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AVALIAÇÃO DO PODER ANTIOXIDANTE DO CHOCOLATE AMARGO – UM COMPARATIVO COM O VINHO TINTO

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Dissertação apresentada ao Curso de Pós Graduação em Ciência e Tecnologia de Alimentos como um dos requisitos para obtenção do grau de Mestre em Ciência e Tecnologia de Alimentos.

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Submetida como parte dos requisitos para obtenção do grau de

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RESUMO

Um chocolate amargo com alto teor de cacau (71%) foi desenvolvido e codificado como D71. Previamente, foi realizada a seleção da amostra de liquor de cacau mais rica em polifenóis e flavonóides, dentre três amostras recebidas de fornecedores diferentes. Como comparativo, quatro variedades de vinho tinto foram estudadas (Merlot, Cabernet Sauvignon, Pinot-Noir e Tannat) e o vinho Tannat selecionado. Para caracterizar o produto D71 sua quantidade de polifenóis e flavonóides foi comparada a amostras de chocolate amargo comerciais contendo diferentes percentuais de cacau: 50%, 70%, 72%, 86% e 99%. Em um segundo passo, efetuou-se a comparação do D71 com diferentes tipos de chocolate comerciais: branco (WC), ao leite (MC) e meio amargo (D40). O produto D71 demonstrou menor quantidade de polifenóis em relação às amostras comerciais de chocolate amargo, porém similar quantidade de flavonóides. Quando as amostras de WC, MC e D40 foram comparadas ao D71, este produto mostrou superioridade significativa em relação aos teores de polifenóis e flavonóides. Comparando a quantidade de flavonóides presentes no D71 e no vinho tinto Tannat verificou-se que para obtenção da mesma quantidade de flavonóides de 196 mL deste vinho seria necessário o consumo de 49g do chocolate desenvolvido (D71). Esta relação foi utilizada para o ensaio *in vivo* com ratos da raça Wistar, realizado por um período de 45 dias. Os resultados obtidos nas análises do sangue demonstraram que as taxas de LDL oxidado (ox-LDL) nos ratos que consumiram vinho tinto puro ou vinho tinto combinado com o chocolate foram superiores às dos ratos que consumiram somente chocolate. Quando o efeito sinérgico das duas fontes de antioxidantes foi avaliado, o ox-LDL mostrou-se ainda mais elevado sugerindo uma interação negativa entre os antioxidantes do chocolate D71 com o vinho tinto Tannat. A análise do colesterol total no fígado mostrou níveis reduzidos nos grupos que ingeriram vinho tinto sendo coerente com os resultados sangüíneos. Os dados obtidos neste trabalho permitem sugerir que estes compostos presentes no D71, quando ingeridos na ausência do vinho tinto (Tannat), apresentam melhor capacidade antioxidante; demonstrando o potencial efeito benéfico dos polifenóis e flavonóides do chocolate amargo (D71) à saúde.

Palavras-chave: Chocolate Amargo, Vinho Tinto, Polifenóis, Flavonóides, Ratos Wistar , LDL Oxidado

ABSTRACT

The evaluation of potential benefits to health of dark chocolate in a comparison with red wine was studied. For that, a dark chocolate with a high level of cocoa (71%, labeled as D71) was developed and the quantity of polyphenols and flavonoids of the sample was measured. Previous to the manufacture of chocolate, cocoa liquor was selected based on the amount of antioxidants. Red wine polyphenols and flavonoids were also evaluated and Tannat was selected between four varieties (Merlot, Cabernet Sauvignon, Pinot-Noir and Tannat). The quantity of antioxidants of D71 was analyzed and compared with other samples of dark chocolate with different levels of cocoa (50%, 70%, 72%, 86% and 99%) and with different types of chocolate (White, Milk and Dark with 40% of cocoa). D71 was the lowest in polyphenols levels, but similar in flavonoids quantity. When compared with different types of chocolate, D71 showed the highest amount of polyphenols and flavonoids and a statistical difference could be observed. The obtained results of D71 and Tannat showed that to ingest the same amount of flavonoids found in 196 ml of Tannat an adult has to eat 49g of D71. This proportion was used to an *in vivo* assay with Wistar rats for 45 days. Results obtained in blood analysis showed that oxidized LDL (ox-LDL) in rats that ingested red wine was higher than the groups that ingested only chocolate. When synergic effect of these two sources of antioxidants was measured, the ox-LDL showed the highest values, suggesting a competition between these antioxidants. Total cholesterol in liver showed lower levels in groups that ingested red wine that was a consistent result when compared to ox-LDL in blood. This research suggests that antioxidants from D71, in absence of Tannat Red Wine, could contribute to health benefits.

Keywords: Antioxidants, Dark Chocolate, Oxidized LDL, Red Wine, Wistar rats

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1 INTRODUÇÃO

1.1 Doenças Cardiovasculares: o perigo da Aterosclerose.

A aterosclerose é responsável pela morte de cerca 17 milhões de pessoas por ano no mundo. No Brasil este número atinge aproximadamente 300 mil mortes por ano (PEREIRA, 2005).

Esta se caracteriza como uma doença inflamatória crônica que pode causar obstrução das artérias coronárias, cerebral e periféricas. A alteração endotelial é relatada como passo inicial da aterosclerose. O processo de formação de placas ocorre no momento em que, devido a uma disfunção endotelial, partículas de colesterol de baixa densidade (LDL), leucócitos circulantes (especificamente T linfócitos) e monócitos penetram na área sub endotelial e passam a produzir espécies reativas de oxigênio. Estas promovem a oxidação do LDL das camadas sub-endoteliais dos vasos arteriais e o organismo responde através de processos inflamatórios que promovem a penetração de monócitos nas camadas vasculares. Os monócitos evoluem e se transformam em macrófagos, fagocitam o LDL oxidado, porém conduzem o avanço das lesões do endotélio seguindo-se a deposição de plaquetas e formação de trombos. Esta deposição progride lentamente e poderá acarretar a obstrução do vaso, isquemia e outras manifestações clínicas como angina (DA LUZ; COIMBRA, 2004; ENGLER; ENGLER, 2004).

Os radicais livres gerados no processo acima são citotóxicos às células do endotélio vascular e diminuem a disponibilidade do óxido nítrico (NO). Este estresse oxidativo causará danos a moléculas tais como DNA, lipídeos e proteínas ampliando os efeitos deletérios sobre as células endoteliais. Como conseqüência, a mencionada redução de óxido nítrico favorece a uma vasoconstrição local, seguindo-se trombose e inflamações (ENGLER; ENGLER, 2004).

Em relação ao impacto gerado pela formação de LDL oxidado (ox-LDL) no organismo, Johnston et al. (2006) demonstrou que pacientes com níveis de LDL oxidado acima da média demonstraram duas vezes mais chance de sofrer infarto do miocárdio nos 2 anos seguintes a um evento coronariano agudo quando comparados com pacientes com níveis menores do ox-LDL. Desta forma, este valor pode ser utilizado como indicador na prevenção à aterosclerose.

Fatores como hipercolesterolemia, fumo, hipertensão, sedentarismo, obesidade e diabetes são os mais relacionados como desencadeadores do processo aterosclerótico. No entanto, Pereira (2005) reporta a recente pesquisa realizada pelo cardiologista Protásio Lemos da Luz, do InCor, o qual destacou que cerca de 35% dos pacientes com doença das artérias coronárias não estão sujeitos a estas alterações e que pessoas com baixo nível de colesterol LDL podem

apresentar placas ateroscleróticas. A causa, segundo o autor, seria a presença, no sangue, de uma proteína denominada homocisteína que seria a responsável pela alteração endotelial, partindo para lesões dos vasos sangüíneos e surgimento de inflamação decorrente formação de placas de gordura.

Neste contexto de prevenção à aterosclerose, os polifenóis, mais especificamente os flavonóides, têm recebido destaque por evitarem a oxidação LDL e conseqüente processo aterosclerótico.

1.2 Antioxidantes Presentes em Alimentos – Polifenóis e Flavonóides

Os polifenóis têm sido destacados por apresentarem efeito benéfico na prevenção de doenças cardiovasculares, diversos tipos de cânceres, inflamações e na resposta imune que pode envolver o processo de aterogênese. Estes compostos são agentes redutores que tem a capacidade de oferecer proteção contra o estresse oxidativo, portanto podem ser classificados como antioxidantes. Além disto, os polifenóis são os antioxidantes mais abundantes em nossa dieta. No entanto, nem todos os milhares de polifenóis existentes na natureza estão disponíveis. É importante o entendimento da estrutura química dos polifenóis para que se possam avaliar efeitos biológicos como: disponibilidade, atividade antioxidante, interações específicas com receptores celulares e enzimas, dentre outras funções (SCALBERT; WILLIAMSON, 2000; ENGLER; ENGLER, 2004).

As principais classes de polifenóis são definidas de acordo com a natureza de sua cadeia carbonada as quais citam-se: ácidos fenólicos, flavonóides, estilbenos e lignanas (SCALBERT; WILLIAMSON, 2000).

Os flavonóides são os polifenóis mais abundantes. São caracterizados por conterem dois ou mais anéis aromáticos ligados à, pelo menos, uma hidroxila aromática e conectados por uma ponte de carbono (Figura 1) (PETERSON; DWYER, 1998; BEECHER, 2003; ENGLER; ENGLER, 2004).

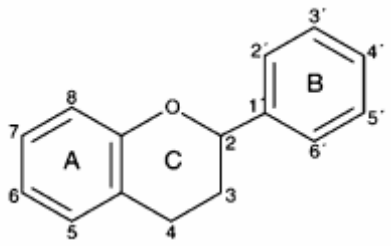

Figura 1: Estrutura monomérica básica dos flavonóides
Fonte: Yilmaz (2006).

A capacidade antioxidante dos compostos fenólicos deve-se à facilidade com a qual um átomo de hidrogênio do grupo hidroxil aromático pode ser doado para um radical livre e à habilidade do grupo fenólico suportar um elétron não pareado. Esta atividade reduz a oxidação do LDL por radicais livres e decresce a possibilidade de aterogenicidade. A Figura 2 mostra a estrutura química da (+)-catequina. Por ser rico em grupos hidroxila, este composto possui a capacidade de doar moléculas de hidrogênio que auxiliam na estabilização de radicais livres (BURNS *et al.*, 2000).

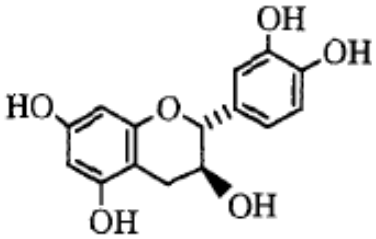

Figura 2: Estrutura (+)-catequina
Fonte: Burns et al. (2000).

Os flavonóides são divididos em diversas classes de acordo com o grau de oxidação do oxigênio heterocíclico, as quais podem ser observadas na Tabela 1. Durante seu metabolismo, os flavonóides são adicionados de grupos hidroxila, metilados, sulfatados e glucuronidados (HEIM; TAGLIAFERRO; BOBILYA, 2002).

Tabela 1: Classes de flavonóides, exemplos de compostos mais encontrados em alimentos e fonte em alimentos.

Classe de Flavonóides	Compostos mais encontrados em alimentos	Fontes em alimentos
Flavanols ou Flavan-3-ols	catequina, epicatequina, galocatequina, epigalocatequina, epicatequina-3-galato, epigalatocatequina-3-galato.	chá, chocolate e vinho tinto
Flavanonas	naringenina, hespertina, eriodictol	frutas cítricas
Flavonas	apigenina, luteolina	aipo e pimenta vermelha
Isoflavonas	daidzeina, genisteina, gliciteina, biochanina, formononentina.	soja
Flavonol	quercetina, kaempferol, miracetina, isorhamnetina	cebolas, maçãs, chá e vinho tinto
Antocianinas	cianidina, malvidina, peonidina, delfinidina, pelargonidina, petunidina.	pigmentos de frutas vermelhas, ex: berries e uvas tintas.

Fonte: Peterson; Dwyer (1998); Scalbert; Williamson (2000); Heim; Tagliaferro; Bobilya (2002); Beecher (2003); Engler; Engler (2004).

