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Cristiane Copetti

ESTUDO COMPARATIVO DE SUCO DE UVA E VINHO TINTO EM PARÂMETROS BIOQUÍMICOS E DE ESTRESSE OXIDATIVO *IN VIVO* E EM HUMANOS

Santa Maria, RS
2017

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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Ciência e Tecnologia dos Alimentos, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de **Doutora em Ciência e Tecnologia dos Alimentos**

Orientadora: Profª. Drª. Neidi Garcia Penna

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2017

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Santa Maria, RS
2017

DEDICATÓRIA

Aos meus pais, Luiz Carlos e Alda
pelos princípios, apoio e orientação,

Aos meus "Antônios", minha razão
de viver,

Com amor, dedico.

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“Foi o tempo que dedicaste a tua rosa que fez a tua rosa tão importante.”
(Trecho de "O Pequeno Príncipe)
Antoine de Saint-Exupéry

RESUMO

CAPACIDADE ANTIOXIDANTE E EFEITO DO CONSUMO AGUDO DE SUCO DE UVA INTEGRAL E VINHO TINTO EM HUMANOS

AUTORA: CRISTIANE COPETTI
ORIENTADORA: NEIDI GARCIA PENNA

Os compostos fenólicos são as maiores fontes de antioxidantes consumidos na dieta humana e estão distribuídos amplamente em uvas e seus produtos derivados. Dentre estes compostos destacam-se os flavonoides (catequina, epicatequina, quercetina, antocianinas e procianidinas) e o resveratrol (3,5,4-trihidroxi-estilbeno), os quais são encontrados, principalmente, em produtos de uva tinta. Diversos estudos *in vitro*, em modelos animais e humanos vêm demonstrando que estes compostos possuem ação antioxidante e melhoram o metabolismo lipídico. No entanto, os compostos fenólicos presentes no vinho tinto e suco de uva apresentam diferenças em sua composição e quantidade. O presente estudo teve por objetivo avaliar o efeito do consumo agudo de suco de uva integral e vinho tinto de duas cultivares sobre os biomarcadores de estresse oxidativo em indivíduos saudáveis e o efeito antioxidante celular *in vitro*. Este estudo de intervenção, caracterizado como um ensaio clínico *crossover*, utilizou o suco de uva integral, vinho tinto e água (como controle), associado ou não à uma refeição, voluntários saudáveis foram avaliados antes e após o consumo. Cada sujeito foi submetido ao procedimento descrito acima, três vezes com intervalo de uma semana entre cada procedimento, recebendo em cada uma das vezes um dos três tratamentos. O sangue foi utilizado para análises bioquímicas e de potencial antioxidante. Em adição a isso, células neurais da linha SH-SY5Y foram induzidas ao estresse oxidativo com a adição de peróxido de hidrogênio e adicionadas de diferentes concetrações de suco e vinho afim de testar o potencial antioxidante das bebidas. Os resultados mostram que a grande quantidade de compostos fenólicos encontrados nas amostras de suco de uva e vinho Bordo contribuíram para o alto potencial antioxidante *in vitro* dessas bebidas. Além disso, a capacidade antioxidante *in vitro* pode ser reproduzida *in vivo* após a ingestão aguda por sujeitos saudáveis, o consumo de suco e vinho Bordo melhorou a capacidade antioxidante e reduziu a oxidação lipídica em voluntários saudáveis. Suco e vinho Bordo foram capazes de diminuir os

níveis de glicose e apenas o vinho aumentou os níveis de ácido úrico. Do mesmo modo, o vinho não teve efeito antioxidante na cultura celular mostrando ser tóxico em alta concentração, enquanto o suco teve efeitos antioxidantes contra o estresse oxidativo celular induzido por H_2O_2. O suco de uva e vinho podem ser utilizados para melhorar a saúde e na prevenção de doenças relacionadas ao estresse oxidativo, mas o vinho deve ser consumido em doses menores devido ao efeito pró-oxidante observado na cultura celular.

Palavras chave: compostos bioativos, atividade antioxidante, estresse oxidativo, ingestão aguda, suco de uva integral, vinho tinto, ensaio celular.

ABSTRACT

ANTIOXIDANT CAPACITY AND ACUTE CONSUMPTION EFFECT OF INTEGRAL GRAPE JUICE AND RED WINE IN HUMANS

AUTHOR: CRISTIANE COPETTI
ADVISER: NEIDI GARCIA PENNA

Phenolic compounds are the major sources of antioxidants consumed in the human diet and are widely distributed in grapes and their by-products. Among these compounds are the flavonoids (catechin, epicatechin, quercetin, anthocyanins and procyanidins) and resveratrol (3,5,4-trihydroxy-stilbene), which are mainly found in grape products. Several *in vitro* studies in animal and human models have been demonstrating that these compounds have antioxidant action and improve lipid metabolism. However, the phenolic compounds present in red wine and grape juice show differences in their composition and quantity. The objective of the present study was to evaluate the effect of the acute consumption of grape juice and red wine from two cultivars on the biomarkers of oxidative stress in healthy individuals and the antioxidant effect *in vitro*. This intervention study, characterized as a crossover clinical trial, used grape juice, red wine and water (control), where healthy volunteers were evaluated before and after consumption, associated or not with a meal. Each subject was submitted to the procedure described above, three times with a one-week interval between each procedure, receiving at each of the three treatments. The blood was used for biochemical analysis and antioxidant potential. In addition to this, SH-SY5Y neural cells were induced to oxidative stress with the addition of hydrogen peroxide and added to different concentrations of juice and wine in order to test the antioxidant potential of the beverages. The results showed that great amount of phenolic compounds found in the samples of grape juice and wine contributed to the high *in vitro* antioxidant potential of these beverages. In addition, *in vitro* antioxidant capacity can be reproduced *in vivo* following acute ingestion by healthy subjects, consumption of Bordo juice and wine improved antioxidant capacity and reduced lipid oxidation in healthy volunteers. Juice and wine were able to lower glucose levels and only wine increased uric acid levels. Likewise, the wine had no antioxidant effect on the cell culture showing to be toxic in high concentration, while the juice had antioxidant effects against the cellular oxidative stress induced by H_2O_2. Grape juice and wine can be

used to improve health and as a preventive for diseases related to oxidative stress, but wine should be consumed in smaller doses due to the pro-oxidant effect observed in cell culture.

Keywords: bioactive compounds, antioxidant activity, oxidative stress, acute intake, whole grape juice, red wine, cell assay.

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LISTA DE ABREVIATURAS E SIGLAS

°C	Graus Celsius
µL	microlitros
AAPH	2,2'-Azobis(2-amidinopropane) dihydrochloride
ABTS	2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium
$AlCl_3$	Cloreto de alumínio
CE	Equivalente em catequina
CO_2	Dióxido de carbono
DNA	Ácido desoxirribonucleico
ERN	Espécies reativas de nitrogênio
ERO	Espécies reativas de oxigênio
$FeCl_3$	Cloreto de Ferro
FRAP	Ferric reducing antioxidant power
GAE	Equivalente em ácido gálico
h	horas
H_2O_2	Peróxido de hidrogênio
HBA	Ácido hidroxibenzóico
HCA	Acido hidroxicinâmico
HCl	Ácido clorídrigo
HDL	Lipoproteína de alta densidade
HPLC	Cromatografia líquida de alta eficiência
L^{-1}	Por litro
LDL	Lipoproteína de baixa densidade
m	metros
M	Molar
M-3-G	Malvidina-3-glicosídeo
ME	Equivalente em malvidina
mg	miligramas
min	Minutos
mL	mililitros
mM	Milimolar
Na_2CO_3	Carbonato de sódio
$NaNO_2$	Nitrito de sódio
NaOH	Hidróxido de sódio
nm	nanometros
ROS	Reactive oxygen species
S	South
SO_2	Dióxido de enxofre
TAC	Capacidade antioxidante
TE	Equivalente em trolox
TPTZ	2,4,6-tris(2-pyridyl)-S-triazine
TxB_2	Thromboxane B2
W	west

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SUMÁRIO

1 APRESENTAÇÃO

Essa tese segue as normas estabelecidas na Estrutura e Apresentação de Monografias, Dissertações e Teses – MDT da UFSM (UFSM, 2015). Os resultados estão apresentados na forma de três artigos científicos que se encontram no ítem **DESENVOLVIMENTO**. As seções Materiais e Métodos, Resultados e Discussão encontram-se nos artigos científicos e representam a íntegra desse trabalho. Ao final dessa tese, encontra-se o item **CONCLUSÕES**, apresentando uma compilação de interpretações e comentários a respeito dos resultados demonstrados nos artigos científicos contidos nesse trabalho. As **REFERÊNCIAS BIBLIOGRÁFICAS** referem-se somente às citações que aparecem no item **INTRODUÇÃO** e **REVISÃO BIBLIOGRÁFICA** dessa tese.

2 INTRODUÇÃO

A uva é uma das frutas mais investigadas no mundo, sua composição e propriedades tem sido extensivamente estudada quanto a presença de grandes quantidades de compostos fenólicos, sendo que a maioria desses compostos encontrados podem atuar como antioxidantes (ROCKENBACH et al., 2011). Os compostos fenólicos são as maiores fontes de antioxidantes consumidos na dieta humana e estão distribuídos amplamente em frutas, hortaliças e bebidas como o chá, a cerveja, o suco de uva e o vinho (AGUDO et al., 2007).

Os vinhos tintos são fontes ricas em compostos fenólicos, e estes exercem um potente aumento da capacidade antioxidante em humanos, ao inibir a oxidação das lipoproteínas de baixa densidade (LDL) humana *in vitro* e reduzir a susceptibilidade da peroxidação lipídica no plasma humano (GOLDE; SLOOTS; VERMEULEN, 1999; DOTAN; LICHTENBERG; PINCHUK, 2004). O suco de uva também é apontado como uma excelente fonte desses compostos. Em estudo realizado por Chou, Keevil e Aeschlimann (2001), estes demonstraram que o consumo de suco reduziu a agregação plaquetária, inibição da oxidação da LDL e agiu como relaxante do endotélio.

No entanto, os compostos fenólicos presentes no vinho tinto e suco de uva apresentam diferenças em sua composição e quantidade (DAS; DAS, 2010). Além do mais, o mecanismo que envolve a proteção do vinho é bastante discutível, devido aos efeitos do álcool e dos componentes não alcóolicos do vinho. O álcool por si só possui efeitos favoráveis nos índices das lipoproteínas de alta densidade (HDL) e agregação plaquetária (ESTRUC; SCANELLA; BADÍA, 2004; IRITI; VARONI, 2014).

Por tudo isso, muitos estudos sugerem os benefícios do consumo do vinho pela presença dos compostos fenólicos, como também sua associação ao álcool; por outro lado, alguns estudos ainda sugerem o consumo do suco de uva como fonte de compostos fenólicos e alternativa não alcóolica, e disponível para uma ampla faixa da população. Contudo, não foram encontrados na literatura, estudos conclusivos comparando o consumo do vinho e do suco integral (Tabela 1). Este estudo propõe também a correlação entre o potencial antioxidante *in vitro* e *in vivo* das bebidas, a fim de demonstrar se os benefícios são reprodutíveis em modelo humano.

Tabela 1 - Estado da arte referente a estudos que utilizaram como variáveis o efeito do consumo de suco e vinho sobre a atividade antioxidante de indivíduos saudáveis.

Autores/Local	Objetivos	Ensaio experimental e parâmetros avaliados	Conclusão
Bub e colaboradores, 2001 - Alemanha	Comparar as alterações na malvidina-3-glucósido do plasma (M-3-G) e sua excreção urinária após ingestão de vinho tinto, vinho tinto desalcolizado e suco de uva tinto.	Seis homens saudáveis consumiram 500 mL de cada bebida. M-3-G no plasma e na urina foram mensurados por HPLC.	M-3-G é mal absorvido após uma única ingestão. Outros compostos polifenólicos podem ser responsáveis pela atividade antioxidante *in vivo*.
Frank e colaboradores, 2003 - Alemanha	Investigar os parâmetros farmacocinéticos após o consumo de vinho e suco de uva tinto.	Nove voluntários saudáveis ingeriram 400 mL de cada bebida. Antocianinas no plasma e urina foram mensuradas por HPLC.	Uma baixa biodisponibilidade não conseguiu explicar o efeito antioxidante das antocianinas.
Coimbra e colaboradores, 2005 - Brasil	Comparar os efeitos de suco de uva e vinho tintos sobre a reatividade arterial, agregação plaquetária, moléculas de adesão, glicose e lipídios plasmáticos em indivíduos hipercolesterolêmicos.	Dezesseis indivíduos hipercolesterolêmicos ingeriram 500 mL de suco de uva ou 250 mL de vinho tinto por dia durante 14 dias. Análises de perfil lipídico e de agregação plaquetária.	O suco protegeu contra a doença arterial coronariana sem os efeitos negativos adicionais do álcool.
Bitsch e colaboradores, 2004 - Alemanha	Investigar a biodisponibilidade e biocinética após o consumo de vinho e suco de uva tinto.	Nove indivíduos saudáveis ingeriram 400 mL de suco de uva ou vinho tinto. Antocianinas foram avaliadas no plasma e urina por HPLC.	A atividade antioxidante plasmática aumentou para níveis mais altos após a ingestão de suco em comparação com o vinho

Desta forma elaborou-se a seguinte pergunta de partida: Qual é o efeito do consumo agudo de suco e vinho na atividade antioxidante e biomarcador de dano oxidativo em indivíduos saudáveis?

3 OBJETIVOS

3.1.1 Objetivo geral

Comparar os efeitos do consumo de suco de uva integral e vinho tinto sobre os marcadores de estresse oxidativos em humanos.

3.1.2 Objetivos específicos

- Avaliar o efeito neuroprotetor de suco e vinho de uvas Cabernet Sauvignon em células SH-SY5Y.
- Avaliar o efeito de suco e vinho de uvas Bordo sobre o *status* oxidativo *in vitro* e em humanos.
- Avaliar o efeito de suco e vinho de uvas Bordo sobre alterações pós prandiais em parâmetros bioquímicos e oxidativos em humanos.

4 REVISÃO BIBLIOGRÁFICA

4.1 VITICULTURA

A videira pertence ao gênero *Vitis,* família *Vitaceae*. O gênero *Vitis* é composto por mais de 60 espécies, cuja distribuição geográfica espontânea contempla os continentes asiático, europeu e americano. A espécie mais cultivada no mundo é a *Vitis vinifera*, apresentando grande número de cultivares, tanto de uvas para vinho como também de uvas de mesa e de uvas para a produção de passas. As cultivares desta espécie também são conhecidas como uvas europeias ou uvas finas. A segunda espécie em importância pela área cultivada no mundo é a *Vitis labrusca* e o número de variedades cultivadas desta espécie limita-se a algumas dezenas. As uvas de *V. labrusca* são utilizadas para o consumo *in natura* e processamento, em especial para a elaboração de suco de uva; em alguns países da América e da Ásia também são elaborados vinhos com uvas labruscas (CAMARGO, 2017).

As uvas finas (*V. vinifera*) são usadas em todo o mundo para consumo *in natura* e processamento. Aproximadamente 70 cultivares compõem o elenco varietal brasileiro de uvas finas para processamento. As principais cultivares tintas, pelo volume processado, são Cabernet Sauvignon, Merlot, Cabernet Franc, Tannat, Ancellota, Pinot Noir e Egiodola, mais expressivas no sul do país, e as cvs. Syrah e Alicante Bouschet, mais importantes na Região Nordeste. As uvas comuns representam mais de 80% da produção brasileira de uvas para processamento e têm significativa importância também como uvas de mesa. Cerca de 40 cultivares entre labruscas, bourquinas e híbridas interespecíficas compõem o elenco varietal brasileiro. As principais cultivares tintas são Isabel, Bordô, Concord, pertencentes à espécie *V. labrusca*, com grande aptidão para a elaboração de suco, mas também utilizadas para a produção de vinhos (CAMARGO, 2017).

No Brasil as variedades de uvas americanas *Vitis labrusca* L. são amplamente cultivadas, principalmente para a elaboração de sucos e vinhos. A variedade Bordô é uma das mais cultivadas nacionalmente e se destaca por sua excelente adaptação às condições climáticas brasileiras, apresentando em sua composição alta concentração de matéria corante e originando bebidas com intensa coloração (MAIA; CAMARGO, 2005). Embora a cultivar Cabernet Sauvignon tenha sido introduzida no Brasil em 1921, foi somente depois de 1980 que houve incremento de seu plantio na Serra

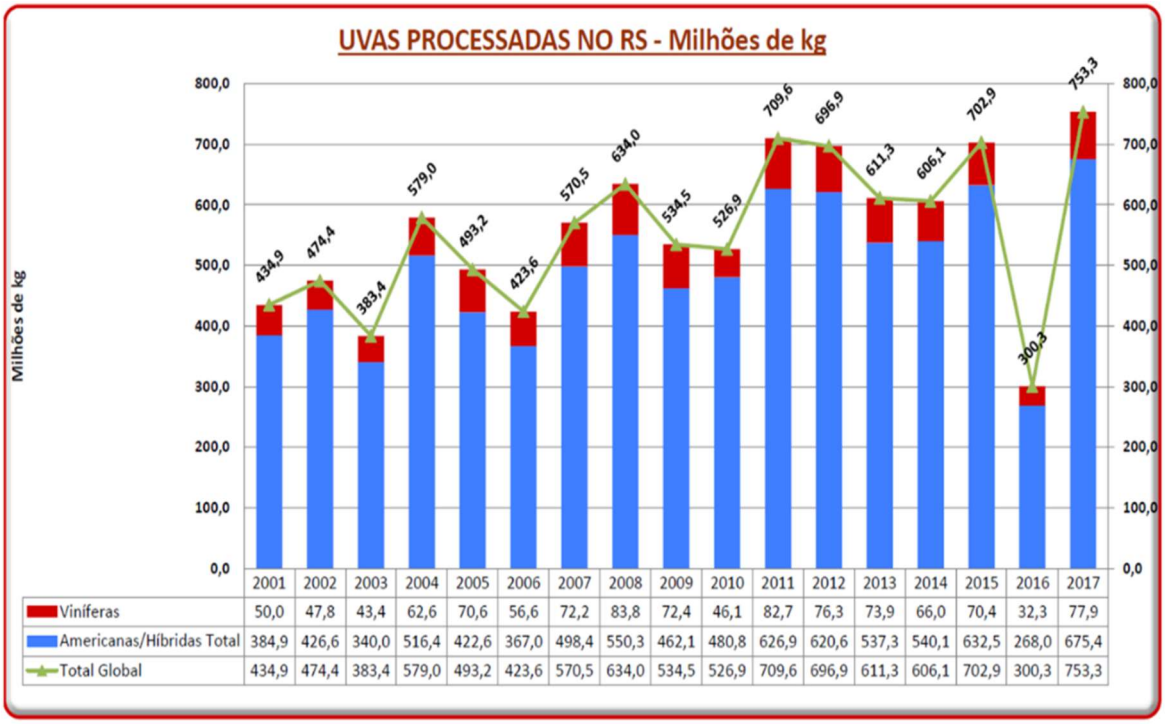
Gaúcha e na Fronteira Oeste do Rio Grande do Sul. Atualmente, é a cultivar de *Vitis vinifera* com maior demanda para a implantação de novos vinhedos (CAMARGO, 2017).

A viticultura, ciência que estuda a produção da uva, é uma atividade tradicional, a qual se desenvolveu, no Brasil, com base em uvas americanas da variedade *Vitis labrusca* e *Vitis bourquina*, usadas para a elaboração de vinho de mesa. Só a partir do século XX, passou a produzir vinhos finos, com uvas da variedade *Vitis vinífera* (CAMARGO, 2009).

