A seleção do microrganismo foi decorrente de resultados obtidos por estudos anteriores do grupo (BATTESTIN, PASTORE, MACEDO, 2005; BATTESTIN & MACEDO 2007; BATTESTIN, MACEDO, FREITAS 2008).

Desta forma, este estudo teve como objetivo avaliar um extrato de resíduo de cítricos biotransformado quanto a sua atividade antioxidante, antiinflamatoria, capacidade de reduzir a lipogênese e induzir a lipólise utilizando testes *in vitro*.

O trabalho apresentado nessa tese está organizado em três capítulos, sendo o primeiro um artigo de revisão bibliográfica, intitulado “Citrus bioactive phenolics: role in the obesity treatment”, publicado na revista LWT-Food Science and Technology (Apêndice 1); o segundo, baseado no artigo “Biotransformation effects on anti lipogenic activity of citrus extracts”, publicado na revista Food Chemistry, que trata da avaliação do potencial antiobesogênico do extrato desenvolvido (Apêndice 2); e o terceiro baseado no artigo

“Biotransformed citrus extract as a source of anti-inflammatory polyphenosl: effects in macrophages and adipocytes”, submetido a revista Journal of Nutritional Biochemistry, que contém os resultados indicativos do potencial anti-inflamatório do extrato. O Apêndice 3 contém alguns resultados não incluídos nos artigos (ensaio MTT nos adipócitos diferenciados, ensaio de coloração por Oil Red O em células que receberam tratamento com os extratos após a diferenciação, Western blot de PPAR-alfa em células 3T3-L1); o Apêndice 4 o artigo do grupo de trabalho intitulado “Rich bioactive phenolic extract production by microbial biotransformation of Brazilian citrus residues” publicado na revista Chemical Engineering Research and Design; o Apêndice 5 o capítulo “Hesperitin: Simple natural compound with multiple biological activity” publicado no livro Fruit and Pomace Extracts: Biological Activity, Potential Applications and Beneficial Health Effects.

INTRODUCTION

Obesity is a disease characterized by excess adipose tissue and it is associated with a subclinical chronic inflammatory condition caused by increased secretion of pro-inflammatory adipokines (BALISTRERI; CARUSO; CANDORE, 2010). In increased fat tissue, in addition to adipocytes, there is an increase in macrophage infiltration, contributing to enhanced secretion of cytokines with pro-inflammatory activity (WEISBERG et al., 2003). This rise in circulating adipokines and cytokines appears to be responsible for the development of chronic diseases associated with obesity, causing insulin resistance, blood pressure increase, change in serum lipids, increased inflammatory response and thrombus formation (GRUNDY et al., 2004).

In addition to many complications associated with obesity, the increased prevalence of obesity made it a public health problem. Thus, various strategies and products have been evaluated in an attempt to prevent and treat excessive body fat and its complications. Among the compounds studied, it is of great importance phenolics present in food.

An interesting source of phenolic compounds are citrus fruits. Among the most commercially important citrus, there is orange, with Brazil being the largest producer in the world, reaching a production of 16.85 million tons in 2013/2014, according to estimates of Food and Agriculture Organization (FAO). On national scene, the state of São Paulo is the major producer with 70% of the volume, followed by Bahia and Minas Gerais. From the total produced, it is estimated that 85% is for juice industry and the rest for *in natura* consumption (CONAB, 2013). In juice production, about 50% of the waste generated is composed of peel and pulp, commonly used as animal feed component.

The main class of citrus flavonoid is flavanone, also having considerable amounts of flavones, flavonols and anthocyanins (BENAVENTE-GARCÍA et al., 1997). The most common flavonoids in citrus are hesperidin, naringin, narirutin, eriocitrin, nobiletin and tangeritin (SUN et al., 2013).

It is important to emphasize that citrus peel have higher content of polyphenols and antioxidant activity than pulp, indicating that citrus residue is a promising source of bioactive compounds (BARROS; FERREIRA; GENOVESE, 2012). Also, the positive effects of citrus flavonoids in treatment of obesity and its complications are demonstrated in various studies in cell culture (KANG et al., 2012; KIM et al., 2012; YOSHIDA et al., 2010, 2013), biological

assays (ALAM; KAUTER; BROWN, 2013; UM et al., 2013), and clinical trials (DALLAS et al., 2008, 2013).

In the early stages of new products development, cell culture use is advantageous due to the need of low quantity of sample for testing, and lower cost. Moreover, in *in vitro* studies it is possible to define the pathways of action that the new product can act. *In vitro* studies associated with obesity use 3T3-L1 pre-adipocytes as a model. To mimic the inflammation in adipose tissue in obesity, the assay is carried out in co-culture of 3T3-L1 and RAW264.7, a macrophage culture that secrets pro-inflammatory cytokines (FREIWALD et al., 2013; YAMASHITA et al., 2007).

Thus, this study intended to assess *in vitro* biological potential of an extract rich in phenolic compounds, obtained by biotechnological processes, from citrus waste of pectin extraction industry. It is noteworthy that the value of this waste is very low, being a by-product of two subsequent industrial processes: first, the orange juice extraction; and second pectin extraction.

In addition to the use of industrial waste with low added value as a source of phenolic compounds, an important innovation proposed by the study was the polyphenols biotransformation from citrus waste, by the action of the microorganism *Paecilomyces variotii* in solid-state fermentation, resulting in a product with polyphenols composition profile different from those obtained by simple extraction. The microorganism selection was based on previous studies from the group (BATTESTIN, PASTORE, MACEDO, 2005; BATTESTIN & MACEDO 2007; BATTESTIN, MACEDO, FREITAS 2008).

Thus, this study aimed to evaluate antioxidant and anti-inflammatory activity, ability to reduce lipogenesis and induce lipolysis of a biotransformed citrus residue extract using *in vitro* tests.

The work presented in this thesis is organized in three chapters, the first being an review article, titled "Citrus bioactive phenolics: role in the obesity treatment", published in LWT-Food Science and Technology journal (Appendix 1); the second, based on the article "Biotransformation effects on anti lipogenic activity of citrus extracts," published in the Food Chemistry, and presents the extract antiobesogenic potential (Appendix 2); and the third based on the article "Biotransformed citrus extract as a source of anti-inflammatory polyphenols:

effects in adipocytes and macrophages," submitted to the Journal of Functional Foods, which contains the results indicating the anti-inflammatory potential of the extract. Appendix 3 contains some results that are not included in the articles (MTT assay in differentiated adipocytes, Oil Red O staining in cells that were treated with the extracts after differentiation, Western blot of PPAR-α in 3T3-L1 cells); Appendix 4 article of the group titled "Rich bioactive phenolic extract production by microbial biotransformation of Brazilian citrus residues" published in the journal Chemical Engineering Research and Design; Appendix 5 the chapter "Hesperitin: Simple natural compound with multiple biological activity" published in the book Fruit and Pomace Extracts: Biological Activity, Potential Applications and Beneficial Health Effects.

CHAPTER 1.CITRUS BIOACTIVE PHENOLICS: ROLE IN THE OBESITY TREATMENT

(REVIEW PAPER PUBLISHED BY LWT – FOOD SCIENCE AND TECHNOLOGY)

Vânia Mayumi Nakajima, Gabriela Alves Macedo, Juliana Alves Macedo.

Abstract

Adipose tissue performs many functions in the body, being considered an endocrine organ due to substances secreted, called adipokines. The excess of adipose tissue is called obesity, and it is associated with a state of chronic subclinical inflammation. Various strategies and products have been evaluated in an attempt to prevent and treat obesity, standing out the importance of polyphenols in citrus fruits. This group of fruits is important source of bioactive compounds, mainly flavonoids. Therefore, this paper aims to review studies developed to evaluate the role of these compounds in the obesity. Despite the difficulties in the comparison of study results, due to the variety of methodologies and samples evaluated, some general trends can be highlighted. The *in vitro* studies indicate that citrus polyphenols could assist in the management of obesity, since they cause a reduction in adipocyte differentiation, lipid content in the cell and may also function in programmed cell death.The results of biological assays are not entirely consistent; however, most of them indicated a reduction in adipose tissue; increased expression of PPARα and its target genes, indicating a stimulus to β-oxidation; improved lipid profile and glycemia; as well as some evidence of improvement in inflammatory status. The effects on total body weight are more evident in the studies that used extracts instead of analytical standards, indicating a possible synergistic effect of extracts. Furthermore, the higher cost of analytical standards limits their use; meanwhile the extracts are generally made from industrial wastes of fruits, a material frequently discarded. Several clinical trials have demonstrated the positive effect of citrus flavonoids in the reduction of pro-inflammatory cytokines in humans, being beneficial to alleviate the complications present in obesity. However, there are few clinical trials developed to examine its role in reducing adiposity, indicating a research field still in expansion.

Keywords: obesity, citrus flavonoids, *in vitro* assay, biological assay, clinical trial.

1. Introduction

Adipose tissue has long been considered only as a site of energy storage, however it is now known that it performs many functions in the body. This tissue is considered an endocrine organ due to paracrine substances secreted, called adipokines (GRUNDY et al., 2004). Also there are other cells present in adipose tissue, besides the adipocytes, that release active substances involved in metabolic pathways, such as macrophages (WEISBERG et al., 2003). In parallel, the adipose tissue has receptors for afferent signals emitted by other endocrine systems, enabling a communication with the central nervous system. This network interactions explains the coordinating activity of adipose tissue in energy metabolism, neuroendocrine and immune function (KERSHAW; FLIER, 2004).

Obesity is a disease characterized by excess body weight, associated with a state of chronic subclinical inflammation, caused by an increased secretion of adipokines that modulate certain responses in the body (BALISTRERI; CARUSO; CANDORE, 2010). Overall, the vast majority of adipokines studied have a role in the development of chronic diseases associated with obesity causing insulin resistance, increased blood pressure, abnormal blood lipids, increased inflammatory response, and thrombus formation (GRUNDY et al., 2004).

In addition to many complications associated with obesity, the high prevalence of the disease made it a public health problem. Accordingly, various strategies and products have been evaluated in an attempt to prevent and treat excessive body weight. Among the compounds studied, stands out the importance of polyphenols in plant food.

A source of polyphenols widely studied is citrus fruits. This group of fruits is important source of bioactive compounds, mainly flavonoids, being target of many studies concerning the adipose tissue and obesity. Therefore, this paper aims to review studies developed to evaluate the role of these compounds in the obesity and associated changes.

2. Phenolics in citrus fruits

Phenolic compounds refers to a group of molecules found in plants, that exert photoprotection function, defense against microorganisms and insects, being responsible for pigmentation and some food organoleptic characteristics (ESCARPA; GONZALEZ, 2001). Among the various classes that comprise the phenolics, flavonoids are considered important for human consumption due to its wide distribution in plant foods.

The flavonoid structure is based on the flavylium nucleus, which consists of three phenolic rings (Figure 1). The first benzene ring (A) is condensed with the sixth carbon of the third ring (C), which in the 2-position carries a phenyl group (B) as a substituent (AHERNE; O'BRIEN, 2002).

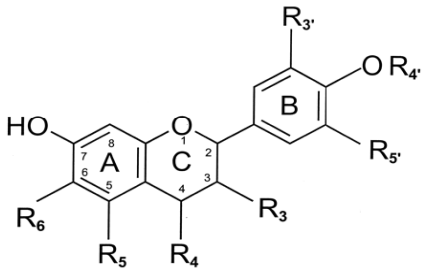


Figure 1.1 General structure of food flavonoids

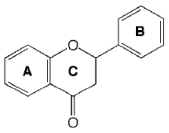
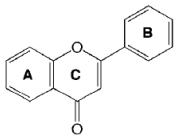
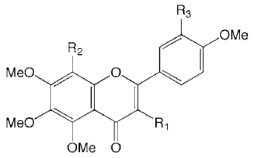
The biochemical activities of flavonoids and their metabolites depend on their chemical structure, which may vary with one or more hydroxyl substituents, including derivatives. Flavonoids and isoflavones commonly occur as esters, ethers or derivatives glycosides, or a mixture of them. Except the group of leucoantocianines, other flavonoids occur in plants usually accompanied by carbohydrates thus receiving the name of glycosylated flavonoids. The glycidic substituents includes: D-glucose, L-rhamnose, glucose-rhamnose, galactose and arabinose (BIRT; HENDRICH; WANG, 2001). When the flavonoid is free of carbohydrates, the structure is called aglycone.

Citrus fruits are rich in various nutrients, such as vitamins A and C, folic acid and dietary fiber. Furthermore, these fruits are source of bioactive compounds, as flavonoids, coumarins, limonoids and carotenoids (DING et al., 2012; TURNER; BURRI, 2013).

Among the flavonoids, citrus present considerable amounts of flavanones, flavones, flavonols and anthocyanins, however the main flavonoid are the flavanones (BENAVENTE-GARCÍA et al., 1997). In this class of compounds, the most frequent ones are hesperidin, narirutin, naringin and eriocitrin (GHASEMI; GHASEMI; EBRAHIMZADEH, 2009; SUN et al., 2013). Other phenolics often found in citrus are p-coumaric, ferulic, caffeic and sinapic acids (MANTHEY; GROHMANN, 2001; SUN et al., 2013).

GATTUSO et al. (2007) reviewed the flavonoid composition of citrus, and some of their results are summarized at the Table 1.

Table 1. 1 Reviewed flavonoid composition of some citrus juices

Flavonoid composition of C. sinensis (sweet orange) juice (mg/100 mL)					
	Mean	SD	Median	MIN	MAX
Flavanones	 Flavanone skeleton				
Didymin	1.89	0.92	1.60	0.80	3.10
Eriocitrin	0.31	0.18	0.29	0.11	0.67
Hesperidin	28.6	11.9	28.0	3.51	55.2
Narirutin	5.2	3.1	4.2	0.55	14.2
Flavones	 Flavone skeleton				
Neoeriocitrin	0.59	-	-	-	-
Poncirin	1.04	0.78	1.04	0.49	1.59
6,8-di-C-Glu-Apigenin	5.72	2.02	5.00	4.15	8
6,8-di-C-Glu-Diosmetin	0.35	0.14	0.35	0.25	0.45
Rhoifolin	0.05	-	-	-	-
Isorhoifolin	0.07	-	-	-	-
Diosmin	0.09	-	-	-	-
Neodiosmin	0.08	-	-	-	-
Polymethoxyflavones					
Nobiletin	0.33	0.19	0.33	0.19	0.46
Sinensetin	0.37	-	-	-	-
Tangeretin	0.04	0.04	0.04	0.01	0.07
Flavonoid composition of *C. clementina* juice (mg/100 mL)					
	Mean	SD	Median	MIN	MAX
Flavanones					
Hesperidin	39.9	29.4	34.9	5.21	86.1
Naringin	0.08	0.03	0.08	0.05	0.12
Narirutin	4.64	-	-	-	-
Flavones					
6,8-di-*C*-Glu-Apigenin	0.5	-	-	-	-
6,8-di-*C*-Glu-Diosmetin	0.2	-	-	-	-
Diosmin	1.25	0.51	1.26	0.67	2.12
Flavonoid composition of *C. limon* (lemon) juice (mg/100 mL)					
Flavanones					
Eriocitrin	16.7	10.3	16.55	1.67	39.1

Hesperidin	20.5	12.4	18.85	3.84	41
Flavones					
6,8-di-*C*-Glu-Apigenin	1.17	0.25	1.05	1	1.45
6,8-di-*C*-Glu-Diosmetin	4.95	0.88	5	4.05	5.8
7-*O*-Rut-Luteolin	3.93	2.14	3.5	1.5	6.5
Diosmin	3.12	1.66	3.65	0.51	5.1
Aglycones					
Luteolin	0.08	-	-	-	-
Flavonoid composition of *C. paradisi* (grapefruit) juice (mg/100 mL).					
Flavanones					
Didymin	0.30	0.04	0.30	0.27	0.33
Eriocitrin	0.41	0.19	0.41	0.27	0.54
Hesperidin	0.93	0.58	0.87	0.25	1.79
Naringin	23.0	12.8	21.9	4.5	60.2
Narirutin	7.60	5.80	7.70	2.50	17.0
Neohesperidin	1.21	0.35	1.28	0.67	1.58
Neoeriocitrin	0.32	0.02	0.32	0.30	0.33
Poncirin	1.26	0.35	1.30	0.85	1.58
Flavones					
Rutin	3.26	-	-	-	-
Rhoifolin	0.28	-	-	-	-
Polymethoxyflavones					
Heptamethoxyflavone	0.06	0.07	0.06	0.01	0.11
Nobiletin	0.15	0.04	0.15	0.12	0.17
Tangeretin	0.12	-	-	-	-
Aglycones					
Hesperetin	0.74	-	-	-	-
Naringenin	2.70	2.68	1.70	0.98	8.00
Taxifolin	0.16	-	-	-	-
Quercetin	0.19	0.03	0.19	0.17	0.21
Adapted from Gattuso et al., 2007.					

The genus Citrus comprises several orange species as Citrus sinensis (sweet orange), Citrus aurantium (sour oranges), Citrus reticulata (tangerine or mandarin) e and their hybrids e tangors, which are orange-tangerine hybrids, and tangelos, which are tangerine-grapefruit or tangerine pummelo hybrids. Many of these species or hybrids can have different varieties (GATTUSO et al., 2007).

In general, the data where the specific *C. sinensis* variety analyzed is reported show that different varieties present approximately the same flavonoid composition pattern. Commercial orange juices present a similar composition to freshly squeezed ones, with the appearance of some unexpected compounds. Naringin and diosmin hint at the possibility that some of the samples analyzed are not pure orange juices, or, as in the case of hand-squeezed juices, the presence of polymethoxyflavones (PMFs) in variable quantities suggests that they

could be essentially derived from the flavedo and confirm that the amounts of PMFs found in industrial juices are a consequence of the pressing process used (GATTUSO et al., 2007).

It is also important to consider that the flavanones in citrus can be glycosylated or aglycone. The glycosylated forms are also divided into neohesperidosides that contain a neohesperidose (ramnosil-α-1 ,2 glucose) and have a bitter taste; and rutinosides that contain a flavanone and a disaccharide residue, and do not have taste (MACHEIX; FLEURIET; BILLOT, 1990). Naringin, neohesperidin and neoeriocitrin are examples of neohesperidosides; while hesperidin, narirutin and didymin are examples of rutinosides (TRIPOLI et al., 2007). Naringenin and hesperetin are the most common aglycones, often found in trace concentrations.

Concerning the quantity of the compounds, MILLER; RICE-EVANS (1997) detected the presence of hesperidin (141 ± 49 µmol/L) and narirutin (62 ± 16 µmol/L) in longlife orange juice. KLIMCZAK et al. (2007) also evaluated longlife orange juice, verifying the presence of some hydroycinnamic acids as caffeic (8.2 mg/L), p-coumaric (0.5 mg/L), ferulic (0.6 mg/L) and sinapic (0.7 mg/L). However, as mentioned above, the flavanones were found in greater quantity, being detected the presence of narirutin (70.2 mg/L), hesperidin (76.9 mg/L) and dydymin (9.9 mg/L). Of the flavanones analyzed, naringin and neohesperidin were not detected.

STUETZ et al. (2010) evaluated the polyphenol content of *Citrus reticulata* Blanco cv. Sainampueng, to verify the difference between hand-pressed juice and the peeled fruit. The peeled fruit had low content of PMFs, while the hand-pressed juice presented high content of tangeritin (5.99 – 31.8 mg/L), nobiletin (5.49 – 28.2 mg/L) and sinensetin (0.30 – 2.00 mg/L). The authors observed that the PMFs were present in the peel of the fruit, and a simple squeezing can cause the transfer of these compounds from the peel to the juice. Besides this class of polyphenol, it was also detected the presence of the flavanones didymin (4.44 – 9.50 mg/L), narirutin (17.7 – 43.4 mg/L) and hesperidin (123.3 – 206.7 mg/L) in the hand-pressed juice. On the other side, the peeled fruit had high content of didymin (45 – 112 mg/kg), narirutin (181 – 600 mg/ kg) and hesperidin (841 – 1898 mg/ kg).

Some researchers also study the peels and peels extract of citrus fruits, as RAMFUL et al. (2010) that evaluated orange, clementine, mandarine, tangor, tangelo and pamplemousses peels. The flavonoids detected in this matrix were poncirin (2.49 – 18.85 mg/g FW), rhoifolin (4.54 – 10.39 mg/g FW), didymin (3.22 – 13.94 mg/g FW), rutin (8.16 – 42.13 mg/g FW),

diosmin (4.01 – 18.06 mg/g FW), isorhoifolin (1.72 – 14.14 mg/g FW), neohesperidin 3.20 – 11.67 mg/g FW), hesperidin (83.4 – 234.1 mg/g FW), neoeriocitrin (8.8 – 34.65 mg/g FW) and narirutin (5.05 – 21.23 mg/g FW). Naringin (19.49 mg/g FW) was only detected in mandarine.

LONDOÑO-LONDOÑO et al. (2010) identified using HPLC-MS the presence of hesperidin, neohesperidin, diosmin, nobiletin and tangeritin in orange peel; hesperidin and neohesperidin in tangerine peel; and hesperidin, neohesperidin and diosmin in lime peel. Reinforcing the information above, none of the peels presented the aglycone hesperitin in their compostion.

GHASEMI; GHASEMI; EBRAHIMZADEH (2009) evaluated the total polyphenol and flavonoid content of peels and tissues from three varieties of *Citrus sinensis*, three of *C. reticulata*, three of *C. unshiu*, one of *C. limon*, one of *C. paradisi* and two of *C. aurantium*. For most citrus analyzed the total polyphenols content was higher in the peel (104.2 – 223.2 mg gallic acid equivalent/ g of extract powder) in comparison to tissue (66.5 – 396.8 2 mg gallic acid equivalent / g of extract powder), excepting all *C. retic*ulate varieties, and one *C. sinensis* variety (var. Washington Navel). The total flavonoid content was also higher in the peel (0.3 – 31.1 mg quercetin equivalent/g of extract powder) in relation to tissue (0.3 – 17.1 mg quercetin equivalent/g of extract powder) in most of the samples, excepting four varieties (*C. sinensis* var. Sungin, *C. unshiu* var. Ishikawa, *C. reticulate* var. Clementine, *C. reticulate* var. Page). These results indicate that considerable losses occur with the peel removal before consumption or in industrial process. And besides the information about the content of total polyphenol and flavonoid, several studies have already shown the positive effects of peel extracts in the treatment of chronic non-communicable diseases (DING et al., 2012; FUKUCHI et al., 2008; JUNG, 2011; KANG et al., 2012; KIM et al., 2012; LEE et al., 2011; RAASMAJA et al., 2013).

A factor to consider when talking about flavonoids is whether the compound is in the glycosylated or aglycone form. It has been shown that in rats, after oral consumption of naringin, the sulfate and glucuronate conjugates forms of naringenin are found in the organism, indicating that for naringin absorption the glycoside needs to be released to the formation of the aglycone naringenin, thus depending on one glucosidase for its absorption (WANG et al., 2006). The better absorption of the aglycone form in relation to glycosylated in citrus flavonoids has also been observed in humans using glycosylated forms of eriocitrin and hesperidin compared with the corresponding aglycones eridictiol, homoeridictiol and hesperetin

(MIYAKE et al., 2006). Therefore, the polyphenol structure can modify its bioavailability to the body. This might be the reason why many researchers chose to evaluate the aglycone potential, seen that this form is detected in the organism tissues and blood, and it has higher bioavailability.

The flavonoids found in citrus species act as antioxidants and may protect against oxidative stress-related to inflammation process, thus reducing the risk of macromolecules damage caused by the action of reactive species, conferring protection against several neurodegenerative diseases and reducing the risk of developing cardiovascular disease and cancer (BENAVENTE-GARCÍA et al., 1997).

3. Citrus phenolics effect on cell cultures models of obesity

In vitro studies are useful to understand the mechanisms of action, and guide the decision of which products should be further studied in biological assays and clinical trials. Besides that, they are an alternative when the product is in its early development phase, a moment that the yield is generally low.

Many *in vitro* studies are being conducted with citrus phenolics to evaluate its effects on obesity. One of the mechanisms proposed has been the role of these compounds in the adipocytes apoptosis, because it was observed that the addition of polymethoxyflavones analytical standard (5-hydroxy-3,6,7,8,30,40-hexamethoxyflavone (5-HxMF OH) 3 ,5,6,7,8,30,40-heptamethoxyflavone (HpMF); 5,6,7,30,40 - pentamethoxyflavone (PMTCT), and 30-hydroxy-5,6,7,40-tetramethoxyflavone (30 - OH-TtMF)) of citrus (100 μM) caused an increase in intracellular calcium, which induced the increase of calpain and caspase-12, two proteins associated with programmed cell death (SERGEEV et al., 2009). The reduction in the number of adipose cells due to apoptosis could assist in maintaining weight loss, avoiding the weight cycling.

Another study evaluated the effect of nobiletin analytical standard in 3T3-L1 adipocytes (0 – 100 μM). The treatment of these cells with the citrus phenolic reduced, in a dose-dependent manner, the expression of C/EBPβ and PPAR γ, transcription factors that are associated with differentiation of pre-adipocytes into mature adipocytes. Reinforcing this result, it was also observed lower lipid accumulation in cultured cells when the flavonoid was added (KANDA et al., 2012).

Nowadays, it is considered the importance of toll-like receptors (TLRs) on the association between obesity and other chronic non-communicable diseases, and it is recognized the fact that TLRs are responsible for the activation of inflammatory pathways (SABROE et al., 2008). In a study evaluating the treatment of pre-adipocytes, adipocytes during its differentiation, and differentiated 3T3-L1 cells treated with naringenin analytical standard (100 μM), it was observed an inhibitory effect of the flavonoid on the expression of TLR 2, only during adipocyte differentiation (YOSHIDA et al., 2013), indicating a possible effect on the phase in which the individual is in the process of gaining body fat.

During the obesity development, it is known that in addition to the increase in adipose cells, there is an increase in the macrophages number in adipose tissue (RAMALHO; GUIMARÃES, 2008; WEISBERG et al., 2003). Considering this information, Yoshida et al. (2013) conducted a test with 3T3-L1 adipocytes and macrophages RAW 264 in co-culture. The co-culture showed increased expression of TLR 2, and treatment with naringenin inhibited this increased expression observed. Furthermore, the expression of TLR 2 was increased with TNF-α addition to the culture of mature adipocytes, however naringenin added to this medium was able to inhibit TNF-α-induced TLR 2 expression by inhibiting JNK and NF-kB pathways. Besides, naringenin appears to reduce the expression of TLR 2 via increased activation of PPAR γ, a nuclear transcription factor that could cause greater differentiation of pre-adipocytes into mature adipocytes and increase lipid accumulation in these cells, exactly as was observed on the experiment (YOSHIDA et al., 2013).

Also, YOSHIDA et al. (2010) found that in 3T3-L1 adipocytes cell culture, hesperetin and naringenin analytical standards showed anti-inflammatory effect by inhibiting the activation of NFκB through TNF-α, with a consequent reduction in the secretion of interleukin-6 (IL-6); and anti-lipolytic effect by inhibit ERK(extracellular signal regulated kinase) pathway causing a decreased activation of hormone sensitive lipase (HSL); contributing to reduce the insulin resistance (Figure 2).

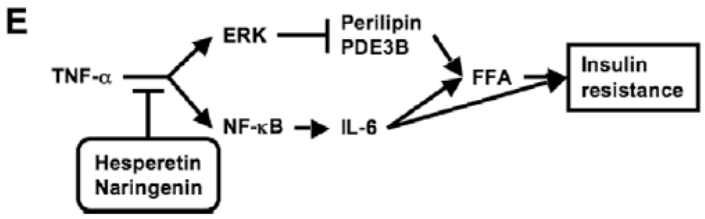


Figure 1.2 Scheme proposed by Yoshida et al. (2010) for the action of hesperetin and naringenin on inhibition of ERK and NFκB pathways, resulting in the reduction of free fatty acids (FFA), and consequently improving insulin resistance.

Other studies, one with orange peel flavonoids ethanol extract rich in hesperidin (13.79 mg / g), narirutin (7 mg / g) and naringin (262.5 mg / g) (JUNG, 2011), and another with *Citrus aurantium* flavonoids extract that contained naringin, hesperidin, poncirin, isosiennsetin, sineesytin, tetramrthnl-o-isoscutellaeein, nobiletin, heptamethoxyflavone, 3-hydoxynobiletin, tangeretin, hydroxypentamethoxyflavone, and hexamethoxyflavone. KIM et al. (2012), also observed a stimulus in lipolysis and lower triglyceride accumulation in 3T3-L1 adipocytes. Still, extracts caused a lower accumulation of total lipids and reduced the expression of C/EBPα, C/EBP β, PPAR γ, aP2 (activating protein 2) and FAS (fatty acid synthase), being the last two ones target genes of C/EBP β and PPAR γ (JUNG, 2011; KIM et al., 2012). The extracts have also generated a smaller amount of Akt (serine / threonine kinase) and phosphorylated GSK3β. The phosphorylated Akt, promotes the phosphorylation of GSK3β, and this phosphorylates C/EBP α and C/EBP β, which become activated (KIM et al., 2012), then acting in adipocyte differentiation.

The orange peel flavonoids ethanol extract evaluated by JUNG (2011) also caused a suppressive effect on the expression of perilipin, indicating another factor that may be associated with the positive effect of citrus flavonoids in obesity. This is a lipid-associated protein secreted only in adipocytes (PERSSON et al., 2007), that controls fatty acid release stimulated by HSL, because it binds and stabilizes lipid droplets in adipose tissue (LE LAY; DUGAIL, 2009).

The treatment of mature 3T3-L1 adipocytes with *Citrus sunki* peel ethanol extract that contained tangeritin (55.13 mg/g), nobiletin (38.83 mg/g), rutin (17.02 mg/g), hesperidin (17.11 mg/g), sinensetin (4.23 mg/g) induced LKB1, AMPK (AMP-activated protein kinase) and ACC (acetyl-CoA carboxylase) phosphorylation in a dose-dependent manner, and also caused an increase in mRNA levels of CPT-1a (carnitine palmitoyl transferase 1a) indicating

the role of this extract to increase the β-oxidation. Furthermore, lipolysis stimulation occurred 24 hours after the extract addition to the cell culture, in a dose dependent manner. Associated with this result, the authors observed that the extract caused phosphorylation of PKA substrate (cAMP-dependent protein kinase) and HSL (KANG et al., 2012).

Besides the effect on adipose tissue, flavonoids can also act in the management of obesity by interfering in the control of hunger and satiety. In this context, hesperetin analytical standard (0.1 – 1.0 mM) has shown to cause an increase in the secretion of cholecystokinin (CCK) in STC-1 cells through increase in intracellular calcium concentration by the TRP (transient receptor potential) and TRP 1 ankirin channels. The addition of hesperidin analytical standard in the same model caused no effect, indicating that only the aglycone form influences hormone secretion (KIM et al., 2013). The increase in CCK would be interesting because this hormone, secreted from endocrine cells in the small intestine, assists in the control of food intake (RAYBOULD, 2009).

Some products have been developed in order to assist in the obesity prevention and treatment, and it could be mentioned Sinetrol, a citrus-based fruits (juice, peels, seeds) extract obtained by physical treatment (crushing of fruits, cold pressure of juice, extraction, centrifugation, filtration, spray drying) of a specific varieties of red orange (*Citrus sinensis L. Osbeck* (*Blood group*)), sweet orange (*Citrus aurantium L. var. sinensis*),bitter orange (*Citrus aurantium L. var.amara*), grapefruit(*Citrus paradise*) and guarana (*Paulinia cupanna*), which contained 60% of polyphenol, 16.7% of flavanones, 2% of anthocyanins and 3.6% of caffeine, studied by DALLAS et al.(2008). The researchers noted that this supplement (20 mg/mL) was able to stimulate lipolysis in human fat cells in a *in vitro* study, verified by the free fatty acids enhancement. The authors suggest that the compounds present in the supplement, especially naringin and cyanidin 3-glycoside, have an effect on the inhibition of cAMP-phosphodiesterase, and thus there is an increase in cAMP and subsequent stimulation of hormone-sensitive lipase (HSL), the enzyme that stimulates lipolysis in human.

4. Evaluation of citric polyphenol effect in biological assay

Despite the important information collected by *in vitro* assays, biological assay helps understand the bioactive compounds effects in the whole body, further illustrating the changes caused due to their consumption.

ALAM; KAUTER; BROWN (2013) evaluated the effect of supplementation with naringin analytical standard (approximately 100 mg/kg diet/day, corresponding to 0.01%) in male Wistar rats fed a diet rich in lipid and carbohydrate. They did not observe the effect of the flavonoid in weight gain. However, supplementation promoted a reduction in retroperitoneal abdominal fat deposition, a better serum lipid profile and oral glucose tolerance. The insulin concentration and pancreas wet weight in rats supplemented was similar to the control group, which received a standard diet, presenting lower values than the high carbohydrate, high fat diet-group without the flavonoid. A high carbohydrate, high fat diet promoted greater inflammatory cell infiltration and accumulation of fat droplets in the liver compared to the control group, however naringin supplementation decreased these two parameters.

In another study, naringenin analytical standard (1%) was supplemented in mice fed a high fat diet, and also no effect was observed in weight gain and food consumption. However, the supplementation improved hyperglycemia, reduced expression of TNF-α (tumor necrosis factor-alpha), MCP-1 (monocyte chemotactic protein-1), and TLR 2 in adipose tissue (YOSHIDA et al., 2013), promoting protection against chronicle non-communicable diseases.

In an experiment conducted with male Long-Evans adult rats fed a semi-purified experimental diet with 16% fat and 45.5% sucrose, supplementation with 0.012% naringenin analytical standard promoted less visceral fat accumulation, and lower triacylglycerol content in the tissue, compared to the control group that did not receive the flavonoid. However, no effect was observed in the total body weight. Food consumption did not differ between the groups. Also, the supplemented group had lower concentration of serum triglycerides, total and free cholesterol in plasma, and lower accumulation of triacylglycerol and cholesterol in the liver. The flavonoid intake caused an increased expression of PPARα, CPT1 (carnitine palmitoyltransferase 1) and UCP2 (uncoupling protein 2) protein, indicating the role of naringenin to increase lipid β-oxidation in animals (CHO et al., 2011).

The administration of 0.05% coumarin analytical standard in C57BL/6J mice receiving a high fat diet also caused less accumulation of visceral fat, and yet caused reduction of total body weight compared to the high fat diet group without the supplement. Still, coumarin supplementation caused less accumulation of lipids, triacylglycerol and cholesterol in the liver; and reduced protein levels of SRBP-1c, FAS, ACC1, PPAR γ and C/EBPα. Histological

analyzes showed a minor adipocyte size by using the phenolic compound, indicating a contribution in the reduction of adipose tissue (UM et al., 2013).

High fructose diets are used in animal experiments to induce hypertriglyceridemia and insulin resistance (BEZERRA et al., 2000; KELLEY; ALLAN; AZHAR, 2004). The supplementation of citrus polymethoxyflavones analytical standard (125 mg / kg body weight/day), mainly containing tangeritin and nobiletin, in hamsters subjected to this modified diet, reduced the weight gain, serum triglyceride, triglyceride in liver and heart, and improved adiponectin levels compared with the group receiving high-fructose diet without the flavonoid. Moreover, a positive effect was observed in the levels of some inflammatory cytokines, reducing TNFα and IFN-γ after the addition of polymethoxyflavones. In this experiment also occurred increased expression of hepatic PPARα and PPAR γ as the effect of supplementation, which according to the authors, would be a major regulatory pathway of the effects observed (LI et al., 2006).

Another experiment with polymethoxyflavones was performed with *Citrus depressa Hayata* peel methanol extract that contained nobiletin and tangeritin (1,5%), in ICR mice consuming a high fat diet. The addition of the extract caused less weight gain, lower weight of white adipose tissue, reduced adipocyte size, and lower serum levels of triglycerides and leptin. There was also a decrease in ACC1, SCD1 (esteroil-CoA desaturase), FATP (transport protein fatty acid), aP2 and DAGT1 (diacylglycerol acyltransferase 1) mRNA in white adipose tissue. All the genes cited are involved in the synthesis of fatty acids and triacylglycerols. Despite the positive effects observed, there was no effect on serum adiponectin nor in the mRNA levels of SREBP1 (binding protein sterol regulatory element 1), FAS and ACC1 in the liver (LEE et al., 2011).

In a study developed by LEE et al. (2013), the administration by gavage of 100 mg/kg of purified nobiletin extracted from *Citrus depressa* peel to male C57BL/6J mice fed a high fat diet, caused less overall weight gain, lower weight of white adipose tissue and serum triglycerides. There was no effect of the extract on hepatic triacylglycerol levels and serum adiponectin. Controversially, there was an increase in the expression of PPAR γ and PPAR α, as well as their target genes SREBP-1c, FAS, SCD-1; and CPT-1, UCP2; respectively. These results indicate that the extract induced lipid accumulation and fatty acid oxidation at the same time, however with a greater catabolic effect seen the less weight gain when compared to the

group receiving high fat diet without the extract. The extract had the positive effect of reducing TNF-α and MCP-1 (LEE et al., 2013), which helps improve insulin sensitivity, as it is known that TNF-α causes a reduction in expression and translocation of GLUT4, the glucose transport protein in insulin-dependent cells (HOTAMISLIGIL; SHARGILL; SPIEGELMAN, 1993). The authors observed an increase in expression of IκBα after the flavonoid use, indicating that the anti-inflammatory effect is possibly through NFκB pathway inactivation (LEE et al., 2013).

The addition of lemon peel polyphenol ethanol extract (0.5%), containing greater amounts of eriocitrin, hesperidin and narirutin; also promoted less total weight and white adipose tissue gain after consumption of a high fat diet in male C57BL/6J mice. Note that polyphenols also caused increased hepatic PPARα mRNA level, and acyl-CoA oxidase in the liver and white adipose tissue, indicating increased peroxisomal fatty acid oxidation (FUKUCHI et al., 2008).

Another extract that showed positive effects of citrus polyphenols was *Citrus ichangensis* peel ethanol extract that contained naringin (8.12 mg/g), hesperidin (0.84 mg/g) and poncirin (1.33 mg/g), administered to female mice fed standard (control), and high-fat diets supplemented with 1% extract. The weight gain in the group that received the high-fat diet alone was greater than the control, and the addition of the extract in the high-fat diet caused less weight gain, being similar to control group. The extract caused a lower fasting glucose and improved glucose tolerance. Also, there was less accumulation of triacylglycerol and cholesterol in the liver due to the extract administration. Moreover, this has caused lower expression of PPAR γ mRNA and lower levels of this transcription factor target genes, including FAS, acyl-CoA oxidase and UCP 2 (DING et al., 2012); in agreement with the *in vitro* studies presented previously in this paper.

KANG et al. (2012) also tested in mice a high-fat diet supplemented by gavage with mature *Citrus sunki* peel extract (150 mg/kg body weight/day), source of the flavonoids tangeritin (55.13 mg/g), nobiletin (38.83 mg/g), rutin (17.02 mg/g), hesperidin (17.11 mg/g), sinensetin (4.23 mg/g). Likewise, the authors found that supplementation reduced weight gain caused by the consumption of a high-fat diet, in addition to promoting lower weight of perirenal and epididymal adipose tissue, as well as smaller size of adipocytes in epididymal tissue. Another positive effect of the supplementation can be observed in the serum levels of total cholesterol and triglycerides, that were lower compared to the group that received only the high-

fat diet. Still, lipid accumulation in the liver was lower, comparable to the control group that received a standard diet. It was also observed increased expression of proteins related to β-oxidation when the extract was added, along with a greater expression of adiponectin gene.

In another study, a water and alcohol extract of *Citrus grandis* whole fruits containing 19% naringin was tested in genetically obese Zucker rats fed with high-fat/high-cholesterol diet. No effect was observed in the body weight, however serum cholesterol and triglyceride were improved when 600 mg/kg of the extract was administered by intragastric gavage for four weeks (RAASMAJA et al., 2013).

SALAMONE et al. (2012) evaluated the effect of *Moro* orange juice, rich in anthocyanins (85 mg / L) in mice C57BL6/J fed a high-fat diet. The juice consumption was *ad libitum*, resulting in a mean intake of 4.1 ± 0.75 mL/day and consequent anthocyanin consumption of about 0.34 mg per day. The group that received the juice had lower levels of triacylglycerol and total lipids in the liver. In addition, there was increased expression of PPAR α and acyl-CoA oxidase, and lower of LXR (liver X receptor), FAS, HMG-CoA reductase; indicating a potential effect in stimulating lipid oxidation and reduction of lipogenesis.

A limitation in the comparison of the studies is the phenols administration form, in some cases the compounds are administered as a dietary ingredient and other by gavage. In studies in which the product is incorporated into the diet, consumption data are not always available, providing only the concentration in the diet; on the other hand in studies that used gavage, the information provided is the total quantity consumed.

5. Evaluation of citric polyphenol effect in clinical trials

Despite the evidence observed in *in vitro* studies and biological assays, clinical trial is essential to the conclusions, since it considers the influence of compounds bioavailability in the human body. Accordingly, AMEER et al. (1996) ascertained the bioavailability of naringin (500 mg) consumed pure, naringin (500 mg) administered with hesperidin (500 mg), grapefruit juice (1250 mL) co administered with orange juice (1250 mL) and grapefruit (1 unit) consumed daily for 4 weeks. The presence of the aglycone hesperetin and naringenin were detected in urine after the consumption of pure hesperidin and naringin, and after consuming grapefruit and orange juice. After 4 weeks of consuming grapefruit, naringenin was present in plasma and urine. The authors conclude that the aglycone forms of the flavonoid were detected, as the

glycoside linkages are not stable to the acidic environment of the stomach, in addition to the possible action of glycosides from intestinal bacteria cleaving the sugar residues.

Another important finding of AMEER et al. (1996) was the observation that the consumption of hesperidin associated with naringin does not affect the urinary recovery of the second, indicating that it does not disturb the bioavailability of the other. Furthermore, these results indicate that the absorption of these two flavonoids occurs in pure form, and also when consumed in a food matrix in the form of juice or fresh fruit. And, as the aglycone form is found after the consumption, maybe its bioavailability is higher since it does not require an enzyme to be absorbed. However, it should be noted that this study was conducted with only 4 volunteers, limiting extrapolation of the results to the general population, and indicating the need for more bioavailability studies in clinical trials.

In addition to assessing the effect of a supplement rich in citrus polyphenols *in vitro*, DALLAS et al. (2008) conducted a double-blind placebo-controlled study evaluating the effect of the supplementation in overweight men, observing a greater weight and body fat loss in the group that consumed the supplement. However, the food habits were not controlled, being only mentioned that the volunteers were not supposed to modify it.

A variation of this supplement was studied in another group of humans. This new product was polyphenolic rich fruit extract (red orange, grapefruit, orange sweet and guarana), that contained at least 90% of polyphenols, at least 20% of flavanones and between 1 and 3 % of natural caffeine. The authors reported a reduction in waist and hip circumferences; in markers of inflammation C-reactive protein and fibrinogen; and improved oxidative stress status, with the reduction in malondialdehyde (MDA) and increase in superoxide dismutase and glutathione levels. There were no adverse effects in liver and kidney. There was an increase in serum free fatty acids, but no change in the serum lipids levels (DALLAS et al., 2013). However, it was not reported in the paper what types of polyphenols and flavonoids were offered with the supplement, and there was no mention about the food habits of the volunteers.

6. Potential of citrus flavonoids produced by biotechnology

Bioprocessing strategies aiming the improvement of the bioaccessibility of phenolic compounds have been investigated in the last years. The use of α-L-rhamnosidases from *Aspergillus aculeatus* was investigated in the transformation of flavonoid rutinosides from fruit

juices (orange and blackcurrant) and green tea into their flavonoid glucoside counterparts in a reaction at 30°C for 10 hours. Aliquots of the controls and the enzyme treated samples were taken at different time points and flavonoids rutinosides (anthocyanins in blackcurrant juice, flavanones in orange in juice, and flavonols in green tea) and glucosides were identified and quantified. Even with the assay conditions in each beverage being different, the enzyme was able to remove terminal rhamnosyl groups in the three beverages. Results showed a decrease in the flavonoid rutinoside and an increase in their flavonoid glucoside counterparts (GONZÁLEZ-BARRIO et al., 2004).

The effects on the bioavailability of hesperitin was investigated in a double-blind, randomized, crossover study, in human subjects. The volunteers consumed orange juice with natural hesperidin (hesperitin-7-*O*-rutinoside), orange juice treated with the enzyme hesperidinase and orange juice fortified to obtain 3 times more hesperidin than naturally present. A significant improvement in the bioavailability of the aglycone hesperetin was observed after enzymatic modification of the orange juice. The peak plasma concentrations of the aglycone when subjects consumed the juice containing hesperetin-7-glucoside, generated after removal of the rhamnose by the hesperidinase, were 4-fold higher compared with the untreated juice and 1.5-fold higher than the fortified juice (NIELSEN et al., 2006).

A study employing orange pomace as substrate for solid-state fermentation by *Paecilomyces variotii* to produce the enzymes tannase and phytase simultaneously, also evaluated the phenolic content and antioxidant capacity of orange pomace during fermentation. The fermentation medium was prepared with the orange pomace, a saline solution and 10% tannic acid and, after inoculation, was incubated at 30°C for 120 hours. In addition to tannase and phytase production at significant levels, results showed no difference in total phenolic content before and after the fermentation processes. However, the antioxidant capacity of orange pomace, tested against the free radical ABTS, increased approximately tenfold after fermentation, potentially enhancing the value of this residue (MADEIRA JR; MACEDO; MACEDO, 2011).

Enzymatic hydrolysis and fermentation appear to be an attractive mean to promote the biotransformation of phenolic glycosides and polymers and to increase the concentration of free phenolics in citrus fruits and agro-industrial wastes. The biotransformation of phenolics improved the antioxidant activity and bioaccessibility of these compounds. Further research is

necessary to explore new substrates, enzymes and microorganisms and to evaluate the use of biotransformed products as natural antioxidants and as food supplements.

7. Conclusion

Despite the difficulties in the comparison of study results, due to the variety of methodologies and samples evaluated, some general trends can be highlighted.

The studies with cells culture indicate that citrus polyphenols could assist in the management of obesity, since they cause a reduction in adipocyte differentiation, lipid content in the cell and may also function in programmed cell death.

The results of biological assays are not entirely consistent, since in some cases the addition of citrus fruit polyphenols caused lower weight gain, and in other studies this effect was not noticed. However, most of them indicated a reduction in adipose tissue; increased expression of PPARα and its target genes, indicating a stimulus to β-oxidation; improved lipid profile and glycemia; as well as some evidence of improvement in inflammatory status due to a reduction in the proinflammatory cytokines levels. The effects on total body weight are more evident in the studies that used extracts instead of analytical standards, indicating a possible synergistic effect among the different phenolics found when using an extract. Furthermore, the higher cost of analytical standards limits their use in biological assay; meanwhile the extracts are generally made from fruits industrial wastes, a material that would be discarded.

Several clinical trials have demonstrated the positive effect of citrus flavonoids in the reduction of pro-inflammatory cytokines in humans (BERNABÉ et al., 2013; BUSCEMI et al., 2012; DEVARAJ et al., 2011; IWAMOTO et al., 2012; MORAND et al., 2011), being beneficial to alleviate the complications present in obesity. However, there are few clinical trials developed to examine its role in reducing adiposity, indicating a research field still in expansion.

8. References

AHERNE, S. A.; O'BRIEN, N. M. Dietary flavonols: chemistry, food content, and metabolism. **Nutrition**, v. 18, n. 1, p. 75–81, jan. 2002.

ALAM, M. A.; KAUTER, K.; BROWN, L. Naringin improves diet-induced cardiovascular dysfunction and obesity in high carbohydrate, high fat diet-fed rats. **Nutrients**, v. 5, n. 3, p. 637–50, mar. 2013.

AMEER, B. et al. Flavanone absorption after naringin, hesperidin, and citrus administration. **Clinical Pharmacology and Therapeutics**, v. 60, n. 1, p. 34–40, jul. 1996.

BALISTRERI, C. R.; CARUSO, C.; CANDORE, G. The role of adipose tissue and adipokines in obesity-related inflammatory diseases. **Mediators of Inflammation**, v. 2010, p. 1–19, jan. 2010.

BENAVENTE-GARCÍA, O. et al. Uses and properties of Citrus flavonoids. **Journal of Agricultural and Food Chemistry**, v. 45, n. 12, p. 4505–4515, 1997.

BERNABÉ, J. et al. Effects of a citrus based juice on biomarkers of oxidative stress in metabolic syndrome patients. **Journal of Functional Foods**, v. 5, n. 3, p. 1031–1038, jul. 2013.

BEZERRA, R. M. N. et al. A high fructose diet affects the early steps of insulin action in muscle and liver of rats. **The Journal of Nutrition**, v. 130, p. 1531–1535, 2000.

BIRT, D. F.; HENDRICH, S.; WANG, W. Dietary agents in cancer prevention: flavonoids and isoflavonoids. **Pharmacology & Therapeutics**, v. 90, n. 2–3, p. 157–177, 2001.

BUSCEMI, S. et al. Effects of red orange juice intake on endothelial function and inflammatory markers in adult subjects with increased cardiovascular risk. **American Journal of Clinical Nutrition**, v. 95, p. 1089–1095, 2012.

CHO, K. W. et al. Dietary naringenin increases hepatic peroxisome proliferators-activated receptor α protein expression and decreases plasma triglyceride and adiposity in rats. **European Journal of Nutrition**, v. 50, n. 2, p. 81–8, mar. 2011.

DALLAS, C. et al. Lipolytic effect of a polyphenolic citrus dry extract of red orange, grapefruit, orange (SINETROL) in human body fat adipocytes. Mechanism of action by inhibition of cAMP-phosphodiesterase (PDE). **Phytomedicine**, v. 15, p. 783–792, out. 2008.

DALLAS, C. et al. Clinical study to assess the efficacy and safety of a citrus polyphenolic extract of red orange, grapefruit, and orange (Sinetrol-XPur) on weight management and metabolic parameters in healthy overweight individuals. **Phytotherapy Research**, p. 1–7, 3 abr. 2013.

DEVARAJ, S. et al. Effect of orange juice and beverage with phytosterols on cytokines and PAI-1 activity. **Clinical Nutrition**, v. 30, n. 5, p. 668–671, out. 2011.

DING, X. et al. Citrus ichangensis peel extract exhibits anti-metabolic disorder effects by the inhibition of PPARγ and LXR signaling in high-fat diet-induced C57BL/6 mouse. **Evidence-Based Complementary and Alternative Medicine**, v. 2012, p. 1–10, jan. 2012.

ESCARPA, A.; GONZALEZ, M. C. An overview of analytical chemistry of phenolic compounds in foods. **Critical Reviews in Analytical Chemistry**, v. 31, n. 2, p. 57–139, abr. 2001.