Muitos flavonóides, durante o beneficiamento de alimentos ou através do próprio metabolismo das plantas, sofrem um processo de polimerização gerando moléculas maiores.

Estes polímeros são denominados de taninos e são subdivididos em diversas classes, das quais as de maior importância são: taninos condensados (proantocianidina), taninos derivados e taninos hidrolisados (HEIM; TAGLIAFERRO; BOBILYA, 2002; BEECHER, 2003).

Os taninos condensados (ou proantocianidinas) recebem destaque devido aos seus possíveis efeitos à saúde. Existem 15 subclasses, porém somente três são de real importância para alimentos que são as procianidinas (ou catequina-(4α(8)-catequina-3-O-galato), prodelfidinas e propelargonidinas. Dentre estas, o maior destaque é dado à procianidina considerada a mais relevante à dieta humana sendo formada por monômeros de (+)-catequina e (-) epicatequina. Estes monômeros são agrupados por ligações carbono-carbono. A condensação oxidativa ocorre entre o C4 do heterociclo e C6 ou C8 das unidades adjacentes. As proantocianidinas são flavanois poliméricos que se apresentam em misturas complexas de polímeros podendo se arranjar de dímeros até grandes polímeros, com uma média de polimerização entre 4 e 11. São encontradas em ampla variedade de alimentos, sendo responsáveis pela adstringência em vinhos tintos e chocolate (BEECHER, 2003; PETERSON; DWYER, 1998; HEIM; TAGLIAFERRO; BOBILYA, 2002; SCALBERT; WILLIAMSON, 2000; DELL'AGLI; BUSCIALÀ; BOSISIO, 2004).

As células do organismo humano se protegem contra os danos oxidativos por uma série de mecanismos. Dentre estes estão a utilização das vitaminas C e E e dos polifenóis, mais especificamente os flavonóides, como os principais antioxidantes derivados da dieta (GELETTI et al., 2002).

Alguns flavonóides, como epicatequina, apresentam não só um efeito antioxidante direto, mas também são capazes de poupar outros antioxidantes como as vitaminas C e E. Relata-se que pequenas quantidades, como 1μM de epicatequina, já são suficientes para que as vitaminas C e E sejam protegidas da oxidação (AUGER et al., 2004; KEEN, 2001).

Os flavonóides, dentre os antioxidantes naturais, estão entre os de maior caráter lipofílicos, perdendo apenas para o α-tocoferol. Catequinas e procianidinas têm demonstrado serem mais potentes como inibidores da oxidação de LDL do que o α-tocoferol. Dentre os fatores que explicam esta observação está a maior facilidade dos flavonóides em capturar os radicais livres presentes no sangue. Isto parece ser devido ao fato de que os α-tocoferóis são menos acessíveis por se localizarem na membrana lipídica dentro da bicamada dos fosfolipídeos, enquanto os flavonóides, em sua maioria, se localizam na superfície polar da bicamada da membrana. Além disso, se a peroxidação lipídica tem início, ou está ocorrendo, os flavonóides podem impedir o curso da reação pela limitação da fase inicial (AUGER *et al.*, 2004; TEISSEDRE *et al.*, 1996).

Uma série de estudos epidemiológicos tem apresentado uma relação inversamente proporcional entre doenças cardíacas e o consumo de vinho, de flavonóides e de outros polifenóis presentes no vinho, frutas e vegetais. Dentre estes, encontra-se o cacau que é um produto rico em flavonóides responsáveis por efeitos cardioproteores principalmente devido à presença das procianidinas (KEEN, 2001).

1.2.1 Capacidade antioxidante do cacau

Os benefícios à saúde provenientes do cacau e chocolate são conhecidos há centenas de anos. Quando Hernán Cortez primeiramente visitou a América Central, uma de suas primeiras observações foi o uso rotineiro do chocolate. Naquela época o produto à base de cacau era consumido como medicamento sendo usado no tratamento de uma série de desordens como angina e “dores do coração”. Este conceito, de que bebidas de cacau poderiam oferecer benefícios à saúde foi amplamente divulgado entre os anos de 1850 e 1900. Somente passados cerca de 30 a 40 anos é que estas percepções foram alteradas e o chocolate deixou de ser um alimento medicinal para ser classificado como uma guloseima podendo, inclusive, prejudicar a saúde (KEEN, 2001).

O chocolate e o cacau são únicos nos tipos de flavonóides presentes. O cacau é rico em polifenóis, particularmente em flavan-3-ols ou flavanois, como formas monoméricas (-)-epicatequina e (+)-catequina, e as formas oligoméricas como as procianidinas. Estes flavonóides são comuns nas sementes de cacau utilizadas na fabricação de chocolate e produtos de cacau. A estrutura destes compostos os vinculam a uma capacidade poder antioxidante. Unidades monoméricas simples, quando presentes nos alimentos, são mais rapidamente absorvidas pelo organismo do que produtos com unidades poliméricas, sendo assim epicatequinas e catequinas apresentam resposta mais rápida. Dentre os flavonóides do cacau/chocolate a epicatequina é a de maior destaque visto que representa quase 60% do total de catequinas. Pesquisas apontam que o aumento da habilidade do plasma em se proteger contra os danos oxidativos e inibir a peroxidação lipídica após o consumo de cacau/chocolate é devido a flavonóides como epicatequina, mas outros componentes não podem ser descartados.

Estudos *in vitro* demonstraram que os flavonóides do cacau inibem a oxidação de LDL e sugerem que alta ingestão de flavonóides na dieta pode reduzir o risco de doenças coronarianas. Pó de cacau e chocolate tem demonstrado poder antioxidante e inibição da oxidação de LDL *in vitro*. Estudos prévios também demonstram que a ingestão de cacau ou chocolate pode aumentar a capacidade antioxidante do plasma e inibir a oxidação de LDL

(SERAFINI *et al.*, 2000; SCALBERT; WILLIAMSON, 2000; KEEN, 2001; ENGLER; ENGLER, 2004; MURSU *et al.*, 2004).

O chocolate é um produto que possui alto teor de gordura saturada sendo considerado, por muitos, como gerador de efeitos como hipercolesterolemia. No entanto, experimentos clínicos realizados demonstram que o consumo de chocolate tem efeitos neutros no colesterol LDL. A justificativa está no alto conteúdo de ácido esteárico (~30% de ácidos graxos), considerado neutro no que se refere a este tipo de colesterol. Estudos recentes demonstram que o consumo de chocolate amargo aumenta a concentração de colesterol HDL no plasma em 4% (MURSU *et al.*, 2004).

Mesmo destacando os benefícios apresentados pelo consumo de produtos à base de pasta de cacau é importante ressaltar o fato de que o chocolate tem alto conteúdo de gordura, conseqüentemente de energia, e seu consumo diário, em grandes quantidades, por um longo período, pode causar aumento de peso (MURSU *et al.*, 2004).

A composição de polifenóis difere entre o chocolate e o vinho tinto. A maior diferença é na fração procianidina que o vinho possui em pequena quantidade. Além disso, há no cacau alto teor de teobromina que é eficientemente absorvida e rapidamente metabolizada (REIN *et al.*, 2000).

1.2.2 Os benefícios do vinho tinto

Existe grande diversidade de polifenóis nos vinhos os quais podem ser divididos em: **não flavonóides** (ácidos hidroxibenzóico e hidroxicinâmico e seus derivados, estilbenos e alcóois fenólicos) e **flavonóides** (antocianinas, flavanols, flavonóis e dihidroflavonois). Durante o processo de maturação do vinho os compostos fenólicos são envolvidos em uma série de reações. As antocianinas, que existem em grande quantidade no vinho tinto, são transformadas em pigmentos mais estáveis modificando significativamente a cor e a adstringência do produto. Os flavanóis são envolvidos em reações de escurecimento enzimático e sua interação com proteínas resulta no turvamento da bebida. Sendo assim, os compostos fenólicos estão relacionados diretamente com parâmetros organolépticos do produto como cor, adstringência e amargor (MONAGAS; GÓMÉZ-CORDOVÉS; BARTOLOMÉ, 2005).

A maioria dos fenóis existentes no vinho são taninos dos quais podem ser citados os taninos condensados (procianidina, (+)-catequina e (-)-epicatequina) e os taninos hidrolisados (ácido gálico e derivados). Segundo Meyer et al. (1997) extratos com uvas mais maduras tem maior conteúdo total de fenóis e nível absoluto maior de antocianinas, flavan-3-ols e flavonóis (BURNS *et al.*, 2000; MEYER *et al.*, 1997).

Há quem creia que os efeitos benéficos atribuídos ao consumo de vinhos sejam devido ao etanol, no entanto, o fato de que cerveja não apresenta estes efeitos sugere que esta não seja a principal razão (DONOVAN *et al.*, 1999; VINSON; TEUFEL; WU, 2001).

Segundo Vinson et al. (2001), tanto o vinho tinto quanto o vinho tinto dealcoolizado possuem efeito significativamente superior na redução da aterosclerose quando comparado com o etanol sozinho. Este fato indica que existe algo a mais nestas bebidas, possivelmente os polifenóis. Com relação à redução do LDL, o vinho demonstrou-se significativamente melhor que o etanol ou o vinho dealcoolizado. Estudos realizados, recentemente pelo mesmo autor, demonstraram que 0,36 mL/kg de etanol proveniente do vinho tinto por dia reduz significativamente a aterosclerose, o que representa o consumo de 196 mL de vinho com cerca de 13° GL (aproximadamente 1 copo) por uma pessoa de 70kg.

A interação entre componentes fenólicos e outros nutrientes das bebidas é um fator importante. Conforme verificado por Serafini et al. (2000) a redução dos teores de álcool do vinho produz aumento linear na interação entre proteínas e taninos e paralelamente decréscimo da capacidade antioxidante dos vinhos. A interação entre proteínas e taninos forma complexos insolúveis que afetam a disponibilidade dos fenóis. Além disso, pesquisas demonstraram que mais fenóis são absorvidos do whisky do que do vinho devido ao alto teor de álcool presente naquele. Isto demonstra um possível efeito protetor do álcool em relação aos fenóis presentes nos alimentos. Sendo assim, seriam esperados resultados maiores de fenóis no plasma quando a amostra de vinho não é dealcoolizada.

Quanto à sua atividade antioxidante, os vinhos tintos demonstraram possuir poder de inibição da oxidação de LDL bastante superior ao do vinho branco. Estima-se que a inibição da oxidação de LDL varie de 46% a 100% para vinhos tintos e apenas de 3% a 6% para vinhos brancos (FRANKEL; WATERHOUSE; TEISSEDRE, 1995).

Segundo Serafini et al. (2000) o vinho tinto é rico em antocianinas e em taninos solúveis e hidrolisáveis que representam uma forma condensada dos flavonóides, enquanto o vinho branco não contém antocianinas. O vinho branco não apresenta nenhum efeito em relação a oxidação LDL o que pode ser explicado pelo baixo teor de componentes fenólicos. Esta bebida só passa a ter efeito protetor quando consumida em quantidades 10 vezes maiores do que o vinho tinto sugerindo que o conteúdo fenólico das bebidas apresenta importante papel na prevenção a oxidação LDL (SERAFINI *et al.*, 2000).

Teissedre *et al*. (1996) demonstraram que dentre os vários compostos fenólicos existentes nos vinhos, a maior atividade antioxidante na prevenção a oxidação LDL foi apresentada pelos flavan-3-ols como catequina, epicatequina, dímeros e trímeros de procianidinas, sendo as catequinas as de maior atividade antioxidante (TEISSEDRE *et al.*, 1996).

Devido à presença destes compostos os vinhos tintos e uvas possuem a característica de promover relaxamento dos vasos sangüíneos através do aumento da atividade biológica do óxido nítrico. A amplitude de vasorelaxamento é função da variabilidade dos constituintes do vinho de acordo com o tipo de uva, a área de cultivo, tempo de exposição ao sol, idade do vinho e do processamento utilizado (BURNS *et al.*, 2000; DELL'AGLI; BUSCIALÀ; BOSISIO, 2004).