A viticultura brasileira se destaca em 13 regiões brasileiras, sendo subdivididas quanto as condições climáticas, zona temperada, subtropical e tropical. A zona temperada é composta por 9 regiões, Fronteira, Serra do Sudeste, Centro e Norte do RS, Serra Gaúcha, Campos de cima da Serra, Planalto Norte e Carbonífera, Planalto Serrano e Vale do Rio do peixe, Sudeste de São Pauto e Sul de Minas. A região do Norte do Paraná é a única integrante da Zona Subtropical. E as regiões Noroeste de SP, Norte de Minas e Vale do Submédio do São Francisco (Pernambuco e Bahia) caracterizam-se como zonas tropicais, com sistemas de manejo adaptado as suas condições ambientais específicas (IBRAVIN, 2012).

A produção de uvas no Brasil vem aumentando a cada ano, apenas no Rio Grande do Sul, responsável por cerca de 90% da produção nacional de vinhos, sucos e derivados, a quantidade de uvas processadas passou de 434,9 para 753,3 milhões de Kg nos últimos 10 anos. Só no ano de 2017 obteve-se uma produção de 77,9 milhões de Kg de uvas viníferas, enquanto que a produção de uvas híbridas americanas atingiu a marca de 675,4 milhões de Kg (Figura 1) (IBRAVIN, 2017). A produção de uvas é especialmente importante na serra gaúcha pois, quase a totalidade da produção se destina à agroindústria do suco e do vinho e é essencialmente produzida por pequenos agricultores de agricultura familiar. Nos últimos anos, com a implementação das Indicações Geográficas no Brasil, a viticultura tem contribuído fortemente para o desenvolvimento dos territórios envolvidos, promovendo agregação de valor aos produtos e a valorização de seus respectivos fatores naturais e culturais (EMBRAPA, 2016).

Figura 1 - Dados da evolução de quantidade de uvas processadas pelas empresas do RS (milhões de Kg).



Fonte: IBRAVIN/MAPA/SEAPI-RS Cadastro Vinícola, 2017.

Segundo dados do informativo da EMBRAPA no ano de 2016, os vinhos de mesa, elaborados com uvas americanas e híbridas, mostraram aumento de 7,21% na sua produção, com alta de 7,63% para os tintos e de 5,66% para os brancos. O suco de uva apresentou incremento de produção de 9,63%, sendo o maior aumento de suco de uva integral (20,54%). A produção de suco concentrado aumentou em 6,79%. Cabe destacar também o aumento de produção de mosto simples em 75,24%, o qual pode ser usado tanto para vinificação quanto para a elaboração de suco e outros derivados. O segmento de suco tem sido uma alternativa para a sustentabilidade da vitivinicultura gaúcha pois tem absorvido uma boa parte da produção de uvas americanas e híbridas que tradicionalmente eram absorvidas pelos vinhos de mesa (MELLO, 2015). Além do mais, dados desse ano mostraram que a produção de vinhos, sucos e derivados no Rio Grande do Sul, foi de 485,44 milhões de litros, cerca de 2,4 vezes maior que no ano anterior (Figura 2).

Figura 2 - Tabela da Elaboração de Vinhos e Derivados no Rio Grande do Sul - 2006 a 2017

ANO	MILHÕES DE LITROS			
	Vinhos Viníferas	Vinhos Comuns	Outros derivados da uva e do vinho	TOTAL
2006	32,12	185,08	59,13	276,33
2007	43,18	275,25	70,89	389,32
2008	47,33	287,44	93,19	427,97
2009	39,90	205,42	96,50	341,82
2010	27,85	195,25	98,96	321,21
2011	52,20	258,73	151,15	461,07
2012	48,60	213,10	167,28	428,98
2013	48,40	197,90	125,15	371,45
2014	38,46	196,07	140,19	374,72
2015	39,20	210,30	193,00	442,50
2016	20,60	86,41	93,72	200,73
2017	49,31	254,15	181,98	485,44

Fonte: IBRAVIN/MAPA/SEAPI-RS - Cadastro Vinícola (2017)

4.2 POLIFENÓIS NA UVA, SUCO E VINHO

Os compostos fenólicos são importantes metabólitos de plantas, os quais estão presentes em uvas e derivados como o vinho e o suco e desempenham um papel importante nas características sensoriais destes, pois são responsáveis por algumas propriedades organolépticas, tais como aroma, cor, sabor, amargura e astringência (LINSKENS; JACKSON, 1988; SCALBERT et al., 1993). Além das características sensoriais, essas substâncias estão relacionadas com a estabilidade química que proporcionam diversos benefícios para a saúde (CAMARGO et al., 2014; GARRIDO; BORGES, 2013; TOALDO, 2015).

Estes compostos podem se dividir em dois grandes grupos: flavonóides e não flavonóides. Em termos de quantidade, os principais fenólicos presentes em vinhos e sucos pertencem às famílias dos flavonóides, tais como flavanóis, antocianinas e ácidos fenólicos (GRANATO et al., 2015; LEEUW et al., 2014; LIMA et al., 2014). Os principais flavanóis encontrados em vinhos e sucos de uva são as catequinas, epicatequinas, epigalocatequinas e procianidinas, compostos estes associados ao sabor e várias propriedades antimicrobianas, antiinflamatórias e bioativas como atividade antioxidante *in vitro* e *in vivo* (GRANATO et al., 2016; LEEUW et al., 2014;

SCOLA et al., 2010). As antocianinas são as substâncias responsáveis pela cor característica de vinhos e sucos de uva, e as principais encontradas são: malvidina, cianidina, peonidina, delfinidina, petunidina e pelargonidina, as quais nas variedades *Vitis vinifera L.* e *Vitis labrusca L*. predominam nas formas de 3-monoglicosideo e 3,5-diglicosideo, respectivamente (GARRIDO; BORGES, 2013; LAMBRI ET AL., 2015; NIXDORF; HER MOSÍN-GUTIÉRREZ, 2010).

Os principais compostos não flavonóides em vinho são os ácidos fenólicos e são divididos em hidroxibenzóico (HBA) e hidroxicinâmico (HCA). Os principais ácidos HBA presentes em sucos e vinhos são protocatequina, vanilina, ácido gálico e siríngico, e os principais HCA são q-cumárico, cafeína, ferulico, ácido cis e trans cinâmico (GARRIDO; BORGES, 2013; GRANATO et al., 2016; LEEUW, et al., 2014; TOALDO, 2015). Estes compostos desempenham um papel primordial na definição das características sensoriais dos vinhos, dando o sabor de "madeira de carvalho" típico dos vinhos envelhecidos, além de serem em grande parte responsáveis pela adstringência e amargor dos vinhos jovens (MONAGAS; BARTOLOMÉ; GÓMEZ-CORDOVÉS, 2005; SOMERS; EVANS, 1987; VRHOVSEK, 1998). Os ácidos hidroxicinâmicos e os seus ésteres tartáricos são a principal classe de fenólicos não flavonóides nos vinhos tintos. Eles estão envolvidos nas reações de ressonância de mosto e vinho e são precursores de fenóis voláteis (VRHOVSEK, 1998). O ácido gálico é o principal ácido hidroxibenzóico no vinho tinto. Vinhos envelhecidos em carvalho apresentam altos níveis de derivados de ácido hidroxibenzóico, principalmente ácido elágico (LOPES et al., 2006).

O conhecimento da relação entre a qualidade de um determinado vinho e a sua composição fenólica são, no presente, um dos maiores desafios na pesquisa enológica. O perfil de antocianinas, por exemplo, foi proposto como uma ferramenta analítica na certificação de varietais de vinhos (KENNEDY, 2008; KONTOUDAKIS et al., 2011). É conhecido, também, que os padrões de algumas classes de flavonóides, como antocianinas, estão sob rigoroso controle genético e que sua distribuição varia consideravelmente entre diferentes cultivares de uva (REVILLA et al, 2001). Pode, ainda, ajudar a avaliar a autenticidade dos produtos regionais e a predição das propriedades sensoriais e estabilidade oxidativa do vinho (LOPES et al., 2006). Além disso, os fenólicos são utilizados como marcadores da tecnologia de processamento de vinhos ou envelhecimento do vinho (RIBÉREAU-GAYON et al., 1998; VRHOVSEK, 1998).

A composição fenólica não volátil do vinho depende de inúmeras fatores como variedade de uva e maturidade, fatores ambientais nos vinhedos (clima, solo e estágio sanitário) e a tecnologia vinícola, bem como condições de fermentação e envelhecimento (FANG et al., 2008). Práticas de pré-fermentação, como adição de SO_2 e ácido ascórbico antes do esmagamento de uvas ou operações como maceração, fermentação alcoólica, inoculação de diferentes cepas de fermento, fermentação maloláctica, fenômenos de precipitação, oxidação ou adsorção, em conjunto com atividade da β-glucosidase e esclarecimento com alguns agentes de compensação (usado nas operações de esclarecimento e filtração do vinho) também podem influenciar o níveis de compostos fenólicos durante o processo de vinificação (BALÍK, et al, 2008; KENNEDY, 2008; LINSKENS; JACKSON, 1988; SAUCIER, 2010; SCALBERT et al., 1993).

4.3 POTENCIAL ANTIOXIDANTE *IN VIVO* E *IN VITRO* DO SUCO E DO VINHO

A uva e seus derivados, conforme já visto, são ricos em compostos fenólicos e diferentes estudos vêm demonstrando que essas substâncias possuem atividade biológica benéfica para a saúde dos consumidores (KRIKORIAN et al., 2012; VAUZOUR et al., 2010). Os compostos fenólicos, principalmente flavonóides (flavanóis, flavonóis e antocianinas) estão associados com uma melhora na saúde, juntamente com outros compostos que não são flavonóides, como os ácidos fenólicos e o estilbeno resveratrol (ALI et al., 2010; KRIKORIAN et al., 2012; SAUTTER et al., 2005; XIA et al., 2010).

Os flavonóis receberam interesse considerável devido às suas propriedades antioxidantes (MUDNIC et al., 2010). Entre eles, (+)-catequina, (-)-epicatequina e as procianidinas ganharam atenção devido a sua atividade antioxidante, antimicrobiana e bactericida (XIA et al., 2010). As principais antocianinas encontradas, malvidina, cianidina, delfinidina, petunidina, peonidina e pelargonidina e seu consumo está associado a atividades biológicas, como capacidade antioxidante e prevenção de doenças cardiovasculares (XIA et al., 2010). Ácidos fenólicos, como o gálico, caféico e clorogênico, foram estudados por sua capacidade antioxidante e atuação como dilatadores venosos (MUDNIC et al., 2010). Além disso, estilbenos, particularmente o trans-resveratrol (trans-3,5,40-trihydroxystilbene), tem sido associado com muitos benefícios para a saúde incluindo ação bactericida, fungicida, cardio-protetora,

atividade anticancerígena, assim como um aumento na longevidade em humanos (ALI et al., 2010).

Estudos *in vitro* mostraram que o suco de uva tem significativa atividade antioxidante e pode inibir a oxidação de LDL (ABU-AMSHA et al., 1996; WANG, 1996; DURAK et al., 1999). Estudos em humanos mostraram resultados promissores, mas foram limitados pela curta duração de suplementação (MIYAGI, 1997; DAY, et al, 1997; FREEDMAN et al., 2001; STEIN et al., 1999), até o presente momento, existem poucos estudos sobre os efeitos de consumo crônico de suco de uva em estado antioxidante e marcadores de danos oxidativos em lipídios e proteínas. Estudos *in vitro*, conforme descrito por O'byrne et al. (2002), sugerem que os flavonóides de suco de uva fornecem uma proteção antioxidante in vivo mais potente que antioxidantes lipofílicos, tal como o tocoferol. O estudo comprovou que a suplementação com 400 µL de tocoferol ao dia é suficiente para diminuir a oxidação de LDL e F2-isoprostanos urinários que são marcadores de peroxidação lipídica.

O consumo moderado de vinho tinto, em particular, demonstrou ter um efeito positivo sobre risco de doença cardiovascular, que não pode ser meramente atribuído ao seu teor de etanol (DELL'AGLI, 2004). Para vinho tinto e suco de uva (STEIN et al., 1999; PARK, 2004; PAPAMICHAEL et al., 2004; LOPEZ-SEPULVEDA et al., 2008), vários estudos de intervenção apoiam os efeitos benéficos sobre os parâmetros relacionados à função cardiovascular como vasodilatação, pressão sanguínea, resistência à insulina e lipídios plasmáticos. A composição de polifenóis do vinho tinto e das uvas é complexa e está cada vez mais tornando-se claro que em seres humanos e animais a biodisponibilidade de muitos polifenóis alimentares é baixa, o que foi sugerido como verdadeiro para proantocianidinas e antocianinas presentes em uva e vinho tinto (VAN DORSTEN et al., 2010).

4.4 ESTRESSE OXIDATIVO E ESTADO PÓS-PRANDIAL

As espécies reativas (ER) são produzidas nos organismos vivos como resultado do metabolismo celular normal. Estas espécies constituem substâncias reativas e instáveis, geradas *in vivo,* tanto em condições fisiológicas quanto em condições patológicas. Em concentrações baixas a moderadas, funcionam em condições celulares fisiológicas, mas em altas concentrações, elas produzem

modificações adversas aos componentes celulares. A proteção do organismo contra os danos oxidativos abrange um sistema de defesa antioxidante, que pode ser produzido pelo próprio corpo ou absorvido através da dieta, atuando contra o excesso de espécies reativas de oxigênio (ERO) e de espécies reativas de nitrogênio (ERN) (HALLIWELL, 2012).

O termo estresse oxidativo é caracterizado pelo desequilíbrio entre as concentrações das espécies reativas e os mecanismos de defesa antioxidante do organismo, que resulta em dano oxidativo a biomoléculas como DNA, lipídeos e proteínas. Esta situação pode ocorrer pelo aumento dos agentes pró-oxidantes (ERO e ERN) sem simultâneo aumento das defesas antioxidants. Porém em outras ocasiões, os antioxidantes podem apresentar-se em concentrações diminuídas sem elevação das ERO e ERN; ou ainda, os fatores anteriormente citados podem ocorrer de forma concomitante, denotando situação mais severa (SIES, 1986; DOTAN; LICHTENBERG; PINCHUK, 2004).

O estresse oxidativo está associado a diversas doenças tais como a carcinogênese, mutagênese, doenças crônicas, entre outros (FANG; YANG; WU, 2002). O papel de espécies reativas na origem e/ou progressão da maioria das doenças humanas ainda não é claro, apesar de serem importantes no processo de carcinogênese e doenças neurodegenerativas (HALLIWELL, 2012).

O estado pós-prandial é um período do metabolismo oxidativo ativo e formação de ROS. A hiperlipidemia e hiperglicemia pós-prandial induzidas por refeições ricas em lipídios e carboidratos induz um estresse oxidativo relativo e que é exagerado e prolongado em indivíduos obesos ou diabéticos (CHUNG et al., 1998). O estresse oxidativo pós-prandial geralmente é acompanhado de inflamação e alteração da função endotelial. Hiperlipidemia e hiperglicemia pós-prandiais são fatores de risco para doença cardio-metabólica, que está fortemente associada a desequilíbrio oxidativos (CERIELLO et al., 2002).

A fim de amenizar o quadro de estresse oxidativo, antioxidantes provenientes de determinados alimentos vêm sendo estudados, dentre eles destacam-se os compostos fenólicos. O consumo de alimentos ricos polifenóis concomitante com as refeições podem ter várias vantagens no estado pós-prandial, em primeiro lugar, através de suas propriedades antioxidantes inerentes e potencial para modular o equilíbrio oxidativo (redox) celular (BOURTON-FREEMAN, 2010).

5 DESENVOLVIMENTO

5.1 Manuscrito 1

NEUROPROTECTIVE EFFECT OF CABERNET SAUVIGNON GRAPE JUICE AND WINE AGAINST HYDROGEN PEROXIDE-INDUCED OXIDATIVE STRESS IN HUMAN NEURON-LIKE CELLS (SH-SY5Y)

Este manuscrito está em processo de revisão para submissão no periódico *Free Radical Biology Medicine*

NEUROPROTECTIVE EFFECT OF CABERNET SAUVIGNON GRAPE JUICE AND WINE AGAINST HYDROGEN PEROXIDE-INDUCED OXIDATIVE STRESS IN HUMAN NEURON-LIKE CELLS (SH-SY5Y)

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Abstract: The antioxidant capacity of grape juice and wine has been demonstrated in biological systems *in vitro* and *in vivo*, being usually attributed to some bioactive compounds present in beverages, such as polyphenols, since they behave as reactive oxygen species-scavengers and metal-chelators. However, there have been few reports comparing and discussing the antioxidant capacity effects of developed in juice and wine from the same species and cultivar. Therefore, the objectives of this study were to evaluate the total phenolic, flavonoid and anthocyanin contents as well as total antioxidant capacity of Cabernet Sauvignon juice and wine. In addition, the neuroprotective effects using SH-SY5Y cells insulted with H_2O_2 *in vitro*. Cellular-based measurements of antioxidante capacity exhibited that the juice and wine extracts had cellular antioxidant capacities. Furthermore, juice increased the viability of SH-SY5Y cells, in contrast with wine that as the doses increased the cell viability decreased. Our findings suggest that juice and wine are potential antioxidant and have positive effect against reactive species generated in SH-SY5Y cells, suggesting a neuroprotective effect.

Keywords: *Vitis vinifera*, antioxidant capacity, dichlorofluorescein-diacetate assay, metabolic mitochondrial viability assay, reactive oxygen species.

1 INTRODUCTION

Oxidative stress is caused by the insufficient capacity of biological systems to neutralize reactive species produced in excess. A serious imbalance between the generation of reactive oxygen species (ROS) and antioxidant protection in favor of the former causes excessive oxidative damage in cells and tissues (HALLIWELL, 2011) because the ROS excessive production is associated with disruption of cell cycle regulatory mechanisms (GASPAROTTO et al., 2014). Furthermore, excessive or prolonged ROS generation cause various health problems, such as cardiovascular disease, insulin resistance, type 2 diabetes, osteoporosis, arthritis, asthma, and inflammatory bowel disease (HALLIWELL et al., 1995; DRÖGE, 2002; RANKIN, 2004), therefore, regulation of ROS levels is critical for reducing the risk of related chronic diseases (WANG; CAO; PRIOR, 1996).

Towards the end of 20th century, epidemiological studies and associated meta-analyses strongly suggested that long term consumption of diets rich in plant polypehonols offered some protection against development of cancers, cardiovascular diseases, diabetes, osteoporosis and neurodegenerative diseases (PANDEY; RIZVI, 2009). Polyphenols are naturally occurring compounds found largely in the fruits, vegetables, cereals and beverages because they are secondary metabolites of plants and are generally involved in defense against ultraviolet radiation or aggression by pathogens (BECKMAN, 2000).

In food, polyphenols may contribute to the bitterness, astringency, color, flavor, odor and oxidative stability. Fruits and vegetables are rich in dietary antioxidants, such as vitamins and phenolics (WANG; CAO; PRIOR, 1996). Fruits like grapes, apple, pear, cherries and berries contains up to 200–300 mg polyphenols per 100 grams fresh weight. The products manufactured from these fruits, also contain polyphenols in significant amounts, typically a glass of red wine or a cup of tea or coffee contains about 100 mg of polyphenols (SCALBERT et al., 2005; SPENCER et al., 2008).

In this sense, grapes and their products are rich in antioxidant compounds such as flavonoids, anthocyanins, tannins, phenolic acids, among others (ABE et al., 2007; CAPANOGLU et al., 2013). Grapes are rich in phenolic compounds and different studies have demonstrated that these substances possess biological activity related to health benefits for the consumers (KRIKORIAN et al., 2012; VAUZOUR et al., 2010) and its bioactive compounds can reduce the damage caused by oxidative stress,

helping to the prevention of many chronic and neurological diseases (CASTILLA et al., 2006; DANI, 2007; CANTOS et al., 2002; LACERDA et al, 2014). The phenolic compounds in grapes and derivatives, mainly the flavonoids, flavanols, flavonols and anthocyanins, are associated with improved health, along with other compounds which are not flavonoids, such as phenolic acids and the stilbene resveratrol (SAUTTER et al., 2005; XIA et al., 2010; ALI et al., 2010; KRIKORIAN et al, 2012; GRIS, 2013).

