CARACTERIZACIÓN DE LAS FRACCIONES POLISACARÍDICA Y POLIFENÓLICA DE VINOS Y SU RELACIÓN CON PROPIEDADES ORGANOLÉPTICAS Y FARMACOLÓGICAS

CARACTERIZAÇÃO DAS FRAÇÕES POLISACARÍDICAS E POLIFENÓLICAS DE VINHOS E SUA RELAÇÃO COM PROPRIEDADES ORGANOLÉPTICAS E FARMACOLÓGICAS

CHARACTERIZATION OF THE POLYSACCHARIDE AND POLYPHENOLIC FRACTIONS OF WINES AND THEIR RELATIONSHIP WITH ORGANOLEPTIC AND PHARMACOLOGICAL PROPERTIES

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A minha família,

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RESUMEN

El vino es una de las bebidas más consumidas y económicamente más importantes, con una facturación de aproximadamente € 30 mil millones por año en el mercado debido a una producción mundial de 292 millones de hectolitros. Los enólogos buscan continuamente la producción de vinos de alta calidad, por lo tanto, hay mucho interés en la investigación dirigida a identificar los componentes del vino y comprender mejor su efecto durante la vinificación, lo que puede resultar de ayuda para el avance del sector del vino. Los polisacáridos y los compuestos fenólicos son compuestos altamente relacionados con la calidad del vino, debido a su impacto no sólo en sus propiedades organolépticas (color, sabor, astringencia) sino también en sus propiedades saludables. Por lo tanto, este trabajo tuvo como objetivo identificar, caracterizar y cuantificar los polisacáridos y compuestos fenólicos encontrados en los vinos y evaluar la interacción entre ellos. Además, pretende evaluar la similitud entre los polisacáridos extraídos de vinos comerciales de diferentes añadas y estudiar las propiedades antiinflamatorias de los polisacáridos *in vitro* en células RAW 264.7 estimuladas con LPS e *in vivo* por el modelo de septicemia inducida por cirugía de ligadura y perforación del ciego en ratones, así como la biodistribución de estos polisacáridos durante el proceso inflamatorio. Para este propósito, se eligieron vinos comerciales elaborados con uvas *Vitis vinífera* de diferentes variedades. Los polisacáridos fueron extraídos, caracterizados y cuantificados por GC-MS y RMN. Se identificó la presencia de manano, arabinogalactano tipo II y ramannogalactonano tipo I y II. También se ha demostrado que los vinos producidos a partir de la misma variedad de uva y de la misma bodega tienen patrones de polisacáridos similares, aunque hayan sido producidos en años diferentes. Los compuestos fenólicos fueron identificados y cuantificados por HPLC-DAD-MS. El análisis mostró una prevalencia del contenido de procianidinas (entre 58 y 275 mg / L) frente al de las prodelfinidinas (entre 4 y 8 mg / L). El análisis de antocianinas mostró la mayoría de los derivados de monoglucósidos y sus derivados acetilados. Los análisis estadísticos revelaron la existencia de relaciones entre la estructura de los polisacáridos y su capacidad para precipitar procianidinas, lo que explicaría la relación observada en los vinos entre la composición de polisacáridos y la concentración de flavanoles. Las fracciones de polisacárido inhibieron la producción de citoquinas inflamatorias (TNF-α e IL-1β) y mediador (NO) en células RAW 264.7. La administración subcutánea y oral de polisacáridos redujo la tasa de mortalidad de los ratones. El tratamiento redujo la migración de leucocitos, inhibió las citocinas proinflamatorias y aumentó la producción de la citocina antiinflamatoria IL-10. También redujeron los niveles plasmáticos de AST, ALT, bilirrubina, urea y creatinina, con la consiguiente protección contra el daño

tisular. Además, la biodistribución de polisacáridos por el cuerpo cuando se administra por vía subcutánea parece ocurrir a través del sistema inmune. Los polisacáridos han demostrado un potente efecto antiinflamatorio in vitro e in vivo, lo que puede indicar efectos beneficiosos del consumo moderado de vino en la salud humana.

Palabras clave: vinos, polisacáridos, compuestos fenólicos, RMN, HPLC, inflamación.

RESUMO

O vinho é uma das bebidas mais consumidas e de maior importância econômica, movimentando por ano aproximadamente 30 bilhões de euros no mercado decorrentes de uma produção mundial de 292 milhões de hectolitros. Os produtores de vinho buscam continuamente a produção de vinhos de alta qualidade; portanto, há muito interesse em pesquisas destinadas a identificar os componentes do vinho e a entender melhor seu efeito durante a vinificação, o que pode ser útil para o avanço do setor vitivinícola. Polissacarídeos e compostos fenólicos são compostos altamente relacionados à qualidade do vinho, devido ao seu impacto não apenas em suas propriedades organolépticas (cor, sabor, adstringência), mas também em suas propriedades saudáveis. Portanto, este trabalho teve como objetivo identificar, caracterizar e quantificar os polissacarídeos e compostos fenólicos encontrados nos vinhos e avaliar a interação entre eles. Além disso, avaliar a semelhança entre os polissacarídeos extraídos de vinhos comerciais de diferentes safras e estudar as propriedades anti-inflamatórias dos polissacarídeos *in vitro* em células RAW 264.7 estimuladas com LPS e *in vivo* pelo modelo de septicemia induzido pela ligação e perfuração do ceco em camundongos, bem como a biodistribuição desses polissacarídeos durante o processo inflamatório. Para isso, foram escolhidos vinhos comerciais feitos com uvas *Vitis vinífera* de diferentes variedades. Os polissacarídeos foram extraídos, caracterizados e quantificados por GC-MS e RMN. Foi identificada a presença de manana, arabinogalactana tipo II e ramannogalactonana tipo I e II. Também foi demonstrado que os vinhos produzidos a partir da mesma variedade de uva e da mesma vinícola têm padrões de polissacarídeos semelhantes, embora tenham sido produzidos em anos diferentes. Os compostos fenólicos foram identificados e quantificados por HPLC-DAD-MS. A análise mostrou uma prevalência de conteúdo de procianidina (entre 58 e 275 mg/L) versus a de prodelfinidinas (entre 4 e 8 mg / L). A análise de antocianina mostrou a maioria dos derivados monoglicósidos e seus derivados acetilados. As análises estatísticas revelaram a existência de relações entre a estrutura dos polissacarídeos e sua capacidade de precipitar procianidinas, o que explicaria a relação observada nos vinhos entre a composição dos polissacarídeos e a concentração de flavanóis. As frações de polissacarídeos inibiram a produção de citocinas inflamatórias (TNF-α e IL-1β) e mediador (NO) em células RAW 264.7. A administração subcutânea e oral de polissacarídeos reduziu a taxa de mortalidade dos camundongos. O tratamento reduziu a migração de leucócitos, inibiu citocinas pró-inflamatórias e aumentou a produção da citocina anti-inflamatória IL-10. Eles também reduziram os níveis plasmáticos de AST, ALT, bilirrubina, uréia e creatinina, com a consequente proteção contra danos nos tecidos. Além

disso, a biodistribuição de polissacarídeos pelo organismo, quando administrada via subcutânea, parece ocorrer através do sistema imunológico. Os polissacarídeos demonstraram um potente efeito anti-inflamatório *in vitro* e *in vivo*, o que pode indicar efeitos benéficos do consumo moderado de vinho na saúde humana.

Palavras-chave: vinhos, polissacarídeos, compostos fenólicos, NMR, HPLC, inflamação.

ABSTRACT

Wine is one of the most consumed and economically important beverages, handling approximately € 30 billion a year on the market resulting from a worldwide production of 292 million hectoliters. Wine producers continually seek to produce high quality wines; therefore, there is much interest in research aimed at identifying the components of wine and better understanding its effect during winemaking, which can be useful for the advancement of the wine sector. Polysaccharides and phenolic compounds are compounds highly related to wine quality, due to their impact not only on their organoleptic properties (color, flavor, astringency), but also on their healthy properties. Therefore, this work aimed to identify, characterize and quantify the polysaccharides and phenolic compounds found in wines and to evaluate the interaction between them. In addition, to evaluate the similarity between the polysaccharides extracted from commercial wines from different vintages and to study the anti-inflammatory properties of the polysaccharides *in vitro* in RAW 264.7 cells stimulated with LPS and *in vivo* by the septicemia model induced by the cecum binding and perforation in mice, as well as the biodistribution of these polysaccharides during the inflammatory process. For this, commercial wines made with *Vitis vinífera* grapes of different varieties were chosen. Polysaccharides were extracted, characterized and quantified by GC-MS and NMR. The presence of mannan, type II arabinogalactans and type I and II rhamannogalactonans were identified. It has also been shown that wines produced from the same grape variety and from the same winery have similar polysaccharide patterns, although they have been produced in different years. The phenolic compounds were identified and quantified by HPLC-DAD-MS. The analysis showed a prevalence of procyanidin content (between 58 and 275 mg/L) versus that of prodelphinidin (between 4 and 8 mg/L). The anthocyanin analysis showed the majority of monoglycoside derivatives and their acetylated derivatives. Statistical analyzes revealed the existence of relationships between the structure of polysaccharides and their ability to precipitate procyanidins, which would explain the relationship observed in wines between the composition of polysaccharides and the concentration of flavanols. The polysaccharide fractions inhibited the production of inflammatory cytokines (TNF-α and IL-1β) and mediator (NO) in RAW 264.7 cells. Subcutaneous and oral administration of polysaccharides reduced the mortality rate of mice. The treatment reduced leukocyte migration, inhibited pro-inflammatory cytokines and increased production of the anti-inflammatory cytokine IL-10. They also reduced plasma levels of AST, ALT, bilirubin, urea and creatinine, with consequent protection against tissue damage. In addition, the polysaccharide biodistribution by the body, when administered subcutaneously, seems to

occur through the immune system. Polysaccharides have demonstrated a potent anti-inflammatory effect in vitro and in vivo, which may indicate beneficial effects of moderate wine consumption on human health.

Keywords: wines, polysaccharides, phenolic compounds, NMR, HPLC, inflammation.

SUMÁRIO

1. INTRODUÇÃO

O vinho é produzido a partir de uvas das variedades (ou castas) da espécie *Vitis vinifera* utilizada para produção de vinhos finos e da espécie *Vitis labrusca* utilizada para produção de vinhos de mesa (comuns). A constituição química das uvas permite que elas fermentem durante a vinificação sem que lhes sejam adicionados açúcares, ácidos, enzimas ou outros nutrientes. Essa fermentação é feita por leveduras que consomem os açúcares presentes nas uvas transformando-os em álcool (JOHNSON, 1989; (CRUZ, 2006). As principais macromoléculas encontradas na bebida são polissacarídeos (até 15%) e polifenóis (até 35%) (CARPITA *et al*., 1993).

Os polissacarídeos presentes nos vinhos possuem influência importante em várias etapas do processo de vinificação, incluindo fermentação, filtração, estabilização e são parcialmente responsáveis pelas propriedades organolépticas dos vinhos (GERBAUD *et al*., 1997; MOINE-LEDOUX & DUBOURDIEU, 1999). No entanto, estudos já mostraram que nem todos os polissacarídeos apresentam o mesmo comportamento em relação aos vinhos, e sua influência no processamento e nas propriedades sensoriais depende da quantidade, classe e características estruturais (RIOU *et al*, 2002; VIDAL *et al.*, 2003; GUADALUPE & AYESTARÁN, 2007).

Essas macromoléculas são provenientes de bagas da uva, de leveduras, de bactérias e da contaminação por fungos. Do ponto de vista enológico e quantitativo, os polissacarídeos de uvas e de leveduras são os mais importantes. A estrutura dos polissacarídeos e suas concentrações dependem de muitos parâmetros, tais como cultivo, estágio de maturação, técnicas de vinificação e os tratamentos para aumento da solubilização dos componentes macromoleculares das paredes celulares de bagas de uva (DOCO *et al.*, 1999; CABANIS & CABANIS, 2000; AYESTARAN *et al.*, 2004). Arabinose e polissacarídeos ricos em galactose (AGs), como as arabinogalactanas-proteínas tipo II e arabinanas, ramnogalacturonanas tipo I e tipo II, e homogalacturonanas são provenientes de bagas de uva, enquanto as glucanas, mananas e manoproteínas são liberadas da levedura durante a fermentação ou pela atividade enzimática da levedura por autólise durante o envelhecimento em barris (PELLERIN *et al*., 1995; VIDAL *et al*., 2003; AYESTARÁN *et al*., 2004). Esses açúcares parecem ser uma das moléculas mais interessantes em enologia devido aos seus efeitos na qualidade final do vinho (FOURNAIRON *et al*., 2002; DOCO *et al.*, 2003), no entanto, informações detalhadas sobre composição e caracterização estrutural dos

polissacarídeos são relativamente escassas, o que torna importante o estudo desses polissacarídeos encontrados nos vinhos como proposto nesse trabalho.

Os polifenóis presentes no vinho são provenientes das uvas, do metabolismo dos microrganismos e também da madeira dos barris utilizados durante o amadurecimento (JACKSON, 2008). Portanto, sua composição e concentração também dependem da variedade de uva usada na vinificação, do procedimento usado para a vinificação e das reações químicas que ocorrem durante o envelhecimento (RIBÉREAU-GAYON *et al.*, 2006; RECAMALES *et al*., 2006).

Os compostos fenólicos derivados de uvas, de grande importância para a enologia, são encontrados principalmente em sementes e peles (ESCRIBANO-BAILÓN *et al*, 1992; 1995 HANLIN *et al*., 2011). Esses compostos estão envolvidos na estabilidade e nas propriedades organolépticas, como cor e adstringência, ou seja, na qualidade do vinho (RIBÉREAU-GAYON *et al.,* 2006; QUIJADA-MORÍN *et al.,* 2014). Nesse sentido, é amplamente reconhecido que os vinhos tintos de alta qualidade têm um nível equilibrado de adstringência, ou seja, possuem níveis equilibrados de compostos fenólicos e polissacarídeos (BATE-SMITH, 1954; GAWEL *et al.,* 2000; BROSSAUD *et al.,* 2001; PREYS *et al*., 2006; QUIJADA-MORÍN *et al.*, 2014).

Estudos mostram que os polissacarídeos podem afetar as propriedades organolépticas do vinho (OZAWA *et al,* 1987; QUIJADA-MORÍN *et al.,* 2014; OSETE-ALCARAZ *et al*., 2019). Polissacarídeos podem limitar a concentração de proantocianidinas disponíveis e reduzir a adstringência do vinho (ESCOT *et al*., 2001; MATEUS *et al.*, 2004; CARVALHO *et al*., 2006). A falta de equilíbrio entre os compostos fenólicos e polissacarídeos e a consequente alteração das propriedades organolépticas leva a uma diminuição na qualidade e estabilidade do vinho (COOMBE, 1973; ORDUÑA, 2010;). Portanto, o estudo desses compostos e a relação entre eles são extremamente importantes para a produção de um vinho de qualidade e competitivo no mercado.

Além disso, há um grande interesse em conhecer os benefícios que o consumo moderado de vinho pode trazer à saúde. Alguns autores descreveram importantes efeitos imunomoduladores, antioxidantes, anti-septicêmicos, anti-inflamatórios, antineoplásicos e gastroprotetores dos polissacarídeos extraídos de plantas, frutas e vinho (MUELLER & ANDERER, 1990; MELLINGER *et al.,* 2008; CIPRIANI *et al*., 2009; NASCIMENTO *et al*., 2013; PARK *et al.,* 2013; DARTORA *et al*., 2013; INNGJERDINGEN *et al.,* 2014; STIPP *et al.,* 2016; CAILLOT *et al*., 2018). Alguns efeitos benéficos do vinho na saúde têm sido atribuídos aos polifenóis presentes na pele das uvas (DIAZ-GEREVINI *et al.,* 2016; GOJKOVIC-BUKARICA *et al.,* 2018). No entanto, existem poucos estudos sobre atividades

biológicas e modulação de mediadores inflamatórios por polissacarídeos de vinho o que torna importante o estudo dos possíveis benefícios dos polissacarídeos encontrados nos vinhos.