Tendo em vista que os benefícios cardíacos atribuídos ao vinho podem ser oriundos do consumo moderado de etanol é necessário que se comprove a existência de outros compostos responsáveis pelos efeitos benéficos desta bebida. Neste contexto foi descoberto, por Corder et al. (2001), que os polifenóis existentes no vinho tinto decrescem a síntese de endotelina - 1 (ET-1), um potente peptídeo vasoconstritor que está relacionado com o desenvolvimento de doenças cardiovasculares e aterosclerose. Em um experimento com frações de vinho tinto dealcoolizadas, vinho branco, vinho rose e suco de uva, o resultado demonstrou que tanto as frações de vinho tinto quanto as de suco de uva apresentaram efeito inibidor na produção de ET-1. Para vinho branco e rose nenhum efeito na síntese da ET-1 foi verificado. Como o vinho rose é elaborado a partir de uvas tintas, parece que o princípio ativo do vinho tinto deriva-se da pele das uvas tintas ou de algum outro componente extraído durante o processo de vinificação.

1.3 Efeitos do Processamento sobre a Disponibilidade de Antioxidantes

Durante o processo de transformação, os alimentos passam por diversas modificações físico-químicas que podem ser benéficas ou não à manutenção de nutrientes. Dentro deste contexto fatores que influenciam na disponibilidade dos flavonóides em alimentos têm sido estudados.

Na fabricação do chocolate, ressalta-se o fato de que o processamento das sementes de cacau pode afetar a quantidade de flavonóides retidos e, conseqüentemente, sua capacidade antioxidante. Os níveis de flavonóides no chocolate são preservados aproximadamente em 70 a 95% quando calor e alcalinização são reduzidos durante o processamento. O processo tradicional conserva somente 50 a 75% do total de flavonóides do cacau (ENGLER; ENGLER, 2004).

Para os vinhos, o processamento beneficia a extração dos compostos fenólicos através da extração destes das sementes e da pele da uva. Segundo estudo realizado por Bourzeix (1986) sobre a presença de catequinas em uvas verificou-se que, em média, as uvas apresentam 377mg/kg de catequinas, sendo as da variedade pinot-noir as que apresentaram a maior quantidade, com 1165mg/kg seguidas pelas uvas colobel (híbridas) com 862mg/kg e merlot com 601mg/kg. Com relação à distribuição dos polifenóis na fruta, destaca-se que cerca de

65% das catequinas se encontram nas sementes, 20% no engace, 14% na pele e apenas 1% na polpa. As técnicas de elaboração do vinho, assim como a variedade das uvas e a área de produção são fatores determinantes na quantidade final de flavonóides na fruta (FRANKEL; WATERHOUSE; TEISSEDRE, 1995; BOURZEIX *apud* AUGER *et al.*, 2004).

Durante o processo de vinificação as catequinas se transferem para o mosto na etapa de maceração, sendo esta a que constitui a maior diferença entre a vinificação tinta e branca, já que durante a fabricação do vinho branco ocorre apenas uma rápida prensagem para separar casca e polpa. Isto explica o fato da baixa quantidade de fenólicos existente no vinho branco (AUGER *et al.*, 2004).

Segundo Monagas et al. (2005), foi verificado aumento da concentração de catequinas nos vinhos após 12 meses de engarrafado. No entanto, este aumento além do tempo de permanência na garrafa também ocorre em função do tipo de uva.

1.4 Objetivo

Diante do exposto, o presente trabalho foi realizado com o objetivo de avaliar os potenciais benefícios à saúde provenientes dos antioxidantes presentes no chocolate amargo. Para isto, um chocolate com alto teor de cacau foi desenvolvido e seus teores de polifenóis e flavonóides foram quantificados. Tendo em vista o fato de que o vinho tinto é atualmente reconhecido como benéfico à saúde, devido aos antioxidantes presentes em sua composição, esta bebida teve seus polifenóis e flavonóides mensurados sendo estes resultados utilizados como comparativo de quantidade destes compostos. A fim de se verificar o poder antioxidante *in vivo* foi realizado um experimento com animais.

A seguir, são apresentados três artigos científicos que relatam as etapas de quantificação e qualificação do teor antioxidante do chocolate amargo demonstrando os resultados e as conclusões obtidas no desenvolvimento deste projeto.

2 ARTIGOS CIENTÍFICOS

2.1 Evaluation of Polyphenols and Flavonoids Content in Samples of Cocoa Liquor to Develop a Local Dark Chocolate. Será submetido à Revista *Food Chemistry*.

2.2 Chocolate and Red Wine - A Comparative of Polyphenols and Flavonoids Content. Será submetido à Revista *Food Chemistry*.

2.3 Antioxidants from Dark Chocolate and Red Wine – An *In Vivo* Comparative. Será submetido à Revista *Nutrition Research*.

2.1 Evaluation of Polyphenols and Flavonoids Content in Samples of Cocoa Liquor to Develop a Local Dark Chocolate

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Abstract

Polyphenol and flavonoid contents were evaluated in samples of cocoa liquor from different suppliers in Brazil. After this, cocoa liquor was selected to develop a local dark chocolate and, further, to compare the phenolic content of this product with dark chocolates available in international market. Results showed a significant difference between cocoa liquor from different suppliers, Brazilian liquor had the lowest quantity of polyphenols and flavonoids when compared to African cocoa. The relationship between defatted cocoa content and the quantity of polyphenols and flavonoids in chocolate could be seen in the results of foreign dark chocolates, but an organic sample showed a different profile. The developed sample D71 was poor in polyphenols but showed a significant quantity of flavonoids and could be considered as a good source of these components.

Keywords: Cocoa; Dark Chocolate; Polyphenols; Flavonoids.

1. Introduction

Cocoa is recognized as a food rich in (-)-epicatechin and (+)-catechin, which represents around 60% of total polyphenols (Dreosti, 2005; Keen, 2001; Scalbert & Williamsom, 2000).

Concentration of polyphenols in cocoa is affected by a variety of biological and processing conditions of cocoa crops, which could affect its antioxidant capacity. The phenolic content in cocoa liquor depends on the origin of the crops, fermentation, drying, roasting and the manufacture of chocolate (Counet Ouwerx, Rosoux & Collin, 2004; Miller et al, 2006; Othman, Ismail, Ghani & Adenan, 2007).

For many years, cocoa was an important crop in Brazil's economy, which was the number one cocoa manufacturer around the world. Nowadays, Brazil is the seventh world producer of cocoa, as in regard to harvesting area. This decrease in production led chocolate factories to import cocoa, since 1992, from Indonesia and Ivory Coast, to make blends (Cuenca & Nazário, 2004). Miller et al. (2006), reported that these blends are interesting because they could reduce the variability between cocoa from different countries. However, each factory could choose a different blend and the final cocoa liquors could have significantly difference among them. As a consequence it is very important to evaluate polyphenols and flavonoids content on available cocoa liquors in Brazil, as well as to develop a local dark chocolate. This is even more important when recent studies are showing a relationship between cocoa and health and many consumers would like to buy this kind of product, but they can only find it on the imported sections of the markets for a very high price.

This work evaluated polyphenols and flavonoids content in cocoa liquor from three different cocoa suppliers in Brazil and in specially treated cocoa liquor from a Brazilian company; following that a brazilian dark chocolate was developed and its phenolic content compared to dark chocolates available at the international market.

2. Materials and Methods

2.1 Cocoa Liquor Samples

Cocoa Liquor from three different suppliers (A, B and C) were provided by Florestal Alimentos S/A - Chocolates Neugebauer (Porto Alegre, Brazil). These three are the main suppliers of the Company.

Supplier B offered three special cocoa liquors to be included in this research, labeled as B1, B2 and B3.

2.2 Chocolate Samples

Five dark chocolate samples, with different cocoa contents (99% (D99), 86% (D86), 72% (D72), 70% (D70) and 50% (D50)), were bought in supermarkets in Toulouse (France).

2.3 Development of Dark Chocolate

A sample of dark chocolate with 71% of cocoa (D71) was developed at R&D Department of Florestal Alimentos S/A especially for this project. This development followed the cocoa liquor evaluation from different suppliers and was carried out with the product which presented the highest quantity of polyphenols and flavonoids. Several pilot tests were run to develop the final formulation.

An industrial test was done following the same steps of the Company's chocolate production. This procedure follows the traditional process, consisting of mixing the ingredients (cocoa liquor, cocoa butter and sugar), refining and conching. Then the dark chocolate was tempered and molded.

2.4 Determination of Polyphenols Content

2.4.1 Chocolate and Cocoa Liquor Preparation

The technique described for Vinson, Hao, Su and Zubik (1998) with some modifications was used for preparation of the samples.

The analysis was done in triplicate. Each chocolate or cocoa liquor were weighed (100mg), grinded and defatted by multiple extractions with hexane. Samples were dried overnight (T= 35°C) and 50 mg were weighed, mixed with 5 ml of 1.2M HCl in 50% aqueous methanol and heated for 2h at 90°C with vortexing every 30 min.

The extracts were filtered with a 0.45 μm filter and immediately used to measure the total polyphenols.

2.4.2 Determination of Total Polyphenols

According to Vinson, Proch and Bose (2001), 100 μl of an aliquot of blank or extract of chocolate were reacted with 1000 μl of Folin Ciocalteau reagent diluted 1:9 with distilled water. After 20 minutes at

room temperature the developed color was measured at 750 nm using a Hitachi U1100 Spectrophotometer. A standard curve was developed using a 1000 μM solution of (-)-catechin (Sigma-Aldrich).

2.5 Determination of Flavonoids Content

The method published by Lee, W.; Kim; Lee, J. and Lee, Y. (2003) was adapted for flavonoids determination.

2.5.1 Preparation of the samples

Grinded chocolate or cocoa liquor (7.3g) were dissolved in 200 ml of distilled water at 100°C and mixed to melt the fat. The mixture was then centrifuged in a Hermle Z323K refrigerated centrifuge for 5 minutes at 4°C and 12000 rpm of rotation. The defatted supernatant was analyzed in triplicate.

2.5.2. Analysis

Diluted samples (1.0 ml) was mixed with 4.0 ml of distilled water in a 10.0 ml volumetric flask. At time zero, 0.3 ml of 5% $NaNO_2$ was added to each flask; at 5min, 0.3 ml of 10% $AlCl_3$ was added; at 6min, 2.0 ml of NaOH 1M was added and the solution was immediately dilluted with distilled water to reach the final volume of 10.0 ml. The solution was thoroughly mixed and the absorbance was measured against a blank at 510 nm using a Hitachi U1100 Spectrophotometer.

A standard curve was developed using a 1000 μM solution of (-)-catechin (Sigma-Aldrich).

2.6 Fat Content

Fat content was measured by Soxhlet extraction, following acid hydrolysis (AOAC, 1990).

2.7 Deffated Cocoa (DC) Content

The Deffated Cocoa (DC) Content was evaluated with the equation:

$$\%DC = \%\text{ of cocoa on the sample}^* - \%\text{ fat} \quad (1)$$

*declared by the manufacturer.

2.8 Statistical Analysis

Statistical significance between the samples was examined using one-way-ANOVA. When appropriate, multiple comparisons were made using Tukey-Kramer corrections. Results considered 5% of level of significance.

3. Results and Discussion

During cocoa processing and chocolate manufacturing there are some steps that might contribute with the lost of polyphenols and flavonoids. There are many suppliers of these raw materials around the world, but the processes used by them to obtain cocoa derivates usually have some particularities. The variety of cocoa, the country were it is cultivated, the characteristics of the soil, the temperatures of the fermentation and roasting are some of factors that has influence in the concentration of these compounds in the final product. Because of these influences, it is important to select a cocoa liquor supplier with the highest polyphenols and flavonoids content to produce a chocolate with considerable amount of these desirable compounds.

Table 1 shows a comparison between three different cocoa liquor suppliers. One lot of each product was received (n=1).

It is interesting to observe that although almost all suppliers uses Brazilian cocoa to produce cocoa liquor, there was a significant difference in polyphenols and flavonoids results between the samples. Three hypotheses could be considered to explain this: blends of Brazilian cocoa with cocoa from other countries, different cultivated area in Brazil and differences in manufacturing process of the companies.