The phenolic content of wine has been extensively studied, mainly in relation to providing beneficial effects on health, since in addition to antioxidante capacity it also has anti-inflammatory and anticarcinogenic effects, among others. Polyphenols from wine can be classified into two groups: non-flavonoid compounds (hydroxycinnamic and hydroxybenzoic acids and their derivatives and stilbenes) and flavonoid compounds (anthocyanins, flavanols and flavonols) (FRANKEL et al., 1998; BURIN et al., 2011). The flavonoid composition of red wines includes anthocyanins, catechins, and flavonols. The main flavonols are myricetin, quercetin, kaempferol, syringetin and laricitrin (MATTIVI et al., 2006). Anthocyanins are the main phenolic compounds associated with the color of red wines and are antioxidants (RICE-EVANS, MILLER, PAGANGA, 1996; ROSSETTO et al., 2004).

Besides these functions, the chemical structure of polyphenols, mainly flavonoids and stilbenes (resveratrol), makes them suitable to act as antioxidants, trapping and neutralizing free radicals. Among these derivatives, the grape juice can be highlighted because it is a product that can be included in the diet of all people, from children to elderly, where the wine is not indicated. However, there have been few reports comparing and discussing the antioxidant capacity effects of developed in juice and wine from the same species and cultivar. Therefore, the objectives of this study were to evaluate the total phenolic and flavonoid contents, and antioxidante capacity of juice and wine from cultivar Cabernet Sauvignon, *Vitis vinifera*, and to investigate the intracellular antioxidant capacities of juice and wine using SH-SY5Y neuron-like cells.

2 MATERIALS AND METHODS

2.1 SAMPLES

The commercial samples of grape juice and wine cultivar Cabernet Sauvignon were produced by a winemaker (Casa Valduga, Bento Gonçalves, RS, Brazil). The grape fruits used to prepare juice and wine were harvested in Bento Gonçalves (29° 10’ 17” S, 51° 31’ 09” W, altitude 691 m), in the State of Rio Grande do Sul, Brazil, on January 2014. Cabernet grape juice was prepared by the enzymatic method, in which grape is crushed and then heated to at least 65°C in a hot macerator. Next, commercial pectolytic enzymes are added and must is kept between 55 and 60 °C during 1-2 h. The extracted juice is then clarified, pasteurized and bottled (RIZZON; LINK, 2006). Cabernet wine was obtained from vinification process by the coupled dispositive to the crushing machine that is called dewaxing. In winemaking of red wine, grape skin remains inside tanks during fermentation for extraction of phenolic pigments (PSZCZÓLKOWSKI; LECCO, 2011).

2.2 BIOACTIVE COMPOUNDS

2.2.1 Determination of Total Phenolic Content

The total phenolic content of juice and wine was measured using a colorimetric method with Folin-Ciocalteu’s phenol reagent (SINGLENTON; ROSSI, 1965). Each extract (200 µl) was diluted by mixing with 2.6 mL of deionized water followed by adding 200 µl of Folin-Ciocalteu’s phenol reagent. After a 6 min in incubation, 2.0 mL of 7% (w/v) Na_2CO_3 solution was added to the reaction mixture. At 90 min, absorbance was measured at 760 nm. Total phenolic content was expressed as mg of gallic acid equivalents (GAE) L^{-1}.

2.2.2 Determination of Total Flavonoid Content

The total flavonoid content of the juice and wine was measured using a modified method of Zhishen et al. (1999). Briefly, 500 µl of beverages or catechin standards were mixed with 3.2 mL of deionized water. Five minutes after adding 150 µl of 5%

(w/v) $NaNO_2$, an equal volume of 10% (w/v) $AlCl_3$ was added. After 6 min of incubation, the reaction was stopped by adding 1 mL of 1 M NaOH. The absorbance of the solution was measured immediately at 510 nm. Total flavonoid content was expressed as mg catechin equivalents (CE) 100 L^{-1}.

2.2.3 Determination of Total Anthocyanin Content

The total anthocyanin content was assessed by the difference of absorbance before and after sample decoloration (RIBÉREAU-GAYON; STONESTREET, 1965). Two samples were prepared, each containing 1 ml of the sample to 1 ml of ethanol acidified with 0.1% HCl. Briefly, 10 mL of 2% HCl (pH 0.7) was added to the first sample and 10 ml of pH 3.45 buffer solution was added to another sample. After 15 minutes the absorbance of the solution was measured immediately at 520 nm. Total anthocyanin content in juice and wine was expressed as mg malvidin equivalents (ME) 100 L^{-1}.

2.3 ANTIOXIDANT CAPACITY ASSAYS

2.3.1 Determination of Antioxidant Capacity using ABTS assay

The total antioxidant capacity of juice and wine was evaluated using ABTS radicals (RE et al., 1999). Fresh ABTS radical solution was prepared by dissolving 1.0 mM of AAPH and 2.5 mM of ABTS in 100 ml of phosphate buffer solution, pH 7.4, and allowing the mixture to react for 30 min at 70°C. To measure the antioxidante capacity of beverages, diluted samples (10 µl) were reacted with the ABTS radical working solution (990 µl) at 37°C for 10 min. The absorbance of the mixture was measured at 734 nm. The results were expressed as Trolox equivalents (TE) L^{-1}.

2.3.2 Determination of Antioxidant Capacity using FRAP assay

The antioxidant capacity of juice and wine was determined using the ferric reducing antioxidant power (FRAP) assay as described by Benzie and Strain (1996). In this procedure, the antioxidants present in the serum are evaluated as reducers of Fe^{3+} to Fe^{2+}, which is chelated by 2,4,6-tris(2-pyridyl)-S-triazine (TPTZ) to form a

complex (Fe^{2+} –TPTZ) with maximum absorbance at 593 nm. Beverages samples were mixed with 1 mL of reagent containing 1.7 mM $FeCl_3$ and 0.8 mM TPTZ, prepared in 300 mM sodium acetate, pH 3.6. The samples were incubated for 15 min at 37 °C and the absorbance was measured at 593 nm. The results were expressed as μmol Trolox equivalents (TE) L^{-1}.

2.4 CELL CULTURE ASSAYS

Human neuron-like cell line SH-SY5Y obtained from the European Collection of Authenticated Cell Cultures (ECACC) were maintained in 75-cm^2 flasks containing DEMEM/F12 medium (1:1) supplemented with 10% fetal bovine serum (FBS) and 1× antibiotic/antimycotic solution (Sigma-Aldrich). Cells were cultured in a humidified incubator set at 37°C with 5% CO_2. When cultures reached confluence, cells were trypsinized and seeded at a density of 30×10^3 cells/cm^2 in 96-well culture plates. Treatments started 24 horas after seeding. All treatments were performed using 1% FBS supplemented medium. Juice and wine were freeze-dried to remove water and alcohol and then dissolved in culture medium at the desired concentration (w/v). Cells were exposed to these juice and wine solutions or vehicle (culture medium).

2.4.1 Determination of Intracellular ROS Production

Intracellular ROS production was detected using the 2',7'-dichlorofluorescein diacetate (DCFH-DA, Sigma) as described by Wang and Joseph (1999). Cells were pre-treated with Cabernet juice or wine (solutions in culture medium, see section 2.4) or vehicle (culture medium) during 2 h and then incubated in the absence (control) or presence of H_2O_2 (100μM) for 3h before monitoring DCF fluorescence. H_2O_2 was used as a positive control to induce ROS generation (RABELO et al., 2012). DCFH-DA stock solution was dissolved in DMSO at a final concentration of 10 mM and stored at −20°C protected from light. Before cells were treated, DCFH-DA was diluted to 100 μM using 1% FBS supplemented medium solution. After addition of DCFH-DA, cells were incubated at 37°C, with 5% CO_2, and protected from light exposure for 1 h. After DCFH internalization, the medium was replaced by fresh 1% FBS supplemented medium solution. When internalized, ROS cause DCFH oxidation and it becomes a fluorophore (DCF), which was quantified using a SpectraMAX i3 (Molecular Devices) fluorescence

plate reader (Ex/Em = 485/532 nm). Fluorescence was monitored and the area under the curve (AUC) of fluorescence vs. time was calculated. The results are expressed as the percentage of DCF fluorescence. Trolox_ (250 lM) dissolved in dimethyl sulfoxide was used as standard antioxidant.

2.4.2 Metabolic Mitochondrial Viability

Metabolic mitochondrial viability was assessed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay as previously described by Gelain and Moreira (2008). SH-SY5Y cells were plated onto 96-well plates and exposed to Cabernet juice or wine (solutions in culture medium, see section 2.4) or vehicle (culture medium) during 24 h. Parallel sets of wells were run in the absence or presence of H_2O_2 (100 μM) (co-exposure scheme with juice/wine), which was used as a positive control to induce cell death (Ferrari et al., 1990). Then, cells were incubated with MTT for 45 min at 37°C in a humidified 5% CO_2 atmosphere. The medium was then removed and plates were shaken with DMSO for 30 min. The optical density of each well was measured at 550 nm (test) and 690 nm.

2.5 STATISTICAL ANALYSIS

All the measurements for the levels of bioactive compounds were carried out in triplicate and the results are expressed as mean ± standard deviation. The data were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's test. The cell culture experiments were performed with n=6 (6 wells per group). The experiments were repeated thrice and the results are expressed as mean ± standard error of the mean (SEM). The diferences among the data were evaluated by the analysis of variance (ANOVA) followed by Tukey's test. In addition, the Principal Component Analysis (PCA) was applied to verify the association between the anti-oxidative stress responses and the phenolic composition of juice and wine. In all cases, the differences were considered significant when $p<0.05$. Data analyses were performed using Statistica 7.0 software (Statsoft Inc.,Tulsa, USA).

3 RESULTS AND DISCUSSION

3.1 BIOACTIVE COMPOUNDS AND ANTIOXIDANT CAPACITY

The contents of phenolics, flavonoids and anthocyanins and the antioxidant activity (measured through ABTS and FRAP assays) in the Cabernet Sauvignon juice and wine sample are presented in Table 1.

Table 1 - Phenolic composition and *in vitro* antioxidant activity of the grape juice and wine.

Cabernet Sauvignon *cultivar*	Total Phenolics (GAE mg L^{-1})	Total Flavonoids (CE mg L^{-1})	Total Anthocyanins (ME mg L^{-1})	Total Antioxidant Capacity (µmol TE L^{-1})	
				ABTS	FRAP
Juice	466.88±51.6^{b}	245.13±1.4^{b}	2.73±0.2^{b}	1.67±0.3^{b}	21.18±0.1^{b}
Wine	4448.55±8.5^{a}	6205.66±49.9^{a}	101.61±2.6^{a}	3.54±0.3^{a}	265.00±0.1^{a}

Data are presented as the mean ± standard deviation (n = 3). GAE, CE, ME, and TE stand for gallic acid equivalents, catechin equivalents, malvidin equivalents and trolox equivalents, respectively. Different superscripts in the same column indicate significant differences by Tukey's post hoc test (p<0.05).

Significant differences in polyphenol content and antioxidant activity were found between juice and wine (p<0.001) and the highest total phenolic content was found in the wine. According to other previous study (BURIN et al., 2011), the total phenolic content of different clones of Cabernet Sauvignon wine, showed values between 2111.95 – 2569.81 mg GAE L^{-1}, which corroborates with the hight phenolic concentration in Cabernet Sauvignon wine in the present study, since different clones, crop year and type of cultivation can vary the phenolic composition. In order to compare the results in the juice, we searched for references of rosé and white juice, according to the method used to elaborate the juice studied. Dani et al. (2009) found in rosé Goethe juice, 156.60 mg CE L^{-1} of total phenolics. In the other study, 487,3 mg EAG L^{-1} of total phenolic were found in white juice without varietal identification (VARGAS; HOELZEL; ROSA, 2008).

The wine showed the highest total flavonoid and anthocyanins content compared to juice (Table 1). Similars results also were finding by Burin et al. (2011) of total monomeric anthocyanins in different clones of Cabernet Sauvignon wine (164.08 – 209.33 mg L^{-1}). Anthocyanins are flavonoids responsible for the pigmentation in

several different fruits. In grapes, they are found almost exclusively in the skins (LIAZID et al., 2011). Choi et al. (2010) showed that anthocyanins are primarily responsible for the antioxidant activity of this grape variety, which was also reported in other grape varieties. In animal studies, anthocyanins have been shown to cross the blood−brain barrier (KALT et al., 2008) to accumulate in a number of brain regions including those essential to cognitive function, and to enhance memory performance (ANDRES-LACUEVA et al., 2005).

The wine had the highest antioxidant capacity (Table 1). We hypothesize that the main reason for this significative difference is due to the different processing to obtain the beverages. The juice manufacturing process originated a rosé product, which there isz less contact of the grape skin with the must. Furthermore, this difference occurs because phenolic compounds are secondary metabolites produced and accumulated in plant tissues, and changes in phytopathogenesis, among other factors, may result in different concentrations of these compounds in plant organs (FERGUSON, 2001, DANI et al., 2009).

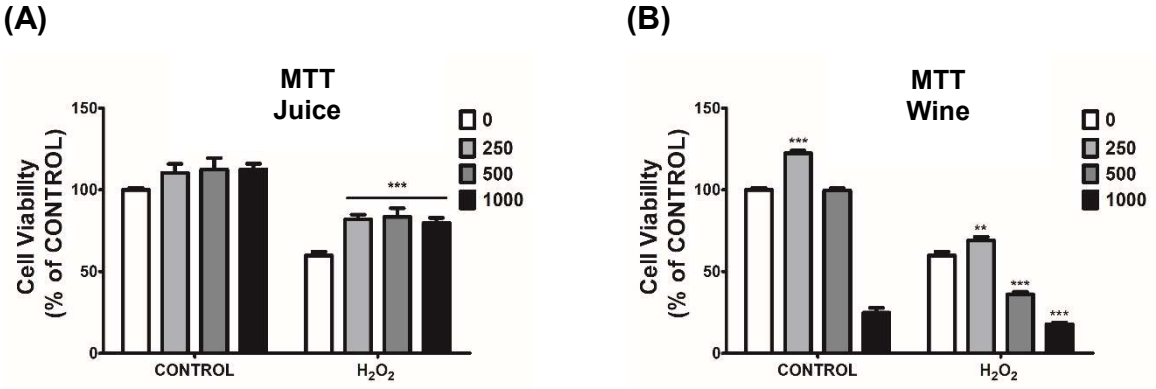
3.2 NEUROPROTECTIVE EFFECTS OF CABERNET SAUVIGNON JUICE AND WINE

Cell culture has often been used to study the cellular effects of reactive species and of antioxidants, and many useful data have resulted (HALLIWELL, 2011). Hydrogen peroxide is a physiological constituent of living cells and is continuously produced via diverse cellular pathways. Intracellular steady-state concentrations of H_2O_2 above 1 μM are considered to cause oxidative stress inducing growth arrest and cell death (ANTUNES; CADENAS, 2001; STONE; YANG, 2006). In experimental models used to investigate physiological functions and toxic effects of H_2O_2, oxidative stress responses of cells, or cytoprotection by antioxidant agents, cultured cells are often exposed to H_2O_2 added as a bolus into the culture medium (GÜLDEN et al., 2010). We investigated whether grape juice and wine could prevent H_2O_2-induced (100 μM) intracellular ROS production in SH-SY5Y cells and promote neuroprotective actions (Figures 1 and 2).

To determine whether Cabernet Sauvignon juice and wine could protect against oxidative stress-induced cell death, the SH-SY5Y cell line was used as an *in vitro* model and H_2O_2 as pro-oxidant insult. After 24 h of H_2O_2 exposure in combination with

Cabernet juice we observed that all tested concentrations (250-1000 µg/mL) were able to increase the cellular viability in relation to the 0 µg/mL concentration (Figure 1A; p<0.001). Cabernet wine at 250 µg/mL significantly increase the cell viability in the absence of H_2O_2, compared to 0 µg/mL, whereas 1000 µg/mL significantly increase the cell death (Figure 1B; p<0.001). In the presence of H_2O_2, only 250 µg/mL of Cabernet wine protected against cytotoxicity (p<0.01), while 500 and 1000 µg/mL induced further cytotoxicity compared to vehicle-H_2O_2 (Figure 1B, p<0.001).

Figure 1 - Effect of Cabernet Sauvignon juice and wine on H_2O_2-induced cytotoxicity in SH-SY5Y cells.



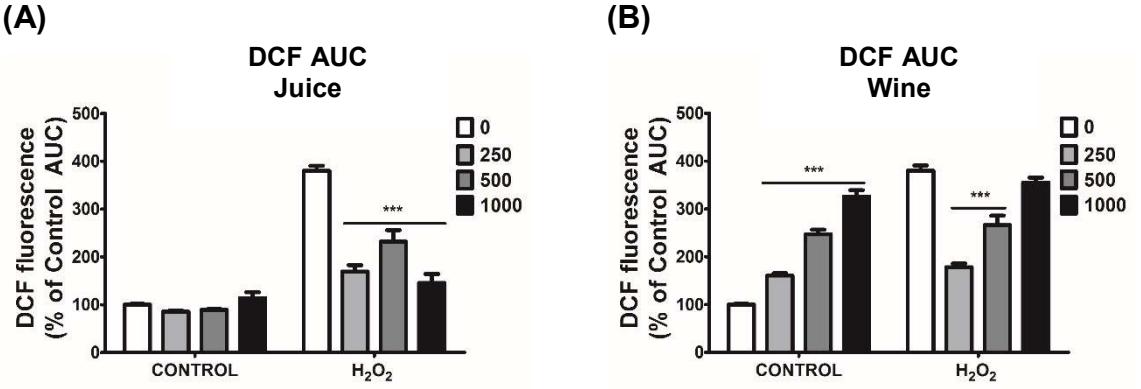
(A): Cell viability of cells treated with juice. **(B)**: Cell viability of cells treated with wine. Cells were exposed to 0 (vehicle), 250, 500 and 1000 µg/mL of juice or wine during 24 h. Two-way ANOVA was applied to all data. *p<0.05, **p<0.01 and ***p<0.001; vs. the respective vehicle group. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide).

Similar results were found by Xiang et al. (2014), when SH-SY5Y cells were treated with 100 µM H_2O_2, a significant loss in cell viability was observed as compared with control. However, when cells were treated with 4 mg mL^{-1} red wine extracts or red wine adding 10-fold resveratrol, all wine varieties showed significant neuroprotective effect against H_2O_2-induced oxidative stress. Furthermore, no significant differences on cell viability were observed between those cells treated with red wine adding 10-fold resveratrol extra and red wine separately in all varieties.

Results of the intracellular antioxidant capacity measurements of the juice and wine samples using the DCFH-DA assay were showed in figure 2. In Cabernet Sauvignon juice, all tested concentrations (250-1000 µg/mL) reduced the H_2O_2-induced intracellular ROS production (Figure 2A; p<0.001). In the other hand, Cabernet wine had a pro-oxidant effect per se by increasing the DCF levels in the absence of H_2O_2 (Figure 2B; p<0.001). Whereas, 250 and 500 µg/mL of wine significantly reduced

H_2O_2-induced production of ROS (Figure 2B; $p<0.001$). According Torma et al. (2017), no protective effects of açaí extracts on reactive species generation were observed in the absence of a pro-oxidant agent (H_2O_2). However, when cells were insulted with H_2O_2, there was an increase in the intracelular reactive species generation compared to untreated cells. These authors believe that H_2O_2 exhibits good permeability within and between cells, and in combination with metal ions (Fe and Cu), generates hydroxyl radicals (HALLIWELL, 2015).