2. OBJETIVOS

2.1. OBJETIVO GERAL

Extração, caracterização, quantificação dos polissacarídeos e polifenóis de vinhos e avaliação da similaridade entre polissacarídeos extraídos de vinhos comerciais de diferentes safras. Como também, investigar interação entre essas moléculas e os benefícios dos polissacarídeos para a saúde, através da avaliação da propriedade anti-inflamatória em ensaios *in vitro* e *in vivo*.

2.2. OBJETIVOS ESPECÍFICOS

- Extrair, caracterizar e quantificar os polissacarídeos de vinhos.
- Avaliar a similaridade entre polissacarídeos extraídos de vinhos comerciais de diferentes safras.
- Caracterizar e quantificar os compostos fenólicos extraídos de vinhos e estabelecer uma possível relação com polissacarídeos.
- Analisar o efeito anti-inflamatório *in vitro* dos polissacarídeos extraídos dos vinhos utilizando células RAW 264.7 estimuladas com lipopolisacarídeos (LPS).
- Avaliar os efeitos anti-inflamatórios das frações polissacarídicas obtidas dos vinhos, através do modelo de septicemia induzida pela cirurgia de ligadura e perfuração do ceco (CLP) e verificar a taxa de sobrevida dos camundongos tratados com os polissacarídeos.

3. REVISÃO BIBLIOGRÁFICA

3.1. UVA

A baga da uva é composta pela casca, polpa e sementes (Figura 1) e a estrutura do cacho de uva é fornecida pela ráquis, a qual tem a função de sustentar as bagas. As cascas ou películas contêm matéria corante e compostos odoríferos, além de polifenóis. Na superfície externa da casca está a pruína que é uma camada de substância cerosa onde ficam aderidos micro-organismos (exemplo, leveduras alcoolgênicas e bactérias lácticas), os quais conduzem a fermentação espontânea do mosto (KUHN, 2003).

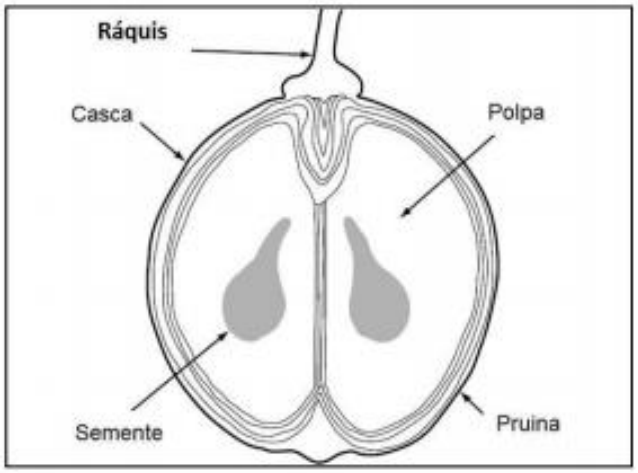

FIGURA 1: Estrutura da baga da uva. FONTE: GRAINGER & TATTERSALL, 2005.

A polpa representa 4/5 do peso da baga e é composta por água, açúcares, ácidos, proteínas e minerais. Os principais açúcares encontrados na uva são a frutose e a glucose. Já os ácidos mais importantes encontrados na baga verde são o ácido tartárico e o málico, sendo que durante o amadurecimento da fruta o ácido tartárico torna-se o principal. Quanto aos minerais, o potássio é o que se apresenta em maior quantidade (2.500 mg/kg), já a concentração dos demais (exemplo, cálcio e magnésio) não ultrapassa 200 mg/kg cada (GUERRA, 2003; GRAINGER & TATTERSALL, 2005).

À medida que amadurecem, as uvas passam por mudanças de forma e de composição, como o ganho de açúcar, a queda da acidez, o aumento de volume e de peso, a mudança de coloração e o enriquecimento dos aromas. O tempo da colheita é provavelmente a mais importante decisão na viticultura, pois a qualidade da uva é indispensável para a qualidade do vinho (GUERRA, 2003).

3.1.1. Tipos de videira

Mundialmente existem milhares de variedades de uva. A maioria delas pertence à espécie *Vitis vinifera*, originária do Cáucaso entre 7.000 e 5.000 a.C., de onde foi difundida por toda a costa mediterrânea. Entre as variedades dessa espécie estão as conhecidas uvas tintas Cabernet Sauvignon, Cabernet Franc, Merlot, Pinot Noir, Tempranillo, Syrah e Tannat e, entre as uvas brancas, destacam-se a Chardonnay, Sauvignon Blanc, Prosecco e Riesling. Algumas delas se difundiram pelo mundo em virtude da sua capacidade de adaptação e pelas características dos vinhos que originam. Outras, de adaptação mais restrita, permaneceram em suas regiões de origem, proporcionando aos seus habitantes a oportunidade de elaboração de produtos típicos e exclusivos (GUERRA *et al*., 2009).

As uvas oriundas de videiras *Vitis vinifera*, cultivares europeus, são comumente usadas na elaboração de vinho pelo mundo, principalmente na Europa. Nos Estados Unidos, espécies como a *Vitis labrusca*, *Vitis riparia*, *Vitis aestivalis*, *Vitis rupestris* e *Vitis rotundifolia* são as mais usadas para a vinificação (YANG, MARTINSON & LIU, 2009). As frutas destas últimas espécies contêm menos açúcar que as oriundas de espécies *Vitis vinifera*. Desta forma, seus vinhos também não apresentam uma alta qualidade (ESTREICHER, 2006).

3.2. VINHO

O vinho é uma das primeiras criações da humanidade e ocupou um lugar privilegiado em inúmeras civilizações. Há cerca de 60 milhões de anos, surgiram as espécies *Vitis vinifera* e as genericamente chamadas de americanas, como *Vitis labrusca* e *Vitis riparia*. Desde há muito que esta bebida vem acompanhando ascensões e declínios de civilizações e impérios, estando a sua produção fortemente interligada com a agricultura ao longo dos anos. O cultivo de vinha é realizado entre os povos europeus desde os anos 12.000 a.C. no entanto na há qualquer registro de produção de vinho que remonte a essas dadas. Pensa-se ter sido na Ásia há 9000 anos que a utilização de uva fermentada terá tido o seu início e em 4000 a.C. a produção de vinho terá chegado aos povos europeus (ROSADO, 2013; LAROUSSE DO VINHO, 2007).

Com crescimento do comércio do vinho, a ciência passou a ocupar papel importante na vinicultura, e desenvolveram-se programas de pesquisa sobre a vinha, a fermentação e o envelhecimento em adega. Paralelamente, o consumo do vinho disseminou para o mundo

inteiro. Com isso, novos países produtores chegaram ao mercado, ocorrendo a ascensão de países do Novo Mundo, que atualmente competem em qualidade e quantidade com os vinhos europeus (LAROUSSE DO VINHO, 2007).

Dados mostram que o vinho é uma das bebidas mais consumidas atualmente e de maior importância econômica. O nível de produção global em 2018 foi de 292 milhões de hectolitros de vinho, sendo França, Itália e Espanha os três maiores produtores de vinho do mundo, movimentando em torno de 30 bilhões de euros no mercado mundial (fig. 2) (dados da Organização Internacional da Vinha e do Vinho - OIV). O Brasil vem se destacando nesse mercado e, produziu 3,1 milhões de litros em 2018, assumindo a 15ª posição no ranking de maiores produtores de vinho segundo a OIV.

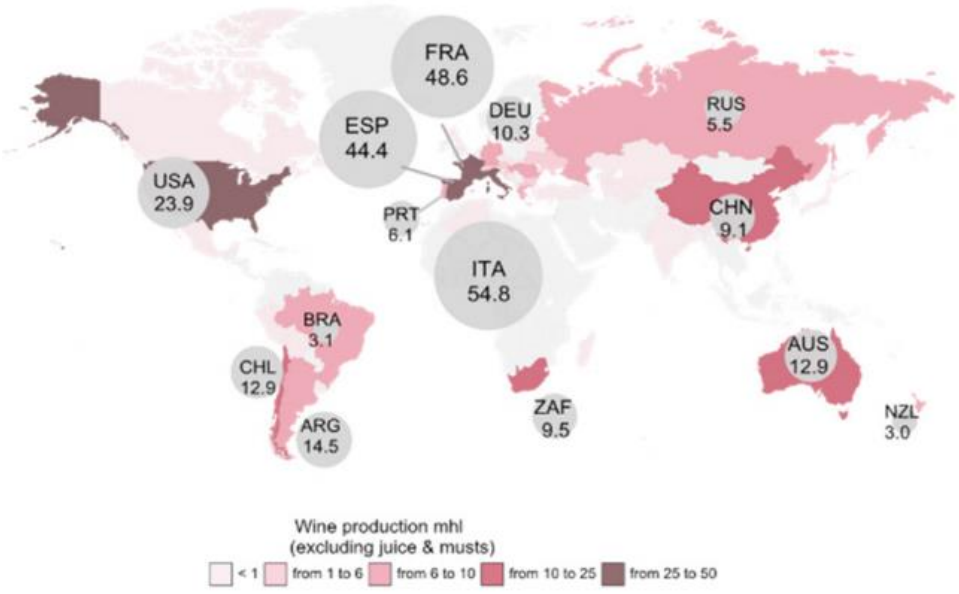

FIGURA 2: Produção mundial de vinho em 2018. FONTE: 2019 Statistical Report on World Vitiviniculture. International Organisation of Vine and Wine.

3.2.1. Processo de produção do vinho

Os vinhos tintos são produzidos a partir de uvas tintas, já os vinhos brancos, são geralmente elaborados a partir de uvas brancas, mas também podem ser produzidos com uvas rosadas e tintas. Para isso, é necessário separar imediatamente a fase líquida da sólida, que contém os pigmentos responsáveis pela cor (CRUZ, 2006; HOFFMAN, 2009).

O processo de produção do vinho inicia-se com a colheita seguida da recepção das uvas, esmagamento e desengace, fermentação, clarificação, filtração, envelhecimento,

engarrafamento e rotulagem, análises do vinho e resíduos (figura 3) (CRUZ, 2006; HOFFMAN, 2009). Os vinhos apresentam diferenças ao nível das operações tecnológicas dependendo da vinícola. No processo de fabricação do vinho, as etapas mais importantes descritas detalhadamente são (GRAINGER, *et. al*., 2005):

- Recepção e Descarga no Tegão: Na recepção da uva ocorre o primeiro controle de qualidade para a produção de vinho. Este controle define a ordem de descarga e o tipo de vinho que as uvas irão dar origem e baseia-se no grau alcoólico provável e peso das uvas bem como no resultado da inspecção visual à presença de corpos estranhos e do estado sanitário das uvas.

- Desengace e Esmagamento: Após a descarga no tegão, as uvas sofrem um processo de desengace para remoção da parte lenhosa do cacho e um processo de esmagamento para quebrar a película da uva e promover o arejamento que facilita a multiplicação de leveduras. Os vinhos são trasfegados para depósitos onde são mantidos a temperaturas entre 14 e 16ºC. Pode ser adicionado SO_2 para proteger de contaminações microbianas e também enzimas de extracção aromática e ácido tartárico.

- Fermentação alcoólica. A fermentação é um processo natural de transformação dos açúcares das uvas em etanol. Este processo ocorre a menos de 18 ºC, permitindo assim manter os aromas característicos das uvas, e dura 10 a 15 dias. Nesta fase podem ser adicionadas leveduras, de acção conhecida.

- Prensagem: Após o esmagamento, o sumo passa pela prensa pneumática para separar a parte sólida da parte líquida.

- Fermentação malolática: Nessa etapa o ácido málico é convertido em ácido lático, menos ácido e menos agressivo, dando sabore mais agradável ao vinho. Geralmente é feita apenas nos vinhos tintos.

- Trasfega: Processo de transferência do vinho para um novo depósito. Após a fermentação alcoólica, o vinho é transferido para um depósito de armazenamento e decantação e mantido entre 15 e 17 ºC. Depois, é novamente trasfegado permitindo a remoção das partículas que se encontram no fundo – borras.

- Escolha e estudo do lote: Permite a uniformização das características dos vinhos de acordo com as especificações. Nesta fase o vinho pode sofrer vários processos de alteração do aroma como, por exemplo, a adição de madeiras, mistura de vinhos de castas diferentes, etc.

- Clarificação. Este processo tem como propósito a eliminação de impurezas em suspensão por coagulação e formação de partículas mais densas que sedimentam.

- Envelhecimento: Essa etapa é realizada apenas com vinho tinto. O envelhecimento do vinho pode ser feito em tanque de inox, em barrica de carvalho e na garrafa. O tempo e a forma de envelhecimento do vinho dependem da variedade da uva, de seu processo de elaboração, do tipo e da estrutura do produto que se deseja, da safra e do valor agregado que terá esse vinho.
- Filtração e homogeneização: O vinho passa por uma série de filtrações e homogeneizações de modo a reter a maior quantidade possível de elementos em suspensão e impurezas.
- Engarrafamento: o vinho é engarrafado em máquinas especiais que impedem o contato com ar, evitando oxidações e contaminações.

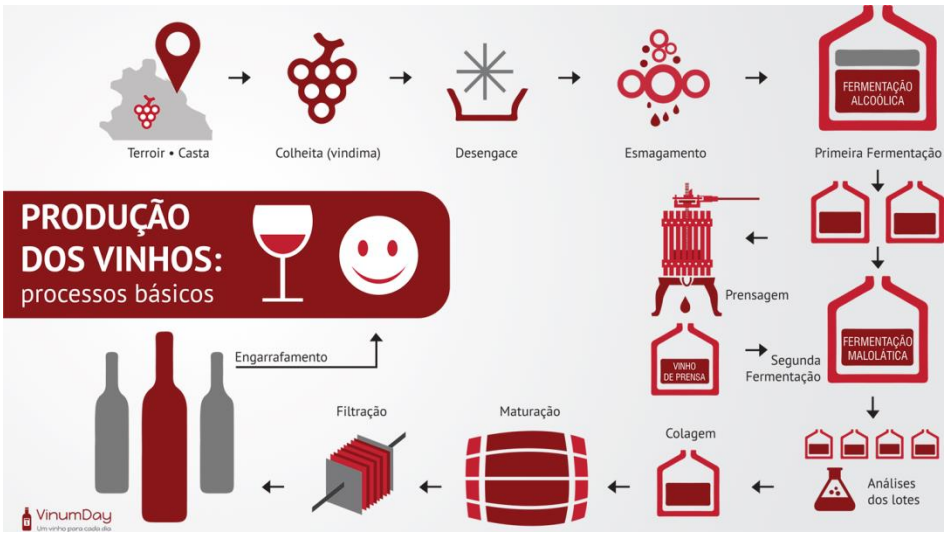

FIGURA 3: Fluxograma da produção do vinho tinto e branco. http://blog.vinumday.com.br/como-e-feito-o-vinho/ (acesso em 2 de fevereiro de 2020).

3.2.2. Benefícios do vinho

O vinho é uma bebida obtida exclusivamente a partir da fermentação alcoólica do mosto da uva. Apresenta grande complexidade química devido à natureza dos seus constituintes. O hábito do consumo moderado de vinho é benéfico à saúde do consumidor, e participa da dieta da população de muitos países (AMERINE *et al.*, 1977; ABAD *et al.*, 1993; NDIAYE *et al* ., 2004).

Entre os efeitos benéficos do vinho destaca-se a capacidade de favorecer a formação em maior quantidade do “bom colesterol” (HDL), o efeito para combater os radicais livres, o

efeito antimicrobiano, diurético, anti-inflamatório e antineoplásico (ABAD *et al*., 1993; STIPP *et al.*, 2017; BEZERRA *et al*., 2018).

Os taninos, flavonóis e estilbenos são compostos fenólicos presentes em maior quantidade nos vinhos envelhecidos. Os taninos favorecem a digestão e os flavonóis têm sido estudados devido ao seu poder antioxidante e efeito protetor em relação aos radicais livres. No grupo dos estilbenos encontra-se o resveratrol, que é uma fitoalexina. O resveratrol tem sido muito estudado devido ao provável efeito protetor contra doenças cardiovasculares. Na uva, o resveratrol encontra-se na película, e o vinho tinto apresenta maior quantidade, devido ao processo de elaboração. A concentração de resveratrol no vinho varia de 1,3 a 7,0 mg/L (BOURZEIX *et al*., 1989; ADRIAN *et al*., 2000; JANNIN *et al*., 2001). Muitos efeitos benéficos do vinho na saúde têm sido atribuídos ao resveratrol, um polifenol presente na pele das uvas (DIAZ-GEREVINI *et al*., 2016; GOJKOVIC-BUKARICA *et al.,* 2018). No entanto, existem poucos estudos sobre atividades biológicas e modulação de mediadores inflamatórios por outros componentes do vinho, como os polissacarídeos de vinho.