In Brazil, Bahia has the biggest producing area (with 84% of the harvesting area of the country, in 2002) but there are other states, like Para, Espírito Santo, Rondônia, Mato Grosso, Minas Gerais and Amazonas that also cultivate cocoa beans (Cuenca & Nazário, 2004). These states are located in different regions of the country with characteristics of soil, temperature, relative humidity, rainy period as different as the bean would be cultivated in different countries. Therefore, the percentage of each kind of cocoa used to make the cocoa liquor is important to the final amount of antioxidants. Blends used for each supplier is described on Table 2.

Samples A, B and C are the traditional product of the factories with no special control to maintain polyphenols and flavonoids during the manufacture. Nowadays, based on reports that indicate cocoa as a good source of antioxidants, some companies are developing strict controlled parameters, from the farm to the industry, to produce special types of cocoa liquor with higher concentration of polyphenols than traditional cocoa liquor.

Supplier B has provided three cocoa liquor samples that were compared with traditional cocoa samples received. Results are shown on Table 1.

The difference between samples B1 and B2 was the roasting process. B1 uses continuous roasters and B2 is roasted in batches. It seems that the continuous process is less aggressive to flavonoids than the batch roaster. One hypothesis is that in batch roasters the beans are heated in a non uniform way what damages more the flavonoids than other polyphenols that are on the samples.

Sample B3 was analyzed to evaluate the influence of cocoa from different countries in blends. While the traditional blend (B) uses a mix of brazilian and indonesian cocoa, sample B3 is made only with cocoa from Africa. According to Counet et al. (2004), African cocoa beans, from Ivory Coast and Ghana, are recognized as well fermented thus having a lower quantity of polyphenols. It is important to observe that no statistical difference in the flavonoid content was found when sample B and B3 were compared; in relation to polyphenols, B has a significantly higher amount. Moreover, the results of A, B and C and the analysis of manufacturer's information make us suggest that if the percentage of brazilian cocoa is higher, the polyphenols and flavonoids content are lower. This is in disagreement with results obtained from Natsume et al. (2000) that evaluated cocoa liquor from Ecuador, Venezuela, Ghana, Colombia, Ivory Coast and Brazil and found that brazilian cocoa has the largest percentage of polyphenols with a higher concentration of epicatechin monomers than any other. However, when B3 was compared with C, which is made only with brazilian cocoa, the african beans showed a significantly higher quantity of polyphenols and flavonoids. This suggests that cocoa liquor in Brazil have different profiles that should be better studied.

As a result of these analysis, supplier A was selected to produce the dark chocolate with high amount of cocoa, because it showed the best performance among those going through a traditional process and similar results to the special ones.

A dark chocolate with 71% of A cocoa was developed and compared with similar products available in international market. Table 3 shows the relationship between the D71 and dark chocolates from international brands. Products with different amounts of cocoa in their composition were selected to evaluate the relationship between the amount of cocoa and the presence of antioxidants. After the analysis, it was confirmed that the increase of cocoa percentage in the formulation is related to the increase of polyphenols / flavonoids in the chocolate. However, the linear relationship published by Miller

et al. (2006), with a strong association between the quantity of polyphenols, procyanidins, ORAC (Oxygen Radical Absorbance Capacity) activity and the percentage of Non Fat Cocoa Solids (NFCS) in chocolate and cocoa was not observed.

To determinate the percentage of defatted cocoa (%DC) of D50 the information present in wrapping (that it has minimum of 50% of cocoa) was used, because this chocolate has other ingredients that could contribute to fat content (Table 4).

Statistical comparison of D71 and international chocolate with similar amount of cocoa showed that D71 is the poorest for polyphenols. This could be explained because the source of cocoa in D71 and D72 is cocoa liquor and cocoa butter, whilst D70 has only cocoa liquor in its formulation. The use of cocoa liquor contributes to increase the quantity of cocoa solids and consequently, the quantity of polyphenols, as Miller et al. (2006) reported. As cocoa butter is taken into account on total percentage of cocoa in D71 and D72, this results in a lower quantity of solids on this samples and consequently lower quantity of polyphenols than in D70 (Table 3). Other important information is that D70 is an organic product which suggests a special process to obtain cocoa from the farm to final product and could result in a bigger amount of antioxidants. The evaluation of deffated cocoa showed that D70, between the ones which has similar amounts of cocoa, has the biggest quantity of solids and could be compared with D86 (Table 3).

As regard to flavonoids, it can be observed that D71 doesn't show statistical difference when compared to D86, D72 and D50 and has a lower concentration than D70. D70 showed no flavonoids statistical difference when compared to D99, probably because both are manufactured only with cocoa liquor and sugar and do not have interference from other ingredients (as whey, lactose or anhydrous milkfat) or have their total cocoa content including cocoa butter (Table 4).

As catechin is a flavonoid, the technique to measure flavonoids should be more specific to determinate this compound. It is interesting to observe that although D71 was the poorest chocolate sample when polyphenols was concern, its flavonoids content seems to be similar to D86, D72 and D50. As discussed by Osman et al. (2004) this could be due to the presence of other phenols that are not accounted on flavonoids technique, but are detected on polyphenols method. Phenolic compounds like metilxanthine (theobromine and caffeine) and anthocyanins in cocoa beans might influence the antioxidant capacity (Othman et al, 2007) and could be measured by polyphenols technique.

Based on results showed on Table 3, it can be said that although D71 does not have the highest quantity of flavonoids, it is the sample with the lowest difference between the results of polyphenols and flavonoids. This suggests that this sample has the highest percentage of flavonoids in relationship to the overall level of antioxidants. This difference could be explained because dark chocolate developed in Brazil (with brazilian cocoa liquor) is compared to dark chocolate made in France and Belgium, which probably use cocoa liquor from other producers. As it can be seen in Table 1 the selected cocoa (Supplier A) has a significant difference in the polyphenols and flavonoids content when compared with the african sample (B3), which is probably the cocoa supplier for France and Belgium producers, as the Ivory Coast is the biggest producer around the world, as reported for Cuenca and Nazário (2004).

It is interesting to evaluate the relationship between the quantity of flavonoids in selected cocoa liquor sample (A) and in D71. Based on the percentage of this raw material on the developed chocolate it can be observed that almost 50% of flavonoids were lost during the industrial manufacture (mixture, refining and conching). This is in accordance with results obtained from Engler et al. (2004) that estimated that the levels of preserved flavonoids on chocolate could be between 50 and 75% when traditional method is used. If the loss of polyphenols is evaluated this value increases to more than 70%. This suggests that a better control should be done for the production of special chocolates with high amount of antioxidants to guarantee its quality.

4. Conclusion

Brazilian cocoa liquor showed a lower quantity of polyphenols than cocoa liquor made with African beans which is in agreement with the increase of polyphenols and flavonoids in samples that have a bigger amount of foreign cocoa.

As there is no concern to produce chocolate with high amount of antioxidants in Brazil, there is a large difference among cocoa producers around the country, with only a few companies trying to develop a process to preserve antioxidants in cocoa. The analysis of special samples showed that roasting process affects the maintenance of antioxidants.

A relationship between defatted cocoa content and the quantity of polyphenols and flavonoids in the chocolate were detected. However the sample D70, which is made with organic cocoa, showed a better performance than other samples that has around 70% of cocoa. This suggests that the harvesting of cocoa is also an important factor.

As regard to flavonoids, the developed chocolate D71 showed a significant quantity and could be considered as a good source of this component.

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Table 1

Comparative between Cocoa Liquor from different Suppliers and Processes

	Polyphenols*	Flavonoids*	% fat	%DC	Observation
Traditional Cocoa Liquor					
A	318.2 ± 8.9^{a}	56.3 ± 1.4^{b}	50.7	49.3	Brazilian + Imported Beans
B	250.5 ± 8.5^{b}	37.6 ± 0.5^{d}	52.6	47.4	Brazilian + Imported Beans
C	183.6 ± 9.2^{d}	26.9 ± 0.0^{e}	52.1	47.9	Brazilian Beans
Special Types of Liquor					
B1	238.1 ± 8.7^{bc}	62.9 ± 1.9^{a}	52.6	47.4	Continuos Roaster
B2	339.1 ± 12.0^{a}	47.9 ± 2.7^{c}	52.8	47.2	Batch Roaster
B3	220.8 ± 8.9^{c}	33.7 ± 0.6^{d}	53.5	46.5	African Beans

* μmol of catechin equivalents per gram

Values in same column followed by different letters were significantly different ($p<0.05$).

Table 2

Blends of cocoa beans from different suppliers

Supplier	Blend	Brazilian Cocoa	Foreign Cocoa
A	60 % brazilian cocoa 40% imported cocoa	90% from Bahia 10% divided between Rondônia, Acre and Espírito Santo.	Mainly from Indonesia, but also from Malaysia and Ghana.
B	80% brazilian cocoa 20% indonesian cocoa*	Bahia and Pará - Quantity of brazilian cocoa depends on the period of harvesting.	*Maximum percentage is around 30%.
C	100% brazilian cocoa	Bahia, Pará, Espírito Santo and Rondônia. Main blend: 60% Bahia and 40% Pará, but also mixture Bahia with Rondônia and Espírito Santo with Pará.	-

Table 3

Comparative between developed dark chocolate and similar samples available on the Market

	Polyphenols*	Flavonoids*	%fat	%DC
Dark Chocolate 99% (D99)	142.0±1.6[a]	32.2±0.4[a]	44.9	54.1
Dark Chocolate 86% (D86)	135.8±2.9[a]	25.0±0.2[b]	53.4	32.6
Dark Chocolate 72% (D72)	85.5±1.8[c]	22.5±0.7[bc]	39.8	32.2
Dark Chocolate 71% (D71)**	62.9±0.1[e]	21.6±2.4[bc]	42.1	28.9
Dark Chocolate 70% (D70)	120.0±3.1[b]	33.2±1.0[a]	37.0	33.0
Dark Chocolate 50% (D50)	76. 1±6.2[d]	19.0±0.9[c]	26.0	25.0

* μmol of catechin equivalents per gram

**developed sample

Values in same column followed by different letters were significantly different ($p<0.05$).

Table 4

Ingredients and Place of Origin of analyzed dark chocolate samples.

Sample	Ingredients	Made in
D 99	Cocoa and Sugar	France
D 86	Cocoa Liquor, Cocoa Butter, Sugar and Deffated Cocoa.	Belgium
D 72	Cocoa Liquor, Sugar, Cocoa Butter, Emulsifier and Vanilin.	Belgium
D 71	Cocoa Liquor, Sugar, Cocoa Butter, Emulsifier and Vanilin.	Brazil
D 70	Cocoa Liquor, Sugar, Emulsifier and Vanilin.	France
D 50	Cocoa Liquor, Sugar, Lactose, Anydrous Milkfat, Emulsifiers.	France

2.2 Chocolate and Red Wine - A Comparative of Polyphenols and Flavonoids Content

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Abstract

Polyphenols and flavonoids content of four different types of chocolate (White, Milk, Dark with 40% of cocoa and Dark with 71% of cocoa) and four varieties of red wine (Pinot-Noir, Cabernet Sauvignon, Merlot and Tannat) were evaluated. Samples of dark chocolate with 71% of cocoa (D71) and Tannat red wine showed the biggest quantities. Flavonoids method demonstrated interference of vanillin flavor when color development was measured. In polyphenols determination, the use of chocolate samples without lyophilization is proposed, because no statistical difference was found between the results of samples with and without water. According to obtained results, 49g of chocolate sample D71 has the same quantity of flavonoids that 196 ml of Tannat red wine, which is the daily wine dose recommended to bring health benefits to an adult of 70 kg of body weight.

Keywords: Dark Chocolate; Red Wine; Polyphenols; Flavonoids.

1. Introduction

Flavan-3-ols or flavanols, such as catechin, epicatechin and procyanidins, contributes to the major antioxidant activity of red wines in the prevention of LDL oxidation (Teissedre, Frankel, Waterhouse, Peleg and German, 1996). As reported for Auger et al. (2004) around 65% of catechins found on grapes are in the seeds so the wine-making technique seems to be an important step to flavonoids content of the wine. Other factors as variety of the grapes, cultivation area, period of sun exposure and age of the wine have to be also observed (Burns et al., 2000; Dell'Agli et al., 2004; Frankel, Waterhouse & Teissedre, 1995).

As red wine, cocoa has been published as a good source of flavonoids. In this context, dark chocolate with high amounts of cocoa are been recognized as an important antioxidant alternative to the diet (Serafini, Bugianesi, Maiani, Valtuena, De Santis and Croizer, 2003).