Figure 2 - Effect of Cabernet Sauvignon juice and wine on H_2O_2-induced cytotoxicity in SH-SY5Y cells.

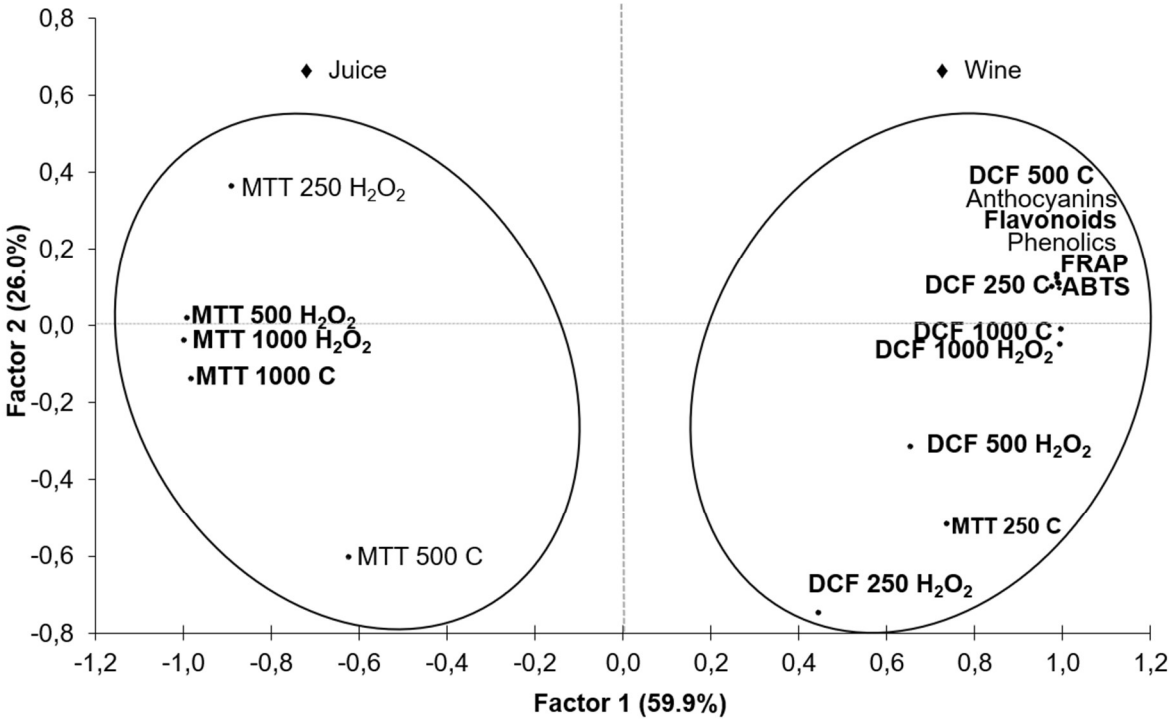


(A): DCF fluorescence measured of cells treated with juice. **(B):** DCF fluorescence of cells treated with wine. Cells were exposed to 0 (vehicle), 250, 500 and 1000 µg/mL of juice or wine during 5 h. Two-way ANOVA was applied to all data. *$p<0.05$, **$p<0.01$ and ***$p<0.001$; vs. the respective vehicle group. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide).

There are two possible mechanisms for the intracellular antioxidant capacity of Cabernet juice and wine. First, the beverages extract could directly eliminate ROS generated from cells macrophages through radical scavenging activity, as was previously shown by Sittisart and Chitsomboon (2014). Non-fluorescent DCFH-DA crosses cell membranes and is hydrolyzed by intracellular esterase to non-fluorescent DCFH, which can be further oxidized to fluorescent DCF by hydroxyl radicals converted from hydrogen peroxide (GIRARD-LALANCETTE; PICHETTE; LEGAULT, 2009). Based on our results, the juice and wine extracts may have been absorbed into the cells and removed cellular hydroxyl radicals so that the production of fluorescent DCF was inhibited. These cells elicit a functional response similar to human neurons, such as outgrow neurites and undergo morphological changes when challenged by oxidative stress *in vitro* (RABELO et al., 2012).

In order to evaluate the association of the phenolic composition, *in vitro* antioxidant capacity and in SH-SY5Y cells H_2O_2-induced of the Cabernet Sauvignon juice and wine were assessed by principal components analysis (PCA) as demonstrated in Figure 3. The first two principal components explained 85.9% of the total variance, whereas Factor 1 and Factor 2 explained 59.9% and 26.0% of the total variability, respectively. The first principal component (PC1) separated juice from wine sample, perfectly distinguishing between cell viability (MTT assay) and antioxidant activity (DCFH-DA assay). Also, the phenolic composition and *in vitro* antioxidante capacity of wine and the antioxidante acvity in cell response showed similar scores, whereas, the cell viability in juice showed similar scores as described by the PC1. The second principal component (PC2) separated the cell viability (MTT assay) and antioxidant activity (DCFH-DA assay) associated the control and H_2O_2 group, thus corroborating with the results from analysis previously described (Figures 1 and 2).

Figure 3 - Principal componente analysis plot of Cabernet Sauvignon juice and wine sample over the phenolic composition, the *in vitro* antioxidant activity and in SH-SY5Y cells.



H_2O_2, C and values (250, 500 and 1000) means the hydrogen peroxide, control and concentrations in μg mL^{-1}

The phenolic composition and *in vitro* antioxidant capacity were positively scored in PC1 and were associated with the wine. In addition, the improvement on the anti-oxidative responses, verified by the decrease on EROs production, were associated with the phenolic compounds in the PCA plot, such as flavonoids, phenolics and anthocyanins, as well as the ABTS and FRAP assay. In the other hand, the cell viability can be better explicated by juice, which were quantified in juice and wine.

4 CONCLUSIONS

This study analysed the phenolic composition and antioxidant activity of juice and wine from *Vitis vinifera*, cultivar Cabernet Sauvignon, and demonstrated the significantly higher concentration of phenolic constituents in wine, as well as, in the antioxidant activity (mainly in FRAP and DCFH-DA assays) and neuroprotective effects on SH-SY5Y cells. However, despite the low concentration of phenolics in the juice, it showed an important response in cell viability, which can be explained by other compounds present in the grape and not found only in the skin. These results incite further *in vitro* and *in vivo* research in order to elucidate the therapeutic potential of these beverages, especially the Cabernet Sauvignon juice, still little explored by the wineries.

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5.2 Manuscrito 2

ACUTE CONSUMPTION OF BORDO GRAPE JUICE AND WINE IMPROVE SERUM ANTIOXIDANT STATUS IN HEALTHY INDIVIDUALS AND INHIBIT REACTIVE OXYGEN SPECIES PRODUCTION IN HUMAN NEURON-LIKE CELLS

Este artigo foi aceito para publicação no periódico *Journal of Nutrition and Metabolism* (ANEXO 3)

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Abstract: Few studies investigated the biological effects of American grape cultivars. We investigated the metabolic response after acute consumption of grape juice or wine from Bordo grapes (*Vitis labrusca*) in a placebo-controlled cross-over study with fifteen healthy volunteers. Blood samples were collected 1 hour after the intake of 100mL of water, juice or wine to measure TBARS, ABTS, FRAP, glucose and uric acid levels. To evaluate differences in cellular response, intracellular reactive species production (DCFH-DA) and metabolic mitochondrial viability (MTT) were assessed after exposure of human neuron-like cells (SH-SY5Y) to juice or wine. Glycemia was reduced after juice or wine consumption, whereas blood levels of uric acid were reduced after juice consumption but increased after wine consumption. Juice and wine consumption reduced plasma lipid peroxidation and increased plasma antioxidant capacity (ABTS and FRAP assay). Furthermore, juice inhibited H_2O_2-induced intracellular production of reactive species (RS) and increased the viability of SH-SY5Y cells. In contrast, wine (dealcoholized) exhibited a per se effect by inducing the production of RS and reducing cell viability. These results indicate a positive impact of acute consumption of Bordo juice and wine on human oxidative status, whereas only juice had protective effects against oxidative stress-induced cytotoxicity.

Keywords: *V labrusca* L.; polyphenols; antioxidant capacity; oxidative stress; clinical trial.

1 INTRODUCTION

Oxidative stress is caused by the insufficient capacity of biological systems to neutralize the excessive production of reactive species (HALLIWELL, 2011), which leads to oxidative damage in cells. Neuronal cells are particularly susceptible to reactive oxygen species (ROS) and reactive nitrogen species (RNS) due to their high metabolic activity, low antioxidant capacity and their non-replicative nature. Furthermore, the abundance of mitochondria in brain cells increases the generation of reactive species (LEE; GIORDANO; ZHANG, 2012).

Fruits and vegetables have many bioactive compounds such as polyphenols, which have antioxidant properties with a role in the protection of cellular macromolecules against oxidative damage induced by ROS and RNS (HALLIWEL, 2006; VALKO et al., 2007; KARDUM, 2014). There is increasing evidence that polyphenols may protect cell constituents against oxidative damage and, therefore, limit the risk of various degenerative diseases associated with oxidative stress (CROWE, 2011). Studies have repeatedly shown an inverse association between the risk of several chronic human diseases and the consumption of polyphenol-rich diet (GUERRA, 2015). The phenolic group of polyphenols can accept an electron to form relatively stable phenoxyl radicals, thereby disrupting chain oxidative reactions in cellular components. It is well established that polyphenol-rich foods and beverages may increase plasma antioxidant capacity (CLIFFORD, 2000; PANDEY; RIZVI, 2010).

Grapes contain high levels of polyphenols, which have been demonstrated to reduce oxidative stress, inflammatory response, and the oxidation of low density lipoprotein cholesterol (LDL-c), while inhibiting platelet aggregation and improving protection against atherothrombotic episodes. Such actions promote beneficial effects on coronary heart disease (CHD) and atherosclerosis (BAGCHI, 2000; SANO, 2007; ARTS; HOLLMAN, 2005). Red wines are rich in polyphenols, such as phenolic acids (gallic acid, caffeic acid, p-coumaric acid, and others), stilbenes (trans-resveratrol), and flavonoids (catechin, epicatechin, quercetin, rutin, myricetin, and others) (KAMMERER, 2004). Therefore, a regular consumption of red wine has been linked with the “French paradox”, which explains the apparent compatibility of a high-fat diet with a low mortality from CHD. Also, current evidence suggests that wine consumption is correlated with a reduction in the incidence of neurodegenerative diseases associated to oxidative stress such as Alzheimer’s and Parkinson’s disease (SUN,

2008). Grape juice is a natural and non-alcoholic beverage that contains sugars, minerals, and phenolic compounds like anthocyanins, among which malvidin 3,5-diglucoside is the major one (TOALDO et al., 2015). This beverage has been shown to exert antioxidant activity *in vitro* and *in vivo*, as well as hypolipidemic and anti-inflammatory effects in rats and humans (CASTILLA et al., 2006; DANI, 2007; TOALDO; CRUZ; DA SILVA, 2007).

However, few studies have compared the effects of wine and juice consumption in biological parameters of humans and these studies used European grape species (Vitis vinifera) (PACE-ASCIAK, 1996; COIMBRA, 2005; VAN DORSTEN et al., 2010). In contrast, the biological effects of wine and juice from American grape species (Vitis labrusca) have not been compared. This investigation is particularly interesting as the red grape cultivar ‘Bordo’ (V. labrusca), which is the most important grape cultivated in Brazil (TOALDO et al., 2015), has been recently demonstrated to exhibit higher content of phenolic compounds and *in vitro* antioxidant capacity than V. vinifera species (BURIN, 2014). In the present study, we compared the biological effects of juice and wine from ‘Bordo’ grapes (*V. labrusca L*) by assessing blood antioxidant response after human consumption and the oxidative cellular response in human neuron-like cells (SH-SY5Y).

2 MATERIALS AND METHODS

2.1 BORDO GRAPE JUICE AND WINE

The commercial samples of Bordo grape juice and Bordo wine were produced by a winemaker (Casa Perini, Farroupilha, RS, Brazil). The grape fruits used to prepare juice and wine were harvested in Farroupilha (29° 13’ 30” S, 51° 20’ 52” W, altitude 783 m), in the State of Rio Grande do Sul, Brazil, on January 2014. Bordo grape juice was prepared by the enzymatic method, in which grape is crushed and then heated to at least 65°C in a hot macerator. Next, commercial pectolytic enzymes are added and must is kept between 55 and 60°C during 1-2 h. The extracted juice is then clarified, pasteurized and bottled (RIZZON, LINK, 2006). Bordo wine was obtained from vinification process by the coupled dispositive to the crushing machine that is called dewaxing. In winemaking of red wine, grape skin remains inside tanks during

fermentation for extraction of anthocyanin pigments (PSZCZÓLKOWSKI; LECCO, 2011).

2.2 DETERMINATION OF BIOACTIVE COMPOUNDS IN BORDO JUICE AND WINE

The total phenolic content was determined at 760 nm using the Folin-Ciocalteu method and gallic acid as standard (SINGLETON; ROSSI, 1965). Total anthocyanin content was assessed at 520 nm as the difference of absorbance before and after sample decoloration using sodium bisulfite at pH 0.8, and was expressed as mg of malvidin-3-glucoside L^{-1} (RIBÉREAU-GAYON; STONESTREET, 1965). The total flavonoid content was estimated at 510 nm using a standard curve of catechin (0-200 mg L^{-1}) (ZHISHEN; MENGCHENG; JIANMING, 1999).

2.3 ANTIOXIDANT CAPACITY OF BORDO JUICE AND WINE

The antioxidant capacity of grape juice and red wine were determined using the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and ferric reducing antioxidant power (FRAP) methods as described by Re et al. (1999) and Benzie and Strain (BENZIE; STRAIN, 1996), respectively. The ABTS assay is assessed at 764 nm and is based on the ability of sample to scavenge the cation radical ABTS•+. The FRAP assay is assessed at 620 nm and is based on the reduction of ferric-tripyridyltriazine (Fe III -TPTZ) by antioxidants present in the samples forming ferrous-tripyridyltriazine (Fe II -TPTZ), a blue-colored product. Trolox was used in the calibration curve.

2.4 *IN VIVO* STUDY

2.4.1 Participants

The study design was approved by the Ethics Committee of Federal University of Santa Maria (CAAE 39197614.3.0000.5346) and all subjects signed a written agreement before participating. Fifteen healthy volunteers, with mean age 24.0±3.6, were recruited from the University staff. The health status and medical history of volunteers were examined by a structured interview for inclusion or exclusion according to the criteria shown in Table 1.

Table 1 - Selection criteria of study participants.

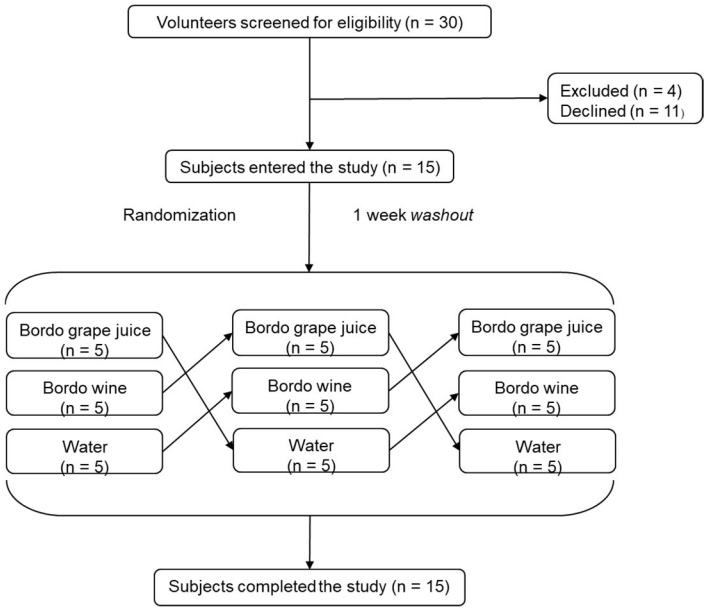
Inclusion criteria	Exclusion criteria
Apparently healthy individuals	Pregnant and lactating women
Age 18-35 years old	Alcoholic and smokers
BMI between 18.5 and 29.9 kg/m^2	Vegetarian diet
SBP <140mmHg and DBP ≤90mmHg	Regular use of antioxidants or vitamin supplements
	Chronic diseases (cardiovascular diseases, hypertension, diabetes, liver diseases, cancer or allergy); gastrointestinal disorders or known metabolic diseases, infections or inflammatory processes visible or known in the three months prior to the study

BMI = body mass index, SBP = Systolic blood pressure, DBP = Diastolic blood pressure.

2.4.2 Study design

In this cross-over controlled clinical study, 15 volunteers were included, being 10 women (67%) and 5 men (33%). All participants received the three treatments, namely Bordo grape juice, Bordo wine and water (control) with a washout period of 1 week between treatments. The sequence of the treatments was randomized among the participants as depicted in Figure 1.

Figure 1 -Flow chart of the selection of subjects in the controlled intervention study



Participants were oriented to follow a low-antioxidant diet for 48 h prior the day of intervention, avoiding some fruits, vegetables and juices, mainly rich in anthocyanins, tea, coffee, cocoa foodstuffs and alcoholic beverages. This dietary

restriction was aimed to reduce dietary phenolic compounds from blood as these compounds are typically cleared within 48 h of consumption (MANACH, 2004). The intake of energy, macronutrients, dietary fiber and antioxidants before the intervention was monitored using a prospective 48 h dietary record. Each participant served as his own control because we compared data obtained after either juice, wine or water consumption with the respective baseline values before consumption. In the day of intervention, baseline blood samples were collected after overnight fasting (12 h), then subjects consumed 100 mL of Bordo grape juice, Bordo wine or water. One hour after drinking, test blood samples were collected. This protocol was chosen based on a previous study that revealed maximal antioxidant capacity and phenolic concentration in serum 1 h after the intake of fruit or beverage (MANACH, 2004). No food was provided during this period.

2.4.3 Blood collection and analyses

Fasting venous blood samples were collected through aseptic venipuncture into heparinized tubes and EDTA-containing tubes that were centrifuged (1500 x g, 10 min) to yield plasma for thiobarbituric acid reactive species (TBARS), ABTS and FRAP analysis. Blood collected in tubes without additives was centrifuged (1500 x g, 10 min) to yield serum for analysis of uric acid and glucose. Serum and plasma samples were stored at -80°C until analysis.

Uric acid and glucose were determined in serum using commercially available enzymatic kits (Bioclin, Belo Horizonte, Brazil). Lipid peroxidation was determined by measurement of TBARS at 535 nm in plasma (OHKAWA; OHISHI; YAGI, 1979). The antioxidant capacity of plasma was assessed by the ABTS (RE et al, 1999) and FRAP assays (BENZIE; STRAIN, 1996).

2.4.4 Cell culture assays

Human neuron-like cell line SH-SY5Y obtained from the European Collection of Authenticated Cell Cultures (ECACC) were maintained in 75-cm2 flasks containing DEMEM/F12 medium (1:1) supplemented with 10% fetal bovine serum (FBS) and 1× antibiotic/antimycotic solution (Sigma-Aldrich). Cells were cultured in a humidified incubator set at 37°C with 5% CO_2. When cultures reached confluence, cells were

trypsinized and seeded at a density of 30 × 103 cells/cm^2 in 96-well culture plates. Treatments started 24 horas after seeding. All treatments were performed using 1% FBS supplemented medium. Bordo juice and wine were freeze-dried to remove water and alcohol and then dissolved in culture medium at the desired concentration (w/v). Cells were exposed to these juice and wine solutions or vehicle (culture medium).