3.2.3. Carboidratos dos vinhos

Os carboidratos são um dos principais constituintes químicos da maioria dos tecidos e células dos organismos (SIMÕES *et al.,* 2003). Eles existem como monossacarídeos, oligossacarídeos, polissacarídeos e seus derivados (AVIGAD & DEY, 1997). De acordo com a sua função, os carboidratos podem ser classificados em dois grandes grupos: estruturais e de reserva. Os carboidratos estruturais são responsáveis pela formação da parede celular e de outras estruturas, sendo considerados os compostos orgânicos mais abundantes da Terra (REID, 1997).

Os carboidratos presentes no vinho são derivados tanto das paredes das células de micro-organismos quanto das uvas (DOCO *et al.*, 1999; CABANIS & CABANIS, 2000b; VIDAL et al., 2003; AYESTARAN *et al*., 2004).). Os originários das paredes celulares de uva incluem as arabinogalactanas, ramnogalacturonanas e homogalacturonanas, enquanto que aquelas originadas a partir de paredes das células de levedura são principalmente glucanas, mananas e manoproteínas (WATERS et al., 1994; PELLERIN et al., 1995; MARTÍNEZ-LAPUENTE et al., 2013).

3.2.3.1. Parede celular vegetal e polissacarídeos

A parede celular é dividida em: primária, não lignificada, com células jovens que ainda mantém a capacidade de divisão e alongamento, apresentam uma parede celular muito fina, com 0,1 a 1 µm de espessura; e secundária, lignificada, com células sem capacidade de crescimento e divisão, que conferem rigidez aos tecidos (REID, 1997).

A parede celular dos vegetais é altamente organizada e constituída de polissacarídeos, proteínas e substâncias aromáticas. Os polissacarídeos são os principais componentes da parede celular e são divididos em pectinas, hemiceluloses e celulose, como pode ser observado na figura 4 (CARPITA & McCANN, 2000). A parede celular primária é composta de 25-40% celulose, 15-25% hemicelulose, 15-40% pectina e 5-10% proteínas e proporções muito pequenas de compostos fenólicos. Já a parede celular secundária, muito mais espessa que a parede primária, possui de 40-45% de celulose, 15-35% de hemicelulose, 15-30% de lignina e quantidade mínima de pectina (DEY *et al*., 1997).

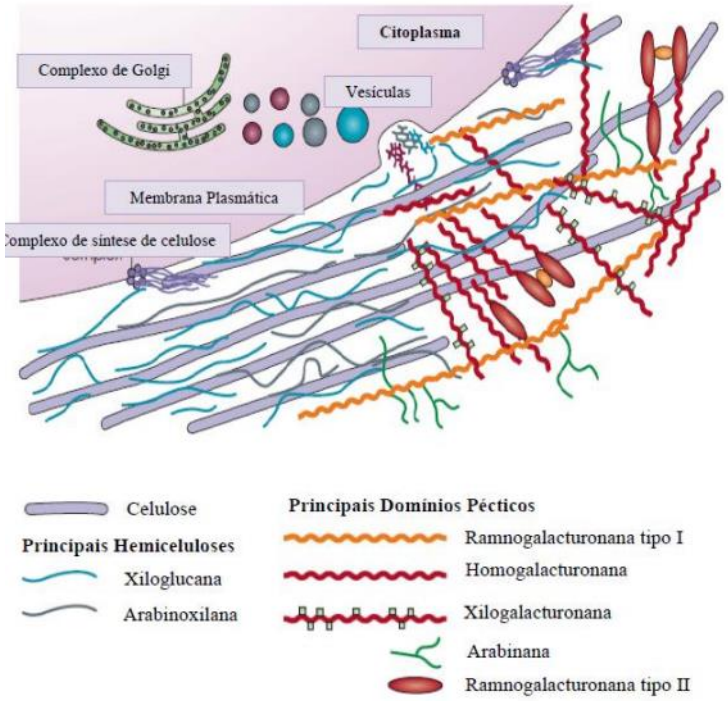

FIGURA 4: Modelo da estrutura da parede celular primária de vegetal. FONTE: Adaptado de COSGROVE, 2005.

As hemiceluloses são polissacarídeos que ocorrem juntamente com a celulose, são constituídas de unidades monossacarídicas de D-manose, D-galactose, D-glucose, L-arabinose, D-xilose, ácido D-galacturônico, ácido D-glucurônico e ácido 4-*O*-metilglucurônico e podem apresentar ainda grupamentos *O*-acetil ligados às unidades pertencentes às cadeias principal e/ou lateral (ASPINALL, 1959; FENGEL & WEGNER, 1989). Esses monossacarídeos formam diferentes polissacarídeos que podem ser incluídos nas classes arabinogalactanas,

glucuronoxilanas arabinoxilanas, xilanas, galactoglucomananas, galactomananas, glucuronomananas, glucomananas, mananas e xiloglucanas (ASPINALL, 1980; BRETT & WALDRON, 1990; PULS & SCHUSEIL, 1993; KACURÁCOVÁ *et al.,* 2000). Alguns estudos já relataram a presença de arabinogalactanas e mananas no vinho (PELLERIN et al., 1995; MARTÍNEZ-LAPUENTE *et al*., 2013).

As pectinas são polissacarídeos ricos em ácido galacturônico, ramnose, arabinose e galactose. O termo pectina refere-se a polissacarídeos complexos que contém unidades de ácido α-D-galacturônico (GalA). Os principais representantes são: ramnogalacturonas do tipo I (RG I) e ramnogalacturonanas do tipo II (RG II) e homogalacturononas (ASPINALL, 1980; BRETT & WALDRON, 1990; CARPITA & GIBEAUT, 1993). Dentre elas, as RG I e RG II já foram relatadas em estudos feitos com o vinho (PELLERIN *et al.,* 1995; MARTÍNEZ-LAPUENTE *et al.,* 2013).

3.2.3.1.1. Arabinogalactanas

As arabinogalactanas podem ser classificadas em dois grupos de acordo com as ligações químicas da cadeia principal: arabinogalactanas do tipo I (AG I) e arabinogalactanas do tipo II (AG II) (ASPINAL, 1973).

As arabinogalactanas do tipo I apresentam cadeia principal formada por (1→4)-β-D-galactanas. São pouco ramificadas e, na maioria das vezes, apresentam unidades de arabinose ligadas no *O*-3 das unidades de galactose da cadeia principal (CARPITA & GIBEAUT, 1993).

As arabinogalactanas do tipo II são altamente ramificadas, possuem cadeia principal formada por (1→3) e/ou (1→6)-β-D-galactanas ligadas umas as outras por pontos de ramificação em *O*-3 e *O*-6 e apresentam a maior parte das posições *O*-3 e *O*-6 restantes ocupadas por unidades de arabinose (CARPITA & GIBEAUT, 1993). Embora apresentem uma estrutura geral semelhante, a estrutura fina das arabinogalactanas do tipo II varia muito entre as espécies (CARPITA & GIBEAUT, 1993; ALBERSHEIM *et al*., 1996).

3.2.3.1.2. Ramnogalacturononas

As ramnogalacturonanas são polissacarídeos ricos em ácido galacturônico e ramnose (REID, 1997). Esses polímeros podem ser classificados em dois grupos: ramnogalacturonana do tipo I (RG I) e ramnogalacturonana do tipo II (RG II).

As ramnogalacturonanas do tipo I são heteropolímeros formados por cadeia principal constituída de unidades alternadas de ácido α-D-galacturônico ligadas α-(1→4) e unidades de α-L-ramnose ligadas α-(1→2). Esses polímeros podem ser estendidos por ácido poligalacturônico em seus terminais e as unidades ramnosil podem interromper longos períodos de ácido poligalacturônico (CARPITA & GIBEAUT, 1993).

A ramnogalacturonana do tipo II é o polissacarídeo péctico mais complexo, porém o que apresenta a estrutura mais conservada. A característica mais marcante dessa molécula é possuir açúcares raros. São polímeros altamente substituídos que apresentam cadeia principal constituída por unidades de ácido α-D-galacturônico unidas por ligação do tipo (1→4) (O´NEILL *et al.*, 1990). Esses polissacarídeos apresentam cadeias laterais geralmente substituídas em C-2 das unidades de α-D-Gal*p*A e seus substituintes são formados por oligossacarídeos contendo monossacarídeos raros como apiose, 2-*O*-metil-fucose, 2-*O*-metil-xilose, ácido acérico, Kdo (ácido 2-ceto-3-deoxi-D-mano-octulosônico), Dha (ácido 3-deoxi-D-lixo-2-heotulosárico) entre outros, formando estruturas altamente complexas (CARPITA & GIBEAUT, 1993; REID, 1997; PÉREZ, MAZEAU & PENHOAT, 2000; PAULSEN & BARSETT, 2005; ATMODJO *et al.*, 2013).

3.2.3.2. Parede celular de leveduras e polissacarídeos

Leveduras são fungos que possuem a mesma estrutura subcelular de células animais e vegetais (OSUMI, 1998). São micro-organismos unicelulares, com tamanho que varia de 5 a 10 microns. As espécies variam entre si segundo a morfologia ou forma, metabolismo com relação a diferentes substratos, modo de reprodução e onde são encontrados. Enquanto existem aproximadamente 50.000 espécies de fungos, existem apenas 60 gêneros diferentes de leveduras, com aproximadamente 500 espécies diferentes (STONE & MILLS, 2006).

A parede celular da levedura é constituída por aproximadamente 40% de β-glucanas, 40% de α-mananas, 8% de proteínas, 7% de lipídios, 3% de substâncias inorgânicas e 2% de hexosaminas e quitina (HOUGH, 1990). Os principais monossacarídeos presentes nos polímeros são a glucose e a manose, seguidos pela galactose, xilose, N-acetil-D-glucosamina, ácidos urônicos e outros componentes secundários. A composição química qualitativa da parede celular é característica de cada espécie, podendo ser empregada como marcador taxonômico (FARKAS, 1989).

A principal levedura responsável pela fermentação na produção do vinho é a Saccharomyces cerevisiae, a qual possui em sua parede celular cerca de 10-20% de proteínas e lipídios e 80-90% de carboidratos, dos quais 50% são glucanas e mananas (NORTHCOTE & HORNE, 1952; BARTNICKI-GARCIA & LIPPMAN, 1982; VUKOVIC *et al.*, 1994). Segundo Vukovic (1994), a *S. cerevisiae* apresenta a parede celular em camadas, sendo as cadeias de mananas externas e as glucanas, internas. Já foi demonstrada a presença de mananas entre os polissacarídeos encontrados no vinho (MARTÍNEZ-LAPUENTE *et al.*, 2013).

3.2.3.2.1. Mananas

As mananas são um dos polissacarídeos de maior importância da parede celular da levedura, responsáveis por eventos de reconhecimento célula-célula, além de limitar o acesso de agentes líticos às porções mais internas da parede celular e à membrana celular (KLIS, *et al*., 2002).

Há evidências de que as mananas juntamente com os peptídeos complexos constituem a camada mais externa da parede celular das leveduras (BALLOU, 1970; CABIB, 1975). Esses polissacarídeos são altamente ramificado, contendo uma cadeia principal constituída por ligações α-(1→6) substituída nas posições *O*-2 por cadeias laterais de unidades de α-D-manopiranose ou oligossacarídeos com unidades de manose (1→2) e (1→3) ligadas (STEWART & BALLOU, 1968; BARRETO BERTGER & GORIN, 1983; HALÁSZ & LÁSZTITI, 1991).

3.2.4. Polifenóis do vinho

Os compostos fenólicos constituem um grupo diversificado de metabólitos secundários presentes nas uvas e no vinho. O conteúdo fenólico e a composição dos produtos processados da uva (vinho) são grandemente influenciados pela prática tecnológica à qual as uvas estão expostas. Os polifenóis são componentes intrínsecos das uvas e produtos relacionados, particularmente o vinho (LINSKENS & JACKSON, 1988; SCALBERT, 1993). Os compostos fenólicos desempenham um papel importante nas características sensoriais do vinho, são responsáveis por algumas das propriedades organolépticas como: aroma, cor, sabor e adstringência (LINSKENS & JACKSON, 1988; SCALBERT, 1993).

Existe uma grande diversidade química na composição fenólica das uvas e do vinho. Isto se deve não apenas às diferentes variedades de uvas, como também ao fato de que esse tipo de compostos existe em ambas as formas livre e conjugada, pois podem estar ligadas ao ácido quínico ácido ou a uma ou mais moléculas de açúcar (glicose, galactose, sacarose e manose) produzindo mono-, di-, tri- ou mesmo tetraglicósidos (CHEYNIER *et al*., 2010; LINSKENS & JACKSON, 1988; SCALBERT, 1993).

Os componentes fenólicos não voláteis das uvas e do vinho compreendem diversas classes: ácidos fenólicos, flavonóides, taninos, estilbenos, cumarinas, derivados de fenilpropanol, lignanas e neolignanas (LINSKENS & JACKSON, 1988; SCALBERT, 1993).. Os polifenóis econtrados no vinho são provenientes da uva que estão localizados principalmente nas partes sólidas: pele, semente e tecido vascular. Os flavonóis e antocianinas são encontrados na pele e sementes da uva, já as antocianinas são encontradas apenas na pele da uva de tinta, sendo responsável pela cor vermelha característica dos vinhos tintos (KOSIR & KIDRIC, 2002; FLAMINI, 2003).

Os flavonóides pertencem a uma classe química que exibe uma estrutura básica de 15 átomos de carbono compreendendo dois anéis aromáticos ligados através de uma cadeia de 3 carbonos (C6 - C3 - C6), que pode ou não fazer parte de um terceiro anel. Esse esqueleto de carbono é responsável pela diversidade química dessa família de compostos. Os flavonóides são geralmente agrupados em várias classes, que diferem principalmente no grau de oxidação do anel pirano central, exceto no caso das chalconas. Eles compreendem diferentes tipos de compostos tais como flavonas, flavonóis, flavanonas, flavononóis, flavanos, flavanóis, antocianidinas e antocianinas, chalconas e di-hidrochalcones (BADERSCHNEIDER & WINTERHALTER, 2001).

3.2.4.1. Flavanóis

Flavanóis são benzopiranos que possuem carbono saturado cadeia entre C2 e C3, uma função hidroxila em C3 e nenhuma carbonila grupo em C4. Tanto o flavan-3-ols quanto o flavan-3,4-dióis podem ser encontrado na natureza, sendo este último frequentemente presente em madeira e casca de árvore, mas raramente encontrado em frutas. Flavan-3,4-dióis também são freqüentemente chamados de leucoantocianidinas (GARRIDO & BORGES, 2013).

Os flavanoís mais abundantes na natureza são a catequina e seu enantiômero epicatequina. Estes compostos estão presentes na casca e sementes de uvas, bem como no vinho. Nos vinhos brancos produzidos em condições especiais, evitando contato prolongado

com a pele das uvas, a catequina foi considerada a mais abundante flavonóide, sendo amplamente responsável pelo sabor característico do vinho (LUNTE, BLANKENSHIP, & READ, 1988). Alguns derivados da catequina, nomeados galocatequina, epigalocatequina, e epicatequina galato foram identificados em uvas e vinho (DECENDIT *et al*., 2002; MATTIVI *et al*, 2009).