Recently, Counet, Callemien and Collin (2006) identified the presence of *trans*-resveratrol in samples of cocoa and chocolate; this stilben, present in red wines, is postulated as one of the compounds responsible for the French Paradox. However, the procyanidins content seems to be more significant and could be related to the antioxidant power of chocolate.

Even though cocoa and red wine are important sources of antioxidants; in Brazil, they do not represent the main sources of these compounds. The consume per capita of chocolate, in 2004, was 2.07kg, in Brazil, while in countries like German and Switzerland it was 11.1 and 10.8 kg/ per capita, respectively (Abicab, 2006). As regard to wine, per capita Brazil ingestion was 1.76 liters, in 2004, considering red and white varieties. In Brazil, the main wine manufacturer is the state of Rio Grande do Sul (RS), were this research was carried out, with 55% of grape production and about 95% of wine production of the country (Hoffmann, Camargo & Maia, 2006). As it can be observed there is a great market to functional foods to be explored in Brazil.

Therefore, based on the fact that cocoa and red wine have very similar groups of antioxidants (specially catechins) and red wine is worldwide recognized as a healthy beverage, red wine was chosen as a comparative of polyphenols and flavonoids content to a special dark chocolate developed to this project.

2. Materials and Methods

2.1 Chocolate Samples

Three types of chocolate were gently provided by Florestal Alimentos S/A - Chocolates Neugebauer (Porto Alegre, Brazil), which were white chocolate (WC), milk chocolate (MC) and dark chocolate with 40% of cocoa (D40). From each chocolate three different lots were collected (n=3). The samples were kept on a cool and dry place at temperatures between 18°C and 22°C to guarantee the quality.

A dark chocolate with 71% of cocoa (D71) was developed at R&D Department of Florestal Alimentos S/A - Chocolates Neugebauer exclusively for this project (Pimentel et al., 2007).

2.2 Red Wine Samples

Four samples of red wine were provided by a local wine-manufacturer (Bento Gonçalves, RS, Brazil). The varieties were Pinot-Noir, Cabernet Sauvignon, Merlot and Tannat. From each one three different lots produced in 2004 were collected (n=3). All the varieties have the same year of production, because according to Monagas, Góméz-Cordovés and Bartolomé (2006) during the ageing, the wine in the bottle has an increase on the degree of polymerization of the polyphenols.

2.3 Determination of Polyphenols Content

2.3.1 Chocolate Preparation

The technique described for Vinson, Hao, Su and Zubik (1998) with some modifications, was used for preparation of samples of chocolate (WC, MC, D40 and D71).

One lot of each type was freeze-dried and freezer stored. Other two samples of each type were analyzed without this treatment.

Each triplicate sample (100 mg) was grinded and defatted by multiple extractions with hexane, and dried, 35°C, overnight; therefore 50 mg were weighed and heated with 5 ml of 1.2M HCl in 50% aqueous methanol for 2h at 90°C with vortexing every 30 min.

The extracts were filtered with a 0.45 μm filter and immediately used to measure the total polyphenols.

2.3.2 Red Wine Preparation

Samples were prepared according to Vinson and Hontz (1995). 100 ml of each sample were dealcoholized in a rotatory evaporator with vacuum at 50ºC and diluted to the original volume with distilled water.

Previously to the quantification, samples of wine should have the elimination of sulfites. This component, present in the wine, reacts with the Folin reagent to give a color. To avoid this interference, the technique described for Vinson, Proch and Bose (2001a) was used. Briefly 1 ml of each dealcoholized sample was mixed with 1ml of acetate buffer to adjust pH to 3.0. After, 20 μl of acetaldehyde were added and the treated samples reacted for 30 minutes at room temperature. The treatment was done in triplicate.

2.3.3 Determination of Total Polyphenols

Procedure according Vinson et al. (2001a), 100 μl of an aliquot of blank or the extract of chocolate or red wine were reacted with 1000 μl of Folin Ciocalteau reagent diluted 1:9 with distilled water. After 20 minutes at room temperature the developed color was measured at 750 nm using a Hitachi U1100 Spectrophotometer. A standard curve was developed using a 1000 μM solution of (-)-catechin (Sigma-Aldrich).

2.4 *Determination of Flavonoids Content*

Flavonoids were evaluated according Lee, K; Kim, Y; Lee, H and Lee, C. (2003) with slight modifications.

For chocolate, 7.3g of grinded samples were dissolved in 200 ml of distilled water at 100°C and mixed until the melting of the fat. The mixture was then centrifuged in a Hermle Z323K refrigerated centrifuge for 5 minutes at 4°C and 12000 rpm of rotation. The defatted supernatant was analyzed in triplicate.

For red wine, samples (1 ml) were diluted in 4ml of distilled water in a 10 ml volumetric flask. At time zero, 0.3 ml of 5% $NaNO_2$ was added to each flask; at 5min, 0.3 ml of 10% $AlCl_3$ was added; at 6min, 2.0 ml of NaOH 1M were added and the solution was immediately dilluted with distilled water to reach the final volume of 10 ml. The solution was well mixed and the absorbance was measured against a blank at 510 nm using a Hitachi U1100 Spectrophotometer.

A standard curve was developed using a 1000 μM solution of (-)-catechin (Sigma-Aldrich).

2.5 Water Content

Samples of chocolate were grinded to a powder and dried until constant weight in platinum dish in air oven at 100°C. The water content was calculated by the loss of weight (AOAC, 1990b).

2.6 Fat Content

Fat content was measured by Soxhlet extraction, following acid hydrolysis (AOAC, 1990a).

2.7 Determination of Vanillin Interference

A solution of 0.1g/l vanillin diluted in ethanol/water was used to test the interference of this ingredient on the Folin Ciocalteau and flavonoids techniques. A standard curve was done with intervals of 0.02g/l.

2.8 Statistical Analysis

Statistical significance between the samples was examined using one-way-ANOVA. When appropriate, multiple comparisons were made using Tukey-Kramer corrections. Results considered 5% of level of significance.

3. Results and Discussion

3.1 Chocolate

Polyphenols and flavonoids of different chocolates are on Table 1.

The highest and significant quantity of polyphenols of D71 (dark chocolate with 71% of cocoa) could be attributed to the percentage of cocoa liquor in formulation. Although there is 30% of cocoa in WC (white chocolate) all of it derives from cocoa butter that does not have enough quantity of these compounds, because, as described by Miller et al. (2006), polyphenols are hydrophilic and tend to be found in the non fat fraction of cocoa and chocolate. This could be an explanation for the significant difference between MC and D40, although they have almost the same quantity of cocoa. According to Miller et al. (2006) there is a strong relation between the amount of non fat cocoa solids (NFCS) and the polyphenols, however polyphenols and flavonoids were found in WC that does not have cocoa solids on its formulation. The WC result (Table 1) is different from obtained by Mursu et al. (2004) that quantified antioxidants in white chocolate using HPLC method and found 0.3mg of gallocatechins per 100g of product, but did not found significant amounts of catechin and procyanidins in the samples. One explanation of the quantity of polyphenols/flavonoids found could be the interference of some ingredient in the quantification method.

The ingredients of samples of WC are sugar, cocoa butter, whole milk powder, skimmed milk powder, emulsifier soybean lecithin and vanillin flavor. Vanillin chemical structure has a phenol ring that could contribute to develop color with reagents used to quantify some compounds like catechins (Sun, Ricardo-Da-Silva & Spranger, 1998) and flavanols (Sarkar & Howarth, 1976). A maximum concentration of 0.1% was chosen because it is the quantity that is generally used on chocolate formulation. The result showed no interference in the Folin Ciocalteau technique but a strong linear relation in Flavonoids method (r^2=0.9597).

However, this interference does not justify all the developed color during the analysis. WC samples had 0.05% of vanillin and this could contribute with 0.01nm which is much lower than the resultant absorbance. It is important to observe that flavonoids content are in agreement with results found by Lee et al. (2003) that showed antioxidant capacity when there is no epicatechin detected.

Other ingredient that could contribute to the development of color is sugar. According to Vinson et al. (2001a), sugars do not interfere with acidic Folin reaction at concentrations found in high sugar foods, like

chocolate; but they interfere in alkaline Folin method if it is in high concentrations. So, sugar could have an influence in the final results obtained.

Another consideration is that although Miller et al. (2006) described flavonoids as hydrophilic, Auger et al. (2004) published that flavonoids are the most lipophilic of the natural antioxidants. Based on this, the WC results could be derived from antioxidants of cocoa butter. More studies should be done to better understand this effect.

Although the results suggests that there is some kind of interference and super estimation of the results, for MC and D71 the amount of polyphenols was lower as regarding to Vinson, Proch and Zubik (1999) which found an average of 52.2 μmol catechin/g for MC and 126.0 μmol catechin/g for dark chocolate with a high content of cocoa.

The impact of water content in determination of polyphenols was evaluated and no statistical difference was found between lots with or without lyophilization so it can be suggested that for foods with very low water content the method of determination of total polyphenols could be adapted with no need of lyophilyzation during sample preparation.

3.2 Red Wine

Table 3 shows the quantification of antioxidants in red wine. A statistical difference in flavonoids content can be seen between variety Tannat and others.

Gonzáles-Neves et al. (2004) studied the phenolic potential of Tannat, Cabernet Sauvignon (CS) and Merlot grapes and their correspondent wine, in Uruguay. This research showed that Tannat variety had the biggest amount of polyphenols in grapes and of polyphenols, catechins and procyanidins in red wines. It shows statistical difference from CS and Merlot. These last varieties did not show statistical difference between them although CS had bigger amounts of antioxidants. As pointed out by Meyer, Yi, Pearson, Waterhouse and Frankel (1997) regional and climatic differences influence the amount of antioxidants in wine. The wines evaluated were from the South of Brazil which is geographically near to Uruguay thus presenting some similarities of soil and climate that confirm these convergent results.

Based on the obtained results Tannat variety, harvest 2004, was chosen as the best red wine to be used as a comparative of polyphenols/flavonoids to D71.

3.3 Dark Chocolate and Red Wine

According to Vinson, Teufel and Wu (2001b), the consumption of 196 ml of red wine with 13° GL per day is enough to reduce significantly the risk of atherosclerosis, in an adult with 70kg of body weight. As Tannat was the best red wine evaluated, in relation to antioxidant compounds, it was chosen as a reference to approximate how many grams of chocolate would have to be eaten to ingest the same amount of flavonoids of one portion of this wine. For this comparison, flavonoids results was chosen because they represents a better approximation of the quantity of catechins in the sample; flavonoids are a polyphenols subgroup and on determination of total polyphenols other compounds like phenolic acids, lignans and stilbenes could be measured.

Table 4 shows the quantity of chocolate that and adult of 70kg should eat to ingest the same quantity of flavonoids that is present in one glass of Tannat red wine.

3.4 Chocolate and Nutrition Facts

As it can be seen in Table 4, to ingest the same amount of flavonoids of 49g of D71 an adult of 70kg should eat 126g of MC, which represents 250% more chocolate. In Brazil, the recommended ingest of calories per day to an adult of 70 kg of body weight is 2000 kcal (Brasil, 2006). This quantity of MC represents 35% of the needed calories of the day. Another interesting observation is that there is a small difference, in grams, between what should be ingested from D71 and D40 and it represents 14% and 16% of the necessary calories of a day, respectively. This suggests that both samples could be used as a good source of dietary antioxidants.

In regard to fat content, the recommendation of Brazilian agency is a maximum ingestion of 55g of total fat per day (Brasil, 2006). The Food and Drug Administration, recommends 65g of total fat considering an ingestion of 2000 calories per day (FDA, 2007). Using the results obtained from fat content analysis (Table 4) and the Brazilian legislation, D40 is the chocolate which showed the lowest contribution to dietary fat with 30% of daily value. D71 contributes with 38% of daily fat and MC with 74%. This brings another important discussion about the contribution of chocolate in weight increase. Although chocolate has a significant contribution to total fat ingested per day, many studies showed that the ingestion of dark chocolate contribute to reduce the LDL cholesterol. Mursu et al. (2004) showed that ingestion of dark chocolate with high levels of polyphenols could contribute to increase the HDL cholesterol and, after 3 weeks eating 75 grams of chocolate per day, the studied volunteers have no weight increase. This could

be due to cocoa butter absorption. Kubow (1996) reported that the total fat absorption is influenced for the total stearic acid content in the fat and the amount of palmitic acid in the 1 and 3 positions. Even though cocoa butter contains more than 50% of saturated fatty acids, it possesses a high unsaturated character on 2-position (mainly oleic – 87%). In cocoa butter the stearic acid is in 1 and 3 position and this distribution has been suggested as the possible mechanism for neutral effect of cocoa butter in LDL cholesterol, different to highly saturated fats that have saturated fatty acids on 2-position of glycerol.

