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COMPOSTOS BIOATIVOS EM UVAS 'ISABEL': INFLUÊNCIA DA RADIAÇÃO UV-C E POTENCIAL PROTETOR EM MODELO DE COLITE ULCERATIVA EM RATOS

Santa Maria, RS

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

Orientadora: Profª. Drª. Tatiana Emanuelli

Co-orientadora: Profª. Drª. Claudia Kaehler Sautter

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Santa Maria, RS
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Dedico este trabalho à minha mãe Maristella, minha maior incentivadora e meu maior exemplo de força, honestidade e dedicação.

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“There's always gonna be another mountain
I'm always gonna want to make it move
Always gonna be an uphill battle
Sometimes I'm going to have to lose
Ain't about how fast I get there
Ain't about what's waiting on the other side
It's the climb.”
(The climb, M. Cyrus)

RESUMO

COMPOSTOS BIOATIVOS EM UVAS 'ISABEL': INFLUÊNCIA DA RADIAÇÃO UV-C E POTENCIAL PROTETOR EM MODELO DE COLITE ULCERATIVA EM RATOS

AUTORA: Luana Haselein Maurer
ORIENTADORA: Tatiana Emanuelli

Os objetivos deste estudo foram avaliar o efeito da irradiação UV-C pós-colheita sobre marcadores de defesa antioxidante de uvas 'Isabel' e estudar o potencial protetor do pó da casca de uva (PC) e suas frações bioativas (compostos fenólicos livres, PE; fenólicos associados à fibra, RE; e fibra alimentar, RF) em um modelo de colite em ratos. Uvas 'Isabel' foram tratadas com diferentes doses de UV-C (0, 0,5, 1, 2 e 4 kJ/m^2) e armazenadas por 1, 3 ou 5 dias para avaliação da sua capacidade antioxidante enzimática e não-enzimática através da determinação da atividade da superóxido dismutase (SOD), catalase (CAT), glutationa redutase (GR), do conteúdo de tiois e da capacidade de remoção de radicais superóxido e peroxil. No dia seguinte à irradiação, houve aumento dos níveis de tiois e da atividade das enzimas antioxidantes, especialmente quando doses intermediárias (1 e 2 kJ/m^2) foram usadas. Estas mesmas doses também aumentaram os níveis de fenólicos, enquanto que as doses de 0,5 e 4 kJ/m^2 não apresentaram efeito. Os níveis de antocianinas aumentaram cerca de 35% após irradiação com 1 kJ/m^2, considerada a dose hormética, porém não houve alteração no perfil de antocianinas. No experimento envolvendo a indução de colite, ratos Wistar receberam dietas suplementadas com 8% de pó da casca de uva (PC) ou suas frações bioativas (PE, RE e RF) por 15 dias antes da indução da colite (administração intraretal de 2,4,6-ácido trinitrobenzenosulfônico, TNBS, 10 mg/animal) e por mais 7 dias após a indução. PE, RE e RF foram adicionadas em quantidades equivalentes às encontradas no PC. PC apresentou 25% de fibra alimentar e 776 mg de compostos fenólicos/100 g. PE apresentou 80% de antocianinas e RE um teor de 78% de fibra alimentar e 7,7% de fenólicos ligados à fibra. O ganho de peso prévio à indução da colite não diferiu entre os grupos. O consumo de ração diminuiu após a colite e apenas o grupo PE não restabeleceu a ingestão alimentar, além de apresentar efeitos adversos após a exposição ao TNBS. Tais danos não foram observados quando os fenólicos estavam ligados à matriz, como em PC. A colite aumentou a peroxidação lipídica, oxidação proteica, níveis de óxido nítrico (NO) e citocinas inflamatórias a nível tecidual e sistêmico, além de ativar a via do NF-κB. A dieta PC reduziu os marcadores inflamatórios, restaurou a atividade da SOD e CAT e diminuiu a oxidação tecidual e os níveis de NO. Apenas RE reduziu a expressão proteica de *p*NF-κB e a infiltração de neutrófilos. A colite reduziu os níveis de tiois e a atividade daGR, SOD, CAT, glutationa peroxidase (GPx) e glutationa-S-transferase (GST) no cólon, além de aumentar a expressão gênica de ambas as subunidades da glutamato-cisteína ligase (GCL). PC e RE restauraram a atividade da GPx. Todas as dietas experimentais reduziram a expressão da proteína IKK-β e os níveis de NO no cólon, além de terem aumentado parcialmente os níveis de tiois e restaurado as atividades da GR e da GST. O efeito sobre o sistema da glutationa foi atribuído à reciclagem da glutationa ao invés da sua síntese *de novo.* A colite também induziu apoptose no tecido colônico e RE e RF reduziram este efeito. As frações bioativas do PC não protegeram contra os danos macroscópicos e o PE agravou a lesão. PC, PE e RE reduziram a expressão gênica de claudina-2 e a fração RF aumentou a expressão gênica de zônula ocludens e ocludina. A colite reduziu cerca de 30% a produção de ácidos graxos de cadeia curta (AGCC) e PC e RE reverteram este efeito, enquanto RF foi ineficaz. Os efeitos protetores do PC no cólon foram associados à inibição da via NF-κB, à redução dos níveis de NO e citocinas inflamatórias, à melhora da atividade das enzimas antioxidantes, da função de barreira intestinal e da produção de AGCC. A fibra dietética e os fenólicos ligados à fibra foram mais eficazes do que os fenólicos solúveis na proteção contra a colite e o consumo de pó de casca de uva pode ser investigado como uma alternativa benéfica para pacientes com inflamação intestinal.

Palavras-chave: Irradiação. Hormese. Inflamação. Compostos fenólicos. Fibra alimentar. Cólon.

ABSTRACT

BIOACTIVE COMPOUNDS IN 'ISABEL' GRAPES: INFLUENCE OF UV-C IRRADIATION AND PROTECTIVE POTENTIAL IN A RAT MODEL OF ULCERATIVE COLITIS

AUTHOR: Luana Haselein Maurer
ADVISOR: Tatiana Emanuelli

The aims of this study were to evaluate the effect of post-harvest UV-C irradiation on antioxidant markers of 'Isabel' grapes and to study the protective potential of grape peel powder (GPP) and its bioactive fractions (free phenolic compounds, EP; fiber-bound phenolics, NEP-F; and dietary fiber, F) in a rat model of colitis. 'Isabel' grapes were treated with different doses of UV-C (0, 0.5, 1, 2, and 4 kJ/m^2) and stored for 1, 3 or 5 days to evaluate their enzymatic and non-enzymatic antioxidant capacity by determining the activity of superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR), the content of thiols, and the ability to remove superoxide and peroxyl radicals. One day after irradiation with UV-C, thiol levels and antioxidant enzyme activities increased, especially when the intermediate doses (1 and 2 kJ/m^2) were used. The same doses also promoted an increase in the total phenolic content whereas 0.5 and 4 kJ/m^2 had no effect. Anthocyanin levels increased by ~35% after irradiation with 1 kJ/m^2 of UV-C, which was considered the hormetic dose, however there was no change in the anthocyanin profile. In the experiment involving the induction of colitis, Wistar rats were fed with diets supplemented with 8% of GPP or its bioactive fractions (EP, NEP-F, and F) for 15 days before colitis induction (intrarectal administration of 2,4,6-trinitrobenzenesulfonic acid, TNBS, 10 mg/animal) and for a further 7 days after induction. EP, NEP-F, and F were added at amounts equivalent to those found in the GPP. GPP had 25% of fiber and 776 mg phenolic compounds/100g. EP had 80% anthocyanins, whereas NEP-F had 78% of fiber and 7.7% of fiber-bound phenolics. The weight gain prior to the induction of colitis did not differ among groups. Feed intake was reduced after colitis and only the EP group did not reestablish feed intake, beyond exhibiting adverse effects after TNBS exposure. Such damages were not observed when phenolics were bound to the matrix, as in GPP. Colitis increased lipid peroxidation, protein oxidation, nitric oxide (NO) levels, and inflammatory cytokines at tissue and systemic level, beyond activating the NF-κB pathway. GPP reduced inflammatory markers, restored SOD and CAT activities, and decreased tissue oxidation and NO levels. Only NEP-F reduced protein expression of *p*NF-κB and neutrophil infiltration. Colitis reduced the thiol levels and the activity of GR, SOD, CAT, glutathione peroxidase (GPx), and glutathione-S-transferase (GST) in the colon, in addition to increasing the mRNA expression of both subunits of glutamate-cysteine ligase (GCL). GPP and NEP-F restored GPx activity. All experimental diets reduced the protein expression of the IKK-β, and NO levels in the colon, in addition to partially increasing thiol levels and restoring GR and GST activities. The effect about GSH system was attributed to the GSH recycling rather than the *de novo* synthesis. Colitis also induced apoptosis in colonic tissue and NEP-F and F reduced this effect. The bioactive fractions of GPP did not protect against macroscopic damage and EP aggravated the lesion. GPP, EP, and NEP-F reduced the mRNA expression of claudin-2, whereas F fraction increased the mRNA expression of zonula occludens and occludin. Colitis reduced by ~30% the short-chain fatty acids (SCFA) production and GPP and NEP-F reversed this effect, while F fraction was ineffective. The protective effects of GPP in the colon were associated with inhibition of the NF-κB pathway, reduction of NO and inflammatory cytokines, improvement of the antioxidant enzymes activity, intestinal barrier function, and SCFA production. Dietary fiber and fiber-bound phenolics were more effective than soluble phenolics to protect against colitis and the consumption of grape peel powder could be investigated as a benefic alternative for patients with intestinal inflammation.

Keywords: Irradiation. Hormesis. Inflammation. Phenolic compounds. Dietary fiber. Colon.

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LISTA DE ABREVIATURAS

AGCC – Ácidos graxos de cadeia curta
ANOVA – Análise de variância
AOPP – Produtos avançados de oxidação proteica
CAT – Catalase
CC – *Control colitis*
cDNA – DNA complementar
CDNB - 1-cloro-2,4-dinitrobenzeno
CLAE-EM - Cromatografia líquida de alta eficiência acoplada a espectrômetro de massas
CM – *Control mesalamine*
COX-2 – Ciclo-oxigenase 2
DII – Doença inflamatória intestinal
DNA – *Deoxiribonucleotide acid*
DPPH – 1,1 difenil-2-picrilhidrazina
DSS – Dextran sulfato de sódio
DTNB – 5,5-di-tiobis-2-ácido nitrobenzoico
ELISA – Ensaio de imunoprecipitação enzimático
EP - *Extractable polyphenols-rich fraction*
EROs – Espécies reativas de oxigênio
ESI – *Electrospray ionization*
F - *Polyphenols-poor, fiber-rich fraction*
FRAP – *Ferric reducing antioxidant power*
GAPDH – Gliceraldeído 3-fosfato desidrogenase
GC- *Gas chromatography*
GCL – Glutamato cisteína ligase
GCLc - Glutamato cisteína ligase subunidade catalítica
GCLm - Glutamato cisteína ligase subunidade modificadora
G-POD – Guaiacol peroxidase
GPx – Glutationa peroxidase
GR – Glutationa redutase
GS – Glutationa sintase
GSH – Glutationa
GSSG – Glutationa oxidada
GST – Glutationa-S-transferase
GPP – *Grape peel powder*
IAD – Índice de atividade de doença
IκB – *Inhibitor of nuclear fator kappa B*
IKK-β – *Inhibitor of nuclear factor kappa B quinase subunit beta*
IL-10 – Interleucina 10
IL-1β – Interleucina 1 beta
IL-6 – Interleucina 6
iNOS – Óxido nítrico sintase induzível
LC – *Liquid chromatography*
MDA – Malonaldialdeído
MMP-9 – Metaloproteinase de matriz 9
MPO – Mieloperoxidase
NADPH – *Nicotinamide adenine dinucleotide phosphate*
NEP-F - *Non-extractable polyphenols-rich fraction*

NF-κB – *Nuclear factor kappa B*
NO – Óxido nítrico
$ONOO^-$ - Peroxinitrito
ORAC – *Oxygen removal antioxidant capacity*
PAMPs – Padrões moleculares associados a patógenos
PC – Pó da casca de uva
PCR – Reação da polimerase em cadeia
PDA - *Photodiode array detector*
PE – Extrato rico em compostos fenólicos extraíveis
PGE-2 – Prostaglandina E 2
RE – Fração rica em fibras e em fenólicos não-extraíveis
RF - Fração rica em fibras e pobre em fenólicos
RNA – *Ribonucleic acid*
RNAm – RNA mensageiro
RT-qPCR – PCR em tempo real
SDS-PAGE – *Sodium dodecyl sulphate – polyacrylamide gel electrophoresis*
SOD – Superóxido dismutase
TBARS – Substâncias reativas ao ácido tiobarbitúrico
TLR – Receptor tipo *toll*
TNBS – Ácido 2,4,6-trinitrobenzenosulfônico
TNF-α – Fator de necrose tumoral alfa
UC – *Ulcerative colitis*
UV-C – Ultravioleta do tipo C

SUMÁRIO

1 INTRODUÇÃO

Os compostos fenólicos são oriundos do metabolismo secundário das plantas, onde desempenham funções relacionadas à defesa contra patógenos e à proteção contra a luz ultravioleta (UV) (KOYAMA et al., 2012; LIU, 2013). Devido às suas propriedades antioxidantes, antimicrobianas e anti-inflamatórias observadas em estudos *in vitro* e *in vivo*, esses compostos são associados a diversos benefícios à saúde, incluindo a prevenção de doenças crônicas como doenças cardiovasculares, doenças neurodegenerativas, diabetes e câncer (DEL RIO et al., 2013; XIA et al., 2013; ZHANG; TSAO, 2016).

Nesse sentido, o aumento do consumo de alimentos ricos em compostos fenólicos, tais como frutas e vegetais, tem sido bastante incentivado, assim como tecnologias e práticas agrícolas que visam estimular a síntese desses compostos (AMAROVICZ et al., 2009; BOUE et al., 2009; TREUTTER, 2010). Tratamentos pós-colheita são usados para preservar a qualidade de produtos frescos visando o combate ao crescimento microbiano e o aumento da vida de prateleira (COSTA et al., 2006; LI et al., 2010). No entanto, tem-se observado uma melhora no sistema antioxidante em resposta a certas condições de estresse usadas como tratamento pós-colheita (CISNEROS-ZEVALLOS, 2003; GONZÁLEZ-AGUILAR et al., 2010). O uso de agentes estressores abióticos, como a irradiação com luz ultravioleta do tipo C (UV-C), é uma forma de induzir a síntese de compostos fenólicos (URBAN et al., 2016). Diversos estudos têm demonstrado sua eficácia em aumentar o conteúdo de compostos fenólicos em frutas e vegetais (WANG; CHEN; WANG, 2009; JIANG et al., 2010; LIU et al., 2012; SHEN et al., 2013), no entanto é preciso conhecer o metabolismo de cada espécie a fim de determinar as condições de tratamento e armazenamento para otimizar sua síntese.

Quando ingeridos, os compostos fenólicos das frutas e vegetais são pouco absorvidos ao longo do trato gastrointestinal e a maioria chega intacta ao cólon, onde será biotransformada pela microbiota intestinal (CARDONA et al., 2013; CELEP; RASTMANESH; MAROTTA, 2013). Devido a essa característica, o consumo de compostos fenólicos poderia ser uma alternativa para a prevenção de doenças inflamatórias intestinais e câncer de cólon (KAULMANN; BOHN, 2016). A casca de uva é rica em compostos fenólicos e possui alto percentual de fibra (BERES et al., 2016). Além disso, é uma fruta bastante popular e de alta taxa de produção no Brasil, especialmente no estado do Rio Grande do Sul (MELLO, 2015).

Nos últimos anos, tem-se percebido que o conteúdo de compostos fenólicos na matriz alimentar está sendo subestimado à medida em que se avaliam apenas os compostos solúveis presentes nos extratos, enquanto boa parte do conteúdo de fenólicos permanece associado à matriz insolúvel, composta especialmente por proteínas e fibras (PÉREZ-JIMÉNEZ; ARRANZ; SAURA-CALIXTO, 2009). Uma vez que as fibras colaboram no carreamento dos compostos fenólicos ao longo do trato gastrointestinal (SAURA-CALIXTO, 2011) e que o consumo de alimentos ricos em fibras também é preconizado na prevenção de doenças intestinais (ROSE et al., 2007), é importante estudar a participação de cada uma dessas frações bioativas, quando parte de uma matriz alimentar complexa, em modelos de doença intestinal.

1.1 COMPOSTOS FENÓLICOS

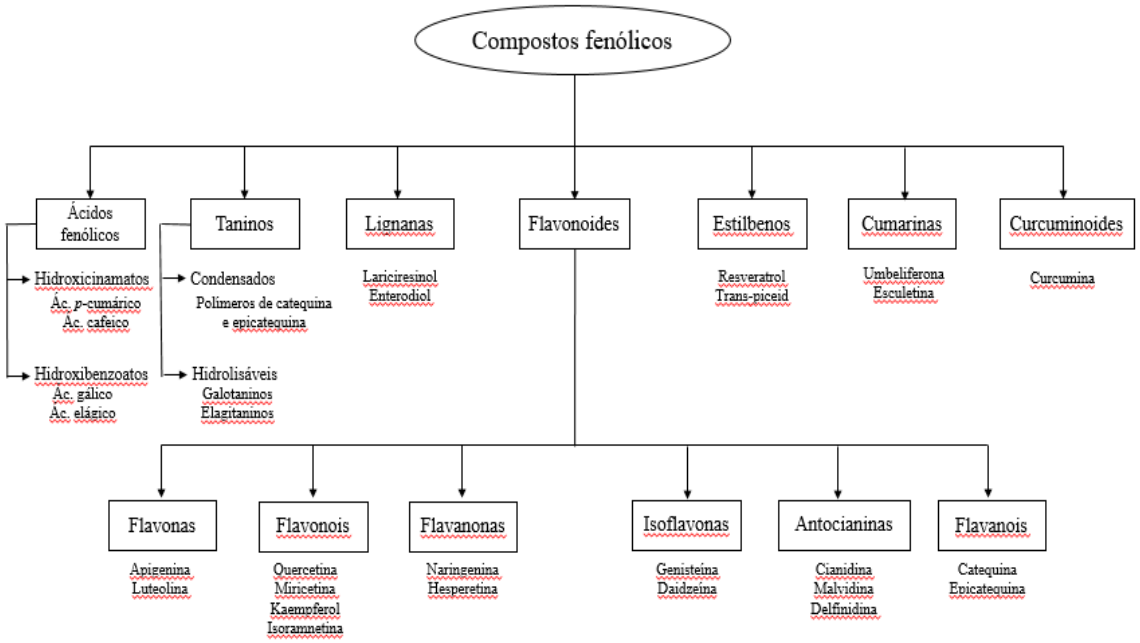
Os compostos fenólicos são definidos como moléculas que contém pelo menos um anel aromático com uma ou mais hidroxilas. Estão presentes em altas concentrações nas folhas, cascas e sementes de frutas e vegetais e conferem características atrativas como cor e sabor a esses alimentos e a produtos derivados, como chás e vinhos (DEL RIO et al., 2013). Esses compostos podem ser classificados em dois grupos: flavonoides e não-flavonoides (Figura 1). Os flavonoides incluem as flavonas, os flavanois, as flavanonas, os flavonois, as isoflavonas e as antocianinas, enquanto os não-flavonoides incluem os ácidos fenólicos derivados do ácido hidroxicinâmico e hidroxibenzoico, os taninos condensados e hidrolisáveis, as lignanas, as cumarinas, os curcuminoides e os estilbenos, como o resveratrol (GEORGIEV; ANANGA; TSOLOVA, 2014).

A maior parte dos efeitos benéficos atribuídos aos compostos fenólicos decorre da sua ação antioxidante, proporcionada pela capacidade de seu grupamento fenol deslocalizar um par de elétrons da sua estrutura a fim de estabilizar radicais livres e quelar metais (CÖMERT; GÖKMEN, 2017).

De acordo com sua localização celular e interação com outros componentes dos alimentos, os compostos fenólicos podem estar sob a forma livre ou ligada (CÖMERT; GÖKMEN, 2017). A maior parte dos estudos que determina a quantidade de compostos fenólicos presente nos alimentos leva em conta apenas os fenólicos extraíveis, ou seja, fenólicos solubilizados durante os processos convencionais de extração que utilizam misturas de solventes aquoso-orgânicos (ARRANZ et al., 2009; PÉREZ-JIMÉNEZ; DÍAZ-RUBIO;

SAURA-CALIXTO, 2013). Flavonois, antocianinas, ácidos fenólicos, flavanois e estilbenos são exemplos de fenólicos solúveis (SAURA-CALIXTO, 2011).

Figura 1 - Classificação dos compostos fenólicos.



Fonte: autoria pessoal.

No entanto, têm-se demonstrado que grande parte dos compostos fenólicos permanece nos resíduos de extração e são, por isso, chamados de fenólicos não-extraíveis por alguns autores. Esses compostos fenólicos não-extraíveis estão ligados a proteínas e fibras e são representados por moléculas de alto peso molecular, como os taninos condensados (ou proantocianidinas), os taninos hidrolisáveis (elagitaninos e galotaninos) e os fenólicos hidrolisáveis (ácidos fenólicos associados a polissacarídeos) (Figura 1) (ARRANZ; SILVÁN; SAURA-CALIXTO, 2010; MATEOS-MARTÍN et al., 2012; PÉREZ-JIMÉNEZ; DÍAZ-RUBIO; SAURA-CALIXTO, 2013; ZURITA; DÍAZ-RUBIO; SAURA-CALIXTO, 2012). Os taninos condensados são oligômeros e polímeros de flavanois que apresentam alta capacidade antioxidante e são encontrados especialmente no cacau, no vinho e em chás (SHAHIDI; AMBIGAIPALAN, 2015). Já os taninos hidrolisáveis são moléculas de ácidos

fenólicos simples, como ácido gálico e ácido elágico, associadas a uma molécula de açúcar (DOMÍNGUEZ-RODRÍGUEZ; MARINA; PLAZA, 2017).

1.2 IRRADIAÇÃO COM LUZ ULTRAVIOLETA DO TIPO C (UV-C)

A radiação ultravioleta do tipo C (UV-C) abrange os comprimentos de onda entre 200 e 280 nm e é usada na indústria alimentícia principalmente para fins de controle da contaminação de superfícies e embalagens devido ao seu potencial germicida (BEGUM; HOCKING; MISKELLY, 2009).

É considerada um estressor abiótico, uma vez que pode ativar mecanismos de defesa, como o acúmulo de fitoalexinas (compostos produzidos pelas plantas em resposta ao estresse) e o aumento da atividade de enzimas antioxidantes a fim de proteger as células dos efeitos citotóxicos das espécies reativas de oxigênio (EROs) geradas durante o processo de irradiação (ALOTHMAN; BHAT; KARIM, 2009; GONZÁLEZ-AGUILAR et al., 2010; GONZÁLEZ-AGUILAR; ZAVALETA-GATICA; TIZNADO-HERNÁNDEZ, 2007). Essa resposta de adaptação às condições de estresse contribui para a manutenção da qualidade da fruta e para o aumento do potencial antioxidante (LIM; LIM; TEE, 2007).

1.3 UVA

A uva é uma fruta que apresenta grande variedade de compostos fenólicos, principalmente flavonoides (como as antocianinas), estilbenos e proantocianidinas. Isso faz com que a uva, especialmente as variedades tintas, seja considerada uma das mais ricas fontes de compostos fenólicos da nossa dieta (GEORGIEV; ANANGA; TSOLOVA, 2014; MARCHI et al., 2014).

A uva Isabel é uma cultivar não-vinífera de alta produtividade, comumente encontrada ao alcance do público consumidor. É bastante utilizada para a produção de sucos, geleias e vinhos, podendo ser misturada a outras cultivares como Concord e Bordô (MELLO, 2015).

O uso de UV-C em uvas tem mostrado bons resultados sobre o aumento da síntese de antocianinas e de resveratrol (CANTOS; ESPÍN; TOMÁS-BARBERÁN, 2001; GUERRERO et al., 2010; PINTO et al., 2016). No entanto, se desconhecem seus efeitos sobre o metabolismo antioxidante enzimático.

1.4 METABOLISMO DOS COMPOSTOS FENÓLICOS

No alimento, os compostos fenólicos apresentam-se sob a forma de glicosídeos, ésteres ou polímeros, os quais são pouco absorvidos em sua forma nativa e precisam ser hidrolisados para serem absorvidos (CARDONA et al., 2013; CHIOU et al., 2014; DEL RIO; BORGES; CROZIER, 2010). Apesar dos compostos fenólicos da dieta serem modificados ao longo do intestino delgado, a maioria não é eficientemente absorvida (DALL'ASTA et al., 2012). Estima-se que apenas 5-20% dos compostos fenólicos consumidos são absorvidos no estômago e intestino delgado e que os 80-95% restantes chegam intactos ao cólon (CARDONA et al., 2013; CELEP; RASTMANESH; MAROTTA, 2013; CHIOU et al., 2014).

Além disso, estudos recentes revelaram que os compostos fenólicos interagem com a microbiota intestinal, modulam o microbioma e são metabolizados até produtos de menor peso molecular (DALL'ASTA et al., 2012; CHIOU et al., 2014; MACDONALD; WAGNER, 2012). Esses metabólitos, uma vez absorvidos, podem apresentar bioatividade superior à de seus precursores e serem os responsáveis pelos efeitos benéficos à saúde atribuídos aos alimentos ricos em fenólicos (CARDONA et al., 2013; DALL'ASTA et al., 2012; ETXEBERRIA et al., 2013; PARKAR; TROWER; STEVENSON, 2013; TUOHY et al., 2012).

1.5 FIBRA ALIMENTAR

Assim como a maioria dos compostos fenólicos, as fibras são componentes dos vegetais resistentes à digestão e absorção no intestino delgado que chegam intactas no cólon, onde serão fermentadas parcial ou totalmente (DEVRIES et al., 2001). De acordo com a definição, polissacarídeos, oligossacarídeos, lignina e outras substâncias vegetais associadas são considerados fibra alimentar, além de incluir também o amido resistente e os compostos fenólicos (MACAGNAN; DA SILVA; HECKTHEUER, 2016). As fibras são classificadas de acordo com a solubilidade em água em fibras solúveis (pectina, gomas, mucilagens e algumas hemiceluloses, oligossacarídeos, inulina) e fibras insolúveis (celulose, outros tipos de hemiceluloses e lignina) (AJILA; PRASADA RAO, 2013; ELLEUCH et al., 2011).

Enquanto a fração insolúvel é associada com a formação do bolo fecal, regulação intestinal e absorção de água, a fração solúvel é associada à redução da absorção de colesterol

e glicose no intestino delgado (MUDGIL; BARAK, 2013; AJILA; PRASADA RAO, 2013). Recomenda-se que a proporção ideal entre as duas varie entre 70-50% de fração insolúvel para 30-50% de fração solúvel. Essa proporção, aliás, é encontrada de forma mais balanceada em frutas do que em cereais (AJILA; PRASADA RAO, 2013).

Além disso, as fibras também são associadas à redução do tempo de trânsito intestinal, à estimulação seletiva da microbiota saudável e à melhora do sistema imunológico (LIZARRAGA et al., 2011; SAURA-CALIXTO, 2011). Com a fermentação das fibras, são produzidos vários compostos, como aminas, fenois e ácidos graxos de cadeia curta (AGCC), como acetato, butirato e propionato (SAURA-CALIXTO, 2011). As fibras solúveis apresentam maior fermentabilidade que as fibras indolúveis e, portanto, levam a uma maior produção de AGCC no cólon (SIVAPRAKASAM et al., 2016). Aos AGCC são atribuídas funções como regulação hormonal, controle da saciedade, proteção da barreira intestinal, regulação da inflamação, da diferenciação celular e da apoptose (CLEMENTE et al., 2012; RUSSELL et al., 2013; TUOHY; FAVA; VIOLA, 2014). Enquanto o butirato é usado como fonte de energia primária pelos colonócitos, o propionato é metabolizado pelo fígado e o acetato atinge as maiores concentrações plasmáticas. Ao butirato, em especial, é associada a redução da inflamação intestinal e a prevenção do câncer de cólon (RUSSELL et al., 2013; SIVAPRAKASAM et al., 2016).

Resíduos de vinificação são compostos especialmente pelas cascas das uvas após a fermentação e apresentam teores de fibra variável, de 17% até quase 80% em base seca, dependendo da cultivar estudada (DENG; PENNER; ZHAO, 2011; POZUELO et al., 2012). A fração insolúvel é predominante em relação à fração solúvel, representando até 80% da fibra alimentar total (POZUELO et al., 2012).

1.6 INFLAMAÇÃO INTESTINAL

As doenças inflamatórias intestinais (DIIs) são desordens inflamatórias crônicas que afetam o trato gastrointestinal e possuem etiologia desconhecida e multifatorial, onde há a interação de fatores genéticos, imunes e ambientais (SOBCZAK et al., 2014). Os exemplos mais comuns de DIIs são a doença de Chron e a colite ulcerativa. Enquanto a doença de Chron tem características de inflamação transmural e pode afetar todo o trato gastrointestinal, especialmente íleo e cólon, a colite ulcerativa é mais restrita às camadas mucosa e submucosa do cólon e do reto (CORRIDONI; ARSENEAU; COMINELLI, 2014; SOBCZAK et al., 2014; SOMANI et al., 2015). Os principais sintomas observados são perda de peso, fezes

diarreicas e sanguinolentas, dor abdominal recorrente, febre, perda de apetite e anorexia (HEAD; JURENKA, 2004; SOMANI et al., 2015). Além disso, há maior risco para desenvolvimento de câncer gastrointestinal e colorretal (MACDONALD; WAGNER, 2012; ROGLER, 2014; SINGH et al., 2012a).

A maior incidência das DIIs ocorre em países industrializados da América do Norte e Europa, mas vem aumentando ao longo das últimas décadas em países em desenvolvimento da América Latina, da Ásia e do Leste Europeu (DA SILVA et al., 2014; MOLODECKY et al., 2012; SOBCZAK et al., 2014). Há pouco mais de dez anos atrás, estimava-se que as DIIs afetavam cerca de 4 milhões de pessoas em todo o mundo, sendo 2,2 milhões de europeus e 1,4 milhões de americanos, principalmente dentro da faixa etária de 15 a 30 anos de idade (LOFTUS, 2004). Segundo a Fundação de Doença de Chron e Colite da América (2014), atualmente, cerca de 1,6 milhões de americanos possuem doença de Chron ou colite ulcerativa e cerca de 70.000 novos casos são diagnosticados a cada ano (SHIVASHANKAR et al., 2014). Nos Estados Unidos, estima-se que sejam gastos de 8 a 15 bilhões de dólares ao ano com a colite ulcerativa (UNGARO et al., 2016). O Brasil ainda é considerado um país de baixa prevalência de DIIs, apesar do aumento significativo na incidência dessas doenças (DE BARROS; DA SILVA; NETO, 2014; VICTORIA; SASSAKI; NUNES, 2009).

A patogênese da inflamação intestinal inclui disfunção da barreira epitelial intestinal, aumento da permeabilidade intestinal, invasão microbiana e ativação da resposta imune, acompanhada da excessiva produção e liberação de mediadores pró-inflamatórios, como citocinas, moléculas de adesão e EROs e espécies reativas de nitrogênio (ERNs), que contribuem para a perpetuação da resposta inflamatória no intestino (BRENNA et al., 2013; LEE et al., 2014b; MARCHI et al., 2014; MOURA et al., 2015). Além disso, há fortes evidências de que alterações na microbiota intestinal também façam parte da patogênese dessa doença (CAMMAROTA et al., 2015; MITSUYAMA; SATA, 2008).

A colite ainda é considerada incurável e os alvos terapêuticos baseiam-se na redução dos sintomas, na remissão da doença, na prevenção e tratamento de complicações associadas e na correção de déficits nutricionais (COSNES et al., 2011; SOMANI et al., 2015). A farmacoterapia consiste na administração de aminosalicilatos (sulfasalazina, mesalazina), corticosteroides (prednisolona, budesonida), antibióticos, supressores de resposta imune (6-mercaptopurina, metotrexato) e agentes biológicos anti-TNF-α (infliximabe, adalimumabe) (HEAD; JURENKA, 2004; SAXENA et al., 2014; SOBCZAK et al., 2014; SOMANI et al., 2015). No entanto, essas drogas possuem diversos efeitos colaterais a longo prazo e nem

sempre são efetivas, fazendo com que grande parte dos pacientes recorra a tratamentos alternativos ou à intervenção cirúrgica (COSNES et al., 2011; DE BARROS; DA SILVA; NETO, 2014; MOURA et al., 2015; SOBCZAK et al., 2014; SOMANI et al., 2015).

Estima-se que cerca de 40% dos pacientes com DII faz uso de algum tratamento alternativo, como vitaminas, suplementos naturais e derivados de plantas (HEAD; JURENKA, 2004). De fato, sabe-se que os fitoquímicos, apesar de não apresentarem propriedades nutricionais, são capazes de atuar na redução do estresse oxidativo, na supressão de processos inflamatórios e de proliferação celular e na modulação da sinalização celular (HUR et al., 2012; SOMANI et al., 2015).

Nesse contexto, a inserção de alimentos com propriedades antioxidantes, prebióticos, probióticos e fitoquímicos (como os polifenois) na dieta de pacientes com DII vem sendo sugerida como forma de compensar os processos inflamatórios e pró-oxidantes envolvidos na patogênese da doença, modular a resposta imune e a atividade e a composição da microbiota intestinal (SAXENA et al., 2014; SINGH et al., 2012b; SOMANI et al., 2015).

Produtos derivados de uvas, como sementes, resíduo de vinificação ou suco apresentaram efeitos protetores, incluindo modulação da resposta inflamatória, redução do estresse oxidativo e da gravidade e extensão do dano em modelos animais de colite (BOUSSENNA et al., 2016a; LI et al., 2017; MARCHI et al., 2014; WANG et al., 2011). Os benefícios observados geralmente são atribuídos aos compostos fenólicos, especialmente às antocianinas ou às proantocianidinas, enquanto a participação da fibra presente na casca ou de sua associação com os compostos fenólicos é pouco discutida. Alguns trabalhos também avaliaram o efeito de compostos isolados presentes nas uvas, como o resveratrol, em modelos de colite e também observaram resultados benéficos (LARROSA et al., 2009).

Diferentes modelos animais de DIIs têm sido desenvolvidos para estudar o envolvimento dos diversos fatores na patogênese da doença e testar novas estratégias terapêuticas (CORRIDONI; ARSENEAU; COMINELLI, 2014). Os modelos experimentais de DII mais usados incluem a colite induzida quimicamente (dextran sulfato de sódio [DSS] e ácido 2,4,6-trinitrobenzenosulfônico [TNBS]) e o desenvolvimento espontâneo da inflamação intestinal em animais geneticamente modificados (como os animais *knock-out* para a expressão de IL-10) (ZHU; LI, 2012).

A administração do TNBS por via intracolônica é um modelo comumente usado em ratos e camundongos para induzir inflamação intestinal aguda ou crônica e testar o mecanismo e a eficácia de diversas terapias farmacológicas, além da influência da composição da dieta sobre a inflamação (BRENNA et al., 2013). Apresenta como vantagens a

simplicidade e a reprodutibilidade, o tempo experimental curto e a capacidade de mimetizar tanto a fase aguda como a fase crônica da colite ulcerativa, assim como observado em humanos (MOTAVALLIAN-NAEINI et al., 2012).

1.7 MECANISMOS MOLECULARES ASSOCIADOS À COLITE INDUZIDA POR TNBS

Diversos mecanismos celulares e moleculares que estão envolvidos na regulação da homeostase intestinal encontram-se alterados nas DIIs, dentre eles: formação de EROs e ERNs, manutenção da função de barreira e restituição da mucosa epitelial, regulação da resposta imune e da resposta inflamatória, apoptose, autofagia e estresse do retículo endoplasmático (KHOR, GARDET e XAVIER, 2011).

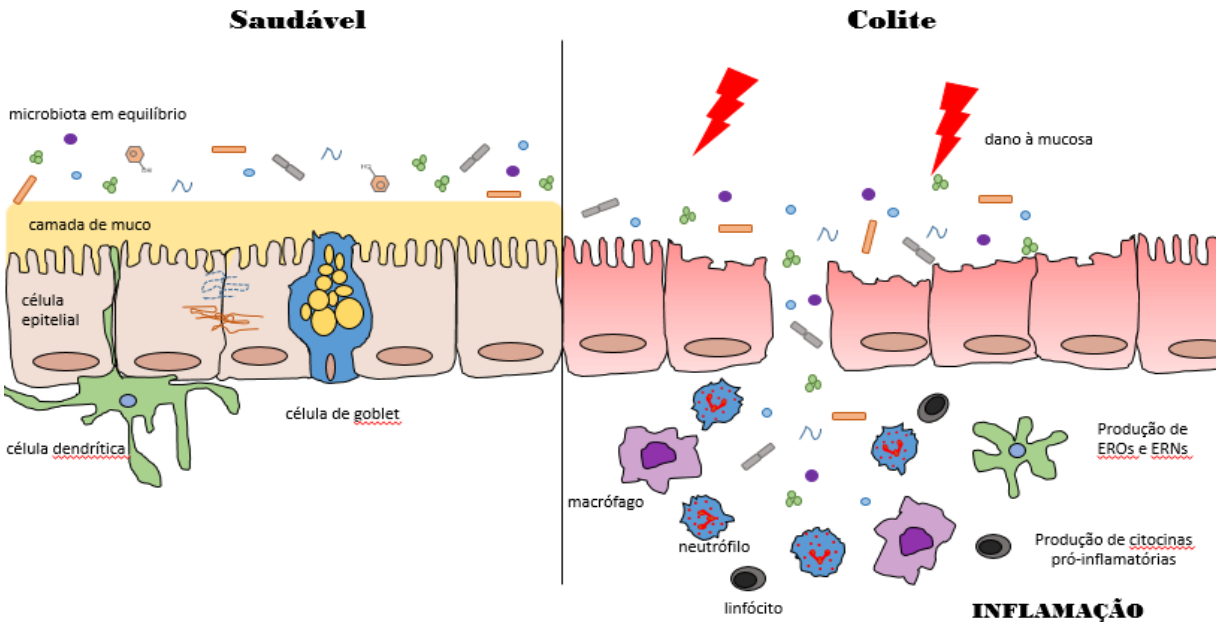
No modelo de colite experimental induzida por TNBS, o rompimento da barreira intestinal é facilitado pela ação do etanol, veículo no qual o TNBS é diluído. Com o dano causado à mucosa, o TNBS é reconhecido pelas células intestinais e ocorre ativação de linfócitos T, os quais interagem com células apresentadoras de antígenos (células dendríticas), que, por sua vez, induzem uma resposta inflamatória mediada por linfócitos T *helper* 1 (Th1). Há liberação de interferon gama (IFN-γ) e os macrófagos passam a produzir fator de necrose tumoral (TNF-α) e outras citocinas pró-inflamatórias, como IL-1β e IL-6 (RANDHAWA et al., 2014; SADAR; VYAWAHARE; BODHANKAR, 2016).

O trato intestinal contém o maior número de células imunes no organismo e a microbiota intestinal opera em um delicado equilíbrio com o sistema imunológico (CHOY et al., 2014). A lesão da mucosa intestinal pode desencadear uma resposta anormal das células imunes locais contra componentes da microbiota comensal resultando em inflamação intestinal crônica (DEMON et al., 2014).

Receptores do tipo *toll* (TLRs) são expressos no epitélio do intestino delgado e do cólon e fazem parte da resposta imune inata. Os TLRs, particularmente TLR-4 e TLR-2, estão implicados na iniciação, curso e resolução da inflamação intestinal (TERÁN-VENTURA et al., 2014). Pacientes com inflamação intestinal crônica ou neoplasia intestinal têm expressão aumentada desses receptores, especialmente do TLR-4, ativado por lipopolissacarídeos (LPS) de bactérias gram-negativas (SANTAOLALLA; SUSSMAN; ABREU, 2011; TERÁN-VENTURA et al., 2014). Esses receptores reconhecem padrões moleculares associados a patógenos (PAMPs) e padrões moleculares associados a dano (DAMPs) que ativam a

sinalização intracelular e a resposta inflamatória com o recrutamento de células inflamatórias (SANTAOLALLA; SUSSMAN; ABREU, 2011).

Figura 2 – Patogênese da inflamação intestinal.



Fonte: autoria pessoal. EROs = espécies reativas de oxigênio; ERNs = espécies reativas de nitrogênio.

A ativação da resposta imune aumenta a produção de citocinas pró-inflamatórias e promove o aumento da permeabilidade intestinal e o recrutamento de macrófagos e neutrófilos para o tecido (MARCHI et al., 2014). A elevada infiltração de neutrófilos leva a uma produção excessiva de EROs, óxido nítrico (NO) e prostaglandina E2 (PGE-2), que podem resultar na ruptura da barreira intestinal, além de aumento na atividade da mieloperoxidase (MPO), uma enzima presente nos grânulos dos neutrófilos e indicativa da atividade pró-inflamatória (SOCCA et al., 2014).

A geração excessiva de EROs e citocinas está associada à ativação de diversos fatores de transcrição que regulam a resposta inflamatória. Dentre as principais cascatas de sinalização intracelular envolvidas na inflamação intestinal, a via do fator nuclear kappa B (NF-κB), por exemplo, regula a expressão de genes pró-inflamatórios, como os que codificam para a síntese de TNF-α, IL-1β e IL-6, cicloxigenase 2 (COX-2), óxido nítrico sintase induzível (iNOS) e ainda moléculas de adesão, proteínas imunoregulatórias e proteínas de resposta de fase aguda (ARAB et al., 2014; GINER et al., 2011; MARÍN et al., 2013).

O NF-κB é um heterodímero de subunidades p65 e p50 que fica retido em uma forma inativa associada à proteína inibitória IκB no citosol. Quando as células são expostas a condições de estresse, como agentes inflamatórios ou EROs, as quinases IKK-α e IKK-β são ativadas e vão fosforilar a IκB, levando à liberação do NF-κB, que, por sua vez, é translocado até o núcleo para controlar a expressão de genes-alvo (ARAB et al., 2014; LEE et al., 2014a; VENDRAME; KLIMIS-ZACAS, 2015). Uma vez que as EROs podem ativar o NF-κB e induzir a expressão de citocinas pró-inflamatórias, a relação entre o estresse oxidativo e o processo inflamatório é bastante próxima (ARAB et al., 2014; LEE et al., 2014a; VENDRAME; KLIMIS-ZACAS, 2015).

O epitélio intestinal é uma barreira indispensável na interface entre o interstício gastrointestinal e o meio luminal, na regulação do transporte e absorção de íons, água e nutrientes e ainda na atuação como barreira contra toxinas, patógenos e antígenos (HU et al., 2015). Junto com outras proteínas, as junções de oclusão (*tight junctions*) fazem parte do mecanismo de regulação da permeabilidade do epitélio intestinal e são compostas por proteínas transmembranares e *scaffold* intracelulares como as claudinas e as ocludinas (HU et al., 2015). Pacientes com colite ulcerativa tendem a apresentar defeitos nesse mecanismo de barreira, aumento da permeabilidade intestinal, comprometimento da camada de muco, maior entrada de antígenos e ativação da resposta inflamatória intestinal e da apoptose, além da maior secreção de água e solutos, levando ao quadro de diarreia (HU et al., 2015; KHOR; GARDET; XAVIER, 2011).

2 OBJETIVOS

2.1 OBJETIVO GERAL

Estudar os efeitos do uso da radiação UV-C sobre o perfil de antocianinas e sobre o sistema antioxidante enzimático e não-enzimático de uvas e investigar o potencial dos compostos bioativos da casca de uva na prevenção e tratamento da inflamação intestinal em modelo animal.

2.2 OBJETIVOS ESPECÍFICOS

- Avaliar o efeito da UV-C sobre a composição individual de antocianinas e a atividade de enzimas antioxidantes envolvidas na defesa contra o estresse em uvas ‘Isabel’;
- Obter e caracterizar a casca da uva ‘Isabel’ e frações bioativas da casca (polifenois extraíveis, polifenois associados à fibra alimentar e fibra livre de compostos fenólicos);
- Estudar o efeito da casca da uva e suas frações sobre o processo oxidativo e inflamatório em modelo de colite experimental induzida por TNBS em ratos Wistar;
- Estudar o efeito da casca da uva e suas frações sobre o metabolismo da glutationa e o processo de apoptose em modelo animal (ratos Wistar) de colite experimental induzida por TNBS;
- Estudar o efeito da casca da uva e suas frações sobre a função de barreira epitelial intestinal em modelo animal (ratos Wistar) de colite experimental induzida por TNBS.

3 MATERIAL E MÉTODOS

Esta tese é composta por dois experimentos principais. No primeiro experimento foi avaliado o efeito de diferentes doses de UV-C e tempos de armazenamento pós-irradiação sobre a atividade antioxidante enzimática e não-enzimática de uvas 'Isabel' (artigo 1). No segundo experimento, foram estudadas a composição e a aplicação das diferentes frações de compostos bioativos presentes na casca de uvas 'Isabel' em modelo animal de inflamação intestinal (manuscritos 1, 2 e 3).

3.1 EFEITO DA UV-C SOBRE O METABOLISMO ANTIOXIDANTE ENZIMÁTICO E NÃO-ENZIMÁTICO DE UVAS 'ISABEL'

3.1.1 Amostras

As amostras de uva 'Isabel' (safra 2013) foram colhidas no município de Itaara (RS) e levadas imediatamente ao laboratório para corte das bagas na altura do pedúnculo e formação das unidades amostrais (300-400g).

3.1.2 Irradiação das amostras com UV-C

O delineamento experimental foi inteiramente casualizado, constituído de cinco tratamentos (doses de irradiação UV-C: 0, 0.5, 1, 2 e 4 kJ/m^2), com quatro repetições por tratamento, que foram avaliadas ao longo do tempo (1, 3 e 5 dias após a irradiação).

As bagas foram dispostas em calhas de alumínio e submetidas à radiação UV-C através de fonte de radiação com comprimento de onda de 254 nm (Philips® modelo TUV-30W/G30T8) distante 30±1 cm. A intensidade da radiação foi mensurada previamente com o uso de espectroradiômetro (International Light®, modelo RPS900). Os tempos de exposição foram calculados a partir da dose requerida e da irradiância conhecida.

3.1.3 Determinação das atividades enzimáticas

Para determinação da atividade das enzimas, as bagas foram congeladas em nitrogênio líquido e mantidas a -25°C até o momento da análise. Sob nitrogênio líquido, as bagas inteiras sem sementes foram reduzidas a pó em gral com pistilo, para extração enzimática.

A extração para análise da enzima superóxido dismutase (SOD) foi feita de acordo com o proposto por Li et al. (2010) e sua atividade foi avaliada conforme Spitz & Oberley

(1989). A extração para análise da enzima catalase (CAT) foi feita conforme Erkan et al. (2008) e sua atividade foi determinada conforme Aebi (1984). O procedimento para extração da enzima guaiacol peroxidase (G-POD) foi o mesmo já mencionado para a extração da enzima CAT e a atividade foi mensurada conforme Andrade Cuvi et al. (2011). A extração para análise da enzima glutationa redutase (GR) foi a mesma mencionada para a SOD e a atividade foi determinada conforme Carlberg & Mannervick (1979). O conteúdo de proteínas das amostras usadas para avaliação da atividade enzimática foi determinado conforme Bradford (1976) usando albumina bovina como padrão.

3.1.4 Determinação do conteúdo de tiois totais e não-proteicos

A extração para análise de tiois foi feita com tampão fosfato de potássio 0,1M pH 7,8 seguida de centrifugação a 13000 *x* g a 4°C por 20 min. O sobrenadante foi usado para determinação de tiois totais a 412 nm, conforme descrito por Ellman (1959). Para determinação dos tiois não-proteicos, o sobrenadante foi misturado (1:1) com ácido tricloroacético 4% e centrifugado novamente. O conteúdo de tiois não-proteicos foi mensurado nesse novo sobrenadante conforme já mencionado para os tiois totais.

3.1.5 Extração dos compostos fenólicos

Os compostos fenólicos da casca foram extraídos com solução água:etanol:ácido fórmico (27:70:3, v/v/v) numa proporção 1:3 (m/v). A mistura foi homogeneizada em Ultraturrax por 2 min a 12000 rpm e centrifugada a 2300 *x* g por 10 min. O sobrenadante foi coletado e utilizado para determinação da capacidade antioxidante e análise do perfil de antocianinas.