3.2.4.2. Pigmentos antociânicos

Nos últimos anos, várias famílias de derivados de antocianinas têm sido relatadas em uvas e vinho. Pigmentos antociânicos são responsáveis pela cor das uvas e vinho, uma característica determinada pela sua estrutura química, grau de hidroxilação, metilação e/ou glicosilação (HE *et al*., 2010). Em uvas vermelhas e vinho, seis antocianidinas foram identificadas: cianidina, peonidina, delfinidina, pelargonidina, petunidina e malvidina (HE *et al*., 2010; KOPONEN et al, 2007). Este último é considerado o composto mais representativo em uvas *Vitis vinifera* (CASTILLO-MUÑOZ *et al*., 2010). As antocianidinas são geralmente encontradas na natureza nas formas glicosiladas: 3-monoglucósidos 3,5 e 3,7-diglucósidos (KOPONEN *et al*., 2007). São encontradas apenas na pele da uva, a quantidade de antocianinas e flavonóides extraídos após a vinificação dependem da duração do processo e das condições em que ele ocorre, como a temperatura ou a extensão da ruptura das uvas (ZOECKLEIN *et al.,* 1995).

3.2.4.3. Compostos fenólicos e índice de cor

A coloração dos vinhos está estreitamente relacionada com a sua composição em pigmentos fenólicos (MAZZA *et al*., 1999). Esses pigmentos apresentam uma variação de cor de acordo com certos parâmetros físico-químicos do meio onde se encontram. Um dos aspectos principais a se ter em conta é o pH do vinho, uma vez que, em meio ácido, as antocianinas apresentam uma coloração vermelha que se dissipa à medida que sobe o pH, apresentando uma cor azul-arroxeada quando o pH é superior a 4 e uma cor amarela quando o pH é neutro ou básico (RIBÉREAU-GAYON, *et al*. 2006b). Algumas classes de pigmentos antociânicos apresentam maior estabilidade com mudança de pH e com evelhecimento.

Polifenóis são compostos instáveis e suas reações começam assim que a uva é esmagada ou prensada e continuam durante o processo de vinificação e envelhecimento, resultando uma diversidade grande de novos produtos. Tais produtos apresentam propriedades

organolépticas específicas, frequentemente diferentes daquelas de seus precursores. A composição fenólica nos vinhos depende da uva utilizada e das condições de vinificação que influenciam na extração de vários compostos da uva e das subsequentes reações (CHEYNIER *et al*. 2006). Durante o armazenamento, os pigmentos antociânicos podem provocar mudança de coloração (CEJUDO-BASTANTE *et al*. 2011). O aumento da concentração dos compostos fenólicos ao longo do tempo é atribuído às reações de oxidação e polimerização, onde também ocorrer um aumento na absorbância de 420 nm, diretamente relacionada com o escurecimento dos vinhos (BARÓN *et al.* 1997). Por tanto, os compostos fenólicos são meléculas de grande importância para a enologia, sendo um dos principais responsáveis pelas porpriedades organolépticas do vinho.

3.3. PROCESSO INFLAMATÓRIO E POLISSACARÍDEOS

A reação da imunidade natural, ou inflamação, é uma resposta do organismo a estímulos nocivos. Durante uma invasão por patógeno ou danos nos tecidos do hospedeiro, a imunidade natural é a primeira linha de defesa do organismo (WANG *et al.,* 2002). Essa ação protetora é obtida principalmente pelo recrutamento de leucócitos, seguida por uma cascata de reações bioquímicas que se propagam e iniciam a resposta inflamatória (NAKAMURA *et al*., 1992; VARKI, 1997; SIMON *et al.,* 1999; MULLER, 2003; YADAV *et al*., 2003). Essa migração leucocitária ocorre do compartimento intravascular, principalmente de vênulas pós-capilares, para o tecido onde ocorreu o estímulo nocivo, sendo denominado de extravasamento ou diapedese. Esse mecanismo é constituído de várias etapas: marginação, ligação inicial e rolamento dos leucócitos no endotélio inflamado, ativação dos leucócitos, firme aderência à parede vascular, degradação da membrana basal e migração dos leucócitos para o sítio de inflamação (Figura 4) (SEELY *et al*., 2003; PARISH, 2006; SHERWOOD *et al.*, 2004).

Os indutores inflamatórios no sítio específico levam à produção de citocinas pró-inflamatórias, TNF (Fator de Necrose Tumoral) e IL-1 (Interleucina-1) pelas células locais (BARRINGTON *et al.,* 2001; NAKAMURA *et al.,* 1992; SIEDLE *et al.,* 2003). Essas substâncias irão agir no endotélio vascular, causando alterações intracelulares que culminam na expressão de moléculas de adesão e citocinas, seguida pela modificação do seu citoesqueleto de retração. Isso garantirá a passagem dos leucócitos da circulação para o local da lesão (SIMON *et al*., 1999; HOGG *et al.,* 1995; LASKY, 1992; VARKY, 1997). A aderência e o rolamento de leucócitos no endotélio ativado pelo estímulo inflamatório envolvem selectinas de linfócitos, plaquetas e células endoteliais (L-, P-, e E-selectinas,

respectivamente) e seus ligantes (figura 5) (PARISH, 2006). A L-selectina é expressa constitutivamente por leucócitos e se liga ao endotélio inflamado através das cadeias de heparam sulfato (HS). P- e E-selectina são expressas nas células endoteliais após ativação por citocinas pró-inflamatórias (LOWE, 2003).

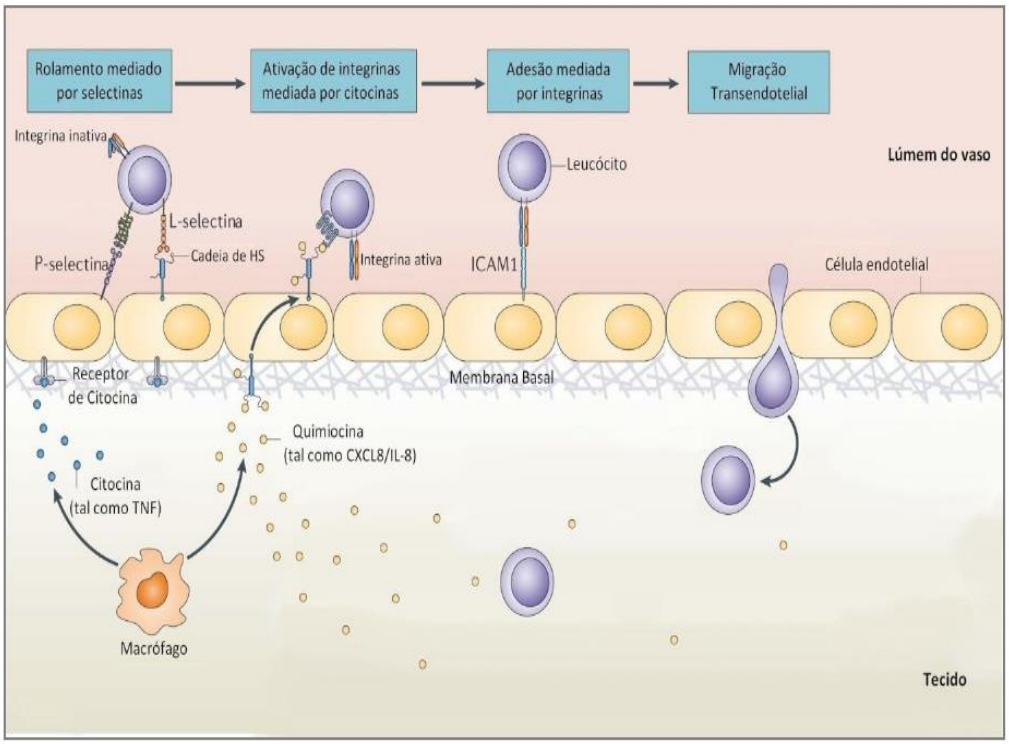

FIGURA 5: Recrutamento de leucócitos e processo inflamatório. FONTE: Adaptada de PARISH, 2006.

Dessa forma, as primeiras células a chegarem ao sítio de injúria são os leucócitos polimorfonucleares (PMNs). Em seguida, aparecem os monócitos, que se transformam em macrófagos nos tecidos e exercem importante papel na fagocitose e eliminação dos antígenos através dos mecanismos microbicidas, inerentes a essas células (ZARBOCK *et al*., 2008). Em um período mais tardio, migram as células da imunidade adquirida, os linfócitos, que auxiliam de modo direto ou indireto as células da imunidade inata em um mecanismo de amplificação da resposta imune.

Quando a resposta inflamatória torna-se exagerada pode culminar em doenças inflamatórias graves como a sepse (SERHAM *et al.*, 2008), sendo necessária a intervenção com uso de moléculas que possam modular o processo inflamatório.

3.3.1. Sepse

A inflamação é considerada uma resposta normal e essencial do hospedeiro que pode ocorrer na presença de agentes infecciosos. Alguns estudos sugerem que o início e a progressão da sepse se devem a uma "desregulação" da resposta normal, com uma ativação maciça e descontrolada de células inflamatórias (PECK, 2007). Em indivíduos que evoluem para esse quadro, observa-se uma completa ativação da resposta imune e isso ocorre devido à liberação de altos níveis de padrões moleculares associados ao dano celular dos micro-organismos invasores e/ou tecido do hospedeiro lesado levando a estimulação de células do sistema imune.

A fisiopatologia da sepse é extremamente complexa e ainda bastante investigada. Seu principal evento é a resposta inflamatória sistêmica ao agente infeccioso (CINEL *et al.,* 2009) após o reconhecimento dos constituintes expressos pelo micro-organismo invasor e os receptores de reconhecimento padrão (RRP), como os receptores Toll-like (TLR) e o CD14, que reconhecem os patógenos ou seus produtos, identificados como PAMPs (padrões moleculares associados a patógenos). As células hospedeiras sintetizam uma série de proteínas incluindo citocinas pró-inflamatórias que, juntamente com outros mediadores gerados pela cascata inflamatória, agem contra os micro-organismos desencadeando distúrbios hemostáticos e disfunção orgânica (LEVI *et al.,* 2010; SEMERARO *et al*., 2010). A inflamação pode também resultar em apoptose ou necrose de células (REETTA *et al*., 2011;CINEL *et al.,* 2009). Evidências recentes indicam que produtos liberados a partir de células mortas, tais como as proteínas nucleares, são capazes de propagar ainda mais inflamação, morte celular e falência de órgãos (CINEL *et al.,* 2009; XU *et al*., 2009) .

A falência de órgãos, também conhecida como síndrome da disfunção de múltiplos órgãos (MODS), provocada pela sepse grave representa a principal causa da elevada taxa de mortalidade nessas condições, causada pela hiper-resposta inflamatória generalizada desencadeada pela invasão de um patógeno (WANG *et al*., 2008). Estudos sugerem que a MODS durante a sepse pode ser causada, também, por espécies reativas de oxigênio e enzimas proteolíticas liberadas pelos neutrófilos recrutados para os tecidos, além disso, a presença de altas concentrações de citocinas no espaço intersticial pode ser tóxica e desempenha importante papel na sepse com leucocitose severa (WANG *et al*., 2008).

Os pulmões, o fígado e os rins são os primeiros órgãos afetados pela MODS na sepse, por esse motivo se faz necessária uma avaliação das funções desses órgãos no estágio inicial da doença, a qual na prática clínica, além do estudo histopatológico, é realizada também por meio de avaliação de parâmetros bioquímico como análises das enzimas aminotransferases e da bilirrubina para avaliação da disfunção hepática e as análises de creatinina sérica e ureia para avaliação da disfunção renal (PRATT *et al.,* 2010).

Diversos trabalhos foram realizados e as atividades biológicas mais comumente atribuídas aos carboidratos de diversas fontes são: atividade anti-inflamatória, antitumoral, antiviral e anticoagulante (BOHN & MILLER, 1995; CAPEK *et al.*, 2003; RENARD *et al.*, 2005, CIPRIANI *et al.*, 2006, 2009; SIMAS-TOSIN *et al.*, 2012; DARTORA *et al.*, 2013).

Polissacarídeos, como ramnogalactofuranana e arabinogalactana de várias fontes apresentaram atividade anti-inflamatória e redução da letalidade de camundongos quando submetidos à sepse polimicrobiana induzida por ligadura e punção cecal (CLP) (SCOPARO *et al.*, 2013). Esse modelo biológico mimetiza a sepse em humanos, causada por patógenos derivados do trato intestinal e é considerada como uma simulação próxima da situação clínica (RITTIRSCH, HUBER-LANG, FLIERL, & WARD, 2009).

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CAPITULO I

Structural Characterization of Polysaccharides from Cabernet Franc, Cabernet Sauvignon and Sauvignon Blanc wines: anti-inflammatory activity in LPS stimulated RAW 264.7 cells

Polysaccharides are present in wines and have important influence on several stages of the winemaking process, including fermentation, filtration, stabilization and are partially responsible for the organoleptic properties of wines (Gerbaud, Gabas, Blounin, Pellerin & Moutounet, 1997; Moine-Ledoux & Dubourdieu, 1999; Vernhet, Pellerin, Belleville, Planque & Moutounet, 1999). However, it has been shown that not all polysaccharides have the same behavior regarding wines. Their influence on wine processing and sensory properties depend on their quantity, class and structural features (Del Barrio-Galan, Pérez-Magarino, Ortega-Heras, Williams & Doco, 2011; Guadalupe & Ayestaran, 2007; 2008; Riou, Vernhet, Doco & Moutounet, 2002; Vidal et al., 2004).

The polysaccharides come from grape berries, yeast, bacterial and fungal grape microbiome. From the enological and quantitative perspective, polysaccharides from grapes and yeast are the most important. Their concentrations depend on many parameters, such as, cultivation, stage of maturity, wine-making techniques and the treatments leading to increased solubilization of the macromolecular components of the grape berry cell walls (Pellerin & Cabanis, 1998). Arabinose and galactose rich polysaccharides (AGs), such as type II arabinogalactan-proteins (AGPs) and arabinans, rhamnogalacturonans type I (RG-I) and type II (RG-II), and homogalacturonans (HLs) come from grape berries, while glucans (GLs), mannans and mannoproteins (MPs) are released by yeast either during fermentation or by enzymatic activity during aging on yeast lees by autolysis (Ayestarán, Guadalupe, León, 2004; Belleville, Brillouet, Tarodo de la Fuente & Moutounet, 1991; Brillouet, Bosso & Moutounet, 1990; Doco & Brillouet, 1993; Doco, Vuchot, Cheynier & Moutounet, 2003; Pellerin, Vidal, Williams & Brillouet, 1995; Vidal, Williams, Doco, Moutounet & Pellerin, 2003).

Nowadays one of the main targets of the wine sector is to improve wine quality, elaborating wines that satisfy consumer demand, and expand the offer of quality wines. Cabernet Franc, Cabernet Sauvignon and Sauvignon Blanc wines, widely produced from grapes of *Vitis vinifera* originating in France, are very popular and quite consumed worldwide. Curiously, there is no information regarding their polysaccharides composition

and structural characterization. Despite this, polysaccharides appear to be the most interesting molecules in enology due to their positive effects on the final quality of the wine (Doco et al., 2003; Fournairon, Camarasa, Moutounet, & Salmon, 2002).

Besides concerns related to the wine quality, there is also great interest in knowing the benefits that moderate consumption of wine can bring to health. So far, many other polysaccharides presented reported biological activities, such as antiviral, antitumor, immunostimulatory, anti-inflammatory, anticomplementary, anticoagulant, hypoglycemic, and anti-ulcer (Capek et al., 2003; Cipriani et al., 2008, 2009; Nergard et al., 2005; Srivastava & Kulshveshtha, 1989; Yamada, 1994). Nevertheless, there are very few reports regarding biological activities and modulation of inflammatory mediators by polysaccharides from wines.

The pathology of inflammation is a complicated process triggered by microbial pathogens, such as: viruses, bacteria, prion, and fungi (Vitaliti et al., 2014). Macrophages account for the first defense line of human body. LPS is usually employed as a model for inflammation due to its ability to stimulate macrophages. Different inflammatory mediators are secreted by macrophages induced by LPS: cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β), and inflammatory mediator nitric oxide (NO) (Agarwal, Piesco, Johns & Riccelli, 1995; Lee et al., 2014). The LPS induced RAW 264.7 (mouse macrophages cell lines) is commonly employed as the anti-inflammation model for anti-inflammation candidate screening *in vitro* (Zhang et al., 2015).