FUKUCHI, Y. et al. Lemon polyphenols supress diet-induced obesity by up-regulating of mRNA levels of the enzymes involved in beta-oxidation in mouse white adipose tissue. **Journal of Clinical Biochemistry and Nutrition**, v. 43, n. November, p. 201–209, 2008.

GATTUSO, G. et al. Flavonoid composition of citrus juices. **Molecules**, v. 12, n. 8, p. 1641–1673, 2007.

GHASEMI, K.; GHASEMI, Y.; EBRAHIMZADEH, M. A. Antioxidant activity, phenol and flavonoid contents of 13 citrus species peels and tissues. **Pakistan Journal of Pharmaceutical Sciences**, v. 22, n. 3, p. 277–281, jul. 2009.

GONZÁLEZ-BARRIO, R. et al. Production of bioavailable flavonoid glucosides in fruit juices and green tea by use of fungal alpha-L-rhamnosidases. **Journal of Agricultural and Food Chemistry**, v. 52, n. 20, p. 6136–6142, 6 out. 2004.

GRUNDY, S. M. et al. Definition of Metabolic Syndrome: Report of the National Heart, Lung, and Blood Institute/American Heart Association Conference on Scientific Issues Related to Definition. **Circulation**, v. 109, p. 433–438, 2004.

HOTAMISLIGIL, G. S.; SHARGILL, N. S.; SPIEGELMAN, B. M. Adipose expression of tumor necrosis factor-alfa : direct role in obesity-linked insulin resistance. **Science**, v. 259, n. January, p. 87–91, 1993.

IWAMOTO, M. et al. Supplementation of highly concentrated β-cryptoxanthin in a satsuma mandarin beverage improves adipocytokine profiles in obese Japanese women. **Lipids in Health and Disease**, v. 11, n. 52, p. 1–4, jan. 2012.

JUNG, H. K. Inhibitory Effect of Citrus Peel Extract on Lipid Accumulation of 3T3-L1 Adipocytes. **Journal of the Korean Society for Applied Biological Chemistry**, v. 54, n. 2, p. 169–176, 30 abr. 2011.

KANDA, K. et al. Nobiletin suppresses adipocyte differentiation of 3T3-L1 cells by an insulin and IBMX mixture induction. **Biochimica et Biophysica Acta**, v. 1820, p. 461–468, abr. 2012.

KANG, S.-I. et al. Immature Citrus sunki peel extract exhibits antiobesity effects by β-oxidation and lipolysis in high-fat diet-induced obese mice. **Biological & Pharmaceutical Bulletin**, v. 35, n. 2, p. 223–230, jan. 2012.

KELLEY, G. L.; ALLAN, G.; AZHAR, S. High dietary fructose induces a hepatic stress response resulting in cholesterol and lipid dysregulation. **Endocrinology**, v. 145, n. 2, p. 548–55, fev. 2004.

KERSHAW, E. E.; FLIER, J. S. Adipose Tissue as an Endocrine Organ. **The Journal of Clinical Endocrinology & Metabolism**, v. 89, n. 6, p. 2548–2556, 2004.

KIM, G.-S. et al. Citrus aurantium flavonoids inhibit adipogenesis through the Akt signaling pathway in 3T3-L1 cells. **BMC Complementary and Alternative Medicine**, v. 12, n. 31, p. 1–10, jan. 2012.

KIM, H. Y. et al. Hesperetin stimulates cholecystokinin secretion in enteroendocrine STC-1 cells. **Biomolecules & Therapeutics**, v. 21, n. 2, p. 121–125, 2013.

KLIMCZAK, I. et al. Effect of storage on the content of polyphenols, vitamin C and the antioxidant activity of orange juices. **Journal of Food Composition and Analysis**, v. 20, n. 3–4, p. 313–322, maio 2007.

LE LAY, S.; DUGAIL, I. Connecting lipid droplet biology and the metabolic syndrome. **Progress in Lipid Research**, v. 48, p. 191–195, 2009.

LEE, Y.-S. et al. Effects of a Citrus depressa Hayata (shiikuwasa) extract on obesity in high-fat diet-induced obese mice. **Phytomedicine**, v. 18, p. 648–654, 15 jun. 2011.

LEE, Y.-S. et al. Nobiletin improves obesity and insulin resistance in high-fat diet-induced obese mice. **The Journal of Nutritional Biochemistry**, v. 24, p. 156–162, jan. 2013.

LI, R. W. et al. Citrus polymethoxylated flavones improve lipid and glucose homeostasis and modulate adipocytokines in fructose-induced insulin resistant hamsters. **Life Sciences**, v. 79, p. 365–373, 20 jun. 2006.

LONDOÑO-LONDOÑO, J. et al. Clean recovery of antioxidant flavonoids from citrus peel: Optimizing an aqueous ultrasound-assisted extraction method. **Food Chemistry**, v. 119, n. 1, p. 81–87, mar. 2010.

MACHEIX, J.-J.; FLEURIET, A.; BILLOT, J. **Fruit Phenolics**. Florida: CRC Press, 1990.

MADEIRA JR, J. V.; MACEDO, J. A.; MACEDO, G. A. Detoxification of castor bean residues and the simultaneous production of tannase and phytase by solid-state fermentation using Paecilomyces variotii. **Bioresource Technology**, v. 102, p. 7343–7348, 2011.

MANTHEY, J. A.; GROHMANN, K. Phenols in citrus peel byproducts. Concentrations of hydroxycinnamates and polymethoxylated flavones in citrus peel molasses. **Journal of Agricultural and Food Chemistry**, v. 49, p. 3268–3273, jul. 2001.

MILLER, N. J.; RICE-EVANS, C. A. The relative contributions of ascorbic acid and phenolic antioxidants to the total antioxidant activity of orange and apple fruit juices and blackcurrant drink. **Food Chemistry**, v. 60, n. 3, p. 331–337, nov. 1997.

MIYAKE, Y. et al. Difference in plasma metabolite concentration after ingestion of lemon flavonoids and their aglycones in humans. **Journal of Nutritional Science and Vitaminology**, v. 52, p. 54–60, fev. 2006.

MORAND, C. et al. Hesperidin contributes to the vascular protective effects of orange juice : a randomized crossover study in healthy volunteers. **American Journal of Clinical Nutrition**, v. 93, p. 73–80, 2011.

NIELSEN, I. L. F. et al. Bioavailability is improved by enzymatic modification of the citrus flavonoid hesperidin in humans: a randomized, double-blind, crossover trial. **The Journal of Nutrition**, n. October 2005, p. 404–408, 2006.

PERSSON, J. et al. Perilipin and adipophilin expression in lipid loaded macrophages. **Biochemical and Biophysical Research Communications**, v. 363, p. 1020–1026, 30 nov. 2007.

RAASMAJA, A. et al. A water-alcohol extract of Citrus grandis whole fruits has beneficial metabolic effects in the obese Zucker rats fed with high fat/high cholesterol diet. **Food Chemistry**, v. 138, p. 1392–1399, 1 jun. 2013.

RAMALHO, R.; GUIMARÃES, C. Papel do tecido adiposo e dos macrófagos no estado de inflamação crónica associada à obesidade- Implicações Clínicas. **Acta Medica Portuguesa**, v. 21, p. 489–496, 2008.

RAMFUL, D. et al. Bioactive phenolics and antioxidant propensity of flavedo extracts of Mauritian citrus fruits: potential prophylactic ingredients for functional foods application. **Toxicology**, v. 278, n. 1, p. 75–87, 28 nov. 2010.

RAYBOULD, H. E. Mechanisms of CCK signaling from gut to brain. **Current Opinion in Pharmacology**, v. 7, n. 6, p. 570–574, 2009.

SABROE, I. et al. The role of TLR activation in inflammation. **The Journal of Pathology**, p. 126–135, 2008.

SALAMONE, F. et al. Moro orange juice prevents fatty liver in mice. **World Journal of Gastroenterology**, v. 18, n. 29, p. 3862–3868, 7 ago. 2012.

SERGEEV, I. N. et al. Polymethoxyflavones activate Ca2+-dependent apoptotic targets in adipocytes. **Journal of Agricultural and Food Chemistry**, v. 57, p. 5771–5776, 8 jul. 2009.

STUETZ, W. et al. Polymethoxylated flavones, flavanone glycosides, carotenoids, and antioxidants in different cultivation types of tangerines (Citrus reticulata Blanco cv. Sainampueng) from Northern Thailand. **Journal of agricultural and food chemistry**, v. 58, n. 10, p. 6069–74, 26 maio 2010.

SUN, Y. et al. Phytochemical profile and antioxidant activity of physiological drop of citrus fruits. **Journal of Food Science**, v. 78, n. 1, p. C37-42, jan. 2013.

TRIPOLI, E. et al. Citrus flavonoids: Molecular structure, biological activity and nutritional properties: A review. **Food Chemistry**, v. 104, n. 2, p. 466–479, jan. 2007.

TURNER, T.; BURRI, B. J. Potential nutritional benefits of current citrus consumption. **Agriculture**, v. 3, p. 170–187, 2013.

UM, M. Y. et al. Coumarin attenuates hepatic steatosis by down-regulating lipogenic gene expression in mice fed a high-fat diet. **The British Journal of Nutrition**, v. 109, p. 1590–1597, maio 2013.

WANG, M.-J. et al. Pharmacokinetics and conjugation metabolism of naringin and naringenin in rats after single dose and multiple dose administrations. **Journal of Food and Drug Analysis**, v. 14, n. 3, p. 247–253, 2006.

WEISBERG, S. P. et al. Obesity is associated with macrophage accumulation in adipose tissue. **The Journal of Clinical Investigation**, v. 112, n. 12, p. 1796–1808, 2003.

YOSHIDA, H. et al. The citrus flavonoids hesperetin and naringenin block the lipolytic actions of TNF-alpha in mouse adipocytes. **Biochemical and Biophysical Research Communications**, v. 394, p. 728–732, 9 abr. 2010.

YOSHIDA, H. et al. Citrus flavonoid naringenin inhibits TLR2 expression in adipocytes. **The Journal of Nutritional Biochemistry**, v. 24, n. 7, p. 1276–1284, jul. 2013.

CHAPTER 2.BIOTRANSFORMATION EFFECTS ON ANTI LIPOGENIC ACTIVITY OF CITRUS EXTRACTS

(ORIGINAL RESEARCH PAPER PUBLISHED BY FOOD CHEMISTRY)

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Abstract

Citrus peel is a good source of flavonoids, with higher content in relation to pulp. This study proposed to investigate the anti-lipogenic potential of a newly developed citrus flavonoids extract, obtained from citrus industrial residue, bioprocessed in order to generate a commercial source of some flavonoids naturally found in low quantity. The results showed that the citrus peel extract obtained after biotransformation was a good source of hesperitin and naringenin, flavonoids that has no source for production on a large scale, as in supplements or medicines. Still, the results showed that all extracts could be used in obesity treatment. The original extract, "In Natura", would be useful to reduce new adipocytes synthesis and lipid accumulation, and the extract bioprocessed, "Biotransformed" extract could be used to induce lipolysis on fat tissue.

KEYWORDS: citrus peel extract; biotransformation; hesperitin; hesperidin; naringenin; naringin; adipocytes; lipolysis

1. Introduction

Citrus fruits are source of many bioactive compounds, as flavonoids, coumarins, limonoids and carotenoids (TURNER; BURRI, 2013). The main class of citrus flavonoid are the flavanones, but there are also considerable amounts of flavones, flavonols and anthocyanins (BENAVENTE-GARCÍA et al., 1997). The most frequent types of flavonoids found in citrus are hesperidin, naringin, narirutin, eriocitrin, nobiletin and tangeritin (SUN et al., 2013).

The positive effects of citrus flavonoids in obesity treatment (inducing lipolysis and reducing lipid accumulation), and its complications (causing anti-inflammatory response, reducing serum lipids, and improving blood pressure) are demonstrated in several studies in cell culture (KIM et al., 2012; YOSHIDA et al., 2010, 2013), biological assays (ALAM;

KAUTER; BROWN, 2013; UM et al., 2013) and clinical trials (DALLAS et al., 2008, 2013). It is noteworthy that citrus peel has higher content of polyphenols and antioxidant activity in comparison to pulp, indicating that citrus residues are a promising source of bioactive compounds (BARROS; FERREIRA; GENOVESE, 2012).

In most of the studies, citrus peel is obtained from the fruit acquired particularly for the research, and we aimed to evaluate the potential of a citrus residue from industrial waste as a commercial source of bioactives. In this context, Brazil is the world's largest orange producer, according to estimates from the Food and Agriculture Organization (FAO). Of the total produced, it is estimated that 85% is destined for juice industry (CONAB, 2013). In juice production, about 50% of the waste generated is composed of peel and pomace, indicating that there is a rich source of this raw material.

Still, citrus extracts commonly used in researches are rich in hesperidin and naringin, with low amount of aglycones. Studies developed to test the aglycones forms commonly use high cost analytical standards. Thus, a residue extract containing the biotransformed polyphenols on a unique composition with biological potential would be an innovation with commercial interest.

Our research group have been studying alternatives of bioprocesses to increase the production of more bioactive polyphenols from these industrial arrange residues. MADEIRA et al. (2014) observed that the fermentation process of citrus peel resulted in an extract rich in flavanones aglycones, often found in low amounts in the nature. This is an advantage because some evidence have shown that the aglycones form have higher antioxidant capacity (HIRATA et al., 2005; SILVA et al., 2013), and higher bioavailability (LI et al., 2008) in comparison to glycosides. Besides, recent evidences are highlighting the importance of synergism among bioactive compounds in complex matrix with better effect than isolated compounds.

These polyphenols from plant material are commonly extracted with methanol (HAYAT et al., 2010; RAMFUL et al., 2010; SINGH; SOOD, 2011). However this is a toxic solvent (TEPHLY, 1991), being of interest the development of a extraction procedure using a food grade solvent.

Considering these, the study aimed to test a biotransformed citrus peel extract for its antioxidant activity *in vitro*, and the ability to reduce lipogenesis and induce lipolysis in adipocyte cell culture.

2. Materials and methods

2.1. Chemicals

Gallic acid, Folin–Ciocalteu reagent, 2,2′-azobis(2-methylpropionamidine) (97%) (AAPH), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Trolox®, analytical standards hesperidin, hesperitin, naringin and naringenin, insulin, dexamethasone (DEX), 3-isobutyl-1-methylxanthine (IBMX), Oil Red O were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO). Fluorescein was purchased from ECIBRA. All the other chemicals used were in an analytical grade.

2.2. Biotransformed citrus residue

The citrus residue was supplied by CP Kelco Industry Headquarters, from Limeira - SP - Brazil, specialized in pectin production. The residue was dry and contained citrus peel (flavedo and albedo). The material was crushed, and passed through a 10 mesh sieve (Bertel Metallurgical Industries LT). The residue was biotransformed by solid-state fermentation using the microorganism *Paecilomyces variotii* (Brazilian Collection of Environmental and Industrial Microorganisms-CBMAI 1157) according to MADEIRA et al. (2014). Briefly, the fermentation medium was prepared in 250 ml Erlenmeyer flasks containing 10 g of the residue and 10 ml of water. The medium was sterilized by autoclaving for 15 minutes at 121 °C. After cooling, the flasks were inoculated with 1 mL of the microorganism spore suspension (9×10^6 spores/mL) and incubated at 30 °C with 90 % relative humidity (Climate Camera 420 CLD – Nova Ética, SP, Brazil) for 48 hours.

2.3. Preparation of polyphenols extracts from citrus residue

The extraction of phenolic compounds was carried out according to a process adapted from HAYAT et al. (2010). One gram of the biotransformed material was mixed with 25 mL 70% methanol. The solution was treated in ultrasonic bath at 30°C for 15 min, in shaker at 200 rpm for 15 min at room temperature, and then filtered on Whatman paper (No. 1). Different extraction solvents were tested instead of 70% methanol, in order to reduce costs and toxicity of the final extract. The tested extraction solvents were: 70% ethanol (v/v), 70% ethanol (v/v) acidified with 1% HCl (v/v), 50% ethanol (v/v) and water.

After the definition of the extraction solution, the extracts were prepared from the "Biotransformed" residue and two control residues. The first control was the unfermented residue consisting of the product without any processing ("In Natura"), and the second control was the sterilized residue ("Autoclaved"). The sterilized residue was used as a control of process to verify the modifications that occurred in the extract after the sterilization by autoclaving.

After filtration, the product obtained was concentrated on a rotary evaporator at 40 °C to remove the organic solvent. Then the aqueous solution was frozen and freeze-dried.

2.4. Extracts characterization

2.4.1. Total phenolic content

Total phenolic contents of the extracts were measured using the Folin–Ciocalteu assay according to SINGLETON; ORTHOFER; LAMUELA-RAVENTÓS (1999). Gallic acid was used as a standard and a calibration curve was plotted in a concentration range of 25–200 µg/mL. All analyses were performed in triplicate and results were expressed as mg of gallic acid equivalents (GAE) / mL of extract or mg of lyophilized extract (LE).

2.4.2. Determination of main flavanone compounds by High Performance Liquid Chromatography (HPLC)

A DionexUltiMate 3000 (Germany) liquid chromatography, equipped with a C-18 Acclaim® 120 column (Dionex, 3µm, 4.6×150 mm) maintained at 30 °C by a thermostat, was used. The detection was carried out using a UV/VIS (DAD-3000). The method was adapted from CARIDI et al. (2007), and DE MEJÍA et al. (2010). The solvents were: A (water/formic acid, 99.9:0.1 v/v) and B (methanol/formic acid, 99.9:0.1 v/v), with a flow rate of 0.6 mL/min. The spectra absorption were obtained at 190 and 480 nm, and the chromatograms were processed at 280 nm. The standard flavanones detected and quantified were naringin, naringenin, hesperidin and hesperitin.

2.4.3. DPPH radical-scavenging activity

The potential antioxidant activity of the extracts was assessed based on the scavenging activity of the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical, as

described by MACEDO et al. (2011). The reaction mixtures, consisting of 50 µl of test samples and 150 µl of 0.2 mM DPPH in methanol, were carried out on a NovoStarMicroplate reader (BMG LABTECH, Germany) with absorbance filters for a wavelength of 520 nm. The decolorizing process was recorded after 90 min of reaction. The DPPH solution and reaction medium were freshly prepared and stored in the dark. The measurement was performed in triplicate. The antioxidant activity was calculated from the equation obtained by the linear regression after plotting known concentration solutions of Trolox®. Antiradical activity was expressed as µmol of Trolox® equivalent/mg of extracts.

2.4.4. ORAC

The ORAC (Oxygen Radical Absorbance Capacity) assay was performed using fluorescein (FL) as the fluorescent probe, as described by DÁVALOS; GÓMEZ-CORDOVÉS; BARTOLOMÉ (2004), and adapted by FERREIRA et al. (2013). Briefly, 20 µL aliquots of the sample, Trolox® solution or buffer (blank) were distributed in black-walled 96-well plate, followed by the addition of 120 µL fluorescein sodium salt solution 0.38 µg/mL (Ecibra, São Paulo, Brazil) diluted in sodium phosphate buffer 75 mM (pH 7.4). The reaction was initiated by addition of 60 µL AAPH solution (Sigma-Aldrich, Steinheim, Germany) at a concentration of 108 mg/mL dissolved in sodium phosphate buffer 75 mM (pH 7.4). The fluorescence was monitored every 56 seconds during 75 min using a Novo Star Microplate Reader (BMG LABTECH, Germany) at 37 ° C with excitation filter 485 nm and emission filter 520 nm. The measurements were performed in triplicate. ORAC values were defined as the difference between the area under the FL decay curve of the samples and the blank (net AUC). Regression equations between net AUC and antioxidant concentration were calculated for all of the samples and Trolox® (control). ORAC-FL values were expressed as µmol of Trolox®/mg of extracts.

2.5. Cell culture assay

2.5.1. Cell culture

3T3-L1 murine pre-adipocytes were cultured in Dulbecco's modified Eagle's medium (DMEM) at 37°C in a humidified atmosphere with 5% CO^2. All media contained 10% fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (100 µg/ml).

2.5.2. MTT Assay

The 3T3-L1 cells (1.0 x 105 cells/mL) were seeded in 96-well plates and incubated for 24 h at 37°C in a humidified atmosphere with 5% CO^2. Then the cells were treated with the samples (0.01 mg/mL – 1.00 mg/mL). After 24 h of incubation, all media was removed and 10 µL of MTT solution (5 mg/mL) was added to the cell culture. The cells were further incubated at 37°C in a humidified atmosphere with 5% CO^2 for 4 h. The MTT formazan crystals were dissolved in SDS 10% in HCl 0.01M for 18 hours. The optical density of formazan solution was measured with a microplate reader at 540 nm. The results are expressed as a % of control cells, that are cells without any sample treatment

2.5.3. Pre-adipocytes differentiation

The 3T3-L1 cells (2.0 x 104 cells/mL) were seeded in 24-well plates and grown until confluence. Two days after confluence, designated as day 0, the cells were switched to differentiation medium containing 10µg/mL insulin, 0.5 mM isobutylmethylxanthine (IBMX), and 1µM dexamethasone (DEX) in DMEM for another 3 days. Then, the cell culture medium was replaced with maturation medium containing 10µg/mL insulin in DMEM. The maturation medium was changed every 2 days, until day 12, after which mature adipocytes containing lipid droplets were formed.

2.5.4. Oil Red O staining

The cells were submitted to two different treatments. First, cells were exposed to the extracts sample (0.05 mg/mL, 0.20 mg/mL, 0.50 mg/mL, 1.00 mg/mL) in the differentiation medium followed by maturation medium without the samples. Using this treatment it is possible to see if the extracts could impair the pre-adipocytes differentiation.

The second treatment consisted in the addition of the extracts only in the maturation medium. This procedure intended to verify if the extracts could reduce triglyceride accumulation in mature adipocytes.

In both cases, on day 12, the 3T3-L1 mature adipocytes plated onto 24-well plates were washed once with formaldehyde 10% in PBS, and fixed with formaldehyde 10% in PBS for 60 min. After replacement of formaldehyde 10% in PBS with 60% isopropanol, the cells were stained for 30 min in freshly diluted Oil red O (Sigma) solution (2.1 mg/ml) with 60%

isopropanol. Thereafter, the cells were washed four times with water and the wells were dried at room temperature. Subsequently, the Oil Red O in the stained cells was eluted with 100% isopropanol. The absorbance was measured with a microplate reader at 492 nm. Each treatment was performed in triplicate. The results are expressed as a percentage of control cells, that are fully differentiated cells without any sample treatment, according to the equation bellow:

% of Oil Red O staining = (Abs Sample/Abs Control) x 100

2.5.5. Glycerol assay

On day 12 of the maturation sequence, cells were treated with the samples for 18 hours, and the supernatant was collected. The amount of glycerol in the medium was determined using a Glycerol Assay Kit (Cayman, CO, U.S.A.) in accordance with the manufacturer's instructions.

2.6. Statistical analysis

Results were expressed as means ± standard deviation (SD). The statistical difference between the groups was analyzed using analysis of variance (ANOVA). Post hoc comparison was performed by Tukey's test. Differences were considered significant when $p \leq 0.05$. All analyses were performed using the software GraphPad Prism 5 for Windows version 5.00 (GraphPad Software Inc.).

3. Results

3.1. Extraction Solution Selection

The total phenolic content of the extracts obtained by using different extraction solvents varied from 72.29 ± 4.83 to 90.45 ± 5.44 mg of Gallic Acid Equivalent/ml of extract for water and ethanol 70% HCl 1%, respectively. There was no statistical difference between the samples, not being possible to use this parameter to determine the best extraction solution (data not shown).

The HPLC analysis showed that the extraction with 70% methanol, 70% ethanol acidified with 1% HCl and 50% ethanol resulted in higher content of the quantified flavanones (Figure 2.1). Due to the lack of difference in methanol or ethanol as extraction solvent, it is justified the use of solutions with ethanol, since it is a food grade solvent. Still, aiming the lower

solvent use, and considering the statistical similarity between the results, we selected the solution of 50% ethanol for extraction of flavanones from the "Biotransformed" residue.

Despite the widespread use of Folin Ciocalteau assay, this was not a good method for screening the best extracting solution. There was no significant difference between the samples according to this analysis, even though the HPLC results clearly indicating smaller potential of water as an extracting solvent for this system.

3.2. Characterization of "Biotransformed" residue extract obtained from the 50% ethanol solution

There was no difference in the content of total polyphenols by Folin Ciocalteau assay between the "Biotransformed" residue and the controls (Table 2.1).

However, once again the HPLC analysis showed difference between the samples. The "In Natura" residue had higher content of glycosides flavanones, naringin and hesperidin, while the "Biotransformed" residue had higher level of the aglycone flavanones, naringenin and hesperitin (Figure 2.2). These results demonstrate that the fermentation process caused the biotransformation of the flavanones, increasing the amount of flavonoids free form.

The results in Figure 2.2 indicate that the sterilization process by autoclaving degraded a certain amount of flavanones. However, this is a necessary step in the fermentation process to eliminate any microbial contamination present in the residue and allow only the reaction by the inoculated one.

Despite the fact that the "Biotransformed" residue extract presented a much smaller amount of total flavanones than the controls (Figure 2.2), the antioxidant capacity of the extracts was similar according to DPPH and ORAC assays (Table 2.1), indicating that the flavanones presented in the "Biotransformed" residue had higher antioxidant potential.

Table 2.1 Total polyphenols, ORAC and DPPH radical-scavenging activity of the extracts.

	Total Polyphenol (mg GAE/mg of LE)	DPPH (μmol equivalent Trolox/mg of LE)	ORAC (μmol equivalent Trolox/mg of LE)
Biotransformed	35.04 ± 2.36^{a}	136.77 ± 5.41^{a}	542.93 ± 78.04^{a}
In Natura	36.23 ± 3.01^{a}	130.80 ± 11.17^{a}	666.99 ± 110.54^{a}
Autoclaved	33.31 ± 1.03^{a}	129.17 ± 6.71^{a}	658.38 ± 70.87^{a}

Different letters in the column indicate significant differences by Tukey's test ($p \leq 0,05$).

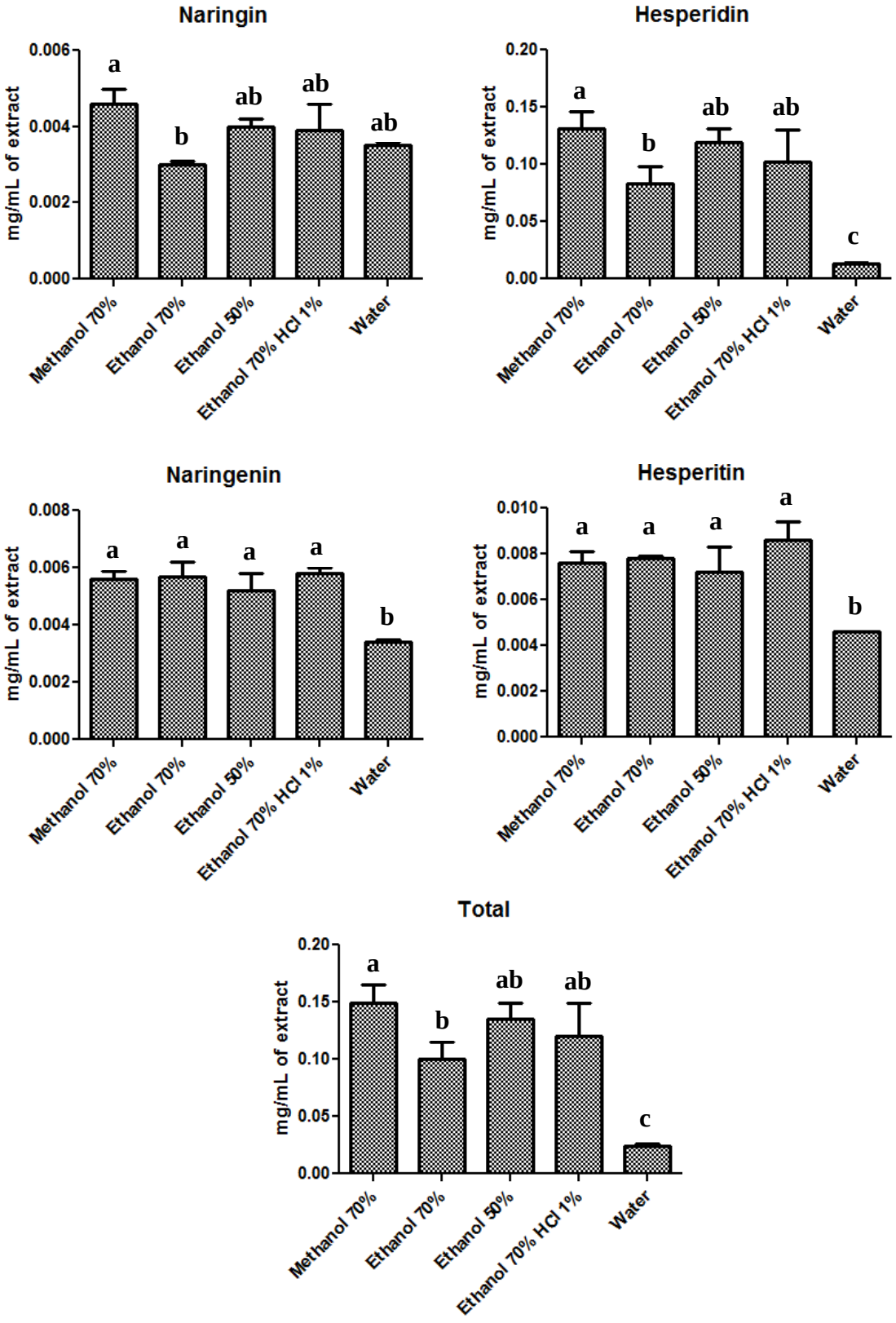

Figure 2.1 Flavonoids of interest quantified by High Performance Liquid Chromatography (mg/mL of extract). Different letters indicate significant differences by Tukey's test ($p \leq 0.05$).

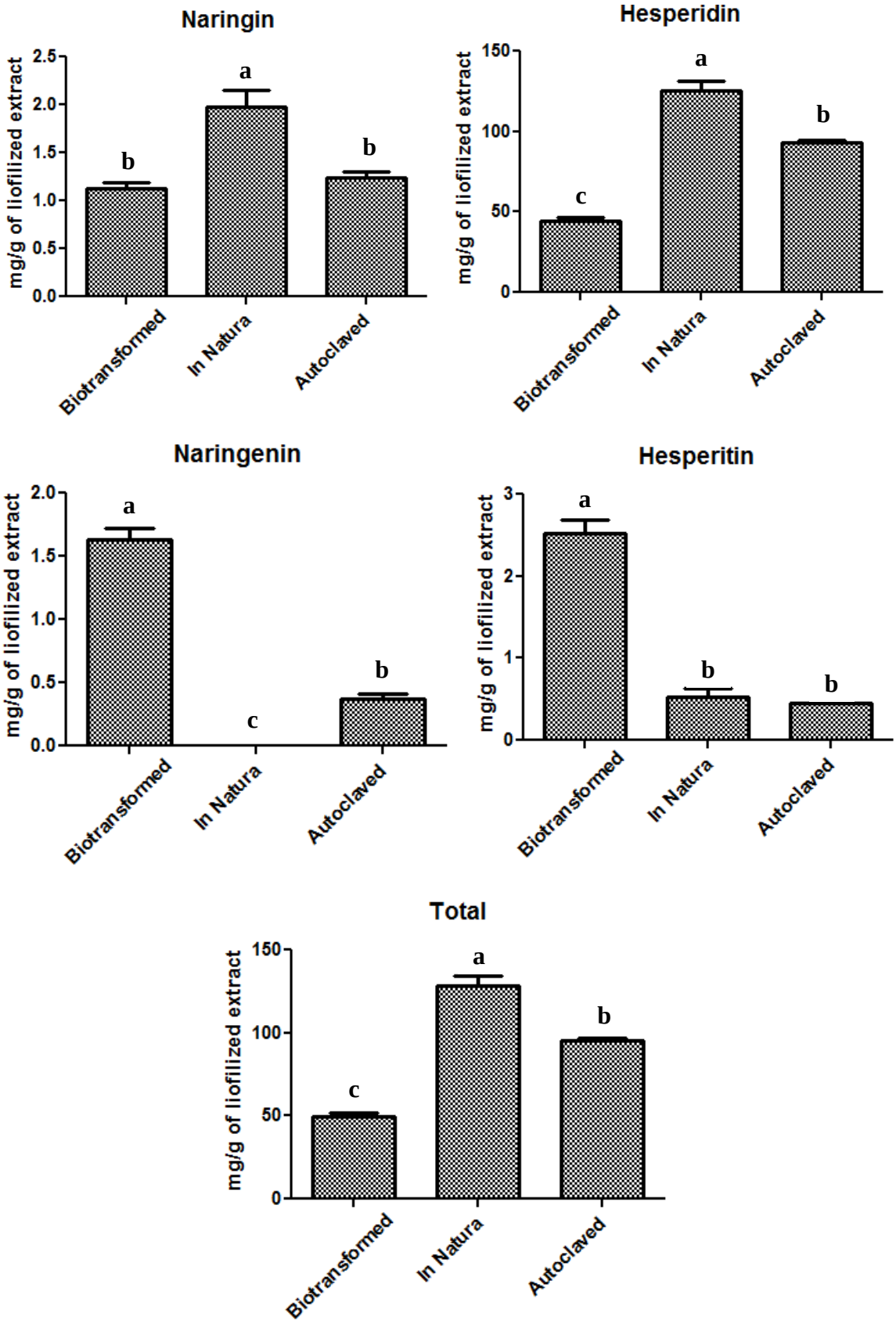

Figure 2.2 Flavonoids of interest quantified by High Performance Liquid Chromatography (mg/g of liofilized extract). Different letters indicate significant differences by Tukey's test ($p \leq 0.05$).

3.3.Cell Assays

According to MTT assay, none of the extracts were toxic to 3T3-L1 cell line in the concentrations tested (Figure 2.3). Since no loss of cell viability was observed in the concentration range that the cells were exposed, it is considered safe to continue with the following cellular assays within the concentrations tested.

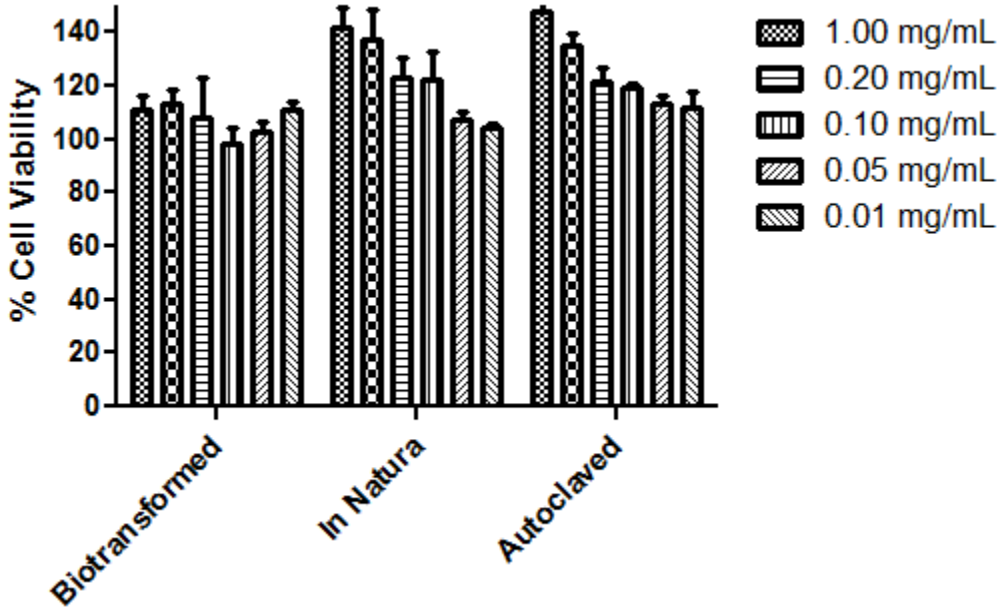

Figure 2.3 Cell viability of 3T3-L1 cell line according to MTT assay.

The analysis Oil Red O staining showed that when the samples were added in the differentiation medium, there was little effect in the total lipid accumulation (Table 2.2). The best result was observed with the addition of “In Natura” extract in the concentration of 0.5 mg/mL, presenting a lipid accumulation reduction of about 19% in relation to control cells with no treatment.

Table 2.2 Oil Red O staining with samples added in the Differentiation Medium, % in relation to control.

Samples	Concentration			
	0.05 mg/mL	0.20 mg/mL	0.50 mg/mL	1.00 mg/mL
Biotransformed	101.52±10.00[a]	100.00±4.50[a]	103.21±4.55[b]	107.22±4.03[b]
In Natura	95.59±1.52[a]	93.98±3.94[a]	80.96±4.19[a]	90.46±4.61[a]
Autoclaved	103.45±3.71[a]	99.17±3.26[a]	96.05±2.79[b]	98.39±2.22[a]

Different letters in the comlun indicate significant difference between the samples in the same concentration by Tukey's test ($p \leq 0,05$).

On the other hand, the addition of the extracts in the maturation medium caused a decrease in the lipid accumulation (Table 2.3). This reduction was dose-dependent for all the samples, reaching a reduction of 22%, 38% and 48% for "Biotransformed", "Autoclaved" and "In Natura" extracts, respectively.

Table 2.3 Oil Red O staining with samples added in the Maintenance Medium, % in relation to control.

Samples	Concentration			
	0.05 mg/mL	0.20 mg/mL	0.50 mg/mL	1.00 mg/mL
Biotransformed	97.56±1.15[b]	84.67±5.37[b]	89.45±7.70[c]	78.18±6.50[b]
In Natura	86.91±3.55[a]	65.67±4.30[a]	55.96±3.50[a]	52.89±2.04[a]
Autoclaved	103.59±1.11[c]	88.86±2.14[b]	75.65±4.12[b]	62.66±2.93[a]

Different letters in the column indicate significant difference between the samples in the same concentration by Tukey's test ($p \leq 0,05$).

The total glycerol concentration was below the limit of detection for the cells treated with "In Natura" and "Autoclaved" extracts. Thus, the "Biotransformed" residue extract was the only that presented some effect on the amount of glycerol released, and the values observed presented dose dependent behavior (2.39 ± 0.17 - 5.24 ± 0.29 mg/mL of glycerol)

4. Discussion

The Folin Ciocalteu assay was not useful to distinguish the samples. It is noteworthy that the color reaction the Folin Ciocalteau assay is based on not only occurs due

to the presence of polyphenols, but can also be caused by other compounds with reducing power (HUANG; OU; PRIOR, 2005).

The total amount of flavanones was statistically similar when using methanol or ethanol as extraction solvents. Solutions containing methanol are the most used in the extraction of polyphenols of solid materials (HAYAT et al., 2010; RAMFUL et al., 2010; SINGH; SOOD; MUTHURAMAN, 2011). However, due to its toxicity (LIESIVUORI; SAVOLAINEN, 1991; TEPHLY, 1991), it is interesting the development of a process using more friendly solvents. The results presented indicate the potential of 50% ethanol solution in extraction of the flavanones naringin, hesperidin, naringenin and hesperitin from fermented citrus residue. Thereby, this process used a solvent less harmful to health, increasing the feasibility of the extract in studies with cell culture, animal models and humans. Still, using ethanol instead of methanol takes into account economic considerations imposed by the industrial context.

The HPLC analysis clearly showed the change in flavanones profile in "Biotransformed" extract. According to MADEIRA et al. (2014) the microorganism employed in this process probably uses naringin and hesperidin as source of carbon and energy during fermentation. In previous study of our group, it was observed that the tannase produced by *Paecilomyces variotii* strain during fermentation has the ability to catalyze the flavanones deglycosylation, such as hesperidin (FERREIRA et al., 2013). Also, in microbial fermentation the compounds like hesperitin and naringenin are transformed in other chemical particles, with lower molecular weight, that are used in microorganism metabolism (JUSTESEN et al., 2000). AGUILAR; AGUILERA-CARBO; ROBLEDO (2008) observed that the solid-state fermentation of creosote bush leaves and pomegranate peels transformed the tannins present in the substrate into lower molecular weight phenolics, confirming the fact that the fermentation process is able to change the phenolic profile of a product.

Even using a citrus residue from pectin industry waste, the quantity of flavanones extracted was comparable to other studies. YU et al. (2014) and HO; SU; LIN (2013) performed the extraction of flavonoids from fruits acquired for the research, taking care in the acquisition, transport and storage of raw materials. The first authors were able to extract 1.278 mg/g FW (fresh weight) of naringin and 1.480mg/g FW of hesperidin, indicating values comparable to the present work for hesperidin. In the second study, the extraction was performed in nine different citrus fruits, and for five of them the amount of hesperidin and naringin was lower

than that obtained in the present study. This data reinforces the advantage of the produced extract, once it was obtained from low cost industrial waste residue, commonly used for animal feed, presenting a possibility to increase the commercial value of this product.

Despite the lower content of total flavanones in "Biotransformed" extract, antioxidant activity by DPPH and ORAC was similar between the samples. The literature indicates that in some cases hesperitin and naringenin have higher biological potential than hesperidin and naringin. LONDOÑO-LONDOÑO et al. (2010) demonstrated that hesperitin was more active than hesperidin in reducing lipid peroxidation in hepatic microsomes, with lower amount of TBARS. Moreover, these authors observed that at low concentrations (10 mg/mL) hesperitin was more effective in reducing oxidized LDL by peroxynitrite-oxidized LDL model. SILVA et al. (2013) biotransformed orange and lime juices by enzymatic de-glycosylation and observed higher antioxidant activity by DPPH method and FRAP assay after the biotransformation, indicating the higher antioxidant activity of the aglycones obtained.

According to the results, all extracts were able to reduce lipogenesis *in vitro*, however, the data found seem to indicate that each extract have a different mechanism of action. Only the "In Natura" extract showed some effect on the inhibition of pre-adipocytes differentiation (Table 2.2), and may have a promising application in preventing the formation of new mature adipocytes. KIM et al. (2012) also observed that the addition of *Citrus aurantium* Flavonoids extract inhibited 3T3-L1 differentiation with a reduction in the amount of lipid droplets, confirming this positive effect of flavonoids from citrus.

When the extracts were added to the maturation medium, all samples were able to reduce lipid accumulation, with a greater effect of "In Natura" extract. However, only "Biotransformed" extract, with higher content of hesperitin and naringenin, caused induction of lipolysis, observed by higher amount of free glycerol on the supernatant of the culture. It is noteworthy that "Biotransformed" extract had lower amount of flavanones per gram of liofilized extract in comparison to "In Natura" extract (Figure 2), and despite this great difference, the "Biotransformed" extract was able to reduce lipids content in the cells as "In Natura" extracts, and it was the only one able to cause lipolysis.

SUBASH-BABU; ALSHATWI (2014) studied the effects of 20 µM hesperitin analytical standard in immortalized human bone marrow mesenchymal stem-cell (TERT20) differentiated with dexamethasone, IBMX, indomethacin and insulin. Hesperitin was added in

two different situations: in group 1 the flavanone was administered in the differentiation medium; in group 2 the compound was added after the differentiation in the maintenance medium. In both cases, there was a reduction on lipid accumulation according to staining with Oil Red O, even though the effect was more pronounced in group 2, similar to our results. They also observed a slight stimulation of lipolysis, confirming the lipolytic activity of hesperitin.

Some studies have been shown that hesperitin (GAMO et al., 2014) and naringenin (YOSHIDA et al., 2013) seems to act as PPAR γ agonist. PPARγ is a nuclear transcription factor that induce adipocyte differentiation (KUBOTA et al., 1999), causing greater differentiation of preadipocytes to mature adipocytes when it is activated. This could explain why the "Biotransformed" extract were not able to reduce the differentiation process. Some researchers have found that when these flavonoids were added to 3T3-L1 cell culture, it was observed a greater accumulation of lipid droplets possibly due to its agonist role (GAMO et al., 2014; MORIKAWA et al., 2008; YOSHIDA et al., 2013). However, in our study, despite the possible action of hesperitin and naringenin as PPAR γ agonist, there was an expressive reduction in lipid accumulation, which can be explained by the lipolytic role played by these aglycones. Still, we must consider that the afore mentioned studies were done with analytical standards, evaluating each compound alone. In the present work, we used a crude extract, and differences in response can occur due to the synergistic effect that compounds together may cause.

There are many studies indicating the potential of citrus extract in obesity treatment (KANG et al., 2012; KIM et al., 2012), however for the first time it is documented promising results with an extract rich in aglycones. The studies using aglycones usually test high cost analytical standards isolated, missing the synergistic effects of the natural extracts we propose.

Still, some authors indicate other advantages of aglycone forms in obesity treatment. KIM et al. (2013) found that hesperitin caused higher secretion of cholecystokinin (CCK) in STC-1 cells in comparison to hesperidin, indicating a possible role of this aglycone flavanone in food intake control since CCK is an anorexigen hormone (RAYBOULD, 2009). Thus, these other information suggests the potential of the extract produced by biotransformation to other biological activities, being suitable for further studies.

5. Conclusions

Ethanol can replace methanol as extraction solvent of flavanones from biotransformed citrus residue. The biotransformation was able to modify the flavanones profile of the citrus residue extract, increasing the content of hesperitin and naringenin that naturally occur in low quantities in citrus fruits. Still, all extracts could be used in obesity treatment, however aiming different targets. The “In Natura” extract would be useful to reduce new adipocytes synthesis and lipid accumulation, and “Biotransformed” extract could be used to induce lipolysis on fat tissue.

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6. References

AGUILAR, C. N.; AGUILERA-CARBO, A.; ROBLEDO, A. Production of Antioxidant Nutraceuticals by Solid-State Cultures of Pomegranate (Punica granatum) Peel and Creosote Bush (Larrea tridentata) Leaves. **Food Technology and Biotechnology**, v. 46, n. 2, p. 218–222, 2008.

ALAM, M. A.; KAUTER, K.; BROWN, L. Naringin improves diet-induced cardiovascular dysfunction and obesity in high carbohydrate, high fat diet-fed rats. **Nutrients**, v. 5, n. 3, p. 637–50, mar. 2013.

BARROS, H. R. D. M.; FERREIRA, T. A. P. D. C.; GENOVESE, M. I. Antioxidant capacity and mineral content of pulp and peel from commercial cultivars of citrus from Brazil. **Food Chemistry**, v. 134, n. 4, p. 1892–8, 15 out. 2012.

BENAVENTE-GARCÍA, O. et al. Uses and properties of Citrus flavonoids. **Journal of Agricultural and Food Chemistry**, v. 45, n. 12, p. 4505–4515, 1997.

CARIDI, D. et al. Profiling and quantifying quercetin glucosides in onion (Allium cepa L.) varieties using capillary zone electrophoresis and high performance liquid chromatography. **Food Chemistry**, v. 105, n. 2, p. 691–699, 2007.

DALLAS, C. et al. Lipolytic effect of a polyphenolic citrus dry extract of red orange, grapefruit, orange (SINETROL) in human body fat adipocytes. Mechanism of action by inhibition of cAMP-phosphodiesterase (PDE). **Phytomedicine**, v. 15, p. 783–792, out. 2008.

DALLAS, C. et al. Clinical study to assess the efficacy and safety of a citrus polyphenolic extract of red orange, grapefruit, and orange (Sinetrol-XPur) on weight management and metabolic parameters in healthy overweight individuals. **Phytotherapy Research**, p. 1–7, 3 abr. 2013.

DÁVALOS, A.; GÓMEZ-CORDOVÉS, C.; BARTOLOMÉ, B. Extending applicability of the oxygen radical absorbance capacity (ORAC-fluorescein) assay. **Journal of Agricultural and Food Chemistry**, v. 52, n. 1, p. 48–54, 14 jan. 2004.

DE MEJÍA, E. G. et al. Yerba mate tea (Ilex paraguariensis): Phenolics, antioxidant capacity and *in vitro* inhibition of colon cancer cell proliferation. **Journal of Functional Foods**, v. 2, n. 1, p. 23–34, jan. 2010.

FERREIRA, L. R. et al. Improving the chemopreventive potential of orange juice by enzymatic biotransformation. **Food Research International**, v. 51, n. 2, p. 526–535, maio 2013.

GAMO, K. et al. Mechanism of Peroxisome Proliferator-Activated Receptor Gamma (PPAR γ) Transactivation by Hesperetin Glucuronides Is Distinct from That by a Thiazolidine-2 , 4-dione Agent. **Chemical and Pharmaceutical Bulletin**, v. 62, n. May, p. 491–493, 2014.

HAYAT, K. et al. Effect of microwave treatment on phenolic content and antioxidant activity of citrus mandarin pomace. **Food Chemistry**, v. 123, n. 2, p. 423–429, nov. 2010.

HIRATA, A. et al. Kinetics of radical-scavenging activity of hesperetin and hesperidin and their inhibitory activity on COX-2 expression. **Anticancer Research**, v. 25, n. 5, p. 3367–3374, 2005.

HO, S.-C.; SU, M.-S.; LIN, C.-C. Comparison of Peroxynitrite-Scavenging Capacities of Several Citrus Fruit Peels. **International Journal of Food Properties**, v. 17, n. 1, p. 111–124, 2013.

HUANG, D.; OU, B.; PRIOR, R. L. The chemistry behind antioxidant capacity assays. **Journal of Agricultural and Food Chemistry**, v. 53, n. 6, p. 1841–56, 23 mar. 2005.

JUSTESEN, U. et al. Degradation of Flavonoid Glycosides and Aglycones During *in vitro* Fermentation with Human Faecal Flora. **LWT - Food Science and Technology**, v. 33, n. 6, p. 424–430, 2000.

KANG, S.-I. et al. Immature Citrus sunki peel extract exhibits antiobesity effects by β-oxidation and lipolysis in high-fat diet-induced obese mice. **Biological & Pharmaceutical Bulletin**, v. 35, n. 2, p. 223–230, jan. 2012.

KIM, G.-S. et al. Citrus aurantium flavonoids inhibit adipogenesis through the Akt signaling pathway in 3T3-L1 cells. **BMC Complementary and Alternative Medicine**, v. 12, n. 31, p. 1–10, jan. 2012.

KIM, H. Y. et al. Hesperetin stimulates cholecystokinin secretion in enteroendocrine STC-1 cells. **Biomolecules & Therapeutics**, v. 21, n. 2, p. 121–125, 2013.

KUBOTA, N. et al. PPARγ mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance. **Molecular Cell**, v. 4, n. 4, p. 597–609, 1999.

LI, Y. et al. In vivo pharmacokinetics of hesperidin are affected by treatment with glucosidade-like Bg1A protein isolated from yeasts. **Journal of Agriculture and Food Chemistry**, v. 56, n. 14, p. 5550–5557, 2008.