2.4.5 Determination of intracellular ROS production

Intracellular ROS production was detected using the 2',7'-dichlorofluorescein diacetate (DCFH-DA, Sigma) as described (WANG; JOSEPH, 1999). Cells were pre-treated with Bordo juice or wine (solutions in culture medium, see section 2.3) or vehicle (culture medium) during 2 h and then incubated in the absence (control) or presence of H_2O_2 (100μM) for 3h before monitoring DCF fluorescence. H_2O_2 was used as a positive control to induce ROS generation (RABELO, 2012). DCFH-DA stock solution was dissolved in DMSO at a final concentration of 10 mM and stored at −20°C protected from light. Before cells were treated, DCFH-DA was diluted to 100 μM using 1% FBS supplemented medium solution. After addition of DCFH-DA, cells were incubated at 37°C, with 5% CO_2, and protected from light exposure for 1 h. After DCFH internalization, the medium was replaced by fresh 1% FBS supplemented medium solution. When internalized, ROS cause DCFH oxidation and it becomes a fluorophore (DCF), which was quantified using a SpectraMAX i3 (Molecular Devices) fluorescence plate reader (Ex/Em = 485/532 nm). Fluorescence was monitored and the area under the curve (AUC) of fluorescence vs. time was calculated.

2.4.6 Metabolic mitochondrial viability

Metabolic mitochondrial viability was assessed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay as previously described (GELAIN; MOREIRA, 2008). SH-SY5Y cells were plated onto 96-well plates and exposed to Bordo juice or wine (solutions in culture medium, see section 2.3) or vehicle (culture medium) during 24 h. Parallel sets of wells were run in the absence or presence of H_2O_2 (100 μM) (co-exposure scheme with juice/wine), which was used as a positive control to induce cell death (FERRARI; FORNASIERO; ISETTA, 1990). Then, cells were incubated with MTT for 45 min at 37°C in a humidified 5% CO_2

atmosphere. The medium was then removed and plates were shaken with DMSO for 30 min. The optical density of each well was measured at 550 nm (test) and 690 nm.

2.5 STATISTICAL ANALYSIS

All the analyses were performed in triplicate. Results were analyzed using the Statistica software package (StatSoft Inc., Tulsa, Okla, USA) and expressed as mean ± SEM. The parameters of the juice and wine were compared by the Student's t test. The effects of wine, juice and water intake on blood parameters were evaluated by the paired t test to compare baseline vs. test data (intragroup comparison) and by analyses of variance followed by Tukey's test for intergroup comparison. Significance was set at $p<0.05$.

3 RESULTS

3.1 CHARACTERISTICS OF THE SUBJECTS

General characteristics of the study group are presented in Table 2.

Table 2 - Baseline characteristics of subjects enrolled in the study

	Participants (n=15)	
	Male (n=5)	Female (n=10)
Age (years)	23.8 ± 4.0 (19 - 30)	24.3 ± 4.0 (22 - 33)
Weight (kg)	79.0 ± 14.7 (65 - 95)	61.0 ± 5.8 (54 - 70)
Height (cm)	180.6 ± 0.1 (169 - 191)	160.0 ± 0.1 (154 - 172)
BMI (kg/m^2)	24.3 ± 4.5 (20.1 - 30.3)	23.4 ± 2.5 (20.2 - 28.7)
SBP (mm Hg)	117.2 ± 13.6 (110 - 132)	115.8 ± 9.8 (100 - 130)
DBP (mm Hg)	81.6 ± 8.2 (70 - 90)	76.9 ± 4.7 (70 - 80)
Practice of physical activity at least once a week (%)	2 (40%)	3 (30%)
Physical inactivity (%)	3 (60%)	7 (70%)

Data are expressed as means ± SEM (minimum-maximum), except for the physical activity/inactivity that were expressed as the number of participants (%). BMI = body mass index, SBP = systolic blood pressure, DBP = diastolic blood pressure.

Fifteen apparently healthy individuals, 5 men and 10 women, respectively with mean age 24.1±3.7 and body mass index of 23.7±3.2 kg/m2 were included. The systolic and diastolic blood pressures of participants were within the intervals of optimal

and normal blood pressures according to the Brazilian Society of Hypertension, Brazilian Society of Cardiology and Brazilian Society of Nephrology (MALACHIAS, 2016) and according to US-American Hypertension Guideline (JAMES, 2014).

3.2 BORDO GRAPE JUICE AND WINE ANTIOXIDANT ACTIVITY *IN VITRO*

The chemical composition of Bordo grape juice and wine in the same serving size (portion) administered to healthy individuals in this study is shown in Table 3. Grape juice and wine showed high amounts of total phenolic content, but wine had higher amount than grape juice (Table 3, $p<0.05$). The concentration of total monomeric anthocyanins and total flavonols was also higher in wine compared to grape juice (Table 3, $p<0.05$).

Table 3 - Phenolic composition and in vitro antioxidant activity of the Bordo grape juice and wine

Parameter	Bordo juice	Bordo wine
Total polyphenol index (µmol GAE/100mL)	184.2 ± 13.1[b]	371.3 ± 9.6[a]
Total anthocyanins (µmol malvidin-3-glucoside/100ml)	17.5 ± 22.5[b]	66.74 ± 10.2[a]
Total flavonols (µmol catechin/100mL)	84.5 ± 9.7[b]	93.6 ± 4.6[a]
Total antioxidant activity		
ABTS (µmol TEAC/100mL)	316.5 ± 14.6[b]	448.7 ± 12.1[a]
FRAP (µmol TEAC/100mL)	234.6 ± 9.5[a]	234.9 ± 7.1[a]

Values are means ± SEM of determinations in triplicate. Different superscript letters [a,b]denote significant differences (Tukey's test, $p<0.05$). GAE= gallic acid equivalent; TEAC= Trolox equivalent antioxidant capacity.

The antioxidant activities were elevated in the two grape beverages used in this study. Bordo wine showed higher antioxidant capacity by the ABTS method, determined by the decolorization of the ABTS•+, through measuring the reduction of the radical cation as the percentage inhibition of absorbance at 734 nm, when compared with grape juice (Table 3, $p<0.05$). On the other hand, the wine antioxidant capacity assessed by the FRAP method, based on the ferric ion reduction (Fe^{+3}) capacity did not differ from juice (Table 3, $p<0.05$).

3.3 ACUTE CONSUMPTION OF BORDO JUICE AND WINE

After the consumption of Bordo grape juice and wine serum levels of TBARS were respectively decreased by 22.3% and 25.7% compared to baseline values

($p<0.001$), but no significant differences were observed between Bordo juice and wine (Figure 2A). Changes in TBARS levels after juice and wine intake were significantly different from changes observed after water intake ($p<0.05$), which increased (13.6%) TBARS levels compared to baseline values ($p<0.001$).

A significant increase in the antioxidant capacity levels, measured by ABTS and FRAP assays, was found 1 h after the consumption Bordo juice (9.1% and 14.1%, respectively) and wine (7.8% and 12.5%, respectively), compared to baseline values (Figure 2B and C; $p<0.05$). Changes in ABTS and FRAP levels after juice and wine intake were significantly ($p<0.05$) different from changes observed after water intake, which decreased ABTS (9.7%; $p<0.001$) and FRAP values (9.8%; $p<0.05$) compared to baseline values.

Table 4 - Glucose and uric acid levels in healthy individuals at baseline and after the interventions with Bordo grape juice, Bordo wine and water (control).

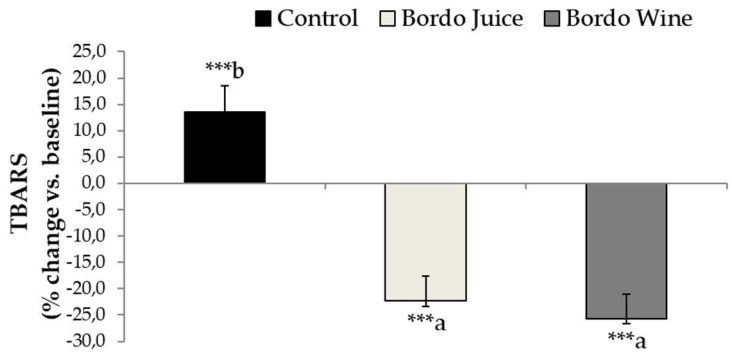
Biochemical Parameters	Intervention samples		
		Bordo grapes	
	Control	Bordo juice	Bordo wine
Serum glucose (mg/dL)			
Baseline	84.1 ± 5.8	74.4 ± 5.5	82.2 ± 7.1
1h after intervention	82.3 ± 5.5	69.3 ± 6.2	74.8 ± 7.4
Change vs. baseline (%)	-2.0 ± 1.5^{b}	-6.7 ± 1.7^{ab**}	-8.8 ± 2.0^{a**}
Uric Acid (mg/dL)			
Baseline	4.8 ± 1.9	4.8 ± 1.6	4.4 ± 1.2
1h after intervention	4.6 ± 1.8	4.6 ± 1.5	4.6 ± 1.2
Change vs. baseline (%)	-4.6 ± 1.4 ^{b**}	-4.1 ± 1.1 ^{b**}	4.2 ± 1.2 ^{a*}

Results are expressed as means ± SEM (n = 15). *Significantly different from baseline (paired Student's t test; *p<0.05; **p<0.01). ^{a,b}Different letters indicate significant difference among interventions (Tukey's test, p<0.05).

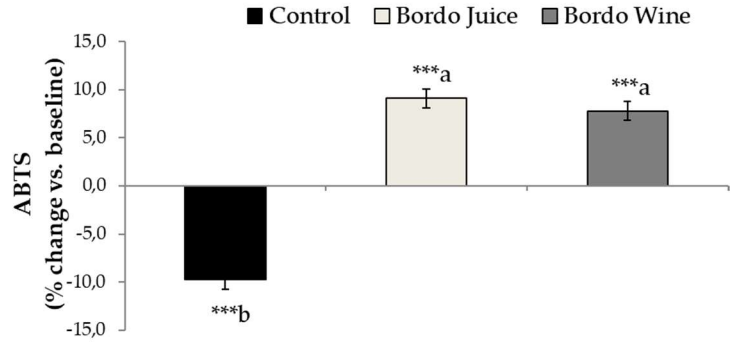
Significant changes were detected in the mean values of serum glucose and uric acid after the intake of the Bordo grape juice and wine (Table 4). Blood glucose was reduced after consumption of Bordo juice and wine compared to baseline values ($p<0.01$). Furthermore, wine triggered a greater decrease in blood glucose levels compared with water intake (-8.8% vs. -2.0%; $p<0.05$). Consumption of wine had a different effect in blood uric acid levels compared to water and juice ($p<0.05$; Table 4). Compared to baseline values, blood uric acid levels were increased after the consumption of Bordo grape wine ($p<0.05$) but decreased after the consumption of water and Bordo grape juice ($p<0.01$).

Figure 2 - Changes in serum TBARS levels (A) and plasma antioxidant capacity assessed by the ABTS (B) and FRAP assays (C) in humans after consumption of Bordo juice, Bordo wine or water (control)

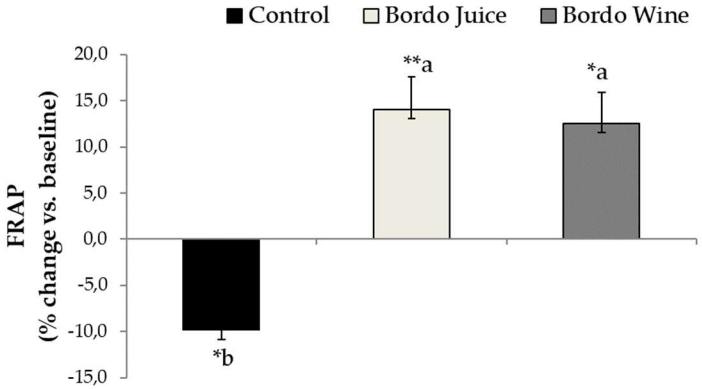
(A)



(B)



(C)



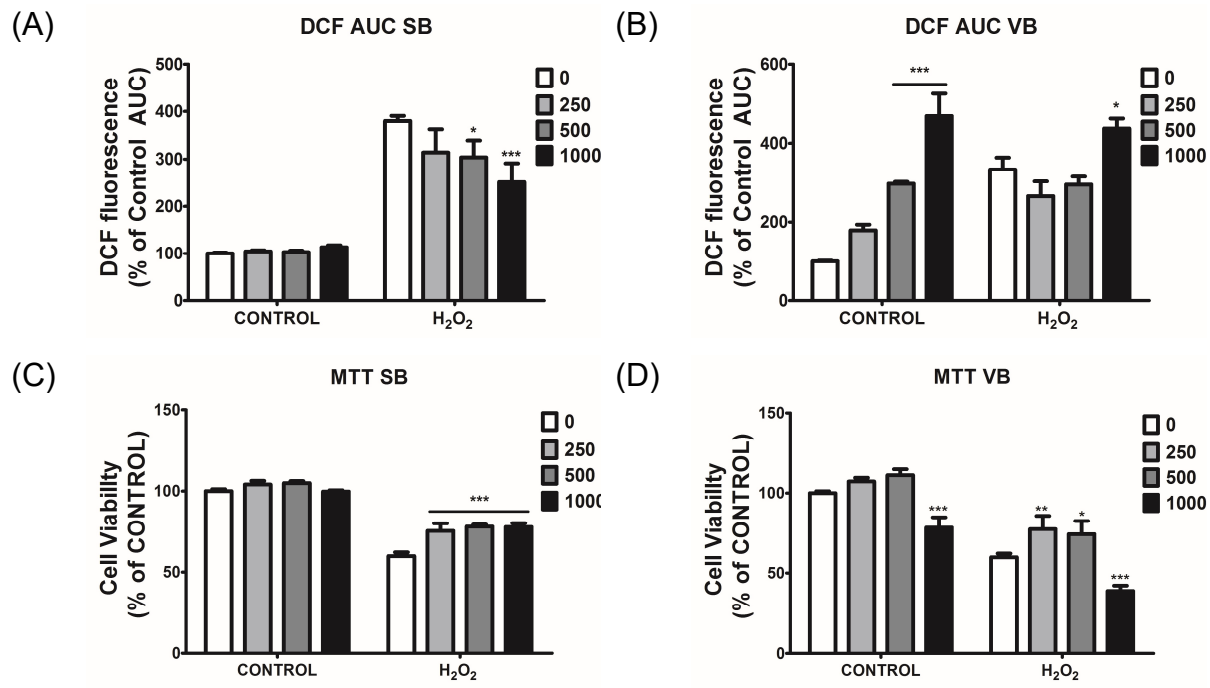
Results are expressed as percentage of baseline values for each group (means ± SEM, n = 15). *Significantly different from baseline (paired Student's t test; *p<0.05; **p<0.01; p<0.001). a,bDifferent letters indicate significant difference among interventions (Tukey's test, p<0.05).

3.4 NEUROPROTECTIVE EFFECTS OF BORDO JUICE AND WINE

We investigated whether Bordo grape juice and wine could prevent H_2O_2-induced intracellular ROS production in SH-SY5Y cells and promote neuroprotective

actions (Figure 3). Our results showed that exposure to H_2O_2 increased the intracellular ROS production (Figure 3A and B). However, 500 and 1000 µg/mL of Bordo grape juice significantly ($p<0.05$ and $p<0.001$) reduced H_2O_2-induced production of ROS (Figure 3A), whereas 1000 µg/mL of Bordo grape wine had a pro-oxidant effect per se by increasing the DCF levels in the absence of H_2O_2 ($p<0.001$; Figure 3B). Bordo grape wine was unable to prevent the increase in ROS induced by H_2O_2 and only 1000 µg/mL of Bordo grape wine induced further increase in ROS levels compared to H_2O_2 (Figure 3B, $p<0.05$).

Figure 3 - Effect of Bordo grape juice and wine on H_2O_2-induced cytotoxicity in SH-SY5Y cells.



(A): DCF fluorescence of cells treated with Bordo grape juice. (B): DCF fluorescence of cells treated with Bordo grape wine. (C): Cell viability of cells treated with Bordo grape juice. (D): Cell viability of cells treated with Bordo grape wine. Cells were exposed to 0 (vehicle), 250, 500 and 1000 µg/mL of Bordo juice or Bordo wine during 5 h (panels A and B) or 24 h (panels C and D). Two-way ANOVA was applied to all data. *$p<0.05$, **$p<0.01$ and ***$p<0.001$; vs. the respective vehicle group.

To determine whether Bordo grape juice and wine could protect against oxidative stress-induced cell death, the SH-SY5Y cell line was used as an *in vitro* model and H_2O_2 as pro-oxidant insult. After 24 h of H_2O_2 exposure in combination with Bordo grape juice, we observed that all tested concentrations (250-1000 µg/mL) of grape juice protected against H_2O_2 -induced cell death (Figure 3C; $p<0.001$). However,

Bordo wine at 1000 µg/mL significantly ($p<0.001$) reduced cell viability in the absence of H_2O_2 (Figure 3D). In the presence of H_2O_2, only 250-500 µg/mL of Bordo wine protected against cytotoxicity ($p<0.01$ and $p<0.05$), whereas 1000 µg/mL of Bordo grape wine induced further cytotoxicity compared to vehicle- H_2O_2 (Figure 3D, $p<0.001$).

4 DISCUSSION

Polyphenols, which have high antioxidant capacity and exhibit strong protective effect against cellular oxidative damage are the most abundant secondary metabolites in plants and antioxidants in human diet (MANACH; MAZUR; SCALBERT et al., 2005). Grapes and derivatives contain high amounts of phenolic compounds, mainly flavonoids. In fact, high levels of phenolic compounds were found in samples of Bordo grape juice and wine used in the present study, which may contribute to the high antioxidant potential of those beverages. Furthermore, many of these compounds exhibit multiple biological activities and these functions are mainly attributed to their antioxidant and antiradical activity (SERUGA; NOVAK; JAKOBEK, 2005; BARONI, 2012). The main finding of our study is that the consumption of Bordo grape juice and wine yielded similar antioxidant effects by increasing total antioxidant capacity and reducing lipid oxidation, despite the higher content of phenolic compounds and *in vitro* antioxidant activity of Bordo wine compared to juice.

Concerning the study of antioxidant effectiveness, the use of different *in vitro* models has been recommended, due to the differences between the various free radical scavenging assays (RUBERTO, 2007; ROCKENBACH et al., 2011). Thus, antioxidant activity of Bordo juice and wine were assessed using the ABTS method, which measures the scavenging of the ABTS radical cation, and the FRAP method, which measures the ability to reduce the ferric-tripyridyl triazine complex (Fe III-TPX) to ferrous complex (FeII-TPZ) under acidic conditions. In our study, the ABTS assay showed higher and significantly values compared to FRAP values, mainly for wine. However, the reaction of FRAP method may not be complete even several hours after the initiation of the reaction, mainly because of subsequent dimerizations and polymerizations (NENADIS; LAZARIDOU; TSIMIDOU, 2007). Drawbacks of this method are concerned with compounds that have low redox potential and can reduce the Fe III even though they do not behave as antioxidants *in vivo* (PÉREZ-JIMÉNEZ,

2008; DOTAN, LICHTENBERG, 2008), interfering compounds that can absorb at the same wavelength and the assay being performed at a non-physiological pH.