3.1.6 Perfil de antocianinas por CLAE-DAD e CLAE-EM

A análise de antocianinas individuais foi feita em cromatógrafo líquido de alta eficiência (CLAE) (Shimadzu®, Japão) utilizando coluna de fase reversa C18 CLC-ODS (250 mm x 4,6 mm; 5 μm de tamanho de partícula; Shimadzu®, Japão) à temperatura de 40°C e fluxo de 0,8 mL/min de fase móvel composta por água:ácido fórmico (97:3, v/v) (fase móvel A) e acetonitrila (fase móvel B) em modo gradiente: 0-3 min: 0-8% de B; 3-13 min: 8% de B; 13-37 min: 8-20% de B; 37-57 min: 20% de B; 57-58 min: 20-50% de B; 58-63 min: 50% de B; 63-65 min: 50-90% de B; 65-75 min: 90% de B; 75-80 min: 90-0% de B; 80-85 min: 0% de B. O espectro ultravioleta-visível foi registrado de 200-800 nm e os cromatogramas obtidos em 520 nm utilizando detector de arranjo de diodos (DAD) (Shimadzu®, Japão).

Previamente à etapa de identificação, os extratos foram purificados utilizando cartuchos de extração em fase sólida (C18, Merck®, Alemanha) para obtenção da fração antociânica conforme descrito por Rodriguez-Saona e Wrolstad (2001) e adaptado por Bochi et al. (2014). A identificação foi realizada em sistema de CLAE (Agilent®, Estados Unidos) acoplada a espectrômetro de massas (EM) (5500QTRAP, AB Sciex®) equipado com fonte de ionização em eletrospray operada em modo positivo. As condições cromatográficas foram as mesmas descritas anteriormente para o sistema CLAE-DAD. Os íons foram analisados numa faixa de razão massa/carga (m/z) de 100 a 900 e os fragmentos correspondentes às antocianidinas (m/z 271, m/z 287, m/z 301, m/z 303, m/z 317 e m/z 331 para pelargonidina, cianidina, peonidina, delfinidina, petunidina e malvidina, respectivamente) foram pesquisados. A identificação foi feita a partir da combinação das informações obtidas com o espectro UV-Vis do sistema CLAE-DAD, com a ordem de eluição em coluna de fase reversa e de acordo com o padrão de fragmentação obtido no sistema CLAE-EM.

3.1.7 Ensaio de remoção do radical superóxido

A capacidade dos extratos de remover o radical superóxido ($O_2^{-\cdot}$) formado pelo sistema xantina-xantina oxidase foi avaliada indiretamente através da inibição da reação de redução do nitroazul de tetrazólio até formazan azul ocorrida na presença de radical superóxido em 560 nm (SPITZ; OBERLEY, 1989). A metodologia de análise foi a mesma utilizada para avaliação da atividade da enzima superóxido dismutase, porém, utilizando os extratos ricos em compostos fenólicos.

3.1.8 Análise estatística

Os dados foram submetidos à análise de variância (ANOVA) de duas vias, seguida de comparação de médias através do teste de Tukey, com resultados considerados estatisticamente significativos quando $p<0,05$.

3.2 CARACTERIZAÇÃO DA CASCA DE UVA, ISOLAMENTO DAS FRAÇÕES BIOATIVAS E AVALIAÇÃO DO EFEITO PROTETOR EM MODELO DE COLITE EXPERIMENTAL EM RATOS

3.2.1 Obtenção do pó da casca de uva (PC)

As cascas das uvas 'Isabel' (*V. labrusca* x *V. vinifera* L., safras 2013 e 2014) foram removidas manualmente, liofilizadas e pulverizadas em micromoinho. O pó da casca foi avaliado quanto à sua composição centesimal (umidade, proteína, cinzas, lipídios, fibra) e fitoquímica (compostos fenólicos, antocianinas monoméricas, proantocianidinas) e quanto à sua capacidade antioxidante.

3.2.2 Extrato rico em compostos fenólicos extraíveis (PE)

Para obtenção do extrato contendo os fenólicos solúveis/extraíveis, o pó da casca foi extraído (1:40, m/v) com solução metanol:água (50:50, v/v, pH 2) sob agitação constante, por 1 h e à temperatura ambiente. A mistura foi centrifugada a 2700 *x* g por 15 min e o sobrenadante foi coletado. Ao sedimento adicionou-se uma solução de acetona:água (70:30, v/v), o processo de extração foi repetido e os sobrenadantes obtidos foram combinados (ZURITA; DÍAZ-RUBIO; SAURA-CALIXTO, 2012). Esse extrato foi concentrado em rotaevaporador a 39°C antes de ser incorporado na dieta e teve sua composição fitoquímica e capacidade antioxidante avaliada por métodos espectrofotométricos (compostos fenólicos, antocianinas monoméricas, proantocianidinas solúveis, capacidade de remoção do radical peroxil e do radical sintético DPPH, capacidade de redução de íons férricos) e por cromatografia líquida de alta eficiência acoplada a espectrômetro de massas (CLAE-EM) (ácidos fenólicos, flavonois, antocianinas, resveratrol).

Previamente à análise cromatográfica da fração PE, um volume conhecido de extrato foi purificado em cartucho SPE-C18 (1 g, Phenomenex®, Estados Unidos) para obtenção das frações antociânica e não-antociânica, conforme descrito por Rodriguez-Saona e Wrolstad (2001) e adaptado por Bochi et al. (2014). Cada fração foi rotaevaporada, ressuspensa num volume conhecido e filtrada em filtros de membrana de PTFE (tamanho de poro 0,22 μm) antes de ser injetada no sistema CLAE.

A fração antociânica foi separada utilizando CLAE (Shimadzu®, Japão) acoplado a detector UV-Vis (Shimadzu®, Japão) utilizando coluna C18 Kinetex 100-A (100 mm x 4,6 mm; 2,6 μm de tamanho de partícula; Phenomenex®, Estados Unidos) à temperatura de 38°C e fluxo de 0,9 mL/min de fase móvel composta por água:ácido fórmico (97:3, v/v) (fase

móvel A) e acetonitrila (fase móvel B) em modo gradiente: 0 min (10% de B), 0-20 min (30% de B), 20-25 min (80% de B), 25-27 min (80% de B), 27-29 min (10% de B) e 29-34 min (10% de B). Foi obtido o espectro UV-visível e os cromatogramas foram analisados em 520 nm. A quantificação foi feita utilizando curva de malvidina-3-glicosídeo (Sigma-Aldrich®, Estados Unidos) como padrão (0,05-60 mg/L, R^2 = 0,9924) e os resultados expressos em mg equivalentes a malvidina-3-glicosídeo/100 g casca seca.

Já os compostos fenólicos não-antociânicos foram separados em CLAE (Shimadzu®, Japão) utilizando coluna C18 Hypersil Gold (150 mm, 4,6 mm; 5 μm de tamanho de partícula, Thermo Fischer Scientific®, Estados Unidos) a 38°C, num fluxo de 1 mL/min e fase móvel composta por água:ácido fórmico (99,9:0,1 v/v) contendo 5% de metanol (fase móvel A) e acetonitrila:ácido fórmico (99,9:0,1 v/v) em modo gradiente: 0-10 min (0-4% de B), 10-21 min (4% de B), 21-55 min (4-16% de B), 55-70 min (16-50% de B), 70-72 min (50-100% de B), 72-80 min (100% de B), 80-83 min (100-0% de B) e 83-92 min (0% de B). Os espectros UV-visível foram obtidos em detector DAD numa faixa de 200 a 800 nm e os cromatogramas foram analisados em 280 nm (derivados do ácido hidroxibenzoico e flavanois), 305 nm (resveratrol), 320 nm (derivados do ácido hidroxicinâmico) e 360 nm (flavonois). A quantificação dos derivados do ácido hidroxicinâmico, derivados do ácido hidroxibenzoico, flavanois, estilbenos e flavonois foi feita utilizando os padrões de ácido cafeico (0,02 – 60 ppm; R^2 = 0,9982), ácido gálico (0,04 – 60 ppm; R^2 = 0,9979), catequina (0,08 – 60 ppm; R^2 = 0,9981), resveratrol (0,044 – 60 ppm; R^2 = 0,9954) e kaempferol-3,7-diglicosídeo (0,1 – 60,3 ppm; R^2 = 0,9976), respectivamente.

Para identificação, o espectro de massas dos compostos antociânicos e não-antociânicos foi obtido de 100 a 1000 m/z em espectrômetro de massas (Bruker Daltonics®, Estados Unidos) com fonte de ionização por eletrospray e analisador por tempo de voo (micrOTOF-QIII). O eluato da coluna foi diminuído para metade do fluxo inicial antes da ionização. Os parâmetros utilizados foram os seguintes: ionização no modo positivo para antocianinas e negativo para fenólicos; 3000V de voltagem de capilar; temperatura do gás de secagem (N_2) 310 °C; fluxo 8 L/min; pressão do gás nebulizador 4 bar. Os compostos não-antociânicos e antociânicos foram identificados de acordo com a ordem de eluição e tempo de retenção em coluna de fase reversa C18, com os espectros UV-visível e de massas obtidos, por comparação com padrões analisados sob as mesmas condições e ainda com dados disponíveis na literatura.

3.2.3 Fração rica em fibras e fenólicos não extraíveis (resíduo RE)

O resíduo obtido com a extração dos fenólicos solúveis constituiu a fração rica em fibras e fenólicos ligados (Esquema 1). Esse resíduo de extração foi seco em capela de exaustão *overnight* em temperatura ambiente e foi avaliado quanto à sua composição centesimal (umidade, fibra, cinzas, lipídios, proteínas) e fitoquímica (proantocianidinas ligadas).

3.2.4 Fração rica em fibras e pobre em fenólicos (resíduo RF)

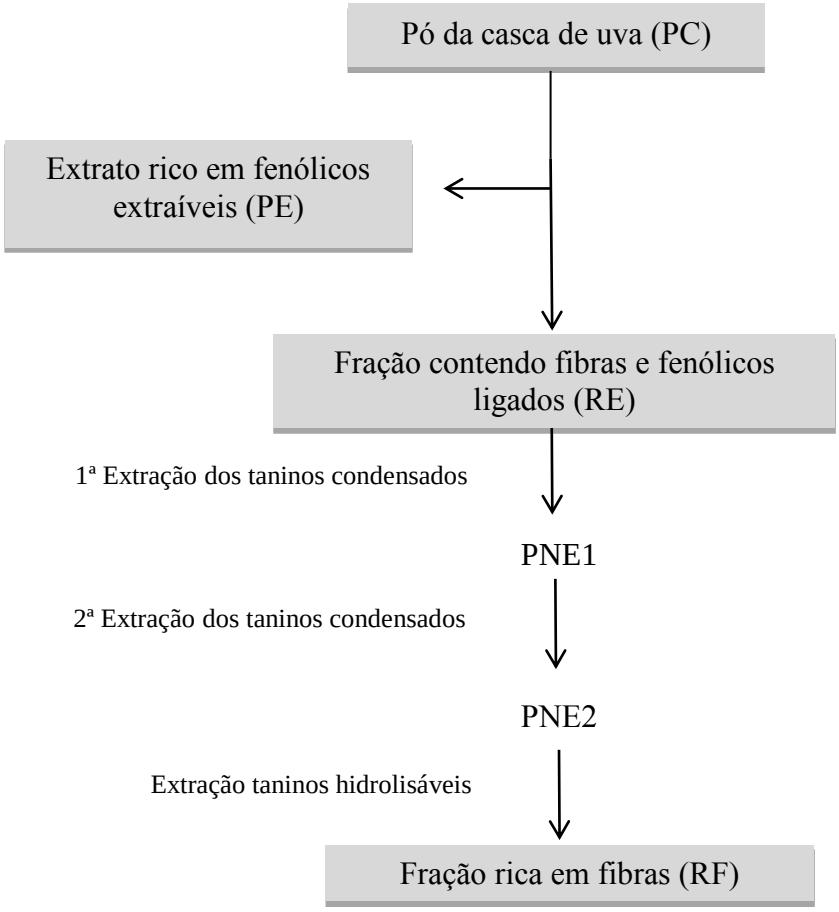
A fração com teor reduzido de fenólicos foi obtida após a extração dos fenólicos ligados do resíduo obtido na extração dos fenólicos solúveis do pó da casca de uva (Esquema 1). Para isso, o resíduo RE foi submetido à extração das proantocianidinas ligadas com solução de HCl:butanol (5:95, v/v) contendo $FeCl_3$ (0,7 g/L) a 100°C por 3 h (ZURITA; DÍAZ-RUBIO; SAURA-CALIXTO, 2012). O resíduo obtido dessa extração (PNE1) foi submetido a uma nova extração para máxima remoção. A cada etapa, o sobrenadante foi coletado até um volume final conhecido para determinação espectrofotométrica do teor de proantocianidinas (ZURITA; DÍAZ-RUBIO; SAURA-CALIXTO, 2012). O último resíduo (PNE2) foi então submetido à extração dos taninos hidrolisáveis residuais utilizando o método proposto por Hartzfeld et al. (2002), com uma solução de metanol:H_2SO_4 (90:10, v/v) a 85°C por 20 h. Após a extração dos taninos hidrolisáveis, o resíduo obtido (RF) foi lavado com água até remoção do ácido e seco em estufa a 55°C por 48 h para remoção da umidade e de qualquer solvente residual. Depois de seco, o resíduo RF teve seu conteúdo de fibra alimentar e de umidade avaliados.

3.2.5 Composição centesimal e características físico-químicas do pó da casca de uva e das suas frações

Umidade, cinzas e proteína bruta foram determinados segundo a *Association of Official Analytical Chemists* (AOAC, 2006). O conteúdo de fibra alimentar total e a fração insolúvel foram determinados por método gravimétrico (PROSKY et al., 1988) e a fração solúvel foi calculada a partir da diferença entre a fibra alimentar total e a fração insolúvel. Os lipídios totais foram extraídos usando clorofórmio e metanol e quantificados por gravimetria (BLIGH; DYER, 1959). Os carboidratos não fibrosos foram calculados por diferença. Os açúcares solúveis foram extraídos usando álcool etílico 80% e determinados espectrofotometricamente através do ensaio fenol-ácido sulfúrico (HALL, 2000). O

rendimento de cada fração a partir do pó da casca de uva e a composição centesimal do pó da casca e de suas frações estão dispostos nas tabelas 1 e 2, respectivamente.

Esquema 1 - Obtenção das diferentes frações bioativas a partir de cascas de uva 'Isabel'.



Fonte: autoria pessoal. PNE1= resíduo da primeira extração dos taninos condensados; PNE2= resíduo da segunda extração dos taninos condensados.

3.2.6 Composição fitoquímica e capacidade antioxidante do pó da casca de uva e das suas frações

O conteúdo de fenólicos totais foi determinado pelo método de Folin-Ciocalteau (SINGLETON E ROSSI, 1965) e os resultados expressos como mg equivalentes ao ácido gálico por 100 g de casca. O conteúdo de antocianinas totais foi determinado pelo método do pH diferencial (WROLSTAD; GIUSTI, 2001) e expresso como mg equivalentes a malvidina-3-glicosídeo por 100 g de casca. O conteúdo de proantocianidinas solúveis foi mensurado de

acordo com o proposto por Wallace e Giusti (2010) e os resultados foram expressos como mg equivalentes a catequina por 100 g de casca.

A capacidade antioxidante foi avaliada pelos métodos de redução de íons férricos - FRAP (BENZIE E STRAIN, 1996), remoção do radical livre DPPH (BRAND-WILLIAMS, CUVELIER, BERSET, 1995) e remoção do radical peroxil - ORAC (OU, HAMPSCH-WOODILL, PRIOR, 2001). Os resultados foram expressos em mmol equivalentes a Trolox por 100 g de casca.

Tabela 1 - Rendimento (%) obtido na extração das frações do pó da casca de uva 'Isabel'.

	Rendimento cumulativo (%)	Rendimento sequencial (%)
Pó da casca de uva	100	100
Resíduo RE	27,2 ± 1,8	27,2 ± 1,8
Resíduo PNE1	14,1 ± 1,5	51,9 ± 5,5
Resíduo PNE2	12,0 ± 0,5	85,0 ± 3,7
Resíduo RF	4,4 ± 0,3	36,5 ± 2,3

Resultados apresentados como média ± desvio padrão. RE= fração rica em fenólicos não-extraíveis; PNE1= resíduo da 1ª extração dos taninos condensados; PNE2= resíduo da 2ª extração dos taninos condensados; RF= fração rica em fibra e pobre em fenólicos.

3.2.7 Animais e dieta

Foram utilizados 56 ratos machos adultos HanUnib:WH (Wistar) com peso inicial entre 180 e 200 g provenientes do CEMIB/UNICAMP. O estudo foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas (CEUA/UNICAMP) (protocolo n° 3815-1/2015) (Anexo 1). Os animais foram abrigados em gaiolas metálicas individuais, sob ciclo claro/escuro de 12h, com temperatura e umidade controladas, e passaram por período de adaptação de 7 dias, durante o qual receberam ração AIN-93G (REEVES, 1997) sob a forma peletizada (Tabela 3).

Tabela 2 - Composição centesimal e características físico-químicas do pó da casca de uva 'Isabel' e de suas frações bioativas.

	Pó da casca	Extrato PE	Resíduo RE	Resíduo RF
	%, peso seco			
Cinzas	6,11 ± 0,77	n.a.	4,53 ± 0,14	0,17 ± 0,02
Extrato etéreo	3,62 ± 0,26	n.a.	6,27 ± 0,07	n.a.
Proteínas	6,12 ± 0,08	n.a.	14,95 ± 0,11	4,12 ± 0,02
Fibra alimentar	26,37 ± 2,86	n.a.	77,55 ± 1,70	96,88 ± 0,87
Fibra solúvel	8,36 ± 0,57	n.a.	15,05 ± 0,73	1,52 ± 0,70
Fibra insolúvel	16,38 ± 1,81	n.a.	62,50 ± 1,36	95,36 ± 0,21
Carboidratos não-fibrosos [&]	57,78	n.a.	n.d.	n.d.
Açúcares solúveis [$]	58,22 ± 6,83	15,96 ± 1,27	1,68 ± 0,18	n.a.
pH	3,30 ± 0,06	n.a.	3,17 ± 0,02	n.a.
Acidez (%)	0,92 ± 0,02	n.a.	0,67 ± 0,01	n.a.

Resultados apresentados como média ± desvio padrão. PE = fração rica em fenólicos extraíveis; RE= fração rica em fenólicos não-extraíveis; RF= fração rica em fibra e pobre em fenólicos. n.d. = não detectado. n.a. = não avaliado. [&] Calculados por diferença entre 100 e a soma dos demais componentes. [$] g de sacarose por 100 g (peso seco).

Após o período de adaptação, os animais receberam as dietas experimentais (dieta AIN-93G adicionada ou não de casca de uva ou suas frações) por um período de 15 dias (Esquema 2). A quantidade de pó da casca de uva adicionada na dieta (8%) foi escolhida para resultar em oferta de cerca de 40 mg de fenólicos/kg/dia, considerando que foram ofertados 20 g de ração/animal/dia e água *ad libitum* durante o período experimental. O consumo foi monitorado diariamente e o peso corporal a cada dois dias. Para identificar a fração de compostos bioativos responsável pelos efeitos benéficos da casca de uva na prevenção/tratamento da colite, foram utilizadas 5 dietas: dieta controle (ração AIN-93G), dieta com pó da casca de uva (ração AIN-93G contendo 8% de pó da casca de uva), dieta com fenólicos extraíveis (ração AIN-93G contendo extrato PE em quantidade equivalente à concentração desta fração na dieta com pó da casca de uva), dieta com fibra e fenólicos ligados a fibra (ração AIN-93G contendo resíduo RE em quantidade equivalente à concentração desta fração na dieta do grupo pó da casca de uva), dieta com fibra e pobre em fenólicos (ração AIN-93G contendo resíduo RF em quantidade equivalente à concentração desta fração na dieta do grupo pó da casca de uva).

Os conteúdos de celulose e de sacarose adicionados nas formulações foram corrigidos de acordo com a composição centesimal de cada fração para manter constantes as concentrações de fibra e açúcares em todas as dietas (Tabela 3).

No 16º dia foi induzida a colite (descrito no item 3.2.8) e após a indução, os animais ntinuaram recebendo as dietas experimentais por mais 7 dias até a eutanásia (Esquema 2).

Tabela 3 - Composição da dieta padrão AIN-93G e das dietas experimentais.

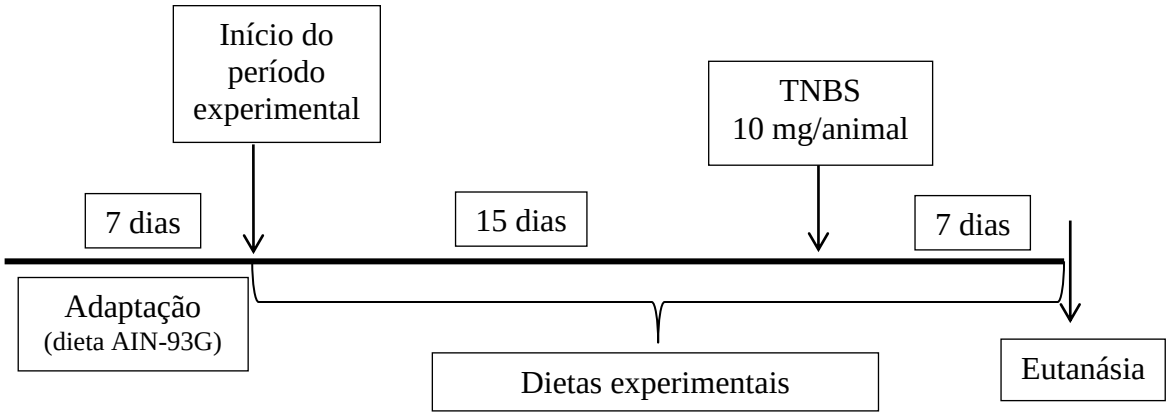
	AIN-93G	PC	PE	RE	RF
Ingredientes					
Amido de milho (g)	440,9	420,2	440,9	433,4	440,4
Caseína (g)	156,6	156,6	156,6	156,6	156,6
Maltodextrina (g)	132,0	132,0	132,0	132,0	132,0
Sacarose (g)	100,0	57,8	68,3	100,0	100,0
Óleo de soja (g)	70,0	70,0	70,0	70,0	70,0
Celulose (g)	50,0	33,1	50,0	35,8	47,0
Mix de minerais (g)	35,0	35,0	35,0	35,0	35,0
Mix de vitaminas (g)	10,0	10,0	10,0	10,0	10,0
L-cistina (g)	3,0	3,0	3,0	3,0	3,0
Bitartarato de colina (g)	2,5	2,5	2,5	2,5	2,5
Terc-butilhidroquinona (g)	0,014	0,014	0,014	0,014	0,014
Pó de casca de uva (g)		80,0			
Extrato PE (mL)			198,0		
Fração RE (g)				21,7	
Fração RF (g)					3,5
Total (g)	1000	1000	1000	1000	1000
Valor energético#					
Valor energético (kcal/kg)	3948,0	3928,7	3948,0	3943,3	3946,6
Composição centesimal					
Cinzas*	2,9 ± 0,2	3,6 ± 0,1	3,9 ± 0,1	2,9 ± 0,1	2,9 ± 0,1
Proteínas*	15,2 ± 0,5	17,8 ± 1,5	17,9 ± 0,6	15,5 ± 0,5	14,8 ± 1,0
Lipídeos*	14,5 ± 1,0	15,6 ± 0,2	15,9 ± 0,2	14,8 ± 1,0	14,5 ± 0,2
Carboidratos totais&*	67,4	63,0	62,3	66,8	67,8

Resultados apresentados como média ± desvio padrão. # Calculado a partir da contribuição energética de cada ingrediente adicionado a ração. * % em peso seco & Calculados por diferença entre 100 e a soma dos demais constituintes. PC = pó de casca de uva; PE = fração rica em fenólicos extraíveis; RE = fração rica em fenólicos não-extraíveis; RF = fração rica em fibra e pobre em fenólicos.

3.2.8 Indução da colite

Para a indução da colite, os animais foram deixados em jejum e levemente sedados com isoflurano para administração de dose única de 10 mg de ácido 2,4,6 trinitrobenzenosulfônico (TNBS) (diluído em 0,25 mL de etanol 50%, v/v) por via intraretal (MORRIS et al., 1989) através de uma sonda uretral de PVC flexível (2 mm de diâmetro) cuidadosamente introduzida até 8 cm a partir do ânus. Após a administração do TNBS, os ratos foram mantidos por alguns segundos de cabeça para baixo a fim de garantir que a droga não fosse eliminada. Os ratos que não foram submetidos à indução da colite (grupo controle) receberam apenas o veículo (etanol 50%) por via intraretal. O grupo controle positivo recebeu mesalazina (25 mg/animal/dia) por gavagem durante os 7 dias subsequentes à indução da colite (Esquema 2).

Esquema 2 - Protocolo de tratamento e indução da colite.



Fonte: autoria pessoal. TNBS = ácido 2,4,6-trinitrobenzenosulfônico.

3.2.9 Desenho experimental

Os animais foram divididos ao acaso em 7 grupos experimentais (Tabela 4):

1. **Grupo controle (C) (n=8):** receberam dieta AIN-93G e veículo etanol 50% por via intraretal.
2. **Grupo colite (CC) (n=8):** receberam dieta AIN-93G e 10 mg de TNBS por via intraretal;
3. **Grupo colite + mesalazina (CM) (n=8):** receberam dieta AIN-93G, 10 mg de TNBS por via intraretal e mesalazina (25 mg/animal/dia) por gavagem nos 7 dias subsequentes à indução da colite;
4. **Grupo colite + pó da casca (PC) (n=8):** receberam dieta AIN-93G contendo 8% de pó de casca de uva e 10 mg de TNBS por via intraretal;
5. **Grupo colite + fenólicos extraíveis (PE) (n=8):** receberam dieta AIN-93G adicionada de extrato de fenólicos solúveis da casca de uva (em quantidade equivalente à concentração desta fração na dieta do grupo 4) e 10 mg de TNBS por via intraretal;
6. **Grupo colite + resíduo de extração dos fenólicos extraíveis (RE) (n=8):** receberam dieta AIN-93G adicionada do resíduo da extração dos fenólicos solúveis, que consiste na fibra e fenólicos associados, em quantidade equivalente à concentração desta fração na dieta do grupo 4) e 10 mg de TNBS por via intraretal;

7. **Grupo colite + fração rica em fibras (RF) (n=8):** receberam dieta AIN-93G adicionada do resíduo da extração dos fenólicos associados à fibra (em quantidade equivalente à concentração desta fração na dieta do grupo 4) e 10 mg de TNBS por via intraretal.

Tabela 4 - Distribuição das dietas e tratamentos entre os grupos experimentais.

Grupos	Indução da colite	Tratamento de referência	Compostos bioativos da casca de uva		
	TNBS	Mesalazina	Fibra da casca	Fenólicos extraíveis	Fenólicos ligados
Controle					
CC	X				
CM	X	X			
PC	X		X	X	X
PE	X			X	
RE	X		X		X
RF	X		X		

CC = colite não tratado; CM = colite tratado com mesalazina; PC = pó de casca de uva; PE = fração rica em fenólicos extraíveis; RE = fração rica em fenólicos não-extraíveis; RF = fração rica em fibra e pobre em fenólicos; TNBS= ácido 2,4,6 trinitrobenzenosulfônico.

3.2.10 Índice de gravidade da colite

A gravidade da colite foi avaliada diariamente nos 7 dias subsequentes à sua indução através do índice de atividade de doença (IAD) utilizando a soma dos escores de dano para os 3 critérios apresentados na Tabela 5 (ARAB et al., 2014; HÅKANSSON et al., 2009). A perda de peso após a indução da colite foi calculada em relação ao peso inicial de cada animal antes da indução.

3.2.11 Coleta do material biológico

Depois de decorrido o período experimental, os animais foram anestesiados com injeção intraperitoneal de quetamina e xilazina (90 e 5 mg/kg de peso corporal, respectivamente) e eutanasiados por exsanguinação através de punção cardíaca. O sangue coletado foi disposto em tubos contendo gel acelerador de coagulação para obtenção do soro após centrifugação a 2000 *x* g por 15 min. O soro foi armazenado a -80°C até a avaliação do status antioxidante e dos marcadores inflamatórios.

Tabela 5 - Escores de dano para avaliar a gravidade da colite induzida por TNBS.

Critério de dano	Escore de dano				
	0	1	2	3	4
Perda de peso	<1%	1 - 5%	5 - 10%	10 - 15%	> 15%
Consistência das fezes	Fezes normais, com *pellets* bem formados		Fezes moles/ pastosas, que não se aderem ao ânus		Diarreia, fezes líquidas que se aderem ao ânus
Sangramento retal	Negativo		Positivo		Sangramento intenso

O baço foi removido e pesado. O cólon foi removido, lavado com NaCl 0,9% (m/v) gelado e seco em gaze para avaliação do peso e comprimento. Após, o cólon foi aberto longitudinalmente para avaliação macroscópica da lesão intestinal. Para análise histopatológica, parte do cólon representativa das áreas lesionada e não lesionada foi excisada (a distância para coleta do cólon lesionado foi padronizada para todos os grupos em cerca de 4 cm a partir do reto) e imersa em formaldeído 6%. Após, o restante do cólon foi cortado em pequenos pedaços e fracionado em 3 partes (Western blotting, expressão gênica e homogeneizado) e imediatamente congelado em nitrogênio líquido. O homogeneizado foi feito com tampão fosfato 0,1 M, pH 7,4, usando politron e centrifugado a 1500 *x* g por 15 min a 4°C para coleta do sobrenadante, que foi utilizado para determinação da atividade das enzimas antioxidantes, conteúdo de tiois totais, quantificação das substâncias reativas ao ácido tiobarbitúrico (TBARS) e determinação de citocinas inflamatórias.

O ceco foi removido, pesado e seu conteúdo fecal coletado e imediatamente congelado em nitrogênio líquido e armazenado a -80°C para posterior análise do pH e dos ácidos graxos de cadeia curta.

3.2.12 Análises do soro

3.2.12.1 Marcadores do status antioxidante

Para análise do status antioxidante, amostras de soro foram desproteinizadas com etanol/água ultrapura e ácido metafosfórico 0,75 mM (LEITE et al., 2011), homogeneizadas em vortex e centrifugadas a 20000 *x* g por 10 min a 4°C. O sobrenadante foi coletado e usado para determinar a capacidade antioxidante pelos métodos de remoção do radical peroxil (ORAC) (OU, HAMPSCH-WOODILL, PRIOR, 2001) e capacidade de redução de íons

férricos (FRAP) (BENZIE E STRAIN,1996). Os resultados foram expressos como nmol equivalentes a Trolox por mg de proteína.

3.2.12.2 Marcadores de estresse oxidativo

A peroxidação lipídica foi avaliada pela determinação das substâncias reativas ao ácido tiobarbitúrico (TBARS) em 532 nm conforme descrito por Ohkawa, Ohishi e Yagi (1979) e os resultados foram expressos como nmol de malonaldialdeído (MDA) por mL de soro.

A oxidação proteica foi avaliada através da determinação dos produtos avançados de oxidação proteica (AOPP) a 340 nm (HANASAND et al., 2012) e os resultados foram expressos em µmol de AOPP por mg de proteína.

Os níveis de óxido nítrico (NO) foram mensurados de forma indireta através da determinação dos seus metabólitos estáveis, especialmente nitrato e nitrito (MIRANDA; ESPEY; WINK, 2001) e os resultados expressos como nmol de nitrito por mL de soro.

Os níveis de tiois totais foram determinados a 412 nm após reação com 5,5-ditiobis-2 ácido nitrobenzoico (DTNB), conforme descrito por Ellman (1959) e os resultados expressos como nmol de GSH por mL de soro.

3.2.12.3 Marcadores inflamatórios

Os níveis de interleucina 1β (IL-1β) e do fator de necrose tumoral alfa (TNF-α) foram determinados pelo método de ELISA (PeproTech®, Brasil), seguindo as instruções do fabricante.

3.2.13 Análises do cólon

3.2.13.1 Análise macroscópica

O cólon foi medido, pesado e avaliado macroscopicamente de acordo com protocolo adaptado de Bell, Gall e Wallace (1995) e Marchi et al. (2014) (Tabela 6). O tecido foi mantido sobre gelo durante a avaliação, fotografado e logo após cortado e congelado em nitrogênio líquido.

Tabela 6 - Critérios para avaliação do dano macroscópico do cólon.

Escore	Critério de dano
	Quanto à presença de hiperemia e úlceras
0	Normal, sem qualquer dano aparente
1	Hiperemia, ausência de úlceras
2	Úlcera sem inflamação significativa
3	Úlcera com inflamação em um local
4	2 ou mais sítios de ulceração/inflamação
5	2 ou mais grandes sítios de ulceração e inflamação ou um sítio de ulceração/inflamação, com extensão maior que 1 cm ao longo do comprimento do cólon
6 -10	Se o dano cobre mais que 2 cm ao longo do comprimento do cólon, o escore aumenta em uma unidade a cada centímetro adicional de lesão
	Quanto à presença de adesões
0	Sem adesões
1	Aderências leves, facilmente separadas de outros tecidos
2	Adesões severas

3.2.13.2 Análise microscópica

As amostras foram fixadas em formaldeído 6%, embebidas em parafina e seccionadas a 6 µm em micrótomo rotativo. As secções foram então coradas com hematoxilina e eosina e analisadas em microscópio por um patologista sem conhecimento da identificação dos grupos. O escore de dano histopatológico do cólon foi adaptado de Krause et al. (2014) (Tabela 7).

3.2.13.3 Marcadores inflamatórios

O homogeneizado do cólon foi usado para avaliação dos níveis de IL-1β, interleucina-10 (IL-10) e TNF-α pelo método de ELISA (PeproTech®, Brasil), conforme instruções do fabricante.

A atividade da enzima mieloperoxidase (MPO) foi medida através da oxidação da o-dianisidina dependente de peróxido, conforme Krawisz, Sharon e Stenson (1984).

A expressão gênica de marcadores inflamatórios no cólon foi feita após extração do RNA total com Trizol (InvitrogenCorp., Carlsbad, CA), síntese do cDNA usando kits de transcrição reversa e amplificação usando primers específicos para os genes de interesse (IL-6, IL-1β, IL-10, TNF-α, NF-κB p65, MMP-9) e reação em cadeia da polimerase em tempo

real (RT-qPCR). A expressão dos genes de interesse foi normalizada em relação ao gene estrutural GAPDH.

Tabela 7 - Critérios para avaliação do dano microscópico do cólon.

Gravidade da inflamação		Extensão da inflamação		Dano às criptas		Envolvimento, %	
0	Nenhuma	0	Nenhuma	0	Nenhum	0	0 %
1	Leve	1	Mucosa	1	1/3 Basal	x 1	1 – 25 %
2	Moderada	2	Mucosa e submucosa	2	2/3 Basal	x 2	26 –50 %
3	Severa	3	Transmural	3	Perda das criptas, superfície epitelial presente	x 3	51 –75 %
				4	Perda das criptas e da superfície epitelial	x 4	> 75 %

Escore total = (gravidade da inflamação x % de envolvimento) + (extensão da inflamação x % de envolvimento) + (dano às criptas x % de envolvimento).

A expressão proteica dos marcadores inflamatórios COX-2, IKK-β, *p*NF-κB p50 e receptor *toll-like* TLR-4 foi avaliada por Western blotting. As proteínas foram extraídas e submetidas à eletroforese em gel SDS-PAGE. Após separação, as proteínas foram transferidas para uma membrana de nitrocelulose e incubadas com anticorpos primários específicos para as proteínas de interesse. Após, foi realizada a reação com o anticorpo secundário conjugado à peroxidase e as bandas foram visualizadas em sistema de detecção e fotodocumentadas. A expressão das proteínas de interesse foi normalizada em relação à β-actina.

3.2.13.4 Marcadores de estresse oxidativo

As amostras de cólon foram homogeneizadas em tampão fosfato 0,1 M pH 7,4 (1:4) e o sobrenadante utilizado para determinação de peroxidação lipídica e proteica, níveis de óxido nítrico, tiois totais (metodologias já mencionadas no item 3.2.12.2) e para a atividade das enzimas antioxidantes.

A atividade da enzima superóxido-dismutase (SOD) foi avaliada colorimetricamente conforme Spitz e Oberley (1989). A atividade da enzima catalase (CAT) foi avaliada de acordo com o método de Aebi (1984). A atividade da enzima glutationa redutase (GR) foi determinada utilizando glutationa oxidada (GSSG) e NADPH conforme descrito por Carlberg e Mannervick (1979), enquanto a da glutationa-S-transferase (GST) foi determinada usando

1-cloro-2,4-dinitrobenzeno (CDNB) e GSH como substratos em pH neutro a 340 nm (HABIG; PABST; JAKOBY, 1974). Já a atividade da glutationa peroxidase (GPx) foi avaliada de acordo com o método de Paglia e Valentine (1967) baseado na capacidade de a enzima oxidar a glutationa. O conteúdo de proteína das amostras usadas para avaliação da atividade enzimática foi determinado pelo método de Bradford, usando albumina bovina como padrão (BRADFORD, 1976).

A expressão gênica das enzimas glutationa sintase e glutamato cisteína ligase (subunidades catalítica e modificadora), responsáveis pela síntese de glutationa, foi feita após extração do RNA total com Trizol (InvitrogenCorp., Carlsbad, CA), transcrição do cDNA e amplificação usando primers específicos e reação em cadeia da polimerase em tempo real (RT-qPCR). A expressão dos genes de interesse foi normalizada em relação ao gene estrutural β-actina.

3.2.13.5 Marcadores de apoptose

A expressão gênica da caspase-3 e da caspase-9 foi feita após extração do RNA total com Trizol (InvitrogenCorp., Carlsbad, CA), transcrição do cDNA e amplificação usando primers específicos para os genes de interesse e reação em cadeia da polimerase em tempo real (RT-qPCR). A expressão dos genes de interesse foi normalizada em relação ao gene estrutural β-actina.

3.2.13.6 Marcadores da integridade da barreira epitelial intestinal

A expressão dos genes que codificam as proteínas envolvidas na formação das *tight junctions* (ocludina-1, claudina-2 e zônula ocludens-ZO-1) foi feita após extração do RNA total com Trizol (InvitrogenCorp., Carlsbad, CA), transcrição do cDNA e amplificação usando primers específicos para os genes de interesse e reação em cadeia da polimerase em tempo real (RT-qPCR). A expressão dos genes de interesse foi normalizada em relação ao gene estrutural GAPDH.

3.2.14 Análises das fezes

3.2.14.1 Ácidos graxos de cadeia curta (AGCC)

Os AGCC foram extraídos conforme Zhao, Nyman e Jonsson (2006) e analisados por cromatografia gasosa utilizando um cromatógrafo (Agilent 6890®, Estados Unidos) com detector de ionização em chama (DIC) e auto-injetor.

3.2.14.2 pH

Uma porção de 0,1 g de fezes cecais foram homogeneizadas com 10 mL de água deionizada e o pH foi mensurado em peagâmetro de bancada.

3.2.15 Análise estatística

Os dados referentes ao consumo de ração e perda de peso foram avaliados por análise de variância utilizando o tempo como medida repetida. Os dados referentes ao índice de severidade da colite, dano macro e microscópico do cólon e outros dados que não atenderam aos pressupostos da análise de variância (ANOVA) foram submetidos à análise de variância de Kruskal-Wallis, seguida de teste não-paramétrico de comparação múltipla. Os demais dados foram submetidos à ANOVA de uma via, seguida do teste de Duncan para comparação das médias. Os resultados foram considerados significativos quando $p<0,05$.

4 DESENVOLVIMENTO

4.1 ARTIGO 1

Postharvest UV-C irradiation stimulates the non-enzymatic and enzymatic antioxidant system of 'Isabel' hybrid grapes (*Vitis labrusca* × *Vitis vinifera* L.)

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Postharvest UV-C irradiation stimulates the non-enzymatic and enzymatic antioxidant system of 'Isabel' hybrid grapes (*Vitis labrusca* × *Vitis vinifera* L.)

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ABSTRACT

Ultraviolet light type C (UV-C) was studied as a tool to increase enzymatic and non-enzymatic antioxidant defenses and phytochemical levels in 'Isabel' grapes (*Vitis labrusca* × *Vitis vinifera* L.). Grapes were exposed to 0, 0.5, 1.0, 2.0, or 4.0 kJ m^{-2} UV-C and stored for 1, 3, or 5 days post-treatment. One day after UV-C irradiation, the activities of grape antioxidant enzymes and thiols were increased, especially at 1.0 and 2.0 kJ m^{-2}. These doses increased total phenolic content by almost 20%, while 0.5 and 4.0 kJ m^{-2} had no effects. Total monomeric anthocyanin content was increased by > 35% by UV-C at 1.0 kJ m^{-2}; however, anthocyanin profile was unchanged. Grape skin antioxidant capacity was also improved by UV-C irradiation. The 1.0 kJ m^{-2} UV-C was considered the hormetic dose. Postharvest UV-C had an elicitor effect on 'Isabel' grapes, positively impacting the antioxidant capacity and phytochemical content.

1. Introduction

Polyphenols in grapes have been already reported as having cardioprotective, anticancer, anti-inflammatory, antiaging, and antimicrobial properties (Xia, Deng, Guo, & Li, 2010). 'Isabel' (syn. 'Isabella') is a hybrid black grape cultivar (*Vitis labrusca* × *Vitis vinifera* L.) and is the most cultivated grape in Brazil. It represents 35% of the total grape production in Rio Grande do Sul, the main state for grape production in Brazil (Mello, 2015). Despite its high productivity, this cultivar generally has a less intense skin color than other black grape varieties. Thus, in general, it is blended with other pigment rich-grapes to achieve satisfactory quality parameters during juice production (Koyama, De Assis, Yamamoto, Prude, & Roberto, 2014). Therefore, pre- or postharvest technologies that increase the concentration of natural pigments and antioxidants in berry skin are particularly relevant for the functional quality of grapes.

The quantity and quality of phytochemicals, such as non-enzymatic plant antioxidants can be improved by exposure to biotic or abiotic stressors (Gill & Tuteja, 2010). Ultraviolet type C (UV-C) light is an abiotic stressor that can induce an adaptive plant defense response by increasing the synthesis of phenolic compounds (Urban, Charles, de Miranda, & Aarrouf, 2016; Wrzaczek, Vainonen, Gauthier, Overmyer, & Kangasjärvi, 2011). However, exposure to high doses of UV-C can also induce an overproduction of reactive oxygen species (ROS), which has the potential to ultimately cause plant damage. Thus, determining the hormetic dose of UV-C, which means the one that elicits a defense response with no plant damage, is a requirement to make UV-C irradiation a feasible post-harvest technology to improve grape's quality.

Most studies on grape irradiation by UV-C have focused on wine varieties (Cantos, Espín, & Tomás-Barberán, 2001; Cantos, Tomás-Barberán, Martínez, & Espín, 2003; Guerrero, Puertas, Fernández, Palma, & Cantos-Villar, 2010; Wang et al., 2010) or have evaluated only the resveratrol levels of irradiated table grapes (Cantos, Espín, & Tomás-Barberán, 2002; Cantos, Garcia-Viguera, De Pascual-Teresa, & Tomás-

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Barberán, 2000). A recent study reported an increase in antioxidant capacity and anthocyanin content after UV-C irradiation in 'Concord' grapes (Pinto et al., 2016). However, no reports have been found on the effect of UV-C on the non-enzymatic antioxidant defense system of 'Isabel' grapes. In addition, previous reports detailing the anthocyanin composition of 'Isabel' grapes focused on grape derived products, as wine and juice (Cazarin et al., 2013; Nixdorf & Hermosín-Gutiérrez, 2010); thus, this is the first report on the grape skin itself.

The elicitor effects of UV-C on plant secondary metabolism have been attributed to the stimulation of reactive oxygen species (ROS) production by this light (Urban et al., 2016). Thus, to avoid cell damage or even death, it is imperative that the enzymatic antioxidant machinery counterbalance any excess ROS generated. The enzymatic plant antioxidant system includes catalase (CAT), superoxide dismutase (SOD), several peroxidases (PODs), and the glutathione system (reduced glutathione, GSH; glutathione peroxidase, GPx; glutathione reductase, GR) (Sharma, Jha, Dubey, & Pessarakli, 2012). Upregulation of these enzymes has been observed in response to the generation of ROS by abiotic stressors in strawberries, shiitake mushrooms, and broccoli (Erkan, Wang, & Wang, 2008; Gill & Tuteja, 2010; Jiang, Jahangir, Jiang, Lu, & Ying, 2010; Lemoine, Chaves, & Martínez, 2010). However, the behavior of grape antioxidant enzymes after UV-C exposure has been not explored. To the best of our knowledge, this is the first time that the behavior of the antioxidant enzymatic system has been evaluated in grapes after UV-C irradiation.

Since the antioxidant defense system plays a crucial role in the cellular defense against abiotic stressors, we hypothesized that exposure to UV-C light can upregulate the synthesis of not only non-enzymatic antioxidants but also enzymes linked to ROS neutralization in grapes. Moreover, knowledge of the whole system can more clearly show which UV-C dose and post-treatment storage time is capable of producing an increase in phytochemicals with minimal plant cell damage. As the nutraceutical properties of grapes are linked to their phytochemical content (such as anthocyanin and non-anthocyanin phenolic compounds) it is highly important to study these compounds and to understand their alterations in stressful conditions such as UV-C irradiation.

Finally, the aims of this study were to determine the effects of postharvest UV-C irradiation on the enzymatic and non-enzymatic antioxidant defense systems of 'Isabel' grapes, to establish the hormetic dose of UV-C, and to identify the optimal storage time after UV-C exposure. Since anthocyanin is the major polyphenol class in grapes and it is responsible for the color of grapes and grape foods, anthocyanin composition was also assessed using liquid chromatography coupled to mass spectrometry.

2. Materials and methods

2.1. Fruit samples

'Isabel' grapes (*Vitis vinifera* × *Vitis labrusca* L.) were hand-harvested in Itaara (29°35′55″ S and 53°46′13″ W, Brazil) from different plants (during January 2013). Ruptured, injured, and unripe grapes were discarded. The samples selected for this study had the following initial quality parameters: a pH of 3.32 ± 0.02 with titratable acidity of 0.62 ± 0.03 mg tartaric acid per 100 g^{-1} fresh weight (FW) and a total soluble solid (TSS) content of 14.2 ± 0.3°Brix. Grapes were cut at the stem and randomized to evaluate the effects of UV-C irradiation on postharvest grape metabolism.

2.2. UV-C irradiation

Grape berries were placed in aluminum trays and subjected to 0.5, 1.0, 2.0, or 4.0 kJ m^{-2} doses of UV-C irradiation (corresponding to 2.68, 5.36, 10.72, or 21.46 min of exposure to UV-C, respectively) using a Philips® lamp at 254 nm (TUV-30W/G30T8; USA). Irradiation intensity was confirmed with a wide band spectroradiometer RPS900 (International Light®, Peabody, MA, USA) and the lamp was set at a distance of 30 ± 1 cm from samples. Non-irradiated grapes were used as the control. All samples were stored for 1, 3, or 5 days in separate tulle bags, under natural light at a controlled temperature of 20 ± 1 °C. At the end of the storage time, samples were immediately frozen in liquid nitrogen and stored (−20 °C) until further analysis. Each treatment time point was composed of four replicates (approximately 300 g of grape each).

2.3. Enzymatic antioxidant assays

Frozen grapes without seeds were pulverized with a cold mortar and pestle in liquid nitrogen, and this powder was used for enzyme and thiol extraction. All enzymatic antioxidant assays were run in duplicate.

SOD extraction was performed as described by Li et al. (2010). SOD activity was measured in a microplate reader (SpectraMax M5) by an indirect assay based on the competitive reaction between grape extract SOD and nitroblue tetrazolium (NBT) chloride, observed at 560 nm (Spitz & Oberley, 1989). In this assay, xanthine/xanthine oxidase generates a superoxide anion radical that reduces NBT to blue formazan. One unit of activity was defined as the amount of protein (U mg^{-1}) to achieve half-maximal inhibition of NBT reduction. A control reaction mixture of grape sample and NBT without xanthine oxidase was performed, and it revealed that the grape sample alone did not reduce NBT to blue formazan (data not shown).