Given the importance of polysaccharides in the wine making process and sensory properties of the beverage, understanding their content, identification and quantification is essential. Different analytical methodologies were developed to determine wine polysaccharides in this study. This preliminary work was aimed at characterizing the spectra of the purified polysaccharides. Such spectral identification of each polysaccharide family (AGs, MPs, and RGs) in Cabernet Franc, Cabernet Sauvignon and Sauvignon Blanc wines have not been reported previously as well as their *in vitro* involvement in the inflammatory process.

2. Materials and methods

2.1. Materials

Cabernet Franc, Cabernet Sauvignon and Sauvignon Blanc wines used in this study belonged to four vintages (2011, 2012, 2013 and 2014). All the wines were made from *Vitis*

vinifera var. and that have evolved during the ageing time. They were stored under cellar conditions before the analyses were conducted. Oenological parameters such as pH or ethanol content were similar across the studied wines, and they were not included in the statistical data treatment.

2.2. Polysaccharides extraction and purification

The polysaccharides were precipitated by addition of cold EtOH (3 vol.), and separated by centrifugation (8.000 rpm at 4 °C, 20 min). The sediment was dissolved in H_2O, dialyzed against water for 72 h to remove the remaining low-molecular weight compounds, giving rise to a crude polysaccharide fraction: WCF (Cabernet Franc), WCS (Cabernet Sauvignon) and WSB (Sauvignon Blanc). For the purification of the polysaccharides, it was chosen the fraction with the highest yield. This fraction was frozen and then allowed to unfreeze at room temperature (Gorin & Iacomini, 1984), resulting in soluble and insoluble fractions which were separated by centrifugation as previously described. The insoluble fraction was not analyzed in this study due to its lower yield and difficult solubilization. The water-soluble fraction was treated with α-amylase and then with Fehling solution (Jones & Stoodley, 1965). The soluble fraction (FCF) was isolated from the insoluble fraction (PCF) by Cu^{2+} complex (Fehling solution) and centrifugation under the same conditions previously described. The respective fractions were both neutralized with HOAc, dialyzed against water and deionized with mixed ion exchange resins and then freeze dried.

2.3. Monosaccharide analysis

WCF, WCS, WSB, FCF and PCF fractions (2 mg) were hydrolyzed with 1 M TFA at 100 °C for 14 h, the solution was then evaporated, and the residue dissolved in water (2 mL). The resulting monosaccharide mixture was examined by thin layer chromatography (TLC) silica-gel 60 (Merck), with ethyl acetate:acetic acid:n-propanol:water (4:2:2:1, v/v), then stained with orcinol-sulfuric acid (Sassaki, Souza, Cipriani, & Iacomini, 2008; Skipski, 1975). The monosaccharides were then reduced with 2 mg $NaBH_4$, yielding alditols, which were acetylated in Ac_2O-pyridine (1:1, v/v, 0.5 mL) at room temperature for 12 h (Wolfrom & Thompson, 1963a; Wolfrom & Thompson, 1963b). The resulting alditol acetates were extracted with $CHCl_3$ and analyzed by gas chromatography–mass spectrometry (GC–MS – Varian, Saturn 2000R, Ion-Trap detector), using a DB-225-MS column (30 m × 0.25 mm ×

0.25 µm), programmed from 50 to 220 ˚C at 40 ˚C/min, with he as carrier gas. Components were identified by their typical retention times and electron ionization (EI 70 eV) spectra. The uronic acids content of the fractions were determined using the colorimetric m-hydroxybiphenyl method (Filisetti-Cozzi & Carpita, 1991).

Carboxy-reduction of FCF (10 mg) was carried out by the carbodiimide method (Taylor & Conrad, 1972), using $NaBH_4$ as the reducing agent, having its uronic acid carboxyl groups reduced to primary alcohols and the results were given as mol % (Pettolino, Walsh, Fincher & Bacic, 2013).

2.4. Methylation analysis

Per-*O*-methylation of each isolated polysaccharide (10 mg) was carried out using NaOH-Me_2SO-MeI as described by Ciucanu and Kerek (1984). The process, after isolation of the products by neutralization (HOAc), dialysis and evaporation, was repeated, and the methylation was complete. The per-*O*-methylated derivatives were hydrolyzed with 72% (v/v) aq. H_2SO_4 (0.5 mL, v/v, 1 h, 0 ˚C), followed by dilution to 8% (v/v). The solution was kept at 100 ˚C for 17 h, then neutralized with $BaCO_3$, filtered and evaporated to dryness (Saeman, Moore, Mitchell, & Millet, 1954). The hydrolyzate was reduced with $NaBD_4$ (sodium borodeuteride) and then acetylated, giving rise to partially *O*-methylated alditol acetates and analyzed by GC–MS, as the previously described, but with a final temperature of 215 ˚C. The compounds were identified by their typical retention times and electron impact spectra (Sassaki, Gorin, Souza, Czelusniak, & Iacomini, 2005).

2.5. Nuclear magnetic resonance (NMR) spectroscopy

1D and 2D NMR spectra were obtained with a 400 or 600 MHz Bruker spectrometer using 5 mm direct or inverse probeheads (Avance III and HD, Bruker, Billerica, Massachusetts, USA). 1D ^{1}H and ^{13}C-NMR at 600 MHz were collected after a 90° (p1) pulse calibration at 10.75 µs. The ^{1}H and ^{13}C chemical shifts were determined after ^{1}H assignments trough 2D COSY and TOCSY analysis; bonding connections were performed by HMBC and $^{1}H/^{13}C$ chemical shift correlation mapping was finally determined by HSQCed (Heteronuclear Single Quantum Coherence edited spectroscopy CH, CH_3 positive phase CH_2 negative phase) performed at 30 ºC in deuterium oxide (D_2O). 2D NMR experiments were recorded using quadrature detection at indirect dimension and acquired using 24 scans per series of 1024x320

data points, with zero filling in F1 (4096) prior to Fourier transformation, using the Software TOPSPIN version 3.2 pl6 (Bruker Biospin, Rheinstetten, Germany). The chemical shifts of the polysaccharide were expressed in δ (ppm) relative to trimethylsilyl propionic acid (TMSP). The ratios of the polysaccharides were calculated by integrating the area of specific anomeric signals corresponding to each cross-peak assignment on the 2D-HSQCed spectra, the volumes were normalized and calibrated to 1.00, using the most intense volume. The structural model was designed using the software ChemSketch v. 12.0 (ACD/Labs, Toronto, Ontario, Canada).

2.6. Determination of homogeneity and molar mass

The homogeneity and molar mass of the purified polysaccharide was determined by high performance steric exclusion chromatography (HPSEC), using a refractive index (RI) detector. Four columns were used in series, with exclusion sizes of 7 x 10^6 Da (Ultrahydrogel 2000, Waters), 4 x 10^5 Da (Ultrahydrogel 500, Waters), 8 x 10^4 Da (Ultrahydrogel 250, Waters) and 5 x 10^3 Da (Ultrahydrogel 120, Waters). The eluent was 0.1 M $NaNO_3$, containing 0.5 g/L NaN_3. The solutions were filtered through a membrane of 0.22 µm pore size (Millipore) and loaded (100 µL, loop) at a concentration of 1 mg/mL. The molar mass of the polymer was estimated using Astra software 4.70.

2.7. Cell Culture

RAW 264.7 macrophages were maintained at 37 °C in humidified atmosphere of 5% CO_2 in DMEM medium supplemented with 10% fetal bovine serum and penicillin/streptomycin/normocin (50 U/mL, 50 g/mL and 100 g/mL, respectively; InvivoGen, San Diego,CA, USA). Exponential phase cells were used throughout the experiments.

2.8. Analysis of cell viability

RAW 264.7 cells in exponential growth phase were seeded in 24-well plates at a density of 4.8 x 10^5 cells/well. The WCF, WCS, WSB, FCF and PCF were added at indicated concentrations (0.1, 1, 10 and 100 µg/mL). The mitochondrial-dependent reduction of 3- (4,5-dimethylthizaol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) to formazan was used to measure cell respiration as an indicator of cell viability (Mosmann, 1983). Briefly, after 24 h

incubation with or without polysaccharides, 350 µL/well of MTT solution (1 mg/mL) was added and the cells were incubated for another 4 h at 37 ºC. After removing the supernatant, 500 µL of DMSO (dimethyl sulfoxide) was added to the cells to dissolve the formazan. The absorbance of each group was measured by a microplate reader at wavelength of 570 nm. The control group consisted of untreated cells was considered as 100% of viable cells. Results were expressed as percentage of viable cells when compared with control group.

2.9. Measurement of cytokines production and NO production

The analysis was based on exposing cell line to fractions WCF, WCS, WSB, FCF and PCF (0.1, 1, 10 and 100 µg/mL) in the presence of lipopolysaccharide (LPS) in the wells. RAW 264.7 cells (4.8 x 10^5 cells/well) were plated into 96-well plates, then were stimulated with LPS (2 µg/mL) and after 1h, were treated with different concentrations of the mentioned fractions. After 24 h, the culture media were collected. The amount of cytokines in the supernatants for TNF-α and IL-1β, respectively, were determined by enzyme-linked immunosorbent assay kits according to the manufacturer's instructions. Three replicates were carried out for each different treatment. The absorbance of each group was measured by a microplate reader at wavelength of 450 nm. To determine the total concentration of NO in the culture media, Griess reagent was added to 40 mL of supernatant and the absorbance at 545 nm was evaluated with an ELISA kit (BD Biosciences, San Jose, CA, USA) following the manufacture manual.

2.10. Statistical analysis

Data were expressed as means ± standard deviation (SD) of five or ten mice examined in each group. Statistical error was determined by one-way ANOVA; the post hoc test was Bonferroni's. Calculations were performed with Graphpad Prism 5.0. *p*-values < 0.001 were considered significant.

3. Results and Discussion

3.1. Isolation and structural analysis of the polysaccharides

Approximately 750 mL of each wine were concentrated and the polysaccharides were recovered by ethanol precipitation followed by centrifugation and dialysis against water (Fig. 1). The solution was then freeze-dried, generating three fractions of polysaccharides namely (WCF) 0.16%, (WCS) 0.05% and 0.02% (WSB).

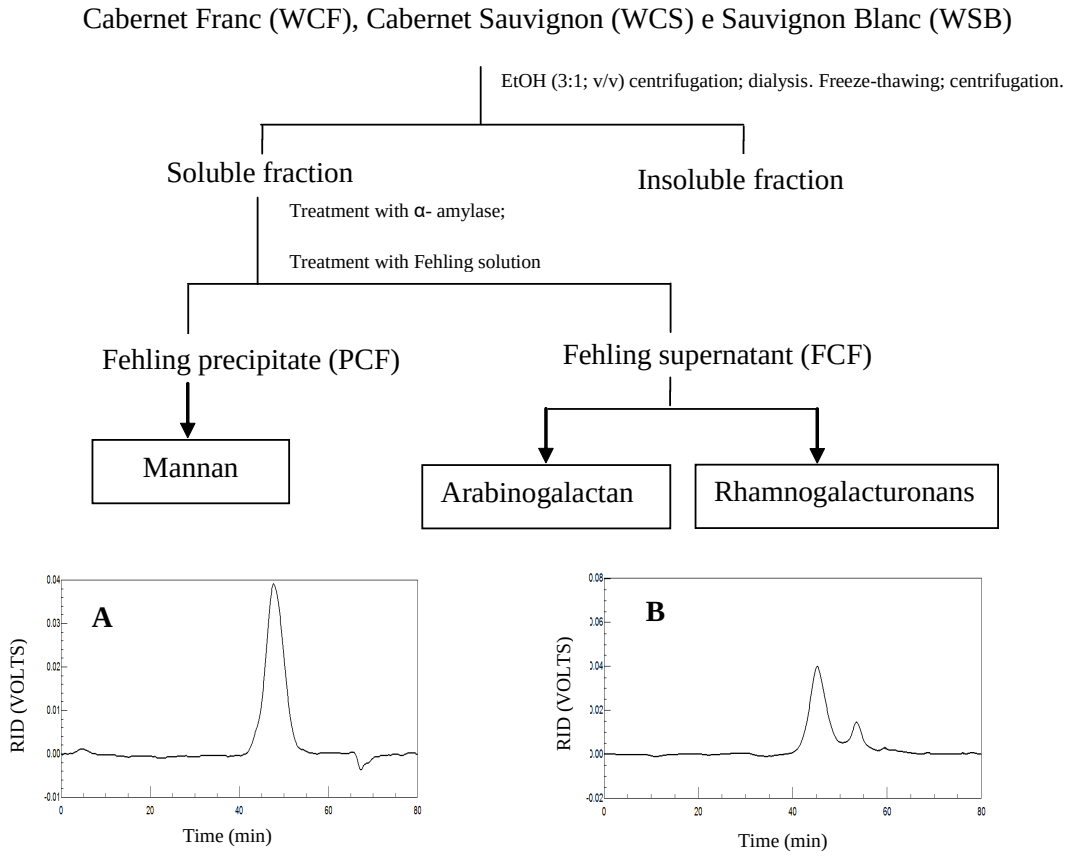
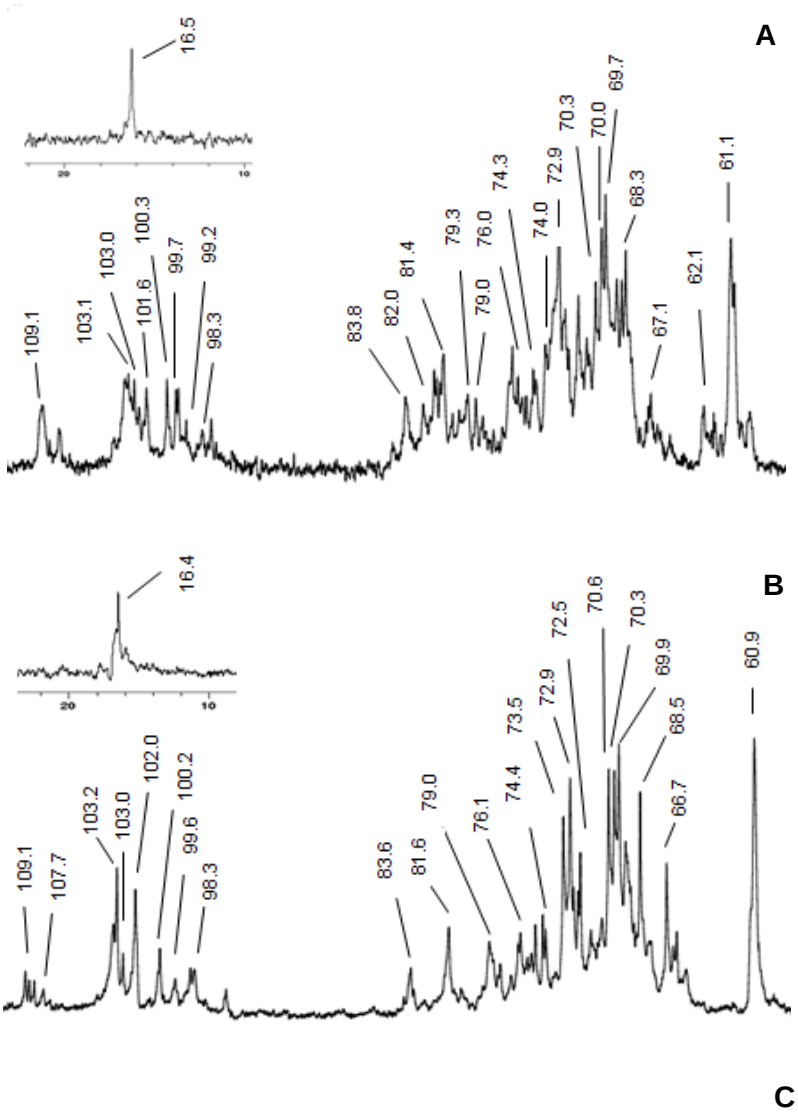


Fig. 1: Scheme of extraction and purification of polysaccharides; Elution profile of (A) PCF and (B) FCF fractions determined by HPSEC using light scattering and refractive index detectors.

The ^{13}C-NMR analysis of the WCF, WCS and WSB fractions showed characteristic signals of polysaccharides (Fig. 2). The anomeric region showed a complex profile, showing many peak overlaps signals due to mixture of polysaccharides.