Numerous indices and methods have been used to assess oxidative stress, defined as an imbalance between the production of ROS and their removal by antioxidants. Among various indices, products of lipid peroxidation are the most common group used to evaluate the individual oxidative (antioxidant/pro-oxidant) status (KARDUM, 2014; DOTAN; LICHTENBERG; PINCHUK, 2004). Lipid peroxidation is a result of complex reactions which yield compounds that can be determined as TBARS (MOORE; ROBERTS, 1998), according to García-Alonso et al. (GARCÍA-ALONSO, 2006) a reduction in the lipid oxidation might be associated with the intake of phenolic beverages. Our results showed a significant ($p<0.05$) decrease in serum lipid peroxidation after the intake of both Bordo juice and wine compared with baseline values and this effect was not observed after water intake. Similar effects were previously reported in human serum or plasma after the intake of polyphenol-rich foods, and according to these studies the decrease in lipid peroxidation probably occurred due to the quick absorption of polyphenols into the bloodstream (TOALDO et al., 2015; VIEIRA et al., 2012; CARDOSO, 2015). These phytochemicals are known to prevent lipid peroxidation by scavenging peroxyl radicals (TOALDO et al., 2015; CARDOSO, 2015; GRIS, 2013). Moreover, evidence from in vitro studies indicates that resveratrol, which is among the most important grape polyphenols (PANDEY; RIZVI, 2009), can be accumulated into erythrocytes and activates the erythrocyte plasma membrane redox system (RIZVI; PANDEY, 2010). Resveratrol may function as an electron donor for this enzymatic system, which reduces extracellular oxidants and recycles oxidized ascorbate, thereby contributing to counteract extracellular oxidative processes (RIZVI; PANDEY, 2010). In addition, in silico studies revealed that other grape polyphenols, namely quercetin, epigallocatechin gallate, catechin, epicatechin, are able to interact and donate protons to the human NADH-cytochrome b5 reductase, which is a component of the erythrocyte plasma membrane redox system (KESHARWANI, 2010). These mechanisms may underline the antioxidant effect of Bordo juice and wine in serum as observed in the present study.

Short-term studies involving the consumption of polyphenol beverages have reported acute increases in the antioxidant capacity of plasma or serum, which have usually been attributed to the high levels of polyphenolic antioxidants provide by plants (KARDUM, 2014; TOALDO; CRUZ; DA SILVA, 2018; BOAVENTURA, 2013;

O'BYRNE et al, 2002). Our findings showed significant ($p<0.05$) improvement in antioxidant status after the consumption of Bordo grape juice and wine, in opposite to the ingestion of control beverage (water), confirming therefore our hypothesis that polyphenols present in the Bordo juice and wine favorably influence the antioxidant capacity *in vivo*. Malvidin-3-glucoside (M-3-G), which is the most abundant anthocyanin in grapes and grape products, has similar bioavailability after the ingestion of red wine or dealcoholized red wine, indicating that ethanol in red wine does not seem to affect the absolute uptake and plasma concentrations of M-3-G (BUB, 2001). Furthermore, increases in plasma anthocyanin concentrations after the consumption of either red wine or dealcoholized red wine were about two times lower than those measured after consumption of red grape juice. These authors did not measure the antioxidant capacity after beverages intake. We found that anthocyanin concentration in Bordo wine was 3 times higher than in Bordo grape juice but both beverages were similarly effective to increase blood antioxidant capacity and reduce lipid oxidation in humans after consumption.

The hypothesis that flavonoids are responsible for the increase in plasma antioxidant capacity after the intake of flavonoid-rich foods has been disputed by evidence that such effect could be a consequence of increased uric acid levels (LOLITO; FREI, 2006). Uric acid has been demonstrated to be one of the major contributors to the antioxidant capacity in human sérum (VIEIRA et al., 2012) and particularly contributes to the antioxidant capacity of serum assessed by the FRAP assay (BENZIE; STRAIN, 1996; TOALDO; CRUZ; DA SILVA, 2016). Fructose from flavonoid-rich fruits has been demonstrated to be responsible for increasing plasma uric acid levels (LOLITO; FREI, 2006). However, in the present study the consumption of 100 mL of Bordo juice or wine did not increase glycemia. Moreover, we demonstrated that Bordo grape juice decreased blood uric acid levels, indicating that the increase in antioxidant capacity of serum was promoted by grape juice antioxidants and not by urate. Similar results were recently found after acute consumption of grape juices (TOALDO; CRUZ; DA SILVA, 2016). On the other hand, we found an increase in serum levels of uric acid after Bordo wine consumption that was parallel to the increase in plasma antioxidant capacity (FRAP and ABTS assay) and to the decrease in plasma lipid oxidation. Similar results were found for port wine consumption (DAY; STANSBIE, 1995).

Assays using living cells have proven to be useful for routine testing of various products, producing reliable results for the identification of biological activities, including antioxidant capacity (CYBORAN, 2012). Excessive ROS production is associated with disruption of cell cycle regulatory mechanisms. In the present study, we used the human neuron-like cells SH-SY5Y, which were challenged with H_2O_2 that is among the major physiologically relevant ROS species (BIERBEN, 2012; ANASTASIADI, 2010). Bordo juice inhibited the production of RS and the loss of cell viability induced by H_2O_2. In contrast, Bordo wine had only a small protective effect against the loss of cell viability at intermediate concentrations but increased RS production and promoted loss of cell viability per se at the highest concentration. Such effect was not related to the ethanol content of wine as ethanol was removed by freeze-drying before the experiment.

The direct radical scavenging action of polyphenols requires the presence of the antioxidant at the exact place where such radicals are formed. Polyphenols protect biological membranes from oxidation as they interact with the lipid phase of membrane with a tendency to incorporate into the outer hydrophilic portion of the phospholipid bilayer (BIRBEN, 2012). The antioxidant components of fruits and vegetables, such as polyphenols have been found to possess properties which play a role in protecting cellular macromolecules from ROS-induced damage (HALLIWELI, 2006; VALKO et al., 2007). Many grape compounds could be responsible for the grape juice antioxidant activity against H_2O_2-induced damage in SH-SY5Y cells. Polyphenol composition of wines shows higher complexity when compared to their corresponding juice berries because during the wine making and maturation processes there are numerous reactions involving phenolic compounds (enzymatic and chemical oxidation reactions, condensation reactions, hydrolysis, etc.).

We propose that wine fermentation process generates compounds that exhibit pro-oxidant effects at high concentrations and would be responsible for the overproduction of RS induced by the highest wine concentration (1 mg L^{-1}) in SH-SY5Y cells. Conversely, commercial red wine from China exhibited neuroprotective effects against H_2O_2-induced oxidative stress in SH-SY5Y cells up to 4 mg mL^{-1} (XIANG et al., 2014). This discrepancy may be attributed to differences in the cultivars used to prepare the wines. Another explanation for the different effect of Bordo wine and juice in the culture assays could be the higher concentration of phenolic compounds in Bordo wine compared to Bordo juice, which could exert a pro-oxidant effect. In fact,

Long et al. Long, Clement and Halliwell (2000) showed that addition of phenolic compounds, especially epigallocatechin and epigallotechin gallate, to the cell culture media rapidly generates substantial amounts of H_2O_2. This effect was dose-dependent and significant amounts of H_2O_2 (200 – 400 μM) have been shown to be formed after the addition of phenolics at concentrations ≥ 100 μM.

The small number of individuals studied may be considered a limitation of the present study. However, it should be noted that all the analyses were paired comparisons, which has strong statistical power. In conclusion, the high amount of phenolic compounds found in samples of Bordo grape juice and wine used in the present study, may contribute to the high *in vitro* antioxidant potential of those beverages. Furthermore, the *in vitro* antioxidant capacity can be reproduced *in vivo* antioxidant after acute human intake because the consumption of Bordo grape juice and wine improved antioxidant capacity and reduced lipid oxidation in healthy volunteers. In addition, Bordo juice and wine were able to decrease glucose levels and only wine increased uric acid levels. The same way, wine did not have antioxidant effect in cell culture showing to be toxic at high concentration, whereas juice had antioxidant effects against H_2O_2-induced cellular oxidative stress. Bordo grape juice and wine can be used for improving health and as a preventive for oxidative stress-related diseases, but wine should be consumed in smaller doses due to the pro-oxidant effect observed in cell culture.

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5.3 Manuscrito 3

COMPARISON OF BORDO GRAPE JUICE AND WINE ANTIOXIDANT EFFECTS ON MARKERS OF OXIDATIVE STRESS IN HEALTHY ADULTS

Este manuscrito está em processo de revisão para submissão no peródico *Nutrition Research*

Comparison of Bordo grape juice and wine antioxidant effects on markers of oxidative stress in healthy adults

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Abstract: Grape juice and wine are a rich source of phenolic compounds with a powerful antioxidant efficacy *in vitro* and *in vivo*, however, the efficacies in a post prandial intervention were not yet compared. This cross-over study evaluated the effects of the acute consumption of Bordo grape juice and wine with a meal intervention in ten healthy volunteers. Blood samples were collected before and 30, 60, and 120 minutes after juice, wine or water (control) intake to measure the biochemical and oxidative parameters in serum. We also observed that the acute intake of a single dose of wine was able to decrease the oxidation protein products of healthy individuals through a significant interaction between treatment and time. These results indicate a positive effect of Bordo juice and wine consumption on the antioxidant status and cellular oxidative damage of healthy individuals.

Keywords: Bordo juice, Bordo wine, clinical trial, post prandial response, anthocyanins, antioxidante status.

1 INTRODUCTION

Several epidemiologic studies with alcohol consumption have demonstraded a reduced in risk of many chronic human diseases, with special atention to red wine (CACCETTA et al., 2000). This beverage contains many phenolic compounds such as phenolic acids (gallic acid, caffeic acid, p-coumaric acid, and others), stilbenes (trans-resveratrol), and flavonoids (catechin, epicatechin, quercetin, rutin, myricetin) with special attention to anthocyanins, the main phytochemicals presents, responsible for the color of grapes and wines (KAMMERER et al., 2004).

To form stable phenoxyl radicals, polyphenols can accept an electron, disrupting chain oxidative reactions in cellular components, that may increase plasma antioxidant capacity. In the case of anthocyanins, due to its structure, the antioxidant activity occurs through the donation of electrons or hydrogen atoms from its hydroxyl to free radicals (MOTOHASHI; SAKAGAMI, 2009).

Anthocyanins, which are pigments of the flavonoids family, are the main phytochemicals present in grapes and derivatives (GARRIDO, 2013). Over the last years several families of anthocyanin derivatives have been reported in grapes, wine and wine-like solutions. Anthocyanic pigments are responsible for the color of grapes and wine, a characteristic that is determined by their chemical structure, namely their degree of hydroxylation, methylation and/or glucosilation (HE, 2005). The antioxidant activity of an anthocyanin occurs through the donation of electrons or hydrogen atoms from its hydroxyl to free radicals (MOTOHASHI; SAKAGAMI, 2009).

The postprandial state is a pro-oxidant state. The postprandial period is a time of active oxidative metabolism and formation of ROS (reactive oxygen species). An imbalance between oxidant generation and antioxidant defence in favour of oxidants potentially leading to biological dysfunction and/or damage is referred to as oxidative stress. Therefore, consuming fruits rich in phenolic compounds with meals may have several advantages in the postprandial state, first and foremost, through their inherent antioxidant properties and potential to modulate cellular reductive–oxidative (redox) balance (BURTON-FREEMAN, 2010).

In the present study, we examined the effect of Bordo grape juice and wine with a meal on postprandial biochemical parameters and markers of oxidative stress response in healthy subjects.

2 MATERIALS AND METHODS

2.1 SAMPLES

The commercial samples of Bordo grape juice and Bordo wine were produced by a winemaker (Casa Perini, Farroupilha, RS, Brazil). The grape fruits used to prepare juice and wine were harvested in Farroupilha (29° 13’ 30” S, 51° 20’ 52” W, altitude 783 m), in the State of Rio Grande do Sul, Brazil, on January 2014. Bordo grape juice was prepared by the enzymatic method, in which grape is crushed and then heated to at least 65°C in a hot macerator. Next, commercial pectolytic enzymes are added and must is kept between 55 and 60°C during 1-2 h. The extracted juice is then clarified, pasteurized and bottled (RIZZON, LINK, 2006). Bordo wine was obtained from vinification process by the coupled dispositive to the crushing machine that is called dewaxing. In winemaking of red wine, grape skin remains inside tanks during fermentation for extraction of anthocyanin pigments (PSZCZÓLKOWSKI; LECCO, 2011).

2.2 SUBJECTS

The study was approved by the Federal University of Santa maria Ethics Committee (protocol number 39197614.3.0000.5346) prior to initiation of the study. Informed consent was obtained from all healthy volunteers. Inclusion criteria for the volunteers were: no clinical disease condition, no gastrointestinal disorders or known metabolic diseases, no infections or inflammatory processes visible or known three months prior to the study, age between 18 and 30 years, non-smoking, non-alcoholic, body mass index (BMI) <30 kg/m^2, non-user of drugs or dietary supplements, and no gastric intolerance or complications associated with the ingestion of grape juice and wine. These criteria were evaluated by a clinical history questionnaire. The nutritional characteristics of the participants were recorded with data on age, weight, height and food consumption using a 3-day food record. Weight and height measurements were used to calculate the body mass intake (BMI) (WORLD HEALTH ORGANIZATION, 2000).

2.3 STUDY DESIGN

In this cross-over clinical study, participants were randomly divided into three groups, the juice group (JG), the wine group (WG) and the control group (CG), receiving 100 mL of juice, wine or water according to their allocated group. Immediately the participants also received a meal consisting of a hamburger to be consumed along with the beverages. The nutritional value of meal is described in table 1. One week after the washout period, treatments were crossed for the participants of each group (JG, WG or CG). In order to better observe the effect of the acute consumption of beverages on *in vivo* antioxidant activity, participants were oriented to follow a low-antioxidant diet for 48 h prior to the day of intervention, avoiding phenolic-rich foods or beverages (fruits, vegetables, fruit juices, tea, coffee, wine or chocolate) and alcohol in general. Food allowed during this period was: beef, pork, chicken, cheese, white bread, white rice, regular pasta (not whole-wheat), butter and milk (MAFFEI et al., 2007). The intake of energy, macronutrients, dietary fiber and antioxidants on the days before the study was monitored using a prospective 48 h dietary record. If the subjects reported symptoms of the gastrointestinal tract or any other discomfort on the experiment day, the examination was postponed. This post prandial experiment protocol was based on an earlier study by Roehrs (2017), with some modifications.

Table 1 - Nutrition composition and caloric value of test meal

Portion (130g)		Daily intake (%)*
Caloric value (Kcal)	362	18
Carbohydrate (g)	34	11
Protein (g)	19	25
Total fat (g)	17	31
Saturated fat (g)	5,7	26
Trans fat (g)	1,5	-
Food fiber (g)	1,7	7
Sodium (mg)	1.168	49

*Calculated based on the recommended daily amounts from FAO/OMS - 2003.

2.4 BLOOD COLLECTION AND ANALYSES

All blood collections (10 ml each one) were made by puncturing the median cubital vein or basilic vein with three vacuum system tubes containing no anticoagulant (to obtain serum). The first blood sample collection (baseline collection before the meal ingestion) was made between 8 and 9 h in the morning, after the blood pressure measurement of fasted subjects (12 h fasting). Immediately after baseline blood collection, subjects were randomly assigned to one of the test beverages (control, Bordo juice or Bordo wine) concomitant with the meal that was consumed within 20 min. Blood samples were subsequently collected at 30, 60 and 120 minutes after ingestion. Each subject was submitted to this procedure at three different occasions with an interval of least 1 week, receiving one of the three intervention at each occasion.

2.5 BIOCHEMICAL ANALYSIS

The serum levels of triglycerides (TG), total cholesterol (TC), high density lipoprotein cholesterol (HDL), low density lipoprotein cholesterol (LDL), glucose, uric acid and albumin were measured by colorimetric kits (Bioclin, Belo Horizonte, MG, Brazil).

2.6 ANTIOXIDANT CAPACITY AND OXIDATIVE STRESS ASSAYS

The serum total antioxidant capacity (TAC) was assessed at 660 nm as the capacity to reduce the 2,2´-azinobis (3-ethylbenzothiazoline-6-sulfonate) ($ABTS^{\bullet+}$) to its colourless form (EREL, 2004), the serum antioxidant capacity was determined using the FRAP assay (BENZIE; STRAIN, 1996).

2.7 STATISTICAL ANALYSIS

Data are presented as mean ± standard deviation (SD) or standard error (SEM) was verified by the Bonferroni Post Hoc test. Potential differences caused by the application of the treatments and the analysis times were detected by the repeated measures analysis of generalized equations, taking into account the model, type of

treatment, time and interaction between type of treatment and time. In addition, relative changes in the post consumption data compared with baseline were determined in order to evaluate the difference between the three treatments. To compare the relative changes between the Bordo juice, wine and the control groups, after 30, 60, and 120 h of consumption, a paired test with non-parametric measures (Wilcoxon signed rank test) was used. Pearson or Spearman correlations were performed according to the data symmetry. Data were analyzed using Stat version 11.0 for Windows (Stata Corporation, College Station, TX, USA), and a significance level of less than 5% ($p < 0.05$) was considered.

3 RESULTS AND DICUSSION

There is already evidence of the antioxidant activity of juice and wine *in vitro*, animal and human studies; however to the best of our knowledge, this is the first clinical investigation evaluating the effects of Bordo juice and wine (*Vitis labrusca*) post prandial intake on endogenous antioxidant markers and biochemical parameters in healthy individuals. Table 2 shows the absolute changes in antioxidant activity and biochemical parameters over time. Relative changes in TAC, FRAP, AOPP, glucose, triglycerides, total cholesterol, HDL and LDL, uric acid and albumin outcomes are shown in Figures 1, 2 and 3.

In this study, we observed a significant variation (p<0,001) in beverages according to the time in their respective groups. Juice and wine had the highest glucose peaks after 30 minutes of their consumption, however they significantly reduced their contents after 120 minutes, not differing from each other, but differing significantly from the water that obtained a reduction only after 120 minutes of its consumption. One can conclude that juice and wine, although they had higher blood glucose peaks, were able to reduce and stabilize their contents in a shorter time, while water took another 1 hour to achieve this effect. The triglycerides (TG) contents of all beverages promoted variation after 60 and 120 minutes of consumption, and for juice and wine the highest peaks were observed after 120 minutes. These results demonstrate that the meal was probably responsible for increasing the TG levels and that the consumption of these beverages was not able to protect in the variation of their blood levels.

Table 2 - Biochemical parameters, total antioxidant and oxidant status of healthy individuals after meal with Control (water), Bordo juice or Bordo wine.