Guaiacol peroxidase (G-POD) extraction was performed as previously described (Erkan et al., 2008). G-POD catalyzes the oxidation of colorless reduced guaiacol to amber oxidized guaiacol in order to reduce H_2O_2. Enzyme activity was assessed by the increase in absorbance at 470 nm due to guaiacol oxidation. The results are expressed as the change in optical density (OD) during the reaction ($1 \times 10^3\ \Delta OD_{470\ nm}\ min^{-1}\ mg^{-1}$ protein) (Andrade Cuvi, Vicente, Concellón, & Chaves, 2011).

CAT was extracted as described for G-POD and assessed according to the protocol by Aebi (1984). In this assay, the decomposition of H_2O_2 by CAT is followed directly as the decrease in absorbance at 240 nm, and the activity is expressed as the rate constant of the reaction ($k_{60s}\ mg^{-1}$ protein).

GR activity was extracted as described for SOD and determined by the consumption of NADPH at 340 nm, which is coupled to the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) (Carlberg & Mannervick, 1979). The results are expressed as μmol NADPH $min^{-1}\ mg^{-1}$ protein.

The protein content of the enzymatic extracts was measured with bovine serum albumin as the standard (Bradford, 1976) and the resulting concentrations were used to calculate enzyme activities.

2.4. Thiol content

Total thiol content was measured at 412 nm after reacting with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) (Ellman, 1959). Non-protein thiols were assessed using the same method except that samples were pretreated with 4% trichloroacetic acid (TCA) to remove protein thiols. Analyses were performed in duplicate, and a standard curve of cysteine was used for calculation. The results are expressed as nmol GSH g^{-1} grape (FW).

2.5. Extraction of grape skin polyphenols

Five grams (FW) of grape skin were homogenized with 15 mL of water:ethanol:formic acid solution (27:70:3, v/v/v) for 2 min at 12,000 rpm using an Ultra Turrax homogenizer. The mixture was centrifuged at 2300 × *g* for 10 min, and the total phenolic compound content and antioxidant capacity of the supernatant were determined.

2.6. Colorimetric measurement of phenolic compounds in grape skins

Total phenolic compound content was measured as previously reported by Singleton and Rossi (1965) using a calibration curve of gallic acid ranging from 10 to 50 mg L^{-1} (R^2 = 0.982).

Total flavonol content was quantified as detailed by Zhishen, Mengcheng, and Jianming (1999) using a calibration curve of quercetin-3-rutinoside (120 to 960 mg L^{-1}; R^2 = 0.990).

The proanthocyanidin content was determined as described by Wallace and Giusti (2010) using the 4-(dimethylamino) cinnamaldehyde (DMAC) reagent and a calibration curve of catechin (12.5 to 500 mg L^{-1}; R^2 = 0.994).

The pH-differential method was used for quantification of the total monomeric anthocyanin content using the molecular weight (493.5) and molar extinction coefficient of malvidin-3-glucoside (29,500) as described by Wrolstad and Giusti (2001).

2.7. Total antioxidant capacity of grape skins

All antioxidant assays were performed in triplicate in grape skin extracts obtained as described in Section 2.5.

The DPPH radical scavenging assay was carried out as described by Brand-Williams, Cuvelier, and Berset (1995) and Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as a standard for the calibration curve (0–1.75 mM; R^2 = 0.988).

The ferric reducing antioxidant power (FRAP) was assessed as described by Benzie and Strain (1996). A Trolox calibration curve was used for quantification (0–250 μM; R^2 = 0.995).

Antioxidant capacity was assessed by the oxygen radical absorbance capacity (ORAC) assay according to Ou, Hampsch-Woodill, and Prior (2001) with modifications as described by Rodrigues, Mariutti, Faria, and Mercadante (2012). Fluorescence (λ_{exc} = 485 nm and λ_{em} = 528 nm) was measured for 90 min using a SpectraMax M5 plate reader (Molecular Devices, USA). A calibration curve of Trolox (0–96 μM; R^2 = 0.987) was used to express ORAC equivalent values.

The superoxide anion radical ($O_2^{-\cdot}$) scavenging activity of grape skin extracts was assessed as described for SOD activity (Spitz & Oberley, 1989), except that grape samples were extracted with organic solvents (see Section 2.5), which remove the protein fraction, allowing for the assessment of the non-enzymatic $O_2^{-\cdot}$ scavenging potential. Parallel controls confirmed that the grape extracts did not inhibit xanthine oxidase at the tested concentrations. A Trolox calibration curve was used (0–100 μM; R^2 = 0.950).

2.8. Identification of anthocyanin profile by HPLC-PDA and HPLC-MS/MS

The analysis of 'Isabel' grape skin anthocyanins was performed using a HPLC system (CBM-20A prominence, Shimadzu®, Japan) equipped with degasser (DGU20A5 prominence, Shimadzu®, Japan), column oven (CTO-20A prominence, Shimadzu®, Japan) and coupled to a PDA detector (SPDM-20A prominence, Shimadzu, Japan). Skin extracts were obtained as described in Section 2.5, dried at 38 ± 2 °C (Buchi® rotatory evaporator, Switzerland), and adjusted to a known volume using a water/formic acid (97:3, v/v) solution. Samples were filtered (0.45 μm, 13 mm hydrophilic PTFE; Restek®, China) and injected in a reverse-phase column C-18 (CLC-ODS, 250 mm, 4.6 mm, 5 μm particle size; Shimadzu®, Japan) thermostated at 40 °C. Mobile phase A consisted of 3% (v/v) formic acid solution (88%) in ultrapure water. Mobile phase B was pure acetonitrile. The flow rate was set at 0.8 mL min^{-1}. The gradient elution was as follows: from the initial time to 3 min: 0 to 8% of B; from 3 to 13 min: 8% of B; from 13 to 37 min: 8 to 20% of B; from 37 to 57 min: 20% of B; from 57 to 58 min: 20 to 50% of B; from 58 to 63 min: 50% of B; from 63 to 65 min: 50 to 90% of B; from 65 to 75 min: 90% of B; from 75 to 80 min: 90 to 0% of B, and from 80 to 85 min: 0% of B. Quantification was performed by area normalization on PDA-chromatograms obtained at 520 nm, and spectral information collected from 200 to 800 nm was used for identification.

Before LC-MS/MS analysis, whole polyphenol extracts were dried using a rotatory evaporator and applied to solid phase extraction cartridges (SPE-C18 cartridges, Merck®, Germany) to obtain a semi-purified fraction of anthocyanin, which was dried in rotatory evaporator and made up to a known volume using a water solution of 0.35% formic acid (v/v) as proposed by Rodriguez-Saona and Wrolstad (2001) with the modifications described by Bochi et al. (2014).

HPLC-MS/MS analysis was performed in 1290 Agilent® technologies HPLC system coupled to the QTrap mass spectrometer (5500QTRAP, AB Sciex®) equipped with electrospray ionization (ESI) source. Separation was performed using the same chromatographic conditions described for HPLC-PDA analysis. Ionization was performed on positive ion mode controlled by Analyst 1.5 software (AB Sciex®). The ESI source operation parameters were as follows: source temperature 350 °C; ion spray voltage (IS) 5000 V; ion source gas I, gas II, and curtain gas set at 50, 50, and 15 psi, respectively. Collision gas was set at medium. Precursor ion scan analysis (m/z scan range from 100 to 900) was performed for major anthocyanidin fragments (m/z 271, m/z 287, m/z 301, m/z 303, m/z 317, and m/z 331 for pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin, respectively).

Identification was obtained by combining information from UV to visible spectra obtained from PDA detector, the elution order in reverse phase, and the fragmentation pattern in LC-MS/MS analysis.

2.9. Statistical analyses

All data were submitted to factorial analyses of variance (5 UV-C doses × 3 storage times), followed by Tukey's HSD test when appropriate. For each treatment and time point, the data are expressed as the means ± standard error of four replicates for each treatment and time point. Results were considered significantly different when $p < 0.05$. Statistica 9.0 software (StatSoft®, Inc., Tulsa, OK, USA) was used for analysis.

3. Results and discussion

3.1. Effect of UV-C and post-treatment storage time on the enzymatic antioxidant system of 'Isabel' grapes

ANOVA revealed a significant interaction between UV-C dose x storage time in the activities ($p < 0.05$) of SOD (Fig. 1A), G-POD (Fig. 1B), CAT (Fig. 1C), and GR (Fig. 1D).

SOD is essential to minimize the accumulation of $O_2^{-\cdot}$ during fruit storage. SOD activity has been shown to be increased by numerous ROS-generating stressors and it is also correlated with increased tolerance to abiotic stress (Gill & Tuteja, 2010; Sharma et al., 2012). SOD activity was increased only by the 1.0 kJ m^{-2} UV-C irradiation dose and only at 1 and 3 days of post-treatment storage ($p < 0.05$). SOD activity decreased over the entire storage time for all experimental groups ($p < 0.05$). At 5 days post-treatment, differences in SOD activity due to irradiation were no longer perceived in 'Isabel' grape berries. Similar results have been found in broccoli (Lemoine et al., 2010) and peppers (Promyou & Supapvanich, 2012) exposed to UV-C. In plants, the manganese isoform of SOD (MnSOD) is localized to the mitochondria and peroxisomes (Gill & Tuteja, 2010), which are the main sources of intracellular ROS in plants, as the grapevine, exposed to UV light (Carvalho, Vidigal, & Amâncio, 2015). UV-C irradiation has been shown to promote the proliferation and elongation of peroxisomes in plant tissue (Mittler, 2002). Thus, increased SOD activity in irradiated grapes may be a defense response to counteract the overproduction of ROS.

CAT activity was increased 2-fold after exposure to 1.0 kJ m^{-2} UV-C and after 1 day of post-treatment storage compared with the activity of control (non-irradiated) grapes ($p < 0.05$). However, this increase

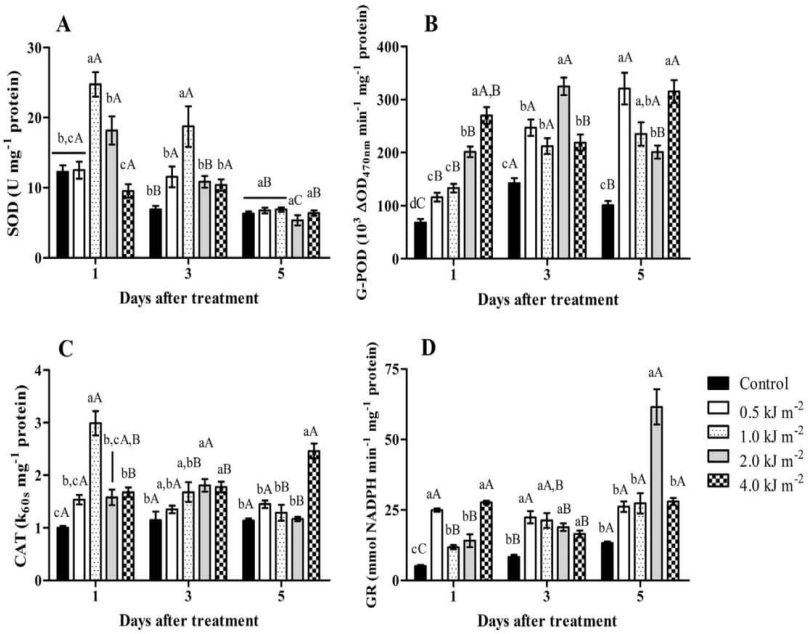

Fig. 1. Effect of UV-C irradiation and storage time on the enzymatic antioxidant system of 'Isabel' grapes: SOD (A), G-POD (B), CAT (C), and GR (D) activities. The results are the average of 4 replicates ± standard error. Bars that have different lower-case letters indicate a significant effect of UV-C irradiation within the same day, and different upper-case letters indicate a significant effect of storage time within the same UV-C dose ($p < 0.05$; Tukey's HSD).

in activity disappeared after the samples had been stored for 3 days. The highest UV-C dose (4.0 kJ m^{-2}) also increased CAT activity after 1 day of post-treatment storage compared to that of control grapes ($p < 0.05$) but to a lower extent than the increase observed with the 1.0 kJ m^{-2} dose. This increase persisted for 3 days of storage but was significant only for the 2.0 and 4.0 kJ m^{-2} doses. At 5 days post-irradiation, only grapes exposed to the highest UV-C dose had higher CAT activity than that of the control samples ($p < 0.05$).

CAT function is closely related to SOD since it removes the excessive peroxide radicals formed during superoxide dismutation (Kurutas, 2016). Grape exposure to 1.0 kJ m^{-2} UV-C combined with 1 day of storage yielded a parallel increase in SOD and CAT activity, suggesting that these antioxidant enzymes are key players in the early hormetic response of grapes against UV-C stress, protecting from further cell damage due to oxidative stress. In fact, peppers submitted to UV-C irradiation presented with an increase in CAT activity and the appearance of crystals of active CAT in peroxisomes (Sarghein Hosseini, Carapetian, & Khara, 2011). Thus, our study demonstrates that 'Isabel' grapes are able to synchronize these two key enzymes in stress management after irradiation with 1.0 kJ m^{-2} UV-C.

Hydrogen peroxide and other peroxides can also be reduced by peroxidases such as G-POD, which use phenolic compounds as electron donors (Sharma et al., 2012). At one day of post-treatment storage time, UV-C irradiation caused an increase in G-POD activity in a dose-dependent manner. Except for those that received the 4 kJ m^{-2} UV-C dose, grapes from all treatments had higher G-POD activity after 3 days of storage than after only one day. The upregulation of G-POD activity by irradiation persisted for 5 days after treatment; however, the effect was not linearly increased by the irradiation dose at this time point (Fig. 1B). The dose-dependent increase in G-POD activity observed during the shorter storage times suggests that exposure to higher UV-C doses (2.0 and 4.0 kJ m^{-2}) yields greater peroxide production and possibly plant cell damage than exposure to the lowest UV-C doses (0.5 and 1.0 kJ m^{-2} UV-C). In fact, the oxidation of fruit phenolic compounds by peroxidase activity could lead to enzymatic browning and may be associated with changes in flavor, texture, color, and nutritive characteristics (Bett-Garber, Lamikanra, Lester, Ingram, & Watson, 2005; Lamikanra, Kueneman, Ukuku, & Bett-Garber, 2005).

CAT and PODs can cooperate with each other to remove the H_2O_2 generated under stressful conditions, CAT removes the excess H_2O_2 produced by photorespiration, and PODs scavenge the low levels of H_2O_2 that are not removed by CAT (Foyer, 2002). We demonstrated that both CAT and G-POD were immediately activated in 'Isabel' grapes after UV-C exposure; however, after 5 days of storage, only G-POD activity remained higher than that of the control grapes for all UV-C doses.

Reduced glutathione (GSH) plays an essential role in the maintenance of redox balance in plant cells (Erkan et al., 2008). When cells are exposed to oxidative conditions GSH is oxidized to its primary oxidized product, glutathione disulfide (GSSG) (Gill & Tuteja, 2010). GSSG can be reduced back to GSH at the expense of NADPH by GR (Erkan et al., 2008; Gill & Tuteja, 2010). All doses of UV-C significantly increased GR activity at 1 and 3 days of post-treatment storage. However, after 5 days of storage, only samples exposed to 2.0 kJ m^{-2} UV-C had increased GR activity compared with that of the control and the activity at all other UV-C doses ($p < 0.05$).

We have described for the first time the effect of UV-C on the enzymatic antioxidant defense system of grapes. UV-C stress caused an early activation of the enzymatic antioxidant defense system in 'Isabel' grapes. SOD and CAT appear to be activated only at hormetic UV-C doses and generally reached peak values the first day after UV-C exposure; G-POD and GR were activated by all UV-C doses and generally reached peak activity at the 3rd or 5th day after irradiation treatment.

3.2. Effect of UV-C and post-treatment storage time on thiol content of 'Isabel' grapes

The total thiol content increased linearly with the UV-C dose ($p < 0.05$) at one day post irradiation (Fig. 2A). Nonetheless, only the grapes receiving 2.0 or 4.0 kJ m^{-2} UV-C had significantly different

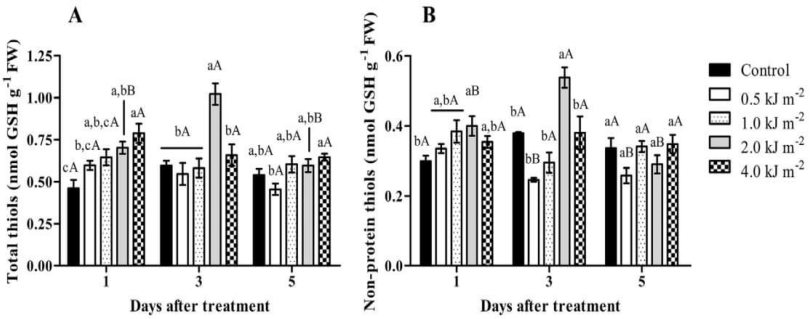

Fig. 2. Effect of UV-C irradiation and storage time on total (A) and non-protein (B) thiol levels of 'Isabel' grapes. The results are the average of 4 replicates ± standard error. Bars that have different lower-case letters indicate a significant effect of UV-C irradiation within the same day, and different upper-case letters indicate a significant effect of storage time within the same UV-C dose ($p < 0.05$; Tukey's HSD).

thiol content compared with that of the control grapes ($p < 0.05$). Only grapes exposed to 2.0 kJ m^{-2} UV-C had a higher total thiol content than that of the control samples after 3 days of storage. After 5 days of storage, the total thiol content was similar in all samples. Non-protein thiols mostly reflect GSH content and similar to the total thiol content, were increased after exposure to 2.0 kJ m^{-2} UV-C after 1 and 3 days of storage (Fig. 2B). After 5 days of storage, there were no significant differences observed between the irradiated and control samples.

This finding is in agreement with the greatly enhanced increase in GR activity (almost 6-times higher after one day of storage) of all UV-C-treated grapes. The greatest increase in GR activity was observed at the 2.0 kJ m^{-2} UV-C dose after 5 days of storage. Interestingly, 'Isabel' grapes treated with 2.0 kJ m^{-2} of UV-C and stored for 3 days had a higher GSH content than that of the control grapes, supporting the essential role of the glutathione system in recovering plant redox balance after exposure to stress. Pears treated with 5.0 kJ m^{-2} UV-C also had an increase in GR activity (Li et al., 2010).

3.3. Effect of UV-C and post-treatment storage time on total concentration of phenolic compounds in 'Isabel' grape skins

Non-enzymatic antioxidants have also been shown to play a protective role against stressful conditions and to improve the antioxidant potential of some fruits (González-Aguilar, Villa-Rodriguez, Ayala-Zavala, & Yahia, 2010). ANOVA results showed that the total content of phenolics, monomeric anthocyanins, and flavonols was significantly affected by storage time and treatment dose ($p < 0.05$), but there was not any significant interaction among these variables (Table 1). All samples showed an increase in total phenolics, flavonols, and monomeric anthocyanins ($p < 0.05$; Table 1) during the storage time. Similar findings have been reported for table grape cv Redglobe (*V. vinifera*) (Crupi, Pichierri, Basile, & Antonacci, 2013) and various berries (Kalt, Forney, Martin, & Prior, 1999). The accumulation of polyphenols along the storage has been suggested to result from the increased supply of carbon skeletons for polyphenol synthesis due to the degradation of organic acids (Kalt et al., 1999).

Exposure to 1.0 or 2.0 kJ m^{-2} UV-C increased the concentration of total polyphenols in grape skins by almost 20% compared to that of control grapes ($p < 0.05$), while the 0.5 and 4.0 kJ m^{-2} doses had no effect (Table 1, $p > 0.05$).

UV-C irradiation had no effect ($p > 0.05$) on the total flavonol content of 'Isabel' grape skins (Table 1), and the flavonol content was only affected by the storage time after irradiation exposure. However, grape exposure to UV-C doses between 0.5 and 2.0 kJ m^{-2} increased the content of anthocyanins in 'Isabel' grape skins by > 35% compared with that of the control group ($p < 0.05$). The UV-C dose of 4.0 kJ m^{-2} did not affect the anthocyanin content compared with that of the control group ($p < 0.05$; Table 1).

There was a significant interaction effect between UV-C dose and post-treatment storage time for total proanthocyanidin content in 'Isabel' grape skin ($p < 0.05$; Table 1). The total proanthocyanidin content increased with the storage time after UV-C exposure (day 1 vs. day 5; $p < 0.05$), except in grapes exposed to the 2.0 kJ m^{-2} UV-C dose, which peaked in proanthocyanidin content after only one day of storage. In fact, excluding the 4.0 kJ m^{-2} UV-C dose, all irradiation doses promoted higher proanthocyanidin content than that of the control group after 1 day of post-treatment storage ($p < 0.05$; Table 1). After 3 and 5 days of storage, no UV-C dose caused an increase in the concentration of proanthocyanidins in grape skins compared to that of the control group (Table 1).

Our study demonstrated that the total content of polyphenols, anthocyanins, and proanthocyanidins was increased in grapes after irradiation at doses ranging from 0.5 to 2.0 kJ m^{-2} UV-C. The highest amount of total proanthocyanidin content was achieved 1 day after exposure to 2.0 kJ m^{-2} UV-C. However, the total content of polyphenols and anthocyanins increased in all samples during the storage time, and therefore, we can assume that a dose of at least 0.5 kJ m^{-2} and storage for at least 5 days are necessary to achieve the highest deposition of polyphenols. Moreover, it is interesting that the concentrations of anthocyanins and total polyphenols in grape skin showed a bell-shaped response to the UV-C doses with peak amounts usually found at the 1.0 kJ m^{-2} dose. This bell-shaped response to UV-C dose has already been reported for phenolics and anthocyanins in strawberries and blueberries (Erkan et al., 2008; Wang, Chen, & Wang, 2009). This phenomenon can be explained by the hormesis response, which can be attributed to intermediate UV-C doses that stimulate the plant defense mechanisms resulting in the increased synthesis of phytochemicals, minimal cellular damage and the consequent sparing of phenolic antioxidants (Rohanie & Ayoub, 2012). In fact, plant cell damage by oxidative stress could be occurring at doses higher than 1.0 kJ m^{-2}, since grapes treated with the highest UV-C dose (4.0 kJ m^{-2}) had a lower anthocyanin content than those treated with 1.0 kJ m^{-2} UV-C and did not show the increase in SOD activity that was observed at the hormetic UV-C dose.

No UV-C effect on flavonol content was observed in our study as previously reported for 'Beiquan' red grapes (*V. vinifera* × *V. amurensis*) (Li et al., 2009), table grapes (Cantos et al., 2002), and 'Monastrell' wine grapes (Cantos, Espín, Fernández, Oliva, & Tomás-Barberán, 2003). These findings suggest that flavonols are less affected by UV-C irradiation than other phenolic compounds. Nevertheless, colorimetric assays do not rule out a possible increase in specific flavonols after UV-C exposure. Thus, more sensitive and specific methods are required to determine the behavior of these compounds in plant-cell homeostasis after UV-C irradiation. Moreover, several studies have suggested that the induction of flavonoid synthesis is a specific adaptive response to

Table 1
Colorimetric measurements of the total polyphenol content by class in the skin of UV-C irradiated 'Isabel' grapes.

	UV-C dose (kJ m^{-2})	Post-treatment storage time (days at 20 °C)			Mean ± SEM (n = 12)
		1	3	5	
Total polyphenols (TP) (mg eq gallic acid 100 g^{-1} skin FW)	Control	686.9 ± 34.9	782.5 ± 32.7	944.3 ± 12.3	804.6 ± 40.2 b
	0.5	849.5 ± 21.7	897.2 ± 62.6	1053.1 ± 43.3	933.3 ± 38.3 a,b
	1.0	846.1 ± 44.3	1022.2 ± 41.2	1094.6 ± 69.3	987.6 ± 45.4 a
	2.0	925.4 ± 45.6	975.9 ± 26.0	975.7 ± 20.2	959.0 ± 18.3 a
	4.0	760.0 ± 22.7	908.6 ± 49.5	1014.1 ± 19.3	894.2 ± 40.4 a,b
	Mean ± SEM (n = 20)	813.6 ± 25.7 C	917.3 ± 27.4 B	1016.4 ± 20.5 A	
Total flavonols (TF) (mg eq quercetin-3-rutinoside 100 g^{-1} skin FW)	Control	293.9 ± 28.2	427.5 ± 40.6	485.3 ± 31.4	402.2 ± 33.0 a
	0.5	381.5 ± 7.1	401.7 ± 68.1	482.6 ± 20.9	422.0 ± 25.8 a
	1.0	393.7 ± 35.7	432.7 ± 40.9	602.6 ± 28.4	476.3 ± 36.6 a
	2.0	339.7 ± 46.9	440.8 ± 33.1	413.9 ± 52.2	398.1 ± 27.0 a
	4.0	346.5 ± 23.8	420.6 ± 10.7	500.1 ± 44.2	422.4 ± 26.7 a
	Mean ± SEM (n = 20)	351.1 ± 15.1 C	424.7 ± 16.6 B	496.9 ± 21.4 A	
Total monomeric anthocyanins (TMA) (mg eq malvidin-3-glucoside 100 g^{-1} skin FW)	Control	164.4 ± 16.3	218.5 ± 14.2	210.5 ± 3.3	197.8 ± 10.5 b
	0.5	222.0 ± 5.7	230.9 ± 12.4	286.9 ± 4.6	245.0 ± 10.4 a
	1.0	251.3 ± 16.7	238.8 ± 15.3	325.2 ± 18.3	268.5 ± 15.1 a
	2.0	230.4 ± 6.2	250.0 ± 5.8	269.6 ± 15.9	250.1 ± 7.7 a
	4.0	208.3 ± 14.3	225.8 ± 16.0	268.7 ± 19.0	233.4 ± 11.7 a,b
	Mean ± SEM (n = 20)	215.3 ± 9.1 C	232.6 ± 6.0 B	272.2 ± 11.2 A	
Total proanthocyanidins (TPC) (mg eq catechin 100 g^{-1} skin FW)	Control	62.4 ± 4.9 dB	91.0 ± 3.3 a,bA	94.4 ± 6.3 aA	83.4 ± 5.2
	0.5	95.1 ± 2.6 a,bB	81.2 ± 5.5 bB	113.4 ± 1.5 aA	96.5 ± 4.4
	1.0	83.3 ± 3.8 b,cB	103.1 ± 4.5 aA,B	114.2 ± 6.7 aA	100.2 ± 4.7
	2.0	110.4 ± 5.5 aA	100.2 ± 4.6 a,bA	97.5 ± 5.6 aA	102.0 ± 3.2
	4.0	76.9 ± 1.1 c,dB	95.4 ± 4.0 a,bA	100.1 ± 2.2 aA	90.8 ± 3.3
	Mean ± SEM (n = 20)	85.5 ± 3.9	94.2 ± 2.5	104.4 ± 2.7	

ANOVA results (*p*-value)

Effects	TP	TMA	TF	TPC
UV-C dose	< 0.001	< 0.001	0.107	< 0.001
Storage time	< 0.001	< 0.001	< 0.001	< 0.001
UV-C dose × storage time	0.155	0.105	0.314	< 0.001

Results are the average of 4 replicates ± standard error. Different lower-case letters indicate significant effect of UV-C irradiation within the column, and different upper-case letters indicate significant effect of storage time within the line ($p < 0.05$; Tukey's HSD).

different stressor agents, which supports the hypothesis that distinct UV-signaling pathways exist in plant tissues (Jiang et al., 2010). Accordingly, UV-C irradiation of 'Isabel' grapes had a greater effect on anthocyanins than flavonols. This finding strongly indicates that UV-C irradiation may be a good alternative for enhancing color and functional quality of grape berries.

3.4. Effect of UV-C on anthocyanin profile of 'Isabel' grapes

UV-C increased the entire content of both non-anthocyanin and anthocyanin phenolic compounds. Anthocyanin is the major phenolic class in grape skin, and it plays an important role in fruit quality. Since not only the concentration but also the profile of anthocyanidins in grapes can be greatly affected by cultivar type and viticultural conditions (He et al., 2010), we wanted to determine if UV-C irradiation can also affect the distribution profile of these compounds. Only grape samples stored for one day were selected for anthocyanin characterization by HPLC-PDA-MS/MS. Table 2 and Fig. 3 show the ultraviolet to visible spectra information and the fragments obtained by mass spectrometry analysis of the chromatographic profile of anthocyanins detected in the extracts of 'Isabel' grape skins.

UV-C irradiation increased anthocyanin content, as perceived by the colorimetric results (Table 1). However, none of the UV-C doses affected the anthocyanin profile in grape skin. Thus, by the identification of each compound and its relative percentage, no differences were observed in the relative percentage of any specific anthocyanin after UV-C irradiation, indicating that all compounds were equally increased in grape skin.

A total of 16 different anthocyanins were tentatively identified in 'Isabel' grape skins (Fig. 3; Table 2). No new compounds were detected after UV-C irradiation. Moreover, the anthocyanin profile showed that the main compounds in 'Isabel' grape skin were malvidin derivatives (52.5% of total anthocyanins), of which malvidin-3-glucoside was the predominant skin pigment (peak 9, 37.5%). Peonidin derivatives amounted to 26.7% of natural pigments in 'Isabel' grapes, of which peonidin-3-glucoside was the dominant one (peak 8, 19.1%). Cyanidin and petunidin derivatives accounted for 2.4% and 8.7% of the total detected compounds, respectively. Pelargonidin derivatives were not found in 'Isabel' grape skins.

In this study, there was a prevalence of non-acylated anthocyanins (approximately 80% of total anthocyanins), and the major aromatic acid that was acylated with a sugar molecule was *p*-coumaric acid (peaks 11, 13, 14, 15, and 16), which was identified by a longer retention time than the glucoside derivatives and by a molecular weight with an additional residue of 146 *m/z*. This acid can be linked in two stereoisomer positions to anthocyanidin-3-*O*-*p*-coumaroyl glucosides (*cis* and *trans* isoforms), such as was observed in this work with peonidin (peaks 14 and 15). Moreover, the acetyl derivatives were also detected (peaks 10 and 12) and identified by their retention times, which were shorter than those of the *p*-coumaroyl derivatives (peaks 14, 15, and 16) but longer than only those of the non-acylated structures (peaks 8 and 9). The final molecular weights of these compounds (527 and 557 for peaks 10 and 12, respectively, Table 2) reflect the aglycone (301 and 331 *m/z*), one hexose moiety (162 *m/z*) and one dehydrated acetyl residue (64 *m/z*).

Most of the previous data on the phenolic composition of 'Isabel' grapes has not investigated the profile of anthocyanins in fresh grapes but has focused on the anthocyanin-derived pigments of wine

Table 2
Chromatographic and spectral data from HPLC–PDA and HPLC-MS/MS analysis of anthocyanins extracted from 'Isabel' grape skins submitted or not (control) to UV-C irradiation and stored for 1 day after treatment.

Peak	HPLC-PDA		MS		Tentative identification		Relative percentage (mean ± standard deviation)				
							UV-C doses				
	Rt (min)	λ_{max} (nm)	Precursor Ion	Product Ion	Aglycone	Anthocyanin	Control	0.5 kJ m^{-2}	1.0 kJ m^{-2}	2.0 kJ m^{-2}	4.0 kJ m^{-2}
1	12.3[a]	n.d.	627	303	Dp	Dp-3,5-diglc	n.d.	n.d.	n.d.	n.d.	n.d.
2	15.2[a]	n.d.	611	287	Cy	Cy-3,5-diglc	n.d.	n.d.	n.d.	n.d.	n.d.
3	26.4	523/517	465/625	303/301	Dp + Pn	Dp-3-glc	7.9 ± 0.3	8.0 ± 0.1	8.2 ± 0.1	8.5 ± 0.1	8.1 ± 0.0
4						Pn-3,5-diglc					
5	29.6	517	449	287	Cy	Cy-3-glc	1.4 ± 0.0	1.5 ± 0.0	1.4 ± 0.1	1.5 ± 0.1	1.5 ± 0.0
6	31.0	517	655	331	Mv	Mv-3,5-diglc	5.7 ± 0.1	6.0 ± 0.0	5.8 ± 0.0	6.3 ± 0.1	5.7 ± 0.0
7	34.0	523	479	317	Pt	Pt-3-glc	8.7 ± 0.2	8.7 ± 0.1	9.0 ± 0.1	8.9 ± 0.0	8.8 ± 0.0
8	37.1	516	463	301	Pn	Pn-3-glc	19.1 ± 0.2	19.5 ± 0.1	18.6 ± 0.1	19.5 ± 0.0	19.0 ± 0.0
9	39.0	526	493	331	Mv	Mv-3-glc	37.5 ± 0.8	35.9 ± 0.5	37.5 ± 0.1	36.0 ± 0.0	38.1 ± 0.0
10	49.5	517	527	301	Pn	Pn-3-ac-glc	0.5 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
11	49.9	529	611	303	Dp	Dp-3-cm-glc	0.9 ± 0.1	0.9 ± 0.2	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1
12	50.8	517	557	331	Mv	Mv-3-ac-glc	1.7 ± 0.1	1.8 ± 0.0	1.7 ± 0.0	1.7 ± 0.0	1.7 ± 0.0
13	53.8	517	595	287	Cy	Cy-3-cm-glc	1.0 ± 0.0	1.1 ± 0.1	1.0 ± 0.0	1.0 ± 0.1	0.9 ± 0.0
14	56.2	523	609–771	301	Pn	Pn-3-*cis*-cm-glc	1.2 ± 0.1	1.2 ± 0.2	1.2 ± 0.3	1.1 ± 0.1	1.2 ± 0.0
15	63.9	520	609	301	Pn	Pn-3-*trans*-cm-glc	5.9 ± 0.7	6.4 ± 0.0	5.8 ± 0.0	5.8 ± 0.0	5.5 ± 0.1
16	67.3	532	639	331	Mv	Mv-3-cm-glc	7.6 ± 0.4	7.6 ± 0.1	7.5 ± 0.1	7.1 ± 0.1	7.3 ± 0.1
17	70.0	531	n.i.	331	Mv	n.i.	0.8 ± 0.1	1.0 ± 0.1	1.0 ± 0.0	1.0 ± 0.0	0.8 ± 0.1

Nomenclature abbreviations: Dp, delphinidin; Cy, cyanidin; Pn, peonidin; Mv, malvidin; Pt, petunidin; diglc: diglucoside; glc: glucoside; ac: acetyl; cm: coumaroyl; n.i.: not identified. n.d.: not detected in the LC-PDA analysis, only detected in LC-MS/MS.
[a] Retention time was obtained from HPLC-MS/MS analysis since these compounds were not detected in HPLC-PDA analysis.

(Nixdorf & Hermosín-Gutiérrez, 2010) and juice (Cazarin et al., 2013). We have described for the first time the anthocyanin composition of the skin of fresh 'Isabel' grapes and have demonstrated that all anthocyanin derivatives are equally increased by UV-C irradiation.

3.5. Effect of UV-C and post-treatment storage time on the antioxidant capacity of 'Isabel' grapes

The DPPH, FRAP, ORAC, and $O_2^{-\cdot}$ scavenging assays determine the activity of the non-enzymatic antioxidants of grape extracts as the extracts are deproteinized. The interaction of the irradiation dose with the storage time had a significant effect on the antioxidant capacity of 'Isabel' grapes, as assessed by the DPPH, FRAP, and $O_2^{-\cdot}$ scavenging assays ($p < 0.05$; Table 3).

After only 1 day of storage, using the DPPH scavenging assay, there was a clear difference among the antioxidant activities of grapes receiving different UV-C doses. At this storage time, nearly all irradiation doses resulted in a higher antioxidant capacity than that of the control grapes ($p < 0.05$; Table 3). The highest UV-C dose (4.0 kJ m^{-2}) did not differ from the control samples at any storage time assessed. No differences were still perceived between control and UV-C-treated grapes when grapes were stored for 3 days. However, there was a higher antioxidant capacity in the 1.0 kJ m^{-2}-dosed group than in the control group after 5 days of storage ($p < 0.05$), as determined by DPPH.

Irradiated grapes showed higher capacity to reduce ferric ion (FRAP assay) than that of control grapes after only 1 day of post-treatment storage ($p < 0.05$; Table 3). In addition, FRAP values for all samples increased during storage, and the highest values were found in grapes stored for 5 days after irradiation ($p < 0.05$; Table 3). The lowest UV-C dose (0.5 kJ m^{-2}) improved the antioxidant capacity only in samples stored for 1 day. All other doses increased the FRAP values at the intermediate post-treatment storage time (3 days, $p < 0.05$; Table 3). At the longest post-treatment storage time tested (5 days), no significant differences in FRAP values were observed among the samples, and all samples exhibited the highest observed FRAP scores (Table 3).

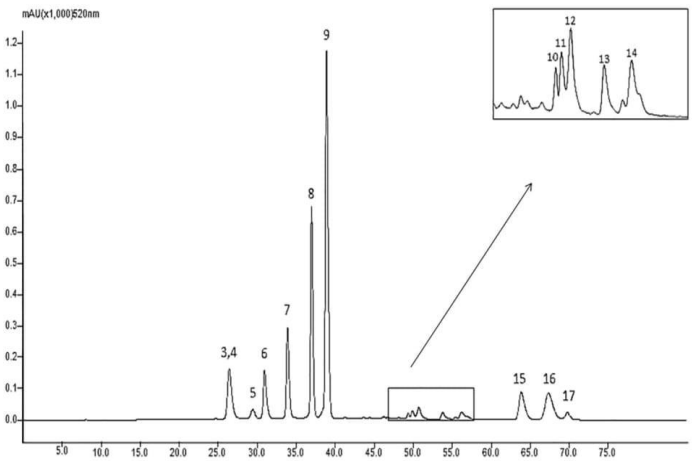

Fig. 3. Chromatographic profile of anthocyanins obtained from 'Isabel' grape skins. The numbers above peaks correspond to the tentative identification reported in Table 2.

Table 3
Effect of UV-C irradiation and storage time on the total antioxidant capacity of 'Isabel' grape skin, as determined by the DPPH, FRAP, ORAC, and superoxide anion radical ($O_2^{-\cdot}$) scavenging assays.

	UV-C dose (kJ m^{-2})	Post-treatment storage time (days at 20 °C)			Mean ± SEM (n = 12)
		1	3	5	
DPPH (mmol eq Trolox 100 g^{-1} skin FW)	Control	4.52 ± 0.47 bA	6.03 ± 0.31 a,bA	5.20 ± 0.52 bA	5.25 ± 0.31
	0.5	6.35 ± 0.22 aA	5.22 ± 0.14 bB	6.31 ± 0.13 a,bA	5.96 ± 0.20
	1.0	6.34 ± 0.48 aA	6.26 ± 0.09 a,bA	7.39 ± 0.46 aA	6.74 ± 0.27
	2.0	6.31 ± 0.43 aA	6.61 ± 0.33 aA	6.83 ± 0.37 a,bA	6.58 ± 0.20
	4.0	5.91 ± 0.18 a,bB	6.26 ± 0.20 a,bA,B	6.91 ± 0.15 a,bA	6.36 ± 0.17
	Mean ± SEM (n = 20)	5.88 ± 0.24	6.08 ± 0.15	6.58 ± 0.24	
FRAP (mmol eq Trolox 100 g^{-1} skin FW)	Control	2.86 ± 0.22 bC	4.52 ± 0.41 cB	6.36 ± 0.04 aA	4.58 ± 0.52
	0.5	4.81 ± 0.25 aB	4.45 ± 0.03 cB	7.40 ± 0.51 aA	5.55 ± 0.49
	1.0	5.07 ± 0.24 aB	5.68 ± 0.16 bB	7.82 ± 0.38 aA	6.19 ± 0.44
	2.0	5.30 ± 0.25 aB	6.85 ± 0.19 aA,B	7.72 ± 0.75 aA	6.62 ± 0.42
	4.0	4.50 ± 0.20 aB	6.59 ± 0.22 a,bA	7.41 ± 0.34 aA	6.29 ± 0.43
	Mean ± SEM (n = 20)	4.51 ± 0.25	5.62 ± 0.28	7.34 ± 0.22	
ORAC (mmol eq Trolox 100 g^{-1} skin FW)	Control	13.73 ± 0.57	15.72 ± 0.93	13.36 ± 0.65	14.35 ± 0.51 b
	0.5	18.18 ± 1.11	16.23 ± 0.77	14.85 ± 0.68	16.42 ± 0.61 a,b
	1.0	18.10 ± 0.93	19.87 ± 0.37	16.47 ± 0.75	18.30 ± 0.57 a
	2.0	16.75 ± 1.64	17.53 ± 0.72	14.49 ± 0.66	16.26 ± 0.69 a,b
	4.0	14.12 ± 0.81	17.17 ± 1.05	15.21 ± 0.35	15.50 ± 0.56 b
	Mean ± SEM (n = 20)	16.17 ± 0.61 A,B	17.30 ± 0.46 A	14.87 ± 0.33 B	
$O_2^{-\cdot}$ scavenging (mmol eq Trolox 100 g^{-1} skin FW)	Control	21.92 ± 1.82 bA	23.18 ± 3.16 a,bA	29.03 ± 1.85 a,bA	24.85 ± 1.39
	0.5	35.83 ± 1.99 a,bA	25.84 ± 2.39 a,bB	32.47 ± 0.42 aA	31.49 ± 1.49
	1.0	40.90 ± 3.55 aA	33.96 ± 3.19 aA,B	23.00 ± 1.42 cB	32.62 ± 2.68
	2.0	37.11 ± 2.49 aA	24.00 ± 2.11 a,bB	25.93 ± 0.98 b,cB	29.01 ± 2.02
	4.0	43.79 ± 4.50 aA	22.95 ± 1.69 bB	31.47 ± 1.70 a,bA,B	32.85 ± 3.26
	Mean ± SEM (n = 20)	35.91 ± 2.20	26.15 ± 1.39	28.22 ± 0.99	

ANOVA results (*p*-value)

Effects	DPPH	FRAP	ORAC	$O_2^{-\cdot}$ scavenging
UV-C dose	< 0.001	< 0.001	< 0.001	0.018
Storage time	0.017	< 0.001	< 0.001	< 0.001
UV-C dose × storage time	0.035	0.015	0.146	< 0.001

Results are the average of 4 replicates ± standard error. Different lower-case letters indicate significant effect of UV-C irradiation within the column, and different upper-case letters indicate significant effect of storage time within the line ($p < 0.05$; Tukey's HSD).

There was a significant effect of UV-C irradiation and storage time on the antioxidant capacity determined by the ORAC assay ($p < 0.05$), but there was no interaction between these two factors. Only grapes exposed to 1.0 kJ m^{-2} UV-C showed a higher capacity to remove peroxyl radicals than that of control grapes ($p < 0.05$; Table 3). The antioxidant capacity assessed by the ORAC assay decreased after 5 days of storage. The highest values of ORAC-determined antioxidant activity (Table 3) occurred after three days of post-irradiation storage.

Superoxide anion radicals generated under stress conditions can be neutralized by plant hydrogen donors such as polyphenols (Sato, Toyazaki, Yoshioka, Yokoi, & Yamasaki, 2010), ascorbate, and tocopherol (Pisoschi & Pop, 2015). Grapes treated with UV-C (except 0.5 kJ m^{-2}) had approximately twice the $O_2^{-\cdot}$ scavenging activity of the control samples one day after irradiation ($p < 0.05$; Table 3). However, this increase was not maintained on the 3rd day of storage. Five days after irradiation, grapes treated with 1.0 kJ m^{-2} showed reduced $O_2^{-\cdot}$ scavenging activity compared to that of the control grapes. While grapes treated with UV-C demonstrated a maximum $O_2^{-\cdot}$ scavenging capacity in the first day after irradiation, control grapes showed lower scavenging activity on the first day and had small changes during the storage.

In this study, we demonstrated an improvement in the total non-enzymatic antioxidant capacity of grapes submitted to UV-C irradiation. This effect was most prominent when 1.0 kJ m^{-2} was used, and this improvement was evident only on the first day after UV-C as control grapes showed an increase in antioxidant capacity along the storage. Therefore, UV-C treatment seems to anticipate the activation of the antioxidant defense systems in 'Isabel' grapes. Likewise, UV-C has been shown to enhance the total antioxidant capacity of organic and conventional 'Concord' grapes (Pinto et al., 2016), blueberries (Perkins-Veazie, Collins, & Howard, 2008), tomatoes (Bravo et al., 2012), mangoes (González-Aguilar, Villegas-Ochoa, Martínez-Téllez, Gardea, & Ayala-Zavala, 2007), and strawberries (Erkan et al., 2008).

Only the doses of 1.0 and 2.0 kJ m^{-2} UV-C simultaneously increased the SOD activity and the non-enzymatic removal of $O_2^{-\cdot}$ at the short storage time point after irradiation, indicating that the adequate removal of superoxide radicals is a requirement for the hormetic response in 'Isabel' grapes. Moreover, it is interesting that the non-enzymatic antioxidant activity of 'Isabel' grape skin extracts had greater potency for removing biologically relevant radicals, namely $O_2^{-\cdot}$ and peroxyl radicals (ORAC assay), than for removing synthetic DPPH radicals or for reducing ferric ions.

The optimal dose and storage time for observing the beneficial effects of UV-C in 'Isabel' grapes were 1.0 kJ m^{-2} and one day, respectively. The lowest and the highest UV-C doses had no effects or even undesirable effects on most measures of the enzymatic and non-enzymatic antioxidant defense system, including the content of phenolic compounds. It is possible that the antioxidant defense system was not sufficiently activated after exposure to 0.5 kJ m^{-2} UV-C or that the effect of this dose was less persistent compared to that of the other doses, as was observed for the antioxidant capacity evaluated by the DPPH, FRAP, and ORAC assays. On the other hand, exposure to 4.0 kJ m^{-2} UV-C seems to be associated with harmful effects, as the low activities of SOD and CAT may result in the accumulation of $O_2^{-\cdot}$ and H_2O_2 in these grapes. The low ORAC values exhibited by grapes irradiated at 4.0 kJ m^{-2} UV-C support this proposal. In agreement,

grapes irradiated with 4.0 kJ m^{-2} UV-C also had a lower content of phenolic compounds than the other treatments, suggesting that they may be consumed to counteract the oxidative stress. These results clearly indicate that 'Isabel' grapes are more sensitive to UV-C damage than Red globe grapes, which have been shown to exhibit the greatest increase in the content of anthocyanin after exposure to 4.1 kJ m^{-2} UV-C dose (Crupi et al., 2013).

Our results clearly show an opposite relationship between the time-course of changes in the SOD activity and in the accumulation of phenolic compounds in grape skin. While SOD activity decreased over the storage time, polyphenols increased. This result possibly indicates that polyphenol deposition in grape skin depends on the upregulation of biosynthetic pathways that are triggered by increased ROS, as has been previously reported in the literature (González-Aguilar et al., 2010; Kovács & Keresztes, 2002; Wrzaczek et al., 2011). The dose of 1.0 kJ m^{-2} of UV-C increased SOD activity and yielded the greatest deposition of total polyphenols and anthocyanins, resulting in an improved antioxidant capacity, as determined by DPPH and ORAC assays. Moreover, proanthocyanidin skin content was also increased by 1.0 kJ m^{-2} UV-C after 3 and 5 days of post-treatment storage.

Since exposure to 1.0 kJ m^{-2} UV-C yielded the greatest increase in grape skin phytochemicals and overall antioxidant capacity, it can be considered as the hormetic dose of UV-C irradiation for 'Isabel' grapes. Storage at room temperature for at least one day after UV-C irradiation yields good results regarding the antioxidant defense system. Thus, UV-C irradiation at this hormetic dose can be used to increase the antioxidant capacity and levels of natural pigments in 'Isabel' grapes yielding improved nutritional and technological quality. This technology is feasible in industrial applications that use grapes to produce wine, juice, jelly, and other grape food products, as a short post-treatment storage time can be used to yield good results.

4. Conclusion

Both enzymatic and non-enzymatic antioxidant systems were stimulated as an adaptive defense response just one day after the exposure of 'Isabel' grapes to UV-C irradiation. SOD and CAT activation appear to play a role in the hormetic grape response against UV-C. The hormetic dose of UV-C established in this work was 1.0 kJ m^{-2}, followed by a short storage of 24 h at room temperature. The use of these controlled conditions may enhance the antioxidant and nutritional potential of 'Isabel' grapes without changing the profile of anthocyanins but by increasing the concentration of these natural pigments. Thus, as long as a suitable dose is applied, UV-C irradiation may be a technological alternative for improving the color characteristics and bioactive compound content of 'Isabel' grapes before processing and may also serve as a marketing tool for consumers of processed grape products such as wines, juices, and jams.