LIESIVUORI, J.; SAVOLAINEN, H. Methanol and formic acid toxicity: biochemical mechanisms. **Pharmacology & Toxicology**, v. 69, n. 3, p. 157–63, set. 1991.

LONDOÑO-LONDOÑO, J. et al. Clean recovery of antioxidant flavonoids from citrus peel: Optimizing an aqueous ultrasound-assisted extraction method. **Food Chemistry**, v. 119, n. 1, p. 81–87, mar. 2010.

MACEDO, J. A. et al. Author ' s personal copy Increasing the antioxidant power of tea extracts by biotransformation of polyphenols. **Food Chemistry**, v. 126, p. 491–497, 2011.

MADEIRA, J. V. et al. Rich bioactive phenolic extract production by microbial biotransformation of Brazilian Citrus residues. **Chemical Engineering Research and Design**, v. 92, n. 10, p. 1802–1810, 2014.

MORIKAWA, K. et al. Naringenin and hesperetin induce growth arrest, apoptosis, and cytoplasmic fat deposit in human preadipocytes. **Journal of Agricultural and Food Chemistry**, v. 56, n. 22, p. 11030–11037, 2008.

RAMFUL, D. et al. Bioactive phenolics and antioxidant propensity of flavedo extracts of Mauritian citrus fruits: potential prophylactic ingredients for functional foods application. **Toxicology**, v. 278, n. 1, p. 75–87, 28 nov. 2010.

RAYBOULD, H. E. Mechanisms of CCK signaling from gut to brain. **Current Opinion in Pharmacology**, v. 7, n. 6, p. 570–574, 2009.

SILVA, C. M. G. et al. Enhancement of the antioxidant activity of orange and lime juices by flavonoid enzymatic de-glycosylation. **Food Research International**, v. 52, n. 1, p. 308–314, jun. 2013.

SINGH, J.; SOOD, S.; MUTHURAMAN, A. In-vitro evaluation of bioactive compounds, anti-oxidant, lipid peroxidation and lipoxygenase inhibitory potential of Citrus karna L. peel extract. **Journal of Food Science and Technology**, 17 ago. 2011.

SINGLETON, V. L.; ORTHOFER, R.; LAMUELA-RAVENTÓS, R. M. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. In: PACKER, L. (Ed.). . **Oxidants and Antioxidants Part A**. Methods in Enzymology. [s.l.] Academic Press, 1999. v. 299, p. 152–178.

SUBASH-BABU, P.; ALSHATWI, A. A. Hesperitin inhibit adipocyte differentiation and enhance Bax- and p21-mediated adipolysis in human mesenchymal stem cell adipogenesis. **Journal of Biochemical Molecular Toxicology**, v. 0, p. 1–10, 2014.

SUN, Y. et al. Phytochemical profile and antioxidant activity of physiological drop of citrus fruits. **Journal of Food Science**, v. 78, n. 1, p. C37-42, jan. 2013.

TEPHLY, T. R. The toxicity of methanol. **Life Sciences**, v. 48, n. 6, p. 1031–1041, 1991.

TURNER, T.; BURRI, B. J. Potential nutritional benefits of current citrus consumption. **Agriculture**, v. 3, p. 170–187, 2013.

UM, M. Y. et al. Coumarin attenuates hepatic steatosis by down-regulating lipogenic gene expression in mice fed a high-fat diet. **The British Journal of Nutrition**, v. 109, p. 1590–1597, maio 2013.

YOSHIDA, H. et al. The citrus flavonoids hesperetin and naringenin block the lipolytic actions of TNF-alpha in mouse adipocytes. **Biochemical and Biophysical Research Communications**, v. 394, p. 728–732, 9 abr. 2010.

YOSHIDA, H. et al. Citrus flavonoid naringenin inhibits TLR2 expression in adipocytes. **The Journal of Nutritional Biochemistry**, v. 24, n. 7, p. 1276–1284, jul. 2013.

YU, E. A. et al. Flavonoid profile and biological activity of Korean citrus varieties (II): Pyunkyul (Citrus tangerina Hort. ex Tanaka) and overall contribution of its flavonoids to antioxidant effect. **Journal of Functional Foods**, v. 6, n. 1, p. 637–642, 2014.

CHAPTER 3. BIOTRANSFORMED CITRUS EXTRACT AS A SOURCE OF ANTI-INFLAMMATORY POLYPHENOLS: EFFECTS IN MACROPHAGES AND ADIPOCYTES

(ORIGINAL RESEARCH PAPER SUBMITTED TO JOURNAL OF NUTRITIONAL BIOCHEMISTRY)

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Abstract

In obesity, due to increased macrophage infiltration on adipose tissue, there is greater secretion of pro-inflammatory cytokines, contributing to development of chronic non-communicable diseases. We evaluated the anti-inflammatory potential of a unique phenolic extract, obtained from bioprocessed citrus residue, on a cellular model with RAW264.7 and 3T3-L1 cells. Stimulated RAW 264.7 cells treated with the extract presented lower secretion of TNF-α and NO and lower protein expression of NFκB. In RAW264.7 and 3T3-L1co-culture, treatment with 1.0mg/mL of the extract reduced secretion of TNF-α (30.7%) and IL-6 (43.4%). Still, the "Biotransformed" extract caused greater increase in adiponectin in relation to control extracts. When the co-culture received LPS stimulus, the sample at 0.2mg/mL reduced IL-6 and TNF-α concentration; and caused greater increase in adiponectin. The citrus extract evaluated in this study showed anti-inflammatory activity in macrophages and in co-culture, indicating that bioprocess of citrus residue can contribute to new product development with anti-inflammatory potential.

Keywords: citrus residue extract, polyphenols, obesity, anti-inflammatory activity, cell culture

1. Introduction

Obesity is a disease characterized by excess body fat, associated with a chronic subclinical inflammatory condition caused by increased secretion of pro-inflammatory adipokines (BALISTRERI; CARUSO; CANDORE, 2010). This adipokines circulation

increase also appears to be responsible for the development of chronic non-communicable diseases associated with obesity, causing insulin resistance, blood pressure increase, change in serum lipids, higher inflammatory response and thrombus formation (GRUNDY et al., 2004). The phenolic compounds are a class of substances that has been investigated for use in prevention and treatment of these diseases. An interesting source of phenolic compounds is citrus fruits.

Among the most commercially important citrus, there is orange, with Brazil being the largest producer in the world, reaching a production of 18,012,560 megatons in 2012, according to estimates of the Food and Agriculture Organization (FAO). However, it is important to observe that most of oranges are destined to juice production, and about 50% of the waste generated is composed of peel and pulp, commonly used as animal feed component. However, it is known that citrus peel has a high content of polyphenols, and several studies have shown the positive effects of peel extracts in the treatment of chronic non-communicable diseases (DING et al., 2012; KANG et al., 2012; KIM et al., 2012; RAASMAJA et al., 2013). Still, some studies have demonstrated the anti-inflammatory role of citrus flavonoids, mainly hesperetin (GIMÉNEZ-BASTIDA et al., 2016; MA; FENG; DING, 2015; REN et al., 2016) and naringenin (KARUPPAGOUNDER et al., 2016; YU et al., 2014). However, these studies often test analytical standards of high cost.

Researches with citrus extracts commonly use samples rich in hesperidin and naringin, with low amount of aglycones. However, some evidence have shown that the aglycones form have higher antioxidant capacity (HIRATA et al., 2005; SILVA et al., 2013), and higher bioavailability (LI et al., 2008) in comparison to glycosides. Thus, a residue extract containing glycosides and aglycones polyphenols on a unique composition with biological potential would be an innovation with commercial interest.

Biotransformation by fermentation process is one way to produce an extract with higher content of aglycones. Our research group have been studying biotransformation processes to increase the production of more bioactive polyphenols from these industrial arrange residues (FERREIRA et al., 2013; MADEIRA et al., 2014; MADEIRA JR; SPERANZA; MACEDO, 2012; NAKAJIMA et al., 2016). Among the extracts already developed, we observed that the biotransformation produced an extract with lipolitic activity *in vitro*, whereas the unprocessed control extract showed no activity (NAKAJIMA et al., 2016).

In the present work, we intended to determine whether the fermentative biotransformation of this residue could result an extract with improved biological profile. Thus, this study aimed to evaluate the anti-inflammatory potential on obesity scenarios of different citrus residue extracts in RAW 264.7 macrophages and in co-culture of RAW 264.7 macrophages and 3T3-L1 adipocytes.

2. Materials and methods

2.1. Chemicals

Insulin, dexamethasone (DEX), 3-isobutyl-1-methylxanthine (IBMX), Lipopolysaccharide (LPS) were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO). All the other chemicals used were in an analytical grade.

2.2. Biotransformed citrus residue

The citrus residue was supplied by CP Kelco Industry Headquarters, from Limeira - SP - Brazil, specialized in pectin production. The residue was dry and contained citrus peel (flavedo and albedo). The material was crushed, and passed through a 10 mesh sieve (Bertel Metallurgical Industries LT). The residue was biotransformed by solid-state fermentation using the microorganism *Paecilomyces variotii* (Brazilian Collection of Environmental and Industrial Microorganisms-CBMAI 1157) according to MADEIRA et al. (2014). Briefly, the fermentation medium was prepared in 250 ml Erlenmeyer flasks containing 10 g of the residue and 10 ml of water. The medium was sterilized by autoclaving for 15 minutes at 121 °C. After cooling, the flasks were inoculated with 1 mL of the microorganism spore suspension (9×10^6 spores/mL) and incubated at 30 °C with 90 % relative humidity (Climate Camera 420 CLD – Nova Ética, SP, Brazil) for 48 hours.

2.3. Preparation of polyphenols extracts from citrus residue

The extraction of phenolic compounds was carried out according to a process adapted from HAYAT et al. (2010). One gram of the biotransformed material was mixed with 25 mL of 50% ethanol. The solution was treated in ultrasonic bath at 30°C for 15 min, in shaker at 200 rpm for 15 min, and then filtered on Whatman paper (No. 1).

The extracts were prepared from the “Biotransformed” residue and two control residues. The first control was the unfermented residue consisting of the product without any processing (“In Natura”), and the second control was the sterilized residue (“Autoclaved”). The sterilized residue was used as a control of process to verify the modifications that occurred in the extract after the sterilization by autoclaving.

After filtration, the product obtained was concentrated on a rotary evaporator at 40 °C to remove the organic solvent. Then the aqueous solution was frozen and freeze-dried.

These extracts were previously analyzed and the polyphenols profile already published (NAKAJIMA et al., 2016). In summary, the “Biotransformed” extract had higher content of the aglycones hesperetin and naringenin, the “In Natura” presented higher amount of the glycosides hesperidin and naringin, while the “Autoclaved” consisted in a mixture of the four mentioned flavonoids, however with lower concentrations of all.

2.4. Cell culture assay

2.4.1. Cell culture

RAW 264.7 murine macrophages and 3T3-L1 murine pre-adipocytes were cultured in Dulbecco’s modified Eagle’s medium (DMEM) at 37°C in a humidified atmosphere with 5% CO^2. All media contained 10% fetal bovine serum (FBS), penicillin (100 units/mL) and streptomycin (100 µg/mL).

2.4.2. MTT Assay

RAW 264.7 cells (1.0 x 10^5 cells/mL) were seeded in 96-well plates and incubated for 24 h at 37°C in a humidified atmosphere with 5% CO2. Then the cells were treated with the samples (0.01 mg/mL – 1.00 mg/mL). After 24 h of incubation, all media was removed and 10 µL of MTT solution (5 mg/mL) was added to the cell culture. The cells were further incubated at 37°C in a humidified atmosphere with 5% CO^2 for 3 h. The MTT formazan crystals were dissolved in SDS 10% in HCl 0.01M for 18 hours. The optical density of formazan solution was measured with a microplate reader at 540 nm. The results are expressed as a % of control cells, that are cells without any sample treatment.

The same protocol was used for 3T3-L1 cells. However, after addition of MTT solution, the cells were further incubated for 4 h.

2.4.3. RAW 264.7 inflammatory assay

RAW 264.7 macrophages were grown in 24-well plates (1×10^5 cells/well) for 24 h. Extracts (0.2 mg/mL and 1.0mg/mL) plus LPS (100 ng/ml) was added to the treatment group of plates, while medium or LPS alone was added to the control group of plates. After LPS-stimulation for 24 h, the cell-free supernatants were collected and assayed for TNF-αlevels using the enzyme-linked immunosorbent assay (ELISA) kit Mouse TNF (Mono/Mono) ELISA Set (BD OptEIA™), in accordance with the manufacturer's instructions. The optical density of each well was read at 450 nm.

The cell free supernatant was also used to measure nitric oxide concentration. The nitric oxide (NO) determination was carried out according to GREEN et al. (1982). Nitrite concentration was measured as an indicator of NO production using Griess reagent (1% sulfanilamide, 5% phosphoric acid, 0.1% N - (1-naphthyl) ethylenediamine. The absorbance was measured at 540 nm, and the nitrite concentration in the samples was determined by comparison to a standard curve of sodium nitrite (5-320 mM).

For Western Blotting, RAW 264.7 macrophages were grown in 6-well plates (1×10^5 cells/well) for 24 h. Extracts (0.2 mg/mL and 1.0mg/mL) plus LPS (100 ng/ml) were added to the treatment group of plates, while medium or LPS alone was added to the control group of plates. After LPS-stimulation for 18 h, the cells were washed with ice-cold PBS and then lysed with 150µl cell lysis buffer [100 mM Tris–HCl, 10 mM EDTA, 1% Triton X-100, 10mM sodium pyrophosphate, 100 mM sodium fluorete, 10 mM sodium orthovanadate, 2 mM PMSF, 0.1 mg/ml aprotinin] per well of a six-well plate for 40 minutes. The lysate was centrifuged at 12,000 rpm for 15 min at 4 °C, and the protein quality of each sample was determined using Lowry protein assay. Samples of 20 µg protein were electrophoresed by SDS–PAGE and transferredto nitrocellulose membrane. After blocking the non specific site with blocking solution (5% BSA for HSP70 and 5% non-fat milk for NFκB) for 2 h, the membrane was incubated with antibodies (1:1000) against HPS70 and NF-κB at 4 °C overnight and then incubated with secondary antibodies (1:2000) at room temperature for 2 h. Protein detection was visualized with the Amersham ECL Western Blotting Detection Reagent (GE Amersham Biosciences) and UVITEC Cambridge instrument (model Alliance LD2). Each band was

quantitatively determined using the ImageJ software and β-actin (ADI905733 - Enzo Life Science) was used as the loading control. The experiments were performed in triplicate.

2.4.4. RAW 264.7 and 3T3-L1 co-culture assay

2.4.4.1. Pre-adipocytes differentiation

The 3T3-L1 cells (2.0 x 10^4 cells/mL) were seeded in 24-well plates and grown until confluence. Two days after confluence, designated as day 0, the cells were switched to differentiation medium containing 10 µg/mL insulin, 0.5 mM isobutylmethylxanthine (IBMX), and 1µM dexamethasone (DEX) in DMEM with 10%FBS for another 3 days. Then, the cell culture medium was replaced with maturation medium containing 10 µg/mL insulin in DMEM with 10%FBS. The maturation medium was changed every 2 days, until day 12, after which mature adipocytes containing lipid droplets were formed.

2.4.4.2. Co-culture

On day 12 of the maturation sequence, transwell inserts (0.45 µm - Millipore, Ireland) were placed in each well and RAW 264.7 (1 x 10^5 cells/well) were inoculated in the upper layer. After 24 hours, cells were treated with extracts (0.2 mg/mL and 1.0mg/mL) or extracts (0.2 mg/mL and 1.0mg/mL) plus LPS (100 ng/mL) for 24 hours, and the supernatant was collected. The amount of adiponectin, IL-6 and TNF-α in the medium was determined using a Milliplex® MAP Mouse Adipocyte Luminex assay in accordance with the manufacturer's instructions.

The cell free supernatant was also used to measure nitric oxide concentration, according to the protocol described above.

2.5. Statistical analysis

Results were expressed as means ± standard deviation (SD). The statistical difference between the groups was analyzed using analysis of variance (ANOVA). Post hoc comparison was performed by Dunnet's and Tukey's test. Differences were considered significant when $p \leq 0.05$. All analyses were performed using the software GraphPad Prism 5 for Windows version 5.00 (GraphPad Software, Inc.).

3. Results

3.1. Inflammatory assay with RAW 264.7 stimulated by LPS

According to MTT assay, none of the extracts were toxic to RAW 264.7 cells in concentrations varying from 0.01 to 1.0 mg/mLl (Figure3.1). Thus, the following tests were carried out using 0.2 and 1.0 mg/mL concentrations.

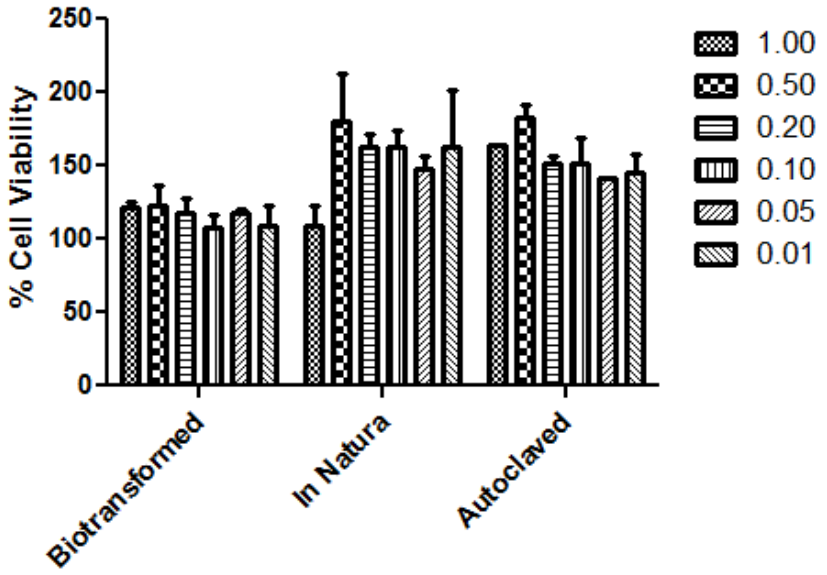

Figure 3.1 Cell viability of RAW264.7 cell line according to MTT assay.

The addition of LPS in RAW 264.7 cells caused an increase in TNF-α and NO concentration, indicating the occurrence of the inflammatory stimulus. The macrophages treatment with the extracts caused a reduction in TNF-α in comparison to control group (cells without any treatment), being significant only with the addition of 1.0 mg/mL. There was no significant difference between samples (Figure 3.2).

The “Biotransformed” extract was the only one able to reduce the amount of NO in the lowest concentration tested, being significantly lower than the Positive Control. The addition of 1.0 mg/mL of extracts significantly reduced the NO concentration in comparison to Positive Control; however the best results were for “Biotransformed” and “Autoclaved” extracts that were significantly lower than “In Natura” (Figure 3.2).

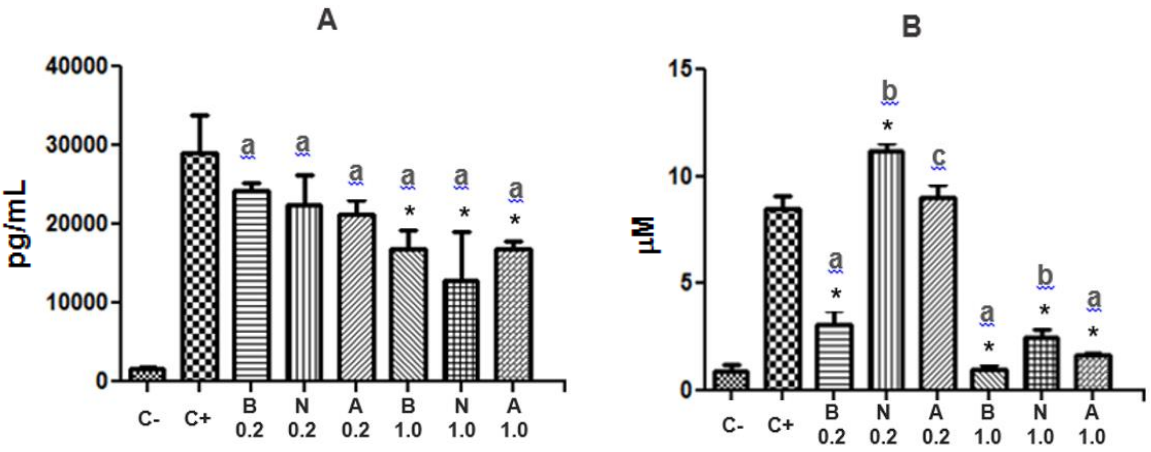

Figure 3.2 A: TNF-α (pg/mL) and B: NO (µM) concentrations in RAW 264.7 supernatant after 24 hours of treatment. C-: Cells without LPS stimulus or sample, C+: Cells with LPS stimulus without sample, B: Biotransformed, N: In Natura, A: Autoclaved. 0.2 indicates 0.2 mg/mL of extract and 1.0 indicates 1.0 mg/mL of extract. All cells with samples treatment received LPS stimulus. Asterisk indicates the statistical significant difference compared to Positive Control by Dunnett's test ($p \leq 0.05$). Different small letters indicate significant difference between the samples in the same concentration by Tukey's test ($p \leq 0.05$).

All extracts were able to reduce HSP70 protein expression in the higher concentration tested, however there was only statistical difference for "In Natura" extract (Figure 3.3A). Despite the lack of statistical significance, cell treatment with 1.0 mg/mL of extacts presented lower NFκB protein expression in comparison to Control, with greater reduction with "In Natura" extract (Figure 3.3B). There was a positive significant correlation between TNF-α and protein expression of HSP70 and NFκB (Figure 3.3C and 3.3D).

These results indicate that the samples were able to attenuate some parameters of inflammation induced by LPS addition. At the highest concentration tested, "In Natura" extract showed better results.

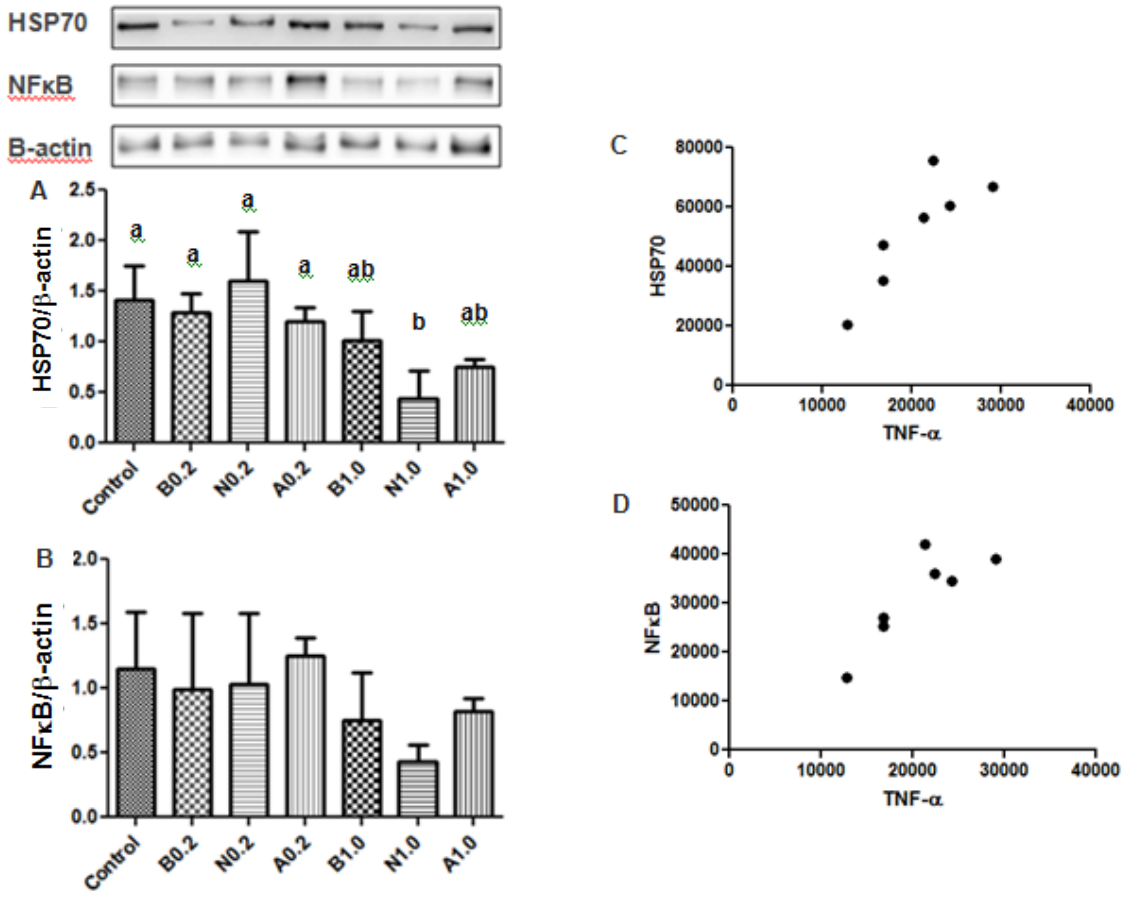

Figure 3.3 Extracts effect on protein expression of A: HSP70 and B: NFκB evaluated by Western Blot. Control: Cells with LPS stimulus without sample, B: Biotransformed, N: In Natura, A: Autoclaved. 0.2 indicates 0.2 mg/mL of extract and 1.0 indicates 1.0 mg/mL of extract. All cells with sample treatment received LPS stimulus. Different small letters indicate significant difference between the samples in the same concentration by Tukey's test ($p \leq 0.05$). Pearson Correlation between TNF-α and C: HSP (r 0.8526, p 0.0148) and D: NFκB (r 0.8503, p 0.0153).

3.2. Inflammatory assay in 3T3-L1 and RAW 264.7 co-culture

3.2.1. Inflammation induced by 3T3-L1 and RAW 264.7 co-culture

As for RAW 264.7 cell line, according to MTT assay, none of the extracts were toxic to 3T3-L1 cells in concentrations varying from 0.01 to 1.0 mg/ml (data not shown). Thus, the following tests were carried out using 0.2 and 1.0 mg/ml concentrations.

The increased concentration of IL-6 and NO in co-culture compared to isolated culture of 3T3-L1 and RAW-264.7, indicates that an inflammatory stimulus occurred when the

two cells shared the same environment. Moreover, the co-culture caused a reduction in adiponectin concentration compared to 3T3-L1 grown alone (Table 3.1).

Table 3.1 Adipokines and NO concentration in isolated 3T3-L1 cell culture and in co-culture.

	IL-6 (pg/mL)	TNF-α (pg/mL)	NO (µM)	Adiponectin (pg/mL)
3T3-L1	271.2±25.48	BLD	1.80±0.55	2610.79±771.15
RAW 264.7	13.41±0	2886.67±1716.53	0.88±0.18	BLD
Co-culture	2190.54±1061.05	529.29±56.48	56.67±11.30	1391.95±434.80

BLD- Bellow limit of detection

Cell treatment with all extracts increased the amount of adiponectin compared to Control, being significant when the cell were treated with "Autoclaved" extract (Table 3.2).

Although the "Autoclaved" extract caused a greater increase in adiponectin concentration, it had no effect on IL-6 and even caused a slight increase in NO, in the two concentrations tested, not showing improvement in inflammatory status. On the other side, the addition of 1.0 mg/mL of "Biotransformed" and "In Natura" extracts reduced the concentration of IL-6 and TNF-α. Despite the lack of statistical significance, it is noteworthy that "Biotransformed" and "In Natura" reduced IL-6 concentration in relation to control by 43.4% and 42.7%, respectively. Evaluating TNF-α secretion, the addition of "Biotransformed" extract caused a reduction of 30.7%, and "In Natura" a reduction of 14.9%. These extracts also caused a slight reduction in NO concentration (Table 3.2).

Since only "Biotransformed" and "In Natura" extracts at a concentration of 1.0 mg/ml were able to reduce the secretion of pro-inflammatory cytokines, and at this concentration "Biotransformed" extract caused greater increase in adiponectin secretion, we can conclude that "Biotransformed" extract showed better anti-inflammatory activity in adipose tissue stimulated by the presence of macrophages.

Table 3.2 Cytokines and NO concentration in 3T3-L1 and RAW 264.7 co-culture without LPS stimulus.

	Control	Conc mg/mL	Biotransformed	In Natura	Autoclaved
Adiponectin (pg/mL)	1391.95±434.80	0.2	1937.69±312.13^{a}	2246.59±318.04^{ab}	2926.31±421.84*^{b}
		1.0	2311.23±554.79^{a}	1883.43±139.61^{a}	2691.94±606.68*^{a}
IL-6 (pg/mL)	2190.54±1061.05	0.2	2174.09±721.92^{a}	2172.84±180.38^{a}	2237.48±284.59^{a}
		1.0	1239.58±728.43^{a}	1255.74±189.67^{a}	2187.16±807.58^{a}
TNF-α (pg/mL)	529.29±56.48	0.2	488.57±49.60^{a}	767.87±136.78^{b*}	680.64±113.93^{ab}
		1.0	366.73±44.77^{a}	450.65±95.61^{a}	452.60±118.43^{a}
NO (µM)	56.67 ± 11.30	0.2	56.93 ± 15.71 ^{a}	58.47 ± 8.54 ^{a}	61.56 ± 5.10 ^{a}
		1.0	48.53 ± 5.32 ^{a}	48.78 ± 9.57 ^{a}	62.46 ± 3.48 ^{a}

Asterisk indicates the statistical significant difference compared to control by Dunnett's test ($p \leq 0.05$).Different small letters indicate significant difference between the samples in the same concentration by Tukey's test ($p \leq 0.05$).

As expected, there was a positive correlation between NO and the proinflammatory cytokines IL-6 and TNF-α (Figure 3.4).

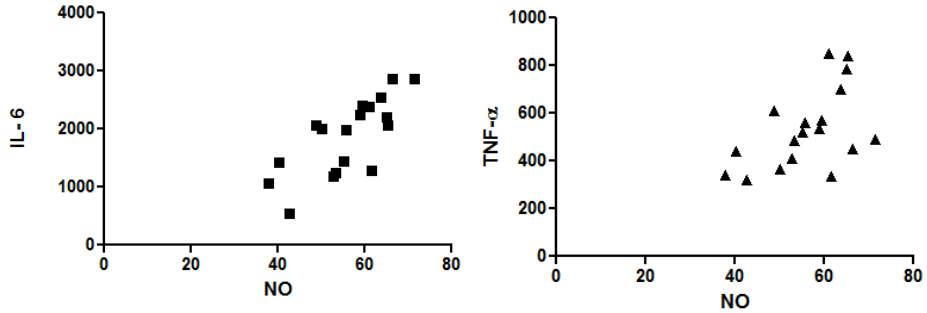

Figure 3.4 Pearson Correlation between NO and IL-6 (r 0.7527, p 0.0003) and TNF-α (r 0.5327, p 0.0228).

3.2.2. Inflammation induced by LPS stimulus in 3T3-L1 e RAW 264.7 co-culture

The results for this co-culture assay, in which cells suffered LPS inflammatory stimulus, all samples were capable of increasing adiponectin concentration, however only

"Autoclaved" extract had a significant difference compared to Control at lower concentration evaluated (Table 3.3).

At 0.2 mg/mL concentration, "Biotransformed" and "In Natura" extracts reduced IL-6 and TNF-α secretion compared to Control. Still, compared to "Autoclaved" extract, "Biotransformed" showed significant lower secretion of IL-6 and "In Natura" lower secretion of TNF-α. Between these two extracts, "Biotransformed" presented better anti-inflammatory results because also promoted increased secretion of adiponectin and lower of NO (Table 3.3).

At the concentration of 1.0mg/mL the autoclaved extract was the most positive because it promoted greater reduction of IL-6, TNF-alpha and NO; also causing a significant increase in adiponectin concentration when compared to Control (Table 3.3).

In this assay, there was a positive significant correlation between NO and IL-6 (Figure 3.5).

Table 3.3 Cytokines and NO concentration in 3T3-L1 and RAW 264.7 co-culture LPS stimulated.

	Control	Conc mg/mL	Biotransformed	In Natura	Autoclaved
Adiponectin	1577.87±610.07	0.2	2568.09±959.92^{a}	1899.24±150.24 ^{a}	2899.52±291.63*^{a}
		1.0	1895.89±362.28 ^{a}	2548.96±506.07 ^{a}	1912.84±525.65 ^{a}
IL-6	2221.71±856.35	0.2	1343.50±231.54^{a}	1535.98±246.89^{ab}	2206.21±421.02^{b}
		1.0	1154.57±241.20*^{a}	1801.72±273.34^{b}	818.09±235.78*^{a}
TNF-α	737.86±74.02	0.2	624.88±88.30^{ab}	613.91±30.37^{a}	777.11±60.12^{b}
		1.0	608.44±73.35 ^{a}	656.79±78.73^{a}	519.85±67.54*^{a}
NO	58.70 ± 9.46	0.2	47.64 ± 9.23 ^{a}	65.25 ± 9.21 ^{a}	55.16 ± 10.57 ^{a}
		1.0	55.44 ± 2.66^{a}	75.45 ± 12.95^{b}	47.55 ± 3.78^{a}

Asterisk indicates the statistical significant difference compared to control by Dunnett's test ($p \leq 0.05$).Different small letters indicate significant difference between the samples in the same concentration by Tukey's test ($p \leq 0.05$).

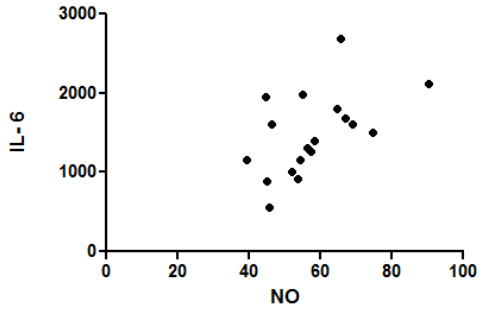

Figure 3.5 Pearson Correlation between NO and IL-6 (r 0.5258, p 0.025).

4. Discussion

4.1. Inflammatory assay with RAW 264.7 stimulated by LPS

The inflammatory stimulus of RAW264.7 with LPS causes increase in TNF-α concentration (LICHTMAN; WANG; LEMASTERS, 1998; WULSTER-RADCLIFFE et al., 2004). This cytokine acts in host defense, however it is also known for its pro-inflammatory activity (BEUTLER; CERAMI, 1986; CARSWELL et al., 1975). In some situations, such as sepsis, secretion of this cytokine is increased, causing an inflammatory state damaging to the organism, which can lead to death (KOTHARI et al., 2013; TRACEY; CERAMI, 1994). Thus, substances that help control this secretion could contribute to a better prognosis in these situations. In our study, we also observed an increase in TNF-α secretion after LPS stimulus. However all extracts in the higher concentration tested were able to reduce significantly its concentration compared to Control.

Treatment of RAW264.7 cells with the extracts at a concentration of 1.0 mg/mL resulted in inhibition of NFκB protein expression. According to WULSTER-RADCLIFFE et al. (2004) the increased secretion of TNF-α after LPS stimulus in pig macrophages is caused by ERK1/2 and NFκB pathway activation. NFκB is the most known pathway of inflammation, and when activated, it translocates to the nucleus, stimulating the synthesis of pro-inflammatory cytokines such as IL-6, TNF-α e IL-1 (GILMORE, 2006). Thus, inhibition of this pathway by the extracts can result in inflammatory profile improvement.

Intracellular HSP70 has anti-apoptotic and cytoprotective activity (BEERE, 2004), whereas the extracellular has immunomodulatory action being an endogenous TLR ligand, that activates the Toll/IL-1 receptor signal pathway (VABULAS et al., 2002). Thus, increased expression of HSP 70, with subsequent secretion of the cell may have pro-inflammatory effect.

GUPTA et al. (2013) studied RAW 264.7 cells and observed that the presence of LPS with fever increases the expression of HSP70 and its secretion in the extracellular medium, being a risk factor for inflammation. Hence, in presence of LPS, as in the experiment performed in the present study, the reduction in HSP70 expression caused by the presence of the extracts in highest concentration tested, can have beneficial effects on inflammatory process.

Besides the citokines, nitric oxide (NO) is also implicated in cell inflammatory response. NO is a free radical and internal messenger that participates in vascular homeostasis and host defense. It is synthesized by nitric-oxide synthase (NOS). There are three isoforms of NOS (MARLETTA, 1993), but for macrophage bactericidal and tumoricidal activities the inducible NOS (iNOS) is the most important one (STUEHR; NATHAN, 1989). The excessive iNOS activity is associated with inflammation tissue damage (GUZIK; KORBUT; ADAMEK-GUZIK, 2003). LPS is a potent stimulator of NO production in macrophages (WU et al., 2003), behavior observed in the present study (Figure 3.2B). The extracts were able to reduce the secretion of this compound, especially the "Biotransformed" extract that promoted the greatest reduction in the concentration of NO in the two concentrations tested, and in the highest concentration its value was close to the cells without inflammatory stimulus. The reduced production of NO may be beneficial to protect cell and tissue against injury induced by inflammation acting on oxidative stress induced by NO, or indirectly causing reduced levels of anti-inflammatory cytokines such as TNF-α (WU et al., 2003).

4.2. Inflammatory assay in 3T3-L1 and RAW 264.7 co-culture

In obesity, besides the increasing number and size of adipocytes, there is a macrophage infiltration, causing increased cytokine secretion, contributing to the subclinical inflammatory state present in this disease. Among the cytokines that contribute to this pro-inflammatory condition, there are TNF-α and IL-6, that are associated with insulin resistance found in that situation (WEISBERG et al., 2003). Thus, compounds capable of regulating cytokines secretion after an inflammatory stimulus could be effective in treating certain conditions associated with excess weight.

3T3-L1 and RAW264.7 co-culture is widely used to mimic this situation. The results presented in Table 1 show that cultivation of these two cells in the same environment caused changes in cytokine secretion profile, being a good model for studying the inflammatory

changes present in obesity. In co-culture, the inflammation was possibly initiated by TNF-α secreted from RAW264.7. ARAKI et al. (2006) stimulated 3T3-L1 with TNF-α and observed an increase in NFκB activity and in IL-6 secretion, and reduction in adiponectin concentration; confirming that the presence of TNF-α is capable of causing an inflammatory stimulus in adipocytes.

Despite the evidence of inflammatory stimulus in co-culture, TNF-α secretion was lower in this assay than in isolated RAW 264.7 culture. Adiponectin from 3T3-L1 may have inhibited macrophage secretion. Adiponectin is an adipokine secreted by adipocytes that presents protective role, with anti-inflammatory activity and capacity of improving insulin sensitivity (AJUWON; SPURLOCK, 2005; ZOICO et al., 2009). WULSTER-RADCLIFFE et al. (2004) observed that pre-treatment of pig macrophages with adiponectin inhibited the activation of NFκB. Thus, reduction of TNF-α and IL-6 would be caused by adiponectin via inhibition of NFκB nuclear translocation, and ERK1/2 activity. YOKOTA et al. (2000) also showed that adiponectin inhibits TNF-α secretion by macrophages stimulated with LPS. Moreover, YEN et al. (2011) demonstrated that treatment of 3T3-L1 with TNF-α causes a decrease in adiponectin secretion. Thus, possibly in co-culture, adiponectin reduced TNF-αsecretion, and TNF-α also influenced adiponectin secretion (Table 3.1). The LPS stimulation was only 24 hours after the macrophages were inoculated in the well with 3T3-L1, then maybe adiponectin from adipocytes had been a pre-treatment for macrophages, which may not have been converted in the most inflammatory state. If this occurred, the increased secretion of IL-6 and NO was mainly from 3T3-L1, and not from RAW264.7; and perhaps LPS acted mainly stimulating 3T3-L1.

It is noteworthy that the inflammatory stimulus with or without LPS in co-culture did not cause major differences in cytokines concentrations between the Control groups (Table 3.2 and Table 3.3). However, the different responses observed when the samples were added to groups stimulated or not with LPS, indicates that there was possibly a different mechanism of action in the pro-inflammatory model. Possibly, in the co-culture, TNF-α secreted by RAW 264.7 was the original stimulator of the inflammatory response; and in the co-culture with LPS, both LPS and TNF-α acted in the stimulus.

3T3-L1 treatment with LPS stimulates ERK phosphorylation and mRNA expression of IL-6, TNF-α and MCP-1 (PARK; MUN, 2014). Moreover, the presence of LPS

stimulates higher protein expression of TLR4, MyD88 and TRAF6 (induces phosphorylation of IκB), resulting in increased activation of NFκB (PARK; MUN, 2014). TNF-α has its action mediated by TNF receptor (TNF-r). Despite this difference, it has been shown that both stimulate NFκB activation. However, TNF-α has rapid action with IKK activation between 10 and 15 minutes, NFκB activation with a peak at 45 minutes and returning to pre-stimulus baseline levels after 90 minutes; while with LPS IKK activity is greater at 45 minutes, NFκB activation peak is at 105 minutes, still maintaining high activation at 120 minutes (WERNER, 2005). These results indicate that, despite the two substances action in NFκB, the pathways to occur this activation are distinct.

The results indicate that the samples at 0.2 mg/mL concentration were not effective in inhibiting the inflammation induced by TNF-α. On the other side, cell treatment at 1.0 mg/mL concentration with "Biotransformed" and "In Natura" extracts, inhibited inflammation induced by TNF-α (Table 3.2). "Biotransformed" extract also caused greater increase in adiponectin secretion. In the inflammation induced by LPS, cell treatment with "Biotransformed" and "In Natura" extracts in the lower concentration were able to reduce pro-inflammatory cytokines secretion, especially again "Biotransformed" extract because it caused increased secretion of adiponectin. In the higher concentration tested, "Autoclaved" extract showed the best results reducing all inflammatory cytokines (Table 3.3).

YEN et al. (2011) evaluated the effect of 21 phenolic analytical standards including hesperidin, naringin and naringenin, in IL-6 secretion by 3T3-L1 stimulated with TNF-α. To achieve this purpose, the cells were pretreated for 24 hours with analytical standards and after that they were stimulated with 5 ng/ml TNF-α for 12 hours. The authors reported that naringenin was able to reduce 27% IL-6 secretion after stimulation with TNF-α, while reduction observed for naringin and hesperidin were 30.4% 32.6% respectively; indicating the anti-inflammatory role of these compounds. However, it is important to note that in this study, using extracts with phenolics combination, the effects on IL-6 secretion were more pronounced, achieving reduction of 43.4% when "Biotransformed" extract was added to co-culture without LPS stimulation and 48.0% in the co-culture with LPS stimulation. These results indicate positive synergistic effect when using an extract with phenolic mixture.

Some researchers have shown that hesperitin (GAMO et al., 2014b) and naringenin (YOSHIDA et al., 2013) seems to act as PPAR γ agonist. PPARγ is a nuclear transcription

factor that participates in the suppression of NFκB activation and IL-6 production, and can be induced by adiponectin (AJUWON; SPURLOCK, 2005). So, the presence of the extracts with hesperitin and naringenin ("Biotransformed") and the induction of adiponectin secretion by the extract could contribute to the anti-inflammatory activity via PPAR γand NFκB.

Despite the fact that the extracts did not influenced nitrite concentration in co-culture, there was a positive correlation between this compound and pro-inflammatory cytokines. This was expected because NO activates JNK and NFκB pathways, resulting in increased production of cytokines (NOZAKI et al., 2007).

The citrus extracts used in this study have potential anti-inflammatory activity since they were able to reduce TNF-α secretion and nitric oxide in macrophages culture; in addition to inhibiting TNF-α, IL-6, nitric oxide secretion and increasing adiponectin concentration in co-cultures of adipocytes and macrophages. It is noteworthy that "Biotransformed" and "Autoclaved" extracts showed some advantages in certain circumstances compared to "In Natura", indicating that bioprocess of citrus residue can contribute to the development of a new product with anti-inflammatory potential.

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5. References

AJUWON, K. M.; SPURLOCK, M. E. Adiponectin inhibits LPS-induced NF-kappaB activation and IL-6 production and increases PPARgamma2 expression in adipocytes. **American Journal of Physiology, Regulatory, Integrative and Comparative Physiology**, v. 288, n. 5, p. R1220–R1225, 2005.

ARAKI, S. et al. N-acetylcysteine attenuates TNF-?? induced changes in secretion of interleukin-6, plasminogen activator inhibitor-1 and adiponectin from 3T3-L1 adipocytes. **Life Sciences**, v. 79, n. 25, p. 2405–2412, 2006.

BALISTRERI, C. R.; CARUSO, C.; CANDORE, G. The role of adipose tissue and adipokines in obesity-related inflammatory diseases. **Mediators of Inflammation**, v. 2010, p. 1–19, jan. 2010.

BEERE, H. M. "The stress of dying": the role of heat shock proteins in the regulation of apoptosis. **Journal of Cell Science**, v. 117, n. Pt 13, p. 2641–2651, 2004.

BEUTLER, B.; CERAMI, A. Cachectin and tumor necrosis factor as two sides of the same biological coin. **Nature**, v. 320, p. 584–588, 1986.

CARSWELL, E. A. et al. An endotoxin-induced serum factor that causes necrosis of tumors. **Proceedings of the National Academy of Sciences of the United States of America**, v. 72, n. 9, p. 3666–70, 1975.

DING, X. et al. Citrus ichangensis peel extract exhibits anti-metabolic disorder effects by the inhibition of PPARγ and LXR signaling in high-fat diet-induced C57BL/6 mouse. **Evidence-Based Complementary and Alternative Medicine**, v. 2012, p. 1–10, jan. 2012.

FERREIRA, L. R. et al. Improving the chemopreventive potential of orange juice by enzymatic biotransformation. **Food Research International**, v. 51, n. 2, p. 526–535, maio 2013.

GAMO, K. et al. Mechanism of Peroxisome Proliferator-Activated Receptor Gamma (PPAR γ) Transactivation by Hesperetin Glucuronides Is Distinct from That by a Thiazolidine-2 , 4-dione Agent. **Chemical and Pharmaceutical Bulletin**, v. 62, n. May, p. 491–493, 2014.

GILMORE, T. D. Introduction to NF-kappaB: players, pathways, perspectives. **Oncogene**, v. 25, n. 51, p. 6680–4, 30 out. 2006.

GIMÉNEZ-BASTIDA, J. A. et al. Hesperetin and its sulfate and glucuronide metabolites inhibit TNF-α induced human aortic endothelial cell migration and decrease plasminogen activator inhibitor-1 (PAI-1) levels. **Food Funct.**, v. 7, n. 1, p. 118–126, 2016.

GREEN, L. C. et al. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. **Analytical Biochemistry**, v. 126, n. 1, p. 131–138, 1982.

GRUNDY, S. M. et al. Definition of Metabolic Syndrome: Report of the National Heart, Lung, and Blood Institute/American Heart Association Conference on Scientific Issues Related to Definition. **Circulation**, v. 109, p. 433–438, 2004.

GUPTA, A. et al. Toll-like receptor agonists and febrile range hyperthermia synergize to induce heat shock protein 70 expression and extracellular release. **Journal of Biological Chemistry**, v. 288, n. 4, p. 2756–2766, 2013.

GUZIK, T. J.; KORBUT, R.; ADAMEK-GUZIK, T. Nitric oxide and superoxide in inflammation and immune regulation. **Journal of Physiology and Pharmacology : an official journal of the Polish Physiological Society**, v. 54, n. 4, p. 469–487, 2003.

HAYAT, K. et al. Effect of microwave treatment on phenolic content and antioxidant activity of citrus mandarin pomace. **Food Chemistry**, v. 123, n. 2, p. 423–429, nov. 2010.

HIRATA, A. et al. Kinetics of radical-scavenging activity of hesperetin and hesperidin and their inhibitory activity on COX-2 expression. **Anticancer Research**, v. 25, n. 5, p. 3367–3374, 2005.

KANG, S.-I. et al. Immature Citrus sunki peel extract exhibits antiobesity effects by β-oxidation and lipolysis in high-fat diet-induced obese mice. **Biological & Pharmaceutical Bulletin**, v. 35, n. 2, p. 223–230, jan. 2012.

KARUPPAGOUNDER, V. et al. Naringenin ameliorates skin inflammation and accelerates phenotypic reprogramming from M1 to M2 macrophage polarization in atopic dermatitis NC/Nga mouse model. **Experimental Dermatology**, n. 2, p. 404–407, 2016.

KIM, G.-S. et al. Citrus aurantium flavonoids inhibit adipogenesis through the Akt signaling pathway in 3T3-L1 cells. **BMC Complementary and Alternative Medicine**, v. 12, n. 31, p. 1–10, jan. 2012.

KOTHARI, N. et al. Tumor Necrosis Factor gene polymorphism results in high TNF level in sepsis and septic shock. **Cytokine**, v. 61, n. 2, p. 676–681, 2013.

LI, Y. et al. In vivo pharmacokinetics of hesperidin are affected by treatment with glucosidade-like Bg1A protein isolated from yeasts. **Journal of Agriculture and Food Chemistry**, v. 56, n. 14, p. 5550–5557, 2008.

LICHTMAN, S. N.; WANG, J.; LEMASTERS, J. J. LPS receptor CD14 participates in release of TNF-alpha in RAW 264.7 and peritoneal cells but not in Kupffer cells. **Am J Physiol Gastrointest Liver Physiol**, v. 275, n. 1, p. G39-46, 1998.

MA, H.; FENG, X.; DING, S. Hesperetin attenuates ventilator-induced acute lung injury through inhibition of NF-κB-mediated inflammation. **European Journal of Pharmacology**, v. 769, p. 333–341, 2015.

MADEIRA, J. V. et al. Rich bioactive phenolic extract production by microbial biotransformation of Brazilian Citrus residues. **Chemical Engineering Research and Design**, v. 92, n. 10, p. 1802–1810, 2014.

MADEIRA JR, J. V.; SPERANZA, P.; MACEDO, G. A. Phenolic compounds and tannase production by solid-state fermentation in orange pomace. **New Biotechnology**, v. 29, n. Supplement, p. S94, 2012.

MARLETTA, M. A. Nitric oxide synthase structure and mechanism. **The Journal of Biological Chemistry**, v. 268, n. 17, p. 12231–12234, 1993.

NAKAJIMA, V. M. et al. Biotransformation effects on anti lipogenic activity of citrus extracts. **Food Chemistry**, v. 197, p. 1046–1053, 2016.

NOZAKI, M. et al. Nitric oxide dysregulates adipocytokine expression in 3T3-L1 adipocytes. **Biochemical and Biophysical Research Communications**, v. 364, n. 1, p. 33–39, 2007.

PARK, M. Y.; MUN, S. T. Carnosic acid inhibits TLR4-MyD88 signaling pathway in LPS-stimulated 3T3-L1 adipocytes. **Nutrition Research and Practice**, v. 8, n. 5, p. 516–520, 2014.

RAASMAJA, A. et al. A water-alcohol extract of Citrus grandis whole fruits has beneficial metabolic effects in the obese Zucker rats fed with high fat/high cholesterol diet. **Food Chemistry**, v. 138, p. 1392–1399, 1 jun. 2013.