Parameters	Time (m)	Control	Bordo juice	Bordo wine	Value		
					p*	p**	p***
Glucose (mg/dL)	0	84.90±1.31^{bB}	88.50±2,17^{bBCD}	87,70±1.44^{abBC}	<0,001	0,625	0,036
	30	98.60±2.68^{aAD}	109.90±4,43^{aA}	103.40±4.50^{aAC}			
	60	86.40±3.73^{abBCD}	84.90±2,86^{bB}	82.60±3.88^{bB}			
	120	83.11±2.73^{bB}	80.20±2,28^{bB}	81.90±2.38^{bB}			
Total cholesterol (mg/dL)	0	186.30±7.83	174.50±9.76	179.60±9.14^{a}	0,010	0,673	0,273
	30	182.20±7.74	171.70±8.68	176.70 ±8.19^{bc}			
	60	183.40±8.38	173.70±8.23	180.40±8.76^{ac}			
	120	182.77±8.09	174.00±7.50	183.90±9.17^{a}			
HDL (mg/dL)	0	68.70±6.52^{a}	70.70±6.93	67.20±5.53^{b}	0,005	0,931	0,785
	30	66.40±6.97^{b}	69.10±7.54	64.90±5.32^{a}			
	60	67.30±7.26^{ab}	69.90±7.12	65.40±5.25^{ab}			
	120	68.44±7.38^{ab}	68.88±6.60	67.40±6.63^{ab}			
LDL (mg/dL)	0	101.22±7.30^{a}	85.56±7.79	91.92±6.56	<0,001	0,424	0,590
	30	98.78±6.81^{b}	84.38±7.25	90.14±6.18			
	60	95.74±6.75^{b}	83.70±6.98	90.32±5.99			
	120	93.00±5.99^{b}	82.54±7.12	88.56±6.30			
Triglycerides (mg/dL)	0	81.90±5.94^{b}	91.20±11.02^{c}	102.40±12.15^{c}	<0,001	0,333	0,284
	30	85.10±8.75^{b}	91.10±9.59^{c}	108.30±14.43^{c}			
	60	101.80±8.9^{a}	100.50±10.49^{b}	123.40±15.76^{b}			
	120	106.68±9.8^{a}	113.30±12.20^{a}	139.70±17.27^{a}			
Uric acid (mg/dL)	0	2.08±0.10^{b}	2.13±0.11^{b}	1.94±0.12^{b}	<0,001	0,717	0,570
	30	2.22±0.12^{ab}	2.28±0.14^{a}	2.02±0.14^{b}			
	60	2.32±0.98^{a}	2.23±0.91^{a}	2.20±0.20^{b}			
	120	2.49±0.13^{a}	2.40±0.85^{a}	2.45±0.16^{a}			
Albumin (g/L)	0	4.81±0.83^{ab}	4.82±0.83	4.90±0.53	0,004	0,565	0,982
	30	4.72±0.83^{b}	4.78±0.69	4.86±0.81			
	60	4.78±0.64^{ab}	4.82±0.87	4.89±0.89			
	120	4.84±0.89^{a}	4.86±0.86	4.94±0.93			
TAC (mmol/L)	0	0.960±0.028^{ab}	0.916±0.308	0.944±0.025^{b}	0.046	0,406	0,058
	30	0.958±0.029^{b}	0.836±0.880	0.970±0.023^{a}			
	60	0.972±0.029^{a}	0.944±0.212	0.969±0.020^{a}			
	120	0.966±0.033^{ab}	0.948±0.209	0.977±0.027^{a}			
FRAP (µmol/L)	0	195.60±17.38	234.70±32.42^{b}	275.90±43.76	0,004	0,135	0,856
	30	201.60±17.25	243.00±34.18^{a}	289.40±48.21			
	60	205.20±17.85	241.50±32.90^{ab}	282.90±36.80			
	120	208.33±20.73	239.40±30.60^{ab}	295.20±30.60			
AOPP (µmol/L)	0	18.50±1.87^{AD}	18.30±1.56^{ABD}	15.30±1.17^{BD}	0,385	0,843	<0,001
	30	17.30±1.35^{AD}	17.10±1.34^{ABD}	16.40±1.66^{ABD}			
	60	17.10±1.66^{AD}	17.10±1.34^{ABD}	17.10±1.01^{AC}			
	120	14.78±1.19^{BC}	17.50±1.43^{ABD}	17.10±1.39^{ABD}			

Results are expressed as means ± SEM (n = 10). ^{a,b}Different letters indicate significant difference among time interventions; ^{A,B}Different letters indicate significant difference among time vs group (Bonferroni Post Hoc test; p*: *p* value to "Time"; p: *p* value to "Group", p***: *p* value to Time vs. Group). HDL, high density lipoprotein; LDL, low density lipoprotein; TAC, total antioxidant capacity; FRAP, ferric reducing antioxidant power; AOPP, advanced oxidation protein products.**

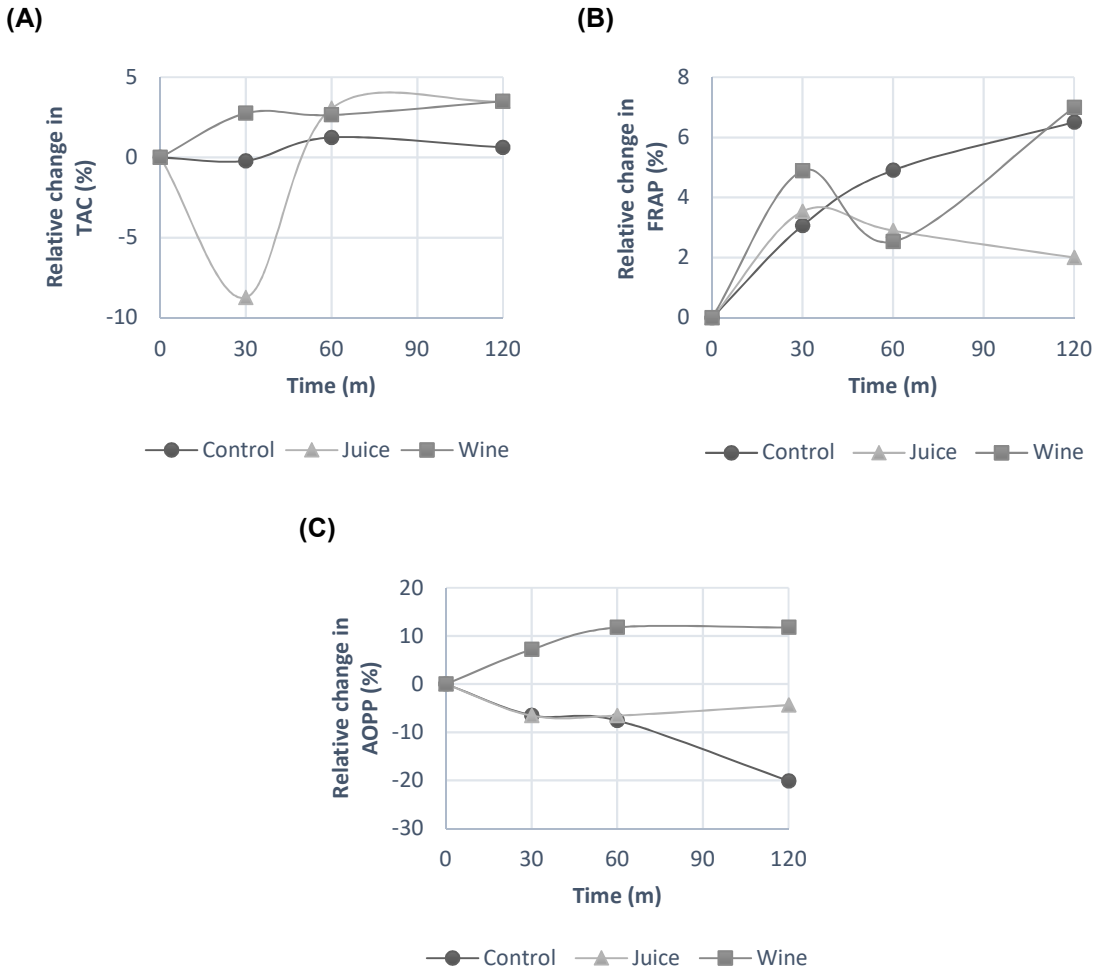
For the total cholesterol levels, only the wine was able to reduce the blood levels after its consumption, and after 30 minutes a significant difference was obtained, remaining stable until the 120 minutes, where again it obtained increase, to fasting. Water and wine groups were able to significantly vary the blood levels of high-density lipoprotein (HDL) after consumption. Since juice maintained HDL-c levels at all times, it may be suggested that this beverage behaved as protective of variations in HDL blood levels. For the group that consumed water there was a reduction in the blood levels of LDL-c, and the groups that consumed juice and wine maintained their serum levels stable.

Serum levels of uric acid after juice consumption increased after 30 and 60 minutes for the group that consumed water, whereas for the wine it took 120 minutes to observe this change. Some studies suggest that uric acid increase is related to the increase of serum antioxidant activity, in this case it can be said that there is a strong correlation between these parameters when we observed the juice contents, since the uric acid contents increased earlier and remained for all measured time. In the correlation analysis, a moderate positive correlation was observed between FRAP and uric acid ($r = 0.31$, $p = 0.03$).

Studies with other food sources rich in polyphenols have also found a correlation between uric acid concentrations and FRAP values (FERNÁNDEZ-PACHÓN et al., 2005; GODYCKI-CWIRKO et al., 2010; JIN et al., 2011), suggesting that antioxidant activity determined by FRAP assay can be partly explained by an increase in uric acid concentrations. Considering the maximum increase in FRAP and uric acid concentrations 1 h after juice consumption, it was noted that the FRAP values exceed the increase in uric acid, suggesting that other components such as phenolic compounds influence the serum antioxidant potential.

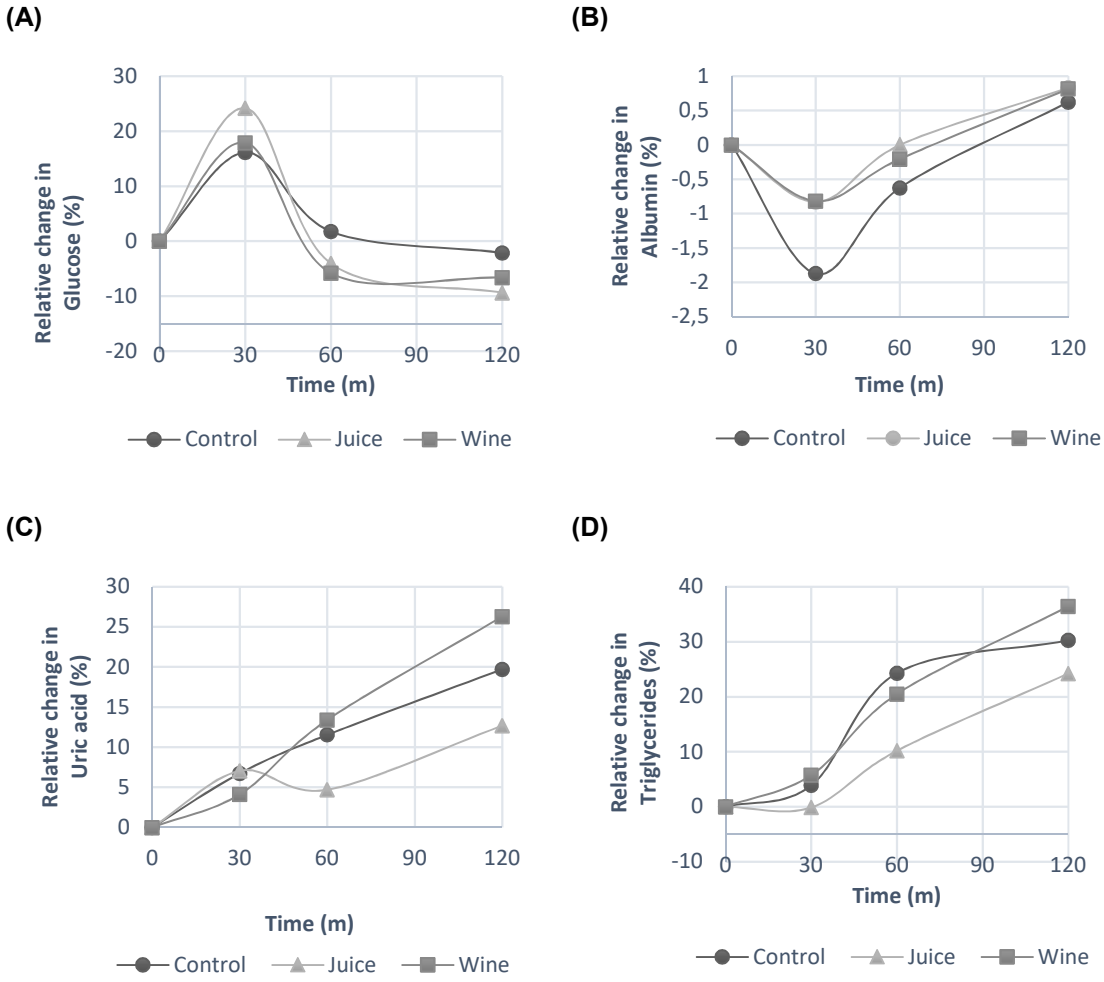
In the group that consumed water, there was an increase in albumin from the time of 30 minutes to 120 minutes. Other comparisons showed no difference. These results suggest that juice and wine were able to maintain serum albumin levels.

Figure 1 - Relative changes in the parameters TAC (A), FRAP (B) and AOPP (C) after juice and wine consumption.



Data are expressed as mean ± SEM. TAC, total antioxidante activity; FRAP, ferric reducing potential; AOPP, advanced oxidation protein products. (n=10).

Figure 2 - Relative changes in the parameters Glucose (A), Albumin (B), Uric Acid (C) and Triglycerides (D) after juice and wine consumption.

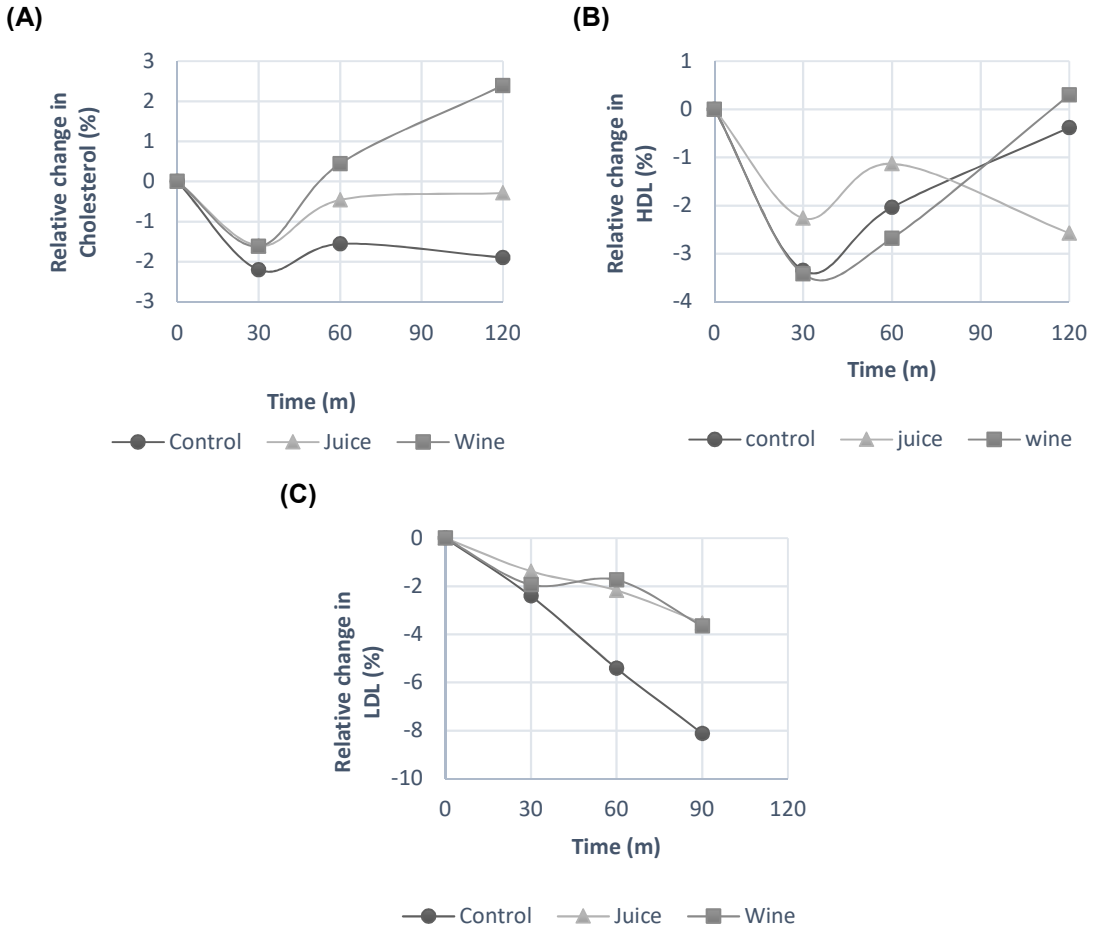


Data are expressed as mean ± SEM. (n=10).

For the group that consumed wine the serum levels of TAC increased after 30 minutes of its consumption and remained so throughout the measured period (120 minutes). While the water provided an increase in the TAC levels after 60 and after 120 minutes did not differ more of the contents without the ingestion. This suggests a significant antioxidant power for wine. On the other hand, for the FRAP levels only the group that consumed juice showed a significant increase after its consumption, which corroborates with the increase of uric acid in the serum levels after the consumption of this same beverage. In the group that consumed water we observed a significant reduction in serum protein oxidation products after 120 minutes of consumption of this

beverage, while for the group that consumed wine, after 60 minutes the blood levels of AOPP increased in relation to time without drinking.

Figure 3 - Relative changes in the parameters Cholesterol (A), HDL (B) and LDL (C) after juice and wine consumption.



Data are expressed as mean ± SEM. HDL, Hight Density Lipoprotein; LDL, Low Density Lipoprotein. (n=10).

The *in vivo* antioxidant findings of our study (Table 2) may be associated with the high amounts of phenolic compounds, mainly anthocyanins, in juice and wine. It is noteworthy that anthocyanins appear to be rapidly absorbed and eliminated by the organism, reaching low maximal concentrations in plasma and urine, with the maximum plasma concentrations observed between 0.5 and 4 h after consumption (FERNANDES et al., 2014; KAY, 2006; POJER et al., 2013). In the colon, anthocyanins are metabolized by bacteria, originating more simple compounds (FERNANDES et al., 2014). The colonic microbiota are responsible for the extensive breakdown of the original polyphenolic structures into a series of low-molecular-weight phenolic

metabolites that, being absorbable, may actually be responsible for the health effects that result from polyphenol-rich food consumption (CARDONA et al., 2013). Furthermore, some authors have reported that phenolic acids can be metabolites derived from anthocyanins, such as protocatechuic acid, syringic acid, vanillic acid and gallic acid (FORESTER; WATERHOUSE, 2010; HE et al., 2005; KAY et al., 2009). The health benefits associated with anthocyanin-rich foods may be explained by a slow and continuous release of these phenolic compounds through the gut and into the bloodstream (FERNANDES et al., 2014).

A limitation of the present study is the non-assessment of plasmatic circulating compounds such as phenolic acids and anthocyanins. It should be noted that the assessment of phenolic metabolites is complex (CROZIER et al., 2009), and many factors can interfere with and limit the absorption or bioavailability of these compounds. Notable factors in this regard are the food matrix, other food components, micronutrients and macronutrients (SANTOS-BUELGA et al., 2014; YANG et al., 2011). In addition, inter-individual differences in the composition of the human microbiota and inter-individual variations in the daily intake of polyphenols may lead to differences in the bioavailability and bioefficacy of polyphenols and their metabolites (CARDONA et al., 2013; DORSTEN et al., 2010). Factors such as the absorption and biotransformation of anthocyanins, the exact nature and amount of circulating metabolites and their activity are still not well understood (SANTOS-BUELGA et al., 2014). In addition, the lack of conclusive results for some parameters may be due to other factors such as the small number of people surveyed; considering the high intra- and intervariability of individuals, it would be of interest to test the effect of Bordo juice and wine in a study with a higher number of participants. Moreover, the participants in our study were relatively young and healthy volunteers, while future studies should include individuals with greater susceptibility to oxidative stress.

4 CONCLUSION

The results of this study verify the antioxidant activity of juice and wine in humans. To the best of our knowledge, this is the first clinical trial in which the effects of the acute intake of Bordo juice and wine in a post prandial protocol on the endogenous antioxidant and biochemical biomarkers of healthy subjects have been investigated. We found a significant increase in the FRAP values after 1 h of juice

intake, with a concomitant significant treatment effect on both the FRAP and uric acid results. We also observed that the acute intake of a single dose of wine was able to decrease the oxidation protein products of healthy individuals through a significant interaction between treatment and time. These results indicate a positive effect of Bordo juice and wine consumption on the antioxidant status and cellular oxidative damage of healthy individuals. The prevention of disease has become increasingly important in modern society. It is generally understood that certain food sources and compounds found naturally in foods can play a role in helping an organism to remain healthy. Thus, there is a need to confirm these health-promoting effects observed using anthocyanin rich foods and beverages. Further studies are needed to evaluate the bioavailability in humans of the bioactive compounds present in juice and wine comparatively.

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6 CONCLUSÕES

- O tratamento celular com suco e vinho da cultivar Cabernet Sauvigon demostrou efeito protetor em células neurais, impedindo a toxicidade e consequentemente mantendo a viabilidade celular (SH-SY5Y) no suco. O vinho, no entanto, em altas doses teve efeito tóxico e gerou um aumento da morte celular.

- Após consumo agudo por humanos, suco e vinho Bordo demonstraram reduções na peroxidação lipídica e aumento na atividade antioxidante dos níveis séricos em indivíduos saudáveis. No ensaio em células, o suco manteve a viabilidade celular e impediu os danos oxidativos induzidos. O vinho em altas doses proporcionou efeito tóxico as células neurais.