Conflict of interest

All authors declare that there is no funding sources, employment or personal financial competing interests that could influence in this manuscript.

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4.2 MANUSCRITO 1

Grape peel powder attenuates the inflammatory and oxidative response of TNBS-colitis in rats by down-regulating the NF-κB pathway and up-regulating antioxidant enzymes

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Grape peel powder attenuates the inflammatory and oxidative response of TNBS-colitis in rats by down-regulating the NF-κB pathway and up-regulating antioxidant enzymes

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Abstract

The aim of this study was to evaluate the protective effect of bioactive fractions extracted from whole grape peel powder (GPP) in a diet of rats with experimental colitis. Colitis was induced intrarectally using 2,4,6-trinitrobenzenesulfonic acid (TNBS) after 15 days of dietary supplementation. Bioactive fractions (extractable polyphenols –EP, fiber-bound polyphenols –NEP-F, and dietary fiber –F) were added to the diets at amounts equivalent to the GPP group. EP extract was composed of 80% anthocyanins and NEP-F was composed of 78% of fiber and 7.7% of bound proanthocyanidins. The decrease in feed intake triggered by the TNBS-colitis was reversed in all groups excepting for the EP group. The EP group worsened the colon shortening and increased the spleen weight but these effects were not observed for the GPP group that has phenolics associated to the food matrix. Colitis induced a significant increase of lipid peroxidation, protein oxidation, nitric oxide (NO) levels, and pro-inflammatory cytokines in serum and colon tissue. The activity of antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) and protein expression of pNF-κB was impaired in the colon tissue after colitis. GPP restored the activity of antioxidant enzymes and decreased colon oxidation and NO levels. All grape fractions reduced IKK-β protein expression and NO levels in colon tissue but only NEP-F reduced the expression of *p*NF-κB and myeloperoxidase activity. The results of this study suggested that the consumption of whole grape peel powder may be a therapeutic alternative in the prevention and treatment of colitis.

Keywords: bound polyphenols; inflammation; dietary fiber; peroxynitrite; IKK-β; superoxide dismutase**.**

1 Introduction

Inflammatory bowel diseases (IBD) such as ulcerative colitis (UC) and Crohn's disease are chronic disorders that affects the mucosa and the submucosa of the colon tissue [1]. During colitis development, there is a disruption of the intestinal epithelial barrier, which favors the entry of pathogens from the intestinal lumen to tissue [2]. Then, inflammatory cells are recruited to the impaired colonic mucosa, where these cells release pro-inflammatory cytokines and extinguish the invasive pathogens mainly by producing reactive species [1]. These oxidizing species and the inflammatory cascade promotes further damage of the colonic tissue and magnification of this vicious cycle [3].

The usual pharmacological therapies for colitis treatment have several long-term side effects and poor responsiveness [4,5]. Moreover, IBD patients have increased risk for developing colorectal cancer [6], which motivates the search for alternative therapeutic strategies.

In this context, dietary supplementation with polyphenols [7,8] or dietary fiber [9] has been shown to be effective for modulating the immune response and compensating the inflammatory and pro-oxidant processes in colitis models. Food phenolic compounds are poorly absorbed in the stomach and small intestine [10], which can be beneficial in the case of intestinal inflammation since greater amount of polyphenols will reach the colon [11].

Free or soluble phenolics are those extracted with the conventional techniques using aqueous-organic solvents and include flavonols, anthocyanins, phenolic acids, soluble proanthocyanidins, stilbenes, and lignans [12]. However, a large amount of polyphenols remains in the residues of extraction of these extractable polyphenols being therefore called non-extractable polyphenols (NEPs) [13]. NEPs, which are high molecular weight compounds bound to fiber and protein, are represented by condensed tannins (or proanthocyanidins), hydrolysable tannins (ellagitannins and gallotannins), and hydrolysable polyphenols (phenolic

acids associated to fiber polysaccharides) [14]. These bound polyphenols can be released from food matrix by intestinal bacterial enzymes generating small molecules that can have greater biological activity than their precursors [15].

Similarly, dietary fibers are indigestible components that arrive intact in the colon, where they will be fermented yielding amines, phenols and short chain fatty acids, such as acetate, butyrate, and propionate [12]. In addition, dietary fiber intake has been also associated with selective stimulation of the healthy microbiota and immune system enhancement [12,16].

Recent studies revealed that grape juice and extracts from grape, wine and grape pomace, as well as the isolated grape compound resveratrol have beneficial effects in UC models [17–20]. However, it is not known whether the dietary fiber fraction or the fiber-associated phenolic compounds from grape peel powder (GPP) could also have beneficial effects against colitis.

Thus, the objective of this research was to investigate the effect of GPP as well as the individual or combined effect of its major bioactive fractions (extractable phenolics, fiber-bound phenolics, and dietary fiber) in Wistar rats with colitis induced by 2,4,6-trinitrobenzenesulfonic acid (TNBS). Moreover, the molecular mechanisms involved in the therapeutic effects of these bioactive fractions were also evaluated.

2 Material and methods

2.1 Grape bioactive fractions

2.1.1 Grape peel powder (GPP)

Grapes (*Vitis labrusca* x *Vitis vinifera* L. cv. Isabel) were hand-harvested at Itaara, RS, Brazil (29°35’55” S and 53°46’13” W) and their peels were freeze-dried and pulverized to

obtain the GPP. GPP composition including the content of dietary fiber, extractable and non-extractable polyphenols, and antioxidant capacity was evaluated.

2.1.2 Extractable polyphenols-rich fraction (EP fraction)

The extractable polyphenols-rich fraction was obtained after exhaustive extraction of GPP with methanol:water:acetone [21] (Scheme 1). One gram of GPP was extracted with 40 mL of methanol:water solution (50:50, v/v, pH 2.0 adjusted with HCl) for 60 min at room temperature under stirring. The supernatant was collected after centrifugation at 2500 *x* g for 10 min. The residue was then extracted with 40 mL of acetone:water solution (70:30, v/v) as described in the first extraction. The supernatants of both extractions were combined, concentrated under vacuum at 39°C and had the phenolic composition evaluated by spectrophotometric methods and high-performance liquid chromatography coupled to mass spectrometry (HPLC-MS/MS).

2.1.3 Non-extractable polyphenols-rich fraction (NEP-F fraction)

The NEP-F fraction was the solid residue obtained after extracting the extractable polyphenols (section 2.1.2; Scheme 1). This residue was dried in an exhaustion hood overnight at room temperature and had its chemical composition, including the content of bound condensed tannins evaluated.

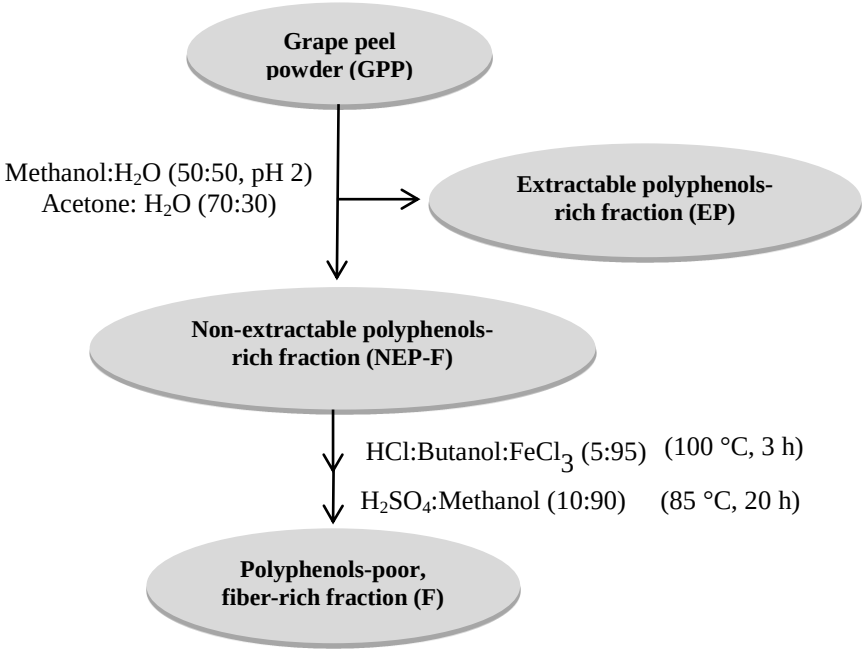
2.1.4 Polyphenols-poor, fiber-rich fraction (F fraction)

The polyphenols-poor, fiber-rich fraction was the solid residue obtained after the extraction of fiber-bound polyphenols from the NEP-F fraction using acid and heating treatment (Scheme 1). For extracting fiber-bound condensed tannins, 200 mL of HCl:butanol solution (5:95, v/v, containing 0.7 g/L of $FeCl_3$) was added to 4 g of the NEP-F fraction and

heated in sealed glass bottles for 3 h in a boiling water bath with agitation [21]. The resultant solid residue was washed two times with HCl:butanol solution (5:95, v/v), dried in an exhaustion hood for about 36 h, pulverized and extracted again to achieve an exhaustive condensed tannin extraction. The extracts obtained were combined and used to quantify the fiber-bound proanthocyanidin content of GPP and NEP-F by the spectrophotometric method previously described [21]. Thereafter, the resultant solid residue was dried, pulverized and submitted to the extraction of hydrolysable tannins. For the hydrolysable tannins extraction, 200 mL of a methanol:H_2SO_4 (90:10, v/v) solution was added to 2 g of the residue from condensed tannin extraction and heated in sealed glass bottles at 85°C under slight agitation for 20 h [22]. The solid residue was washed several times with distilled water (up to 250 mL) and the solid residue was dried in a forced air-drying oven (50°C).

2.2 Chemical composition of grape fractions

Moisture, ash, and crude protein were analyzed according to the Association of Official Analytical Chemists [23]. Total and insoluble dietary fiber were determined by the enzymatic-gravimetric method [24] and the soluble fiber was calculated by the difference between total dietary fiber and insoluble fiber. Lipids were extracted using chloroform and methanol and quantified by gravimetry [25]. Non-fibrous carbohydrates were calculated by difference. Soluble sugars were extracted using ethyl alcohol 80% e were determined spectrophotometrically by phenol-H_2SO_4 assay [26].



Scheme 1. Obtainment of bioactive fractions from grape peel powder.

2.3 LC-PDA-MS/MS of extractable anthocyanins and non-anthocyanic phenolics of GPP

The composition of extractable anthocyanins and non-anthocyanin phenolics of GPP was assessed in the EP fraction. Chromatographic analyses were carried out using a Shimadzu® HPLC (Kyoto, Japan) equipped with a binary pump, on-line degasser, column oven, connected to a photodiode array detector (PDA), and a mass spectrometer with an electrospray ionization (ESI) source and a Q-TOF analyzer (Bruker Daltonics®, micrOTOF-QIII, Bremen, Germany).

Prior to HPLC analysis, EP extract was purified in a SPE-C18 cartridge (Phenomenex®, 1 g) in order to obtain a non-anthocyanic and an anthocyanic fraction. Each fraction was filtered through a PTFE membrane filter 0.22 µm before HPLC injection.

Phenolic compounds were separated using a Hypersil Gold C18 column (5 µm, 150 x 4.6 mm) at a flow rate of 1 mL/min, column temperature at 38°C, using a mobile phase composed by water:formic acid (99.9:0.1, v/v) containing 5% of methanol (mobile phase A)

and acetonitrile:formic acid (99.9:0.1, v/v) (mobile phase B) in a gradient mode: 0-10 min (4% B), 10-21 min (4% B), 21-55 min (16% B), 55-70 min (50% B), 70-72 min (100% B), 72-80 min (100% B), 80-83 min (0% B) and 83-92 min (0% B). The UV-vis spectra was obtained from 200 to 800 nm and chromatograms were analyzed at 280 nm (flavanols and hydroxybenzoic derivatives), 305 nm (stilbenes), 320 nm (hydroxycinnamic acid derivatives), and 360 nm (flavonols). The column eluate was splitting to 0.5 mL/min prior to electrospray ionization (ESI) interface.

Anthocyanins were separated using a Kinetex C18 100-A column (2.6 µm, 100 x 4.6 mm) at a flow rate of 0.9 mL/min at 38°C, using a mobile phase composed by water:formic acid (97:3, v/v) (mobile phase A) and acetonitrile (mobile phase B) in a gradient mode: 0 min (10% B), 0-20 min (30% B), 20-25 min (80% B), 25-27 min (80% B), 27-29 min (10% B), 29-34 min (10% B). The UV-vis spectra and chromatograms were obtained at 520 nm. The column eluate was splitting to 0.45 mL/min prior to ESI interface.

Mass spectra were recorded with a scan range from 100 to 1000 *m/z*. The MS parameters were set as follows: ESI source in negative ion mode for the non-anthocyanic phenolics and in the positive ion mode for anthocyanins; capillary voltage, 3000 V; dry gas (N_2) temperature, 310 °C; flow rate, 8 L/min; nebulizer gas, 4 bar. Phenolic compounds and anthocyanins were identified on the basis of the following information: elution order and retention time in the reversed phase column, UV–vis and MS spectra features compared to data available in the literature and to authentic standards analyzed under the same conditions.

2.4 *In vivo* experimental design and induction of colitis

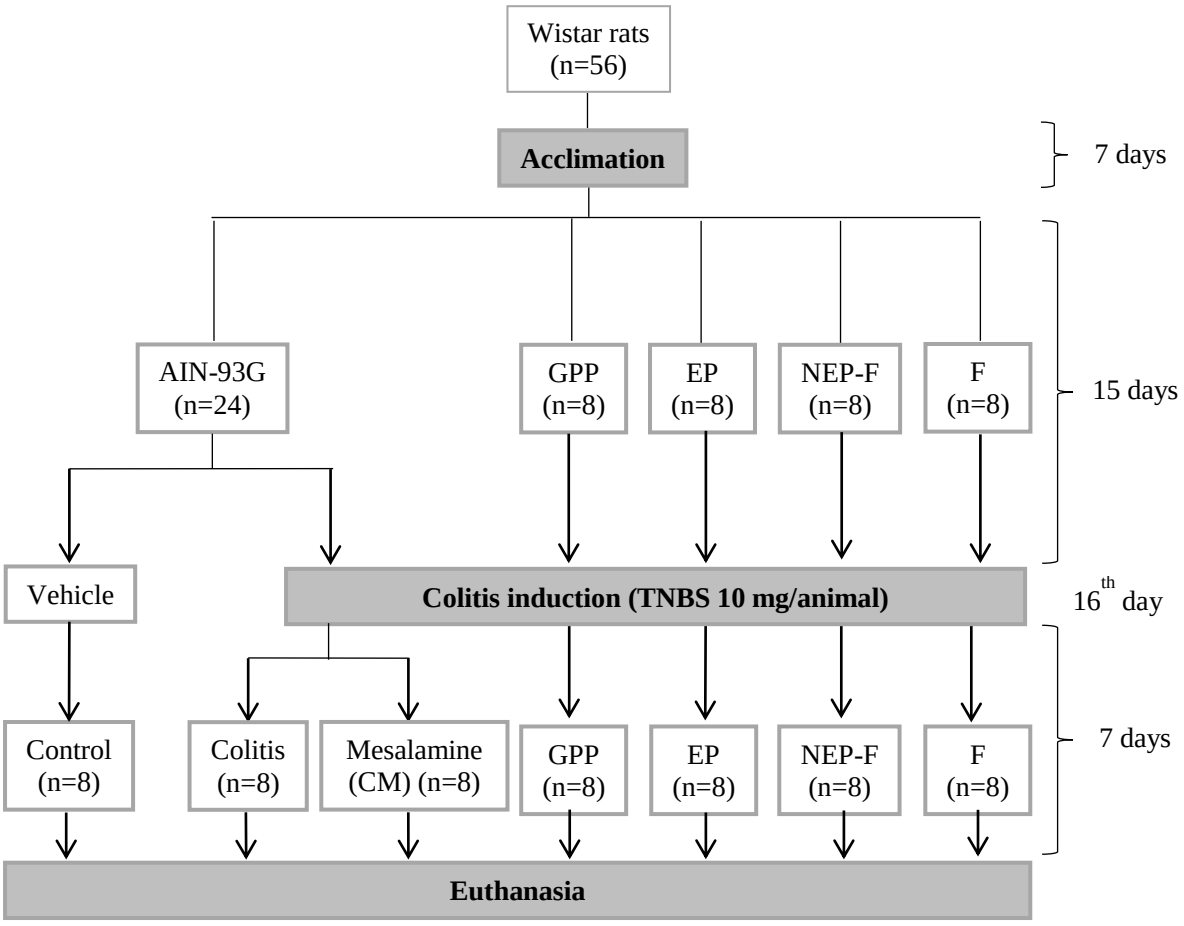
This study was approved by the Animal Research and Ethics Committee of the University of Campinas (Brazil) (protocol 3815-1/2015). Adult male Wistar rats (200 ± 20 g) were allocated in individual cages and maintained under controlled environmental conditions:

22 ± 2 °C, 65 ± 5 % humidity, and 12/12 h light–dark cycle. Animals were divided into seven groups (n=8 per group): control (C), colitis (CC), mesalamine (CM), 8% grape peel powder (GPP), extractable polyphenols (EP), non-extractable polyphenols (NEP-F), and polyphenols-poor residue (F). Groups C, CC, and CM received AIN-93G diet [27], whereas the GPP, EP, NEP-F, and F groups received AIN-93G supplemented with each grape fraction (Scheme 2).

Twenty grams of experimental diets were offered to each animal per day and the intake was daily assessed. The dose of 8% GPP was chosen to provide approximately 12.4 mg of phenolics/animal/day (approximately 40 mg of phenolics/kg body weight/day). Similar doses have been shown to be safe in an UC model [28]. The amount of the EP and NEP-F fractions were calculated to provide extractable and non-extractable polyphenols equivalent to the GPP diet. The amount of F fraction included in the F diet was equivalent to the residue obtained after extraction (extractable and bound-phenolics extraction) of the same amount of GPP used in the GPP diet. As the method used to obtain the F fraction had a very low yield (4.4% from the GPP, data not shown), the F diet had much lower grape fiber content than the GPP and the NEP-F diets. This amount was chosen due to the limited availability of grape peel to produce enough F fraction to achieve grape fiber content equivalent to the GPP diet. The amount of cellulose and sucrose added to the diets supplemented with GPP and its fractions were reduced to ensure that all diets would provide dietary fiber and soluble carbohydrates at equivalent amounts to the AIN-93G diet.

After a 7-days acclimation period, animals received the experimental diets for 15 days before colitis induction and thereafter continued to receive the experimental diets for 7 days (Scheme 2). After slight sedation with isoflurane, colitis was induced in the 16th day by intracolonic administration of 10 mg of 2,4,6-trinitrobenzenesulfonic acid (TNBS) dissolved in 0.25 mL of 50% ethanol (v/v) and administered by a PVC flexible cannula inserted 8 cm into the anus. The same procedure was conducted with the control group by intracolonic

administration of 0.25 mL of 50% ethanol (v/v). After colitis induction, rats were maintained under the same diets for 7 days until the euthanasia. The CM group received the reference therapeutic drug mesalamine by gavage (25 mg/animal/day) in the 7 days following the induction of colitis.



Scheme 2. Dietary treatment and protocol of ulcerative colitis induction.

2.5 Collection of biological material

Rats were anesthetized with ketamine and xylazine (90 and 5 mg/kg body weight) and killed by exsanguination with a cardiac puncture. Blood was collected into tubes containing clot accelerator gel and centrifuged (2000 *x* g, 15 min). Serum samples were stored at -80°C until analyses.

Colonic tissue (from the ileocecal junction to the anal verge) was quickly removed, rinsed with cold saline solution (0.9% NaCl) and blotted dry. Then, the colon was longitudinally opened, the adhering fat tissue was gently removed, and colon was weighed and measured. Spleen and cecum were also collected and weighed. All tissues were frozen in liquid nitrogen and stored at -80°C until analyses.

2.6 Serum analyses

The non-enzymatic antioxidant capacity of serum was analyzed after treatment of samples with ethanol, water and metaphosphoric acid [29] and centrifugation (21000 *x* g, 10 min, 4°C). The supernatant obtained was submitted to the ferric reducing antioxidant power (FRAP) [30] assay. Results were expressed as nmol equivalents of Trolox (TE) per mg of protein. Serum proteins were determined using bovine serum albumine as standard [31].

Lipid peroxidation of serum was evaluated by thiobarbituric acid reactive substances (TBARS) assay [32].

Protein oxidation was determined by the advanced oxidation protein products (AOPP) assay [33] using chloramin-T as standard.

Serum was deproteinized with 300 g/L of $ZnSO_4$ for determination of nitric oxide (NO) levels. NO was indirectly evaluated by determination of its stable metabolites (nitrite and nitrate) as previously described [34].

The levels of inflammatory cytokines IL-1β and TNF-α were determined by ELISA kits according to the manufacturer's instructions (Peprotech, Ribeirão Preto, SP, Brazil).

2.7 Colon tissue analyses

Tissue was homogenized in 100 mM phosphate buffer pH 7.4 using a Polytron homogenizer, centrifuged (1500 *x* g, 15 min, 4°C) and the supernatant was maintained at –

80°C until analyses.

Lipid peroxidation [32] of colon tissue was determined in the crude homogenate, whereas the supernatant was used to evaluate NO [34] and AOPP levels [33]. The supernatant was also used for determining the activity of antioxidant enzymes catalase (CAT) [35] and superoxide dismutase (SOD) [36], which had the activity of the cytoplasmic (CuZnSOD) and mitochondrial (MnSOD) isoforms assessed.

Inflammatory cytokines (TNF-α, IL-1β, IL-10) were determined by ELISA protocol according to the instructions of the manufacturer (Peprotech, Ribeirão Preto, SP, Brazil).

For myeloperoxidase (MPO) extraction, about 50 mg of colon tissue were homogenized with 1 mL of 0.05 M phosphate buffer, pH 6.0 containing 0.5% of hexadecyltrimethylammonium bromide (HTAB). The homogenates were subjected to 3 freeze-thaw cycles, sonicated, and centrifuged at 4°C for 15 min at 13150 *x* g. The supernatants were used to measure MPO activity using H_2O_2 and o-dianisidine dihydrochloride [37].Measurements were made at 450 nm and results were expressed as the area under the curve (AUC) normalized by AUC of the control group.

2.8 Real-time reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA from colon tissue was extracted using Trizol (InvitrogenCorp., Carlsbad, CA) as recommended by the manufacturer. High Capacity cDNA Reverse Transcription kit (#4368813, Applied Biosystems, USA) was used to reverse transcribe cDNA. The expression of mRNA was determined by Real Time PCR (ABI Prism 7500, Applied Biosystems, USA) using primers for inflammatory and anti-inflammatory genes (Table 1) (Integrated DNA Technologies, USA). GAPDH (Applied Biosystems, USA) was used as a housekeeping gene. Each reaction consisted in 50 ng of cDNA, 0.25 μL of primer, 3 μL of TaqMan Fast Advanced Master Mix (Applied Biosystems, USA), and RNase free water to a final volume of

10 μL. Data were analyzed using the Sequence Detector System 7500 (Applied Biosystems, USA).

Table 1

2.9 Western blotting analysis

About 0.1 g of colonic tissue were homogenized with cold 0.1 M Tris-HCl buffer, pH 7.5 containing 10 mM etilenodiaminotetracetic acid (EDTA), 100 mM NaF, 10 mM $Na_4P_2O_7$, 10 mM Na_3VO_4, 2 mM phenylmethylsulfonyl fluoride (PMSF), 10% Triton X-100, and 0.8% aprotinin (from bovine lung). Lysates were centrifuged at 4°C for 20 min at 13150 *x* g and the supernatants were collected and stored at -20°C until electrophoresis separation. The quantification of protein was made using Bradford's reagent (Biorad Protein Assay Dye Reagent Concentrate). Lysate samples (75 μg of protein per lane) were separated by 8-12% SDS-PAGE and transferred to 0.45 μm nitrocellulose membranes (Biorad) by electroblotting. Membranes were incubated with primary antibodies for COX-2 (Santa Cruz Biotechnology, INC.), *p*NF-κB p50 (Santa Cruz Biotechnology, INC.), TLR-4 (Santa Cruz Biotechnology, INC.), and IKK-β (Santa Cruz Biotechnology, INC.) and then incubated with secondary antibodies coupled to horseradish peroxidase. β-actin (Santa Cruz Biotechnology, INC.) was used as the reference protein. Protein bands were revealed with the chemiluminescence detection kit (Biorad) and band intensity was detected by the Gene Genome (GE Healthcare, Little Chalfont, UK) equipment associated with image acquisition software GeneSys (Syngene BioImaging, Cambridge, UK) and quantified using the ImageJ software.

2.10 Statistical analyses

Feed intake and weight data were evaluated by analysis of variance (ANOVA) using

time as a repeated measure. Other data were analyzed by one-way ANOVA, followed by Duncan's test for means comparison. Data were expressed as mean ± SEM and results were considered significant when $p<0.05$.

3 Results

3.1 Chemical composition of grape fractions and diets

GPP was rich in non-fibrous carbohydrates (about 58%) and dietary fiber (about 26%), being the last one mostly composed by insoluble fiber (67% of total dietary fiber) (Table 2). After extracting soluble polyphenols to obtain the EP fraction, the residue consisted in the non-extractable polyphenols-rich fraction (NEP-F), which showed greater content of dietary fiber (about 78%) and fiber-bound proanthocyanidins (7.7%, Table 2) compared to the GPP. The condensed and hydrolysable tannins from NEP-F fraction were chemically removed to obtain a polyphenol-poor and fiber-rich fraction (F), which was almost entirely composed by dietary fiber (about 97%) (Table 2). However, the chemical treatment also changed the composition of the dietary fiber, as some soluble dietary fiber was also removed, yielding an insoluble:soluble fiber ratio of 63:1 in the F fraction, whereas this ratio was 4:1 in the NEP-F fraction and 2:1 in the GPP (Table 2).

Table 2

Since the purpose of this work was to determine the individual contribution of grape extractable polyphenols, fiber-bound proanthocyanidins and dietary fiber for the benefits of GPP supplementation in UC, the amount of EP, NEP-F, and F fraction added to the diets was calculated to provide extractable and non-extractable polyphenols equivalent to the GPP diet (Table 3).

The amount of cellulose and sucrose added to the diets supplemented with GPP and its fractions were reduced to ensure that these diets would provide dietary fiber and soluble carbohydrates at equivalent amounts to the AIN-93G diet. In addition to the extractable polyphenols, the EP extract had soluble sugars (15.96 ± 1.27 g% equivalents to sucrose). Thus, the EP diet contained only the soluble sugars and the extractable polyphenols from GPP and was formulated to provide these constituents at an amount equivalent to that provided by the GPP diet (Table 3). In addition, AIN-93G and EP diet contained only insoluble fiber (cellulose), while the other experimental diets had a variable ratio of insoluble:soluble fiber depending on the grape fraction. The NEP-F diet had non-extractable polyphenols (fiber-bound proanthocyanidins) at an amount equivalent to that provided by the GPP diet (Table 3).

Table 3

3.2 Identification and quantification of extractable phenolic compounds of GPP by LC-PDA-ESI-MS-qTOF

The EP fraction was analyzed to obtain the composition of extractable anthocyanic and non-anthocyanic phenolic compounds (see Supplementary material). The total amount of extractable phenolic compounds in ‘Isabel’ grape peel powder was 776.14 mg/100 g (Table 4 and 5). A total of 10 anthocyanins were identified and the major anthocyanin was malvidin-3-glucoside, which accounts for 52.4% of total anthocyanins (Table 4). The amount of non-anthocyanic phenolics in GPP was 162.50 mg/100 g (Table 5) and LC-PDA-ESI-qTOF revealed that major non-anthocyanic phenolics found in GPP were quercetin-3-hexoside, quercetin-3-glucuronide, *trans*-caftaric acid, catechin, myricetin-3-hexoside, kaempferol-3-hexoside, and *trans*-resveratrol. Anthocyanins accounted for about 80% of the total extractable phenolic compounds from GPP, whereas flavonols, phenolic acids,

proanthocyanidins, and stilbenes accounted for 12.5, 6, 1.7, and 0.6%, respectively (Tables 4 and 5).

Table 4

Table 5

3.3 Body weight, diet intake, weight gain, and phenolic consumption

All experimental groups had a linear increase in the body weight during the sixteen days that animals were fed the experimental diets before the induction of colitis, and there was no difference in the body weight among the different groups in this period (Figure 1A). In addition, before colitis induction (day 0), the daily feed intake of diets containing grape fractions was similar to the control group (AIN93-G diet) (Figure 1B, day -1) and no diarrhea or weight loss were observed before colitis induction (data not shown).

After the induction of colitis (indicated by arrows in Figure 1), there was a significant interaction effect between time and treatment on the daily feed intake ($p<0.05$). On the first day after colitis induction, diet consumption was reduced for all groups, including the control group that received vehicle (ethanol) instead of TNBS administration (Figure 1B). In addition, all groups, excepting for the NEP-F and F groups, had lower feed intake than the control group on the 1st day after TNBS administration ($p<0.05$). Usual feed intake began to be restored after the 3rd day but not for the EP group that showed lower feed intake than the control group ($p<0.05$) along the 7 days after TNBS administration and also lower feed intake than the colitis group between the 4th and 7th days ($p<0.05$; Figure 1B). On the 7th day, only the colitis and EP groups did not reestablish feed intake to the levels before colitis induction ($p<0.05$; Figure 1B).

The daily weight gain before colitis ranged between 1.25 and 1.79% of total body weight and was not different among groups (Figure 1A). After colitis induction, only the time had a significant effect on the weight gain ($p<0.05$). There was significant weight loss in the 1st and 2nd days after TNBS administration and rats started to gain weight from the 3rd day onwards (Figure 1C). From the 5th day onwards, all groups except those fed the EP fraction, exhibited body weight equal to or higher than before TNBS administration (Figure 1C). This finding is in agreement with the lower feed intake of the EP group after colitis induction (Figure 1B).

Before colitis, the daily consumption of extractable phenolics was similar for the GPP and EP groups (40.9 and 41.1 mg/kg body weight, respectively) (Figure 1D). The decrease in daily feed intake after colitis induction caused a concomitant decrease in the intake of extractable phenolics in the GPP and EP groups. In line with its lower feed intake, the EP group also had lower phenolic consumption than the GPP group along the 7 days after colitis induction ($p<0.05$; Figure 1D). Before colitis, the daily consumption of non-extractable polyphenols (bound proanthocyanidins) for the GPP and NEP-F groups was 117.3 and 134.9 mg/kg body weight, respectively (Figure 1E). After colitis, the intake of non-extractable polyphenols was reduced due to the reduced feed intake. GPP and NEP-F groups had similar intake of bound proanthocyanidins for most of the time, excepting in days 0, 3, and 7 after TNBS administration.

Figure 1

3.4 Colon and spleen measurements

All animals treated with TNBS had lower colon length/weight ratio than control ($p<0.05$; Figure 2A) demonstrating that colitis induction led to colon shortening with an

increase in weight and thickness of colonic tissue, which was probably caused by the edema that is associated to the inflammatory process. None of the diets containing grape fractions was able to prevent such macroscopic changes. On the contrary, the EP group had greater colon shortening (lower length/weight ratio) compared to the colitis group ($p<0.05$; Figure 2A). Colitis did not change the relative weight of spleen but rats that received the EP diet had increased spleen weight when compared to the control and colitis non-treated groups (Figure 2C, $p<0.05$). The increase in spleen weight observed in the EP group may indicate an excessive local immune response.

Figure 2

3.5 Oxidative and inflammatory serum status

There was a significant increase of lipid peroxidation (TBARS levels), protein oxidation (AOPP levels), and NO levels in serum after colitis induction ($p<0.05$; Figure 3). Only the mesalamine and F groups were able to restore serum lipid peroxidation to control levels ($p<0.05$; Figure 3A), whereas mesalamine and all experimental diets restored the oxidized protein content to basal levels ($p<0.05$; Figure 3B). Only the mesalamine treatment, GPP and F diets restored serum NO content to control levels ($p<0.05$; Figure 3C).

The non-enzymatic antioxidant status of serum evaluated by FRAP (Figure 3D) or ORAC assay (data not shown) was not modified by the induction of colitis. However, diets containing the NEP-F and EP fractions improved the serum FRAP values compared to the control group ($p<0.05$; Figure 3D), whereas the ORAC assay was not affected by any treatment (data not shown).

The colonic inflammation also induced a significant increase in the levels of serum pro-inflammatory cytokines, TNF-α and IL-1β, when compared to the control group ($p<0.05$;

Figures 3E and F). The serum levels of TNF-α and IL-1β were decreased by the treatment with GPP and NEP-F diets, whereas the mesalamine treatment decreased only TNF-α levels (Figures 3E and F, $p<0.05$).

Figure 3

3.6 Markers of oxidative damage in colon tissue

As observed in the serum, colitis promoted a significant increase in lipid (TBARS levels) and protein oxidation (AOPP levels) and in the NO levels in the colon tissue ($p<0.05$; Figure 4). Lipid peroxidation was completely prevented by diets containing NEP-F and F fraction, whereas GPP and EP diet and mesalamine treatment caused only a partial reduction in TBARS levels ($p<0.05$; Figure 4A). Protein oxidation was restored to basal levels in the groups that received GPP, EP, NEP-F or F diets ($p<0.05$; Figure 4B). The increase in the colonic levels of NO were restored to basal levels by all treatments ($p<0.05$; Figure 4C).

The induction of colitis decreased the activity of CuZnSOD and CAT ($p<0.05$) but did not change the activity of MnSOD (Figure 4D, E, and F). Mesalamine, GPP, EP, and NEP-F diets were able to restore the activity of CuZnSOD to control levels (Figure 4D) but only GPP diet restored CAT activity to control levels (Figure 4F).

Figure 4

3.7 Inflammatory colon markers

Colitis induced an increase in gene and protein expression of pro-inflammatory markers in colonic tissue (Figure 5). The increase of TNF-α protein observed in the colitis group was reversed by all experimental diets and mesalamine (Figure 5B, $p<0.05$). This

increment was mediated by the up-regulation of mRNA expression of TNF-α (Figure 5A, $p<0.05$), which was reduced by all experimental diets and mesalamine treatment.

Figure 5

In addition, colitis significantly increased the mRNA expression of IL-1β in the colonic tissue ($p<0.05$) and the pharmacological treatment with mesalamine was able to reduce the expression of IL-1β to the control level (Figure 5C). GPP and EP diet reduced the mRNA expression of IL-1β compared to the colitis group but levels were still higher than those found in the control group ($p<0.05$; Figure 5C). In agreement with the increased gene expression, there was also a large increase in the protein levels of IL-1β in colonic tissue after colitis induction ($p<0.05$) and only the mesalamine treatment was able to reduce IL-1β levels in the colonic tissue ($p<0.05$; Figure 5D).

The mRNA expression of the anti-inflammatory cytokine IL-10 was not affected by colitis induction. However, mesalamine treatment and diets supplemented with EP or NEP-F fractions promoted a great increase in the mRNA expression of IL-10 in colonic tissue ($p<0.05$; Figure 5E). Despite this, the protein levels of IL-10 were not changed by colitis induction or any treatment ($p>0.05$; Figure 5F).

Colitis also induced a significant increase in the mRNA expression of IL-6 in colonic tissue (Figure 5G, $p<0.05$). GPP diet partially decreased IL-6 mRNA expression, whereas the pharmacological treatment with mesalamine or dietary supplementation with NEP-F or F fractions was able to reduce the levels of IL-6 mRNA (Figure 5G, $p<0.05$). In contrast, dietary supplementation with the EP fraction had no effect on IL-6 mRNA expression.

TLR-4 activation promotes the activation of pro-inflammatory pathways related to inflammatory cytokines, NF-κB, and mitogen-activated protein kinases (MAPKs) [37]. As

expected, we observed that TLR-4 protein expression was significantly enhanced by colitis (Figure 6A, $p<0.05$). Mesalamine and EP diet partially reduced TLR-4 expression (Figure 6A, $p>0.05$). Thus, the anti-inflammatory effects observed were not associated with the inactivation of TLR-4 pathway. The induction of colitis increased by 4-fold the protein expression of IKK-β ($p<0.05$; Figure 6B). The treatment with mesalamine or all experimental diets were able to decrease IKK-β protein to control levels (Figure 6B).

Figure 6

Although NF-κB is an important marker of the activation of pro-inflammatory cascade in cells, its gene expression was not enhanced by colitis induction (Figure 6C). Nevertheless, the induction of colitis increased by 1.6-fold the protein expression of the phosphorylated NF-κB p50 subunit ($p<0.05$; Figure 6D). Mesalamine and GPP, EP, and NEP-F diets decreased *p*NF-κB protein expression to control levels ($p<0.05$; Figure 6D).

MPO activity, which is assessed as a marker of neutrophil infiltration in the colonic tissue, had a 4-fold increase after colitis induction ($p<0.05$; Figure 7A). Only the diet supplemented with the NEP-F fraction reduced MPO activity compared to the colitis group ($p<0.05$) but did not restore the MPO activity to the control level. The protein expression of COX-2 in colon tissue was not changed by colitis induction, grape peel fractions or mesalamine (Figure 7B, $p>0.05$).

Figure 7

4 Discussion

We demonstrated that dietary supplementation with grape peel powder, the extractable

or the fiber-bound polyphenol grape fraction reduced the inflammatory response and oxidative stress in the colon of TNBS-exposed rats. However, no grape fraction or the treatment with the standard therapeutic drug mesalamine were able to attenuate the colonic shortening induced by TNBS. In addition, dietary supplementation with the EP fraction induced adverse effects as it reduced feed intake and increased body weight loss, spleen weight, and the colonic shortening after TNBS-exposure. Although the EP and GPP diets had similar amounts of extractable polyphenols and the EP group had lower daily intake of extractable polyphenols than the GPP group (due to the lower feed intake after TNBS exposure), no adverse effect was observed in the GPP group. This result indicates an interaction between the bioactive fractions of GPP that can protect against the adverse effects of extractable polyphenols either by reducing their bioavailability or by direct mechanisms.

Some studies have shown that high doses of free grape polyphenols have no protective effect or even led to adverse effects in rats with DSS-induced colitis, although low doses have reduced the severity of colonic inflammation in this model [17,39]. In fact, an excess of isolated or soluble polyphenols has been shown to act as a pro-oxidant instead of antioxidant and worse the inflammation process in a colon carcinogenesis model associated to DSS-colitis [40].

Since an overproduction of $O_2^{-\bullet}$ and H_2O_2 together with a decrease in the levels of antioxidants in the inflamed colon are associated with the pathogenesis of IBD, antioxidant enzymes like SOD and CAT play an important role in the protection of intestinal mucosa against oxidative stress [3,41]. We observed a significant inhibition of CuZnSOD and CAT after colitis induction. This decreased ability to remove $O_2^{-\bullet}$ combined with the increased NO levels leads to generation of the powerful oxidant peroxynitrite [42], which may be responsible for the increased lipid and protein oxidation in the colon mucosa of colitic rats. In addition, peroxynitrite has been shown to activate the NF-κB inflammatory pathway [43].

GPP and all grape peel fractions were able to decrease lipid and protein oxidation, as well as the NO levels in the colon. In addition, GPP, as well as the EP and NEP-F fractions, ameliorated CuZnSOD activity, indicating that extractable and fiber-bound grape polyphenols up-regulate colon antioxidant enzymes. Moreover, both the extractable and fiber-bound polyphenols were able to improve the non-enzymatic serum antioxidant status (FRAP values) suggesting that they could directly scavenge reactive species. In fact, growing evidence supports an antioxidant role for fiber-bound polyphenols [44] and there is evidence that fiber-bound polyphenols may have even higher antioxidant capacity compared to free and soluble conjugated polyphenols [45]. Moreover, we observed a synergistic effect between the bioactive compounds of grape, as only GPP was able to simultaneously recover CuZnSOD and CAT activity. A significant increase in CAT and SOD activities has been also noticed when DSS-colitic mice were treated with blueberry extract [46]. The fiber (F) fraction was efficient to reduce lipid and protein oxidation in serum and colon tissue. However, these antioxidant properties cannot to be attributed to the phenolic associated compounds since these compounds were removed and this fraction was poor in polyphenols. Thus, the antioxidant effects observed for F fraction could be related to the capacity of fiber to quench free radicals formed in the gastrointestinal tract [47]. However, it is important to notice that the F fraction obtained is not completely equivalent to the grape peel fiber since chemical modification may have occurred in the dietary fiber structure during the extraction process.

TNF-α is synthesized by macrophages, lymphocytes, and polymorphonuclear cells and plays a key role in the initiation of inflammatory processes [28]. GPP was able to inhibit the expression of TNF-α and partially reduced the expression of IL-6 in the colon. All grape fractions studied were able to reduce the levels of TNF-α in the colonic tissue. NEP-F and F reduced IL-6 expression. TNF-α is mostly associated with the early inflammatory response, whereas IL-6 is associated with the progression of the inflammatory process [48]. While all

grape fractions seems to be effective in blocking the secretion of TNF-α in the initial steps of the inflammatory cascade, only NEP-F and F fractions seems to attenuate the progression of the inflammatory process. These findings suggest that different mechanisms underline the anti-inflammatory effects of grape peel phytochemicals. Moreover, dietary fiber seems to be responsible for the reduction of IL-6 expression as the NEP-F and F fraction (but not GPP or soluble polyphenols) were able to diminish this inflammatory marker in colon tissue.

TLR-4 is a transmembrane protein that is predominant in cells from the innate immune system and responds to molecular stimuli associated with pathogens (PAMPs), such as lipopolysaccharides (LPS) from gram-negative bacteria [38]. In fact, the dysregulation of the TLR pathway has been associated with the initiation, progression, and resolution of intestinal inflammation and the pathogenesis of UC [49,50]. Soluble polyphenols seems to have their anti-inflammatory effects at least in part mediated by the inhibition of TLR-4 signaling. However, we cannot rule out that the decrease of TLR-4 expression triggered by soluble phenolics (EP diet) is secondary to an antimicrobial effect against the colonic pathogenic microbiota.

The anti-inflammatory effect of grape fractions in the colon tissue also seems to be associated to the inactivation of the NF-κB pathway. As the activation of TLR-4, the NF-κB cascade may also be initiated by ROS or inflammatory cytokines such as IL-1β [28], and comprises the translocation of NF-κB to the nucleus to promote the expression of pro-inflammatory genes, including further expression of IL-1β [28] and enzymes associated with inflammation, such as iNOS and COX-2 [7]. Although mRNA levels of NF-κB p65 have not been altered by colitis, the protein expression of the *p*NF-κB and IL-1β were significantly increased in the whole cell lysate of colitic rats. No grape fraction was able to reduce the protein level of IL-1β but GPP and NEP-F fraction decreased protein levels of *p*NF-κB, whereas EP caused a partial decrease of *p*NF-κB. IKK-β is a protein responsible for

phosphorylating the inhibitor of NF-κB (IκB) thereby promoting the activation of the NF-κB pathway [7]. GPP and all grape fractions were able to reduce IKK-β protein expression but only GPP, EP, and NEP-F fractions were able to reduce the protein levels of *p*NF-κB. Thus, the extractable and fiber-bound grape polyphenols appear to have similar ability to modulate the NF-κB pathway in the colon. This effect appears to be related to the modulation of IKK-β levels rather than to the decrease in the colonic levels of IL-1β, which is located upstream in this inflammatory cascade. The upregulation of CuZnSOD activity may also contribute to the protective effect of GPP against the colitis-induced activation of the NF-κB pathway as GPP, EP, and NEP-F fractions had parallel effects in upregulating CuZnSOD activity and decreasing *p*NF-κB. SOD can decrease $O_2^{-\bullet}$ levels, thereby preventing peroxynitrite formation and the activation of NF-κB pathway triggered by peroxynitrite [43].

The anti-inflammatory cytokine IL-10 plays an essential role in the maintenance of intestinal homeostasis and its silencing has been shown to induce colitis [51]. Although the protein level of IL-10 in colon was not altered by colitis or experimental diets, the intake of EP and NEP-F fractions have increased the gene expression of IL-10.

Myeloperoxidase (MPO), which is located to neutrophil granules, has been used as an indicative of neutrophil infiltration into damaged tissue [5]. The neutrophil recruitment into a damaged tissue has been shown to be dependent on the release of cytokines and histamine by macrophages and mast cells [52]. We observed that only the NEP-F fraction was able to reduce MPO activity, suggesting that only this fraction was effective for reducing the infiltration of inflammatory cells in the colon tissue. This effect can reflect the role of fiber-bound polyphenols such as non-extractable polymeric proanthocyanidins derived from catechin, which have been described at appreciable amounts in grape pomace [53]. In fact, epigallocatechin-3-gallate, a catechin-based polymeric polyphenol, has been demonstrated to decrease colon injury by modulating the recruitment of macrophages and neutrophils, the

release of histamine and decreasing MPO activity [52].

Our data demonstrated that different mechanisms are implicated in the anti-inflammatory effects of the bioactive fractions from grape peel in the colon. The anti-inflammatory effects of NEP-F fraction may be explained at least in part by the decrease in the colonic levels of IKK-β, the attenuation of the NF-κB pathway and reduced infiltration of neutrophils, as indicated by the reduced MPO activity. On the other hand, the anti-inflammatory effects of the EP fraction were not associated to a decrease in neutrophil infiltration but only to the attenuation of the NF-κB pathway.

Colon inflammation affected not only the colon homeostasis, but also had harmful systemic effects, as can be seen in serum oxidative and inflammatory markers. TNF-α is associated with the initiation of pro-inflammatory processes and can activate routes that lead to the perpetuation of inflammation [28]. IL-1β is involved in the progression of several inflammatory disorders including colitis, being produced by macrophages and dendritic and epithelial cells [54]. Serum levels of TNF-α and IL-1β were increased with colitis development. Only the GPP and NEP-F fractions reversed this increment in serum indicating that the serum anti-inflammatory effects of GPP, appear to be associated to the fiber-bound phenolics rather than to the free phenolics or dietary fiber alone. Fiber-bound proanthocyanidins from grapes have been shown to undergo colonic fermentation releasing catechin and other metabolites with potential healthy effects [55].

5 Conclusion

The intake of whole grape peel powder was effective to attenuate the oxidative and inflammatory response in a rat model of TNBS-induced colitis. The up-regulation of antioxidant enzymes and down-regulation of pro-inflammatory markers appear to underline such effects. The extractable and fiber-bound polyphenols as well as the dietary fiber fraction

exhibit antioxidant effects in the serum and colon tissue in the TNBS model. However, only the fraction rich in fiber-bound polyphenols had anti-inflammatory effects in the serum and was able to reduce neutrophil infiltration in the colonic tissue. In addition, the fiber-bound polyphenols from grape peel had anti-inflammatory effects in the colon, similar to the extractable polyphenols and to the grape peel powder but the extractable polyphenols promoted adverse effects on body weight and colon shortening. Some anti-inflammatory and antioxidant mechanisms appear to depend on the synergic effect among grape bioactive compounds as they were observed after treatment with the whole peel powder but not after treatment with the individual grape peel fractions.

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Table 1. Primer sequences of genes used in qRT-PCR.

Genes	Primer sequences (5' to 3')	
	Forward	Reverse
Tnf	AGACCCTCACACTCAGATCA	GTCTTTGAGATCCATGCCATTG
Il1b	GTGCTGTCTGACCCATGT	TTGTCGTTGCTTGTCTCTCC
Il10	CGACGCTGTCATCGATTTCTC	TGGCCTTGTAGACACCTTTG
Il6	CAGAGCAATACTGAAACCCTAGT	CCTTCTGTGACTCTAACTTCTCC
Rela (NF-κB p65)	GACTCTTCTTCATGATGCTCTTG	GAGTTCCAGTACTTGCCAGAC
Gapdh	AGTGCCAGCCTCGTCTCATA	GATGGTGATGGGTTTCCCGT

Tnf = tumor necrosis factor; *Il1b* = interleukin 1 beta; *Il10* = interleukin 10; *Il6* = interleukin 6; *Rela* (*NFκB-p65) = fator nuclear kappa B subunit 65; Gapdh* = glyceraldehyde 3-phosphate dehydrogenase.

Table 2. Chemical and phytochemical composition of grape peel powder fractions.