Since all the polysaccharide fractions showed the same monosaccharide composition, but with different yields, the highest one was chosen for isolation of polysaccharides (WCF). The fractionation and purification of polysaccharides were carried out by a freeze-thawing procedure (Gorin & Iacomini, 1984), resulting in a cold water-soluble fraction. Therefore, this fraction was submitted to complexation with copper, resulting in insoluble (PCF) and soluble (FCF) fractions.



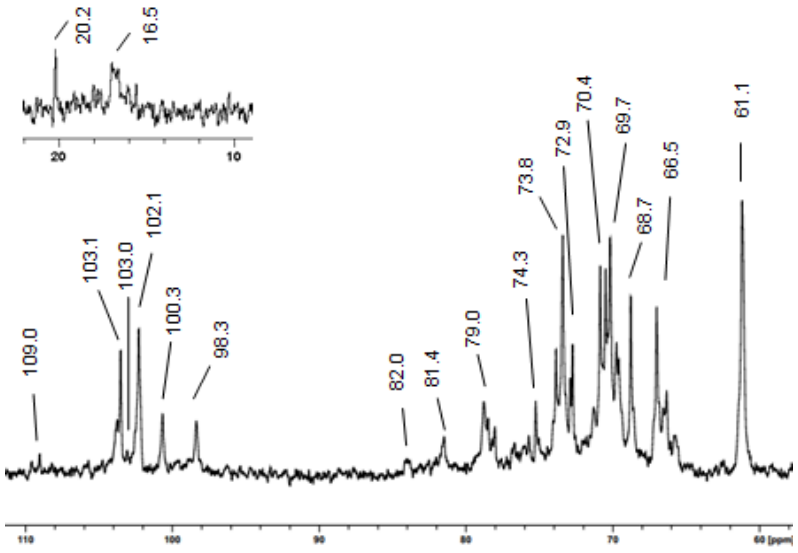


Fig. 2: ^{13}C-NMR spectra of (A) WCF, (B) WCS and (C) WSB in D_2O at 30 ˚C (chemical shifts are expressed in δ ppm).

HPSEC analysis of PCF presented a homogeneous peak (Fig. 1a), with Mw 42,800 g/mol (dn/dc = 0.156). Monosaccharide composition exhibited exclusively mannose, as shown in the table S1 (supporting information), and absence of residual protein, indicating that the polysaccharide found in this sample is a mannan. In order to characterize the glycosidic linkages of the isolated polysaccharide, a methylation analysis was performed. The analysis showed a branched polysaccharide due the presence of non-reducing end units, as 2,3,4,6-Me$_4$-Man*p* and the derivatives 3,4-Me$_2$-Man*p*, 2,3,4-Me$_2$-Man*p* and 3,4,6-Me$_3$-Man*p* (table S2). Some mannose units were 6-*O* and 2,6-di-*O*- substituted (Komura et al., 2010). According to the methylation data, the branching points may be situated mainly at *O*-2 position, suggesting (1→2)-linked-Man*p* units, shown by the presence of the derivative 3,4,6-Me$_3$-Man*p*.

The presence of mannan was commonly found in wines deriving from yeast fermentation (Martínez, Guadalupe, Ayestarán & Perez, 2013), mainly presenting (1→2)-linked-α-D-Man*p* and main chain of *O*-6-substituted α-D-Man*p* units (Kobayashi et al., 1995; Vinogradov, Petersen & Bock, 1998).

The 2D NMR analysis of the mannan (Fig. 3) corroborated with monosaccharide composition (table S1 - supporting information) and with methylation data (table S2 – supporting information). The ^{1}H/^{13}C HSQC spectrum of PCF contain signals at δ 99.2/5.09 and 99.0/5.06 (C-1/H-1), δ 79.5/4.00 and 78.9/3.93 (C-2/H-2) typical of →2,6)-α-D-Man*p*-

(1→. Also, the presence of signals at δ 101.4/5.27, 103.0/5.02 and 103.1/5.12 (C-1/H-1) and δ 79.3/4.09 (C-2/H-2) typical of →2)-α-D-Man*p*-(1→ (Kobayashi et al., 1995; Vinogradov, Petersen & Bock, 1998). In agreement, NMR results and methylation analysis suggest that PCF is a mannan formed by large sequences α-D-Man*p* (1→6) linked and side chains *O*-2 substituted for α-D-mannan (1→2) linked (Fig. S1 – supporting information).

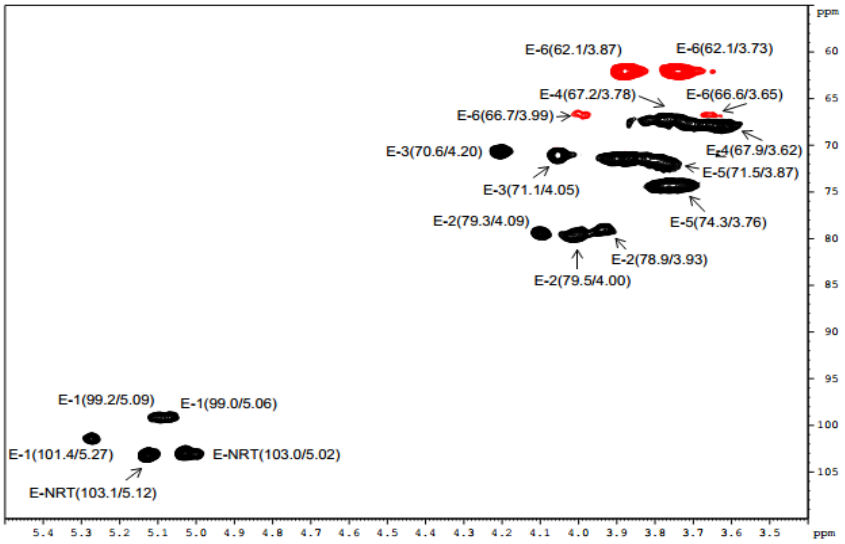


Fig. 3: $^{1}H/^{13}C$ HSQC NMR spectrum of PCF. Solvent D_2O at 30 ºC; numerical values are in δ ppm. E (Man*p*); NRT (non-reducing end units); The letters are followed by the carbon number of the monosaccharide unit. Chemical shifts were determined by HSQCed, COSY, TOCSY and HMBC experiments.

FCF fraction showed a heterogeneous profile (Fig. 1b). It was treated with α-amylase for purification. After this procedure, the monosaccharide composition showed the presence of galactose (62.5%), arabinose (20.8%), rhamnose (8.2%) and galacturonic acid (7.5%) (table 1). Methylation analysis was performed with the carboxy-reduced FCF (Taylor & Conrad, 1972) and the uronic units of FCF were converted into the reduced form.

The methylation analysis of FCF presented a complex and branched polysaccharide by the presence of non-reducing end units of Gal*p*, Ara*f* and the derivatives 2,4-Me_2-Gal*p*, 2,4,6-Me_3-Gal*p* and 2,3,4-Me_3-Gal*p* (table S3 – supporting information). The main chain is probably composed of β-D-Gal*p* (1→3)-linked, due the presence of high amounts of 2,4,6-Me_3Gal*p*. According to the methylation data, the branching points may be situated mainly at

the *O*-6 position of galactose residues. The side chains may be replaced at the *O*-3 position of non-reducing end units of α-L-Ara*f*.

The polysaccharides are derived from grape berries, yeast, bacterial and fungal microbiome, thus the arabinogalactan is derived from the berries of grapes (Doco, Quellec, Moutounet & Pellerin, 1999; Doco, Willams & Cheynier, 2007; Martínez, Guadalupe, Ayestarán & Perez, 2013). These heteropolysaccharides of FCF presented mainly β-D-Gal*p* (1→3)-linked as main chain *O*-6-substituted by β-D-Gal*p* (1→6)-linked units. The side chains may be replaced at the *O*-3 position of non-reducing end units of α-L-Ara*f* (Carpita & Gibeaut, 1993).

In order to resolve the peak overlaps, edited HSQC experiment was performed. FCF showed cross peaks at δ 102.8/4.51 C-1/H-1 of β-D-Gal*p* units (Fig. 4). The signals of C-3/H-3 at δ 81.9/3.93 and C-6/H-6 at δ 69.0/3.80 are from →3,6)-β-D-Gal*p*-(1→ units, whereas the resonances at δ 72.6/3.76 (C-3/H-3) and 61.1/3.76 (C-6/H-6) are from β-D-Gal*p* units 3-*O*- and 6-*O*-substituted, respectively (Cipriani et al., 2006, 2009a; Delgobo, Gorin, Tischer & Iacomini,1999; Fransen et al., 2000; Gorin & Mazurek, 1975; Renard, Lahaye, Mutter, Voragen & Thibault, 1998; Tischer, Gorin & Iacomini, 2002). Typical signals of nonreducing end of α-L-Ara*f* of C-1/H-1 and C-5/H-5 were observed at δ 109.0/5.25 and δ 74.0/4.14, respectively (Delgobo, Gorin, Jones & Iacomini, 1998; Renard et al., 1998).

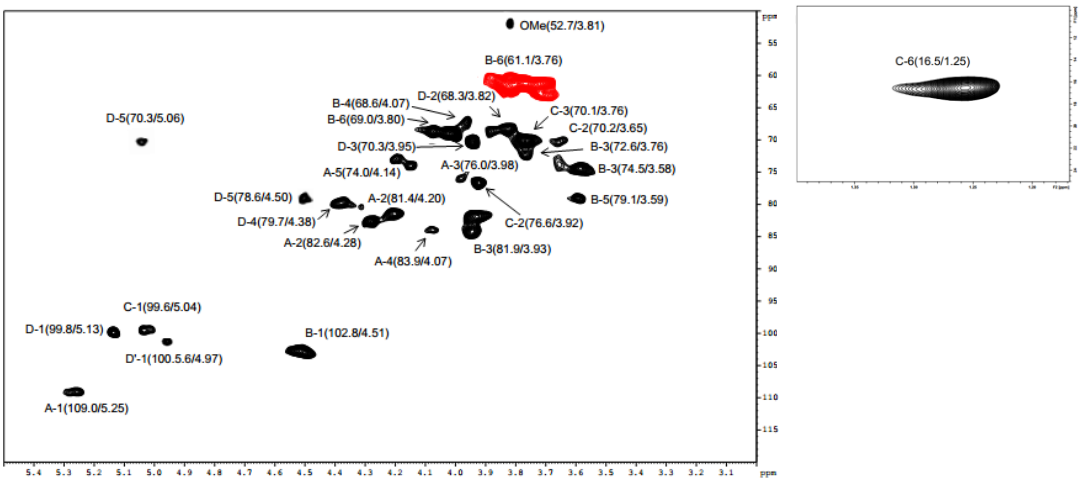


Fig. 4: $^{1}H/^{13}C$ HSQC NMR spectrum of FCF. Solvent D_2O at 30 ºC; numerical values are in δ ppm. A (α-L-Ara*f*); B (β-D-Gal*p*); C (α-L-Rha*p*); D (α-D-Gal*p*A); D' (6-*O*Me-α-D-Gal*p*A). The letters are followed by the carbon number of the monosaccharide unit. Chemical shifts were determined by HSQCed, COSY, TOCSY and HMBC experiments.

The arabinogalactans are classified according to their main chains. Therefore, the NMR result is in agreement with methylation analysis (table S3 – supporting information) and with monosaccharide composition shown in the table S1 (supporting information) that indicated the presence of galactose and arabinose in this fraction, suggesting that the polysaccharide FCF is a type II arabinogalactan, formed by a (1→3)-linked β-D-Gal*p* main chain, substituted at HO-6 by (1→6)-linked β-D-Gal*p* side chains and substituted by nonreducing end-units of arabinose 3-*O*-substituted α-L-Ara*f* chains (Fig. S2).

Moreover, carboxy-reduced derivatives from FCF showed non-reducing end units of Rha*p* and the presence of Gal*p*A (1→4)-linkages, due the derivative 2,3,6-Me_3-Gal*p* (table S3 – supporting information) (Sassaki, Gorin, Souza, Czelusniak & Iacomini, 2005). The presence of 3,4-Me_2-Rha*p*, indicates *O*-2-substitution, which is often found in type I rhamnogalacturonan. According to the methylation data, this polysaccharide is formed by a chain of repeat units (1→4)-linked α-D-Gal*p*A and (1→2)-linked α-L-Rha*p* (Fig. S3) (Carpita & Gibeaut, 1993).

The presence of pectins was confirmed by detection of the sequence of (1 → 4)-linked α- galacturonic acid residues, which gives a fingerprint cross peaks at δ 99.8/5.13 and 100.5/4.97 attributed to C-1/H-1(α-D-Gal*p*A and 6-*O*Me-α-D-Gal*p*A, respectively) and δ 79.7/4.38 (C-4/H-4), indicating this type of linkage (Fig. 4). The latter was confirmed by complete signaling of Gal*p*A units at δ 68.3/3.82 (C-2/H-2), 70.3/3.95 (C-3/H-3), 70.3/5.06 (C-5/H-5 substituted) and 78.6/4.50 (C-5/H-5). Also, the methyl esterification of galacturonic acid is commonly found in type II ramnogalacturonans, being confirmed by the presence of a typical CO_2CH_3 cross peak at δ 52.7/3.81 (Renard et al., 1998; Ovodova et al., 2009; Popov et al., 2011). C-1/H-1 and C-6/H-6 of Rha*p* units (1→2)-linked were shown by resonances at δ 99.6/5.04 and 16.5/1.25, respectively (Renard et al., 1998). The combination of NMR data and methylation analysis suggests that the FCF also presents a type I rhamnogalacturonan, formed by large sequences of →4)-6-*O*Me-α-D-Gal*p*A-(1→ units, interspersed with a few α-L-Rha*p* units, that was also confirmed by the presence of rhamnose and galacturonic acid in the monosaccharide composition (table S1 – supporting information).

Further, the monosaccharide composition analysis of the FCF fraction by GC-MS revealed the presence of units of 2-*O*-methyl-xylose and 2-*O*-methyl-fucose (table S1 – supporting information), which are considered markers of type II rhamnogalacturonan. They also suggest a type II rhamnogalacturonan, although these signals were not observed in NMR spectrum, because of their small amounts in this polysaccharide, corresponding to less than 1% of the total. The 2-*O*-methyl substitution was characterized by electron ionization mass

spectrometry, due the identification of fragments at *m*/*z* 117, 127, 243 and 289 from 2-*O*-methyl-xylitol and at *m*/*z* 117,225, 229 and 275 from 2-*O*-methyl-fucitol (fig. S4 – supporting information).

3.2. Biological experiments

3.2.1. Analysis of cell viability

Macrophages are critical for the natural immune defense system of hosts, which has various immune regulatory functions (Del Carmen Juárez-Vázquez, Alonso-Castro & García-Carrancá, 2013; Schepetkin & Quinn, 2006). Therefore, it was important to evaluate the effects of WCF, WCS, WSB, FCF and PCF fractions on the viability of RAW 264.7 macrophages at the indicated concentrations of 0.1, 1, 10 and 100 μg/mL for 24 h (Fig. 5). Results showed that RAW 264.7 macrophage cells viability was not significantly influenced by fractions at the indicated concentrations of 0.1, 1, 10 and 100 μg/mL (p>0.001). Results indicated that all the fractions were safe up to 100 μg/mL to conduct the assay of anti-inflammatory activity.

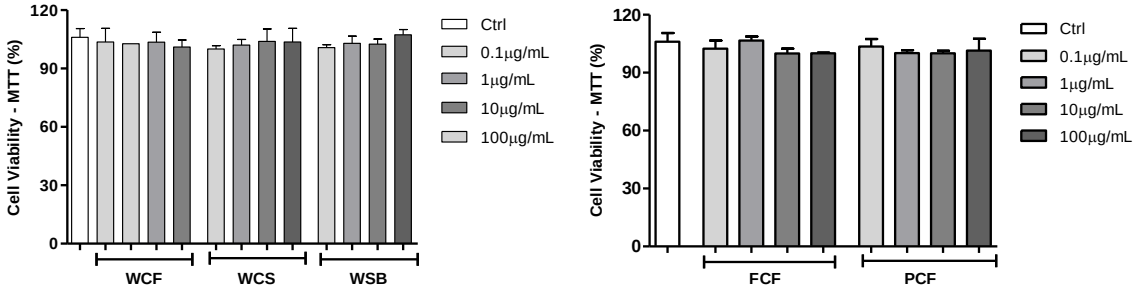


Fig. 5: Effect of WCF, WCS and WSB mixed fractions and the isolated fractions FCF and PCF on the viability of RAW 264.7 cells. The viability was measured by MTT assay. The values for each concentration tested (0.1, 1, 10 e 100 μg/mL) represent the average (mean ± S.D.); Ctrl (control).