As the formulations of these chocolates are known, the contribution of cocoa on the total calories of the product can be calculated. The ingredients that most contribute to total calories are cocoa and sugar. While in MC and D40 47% and 43% of the calories, respectively, derived from cocoa butter in D71, 58% came from this ingredient. As described above, the lipid absorption of cocoa is lower than from other fats, and a higher amount of cocoa butter could contribute to a lower absorption. Moreover, higher quantities of cocoa in the formulation represent lower amounts of sugar, which is easily absorbed. Therefore this high percent of cocoa butter in dark chocolate could collaborate to a small contribution of this product in weight gain.

4. Conclusion

Polyphenols and Flavonoids demonstrate a statistical difference when evaluated from different types of chocolate. D71, as expected, had the highest polyphenols and flavonoids contents, but results showed quantities of these antioxidants in WC, what suggests interference of some chocolate ingredient in the method. Among red wines samples, Tannat variety had the highest values for flavonoids.

A comparison between chocolate and red wine showed that 49g of D71 should be eaten to ingest an equivalent amount of flavonoids there is in 196 ml of Tannat red wine. Results of D40 were similar to D71, making it also an important alternative for antioxidants ingestion.

More studies should be done to evaluate the functionality of these compounds *in vivo* and observe if the same amount of antioxidants, from different sources, are able to give similar health benefits to humans.

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Table 1

Comparative between different kinds of Chocolate

Samples	Polyphenols*	Flavonoids*
Dark Chocolate 71% (D71)	62.9±0.1[a]	21.6±2.4[a]
Dark Chocolate 40% (D40)	49.9±1.8[b]	17.2±1.3[b]
Milk Chocolate 34% (MC)	39.2±3.8[c]	8.4±0.7[c]
White Chocolate 30% (WC)	28.3±2.6[d]	3.4±1.3[d]

* μmol of catechin equivalents per gram

Values in same column followed by different letters were significantly different ($p<0.05$).

Table 2

Water and Polyphenols Content of Chocolate Samples

	Lyophilysate		Non Lyophilysate		
	Water (%)	Polyphenols*(lot 1)	Water (%)	Polyphenols*(lot 2)	Polyphenols*(lot 3)
D71	0.037	63.0 [a]	0.061	62.8 [a]	62.9 [a]
D40	0.024	48.0 [b]	0.037	50.1 [b]	51.5 [b]
MC	0.017	42.3 [c]	0.031	40.3 [c]	35.0 [c]
WC	0.013	31.2 [d]	0.059	26.4 [d]	27.3 [d]

* μmol of catechin equivalents per gram

Values in same column followed by different letters were significantly different ($p<0.05$).

Table 3

Comparative between different varieties of Red Wine

Samples	Polyphenols*	Flavonoids*
Tannat	14.3 ± 1.1^{a}	5.4 ± 0.1^{a}
Cabernet Sauvignon	13.3 ± 0.7^{a}	4.8 ± 0.1^{b}
Merlot	11.6 ± 1.1^{a}	4.6 ± 0.0^{c}
Pinot Noir	8.4 ± 1.2^{b}	4.0 ± 0.1^{c}

* μmol catechin per ml

Values in same column followed by different letters were significantly different ($p<0.05$).

Table 4

Quantity of chocolate (g) that should be ingested to have the same quantity of flavonoids than in 196 ml of Tannat Red Wine and their content of fat

Sample	g	% fat	g of Fat
Dark Chocolate 71% (D71)	49	42.1	20.6
Dark Chocolate 40% (D40)	61	27.4	16.7
Milk Chocolate (MC)	126	32.2	40.6

2.3 Antioxidants from dark chocolate and red wine – an *in vivo* comparative

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List of Abreviations

ROS: Reactive Oxigen Species

Ox-LDL: Oxidized Low-Density Lipoprotein

CVD: Cardiovascular Disease

LDL: Low Density Lipoprotein

HDL: High Density Lipoprotein

Abstract

The objective was to compare the benefits to health of red wine and dark chocolate by an *in vivo* experiment. Six groups of male Wistar rats (n=36) were divided and received per day doses of chocolate or red wine and both chocolate plus red wine. These doses were a supplement of the standard feed. After 45 days, blood and liver were collected. Total cholesterol, HDL, LDL, triglycerides and ox-LDL were measured in blood. Fat, total cholesterol and weight were evaluated in livers. Results of blood ox-LDL showed statistical difference between chocolate groups (D71 and D40) and the others, suggesting that cocoa could be a better source of antioxidants than red wine. Red wine groups showed the highest ox-LDL when compared to chocolate groups. This result could be related to alcohol ingestion on the organism of the rats. Altogether effect, from dark chocolate plus red wine ingestion, was not observed. Rats that ingested chocolate plus red wine showed the highest levels of ox-LDL, suggesting a competitive action between these constituents. Liver analysis showed a statistical difference in total cholesterol, which was in agreement with results obtained in blood.

Keywords: Antioxidants, Dark Chocolate, Oxidized LDL, Red Wine, Wistar rats.

1. Introduction

One of the most dangerous results of ROS to the body is the atherosclerosis. This is an inflammatory disease that contributes to the obstruction of coronary, cerebral and peripheral arteries. The production of ox-LDL, the deposition of platelets and the formation of thrombus will be responsible for the obstruction of the vessels [1,2]. According to Lapointe et al. [3], high circulating ox-LDL concentrations have been related to an increase of CVD. So ox-LDL is being recognized as a good marker to evaluate the risk of CVD.

In this context, diets rich in plants have been studied due to their postulated role in the maintenance of health, mainly, because their antioxidant power. In fact, food for health is becoming one the main trends in the food industry nowadays. The ingestion of beneficial compounds available in foods becomes even more important because humans do not produce enough endogenous antioxidants to act against ROS [4].

One of these compounds is flavonoids, more specifically the catechins, which are said to prevent many degenerative diseases related to free radical tissue damage [5].

Red wine was the first polyphenol-rich beverage that was recognized as a protector against ischemic heart disease and nowadays has become a reference to the functional food market. Similar potential is being demonstrated by cocoa, because of its quantity of flavonoids, mainly catechins and epicatechins [6].

As regard to cocoa, many studies have been demonstrating the beneficial effect of this food in humans. Engler et al. [2] observed an increase of epicatechin on plasma after 2 weeks ingestion of high flavonoids dark chocolate. However the ox-LDL and total antioxidant capacity showed no significant difference. Wang et al. [7] tested polyphenol-rich chocolate in 20 volunteers and detected a rapid absorption of epicatechin. They also observed an increase in plasma antioxidant capacity and reduction in plasma lipid peroxidation. All these results demonstrate the potential benefits of cocoa to health and justify more studies about the mechanisms of action of these compounds.

It is important to understand that not only one specific compound, but combinations of dietary antioxidants have beneficial effects on prevention of LDL oxidization [3]. Based on these synergic effects, this study has the purpose of evaluate the action of dark chocolate and red wine antioxidants in the organism of Wistar rats.

2. Materials and Methods

2.1 Materials

A dark chocolate sample with 40% of cocoa (D40) was gently provided by Florestal Alimentos S/A - Chocolates Neugebauer (Porto Alegre, Brazil). A dark chocolate with 71% of cocoa (D71) was developed at R&D Department of Florestal Alimentos S/A exclusively for this project [8]. Samples were kept on a cool and dry place at temperatures between 18°C and 22°C to guarantee its quality.

A Tannat red wine sample was selected as the variety with the highest amounts of polyphenols and flavonoids among four varieties of red wine (Cabernet Sauvignon, Pinot Noir, Tannat and Merlot) by Pimentel et al. [8] and was provided by a local wine-manufacturer (Bento Gonçalves, Brazil).

Determination of total cholesterol in blood and liver and LDL, HDL and triglycerides in blood, was done with specific kits from Laborclin Produtos para Laboratório Ltda.

2.2 Experimental Study

After a habituation period of 3 days, adult male Wistar rats (n=36) that was born 40 days before the experiment, weighing about 115g purchased from Biotery Laboratory of Federal University of Rio Grande do Sul were randomly divided in 6 groups, put into individual cages, with water *ad libitum*. Adult rats were chosen to reduce undesirable effects of alcohol in the growth of the animal. Room temperature was 22 ± 2ºC and a light/darkness cycle of 12 hours was provided. The period of study was 45 days.

Every day, animals were submitted to a feeding cycle of 12 hours (from 7 am to 7 pm) to induce stress, as described by Baker, Lindsey and Weisbroth [9]. The purpose of this procedure was to produce a bigger quantity of ROS that would probably be impaired by antioxidants of the diet. At 7 am, rats received food in pellets *ad libitum* and at 7 pm 10g of pellets grinded to a powder mixed with chocolate and/or red wine were offered (Table 1). This cycle was defined on a pilot test that was previously done. Food enriched with polyphenols was offered at the night shift. The quantity of 10g was not enough for the whole night, thus rats spent part of the night without food, which stimulated a stress situation in the animals and guaranteed that they would have ingested all the antioxidants needed.

Food in pellets was a standard one, developed specially for rats to provide all needed nutrients to growth (Nuvilab). Centesimal composition was 50.5% of carbohydrates, 22.3% of protein, 10.1% of water, 8.0% of minerals, 4.8% of dietary fiber and 4.3% of fat.

The experiment performed in this study was approved by the Ethical Committee of the Institution.

Every two days rats were weighed at night and at morning to observe weight looses caused by the low quantity of food offered. Based on the growth of the animals, the amount of chocolate and red wine was adjusted to maintain the proportion between the animal's weight and the quantity of supplied antioxidants.

According to Vinson, Teufel and Wu [10], 0.36 ml/kg per day of ethanol from red wine is enough to significantly reduce atherosclerosis. This is equivalent to an ingestion of 196 ml of red wine with 13°GL by a person of 70 kg. In this experiment, this information was used to determinate the amount of flavonoids the rats received daily. So, animals with 115g of body weight received 0.32 ml of Tannat red wine. The amount of flavonoids and polyphenols in samples of chocolate and red wines was evaluated previously [8]. Based on these results and on the proportion of red wine, described above, the chocolate dose was calculated. Groups 5 and 6 received the same amount of antioxidants, equally divided between the two sources.

On the last day of the experiment the polyphenols enriched food was offered at night (9 pm), and after eating all the food rats spent 12 hours fasting. Blood was collected after this time by decapitation according to Rodrigues et al. [11]. Livers were immediately removed, weighed and freeze until the analysis. Previously to the analysis, livers were left overnight on a refrigerator to de-freeze.

2.3 Statistical Analysis

All the results are expressed in average with the standard deviation (SD) to each experimental group and analyzed using two-way analysis of variance (ANOVA). Significance was indicated when $P \leq 0.05$. The differences between averages were determinate using the Tukey Test with 5% of significance.

2.4 Blood Analysis

Blood was put into cooled glass tubes and then taken to an external laboratory of analysis to evaluate Total Cholesterol, LDL, HDL, triglycerides and ox-LDL. To determinate Total Cholesterol, HDL and triglycerides specific kits (Laborclin Produtos para Laboratório Ltda.) were used. LDL was calculated by the equation of Friedewald; Levy; Fredrickson [12]:

$$C_{LDL} = C_{TOTAL} - C_{HDL} - TG/5 \quad (1)$$

The determination of ox-LDL used malondialdeyde (MDA) as a marker of lipid peroxidation.