REN, H. et al. Hesperetin Suppresses Inflammatory Responses in Lipopolysaccharide-Induced RAW 264.7 Cells via the Inhibition of NF-κB and Activation of Nrf2/HO-1 Pathways. **Inflammation**, v. 39, n. 3, p. 964–973, 2016.

SILVA, C. M. G. et al. Enhancement of the antioxidant activity of orange and lime juices by flavonoid enzymatic de-glycosylation. **Food Research International**, v. 52, n. 1, p. 308–314, jun. 2013.

STUEHR, D. J.; NATHAN, C. F. Nitric Oxide. **The Journal of Experimental Medicine**, v. 169, p. 1543–1555, 1989.

TRACEY, K. J.; CERAMI, A.; PH, D. TUMOR NECROSIS FACTOR : A Pleiotropic Cytokine and Therapuetic Target. 1994.

VABULAS, R. M. et al. HSP70 as endogenous stimulus of the toll/interleukin-1 receptor signal pathway. **Journal of Biological Chemistry**, v. 277, n. 17, p. 15107–15112, 2002.

WEISBERG, S. P. et al. Obesity is associated with macrophage accumulation in adipose tissue. **The Journal of Clinical Investigation**, v. 112, n. 12, p. 1796–1808, 2003.

WERNER, S. L. Stimulus Specificity of Gene Expression Programs Determined by Temporal Control of IKK Activity. **Science**, v. 309, n. 5742, p. 1857–1861, 2005.

WU, C.-H. et al. Nitric Oxide Modulates Pro- and Anti-inflammatory Cytokines in Lipopolysaccharide-Activated Macrophages. **The Journal of Trauma: Injury, Infection, and Critical Care**, v. 55, n. 3, p. 540–545, 2003.

WULSTER-RADCLIFFE, M. C. et al. Adiponectin differentially regulates cytokines in porcine macrophages. **Biochemical and Biophysical Research Communications**, v. 316, n. 3, p. 924–929, 2004.

YEN, G. C. et al. Effects of polyphenolic compounds on tumor necrosis factor-α (TNF-α)-induced changes of adipokines and oxidative stress in 3T3-L1 adipocytes. **Journal of Agricultural and Food Chemistry**, v. 59, n. 2, p. 546–551, 2011.

YOKOTA, T. et al. Adiponectin, a new member of the family of soluble defense collagens, negatively regulates the growth of myelomonocytic progenitors and the functions of macrophages. **Blood**, v. 96, n. 5, p. 1723–32, 2000.

YOSHIDA, H. et al. Citrus flavonoid naringenin inhibits TLR2 expression in adipocytes. **The Journal of Nutritional Biochemistry**, v. 24, n. 7, p. 1276–1284, jul. 2013.

YU, D. H. et al. Protective Effect of Naringenin Against Lipopolysaccharide-Induced Injury in Normal Human Bronchial Epithelium via Suppression of MAPK Signaling. **Inflammation**, v. 38, n. 1, p. 195–204, 2014.

ZOICO, E. et al. The effects of adiponectin on interleukin-6 and MCP-1 secretion in lipopolysaccharide-treated 3T3-L1 adipocytes: Role of the NF-κB pathway. **International Journal of Molecular Medicine**, v. 24, p. 847–851, 2009.

GENERAL DISCUSSION

1. Extraction Solution Selection

The total phenolic content of the extracts obtained by using different extraction solvents varied from 72.29 ± 4.83 to 90.45 ± 5.44 mg of Gallic Acid Equivalent/ml of extract with no statistical difference between the samples. Thus, Folin Ciocalteu assay was not useful to distinguish the extracts. It is noteworthy that the color reaction the Folin Ciocalteau assay is based on not only occurs due to the presence of polyphenols, but can also be caused by other compounds with reducing power (HUANG; OU; PRIOR, 2005).

The HPLC analysis showed that the extraction with 70% methanol, 70% ethanol acidified with 1% HCl and 50% ethanol resulted in same content of the quantified flavanones, being similar when using methanol or ethanol as extraction solvents. Solutions containing methanol are the most used in the extraction of polyphenols of solid materials (HAYAT et al., 2010; RAMFUL et al., 2010; SINGH; SOOD; MUTHURAMAN, 2011). However, due to its toxicity (LIESIVUORI; SAVOLAINEN, 1991; TEPHLY, 1991), it is interesting the development of a process using more friendly solvents. The results presented indicate the potential of 50% ethanol solution in extraction of the flavanones naringin, hesperidin, naringenin and hesperitin from fermented citrus residue. Thereby, this process used a solvent less harmful to health, increasing the feasibility of the extract in studies with cell culture, animal models and humans. Still, using ethanol instead of methanol takes into account economic considerations imposed by the industrial context.

Despite the widespread use of Folin Ciocalteau assay, this was not a good method for screening the best extracting solution. There was no significant difference between the samples according to this analysis, even though the HPLC results clearly indicating smaller potential of water as an extracting solvent for this system.

2. Characterization of “Biotransformed” residue extract obtained from the 50% ethanol solution

There was no difference in the content of total polyphenols by Folin Ciocalteau assay between the “Biotransformed” residue and the controls (“In Natura” and “Autoclaved”). However, once again the HPLC analysis showed difference between the samples. “In Natura” residue had higher content of glycosides flavanones, naringin and hesperidin, while the

“Biotransformed” residue had higher level of the aglycone flavanones, naringenin and hesperitin. Thus, HPLC analysis clearly showed the change in flavanones profile in “Biotransformed” extract. According to MADEIRA et al. (2014) the microorganism employed in this biotransformation probably uses naringin and hesperidin as source of carbon and energy during fermentation. In previous study of our group, it was observed that the tannase produced by *Paecilomyces variotii* strain during fermentation has the ability to catalyze the flavanones deglycosylation, such as hesperidin (FERREIRA et al., 2013). Also, in microbial fermentation the compounds like hesperitin and naringenin are transformed in other substances, with lower molecular weight, that are used in microorganism metabolism (JUSTESEN et al., 2000).

Even using a citrus residue from pectin industry waste, the quantity of flavanones extracted was comparable to other studies. YU et al. (2014) and HO; SU; LIN (2013) performed the extraction of flavonoids from fruits acquired for the research, taking care in the acquisition, transport and storage of raw materials. The first authors were able to extract 1.278 mg/g FW (fresh weight) of naringin and 1.480mg/g FW of hesperidin, indicating values comparable to the present work for hesperidin. In the second study, the extraction was performed in nine different citrus fruits, and for five of them the amount of hesperidin and naringin was lower than that obtained in the present study. These data reinforces the advantage of the produced extract, once it was obtained from low cost industrial waste residue, commonly used for animal feed, presenting a possibility to increase the commercial value of this product. “Biotransformed” extract presented lower amount of flavanones in comparison to controls, however antioxidant activity by DPPH and ORAC was similar between the samples. The literature indicates that in some cases hesperitin and naringenin have higher biological potential than hesperidin and naringin. LONDOÑO-LONDOÑO et al. (2010) demonstrated that hesperitin was more active than hesperidin in reducing lipid peroxidation in hepatic microsomes, with lower amount of TBARS. Moreover, these authors observed that at low concentrations (10 mg/mL) hesperitin was more effective in reducing oxidized LDL by peroxynitrite-oxidized LDL model.

3. Cell Assays

According to MTT assay, none of the extracts were toxic to 3T3-L1 and RAW264.7 cell lines in the concentrations tested (0.01 – 1.00 mg/mL). These results indicate that the use of extracts is safe in these concentrations.

3.1.Lipogenesis and lipolysis assays in 3T3-L1 cell line

All extracts were able to reduce lipogenesis *in vitro,* however, the data found seem to indicate that each extract have a different mechanism of action. Only the "In Natura" extract showed some effect on the inhibition of pre-adipocytes differentiation, and may have a promising application in preventing the formation of new mature adipocytes. KIM et al. (2012) also observed that the addition of *Citrus aurantium* flavonoids extract inhibited 3T3-L1 differentiation with a reduction in the amount of lipid droplets, confirming this positive effect of flavonoids from citrus.

When the extracts were added to the maturation medium, all samples were able to reduce lipid accumulation, with a greater effect of "In Natura" extract. However, only "Biotransformed" extract, with higher content of hesperitin and naringenin, caused induction of lipolysis, observed by higher amount of free glycerol on the supernatant of the culture. It is noteworthy that "Biotransformed" extract had lower amount of flavanones per gram of liofilized extract in comparison to "In Natura" extract (Figure 2.2), and despite this great difference, the "Biotransformed" extract was able to reduce lipids content in the cells as "In Natura" extracts, and it was the only one able to cause lipolysis.

Some studies have shown that hesperitin (GAMO et al., 2014) and naringenin (YOSHIDA et al., 2013) seems to act as PPAR γ agonist. PPARγ is a nuclear transcription factor that induce adipocyte differentiation (KUBOTA et al., 1999), causing greater differentiation of preadipocytes to mature adipocytes when it is activated. This could explain why the "Biotransformed" extract were not able to reduce the differentiation process. Some researchers have found that when these flavonoids were added to 3T3-L1 cell culture, it was observed a greater accumulation of lipid droplets possibly due to its agonist role (GAMO et al., 2014; MORIKAWA et al., 2008; YOSHIDA et al., 2013). However, in our study, despite the possible action of hesperitin and naringenin as PPAR γ agonist, there was an expressive reduction in lipid accumulation, which can be explained by the lipolytic role played by these

aglycones. Still, we must consider that the afore mentioned studies were done with analytical standards, evaluating each compound alone. In the present work, we used a crude extract, and differences in response can occur due to the synergistic effect that compounds together may cause.

There are many studies indicating the potential of citrus extract in obesity treatment (KANG et al., 2012; KIM et al., 2012), however for the first time it is documented promising results with an extract rich in aglycones. The studies using aglycones usually test high cost analytical standards isolated, missing the synergistic effects of the natural extracts we propose.

Still, some authors indicate other advantages of aglycone forms in obesity treatment. KIM et al. (2013) found that hesperitin caused higher secretion of cholecystokinin (CCK) in STC-1 cells in comparison to hesperidin, indicating a possible role of this aglycone flavanone in food intake control since CCK is an anorexigen hormone (RAYBOULD, 2009). Thus, these other information suggests the potential of the extract produced by biotransformation to other biological activities, being suitable for further studies.

3.2.Inflammatory assay with RAW 264.7 stimulated by LPS

The addition of LPS in RAW 264.7 cells caused an increase in TNF-α and NO concentration, indicating the occurrence of the inflammatory stimulus. The macrophages treatment with the extracts caused a reduction in TNF-α in comparison to Control group (cells without any treatment), being significant with the addition of 1.0 mg/ml (Figure 3.2A). There was no significant difference between samples. It is evidenced that the inflammatory stimulus of RAW264.7 with LPS causes increase in TNF-α concentration (LICHTMAN; WANG; LEMASTERS, 1998; WULSTER-RADCLIFFE et al., 2004). This cytokine acts in host defense, however it is also known for its pro-inflammatory activity (BEUTLER; CERAMI, 1986; CARSWELL et al., 1975). In some situations, such as sepsis, secretion of this cytokine is increased, causing an inflammatory state damaging to the organism, which can lead to death (KOTHARI et al., 2013; TRACEY; CERAMI, 1994).

Treatment of RAW264.7 cells with the extracts at a concentration of 1.0mg/mL resulted in inhibition of NFκB protein expression, however with no statistical difference. According to WULSTER-RADCLIFFE et al. (2004) the increased secretion of TNF-α after LPS stimulus in pig macrophagesis caused by ERK1/2 and NFκB pathway activation. NFκB is

the most known pathway of inflammation, and when activated, it translocates to the nucleus, stimulating the synthesis of pro-inflammatory cytokines such as IL-6, TNF-α e IL-1 (GILMORE, 2006). Thus, inhibition of this pathway by the extracts can result in inflammatory profile improvement.

Intracellular HSP70 has anti-apoptotic and cytoprotective activity (BEERE, 2004), whereas the extracellular has immunomodulatory action being an endogenous TLR ligand, that activates the Toll/IL-1 receptor signal pathway (VABULAS et al., 2002). Thus, increased expression of HSP 70, with subsequent secretion of the cell may have pro-inflammatory effect. GUPTA et al. (2013) studied RAW 264.7 cells and observed that the presence of LPS with fever increases the expression of HSP70 and its secretion in the extracellular medium, being a risk factor for inflammation. Hence, in presence of LPS, as in the experiment performed in this study, reduction in HSP70 expression caused by the presence of the extracts in highest concentration tested, can have beneficial effects on inflammatory process.

The “Biotransformed” extract was the only one able to reduce the amount of NO in the lowest concentration tested, being significantly lower than the Positive Control. The addition of 1.0 mg/ml of extracts significantly reduced the NO concentration in comparison to Positive Control; however the best results were for “Biotransformed” and “Autoclaved” extracts that were significantly lower than “In Natura”. Besides the citokines, nitric oxide (NO) is also implicated in cell inflammatory response. NO is a free radical and internal messenger that participates in vascular homeostasis and host defense. It is synthesized by nitric-oxide synthase (NOS). There are three isoforms of NOS (MARLETTA, 1993), but for macrophage bactericidal and tumoricidal activities the inducible NOS (iNOS) is the most important one (STUEHR; NATHAN, 1989). The excessive iNOS activity is associated with inflammation tissue damage (GUZIK; KORBUT; ADAMEK-GUZIK, 2003). LPS is a potent stimulator of NO production in macrophages (WU et al., 2003), behavior observed in the present study. The reduced production of NO may be beneficial to protect cell and tissue against injury induced by inflammation acting on oxidative stress induced by NO, or indirectly causing reduced levels of anti-inflammatory cytokines such as TNF-α (WU et al., 2003).

3.3.Inflammatory assay in 3T3-L1 and RAW 264.7 co-culture

In obesity, besides the increasing number and size of adipocytes, there is a macrophage infiltration, causing increased cytokine secretion, contributing to the subclinical inflammatory state present in this disease. Among the cytokines that contribute to this pro-inflammatory condition, there are TNF-α and IL-6, that are associated with insulin resistance found in that situation (WEISBERG et al., 2003). Thus, compounds capable of regulating cytokines secretion after an inflammatory stimulus could be effective in treating certain conditions associated with excess weight.

3T3-L1 and RAW264.7 co-culture is widely used to mimic this situation. The increased concentration of IL-6 and NO in co-culture compared to isolated culture of 3T3-L1 and RAW-264.7, indicates that an inflammatory stimulus occurred when the two cells shared the same environment. Moreover, the co-culture caused a reduction in adiponectin concentration compared to 3T3-L1 grown alone. These results indicate that cultivation of these two cells in the same environment caused changes in cytokine secretion profile, being a good model for studying the inflammatory changes present in obesity. In co-culture, the inflammation was possibly initiated by TNF-α secreted from RAW264.7. ARAKI et al. (2006) stimulated 3T3-L1 with TNF-α and observed an increase in NFκB activity and in IL-6 secretion, and reduction in adiponectin concentration; confirming that the presence of TNF-α is capable of causing an inflammatory stimulus in adipocytes.

Despite the evidence of inflammatory stimulus in co-culture, TNF-α secretion was lower in this assay than in isolated RAW 264.7 culture. Adiponectin from 3T3-L1 may have inhibited macrophage secretion. Adiponectin is an adipokine secreted by adipocytes that presents protective role, with anti-inflammatory activity and capacity of improving insulin sensitivity (AJUWON; SPURLOCK, 2005; ZOICO et al., 2009). WULSTER-RADCLIFFE et al. (2004) observed that pre-treatment of pig macrophages with adiponectin inhibited the activation of NFκB. Thus, reduction of TNF-α and IL-6 would be caused by adiponectin via inhibition of NFκB nuclear translocation, and ERK1/2 activity. YOKOTA et al. (2000) also showed that adiponectin inhibits TNF-α secretion by macrophages stimulated with LPS. Moreover, YEN et al. (2011) demonstrated that treatment of 3T3-L1 with TNF-α causes a decrease in adiponectin secretion. Thus, possibly in co-culture, adiponectin reduced TNF-α secretion, and TNF-α also influenced adiponectin secretion. The LPS stimulation was only 24

hours after the macrophages were inoculated in the well with 3T3-L1, then maybe adiponectin from adipocytes had been a pre-treatment for macrophages, which may not have been converted in the most inflammatory state. If this occurred, the increased secretion of IL-6 and NO in co-culture was mainly from 3T3-L1, and not from RAW264.7; and perhaps LPS acted mainly stimulating 3T3-L1.

It is noteworthy that the inflammatory stimulus with or without LPS in co-culture did not cause major differences in cytokines concentrations between the Control groups. However, the different responses observed when the samples were added to groups stimulated or not with LPS, indicates that there was possibly a different mechanism of action in the pro-inflammatory model. Possibly, in the co-culture, TNF-α secreted by RAW 264.7 was the original stimulator of the inflammatory response; and in the co-culture with LPS, both LPS and TNF-α acted in the stimulus.

3T3-L1 treatment with LPS stimulates ERK phosphorylation and mRNA expression of IL-6, TNF-α and MCP-1 (PARK; MUN, 2014). Moreover, the presence of LPS stimulates higher protein expression of TLR4, MyD88 and TRAF6 (induces phosphorylation of IκB), resulting in increased activation of NFκB (PARK; MUN, 2014). TNF-α has its action mediated by TNF receptor (TNF-r). Despite this difference, it has been shown that both stimulate NFκB activation. However, TNF-α has rapid action with IKK activation between 10 and 15 minutes, NFκB activation with a peak at 45 minutes and returning to pre-stimulus baseline levels after 90 minutes; while with LPS IKK activity is greater at 45 minutes, NFκB activation peak is at 105 minutes, still maintaining high activation at 120 minutes (WERNER, 2005). These results indicate that, despite the two substances action in NFκB, the pathways to occur this activation are distinct.

The results indicate that the samples at 0.2 mg/mL concentration were not effective in inhibiting the inflammation induced by TNF-α. On the other side, "Biotransformed" and "In Natura" reduced IL-6 concentration in relation to control by 43.4% and 42.7%, respectively, however there was no statistical significance. Evaluating TNF-α secretion, the addition of "Biotransformed" extract caused a reduction of 30.7%, and "In Natura" a reduction of 14.9%. These extracts also caused a slight reduction in NO concentration. Thus, cell treatment at 1.0 mg/ml concentration with Biotransformed and In Natura extracts, inhibited inflammation

induced by TNF-α. Biotransformed extract also caused greater increase in adiponectin secretion (Table 3.2).

In the inflammation induced by LPS, at 0.2 mg/mL concentration, "Biotransformed" and "In Natura" extracts reduced IL-6 and TNF-α secretion compared to Control. Still, compared to "Autoclaved" extract, "Biotransformed" showed significant lower secretion of IL-6 and "In Natura" lower secretion of TNF-α. Between these two extracts, "Biotransformed" presented better anti-inflammatory results because also promoted increased secretion of adiponectin and lower of NO. In the higher concentration tested, "Autoclaved" extract showed the best results reducing all inflammatory cytokines (Table 3.3).

YEN et al. (2011) evaluated the effect of 21 phenolic analytical standards including hesperidin, naringin and naringenin, in IL-6 secretion by 3T3-L1 stimulated with TNF-α. To achieve this purpose, the cells were pretreated for 24 hours with analytical standards and after that they were stimulated with 5 ng/ml TNF-α for 12 hours. The authors reported that naringenin was able to reduce 27% IL-6 secretion after stimulation with TNF-α, naringin 30.4% and hesperidin 32.6%; indicating the anti-inflammatory role of these compounds. However, it is important to note that in the present study, using extracts with phenolics combination, the effects on IL-6 secretion were more pronounced, achieving reduction of 43.4% when "Biotransformed" extract was added to co-culture without LPS stimulation and 48.0% in the co-culture with LPS stimulation. These results indicate positive synergistic effect when using an extract with phenolic mixture.

As already mentioned hesperitin (GAMO et al., 2014b) and naringenin (YOSHIDA et al., 2013) seems to act as PPAR γ agonist, and besides acting in lipogenesis, they can also participate in the suppression of NFκB activation and IL-6 production (AJUWON; SPURLOCK, 2005). So, the presence of the extracts with hesperitin and naringenin ("Biotransformed"), and the induction of adiponectin secretion by the extract, could contribute to the anti-inflammatory activity via PPAR γand NFκB.

Despite the fact that the extracts did not influenced NO concentration in co-culture, there was a positive correlation between this compound and pro-inflammatory cytokines. This was expected because NO activates JNK and NFκB pathways, resulting in increased production of cytokines (NOZAKI et al., 2007), as observed here.

CONCLUSÃO GERAL

Podemos concluir que o etanol pode substituir o metanol como solvente para extração das flavanonas de resíduos cítricos, contribuindo para o menor uso de um solvente extremamente tóxico. O processo de biotransformação foi capaz de modificar o perfil de flavanonas do extrato de resíduo de cítricos, aumentando o teor de hesperitina e naringenina, compostos que são encontrados em baixas quantidades na natureza.

De acordo com os resultados expostos, todos os extratos produzidos poderiam ser utilizados no tratamento da obesidade, entretanto visando objetivos diferentes. O extrato "In Natura" poderia ser empregado para reduzir a síntese de novos adipócitos e acumulação de lipídeos, e o extrato "Biotransformado" poderia ser utilizado para induzir a lipólise no tecido adiposo.

Considerando que na obesidade, além do aumento no número e tamanho de adipócitos, há a presença de uma inflamação, os resultados indicam o promissor uso desses extratos devido atividade anti-inflamatória observada, uma vez que eles foram capazes de reduzir a secreção de TNF-α e óxido nítrico em cultura de macrófagos. Além disso, as amostras foram capazes de inibir a secreção de TNF-α, IL-6 e óxido nítrico; e aumentar a concentração de adiponectina em co-cultura de adipócitos e macrófagos. É importante considerar que o extrato "Biotransformado" e "Autoclavado" apresentaram algumas vantagens em determinadas circunstâncias em comparação ao "In Natura", indicando que o resíduo bioprocessado de cítricos pode contribuir para o desenvolvimento de um novo produto com potencial uso no tratamento da obesidade e da inflamação associada.

GENERAL CONCLUSION

We can conclude that ethanol can replace methanol as solvent for flavanones extraction from citrus residue, contributing to less use of a highly toxic solvent. The biotransformation was able to modify the flavanones profile of citrus residue extract, increasing the content of hesperetin and naringenin, compounds that are found in low amounts in nature.

According to the results, all extracts produced could be used to treat obesity, however aiming at different goals. "In Natura" extract could be employed to reduce the new adipocytes synthesis and lipids accumulation, and "Biotransformed" extract could be used to induce lipolysis in adipose tissue.

Considering that in obesity, in addition to increased number and size of adipocytes, there is the presence of inflammation, the results show promising use of the extract due to anti-inflammatory activity observed, since they were able to reduce TNF-α and NO secretion in macrophages. In addition, the samples were able to inhibit TNF-α, IL-6 and nitric oxide secretion and increase adiponectin concentration in adipocytes and macrophages co-culture. "Biotransformed" and “Autoclaved” extracts showed some advantages in certain circumstances compared to "In Natura", indicating that the bioprocessed citrus residue may contribute to the development of a new product with potential use in treating obesity and associated inflammation.

REFERENCES

AGUILAR, C. N.; AGUILERA-CARBO, A.; ROBLEDO, A. Production of Antioxidant Nutraceuticals by Solid-State Cultures of Pomegranate (Punica granatum) Peel and Creosote Bush (Larrea tridentata) Leaves. **Food Technology and Biotechnology**, v. 46, n. 2, p. 218–222, 2008.

AHERNE, S. A.; O'BRIEN, N. M. Dietary flavonols: chemistry, food content, and metabolism. **Nutrition**, v. 18, n. 1, p. 75–81, jan. 2002.

AJUWON, K. M.; SPURLOCK, M. E. Adiponectin inhibits LPS-induced NF-kappaB activation and IL-6 production and increases PPARgamma2 expression in adipocytes. **American Journal of Physiology, Regulatory, Integrative and Comparative Physiology**, v. 288, n. 5, p. R1220–R1225, 2005.

ALAM, M. A.; KAUTER, K.; BROWN, L. Naringin improves diet-induced cardiovascular dysfunction and obesity in high carbohydrate, high fat diet-fed rats. **Nutrients**, v. 5, n. 3, p. 637–50, mar. 2013.

AMEER, B. et al. Flavanone absorption after naringin, hesperidin, and citrus administration. **Clinical Pharmacology and Therapeutics**, v. 60, n. 1, p. 34–40, jul. 1996.

ARAKI, S. et al. N-acetylcysteine attenuates TNF-α induced changes in secretion of interleukin-6, plasminogen activator inhibitor-1 and adiponectin from 3T3-L1 adipocytes. **Life Sciences**, v. 79, n. 25, p. 2405–2412, 2006.

BALISTRERI, C. R.; CARUSO, C.; CANDORE, G. The role of adipose tissue and adipokines in obesity-related inflammatory diseases. **Mediators of Inflammation**, v. 2010, p. 1–19, jan. 2010.

BARROS, H. R. D. M.; FERREIRA, T. A. P. D. C.; GENOVESE, M. I. Antioxidant capacity and mineral content of pulp and peel from commercial cultivars of citrus from Brazil. **Food Chemistry**, v. 134, n. 4, p. 1892–8, 15 out. 2012.

BATTESTIN, V., & MACEDO, G. A. Tannase production by Paecilomyces variotii. **Bioresource Technology**, v. 98, p. 1832–1837, 2007.

BATTESTIN, V., MACEDO, G. A., FREITAS, V. A. P. Hydrolyis of epigallocatechin gallate using a tanase from Paecilomyces variotii. **Food Chemistry,** v. 108, p. 228–233, 2008.

BATTESTIN, V., PASTORE, G., MACEDO, G. Otimizing the fermentation broth for tannase production by a new isolated strain Paecilomyces variotii. In: **Abstracts of the 12th European Congress in Biotechnology**. Denmark, Copenhagen. Journalof Biotechnology, v. 118, S49, 2005.

BEERE, H. M. "The stress of dying": the role of heat shock proteins in the regulation of apoptosis. **Journal of Cell Science**, v. 117, n. Pt 13, p. 2641–2651, 2004.

BENAVENTE-GARCÍA, O. et al. Uses and properties of Citrus flavonoids. **Journal of Agricultural and Food Chemistry**, v. 45, n. 12, p. 4505–4515, 1997.

BERNABÉ, J. et al. Effects of a citrus based juice on biomarkers of oxidative stress in metabolic syndrome patients. **Journal of Functional Foods**, v. 5, n. 3, p. 1031–1038, jul. 2013.

BEUTLER, B.; CERAMI, A. Cachectin and tumor necrosis factor as two sides of the same biological coin. **Nature**, v. 320, p. 584–588, 1986.

BEZERRA, R. M. N. et al. A high fructose diet affects the early steps of insulin action in muscle and liver of rats. **The Journal of Nutrition**, v. 130, p. 1531–1535, 2000.

BIRT, D. F.; HENDRICH, S.; WANG, W. Dietary agents in cancer prevention: flavonoids and isoflavonoids. **Pharmacology & Therapeutics**, v. 90, n. 2–3, p. 157–177, 2001.

BUSCEMI, S. et al. Effects of red orange juice intake on endothelial function and inflammatory markers in adult subjects with increased cardiovascular risk. **American Journal of Clinical Nutrition**, v. 95, p. 1089–1095, 2012.

CARIDI, D. et al. Profiling and quantifying quercetin glucosides in onion (Allium cepa L.) varieties using capillary zone electrophoresis and high performance liquid chromatography. **Food Chemistry**, v. 105, n. 2, p. 691–699, 2007.

CARSWELL, E. A. et al. An endotoxin-induced serum factor that causes necrosis of tumors. **Proceedings of the National Academy of Sciences of the United States of America**, v. 72, n. 9, p. 3666–70, 1975.

CHO, K. W. et al. Dietary naringenin increases hepatic peroxisome proliferators-activated receptor α protein expression and decreases plasma triglyceride and adiposity in rats. **European journal of nutrition**, v. 50, n. 2, p. 81–8, mar. 2011.

CONAB – Companhia Nacional de Abastecimento. **Acompanhamento da safra brasileira. Laranja. Safra 2013/2014. Primeira estimativa. São Paulo e Triângulo Mineiro.** Maio, 2013.

DALLAS, C. et al. Lipolytic effect of a polyphenolic citrus dry extract of red orange, grapefruit, orange (SINETROL) in human body fat adipocytes. Mechanism of action by inhibition of cAMP-phosphodiesterase (PDE). **Phytomedicine**, v. 15, p. 783–792, out. 2008.

DALLAS, C. et al. Clinical study to assess the efficacy and safety of a citrus polyphenolic extract of red orange, grapefruit, and orange (Sinetrol-XPur) on weight management and metabolic parameters in healthy overweight individuals. **Phytotherapy Research**, p. 1–7, 3 abr. 2013.

DÁVALOS, A.; GÓMEZ-CORDOVÉS, C.; BARTOLOMÉ, B. Extending applicability of the oxygen radical absorbance capacity (ORAC-fluorescein) assay. **Journal of Agricultural and Food Chemistry**, v. 52, n. 1, p. 48–54, 14 jan. 2004.

DE MEJÍA, E. G. et al. Yerba mate tea (Ilex paraguariensis): Phenolics, antioxidant capacity and *in vitro* inhibition of colon cancer cell proliferation. **Journal of Functional Foods**, v. 2, n. 1, p. 23–34, jan. 2010.

DEVARAJ, S. et al. Effect of orange juice and beverage with phytosterols on cytokines and PAI-1 activity. **Clinical Nutrition**, v. 30, n. 5, p. 668–671, out. 2011.

DING, X. et al. Citrus ichangensis peel extract exhibits anti-metabolic disorder effects by the inhibition of PPARγ and LXR signaling in high-fat diet-induced C57BL/6 mouse. **Evidence-Based Complementary and Alternative Medicine**, v. 2012, p. 1–10, jan. 2012.

ESCARPA, A.; GONZALEZ, M. C. An overview of analytical chemistry of phenolic compounds in foods. **Critical Reviews in Analytical Chemistry**, v. 31, n. 2, p. 57–139, abr. 2001.

FERREIRA, L. R. et al. Improving the chemopreventive potential of orange juice by enzymatic biotransformation. **Food Research International**, v. 51, n. 2, p. 526–535, maio 2013.

FREIWALD, A. et al. Comprehensive proteomic data sets for studying adipocyte-macrophage cell-cell communication. **Proteomics**, v. 13, p. 3424–3428, 31 out. 2013.

FUKUCHI, Y. et al. Lemon polyphenols supress diet-induced obesity by up-regulating of mRNA levels of the enzymes involved in beta-oxidation in mouse white adipose tissue. **Journal of Clinical Biochemistry and Nutrition**, v. 43, n. November, p. 201–209, 2008.

GAMO, K. et al. Hesperetin glucuronides induce adipocyte differentiation via activation and expression of peroxisome proliferator-activated receptor-γ. **Bioscience, Biotechnology, and Biochemistry**, v. 78, n. 6, p. 1052–1059, 2014a.

GAMO, K. et al. Mechanism of Peroxisome Proliferator-Activated Receptor Gamma (PPAR γ) Transactivation by Hesperetin Glucuronides Is Distinct from That by a Thiazolidine-2 , 4-dione Agent. **Chemical and Pharmaceutical Bulletin**, v. 62, n. May, p. 491–493, 2014b.

GATTUSO, G. et al. Flavonoid composition of citrus juices. **Molecules**, v. 12, n. 8, p. 1641–1673, 2007.

GHASEMI, K.; GHASEMI, Y.; EBRAHIMZADEH, M. A. Antioxidant activity, phenol and flavonoid contents of 13 citrus species peels and tissues. **Pakistan Journal of Pharmaceutical Sciences**, v. 22, n. 3, p. 277–281, jul. 2009.

GILMORE, T. D. Introduction to NF-kappaB: players, pathways, perspectives. **Oncogene**, v. 25, n. 51, p. 6680–4, 30 out. 2006.

GIMÉNEZ-BASTIDA, J. A. et al. Hesperetin and its sulfate and glucuronide metabolites inhibit TNF-α induced human aortic endothelial cell migration and decrease plasminogen activator inhibitor-1 (PAI-1) levels. **Food Funct.**, v. 7, n. 1, p. 118–126, 2016.

GONZÁLEZ-BARRIO, R. et al. Production of bioavailable flavonoid glucosides in fruit juices and green tea by use of fungal alpha-L-rhamnosidases. **Journal of Agricultural and Food Chemistry**, v. 52, n. 20, p. 6136–6142, 6 out. 2004.

GREEN, L. C. et al. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. **Analytical Biochemistry**, v. 126, n. 1, p. 131–138, 1982.

GRUNDY, S. M. et al. Definition of Metabolic Syndrome: Report of the National Heart, Lung, and Blood Institute/American Heart Association Conference on Scientific Issues Related to Definition. **Circulation**, v. 109, p. 433–438, 2004.

GUPTA, A. et al. Toll-like receptor agonists and febrile range hyperthermia synergize to induce heat shock protein 70 expression and extracellular release. **Journal of Biological Chemistry**, v. 288, n. 4, p. 2756–2766, 2013.

GUZIK, T. J.; KORBUT, R.; ADAMEK-GUZIK, T. Nitric oxide and superoxide in inflammation and immune regulation. **Journal of Physiology and Pharmacology : an Official Journal of the Polish Physiological Society**, v. 54, n. 4, p. 469–487, 2003.

HAYAT, K. et al. Effect of microwave treatment on phenolic content and antioxidant activity of citrus mandarin pomace. **Food Chemistry**, v. 123, n. 2, p. 423–429, nov. 2010.

HIRATA, A. et al. Kinetics of radical-scavenging activity of hesperetin and hesperidin and their inhibitory activity on COX-2 expression. **Anticancer Research**, v. 25, n. 5, p. 3367–3374, 2005.

HO, S.-C.; SU, M.-S.; LIN, C.-C. Comparison of Peroxynitrite-Scavenging Capacities of Several Citrus Fruit Peels. **International Journal of Food Properties**, v. 17, n. 1, p. 111–124, 2013.

HOTAMISLIGIL, G. S.; SHARGILL, N. S.; SPIEGELMAN, B. M. Adipose expression of tumor necrosis factor-alfa : direct role in obesity-linked insulin resistance. **Science**, v. 259, n. January, p. 87–91, 1993.

HUANG, D.; OU, B.; PRIOR, R. L. The chemistry behind antioxidant capacity assays. **Journal of Agricultural and Food Chemistry**, v. 53, n. 6, p. 1841–56, 23 mar. 2005.

IWAMOTO, M. et al. Supplementation of highly concentrated β-cryptoxanthin in a satsuma mandarin beverage improves adipocytokine profiles in obese Japanese women. **Lipids in Health and Disease**, v. 11, n. 52, p. 1–4, jan. 2012.

JUNG, H. K. Inhibitory Effect of Citrus Peel Extract on Lipid Accumulation of 3T3-L1 Adipocytes. **Journal of the Korean Society for Applied Biological Chemistry**, v. 54, n. 2, p. 169–176, 30 abr. 2011.

JUSTESEN, U. et al. Degradation of Flavonoid Glycosides and Aglycones During *in vitro* Fermentation with Human Faecal Flora. **LWT - Food Science and Technology**, v. 33, n. 6, p. 424–430, 2000.

KANDA, K. et al. Nobiletin suppresses adipocyte differentiation of 3T3-L1 cells by an insulin and IBMX mixture induction. **Biochimica et Biophysica Acta**, v. 1820, p. 461–468, abr. 2012.

KANG, S.-I. et al. Immature Citrus sunki peel extract exhibits antiobesity effects by β-oxidation and lipolysis in high-fat diet-induced obese mice. **Biological & Pharmaceutical Bulletin**, v. 35, n. 2, p. 223–230, jan. 2012.

KARUPPAGOUNDER, V. et al. Naringenin ameliorates skin inflammation and accelerates phenotypic reprogramming from M1 to M2 macrophage polarization in atopic dermatitis NC/Nga mouse model. **Experimental Dermatology**, n. 2, p. 404–407, 2016.

KELLEY, G. L.; ALLAN, G.; AZHAR, S. High dietary fructose induces a hepatic stress response resulting in cholesterol and lipid dysregulation. **Endocrinology**, v. 145, n. 2, p. 548–55, fev. 2004.

KERSHAW, E. E.; FLIER, J. S. Adipose Tissue as an Endocrine Organ. **The Journal of Clinical Endocrinology & Metabolism**, v. 89, n. 6, p. 2548–2556, 2004.

KIM, G.-S. et al. Citrus aurantium flavonoids inhibit adipogenesis through the Akt signaling pathway in 3T3-L1 cells. **BMC Complementary and Alternative Medicine**, v. 12, n. 31, p. 1–10, jan. 2012.

KIM, H. Y. et al. Hesperetin stimulates cholecystokinin secretion in enteroendocrine STC-1 cells. **Biomolecules & Therapeutics**, v. 21, n. 2, p. 121–125, 2013.

KLIMCZAK, I. et al. Effect of storage on the content of polyphenols, vitamin C and the antioxidant activity of orange juices. **Journal of Food Composition and Analysis**, v. 20, n. 3–4, p. 313–322, maio 2007.

KOTHARI, N. et al. Tumor Necrosis Factor gene polymorphism results in high TNF level in sepsis and septic shock. **Cytokine**, v. 61, n. 2, p. 676–681, 2013.

KUBOTA, N. et al. PPARγ mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance. **Molecular Cell**, v. 4, n. 4, p. 597–609, 1999.

LE LAY, S.; DUGAIL, I. Connecting lipid droplet biology and the metabolic syndrome. **Progress in Lipid Research**, v. 48, p. 191–195, 2009.

LEE, Y.-S. et al. Effects of a Citrus depressa Hayata (shiikuwasa) extract on obesity in high-fat diet-induced obese mice. **Phytomedicine**, v. 18, p. 648–654, 15 jun. 2011.

LEE, Y.-S. et al. Nobiletin improves obesity and insulin resistance in high-fat diet-induced obese mice. **The Journal of Nutritional Biochemistry**, v. 24, p. 156–162, jan. 2013.

LI, R. W. et al. Citrus polymethoxylated flavones improve lipid and glucose homeostasis and modulate adipocytokines in fructose-induced insulin resistant hamsters. **Life Sciences**, v. 79, p. 365–373, 20 jun. 2006.

LI, Y. et al. In vivo pharmacokinetics of hesperidin are affected by treatment with glucosidade-like Bg1A protein isolated from yeasts. **Journal of Agriculture and Food Chemistry**, v. 56, n. 14, p. 5550–5557, 2008.

LICHTMAN, S. N.; WANG, J.; LEMASTERS, J. J. LPS receptor CD14 participates in release of TNF-alpha in RAW 264.7 and peritoneal cells but not in Kupffer cells. **Am J Physiol Gastrointest Liver Physiol**, v. 275, n. 1, p. G39-46, 1998.

LIESIVUORI, J.; SAVOLAINEN, H. Methanol and formic acid toxicity: biochemical mechanisms. **Pharmacology & Toxicology**, v. 69, n. 3, p. 157–63, set. 1991.

LONDOÑO-LONDOÑO, J. et al. Clean recovery of antioxidant flavonoids from citrus peel: Optimizing an aqueous ultrasound-assisted extraction method. **Food Chemistry**, v. 119, n. 1, p. 81–87, mar. 2010.

MA, H.; FENG, X.; DING, S. Hesperetin attenuates ventilator-induced acute lung injury through inhibition of NF-κB-mediated inflammation. **European Journal of Pharmacology**, v. 769, p. 333–341, 2015.

MACEDO, J. A. et al. Author ' s personal copy Increasing the antioxidant power of tea extracts by biotransformation of polyphenols. **Food Chemistry**, v. 126, p. 491–497, 2011.

MACHEIX, J.-J.; FLEURIET, A.; BILLOT, J. **Fruit phenolics**. Florida: CRC Press, 1990.

MADEIRA, J. V. et al. Rich bioactive phenolic extract production by microbial biotransformation of Brazilian Citrus residues. **Chemical Engineering Research and Design**, v. 92, n. 10, p. 1802–1810, 2014.

MADEIRA JR, J. V.; SPERANZA, P.; MACEDO, G. A. Phenolic compounds and tannase production by solid-state fermentation in orange pomace. **New Biotechnology**, v. 29, n. Supplement, p. S94, 2012.

MADEIRA JR, J. V.; MACEDO, J. A.; MACEDO, G. A. Detoxification of castor bean residues and the simultaneous production of tannase and phytase by solid-state fermentation using Paecilomyces variotii. **Bioresource Technology**, v. 102, p. 7343–7348, 2011.

MANTHEY, J. A.; GROHMANN, K. Phenols in citrus peel byproducts. Concentrations of hydroxycinnamates and polymethoxylated flavones in citrus peel molasses. **Journal of Agricultural and Food Chemistry**, v. 49, p. 3268–3273, jul. 2001.

MARLETTA, M. A. Nitric oxide synthase structure and mechanism. **The Journal of Biological Chemistry**, v. 268, n. 17, p. 12231–12234, 1993.

MILLER, N. J.; RICE-EVANS, C. A. The relative contributions of ascorbic acid and phenolic antioxidants to the total antioxidant activity of orange and apple fruit juices and blackcurrant drink. **Food Chemistry**, v. 60, n. 3, p. 331–337, nov. 1997.

MIYAKE, Y. et al. Difference in plasma metabolite concentration after ingestion of lemon flavonoids and their aglycones in humans. **Journal of Nutritional Science and Vitaminology**, v. 52, p. 54–60, fev. 2006.

MORAND, C. et al. Hesperidin contributes to the vascular protective effects of orange juice : a randomized crossover study in healthy volunteers. **American Journal of Clinical Nutrition**, v. 93, p. 73–80, 2011.

MORIKAWA, K. et al. Naringenin and hesperetin induce growth arrest, apoptosis, and cytoplasmic fat deposit in human preadipocytes. **Journal of Agricultural and Food Chemistry**, v. 56, n. 22, p. 11030–11037, 2008.

NAKAJIMA, V. M. et al. Biotransformation effects on anti lipogenic activity of citrus extracts. **Food Chemistry**, v. 197, p. 1046–1053, 2016.

NIELSEN, I. L. F. et al. Bioavailability is improved by enzymatic modification of the citrus flavonoid hesperidin in humans: a randomized, double-blind, crossover trial. **The Journal of Nutrition**, n. October 2005, p. 404–408, 2006.

NOZAKI, M. et al. Nitric oxide dysregulates adipocytokine expression in 3T3-L1 adipocytes. **Biochemical and Biophysical Research Communications**, v. 364, n. 1, p. 33–39, 2007.

PARK, M. Y.; MUN, S. T. Carnosic acid inhibits TLR4-MyD88 signaling pathway in LPS-stimulated 3T3-L1 adipocytes. **Nutrition Research and Practice**, v. 8, n. 5, p. 516–520, 2014.

PERSSON, J. et al. Perilipin and adipophilin expression in lipid loaded macrophages. **Biochemical and Biophysical Research Communications**, v. 363, p. 1020–1026, 30 nov. 2007.

RAASMAJA, A. et al. A water-alcohol extract of Citrus grandis whole fruits has beneficial metabolic effects in the obese Zucker rats fed with high fat/high cholesterol diet. **Food Chemistry**, v. 138, p. 1392–1399, 1 jun. 2013.

RAMALHO, R.; GUIMARÃES, C. Papel do tecido adiposo e dos macrófagos no estado de inflamação crónica associada à obesidade- Implicações Clínicas. **Acta Medica Portuguesa**, v. 21, p. 489–496, 2008.

RAMFUL, D. et al. Bioactive phenolics and antioxidant propensity of flavedo extracts of Mauritian citrus fruits: potential prophylactic ingredients for functional foods application. **Toxicology**, v. 278, n. 1, p. 75–87, 28 nov. 2010.

RAYBOULD, H. E. Mechanisms of CCK signaling from gut to brain. **Current Opinion in Pharmacology**, v. 7, n. 6, p. 570–574, 2009.

REN, H. et al. Hesperetin Suppresses Inflammatory Responses in Lipopolysaccharide-Induced RAW 264.7 Cells via the Inhibition of NF-κB and Activation of Nrf2/HO-1 Pathways. **Inflammation**, v. 39, n. 3, p. 964–973, 2016.

SABROE, I. et al. The role of TLR activation in inflammation. **The Journal of Pathology**, p. 126–135, 2008.

SALAMONE, F. et al. Moro orange juice prevents fatty liver in mice. **World Journal of Gastroenterology**, v. 18, n. 29, p. 3862–3868, 7 ago. 2012.

SERGEEV, I. N. et al. Polymethoxyflavones activate Ca2+-dependent apoptotic targets in adipocytes. **Journal of Agricultural and Food Chemistry**, v. 57, p. 5771–5776, 8 jul. 2009.

SILVA, C. M. G. et al. Enhancement of the antioxidant activity of orange and lime juices by flavonoid enzymatic de-glycosylation. **Food Research International**, v. 52, n. 1, p. 308–314, jun. 2013.

SINGH, J.; SOOD, S.; MUTHURAMAN, A. In-vitro evaluation of bioactive compounds, anti-oxidant, lipid peroxidation and lipoxygenase inhibitory potential of Citrus karna L. peel extract. **Journal of Food Science and Technology**, 17 ago. 2011.

SINGLETON, V. L.; ORTHOFER, R.; LAMUELA-RAVENTÓS, R. M. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. In: PACKER, L. (Ed.). . **Oxidants and Antioxidants Part A**. Methods in Enzymology. [s.l.] Academic Press, 1999. v. 299p. 152–178.

STUEHR, D. J.; NATHAN, C. F. Nitric Oxide. **The Journal of Experimental Medicine**, v. 169, p. 1543–1555, 1989.

STUETZ, W. et al. Polymethoxylated flavones, flavanone glycosides, carotenoids, and antioxidants in different cultivation types of tangerines (Citrus reticulata Blanco cv. Sainampueng) from Northern Thailand. **Journal of Agricultural and Food Chemistry**, v. 58, n. 10, p. 6069–74, 26 maio 2010.

SUBASH-BABU, P.; ALSHATWI, A. A. Hesperitin inhibit adipocyte differentiation and enhance Bax- and p21-mediated adipolysis in human mesenchymal stem cell adipogenesis. **Journal of Biochemical Molecular Toxicology**, v. 0, p. 1–10, 2014.

SUN, Y. et al. Phytochemical profile and antioxidant activity of physiological drop of citrus fruits. **Journal of Food Science**, v. 78, n. 1, p. C37-42, jan. 2013.

TEPHLY, T. R. The toxicity of methanol. **Life Sciences**, v. 48, n. 6, p. 1031–1041, 1991.

TRACEY, K. J.; CERAMI, A. TUMOR NECROSIS FACTOR : A Pleiotropic Cytokine and Therapuetic Target. **Annual Review of Medicine**, v. 45, p. 491–503, 1994.

TRIPOLI, E. et al. Citrus flavonoids: Molecular structure, biological activity and nutritional properties: A review. **Food Chemistry**, v. 104, n. 2, p. 466–479, jan. 2007.

TURNER, T.; BURRI, B. J. Potential nutritional benefits of current citrus consumption. **Agriculture**, v. 3, p. 170–187, 2013.

UM, M. Y. et al. Coumarin attenuates hepatic steatosis by down-regulating lipogenic gene expression in mice fed a high-fat diet. **The British Journal of Nutrition**, v. 109, p. 1590–1597, maio 2013.

VABULAS, R. M. et al. HSP70 as endogenous stimulus of the toll/interleukin-1 receptor signal pathway. **Journal of Biological Chemistry**, v. 277, n. 17, p. 15107–15112, 2002.

WANG, M.-J. et al. Pharmacokinetics and conjugation metabolism of naringin and naringenin in rats after single dose and multiple dose administrations. **Journal of Food and Drug Analysis**, v. 14, n. 3, p. 247–253, 2006.

WEISBERG, S. P. et al. Obesity is associated with macrophage accumulation in adipose tissue. **The Journal of Clinical Investigation**, v. 112, n. 12, p. 1796–1808, 2003.

WERNER, S. L. Stimulus Specificity of Gene Expression Programs Determined by Temporal Control of IKK Activity. **Science**, v. 309, n. 5742, p. 1857–1861, 2005.

WU, C.-H. et al. Nitric Oxide Modulates Pro- and Anti-inflammatory Cytokines in Lipopolysaccharide-Activated Macrophages. **The Journal of Trauma: Injury, Infection, and Critical Care**, v. 55, n. 3, p. 540–545, 2003.

WULSTER-RADCLIFFE, M. C. et al. Adiponectin differentially regulates cytokines in porcine macrophages. **Biochemical and Biophysical Research Communications**, v. 316, n. 3, p. 924–929, 2004.

YAMASHITA, A. et al. Macrophage-Adipocyte Interaction: Marked Interleukin-6 Production by Lipopolysaccharide. **Obesity**, v. 15, n. 11, p. 2549, 2007.

YEN, G. C. et al. Effects of polyphenolic compounds on tumor necrosis factor-α (TNF-α)-induced changes of adipokines and oxidative stress in 3T3-L1 adipocytes. **Journal of Agricultural and Food Chemistry**, v. 59, n. 2, p. 546–551, 2011.

YOKOTA, T. et al. Adiponectin, a new member of the family of soluble defense collagens, negatively regulates the growth of myelomonocytic progenitors and the functions of macrophages. **Blood**, v. 96, n. 5, p. 1723–32, 2000.

YOSHIDA, H. et al. The citrus flavonoids hesperetin and naringenin block the lipolytic actions of TNF-alpha in mouse adipocytes. **Biochemical and Biophysical Research Communications**, v. 394, p. 728–732, 9 abr. 2010.

YOSHIDA, H. et al. Citrus flavonoid naringenin inhibits TLR2 expression in adipocytes. **The Journal of Nutritional Biochemistry**, v. 24, n. 7, p. 1276–1284, jul. 2013.

YU, D. H. et al. Protective Effect of Naringenin Against Lipopolysaccharide-Induced Injury in Normal Human Bronchial Epithelium via Suppression of MAPK Signaling. **Inflammation**, v. 38, n. 1, p. 195–204, 2014a.

YU, E. A. et al. Flavonoid profile and biological activity of Korean citrus varieties (II): Pyunkyul (Citrus tangerina Hort. ex Tanaka) and overall contribution of its flavonoids to antioxidant effect. **Journal of Functional Foods**, v. 6, n. 1, p. 637–642, 2014b.

ZOICO, E. et al. The effects of adiponectin on interleukin-6 and MCP-1 secretion in lipopolysaccharide-treated 3T3-L1 adipocytes: Role of the NF-κB pathway. **International Journal of Molecular Medicine**, v. 24, p. 847–851, 2009.