	GPP	NEP-F	F
Proximate composition (% of dry weight)			
Ash	6.1 ± 0.8	4.5 ± 0.14	0.2 ± 0.0
Lipids	3.6 ± 0.3	6.3 ± 0.1	n.d.
Protein	6.1 ± 0.1	15.0 ± 0.1	4.1 ± 0.0
Dietary fiber	25.8 ± 2.5	77.6 ± 1.7	96.9 ± 0.9
Soluble fiber	8.4 ± 0.6	15.1 ± 0.7	1.5 ± 0.7
Insoluble fiber	17.4 ± 1.5	62.5 ± 1.4	95.4 ± 0.2
Non-fibrous carbohydrates*	58.4	0	0
Phytochemical composition (% of dry weight)			
Total extractable polyphenols	0.77 ± 0.02	n.d.	n.d.
Anthocyanins	0.61 ± 0.02	n.d.	n.d.
Non-anthocyanic phenolics	0.16 ± 0.01	n.d.	n.d.
Bound proanthocyanidins	2.5 ± 0.1	7.7 ± 0.4	n.d.

Results are presented as mean ± s.d. *Calculated by difference between 100 and the sum of the other constituents. GPP = grape peel powder; NEP-F = non-extractable polyphenols-rich fraction; F = polyphenols-poor, fiber-rich fraction. n.d. = not determined.

Table 3. Ingredients and composition of experimental diets.

	AIN-93G	GPP	EP	NEP-F	F
Ingredients					
Corn starch (g)	440.9	420.2	440.9	433.4	440.4
Casein (g)	156.6	156.6	156.6	156.6	156.6
Maltodextrin (g)	132.0	132.0	132.0	132.0	132.0
Sucrose (g)	100.0	57.8	68.3	100.0	100.0
Soybean oil (g)	70.0	70.0	70.0	70.0	70.0
Cellulose (g)	50.0	33.1	50.0	35.8	47.0
Mineral mix (g)	35.0	35.0	35.0	35.0	35.0
Vitamin mix (g)	10.0	10.0	10.0	10.0	10.0
L-cysteine (g)	3.0	3.0	3.0	3.0	3.0
Choline (g)	2.5	2.5	2.5	2.5	2.5
tert-butylhydroquinone (g)	0.014	0.014	0.014	0.014	0.014
Grape peel powder (g)		80.0			
EP extract (mL)			198.0		
NEP-F fraction (g)				21.7	
F fraction (g)					3.5
Total (g)	1000	1000	1000	1000	1000
Proximate composition and energetic value					
Ash [#]	2.9 ± 0.2	3.6 ± 0.1	3.9 ± 0.1	2.9 ± 0.1	2.9 ± 0.1
Protein [#]	15.2 ± 0.5	17.8 ± 1.5	17.9 ± 0.6	15.5 ± 0.5	14.8 ± 1.0
Fat [#]	14.5 ± 1.0	15.6 ± 0.2	15.9 ± 0.2	14.8 ± 1.0	14.5 ± 0.2
Total carbohydrates[&]	67.4	63.0	62.3	66.8	67.8
Energetic value (kcal/kg)*	3948.0	3928.7	3948.0	3943.3	3946.6
Dietary fiber (g%, dry weight) and phytochemical composition (mg%, dry weight)*					
Total dietary fiber	5.0	5.0	5.0	5.0	5.0
Insoluble fiber	5.0	4.3	5.0	4.7	5.0
Soluble fiber	-	0.7	-	0.3	<0.1
Extractable polyphenols	-	62.0	62.6	-	-
Bound proanthocyanidins	-	227.0	-	228.0	-

Results are presented as mean ± s.d. [#] g%, dry weight. [&] Calculated by difference between 100 and the sum of the other constituents. * Calculated based on the ingredient composition and the amount of ingredients added to the diet. GPP = grape peel powder; EP = extractable polyphenols-rich fraction; NEP-F = non-extractable polyphenols-rich fraction; F = polyphenols-poor, fiber-rich fraction.

Table 4. Anthocyanin identification and quantification of 'Isabel' grape peel powder (evaluated in the EP extract) using LC-PDA-MS-qTOF.

Peak	RT-PDA (min)	Anthocyanin	λ_{max} (nm)	Experimental MS $[M-H]^+$	Theoretical MS $[M-H]^+$	Error (ppm)	Experimental fragments	mg/100g* (mean ± sd)
1	2.6	Delphinidin-3-glucoside	523	465.1029	465.103	0.21	303.0472	28.93 ± 1.40
2	3.1	Malvidin-3,5-diglucoside	529	655.1852	655.187	2.74	493.1329; 331.0809	9.64 ± 0.28
3	3.7	Cyanidin-3-glucoside	517	449.1066	449.108	3.11	287.0542	12.71 ± 1.28
4	4.7	Petunidin-3-glucoside	526	479.1143	479.119	12.10	317.0640	49.43 ± 1.61
5	6.5	Peonidin-3-glucoside	517	463.1209	463.124	6.69	301.0683	121.38 ± 4.50
6	7.4	Malvidin-3-glucoside	527	493.1339	493.135	2.23	331.0795	321.87 ± 11.38
7	13.1	Delphinidin-3-coumaroyl-glucoside	531	611.1373	611.14	4.41	303.0421	14.21 ± 0.34
8	13.6	Cyanidin-3-coumaroyl-glucoside	532	595.1432	595.145	3.02	287.0747	1.32 ± 0.34
9	16.5	Peonidin-3-coumaroyl-glucoside	522	609.1539	609.161	11.65	301.0668	13.84 ± 2.38
10	16.8	Malvidin-3-coumaroyl-glucoside	532	639.1677	639.171	5.16	331.0780	40.30 ± 3.89
							Total anthocyanins	*613.64 ± 20.73*

* mg equivalents to malvidin-3-glucoside/100 g of grape peel powder.

Table 5. Non-anthocyanic phenolics identification and quantification of 'Isabel' grape peel powder (evaluated in the EP extract) using LC-PDA-MS-qTOF.

Peak	RT-PDA (min)	Phenolic compound	λ máx (nm)	Experimental MS $[M-H]^-$	Theoretical MS $[M-H]^-$	Error (ppm)	Experimental fragments	mg/100g * (mean ± sd)
Hydroxybenzoic acid derivatives								
1	7.3	Protocatechuic acid hexoside	280	315.0730	315.079	-3.8	152.0131; 108.0232	0.09 ± 0.03
12	18.2	Syringic acid	275	197.0500	197.053	15.2		1.25 ± 0.24
Hydroxycinnamic acid derivatives								
2	8.3	*cis*-caftaric acid	327	311.0436	311.048	11.2	149.0094	0.59 ± 0.14
3	9.3	*trans*-caftaric acid	328	311.0428	311.048	14.1	179.0358; 135.0461	15.13 ± 0.70
5	12.1	Caffeoyil hexoside	330	341.0933	341.095	5.0	161.0250; 133.0291	1.24 ± 0.12
6	13.3	*cis*-coutaric acid	309	295.0472	295.0465	-6.8	163.0380; 149.0133; 119.0520	0.70 ± 0.06
7	13.8	*trans*-coutaric acid	312	295.0472	295.0465	-6.4	163.0409; 149.0133; 119.0510	1.97 ± 0.10
9	16.3	Coumaroyl hexoside	313	325.0975	325.1	7.7	145.0311; 117.0362; 163.0415	0.13 ± 0.01
10	17.2	*trans*-fertaric acid	312	325.0957	325.0912	6.5	145.0317; 134.0411; 193.0358	1.96 ± 0.11
11	17.6	Feruloyil hexoside	326	355.1066	355.111	12.4	160.0187; 175.0450	0.64 ± 0.04
15	30.5	Coumaric acid	307	163.0449	163.047	12.9		0.12 ± 0.02
17	41.1	*trans*-ferulic acid	320	193.0545	193.058	18.1		0.60 ± 0.05
Flavanols								
4	11.3	Procyanidin	280	577.1368	577.1313	-9.5	289.0688	0.95 ± 0.27
8	15.1	Catechin	280	289.0747	289.071	-12.8	245.0854; 205.0394	8.26 ± 0.16
13	20.7	Epicatechin	280	289.0700	289.071	3.4	145.0307; 119.0507	3.18 ± 0.61
14	27.8	Epicatechin-glucuronide	293	465.1115	465.111	-1.1	289.0712; 190.0408	1.85 ± 0.34
Flavonols								
16	39.7	Myricetin-3-hexoside	354	479.0888	479.0831	-11.9	316.0255	5.64 ± 0.35
19	46.2	Quercetin-3-rutinoside	354	609.1505	609.1461	-7.2	463.0938; 339.1135; 300.0331	3.74 ± 0.19
20	47.0	Quercetin-3-glucuronide	354	477.0748	477.0675	-15.3	301.0397	32.65 ± 2.44
21	47.7	Quercetin-3-hexoside	355	463.0955	463.0882	-15.7	301.0394	40.46 ± 1.29
22	49.2	Laricitrin-3-hexoside	356	493.0980	493.0988	-13.4	330.0032	3.32 ± 0.23
24	55.8	Kaempferol-3-hexoside	346	447.0974	447.0933	-10.5	285.0375	5.62 ± 0.21
25	56.4	Isorhamnetin-3-hexoside	354	477.1094	477.1038	-11.7	315.0538	2.45 ± 0.15
26	57.7	Isorhamnetin-3-glucuronide	353	491.0899	491.0831	-13.8	300.033	0.21 ± 0.02
Stilbenes								
18	43.0	*trans*-piceid	307	389.1277	389.1242	-9.0	227.073; 163.0315	0.99 ± 0.04
23	54.2	*trans*-resveratrol	305	227.0748	227.0714	-15.0	143.0537; 125.0312	4.02 ± 0.21
27	63.1	Viniferin	323	453.1404	453.142	3.5	360.7407; 257.1404	0.08 ± 0.03
							Total phenolic compounds	*162.50 ± 9.34*

* Hydroxycinnamic acid derivatives (320 nm) expressed as mg equivalents to caffeic acid per 100 g of grape peel powder; Flavanols (280 nm) expressed as mg equivalents to catechin per 100 g grape peel powder; Hydroxybenzoic acid derivatives (280 nm) expressed as mg equivalents to gallic acid per 100 g grape peel powder; Flavonols (360 nm) expressed as mg equivalents to kaempferol-3,7-diglucoside per 100 g grape peel powder; Stilbenes (305 nm) expressed as mg equivalents to resveratrol per 100 g grape peel powder.

Figure captions

Figure 1. Body weight changes along the dietary supplementation before colitis induction (A) and the daily feed consumption (B), weight gain (% change relative to b.w. before colitis induction) (C), extractable polyphenols (D), and bound proanthocyanidin consumption (E) before (day -1) and in the 7 days subsequent to colitis induction. Arrows indicate the day of colitis induction. Results are presented as the mean ± standard error. # Different from day 0 ($p<0.05$); & Significant difference between EP and colitis groups; * Significant difference between EP and GPP groups (panel C) or NEP-F and GPP groups (panel D) ($p<0.05$). GPP = grape peel powder; EP= extractable polyphenols-rich fraction; NEP-F = non-extractable polyphenols-rich fraction; F= polyphenols-poor, fiber-rich fraction.

Figure 2. Colon length/weight ratio (A) and spleen relative weight (B) after colitis induction. Results are presented as the mean ± standard error. # Different from control group ($p<0.05$); * Different from colitis group ($p<0.05$). GPP = grape peel powder; EP= extractable polyphenols-rich fraction; NEP-F = non-extractable polyphenols-rich fraction; F= polyphenols-poor, fiber-rich fraction. TNBS = 2,4,6-trinitrobenzenesulfonic acid.

Figure 3. Serum oxidative and inflammatory status assessed by lipid peroxidation (A), protein oxidation (B), nitric oxide (C), FRAP (D), TNF-α (E), and IL-1β levels (F). Results are presented as the mean ± standard error, except in panel F (median ± interquartile interval). # Different from control group ($p<0.05$); * Different from colitis group ($p<0.05$). GPP = grape peel powder; EP= extractable polyphenols-rich fraction; NEP-F = non-extractable polyphenols-rich fraction; F= polyphenols-poor, fiber-rich fraction. TNBS = 2,4,6-trinitrobenzenesulfonic acid. TBARS = thiobarbituric acid reactive substances; MDA = malondialdehyde; AOPP = advanced oxidation protein products; NO = nitric oxide; FRAP = ferric reducing antioxidant power; TNF-α = tumor necrosis factor alpha; IL-1β = interleukin 1β.

Figure 4. Oxidative status of colonic tissue assessed by the lipid peroxidation (A), protein oxidation (B) and nitric oxide levels (C), and CuZnSOD (D), MnSOD (E), and catalase (F) activities. Results are presented as the mean ± standard error. # Different from control group ($p<0.05$); * Different from colitis group ($p<0.05$). GPP = grape peel powder; EP= extractable polyphenols-rich fraction; NEP-F = non-extractable polyphenols-rich fraction; F= polyphenols-poor, fiber-rich fraction. TNBS = 2,4,6-trinitrobenzenesulfonic acid. TBARS = thiobarbituric acid reactive substances; MDA = malondialdehyde; AOPP = advanced oxidation protein products; NO = nitric oxide; MnSOD = manganese superoxide dismutase; CuZnSOD = copper zinc superoxide dismutase; CAT = catalase.

Figure 5. Inflammatory markers of colonic tissue assessed by TNF-α mRNA expression (A) and ELISA (B), IL-1β mRNA expression (C) and ELISA (D), IL-10 mRNA expression (E) and ELISA (F), and IL-6 mRNA expression (G). Results are presented as the mean ± standard error. # Different from control group ($p<0.05$); * Different from colitis group ($p<0.05$). GPP = grape peel powder; EP = extractable polyphenols-rich fraction; NEP-F = non-extractable polyphenols-rich fraction; F= polyphenols-poor, fiber-rich fraction. TNBS = 2,4,6-trinitrobenzenesulfonic acid; TNF-α = tumor necrosis factor alpha; IL-1β = interleukin 1 beta; IL-10 = interleukin 10; IL-6 = interleukin 6; ELISA = enzyme-linked immunosorbent assay.

Figure 6. Inflammatory markers of colonic tissue assessed by protein expression of TLR-4 (A), IKK-β (B), NF-κB p65 mRNA expression (C), and *p*NF-κB p50 protein expression (D) in the colonic tissue of rats with ulcerative colitis induced by TNBS. Results are presented as the mean ± standard error. # Different from control group ($p<0.05$); * Different from colitis group ($p<0.05$). GPP = grape peel powder; EP = extractable polyphenols-rich fraction; NEP-F = non-extractable polyphenols-rich fraction; F= polyphenols-poor, fiber-rich fraction. TNBS = 2,4,6 trinitrobenzenesulfonic acid; TLR-4 = toll like receptor 4; IKK-β = inhibitor of nuclear factor kappa-B kinase subunit beta; NF-κB p65 = nuclear factor kappa B subunit p65; *p*NF-κB p50 = phosphorylated nuclear factor kappa B subunit p50.

Figure 7. Inflammatory markers of colonic inflammation assessed by MPO activity (A) and protein expression of COX-2 (B) in the colonic tissue of rats with ulcerative colitis induced by TNBS. Results are presented as the mean ± standard error. # Different from control group ($p<0.05$); * Different from colitis group ($p<0.05$). GPP = grape peel powder; EP = extractable polyphenols-rich fraction; NEP-F = non-extractable polyphenols-rich fraction; F= polyphenols-poor, fiber-rich fraction. TNBS = 2,4,6 trinitrobenzenesulfonic acid; COX-2 = cyclooxygenase-2; MPO = myeloperoxidase.

Figure 8. Anthocyanin profile of EP extract from 'Isabel' grape peel powder analyzed by LC-PDA-MS-qTOF.

Figure 9. Non-anthocyanin phenolic profile of EP extract from 'Isabel' grape peel powder analyzed by LC-PDA-MS-qTOF.

Figure 1

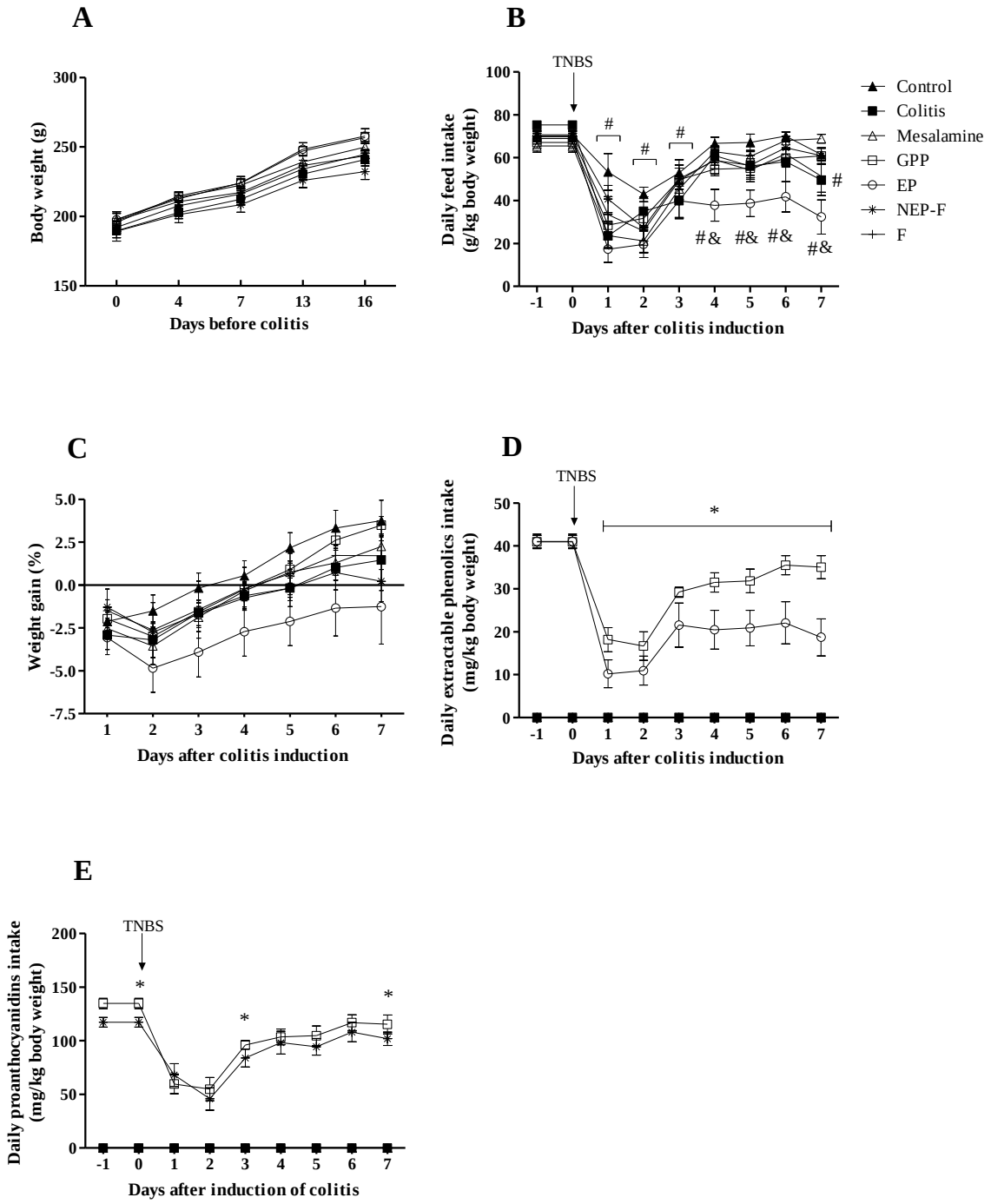

A
Body weight (g)
Days before colitis
B
TNBS
Daily feed intake (g/kg body weight)
Days after colitis induction
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
C
Weight gain (%)
Days after colitis induction
D
TNBS
Daily extractable phenolics intake (mg/kg body weight)
Days after colitis induction
E
TNBS
Daily proanthocyanidins intake (mg/kg body weight)
Days after induction of colitis

Figure 2

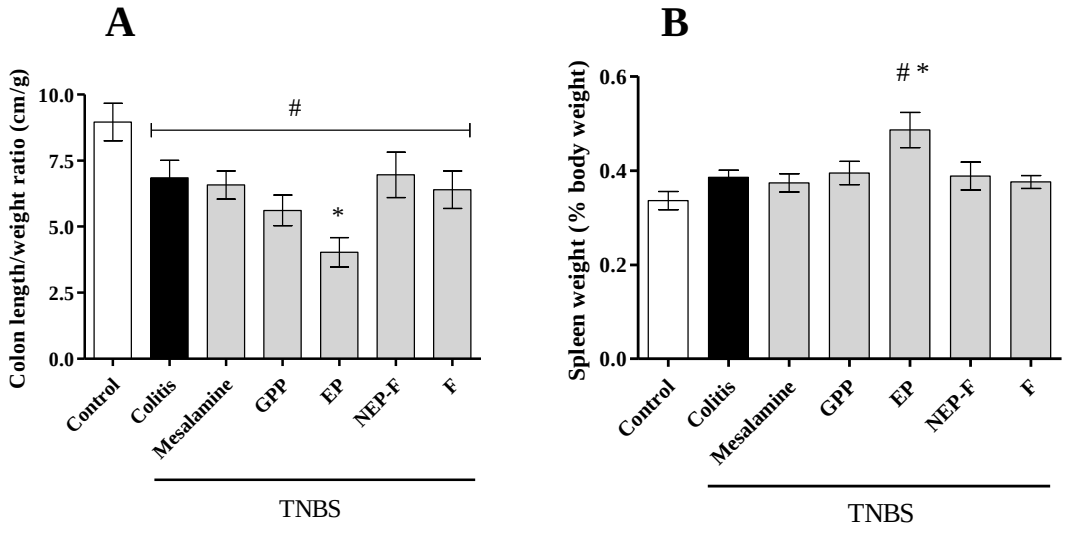

A
Colon length/weight ratio (cm/g)
10.0
7.5
5.0
2.5
0.0
#
*
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS
B
Spleen weight (% body weight)
0.6
0.4
0.2
0.0
*
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS

Figure 3

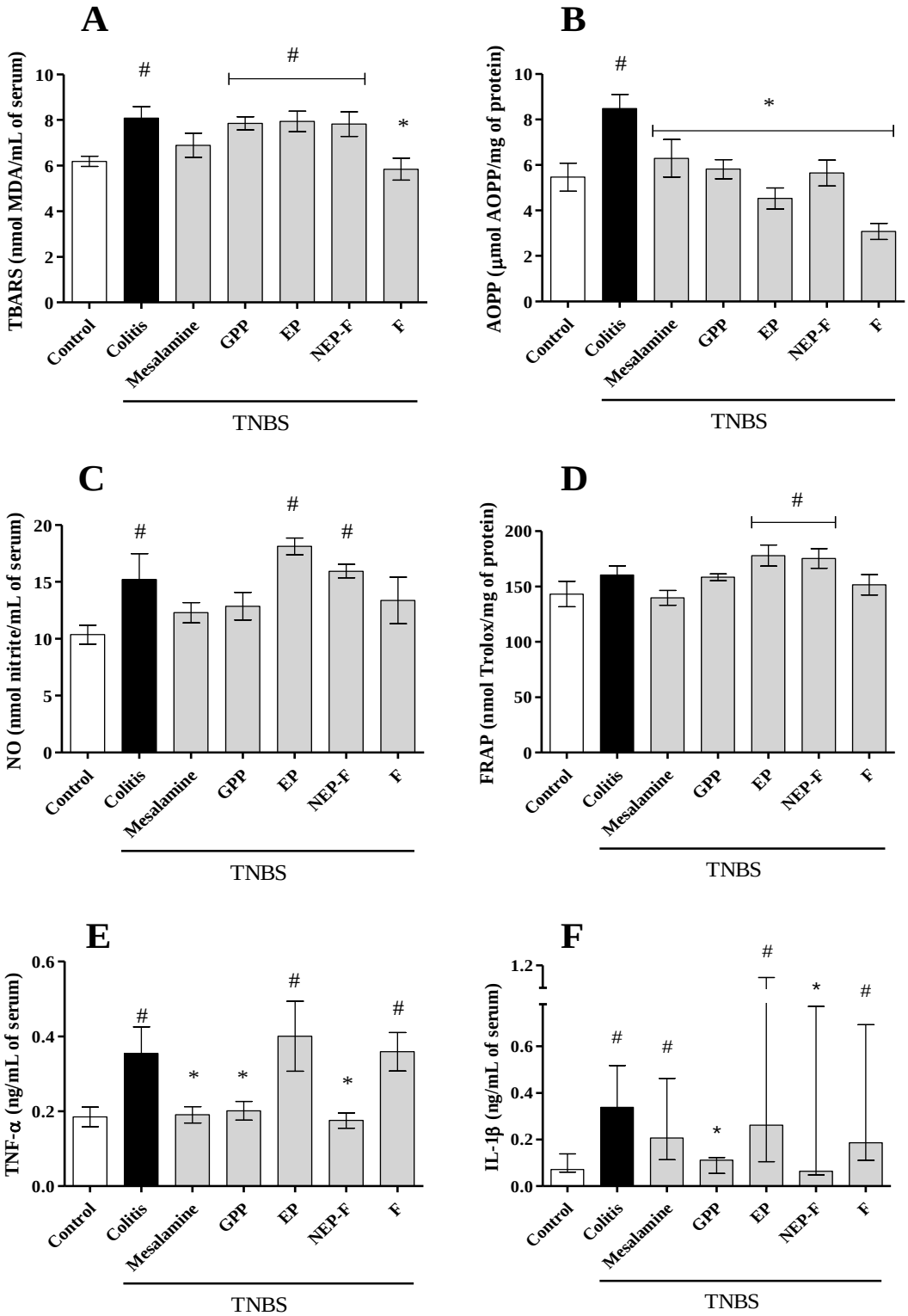

A
TBARS (nmol MDA/mL of serum)
B
AOPP (µmol AOPP/mg of protein)
C
NO (nmol nitrite/mL of serum)
D
FRAP (nmol Trolox/mg of protein)
E
TNF-α (ng/mL of serum)
F
IL-1β (ng/mL of serum)
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS

Figure 4

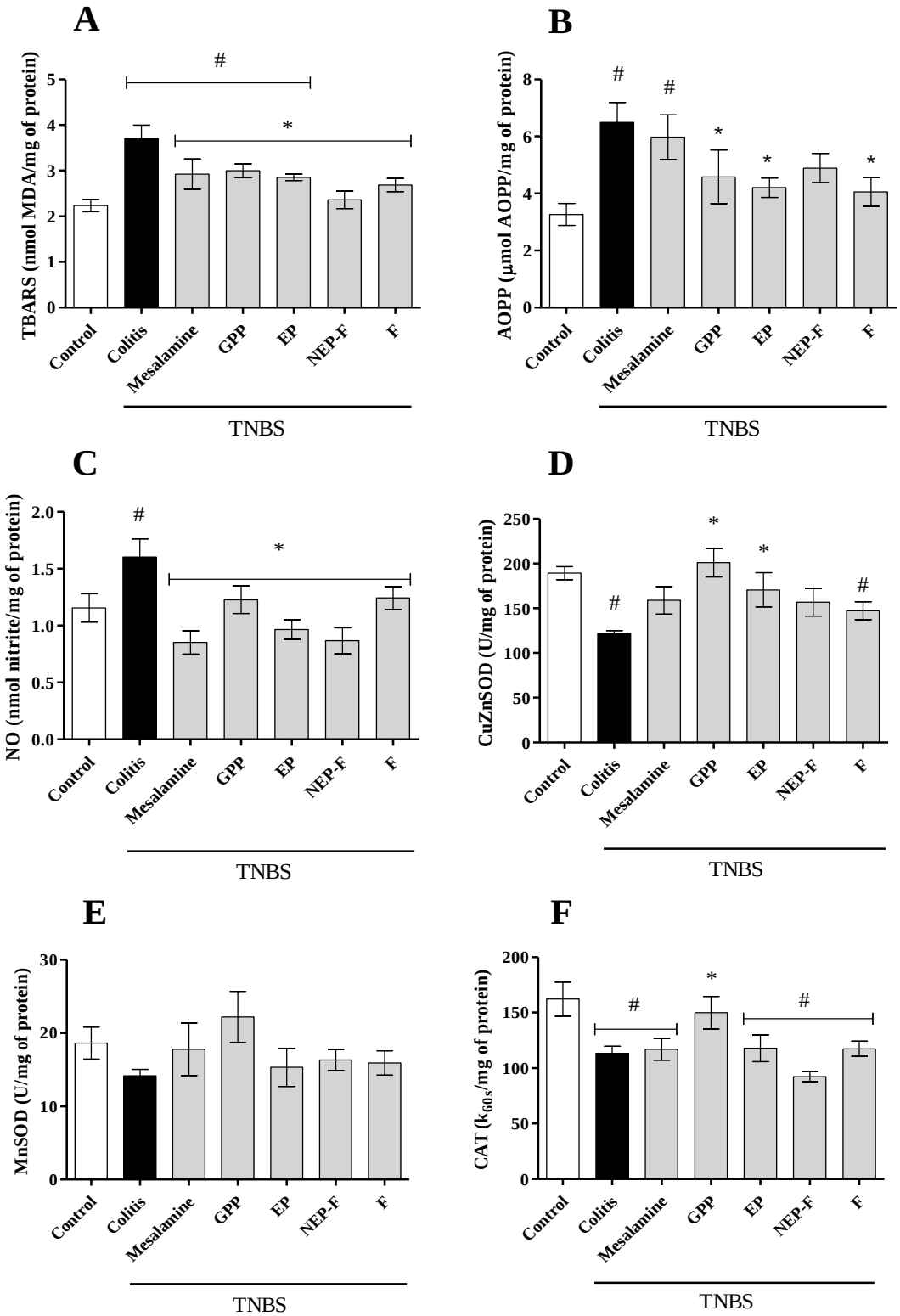

A
TBARS (nmol MDA/mg of protein)
0
1
2
3
4
5
#
*
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS
B
AOPP (µmol AOPP/mg of protein)
0
2
4
6
8
#
#
*
*
*
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS
C
NO (nmol nitrite/mg of protein)
0.0
0.5
1.0
1.5
2.0
#
*
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS
D
CuZnSOD (U/mg of protein)
0
50
100
150
200
250
*
*
#
#
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS
E
MnSOD (U/mg of protein)
0
10
20
30
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS
F
CAT (k_{60s}/mg of protein)
0
50
100
150
200
#
*
#
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS

Figure 5

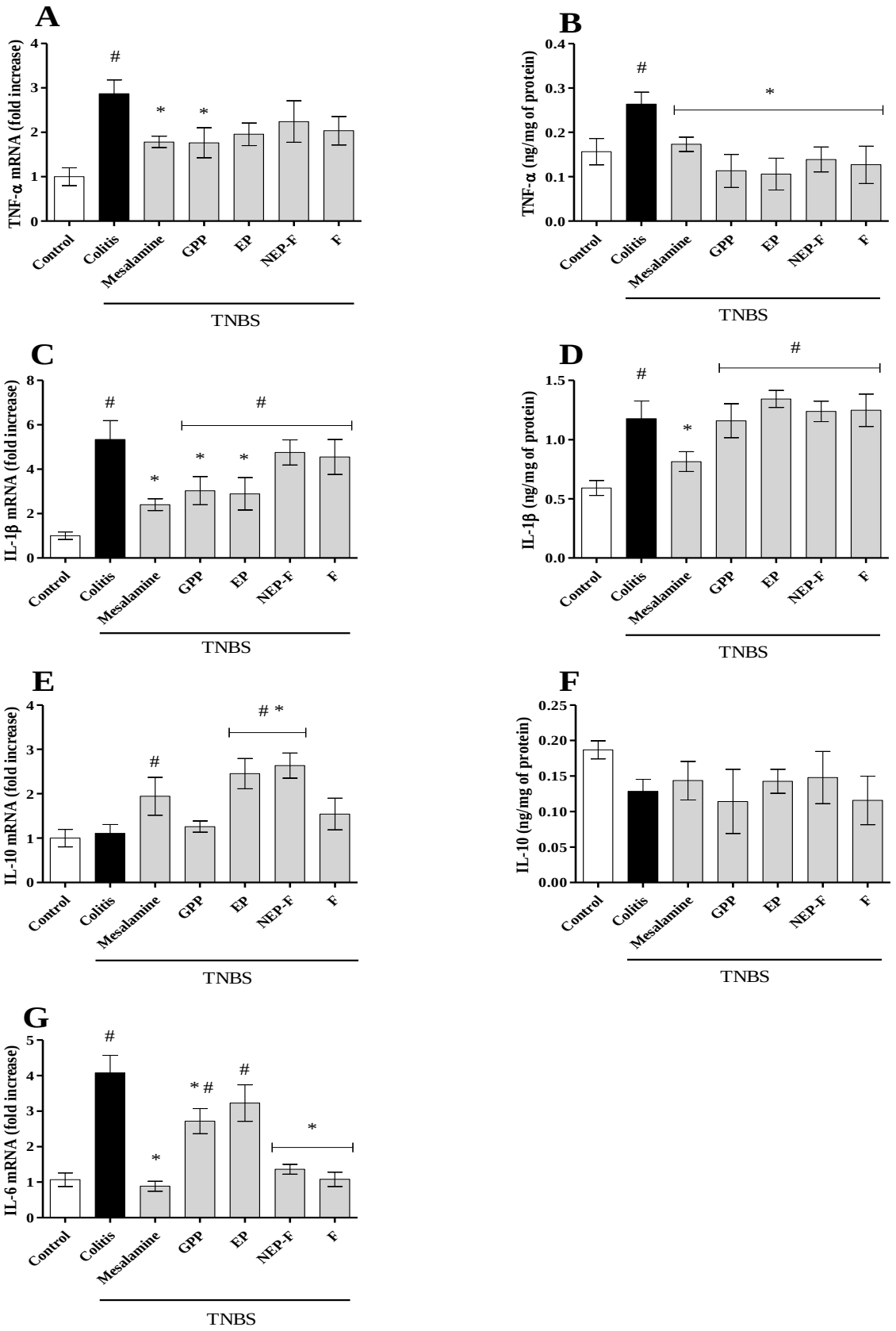

A
TNF-α mRNA (fold increase)
0
1
2
3
4
#
*
*
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS
B
TNF-α (ng/mg of protein)
0.0
0.1
0.2
0.3
0.4
#
*
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS
C
IL-1β mRNA (fold increase)
0
2
4
6
8
#
#
*
*
*
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS
D
IL-1β (ng/mg of protein)
0.0
0.5
1.0
1.5
#
#
*
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS
E
IL-10 mRNA (fold increase)
0
1
2
3
4
*
#
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS
F
IL-10 (ng/mg of protein)
0.00
0.05
0.10
0.15
0.20
0.25
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS
G
IL-6 mRNA (fold increase)
0
1
2
3
4
5
#
*
* #
#
*
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS

Figure 6

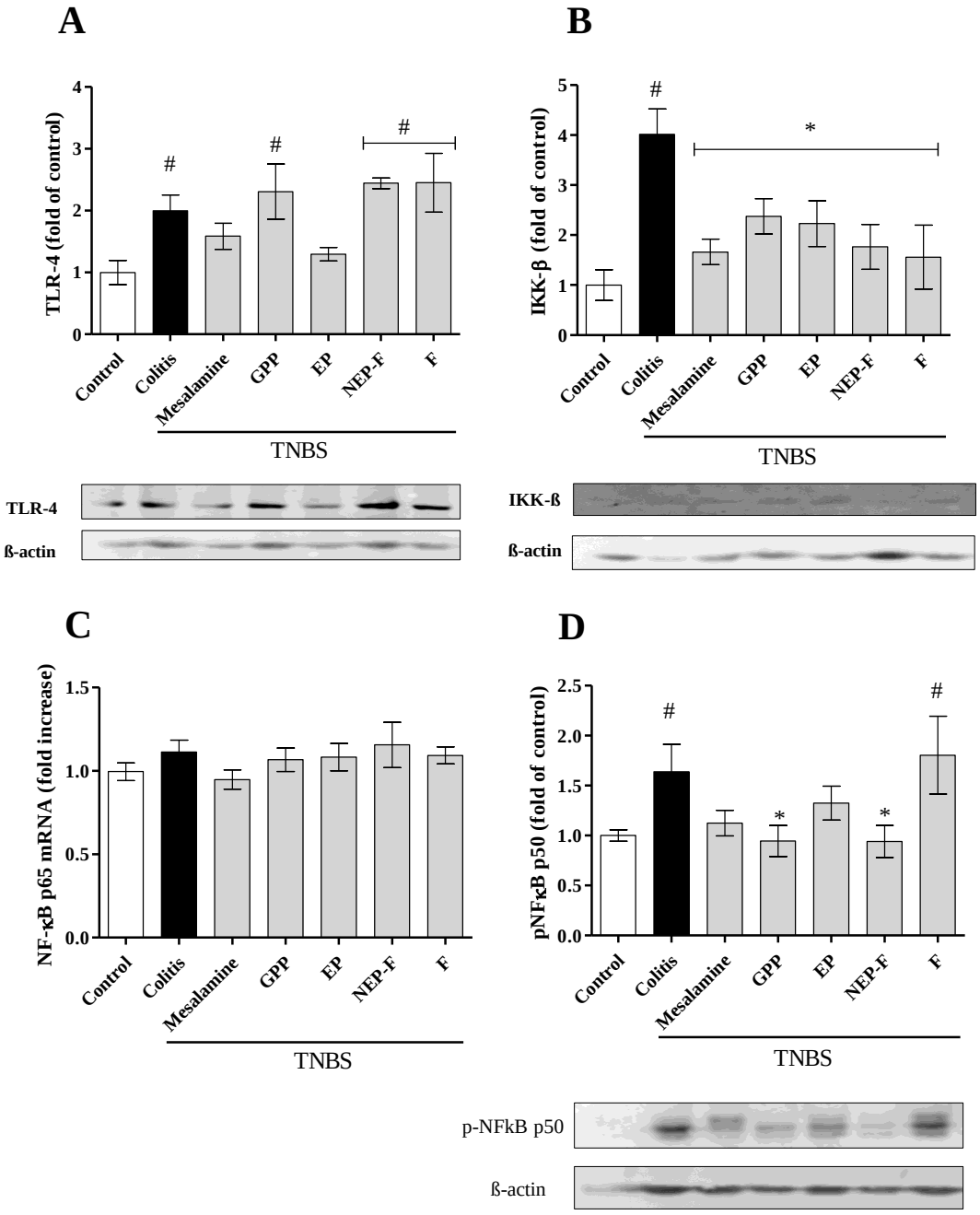
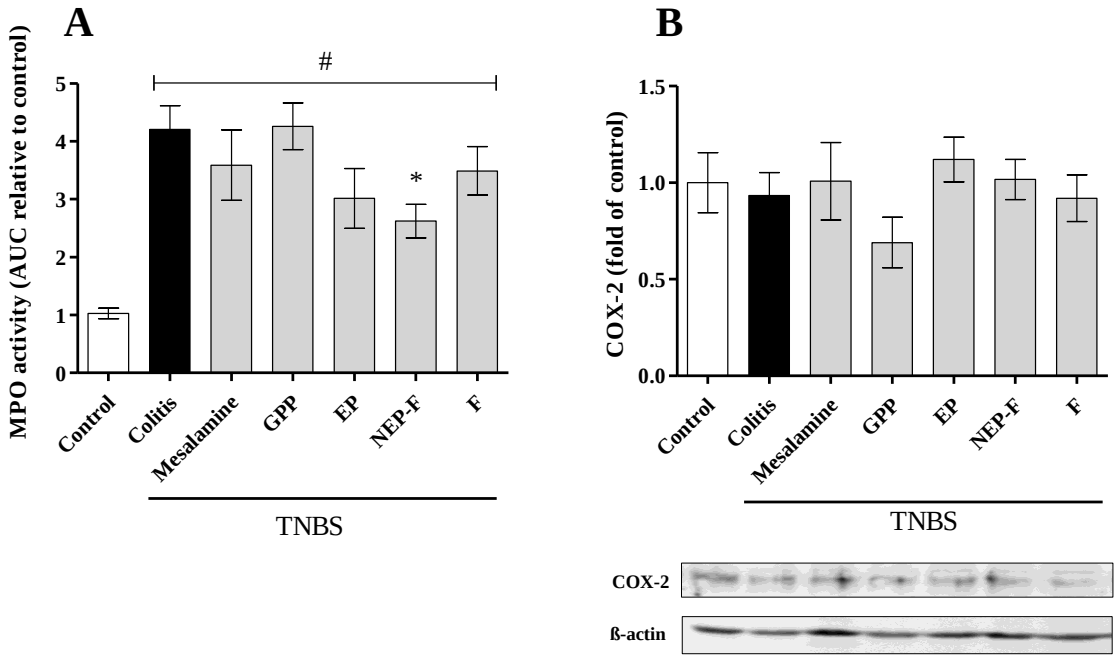
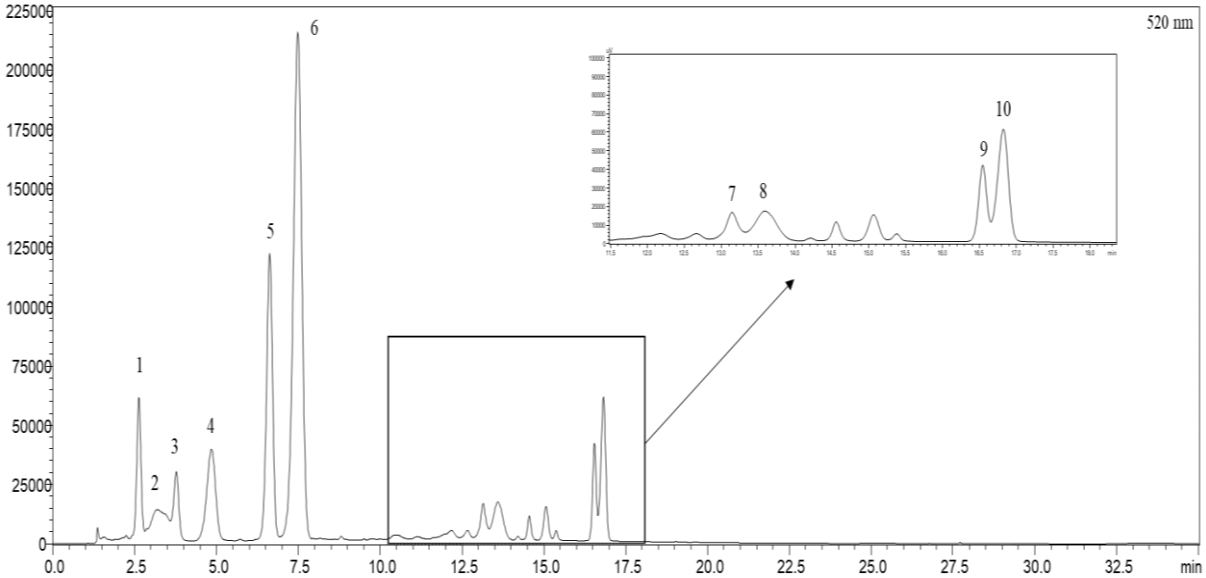

A
TLR-4 (fold of control)
0
1
2
3
4
#
#
#
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS
TLR-4
ß-actin
B
IKK-β (fold of control)
0
1
2
3
4
5
#
*
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS
IKK-ß
ß-actin
C
NF-κB p65 mRNA (fold increase)
0.0
0.5
1.0
1.5
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS
D
pNFκB p50 (fold of control)
0.0
0.5
1.0
1.5
2.0
2.5
#
*
*
#
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS
p-NFkB p50
ß-actin

Figure 7


A
MPO activity (AUC relative to control)
#
*
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS
B
COX-2 (fold of control)
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS
COX-2
ß-actin

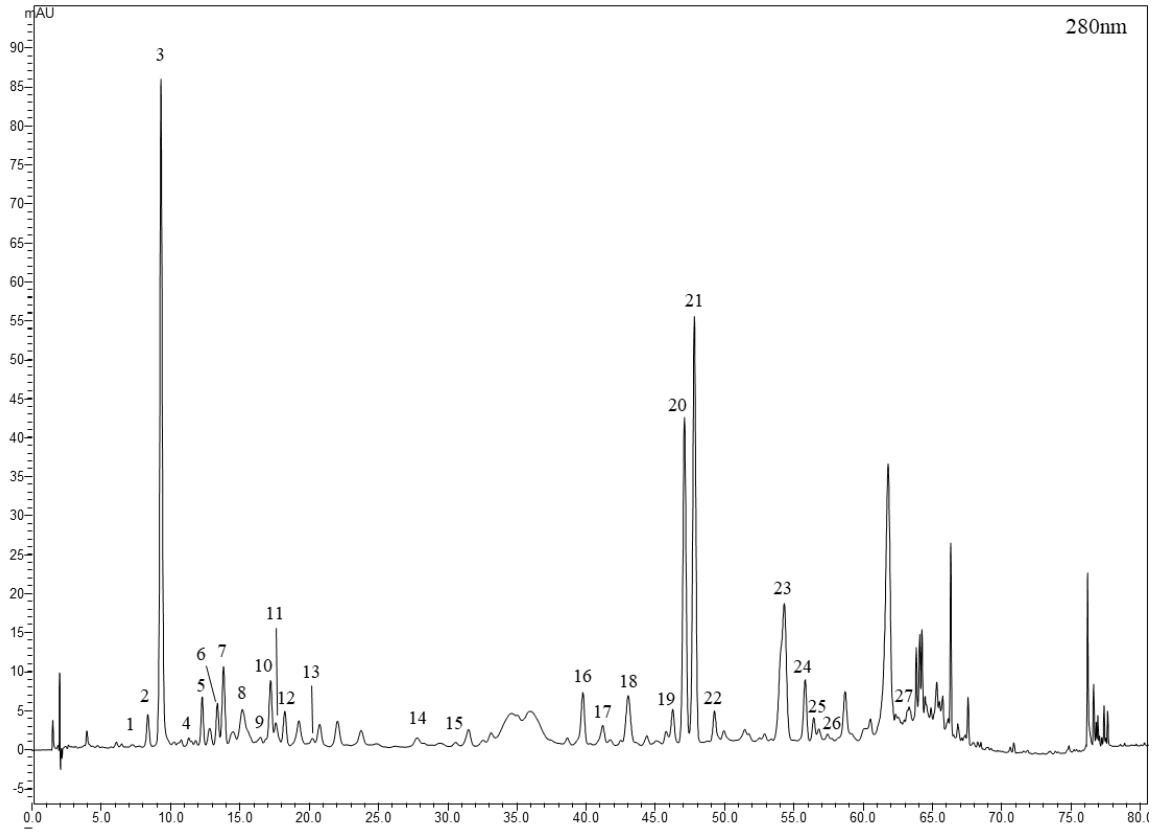
Supplementary material

Figure 8. Anthocyanic phenolics of EP extract from ‘Isabel’ grape peel powder analyzed by LC-PDA-MS-qTOF.



Supplementary material

Figure 9. Non-anthocyanic phenolics of EP extract from ‘Isabel’ grape peel powder analyzed by LC-PDA-MS-qTOF.



4.3 MANUSCRITO 2

Dietary fiber and fiber-bound polyphenols of grape peel powder promote GSH recycling in the colon of rats with TNBS-induced colitis

Manuscrito a ser submetido ao periódico *Molecular Nutrition and Food Research* (FI: 4,323; Qualis A1) (formatado conforme as normas do periódico)

Dietary fiber and fiber-bound polyphenols of grape peel powder promote GSH recycling in the colon of rats with TNBS-induced colitis

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Abstract

Ulcerative colitis (UC) is a chronic inflammatory bowel disease that significantly reduces glutathione (GSH) levels. Our aim was to investigate the effect of the dietary supplementation with grape peel powder (GPP) on the GSH system in an experimental model of colitis and to identify the role of grape bioactive fractions, namely soluble phenolics (EP), bound polyphenols associated to the fiber (NEP-F), and dietary fiber (F). The bioactive fractions were provided at levels equivalent to GPP and colitis was induced using 2,4,6-trinitrobenzenesulfonic acid (TNBS). Colitis reduced GSH levels and glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione-S-transferase (GST) activities in the colon. All diets and mesalamine treatment partially restored colon GSH levels. GPP and NEP-

F restored GPx activity, while all diets restored GR and GST. Colitis increased the mRNA expression of both subunits of glutamate-cysteine ligase and induced apoptosis in colonic tissue. The reestablishment of GSH levels was associated to the recycling of oxidized glutathione via increased GR activity instead of GSH synthesis. NEP-F and F fractions were the responsible for the protective effect of GPP against apoptosis. These data suggest that grape dietary fiber and fiber-bound polyphenols are more effective than the soluble polyphenols to protect against TNBS-induced colitis.