2.5 Liver Analysis

2.5.1 Determination of Cholesterol

Livers were triturated with a Mini Processor HC31 (Black & Decker) and extracted with isopropanol previous to analysis [13]. The obtained extract was used to quantify Total Cholesterol, HDL and triglycerides with specific kits provided by Laborclin Produtos para Laboratório Ltda. The LDL cholesterol was calculated as described on 2.4 [12].

2.5.2 Fat Content

To evaluate fat in liver, the organs were triturated and analyzed by Bligh & Dyer method [14]. Briefly, 3g of samples were treated with 10 ml of chloroform, 20 ml of methanol and 8 ml of distilled water and mixed for 30 minutes. After 10 ml of chloroform and 10 ml of sodium sulfate 1.5% were added and mixed vigorously for 2 minutes. The chloroform extract, in the bottom of the tube, was filtered and 5 ml were poured inside capsules that stayed for 3h in a stove at a temperature of 100°C, until it dried. The fat content is the difference of weight between the capsule with the extract and the capsule after the drying process.

3. Results

After 45 days, no statistical difference was found concerning ingested food and gain of body weight (Table 2). This indicates that all animals received adequate nutrition.

Blood analysis showed statistical differences on ox-LDL. Although control group showed no statistical difference to the others, groups that received red wine presented higher values than those that ingested only chocolate (Table 3).

Liver analysis showed statistical difference in the total cholesterol result (Table 4). It is interesting to observe that the groups that ingested red wine, which had the highest ox-LDL values in blood (Table 3), are the ones which have the lowest values of total cholesterol in liver (Table 4).

Liver's weight in relation to body weight of rats reached a value of 3.2%, which according to Baker, Lindsey and Weisbroth [9] is the expected value to Wistar rats. This indicates that the organs were healthy and the diet did not cause any damage to them.

4. Discussion

A lot of epidemiologic studies showed a strong relationship between CVD and beverages, fruits and vegetables. Red wine is recognized as a good source of antioxidants as well as cocoa that recently received attention due to its antioxidant properties. This study compared the health benefits of red wine and dark chocolate since both are a catechin antioxidant source.

Results showed no statistical difference in gain of weight and ingested food among six studied groups. This is an interesting result since that chocolate represented 0.5% of all ingested food in groups D71 and D40 and suggests that a moderate ingestion of chocolate, which is a food with a high fat content, does not contribute with increase of weight.

In respect to blood analysis, no statistical differences were found on total cholesterol, HDL and LDL values. On triglycerides analysis group 6 (Tannat & D40) demonstrated a significantly lower value. When the ox- LDL was evaluated a significant difference was found between groups that ingested chocolate and red wine, although these groups did not show differences from control group. Those that ingested red wine (groups 4, 5 and 6) demonstrated the highest values of ox-LDL what was not expected. However, according to Roig et al. [15] a moderate consumption of red wine protects Wistar rats against oxidation *in vivo*. But this protection could be observed only after prolonged (six months) consumption. After 45 days, same period of this study, the level of malondialdeyde (MDA), that was used as an index of lipid peroxidation, did not show statistical difference in plasma when red wine and ethanol were compared with the control group. Other important discussion is that in Roig's experiment the animals weighed about 125g and ingested a dose of 2.0 ± 0.2ml/day of red wine and rats, in the present experiment, received and average of 0.3ml/day (Tannat group). Donovan et al. [16] administered different concentrations of catechin to rats and observed that the absorption of catechin is proportional to the offered concentration. It is important to observe that Donovan's experiment used a high dose of pure catechin solution as a source of antioxidants. In the present study, the sources of antioxidants were chocolate and red wine that contribute with a moderate dose of antioxidants. This suggests that rats in this experiment would have to receive studied components for a longer period to demonstrate a significant decrease in lipid peroxidation.

One of the hypotheses to the increase of lipid peroxidation in red wine groups is related to the ingestion of alcohol. A 6 weeks study, with male Wistar rats, was done by Kasdallah-Grissa et al. [17] to evaluate the protective effect of resveratrol on ethanol-induced lipid peroxidation. The group that received

only ethanol in a toxic dose (3g/kg body weight) showed a significant increase on lipid peroxidation in liver. When resveratrol was administered the lipid peroxidation was reduced to the same level of control group. This suggests resveratrol as a good candidate in prevention of alcohol-induced injuries in several organs and helps to explain why groups Tannat, Tannat & D71 and Tannat & D40, that ingested red wine, had higher ox-LDL. Although the values of ox-LDL in blood analysis of these groups were higher than the control group they did not present a significant difference to them. As in this experiment samples of red wine were used, instead of a solution of these antioxidants like Kasdallah-Grissa et al. [17] did, maybe the catechins and resveratrol in the samples were not enough to prevent the alcohol damage.

The comparison between the results obtained with administration of chocolate and red wine showed statistical difference. As described above, the benefit of red wine could be observed generally after a prolonged period. In this experiment the antioxidants from cocoa seemed to be more efficient than those from red wine. In a study about cocoa, red wine, green and black tea potentials, Lee et al. [18] found that cocoa had a bigger amount of phenolic compounds and a higher antioxidant capacity than red wine and teas.

The synergistic effect between Tannat red wine and D71 was also evaluated, but this effect was not found. Tannat & D71 group, that ingested the two main sources of flavonoids, showed significantly higher values of ox-LDL when compared to the pure chocolate group. A similar result was published from Baba et al. [19] that found that when (-) epicatechin and (+)-catechin were administered together their absorption is lower than when this antioxidants are ingested separately suggesting that might be a competitive absorption in the gastrointestinal tract of rats when these antioxidants are taken in combination. McKim et al. [20] evaluated the protection that cocoa extract exerts against alcohol liver injury in rats indicating that cocoa flavonoids extract may be a good source of flavonoids antioxidants. However, they worked with very high levels of alcohol (10-14g/kg per day) and cocoa extract (400mg/kg per day). As the cocoa flavonoids are obtained from an extract it is difficult to determinate the amount of cocoa (chocolate) that a human should have to consume to have the same protection that was observed in rats. The damage caused by the ethanol and the competition between antioxidants could be responsible for the high value of ox-LDL in Tannat & D71 group.

The impact of ethanol could be better evaluated by the results of liver analysis. Although the groups that ingested Tannat red wine had higher values of ox-LDL, the percentage of liver weight in relation to

body weight showed no damage to this organ by the action of ethanol. This may be related to the dose of red wine received for each group that was not toxic to liver but was enough to increase the levels of ox-LDL.

Statistically lower total cholesterol in liver of Tannat red wine groups could be due to the higher values of ox-LDL. Usually LDL is removed from circulation by LDL receptors in the liver (that depend on recognition of apoproteins B and E) [21,22]. When LDL is oxidized it loses the LDL receptor affinity [23] and liver loses the ability of capture this constituent. Bruce et al. [24] reported that this occurs because during LDL oxidation apoprotein B is fragmented. Hence, this ox-LDL attracts monocytes to this part of the arterial wall to engulf the material generating “foam cells”, which are an icon for atherosclerosis [22,24]. This is why, as described for Lapointe et al. [3], the circulating ox-LDL concentrations have been related to the plaque occurrence in arteries.

In conclusion, the contribution and the impact of ingestion of dark chocolate D71 and Tannat red wine could be observed in this study. The impact of ethanol in atherosclerosis, increasing the ox-LDL, and the benefits from cocoa could be also observed. This could suggest that D71 chocolate is more efficient antioxidant than Tannat red wine. However no synergistic effect between these foods was detected, probably because of the low administered doses of antioxidants. Further studies are needed to evaluate the ingestion of dark chocolate with high cocoa content for a prolonged period of time and to better understand the benefits that dietary antioxidants could bring to health.

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Table 1

Diets of each group of rats.

Group	**Diet**
Control	Control Diet
D71	Control Diet + Dark Chocolate with 71% of cocoa (D71)
D40	Control Diet + Dark Chocolate with 40% of cocoa (D40)
Tannat	Control Diet + Red Wine (Tannat)
Tannat & D71	Control Diet + D71 + Tannat
Tannat & D40	Control Diet + D40 + Tannat

Table 2

Consume of Food *versus* Increase of Body Weight during 45 days

	Ingested Food (g)	**Gain of Body Weight (g)**
Control	802.8±60.1[a]	156.5±11.7[a]
D71	809.0±56.7[a]	156.5±12.8[a]
D40	782.7±83.4[a]	149.7±34.3[a]
Tannat	806.1±47.5[a]	150.9±17.9[a]
Tannat & D71	829.4±33.2[a]	162.0±13.9[a]
Tannat & D40	816.3±47.3[a]	149.0±10.3[a]

Values in same column followed by different letters were significantly different ($p<0.05$).

Table 3

Results of Blood Analysis (mg/dL)

	Total Cholesterol	LDL	HDL	Triglycerides	Oxidized LDL
Control	89.2±11.4[a]	20.6±8.4[a]	54.3±3.4[a]	71.2±16.9[a]	0.34±0.07[ab]
D71	78.0±9.6 [a]	12.6±6.0[a]	52.0±4.6[a]	66.8±17.0[a]	0.28±0.06[b]
D40	88.5±15.5[a]	18.8±9.3[a]	56.7±9.2[a]	65.4±20.0[a]	0.29±0.04[b]
Tannat	88.8±11.3[a]	20.6±7.7[a]	57.7±7.8[a]	52.8±14.9[a]	0.41±0.17[ab]
Tannat & D71	79.2±13.7[a]	13.2±9.7[a]	53.3±3.1[a]	63.2±23.6[a]	0.46±0.13[a]
Tannat & D40	75.2±8.9[a]	14.0±9.1[a]	53.5±8.5[a]	38.2±9.0[b]	0.36±0.04[ab]

Values in same column followed by different letters were significantly different ($p<0.05$).

Table 4

Results of Liver Analysis

	% fat	Weight (% of body wt.)	Total Cholesterol (mg/dL)
Control	4.7±0.5[a]	3.2±0,2[a]	33.5±5.7[a]
D71	4.6±0.6[a]	3.2±0.2[a]	30.1±4.4[ab]
D40	4.4±0.6[a]	3.1±0.2[a]	24.5±5.5[b]
Tannat	4.6±0.4[a]	3.1±0.1[a]	16.2±3.0[c]
Tannat & D71	4.8±0.3[a]	3.1±0.2[a]	17.1±3.8[c]
Tannat & D40	4.5±0.4[a]	3.1±0.2[a]	15.0±1.9[c]

Values in same column followed by different letters were significantly different ($p<0.05$).

3 DISCUSSÃO GERAL

No presente trabalho um chocolate amargo com alto teor de cacau (71%) foi desenvolvido no setor de Pesquisa e Desenvolvimento de um fabricante local de chocolates (Florestal Alimentos S/A – Unidade Chocolates Neugebauer) com o objetivo principal de avaliar sua composição de polifenóis e flavonóides e seu potencial antioxidante *in vivo*. O estudo foi realizado, tendo como comparativo amostras de vinho tinto, cedidas por uma vinícola do Estado do Rio Grande do Sul. Os vinhos também passaram por um processo seletivo a fim de escolher aquele que apresentava a maior quantidade dos antioxidantes em estudo.

Prévio ao desenvolvimento do produto, um estudo sobre o perfil polifenólico de amostras de diferentes fornecedores de liquor de cacau foi realizado. Observou-se que, dentre as amostras produzidas em linha normal pelas processadoras, aquela que possuía maior percentual de cacau estrangeiro (fornecedor A) foi a que apresentou a maior quantidade de polifenóis e flavonóides. Quando amostras de cacau 100% brasileiro foram comparadas com cacau proveniente da África, foi verificada a superioridade do cacau africano na quantidade de polifenóis e flavonóides (Tabela 1 - Artigo 1). Este resultado foi diferente do obtido por Natsume et al. (2000) que avaliou amostras de cacau do Equador, Venezuela, Gana, Colômbia, Costa do Marfim e Brasil e verificou que o cacau brasileiro apresentava a maior percentagem de polifenóis e maior concentração de monômeros de epicatequina do que as outras.