SUGESTÃO PARA TRABALHOS FUTUROS

1- A caracterização e quantificação mais precisa do extrato biotransformado desenvolvido, que não foi feito no presente trabalho devido a limitações financeiras para compra de padrões analíticos.

2- A avaliação do potencial do extrato biotransformado na melhora da sensibilidade a insulina, pois há evidência que flavanonas produzidas no bioprocessamento apresentam papel agonista de PPAR-γ, o que contribui na redução da resistência a insulina.

3- Realização de testes em modelos animais, a fim de verificar se os efeitos observados *in vitro* podem ser extrapolados para um modelo mais complexo.

APPENDIX

APPENDIX 1 – Artigo de revisão bibliográfica, intitulado “Citrus bioactive phenolics: role in the obesity treatment”, publicado na revista LWT-Food Science and Technology.

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Citrus bioactive phenolics: Role in the obesity treatment

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ABSTRACT

Adipose tissue performs many functions in the body, being considered an endocrine organ due to substances secreted, called adipokines. The excess of adipose tissue is called obesity, and it is associated with a state of chronic subclinical inflammation. Various strategies and products have been evaluated in an attempt to prevent and treat obesity, standing out the importance of polyphenols from citrus fruits. This paper aims to review studies developed to evaluate the role of these compounds in obesity and some general trends can be highlighted. The *in vitro* studies indicate that citrus polyphenols could assist in the management of obesity, since they cause a reduction in adipocyte differentiation, lipid content in the cell and adipocyte apoptosis. The biological assays are not entirely consistent; however, most of them indicated a reduction in adipose tissue; increased genes expression indicating a stimulus to β-oxidation; improved lipid profile and glycemia; as well as some evidence of improvement in inflammatory status. Several clinical trials have demonstrated the positive effect of citrus flavonoids in the reduction of proinflammatory cytokines in humans, being beneficial to alleviate the complications present in obesity. However, there are few clinical trials developed to examine its role in reducing adiposity.

1. Introduction

Adipose tissue has long been considered only as a site of energy storage, however it is now known that it performs many functions in the body. This tissue is considered an endocrine organ due to paracrine substances secreted, called adipokines (Grundy, Brewer Jr., Cleeman, Smith, & Lenfant, 2004). Also there are other cells present in adipose tissue, besides the adipocytes, that release active substances involved in metabolic pathways, such as macrophages (Weisberg et al., 2003). In parallel, the adipose tissue has receptors for afferent signals emitted by other endocrine systems, enabling a communication with the central nervous system. This network interaction explains the coordinating activity of adipose tissue in energy metabolism, neuroendocrine and immune function (Kershaw & Flier, 2004).

Obesity is a disease characterized by excess body weight, associated with a state of chronic subclinical inflammation, caused by an increased secretion of adipokines that modulate certain responses in the body (Balistreri, Caruso, & Candore, 2010). Overall, the vast majority of adipokines studied have a role in the development of chronic diseases associated with obesity causing insulin resistance, increased blood pressure, abnormal blood lipids, increased inflammatory response, and thrombus formation (Grundy et al., 2004).

In addition to many complications associated with obesity, the high prevalence of the disease made it a public health problem. Accordingly, various strategies and products have been evaluated in an attempt to prevent and treat excessive body weight. Among the compounds studied, stands out the importance of polyphenols in plant food.

A source of polyphenols widely studied is citrus fruits. This group of fruits is important source of bioactive compounds, mainly flavonoids, being target of many studies concerning the adipose tissue and obesity. Therefore, this paper aims to review studies developed to evaluate the role of these compounds in the obesity and associated changes.

2. Phenolics in citrus fruits

Phenolic compounds or polyphenols refers to a group of molecules found in plants, that exert photoprotection function, defense against microorganisms and insects, being responsible for pigmentation and some food organoleptic characteristics (Escarpa & Gonzalez, 2001). Among the various classes that comprise the phenolics, flavonoids are considered important for human consumption due to its wide distribution in plant foods.

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The flavonoid structure is based on the flavylium nucleus, which consists of three phenolic rings (Fig. 1). The first benzene ring (A) is condensed with the sixth carbon of the third ring (C), which in the 2-position carries a phenyl group (B) as a substituent (Aherne & O'Brien, 2002).

The biochemical activities of flavonoids and their metabolites depend on their chemical structure, which may vary with one or more hydroxyl substituents, including derivatives. Flavonoids and isoflavones commonly occur as esters, ethers or derivatives glycosides, or a mixture of them. Except the group of leucoanthocyanins, other flavonoids occur in plants usually accompanied by carbohydrates thus receiving the name of glycosylated flavonoids. The glycidic substituents include: D-glucose, L-rhamnose, glucose-rhamnose, galactose and arabinose (Birt, Hendrich, & Wang, 2001). When the flavonoid is free of carbohydrates, the structure is called aglycone.

Citrus fruits are rich in various nutrients, such as vitamins A and C, folic acid and dietary fiber. Furthermore, these fruits are source of bioactive compounds, as flavonoids, coumarins, limonoids and carotenoids (Ding et al., 2012; Turner & Burri, 2013).

Among the flavonoids, citrus present considerable amounts of flavanones, flavones, flavonols and anthocyanins, however the main flavonoids are the flavanones (Benavente-García, Castillo, Marin, Ortuño, & Del Río, 1997). In this class of compounds, the most frequent ones are hesperidin, narirutin, naringin and eriocitrin (Ghasemi, Ghasemi, & Ebrahimzadeh, 2009; Sun et al., 2013). Other phenolics often found in citrus are p-coumaric, ferulic, caffeic and sinapic acids (Manthey & Grohmann, 2001; Sun et al., 2013).

Gattuso, Barreca, Garguilli, Leuzzi, and Caristi (2007) reviewed the flavonoid composition of citrus, and some of their results are summarized at the Table 1.

The genus *Citrus* comprises several orange species – *Citrus sinensis* (sweet orange), *Citrus aurantium* (sour oranges), *Citrus reticulata* (tangerine or mandarin) – and their hybrids – tangors, which are orange-tangerine hybrids, and tangelos, which are tangerine-grapefruit or tangerine pummelo hybrids. Many of these species or hybrids can have different varieties (Gattuso et al., 2007).

In general, the data where the specific *C. sinensis* variety analyzed is reported show that different varieties present approximately the same flavonoid composition pattern. Commercial orange juices present a similar composition to freshly squeezed ones, with the appearance of some unexpected compounds. Naringin and diosmin hint at the possibility that some of the samples analyzed are not pure orange juices, or, as in the case of hand-squeezed juices, the presence of PMFs in variable quantities suggests that they could be essentially derived from the flavedo and confirm that the amounts of polymethoxyflavones found in industrial juices are a consequence of the pressing process used (Gattuso et al., 2007).

It is also important to consider that the flavanones in citrus can be glycosylated or aglycone. The glycosylated forms are also divided into neohesperidosides that contain a neohesperidose (ramnosil-α-1,2 glucose) and have a bitter taste; and rutinosides that contain a flavanone and a disaccharide residue, and do not have taste (Macheix, Fleuriet, & Billot, 1990). Naringin, neohesperidin and neoeriocitrin are examples of neohesperidosides; while hesperidin, narirutin and didymin are examples of rutinosides (Tripoli, Guardia, Giammanco, Majo, & Giammanco, 2007). Naringenin and hesperetin are the most common aglycones, often found in trace concentrations.

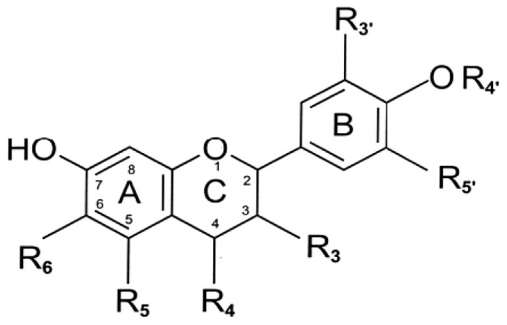


Fig. 1. General structure of food flavonoids.

Concerning the quantity of the compounds, Miller and Rice-Evans (1997) detected the presence of hesperidin (141 ± 49 μmol/L) and narirutin (62 ± 16 μmol/L) in longlife orange juice. Klimczak, Małecka, Szlachta, and Gliszczyńska-Świgło (2007) also evaluated longlife orange juice, verifying the presence of some hydroxycinnamic acids as caffeic (8.2 mg/L), p-coumaric (0.5 mg/L), ferulic (0.6 mg/L) and sinapic (0.7 mg/L). However, as mentioned above, the flavanones were found in greater quantity, being detected the presence of narirutin (70.2 mg/L), hesperidin (76.9 mg/L) and dydymin (9.9 mg/L). Of the flavanones analyzed, naringin and neohesperidin were not detected.

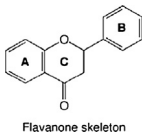
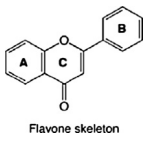
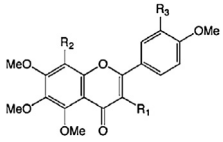
Stuetz, Prapamontol, Hongsibsong, and Biesalski (2010) evaluated the polyphenol content of *C. reticulata* Blanco cv. Sainampueng, to verify the difference between hand-pressed juice and the peeled fruit. The peeled fruit had low content of polymethoxyflavones, while the hand-pressed juice presented high content of tangeritin (5.99–31.8 mg/L), nobiletin (5.49–28.2 mg/L) and sinensetin (0.30–2.00 mg/L). The authors observed that the polymethoxyflavones were present in the peel of the fruit, and a simple squeezing can cause the transfer of these compounds from the peel to the juice. Besides this class of polyphenol, it was also detected the presence of the flavanones didymin (4.44–9.50 mg/L), narirutin (17.7–43.4 mg/L) and hesperidin (123.3–206.7 mg/L) in the hand-pressed juice. On the other side, the peeled fruit had high content of didymin (45–112 mg/kg), narirutin (181–600 mg/kg) and hesperidin (841–1898 mg/kg).

Some researchers also study the peels and peels extract of citrus fruits, as Ramful, Bahorun, Bourdon, Tarnus, and Aruoma (2010) that evaluated orange, clementine, mandarine, tangor, tangelo and pamplemousses peels. The flavonoids detected in this matrix were poncirin (2.49–18.85 mg/g FW), rhoifolin (4.54–10.39 mg/g FW), didymin (3.22–13.94 mg/g FW), rutin (8.16–42.13 mg/g FW), diosmin (4.01–18.06 mg/g FW), isorhoifolin (1.72–14.14 mg/g FW), neohesperidin (3.20–11.67 mg/g FW), hesperidin (83.4–234.1 mg/g FW), neoeriocitrin (8.8–34.65 mg/g FW) and narirutin (5.05–21.23 mg/g FW). Naringin (19.49 mg/g FW) was only detected in mandarine.

Londoño-Londoño et al. (2010) identified using HPLC-MS the presence of hesperidin, neohesperidin, diosmin, nobiletin and tangeritin in orange peel; hesperidin and neohesperidin in tangerine peel; and hesperidin, neohesperidin and diosmin in lime peel. Reinforcing the information above, none of the peels presented the aglycone hesperitin in their composition.

Ghasemi et al. (2009) evaluated the total polyphenol and flavonoid content of peels and tissues from three varieties of *C. sinensis*, three of *C. reticulata*, three of *Citrus unshiu*, one of *Citrus limon*, one of *Citrus paradisi* and two of *C. aurantium*. For most citrus analyzed the total polyphenols content was higher in the peel (104.2–223.2 mg gallic acid equivalent/g of extract powder) in comparison to tissue (66.5–396.8 2 mg gallic acid equivalent/g of extract powder), excepting all *C. reticulata* varieties, and one *C. sinensis* variety (var. Washington Navel). The total flavonoid content was also higher in the peel (0.3–31.1 mg quercetin equivalent/g of extract powder) in relation to tissue (0.3–17.1 mg quercetin equivalent/g of extract powder) in most of the samples, excepting four varieties (*C. sinensis* var. Sungin, *C. unshiu* var.

Table 1
Reviewed flavonoid composition of some citrus juices.

	Mean	SD	Median	MIN	MAX
Flavonoid composition of *C. sinensis* (sweet orange) juice (mg/100 mL)					
Flavanones	 Flavanone skeleton				
Didymin	1.89	0.92	1.60	0.80	3.10
Eriocitrin	0.31	0.18	0.29	0.11	0.67
Hesperidin	28.6	11.9	28.0	3.51	55.2
Narirutin	5.2	3.1	4.2	0.55	14.2
Flavones	 Flavone skeleton				
Neoeriocitrin	0.59	–	–	–	–
Poncirin	1.04	0.78	1.04	0.49	1.59
6,8-di-*C*-Glu-Apigenin	5.72	2.02	5.00	4.15	8
6,8-di-*C*-Glu-Diosmetin	0.35	0.14	0.35	0.25	0.45
Rhoifolin	0.05	–	–	–	–
Isorhoifolin	0.07	–	–	–	–
Diosmin	0.09	–	–	–	–
Neodiosmin	0.08	–	–	–	–
Polymethoxyflavones					
Nobiletin	0.33	0.19	0.33	0.19	0.46
Sinensetin	0.37	–	–	–	–
Tangeretin	0.04	0.04	0.04	0.01	0.07
Flavonoid composition of *C. clementina* juice (mg/100 mL)					
Flavanones					
Hesperidin	39.9	29.4	34.9	5.21	86.1
Naringin	0.08	0.03	0.08	0.05	0.12
Narirutin	4.64	–	–	–	–
Flavones					
6,8-di-*C*-Glu-Apigenin	0.5	–	–	–	–
6,8-di-*C*-Glu-Diosmetin	0.2	–	–	–	–
Diosmin	1.25	0.51	1.26	0.67	2.12
Flavonoid composition of *C. limon* (lemon) juice (mg/100 mL)					
Flavanones					
Eriocitrin	16.7	10.3	16.55	1.67	39.1
Hesperidin	20.5	12.4	18.85	3.84	41
Flavones					
6,8-di-*C*-Glu-Apigenin	1.17	0.25	1.05	1	1.45
6,8-di-*C*-Glu-Diosmetin	4.95	0.88	5	4.05	5.8
7-*O*-Rut-Luteolin	3.93	2.14	3.5	1.5	6.5
Diosmin	3.12	1.66	3.65	0.51	5.1
Aglycones					
Luteolin	0.08	–	–	–	–
Flavonoid composition of *C. paradisi* (grapefruit) juice (mg/100 mL)					
Flavanones					
Didymin	0.30	0.04	0.30	0.27	0.33
Eriocitrin	0.41	0.19	0.41	0.27	0.54
Hesperidin	0.93	0.58	0.87	0.25	1.79
Naringin	23.0	12.8	21.9	4.5	60.2
Narirutin	7.60	5.80	7.70	2.50	17.0
Neohesperidin	1.21	0.35	1.28	0.67	1.58
Neoeriocitrin	0.32	0.02	0.32	0.30	0.33
Poncirin	1.26	0.35	1.30	0.85	1.58
Flavones					
Rutin	3.26	–	–	–	–
Rhoifolin	0.28	–	–	–	–
Polymethoxyflavones					
Heptamethoxyflavone	0.06	0.07	0.06	0.01	0.11
Nobiletin	0.15	0.04	0.15	0.12	0.17
Tangeretin	0.12	–	–	–	–
Aglycones					
Hesperetin	0.74	–	–	–	–
Naringenin	2.70	2.68	1.70	0.98	8.00
Taxifolin	0.16	–	–	–	–
Quercetin	0.19	0.03	0.19	0.17	0.21

Adapted from Gattuso et al., 2007.

Ishikawa, *C. reticulata* var. Clementine, *C. reticulata* var. Page). These results indicate that considerable losses occur with the peel removal before consumption or in industrial process. And besides the information about the content of total polyphenol and flavonoid, several studies have already shown the positive effects of peel extracts in the treatment of chronic non-communicable diseases (Ding et al., 2012; Fukuchi, Hiramitsu, Okada, Hayashi, & Nabeno, 2008; Kang et al., 2012; Kim et al., 2012; Lee et al., 2011; Raasmaja et al., 2013).

A factor to consider when talking about flavonoids is whether the compound is in the glycosylated or aglycone form. It has been shown that in rats, after oral consumption of naringin, the sulfate and glucuronate conjugates forms of naringenin are found in the organism, indicating that for naringin absorption the glycoside needs to be released to the formation of the aglycone naringenin, thus depending on one glucosidase for its absorption (Wang et al., 2006). The better absorption of the aglycone form in relation to glycosylated in citrus flavonoids has also been observed in humans using glycosylated forms of eriocitrin and hesperidin compared with the corresponding aglycones eridictyol, homoeridictyol and hesperetin (Miyake et al., 2006). Therefore, the polyphenol structure can modify its bioavailability to the body. This might be the reason why many researchers chose to evaluate the aglycone potential, seen that this form is detected in the organism tissues and blood, and it has higher bioavailability.

The flavonoids found in citrus species act as antioxidants and may protect against oxidative stress-related to inflammation process, thus reducing the risk of macromolecules damage caused by the action of reactive species, conferring protection against several neurodegenerative diseases and reducing the risk of developing cardiovascular disease and cancer (Benavente-García et al., 1997).

3. Citrus phenolics effect on cell cultures models of obesity

In vitro studies are useful to understand the mechanisms of action, and guide the decision of which products should be further studied in biological assays and clinical trials. Besides that, they are an alternative when the product is in its early development phase, a moment that the yield is generally low.

Many *in vitro* studies are being conducted with citrus phenolics to evaluate its effects on obesity. One of the mechanisms proposed has been the role of these compounds in the adipocytes apoptosis, because it was observed that the addition of polymethoxyflavones analytical standard (5-hydroxy-3,6,7,8,30,40-hexamethoxyflavone (5-HxMF OH) 3,5,6,7,8,30,40-heptamethoxyflavone (HpMF); 5,6,7,30,40-pentamethoxyflavone (PMTCT), and 30-hydroxy-5,6,7,40-tetramethoxyflavone (30-OH-TtMF)) of citrus (100 μM) caused an increase in intracellular calcium, which induced the increase of calpain and caspase-12, two proteins associated with programmed cell death (Sergeev, Li, Ho, Rawson, & Dushenkov, 2009). The reduction in the number of adipose cells due to apoptosis could assist in maintaining weight loss, avoiding the weight cycling.

Another study evaluated the effect of nobiletin analytical standard in 3T3-L1 adipocytes (0–100 μM). The treatment of these cells with the citrus phenolic reduced, in a dose-dependent manner, the expression of C/EBPβ and PPARγ, transcription factors that are associated with differentiation of pre-adipocytes into mature adipocytes. Reinforcing this result, it was also observed lower lipid accumulation in cultured cells when the flavonoid was added (Kanda et al., 2012).

Nowadays, it is considered the importance of toll-like receptors (TLRs) on the association between obesity and other chronic non-communicable diseases, and it is recognized the fact that TLRs are responsible for the activation of inflammatory pathways (Sabroe, Parker, Dower, & Whyte, 2008). In a study evaluating the treatment of pre-adipocytes, adipocytes during its differentiation, and differentiated 3T3-L1 cells treated with naringenin analytical standard (100 μM), it was observed an inhibitory effect of the flavonoid on the expression of TLR 2, only during adipocyte differentiation (Yoshida et al., 2013), indicating a possible effect on the phase in which the individual is in the process of gaining body fat.

During the obesity development, it is known that in addition to the increase in adipose cells, there is an increase in the macrophages number in adipose tissue (Ramalho & Guimarães, 2008; Weisberg et al., 2003). Considering this information, Yoshida et al. (2013) conducted a test with 3T3-L1 adipocytes and macrophages RAW 264 in co-culture. The co-culture showed increased expression of TLR 2, and treatment with naringenin inhibited this increased expression observed. Furthermore, the expression of TLR 2 was increased with TNF-α addition to the culture of mature adipocytes, however naringenin added to this medium was able to inhibit TNF-α-induced TLR 2 expression by inhibiting JNK and NF-κB pathways. However, naringenin appears to reduce the expression of TLR 2 via increased activation of PPARγ, a nuclear transcription factor that could cause greater differentiation of pre-adipocytes into mature adipocytes and increase lipid accumulation in these cells, exactly as was observed on the experiment (Yoshida et al., 2013).

Also, Yoshida et al. (2010) found that in 3T3-L1 adipocytes cell culture, hesperetin and naringenin analytical standards showed anti-inflammatory effect by inhibiting the activation of NFκB through TNF-α, with a consequent reduction in the secretion of interleukin-6 (IL-6); and anti-lipolytic effect by inhibit ERK (extracellular signal regulated kinase) pathway causing a decreased activation of hormone sensitive lipase (HSL); contributing to reduce the insulin resistance (Fig. 2).

Other studies, one with orange peel flavonoids ethanol extract rich in hesperidin (13.79 mg/g), narirutin (7 mg/g) and naringin (262.5 mg/g) (Jung, Jeong, Park, Park, & Hong, 2011), and another with *C. aurantium* flavonoids extract that contained naringin, hesperidin, poncirin, isosiennsetin, sineesytin, tetramrthnl-o-isoscutellaeein, nobiletin, heptamethoxyflavone, 3-hydoxynobiletin, tangeretin, hydroxypentamethoxyflavone, and hexamethoxyflavone (Kim et al., 2012), also observed a stimulus in lipolysis and lower triglyceride accumulation in 3T3-L1 adipocytes. Still, extracts caused a lower accumulation of total lipids and reduced the expression of C/EBPα, C/EBPβ, PPARγ, aP2 (activating protein 2) and FAS (fatty acid synthase), being the last two ones target genes of C/EBPβ and PPARγ (Kim et al., 2012). The extracts have also generated a smaller amount of Akt (serine/threonine kinase) and phosphorylated GSK3β. The phosphorylated Akt, promotes the phosphorylation of GSK3β, and this phosphorylates C/EBPα and C/EBPβ, which become activated (Kim et al., 2012), then acting in adipocyte differentiation.

The orange peel flavonoids ethanol extract evaluated by Jung (2011) also caused a suppressive effect on the expression of perilipin, indicating another factor that may be associated with the positive effect of citrus flavonoids in obesity. This is a lipid-associated protein secreted only in adipocytes (Persson, Degerman, Nilsson, & Lindholm, 2007), that controls fatty acid release stimulated by HSL, because it binds and stabilizes lipid droplets in adipose tissue (Le Lay & Dugail, 2009).

The treatment of mature 3T3-L1 adipocytes with *Citrus sunki* peel ethanol extract that contained tangeritin (55.13 mg/g), nobiletin (38.83 mg/g), rutin (17.02 mg/g), hesperidin (17.11 mg/g), sinensetin (4.23 mg/g) induced LKB1, AMPK (AMP-activated protein kinase) and ACC (acetyl-CoA carboxylase) phosphorylation in a dose-dependent manner, and also caused an increase in mRNA levels of CPT-1a (carnitine palmitoyltransferase 1a) indicating the role of this extract to increase the β-oxidation. Furthermore, a lipolysis stimulation occurred 24 h after the extract addition to the cell culture, in a dose dependent manner. Associated with this result, the authors observed that the extract caused phosphorylation of PKA substrate (cAMP-dependent protein kinase) and HSL (Kang et al., 2012).

Besides the effect on adipose tissue, flavonoids can also act in the management of obesity by interfering in the control of hunger and satiety. In this context, hesperetin analytical standard (0.1–1.0 mM) has shown to cause an increase in the secretion of cholecystokinin (CCK) in STC-1 cells through increase in intracellular calcium concentration by the TRP (transient receptor potential) and TRP 1 ankirin channels. The addition of hesperidin analytical standard in the same model caused no effect, indicating that only the aglycone form influences hormone secretion (Kim, Park, Kim, Lee, & Rhyu, 2013). The increase in CCK would be interesting because this hormone, secreted from endocrine cells in the small intestine, assists in the control of food intake (Raybould, 2009).

Some products have been developed in order to assist in the obesity prevention and treatment, and it could be mentioned Sinetrol, a citrus-based fruits (juice, peels, seeds) extract obtained by physical treatment (crushing of fruits, cold pressure of juice, extraction, centrifugation, filtration, spray drying) of a specific varieties of red orange (*C. sinensis* L. Osbeck (*Blood group*)), sweet orange (*C. aurantium* L. var. sinensis), bitter orange (*C. aurantium* L. var. amara), grapefruit (*C. paradisi*) and guarana (*Paullinia cupana*), which contained 60% of polyphenol, 16.7% of flavanones, 2% of anthocyanins and 3.6% of caffeine, studied by Dallas, Gerbi, Tenca, Juchaux, and Bernard (2008). The researchers noted that this supplement (20 mg/mL) was able to stimulate lipolysis in human fat cells in a *in vitro* study, verified by the free fatty acids enhancement. The authors suggest that the compounds present in the supplement, especially naringin and cyanidin 3-glycoside, have an effect on the inhibition of cAMP-phosphodiesterase, and thus there is an increase in cAMP and subsequent stimulation of hormone-sensitive lipase (HSL), the enzyme that stimulates lipolysis in human.

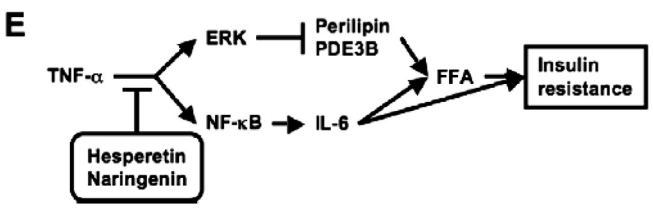


Fig. 2. Scheme proposed by Yoshida et al. (2010) for the action of hesperetin and naringenin on inhibition of ERK and NFκB pathways, resulting in the reduction of free fatty acids (FFA), and consequently improving insulin resistance.

4. Evaluation of citric polyphenol effect in biological assay

Despite the important information collected by *in vitro* assays, biological assay helps understand the bioactive compounds effects in the whole body, further illustrating the changes caused due to their consumption.

Alam, Kauter, and Brown (2013) evaluated the effect of supplementation with naringin analytical standard (approximately 100 mg/kg diet/day, corresponding to 0.01%) in male Wistar rats fed a diet rich in lipid and carbohydrate. They did not observe the effect of the flavonoid in weight gain. However, supplementation promoted a reduction in retroperitoneal abdominal fat deposition, a better serum lipid profile and oral glucose tolerance. The insulin concentration and pancreas wet weight in rats supplemented was similar to the control group, which received a standard diet, presenting lower values than the high carbohydrate, high fat diet-group without the flavonoid. A high carbohydrate, high fat diet promoted greater inflammatory cell infiltration and accumulation of fat droplets in the liver compared to the control group, however naringin supplementation decreased these two parameters.

In another study, naringenin analytical standard (1%) was supplemented in mice fed a high fat diet, and also no effect was observed in weight gain and food consumption. However, the supplementation improved hyperglycemia, reduced expression of TNF-α (tumor necrosis factor-alpha), MCP-1 (monocyte chemotactic protein-1), and TLR 2 in adipose tissue (Yoshida et al., 2013), promoting protection against chronicle non-communicable diseases.

In an experiment conducted with male Long-Evans adult rats fed a semi-purified experimental diet with 16% fat and 45.5% sucrose, supplementation with 0.012% naringenin analytical standard promoted less visceral fat accumulation, and lower triacylglycerol content in the tissue, compared to the control group that did not receive the flavonoid. However, no effect was observed in the total body weight. Food consumption did not differ between the groups. Also, the supplemented group had lower concentration of serum triglycerides, total and free cholesterol in plasma, and lower accumulation of triacylglycerol and cholesterol in the liver. The flavonoid intake caused an increased expression of PPARα, CPT1 (carnitine palmitoyltransferase 1) and UCP2 (uncoupling protein 2) protein, indicating the role of naringenin to increase lipid β-oxidation in animals (Cho, Kim, Andrade, Burgess, & Kim, 2011).

The administration of 0.05% coumarin analytical standard in C57BL/6J mice receiving a high fat diet also caused less accumulation of visceral fat, and yet caused reduction of total body weight compared to the high fat diet group without the supplement. Still, coumarin supplementation caused less accumulation of lipids, triacylglycerol and cholesterol in the liver; and reduced protein levels of SRBP-1c, FAS, ACC1, PPARγ and C/EBPα. Histological analyzes showed a minor adipocyte size by using the phenolic compound, indicating a contribution in the reduction of adipose tissue (Um, Moon, Ahn, & Youl Ha, 2013).

High fructose diets are used in animal experiments to induce hypertriglyceridemia and insulin resistance (Bezerra et al., 2000; Kelley, Allan, & Azhar, 2004). The supplementation of citrus polymethoxyflavones analytical standard (125 mg/kg body weight/day), mainly containing tangeritin and nobiletin, in hamsters subjected to this modified diet, reduced the weight gain, serum triglyceride, triglyceride in liver and heart, and improved adiponectin levels compared with the group receiving high-fructose diet without the flavonoid. Moreover, a positive effect was observed in the levels of some inflammatory cytokines, reducing TNFα and IFN-γ after the addition of polymethoxyflavones. In this experiment also occurred increased expression of hepatic PPARα and PPARγ as the effect of supplementation, which according to the authors, would be a major regulatory pathway of the effects observed (Li et al., 2006).

Another experiment with polymethoxyflavones was performed with *Citrus depressa* Hayata peel methanol extract that contained nobiletin and tangeritin (1.5%), in ICR mice consuming a high fat diet. The addition of the extract caused less weight gain, lower weight of white adipose tissue, reduced adipocyte size, and lower serum levels of triglycerides and leptin. There was also a decrease in ACC1, SCD1 (esteroil-CoA desaturase), FATP (transport protein fatty acid), aP2 and DAGT1 (diacylglycerol acyltransferase 1) mRNA in white adipose tissue. All the genes cited are involved in the synthesis of fatty acids and triacylglycerols. Despite the positive effects observed, there was no effect on serum adiponectin nor in the mRNA levels of SREBP1 (binding protein sterol regulatory element 1), FAS and ACC1 in the liver (Lee et al., 2011).

In a study developed by Lee et al. (2013), the administration by gavage of 100 mg/kg of purified nobiletin extracted from *C. depressa* peel, to male C57BL/6J mice fed a high fat diet, caused less overall weight gain, lower weight of white adipose tissue and serum triglycerides. There was no effect of the extract on hepatic triacylglycerol levels and serum adiponectin. Controversially, there was an increase in the expression of PPARγ and PPARα, as well as their target genes SREBP-1c, FAS, SCD-1; and CPT-1, UCP2; respectively. These results indicate that the extract induced lipid accumulation and fatty acid oxidation at the same time, however with a greater catabolic effect seen the less weight gain when compared to the group receiving high fat diet without the extract. The extract had the positive effect of reducing TNF-α and MCP-1 (Lee et al., 2013), which helps improve insulin sensitivity, as it is known that TNF-α causes a reduction in expression and translocation of GLUT4, the glucose transport protein in insulin-dependent cells (Hotamisligil, Shargill, & Spiegelman, 1993). The authors observed an increase in expression of IκBα after the flavonoid use, indicating that the anti inflammatory effect is possibly through NFκB pathway inactivation (Lee et al., 2013).

The addition of lemon peel polyphenol ethanol extract (0.5%), containing greater amounts of eriocitrin, hesperidin and narirutin; also promoted less total weight and white adipose tissue gain after consumption of a high fat diet in male C57BL/6J mice. Note that polyphenols also caused increased hepatic PPARα mRNA level, and acyl-CoA oxidase in the liver and white adipose tissue, indicating increased peroxisomal fatty acid oxidation (Fukuchi et al., 2008).

Another extract that showed positive effects of citrus polyphenols was *Citrus ichangensis* peel ethanol extract that contained naringin (8.12 mg/g), hesperidin (0.84 mg/g) and poncirin (1.33 mg/g), administered to female mice fed standard (control), high-fat or high-fat diets supplemented with 1% extract. The weight gain in the group that received the high-fat diet alone was greater than the control, and the addition of the extract in the high-fat diet caused less weight gain, being similar to control group. The extract caused a lower fasting glucose and improved glucose tolerance. Also, there was less accumulation of triacylglycerol and cholesterol in the liver due to the extract administration. Moreover, this has caused lower expression of PPARγ mRNA and lower levels of this transcription factor target genes, including FAS, acyl-CoA oxidase and UCP 2 (Ding et al., 2012); in agreement with the *in vitro* studies presented previously in this paper.

Kang et al. (2012) also tested in mice a high-fat diet supplemented by gavage with an immature *C. sunki* peel extract (150 mg/kg body weight/day), source of the flavonoids tangeritin (55.13 mg/g), nobiletin (38.83 mg/g), rutin (17.02 mg/g), hesperidin (17.11 mg/g), sinensetin (4.23 mg/g). Likewise, the authors found that supplementation reduced weight gain caused by the consumption of a high-fat diet, in addition to promoting lower weight of perirenal and epididymal adipose tissue, as well as smaller size of adipocytes

in epididymal tissue. Another positive effect of the supplementation can be observed in the serum levels of total cholesterol and triglycerides, that were lower compared to the group that received only the high-fat diet. Still, lipid accumulation in the liver was lower, comparable to the control group that received a standard diet. It was also observed increased expression of proteins related to β-oxidation when the extract was added, along with a greater expression of adiponectin gene.

In another study, a water and alcohol extract of *Citrus grandis* whole fruits containing 19% naringin was tested in genetically obese Zucker rats fed with high-fat/high-cholesterol diet. No effect was observed in the body weight, however serum cholesterol and triglyceride were improved when 600 mg/kg of the extract was administered by intragastric gavage for four weeks (Raasmaja et al., 2013).

Salamone et al. (2012) evaluated the effect of *Moro* orange juice, rich in anthocyanins (85 mg/L) in mice C57BL6/J fed a high-fat diet. The juice consumption was *ad libitum*, resulting in a mean intake of 4.1 ± 0.75 mL/day and consequent anthocyanin consumption of about 0.34 mg per day. The group that received the juice had lower levels of triacylglycerol and total lipids in the liver. In addition, there was increased expression of PPARα and acyl-CoA oxidase, and lower of LXR (liver X receptor), FAS, HMG-CoA reductase; indicating a potential effect in stimulating lipid oxidation and reduction of lipogenesis.

A limitation in the comparison of the studies is the phenols administration form, in some cases the compounds are administered as a dietary ingredient and other by gavage. In studies in which the product is incorporated into the diet, consumption data are not always available, providing only the concentration in the diet; on the other hand in studies that used gavage, the information provided is the total quantity consumed.

5. Evaluation of citric polyphenol effect in clinical trials

Despite the evidence observed in *in vitro* studies and biological assays, clinical trial is essential to the final conclusions, since it considers the influence of compounds bioavailability in the human body. Accordingly, Ameer, Weintraub, Johnson, Yost, and Rouseff (1996) ascertained the bioavailability of naringin (500 mg) consumed pure, naringin (500 mg) administered with hesperidin (500 mg), grapefruit juice (1250 mL) co administered with orange juice (1250 mL) and grapefruit (1 unit) consumed daily for 4 weeks. The presence of the aglycone hesperetin and naringenin were detected in urine after the consumption of pure hesperidin and naringin, and after consuming grapefruit and orange juice. After 4 weeks of consuming grapefruit, naringenin was present in plasma and urine. The authors conclude that the aglycone forms of the flavonoid were detected, as the glycoside linkages are not stable to the acidic environment of the stomach, in addition to the possible action of glycosides from intestinal bacteria cleaving the sugar residues.

Another important finding of Ameer et al. (1996) was the observation that the consumption of hesperidin associated with naringin does not affect the urinary recovery of the second, indicating that it does not disturb the bioavailability of the other. Furthermore, these results indicate that the absorption of these two flavonoids occurs in pure form, and also when consumed in a food matrix in the form of juice or fresh fruit. And, as the aglycone form is found after the consumption, maybe its bioavailability is higher since it does not require an enzyme to be absorbed. However, it should be noted that this study was conducted with only 4 volunteers, limiting extrapolation of the results to the general population, and indicating the need for more bioavailability studies in clinical trials.

In addition to assessing the effect of a supplement rich in citrus polyphenols *in vitro*, Dallas et al. (2008) conducted a double-blind placebo-controlled study evaluating the effect of the supplementation in overweight men, observing a greater weight and body fat loss in the group that consumed the supplement. However, the food habits were not controlled, being only mentioned that the volunteers were not supposed to modify it.

A variation of this supplement was studied in another group of humans. This new product was polyphenolic rich fruit extract (red orange, grapefruit, orange sweet and guarana), that contained at least 90% of polyphenols, at least 20% of flavanones and between 1 and 3% of natural caffeine. The authors reported a reduction in waist and hip circumferences; in markers of inflammation C-reactive protein and fibrinogen; and improved oxidative stress status, with the reduction in malondialdehyde (MDA) and increase in superoxide dismutase and glutathione levels. There were no adverse effects in liver and kidney. There was an increase in serum free fatty acids, but no change in the serum lipids levels (Dallas et al., 2013). However, it was not reported in the paper what types of polyphenols and flavonoids were offered with the supplement, and there was no mention about the food habits of the volunteers.

6. Potential of citrus flavonoids produced by biotechnology

Bioprocessing strategies aiming the improvement of the bioaccessibility of phenolic compounds have been investigated in the last years. The use of α-L-rhamnosidases from *Aspergillus aculeatus* was investigated in the transformation of flavonoid rutinosides from fruit juices (orange and blackcurrant) and green tea into their flavonoid glucoside counterparts in a reaction at 30 °C for 10 h. Aliquots of the controls and the enzyme treated samples were taken at different time points and flavonoids rutinosides (anthocyanins in blackcurrant juice, flavanones in orange in juice, and flavonols in green tea) and glucosides were identified and quantified. Even with the assay conditions in each beverage being different, the enzyme was able to remove terminal rhamnosyl groups in the three beverages. Results showed a decrease in the flavonoid rutinoside and an increase in their flavonoid glucoside counterparts (González-Barrio et al., 2004).

The effects on the bioavailability of hesperitin was investigated in a double-blind, randomized, crossover study, in human subjects. The volunteers consumed orange juice with natural hesperidin (hesperitin-7-*O*-rutinoside), orange juice treated with the enzyme hesperidinase and orange juice fortified to obtain 3 times more hesperidin than naturally present. A significant improvement in the bioavailability of the aglycone hesperetin was observed after enzymatic modification of the orange juice. The peak plasma concentrations of the aglycone when subjects consumed the juice containing hesperetin-7-glucoside, generated after removal of the rhamnose by the hesperidinase, were 4-fold higher compared with the untreated juice and 1.5-fold higher than the fortified juice (Nielsen et al., 2006).

A study employing orange pomace as substrate for solid-state fermentation by *Paecilomyces variotii* to produce the enzymes tannase and phytase simultaneously, also evaluated the phenolic content and antioxidant capacity of orange pomace during fermentation. The fermentation medium was prepared with the orange pomace, a saline solution and 10% tannic acid and, after inoculation, was incubated at 30 °C for 120 h. In addition to tannase and phytase production at significant levels, results showed no difference in total phenolic content before and after the fermentation processes. However, the antioxidant capacity of orange pomace, tested against the free radical ABTS, increased approximately tenfold after fermentation, potentially enhancing the value of this residue (Madeira Jr., Macedo, & Macedo, 2012).

Enzymatic hydrolysis and fermentation appear to be an attractive mean to promote the biotransformation of phenolic glycosides and polymers and to increase the concentration of free phenolics in citrus fruits and agro-industrial wastes. The biotransformation of phenolics improved the antioxidant activity and bioaccessibility of these compounds. Further research is necessary to explore new substrates, enzymes and microorganisms and to evaluate the use of biotransformed products as natural antioxidants and as food supplements.

7. Conclusion

Despite the difficulties in the comparison of study results, due to the variety of methodologies and samples evaluated, some general trends can be highlighted.

The studies with cells culture indicate that citrus polyphenols could assist in the management of obesity, since they cause a reduction in adipocyte differentiation, lipid content in the cell and may also function in programmed cell death.

The results of biological assays are not entirely consistent, since in some cases the addition of citrus fruit polyphenols caused lower weight gain, and in other studies this effect was not noticed. However, most of them indicated a reduction in adipose tissue; increased expression of PPARα and its target genes, indicating a stimulus to β-oxidation; improved lipid profile and glycemia; as well as some evidence of improvement in inflammatory status due to a reduction in the proinflammatory cytokines levels. The effects on total body weight are more evident in the studies that used extracts instead of analytical standards, indicating a possible synergistic effect among the different phenolics found when using an extract. Furthermore, the higher cost of analytical standards limits their use in biological assay; meanwhile the extracts are generally made from fruits industrial wastes, a material that would be discarded.

Several clinical trials have demonstrated the positive effect of citrus flavonoids in the reduction of proinflammatory cytokines in humans (Bernabé et al., 2013; Buscemi et al., 2012; Devaraj, Jialal, Rockwood, & Zak, 2011; Iwamoto, Imai, Ohta, Shirouchi, & Sato, 2012; Morand et al., 2011), being beneficial to alleviate the complications present in obesity. However, there are few clinical trials developed to examine its role in reducing adiposity, indicating a research field still in expansion.

References

Aherne, S. A., & O'Brien, N. M. (2002). Dietary flavonols: chemistry, food content, and metabolism. *Nutrition, 18*(1), 75–81.

Alam, M. A., Kauter, K., & Brown, L. (2013). Naringin improves diet-induced cardiovascular dysfunction and obesity in high carbohydrate, high fat diet-fed rats. *Nutrients, 5*(3), 637–650. http://dx.doi.org/10.3390/nu5030637.

Ameer, B., Weintraub, R. A., Johnson, J. V., Yost, R. A., & Rouseff, R. L. (1996). Flavanone absorption after naringin, hesperidin, and citrus administration. *Clinical Pharmacology and Therapeutics, 60*(1), 34–40. http://dx.doi.org/10.1016/S0009-9236(96)90164-2.

Balistreri, C. R., Caruso, C., & Candore, G. (2010). The role of adipose tissue and adipokines in obesity-related inflammatory diseases. *Mediators of Inflammation, 2010*, 1–19. http://dx.doi.org/10.1155/2010/802078.

Benavente-García, O., Castillo, J., Marin, F. R., Ortuño, A., & Del Río, J. A. (1997). Uses and properties of citrus flavonoids. *Journal of Agricultural and Food Chemistry, 45*(12), 4505–4515.

Bernabé, J., Mulero, J., Cerdá, B., García-Viguera, C., Moreno, D. A., Parra, S., et al. (2013). Effects of a citrus based juice on biomarkers of oxidative stress in metabolic syndrome patients. *Journal of Functional Foods, 5*(3), 1031–1038. http://dx.doi.org/10.1016/j.jff.2013.02.003.

Bezerra, R. M. N., Ueno, M., Silva, M. S., Tavares, D. Q., Carvalho, C. R. O., & Saad, M. J. A. (2000). A high fructose diet affects the early steps of insulin action in muscle and liver of rats. *The Journal of Nutrition, 130*, 1531–1535.

Birt, D. F., Hendrich, S., & Wang, W. (2001). Dietary agents in cancer prevention: flavonoids and isoflavonoids. *Pharmacology and Therapeutics, 90*(2–3), 157–177.

Buscemi, S., Rosafio, G., Arcoleo, G., Mattina, A., Canino, B., Montana, M., et al. (2012). Effects of red orange juice intake on endothelial function and inflammatory markers in adult subjects with increased cardiovascular risk. *American Journal of Clinical Nutrition, 95*, 1089–1095. http://dx.doi.org/10.3945/ajcn.111.031088.Am.

Cho, K. W., Kim, Y. O., Andrade, J. E., Burgess, J. R., & Kim, Y.-C. (2011). Dietary naringenin increases hepatic peroxisome proliferators-activated receptor α protein expression and decreases plasma triglyceride and adiposity in rats. *European Journal of Nutrition, 50*(2), 81–88. http://dx.doi.org/10.1007/s00394-010-0117-8.

Dallas, C., Gerbi, A., Elbez, Y., Caillard, P., Zamaria, N., & Cloarec, M. (2013). Clinical study to assess the efficacy and safety of a citrus polyphenolic extract of red orange, grapefruit, and orange (Sinetrol-XPur) on weight management and metabolic parameters in healthy overweight individuals. *Phytotherapy Research*, 1–7. http://dx.doi.org/10.1002/ptr.4981.

Dallas, C., Gerbi, A., Tenca, G., Juchaux, F., & Bernard, F.-X. (2008). Lipolytic effect of a polyphenolic citrus dry extract of red orange, grapefruit, orange (SINETROL) in human body fat adipocytes. Mechanism of action by inhibition of cAMP-phosphodiesterase (PDE). *Phytomedicine, 15*, 783–792. http://dx.doi.org/10.1016/j.phymed.2008.05.006.

Devaraj, S., Jialal, I., Rockwood, J., & Zak, D. (2011). Effect of orange juice and beverage with phytosterols on cytokines and PAI-1 activity. *Clinical Nutrition, 30*(5), 668–671. http://dx.doi.org/10.1016/j.clnu.2011.03.009.

Ding, X., Fan, S., Lu, Y., Zhang, Y., Gu, M., Zhang, L., et al. (2012). *Citrus ichangensis* peel extract exhibits anti-metabolic disorder effects by the inhibition of PPARγ and LXR signaling in high-fat diet-induced C57BL/6 mouse. *Evidence-Based Complementary and Alternative Medicine, 2012*, 1–10. http://dx.doi.org/10.1155/2012/678592.

Escarpa, A., & Gonzalez, M. C. (2001). An overview of analytical chemistry of phenolic compounds in foods. *Critical Reviews in Analytical Chemistry, 31*(2), 57–139. http://dx.doi.org/10.1080/20014091076695.

Fukuchi, Y., Hiramitsu, M., Okada, M., Hayashi, S., & Nabeno, Y. (2008). Lemon polyphenols suppress diet-induced obesity by up-regulating of mRNA levels of the enzymes involved in beta-oxidation in mouse white adipose tissue. *Journal of Clinical Biochemistry and Nutrition, 43*, 201–209. November.

Gattuso, G., Barreca, D., Garguilli, C., Leuzzi, U., & Caristi, C. (2007). Flavonoid composition of citrus juices. *Molecules, 12*, 1641–1673.

Ghasemi, K., Ghasemi, Y., & Ebrahimzadeh, M. A. (2009). Antioxidant activity, phenol and flavonoid contents of 13 citrus species peels and tissues. *Pakistan Journal of Pharmaceutical Sciences, 22*(3), 277–281.

González-Barrio, R., Trindade, L. M., Manzanares, P., de Graaff, L. H., Tomás-Barberán, F. A., & Espín, J. C. (2004). Production of bioavailable flavonoid glucosides in fruit juices and green tea by use of fungal alpha-L-rhamnosidases. *Journal of Agricultural and Food Chemistry, 52*(20), 6136–6142. http://dx.doi.org/10.1021/jf0490807.

Grundy, S. M., Brewer, H. B., Jr., Cleeman, J. I., Smith, S. C., & Lenfant, C. (2004). Definition of metabolic syndrome: report of the National Heart, Lung, and Blood Institute/American Heart Association conference on scientific issues related to definition. *Circulation, 109*, 433–438. http://dx.doi.org/10.1161/01.CIR.0000111245.75752.C6.

Hotamisligil, G. S., Shargill, N. S., & Spiegelman, B. M. (1993). Adipose expression of tumor necrosis factor-alfa: direct role in obesity-linked insulin resistance. *Science, 259*, 87–91. January.

Iwamoto, M., Imai, K., Ohta, H., Shirouchi, B., & Sato, M. (2012). Supplementation of highly concentrated β-cryptoxanthin in a satsuma mandarin beverage improves adipocytokine profiles in obese Japanese women. *Lipids in Health and Disease, 11*(52), 1–4. http://dx.doi.org/10.1186/1476-511X-11-52.

Jung, H. K., Jeong, Y. S., Park, C.-D., Park, C.-H., & Hong, J.-H. (2011). Inhibitory effect of citrus peel extract on lipid accumulation of 3T3-L1 adipocytes. *Journal of the Korean Society for Applied Biological Chemistry, 54*(2), 169–176. http://dx.doi.org/10.3839/jksabc.2011.028.

Kanda, K., Nishi, K., Kadota, A., Nishimoto, S., Liu, M.-C., & Sugahara, T. (2012). Nobiletin suppresses adipocyte differentiation of 3T3-L1 cells by an insulin and IBMX mixture induction. *Biochimica et Biophysica Acta, 1820*, 461–468. http://dx.doi.org/10.1016/j.bbagen.2011.11.015.

Kang, S.-I., Shin, H.-S., Kim, H.-M., Hong, Y.-S., Yoon, S.-A., Kang, S.-W., et al. (2012). Immature *Citrus sunki* peel extract exhibits antiobesity effects by β-oxidation and lipolysis in high-fat diet-induced obese mice. *Biological & Pharmaceutical Bulletin, 35*(2), 223–230.

Kelley, G. L., Allan, G., & Azhar, S. (2004). High dietary fructose induces a hepatic stress response resulting in cholesterol and lipid dysregulation. *Endocrinology, 145*(2), 548–555. http://dx.doi.org/10.1210/en.2003-1167.

Kershaw, E. E., & Flier, J. S. (2004). Adipose tissue as an endocrine organ. *The Journal of Clinical Endocrinology & Metabolism, 89*(6), 2548–2556. http://dx.doi.org/10.1210/jc.2004-0395.

Kim, H. Y., Park, M., Kim, K., Lee, Y. M., & Rhyu, M. R. (2013). Hesperetin stimulates cholecystokinin secretion in enteroendocrine STC-1 cells. *Biomolecules & Therapeutics, 21*(2), 121–125.

Kim, G.-S., Park, H. J., Woo, J.-H., Kim, M.-K., Koh, P.-O., Min, W., et al. (2012). *Citrus aurantium* flavonoids inhibit adipogenesis through the Akt signaling pathway in 3T3-L1 cells. *BMC Complementary and Alternative Medicine, 12*(31), 1–10. http://dx.doi.org/10.1186/1472-6882-12-31.

Klimczak, I., Małecka, M., Szlachta, M., & Gliszczyńska-Świgło, A. (2007). Effect of storage on the content of polyphenols, vitamin C and the antioxidant activity of orange juices. *Journal of Food Composition and Analysis, 20*(3–4), 313–322. http://dx.doi.org/10.1016/j.jfca.2006.02.012.

Lee, Y.-S., Cha, B.-Y., Choi, S.-S., Choi, B.-K., Yonezawa, T., Teruya, T., et al. (2013). Nobiletin improves obesity and insulin resistance in high-fat diet-induced

obese mice. *The Journal of Nutritional Biochemistry, 24*, 156–162. http://dx.doi.org/10.1016/j.jnutbio.2012.03.014.

Lee, Y.-S., Cha, B.-Y., Saito, K., Choi, S.-S., Wang, X. X., Choi, B.-K., et al. (2011). Effects of a *Citrus depressa* Hayata (shiikuwasa) extract on obesity in high-fat diet-induced obese mice. *Phytomedicine, 18*, 648–654. http://dx.doi.org/10.1016/j.phymed.2010.11.005.