1 Introduction

Ulcerative colitis (UC) is an inflammatory bowel disease triggered by immunological, genetic, and environmental factors, including diet and free radicals [1]. The mucosal and submucosal layers of colon and rectum tissue are the typically affected areas.

Macrophage and neutrophil infiltration into the colon tissue promotes the overproduction of reactive oxygen species (ROS) and the depletion of glutathione (GSH) levels as observed in the colon tissue of UC patients and in animal models of this disease [2–4]. GSH (L-ɣ-glutamyl-L-cysteinylglycine) is the major redox buffer in most cells [5] as it is able to directly scavenge reactive oxygen and nitrogen species or serve as electron donor for antioxidant enzymes, such as glutathione peroxidase (GPx) and glutathione-S-transferase (GST) [5, 6]. The loss of intracellular redox homeostasis as observed in UC can induce apoptosis by activating caspase cascades [5, 7]. In fact, GSH can regulate the activity of caspases through the maintenance of protein sulfhydryl groups in its appropriate redox state [8].

The loss of intestinal epithelial cells in UC may be linked to the apoptotic process that is exacerbated during inflammation [9]. Apoptosis is a programmed type of cell death triggered by a death receptor at the plasmatic membrane, like TNF-α receptor (extrinsic pathway), or by mitochondria-derived signal (intrinsic pathway) [10]. Among many factors, caspases have been shown to play an important role in the transduction of apoptotic signals [9].

As the treatment for inflammatory bowel diseases is just palliative and the chronic therapy with the recommended drugs (anti-inflammatory agents, corticosteroids, immunosuppressant, antibiotics) may lead to several side effects, nutritional strategies that could contribute to reduce tissue damage or attenuate the symptoms would be welcome [11, 12]. Some studies have proven that dietary antioxidants and phenolic compounds may be an

alternative to recover GSH levels and improve the cellular antioxidant status impaired by colitis development [13–15]. Dietary fiber intake has also shown protective effects in experimental colitis [16–18]. However, when a food matrix rich in both fiber and polyphenols is consumed as part of a whole diet it is not known which compounds are responsible for GSH restitution, which mechanism is involved, and whether these compounds may have synergistic actions. The intracellular levels of reduced glutathione are sustained by the recycling of oxidized glutathione back to the reduced form, which is catalyzed by glutathione reductase (GR) [5] and by *de novo* synthesis of GSH [19]. Two enzymes are involved in *de novo* synthesis of GSH; glutamate cysteine ligase (GCL), which is the rate-limiting enzyme, and GSH synthase (GS) [19].

Grapes are one of the most cultivated fruits around the world and their peels are rich in bioactive compounds such as dietary fiber, phenolic acids, anthocyanins, flavonols, resveratrol, and proanthocyanidins [20]. Some *in vivo* studies have shown evidence that grape bioactive compounds improve the glutathione metabolism and reduce the apoptosis [21–23], but these effects have not been explored in colitis models and the mechanisms responsible for these effects have not been completely elucidated.

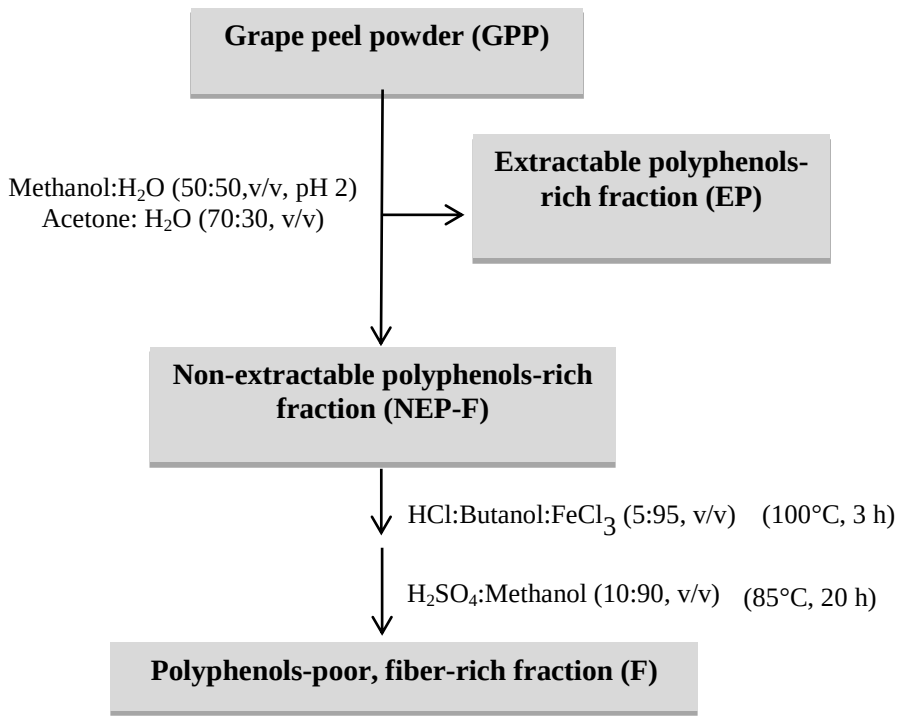
Thus, the objectives of this study were to investigate the effect of grape peel powder on the regulation of glutathione system and apoptosis in an experimental model of colitis and to identify the bioactive fraction (dietary fiber, fiber-bound or soluble polyphenols) responsible for the effect.

2 Material and methods

2.1 Grape peel and its bioactive fractions

Grapes (cv. Isabel *Vitis labrusca* x *Vitis vinifera* L.) were harvested at Itaara, RS, Brazil (29°35’55” S and 53°46’13” W) and peels were manually separated, freeze-dried and

pulverized in order to obtain a grape peel powder (GPP). GPP was extracted to yield fractions that were selectively enriched in soluble polyphenols, fiber-bound polyphenols or dietary fiber (Scheme 1). Extractable polyphenols were extracted using MeOH:water (50:50, v/v, pH 2.0) and acetone:water (70:30, v/v) [24] and this extract (EP) was concentrated under vacuum. The solid residue of this extraction (NEP-F fraction) was rich in fiber and insoluble polyphenols (condensed and hydrolysable tannins and hydrolysable polyphenols) bound to the fiber fraction. Thereafter, the NEP-F fraction was sequentially extracted with butanol:HCl (95:5, v/v) containing 0.7 g/L of $FeCl_3$ at 100°C for 3 h and MeOH:H_2SO_4 (90:10, v/v) at 85°C for 20 h [24, 25] to remove condensed and hydrolysable polyphenols, respectively. The solid residue obtained from this extraction (F fraction) was rich in fiber and poor in polyphenols.

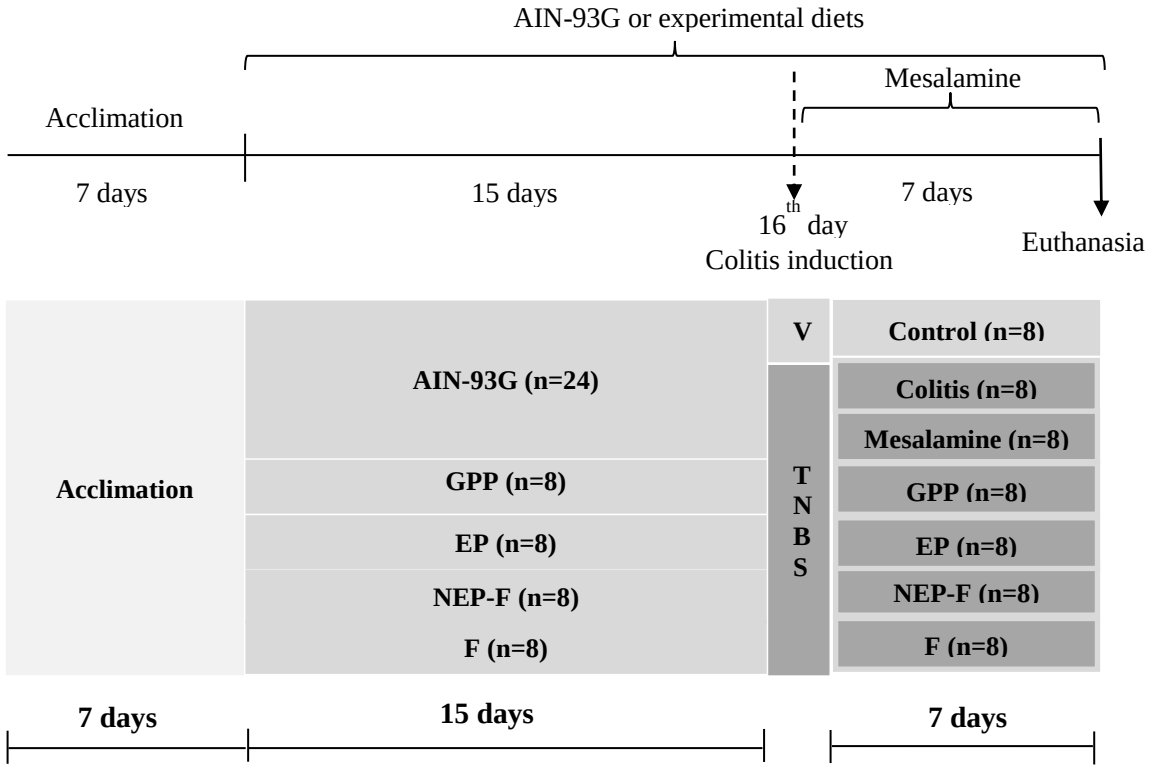


Scheme 1. Fractionation of bioactive compounds from grape peel powder.

2.2 *In vivo* experimental design and colitis induction

This study was approved by the Animal Research and Ethics Committee of the University of Campinas (Brazil) (protocol 3815-1/2015). Adult male Wistar rats (initial weight 200 ± 20 g) were allocated in individual cages and maintained under controlled environment conditions (22 ± 2°C), humidity (60 - 70%), and a light-dark cycle (12/12 h). Animals were divided into seven groups (n=8 per group): control (C), colitis (CC), mesalamine (CM), grape peel powder (GPP), extractable polyphenols (EP), fiber plus non-extractable polyphenols (NEP-F) and fiber-rich and polyphenols-poor residue (F) (Scheme 2). The groups C, CC, and CM received AIN-93G diet [26], whereas groups GPP, EP, NEP-F, and F received AIN-93G diet containing 8% (w/w) GPP, EP, NEP-F, or F fraction, respectively. Twenty grams of experimental diets were offered per day, and the intake was daily monitored. The quantity of GPP supplementation was chosen to provide about 12.4 mg of phenolics/animal/day (or about 40 mg/kg body weight/day). This dose was chosen based on studies that observed beneficial effects of polyphenols in UC models [27]. The amounts of EP, NEP-F, and F were calculated to yield amounts of extractable polyphenols and non-extractable polyphenols equivalent to the GPP group. The amount of F fraction included in the F diet was equivalent to the residue obtained after extraction (extractable and bound-phenolics extraction) of the same amount of GPP used in the GPP diet. As the method used to obtain the F fraction had a very low yield (4.4% from the GPP, data not shown), the F diet had much lower grape fiber content than the GPP and the NEP-F diets. This amount was chosen due to the limited availability of grape peel to produce enough F fraction to achieve grape fiber content equivalent to the GPP diet. The amount of cellulose and sucrose added to the diets supplemented with GPP and its fractions were reduced to ensure that all diets would

provide dietary fiber and soluble carbohydrates at equivalent amounts to the AIN-93G diet (Table 1). The chemical composition of experimental diets was assessed using standard methods [24, 29–31] and is shown in table 1. The phenolic composition of grape peel powder was determined by LC-PDA-MS/MS and is presented in tables 2 and 3.



Scheme 2. Protocol of treatment and colitis induction. GPP = grape peel powder; EP = extractable polyphenols-rich fraction; NEP-F = non-extractable polyphenols-rich fraction; F = polyphenols-poor, fiber-rich fraction; V= vehicle; TNBS= 2,4,6-trinitrobenzenesulfonic acid.

Table 1

Animals received the experimental diets for 15 days before colitis induction on the 16th day. CM group received mesalamine by gavage (25 mg/animal/day) in the seven days following induction of colitis (Scheme 2). After slight sedation with isoflurane, colitis was induced by intracolonic administration of 10 mg of 2,4,6-trinitrobenzenesulfonic acid (TNBS)

dissolved in 0.25 mL of 50% ethanol (v/v) using a PVC flexible cannula that was inserted 8 cm into the anus. The control group received an intracolonic administration of 0.25 mL of 50% ethanol (v/v). After colitis induction animals continued to receive diets for 7 days until the sacrifice.

Table 2

Table 3

2.3 Disease activity index (DAI)

The severity of colitis was assessed daily in the 7 days subsequent to the induction of colitis using the disease activity index (DAI) scoring (Table 4)[2, 32].

Table 4

2.4 Tissue collection and biochemical analysis

Rats were anesthetized with ketamine and xylazine (90 and 5 mg/kg body weight) and killed by exsanguination with a cardiac puncture in the 7th day after colitis induction. Blood was collected into tubes containing clot accelerator gel and centrifuged (2000 *x* g, 15 min). Serum samples were stored at -80°C until analyses.

Colonic tissue (from the ileocecal junction to the anal verge) was quickly removed, rinsed with cold saline solution (0.9% NaCl) and frozen in liquid nitrogen. Tissue was homogenized in 100 mM phosphate buffer pH 7.4 using a Polytron homogenizer, centrifuged (1500 *x* g, 15 min, 4°C) and the supernatant was stored – 80°C until analyses.

Total thiol levels were evaluated in serum and in colon tissue as described by [33]. Results were expressed as nmol of GSH per mL of serum or per mg of protein.

The activity of antioxidant enzymes was determined in the supernatant of colon homogenate using the specific assays previously described for GPx [34], GR [35], and GST [36].

2.5 Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Total RNA from colon tissue was extracted using Trizol (Invitrogen Corp., Carlsbad, CA) as recommended by the manufacturer. High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) was used to reverse transcribe cDNA. The qRT-PCR assay was conducted in the Rotor-Gene Q5plex HRM System (QIAGEN Biotechnology, Germany) with 2x QuantiFast SYBR® Green PCR Master Mix (QIAGEN Biotechnology, Germany) in the following conditions: 95°C for 3 min followed by 40 cycles of 95°C for 10 s, 60°C for 30 s followed by a melting curve of 60°C to 90°C at increments of 0.5°C/5 s. The qRT-PCR reactions of each sample were run in triplicate, using 1.25 µM of each primer, 180 ng/µL of cDNA, RNAase-free water and 2x QuantiFast SYBR® Green PCR Master Mix (QIAGEN Biotechnology**,** Germany), in a final volume of 20 µL. β-actin was used as a housekeeping gene. Gene expression was calculated using the comparative cytosine-thymine (Ct) calculation, normalized to the housekeeping gene and was expressed as the fold expression compared to the control group. The specific primer pairs (Exxtend, Brazil) used are shown in Table 5.

Table 5

2.6 Statistical analysis

The disease activity index (DAI) was submitted to Kruskal-Wallis analysis of variance followed by a nonparametric multiple comparison test. Other data were analyzed by one-way ANOVA, followed by Duncan's test for means comparison. Data were expressed as mean ± SEM and results were considered significant when $p<0.05$.

3 Results

The disease activity index (DAI) reached peak values in the 2nd day after TNBS administration and then regressed from the 3rd day onwards (Table 6). Even the control group, which received only the TNBS vehicle (ethanol), had transitory damage signals between the 1st and the 3rd days but were promptly recovered in the 4th day after TNBS administration. The colitis group exhibited DAI score higher than control group only in the 2nd day after TNBS administration (Table 6; $p<0.05$). DAI scores of TNBS-administered rats were not reduced by the experimental diets or mesalamine, the reference pharmacological treatment.

Table 6

Compared to the control group, TNBS administration (colitis group) significantly reduced serum total thiol levels (by 35%) (Figure 1A). Mesalamine treatment and diet containing 8% of GPP completely restored serum total thiol levels ($p<0.05$), while EP and NEP-F diets partially recovered thiol levels. The fiber-rich fraction (F) did not recover serum thiol levels. As observed in the serum, colitis promoted a significant depletion of total thiol levels also in colon tissue (Figure 1B). All experimental diets and mesalamine treatment partially restored thiol levels in colon, as the levels remained lower than the control group ($p<0.05$).

Figure 1

The induction of colitis also led to a significant decrease in GR (Figure 1B), GPx (Figure 2B), and GST activities (Figure 2C) ($p<0.05$). Mesalamine treatment and the diet containing the non-extractable polyphenols associated to fiber (NEP-F) completely restored GR activity, whereas GPP, EP, and F diet partially recovered GR activity (Figure 2A, $p<0.05$). Only GPP and NEP-F diets completely restored GPx activity, whereas EP and F diets partially recovered GPx activity (Figure 2B; $p<0.05$). All experimental diets completely recovered GST activity, whereas mesalamine treatment partially recovered GST activity (Figure 2C; $p<0.05$).

Figure 2

To investigate how the bioactive fractions of GPP restored thiol levels, we studied the effect of experimental diets in the major genes linked to glutathione synthesis. Colitis induction increased mRNA levels of the modifier and catalytic subunits of GCL but decreased mRNA levels of GS in colon tissue ($p<0.05$; Figure 3).

The increase in the expression of GCLm subunit mRNA induced by colitis was prevented by mesalamine treatment and by GPP, NEP-F, and F experimental diets ($p<0.05$) but not by EP diet (Figure 3A). Diets containing GPP and NEP-F fraction prevented the increase in the expression of GCLc subunit mRNA triggered by colitis ($p<0.05$), while mesalamine treatment or diets containing EP or F fractions had no effect (Figure 3B). No treatment was able to prevent the decrease in GS mRNA expression caused by colitis ($p<0.05$; Figure 3C).

Figure 3

Since depletion of intracellular glutathione, the major intracellular non-protein thiol compound, is an apoptosis triggering factor [6, 10], the mRNA expression of caspase-3 and caspase-9 were studied. The expression of caspase-3 mRNA in colon tissue was increased by 2-fold in the colitis group compared to the control group ($p<0.05$; Figure 4A). Only diets containing GPP, NEP-F, and F fractions were able to prevent this increase, while EP diet and mesalamine treatment had no effect. The mRNA levels of caspase-9 were not modified by colitis induction (Figure 4B). However, diets containing GPP, NEP-F, and F fractions also caused a significant decrease in mRNA levels of caspase-9 (Figure 4B).

Figure 4

4 Discussion

During inflammatory processes, the redox state of intestine is unbalanced and there is an enhancement of the consumption of endogenous antioxidants containing thiol groups, like GSH, which protects the intestinal mucosa from dietary lipid peroxides, helps to maintain the mucus fluidity, and promotes the intestinal mucosal proliferation [37]. Moreover, GSH also plays a key role in the cellular thiol/disulfide redox balance by preserving the reduced state of protein thiol groups [7]. Thus, a decrease in thiol levels facilitates the oxidative and inflammatory damage in gut mucosa [37, 38]. The antioxidant enzymes involved in the GSH cycle, such as glutathione-S-transferases, but especially glutathione peroxidases and glutathione reductase, are highly important to protect the intestinal epithelium against oxidation and inflammation [37].

In fact, depletion of GSH has been reported in the mucosa of patients with UC and in experimental models of this disease [4, 39]. Accordingly, our results revealed that TNBS-induced colitis was characterized by a depletion of total thiol levels both in serum and colon tissue. Such effect was likely caused by the overproduction of ROS generated by colonic inflammation, which led to the impairment of the glutathione system as indicated by inhibition of GR, GPx, and GST activities triggered by colitis (Figure 2). The impairment of GR activity observed in colitis group (~30% of the activity in control group) culminated in the deficiency to restore oxidized glutathione back to GSH and therefore decreased the thiol/disulfide redox state as indicated by the great decrease in total thiol levels in the colon (~30% of control group levels). GPx activity was only slightly decreased in the colitis group but its actual tissue activity was probably lower due to the depletion of GSH. This inefficiency to remove excess peroxides in the colon tissue probably contributes to further GSH oxidation and the perpetuation of colonic injury. GST activity was also impaired in the colitic group indicating a decreased ability of the colon tissue to recover from electrophilic compounds generated during inflammatory damage.

Our findings demonstrate that GPP diet was as effective as mesalamine to restore the thiol/disulfide redox balance in TNBS-treated rats as indicated by the recovery of total thiol levels in the serum and colon tissue. This protective effect of GPP was mostly associated to the extractable polyphenol fraction and to the fraction that was enriched in dietary fiber and fiber-bound polyphenols, which had similar effect to the GPP. In contrast, the fraction that was enriched in fiber but poor in phenolics was unable to recover the serum level of total thiols.

GPP completely prevented the reduction in the activity of GR, GPx, and GST in TNBS-treated rats, indicating that it was more effective than mesalamine to recover the activity of glutathione-related enzymes. Although mesalamine recovered GR activity, it

partially recovered GST activity and was unable to recover GPx activity. The protection against the loss of activity of glutathione-related enzymes, especially GR, is likely to play a pivotal role in the recovery of the thiol/disulfide redox balance as it recycles oxidized glutathione to maintain the levels of GSH. Our results indicate that all grape fractions investigated were effective to restore the loss of activity of glutathione-related enzymes, presenting a similar effect compared to the GPP.

The positive effect of extractable polyphenols on the activity of antioxidant enzymes and the reestablishment of cell redox balance has been already demonstrated for numerous plant sources and in different disease models [40–42]. However, there is scarce evidence that dietary fiber fraction or the polyphenols bound to this fraction could also exhibit such effect. Dietary supplementation with a grape antioxidant dietary fiber, which has a composition similar to our NEP-F fraction, has been demonstrated to enhance the cytosolic GSH:GSSG ratio and to reduce apoptosis in the colonic mucosa of healthy Wistar rats [23]. However, the mechanism responsible for such effect has not been investigated.

Colitis promoted an increase in the gene expression of both GCL subunits and a decrease in the mRNA expression of GS in the colon tissue. In fact, oxidative conditions have been shown to be associated with an increase in gene expression of GCLc subunit and GCL activity to cope with the increased demand of GSH [19, 43]. However, GCLc can be cleaved by caspase-3 during apoptosis induced by cytokines such as TNF-α [19]. UC is characterized by an upregulation of the inflammatory response accompanied by increased levels of TNF-α [1]. Thus, a similar degradation of GCLc protein may have contributed to impair GSH synthesis in our colitis model, despite the increased mRNA expression of GCLc and GCLm. In contrast to GCL, GS expression was decreased after colitis induction. As only GCL is considered the rate-limiting step in GSH synthesis [19], our data indicate that *de novo* synthesis of GSH was activated to cope with the impaired GSH homeostasis induced by

colitis. However, no experimental diets or mesalamine treatments were able to improve GS expression or caused further increase in GCL subunits expression compared to the colitis group. These data reinforce the proposal that the positive effect of GPP on the thiol/disulfide redox balance was triggered by increasing GSH recycling (increased GR activity) rather than by increasing *de novo* synthesis of GSH. Similarly, Kerasioti et al. (2017) observed that sheep fed with grape pomace had higher GST activity and an increased protein expression of GCL in liver tissue but not in spleen, although the latter tissue has shown increased GSH levels. The authors suggested that this effect could be associated to the regeneration of reduced glutathione from oxidized glutathione due to GR activity and not to the *de novo* GSH synthesis although GR activity had not been assessed in this study.

The increased GR activity triggered by dietary supplementation with GPP or its fractions likely contributed to maintain appropriate GSH levels to support GPx and GST activities, which were also recovered by the dietary supplementation with GPP and its fractions. Thus, GPP effects were triggered by the recovering of GSH levels associated to an increased enzymatic capacity to remove ROS. It is important to note that not only the consumption of the entire grape peel (containing dietary fiber, soluble, and fiber-bound polyphenols) or the extractable polyphenols fraction (EP), but also the fractions enriched in dietary fiber and fiber-bound polyphenols were able to recover the impairment in GSH balance triggered by colitis. Dietary fiber has been shown to play an important role in carrying polyphenols (fiber-bound polyphenols) along the gastrointestinal tract until reaching the colon to be fermented and metabolized by colon microbiota [44].

Severe GSH depletion caused by high levels of ROS, decreased GCL activity or enhanced GSH efflux may overload the apoptotic system culminating in the conversion from apoptotic to necrotic cell death [8, 19]. Apoptosis is related to the condensation of chromatin and DNA fragmentation, while necrosis is associated to the rupture of plasmatic membrane

and ATP depletion [8, 19]. GSH modulates both death cell mechanisms due to its ability to regulate the activity and expression of caspases and other proteins related to the cell death [10, 19].

Caspase-3, a cysteine-aspartic acid protease, is the major effector caspase activated in apoptotic cells by extrinsic and intrinsic pathways [45]. Caspase-3 activity and expression have been shown to be increased in UC [2, 46, 47], indicating that apoptotic activity is associated with colitis development and progression [9]. Our findings support this hypotesis since the colitis group had an increased expression of caspase-3. Dietary supplementation with GPP down-regulated colon caspase-3 expression. This effect can be attributed to the fiber-associated polyphenols (NEP-F) and to the dietary fiber (F) fractions, since soluble polyphenols (EP fractions) did not present any effect in caspase-3 expression. It is important to highlight that mesalamine, which is a drug used in colitis management, also had no effect in caspase-3 expression in colon tissue. The relationship of this caspase with GSH depletion can be exemplified by the study of Song et al. (2016), who demonstrated that dietary supplementation with L-cysteine, the thiol aminoacid of GSH, recovered GSH depletion and reduced the activation of caspase-3 in piglets with intestinal disruption.

In addition, le carbone, a charcoal supplement rich in fiber, decreased apoptosis by downregulating caspase-3 expression in an experimental model of colitis induced by dextran sulfate sodium [48]. Similarly, arabinogalactan, a constituent of soluble fiber (pectin), has been shown to abolish the apoptosis cascade in a model of ischemia-reperfusion by inhibiting the conversion of inactive pro-caspase-3 into active caspase-3 [49]. These effects attributed to dietary fiber were correlated to its fermentation into short chain fatty acids. Moreover, proanthocyanidins and ferulic acid (a phenolic acid commonly associated to dietary fiber) also had anti-apoptotic activity by inhibiting caspase-3 activation [40, 50].

Caspase-9 is another indicator of pro-apoptotic activity. However, it belongs to the group of initiator caspases and is associated to the intrinsic pathway of apoptosis, mediated by mitochondria [9, 10]. Although we have not observed a significant effect of colitis in the expression of caspase-9, our findings showed a downregulation of this marker when GPP was added to the diet of colitic rats (Figure 4B). The GPP fractions associated with this effect were once again the fiber-associated polyphenols (NEP-F) and dietary fiber (F). On the other hand, the fraction of extractable polyphenols (EP) had no effect on caspase-3 or -9, despite being as effective as GPP or its fiber fractions to recover total thiol content and the activity of GSH-related antioxidant enzymes. These data suggest the modulation of the apoptotic pathway by GPP and its fiber fractions, which seems to be mediated by other mechanisms than the sole recovery of the GSH homeostasis. However, the modulation of the apoptotic pathway deserves to be further investigated as we have not evaluated the active forms of caspase-3 or -9.

5 Conclusion

GPP was able to promote GSH recycling and recover the activity of GSH-related antioxidant enzymes, contributing to the maintenance of redox homeostasis and protection of intestinal mucosa against damage in an experimental model of ulcerative colitis. While all grape peel fractions were similarly effective to recover GSH homeostasis in the colon tissue, only the NEP-F fraction, rich in dietary fiber and fiber-bound polyphenols, and the fiber fraction poor in polyphenols were able to modulate the caspase expression. Our data suggest that the grape dietary fiber and polyphenols associated to the fiber are more effective than the extractable polyphenols to protect intestinal mucosa from colitis injuries.

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Figure captions

Figure 1. Total thiol levels in serum (A) and colonic tissue (B). # Different from control group ($p<0.05$); *Different from colitis group ($p<0.05$); TNBS= 2,4,6-trinitrobenzenesulfonic acid; GPP = grape peel powder; EP= extractable polyphenols fraction; NEP-F = non-extractable polyphenols-rich fraction; F= polyphenols-poor, fiber-rich fraction.

Figure 2. Activity of GR (A), GPx (B), and GST (C) in the colon tissue of rats with TNBS-induced colitis. # Different from control group ($p<0.05$); *Different from colitis group ($p<0.05$); TNBS= 2,4,6-trinitrobenzenesulfonic acid; GPP = grape peel powder; EP= extractable polyphenols fraction; NEP-F = non-extractable polyphenols-rich fraction; F= polyphenols-poor, fiber-rich fraction; GR= glutathione reductase; GPx= glutathione peroxidase; GST: glutathione-S-transferase.

Figure 3. Quantitative real time RT-PCR (qRT-PCR) of enzymes involved in GSH synthesis in the colon tissue of rats with TNBS-induced colitis. # Different from control group ($p<0.05$); * Different from colitis group ($p<0.05$). TNBS = 2,4,6-trinitrobenzenesulfonic acid; GPP = grape peel powder; EP= extractable polyphenols fraction; NEP-F = non-extractable polyphenols-rich fraction; F= polyphenols-poor, fiber-rich fraction.

Figure 4. Quantitative real time RT-PCR (qRT-PCR) of caspase-3 (A) and caspase-9 (B) genes in the colon tissue of rats with TNBS-induced colitis. # Different from control group ($p<0.05$); * Different from colitis group ($p<0.05$); TNBS = 2,4,6-trinitrobenzenesulfonic acid; GPP = grape peel powder; EP= extractable polyphenols fraction; NEP-F = non-extractable polyphenols-rich fraction; F= polyphenols-poor, fiber-rich fraction.

Figure 1

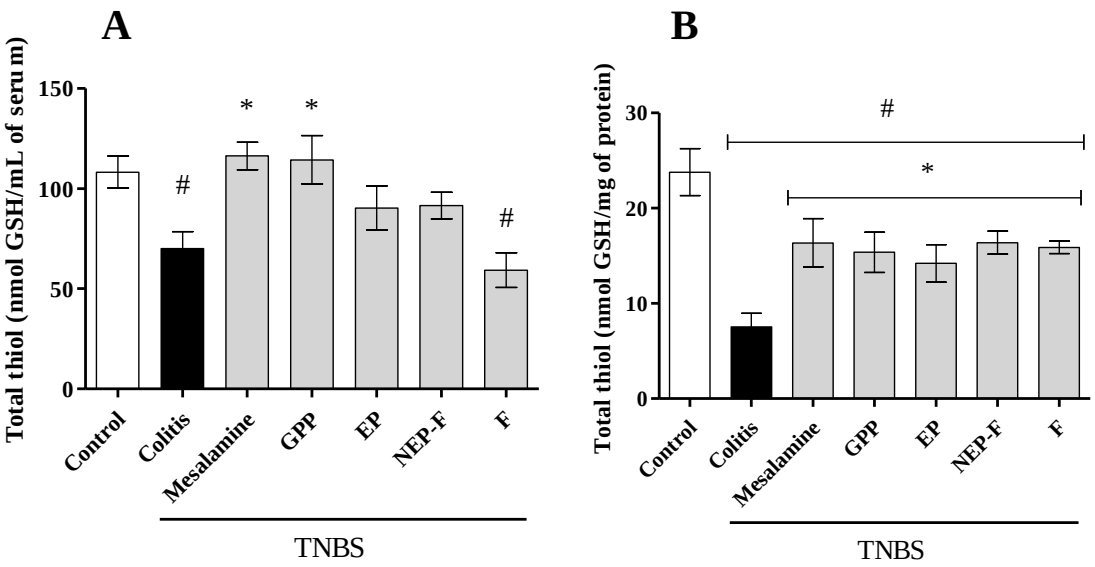
A
Total thiol (nmol GSH/mL of serum)
150
100
50
0
#
*
*
#
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS
B
Total thiol (nmol GSH/mg of protein)
30
20
10
0
#
*
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS

Figure 2

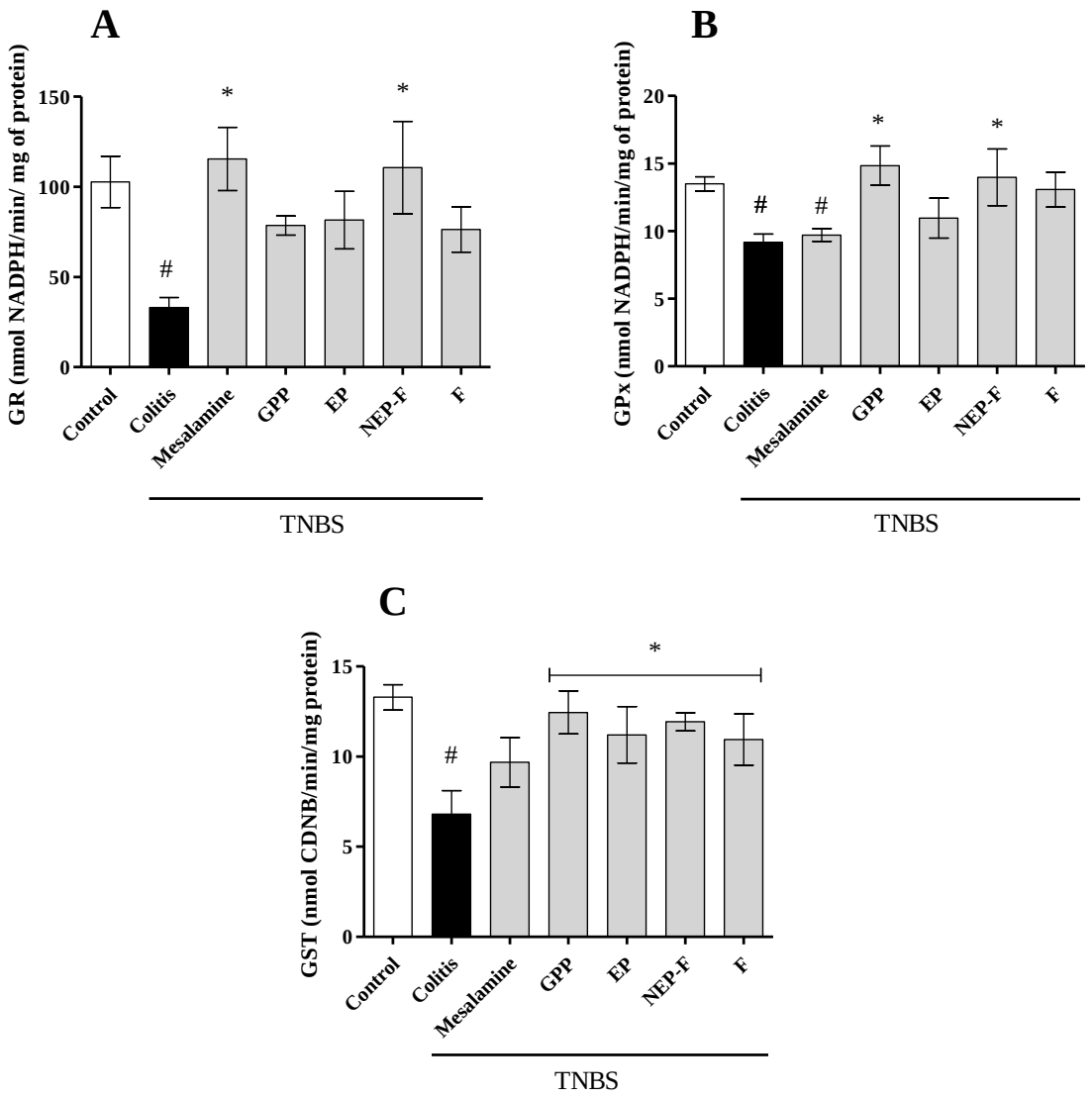
A
GR (nmol NADPH/min/ mg of protein)
150
100
50
0
#
*
*
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS
B
GPx (nmol NADPH/min/mg of protein)
20
15
10
5
0
#
#
*
*
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS
C
GST (nmol CDNB/min/mg protein)
15
10
5
0
#
*
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS

Figure 3

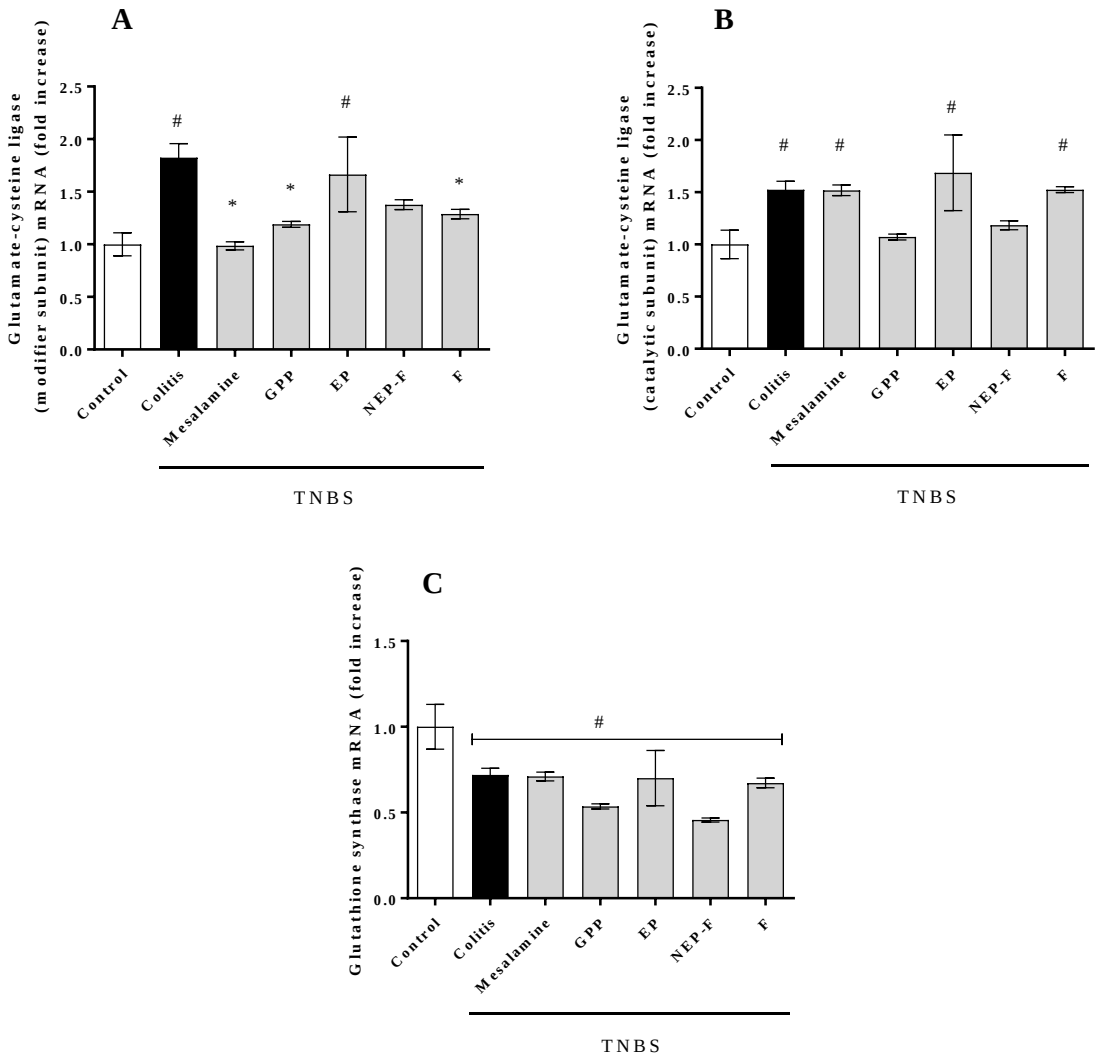
A
Glutamate-cysteine ligase (modifier subunit) mRNA (fold increase)
2.5
2.0
1.5
1.0
0.5
0.0
#
#
*
*
*
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS
B
Glutamate-cysteine ligase (catalytic subunit) mRNA (fold increase)
2.5
2.0
1.5
1.0
0.5
0.0
#
#
#
#
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS
C
Glutathione synthase mRNA (fold increase)
1.5
1.0
0.5
0.0
#
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS

Figure 4

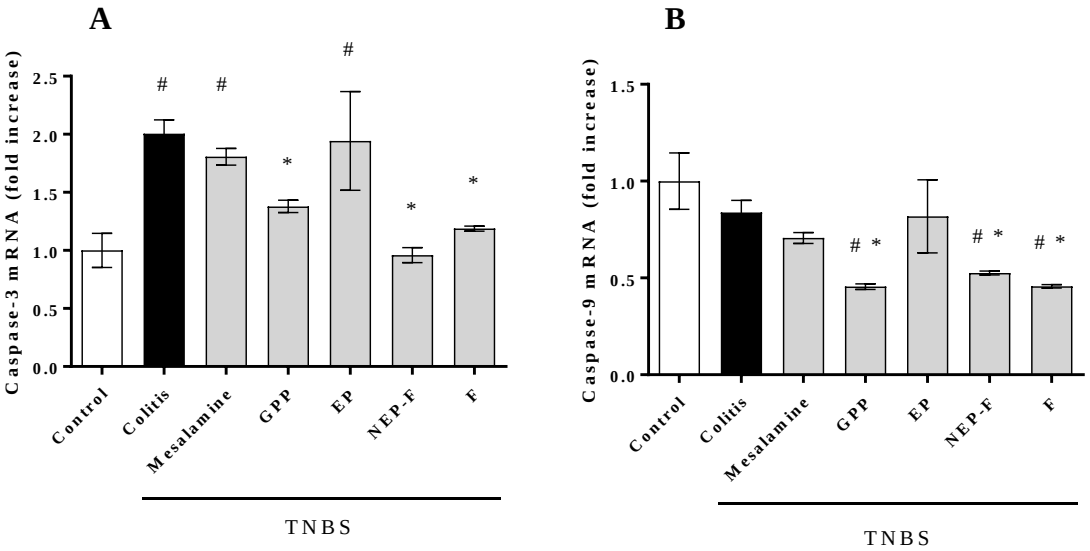
A
Caspase-3 mRNA (fold increase)
2.5
2.0
1.5
1.0
0.5
0.0
#
#
#
*
*
*
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS
B
Caspase-9 mRNA (fold increase)
1.5
1.0
0.5
0.0
*
*
*
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS

Table 1. Ingredients and composition of experimental diets.

	AIN-93G	GPP	EP	NEP-F	F
Ingredients					
Corn starch (g)	440.9	420.2	440.9	433.4	440.4
Casein (g)	156.6	156.6	156.6	156.6	156.6
Maltodextrin (g)	132.0	132.0	132.0	132.0	132.0
Sucrose (g)	100.0	57.8	68.3	100.0	100.0
Soybean oil (g)	70.0	70.0	70.0	70.0	70.0
Cellulose (g)	50.0	33.1	50.0	35.8	47.0
Mineral mix (g)	35.0	35.0	35.0	35.0	35.0
Vitamin mix (g)	10.0	10.0	10.0	10.0	10.0
L-cysteine (g)	3.0	3.0	3.0	3.0	3.0
Choline (g)	2.5	2.5	2.5	2.5	2.5
tert-butylhydroquinone (g)	0.014	0.014	0.014	0.014	0.014
Grape peel powder (g)		80.0			
EP extract (mL)			198.0		
NEP-F fraction (g)				21.7	
F fraction (g)					3.5
Total (g)	1000	1000	1000	1000	1000
Proximate composition and energetic value					
Ash [#]	2.9 ± 0.2	3.6 ± 0.1	3.9 ± 0.1	2.9 ± 0.1	2.9 ± 0.1
Protein [#]	15.2 ± 0.5	17.8 ± 1.5	17.9 ± 0.6	15.5 ± 0.5	14.8 ± 1.0
Fat [#]	14.5 ± 1.0	15.6 ± 0.2	15.9 ± 0.2	14.8 ± 1.0	14.5 ± 0.2
Total carbohydrates[&]	67.4	63.0	62.3	66.8	67.8
Energetic value (kcal/kg)*	3948.0	3928.7	3948.0	3943.3	3946.6
Dietary fiber (g%, dry weight) and phytochemical composition (mg%, dry weight)*					
Total dietary fiber	5.0	5.0	5.0	5.0	5.0
Insoluble fiber	5.0	4.3	5.0	4.7	5.0
Soluble fiber	-	0.7	-	0.3	<0.1
Extractable polyphenols		62.0	62.6	-	-
Bound proanthocyanidins	-	227.0	-	228.0	-

Results are presented as mean ± s.d. [#] g%, dry weight. [&] Calculated by difference between 100 and the sum of the other constituents. * Calculated based on the ingredient composition and the amount of ingredients added to the diet. GPP = grape peel powder; EP = extractable polyphenols-rich fraction; NEP-F = non-extractable polyphenols-rich fraction; F = polyphenols-poor, fiber-rich fraction.

Table 2. Non-anthocyanic phenolic composition of 'Isabel' grape peel powder.

	RT-PDA (min)	λ máx (nm)	Experimental MS $[M-H]^-$	mg/100 g (mean ± SD) *
Phenolic acids				
Protocatechuic acid hexoside	7.3	280	315.0730	0.09 ± 0.03
cis-caftaric acid	8.3	327	311.0436	0.59 ± 0.14
trans-caftaric acid	9.3	328	311.0428	15.13 ± 0.70
Caffeoyil hexoside	12.1	330	341.0933	1.24 ± 0.12
cis-coutaric acid	13.3	309	295.0472	0.70 ± 0.06
trans-coutaric acid	13.8	312	295.0472	1.97 ± 0.10
Coumaroyl hexoside	16.3	313	325.0975	0.13 ± 0.01
trans-fertaric acid	17.2	312	325.0957	1.96 ± 0.11
Feruloyil hexoside	17.6	326	355.1066	0.64 ± 0.04
Syringic acid	18.2	275	197.0500	1.25 ± 0.24
Coumaric acid	30.5	307	163.0449	0.12 ± 0.02
trans-ferulic acid	41.1	320	193.0545	0.60 ± 0.05
Total phenolic acids				*47.82 ± 2.38*
Flavanols				
Procyanidin	11.3	280	577.1368	0.95 ± 0.27
Catechin	15.1	280	289.0747	8.26 ± 0.16
Epicatechin	20.7	280	289.0700	3.18 ± 0.61
Epicatechin-glucuronide	27.8	293	465.1115	1.85 ± 0.34
Total flavanols				*13.50 ± 0.16*
Flavonols				
Miricetin-3-hexoside	39.7	354	479.0888	5.64 ± 0.35
Quercetin-3-rutinoside	46.2	354	609.1505	3.74 ± 0.19
Quercetin-3-glucuronide	47.0	354	477.0748	32.65 ± 2.44
Quercetin-3-hexoside	47.7	355	463.0955	40.46 ± 1.29
Laricitrin-3-hexoside	49.2	356	493.0980	3.32 ± 0.23
Kaempferol-3-hexoside	55.8	346	447.0974	5.62 ± 0.21
Isorhamnetin-3-hexoside	56.4	354	477.1094	2.45 ± 0.15
Isorhamnetin-3-glucuronide	57.7	353	491.0899	0.21 ± 0.02
Total flavonols				*97.28 ± 5.23*
Stilbenes				
trans-piceid	43.0	307	389.1277	0.99 ± 0.04
trans-resveratrol	54.2	305	227.0748	4.02 ± 0.21
Viniferin	63.1	323	453.1404	0.08 ± 0.03
Total stilbenes				*5.07 ± 0.26*
Total soluble non-anthocyanic compounds				*162.50 ± 9.34*

* Hydroxycinnamic acid derivatives (320 nm) expressed as mg equivalents to caffeic acid per 100 g grape peel powder; Hydroxybenzoic acid derivatives (280 nm) expressed as mg equivalents to gallic acid per 100 g grape peel powder; Flavanols (280 nm) expressed as mg equivalents to catechin per 100 g grape peel powder; Flavonols (360 nm) expressed as mg equivalents to kaempferol-3,7-diglucoside per 100 g grape peel powder; Stilbenes (305 nm) expressed as mg equivalents to resveratrol per 100 g grape peel powder.

Table 3. Anthocyanic profile of 'Isabel' grape peel powder (evaluated in the EP extract).

Anthocyanin	**RT-PDA (min)**	**λ_{max} (nm)**	**Experimental MS $[M-H]^+$**	**mg/100g* (mean ± sd)**
Delphinidin-3-glucoside	2.6	523	465.1029	28.93 ± 1.40
Malvidin-3,5-diglucoside	3.1	529	655.1852	9.64 ± 0.28
Cyanidin-3-glucoside	3.7	517	449.1066	12.71 ± 1.28
Petunidin-3-glucoside	4.7	526	479.1143	49.43 ± 1.61
Peonidin-3-glucoside	6.5	517	463.1209	121.38 ± 4.50
Malvidin-3-glucoside	7.4	527	493.1339	321.87 ± 11.38
Delphinidin-3-coumaroyl-glucoside	13.1	531	611.1373	14.21 ± 0.34
Cyanidin-3-coumaroyl-glucoside	13.6	532	595.1432	1.32 ± 0.34
Peonidin-3-coumaroyl-glucoside	16.5	522	609.1539	13.84 ± 2.38
Malvidin-3-coumaroyl-glucoside	16.8	532	639.1677	40.30 ± 3.89
			Total anthocyanins	*613.64 ± 20.73*

* mg equivalents to malvidin-3-glucoside/100 g of grape peel powder.