3.2.2. Anti-inflammatory activity

Inflammation is a host response to foreign pathogens or tissue injury to eliminate harmful stimuli as well as to initiate the healing and repair process of the damaged tissue (Mariathasan & Monack, 2007). LPS is a major constituent of the cell wall of gram-negative bacteria, which can bind to the TLR 4 receptor of macrophages and induce inflammation (Rossol et al., 2011). In response to LPS, macrophages synthesize and release inflammatory mediators such as NO and produce pro-inflammatory cytokines, such as TNF-α and IL-1β, as already mentioned (Pan, Lin-Shiau & Lin, 2000). To test the inhibitory effects of WCF, WCS, WSB, FCF and PCF fractions on the production of the inflammatory cytokines (TNF-α and IL-1β) and mediator (NO) from LPS-stimulated RAW 264.7 cells, RAW 264.7 cells were treated with various concentrations of fractions (0.1, 1, 10 and 100 μg/mL) and then were incubated with or without 2 μg/mL of LPS for another 24 h.

LPS is one of the leading activators of macrophages. The macrophages play important roles in inflammation through the production of several pro-inflammatory molecules, including NO. Production of excessive NO has been associated with a range of inflammatory diseases, including arteriosclerosis, ischemic reperfusion, hypertension and septic shock (Pacher, Beckman & Liaudet, 2007; Terao, 2009). Therefore, inhibition of NO production in LPS stimulated RAW 264.7 cells is one of the possible ways to screen for anti-inflammatory drugs or disease prevention thorough moderate wine intake. In this study WCF, WCS, WSB, FCF and PCF fractions showed the inhibition of NO production in cells, indicating the anti-inflammatory properties. As shown in figure 6 (K-O), while treatment both with the mixed fractions and fractions with isolated polysaccharides induced a significant inhibitory effect and it was observed that this effect was enhanced increasing the concentration of the fractions with a maximal inhibitory effect at the dose of 100 μg/mL.

Pro-inflammatory mediator NO plays a key role in the pathogenesis of many inflammatory diseases. The NO pathway is known to induce ROS production (Jung et al., 2010; Liu, Cheng, Chen & Yang, 2005). ROS is critical for LPS-induced inflammation through the activation of NF-κB related signaling (Asehnoune, Strassheim, Mitra, Kim & Abraham, 2004). Activated NF-κB acts as a transcription factor, leading to increased production of pro-inflammatory cytokines such as TNF-α and IL-1β (Janeway & Medzhitov, 2002). Thus, blocking the effects of pro-inflammatory mediators could be an effective therapeutic strategy. In figure 6 (A-J) it is shown that all fractions tested significantly decreased the TNF-α and IL-1β production at all doses, indicating that the polysaccharides found in wine have anti-inflammatory properties. Thus, blocking the effects of pro-

inflammatory mediators when drinking wine offers an attractive therapeutic strategy or preventive effect.

Statistical analysis was performed comparing the fractions isolated with the mixture fraction to evaluate which fraction would be exerting the anti-inflammatory effect, but no difference was observed between them in any of the doses tested, indicating that all of them had the same action. Our study showed that the mixture and the isolated polysaccharides (type II arabinogalactan, type I and II rhamnogalacturonans and mannan) significantly reduced production of these pro-inflammatory cytokines and mediator in the LPS-induced RAW 264.7 mouse macrophage cells *in vitro* and can contribute to the beneficial properties of wine, as well as resveratrol has been shown to exert its protective effect against cardiovascular disease, ischemia-reperfusion injury and diabetes mellitus through modulation of adipocyte/fibroblast biology, oxidative stress and inflammation (Farghali, Kutinova & Lekic, 2013; Gertz et al., 2012; Lakshminarasimhan et al., 2013). However, recently Chiu-Tsun Tang et al. (2014) reviewed the studies carried out with resveratrol and its biological effects and concluded that after more than 20 years of research there is still no evidence of biological activity of this compound in humans. Therefore, these benefits may be associated with other components of wines, such as polysaccharides.

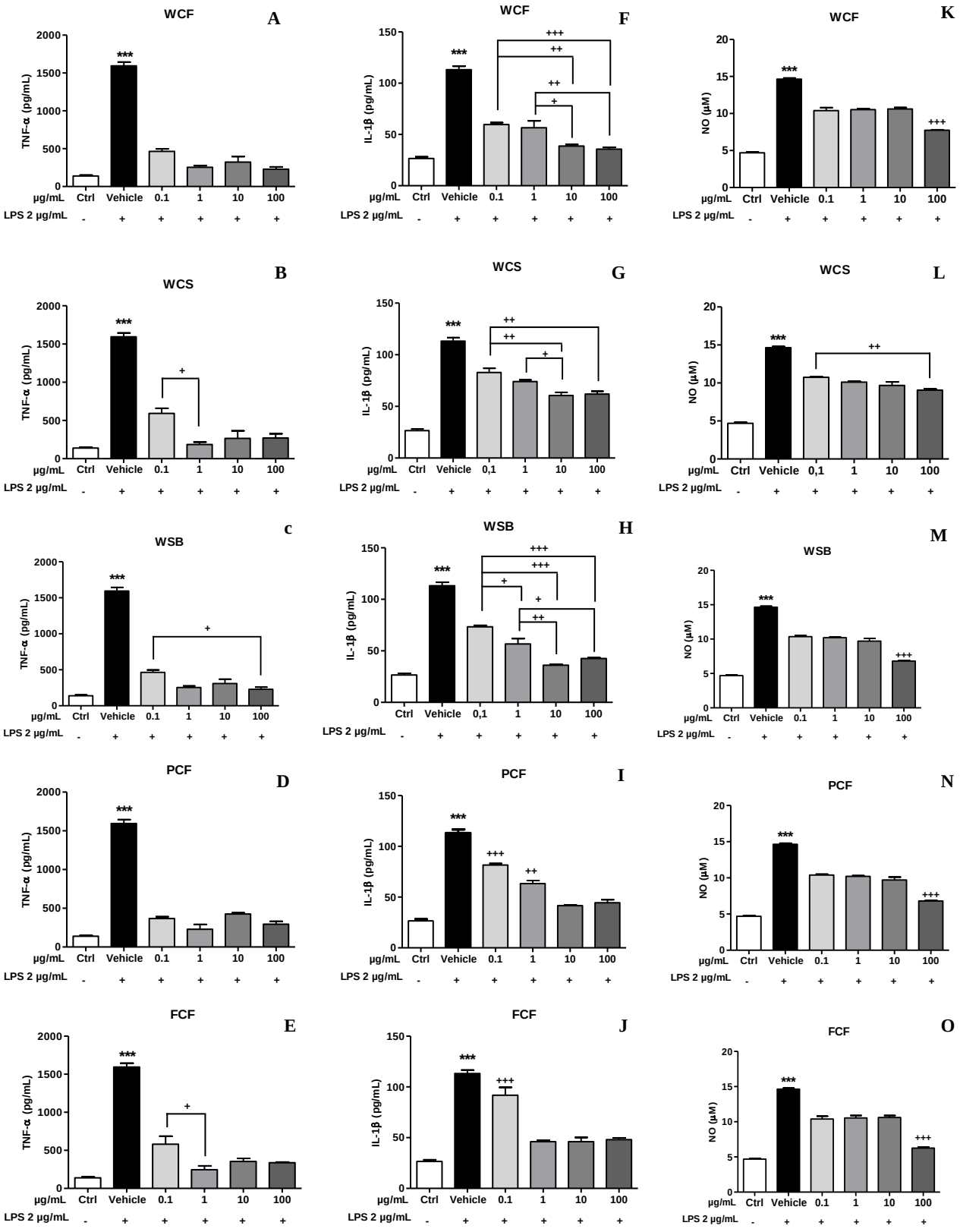


Fig. 6: Anti-inflammatory activities of WCF, WCS, WSB, PCF and FCF on RAW 264.7 cells. (A-E) Expression levels of TNF-α. (F-J) Expression levels of IL-1β. (K-O) NO production levels. Ctrl (control).

4. Conclusions

In conclusion, on the basis of chemical data, the polysaccharides WCF, WCS and WCSB consist of: mannan, formed by large sequences α-D-Man*p* (1→6) linked and side chains *O*-2 substituted for α-D-mannan (1→2) linked; type II arabinogalactan, formed by a (1→3)-linked β-D-Gal*p* main chain, substituted at HO-6 by (1→6)-linked β-D-Gal*p* side chains, respectively and nonreducing end-units of arabinose 3-*O*-substituted α-L-Ara*f* chains; type I rhamnogalacturonan, formed by a chain of repeat units (1→4)-linked α-D-Gal*p*A and (1→2)-linked α-L-Rha*p*; and traces of type II rhamnogalacturonan.

The present study suggests that the mixed fractions (WCF, WCS and WSB) and fractions with isolated polysaccharides FCF (type II arabinogalactan/type I and II rhamnogalacturonans) and PCF (mannan) exerted anti-inflammatory effect on the LPS-induced RAW 264.7 mouse macrophage cells *in vitro*. These activities were mediated by decreasing inflammatory cytokines (TNF-α and IL-1β) and mediator inflammatory (NO).

Supporting Information

Table S1

Monosaccharide composition of fractions WCF, WCS, WSB, PCF and FCF.

Sample	Monosaccharides %							
	Gal	Ara	Rha	Man	Glc	Xyl[1]	Fuc[2]	GalA*
WCF	42.7	11.1	6.7	20.2	13.6	0.4	0.3	5.0
WCS	35.3	10.0	7.0	28.4	12.7	0.3	0.2	6.1
WSB	24.5	9.2	6.2	46.4	7.5	0.2	0.2	5.8
PCF	--	--	--	100	--	--	--	--
FCF	62.5	20.8	8.2	--	--	0.6	0.4	7.5

(1) The xylose found is 2-*O*-methyl-xylose. (2) The xylose found is 2-*O*-methyl-fucose. *The galacturonic acid was determined by the spectrophotometric method of Filisetti-Cozzi and Carpita (1991).

Table S2

Profile of partially *O*-methylated alditol acetates obtained by methylation analysis of PCF.

O-Me-alditol acetate	Structure	Mol%
2,3,4,6-Me_4-Man	Man*p*-(1→	42.0
3,4-Me_2-Man	→2,6)-Man*p*-(1→	42.4
3,4,6-Me_3-Man	→2)-Man*p*-(1→	8.0
2,3,4-Me_3-Man	→6)-Man*p*-(1→	7.6

Table S3

Profile of partially *O*-methylated alditol acetates obtained by methylation analysis of FCF.

O-Me-alditol acetate	Structure	Native	Carboxyl-reduced
2,3,4-Me_3-Rha	Rha*p*-(1→	2.0	1.2
3,4-Me_2-Rha	→2)-Rha*p*-(1→	4.1	3.7
2,3,6-Me_3-Gal	→4)-Galp-(1→	--	4.1
2,4,6-Me_3-Gal	→3)-Gal*p*-(1→	15.1	17.6
2,4-Me_2-Gal	→3,6)-Gal*p*-(1→	38.3	36.2
2,3,4-Me_3-Gal	→6)-Gal*p*-(1→	11.0	9.0
2,3,4,6-Me_4-Gal	Gal*p*-(1→	9.8	10.2
2,3,5-Me_3-Ara	Ara*f*-(1→	19.7	18.0

*The galacturonic acid content of FCF was 1% after carboxy-reduction, according to the spectrophotometric method of Filisetti-Cozzi and Carpita (1991).

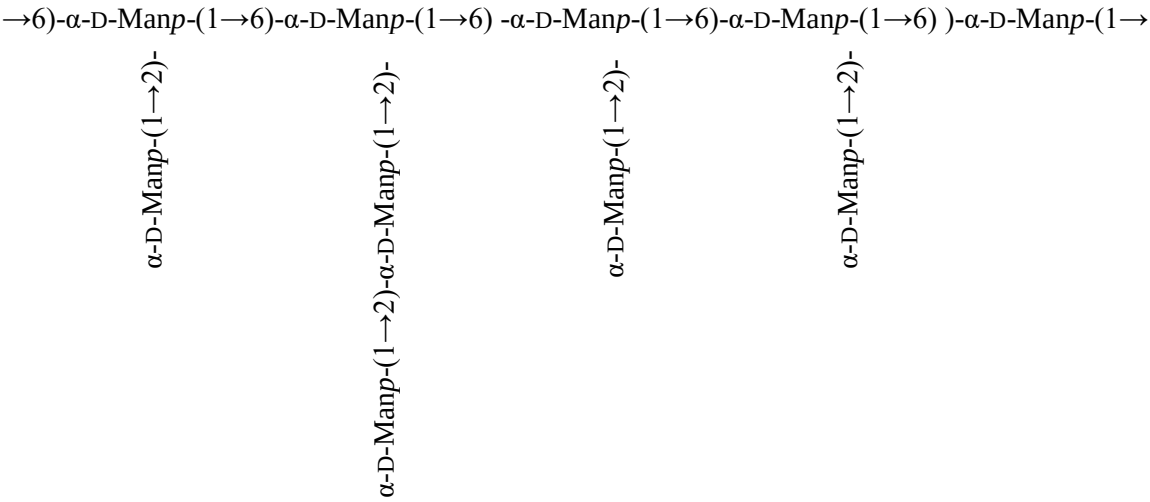


Figure S1: Suggested structure for mannan isolated.

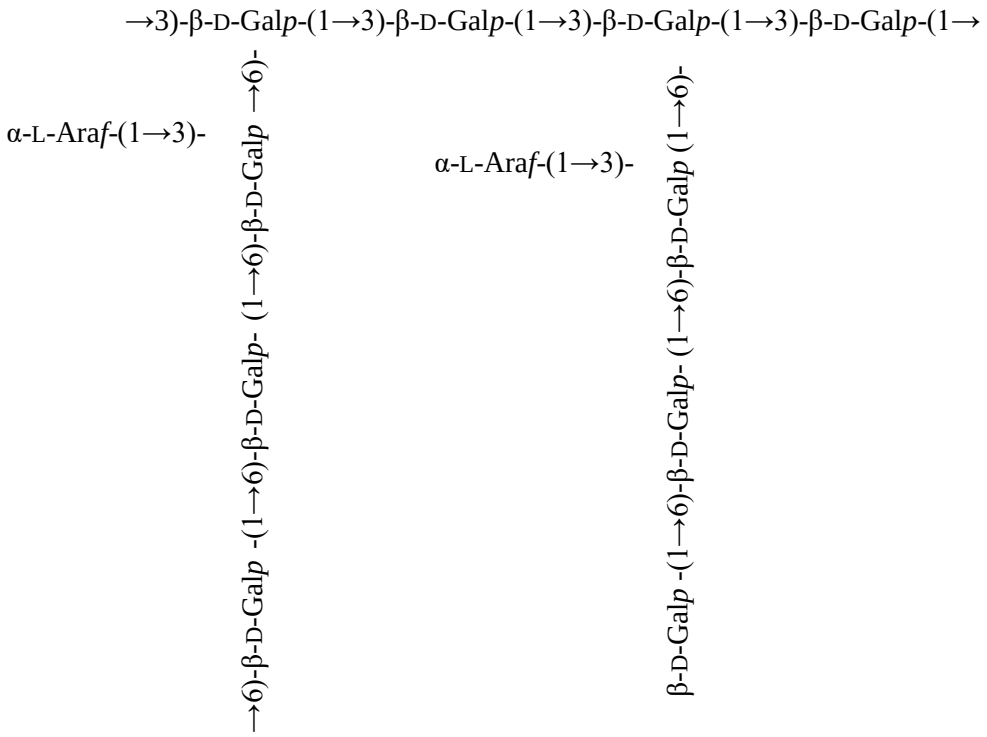


Figure S2: Suggested structure for AGII.