Le Lay, S., & Dugail, I. (2009). Connecting lipid droplet biology and the metabolic syndrome. *Progress in Lipid Research, 48*, 191–195. http://dx.doi.org/10.1016/j.plipres.2009.03.001.

Li, R. W., Theriault, A. G., Au, K., Douglas, T. D., Casaschi, A., Kurowska, E. M., et al. (2006). Citrus polymethoxylated flavones improve lipid and glucose homeostasis and modulate adipocytokines in fructose-induced insulin resistant hamsters. *Life Sciences, 79*, 365–373. http://dx.doi.org/10.1016/j.lfs.2006.01.023.

Londoño-Londoño, J., Lima, V. R. D., Lara, O., Gil, A., Pasa, T. B. C., Arango, G. J., et al. (2010). Clean recovery of antioxidant flavonoids from citrus peel: optimizing an aqueous ultrasound-assisted extraction method. *Food Chemistry, 119*(1), 81–87. http://dx.doi.org/10.1016/j.foodchem.2009.05.075.

Macheix, J.-J., Fleuriet, A., & Billot, J. (1990). *Fruit phenolics*. Florida: CRC Press.

Madeira, J. V., Jr., Macedo, J. A., & Macedo, G. A. (2012). A new process for simultaneous production of tannase and phytase by *Paecilomyces variotii* in solid-state fermentation of orange pomace. *Bioprocess Biosystems Engineering, 8551*, 1–7. http://dx.doi.org/10.1007/s00449-011-0587-y.

Manthey, J. A., & Grohmann, K. (2001). Phenols in citrus peel byproducts. Concentrations of hydroxycinnamates and polymethoxylated flavones in citrus peel molasses. *Journal of Agricultural and Food Chemistry, 49*, 3268–3273.

Miller, N. J., & Rice-Evans, C. A. (1997). The relative contributions of ascorbic acid and phenolic antioxidants to the total antioxidant activity of orange and apple fruit juices and blackcurrant drink. *Food Chemistry, 60*(3), 331–337. http://dx.doi.org/10.1016/S0308-8146(96)00339-1.

Miyake, Y., Sakurai, C., Usuda, M., Fukumoto, S., Hiramitsu, M., Sakaida, K., et al. (2006). Difference in plasma metabolite concentration after ingestion of lemon flavonoids and their aglycones in humans. *Journal of Nutritional Science and Vitaminology, 52*, 54–60.

Morand, C., Dubray, C., Milenkovic, D., Lioger, D., Martin, J. F., Scalbert, A., et al. (2011). Hesperidin contributes to the vascular protective effects of orange juice: a randomized crossover study in healthy volunteers. *American Journal of Clinical Nutrition, 93*, 73–80. http://dx.doi.org/10.3945/ajcn.110.004945.

Nielsen, I. L. F., Chee, W. S. S., Poulsen, L., Offord-Cavin, E., Rasmussen, S. E., Frederiksen, H., et al. (2006). Bioavailability is improved by enzymatic modification of the citrus flavonoid hesperidin in humans: a randomized, double-blind, crossover trial. *The Journal of Nutrition, 136*, 404–408.

Persson, J., Degerman, E., Nilsson, J., & Lindholm, M. W. (2007). Perilipin and adipophilin expression in lipid loaded macrophages. *Biochemical and Biophysical Research Communications, 363*, 1020–1026. http://dx.doi.org/10.1016/j.bbrc.2007.09.074.

Raasmaja, A., Lecklin, A., Li, X. M., Zou, J., Zhu, G.-G., Laakso, I., et al. (2013). A water-alcohol extract of *Citrus grandis* whole fruits has beneficial metabolic effects in the obese Zucker rats fed with high fat/high cholesterol diet. *Food Chemistry, 138*, 1392–1399. http://dx.doi.org/10.1016/j.foodchem.2012.09.140.

Ramalho, R., & Guimarães, C. (2008). Papel do tecido adiposo e dos macrófagos no estado de inflamação crónica associada à obesidade – implicações clínicas. *Acta Medica Portuguesa, 21*, 489–496.

Ramful, D., Bahorun, T., Bourdon, E., Tarnus, E., & Aruoma, O. I. (2010). Bioactive phenolics and antioxidant propensity of flavedo extracts of Mauritian citrus fruits: potential prophylactic ingredients for functional foods application. *Toxicology, 278*(1), 75–87. http://dx.doi.org/10.1016/j.tox.2010.01.012.

Raybould, H. E. (2009). Mechanisms of CCK signaling from gut to brain. *Current Opinion in Pharmacology, 7*(6), 570–574. http://dx.doi.org/10.1016/j.coph.2007.09.006.

Sabroe, I., Parker, L., Dower, S., & Whyte, M. (2008). The role of TLR activation in inflammation. *The Journal of Pathology*, 126–135. http://dx.doi.org/10.1002/path.

Salamone, F., Li Volti, G., Titta, L., Puzzo, L., Barbagallo, I., La Delia, F., et al. (2012). Moro orange juice prevents fatty liver in mice. *World Journal of Gastroenterology, 18*(29), 3862–3868. http://dx.doi.org/10.3748/wjg.v18.i29.3862.

Sergeev, I. N., Li, S., Ho, C.-T., Rawson, N. E., & Dushenkov, S. (2009). Polymethoxyflavones activate Ca2+-dependent apoptotic targets in adipocytes. *Journal of Agricultural and Food Chemistry, 57*, 5771–5776. http://dx.doi.org/10.1021/jf901071k.

Stuetz, W., Prapamontol, T., Hongsibsong, S., & Biesalski, H.-K. (2010). Polymethoxylated flavones, flavanone glycosides, carotenoids, and antioxidants in different cultivation types of tangerines (*Citrus reticulata* Blanco cv. Sainampueng) from Northern Thailand. *Journal of Agricultural and Food Chemistry, 58*(10), 6069–6074. http://dx.doi.org/10.1021/jf904608h.

Sun, Y., Qiao, L., Shen, Y., Jiang, P., Chen, J., & Ye, X. (2013). Phytochemical profile and antioxidant activity of physiological drop of citrus fruits. *Journal of Food Science, 78*(1), C37–C42. http://dx.doi.org/10.1111/j.1750-3841.2012.03002.x.

Tripoli, E., Guardia, M. L., Giammanco, S., Majo, D. D., & Giammanco, M. (2007). Citrus flavonoids: molecular structure, biological activity and nutritional properties: a review. *Food Chemistry, 104*, 466–479. http://dx.doi.org/10.1016/j.foodchem.2006.11.054.

Turner, T., & Burri, B. J. (2013). Potential nutritional benefits of current citrus consumption. *Agriculture, 3*, 170–187. http://dx.doi.org/10.3390/agriculture3010170.

Um, M. Y., Moon, M. K., Ahn, J., & Youl Ha, T. (2013). Coumarin attenuates hepatic steatosis by down-regulating lipogenic gene expression in mice fed a high-fat diet. *The British Journal of Nutrition, 109*, 1590–1597. http://dx.doi.org/10.1017/S0007114512005260.

Wang, M.-J., Chao, P. L., Hou, Y.-C., Hsiu, S.-L., Wen, K.-C., & Tsai, S.-Y. (2006). Pharmacokinetics and conjugation metabolism of naringin and naringenin in rats after single dose and multiple dose administrations. *Journal of Food and Drug Analysis, 14*(3), 247–253.

Weisberg, S. P., Mccann, D., Desai, M., Rosenbaum, M., Leibel, R. L., & Ferrante, A. W., Jr. (2003). Obesity is associated with macrophage accumulation in adipose tissue. *The Journal of Clinical Investigation, 112*(12), 1796–1808. http://dx.doi.org/10.1172/JCI200319246.

Yoshida, H., Takamura, N., Shuto, T., Ogata, K., Tokunaga, J., Kawai, K., et al. (2010). The citrus flavonoids hesperetin and naringenin block the lipolytic actions of TNF-alpha in mouse adipocytes. *Biochemical and Biophysical Research Communications, 394*, 728–732. http://dx.doi.org/10.1016/j.bbrc.2010.03.060.

Yoshida, H., Watanabe, W., Oomagari, H., Tsuruta, E., Shida, M., & Kurokawa, M. (2013). Citrus flavonoid naringenin inhibits TLR2 expression in adipocytes. *The Journal of Nutritional Biochemistry, 24*(7), 1276–1284. http://dx.doi.org/10.1016/j.jnutbio.2012.10.003.

APPENDIX 2 – Artigo original “Botransformation effects on anti lipogenic activity of citrus extracts”, publicado na revista Food Chemistry.

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Biotransformation effects on anti lipogenic activity of citrus extracts

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ABSTRACT

Citrus peel is a good source of flavonoids, with higher content in relation to pulp. This study proposed to investigate the anti-lipogenic potential of a newly developed citrus flavonoids extract, obtained from citrus industrial residue, bioprocessed in order to generate a commercial source of some flavonoids naturally found in low quantity. The results showed that the citrus peel extract obtained after biotransformation was a good source of hesperitin and naringenin, flavonoids that has no source for production on a large scale, as in supplements or medicines. Still, the results showed that all extracts could be used in obesity treatment. The original extract, "In Natura", would be useful to reduce new adipocytes synthesis and lipid accumulation, and the extract bioprocessed, "Biotransformed" extract could be used to induce lipolysis on fat tissue.

1. Introduction

Citrus fruits are source of many bioactive compounds, as flavonoids, coumarins, limonoids and carotenoids (Turner & Burri, 2013). The main class of citrus flavonoid are the flavanones, but there are also considerable amounts of flavones, flavonols and anthocyanins (Benavente-García, Castillo, Marin, Ortuño, & Del Río, 1997). The most frequent types of flavonoids found in citrus are hesperidin, naringin, narirutin, eriocitrin, nobiletin and tangeritin (Sun et al., 2013).

The positive effects of citrus flavonoids in obesity treatment (inducing lipolysis and reducing lipid accumulation), and its complications (causing anti-inflammatory response, reducing serum lipids, and improving blood pressure) are demonstrated in several studies in cell culture (Kang et al., 2012; Kim et al., 2012; Yoshida et al., 2010, 2013), biological assays (Alam, Kauter, & Brown, 2013; Um, Moon, Ahn, & Youl Ha, 2013) and clinical trials (Dallas, Gerbi, Tenca, Juchaux, & Bernard, 2008; Dallas et al., 2013). It is noteworthy that citrus peel has higher content of polyphenols and antioxidant activity in comparison to pulp, indicating that citrus residues are a promising source of bioactive compounds (Barros, Ferreira, & Genovese, 2012).

In most of the studies, citrus peel is obtained from the fruit acquired particularly for the research, and we aim to evaluate the potential of a citrus residue from industrial waste as a commercial source of bioactives. In this context, Brazil is the world's largest orange producer, according to estimates from the Food and Agriculture Organization (FAO). Of the total produced, it is estimated that 85% is destined for juice industry. In juice production, about 50% of the waste generated is composed of peel and pomace, indicating that there is a rich source of this raw material.

Still, citrus extracts commonly used in researches are rich in hesperidin and naringin, with low amount of aglycones. Studies developed to test the aglycones forms commonly use high cost analytical standards. Thus, a residue extract containing the

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biotransformed polyphenols on a unique composition with biological potential would be an innovation with commercial interest.

Our research group have been studying alternatives of bioprocesses to increase the production of more bioactive polyphenols from these industrial arrange residues. Madeira, Nakajima, Macedo, and Macedo (2014) observed that the fermentation process of citrus peel resulted in an extract rich in flavanones aglycones, often found in low amounts in the nature. This is an advantage because some evidence have shown that the aglycones form have higher antioxidant capacity (Hirata, Murakami, Shoji, Kadoma, & Fujisawa, 2005; Silva et al., 2013), and higher bioavailability (Li et al., 2008) in comparison to glycosides. Besides, recent evidences are highlighting the importance of synergism among bioactive compounds in complex matrix with better effect than isolated compounds.

These polyphenols from plant material are commonly extracted with methanol (Hayat et al., 2010; Ramful, Bahorun, Bourdon, Tarnus, & Aruoma, 2010; Singh, Sood, & Muthuraman, 2011). However this is a toxic solvent (Tephly, 1991), being of interest the development of a extraction procedure using a food grade solvent.

Considering these, the study aimed to test a biotransformed citrus peel extract for its antioxidant activity *in vitro*, and the ability to reduce lipogenesis and induce lipolysis in adipocyte cell culture.

2. 2-Materials and methods

2.1. Chemicals

Gallic acid, Folin–Ciocalteu reagent, 2,2′-azobis(2-methylpropionamidine) (97%) (AAPH), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Trolox®, analytical standards hesperidin, hesperitin, naringin and naringenin, insulin, dexamethasone (DEX), 3-isobutyl-1-methylxanthine (IBMX), Oil Red O were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO). Fluorescein was purchased from ECIBRA. All the other chemicals used were in an analytical grade.

2.2. Biotransformed citrus residue

The citrus residue was supplied by CP Kelco Industry Headquarters, from Limeira – SP – Brazil, specialized in pectin production. The residue was dry and contained citrus peel (flavedo and albedo). The material was crushed, and passed through a 10 mesh sieve (Bertel Metallurgical Industries LT). The residue was biotransformed by solid-state fermentation using the microorganism *Paecilomyces variotii* (Brazilian Collection of Environmental and Industrial Microorganisms-CBMAI 1157) according to Madeira et al. (2014). Briefly, the fermentation medium was prepared in 250 ml Erlenmeyer flasks containing 10 g of the residue and 10 ml of water. The medium was sterilized by autoclaving for 15 min at 121 °C. After cooling, the flasks were inoculated with 1 mL of the microorganism spore suspension (9×10^6 spores/mL) and incubated at 30 °C with 90% relative humidity (Climate Camera 420 CLD – Nova Ética, SP, Brazil) for 48 h.

2.3. Preparation of polyphenols extracts from citrus residue

The extraction of phenolic compounds was carried out according to a process adapted from Hayat et al. (2010). One gram of the biotransformed material was mixed with 25 mL 70% methanol. The solution was treated in ultrasonic bath at 30 °C for 15 min, in shaker at 200 rpm for 15 min, and then filtered on Whatman paper (No. 1). Different extraction solvents were tested instead of 70% methanol, in order to reduce costs and toxicity of the final extract. The tested extraction solvents were: 70% ethanol (v/v), 70% ethanol (v/v) acidified with 1% HCl (v/v), 50% ethanol (v/v) and water.

After the definition of the extraction solution, the extracts were prepared from the "Biotransformed" residue and two control residues. The first control was the unfermented residue consisting of the product without any processing ("In Natura"), and the second control was the sterilized residue ("Autoclaved"). The sterilized residue was used as a control of process to verify the modifications that occurred in the extract after the sterilization by autoclaving.

After filtration, the product obtained was concentrated on a rotary evaporator at 40 °C to remove the organic solvent. Then the aqueous solution was frozen and freeze-dried.

2.4. Extracts characterization

2.4.1. Total phenolic content

Total phenolic contents of the extracts were measured using the Folin–Ciocalteu assay according to Singleton, Orthofer, and Lamuela-Raventós (1999). Gallic acid was used as a standard and a calibration curve was plotted in a concentration range of 25–200 μg/mL. All analyses were performed in triplicate and results were expressed as mg of gallic acid equivalents (GAE)/mL or mg of extract.

2.4.2. Determination of main flavanone compounds by High Performance Liquid Chromatography (HPLC)

A DionexUltiMate 3000 (Germany) liquid chromatography, equipped with a C-18 Acclaim® 120 column (Dionex, 3 μm, 4.6 × 150 mm) maintained at 30 °C by a thermostat, was used. The detection was carried out using a UV/VIS (DAD-3000). The method was adapted from Caridi et al. (2007), and De Mejía, Song, Heck, and Ramírez-Mares (2010). The solvents were: A (water/formic acid, 99.9:0.1 v/v) and B (methanol/formic acid, 99.9:0.1 v/v), with a flow rate of 0.6 mL/min. The spectra absorption were obtained at 190 and 480 nm, and the chromatograms were processed at 280 nm. The standard flavanones detected and quantified were naringin, naringenin, hesperidin and hesperitin.

2.4.3. DPPH radical-scavenging activity

The potential antioxidant activity of the extracts was assessed based on the scavenging activity of the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical, as described by Macedo, Battestin, Ribeiro, and Macedo (2011). The reaction mixtures, consisting of 50 μl of test samples and 150 μl of 0.2 mM DPPH in methanol, were carried out on a NovoStarMicroplate reader (BMG LABTECH, Germany) with absorbance filters for a wavelength of 520 nm. The decolorizing process was recorded after 90 min of reaction. The DPPH solution and reaction medium were freshly prepared and stored in the dark. The measurement was performed in triplicate. The antioxidant activity was calculated from the equation obtained by the linear regression after plotting known concentration solutions of Trolox®. Antiradical activity was expressed as μmol of Trolox® equivalent/mg of extracts.

2.4.4. ORAC

The ORAC (Oxygen Radical Absorbance Capacity) assay was performed using fluorescein (FL) as the fluorescent probe, as described by Dávalos, Gómez-Cordovés, and Bartolomé (2004), and adapted by Ferreira, Macedo, Ribeiro, and Macedo (2013). Briefly, 20 μL aliquots of the sample, Trolox® solution or buffer (blank) were distributed in black-walled 96-well plate, followed by the addition of 120 μL fluorescein sodium salt solution 0.38 μg/mL (Ecibra, São Paulo, Brazil) diluted in sodium phosphate buffer 75 mM (pH 7.4). The reaction was initiated by addition of 60 μL AAPH solution (Sigma–Aldrich, Steinheim, Germany) at a concentration of 108 mg/mL dissolved in sodium phosphate buffer 75 mM (pH 7.4). The fluorescence was monitored every 56 s during 75 min using a Novo Star Microplate Reader (BMG LABTECH, Germany)

at 37 °C with excitation filter 485 nm and emission filter 520 nm. The measurements were performed in triplicate. ORAC values were defined as the difference between the area under the FL decay curve of the samples and the blank (net AUC). Regression equations between net AUC and antioxidant concentration were calculated for all of the samples and Trolox® (control). ORAC-FL values were expressed as μmol of Trolox®/mg of extracts.

2.5. Cell culture assay

2.5.1. Cell culture

3T3-L1 murine pre-adipocytes were cultured in Dulbecco's modified Eagle's medium (DMEM) at 37 °C in a humidified atmosphere with 5% CO_2. All media contained 10% fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (100 μg/ml).

2.5.2. MTT Assay

The 3T3-L1 cells (1.0×10^5 cells/mL) were seeded in 96-well plates and incubated for 24 h at 37 °C in a humidified atmosphere with 5% CO_2. Then the cells were treated with the samples (0.01–1.00 mg/mL). After 24 h of incubation, all media was removed and 10 μL of MTT solution (5 mg/mL) was added to the cell culture. The cells were further incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 4 h. The MTT formazan crystals were dissolved in SDS 10% in HCl 0.01 M for 18 h. The optical density of formazan solution was measured with a microplate reader at 540 nm. The results are expressed as a% of control cells, that are cells without any sample treatment.

2.5.3. Pre-adipocytes differentiation

The 3T3-L1 cells (2.0×10^4 cells/mL) were seeded in 24-well plates and grown until confluence. Two days after confluence, designated as day 0, the cells were switched to differentiation medium containing 10 μg/mL insulin, 0.5 mM isobutylmethylxanthine (IBMX), and 1 μM dexamethasone (DEX) in DMEM for another 3 days. Then, the cell culture medium was replaced with maturation medium containing 10 μg/mL insulin in DMEM. The maturation medium was changed every 2 days, until day 12, after which mature adipocytes containing lipid droplets were formed.

2.5.4. Oil Red O staining

The cells were submitted to two different treatments. First, cells were exposed to the extracts sample in the differentiation medium followed by maturation medium without the samples. Using this treatment it is possible to see if the extracts could impair the pre-adipocytes differentiation.

The second treatment consisted in the addition of the extracts only in the maturation medium. This procedure intended to verify if the extracts could reduce triglyceride accumulation in mature adipocytes.

In both cases, on day 12, the 3T3-L1 mature adipocytes plated onto 24-well plates were washed once with formaldehyde 10% in PBS, and fixed with formaldehyde 10% in PBS for 60 min. After replacement of formaldehyde 10% in PBS with 60% isopropanol, the cells were stained for 30 min in freshly diluted Oil red O (Sigma) solution (2.1 mg/ml) with 60% isopropanol. Thereafter, the cells were washed four times with water and the wells were dried in room temperature. Subsequently, the Oil Red O in the stained cells was eluted with 100% isopropanol. The absorbance was measured with a microplate reader at 492 nm. Each treatment was performed in triplicate. The results are expressed as a percentage of control cells, that are fully differentiated cells without any sample treatment, according to the equation bellow:

$$\% \text{ of Oil Red O staining} = (\text{Abs Sample}/\text{Abs Control}) \times 100$$

2.5.5. Glycerol assay

On day 12 of the maturation sequence, cells were treated with the samples for 18 h, and the supernatant was collected. The amount of glycerol in the medium was determined using a Glycerol Assay Kit (Cayman, CO, U.S.A.) in accordance with the manufacturer's instructions.

2.6. Statistical analysis

Results were expressed as means ± standard deviation (SD). The statistical difference between the groups was analyzed using analysis of variance (ANOVA). Post hoc comparison was performed by Tukey's test. Differences were considered significant when $p \leqslant 0.05$. All analyses were performed using the software GraphPad Prism 5 for Windows version 5.00 (GraphPad Software Inc.).

3. Results

3.1. Extraction solution selection

The total phenolic content of the extracts obtained by using different extraction solvents varied from 72.29 ± 4.83 to 90.45 ± 5.44 mg of Gallic Acid Equivalent/ml of extract for water and ethanol 70% HCl 1%, respectively. There was no statistical difference between the samples, not being possible to use this parameter to determine the best extraction solution (data not shown).

The HPLC analysis showed that the extraction with 70% methanol, 70% ethanol acidified with 1% HCl and 50% ethanol resulted in higher content of the quantified flavanones (Fig. 1). Due to the lack of difference in methanol or ethanol as extraction solvent, it is justified the use of solutions with ethanol, since it is a food grade solvent. Still, aiming the lower solvent use, and considering the statistical similarity between the results, we selected the solution of 50% ethanol for extraction of flavanones from the "Biotransformed" residue.

Despite the widespread use of Folin Ciocalteu assay, this was not a good method for screening the best extracting solution. There was no significant difference between the samples according to this analysis, even though the HPLC results clearly indicating smaller potential of water as an extracting solvent for this system.

3.2. Characterization of "Biotransformed" residue extract obtained from the 50% ethanol solution

There was no difference in the content of total polyphenols by Folin Ciocalteu assay between the "Biotransformed" residue and the controls (Table 1).

However, once again the HPLC analysis showed difference between the samples. The "In Natura" residue had higher content of glycosides flavanones, naringin and hesperidin, while the "Biotransformed" residue had higher level of the aglycone flavanones, naringenin and hesperitin (Fig. 2). These results demonstrate that the fermentation process caused the biotransformation of the flavanones, increasing the amount of flavonoids free form.

The results in Fig. 2 indicate that the sterilization process by autoclaving degraded a certain amount of flavanones. However, this is a necessary step in the fermentation process to eliminate any microbial contamination present in the residue and allow only the reaction by the inoculated one.

Despite the fact that the "Biotransformed" residue extract presented a much smaller amount of total flavanones than the controls (Fig. 2), the antioxidant capacity of the extracts was similar according to DPPH and ORAC assays (Table 1), indicating that the flavanones presented in the "Biotransformed" residue had higher antioxidant potential.

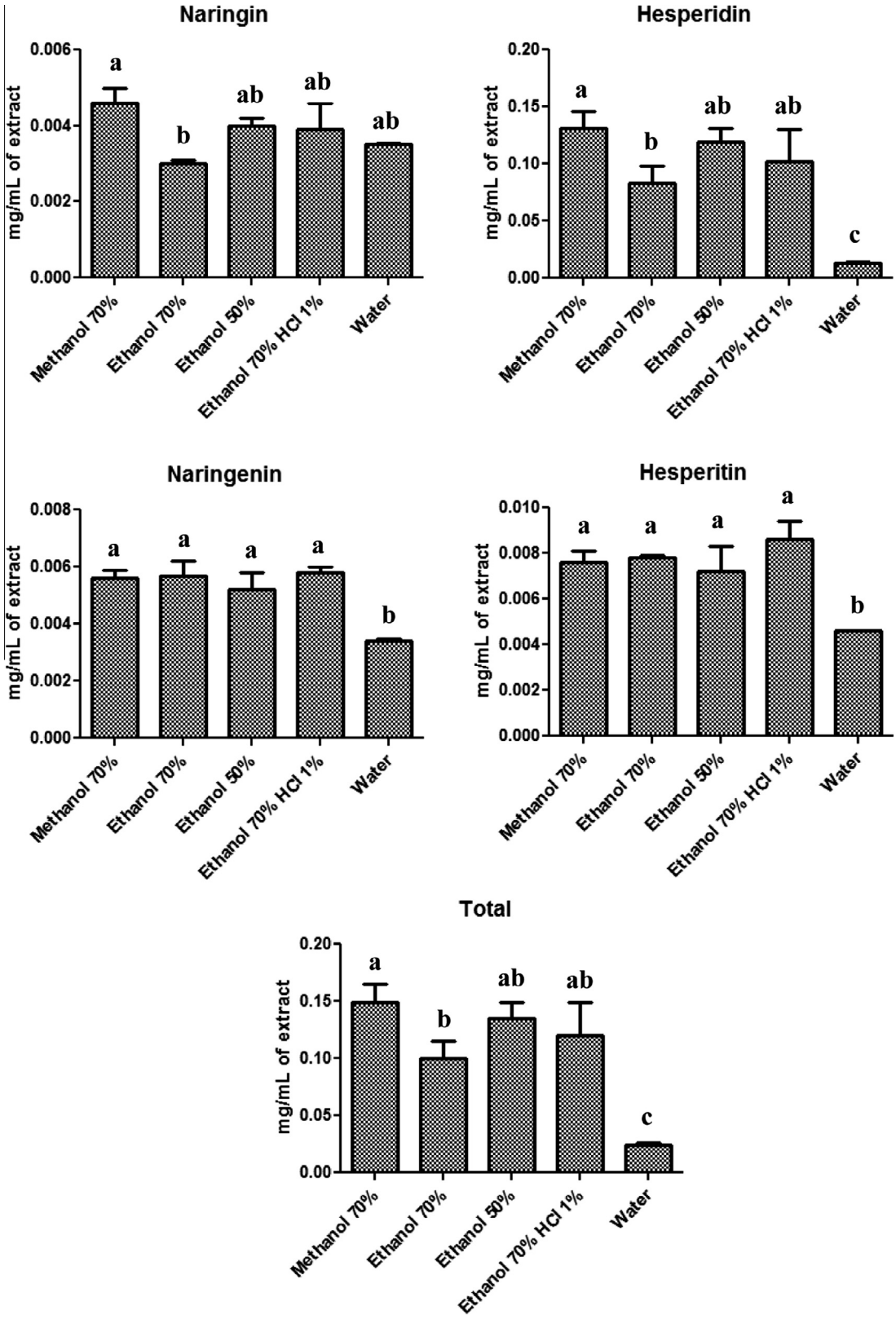

Fig. 1. Flavonoids of interest quantified by High Performance Liquid Chromatography (mg/mL of extract). Different letters indicate significant differences by Tukey's test ($p \leqslant 0.05$).

3.3. Cell assays

According to MTT assay, none of the extracts were toxic to 3T3-L1 cell line in the concentrations tested (Fig. 3). Since no loss of cell viability was observed in the concentration range that the cells were exposed, it is considered safe to continue with the following cellular assays within the concentrations tested.

The analysis Oil Red O staining showed that when the samples were added in the differentiation medium, there was little effect in the total lipid accumulation (Table 2). The best result was observed

Table 1
Total polyphenols, ORAC and DPPH radical-scavenging activity of the extracts.

	Total polyphenol (mg gallic acid equivalent/mg of liofilized extract)	DPPH (μmol equivalent Trolox/mg of liofilized extract)	ORAC (μmol equivalent Trolox/mg of liofilized extract)
Biotransformed	35.04 ± 2.36[a]	136.77 ± 5.41[a]	542.93 ± 78.04[a]
In Natura	36.23 ± 3.01[a]	130.80 ± 11.17[a]	666.99 ± 110.54[a]
Autoclaved	33.31 ± 1.03[a]	129.17 ± 6.71[a]	658.38 ± 70.87[a]

Different letters in the column indicate significant differences by Tukey's test ($p \leqslant 0.05$).

with the addition of "In Natura" extract in the concentration of 0.5 mg/mL, presenting a lipid accumulation reduction of about 19% in relation to control cells with no treatment.

On the other hand, the addition of the extracts in the maturation medium caused a decrease in the lipid accumulation (Table 3). This reduction was dose-dependent for all the samples, reaching a reduction of 22%, 38% and 48% for "Biotransformed", "Autoclaved" and "In Natura" extracts, respectively.

The total glycerol concentration was below the limit of detection for the cells treated with "In Natura" and "Autoclaved" extracts. Thus, the "Biotransformed" residue extract was the only that presented some effect on the amount of glycerol released, and the values observed presented dose dependent behavior (2.39 ± 0.17–5.24 ± 0.29 mg/mL of glycerol).

4. Discussion

The Folin Ciocalteu assay was not useful to distinguish the samples. It is noteworthy that the color reaction the Folin Ciocalteu assay is based on not only occurs due to the presence of polyphenols, but can also be caused by other compounds with reducing power (Huang, Ou, & Prior, 2005).

The total amount of flavanones was statistically similar when using methanol or ethanol as extraction solvents. Solutions containing methanol are the most used in the extraction of polyphenols of solid materials (Hayat et al., 2010; Ramful et al., 2010; Singh et al., 2011). However, due to its toxicity (Liesivuori & Savolainen, 1991; Tephly, 1991), it is interesting the development of a process using more friendly solvents. The results presented indicate the potential of 50% ethanol solution in extraction of the flavanones naringin, hesperidin, naringenin and hesperitin from fermented citrus residue. Thereby, this process used a solvent less harmful to health, increasing the feasibility of the extract in studies with cell culture, animal models and humans. Still, using ethanol instead of methanol takes into account economic considerations imposed by the industrial context.

The HPLC analysis clearly showed the change in flavanones profile in "Biotransformed" extract. According to Madeira et al. (2014) the microorganism employed in this process probably uses naringin and hesperidin as source of carbon and energy during fermentation. In previous study of our group, it was observed that the tannase produced by *P. variotii* strain during fermentation has the ability to catalyze the flavanones deglycosylation, such as hesperidin (Ferreira et al., 2013). Also, in microbial fermentation the compounds like hesperitin and naringenin are transformed in other chemical particles, with lower molecular weight, that are used in microorganism metabolism (Justesen, Arrigoni, Larsen, & Amado, 2000). Aguilar, Aguilera-carbo, and Robledo (2008) observed that the solid-state fermentation of creosote bush leaves and pomegranate peels transformed the tannins present in the substrate into lower molecular weight phenolics, confirming the fact that the fermentation process is able to change the phenolic profile of a product.

Even using a citrus residue from pectin industry waste, the quantity of flavanones extracted was comparable to other studies. Yu et al. (2014) and Ho, Su, and Lin (2013) performed the extraction of flavonoids from fruits acquired for the research, taking care in the acquisition, transport and storage of raw materials. The first authors were able to extract 1.278 mg/g FW of naringin and 1.480 mg/g FW of hesperidin, indicating values comparable to the present work for hesperidin. In the second study, the extraction was performed in nine different citrus fruits, and for five of them the amount of hesperidin and naringin was lower than that obtained in the present study. This data reinforces the advantage of the produced extract, once it was obtained from low cost industrial waste residue, commonly used for animal feed, presenting a possibility to increase the commercial value of this product.

Despite the lower content of total flavanones in "Biotransformed" extract, antioxidant activity by DPPH and ORAC was similar between the samples. The literature indicates that in some cases hesperitin and naringenin have higher biological potential than hesperidin and naringin. Londoño-Londoño et al. (2010) demonstrated that hesperitin was more active than hesperidin in reducing lipid peroxidation in hepatic microsomes, with lower amount of TBARS. Moreover, these authors observed that at low concentrations (10 mg/mL) hesperitin was more effective in reducing oxidized LDL by peroxynitrite-oxidized LDL model. Silva et al. (2013) biotransformed orange and lime juices by enzymatic deglycosylation and observed higher antioxidant activity by DPPH method and FRAP assay after the biotransformation, indicating the higher antioxidant activity of the aglycones obtained.

According to the results, all extracts were able to reduce lipogenesis *in vitro*, however, the data found seem to indicate that each extract have a different mechanism of action. Only the "In Natura" extract showed some effect on the inhibition of pre-adipocytes differentiation (Fig. 3), and may have a promising application in preventing the formation of new mature adipocytes. Kim et al. (2012) also observed that the addition of Citrus aurantium Flavonoids extract inhibited 3T3-L1 differentiation with a reduction in the amount of lipid droplets, confirming this positive effect of flavonoids from citrus.

When the extracts were added to the maturation medium, all samples were able to reduce lipid accumulation, with a greater effect of "In Natura" extract. However, only "Biotransformed" extract, with higher content of hesperitin and naringenin, caused induction of lipolysis, observed by higher amount of free glycerol on the supernatant of the culture. It is noteworthy that "Biotransformed" extract had lower amount of flavanones per gram of liofilized extract in comparison to "In Natura" extract (Fig. 2), and despite this great difference, the "Biotransformed" extract was able to reduce lipids content in the cells as "In Natura" extracts, and it was the only one able to cause lipolysis.

Subash-Babu and Alshatwi (2014) studied the effects of 20 μM hesperitin analytical standard in immortalized human bone marrow mesenchymal stem-cell (TERT20) differentiated with dexamethasone, IBMX, indomethacin and insulin. Hesperitin was added in two different situations: in group 1 the flavanone was administered in the differentiation medium; in group 2 the compound was added after the differentiation in the maintenance medium. In both cases, there was a reduction on lipid accumulation according to staining with Oil Red O, even though the effect was more pronounced in group 2, similar to our results. They also observed a slight stimulation of lipolysis, confirming the lipolytic activity of hesperitin.

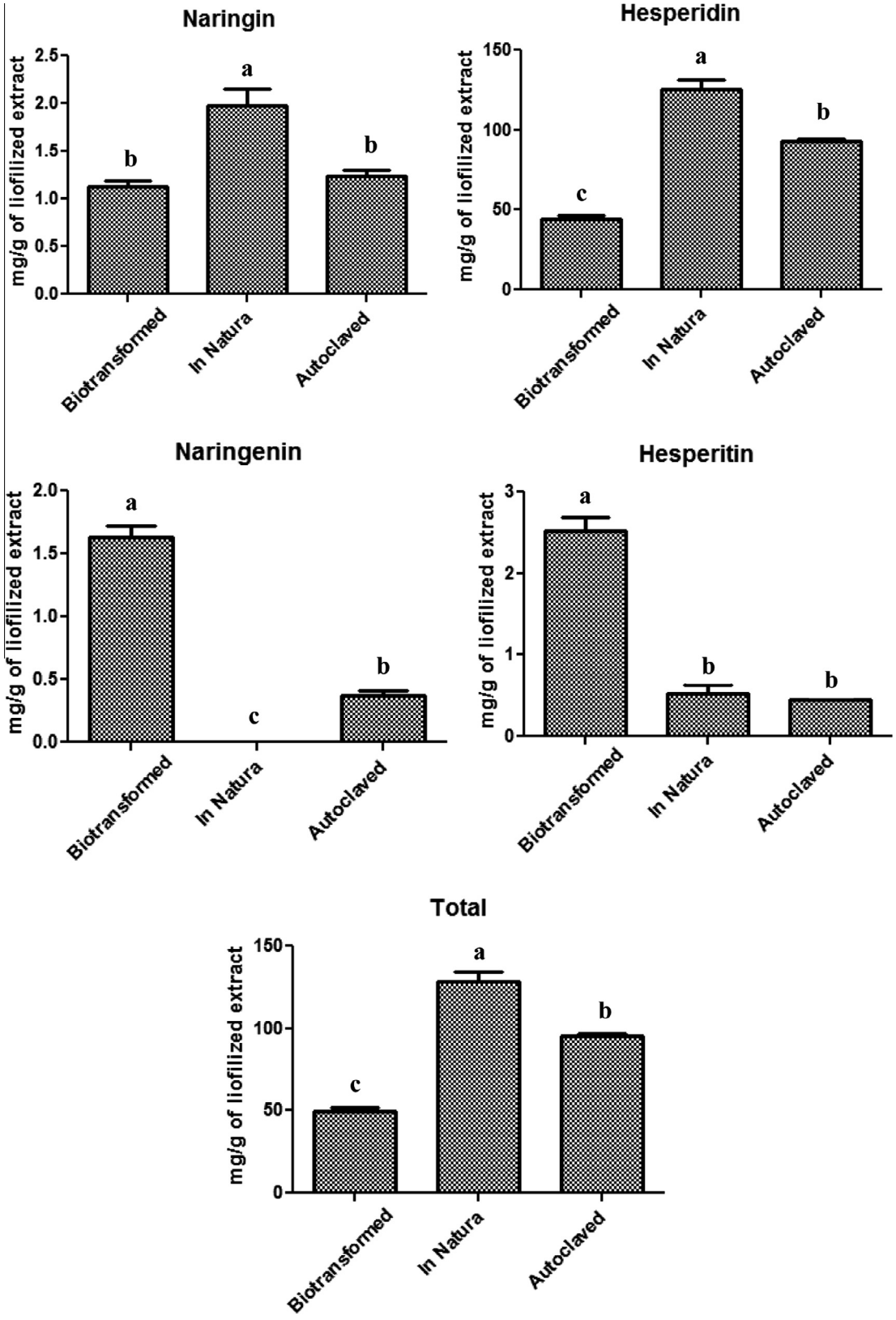

Fig. 2. Flavonoids of interest quantified by High Performance Liquid Chromatography (mg/g of liofilized extract). Different letters indicate significant differences by Tukey's test ($p \leqslant 0.05$).

Some studies have been shown that hesperitin (Gamo, Shiraki, Matsuura, & Miyachi, 2014) and naringenin (Yoshida et al., 2013) seems to act as PPAR γ agonist. PPARγ is a nuclear transcription factor that induce adipocyte differentiation (Kubota et al., 1999), causing greater differentiation of preadipocytes to mature adipocytes when it is activated. This could explain why the "Biotransformed" extract were not able to reduce the differentiation process. Some researchers have found that when these flavonoids

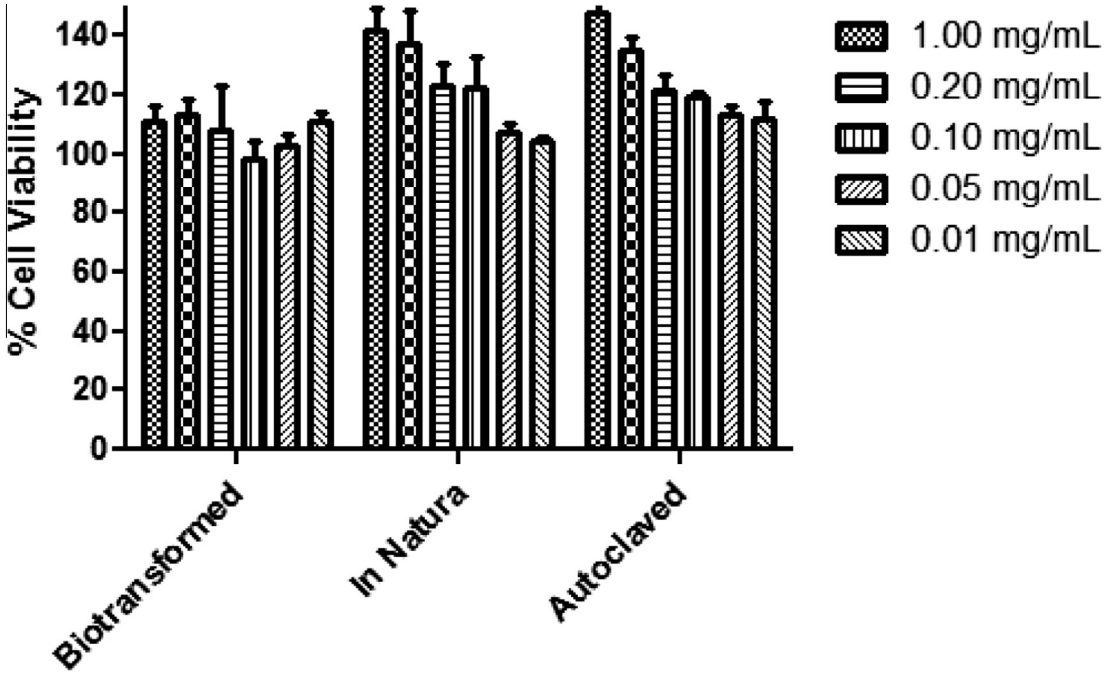

Fig. 3. Cell viability of 3T3-L1 cell line according to MTT assay.

Table 2
Oil Red O staining with samples added in the differentiation medium, % in relation to control.

Samples	Concentration			
	0.05 mg/mL	0.20 mg/mL	0.50 mg/mL	1.00 mg/mL
Biotransformed	101.52 ± 10.00[a]	100.00 ± 4.50[a]	103.21 ± 4.55[b]	107.22 ± 4.03[b]
In Natura	95.59 ± 1.52[a]	93.98 ± 3.94[a]	80.96 ± 4.19[a]	90.46 ± 4.61[a]
Autoclaved	103.45 ± 3.71[a]	99.17 ± 3.26[a]	96.05 ± 2.79[b]	98.39 ± 2.22[a]

Different letters in the column indicate significant difference between the samples in the same concentration by Tukey's test ($p \leqslant 0.05$).

Table 3
Oil Red O staining with samples added in the maintenance medium, % in relation to control.

Samples	Concentration			
	0.05 mg/mL	0.20 mg/mL	0.50 mg/mL	1.00 mg/mL
Biotransformed	97.56 ± 1.15[b]	84.67 ± 5.37[b]	89.45 ± 7.70[c]	78.18 ± 6.50[b]
In Natura	86.91 ± 3.55[a]	65.67 ± 4.30[a]	55.96 ± 3.50[a]	52.89 ± 2.04[a]
Autoclaved	103.59 ± 1.11[c]	88.86 ± 2.14[b]	75.65 ± 4.12[b]	62.66 ± 2.93[a]

Different letters in the column indicate significant difference between the samples in the same concentration by Tukey's test ($p \leqslant 0.05$).

were added to 3T3-L1 cell culture, it was observed a greater accumulation of lipid droplets possibly due to its agonist role (Gamo et al., 2014; Morikawa et al., 2008; Yoshida et al., 2013). However, in our study, despite the possible action of hesperitin and naringenin as PPAR γ agonist, there was an expressive reduction in lipid accumulation, which can be explained by the lipolytic role played by these aglycones. Still, we must consider that the afore mentioned studies were done with analytical standards, evaluating each compound alone. In the present work, we used a crude extract, and differences in response can occur due to the synergistic effect that compounds together may cause.

There are many studies indicating the potential of citrus extract in obesity treatment (Kang et al., 2012; Kim et al., 2012), however for the first time it is documented promising results with an extract rich in aglycones. The studies using aglycones usually test high cost analytical standards isolated, missing the synergistic effects of the natural extracts we propose.

Still, some authors indicate other advantages of aglycone forms in obesity treatment. Kim, Park, Kim, Lee, and Rhyu (2013) found that hesperitin caused higher secretion of cholecystokinin (CCK) in STC-1 cells in comparison to hesperidin, indicating a possible role of this aglycone flavanone in food intake control since CCK is an anorexigen hormone (Raybould, 2009). Thus, these other information suggests the potential of the extract produced by biotransformation to other biological activities, being suitable for further studies.

5. Conclusions

Ethanol can replace methanol as extraction solvent of flavanones from biotransformed citrus residue. The biotransformation was able to modify the flavanones profile of the citrus residue extract, increasing the content of hesperitin and naringenin that naturally occur in low quantities in citrus fruits. Still, all extracts could be used in obesity treatment, however aiming different targets. The "In Natura" extract would be useful to reduce new adipocytes synthesis and lipid accumulation, and "Biotransformed" extract could be used to induce lipolysis on fat tissue.

Acknowledgements

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References

Aguilar, C. N., Aguilera-carbo, A., & Robledo, A. (2008). Production of antioxidant nutraceuticals by solid-state cultures of pomegranate (*Punica granatum*) peel and creosote bush (*Larrea tridentata*) leaves. *Food Technology and Biotechnology, 46*(2), 218–222.

Alam, M. A., Kauter, K., & Brown, L. (2013). Naringin improves diet-induced cardiovascular dysfunction and obesity in high carbohydrate, high fat diet-fed rats. *Nutrients, 5*(3), 637–650. http://dx.doi.org/10.3390/nu5030637.

Barros, H. R. D. M., Ferreira, T. A. P. D. C., & Genovese, M. I. (2012). Antioxidant capacity and mineral content of pulp and peel from commercial cultivars of citrus from Brazil. *Food Chemistry, 134*(4), 1892–1898. http://dx.doi.org/10.1016/j.foodchem.2012.03.090.

Benavente-García, O., Castillo, J., Marin, F. R., Ortuño, A., & Del Río, J. A. (1997). Uses and properties of Citrus flavonoids. *Journal of Agricultural and Food Chemistry, 45*(12), 4505–4515.

Caridi, D., Trenerry, V., Rochfort, S., Duong, S., Laugher, D., & Jones, R. (2007). Profiling and quantifying quercetin glucosides in onion (*Allium cepa* L.) varieties using capillary zone electrophoresis and high performance liquid chromatography. *Food Chemistry, 105*(2), 691–699. http://dx.doi.org/10.1016/j.foodchem.2006.12.063.

Dallas, C., Gerbi, A., Elbez, Y., Caillard, P., Zamaria, N., & Cloarec, M. (2013). Clinical study to assess the efficacy and safety of a citrus polyphenolic extract of red orange, grapefruit, and orange (Sinetrol-XPur) on weight management and metabolic parameters in healthy overweight individuals. *Phytotherapy Research, 1–7*. http://dx.doi.org/10.1002/ptr.4981.

Dallas, C., Gerbi, A., Tenca, G., Juchaux, F., & Bernard, F.-X. (2008). Lipolytic effect of a polyphenolic citrus dry extract of red orange, grapefruit, orange (SINETROL) in human body fat adipocytes. Mechanism of action by inhibition of cAMP-phosphodiesterase (PDE). *Phytomedicine, 15*, 783–792. http://dx.doi.org/10.1016/j.phymed.2008.05.006.

Dávalos, A., Gómez-Cordovés, C., & Bartolomé, B. (2004). Extending applicability of the oxygen radical absorbance capacity (ORAC-fluorescein) assay. *Journal of Agricultural and Food Chemistry, 52*(1), 48–54. http://dx.doi.org/10.1021/jf0305231.

De Mejía, E. G., Song, Y. S., Heck, C. I., & Ramírez-Mares, M. (2010). Yerba mate tea (Ilex paraguariensis): Phenolics, antioxidant capacity and in vitro inhibition of colon cancer cell proliferation. *Journal of Functional Foods, 2*(1), 23–34. http://dx.doi.org/10.1016/j.jff.2009.12.003.

Ferreira, L. R., Macedo, J. A., Ribeiro, M. L., & Macedo, G. A. (2013). Improving the chemopreventive potential of orange juice by enzymatic biotransformation. *Food Research International, 51*(2), 526–535. http://dx.doi.org/10.1016/j.foodres.2013.01.018.

Gamo, K., Shiraki, T., Matsuura, N., & Miyachi, H. (2014). Mechanism of peroxisome proliferator-activated receptor gamma (PPAR γ) transactivation by hesperetin glucuronides is distinct from that by a thiazolidine-2, 4-dione agent. *Chemical & Pharmaceutical Bulletin, 62*(May), 491–493. http://dx.doi.org/10.1248/cpb.c14-00021.

Hayat, K., Zhang, X., Farooq, U., Abbas, S., Xia, S., Jia, C., ... Zhang, J. (2010). Effect of microwave treatment on phenolic content and antioxidant activity of citrus mandarin pomace. *Food Chemistry, 123*(2), 423–429. http://dx.doi.org/10.1016/j.foodchem.2010.04.060.

Hirata, A., Murakami, Y., Shoji, M., Kadoma, Y., & Fujisawa, S. (2005). Kinetics of radical-scavenging activity of hesperetin and hesperidin and their inhibitory activity on COX-2 expression. *Anticancer Research, 25*(5), 3367–3374.

Ho, S.-C., Su, M.-S., & Lin, C.-C. (2013). Comparison of peroxynitrite-scavenging capacities of several citrus fruit peels. *International Journal of Food Properties, 17*(1), 111–124. http://dx.doi.org/10.1080/10942912.2011.614987.

Huang, D., Ou, B., & Prior, R. L. (2005). The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry, 53*(6), 1841–1856. http://dx.doi.org/10.1021/jf030723c.

Justesen, U., Arrigoni, E., Larsen, B. R., & Amado, R. (2000). Degradation of flavonoid glycosides and aglycones during in vitro fermentation with human faecal flora. *LWT – Food Science and Technology, 33*(6), 424–430. http://dx.doi.org/10.1006/fstl.2000.0681.

Kang, S.-I., Shin, H.-S., Kim, H.-M., Hong, Y.-S., Yoon, S.-A., Kang, S.-W., ... Kim, S.-J. (2012). Immature *Citrus sunki* peel extract exhibits antiobesity effects by β-oxidation and lipolysis in high-fat diet-induced obese mice. *Biological & Pharmaceutical Bulletin, 35*(2), 223–230. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/22293353>.

Kim, H. Y., Park, M., Kim, K., Lee, Y. M., & Rhyu, M. R. (2013). Hesperetin stimulates cholecystokinin secretion in enteroendocrine STC-1 cells. *Biomolecules & Therapeutics, 21*(2), 121–125.

Kim, G.-S., Park, H. J., Woo, J.-H., Kim, M.-K., Koh, P.-O., Min, W., ... Cho, J.-H. (2012). *Citrus aurantium* flavonoids inhibit adipogenesis through the Akt signaling pathway in 3T3-L1 cells. *BMC Complementary and Alternative Medicine, 12*(31), 1–10. http://dx.doi.org/10.1186/1472-6882-12-31.

Kubota, N., Terauchi, Y., Miki, H., Tamemoto, H., Yamauchi, T., Komeda, K., ... Kadowaki, T. (1999). PPAR γ mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance. *Molecular Cell, 4*(4), 597–609. http://dx.doi.org/10.1016/S1097-2765(00)80210-5.