Table 4. Scoring of disease activity index (DAI).

	DAI score				
	0	1	2	3	4
Body weight loss	< 1 %	1 – 5 %	5 – 10 %	10 – 15 %	> 15 %
Stool consistency	Normal stool, well-formed pellets		Loose stool, pasty stool that does not stick to the anus		Diarrhea, liquid stool that sticks to the anus
Rectal bleeding	Negative		Positive		Intense

Table 5. Sequence of specific primers used in the qRT-PCR analysis.

Genes	*Primers*	
	Forward	*Reverse*
Actb	GCAGGAGTACGATGAGTCCG	ACGCAGCTCAGTAACAGTCC
Gclc	GTGGACACCCGATGCAGTAT	TCATCCACCTGGCAACAGTC
Gclm	CAGGAGTGGGTGCCACTGT	TTTGACTTGATGATTCCTCTGCTT
Gss	GCAGGAACTGAGCAGGGTG	GCTTCAGCACAAAGTGGCTAG
Casp3	GAGACAGACAGTGGAACTGACGATG	GGCGCAAAGTGACTGGATGA
Casp9	CTGAGCCAGATGCTGTCCCATA	GACACCATCCAAGGTCTCGATGTA

Actb = *β*-actin; *Gclc* = glutamate cysteine ligase catalytic subunit; *Gclm* = glutamate cysteine ligase modifier subunit; *Gss* = glutathione synthase; *Casp3* = caspase 3; *Casp9* = caspase 9

Table 6. Disease activity index (DAI) after colitis induction.

	Time after colitis induction (days)						
Group	1	2	3	4	5	6	7
Control	5 (1-7)	4 (3-5)	3 (0-4)	0 (0-2)	0 (0-0)	0 (0-0)	0 (0-1)
Colitis	3 (2-6)	6 (6-7)[#]	4 (3-5)	3 (2-4)	2 (2-3)	2 (0-4)	2 (0-2)
Mesalamine	5 (5-5)	5 (4-6)	5 (2-7)	2 (2-3)	2 (2-4)	2 (0-2)	2 (0-2)
GPP	4 (3-5)	5 (5-5)	3 (3-4)	2 (2-5)	2 (0-2)	2 (0-2)	0 (0-2)
EP	4 (2-6)	5 (5-6)	3 (2-6)	2 (2-4)	2 (1-4)	2 (0-2)	0 (0-2)
NEP-F	4 (4-5)	5 (5-5)	5 (4-5)	3 (3-3)	2 (2-3)	2 (0-4)	0 (0-0)
F	3 (3-5)	6 (4-6)	4 (4-5)	2 (2-5)	2 (0-3)	2 (0-3)	0 (0-2)

The results are presented as the median of score (Q25-Q75). [#] Different from control ($p<0.05$, Kruskal-Wallis followed by multiple comparison test). GPP = grape peel powder; EP = extractable polyphenols-rich fraction; NEP-F = non-extractable polyphenols-

4.4 MANUSCRITO 3

Grape peel powder promotes the intestinal barrier homeostasis in acute TNBS-induced colitis: a major role for dietary fiber and fiber-bound polyphenols

Manuscrito a ser submetido ao periódico *Food & Function* (FI:3,247; Qualis A1)

(formatado conforme as normas do periódico)

Grape peel powder promotes the intestinal barrier homeostasis in acute TNBS-induced colitis: a major role for dietary fiber and fiber-bound polyphenols

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Abstract

Ulcerative colitis (UC) is characterized by impaired intestinal barrier function. This study aimed to evaluate the effect of grape peel powder (GPP) and its bioactive fractions on the barrier function and colonic injury in a model of colitis induced by 2,4,6 trinitrobenzenesulfonic acid (TNBS). Wistar rats received diets supplemented with GPP (8%), soluble phenolics (EP), fiber-bound polyphenols (NEP-F), or dietary fiber (F) from grapes at amounts equivalent to the GPP group during 15 days before and for 7 days after colitis induction. Grape bioactive fractions did not protect against the macroscopic or microscopic colon damage, although NEP-F decreased the lesion extension. EP diet worsened colitis damage. GPP, EP, and NEP-F reduced claudin-2 mRNA expression, whereas F fraction increased occludin and ZO-1 mRNA expression. The increased MMP-9 mRNA expression was reduced by all experimental diets. Colitis reduced by 30% the production of cecal short-chain fatty acids (SCFA). GPP and NEP-F completely protected against this effect, whereas F fraction was ineffective. Dietary fiber seems to reestablish the intestinal barrier function, whereas fiber-associated phenolics were able to restore cecal metabolism to produce beneficial metabolites like SCFA. Moreover, the consumption of bound phenolics from grape peel was safer than soluble phenolics in this colitis model.

Keywords

Short-chain fatty acid; tight junction; bound polyphenols; inflammation; barrier function; unfolded protein response.

1 Introduction

The intestinal barrier comprises the protective role of the single layer intestinal epithelium in the regulation of immune response and in the defense response against endogenous and exogenous harmful substances including pathogens and other antigens [1]. A physical barrier of mucus is secreted by Goblet cells and helps to protect the intestinal epithelium against the invasion of luminal microorganisms and the attack of chemical agents [2,3]. The tight junctions are a family of intracellular and transmembrane proteins that are responsible for the connection among colonocytes and help to maintain the homeostasis of intestinal barrier and the integrity of intestinal epithelium [2,4]. In fact, disturbances in the production and/or formation of tight junctions and in the protective mucus layer are involved in the pathogenesis of inflammatory bowel diseases [4–7].

Ulcerative colitis (UC) is a chronic inflammatory disorder of gut that reaches the highest rates in developed countries from North America and Northern Europe but has also achieved considerable rates in developing countries [3,8]. The chronic inflammation and the oxidative stress present in UC induce an overproduction of reactive oxygen species (ROS) and pro-inflammatory cytokines, which lead to the disruption of intestinal barrier integrity through the impairment of tight junction's function [9]. This conducts to an extensive infiltration of pathogens and inflammatory cells, and consequently, to a massive ulceration of the colon mucosa [3,10].

The pathogenesis of UC also involves the endoplasmic reticulum (ER) stress, caused by the accumulation of unfolded and misfolded proteins, which culminate in alterations in the intestinal permeability and regeneration. Glucose-regulated proteins (GRPs) such as GRP94 and GRP78 (also known as BIP) are examples of stress-inducible chaperones that play an important role in protein folding, assembly, and degradation [11,12].

The pharmacotherapy of colitis aims to maintain the disease in a remissive state using aminosalicylates, corticosteroids, antibiotics, immunosuppressant, and biologic agents as anti-TNF-α [13]. In addition, the regular consumption of fruits and vegetables rich in phytochemicals has been associated with an improvement in the symptoms of UC and other inflammatory bowel diseases (IBD) [14,15].

It is estimated that about 90% of ingested polyphenols are not absorbed in the upper gastrointestinal system and reach intact in the colon, where these compounds are broken and metabolized into small molecules by enzymes from the gut microbiota [16]. Polyphenol metabolites can act locally in the colon by scavenging free radicals and counteracting the effects of dietary chemicals or pro-oxidants [17], or they can be absorbed and have systemic effects [18].

Dietary fibers include food components resistant to digestion and absorption in the small intestine that are partial or completely fermented in the colon by gut microbiota [19] yielding short-chain fatty acids (SCFAs), such as acetate, propionate, and butyrate [20]. These metabolites have been implicated in the regulation of the gut immune system [21] and in the enforcement of the epithelial barrier by affecting the mucus layer, the tight junction proteins, as well as the epithelial cell survival and proliferation [22,23]. Butyrate is the main source of energy for colonocytes, while acetate and propionate reach the liver. Propionate is metabolized by hepatocytes and acetate remains in the liver or reaches high concentration in the systemic circulation [21,22]. The consumption of high quantity of dietary fiber has been linked to a lower risk to colorectal cancer and IBD [20].

Grape, which is among the richest sources of polyphenols due to the presence of anthocyanins, flavonols, resveratrol, phenolic acids, and procyanidins [24], also has significant amount of dietary fiber [25] and polyphenols bound to the fiber fraction [26]. Grape pomace [27], grape polyphenols extract [28–30], grape seed extract [31] or isolated compounds as resveratrol [32]

have been shown to be good alternatives to protect the colon mucosa in models of intestinal inflammation. However, there is no evaluation on the effect of whole grape peel consumption in experimental colitis or the specific grape fractions (dietary fiber, polyphenols associated to fiber or free polyphenols) responsible for the effect and even the mechanisms involved.

Thus, the aim of this study was to evaluate the impact of dietary supplementation with grape peel powder or its bioactive fractions on the intestinal injury in a colitis model induced by 2,4,6 trinitrobenzenesulfonic acid (TNBS) in rats. The colonic damage was assessed along with the intestinal barrier homeostasis, production of short-chain fatty acids and endoplasmic reticulum dysfunction.

2 Material and methods

2.1 Grape bioactive fractions

Grape peel powder (cv. Isabel *Vitis labrusca x Vitis vinifera* L.) was chemically fractioned to yield fractions selectively enriched in its specific bioactive compounds. Soluble polyphenols (EP fraction) were extracted using MeOH:water (50:50, pH 2.0) and acetone:water (70:30) [33] and this extract was concentrated under vacuum. The residue of this extraction was nominated as the NEP-F fraction, which was rich in fiber and insoluble polyphenols bound to the fiber fraction (condensed and hydrolysable tannins and other hydrolysable polyphenols). The NEP-F fraction was submitted to sequential extraction using butanol:HCl (95:5) at 100°C/3 h and MeOH:H_2SO_4 (90:10) at 85°C/20 h [33,34] in order to remove condensed and hydrolysable polyphenols. The residue obtained in this extraction was nominated the F fraction, which was rich in fiber and poor in polyphenols.

2.2 *In vivo* experimental design and colitis induction

This study was approved by the Animal Research and Ethic Committee of the University of Campinas (Brazil) (protocol 3815-1/2015). Adult male Wistar rats (200 ± 20g) were allocated in individual cages and maintained under controlled temperature (22 ± 2°C), humidity (60-70%), light–dark cycle (12/12 h). Animals were divided into seven groups (n=8 per group): control (C), colitis (CC), mesalamine (CM), 8% grape peel powder (GPP), extractable polyphenols (EP), non-extractable polyphenols (NEP-F), and polyphenols-poor residue (F). Groups C, CC, and CM received the AIN-93G diet [35], whereas GPP, EP, NEP-F, and F groups received the AIN-93G diet containing each fraction (Table 1). Each animal received 20 g of experimental diet per day and the intake was daily monitored. The dose of 8% GPP was chosen to yield about 12.4 mg of phenolics/day/animal (or about 40 mg of phenolics/kg body weight/day). Similar doses of proanthocyanidins from grape seeds have been shown to be beneficial in a colitis model [36]. The amount of the EP and NEP-F fractions were calculated to provide extractable and non-extractable polyphenols equivalent to the GPP diet. The amount of F fraction included in the F diet was equivalent to the residue obtained after extraction (extractable and bound-phenolics extraction) of the same amount of GPP used in the GPP diet. As the method used to obtain the F fraction had a very low yield (4.4% from the GPP, data not shown), the F diet had much lower grape fiber content than the GPP and the NEP-F diets. This amount was chosen due to the limited availability of grape peel to produce enough F fraction to achieve grape fiber content equivalent to the GPP diet. The amount of cellulose and sucrose added to the diets supplemented with GPP and its fractions were reduced to ensure that all diets would provide total dietary fiber and soluble carbohydrates at equivalent amounts to the AIN-93G diet.

Table 1

Animals received diets for 15 days before colitis induction on the 16th day. After slight sedation with isoflurane, colitis was induced by intracolonic administration of 10 mg of 2,4,6-trinitrobenzenesulfonic acid (TNBS) dissolved in 0.25 mL of 50% ethanol (v/v) and administered by a PVC flexible cannula inserted 8 cm into the anus. The same procedure was conducted with the control group by intracolonic administration of 0.25 mL of 50% ethanol (v/v). After colitis induction, animals received diets for more 7 days until the euthanasia. The CM group received mesalamine by gavage (25 mg/animal/day) in the 7 days following colitis induction. Seven days after colitis induction animals were anesthetized with ketamine and xylazine (90 and 5 mg/kg body weight) and exsanguinated by cardiac puncture.

2.3 Assessment of colonic damage

Colonic tissue (from the ileocecal junction to the anal verge) was quickly removed, rinsed with cold saline solution (0.9 % NaCl) and blotted dry. Thereafter, the colon was longitudinally opened, the adhering fat tissue was removed and colon was weighed, measured, and macroscopically evaluated. Soon after being collected, the colons were photographed with a metric reference and the area of colonic lesion was measured using the ImageJ software. The macroscopic damage was evaluated by a protocol adapted from Marchi *et al.* [37] and Bell, Gall, & Wallace [38] (Table 2).

Table 2

Sections of the distal colon were excised and fixed in 6% formaldehyde, embedded in paraffin, sectioned in 6 μm and stained with hematoxylin and eosin for histological analyses. Three histological sections were made for each animal (n=8 per group) and all area of the section was evaluated. Images were made using a microscope Nikon Eclipse E400 coupled to

a digital camera DMX1200C and a 10x objective. Colon histological damage was analyzed by a specialist with no knowledge of the experimental groups using a score damage protocol adapted from Krause *et al.* [39] that has a maximum sum of 40 (Table 3).

Table 3

2.4 Quantitative real time PCR (qRT-PCR) of tight junctions and inflammatory markers in colonic tissue

Total RNA from colon tissue was extracted using Trizol (Invitrogen Corp., USA) as recommended by the manufacturer. High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) was used to reverse transcribe cDNA. The expression of mRNA was determined by Real Time PCR (ABI Prism 7500 – Applied Biosystems) using the primers for MMP-9, claudin-2, zonula occludens (ZO-1), occludin, GRP94, GRP78 (all from Integrated DNA Technologies, USA), and GAPDH (Applied Biosystems, USA) as housekeeping gene. Each reaction consisted in 50 ng of cDNA, primer, TaqMan Fast Advanced Master Mix (Applied Biosystems, USA), and RNase free water to a final volume of 10 µL. Data were analyzed using the Sequence Detector System 7500 (Applied Biosystems, USA).

2.5 Short-chain fatty acids (SCFA) in cecal feces

About 0.5 g of frozen cecal feces were homogenized with 2 mL of ultrapure water, the pH was adjusted to 2-3 using an aqueous solution of 2N HCl and samples were centrifuged at 1700 *x* g for 20 min. The supernatant was collected and the internal standard, 2-ethyl-butyric acid (54.75 mM in 12% formic acid), was added to reach a final concentration of 1 mM. Samples were injected in an Agilent Technologies gas chromatograph (HP 6890 N) equipped with a capillary column Nukol ™ (30 m x 0.25 mm; 0.25 µm) and flame ionization detector

(FID). The temperature of injection port was maintained at 200°C and the carrier gas was nitrogen (1 mL/min). After injection (1 µL, split ratio 10:1), the oven temperature was held at 100°C for 0.5 min, then it was increased to 180°C at 8°C/min and maintained at this temperature for 1 min and then the temperature was increased to 200°C at 20°C/min and maintained at 200°C for 10 min [40]. A standard mix of volatile free fatty acids (46975-U, Sigma-Aldrich, St. Louis, MO, USA) were diluted in a solution of formic acid (12%) and used as external standard.

2.6 Fecal pH

About 0.1 g of cecal feces were mixed with 10 mL of ultrapure water and pH was measured with a pHmeter.

2.7 Statistical analysis

Data were analyzed by one-way ANOVA, followed by Duncan's test for means comparison. Data were expressed as mean ± SEM and results were considered significant when $p<0.05$. Data on the macroscopic and microscopic scores were submitted to Kruskal-Wallis variance analysis, followed by a multiple comparison test.

3 Results and discussion

Before colitis induction, all treatment groups had similar feed intake (data not shown) but the EP group presented higher daily feces production compared to the control group ($p<0.05$; Figure 1A). In addition, the EP group had higher cecum weight than the control and colitis groups after colitis induction ($p<0.05$; Figure 1B), while the other groups were not affected. This result suggests that the EP group had higher production and/or cecal retention of feces regardless of colitis induction, but due to the consumption of a high quantity of free

polyphenols in the diet. This fecal stasis may have contributed to the colon macroscopic damage observed in the EP group, which had higher colonic lesion area (Figure 1C) and macroscopic colon damage score (Figure 1D) compared to the untreated colitis group ($p<0.05$).

Figure 1

The area of injured colon (Figure 1C) and the macroscopic colon damage score (Figure 1D) were significantly higher in the untreated colitis group when compared to control group ($p<0.05$). Dietary supplementation with the NEP-F fraction was as effective as mesalamine to prevent the increase in the area of colonic lesion triggered by TNBS, whereas the other diets had no effect ($p<0.05$; Figure 1C). This effect is likely associated to the fiber-bound proanthocyanidins as the F fraction did not reduce the injured area. In fact, a great part of dietary oligomeric and polymeric proanthocyanidins from grape reach the colon associated to insoluble dietary fiber, and both may interact with bacterial enzymes, affecting the extent of fermentation [41,42]. According to González-Sarrías, Espín, & Tomás-Barberán [43], the metabolites formed during colonic fermentation of non-extractable polyphenols are better absorbed than precursors and can persist up to 3-4 days in the blood stream and exhibit anti-inflammatory and antioxidant systemic effects. The beneficial effects of NEP-F fraction can be due to this prolonged metabolism of the fiber-bound polyphenols. On the contrary, the short-term peaks of free phenolic ingestion (EP fraction) was harmful and exacerbated the colonic lesion.

No experimental diet was able to prevent the increase in the macroscopic damage score triggered by TNBS ($p>0.05$) and dietary supplementation with the EP fraction

exacerbated the macroscopic damage ($p>0.05$; Figure 1D). Only mesalamine therapy was effective for mitigating the macroscopic damage induced by colitis ($p>0.05$; Figure 1D).

Animals that received only TNBS (colitis group) presented severe colitis, evidenced by the absence of crypts and Goblet cells, loss of intestinal mucosal architecture, and infiltration of pro-inflammatory cells (Figure 2A), whereas mesalamine treatment appeared to promote the reconstruction of colonic mucosa and crypt structure, and decreased the infiltration of inflammatory cells (Figure 2A). Although there was no significant difference among groups in the microscopic colon damage score (Figure 2B, $p>0.05$), the histological evaluation of colon tissue shows that EP group displayed similar results to the colitis group (Figure 2A). The addition of GPP, NEP-F, or F fraction in the diet of colitic animals did not ameliorate the histological score. Similarly, the inclusion of passion fruit peel flour in the diet of colitic rats did not improve of colonic macro or microscopic damage scores [44], whereas green banana flour supplementation had only improved the scores of damage when it was accompanied by prednisolone therapy [45].

Figure 2

Diets containing polyphenols may provide protective effects against colitis due to their anti-inflammatory and radical scavenging properties, as well as their ability to modulate gut microbiota [10]. There is no consensus in the literature whether the inflammatory response is the trigger leading to changes in colonic tight junctions (TJ) in UC or whether the changes in TJ are the causal factor that impair barrier integrity leading to a pro-inflammatory response [46]. Some *in vitro* studies have demonstrated that polyphenols can also modulate the transporter function and the TJ in the intestinal epithelial cells [47]. The most studied TJ proteins include claudin-1, claudin-2, zonula occludens, and occludin.

In contrast to other claudins, claudin-2 is a transmembrane pore-forming TJ protein predominantly expressed in leaky gut epithelium and permeable to small cations and water [6]. Its expression in colon tissue was increased by more than 2-fold in the colitis group compared to control group (Figure 3A, $p<0.05$). GPP and both free (EP) and bound polyphenols (NEP-F) were able to reduce this marker of barrier permeability ($p<0.05$), while the fraction composed only by fiber (F) had no effect (Figure 3A). The down-regulation of claudin-2 may be in part due the anti-inflammatory effect of polyphenols, since some pro-inflammatory cytokines such TNF-α and IL-6 have been shown to be involved in the up-regulation of claudin-2 expression and increase of the intestinal permeability [48,49]. However, the attenuation of inflammatory response does not ensure the down-regulation of claudin-2, as the mesalamine therapy, which had anti-inflammatory effect, was not effective to ameliorate this marker of intestinal barrier. This result suggests that another mechanism may be involved in the down-regulation of claudin-2 by polyphenols.

Figure 3

Occludin is a transmembrane protein involved in the sealing of epithelial gut barrier. The mRNA expression of occludin was not affected by colitis (Figure 3B). However, dietary supplementation with GPP or F increased the expression of this tight junction in the colon tissue, indicating that grape insoluble fiber is the fraction responsible for this effect. Similar results have been reported for cocoa powder and cocoa fiber (poor in polyphenols), which are mostly composed by insoluble dietary fiber, and were more effective than inulin (a soluble and highly fermentable fiber) to increase occludin expression in the colon tissue [50]. Since occludin is downregulated in the mucosal tissue of UC patients and its decrease facilitates the

migration of neutrophils [51], we propose that the increased expression of occludin triggered by GPP may help to control the inflammatory process and protect the colonic mucosa.

ZO-1 is an anchoring intracellular protein that binds occludin and claudins to the cytoskeletal actin and maintains the epithelium tightly sealed [46]. ZO-1 mRNA expression was significantly declined with colitis development (Figure 3C, $p<0.05$). However, mesalamine treatment and all experimental diets were effective not only in recovering ZO-1 expression ($p<0.05$ compared to colitis group) but GPP and F fraction also improved the expression of this protein above control values (Figure 3C, $p<0.05$). Similarly, Bibi *et al.* [52] observed that the supplementation with red raspberries powder (composed mostly by insoluble fiber) increased the protein levels of ZO-1 in the colonic tissue of mice with UC.

Matrix metalloproteinases (MMPs) are endopeptidases involved in the control of homeostasis of all extracellular matrix proteins and are able to digest components of the extracellular matrix such as collagen, fibronectin, and laminins [3,53]. Increased expression of MMP-9 has been found in colonic epithelial cells and mediates the inflammation and cell permeability in colitis [54]. The expression of MMP-9 mRNA was greatly enhanced in the colitis group and all experimental diets were able to decrease in MMP-9 mRNA expression (Figure 3D, $p<0.05$). Dietary supplementation with F fraction, however, caused only a partial decrease in MMP-9 expression indicating that the soluble and fiber-bound polyphenols of grape are more effective than the fiber fraction to reduce MMP-9 expression.

In agreement with our findings, UC has been shown to be characterized by an increase of pore-forming TJ proteins such as claudin-2, associated with a decrease and/or redistribution of sealing TJ proteins, such as occludin and ZO-1 [6]. The up-regulation of occludin and ZO-1 and the down-regulation of claudin-2 triggered by polyphenols in the colon tissue seems to involve several signaling pathways, including the reduction in phosphorylation of mitogen-activated protein kinases (MAPK), the inhibition of phosphoinositide-3-kinases (PI3K),

down-regulation of protein kinase C (PKC), tyrosine kinase (TK), and activation of AMP-activated protein kinase (AMPK) [4]. In contrast, the benefit of dietary fiber could be mediated by the metabolites produced during its colonic fermentation rather than by a direct effect. In addition, we cannot despise the effect of polyphenols and dietary fiber on the activity and composition of colon microbiota.

Gut microbiota may be altered in UC and fecal SCFA concentrations can be used as a functional biomarker of changes in microbial metabolism and microbiota composition [10]. SCFAs are believed to be responsible for the improvement in intestinal homeostasis observed after the consumption of dietary fiber and that this effect is mediated by microbiota activity and immune system [21,55]. Since SCFAs are the major energy source for colonocytes, it is reasonable to assume that SCFA deficiency is detrimental to the viability of colonocytes [20].

Six SCFA (acetic, propionic, butyric, valeric acid, isobutyric, and isovaleric) were identified and quantified in the fecal samples (Figures 4 and 5). Acetic acid was quantitatively the major SCFA found in cecal feces (about 40% of total SCFAs), followed by propionic acid (about 25%), butyric acid (about 20%), and valeric acid (about 5%) (Figure 4). Branched-chain fatty acids represented about 10% of total volatile fatty acids (Figure 5).

As can be seen in the figure 4A, the production of all SCFA in cecal feces was reduced (about 30%) by colitis development. The addition of 8% of GPP or NEP-F fraction completely reversed this decrease, whereas mesalamine treatment and the other grape fractions caused only a partial reversal (Figure 4A, $p<0.05$). These findings suggest that the fiber-bound polyphenols are more effective to prevent changes in the metabolism of gut microbiota.

Figure 4

Pectin, cellulose, and xyloglucan-hemicellulose have been shown to be the main components of grape pericarp cell wall [56] and can serve as substrate for microbiota fermentation in colon to produce metabolites such as SCFA. In contrast, grape proanthocyanidins have been shown to inhibit the microbial metabolism and reduce the formation of SCFA *in vitro*, whereas proanthocyanidins associated to the fruit matrix did not exhibit this inhibitory effect [57]. This finding may explain why the fraction containing free phenolics (EP group) promoted only a slight increase in total SCFA and BCFA production compared to colitis group. Similar effect was observed for the F fraction and the type of dietary fiber may help to explain this finding. The high proportion of insoluble fiber, a less fermentable type of dietary fiber [41], in F fraction and the lower proportion of insoluble:soluble fiber present GPP and NEP-F fractions presumably were the responsible for these differences.

Colitis induced a decrease in the production of acetic (Figure 4B), propionic (Figure 4C) and valeric acid (Figure 4E), which were completely recovered by the experimental diets and mesalamine treatment. Butyrate content was also decreased in the feces of colitic rats (Figure 4C) and this effect was reversed by mesalamine, GPP, EP, and NEP-F but not by the F diet (Figure 4C; $p<0.05$). Besides reversing the deficit in the production of acetic acid, the inclusion of GPP in the diet of colitic rats promoted an increase by more than 30% in the production of acetic acid and 20% in the production of butyric acid when compared with control group. Among the SCFA produced during colonic fermentation butyrate has received considerable attention due to its influence on the inflammatory response [20]. Interestingly, GPP contains the major content of soluble fiber compared to the other groups. The fraction composed only by fiber (F) ameliorated the production of SCFA yielding values similar to control group; however, this values did not differ from the colitis group. No significant differences were observed in the fecal pH among groups (Figure 4F), despite the changes in the levels of SCFA.

Since UC patients usually have altered microbiota and lower production of SCFA [58] the improvement in SCFA production is believed to play beneficial effects for UC treatment [22]. The beneficial role of butyrate is particularly relevant as it is the main source of energy for colonocytes, it improves the barrier function and the expression of TJ, and it promotes the immune response and increases the production of mucus [22,59]. Our findings showed that GPP, EP, and NEP-F fractions improved butyrate concentration in cecal feces and ameliorated the barrier function assessed by the expression of TJ proteins. Although F fraction did not enhance fecal butyrate levels, this fraction showed remarkably positive effects in the barrier function. Thus, different mechanisms appear to underline the repair of lesions and maintenance of epithelial barrier integrity triggered by the different grape bioactive fractions.

The two major mechanisms of signaling involving SCFA are the inhibition of histone deacetylases (HDACs) and the activation of G-protein-coupled receptors (GPCRs) [22]. Moreover, SCFAs increase the number of colonic Treg cells by stimulating their proliferation and these Treg cells produce large amount of IL-10, a cytokine that plays a key role in the suppression of intestinal inflammation [60].

Moreover, some of the transporters involved in SCFA absorption are also involved in the phenolic bioavailability and then SCFA could contribute to increase the bioavailability of phenolic compounds derived from microbiota metabolism. Van Rymenant *et al.* [61] demonstrated that chronic exposure of Caco-2 cells to butyrate increases the uptake of ferulic acid into the cells and decrease the efflux of this phenolic to the lumen through the regulation of transporter expression. Ferulic acid is one of hydrolysable phenolic acids covalently bound to the indigestible cell wall polysaccharides, but can be also derived from the metabolism of caffeic acid in the colon. Ferulic acid becomes bioaccessible when released from the food matrix by esterase activity of the intestinal microbiota [19,61].

Our findings suggest that the type of dietary fiber (soluble vs. insoluble fiber), besides the proportion between them, may provide different effect in gut microbial metabolism and colon inflammation injury. However, there are conflicting literature data on the relationship between the type of fiber and the improvement of colitis symptoms. Ritchie *et al.* [10] observed that insoluble fiber of sorghum bran with reduced fermentability was beneficial against the injury induced by colitis. Jiminez *et al.* [59] demonstrated that wheat bran (composed mainly by insoluble fiber) was more effective than resistant starch (soluble and highly fermentable) to reduce infection and colonic inflammation in a bacteria-induced model of colitis. On the other hand, Singh *et al.* [62] observed that pectin was more effective than cellulose to ameliorate colitis development but inulin failed to protect against colitis induced by blocking IL-10 receptor.

Fecal levels of isobutyrate were decreased by colitis development (Figure 5A; $p<0.05$), whereas isovalerate levels remained unchanged (Figure 5B). Mesalamine and all experimental diets prevented the decrease in isobutyrate levels but GPP caused a further increase by approximately 15% in the production of this branched-chain fatty acid compared to control group.

Figure 5

Intestinal epithelial cells secrete high amount of proteins (such as mucins, antimicrobial peptides, and neuropeptides) and, under stress conditions, these cells are highly susceptible to unfolded protein response (UPR) to manage the endoplasmic reticulum (ER) stress [11]. Heat shock proteins, which play a key role in folding, refolding, translocation and degradation of intracellular proteins under normal and stress conditions, have been proposed to regulate the intestinal homeostasis and gut barrier function [1].

GRP78 (also known as BIP or HSP5) senses the presence of misfolded proteins and activates signaling pathways to control the ER stress [11]. Together with BIP/GRP78, GRP94 (a heat shock protein like HSP90, also known as HSP90B1) is the hallmark of the UPR response [12] and an essential chaperone for toll-like receptors (TLRs), especially for TLR4, which remains intracellularly unresponsive to bacterial stimuli in the absence of GRP94 [1].

Colitis induced a great increase in the mRNA expression of GRP94 (Figure 6A, $p<0.05$) and dietary supplementation with the EP or F fractions did not prevent this increase, while the treatment with mesalamine and the inclusion of GPP or NEP-F fraction reduced this effect. GRP78 mRNA expression had a similar pattern compared to GRP94, although changes in the expression of this chaperone among groups did not reach statistical significance (Figure 6B, $p>0.05$).

Figure 6

As part of the diet, polyphenols are associated to carbohydrates, proteins, and lipids forming the food matrix and they are not readily absorbed in the gastrointestinal tract [18]. Thus, it is plausible that the health benefits attributed to diets rich in phytochemicals are not due their individual components but rather due to synergistic effects among them [63]. So, is imperative to identify the dietary bioactive components that are exerting the beneficial effects and to understand the mechanisms that are involved in order to find an alternative and effective treatment based on the diet to alleviate the symptoms of ulcerative colitis.

4 Conclusion

We provided evidence that free grape polyphenols aggravated macroscopic colonic lesion despite some beneficial effects in the intestinal barrier function and SCFA production.

Although the macroscopic damage has not been ameliorated by dietary supplementation with 8% of grape peel powder, we noticed significant improvement of barrier function by recovering TJ, a great enhancement of the SCFA production and a decrease of unfolded protein response markers after GPP supplementation. Fiber fraction was effective for recovering TJ but had a small effect on the production of SCFA. Moreover, the polyphenols associated to dietary fiber appeared to play a remarkable protective effect as it was the sole fraction to reduce the area of colonic lesion and appeared to be the fraction responsible for reducing the activation of the UPR. Future studies are necessary to further elucidate the mechanisms responsible for the protective effects of these grape phytochemicals and understand their interactions to identify the most adequate type of polyphenol or fiber in the diet of patients with intestinal diseases associated to defects on barrier function.

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Table 1. Ingredients and composition of experimental diets.

	AIN-93G	GPP	EP	NEP-F	F
Ingredients					
Corn starch (g)	440.9	420.2	440.9	433.4	440.4
Casein (g)	156.6	156.6	156.6	156.6	156.6
Maltodextrin (g)	132.0	132.0	132.0	132.0	132.0
Sucrose (g)	100.0	57.8	68.3	100.0	100.0
Soybean oil (g)	70.0	70.0	70.0	70.0	70.0
Cellulose (g)	50.0	33.1	50.0	35.8	47.0
Mineral mix (g)	35.0	35.0	35.0	35.0	35.0
Vitamin mix (g)	10.0	10.0	10.0	10.0	10.0
L-cysteine (g)	3.0	3.0	3.0	3.0	3.0
Choline (g)	2.5	2.5	2.5	2.5	2.5
tert-butylhydroquinone (g)	0.014	0.014	0.014	0.014	0.014
Grape peel powder (g)		80.0			
EP extract (mL)			198.0		
NEP-F fraction (g)				21.7	
F fraction (g)					3.5
Total (g)	1000	1000	1000	1000	1000
Proximate composition and energetic value					
Ash [#]	2.9 ± 0.2	3.6 ± 0.1	3.9 ± 0.1	2.9 ± 0.1	2.9 ± 0.1
Protein [#]	15.2 ± 0.5	17.8 ± 1.5	17.9 ± 0.6	15.5 ± 0.5	14.8 ± 1.0
Fat [#]	14.5 ± 1.0	15.6 ± 0.2	15.9 ± 0.2	14.8 ± 1.0	14.5 ± 0.2
Total carbohydrates[&]	67.4	63.0	62.3	66.8	67.8
Energetic value (kcal/kg)*	3948.0	3928.7	3948.0	3943.3	3946.6
Dietary fiber (g%, dry weight) and phytochemical composition (mg%, dry weight)*					
Total dietary fiber	5.0	5.0	5.0	5.0	5.0
Insoluble fiber	5.0	4.3	5.0	4.7	5.0
Soluble fiber	-	0.7	-	0.3	<0.1
Extractable polyphenols	-	62.0	62.6	-	-
Bound proanthocyanidins	-	227.0	-	228.0	-

Results are presented as mean ± s.d. [#] g%, dry weight. [&] Calculated by difference between 100 and the sum of the other constituents. * Calculated based on the ingredient composition and the amount of ingredients added to the diet. GPP = grape peel powder; EP = extractable polyphenols-rich fraction; NEP-F = non-extractable polyphenols-rich fraction; F = polyphenols-poor, fiber-rich fraction.

Table 2. Criteria for the assessment of macroscopic colonic damage.

Score	Criteria
	Criteria relative to ulcers and hyperemia
0	Normal, no damage
1	Hyperemia, no ulcers
2	Ulcer with no significant inflammation
3	Ulcer with inflammation at one site
4	Two or more sites of ulceration/inflammation
5	Two or more major sites of ulceration and inflammation or one site of ulceration/inflammation, extending > 1 cm along the length of the colon
6 -10	If damage covers > 2 cm along the length of the colon, the score is increased by one, for each additional centimeter of involvement
	Criteria relative to the occurrence of adhesions
0	No adhesions
1	Mild adhesions, easily separable from other tissues
2	Severe adhesions

Table 3. Criteria for colon histological damage evaluation.

Inflammation severity		Inflammation extent		Crypt damage		Involvement, %	
0	None	0	None	0	None	0	0 %
1	Mild	1	Mucosa	1	Basal 1/3 damaged	x1	1 – 25 %
2	Moderate	2	Mucosa and submucosa	2	Basal 2/3 damaged	x2	26 –50 %
3	Severe	3	Transmural	3	Crypts lost; surface epithelium present	x3	51 –75 %
				4	Crypts and surface epithelium lost	x4	> 75 %

Score = (inflammation severity x % involvement) + (inflammation extent x % involvement) + (crypt damage x % involvement).

Table 4. Primers sequences used for real-time polymerase chain reaction (qRT-PCR).

Genes	Primer sequence (5' to 3')	
	Forward	Reverse
Cldn2	GCCTCTAATCCCTCATTTCACA	CCCTTCGAGAAAGAACAGCTC
Ocln	GTTGTCTCTGTTGATCTGAAGTG	CAGACCACTATGAAACCGACT
Zo1	ACAGGTAGGACAGACGATCA	TCAGCAGCAACAGAACCAG
Mmp9	GGAGGTCATAGGTCACGTAGG	GAACTCACACAACGTCTTTCAC
Grp94	ACAAGGTATTTGGACCGCAGA	CTTGCCCGTCTGGTATGCTT
Grp78	GGTGGTGGAACCTTCGATGT	ATTTCTTCAGGGGTCAGGCG
Gapdh	AGTGCCAGCCTCGTCTCATA	GATGGTGATGGGTTTCCCGT

Cldn2 = claudin-2; *Ocln* = occludin; *Zo1* = zonula occludens-1; *Mmp9* = matrix metalloproteinase 9; *Grp94* = glucose-regulated protein 94; *Grp78* = glucose-regulated protein 78; *Gapdh* = glyceraldehyde 3-phosphate dehydrogenase.

Figure captions

Figure 1. Daily fecal production before colitis induction (A), cecum plus cecal content weight (B), area of colonic lesion (C), and macroscopic damage score (D) in rats with ulcerative colitis induced by TNBS (2,4,6-trinitrobenzenesulfonic acid). Results presented as mean ± standard error. # Different from control group ($p<0.05$); * Different from colitis group ($p<0.05$). GPP = grape peel powder; EP = extractable polyphenols-rich fraction; NEP-F = non-extractable polyphenols-rich fraction; F = polyphenols-poor, fiber-rich fraction.

Figure 2. Histological evaluation of the colonic sections of rats with ulcerative colitis induced by TNBS (2,4,6-trinitrobenzenesulfonic acid). Results presented as mean ± standard error. C = control; CC = colitis; CM = mesalamine; GPP = grape peel powder; EP = extractable polyphenols-rich fraction; NEP-F = non-extractable polyphenols-rich fraction; F = polyphenols-poor, fiber-rich fraction.

Figure 3. Quantitative mRNA expression (qRT-PCR) of claudin-2 (A), occludin (B), zonula occludens-1 (ZO-1) (C), and matrix metalloproteinase 9 (MMP-9) (D) in colonic tissue of rats with ulcerative colitis induced by TBNS (2,4,6-trinitrobenzenesulfonic acid). Results presented as mean ± standard error. # Different from control group ($p<0.05$); * Different from colitis group ($p<0.05$). GPP = grape peel powder; EP = extractable polyphenols-rich fraction; NEP-F = non-extractable polyphenols-rich fraction; F = polyphenols-poor, fiber-rich fraction.

Figure 4. Short-chain fatty acids and pH in the cecal feces of rats with ulcerative colitis induced by TNBS (2,4,6-trinitrobenzenesulfonic acid). Results presented as mean ± standard error. # Different from control group ($p<0.05$); * Different from colitis group ($p<0.05$). GPP = grape peel powder; EP = extractable polyphenols-rich fraction; NEP-F = non-extractable polyphenols-rich fraction; F = polyphenols-poor, fiber-rich fraction.

Figure 5. Branched-chain fatty acids in cecal feces of rats with ulcerative colitis induced by TNBS (2,4,6-trinitrobenzenesulfonic acid). Results presented as mean ± standard error. # Different from control group ($p<0.05$); * Different from colitis group ($p<0.05$). GPP = grape peel powder; EP = extractable polyphenols-rich fraction; NEP-F = non-extractable polyphenols-rich fraction; F = polyphenols-poor, fiber-rich fraction.

Figure 6. Quantitative expression (qRT-PCR) of unfolded protein response markers in the colonic tissue of rats with ulcerative colitis induced by TNBS (2,4,6-trinitrobenzenesulfonic acid). Results presented as mean ± standard error. # Different from control group ($p<0.05$). GPP = grape peel powder; EP = extractable polyphenols-rich fraction; NEP-F = non-extractable polyphenols-rich fraction; F = polyphenols-poor, fiber-rich fraction.

Figure 1

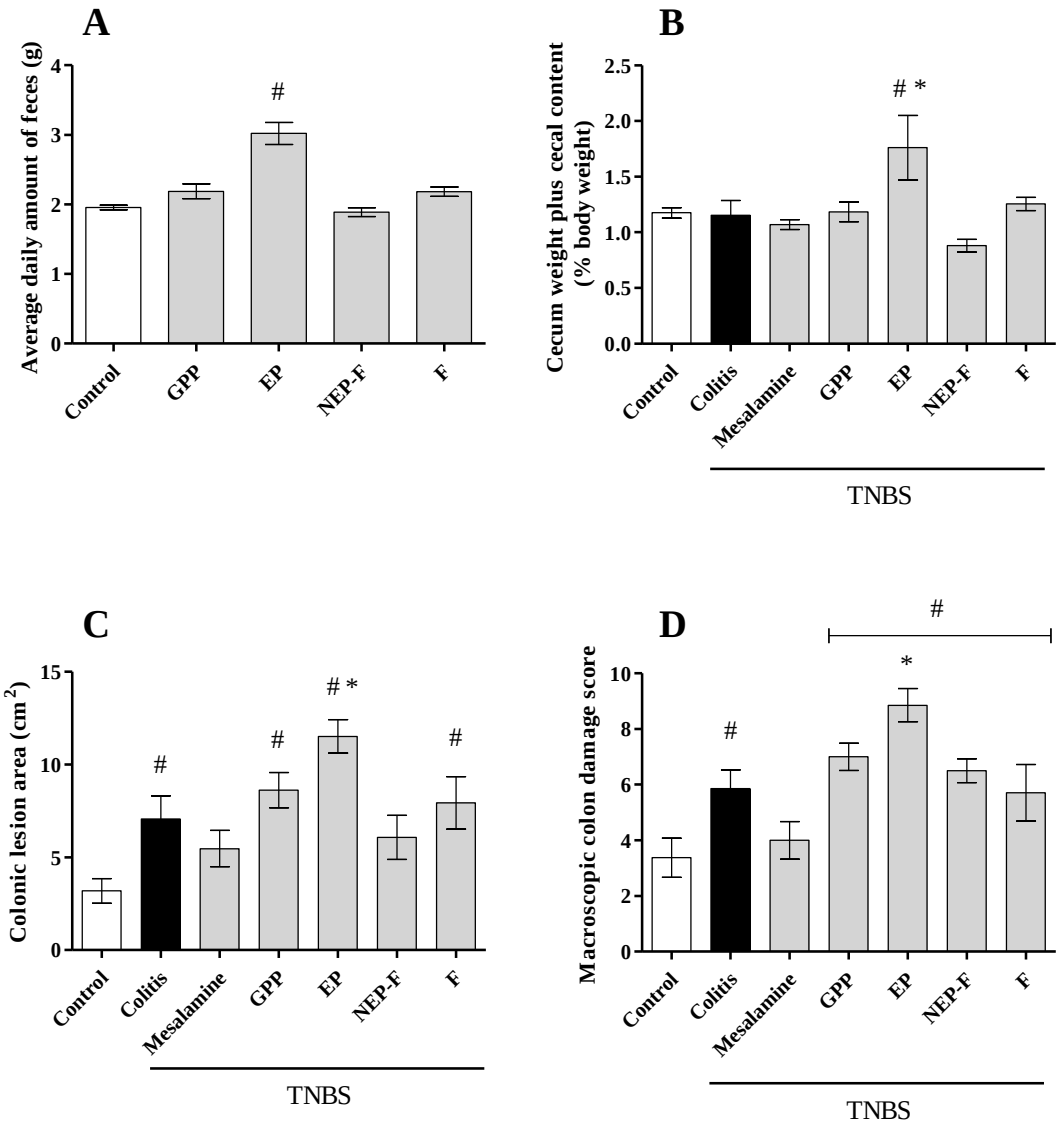
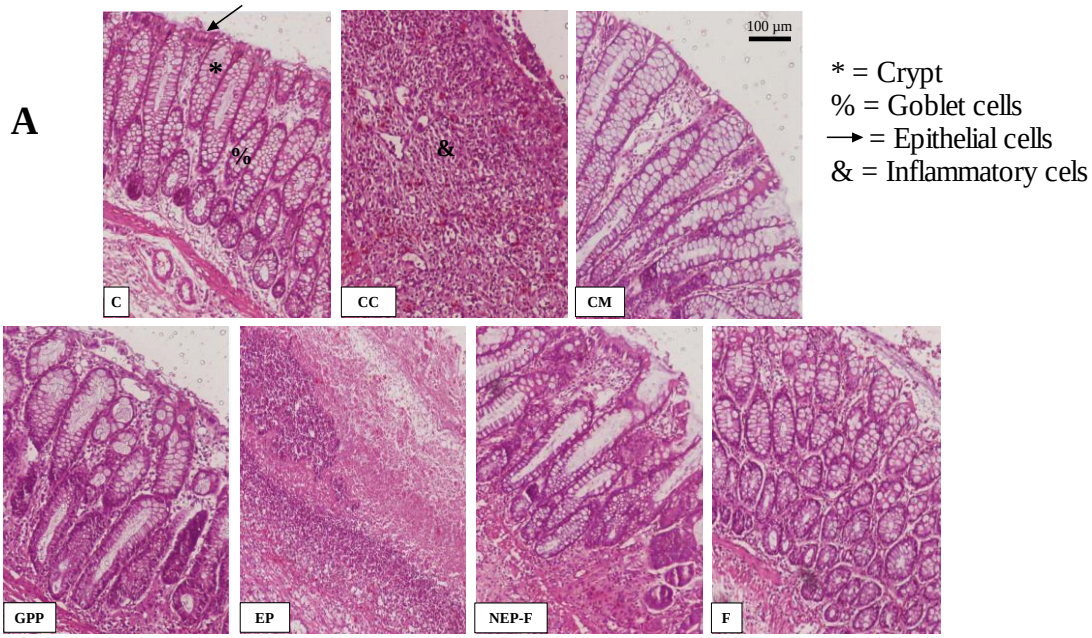

A
Average daily amount of feces (g)
0
1
2
3
4
#
Control
GPP
EP
NEP-F
F
B
Cecum weight plus cecal content
(% body weight)
0.0
0.5
1.0
1.5
2.0
2.5
*
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS
C
Colonic lesion area (cm 2)
0
5
10
15
#
#
*
#
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS
D
Macroscopic colon damage score
0
2
4
6
8
10
#
#
*
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS

Figure 2


A
*
%
&
100 µm
C
CC
CM
GPP
EP
NEP-F
F
* = Crypt
% = Goblet cells
→ = Epithelial cells
& = Inflammatory cels

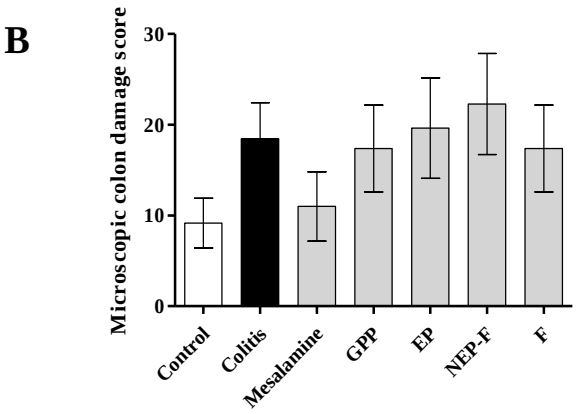

B
Microscopic colon damage score
30
20
10
0
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F