- Em homens e mulheres saudáveis, a ingestão de uma dose única de suco e vinho Bordo associada a refeição, modificou os parâmetros bioquímicos pós-prandiais.

Este trabalho demonstrou que os sucos e vinhos avaliados são ricas fontes de compostos antioxidantes e exercem significativos efeitos *in vitro* e *in vivo.* Sugere-se, no entanto, mais estudos para avaliar a biodisponibilidade, em humanos, dos compostos bioativos presentes no suco e no vinho comparativamente.

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ANEXO 1 **– Parecer do comitê de ética em pesquisas com Seres humanos da Universidade Federal de Santa MARIA (CEP-UFSM)**

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: DETERMINAÇÃO DA CAPACIDADE ANTIOXIDANTE IN VITRO E IN VIVO DE INTEGRAL

Pesquisador: Neidi Garcia Penna

Área Temática: VINHO TINTO E SUCO DE UVA

Versão: 2

CAAE: 39197614.3.0000.5346

Instituição Proponente: Universidade Federal de Santa Maria

Patrocinador Principal: Universidade Federal de Santa Maria/ Pró-Reitoria de Pós-Graduação e Pesquisa

DADOS DO PARECER

Número do Parecer:

914.722

Data da Relatoria:

29/12/2014

Apresentação do Projeto:

Trata-se de um projeto de doutorado que visa avaliar o efeito do consumo agudo de vinho tinto e suco de uva integral sobre os biomarcadores de estresse oxidativo em indivíduos saudáveis.

Este estudo de intervenção, caracterizado como um ensaio clínico crossover, utilizará o vinho tinto, suco de uva e água como controle, onde quinze voluntárias saudáveis serão avaliadas antes e após 1h, 2h e 4h ao consumo do vinho, suco e água. Cada sujeito será submetido ao procedimento descrito acima, 3 vezes com intervalo de 2 semanas entre cada procedimento, recebendo em cada uma das vezes um dos três tratamentos.

O sangue será utilizado para análise do potencial antioxidante redutor férrico (FRAP), ácido

úrico, glutationa reduzida (GSH), glutationa peroxidase (GPx), superóxido dismutase (SOD), catalase (CAT) e hidroperóxidos lipídicos (HL). Espera-se com este trabalho demonstrar o efeito do consumo de vinho tinto e suco de uva integral, a partir de uma

intervenção alimentar aguda, sobre a capacidade antioxidante in vitro e biomarcadores de dano oxidativo in vivo, evidenciando se o potencial antioxidante dessas bebidas está relacionado aos compostos fenólicos, ao álcool ou devido à interação destes.

Objetivo da Pesquisa:

Geral: avaliar os compostos bioativos in vitro em vinho e suco de uva integral e o efeito do consumo, a partir de uma intervenção alimentar aguda, sobre a capacidade antioxidante e biomarcadores de dano oxidativo in vivo.

Específicos:

- Realizar a caracterização físico-química, teor de fenólicos totais, antocianinas monoméricas totais eatividade antioxidante do vinho tinto e suco de uva integral;
- Determinar a atividade antioxidante sérica, ácido úrico e glutationa reduzida eritrocitária do plasmasanguíneo;
- Avaliar a atividade antioxidante enzimática através das dosagens de catalase, superóxido dismutase eglutationa peroxidase eritrocitárias do plasma sanguíneo;
- Mensurar a oxidação lipídica sérica, através da concentração dos hidroperóxidos lipídicos do plasmasanguíneo.

Avaliação dos Riscos e Benefícios:

No TCLE consta a seguinte descrição de riscos e benefícios:

"É possível que aconteçam os seguintes desconfortos ou riscos: Após o jejum de 8 horas, que antecederá a primeira coleta de sangue, algumas pessoas podem apresentar um quadro de hipoglicemia, sendo que nestes casos a pessoa receberá todos os cuidados necessários e será excluída automaticamente do trabalho neste dia. Se a mesma pessoa apresentar este problema novamente será encaminhada a atendimento médico. Você poderá sentir dor no local da picada da agulha, assim como formação de hematoma pela venopunção. Caso ocorram hematomas ou dor será realizada massagem com pomada Hirudoid® no local da punção sem nenhum custo. Apesar da quantidade de vinho a ser administrada ser baixa, os sujeitos serão advertidos a não dirigirem e nem executarem tarefas que exija atenção, pelo fato do álcool prejudicar os sentidos da pessoa. Os sujeitos também serão monitorados quanto à algum tipo de desconforto que o álcool possa causar, como tontura e enjôo. Em caso de ocorrência a pessoa receberá todos os cuidados

necessários e se for preciso serão encaminhados à atendimento médico. Neste dia a pessoa será excluída automaticamente do trabalho. Os benefícios que esperamos

com o estudo será auxiliar os pacientes participantes a monitorar o perfil lipídico e glicose. Em caso de anormalidades nos resultados dos exames, os pacientes serão orientados a procurar tratamento médico. A pesquisa será benéfica no sentido de descobrir quais parâmetros são alterados após ingestão das bebidas, e esperamos que os compostos estudados auxiliem positivamente."

Considerando-se as características do projeto, esta descrição pode ser considerada suficiente.

Comentários e Considerações sobre a Pesquisa:

.

Considerações sobre os Termos de apresentação obrigatória:

Foram apresentados de modo suficiente.

Recomendações:

Veja no site do CEP - http://w3.ufsm.br/nucleodecomites/index.php/cep - na aba "orientações gerais", modelos e orientações para apresentação dos documentos. Acompanhe as orientações disponíveis, evite pendências e agilize a tramitação do seu projeto.

Conclusões ou Pendências e Lista de Inadequações:

As pendências apontadas no parecer anterior foram resolvidas de modo suficiente.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

SANTA MARIA, 15 de Dezembro de 2014

Assinado por:
CLAUDEMIR DE QUADROS
(Coordenador)

ANEXO 2 **– Trabalho parcial apresentado no "XXV Congresso Brasileiro de Ciência e Tecnologia de Alimentos" em Gramado, RS/Brasil.**

XXV Congresso Brasileiro de Ciência e Tecnologia de Alimentos - CBCTA
Alimentação: a árvore que sustenta a vida

X CIGR Section VI International Technical Symposium
Food: the tree that sustains life

Certificado

24 a 27 de outubro de 2016 • FAURGS • GRAMADO/RS

Certificamos que o trabalho intitulado

AVALIAÇÃO DA CAPACIDADE ANTIOXIDANTE E PEROXIDAÇÃO LIPÍDICA EM INDIVÍDUOS SAUDÁVEIS APÓS CONSUMO AGUDO DE SUCO DE UVA INTEGRAL

de autoria de

CRISTIANE COPETTI; NEIDI GARCIA PENNA; EDUARDA DA ROSA MACHADO; FERNANDA WOUTERS FRANCO; MARCELA BROMBERGER SOQUETTA; CLÁUDIA KAEHLER SAUTTER

foi apresentado no formato

PÔSTER

no **XXV Congresso Brasileiro de Ciência e Tecnologia de Alimentos - CBCTA** - Alimentação: a árvore que sustenta a vida e no **X CIGR Section VI International Technical Symposium** - Food: the tree that sustains life, no período de 24 a 27 de outubro de 2016, no Centro de Eventos da FAURGS, em Gramado/RS.

Gramado, 27 de outubro de 2016.

Roberta Thys

Roberta Thys
Presidente do XXV Congresso Brasileiro de Ciência e Tecnologia de Alimentos

Amauri Rosenthal
Presidente do X CIGR Section VI International Technical Symposium

Promoção

sbCTA

ANEXO 3 **- Artigo original "Acute consumption of Bordo grape juice and wine improve serum antioxidant status in healthy individuals and inhibit reactive oxygen species production in human neuron-like cells**

COPETTI et al. Acute consumption of Bordo grape juice and wine improve serum antioxidant status in healthy individuals and inhibit reactive oxygen species production in human neuron-like cells. Journal of Nutrition and Metabolism, 2017.

Gmail Cristiane Copetti <copetti.cris@gmail.com>

4384012: Your manuscript has been accepted

1 mensagem

Michael B. Zemel <jnme@hindawi.com> 30 de novembro de 2017 05:39

Responder a: amira.elbaroudi@hindawi.com
Para: copetti.cris@gmail.com

Cc: mzemel@nusirt.com, fernandafranco8@hotmail.com, dudaerm@hotmail.com, marcelasoquetta@hotmail.com, quatrinandreia@yahoo.com.br, vitorramos1908@gmail.com, 00006866@ufrgs.br, tatiana.emanuelli@ufsm.br, cksautter@gmail.com, ngpenna@gmail.com

Dear Dr. Copetti,

The review process of Research Article 4384012 titled "Acute consumption of Bordo grape juice and wine improve serum antioxidant status in healthy individuals and inhibit reactive oxygen species production in human neuron-like cells" by Cristiane Copetti, Fernanda Wouters Franco, Eduarda da Rosa Machado, Marcela Bromberguer Soquetta, Andréia Quatrin, Vitor Miranda Ramos, José Cláudio F. Moreira, Tatiana Emanuelli, Cláudia Kaehler Sautter and Neidi Garcia Penna submitted to Journal of Nutrition and Metabolism has been completed. I am pleased to inform you that your manuscript has now been accepted for publication in the journal.

The publication process of your manuscript will be initiated upon the receipt of electronic files. Please log in to the Manuscript Tracking System at the link below using your username and password, and upload the electronic files of your final accepted version within the next 2-3 days. http://mts.hindawi.com/author/4384012/upload.files/

The electronic files should include the following:

1- Source file of the final accepted manuscript (Word or TeX/LaTeX).

2- PDF file of the final accepted manuscript.

3- Editable figure files (each figure in a separate EPS/PostScript/Word file) if any, taking into consideration that TIFF,JPG, JPEG, BMP formats are not editable.

Thank you again for submitting your manuscript to Journal of Nutrition and Metabolism. Best regards,

Michael B. Zemel,
Ph.D.

mzemel@nusirt.com

APÊNDICE 1 – Questionário de inclusão dos sujeitos da pesquisa

Nome: _____

Idade:_____ Peso(Kg):_____ Altura(m):_____ IMC(kg/cm^2):____

PAS:_____ PAD:_____ Temperatura(^{o}C):_____

1. Faz exercícios físicos?

( ) Não faço ( ) 1 vez na semana ( ) 2 a 3 vezes na semana ( ) 4 ou mais vezes na semana

2. Teve alguma doença inflamatória, viral ou bacteriana nas últimas semanas?

( ) Sim ( ) Não Qual? Período?

3. Tem alguma doença crônica:

( ) Nenhuma ( ) Diabetes *Mellitus* ( ) Hipertensão ( ) Enfermidade Cardiovascular

( ) Enfermidade Renal ( ) Enteropatias

4. Você tem sentido nos últimos dias os seguintes sintomas (dores de cabeça, náuseas, ansiedade, taquicardia, dores nas juntas, cólicas estomacais, desmaio, convulsão, infecção urinária)?

( ) Sim ( ) Não Qual?

5. Teve febre nos últimos dias?

( ) Sim ( ) Não Quando?

6. Tem alguma alergia?

( ) Sim ( ) Não Qual?

7. Fez algum procedimento cirúrgico nos últimos 12 meses?

( ) Sim ( ) Não Qual?

8. Faz uso de corticoides ou fez nos últimos 12 meses?

( ) Sim ( ) Não

9. Faz uso de analgésicos (paracetamol, dipirona, neosaldina...)?

( ) Sim ( ) Não Quando foi a última vez?

10. Você está em período menstrual?

( ) Sim ( ) Não

11. Usa algum tipo de suplementação vitamínica (vitamina A, E, D, C)?

( ) Sim ( ) Não Qual?

12. Em relação ao consumo de álcool:

( ) Não Bebo ( ) Bebo 1 ou 2X por semana ( ) Bebo 3 ou 4X por semana ( ) Bebo diariamente

13. Você tem hábito de consumir vinho e/ou suco de uva:

( ) Sim ( ) Não ( ) suco ( ) vinho

Com que frequência?

( ) Não bebo 1 vez na semana ( ) 2 a 3 vezes na semana ( ) 4 ou mais vezes na semana ( )

APÊNDICE 2 – Orientações gerais para os sujeitos da pesquisa

ORIENTAÇÕES GERAIS

Orientações gerais quanto à alimentação durante o estudo e quanto aos procedimentos realizados no dia do consumo de suco e vinho.

- ✓ **Não** é permitido ingerir suplementos nutricionais (vitaminas, complexos vitamínicos e outros do gênero);
- ✓ **Durante todo o estudo evitar** o consumo de medicamentos (ex: antiácidos, analgésicos, anti-inflamatórios, anti-histamínicos, diuréticos, entre outros), exceto anticoncepcional oral;
- ✓ **Durante todo o estudo manter** o mesmo estilo de vida quanto à alimentação e à atividade física;
- ✓ **Durante as 48 horas precedentes ao estudo,** não consumir frutas, verduras, sucos de frutas e verduras, óleo de coco, café, capuccino, chás em geral, chá mate, chimarrão, vinho e outras bebidas alcoólicas, energéticos, refrigerantes com cafeína (coca-cola, pepsi), nozes, castanhas, chocolate e medicamentos em geral.
- ✓ **Durante as 24 horas precedentes ao estudo,** não fazer exercício físico;
- ✓ **Realizar jejum alimentar de 12 horas** antes da coleta sanguínea e do consumo das bebidas.
- ✓

Procedimentos no dia da coleta sanguínea:

- ✓ No dia da coleta sanguínea e da ingestão da infusão de erva-mate, dirigir-se ao Núcleo Integrado de Desenvolvimento de Análises Laboratoriais (NIDAL), Departamento de Ciência e Tecnologia de Alimentos, **no horário agendado previamente, ____:____.**
- ✓ Após sua chegada ao laboratório você descansará por 10 minutos para a realização da coleta sanguínea. A coleta sanguínea será realizada de acordo com procedimentos padrões na veia intermédia do braço por profissional treinado;
- ✓ Após esta etapa você consumirá suco ou vinho, de acordo som sorteio prévio;
- ✓ Será realizada uma nova coleta sanguínea 1 hora após o consumo da bebida;
- ✓ Durante o período entre as coletas sanguíneas você deverá permanecer sem fazer atividades exaustivas e sem consumir qualquer alimento ou bebida, inclusive água;
- ✓ Serão coletados 10 mL (2 tubos) em cada coleta sanguínea.

APÊNDICE 3 – TCLE

UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS RURAIS
PROGRAMA DE PÓS GRADUAÇÃO EM CIÊNCIA E TECNOLOGIA DOS ALIMENTOS

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Título do projeto: **Determinação da capacidade antioxidante *in vitro* e *in vivo* de vinho tinto e suco de uva integral**
Pesquisador responsável: Dra. Neidi Garcia Penna
Instituição/Departamento: Universidade Federal de Santa Maria/Departamento de Tecnologia e Ciência dos Alimentos
Telefone para contato: (55) 3220-8254
Pesquisadores participantes: Neidi Garcia Penna, Cristiane Copetti.
Telefones para contato: (55) 3220-8254 e (55) 9973-8910

Você está sendo convidado(a) para participar, como voluntário, em uma pesquisa. Você precisa decidir se quer participar ou não. Por favor, não se apresse em tomar a decisão. Leia cuidadosamente o que se segue e pergunte ao responsável pelo estudo qualquer dúvida que você tiver. Após ser esclarecido(a) sobre as informações a seguir, no caso de aceitar fazer parte do estudo, assine ao final deste documento, que está em duas vias. Uma delas é sua e a outra é do pesquisador responsável. Em caso de recusa você não será penalizado(a) de forma alguma.

♦ O estudo tem como objetivo avaliar o efeito do consumo agudo de vinho tinto e suco de uva integral sobre os biomarcadores de estresse oxidativo em indivíduos saudáveis. Este estudo de intervenção, caracterizado como um ensaio clínico *crossover*, utilizará o vinho tinto, suco de uva e água como controle, onde voluntários saudáveis serão avaliados antes e após 1h ao consumo do vinho, suco e água. Cada sujeito será submetido ao procedimento descrito acima, 3 vezes com intervalo de 2 dias entre cada procedimento, recebendo em cada uma das vezes um dos três tratamentos. A coleta de sangue será realizada por um profissional habilitado.

♦ Após o jejum de 12 horas, que antecederá a primeira coleta de sangue, algumas pessoas podem apresentar um quadro de hipoglicemia, sendo que nestes casos a pessoa receberá todos os cuidados necessários e será excluída automaticamente do trabalho neste dia. Se a mesma pessoa apresentar este problema novamente será encaminhada a atendimento médico. Você poderá sentir dor no local da picada da agulha, assim como formação de hematoma pela venopunção. Caso ocorram hematomas ou dor será realizada massagem com pomada Hirudoid® no local da punção sem nenhum custo. Apesar da quantidade de vinho a ser administrada ser baixa, os sujeitos serão advertidos a não dirigirem e nem executarem tarefas que exija atenção, pelo fato do álcool prejudicar os sentidos da pessoa. Os sujeitos também serão monitorados quanto à algum tipo de desconforto que o álcool possa causar, como tontura e enjôo. Em caso de ocorrência a pessoa receberá todos os cuidados necessários e se for preciso serão encaminhados a atendimento médico. Neste dia a pessoa será excluída automaticamente do trabalho.

♦ Não há benefício direto para o participante. Trata-se de estudo experimental testando a possibilidade de que vinho e suco possam auxiliar no metabolismo do

estresse oxidativo. Somente no final do estudo poderemos concluir a presença de algum benefício.
♦Garantia de acesso: em qualquer etapa do estudo, você terá acesso aos profissionais responsáveis pela pesquisa para esclarecimento de eventuais dúvidas.
♦ Garantia de sigilo: Se você concordar em participar do estudo, seu nome e identidade serão mantidos em sigilo. A menos que requerido por lei ou por sua solicitação, somente o pesquisador, a equipe do estudo, o Comitê de Ética independente terão acesso a suas informações para verificar as informações do estudo.
♦ Período de participação: sua participação na pesquisa terá duração de 1 semana. Você poderá retirar-se da pesquisa em qualquer momento, antes ou durante a mesma, sem penalidades ou prejuízo.
♦ O material coletado e os seus dados serão utilizados somente para esta pesquisa e ficarão guardados com o pesquisador pelo período de cinco anos, após o qual serão destruídos.

Consentimento da participação da pessoa como sujeito:

Eu, _____, abaixo assinado, concordo em participar do presente estudo, como sujeito. Fui suficientemente informado a respeito das informações que li ou que foram lidas para mim, descrevendo o estudo "**DETERMINAÇÃO DA CAPACIDADE ANTIOXIDANTE *IN VITRO* E *IN VIVO* DE VINHO TINTO E SUCO DE UVA INTEGRAL**". Eu discuti com a Nut. Cristiane Copetti sobre a minha decisão em participar nesse estudo. Ficaram claros para mim quais são os propósitos do estudo, os procedimentos a serem realizados, seus desconfortos e riscos, as garantias de confidencialidade e de esclarecimentos permanentes.

Santa Maria, 9 de junho de 2015

_____
Assinatura

Declaro que obtive de forma apropriada e voluntária o Consentimento Livre e Esclarecido deste sujeito de pesquisa ou representante legal para a participação neste estudo.

Santa Maria, 9 de junho de 2015

_____
Pesquisador responsável

Se você tiver alguma consideração ou dúvida sobre a ética da pesquisa, entre em contato: Comitê de Ética em Pesquisa – UFSM - Cidade Universitária - Bairro Camobi, Av. Roraima, nº1000 - CEP: 97.105.900 Santa Maria – RS. Telefone: (55) 3220-9362 – Fax: (55)3220-8009 Email: comiteeticapesquisa@smail.ufsm.br. Web: www.ufsm.br/cep