Li, Y., Li, X., Li, G., Du, W., Zhang, J., Li, W., ... Zhu, Z. (2008). In vivo pharmacokinetics of hesperidin are affected by treatment with glucosidase-like Bg1A protein isolated from yeasts. *Journal of Agriculture and Food Chemistry, 56*(14), 5550–5557. http://dx.doi.org/10.1021/jf800105c.In.

Liesivuori, J., & Savolainen, H. (1991). Methanol and formic acid toxicity: Biochemical mechanisms. *Pharmacology and Toxicology, 69*(3), 157–163. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/1665561>.

Londoño-Londoño, J., De Lima, V. R., Lara, O., Gil, A., Pasa, T. B. C., Arango, G. J., & Pineda, J. R. R. (2010). Clean recovery of antioxidant flavonoids from citrus peel: Optimizing an aqueous ultrasound-assisted extraction method. *Food Chemistry, 119*(1), 81–87. http://dx.doi.org/10.1016/j.foodchem.2009.05.075.

Macedo, J. A., Battestin, V., Ribeiro, M. L., & Macedo, G. A. (2011). Increasing the antioxidant power of tea extracts by biotransformation of polyphenols. *Food Chemistry, 126*, 491–497. http://dx.doi.org/10.1016/j.foodchem.2010.11.026.

Madeira, J. V., Nakajima, V. M., Macedo, J. A., & Macedo, G. A. (2014). Rich bioactive phenolic extract production by microbial biotransformation of Brazilian Citrus residues. *Chemical Engineering Research and Design, 92*(10), 1802–1810. http://dx.doi.org/10.1016/j.cherd.2014.07.014.

Morikawa, K., Nonaka, M., Mochizuki, H., Handa, K., Hanada, H., & Hirota, K. (2008). Naringenin and hesperetin induce growth arrest, apoptosis, and cytoplasmic fat deposit in human preadipocytes. *Journal of Agricultural and Food Chemistry, 56*(22), 11030–11037. http://dx.doi.org/10.1021/jf801965n.

Ramful, D., Bahorun, T., Bourdon, E., Tarnus, E., & Aruoma, O. I. (2010). Bioactive phenolics and antioxidant propensity of flavedo extracts of Mauritian citrus fruits: Potential prophylactic ingredients for functional foods application. *Toxicology, 278*(1), 75–87. http://dx.doi.org/10.1016/j.tox.2010.01.012.

Raybould, H. E. (2009). Mechanisms of CCK signaling from gut to brain. *Current Opinion in Pharmacology, 7*(6), 570–574. http://dx.doi.org/10.1016/j.coph.2007.09.006.

Silva, C. M. G., Contesini, F. J., Sawaya, A. C. H. F., Cabral, E. C., da Silva Cunha, I. B., Eberlin, M. N., & de Oliveira Carvalho, P. (2013). Enhancement of the antioxidant activity of orange and lime juices by flavonoid enzymatic de-glycosylation. *Food Research International, 52*(1), 308–314. http://dx.doi.org/10.1016/j.foodres.2013.03.019.

Singh, J., Sood, S., & Muthuraman, A. (2011). In-vitro evaluation of bioactive compounds, anti-oxidant, lipid peroxidation and lipoxygenase inhibitory potential of *Citrus karna* L. peel extract. *Journal of Food Science and Technology*. http://dx.doi.org/10.1007/s13197-011-0479-9.

Singleton, V. L., Orthofer, R., & Lamuela-Raventós, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin–Ciocalteu reagent. In L. Packer (Ed.). *Oxidants and Antioxidants Part A* (Vol. 299, pp. 152–178). Academic Press. http://dx.doi.org/10.1016/S0076-6879(99)99017-1.

Subash-Babu, P., & Alshatwi, A. A. (2014). Hesperitin inhibit adipocyte differentiation and enhance Bax- and p21-mediated adipolysis in human mesenchymal stem cell adipogenesis. *Journal of Biochemical Molecular Toxicology, 00*, 1–10. http://dx.doi.org/10.1002/jbt.

Sun, Y., Qiao, L., Shen, Y., Jiang, P., Chen, J., & Ye, X. (2013). Phytochemical profile and antioxidant activity of physiological drop of citrus fruits. *Journal of Food Science, 78*(1), C37–C42. http://dx.doi.org/10.1111/j.1750-3841.2012.03002.x.

Tephly, T. R. (1991). The toxicity of methanol. *Life Sciences, 48*(6), 1031–1041.

Turner, T., & Burri, B. J. (2013). Potential nutritional benefits of current citrus consumption. *Agriculture, 3*, 170–187. http://dx.doi.org/10.3390/agriculture3010170.

Um, M. Y., Moon, M. K., Ahn, J., & Youl Ha, T. (2013). Coumarin attenuates hepatic steatosis by down-regulating lipogenic gene expression in mice fed a high-fat diet. *The British Journal of Nutrition, 109*, 1590–1597. http://dx.doi.org/10.1017/S0007114512005260.

Yoshida, H., Takamura, N., Shuto, T., Ogata, K., Tokunaga, J., Kawai, K., & Kai, H. (2010). The citrus flavonoids hesperetin and naringenin block the lipolytic actions of TNF-alpha in mouse adipocytes. *Biochemical and Biophysical Research Communications, 394*, 728–732. http://dx.doi.org/10.1016/j.bbrc.2010.03.060.

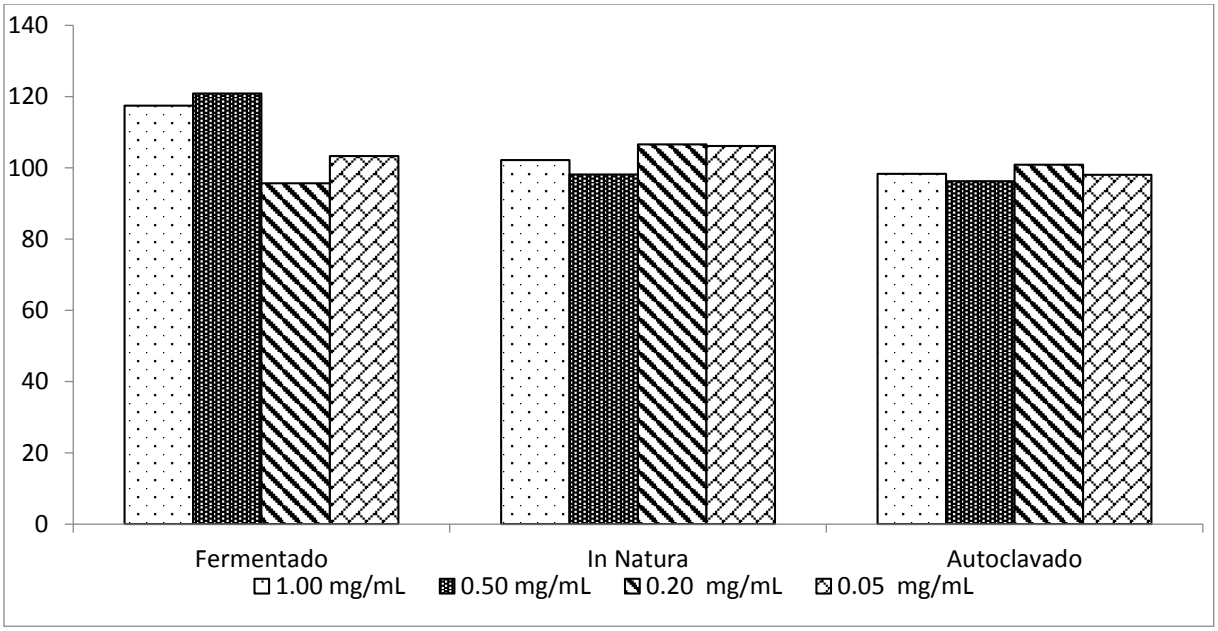
Yoshida, H., Watanabe, W., Oomagari, H., Tsuruta, E., Shida, M., & Kurokawa, M. (2013). Citrus flavonoid naringenin inhibits TLR2 expression in adipocytes. *The Journal of Nutritional Biochemistry, 24*(7), 1276–1284. http://dx.doi.org/10.1016/j.jnutbio.2012.10.003.

Yu, E. A., Kim, G. S., Jeong, S. W., Park, S., Lee, S. J., Kim, J. H., ... Shin, S. C. (2014). Flavonoid profile and biological activity of Korean citrus varieties (II): Pyunkyul (*Citrus tangerina* Hort. ex Tanaka) and overall contribution of its flavonoids to antioxidant effect. *Journal of Functional Foods, 6*(1), 637–642. http://dx.doi.org/10.1016/j.jff.2013.12.007.

APPENDIX 3 - Resultados não incluídos nos artigos.

Os resultados a seguir apresentados não foram incluídos nos artigos publicados/submetidos, entretanto serão apresentados para indicar a execução dos ensaios.

Na figura abaixo está apresentado o resultado de citotoxicidade por MTT nos adipócitos pós-diferenciação. Esse ensaio foi realizado a fim de definir se as amostras, além de não serem tóxicas para os pré-adipócitos, também não seriam para os adipócitos formados. Pelos valores de porcentagem em relação ao controle, podemos observar que nenhum dos extratos foi tóxico para as células, pois em todas as concentrações testadas o valor foi muito próximo ou superior a 100% do controle, que não recebeu nenhum tratamento. Desta forma, comprova-se que os efeitos na redução da concentração de lipídeos pela coloração com Oil Red O realmente se deve a menor quantidade de gordura, e não a morte celular. Esse resultado não foi incluído no artigo original 1 pois o teste foi realizado após a publicação do mesmo.



Ensaio de MTT nos adipócitos pós-diferenciação

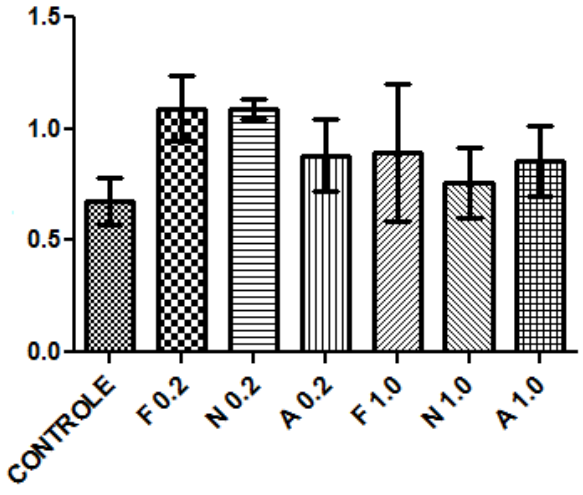
Na tentativa de verificar se os extratos eram capazes de reduzir a quantidade de lipídeo nos adipócitos após a diferenciação completa, ou seja, induzir a lipólise em adipócito maduro, testou-se a adição dos extratos no décimo dia após a diferenciação. Como pode ser observado na tabela a seguir, a adição dos extratos não apresentou influência na concentração de lipídeo pela coloração com Oil Red O. Entretanto, como as células nesse estágio desgrudam

com facilidade (característica do adipócito maduro), não foi possível realizar o tratamento de oito dias, como planejado inicialmente, sendo feito apenas por 4 dias. Esse curto tempo de tratamento pode ter atrapalhado a possível visualização de algum efeito, já que a coloração por Oil Red O não é método muito sensível para detectar pequenas diferenças.

Ensaio de coloração por Oil Red O em células 3T3-L1 que receberam tratamento com os extratos após diferenciação (% em relação ao Controle)

	Concentração	Biotransformado	In Natura	Autoclavado
Sem insulina	1,0 mg/mL	119,35±7,44	117,16±4,51	105,60±2,85
	0,2 mg/mL	113,24±5,43	119,49±7,27	109,31±458
Com insulina	1,0 mg/mL	117,67±11,17	116,82±1,74	106,98±7,20
	0,2 mg/mL	108,83±11,65	112,77±6,87	113,96±5,48

A figura a seguir ilustra os resultados obtidos na análise de expressão proteica de PPAR-α na células 3T3-L1 após tratamento com o extratos adicionados ao meio de maturação. Como pode ser observado, a adição dos extratos houve menor expressão de PPAR-α em relação ao controle, entretanto sem diferença significativa. Isso indica o potencial papel da amostra na indução a lipólise, visto a importância dessa proteína para esse processo. Esse resultado não foi incluído no Artigo Original 1 pois a análise foi realizada após a publicação do mesmo, e isso ocorreu devido a limitações financeiras para a compra dos anticorpos no período de análises do primeiro artigo.



Western blot de PPAR-α em células 3T3-L1.

APPENDIX 4 - Artigo original do grupo de trabalho intitulado “Rich bioactive phenolic extract production by microbial biotransformation of Brazilian citrus residues” publicado na revista Chemical Engineering Research and Design.

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Rich bioactive phenolic extract production by microbial biotransformation of Brazilian *Citrus* residues

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A B S T R A C T

Flavanones in *Citrus* are molecules that play an important role in antioxidant activities in nutraceutical products. Recent studies indicate that molecules of the simplest classes of phenolics have higher biological activity and absorption capacity. However, the molecules that have been shown to be very important bioactive compounds of *Citrus*, such as hesperetin, naringenin and ellagic acid, are found in trace concentrations in the fruit. An interesting environmentally friendly alternative that deserves attention regarding phenolic compound obtaining is the biotransformation of these molecules. The aim of this study was to develop a process of biotransformation of phenolics from Brazilian *Citrus* residues by solid-state fermentation with the microorganism *Paecilomyces variotii*. The optimized fermentation conditions were 10 g of *Citrus* residues (2.0 mm of substrate particle size), 20 mL distilled water, at 32 °C after 48 h of incubation. The development of this process has generated, simultaneously, an increase of 900, 1400 and 1330% of hesperetin, naringenin and ellagic acid concentration, respectively, and an increase of 73% of the antioxidant capacity. These results give strong evidence that microbial biotransformation does not only produce phenolic compounds but also compounds with high biological activity, such as hesperetin and naringenin.

Keywords: Agro-industrial residues; Solid-state fermentation; Tannase; *Paecilomyces variotii*; Flavanones; Antioxidant

1. Introduction

In recent years, *Citrus* flavonoids have gained much interest due to their chemoprotective effects. *Citrus* flavonoids exhibit antioxidant, antimicrobial, anticarcinogenic, antiviral, anti-allergic and anti-inflammatory activities. Through these benefits, there is interest in replacing synthetic food antioxidant substances with natural ones, which has fostered research on vegetable sources and the screening of waste materials aimed at identifying new and/or better antioxidant sources (Ferreres et al., 2012; Lin et al., 2012; Sergent et al., 2012; Tripoli et al., 2007).

There are many classes of flavonoids, flavanones being the most abundant group in *Citrus* fruits (Barros et al., 2012; Ferreira et al., 2013). Flavanones are highly present in plant species from the genus *Citrus*, abundant in the by-products, mostly in peels and pectinolytic material, accounting for 4–12% of the dry weight (Marín et al., 2007). The most prevalent flavanones in tissues and peels of *Citrus* fruits are naringin and hesperidin. Naringin exhibits many health benefits, including

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an ability to prevent cancer by suppression of carcinogenesis and inducing cell apoptosis (Meiyanto et al., 2012). Hesperidin has also been reported to reduce the proliferation of many cancer cells and also possesses an anti-inflammatory effect (Ferreira et al., 2013; Nazari et al., 2011; Park et al., 2008). The glycoside and aglycone forms of flavanones possess several different biological functions. Several studies have revealed that aglycones are superior to glycosides in various bioactivities, due to their effective absorption (Murakami et al., 2008). Only free flavonoids without a sugar molecule were thought to be able to pass through the intestine wall. Hydrolysis only occurs in the colon by microorganisms, which at the same time degrade flavonoids (Hollman and Katan, 1997). According to Nielsen et al. (2006) and Ohguchi et al. (2006), when free phenolics (hesperetin and naringenin) in *Citrus* residues are released from their glycosides (hesperidin and naringin), the photoprotective functionality of these phytochemicals can be improved.

Other important compounds found in *Citrus* fruits are hydroxybenzoic acids, however in lower concentrations than flavanones. Ellagic acid is a very important compound from this category and has a variety of benefits for anti-mutagenic, antimicrobial and antioxidant properties, as well as being an inhibitor of human immunodeficiency viruses (Nutan et al., 2013; Martins et al., 2011; Sepúlveda et al., 2011). The presence of ellagic acid in various functional commercial products is observed. Improving ellagic acid content in *Citrus* residues could provide an interesting source of this compound for the industry. Microbial degradation of tannins is highly documented, and most works report that the selective hydrolysis of galloyl groups from ellagitannins was catalyzed by tannase (Prasad et al., 2012).

Currently, phenolic compounds are obtained by chemical synthesis or extraction. An interesting environmentally friendly alternative that deserves attention regarding phenolic compound production is the solid-state fermentation (Madeira et al., 2013; Banerjee et al., 2012; Martins et al., 2011). The current bioprocess (SSF) has many advantages, such as high concentration, product stability and growth of microorganisms in non-water soluble substrates, the process is usually cheaper with higher productivity than submerged fermentation (Barrios-González, 2012). The SSF on *Citrus* residue using the *Paecilomyces variotii* strain was initially developed by this research group for the production of tannase enzyme. The potential of this enzyme to produce more bioactive forms of the polyphenol molecule extract from vegetables was studied in previous works (Madeira et al., 2012). Georgetti et al. (2009) evaluated the biotransformation of polyphenol glycosides from soybeans to form non-glycosides through solid-state fermentation by *Aspergillus awamori*. The conversion of the glycoside to the form of phenolic non-glycoside was accompanied by production of the enzyme β-glucosidase. The non-glycoside form presents a greater number of free hydroxyl groups in regard to glycoside, thus increasing their biological activity. The microbial biotransformation of phenolic compounds seems to be a promising way to increase the concentration of phenolics with high biological potential.

The present work aimed to optimize some important parameters of this fermentation process in order to produce some phenolic compounds with high bioactivity, such as naringenin, hesperetin and ellagic acid, from *Citrus* residue. These have no viable source of extraction so far, being present in very low concentration in vegetables; however, literature shows their functional potential is increasing more and more every day.

2. Materials and methods

2.1. Materials

Hesperidin, hesperetin, naringin, naringenin, ellagic acid, 2,2′-azobis(2-methylpropionamidine) (97%) (AAPH), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Potato Dextrose Agar (PDA) were purchased from Sigma–Aldrich Co. Fluorescein was purchased from ECIBRA, and Trolox® (97%) was purchased from ACROS Organics. *Citrus* residue (from 5 different cultivars: *Citrus latifolia*, *Citrus sinensis* Hamlin, Valencia, Pera riu and Pera Natal) was kindly donated by CP Kelco industry headquarters (Limeira, SP, Brazil) from juice and pectin extraction, giving origin to a residue of low quality and commercial value.

2.2. Microorganism and inoculum preparation

The *P. variotii* strain was isolated and selected to be the tannase producer and grew on different agro-industrial residues such as castor bean cake, wheat bran and *Citrus* residue (Battestin and Macedo, 2004; Madeira et al., 2011, 2012). The fungus strain was deposited at the Brazilian Collection of Environmental and Industrial Microorganisms (CBMAI) under the number 1157. The *P. variotii* was preserved in PDA medium slants and refrigerated at 4 °C with Vaseline. For sporulation, the fungal strain was inoculated on plates containing PDA medium and incubated at 30 °C for 3 days. The spores were suspended in distilled water at a concentration of 9×10^6 spores/mL (Madeira et al., 2011).

2.3. Culture condition for phenolic compound production

Citrus residue was donated by CP Kelco industry headquarters (Limeira, SP, Brazil) as a dried residue. The residue was ground in a knife mill (Philips, RI 1725) and separated in a sieve shaker (Mesh 10, particle size under 1.86 mm).

The initial fermentation medium established in a previous work (Madeira et al., 2012) for tannase production consisted of the following: 250 mL Erlenmeyer flasks, in which 10 g of the *Citrus* residue was added to 10 mL of distilled water. After sterilization in an autoclave, the flasks were inoculated with 1 mL of spore suspension (9×10^6) and incubated at 30 °C at 90% relative humidity (Climate Chamber 420 CLD – Nova Etica, SP, Brazil) for up to 120 h.

After the incubation period, the tannase extraction was performed by adding 50 mL of acetate buffer (pH 5.5, 0.02 M) to 5 g of fermented substrate. The solution was shaken at 200 rpm for 1 h, and filtered and centrifuged at 10,070 × *g* for 30 min at 4 °C (Centrifuge Beckman J2-21, Beckman-Coulter, Inc., Fullerton, CA, USA). The supernatant was assayed for tannase activity. The phenolic compound extraction was performed by adding 25 mL of methanol 70%, to 1 g of fermented residue. The solution was treated in ultrasonic (Unique UltraSonic Cleaner model USC-1800A) at 40 kHz for 30 min, after being shaken at 200 rpm for 30 min and then passed through a 0.45 μm filter. The filtered extract was assayed for identification and quantification of phenolic compounds by HPLC-DAD (High Pressure Liquid Chromatography-Diode Array Detector).

2.4. Tannase activity assay

Tannase activity was evaluated according to Sharma et al. (2000), adapted using tannic acid as substrate. One unit of activity was defined as the amount of enzyme that released 1 μmol/min of gallic acid. Enzyme activity was expressed as total units (U) per g of dry substrate (gds) of dry solid medium (based on initial mass).

2.5. Identification and quantification of phenolic by HPLC-DAD analysis

HPLC phenolic analysis of the extracts was performed on a Dionex – Ultimate 3000 equipped with a 150 mm × 4.6 mm i.d. reversed phase C18 column (Waters, Massachusetts); detection was carried out at 260, 280 and 330 nm using a diode array detector. The solvents were A, H_2O (0.1% formic acid); and B, Methanol (0.1% formic acid). The gradient elution was as follows: 90% A (0–5 min), 20% A (5–80 min), 90% A (80–85 min), and 90% A at a flow rate of 0.6 mL/min. All HPLC analyses were performed at 30 °C. The Chromeleon software (version 6.8) was used for the data processing. Compounds were identified according to retention time and UV–vis spectra (260 nm of wavelength). A series of standards (hesperidin, hesperetin, naringin, naringenin, ellagic and gallic acid) solutions were used for the calibration curves and quantification of these phenolics on samples, the quantification was carried out at 280 nm using a diode array detector (Ferreira et al., 2013).

2.6. Fermentation parameter optimization for target phenolic production

Phenolic compounds and tannase production by *P. variotii* were observed for 120 h. The time of peak products was determined for the experimental design study.

The fermentation parameters that had the greatest influence on phenolic production were evaluated using the CCD (Central Composite Design) methodology. The independent variables were: particle size substrate (mm), water:substrate ratio (v:w) and temperature of incubation (°C). The water:substrate ratio was determined according to the maximum moisture absorption capacity of the *Citrus* residue. The variables were coded, according to Eq. (1):

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad (1)$$

Here, x_i is the coded variable, X_i is the natural variable of the nutrient factor, X_0 is the value of the natural variable at the center point, and ΔX_i is the step change value. The variables and levels are shown in Table 1.

The experimental design was defined as a full CCD methodology for 3 factors (2^3), consisting of 8 cubic points, 6 star points and 3 replicates at the center point, which served to estimate experimental error and to investigate the suitability of the proposed model, the details of which are presented in Table 1. The experimental results were fitted to a second-order polynomial function, and the Student's *t*-test allowed for checking of the statistical significance of the regression coefficients. Analysis of variance (ANOVA) was performed on the experimental data to evaluate the statistical significance of the model. The response model was expressed in terms of coded variables, ignoring the statistically non-significant terms. Therefore, the optimum conditions of fermentation process were performed to evaluate the productivity of phenolic compounds obtained (hesperetin, naringenin and ellagic acid) in Eq. (2).

$$Y = \left[\frac{PCf - PCi}{PCi}\right] \times 100\% \quad (2)$$

Here, *Y* is the yield of final product, *PCf* is the final phenolic concentration, *PCi* is the initial phenolic concentration (the data were obtained from Table 3).

2.7. Antioxidant potential

2.7.1. ORAC assay

ORAC (Oxygen Radical Absorbance Capacity) assays were performed using fluorescein (FL) as the fluorescent probe, as described by Macedo et al. (2011). The automated ORAC assay was carried out on a NovoStar Microplate reader (BMG LABTECH, Germany) with fluorescence filters for an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The measurements were made in a COSTAR 96 plate. The reaction was performed at 37 °C, having been started by thermal decomposition of AAPH in a 75 mM phosphate buffer (pH 7.4) due to the sensitivity of FL to pH. The measurements were performed in triplicate. ORAC values were defined as the difference between the area under the FL decay curve and the blank (net AUC). Regression equations between net AUC and antioxidant concentration were calculated for all of the samples. A tannase control was performed, and the ORAC value obtained was subtracted from the samples treated with the enzyme. ORAC-FL values were expressed as μmol of Trolox/g of the standards or mL of extracts.

2.7.2. DPPH assay

According to Macedo et al. (2011), the antioxidant capacity of standard and biotransformed hesperidin was assessed on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical. The reaction mixtures, consisting of 50 μl of test samples and 150 μl of 0.2 mM DPPH in methanol, were carried out on a NovoStar Microplate reader (BMG LABTECH, Germany) with absorbance filters for a wavelength of 520 nm. The decolorizing process was recorded after 90 min of reaction and compared with a blank control, instead of DPPH. The DPPH solution and reaction medium were freshly prepared and stored in the dark. The measurement was performed in triplicate. Antiradical activity was calculated from the equation determined from the linear regression after plotting known solutions of Trolox with various concentrations. Antiradical activity was expressed as μMol of Trolox equivalent/g of standards or mL of extracts.

2.8. Statistical analysis

A Statistica 7.0 software (Statsoft, Inc., Tulsa, USA) package was used, and all values reported in the biotransformation of *Citrus* residue represent the mean from three replicates and standard deviation. Significant differences ($p < 0.05$) in times of fermentation for phenolic production were determined by *t*-tests.

Table 1 – Coded levels and actual values (in parentheses) for the experimental design and results of the CCD.

Run	Independent variables[a]			Response (Δμg/g substrate)[b]		
	Substrate particle size (mm)	Water:substrate ratio (v:w)	Temperature (°C)	Hesperetin	Naringenin	Ellagic acid
1	−1 (1.2)	−1 (0.8:1)	−1 (30.0)	21.0	42.0	2320
2	1 (2.8)	−1 (0.8:1)	−1 (30.0)	25.0	42.0	7160
3	−1 (1.2)	1 (1.7:1)	−1(30.0)	50.0	57.0	8950
4	1 (2.8)	1 (1.7:1)	−1 (30.0)	33.0	46.0	13,230
5	−1 (1.2)	−1 (0.8:1)	1 (34.0)	23.0	44.0	2910
6	1 (2.8)	−1 (0.8:1)	1 (34.0)	26.0	47.0	4510
7	−1 (1.2)	1 (1.7:1)	1 (34.0)	38.0	57.0	6710
8	1 (2.8)	1 (1.7:1)	1 (34.0)	35.0	6.0	9000
9	−α (0.7)	0 (1.3:1)	0 (32.0)	68.0	72.0	6030
10	α (3.4)	0 (1.3:1)	0 (32.0)	33.0	31.0	14,540
11	0 (2.0)	−α (0.5:1)	0 (32.0)	9.0	33.0	2120
12	0 (2.0)	α (2.0:1)	0 (32.0)	54.0	70.0	16,570
13	0 (2.0)	0 (1.3:1)	−α (28.5)	30.0	41.0	8220
14	0 (2.0)	0 (1.3:1)	α (35.5)	32.0	36.0	6280
15	0 (2.0)	0 (1.3:1)	0 (32.0)	37.0	54.0	10,610
16	0 (2.0)	0 (1.3:1)	0 (32.0)	44.0	60.0	11,800
17	0 (2.0)	0 (1.3:1)	0 (32.0)	45.0	58.0	11,270

[a] Independents variables: substrate particle size (mm); water:substrate rate (v:w); temperature of incubation (°C).
[b] Response of phenolic compounds production between 0 and 48 h of incubation (μg phenolic/g substrate).

3. Results and discussion

3.1. *Fermentation parameter optimization*

The wild *P. variotii* strain was able to grow in Brazilian *Citrus* residue by solid-state fermentation, which resulted in the production of the extracellular enzyme tannase and phenolic compounds such as hesperetin, naringenin and ellagic acid (Fig. 1). The maximum concentrations of these phenolic compounds occurred after 48 h of fermentation; and the maximum enzymatic activity of tannase was obtained after 96 h of fermentation.

According to the results obtained, the total amount of hesperidin and naringin in the *Citrus* residue extract decreased 50 and 100% after 120 h of incubation, respectively. On the other hand, the initial concentration of hesperetin, naringenin and ellagic acid in the *Citrus* residue were below the detection limit of the method, a practical zero. The three reached their highest value of 100% within 48 h and after that, decreased to 20, 0 and 0% at 120 h of incubation, respectively.

The microorganism probably used hesperidin, naringin and ellagitannins as sources of carbon and energy during the fermentation. In the initial phase of this process, a partial cleavage or change in the phenolic glycosides took place in association with the microbial enzymatic breakdown of the plant cell wall, and hence the aglycones were released (Starzynska-Janiszewska et al., 2008; Vattem and Shetty, 2003). The tannase from *P. variotii* was proven to have the ability to catalyze the deglycosylation of flavanones, such as hesperidin (Ferreira et al., 2013). In this manner, tannase is likely an important enzyme synthesized by *P. variotii*, with the ability of hydrolyzing polyphenolic compounds into their aglycone forms as a first step for the microbial consumption of this compound as an energy source. Although the maximum tannase activity occurs after 96 h, from the beginning of the fermentation process it is possible to observe tannase activity, justifying the early hydrolysis of the hesperidin, naringin and ellagitannins, generating hesperetin, naringenin and ellagic acid, respectively. Numerous studies have been conducted on biodegradation of tannins and on the degradation mechanism of some simple tannins, such as gallotannin. There is less knowledge about the pathways and enzymes involved in breaking down complex tannins, especially regarding the accumulation mechanism of the microorganism for some intermediates. However, some studies have indicated that tannase participates in ellagic acid obtainment from ellagitannins (Prasad et al., 2012). Shi et al. (2005) reported the ellagic acid accumulation by fungal fermentation of valonea (*Quercus aegilops*) tannins. The results showed simultaneous increase in tannase activity, accumulation of ellagic acid and decrease in ellagitannins during fermentation.

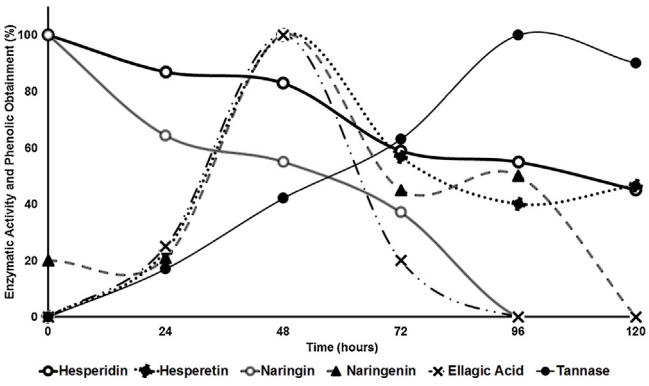

Fig. 1 – Kinetics of fermentation of the *Citrus* residue by *Paecilomyces variotii*: evaluating the tannase production and concentration of hesperidin, hesperetin, naringin, naringenin and ellagic acid.

Consequently, as observed in Fig. 1, the concentration of hesperetin, naringenin and ellagic acid increased during fermentation. After 48 h of incubation, the concentration of these phenolics achieved its maximum and started to decrease. This decrease happened even in the presence of a significantly high concentration of the antecedent hesperidin, naringin and tannase activity. This fact led to the belief that the biotransformation from hesperidin and naringin to hesperetin and naringenin was still happening. However, the microorganism, at this point, probably had its metabolic arsenal ready to consume the hesperetin and naringenin molecules.

Justesen et al. (2000) also evaluated the microbial fermentation of hesperidin and hesperetin, and the results observed seem to corroborate our hypothesis. The results showed similar effects, in which degradation of hesperidin occurred during 72 h of incubation, with high hydrolysis values at 24 h. However, the hesperetin was also used as a substrate by the microorganisms after 24 h, being stored for 24 h and then degraded within 72 h. The hesperetin degradation showed some possible products after 48 h, and this showed that the C ring of the compound was hydrolyzed, releasing compounds such as 4-hydroxyphenyl-propionic acid and 3-hydroxy-4-methoxyphenyl-acetic acid.

After the time-course assay, a CCD was designed to determine the optimal particle substrate size (mm), water:substrate ratio (v:w) and temperature of incubation (°C) for solid-state fermentation, and the results are shown in Table 1. According to previous analyses, in which some conditions were tested in fermentation, these three variables were the most significant; therefore, they were used in the CCD.

The quadratic models used to calculate the hesperetin (Y_a), naringenin (Y_b) and ellagic acid (Y_c) after eliminating the statistically insignificant terms are follows (x_1: particle size; x_2: moisture; x_3: temperature of incubation):

$$Y_a = 44.0 - 5.0x_1 + 10.0x_2 - 6.0x_2^2 - 6.0x_3^2$$

$$Y_b = 54.0 - 5.0x_1 + 8.0x_2 - 5.0x_3^2$$

$$Y_c = 10,300 + 2000x_1 + 3300x_2 - 900x_2^2 - 1600x_3^2$$

The analyses of variance were reproduced and are shown in Table 2. The Fisher *F*-statistic for hesperetin, naringenin and ellagic acid concentrations were higher than the F_t and *p*-value of <0.01, demonstrating that this regression model was statistically significant at a 90% confidence level. Additionally, the R^2 values obtained for the models were 0.77, 0.70 and 0.83, respectively.

The highest hesperetin, naringenin and ellagic acid concentrations obtained in the experimental design tests were 50.0, 60.0 and 10,000 μg/g substrate, under the conditions: 2:1 water:substrate, 32 °C incubation temperature and 1.20 mm particle size substrate (Fig. 2).

Higher temperatures significantly decreased the release of phenolic compounds. The higher amount of phenolic compounds found in the *Citrus* residue fermented by *P. variotii* can be attributed to the ability of this species to hydrolyze structural carbohydrates pectin, cellulose, hemicelluloses and lignin (polyphenolic macromolecule closely bound to cellulose and hemicellulose in cell walls of plants) by way of various glycoside hydrolases (cellulases, hemicellulases). Also, polyphenol compounds such as hesperidin, naringin and ellagitannins can be hydrolyzed by active esterases (β-glucosidase, tannase) present in its metabolism. The higher enzymatic activity arsenal probably occurred at its optimum temperature (32 °C) (Ferreira et al., 2013; Holopainen-Mantila et al., 2013; Pistarino et al., 2013).

The substrate particle size was studied in phenolic compounds obtainment. For ellagic acid, the highest production was obtained with increasing particle size. Increased production for hesperetin and naringenin occurred with decreasing particle size. However, this variable showed less interference in obtaining the latter two phenolics. These effects might be due to the higher superficial area of contact between the substrate and the microorganism. Substrates with intermediate particle size provide a considerable contact area between the fungus and the substrate, favoring its growth; whereas very small particles are more susceptible to compaction and the formation of agglomerates, resulting in decreased oxygen transfer, affecting respiration and fungal development. For some substrates, larger particles had higher porosity than smaller particles; however, particles with larger diameter also presented higher surface area. Despite the larger surface area and pore volume, smaller particles had pores that did not exceed 7 nm. Since the diameter of the fungal hyphae is usually greater than 10 nm, the microorganism would likely grow on the outside of the particles (Schmidt and Furlong, 2012; Membrillo et al., 2011). The more porous particles benefited from aeration, dissipation of gases and heat produced during microbial growth. During the filamentous fungi growth on solid substrates, it is generally accepted that there is a limitation in oxygen supply to the cells that are in close contact with the substrate or that penetrate the substrate (Schmidt and Furlong, 2012; Membrillo et al., 2011).

Higher phenolic compound production was obtained at a maximal water:substrate ratio. Lower production of phenolic compounds at a lower water:substrate ratio might also be due to reduced water availability for biomass growth or reduced mobility of the substrate during solid-state fermentation. During the bioprocess, the water content available to the substrate is extremely important, especially if the substrate has hemicellulose and pectin, which can absorb more water, potentially leading to an increase in microbial growth in the substrate and, consequently, the release of bioactive aglycone phenolic compounds (Madeira et al., 2013).

Thus, in accordance with the optimal conditions of temperature, particle substrate size and water: substrate ratio, the bioprocess obtained an increase of 900, 1400 and 1330% of hesperetin, naringenin and ellagic acid concentration, after 48 h of fermentation, respectively. These results shown in Table 3 are the mean values, where the experimental design was carried out in triplicate. This process used residues from various *Citrus* species (cited in item 2.1), which allows microbial biotransformation to obtain phenolic compounds using any type of *Citrus* as substrate.

Table 3 also shows the decrease in concentration of hesperidin and naringin after fermentation. According to the data, there was a decrease of 45 and 81% of the concentration of naringin and hesperidin, respectively. What leads one to think that the total amount of these glycosylated flavanones were transformed into the aglycone forms, hesperetin and naringenin. However, this affirmation cannot be validated, because in all microbial fermentation, the compounds present in the medium are transformed into different chemical structures that participate in the metabolism of the microorganism. Justesen et al. (2000) concluded that the microbial metabolism of hesperetin included several other compounds of lower molecular weight. Thus, *P. variotii* hydrolyzed naringin and hesperidin into naringenin and hesperetin, respectively, probably, part of these aglycone forms were hydrolyzed to compounds of lower molecular weights, which also have great biological potential.

Therefore, the fermentation showed not only the release of the expected phenolic compounds, but also obtaining phenolic compounds of lower molecular weight which may have greater antioxidant potential in relation to the glycosylated. There are no published studies on hesperetin, naringenin and ellagic acid production by solid-state fermentation or any other biotechnological process. As such, the purpose of this study is to provide extracts with high concentration of

Table 2 – Analysis of variance and regression analyses for the response of the central composite design of phenolic compounds obtainment.

Response	Source of variation	Sum of squares	Degrees of freedom	Mean squares	F_{test}/F_{tab}	p-Value/R^2
Hesperetin	Regression	2.373	4	0.59	9.8/2.5	0.001/0.77
	Residual	0.730	12	0.06		
Naringenin	Regression	1.623	3	0.54	9.0/2.6	0.003/0.69
	Residual	0.746	13	0.06		
Ellagic acid	Regression	240,393	4	60,098	14.8/2.5	0.0001/0.83
	Residual	48,877	12	4073		

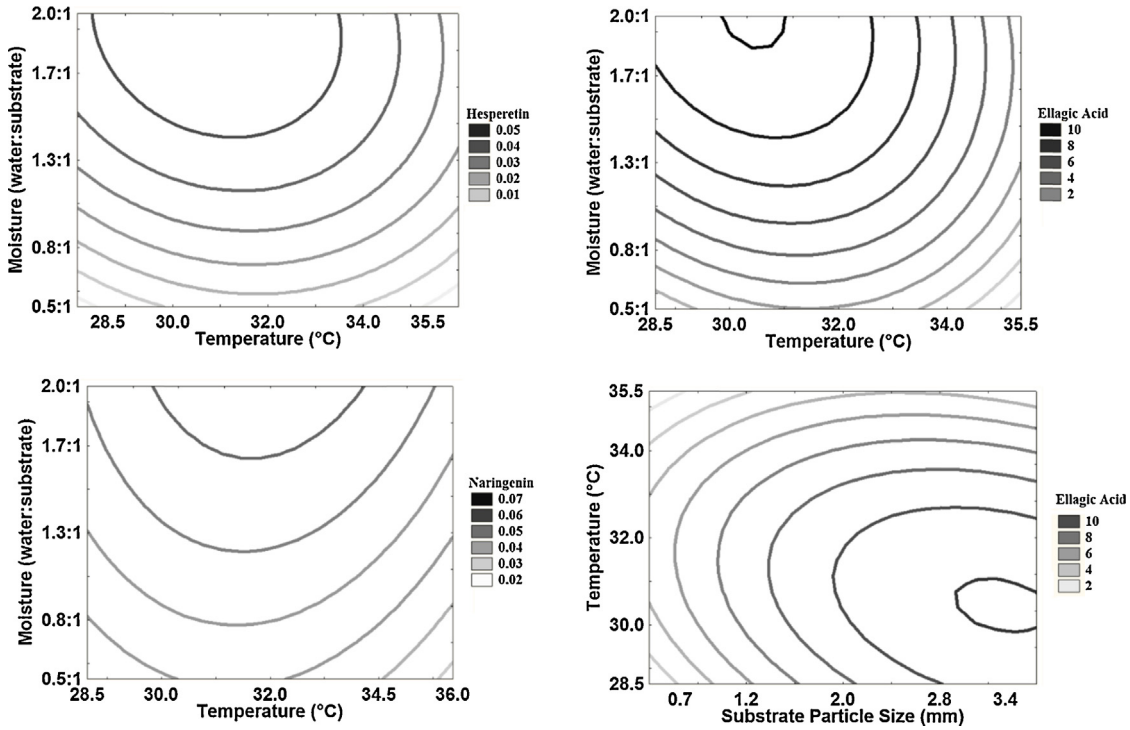

Fig. 2 – A response surface representative of hesperetin, naringenin and ellagic acid production as a function of temperature *vs.* water:substrate ratio *vs.* particle substrate size, according to the CCD.

the bioactive phenolic compounds of interest, which can be obtained by extraction from natural sources.

3.2. *Antioxidant potential*

The results in Table 4 show an increase of 73% in the antioxidant capacity, by the ORAC method, of the residue against the free radical APPH after the fermentation process. One possible reason for this increase is that during the solid-state fermentation process, the biotransformation transformed the phenolics presented in substrate into aglycone phenolic molecules with higher antioxidant capacity.

According to many recent studies, the aglycone form of hesperidin and naringin molecules have more important effects of bioactivity and higher bioavailability than glycoside (Nielsen et al., 2006).

In order to verify if these affirmations would apply to the product of the tannase extract from *P. variotii* catalyzations, commercial isolated hesperidin and naringin were reacted with the tannase extract, and the products of the reactions were assayed for antioxidant capacity. The results obtained for the ORAC and DPPH assays are described in Table 5 and are expressed as a concentration equivalent of Trolox®.

Table 3 – Phenolic compounds productivity in solid-state fermentation.

Sample	Phenolic compounds					
	Hesperidin	Hesperetin	Naringin	Naringenin	Ellagic acid	Gallic acid
Orange residue (μg/g)[a]	3745	5.0	451.0	4.0	700.0	2462
Fermented orange residue (μg/g)[a]	2055	50.0	85.0	60.0	10,000	0.0
Increase of the product (%)[b]	−45	900	−81	1400	1330	–

[a] Mean values of phenolic compounds concentration carried out in triplicate.
[b] Relation between initial and final concentration of compound.

Table 4 – Trolox equivalents and linearity ranges for the ORAC (net AUC *vs.* concentration) and DPPH assay performed on the extract optimal medium samples before and after fermentation.

Sample	ORAC					DPPH
	Trolox equivalents (μmol/mL extract)	Sample concentration range (mL extract)	Slope	Intercept	r^2	Trolox equivalents (μmol/mL extract)
Pre-fermented *Citrus* residue	6538 ± 1253[a]	0.031–0.25	1378	7.4	0.99	260 ± 6[a]
Fermented *Citrus* residue (48 h)	11,287 ± 869[b]	0.016–0.063	2935	4.4	0.99	266 ± 9[a]

[a,b] Results are presented as the mean ($n = 3$) ± SD, and those with different letters are significantly different, with $p < 0.05$.

Table 5 – Trolox equivalents and linearity ranges for the ORAC (net AUC *vs.* concentration) and DPPH assay performed on the phenolic standards.

Sample	ORAC					DPPH
	Trolox equivalents (μmol/g standard)	Sample concentration range (mg/mL)	Slope	Intercept	r^2	Trolox equivalents (μmol/g standard)
Hesperidin	2333 ± 202[a]	0.04–0.08	68.5	1.3	0.99	55 ± 3[a]
Hesperetin	6552 ± 1250[b]	0.04–0.08	110.9	8.1	0.96	865 ± 91[b]
Naringin	7958 ± 610[a]	0.04–0.08	247.5	3.6	0.95	67 ± 2[a]
Naringenin	9955 ± 887[b]	0.04–0.08	293.4	5.4	0.99	428 ± 10[b]
Ellagic acid	1246 ± 226[c]	0.05–0.50	1170.6	0.9	0.99	192 ± 27[c]

[a,b,c] Results are presented as the mean ($n = 3$) ± SD, and those with different letters are significantly different, with $p < 0.05$.

The hesperidin and naringin conversion led to an increase of 180 and 115% in antioxidant activity by the ORAC method, respectively. For the DPPH method, the same conversion led to an increase near 1400 and 540%, respectively (Table 5).

The ellagic acid showed antioxidant potential of 1246 and 192 μmol/g Trolox equivalent by ORAC and DPPH methods, respectively, which is a significant antioxidant potential.

Therefore, it can be assumed that part of the increased antioxidant activity of the fermented extract was associated with the increase of phenolic compounds of lower molecular weight, such as hesperetin, naringenin and ellagic acid.

Additionally, the concentration of phenolics and antioxidant potential showed no relationship before and after microbial biotransformation of the residue of *Citrus*. The values were around 1000% increase in phenolic aglycones (Table 3) and only 73% of the antioxidant activity of *Citrus* residue fermented (Table 4). Other compounds with significant antioxidant capacity, were possibly degraded during fermentation, such as gallic acid (Table 3), and thus diminished protection against oxidative compounds.

Either way, the process of microbial biotransformation produced compounds that are difficult to obtain (hesperetin and naringenin) and have high biological potential.

Madeira et al. (2012) described the fermentation of *Citrus* residue by *P. variotii* for tannase production. After 120 h of incubation, the fermented substrate showed antioxidant activity 10 times greater than the unfermented substrate by the TEAC method. These results strongly indicate that the citrus residue fermented by *P. variotii* was improved in antioxidant compounds, and the amounts of hesperetin, naringenin and ellagic acid are likely responsible for this response.

Barros et al. (2012) studied the antioxidant capacity of pulps and peels of *Citrus* from Brazil. In general, the peels demonstrated higher contents of all phenolic compounds than the pulps. For pulps, the highest value of antioxidant capacity measured using the DPPH assay was 0.46 μmol of Trolox equivalent/g of sample, and for the peels, 0.75 μmol of Trolox equivalent/g of sample. Therefore, the use of the residue for production of antioxidant phenolic compounds seems to provide a major advantage over pulp extraction.

Aguilar et al. (2008) studied the accumulation of antioxidant phenolic compounds by biodegradation of tannins present in creosote bush leaves and pomegranate peels. He observed that the biotransformation during the solid-state fermentation process (by fungal tannase producers) transformed the phenolics present in his substrate into lower molecular weight phenolic molecules with higher antioxidant capacity.

These results show strong evidence that microbial biotransformation is not only able to produce phenolic compounds but compounds with high biological activity, such as hesperetin and naringenin, as well.

4. Conclusion

The *Citrus* residue solid-state fermentation is a clean and viable biotechnology with great potential for application in the obtainment of phenolic resources. The utilization of agro-industrial *Citrus* residues is particularly interesting because of its availability, low cost and features that allow obtaining different bioactive phenolic compounds. The process studied was low cost and used an abundant source of hesperidin and naringin. It provided an interesting commercial source of hesperetin, naringenin and ellagic acid. These molecules

have no commercial source to be extracted from and have been demonstrating much more important bioactivity potential than their glycosylate forms. Also demonstrated was the usefulness of biotechnology on natural food molecules to improve their nutraceutical potential and provide a commercial source of these compounds.

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References

Aguilar, C.N., Aguilera-Carbo, A., Robledo, A., Ventura, J., Belmares, R., Martinez, D., Rodríguez-Herrera, R., Contreras, J., 2008. Production of antioxidant nutraceuticals by solid-state cultures of pomegranate (*Punica granatum*) peel and creosote bush (*Larrea tridentate*) leaves. Food Technol. Biotechnol. 46, 218–222.

Banerjee, S., Singh, S., Rahman, L.U., 2012. Biotransformation studies using hairy root cultures – a review. Biotechnol. Adv. 30, 461–468.

Barrios-González, J., 2012. Solid-state fermentation: physiology of solid medium, its molecular basis and applications. Process Biochem. 47, 175–185.

Barros, H.R.M., Ferreira, T.A.P.C., Genovese, M.I., 2012. Antioxidant capacity and mineral content of pulp and peel from commercial cultivars from Brazil. Food Chem. 134, 1892–1898.

Battestin, V., Macedo, G.A., 2004. Tannase production by *Paecilomyces variotii*. Bioresour. Technol. 98, 1832–1837.

Ferreira, L.R., Macedo, J.A., Ribeiro, M.L., Macedo, G.A., 2013. Improving the chemopreventive potential of orange juice by enzymatic biotransformation. Food Res. Int. 51, 526–535.

Ferreres, F., Gil-Izquierdo, A., Vinholes, J., Silva, S.T., Valentão, P., Andrade, P.B., 2012. *Bauhinia forficata* Link authenticity using flavonoids profile: relation with their biological properties. Food Chem. 134, 894–904.

Georgetti, S.R., Vicentini, F.T.M.C., Yokoyama, C.Y., Borin, M.F., Spadaro, A.C.C., Fonseca, M.J.V., 2009. Enhanced in vitro and in vivo antioxidant activity and mobilization of free phenolic compounds of soybean flour fermented with different β-glucosidase-producing fungi. J. Appl. Microbiol. 106, 459–466.

Hollman, P.C.H., Katan, M.B., 1997. Absorption, metabolism and health effects of dietary flavonoids in man. Biomed. Pharmacother. 51, 305–310.

Holopainen-Mantila, U., Marjamaa, K., Merali, Z., Käsper, A., de Bot, P., Jääskeläinen, A.-S., Waldron, K., Kruus, K., Tamminen, T., 2013. Impact of hydrothermal pre-treatment to chemical composition, enzymatic digestibility and spatial distribution of cell wall polymers. Bioresour. Technol. 138, 156–162.

Justesen, U., Arrigoni, E., Larsen, B.R., Amado, R., 2000. Degradation of flavonoid glycosides and aglycones during in vitro fermentation with human faecal flora. Lebensm. Wiss. Technol. 33, 424–430.

Lin, J.-T., Chen, Y.-C., Lee, Y.-C., Rolis Hou, C.-W., Chen, F.-L., Yang, D.-J., 2012. Antioxidant, anti-proliferative and cyclooxygenase-2 inhibitory activities of ethanolic extracts from lemon balm (*Melissa officinalis* L.) leaves. LWT – Food Sci. Technol. 49, 1–7.

Macedo, J.A., Battestin, V., Ribeiro, M.L., Macedo, G.A., 2011. Increasing the antioxidant power of tea extracts by biotransformation of polyphenols. Food Chem. 126, 491–497.