Foi solicitado que os fornecedores enviassem para avaliação, amostras de cacau tratadas de forma diferenciada com o objetivo de reter maior quantidade de antioxidantes no produto. Foram recebidas amostras torradas de forma contínua e em batelada. As análises indicaram que a amostra torrada em batelada obteve os melhores índices de polifenóis, porém níveis menores de flavonóides. Isto pode ser justificado pelo aquecimento de forma desuniforme nos torradores em batelada, que prejudicaria de forma mais intensa os flavonóides do que os demais polifenóis existentes na amostra. De forma contrária, a amostra torrada de forma contínua protegeu mais eficientemente os flavonóides presentes. Conforme publicado por Kyi et al. (2005), durante o processo de secagem, e de forma similar no processo de torrefação, (-)-epicatequina e procianidinas são oxidadas enzimaticamente produzindo pigmentos poliméricos marrons, reduzindo os níveis destes antioxidantes no produto final.

Desta forma, a amostra de cacau do fornecedor A foi selecionada para o desenvolvimento do chocolate amargo. Esta escolha deveu-se ao fato de este ser o produto de linha do fornecedor, o que não acarretaria em maior custo, e de o mesmo ter resultados similares aos produtos especiais. Para fabricação do chocolate amargo com 71% de cacau, a formulação desenvolvida no setor de P&D foi adaptada para o processo fabril. As etapas de mistura, refino,

conchagem, tempera e molde do chocolate seguiram os padrões de qualidade utilizados rotineiramente pela empresa. A análise comparativa realizada entre os resultados de quantidade de antioxidantes na amostra de liquor de cacau do fornecedor A e no chocolate amargo com 71% mostrou que quase 50% dos flavonóides foram perdidos durante o processamento do chocolate. Este resultado está de acordo com o publicado por Engler & Engler (2004) que encontrou uma perda de cerca de 25 a 50% do total de flavonóides do cacau durante o processamento do chocolate. Em relação aos polifenóis, esta perda foi de mais de 70% dos antioxidantes.

Na etapa de desenvolvimento do chocolate amargo, foi feita uma avaliação do mesmo comparado-o com amostras de diferentes marcas de chocolate amargo disponíveis no mercado e com diferentes tipos de chocolate. O comparativo com chocolates amargo com percentual similar de cacau na formulação demonstrou que o produto desenvolvido era pobre em polifenóis, porém apresentava teores similares de flavonóides (Tabela 2 – Artigo 1). De acordo com Miller et al.(2006) existe uma relação linear entre o percentual de sólidos de cacau não gordurosos e o teor de polifenóis das amostras. No entanto, nos resultados obtidos, a amostra de chocolate amargo com 70% de cacau não apresentou diferença significativa no teor de flavonóides para o chocolate amargo com 99%. Isto pode ser explicado pelo fato de o produto com menor quantidade de cacau ser orgânico, o que sugere controles mais rígidos para a elaboração do chocolate desde a fazenda até o produto final, preservando os antioxidantes presentes. Além disso, este comparativo foi realizado com produtos importados, pois no Brasil não são comercializados produtos nacionais com teores elevados de cacau. Este fator pode ter contribuído para o baixo teor de polifenóis do produto; pois o produto desenvolvido possuia cacau brasileiro em sua composição, o qual demonstrou ter uma menor quantidade de antioxidantes, e os produtos estrangeiros utilizam cacau de outros países, entre eles a África que é o maior produtor mundial conforme publicado por Cuenca e Nazário (2004).

Após, o chocolate amargo desenvolvido foi submetido a uma comparação com outros tipos de chocolate fabricados pela mesma empresa com o intuito de avaliar produtos que sofreram operações unitárias similares. O chocolate branco, ao leite e meio amargo foram avaliados. Nesta análise, a superioridade do chocolate amargo foi verificada estatisticamente no teor de polifenóis e flavonóides (Tabela 1 – Artigo 2). No entanto, o chocolate branco apresentou teores de antioxidantes, o que não era esperado. Dentre os ingredientes deste produto o açúcar e a vanilina foram publicados por outros autores (SUN; RICARDO-DA-SILVA; SPRANGER, 1998; VINSON; PROCH; BOSE, 2001) como possíveis interferentes nas técnicas. Uma solução 0,1% de vanilina foi utilizada para verificar o impacto deste ingrediente nas análises de Folin Ciocalteau (polifenóis) e de flavonóides. Foi verificada uma interação positiva na análise de

flavonóides, porém esta não se mostrou como a única responsável pelo desenvolvimento de coloração nas análises da amostra de chocolate branco. Há contradição quanto à caracterização dos flavonóides. Enquanto Miller et al. (2006) publicaram que estes antioxidantes tendem a ser hidrofílicos e, portanto, a se localizarem na porção desengordurada da amostra, Auger et al. (2004) classifica os flavonóides como os antioxidantes naturais mais lipofílicos existentes devido a sua localização na superfície polar da bicamada lipídica da membrana celular. Havendo esta interação dos flavonóides com a gordura, esta quantidade detectada no chocolate branco pode ser derivada da manteiga de cacau presente no produto (30% da formulação).

Neste trabalho, o vinho tinto foi utilizado como comparativo de qualidade e quantidade de antioxidantes para o chocolate. Para que se estabelecesse esta relação amostras de quatro variedades de vinho tinto foram avaliadas (Pinot Noir, Cabernet Sauvignon, Merlot e Tannat). O vinho Tannat demonstrou-se estatisticamente superior quando o teor de flavonóides foi mensurado (Tabela 3 - Artigo 2), sendo este resultado similar ao encontrado por Gonzáles-Neves et al. (2004) que avaliaram uvas e vinhos produzidos no Uruguai. Por esta razão, o vinho Tannat, da safra 2004, foi selecionado como comparativo para o chocolate amargo D71 nas análises quantitativas e qualitativas (*in vivo*).

Auger et al.(2004) publicaram a pesquisa realizada por Bourzeix na qual a uva do tipo Pinot Noir apresentou maior quantidade de catequina do que a uva Merlot. Este resultado difere do obtido nas análises do presente experimento. Isto pode ser explicado pelo perfil fenólico dos vinhos, o qual difere dos das uvas, visto que ocorrem mudanças na composição fenólica durante o processo de elaboração do vinho como esmagamento, fermentação e maturação (MEYER *et al.*, 1997).

Outro fator bastante discutido em relação ao vinho está na presença de álcool nesta bebida. Serafini et al. (2000) publicaram que bebidas com maior percentual de álcool oferecem maior proteção ao organismo devido a um provável efeito sinérgico entre o álcool e os polifenóis. Este fator pode estar relacionado com a diferença de resultados obtidos para o mesmo tipo de uva em diversos experimentos já que o teor alcoólico dos vinhos pode variar de um fabricante para outro. No entanto, o álcool não é o único responsável pelos efeitos benéficos à saúde, visto que o grau alcoólico de vinhos branco e tinto é bastante similar e somente o último apresenta resultados significativamente importantes.

Mediante estas determinações quantitativas foi possível estabelecer uma relação entre a dosagem recomendada de vinho tinto, para efeito benéfico à saúde, e a quantidade de chocolate amargo que deve ser ingerida. Verificou-se que 196 mL de vinho tinto Tannat forneciam, em flavonóides, o mesmo que 49g de chocolate amargo 71%. O resultado

apresentado pelo chocolate amargo 40% foi de 61g o qual ficou bastante próximo ao D71, por isto as duas amostras de chocolate foram selecionadas para o teste *in vivo*.

Para avaliação da qualidade destes antioxidantes foi realizado um ensaio de 45 dias, com 36 ratos machos da raça Wistar. Os animais foram divididos em 6 grupos e receberam suplementação de chocolate amargo 71% de cacau, chocolate amargo 40% de cacau e vinho tinto Tannat. Ainda, além do grupo controle, foi avaliado o efeito sinérgico dos antioxidantes do vinho tinto com os chocolates (Tabela 1 - Artigo 3).

Os resultados demonstraram que não houve diferença significativa de peso entre os grupos que ingeriram chocolate e o grupo padrão indicando que todos os animais tiveram uma nutrição equilibrada durante o período. A quantidade calculada de chocolate, em relação ao peso corpóreo do animal, era equivalente a 49g de chocolate amargo com 71% de cacau por dia por um adulto de 70 kg o que representa 14% do total de calorias recomendado pela legislação atual da ANVISA que é de 2.000 calorias diárias (BRASIL, 2006). Deste modo, avaliando o chocolate oferecido em relação ao alimento ingerido verificou-se que o chocolate correspondeu a somente 2,0% do total de calorias ingeridas por dia por rato tanto para os ratos do grupo 2 quanto para os do 3. Este pode ser um dos fatores que contribuiu para não ter havido aumento de peso significativo nestes animais.

Em relação às análises do sangue dos animais, Pereira (2005) publicou que a relação triglicérides/HDL é um indicativo do risco de desenvolvimento de aterosclerose, sendo que quanto maior este fator maior o risco (ideal = 3,75, ou seja, 150 mg/dL de triglicérides para 40 mg/dL de colesterol HDL). Na Tabela 2, pode-se observar que, mesmo não havendo diferença significativa entre os grupos, àqueles que ingeriram vinho tinto foram os que apresentaram as menores relações.

Tabela 2: Relação triglicerídeos/HDL e valores ox-LDL no sangue dos animais experimentais.

	Triglicerideos/HDL	**LDL oxidado**
Controle	$1,32 \pm 0,36^{a}$	0.34 ± 0.07^{ab}
D71	$1,30 \pm 0,38^{a}$	0.28 ± 0.06^{b}
D40	$1,20 \pm 0,45^{a}$	0.29 ± 0.04^{b}
Tannat	$0,94 \pm 0,35^{a}$	0.41 ± 0.17^{ab}
Tannat & D71	$1,19 \pm 0,47^{a}$	0.46 ± 0.13^{a}
Tannat & D40	$0,74 \pm 0,23^{a}$	0.36 ± 0.04^{ab}

Médias com letras iguais na mesma coluna indicam que não houve diferença significativa entre os tratamentos (para um nível de significância de 5%).

Observou-se que quando o vinho tinto foi oferecido juntamente com o chocolate D71 os valores da proporção triglicerídeos/HDL e do ox-LDL aumentaram o que, segundo Baba et al.

(2001) pode ser devido a uma absorção competitiva dos antioxidantes no trato gastrointestinal dos ratos. A menor quantidade de cacau do chocolate D40 parece ser favorável a este efeito sinérgico apresentando resultados ainda melhores que os do vinho tinto.

No entanto, a análise destes dois indicadores de aterosclerose se mostrou contraditória, pois enquanto as amostras do vinho tiveram os menores quocientes triglicérides/HDL as mesmas tiveram valores significativamente maiores de LDL oxidado quando comparadas com as amostras de chocolate. Este aumento da peroxidação lipídica em ratos quando ingerem vinho tinto foi verificado por outros autores (KASDALLAH-GRISSA et al.; HÜBSCHER et al., 2005) e foi atribuído ao etanol presente na bebida. Devido ao fato de que as análises de ox-LDL do chocolate foram significativamente diferentes das do vinho tinto, pode-se considerar que o chocolate com teores acima de 40% de cacau apresenta efeito benéfico na redução da taxa de oxidação do LDL que é um dos primeiros estágios no desenvolvimento da doença aterosclerótica. Outro fator importante na ingestão de chocolate é que, segundo Kubow (1996), a absorção da manteiga de cacau no organismo é influenciada pelo conteúdo de ácido esteárico presente na mesma, o que acarreta em um efeito neutro no aumento do colesterol LDL, diferente de outras gorduras saturadas.

Os fígados dos ratos também foram avaliados e, mesmo o órgão não indicando alguma injúria causada pelo álcool, foi verificada diferença estatística entre os grupos na análise de colesterol total. Este resultado foi convergente com o do LDL oxidado no sangue mostrando que os grupos com níveis mais elevados de ox-LDL foram os que tiveram os menores teores de colesterol capturados pelo fígado, deixando esta lipoproteína danosa ao organismo circulante no sangue.

Resumidamente, pode-se dizer que o chocolate amargo desenvolvido com 71% de cacau, comparado ao vinho tinto Tannat, apresentou provável efeito benéfico ao organismo de ratos Wistar. No entanto, estudos complementares devem ser realizados a fim de concentrar o teor de antioxidantes no chocolate durante o beneficiamento e de avaliar possíveis interações negativas entre antioxidantes de diferentes fontes, bem como estudar o benefício dos antioxidantes na redução do impacto do etanol no processo de peroxidação lipídica.

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