Figure 3

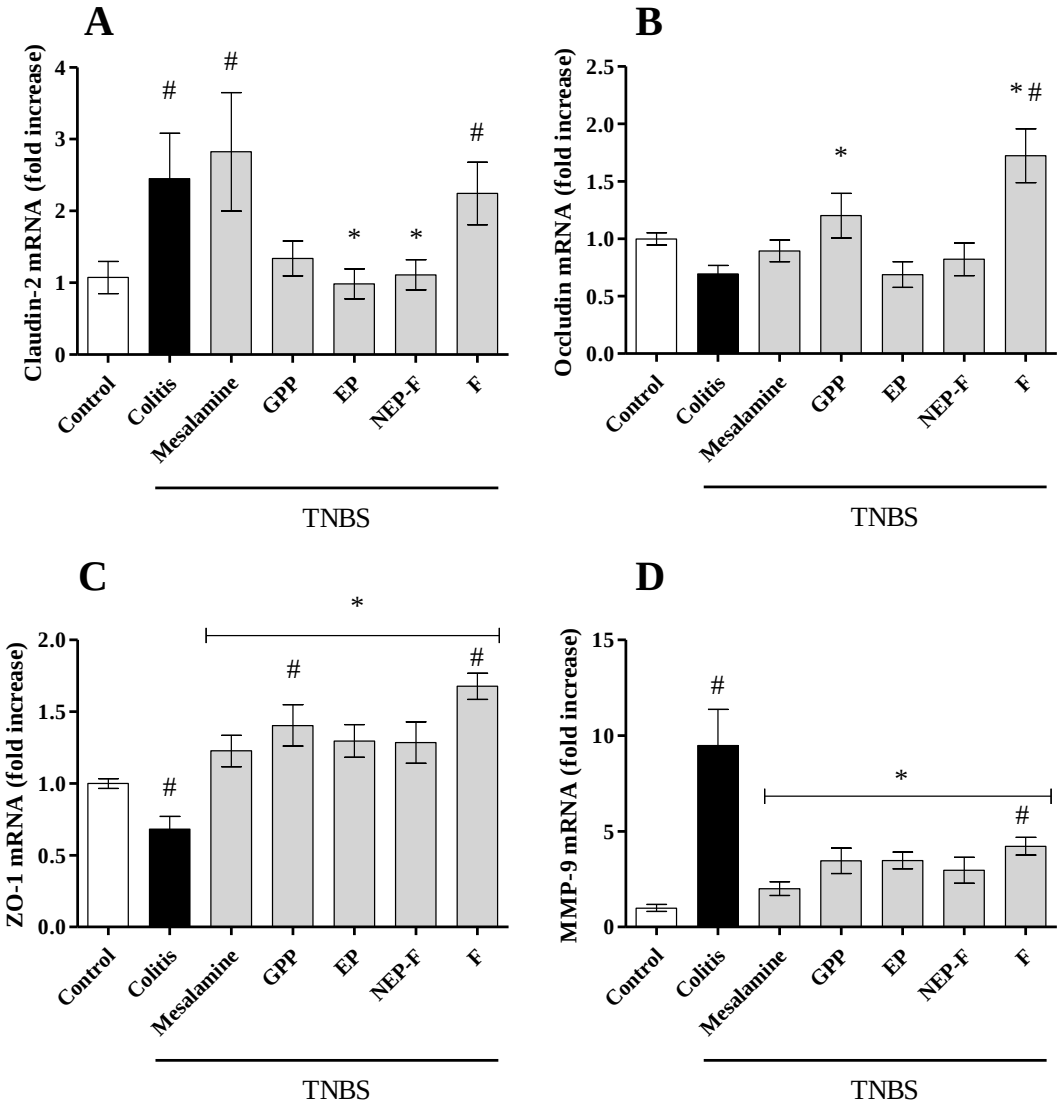

A
Claudin-2 mRNA (fold increase)
0
1
2
3
4
#
#
#
*
*
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS
B
Occludin mRNA (fold increase)
0.0
0.5
1.0
1.5
2.0
2.5
*
* #
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS
C
ZO-1 mRNA (fold increase)
0.0
0.5
1.0
1.5
2.0
*
#
#
#
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS
D
MMP-9 mRNA (fold increase)
0
5
10
15
#
*
#
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS

Figure 4

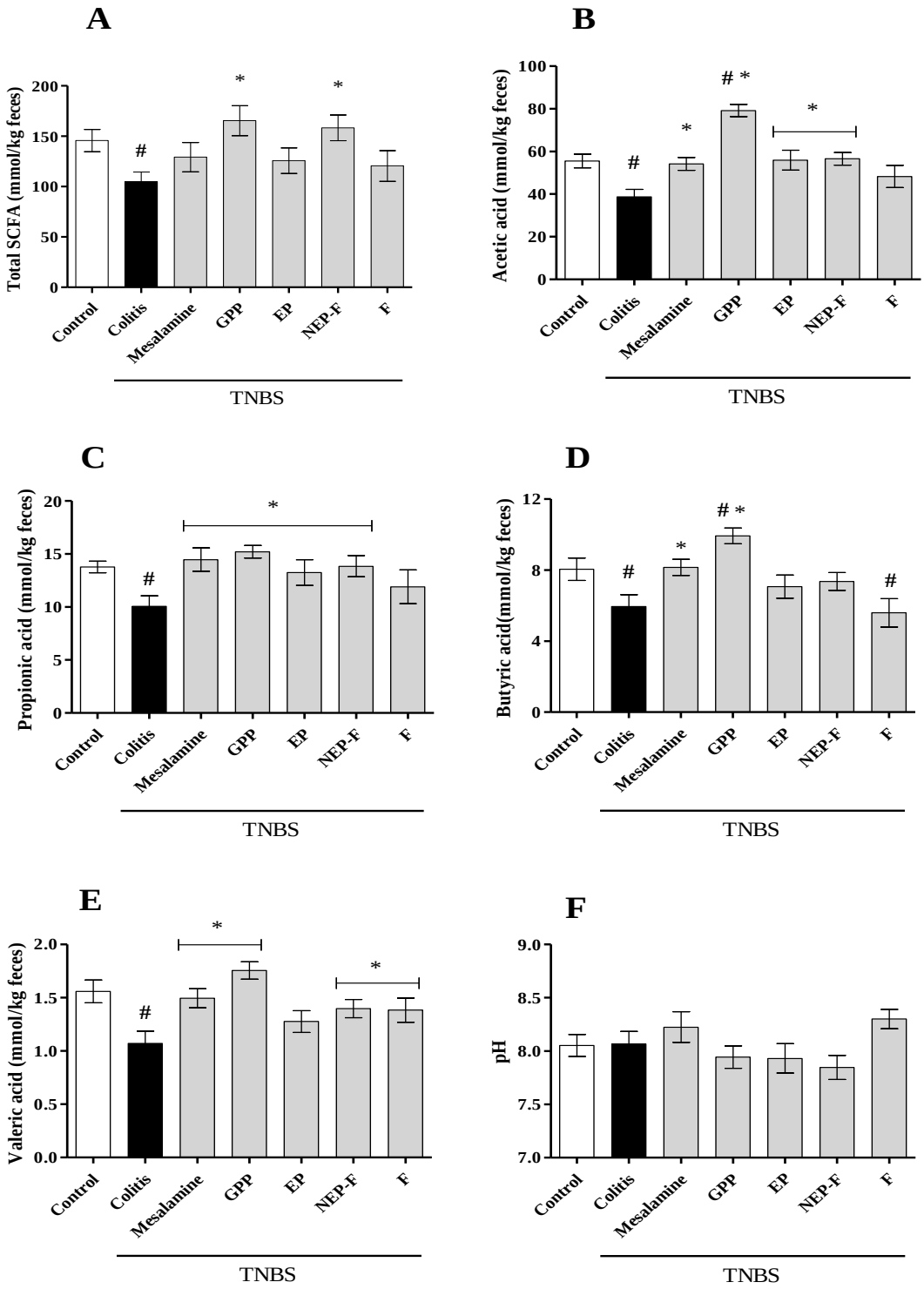

A
Total SCFA (mmol/kg feces)
200
150
100
50
0
#
*
*
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS
B
Acetic acid (mmol/kg feces)
100
80
60
40
20
0
#
*
*
*
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS
C
Propionic acid (mmol/kg feces)
20
15
10
5
0
#
*
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS
D
Butyric acid(mmol/kg feces)
12
8
4
0
#
*
*
#
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS
E
Valeric acid (mmol/kg feces)
2.0
1.5
1.0
0.5
0.0
#
*
*
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS
F
pH
9.0
8.5
8.0
7.5
7.0
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS

Figure 5

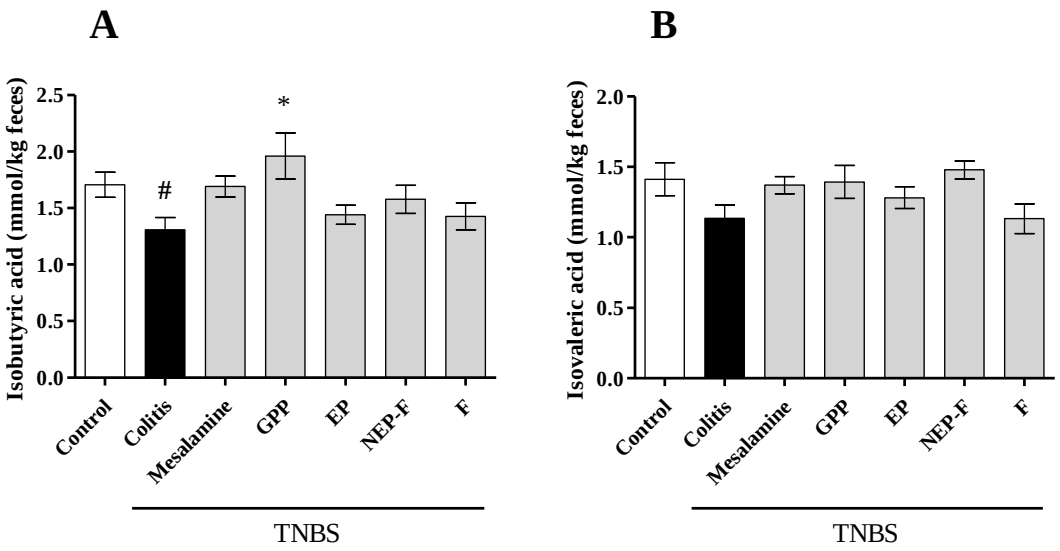
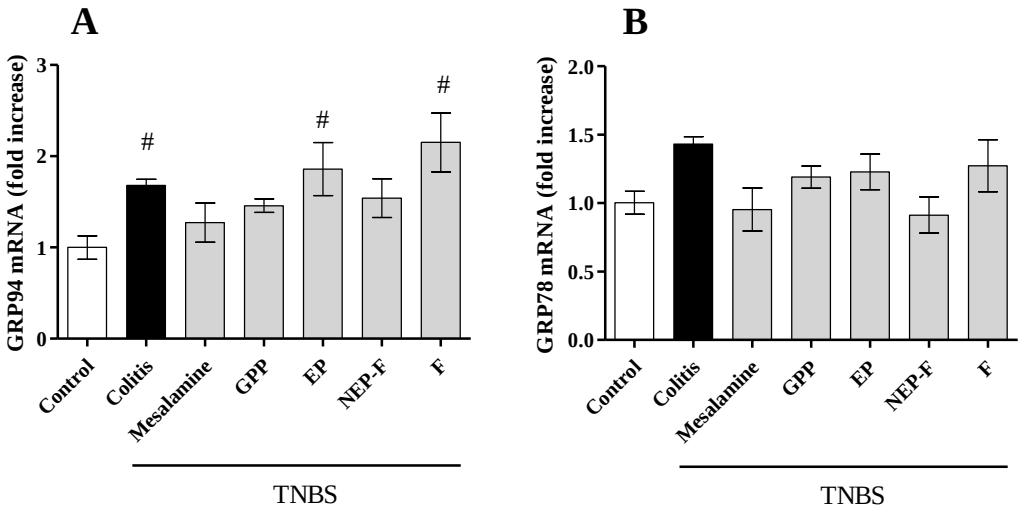

A
B
Isobutyric acid (mmol/kg feces)
Isovaleric acid (mmol/kg feces)
2.5
2.0
1.5
1.0
0.5
0.0
#
*
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS

Figure 6


A
GRP94 mRNA (fold increase)
3
2
1
0
#
#
#
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS
B
GRP78 mRNA (fold increase)
2.0
1.5
1.0
0.5
0.0
Control
Colitis
Mesalamine
GPP
EP
NEP-F
F
TNBS

5 DISCUSSÃO

Tem-se demonstrado que o uso da tecnologia de irradiação com luz UV-C em frutas e hortaliças é uma ferramenta útil na promoção da síntese de compostos fenólicos e que seu uso é seguro (DÜSMAN et al., 2014). No entanto, pouco se sabe sobre como a UV-C atua no metabolismo antioxidante vegetal e quais as recomendações de dose para cada espécie estudada ou por quanto tempo seu efeito é mantido. Em uvas, a aplicação de UV-C na pós-colheita tem sido estudada com o objetivo principal de induzir a síntese de resveratrol (GUERRERO et al., 2015; TANG et al., 2010), porém se desconhecia seu efeito sobre o metabolismo antioxidante enzimático.

Quando aplicada na pós-colheita de uvas 'Isabel', a UV-C foi eficaz no aumento de compostos fenólicos, especialmente de antocianinas (Artigo 1). Seu efeito hormético, isto é, a capacidade de causar efeito benéfico usando baixas doses de um agente estressor, foi claramente demonstrado, uma vez que a maior dose estudada (4 kJ/m^2) não teve efeito ou mesmo reduziu os níveis desses compostos. Apesar dos níveis de antocianinas monoméricas terem aumentado, o perfil se manteve, ou seja, não foi observada síntese de nenhuma antocianina diferente das já existentes e a proporção entre elas manteve-se.

O metabolismo antioxidante enzimático foi ativado logo após a irradiação com UV-C, com a indução da atividade das enzimas superóxido dismutase (SOD), catalase (CAT), guaiacol peroxidase (G-POD) e glutationa redutase (GR). Todas estas enzimas estão envolvidas na remoção de espécies reativas e isto demonstra que a UV-C levou à indução de estresse. Além disso, observou-se um importante papel do sistema glutationa, através da atividade da enzima GR para restituição dos níveis de tiois e redução do estresse gerado (Artigo 1).

O uso de UV-C também aumentou a capacidade antioxidante não-enzimática, inclusive no que se refere à remoção de radicais livres biologicamente relevantes, como demonstrado nos resultados de capacidade de remoção de radical peroxil e radical superóxido (Artigo 1).

A dose de 1 kJ/m^2 foi considerada a dose hormética, por ter induzido a síntese de compostos fenólicos e ter ativado de forma balanceada a atividade das enzimas SOD e CAT. Já a dose de 4 kJ/m^2 foi considerada ineficaz nas condições estudadas para esta cultivar, pois o estresse gerado por essa dose de UV-C não promoveu uma resposta simultânea das enzimas CAT e SOD para remoção das espécies reativas, que provavelmente se acumularam a ponto

de consumir os compostos antioxidantes sintetizados, culminando numa resposta similar a das uvas não irradiadas.

A casca de uva é rica em compostos fenólicos e antocianinas, aos quais é atribuída a capacidade antioxidante observada no artigo 1. A caracterização completa de fenólicos da casca de uva ‘Isabel’ (manuscrito 1) mostrou o predomínio de antocianinas (cerca de 80%) na sua composição. A antocianina majoritária foi a malvidina-3-glicosídeo, que representou mais de 50% do total de antocianinas. Dentre os compostos fenólicos não-antociânicos, o ácido caftárico (um derivado do ácido cafeico associado a uma molécula de ácido tartárico), foi o principal ácido fenólico encontrado, enquanto quercetina-3-glicosídeo e quercetina-3-glicuronídeo foram os flavonois mais abundantes. Além disso, as cascas de uva ‘Isabel’ apresentaram um teor de polifenois ligados de 2,5% e um teor de fibra alimentar (manuscrito 1) de mais de 25% em peso seco, numa proporção de fibra solúvel:fibra insolúvel de cerca de 1:3.

Uma vez que a literatura tem demonstrado que uma fração significativa de compostos fenólicos permanece ligada à matriz alimentar e é subestimada tanto em estudos quantitativos quanto em estudos que avaliam seus efeitos biológicos (PÉREZ-JIMÉNEZ; DÍAZ-RUBIO; SAURA-CALIXTO, 2013; QUIRÓS-SAUCEDA et al., 2014), o resíduo oriundo da extração dos compostos fenólicos extraíveis foi avaliado quanto à sua composição. Observou-se o predomínio de fibra alimentar nessa fração (mais de 77%) e redução da proporção fibra solúvel:fibra insolúvel para 1:4. Além disso, quando submetido a um processo de hidrólise em meio ácido e com uso de alta temperatura (que promove a liberação de compostos fenólicos ligados à matriz) observou-se que esse resíduo possui alto conteúdo de proantocianidinas (7,7%) (manuscrito 1).

Sabendo que, tanto os compostos fenólicos, quanto a fibra são pouco absorvidos ao longo do trato gastrointestinal e que chegam praticamente intactos no cólon para então serem metabolizados (CARDONA et al., 2013), o estudo do seu efeito em doenças intestinais parece oportuno. Esses compostos e seus metabólitos possuem atividade anti-inflamatória comprovada (KIM et al., 2017; TAN et al., 2014) e, nesse sentido, sua incorporação na dieta como forma de prevenir o desenvolvimento de doenças inflamatórias intestinais, como a colite ulcerativa, poderia ser uma alternativa.

O pó de casca de uva foi incorporado na dieta de ratos Wistar numa proporção de 8% e as demais frações (extrato contendo os fenólicos livres, fenólicos associados à fibra e fibra) foram adicionadas em quantidades equivalentes àquelas em que estavam presentes no pó de casca. Durante os 15 dias de consumo das dietas experimentais prévios à indução da colite,

não foram observados sinais como redução de ingesta, perda de peso ou diarreia (manuscrito 1). Apenas o grupo que recebeu os fenólicos livres (grupo PE) apresentou maior quantidade de fezes excretadas por dia (manuscrito 3). Nos 7 dias subsequentes à indução da colite, os animais apresentaram diarreia, sangue nas fezes e perda de peso avaliados a fim de se mensurar o grau de atividade da doença (manuscrito 2). A ocorrência destes sintomas no grupo controle (que não recebeu TNBS) provavelmente foi decorrente da administração do veículo do TNBS (etanol 50%). No entanto, o grau de atividade da doença foi maior no grupo colite do que no grupo controle no 2° dia, e o grupo controle apresentou sinais de recuperação mais rapidamente que o grupo colite não tratado.

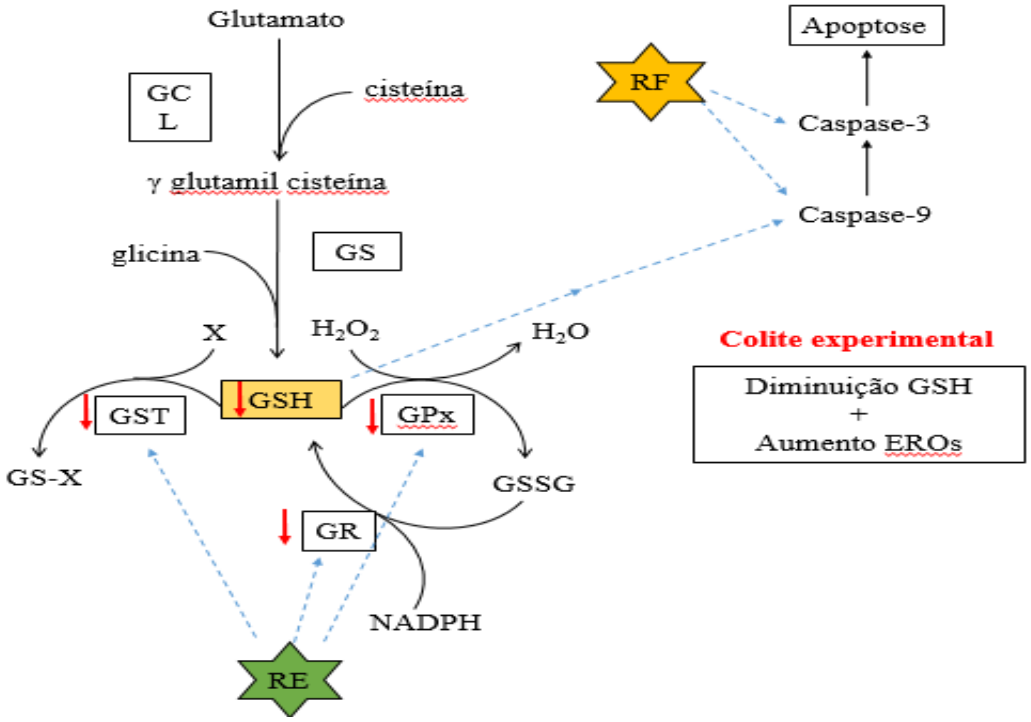
Após a indução da colite, todos os grupos apresentaram um decréscimo significativo no consumo, que só foi restabelecido a partir do 3° dia, com exceção do grupo PE, no qual a redução no consumo de ração foi mais persistente. Mesmo que tenha sido diminuído com a indução da colite, o consumo de fenólicos livres pareceu piorar o quadro da doença, uma vez que parâmetros como encurtamento do cólon, peso do baço (manuscrito 1), área de lesão e dano macroscópico (manuscrito 3) foram iguais ou mesmo superiores aos observados no grupo colite não tratado. O grupo que recebeu os compostos fenólicos extraíveis juntamente com restante da matriz do pó de casca (grupo PC) não apresentou tais sintomas, o que indica que o consumo de fenólicos não prontamente bioacessíveis parece ser benéfico quando já existe um quadro de doença instalado. Boussenna et al. (2016b) avaliaram o efeito da adição de diferentes doses de um extrato obtido a partir do bagaço de vinificação em um modelo de colite em ratos. As doses variaram entre 5 e 50 mg/dia/animal e o percentual de antocianinas correspondia a cerca de 20% do total de compostos fenólicos. Este estudo observou que apenas a menor dose teve efeito protetor, enquanto as demais doses foram ineficazes. De fato, o consumo de altas doses de fenólicos livres pode produzir efeitos pró-oxidantes, devido à estrutura catecol, que favorece a geração de ânion radical superóxido através da formação de quinonas (MURAKAMI, 2014).

Quando o tecido do cólon dos animais com colite foi avaliado microscopicamente, observou-se massiva infiltração de células inflamatórias e perda da estrutura característica composta pela monocamada de células epiteliais e pelas criptas e células produtoras de muco (manuscrito 3). Tais achados foram menos evidentes no grupo tratado com mesalazina e no grupo que recebeu apenas a fração fibra, no entanto não houve diferença significativa entre os tratamentos. A infiltração de células inflamatórias no tecido do cólon também foi confirmada pelo aumento da atividade da enzima mieloperoxidase, presente nos grânulos de neutrófilos

(manuscrito 1). A disfunção da barreira epitelial intestinal (manuscrito 3) observada através da redução da expressão da *tight junction* zônula occludens e do aumento da expressão da claudina-2 (formadora de poros) (Figura 6) pode ter facilitado a infiltração das células inflamatórias devido à maior permeabilidade intercelular.

A indução da colite levou à ocorrência de dano oxidativo, observado pelo aumento da peroxidação lipídica e da oxidação proteica, além da diminuição da atividade de enzimas antioxidantes SOD e CAT (manuscrito 1). Além disso, as atividades das enzimas glutationa redutase (GR), glutationa peroxidase (GPx) e glutationa-S-transferase, todas envolvidas no metabolismo da glutationa (Figura 3), foram significativamente reduzidas com a indução da colite, o que contribuiu para a redução significativa dos níveis de tiois totais observada tanto no soro quanto no cólon (manuscrito 2).

Figura 3 – Mecanismo de ação das frações do pó da casca de uva sobre o metabolismo da glutationa e o processo apoptótico em modelo de colite induzida por TNBS.



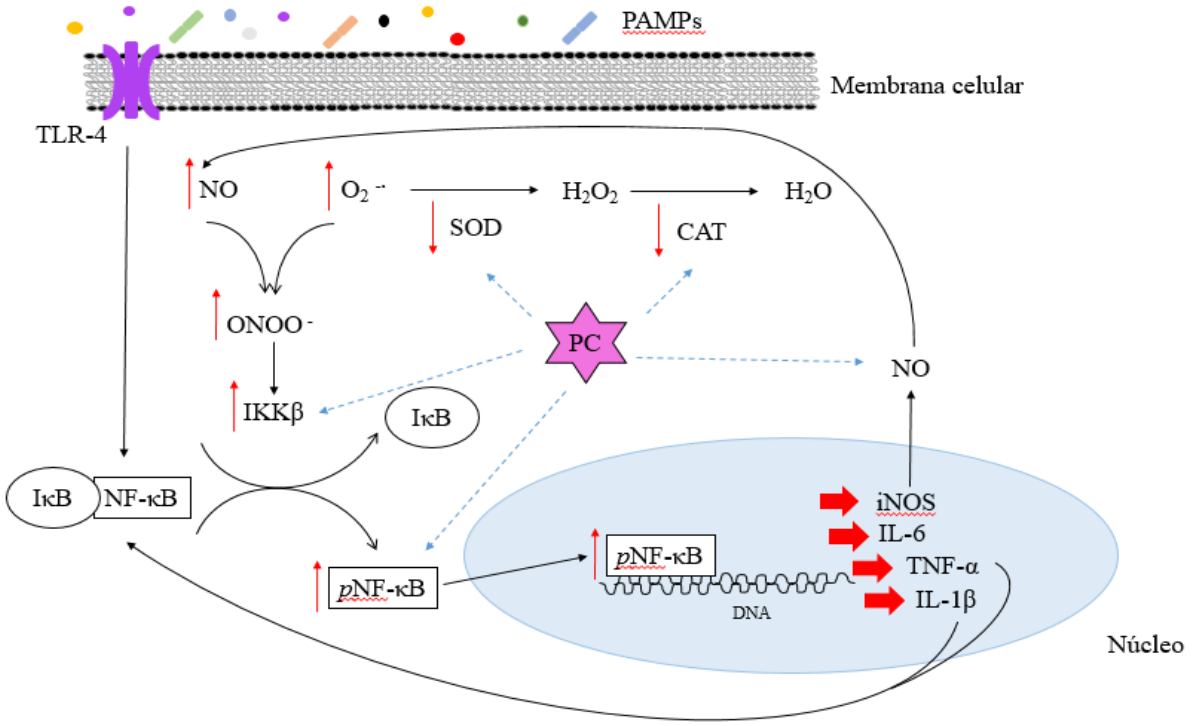
Fonte: autoria pessoal. GCL = glutamato cisteína ligase; GS = glutationa sintase; X = xenobiótico; GSH = glutationa; GST = glutationa S transferase; GPx = glutationa peroxidase; GSSG = glutationa oxidada; GR = glutationa redutase; GS-X = xenobiótico conjugado à glutationa; NADPH = nicotinamida adenina dinucleotídeo fosfato; RE = fração rica em fenólicos não-extraíveis; RF = fração rica em fibra e pobre em fenólicos; EROs = espécies reativas de oxigênio; H_2O_2 = peróxido de hidrogênio. As flechas vermelhas indicam as alterações observadas no modelo de colite experimental. As linhas tracejadas em azul indicam a ação das frações do pó da casca de uva.

Além disso, os níveis de óxido nítrico (NO), aumentaram significativamente em decorrência da colite. O excesso de NO e o acúmulo de radicais superóxido causado pela menor atividade da SOD contribuem para a formação do radical peroxinitrito ($ONOO^-$), que é um dos ativadores de uma das principais vias da resposta inflamatória, a via do NF-κB (Figura 4) (GOCHMAN; MAHAJNA; REZNICK, 2011). A ativação dessa via leva à expressão de genes que promovem a síntese de NO, citocinas pró-inflamatórias e perpetuam a resposta inflamatória.

Outro mecanismo de ativação da via do NF-κB é através dos receptores do tipo *toll* (TLRs), especialmente TLR-4, que é ativado por lipopolissacarídeos e teve aumento significativo na expressão proteica com a indução da colite (manuscrito 1). A expressão proteica da IKK-β, responsável por desligar o NF-κB da sua molécula inibidora, foi significativamente aumentada com a colite (manuscrito 1) (Figura 4). Apesar da expressão gênica do fator de transcrição NF-κB não ter sido alterada com a colite, a expressão proteica da sua forma ativa fosforilada aumentou 1,6 vezes comparada ao grupo controle e, de fato, a indução da colite levou a expressivo aumento nos níveis de citocinas pró-inflamatórias, como TNF-α, IL-6 e IL-1β (manuscrito 1).

Os resultados sugerem que a colite também induziu o início do processo apoptótico das células do cólon (evidenciada pelo aumento da expressão gênica da caspase 3) (manuscrito 2) e induziu a resposta citoprotetora do mal enovelamento de proteínas (*unfolded protein response*) (observada pelo aumento da expressão gênica da chaperona GRP94) (manuscrito 3). No entanto, como não foi avaliada a forma ativa da proteína caspase 3, a ativação da apoptose ainda necessita ser confirmada. A produção de ácidos graxos de cadeia curta (AGCC) foi significativamente reduzida (manuscrito 3) e houve aumento da expressão gênica da metaloproteinase de matriz-9 (MMP-9), uma enzima envolvida na remodelação tecidual.

Figura 4 - Ativação da via do NF-κB pelo ânion peroxinitrito, por receptores do tipo toll 4 e pelas citocinas inflamatórias em modelo de colite induzida por TNBS e provável mecanismo protetor do pó da casca de uva.



Fonte: autoria pessoal. NO = óxido nítrico; $O_2^{\cdot-}$ = ânion radical superóxido; SOD = superóxido dismutase; H_2O_2 = peróxido de hidrogênio; CAT = catalase; PC = pó de casca de uva; $ONOO^-$ = radical peroxinitrito; IKKβ = inibidor do fator nuclear kappa B quinase subunidade beta; IκB = inibidor do fator nuclear kappa B; NF-κB = fator nuclear kappa B; pNF-κB = fator nuclear kappa B fosforilado; iNOS = óxido nítrico sintase induzível; TLR-4 = receptor tipo toll 4; PAMPs = padrões moleculares associados a patógenos; IL-6 = interleucina 6; IL-1β = interleucina 1 beta; TNF-α = fator de necrose tumoral alfa. As flechas vermelhas indicam as alterações observadas no modelo de colite experimental. As linhas tracejadas em azul indicam a ação das frações do pó da casca de uva.

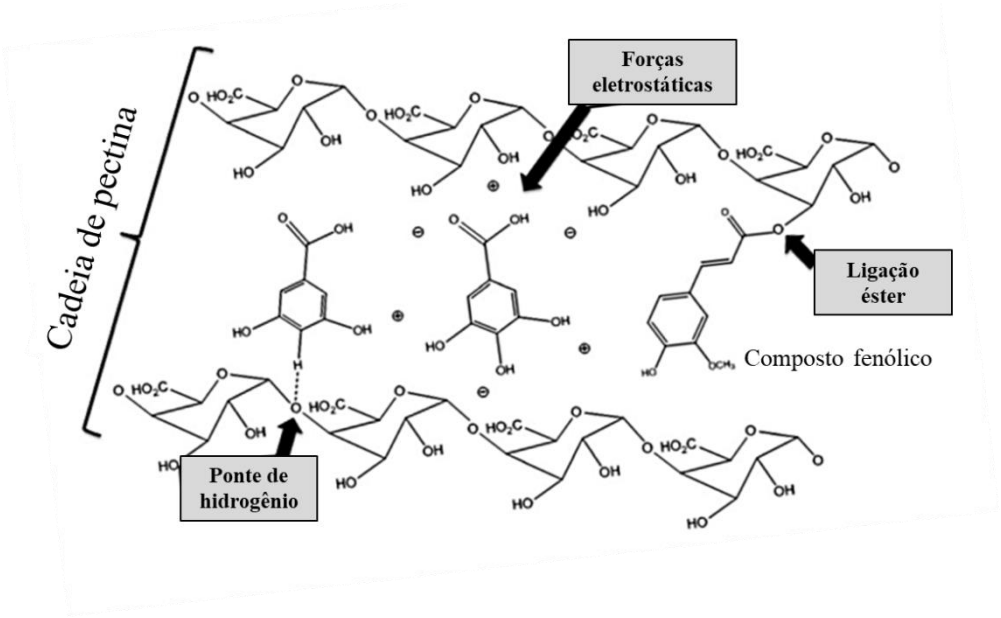
Apesar de não ter promovido redução da lesão macroscópica ou melhora significativa a nível microscópico, a incorporação de 8% de pó de casca de uva na dieta dos animais com colite promoveu diversos efeitos benéficos, tais como redução da resposta inflamatória (evidenciada pela redução da expressão gênica de TNF-α no cólon e redução dos níveis de TNF-α no soro e no tecido, redução da expressão proteica do *p*NF-κB e IKK-β), redução do dano oxidativo (aumento de tiois totais no soro e cólon, redução do níveis de NO, aumento da atividade das enzimas SOD e CAT), reestruturação da função de barreira intestinal (aumento da expressão gênica da *tight junction* oclusiva zônula ocludens e redução da *tight junction* formadora de poros claudina-2), diminuição da expressão gênica da caspase-3 e aumento da síntese de AGCC no ceco (manuscritos 1, 2 e 3). Alguns desses efeitos, tais como a restituição da atividade da CAT e aumento da síntese de ácido butírico e isobutírico, foram decorrentes da atuação sinergística dos compostos fenólicos livres, ligados e da fração fibra,

uma vez que isoladas essas frações não apresentaram efeito significativo. Aprikian et al. (2003) também relatam que o consumo de pectina e fenólicos de maçãs teve efeito mais pronunciado sobre a fermentação cecal quando consumidos juntos do que quando consumidos separadamente. Este resultado corrobora o aumento na produção de ácidos graxos de cadeia curta encontrado nas fezes cecais dos animais que receberam o pó da casca e a fração fibra associada aos compostos fenólicos (manuscrito 3) (Figura 6). A maior proporção de fibra solúvel encontrada no pó da casca pode ser a responsável pelo significativo aumento na síntese dos AGCC, especialmente acetato e butirato. Chen, Lin e Wang (2010) confirmam esta hipótese ao relatar que dietas contendo as fibras solúveis inulina ou pectina, mas não a dieta contendo celulose, foram capazes de aumentar as concentrações de AGCC nas fezes de camundongos comparadas a uma dieta sem fibra. O aumento observado nos níveis de acetato, foi quase o dobro dos níveis observados nos animais que não receberam fibra na dieta, similar ao observado para o grupo PC em relação ao grupo colite.

A ligação dos compostos fenólicos a macromoléculas como proteínas e polissacarídeos se dá por meio de ligações fracas e reversíveis (pontes de hidrogênio, interações hidrofóbicas) ou ligações covalentes do tipo éster (Figura 5) (LE BOURVELLEC; RENARD, 2012). Estas ligações retardam a biodisponibilidade dos compostos fenólicos, que ficam retidos no interior da matriz fibrosa. À medida que estas ligações vão sendo rompidas ao longo do trato gastrointestinal, a interação entre fenólicos e polissacarídeos diminui e os fenólicos tornam-se bioacessíveis, ou seja, disponíveis para serem ou não absorvidos (QUIRÓS-SAUCEDA et al., 2014).

Mesmo assim, fenólicos de alto peso molecular como os taninos condensados e hidrolisáveis não são absorvidos e chegam até o cólon, onde exercem, junto com a fibra alimentar, propriedades biológicas e funcionais, como capacidade antioxidante (QUIRÓS-SAUCEDA et al., 2014). Enzimas que degradam a parede celular vegetal, produzidas pelos microrganismos do cólon, são aptas a liberarem os fenólicos associados à fibra. Esse retardo na bioacessibilidade dos compostos fenólicos quando associados à matriz explica porque a fração contendo a fibra e os fenólicos ligados apresentou resultados protetores na colite e porque o pó da casca de uva não apresentou efeitos deletérios, como os apresentados pelo grupo que recebeu os compostos fenólicos em sua forma livre.

Figura 5 - Interação entre compostos fenólicos e fibra.



Fonte: adaptada de Quirós-Sauceda et al. (2014).

Além disso, a maior proporção entre fibra solúvel e fibra insolúvel no grupo PC também pode responder por parte dos resultados benéficos observados neste grupo. Tanto a fibra insolúvel quanto a solúvel são capazes de se associar com compostos fenólicos, no entanto a pectina demonstra maior afinidade de interação com taninos condensados do que a celulose (LE BOURVELLEC; BOUCHET; RENARD, 2005). Além disso, a pectina é mais facilmente fermentada que fibras insolúveis como a celulose (CHEN; LIN; WANG, 2010) e os metabólitos formados com sua fermentação, como os AGCC, apresentam propriedades anti-inflamatórias (SIVAPRAKASAM; PRASAD; SINGH, 2016).

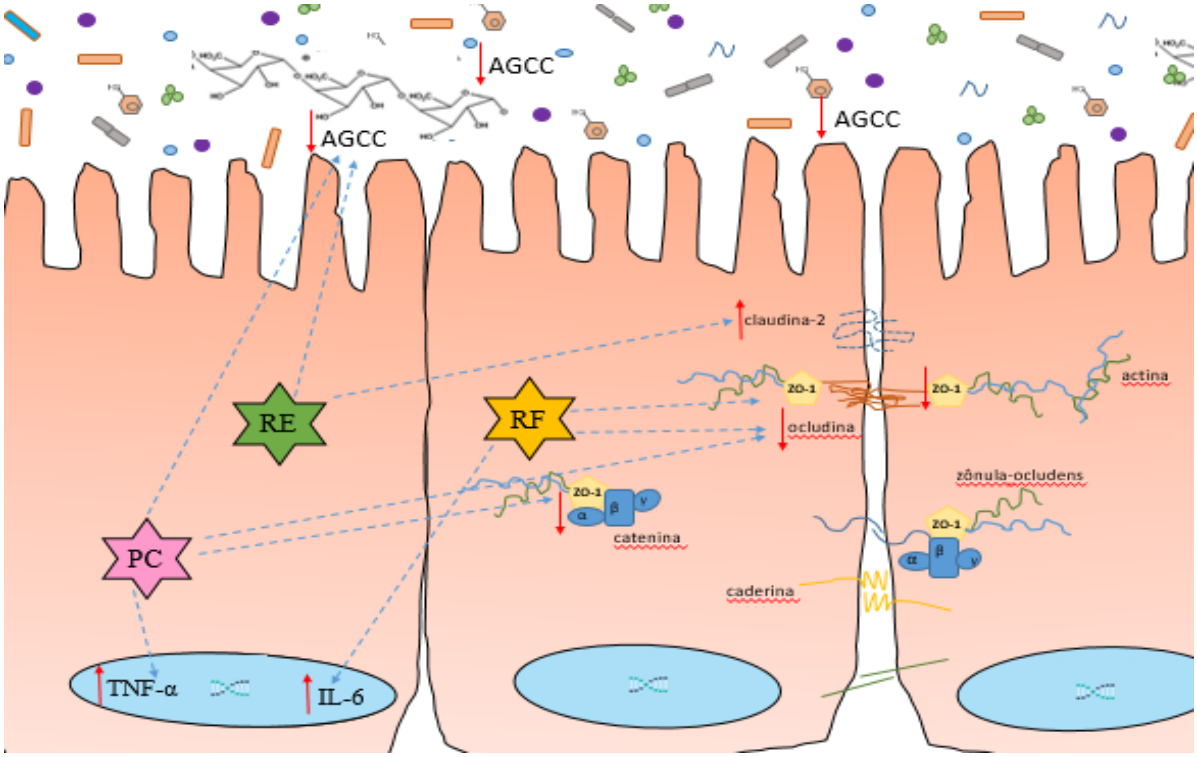
Mesmo tendo provocado efeitos adversos no que se refere a dano macroscópico, redução do ganho de peso, aumento do peso do ceco e baço e de não ter colaborado para redução do dano oxidativo ou inflamatório a nível sistêmico, o consumo de fenólicos livres (grupo PE) apresentou resultados benéficos no que se refere a melhora da barreira intestinal e redução parcial do dano oxidativo e inflamatório a nível tecidual. Isto indica que o efeito dos fenólicos livres seria mais restrito ao local, provavelmente pela capacidade desses compostos atuarem diretamente na remoção de radicais livres e por sua atividade antimicrobiana, ajudando, assim, a controlar a resposta inflamatória tecidual. Por estarem prontamente bioacessíveis, seu efeito seria imediato e de curta duração, ao contrário do observado para a fração de fenólicos associada a fibra (grupo RE), que, por ter metabolização mais lenta,

proporcionou resultados mais duradouros. A diminuição da expressão proteica do receptor TLR-4 causada pelo consumo da fração PE (manuscrito 1) contribui com a resposta anti-inflamatória observada no grupo PE e, de fato, tem sido mostrado que os flavonoides interagem com a microbiota e regulam a resposta inflamatória através da modulação da expressão gênica e proteica desses receptores (PÉREZ-CANO et al., 2014).

O efeito benéfico sobre a colite demonstrado pela fração contendo os fenólicos associados à fibra (resíduo RE) parece estar ligado especialmente à redução da resposta inflamatória, com a inibição da via do NF-κB através da redução da expressão proteica da enzima IKK-β e da expressão gênica da forma ativa fosforilada do NF-κB (Figura 4). Além disso, o efeito do pó da casca em diminuir as citocinas pró-inflamatórias TNF-α e IL-1β a nível sistêmico foi associado a essa fração, assim como apenas esta fração foi capaz de reduzir a infiltração de células inflamatórias no cólon (manuscrito 1).

Em compensação, os animais que receberam a fração fibra oriunda do pó de casca de uva apresentaram redução do dano oxidativo a nível sistêmico (evidenciado pela redução dos níveis de TBARS e AOPP no soro) e aumento da função de barreira intestinal com o aumento da expressão gênica das *tight junctions* no tecido do cólon (Figura 6), sem, no entanto, redução completa da resposta inflamatória e restituição da atividade das enzimas antioxidantes e dos níveis de tiois. É importante salientar que a redução da expressão gênica da caspase 3 observada no grupo PC foi atribuída não aos compostos fenólicos livres mas sim à fração fibra, já que tanto o grupo RE quanto o grupo RF apresentaram redução na expressão gênica das caspases 3 e 9 (Figura 3). Ainda que este resultado sugira atenuação da apoptose, este efeito precisa ser confirmado, visto que avaliou-se apenas a expressão gênica e não a forma ativa da caspase 3. De qualquer forma, existem evidências da literatura de que o consumo de bagaço de uva rico em fibra é capaz de reduzir a apoptose na mucosa do cólon de ratos (López-Oliva et al., 2010). A literatura atribui este feito aos AGCC produzidos com a fermentação da fibra, no entanto, ao contrário do que se esperava, não foi observado efeito dessa fração sobre a produção de AGCC nas fezes. Como esta fração apresentou baixo teor de fibra solúvel, que é mais facilmente fermentável, isso explicaria a ausência de efeito sobre a produção de AGCC.

Figura 6 – Provável mecanismo de ação do pó da casca de uva e suas frações bioativas sobre as junções de oclusão do epitélio intestinal do cólon, a produção de ácidos graxos de cadeia curta e o processo inflamatório em modelo de colite induzida por TNBS.



Fonte: autoria pessoal. ZO-1 = zônula-ocludens; TNF-α = fator de necrose tumoral alfa; IL-6 = interleucina 6; PC = pó da casca de uva; RE = fração rica em fenólicos não-extraíveis; RF = fração rica em fibra e pobre em fenólicos AGCC = ácidos graxos de cadeia curta. As flechas vermelhas indicam as alterações observadas no modelo de colite experimental. As linhas tracejadas em azul indicam a ação das frações do pó da casca de uva.

Uma vez que a quantidade de celulose adicionada na dieta deste grupo (RF) é bastante próxima à adicionada nos grupos controle e colite, o diferencial deste grupo está no percentual de fibra de casca de uva adicionado. É possível que as hidrólises ácidas utilizadas para obtenção dessa fração tenham modificado quimicamente a estrutura da fibra e criado sítios com propriedades antioxidantes que colaboraram em seus efeitos biológicos, no entanto um estudo mais aprofundado da composição e das características dessa fibra deve ser feito para afirmar tal fato.

A dose de compostos fenólicos livres utilizada neste estudo (40 mg/kg de peso corporal no ensaio com ratos) corresponde a uma dose de 4 mg/kg de peso corporal em humanos, considerando a diferença de superfície corporal entre ratos e humanos (10x). Considerando uma pessoa de 70 kg, essa dose corresponde a 280 mg de compostos fenólicos/dia. Em termos quantitativos, essa dose equivale ao consumo de 36 g de pó de casca de uva por dia, a cerca de 1,3 kg de uvas frescas ou a cerca de 1 taça de 300 mL de vinho

tinto. Contudo, no vinho os compostos fenólicos encontram-se sob a forma livre e dissolvidos numa solução alcoólica. Nesse caso, o consumo de vinho poderia, assim como observado no grupo que recebeu o extrato PE, prejudicar a inflamação intestinal em caso de DII já existente. Assim, pacientes com DII devem dar preferência ao consumo dos compostos fenólicos sob a forma de pó de casca de uva, como parte de uma dieta balanceada, para prevenção contra os danos decorrentes da inflamação intestinal.

6 CONCLUSÃO

Evidenciou-se que a UV-C é uma tecnologia promissora para promover o aumento do conteúdo de compostos bioativos em uvas 'Isabel' e essa resposta de defesa envolveu a ativação tanto do sistema antioxidante enzimático, quanto do sistema antioxidante não-enzimático. A dose hormética demonstrada neste estudo foi de 1 kJ/m^2 e o aumento da capacidade antioxidante foi observada já no dia seguinte à irradiação.

A indução da colite em ratos utilizando o modelo TNBS promoveu dano oxidativo lipídico e proteico, tanto a nível tecidual quanto sistêmico, alterações macro e microscópicas, ativação de vias relacionadas à inflamação, apoptose, resposta a proteínas mal enoveladas, além de redução da síntese de AGCC e redução da função de barreira epitelial intestinal. A incorporação de pó de casca de uva na dieta, promoveu diversos benefícios e demonstrou, na maior parte dos resultados, ter efeito protetor superior ao de suas frações isoladas.

A fração contendo a fibra e os compostos fenólicos a ela associados também teve efeito protetor sobre a colite, especialmente na redução da resposta inflamatória. Seu efeito foi superior ao dos fenólicos livres, que pioraram o quadro da doença em alguns marcadores e podem ter promovido efeitos pró-oxidantes. Esta diferença ocorreu provavelmente devido ao metabolismo mais lento e à liberação prolongada dos compostos fenólicos ligados em comparação com a alta oferta de fenólicos livres. A fração fibra reduziu marcadores de dano oxidativo sistêmico e teve importante papel no reequilíbrio da função de barreira intestinal, porém não teve efeito significativo sobre a produção de AGCC no ceco. Cabe salientar também o papel da fibra solúvel, que, presente em maiores proporções no pó da casca e na fração contendo os fenólicos ligados à fibra pareceu responder por boa parte dos resultados benéficos encontrados para esses dois grupos, juntamente com os fenólicos ligados, que não tiveram seu efeito avaliado de forma isolada neste trabalho.

Os resultados obtidos indicam, de maneira geral, que as frações bioativas presentes na casca da uva agem através de mecanismos diferentes sobre os danos causados pela inflamação intestinal e que seu consumo combinado é mais eficaz do que o consumo de fenólicos ou fibra de forma isolada. Isso ressalta para a importância do consumo de compostos bioativos em sua forma natural e alerta para o cuidado com o consumo de fenólicos isolados quando já existe um quadro de doença instalado.

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ANEXO A - APROVAÇÃO DO COMITÊ DE ÉTICA

UNICAMP

CEUA/UNICAMP

C E R T I F I C A D O

Certificamos que o projeto intitulado **"A casca de uva (Vitis labrusca cv. Isabel) como uma estratégia nutricional no tratamento da colite induzido por 2,4,6 trinitrobenzenosulfônico em ratos: identificação da fração bioativa"**, protocolo nº **3815-1**, sob a responsabilidade de **Prof. Dr. Mário Roberto Maróstica / Cinthia Baú Betim Cazarin**, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo *Chordata*, subfilo *Vertebrata* (exceto o homem) para fins de pesquisa científica ou ensino, encontra-se de acordo com os preceitos da **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais e do **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**, e com as normas editadas pelo **Conselho Nacional de Controle da Experimentação Animal - CONCEA**, e foi aprovado pela **Comissão de Ética no Uso de Animais** da **Universidade Estadual de Campinas - CEUA/UNICAMP,** em reunião de **11 de maio de 2015**.

Vigência do projeto: 08/2015-09/2015
Espécie/Linhagem: Rato heterogênico/HanUnib: WH (Wistar)
No. de animais: 58
Peso/Idade: 21 dias/40gr
Sexo: machos
Origem: CEMIB/UNICAMP

A aprovação pela CEUA/UNICAMP não dispensa autorização prévia junto ao **IBAMA, SISBIO** ou **CIBio**.

Campinas, **11 de maio de 2015**.

Prof. Dr. Alexandre Leite Rodrigues de Oliveira
Presidente

Fátima Alonso
Secretária Executiva