Madeira Jr., J.V., Macedo, J.A., Macedo, G.A., 2011. Detoxification of castor bean residues and the simultaneous production of tannase and phytase by solid-state fermentation using *Paecilomyces variotii*. Bioresour. Technol. 102, 7343–7348.

Madeira Jr., J.V., Macedo, J.A., Macedo, G.A., 2012. A new process for simultaneous production of tannase and phytase by *Paecilomyces variotii* in solid-state fermentation of orange pomace. Bioprocess Biosyst. Eng. 35, 477–482.

Madeira Jr., J.V., Teixeira, C.B., Macedo, G.A., 2013. Biotransformation and bioconversion of phenolic compound: an overview. Crit. Rev. Biotechnol., http://dx.doi.org/10.3109/07388551.2013.803020.

Marín, F.R., Soler-Rivas, C., Benavente-García, O., Castillo, J., Pérez-Álvarez, J.A., 2007. By-products from different citrus processes as a source of customized functional fibres. Food Chem. 100, 734–741.

Martins, S., Mussatto, S.I., Martínez-Avila, G., Montañez-Saenz, J., Aguilar, C.N., Teixeira, J.A., 2011. Bioactive phenolic compounds: production and extraction by solid-state fermentation. A review. Biotechnol. Adv. 29, 365–373.

Meiyanto, E., Hermawan, A., Anindyajati, 2012. Natural products for cancer-targeted therapy: citrus flavonoids as potent chemoprotective agents. Asian Pac. J. Cancer Prev. 13, 427–436.

Membrillo, I., Sánchez, C., Meneses, M., Favela, E., Loera, O., 2011. Particle geometry affects differentially substrate composition and enzyme profiles by *Pleurotus ostreatus* growing on sugar cane bagasse. Bioresour. Technol. 102, 1581–1586.

Murakami, A., Ashida, H., Terao, J., 2008. Multitargeted cancer prevention by quercetin. Cancer Lett. 269, 315–325.

Nazari, M., Ghorbani, A., Hekmat-Doost, A., Jeddi-Tehrani, M., Zand, H., 2011. Inactivation of nuclear factor-κB by citrus flavanone hesperidin contributes to apoptosis and chemo-sensitizing effect in Romos cells. Eur. J. Pharmacol. 650, 526–533.

Nielsen, I.L., Chee, W.S., Poulsen, L., Offord-Calvin, E., Rasmussen, S.E., Frederiksen, H., Enslen, M., Barron, D., Horcajada, M.-N., Williamson, G., 2006. Bioavailability is improved by enzymatic modification of the citrus flavonoid hesperidin in humans: a randomized, double-blind, crossover trial. J. Nutr. 136, 404–408.

Nutan, Modi, M., Goel, T., Das, T., Malik, S., Suri, S., Rawat, A.K.S., Srivastava, S.K., Kumar, S., Tuli, R., Malhotra, S., Gupta, S.K., 2013. Ellagic acid & gallic acid from *Lagerstroemia speciosa* L. inhibit HIV-1 infection through inhibition of HIV-1 protease & reverse transcriptase activity. Indian J. Med. Res. 137, 540–548.

Ohguchi, K., Akao, Y., Nozawa, Y., 2006. Stimulation of melanogenesis by the citrus flavonoid naringenin in mouse B16 melanoma cells. Biosci. Biotechnol. Biochem. 70, 1499–1501.

Park, H.J., Kim, M.-J., Ha Chung, J.-H., 2008. Apoptosis effect of hesperidin through caspase 3 activation in human colon cancer cells, SNU-C4. Phytomedicine 15, 147–151.

Pistarino, E., Aliakbarian, B., Casazza, A.A., Paini, M., Cosulich, M.E., Perego, P., 2013. Combined effect of starter culture and temperature on phenolic compounds during fermentation of Taggiasca black olives. Food Chem. 138, 2043–2049.

Prasad, D., Gupta, R.K., Mathangi, G., Kamini, N.R., Gowthaman, M.K., 2012. Advances in production and characteristic features of microbial tannases: an overview. Curr. Trends Biotechnol. Pharm. 6, 145–165.

Sepúlveda, L., Ascacio, A., Rodríguez-Herrera, R., Aguilera-Carbó, A., Aguilar, C.N., 2011. Ellagic acid: biological properties and biotechnological development for production processes. Afr. J. Biotechnol. 10, 4518–4523.

Sergent, T., Vanderstraeten, J., Winand, J., Beguin, P., Schneider, Y.-J., 2012. Phenolic compounds and plant extracts as potential natural anti-obesity substances. Food Chem. 135, 68–73.

Sharma, S., Bhat, T.K., Dawra, R.K., 2000. A spectrophotometric method for assay of tannase using rhodanine. Anal. Biochem. 279, 85–89.

Shi, B., He, Q., Yao, K., Huang, W., Li, Q., 2005. Production of ellagic acid from degradation of valonea tannins by *Aspergillus niger* and *Candida utilis*. J. Chem. Technol. Biotechnol. 80, 1154–1159.

Schmidt, C.S., Furlong, E.B., 2012. Effect of particle size and ammonium sulfate concentration on rice bran fermentation with the fungus *Rhizopus oryzae*. Bioresour. Technol. 123, 36–41.

Starzynska-Janiszewska, A., Stodolak, B., Jamroz, M., 2008. Antioxidant properties of extracts from fermented and cooked seeds of Polish cultivars of *Lathyrus sativus*. Food Chem. 109, 285–292.

Tripoli, E., La Guardia, M., Giammanco, S., Di Majo, D., Giammanco, M., 2007. Citrus flavonoids: molecular structure, biological activity and nutritional properties: a review. Food Chem. 104, 466–479.

Vattem, D.A., Shetty, K., 2003. Ellagic acid production and phenolic antioxidant activity in cranberry residue (*Vaccinium macrocarpon*) mediated by *Lentinus edodes* using a solid-state system. Process Biochem. 39, 367–379.

APPENDIX 5 - Capítulo “Hesperitin: Simple natural compound with multiple biological activity” publicado no livro Fruit and Pomace Extracts: Biological Activity, Potential Applications and Beneficial Health Effects.

Chapter

HESPERETIN: SIMPLE NATURAL COMPOUND WITH MULTIPLE BIOLOGICAL ACTIVITY

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ABSTRACT

Bioactive compounds are extra nutritional constituents that naturally occur in small quantities in plant and food products. Most common bioactive compounds include secondary metabolites, such as antibiotics, mycotoxins, alkaloids, food grade pigments, plant growth factors, and phenolic compounds. Flavonoids constitute the largest group of plant phenolics, accounting for over half of the eight thousand naturally occurring phenolic compounds. Currently, flavanones are obtained by chemical synthesis or extraction from plants, and these processes are only produced in the glycosylated form. However, there are environmentally friendly bioprocesses that deserve attention regarding phenolic compound production, especially in aglycon forms. One of these flavonoids is the hesperetin, that has recently been recognized for their influence on human metabolism, acting in the prevention of some chronic diseases, as well as proving to be an important antioxidant in food. In the last few years, great attention has been paid to bioactive phenolic compounds due to their ability to promote benefits for human health. Hesperetin is reported to be a powerful radical scavenger and a promoter of cellular antioxidant defense-related enzyme activities. This compound exhibited anti-inflammatory activity by inhibiting of LPS-induced expression of the COX-2 gene in RAW 264.7 macrophages. Hesperetin is a potent chemopreventive agent; its supplementation during the initiation, post-initiation, and entire period stages of colon carcinogenesis in the male rat model in vivo significantly reversed these activities. In addition, the aglycon flavanone presents activity against parasites from tropical diseases. Considering the folk claims, several medicinal compounds (including hesperetin) have

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been evaluated for this antifilarial activity. Recent studies showed that hesperetin inhibited (>60%) the adult worms growth (*Wuchereria bancrofti*) at 7.8 and 31.2 μg/ml concentration. The bioactive aglycon phenolic compound demonstrates antiviral activity. Experimental tests showed hesperetin presents inhibition activities of genotype 2 (DENV-2) virus replication. This flavonoid seems to be usefull also in the treatment of some non-communicable diseases, such as cardiac diseases, diabetes, hypertension. A hesperetin suspension administered in adult male C57BL/6 mice inhibited cardiac hypertrophy, fibrosis, oxidative stress and myocytes apoptosis induced by pressure overload and protected against cardiac dysfunction. In another study, hesperetin enhanced ApoA-I-mediated cholesterol efflux in THP-1 macrophages, which was accompanied by an induction of the ABCA1 gene, which is critical for cholesterol metabolism. The effect of hesperetin on ABCA1-dependent cholesterol efflux may be explained by its potency of activation of LXRα and PPARγ enhancers. In a study conducted with Streptozotocin induced diabetic rats, hesperitin reduced vascular leakage, dilatation of retinal vessels and basement membrane thickening. In another study also with Streptozotocin induced diabetic rats, hesperitin treatment rescued retinal neuroinflammation, oxidative stress, apoptosis and oedema as a result of chronic uncontrolled hyperglycaemic state. These studies indicate that hesperitin can be used for the prevention of induced neurovascular complications caused by descompansated diabetes. Intravenous administration of hesperetin-7-O-b-D-glucuronide decreased blood pressure in anesthetized spontaneously hypertensive rat. Furthermore, it enhanced endothelium-dependent vasodilation in response to acetylcholine, decreased hydrogen peroxide-induced intracellular adhesion molecule-1 and monocyte chemoattractant protein-1 mRNA expression in rat aortic endothelial cells. Hesperitin can also be used in management of obesity due to its influence in the control of hunger and satiety. In this context, the flavanone aglycone caused an increase in the secretion of cholecystokinin (CCK) in STC-1 cells through increase in intracellular calcium concentration by the TRP (transient receptor potential) and TRP 1 ankirin channels. The addition of hesperidin analytical standard in the same model caused no effect. The increase in CCK would be interesting because this hormone assists in the control of food intake. The purpose of this chapter is to provide an overview of the study of obtainment and biological properties of hesperetin.

1. INTRODUCTION

Flavonoids correspond to an important group of plant-derived heterocyclic organic compounds. They are divide into 14 different subgroups [1], based on their chemical nature and position of substituents on the A, B and C rings. Their relavance are due to many biological properties that have been reported, including antimicrobial, antioxidant and vascular activities [2]. Flavonoids are usually found in the form of glycosides in foods of plant origin, in particular in vegetables, beverages and citrus fruits [3].

The therapeutical effects of flavonoids are due to their hydrogen-donating antioxidant activity and their capability to complex the divalent transition metal cations involved in processes forming radicals. These compounds have two aromatic rings enclosing a heterocyclic six-membered ring with oxygen. Different classes of flavonoids are based on modifications of this central C-ring: flavones, flavonols, flavanones, isoflavonoids, anthocyanins, flavanols, chalconoids, dihydrochalcones and aurones [4].

This chapter is divided into three parts: sources of hesperetin; methods of extraction/obtantion; and biological potential.

2. SOURCES OF HESPIRITIN

Hesperetin (4′-methoxy-3′,5,7-trihydroxyflavanone), which is a bioactive plant flavonoid belonging to the chemical class ‘flavanone’ (abundantly present in citrus fruits), is rapidly emerging as an especially attractive therapeutic agent with an enormous spectrum of activities. This flavonoid corresponds to the aglycone form of hesperidin. Although hesperetin can be considered much more biologically active, firstly hesperidin is obtained, which is the natural form of these compounds.

Hesperidin (6"-O-(α-L-rahmnopyranosyl)-D-glucose flavonoid) consists of the hesperetin bound at the C-7 position (on ring A) to rutinose ($C_{12}H_{22}O_{10}$), a disaccharide composed of one molecule of rhamnose and one of glucose. However, one important drawback is the limited bioavailability of many flavonoids, and in fact the sugar moiety has been proposed as the major determinant of the absorption of dietary flavonoids in humans, whereas the rutinoside moiety is poorly absorbed in comparison with the aglycone and glucoside forms [5]. Within this context, the enzymatic de-glycosylation of flavonoids has been reported as a good alternative for increasing antioxidant activity of these compounds [6].

Hesperidin is the predominant flavanone glycoside of sweet oranges and is extracted from citrus peel [7] and applied in pharmaceutical industries for its therapeutic importance to many diseased capillary conditions [8]. Orange peel flavedo and albedo are interesting sources of phenolic compounds, more especifically flavonoids including hesperidin and hesperetin. Furthermore, orange peel is the primary waste fraction in the production of orange juice, and therefore it has been used as a source of hesperidin because of its high concentration in this material [9].

Taking into account flavonoids are mainly abundant in plant species from the genus Citrus, they present significant impact on nearly every aspect of citrus fruit production and processing. They are responsible for some unpleasant characteristics of fruit juices, such as turbidity and bitterness [9] and particularly hesperidin clogs the steel pipes of the citrus juice plants. In addition, they are abundant in the by-products, mostly in peels (albedo + flavedo), accounting for 4–12% of the dry weight [10]. Its recovery from citrus industry by-products is attractive because of two main reasons: its bioactive properties and the reduction of the amount of residues. Moreover, worldwide industrial wastes may be estimated at more than 1.5×10^6 tons, as the amount of residue obtained from the fruits accounts for 50% of the original whole fruit mass [11].

From citrus flavonoids, hesperidin is the most abundant in lemons, limes, sweet oranges, tangors and tangelos (~15 mg/100 g edible fruit) [12]. Owing to the importance of hesperidin for food and pharmaceutical industries, several efforts have been made for its extraction and purification.

In the paper of Di Mauro et al. [13] a procedure for recovering hesperidin from the waste water of orange juice processing by concentration of diluted extracts on styrene−divinylbenzene resin was reported, resulting in high concentration of hesperidin in selected fractions (10−78 g/L). On the other side, in the work of Ma et al. [14] hesperidin was extratced from penggan (*Citrus reticulata*) peels by ultrasound-assisted extraction, with interestng results.

Kanaze et al. [15] investigated orange peel (*Citrus sinensis*) cultivated in Greece–Crete as an a new commercial source of hesperidin. The flavonoid content of several methanolic extract

fractions of Navel orange peel (flavedo and albedo of *Citrus sinensis*) cultivated in Greece was first analysed phytochemically and then assessed for its antioxidant activity in vitro. The main flavonoid groups found within the fractions examined were polymethoxylated flavones, O-glycosylated flavones, C-glycosylated flavones, O-glycosylated flavonols, O-glycosylated flavanones and phenolic acids along with their ester derivatives. Furthermore, the quantitative HPLC analysis confirmed that hesperidin is the major flavonoid glycoside found in the orange peel. The authors concluded that quantity of hesperidin at 48 mg/g of dry peel permits the commercial use of orange peel as a source for the production of this compound.

Although the main source of hesperidin is citrus peel, literature reports other different sources of hesperidin, such as Cyclopia species (Fabaceae) [16] and Rosemary (*Rosmarinus officinalis*, Lamiaceae) [17].

3. METHODS OF EXTRACTION/OBTAINTION

The solvent extraction is the most used method for phenolic compounds obtainment from plant tissue. The main factor is the phenolics solubility, which depends on its chemical structure. Plant materials may contain different concentrations of phenolic acids, phenylpropanoids, anthocyanins and tannins. It is possible to occur interactions between phenolics and other plant components, such as carbohydrates and proteins which form complexes responsible for insolubility. Besides that, the polarity of solvent affects the solubility and therefore, it is considered difficult to develop a extraction method suitable for all plant phenolics [18].

Ethanol and methanol are the most used solvents for the citrus flavonoids extraction, as hesperidin, narigenin, narirutin and neohesperidin. It is usually extracted from byproducts residues [19, 20, 21]. Nevertheless, the two solvents present some limitations, such as low efficiency recovery, long extraction time and degradation of unsaturated compounds [21]. To solve this, many technologies have been studied to improve solvent extraction.

Some new "green" extraction techniques, aimed at sparing energy and reducing costs, such as solid state and submerse fermentation, enzymatic, microwave- or ultrasound-assisted extraction, ultrafiltration, flash distillation and controlled pressure drop processing [22, 23] have been studied to improve solvent extraction.

For phenolics extraction it is first necessary to release it from the vegetable matrix. Most of the technologies cited above are used aiming to improve release of phenolics from other compounds complexes as pectin and cellulose; and diffusion of the specific composites into extraction solvent.

3.1. Subcritical Water

Subcritical water has been used to flavonoids extraction, such as citrus flavanones with selectivity capacity modelled by temperature solubility dependence of the phenolics [24, 21]. The subcritical water extraction (SWE) is based on solubility enhacement of phenolics compounds in high temperature water (100-374ºC). To keep the liquid state of water, a high pressure is applied (>40 atm) [25, 21]. With high temperatures it is possible to change the water

polarity, which permits the solubility of not so polar molecules [26, 21]. The performance of SWE in hesperidin extraction from *Citrus unshiu* peel was evaluated by Cheigh *et al.* [21]. Varying the extraction temperature (110–200 °C) and time (5–20 min) under high pressure (100 ± 10 atm), they obtained almost 99% of extraction yield in 10 minutes at 170ºC. When compared with ethanol, methanol and hot water, the extraction yield of SWE was 1.9-, 3.2-, and 34.2-fold higher, respectively.

3.2. Ultrasound

Plant material extraction using ultrasound technique in a laboratory scale has been widely used. Review papers were published dealing with the extraction of plant origin metabolites [27, 28], food flavonoids with different solvents [29, 28] and bioactives from herbs [30, 28]. The ultrasonic technology in food processing has attracted widely attentions nowadays [31] and also many papers had described the ultrasonic extraction of flavonoids from citrus peel [31, 32, 33].

Ultrasound generates energy throught a sound wave which is transferred to the medium resulting in a continuous wave type motion with longitudinal waves creating alternative compression and rarefaction of the medium [34, 35]. This wave type motion forms cavitation bubbles and are classified in two types of cavitation: a stable one with increasing and decreasing size behavior giving rise to the so-called “stable cavitation” generating a micro-agitation of the medium. The second one called “transient cavitation” can also grow and collapse generating very high local temperatures (5000 K) and pressures (1000 atm) with high energy shear waves and turbulence in the cavitation zone [36, 37]. The effects of ultrasound depends on the frequency used and the sound wave amplitude applied contributing to a diverse number of physical, chemical and biochemical effects observed, which permits a variety of applications. Shock waves are generated due to cavitation, which are contributed to the ultrasound effect. Formation and behaviour of the bubble of cavitation upon the propagation of the acoustic waves constitute the essential events which induce the majority of the acoustic effects [38, 39, 36, 40, 35], including the catalysis of solvent extraction, considering that the diffusion through the cell walls and washing out (rinsing) the cell contents are the two types of physical phenomena involved on extraction mechanism. Both phenomena are significantly affected by ultrasonic irradiation [30].

3.3. Microwaves

The Microwave-assisted extraction (MAE) is another technology used to improve solvent extraction of bioactive compounds with high efficiency in extraction time and environmental-friendliness [41, 42]. The mechanism is based on heating dipolar compounds by microwave-irradiation generating a cell wall destruction, release of compounds and diffusion into extraction solvent. Microwaves are transmitted as waves, defunding into biomatrices and interact with polar molecules, such as water in the biomaterials to create heat. Consequently, microwaves can heat a whole material to penetration depth simultaneously. This behavior makes the effect of microwave energy strongly dependent on the dielectric susceptibility of

both solvent and solid plant matrix[41]. The vantages of MAE are the prevention of extracted materials decomposition, reduction in heating period and in volume of solvent demand [42].

MAE is considered a potential technology to improve traditional solid–liquid extraction for the metabolites extraction from plants. Many advantages for MAE application for nutraceuticals includes reduced extraction time, reduced solvent utilization generating improved extraction yield. MAE is also comparable to other modern extraction techniques such as supercritical fluid extraction due to its process simplicity and low cost. By considering economical and practical aspects, MAE is a strong novel extraction technique for the extraction of nutraceuticals. Nevertheless, MAE when compared to SFE, requires an additional filtration or centrifugation to remove the solid residue during MAE. Moreover, the efficiency of microwaves depends on target compounds solvent polarity decreasing its efficiency on bioactive compounds extraction [41].

Previously, [43] employed MAE for extraction of hesperidin from pericarpium citri reticulate (dried pericarp of the ripe fruit of *C. reticulata*), by using 70% aqueous methanol as a solvent, and showed that MAE is a fast, efficient and energy-saving extraction method [42]. In another published study [44] compared MAE, ultrasound and rotary methods to extract phenolic acids from citrus mandarin peels. They concluded that MAE is a better approach showing many advantages, such as shorter time, less solvent, higher extraction rate, savings of energy and better products with lower cost.

3.4. Microbial Transformation

Microbial fermentation has appeared as a biotechnology alternative for biomaterial pre-treat and for obtaining bioproducts metabolized by microorganism. Enzymes present in microbial fermentation are responsible for hydrolysis of glucosidic phenolics, increasing the release with increased solubility.

Comparing with other processes that use high temperatures and therefore generate high energy costs, this could be a great advantage. This difference in clearance of phenolic compounds is due to the metabolic activity of each microorganism. This case is related to various types of microbial enzymes and their activities [45, 46].

Georgetti et al. [47] evaluated the biotransformation of polyphenol glycosides from soybeans to form aglycones through *Aspergillus awamori* solid-state fermentation. This result was direct correlated with β-glucosidase enzyme production. The greater number of free hydroxyl groups present in the non-glycoside form is responsible for the increae on biological activity. The microbial biotransformation of phenolic compounds seems to be a promising way to increase the concentration of phenolics with high biological potential [45].

Madeira et al. [45] developed a bioprocess for phenolics obtainment from Brazilian *Citrus* residues by *Paecilomyces variotii* solid-state fermentation. Using 10g of *Citrus* residues (2.0 mm of substrate particle size), 20mL distilled water, at 32 ºC after 48h of incubation were the optimum conditions which generated, simultaneously, an increase of 900, 1400 and 1330% of hesperetin, naringenin and ellagic acid concentration, respectively, and an increase of 73% of the antioxidant capacity.

3.5. Enzymatic Extraction

Enzyme-assisted extraction has been reported for extraction of carotenoids from marigold flower [48], vanillin from vanilla green pods [49], oil from coconut or seeds [48, 50] and phenols from black currant and herb [51, 52]. The enzyme-assisted extraction mechanism is based on cell wall degrading capacity of enzymes glucanases and pectinases which can weaken or break down the cell wall permitting the intracellular materials release and more accessible for extraction.

The β -glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) catalyzes the hydrolysis of disaccharide glycosides and conjugates from the non-reducing end. It has several applications in the pharmaceutical industries with hydrolysis of cellobiose to glucose. The β-glucosidase enzyme has numerous applications in the food and pharmaceutical industries, working in the hydrolysis of cellobiose to glucose, cellulose to glucose in combination with other cellulolytic enzymes, and the release of aroma compounds in fruit juices and wine. This enzyme is also used in the hydrolysis of cyanogenic compounds present in plants for hormone replacement therapy [53, 54, 46].

Li et al., [20] studied the enzymatic treatment for aqueous extraction of the total phenolic contents of five citrus peels (Yen Ben lemon, Meyer lemon, grapefruit, mandarin and orange). The highest recovery using Celluzyme MX (cellulase) in the enzyme-assisted extraction process was up to 65.5% (about 87.9% of the solvent extraction).

The phenolics in grapefruit peels had the highest total antioxidant activity, followed by Yen Ben lemon, mandarin, orange and Meyer lemon according to the total antioxidant activity (FRAP).

Moreover, Mandalari et al. [55] evaluated the effect of pectinases and cellulases on hydrolysis of hesperidin in Bergamot (*Citrus bergamia Risso*) peel and obtained more than 90% of glycosidic cleavage generating the aglycone form (hesperetin).

4. BIOLOGICAL POTENTIAL

Hesperetin has a variety of biological effects in numerous mammalian cell systems, *in vitro* as well as *in vivo*. They have been shown to exert antimicrobial, antiviral, antiulcerogenic, cytotoxic, antineoplastic, mutagenic, anti-inflammatory, antioxidant, antihepatotoxic, antihipertensive, hypolipidemic and antiplatelet activities. The next topic will discuss the biological potential of hesperetin: combating tropical diseases, anti-tumor, obesity, diabetes and cardiovascular diseases.

Filariasis is an endemic disease in tropical and sub-tropical regions of Asia, Africa, Central, South America and Pacific Island nations. Lymphatic Filariasis is caused by the worms *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori*, which occupy the lymphatic system and in chronic cases lead to the disease Elephantiasis. Flavonoids like naringenin, hesperetin, and naringin were evaluated against the human lymphatic filarial parasite, using an in vitro motility assay with adult worms and microfilariae. Naringenin and hesperetin killed the adult worms and inhibited (>60%) at 7.8 and 31.2 μg/ml concentration, Microfilariae (mf) were killed at 250–500 μg/ml. Thus hesperetin may provide a lead for the design and development of new antifilarial agent [56].

Hesperetin is reported to be a powerful radical scavenger and a promoter of cellular antioxidant defense-related enzyme activities. This compound exhibited anti-inflammatory activity by inhibiting of LPS-induced expression of the COX-2 gene in RAW 264.7 macrophages. Hesperetin is a potent chemopreventive agent; its supplementation during the initiation, post-initiation, and entire period stages of colon carcinogenesis in the male rat model in vivo significantly reversed these activities. Administration of hesperetin to 1,2-dimethylhydrazine (DMH)-treated rats decreased the tumor incidence and the number of aberrant crypt foci with simultaneous enhancement of tissue lipid peroxidation, glutathione S-transferase (GST), GPx, SOD, and CAT activities. Hesperetin induced Notch homolog 1 (NOTCH1) expression in human gastrointestinal carcinoid (BON) cells, subsequently suppressing tumor cell proliferation and bioactive hormone production. Furthermore, results of anti-carcinogenesis experiments indicated that hesperetin inhibited aflatoxin B1-induced carcinogenesis and that hesperetin caused cytotoxicity and apoptosis via a transient induction of caspase-3 activity in HL60 cells. Additionally, it exhibited strong antiproliferative activity in various cancer cells, and its treatment dose showed no toxic effect on normal cells [56, 57].

There are also some evidence that this flavonoid might be usefull in the treatment of some other non-communicable diseases, such as cardiac diseases, diabetes, hypertension.

Considering hypertension, hesperetin and hesperetin-7-O-β-D-glucuronide (HPT7G) enhanced nitric oxide (NO) release by inhibiting NADPH oxidase (nicotinamide adenine dinucleotide phosphate-oxidase) activity in human umbilical vein endothelial cell culture, indicating that hesperetin metabolites in plasma can improve vasodilatation in the vascular system. In the same work, the authors treated women with cold sensitivity, and a single dose of water-dispersible hesperetin was effective on peripheral vasodilatation. These results strongly suggest that hesperetin exert a potential vasodilatation effect by the endothelial action of its plasma metabolites [58].

Another group of researchers investigated the effects of HPT7G and hesperetin-30-O- β-D-glucuronide (HPT30G), which are the predominant hesperetin metabolites in rat plasma, on blood pressure and endothelial function. Intravenous administration of hesperetin and HPT7G (5 mg/kg) decreased blood pressure in spontaneously hypertensive rats (SHRs) compared to the control group. HPT7G enhanced endothelium-dependent vasodilation in response to acetylcholine, but had no effect on endothelium independent vasodilation in response to sodium nitroprusside (SNP) in aortas isolated from SHRs. HPT7G and hesperetin decreased ICAM-1 (intracellular adhesion molecule-1) and MCP-1 (monocyte chemoattractant protein-1) mRNA expression induced by hydrogen peroxide in rat aortic endothelial cells. In contrast, HPT30G had little effect on these parameters. In conclusion, HPT7G exerted hypotensive, vasodilatory and anti-inflammatory activities, similar to hesperetin, indicating that this flavanone could improve hypertension and endothelial dysfunction [59].

A hesperetin [(95 %) from Sigma-Aldrich] suspension was administered for adult male C57BL/6 mice (8–10 weeks old) at a constant volume of 1 ml/100 g body weight by oral gavage once a day. The animals were submitted to aortic banding leading to cardiac remodeling induced by pressure overload. The results indicate that hesperetin inhibited cardiac hypertrophy and myocite cross-sectional area. In response to pressure overload, it was observed the activation of PKCα/βπ, Akt, GSK3β, mTOR, FOXO3a, CaN, GATA4 and JNK. However, hesperetin supplementation almost completely blocked the activation of these factors. Also, aortic banding caused perivascular and intersticial fibrosis that was remarkably reduced in hesperetin-fed mice. mRNA levels of the fibrotic mediators TGFβ1 (transforming growth

factor-β1), CTGF (connective tissue growth factor) and collagen I were high in animals submitted to aortic banding, but hesperetin consumption significantly reduced their expression. The flavanone also attenuated oxidative stress acting in the reduction of NADPH oxidase activity and recovery of SOD1 and SOD2 mRNA expression [60].

In another study, hesperetin enhanced ApoA-I-mediated cholesterol efflux in THP-1 macrophages, probably due to a greater transcription of ABCA1 gene, which is critical for cholesterol metabolism. The effect of hesperetin on ABCA1-dependent cholesterol efflux may be explained in part by its LXRα and PPARγ agonist action. These results indicates the potential of this flavonoid in the prevention and treatment of atherosclerosis [61].

In a study conducted with Streptozotocin induced diabetic rats, hesperetin (200mg/kg body weight by oral gavage) reduced vascular leakage, dilatation of retinal vessels and retinae basement membrane thickening. Diabetic rats treated with hesperetin had lower values of VEGF and PKC-β (angiogenic factors), when compared to untreated diabetic rats. These results indicate that retinal vasoprotective effects of hesperetin are due to its anti-angiogenic properties, preventing early or late stage micro-vasculopathy [62]. In another study developed by the same group also in Streptozotocin induced diabetic rats, hesperetin treatment reduced retinal neuroinflammation with lower levels of TNF-α and IL-1β; reduced oxidative stress with higher levels of glutathione, superoxide dismutase and catalase; inhibited apoptosis via caspase-3 and reduced edema [63]. In both studies, hesperetin treatment in diabetic rats caused a glycaemia reduction, however the glucose levels remained high. These results indicate that hesperetin can be used for the prevention of induced neurovascular complications caused by decompensated diabetes.

Another complication observed in diabetes is the synthesis of advanced glycation endproducts, such as pentosidine. These compounds contribute to the lesions characteristic of microvascular complications and alter glomerular permeselectivity to proteins in diabetes. In a collagen advanced glycation *in vitro* study, hesperetin treatment (250μmol/L in 2% ethanol) inhibited 60% pentosidine formation in collagen incubated with glucose. Aminoguanidine and pyridoxamine, known glycoxidation inhibitors, prevented pentosidine formation by 86% and 89% respectively. These results indicate the promising potential of hesperetin in glycoxidation treatment [64].

Hesperetin can also be used in management of obesity due to its influence in the control of hunger and satiety. In this context, hesperetin analytical standard (0.1 – 1.0 mM) has shown the increase of secretion of cholecystokinin (CCK) in STC-1 cells. This phenomenon was caused by higher intracellular calcium concentration due to the TRP (transient receptor potential) and TRP 1 ankirin channels work. The addition of hesperidin analytical standard in the same model caused no effect, indicating that only the aglycone form influences hormone secretion [65]. The increase in CCK would be interesting because this hormone, secreted from endocrine cells in the small intestine, assists in the control of food intake [66].

Also, Yoshida et al. [67] observed that 3T3-L1 adipocytes cell culture treatment with hesperetin and naringenin analytical standards showed anti-inflammatory effect. NFκB activation through TNF-α was inhibited with a consequent reduction in the secretion of interleukin-6 (IL-6). There was also observed an inhibition of ERK (extracellular signal regulated kinase) pathway causing a decreased activation of hormone sensitive lipase (HSL); contributing to reduce the insulin resistance.

Subash-Babu et al [68] studied the effects of hesperetin in immortalized human bone marrow mesenchymal stem-cell (TERT20) differentiated with dexamethasone, IBMX,

indomethacin and insulin. Hesperetin was added in two different situations: in group 1 the flavanone was administered in the differentiation medium; in group 2 the compound was added after the differentiation in the maintenance medium.

In both cases there were a reduction on lipid accumulation by staining with Oil Red O, even though the effect was more pronounced in group 2, with almost 50% reduction. The glycerol release results shows that the less amount of lipid could be caused by stimulation of lipolysis. Hesperetin treatment also reduced triglyceride levels and GPDH activity, enzyme essential for glycerol-3phosphate synthesis, precursor of triacylglycerol. Only in group 1 there was a reduction in protein expression of PPAR-γ and C / EBPα, transcription factors necessary for the differentiation of pre-adipocytes into mature adipocytes. Adiponectin levels reduced after cell differentiation; however, treatment with hesperetin increased the mRNA expression of this adipokine. Resisitn, TNF-α and LPL mRNA expression reduced with hesperetin treatment. In addition, there was an increase in Bax, Bcl and p21 mRNA expression with hesperetin, especially in group 2, indicating the possible action of the flavanone in programmed cell death of differentiated adipocytes. For the authors, hesperetin could inhibit pre-adipocyte differentiation.

CONCLUSION

Hesperetin belongs to one of the largest group of plant phenolics, accounting for over half of the eight thousand naturally occurring phenolic compounds. Currently, most of phenolic compounds are obtained by chemical synthesis or extraction from plants, and these processes are only produced in the glycosylated form. However, there are environmentally friendly bioprocesses that deserve attention regarding phenolic compound production, especially in aglycon forms. These bioprocesses are clean technologies with great potential for obtaining biologically active compounds from natural sources, such as hesperetin.

The studies performed both *in vitro* and *in vivo* have shown that hesperetin play an important role in the prevention of degenerative and infective diseases, which is related to particular chemical structures. Hesperetin belongs to flavanones, which is a widely distributed group of polyphenolic compounds, called “nutraceutical substances”, with anticancer, anti-atherogenic, antimicrobial and anti-inflammatory properties.

REFERENCES

[1] Cushnie, T.P.T.; Lamb, A.J. Recent advances in understanding the antibacterial properties of flavonoids. *Int. J. Antimicrob. Agents* v. 38, p. 99–107, 2011.

[2] Martini, N.D.; Katerere, D.R.P.; Eloff, J.N. Biological activity of five antibacterial flavonoids from *Combretum erythrophyllum* (*Combretaceae*). *J. Ethnopharmacol.* v. 93, p. 207–212, 2004.

[3] Justesen, U.; Knuthsen, P.; Leth, T. Quantitative analysis of flavonols, flavones, and flavanones in fruits, vegetables and beverages by high- performance liquid chromatography with photo-diode array and mass spectrometric detection. *J. Chrom.* A v. 799, p. 101–110, 1998.

[4] Campillo, N.; Viñas, P.; Férez-Melgarejo, G.;Hernández-Córdoba, M. Dispersive liquid–liquid microextraction for the determination of flavonoid aglycone compounds in honey using liquid chromatography with diode array detection and time-of-flight mass spectrometry. *Talanta*. v. 131, p. 185–191, 2015.

[5] Hollman, P.C.; Bijsman, M.N.; Van Gameren, Y.; Cnossen, E.P.; de Vries, J.H.; Katan, M.B. The sugar moiety is a major determinant of the absorption of dietary flavonoid glycosides in man. *Free Rad. Res*. v. 31, p. 569–573, 1999.

[6] Silva, C.M.G.; Contesini, F.J.; Sawaya, A.C.H.F.; Cabral, E.C.; Cunha, I.B.S.; Eberlin, M.N.; Carvalho, P.O. Enhancement of the antioxidant activity of orange and lime juices by flavonoid enzymatic de-glycosylation. *Food Res. Int.* v. 52, p. 308–314, 2013.

[7] Lopez Sanchez, M. Procedure for obtaining hesperidin from citrus. *Spanish Patent* 545,275, 1986.

[8] Benavente-Garcia, O.; Castillo, J.; Marin, F. R.; Ortuno, A.; Del Rio, J. A. Uses and properties of Citrus flavonoids. *J. Agric. Food Chem*. v. 45, p. 4505−4515, 1997.

[9] Manthey, J.A.; Grohmann, K. Concentrations of hesperidin and other orange peel flavonoids in citrus processing byproducts. *J. Agric. Food Chem*. v. 44, p. 811–814, 1996.

[10] Marín, F.R.; Soler-Rivas, C.; Benavente-García, O.; Castillo, J.; Pérez-Álvarez, J.A. By-products from different citrus processes as a source of customized functional fibres. *Food Chem*. v. 100, p. 734–74, 2007.

[11] Bampidis, V.A.; Robinson, P.H. Citrus by-products as rumiant feeds: A review. *Animal Feed Sc. Technol*. v. 128, p. 175–217, 2006.

[12] Peterson, J.J.; Beecher, G.R.; Bhagwat, S.A.; Dwyer, J.T.; Gebhardt, S.E.; Haytowitz, D.B.; Holden, J.M. Flavanones in grapefruit, lemons, and limes: A compilation and review of the data from the analytical literature. *J. Food Comp. Anal*. v. 19, p. S74–S80, 2006.

[13] Di Mauro, A.; Fallico, B.; Passerini, A.; Maccarone, E. Waste Water from Citrus Processing as a Source of Hesperidin by Concentration on Styrene−Divinylbenzene Resin. *J. Agric. Food Chem*. v. 48, p. 2291–2295, 2000.

[14] Ma, Y.; Ye, X.; Wu, H.; Wang, H.; Sun, Z.; Zhu, P. Evaluation of the effect of ultrasonic variables at locally ultrasonic field on yield of hesperidin from penggan (*Citrus reticulata*) peels. LWT - *Food Sci. Technol.* v. 60, p, 1088–1094, 2015.

[15] Kanaze, F.; Termentzi, A.; Gabrieli, C.; Niopas, I.; Georgarakis, M.; Kokkalou, E. The phytochemical analysis and antioxidant activity assessment of orange peel (Citrus sinensis) cultivated in Greece–Crete indicates a new commercial source of hesperidin. *Biomed. Chrom*. v. 23, p. 239-249, 2009.

[16] Joubert, E.; Beer, D.; Hernández, I.; Munné-Boschc, S. Accummulation of mangiferin, isomangiferin, iriflophenone-3-C-β-glucoside and hesperidin in honeybush leaves (Cyclopia genistoides Vent.) in response to harvest time, harvest interval and seed source. *Ind. Crops Prod*. v. 56, p. 74–82, 2014.

[17] Borrás-Linares, I.; Stojanović, Z. Quirantes-Piné, R.; Arráez-Román, D.; Švarc-Gajić, J.; Fernández-Gutiérrez, A.; Segura-Carretero, A. Rosmarinus Officinalis Leaves as a Natural Source of Bioactive Compounds. *Int. J. Mol. Sci.* v. 15, p. 20585-20606, 2014.

[18] Naczk, M.; Shahidi, F. Phenolics in cereals, fruits and vegetables: Occurrence, extraction and analysis. *J. Pharm. Biom. Anal*. v. 41, p. 1523-1542, 2006.

[19] De Rijke, E.; Out, P.; Niessen, W.M.A.; Ariese, F.; Gooijer, C.; Brinkman, U.A.Th. Analytical separation and detection methods for flavonoids. *J. Chromat.* A v. 1112, p. 31-63, 2006.

[20] Li, B.B.; Smith, B.; Hossain, M.M. Extraction of phenolics from citrus peels: I. Solvent extraction method. Separ. *Purif. Technol*. v. 48, p. 182-188, 2006.

[21] Cheigh, C.-I.; Chung, E.-Y.; Chung, M.-S. Enhanced extraction of flavanones hesperidin and narirutin from Citrus unshiu peel using subcritical water. *J. Food Eng.* v. 110, p. 472-477, 2012.

[22] Chemat, F.; Abert-Vian, M.; Zill-E-Huma. Microwave assisted separations: green chemistry in action. In: Pearlman, J.T. (Ed.). *Green Chemistry Research Trends.* United States: Nova Science Publishers Inc, p. 1-30, 2009.

[23] Khan, M. K.; Zill, E.H.; Dangles, O. A comprehensive review on flavanones, the major citrus polyphenols. *J. Food Comp. Analys*. v. 33, p. 85-104, 2014.

[24] Anderson, T. Parameters affecting the extraction of polycyclic aromatic hydrocarbons with pressurized hot water. Ph.D. Thesis. University of Helsink, Finland. 2007.

[25] King, J. Development and potential of critical fluid technology in the nutraceutical industry. In: P. York, U.B.K., B.V. Shekunov (Ed.). *Drug Delivery and Supercritical Fluid Technology*. New York: Marcel Dekker, 2003. p.579–614.

[26] Hawthorne, S.B.; Yang, Y.; Miller, D.J. Extraction of organic pollutants from environmental solids with sub- and supercritical water. *Analyt. Chem.* v. 66, p. 2912-2920, 1994.

[27] Knorr, D. Impact of non-thermal processing on plant metabolites. *J. Food Engin.* v. 56, p. 131-134, 2003.

[28] Vilkhu, K.; Mawson, R.; Simons, L.; Bates, D. Applications and opportunities for ultrasound assisted extraction in the food industry — A review. *Innov. Food Sc. Emerg. Technol.* v. 9, p. 161-169, 2008.

[29] Yang, Z.R.Y. The extracting technology of flavonoids compounds. *J. Food Machin* v. 1, p. 7, 2003.

[30] Vinatoru, M. An overview of the ultrasonically assisted extraction of bioactive principles from herbs. *Ultrasonics Sonochem*. v. 8, p. 303-313, 2001.

[31] Ma, Y.Q.; Ye, X.Q.; Fang, Z.X.; Chen, J.C.; Xu, G.H.; Liu, D.H. Phenolic compounds and antioxidant activity of extracts from ultrasonic treatment of Satsuma Mandarin (*Citrus unshiu* Marc.) peels. *J. Agric. Food Chem.* v. 23, p. 5682-5690, 2008.

[32] Khan, M.K.; Abert-Vian, M.; Fabiano-Tixier, A.-S.; Dangles, O.; Chemat, F. Ultrasound-assisted extraction of polyphenols (flavanone glycosides) from orange (Citrus sinensis L.) peel. *Food Chem.* v. 119, p. 851-858, 2010.

[33] Londoño-Londoño, J.; de Lima, V.R.; Lara, O.; Gil, A.; Beatriz, T.; Pasa, C.; Arango, G.J.; Pineda, J.R.R. Clean recovery of antioxidant flavonoids from citrus peel: Optimizing na aqueous ultrasound-assisted extraction method. *Food Chem*. v. 119, p. 81-87, 2010.

[34] Povey, M.J.; Mason, T.J. *Ultrasound in food processing*. Springer, 1998. ISBN 0751404292.

[35] Knorr, D.; Zenker, M.; Heinz, V.; Lee, D.-U. Applications and potential of ultrasonics in food processing. *Trends Food Sc. Technol*. v. 15, p. 261-266, 2004.

[36] Leighton, T. 9 The principles of cavitation. *Ultras Food Process*. p. 151, 1998.

[37] Cárcel, J.A.; Pérez-García, J.V.; Benedito, J.; Mulet, A. Food process innovation through new technologies: use of ultrasound. *J. Food Eng*. v. 110, p. 200-207, 2012.

[38] Save, S.; Pandit, A.; Joshi, J. Microbial cell disruption: role of cavitation. *Chem. Engin. J. Biochem. Engin. J.* v. 55, p. B67-B72, 1994.

[39] Thakur, B.; Nelson, P. Inactivation of lipoxygenase in whole soy flour suspension by ultrasonic cavitation. *Food/Nahrung*, v. 41, p. 299-301, 1997.

[40] Dähnke, S.; Swamy, K.M.; Keil, F.J.A. A comparative study on the modeling of sound pressure field distribution in a sonoreactor with experimental investigation. *Ultrasonics Sonochem*. v. 6, p. 221-226, 1999.

[41] Wang, L.; Weller, C.L. Recent advances in extraction of nutraceuticals from plants. *Trends Food Sc Technol*. v. 17, p. 300-312, 2006.

[42] Inoue, T.; Tsubaki, S.; Ogawa, K.; Onishi, K.; Azuma, J.-i. Isolation of hesperidin from peels of thinned Citrus unshiu fruits by microwave-assisted extraction. *Food Chem*. v. 123, p. 542-547, 2010.

[43] Sun, X.L.; Zhang, L.; Qin, P.Y.; Tan, T.W. Microwave-assisted extraction of Hesperidin from pericarpium citri reticulate. *J. Chin. Med. Mater*. v. 30, p. 712-714, 2007.

[44] Hayat, K.; Hussain, S.; Abbas, S.; Farooq, U.; Ding, B.; Xia, S.; Jia, C.; Zhang, X.; Xia, W. Optimized microwave-assisted extraction of phenolic acids from citrus mandarin peels and evaluation of antioxidant activity in vitro. *Sep. Pur. Technol*. v. 70, p. 63-70, 2009.

[45] Madeira Jr., J.V.; Nakajima, V.M.; Macedo, J.A.; Macedo, G.A. Rich bioactive phenolic extract production by microbial biotransformation of Brazilian citrus residues. *Chem. Eng. Res. Design* v. 92, p. 1802-1810, 2014.

[46] Madeira Jr., J.V.; Teixeira, C.B.; Macedo, G.A. Biotransformation and bioconversion of phenolic compounds obtainment: an overview. *Critical Reviews in Biotechnology*, v. 0, n. 0, p. 1-7, 2013.

[47] Georgetti, S.R.; Vicentini, F.T.; Yokoyama, C.Y.; Borin, M.F.; Spadaro, A.C.; Fonseca, M.J. Enhanced in vitro and in vivo antioxidante activity and mobilization of free phenolic compounds of soybean flour fermented with different beta-glucosidase-producing fungi. *J. Appl. Microbiol.* v. 106, p. 459-466, 2009.

[48] Barzana, E.; Rubio, D.; Santamaria, R.I.; Garcia-Correa, O.; Garcia, F.; Ridaura Sanz, V.E.; López-Munguía, A. Enzyme-Mediated Solvent Extraction of Carotenoids from Marigold Flower (*Tagetes erecta*). *J. Agric. Food Chem*. v. 50, p. 4491-4496, 2002.

[49] Ruiz-Terán, F.; Perez-Amador, I.; López-Munguia, A. Enzymatic Extraction and Transformation of Glucovanillin to Vanillin from Vanilla Green Pods. *J. Agric. Food Chem*. v. 49, p. 5207-5209, 2001.

[50] Mcglone, O.C.; Canales, A.L.M.; Carter, J.V. Coconut oil extraction by a new enzymatic process. *Journal of food science*, v. 51, p. 695-697, 1986.

[51] Weinberg, Z.G.; Akiri, B.; Potoyevski, E.; Kanner, J. Enhancement of Polyphenol Recovery from Rosemary (Rosmarinus officinalis) and Sage (Salvia officinalis) by Enzyme-Assisted Ensiling (ENLAC). *J. Agric. Food Chem*. v. 47, p. 2959-2962, 1999.

[52] Landbo, A.-K.; Meyer, A.S. Enzyme-Assisted Extraction of Antioxidative Phenols from Black Currant Juice Press Residues (Ribes nigrum). *J Agricult Food Chem*. v. 49, p. 3169-3177, 2001.

[53] Van Den Brink, J.; De Vries, R.P. Fungal enzyme sets for plant polysaccharide degradation. *Appl. Microbiol. Biotechnol.* v. 91, p. 1477-1492, 2011.

[54] Puri, M.; Sharma, D.; Barrow, C.J. Enzyme-assisted extraction of bioactives from plants. *Trends in Biotechnol*. v. 30, p. 37-44, 2012.
[55] Mandalari, G.; Bennett, R.N.; Kirby, A.R.; Lo Curto, R.B.; Bisignano, G.; Waldron, K.W.; Faulds, C.B. Enzymatic hydrolysis of flavonoids and pectic oligosaccharides from bergamot (*Citrus bergamia* Risso) peel. *J. Agric. Food Chem*. v. 18, p. 8307-13, 2006.
[56] Lakshmi, V.; Joseph, SK.; Srivastava, S.; Verma, S.K.; Sahoo, M.K.; Dube, V.; Mishra, S.K.; Murthy, P.K. Antifilarial activity in vitro and in vivo of some flavonoids tested against *Brugia malayi*. *Acta Tropica*. v. 116, p.127-133, 2010.
[57] Hirata, A.; Murakami, Y.; Shoji, M.; Kadoma, Y.; Fujisawa, S. Kinetics of radical-scavenging activity of hesperetin and hesperidin and their inhibitory activity on COX-2 expression. *Anticancer Res.* v. 25, p. 3367-3374.
[58] Takumi, H.; Nakamura, H.; Simizu, T.; Harada, R.; Kometani, T.; Nadamoto, T.; Mukai, R.; Murota, R.K.; Kawaix, Y.; Terao, J. *Food Funct*. 3, 389 (2012).
[59] M. Yamamoto, H. Jokura, K. Hashizume, H. Ominami, Y. Shibuya, A. Suzuki, T. Hase, A. Shimotoyodome, *Food Funct.* 4, 1346 (2013).
[60] W. Deng, D. Jiang, Y. Fang, H. Zhou, Z. Cheng, Y. Lin, R. Zhang, J. Zhang, P. Pu, Y. Liu, Z. Bian, Q. Tang, *J. Mol. Hist.* 44, 575 (2013).
[61] A. Iio, K. Ohguchi, M. Iinuma, Y. Nozawa, M. Ito, *J. Nat. Prod.* 75, 563 (2012).
[62] B. Kumar, S. K. Gupta, B.P. Srinivasan, T. C. Nag, S. Srivastava, R. Saxena, *Vascular Pharmacology* 57, 201 (2012).
[63] 63.B. Kumar, S. K. Gupta, B.P. Srinivasan, T. C. Nag, S. Srivastava, R. Saxena, K. A. Jha, *Microvascular Research* 87, 65 (2013).
[64] P. Urios, I. Kassab, A.M. Grigorova-Borsos, R. Guillot, P. Jacolot, F. Tessier, J. Peyroux, M. Sternberg, *Diabetes Research and Clinical Practice* 105, 373 (2014).
[65] H. Y. Kim, M. Park , K. Kim, Y. M. Lee, M. R. Rhyu, *Biomolecules & Therapeutics* 21(2), 121–125 (2013).
[66] H. E. Raybould, *Current Opinion in Pharmacology* 7(6), 570 (2009).
[67] H. Yoshida, N. Takamura, T. Shuto, K. Ogata, J. Tokunaga, K. Kawai, H.Kai, *Biochemical and Biophysical Research Communications* 394, 728 (2010).
[68] P. Subash-Babu, .A. A. Alshatwi, *J. Biochem Molecular Toxicology* 00 (2014).

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Vânia Nakajima Good afternoon, Thanks fo Out 5 em 2:02 PM

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> VÃ¢nia Mayumi Nakajima
> Nutricionista - UFV
> Mestre em CiÃªncia da NutriÃ§Ã£o - UFV
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