→4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→

Figure S3: Suggested structure for RGI.

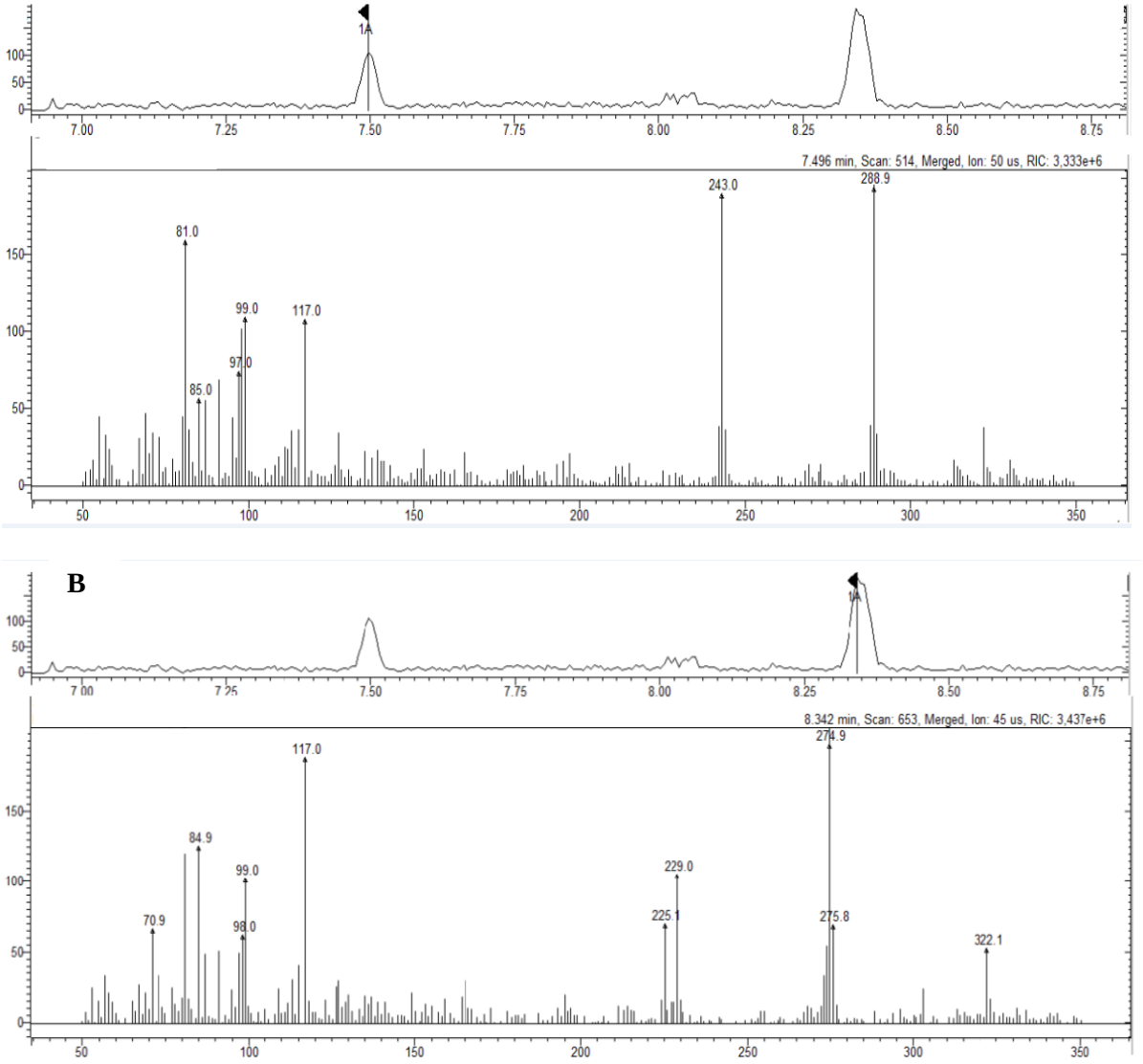


Figure S4: Chromatogram and mass spectra containing retention and fragmentation time from (A) 2-*O*-methyl-xylitol and those from (B) 2-*O*-methyl-fucitol, the identification of fragments at m/z.

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CAPITULO II

Cabernet Sauvignon wine polysaccharides attenuate sepsis inflammation and lethality in mice

Cabernet Sauvignon red wine, produced from *Vitis vinifera* grapes, originally from France, is very popular and quite consumed worldwide. Polysaccharides are one of the main macromolecules found in wine and coming mainly from grape berries, yeast, bacteria and fungal grape microbiome. Their concentrations depend on many parameters, such as, cultivation, stage of maturity, wine-making techniques and the treatments leading to increased solubilization of the macromolecular components of the grape berry cell walls (Carpita & Gibeaut, 1993; Martínez-Lapuente, Guadalupe, Ayestaran, Ortega-Heras & Perez-Magarino, 2013; Pellerin & Cabanis, 1998). Arabinogalactan-proteins, arabinogalactan, rhamnogalacturonans and homogalacturonans come from grape berries, while glucans, mannans and mannoproteins are released by yeast either during fermentation or by enzymatic activity during ageing on yeast lees by autolysis (Ayestarán, Guadalupe, & León, 2004; Brillouet, Bosso, & Moutounet, 1990; Doco & Brillouet, 1993; Doco, Vuchot, Cheynier, & Moutounet, 2003; Quijada-Morín, Williams, Rivas-Gonzalo, Doco & Escribano-Bailón, 2014; Vidal, Williams, Doco, Moutounet, & Pellerin, 2003).

Bezerra et al. 2018 has already demonstrated that the Cabernet Franc, Cabernet Sauvignon and Sauvignon Blanc wines present of mannan, formed by large sequences 1,6-linked α-D-Man*p* and side chains *O*-2 substituted for 1,2-linked α-D-mannan; type II arabinogalactan, formed by a 1,3-linked β-D-Gal*p* main chain, substituted at HO-6 by 1,6-linked β-D-Gal*p* side chains, which are 3-*O*-substituted α-L-Ara*f* chains or nonreducing α-L-Ara*f*; type I rhamnogalacturonan, formed by a chain of repeat units 1,4-linked α-D-Gal*p*A and 1,2-linked α-*L*-Rha*p*; and traces of type II rhamnogalacturonan. In this work was proposed the evaluation of the reproducibility of polysaccharide similarity and its present yields among Cabernet Sauvignon wines from different vintages.

There are few studies regarding wine polysaccharides composition and structural characterization performed by NMR, and very few using the mixture of the polysaccharides. There is a great interest in knowing the benefits that wine moderate consumption can bring to health. Some authors had described important immunomodulatory, antioxidant, anti-septicemic, anti-inflammatory, antineoplastic and gastroprotective effects of polysaccharides (Bezerra et al, 2018; Cipriani et al., 2009; Cordeiro Caillot et al., 2018; Dartora et al., 2013;

Inngjerdingen et al., 2014; Mellinger et al., 2008; Mueller & Anderer, 1990; Nascimento et al., 2013; Park et al., 2013; Stipp et al., 2016). Some beneficial effects of wine on health have been attributed to resveratrol, a polyphenol present in the skin of grapes (Diaz-Gerevini et al 2016; Gojkovic-Bukarica et al., 2018). Nevertheless, there are very few studies regarding biological activities and modulation of inflammatory mediators by wine polysaccharides.

Polysaccharides, such as rhamnogalactofuranan and arabinogalactan from various sources had an anti-inflammatory activity and decreased mice lethality submitted to polymicrobial sepsis induced by Cecal Ligation and Puncture (CLP) (Cordeiro et al., 2012; Scoparo et al., 2013). This biological model mimics sepsis in humans, caused by pathogens derived from the intestinal tract and is considered to closely simulate clinical situation (Rittirsch, Huber-Lang, Flierl, & Ward, 2009). Sepsis represents a state of pro-inflammatory mediators overproduction, a systemic inflammatory response that can cause organ dysfunction, especially in vital organs such as liver, kidney and lung, and consequently, multiple organ failure (Abraham et al, 1997; Angus, Linde-Zwirble, Lidicker, & Clermont, 2001; Cohen, 2002; Hotchkiss e Karl, 2003).

Therefore, this work also was analysis of Cabernet Sauvignon red wine polysaccharides and their anti-inflammatory activity against murine sepsis, evaluating effects on lethality, leukocytes migration, anti- and pro-inflammatory cytokines levels, and biochemical, hematological and histopathological parameters.

1. Materials and methods

1.1. Materials

Cabernet Sauvignon polysaccharides were extracted from commercial wine belonged to three vintages (2016, 2015 and 2014). The wine was made from *Vitis vinifera* cv. Cabernet Sauvignon grapes grown in the Central Valley - Chile, semi-arid Mediterranean climate with strong influence of the Andes Mountain. Maturation of the wines of the three harvests was in French oak barrels. The wines have pH 3.62%, total acidity 3.41 g/L, alcoholic content 12.5% and residual sugar 3.19 g/L. These oenological parameters were similar across the studied wines and were not included in the statistical data treatment.

1.2. Polysaccharides extraction and purification

Polysaccharides were precipitated by addition of cold EtOH (3 vol.) and separated by centrifugation (8.000 rpm at 4 °C, 20 min). The sediment was dissolved in H_2O, dialyzed against distilled water with a 6-8 kDa cut-off membrane for 72 h to remove the remaining low-molecular weight compounds, giving rise to a crude polysaccharide fraction: WCS (Cabernet Sauvignon). This fraction was frozen and then allowed to unfreeze at room temperature (Gorin & Iacomini, 1984), resulting in soluble and insoluble fractions which were separated by centrifugation as previously described. The insoluble fraction in freeze-thawing process was not analyzed in this study due to its lower yield and difficult solubilization. The water-soluble fraction was treated with α-amylase and then with Fehling solution (Jones & Stoodley, 1965).

The fraction treated with Fehling solution was centrifuged under the same conditions previously described and the soluble fraction in Fehling solution (FCS) was isolated from the insoluble fraction in Fehling solution (PCS). The respective fractions were both neutralized with HOAc, dialyzed against distilled water with a 6-8 kDa cut-off membrane for 24 h and submitted to a cathionic exchange resins (Amberlite-IR120-Sigma) and then freeze dried.

1.3. Monosaccharide analysis

WCS, FCS and PCS fractions (2 mg) were hydrolyzed with 1 M TFA at 100 °C for 14 h, the solution was then evaporated, and the residue dissolved in water (2 mL). The monosaccharides were then reduced with 2 mg $NaBH_4$, yielding alditols, which were acetylated in Ac_2O-pyridine (1:1, v/v, 0.5 mL) at room temperature for 12 h (Wolfrom & Thompson, 1963a; 1963b). The resulting alditol acetates were extracted with $CHCl_3$ and analyzed by gas chromatography–mass spectrometry (GC–MS – Varian, Saturn 2000R, Ion-Trap detector), using a DB-225-MS column (30 m × 0.25 mm × 0.25 μm), programmed from 50 to 220 °C at 40 °C/min, with He as carrier gas. Components were identified by their typical retention times and electron ionization (EI 70 eV) spectra. The uronic acid content of the fractions was determined using the colorimetric m-hydroxy biphenyl method (Filisetti-Cozzi & Carpita, 1991).

1.4. Structural identification by NMR

The NMR spectra were obtained with a 400 or 600 MHz Bruker spectrometer using 5 mm direct or inverse probe heads (Avance III and HD, Bruker, Billerica, Massachusetts, USA). 1D ^{1}H and ^{13}C NMR at 600 MHz were acquired after a 90° (p1) pulse calibration at 10.75 μs. $^{1}H/^{13}C$ chemical shift correlation mapping was finally determined by COSY, TOCSY HSQCed (Heteronuclear Single Quantum Coherence edited spectroscopy CH, CH_3 positive phase CH_2 negative phase) performed at 30 °C in deuterium oxide (D_2O) (Sassaki et al., 2013). 2D NMR experiments were conducted using quadrature detection at indirect dimension and acquired using 24 scans per series of 1024 × 320 data points, with zero filling in F1 (4096) prior to Fourier transformation, using the Software TOPSPIN version 3.2 pl6 (Bruker Biospin, Rheinstetten, Germany). The chemical shifts of the polysaccharide were expressed in δ (ppm) relative to trimethylsilyl propionic acid (TMSP).

1.5. *In vivo* experiments

Male Swiss mice (25–35 g) provided by Federal University of Paraná facilities were kept in a temperature-controlled room (20 ± 2 °C) on a 12 h light–dark cycle. Food and water were freely available. Experiments were performed after approval of the protocol by the university Institutional Ethics Committee (sepsis model, CEUA/BIO-UFPR, protocol number 1018). All experiments were conducted in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for the investigation of experimental pain in conscious animals (Zimmermann, 1983). The number of animals and intensities of noxious stimuli used were the minimum necessary to demonstrate consistent effects of the treatment.

1.6. Sepsis induction by cecal ligation and puncture (CLP)

The WCS crude fraction was tested in two routes of administration: subcutaneous (abbreviation s.c.) and the fraction will be called CSS in this route of administration at doses of 0.1, 1 and 10 mg/kg and oral (abbreviation p.o.) and the fraction will be called CSO in this route of administration at doses of 3, 7 and 10 mg/kg. FCS and PCS isolated fractions were tested subcutaneously at doses of 0.1, 1 and 10 mg/kg. All doses (subcutaneous and oral) were prepared using sterile saline solution for solubilization of the polysaccharide fractions.

Mice were randomly divided into groups with 10 animals each: sham-operation, CLP plus saline (10 mL/kg s.c.), CLP plus CSS (0.1 mg/kg s.c.), CLP plus CSS (1 mg/kg s.c.), CLP plus CSS (10 mg/kg s.c.), CLP plus FCS (0.1 mg/kg s.c.), CLP plus FCS (1 mg/kg s.c.), CLP plus FCS (10 mg/kg s.c.), CLP plus PCS (0.1 mg/kg s.c.), CLP plus PCS (1 mg/kg s.c.), CLP plus PCS (10 mg/kg s.c.), CLP plus CSO (3 mg/kg, p.o.), CLP plus CSO (7 mg/kg, p.o.), CLP plus CSO (10 mg/kg, p.o.) and CLP plus dexamethasone - Dex (0.5 mg/kg s.c.). Saline was used as vehicle for dissolving the polysaccharide and dexamethasone was commercially purchased.

Ketamine (80 mg/kg) and xylazine (20 mg/kg) were injected intraperitoneally to anesthetize the mice before surgical procedures. Polymicrobial sepsis was induced by CLP as previously described (Rittirsch, Huber-Lang, Flierl, & Ward, 2009). A midline incision of ~1.5 cm was carried out on the abdomen. The cecum was carefully exposed and 50% of the distal moiety was ligated. The cecum was then punctured thrice with a sterile 16-gauge needle and squeezed to extrude the fecal material from the wounds. The cecum was replaced and the abdomen was stitched surgically. Sham-control animals were treated identically, but no cecal ligation or puncture was carried out. Each mouse received a subcutaneous sterile saline injection (1 mL) for fluid resuscitation after surgery. The mice were then kept on a heating pad (35 °C) until they recovered from the anesthesia. Food and water, *ad libitum*, were provided throughout the experiment. The survival rate was monitored for 7 days, every 12 h. During this period, water (vehicle) and drugs were orally or subcutaneously administered daily. Dexamethasone was commercially purchased and subcutaneously administered at doses of 0.5 mg/kg (Lapa, Silva, Almeida Cabrini, & Santos, 2012; Longhi-Balbinot et al., 2012; Silva et al., 2012).

In this experimental set, mice were orally or subcutaneously treated with vehicle (1 h before surgery), sham-operation, CLP plus saline (10 mL/kg s.c.) and dexamethasone (0.5 mg/kg s.c.). The best dose of each fraction in the survival test was chosen for the anti-inflammatory evaluation tests, as follows: CSS (1 mg/kg, s.c.), CSO (10 mg/kg, p.o.), FCS (1 mg/kg, s.c.) and PCS (1 mg/kg, s.c.). For these doses, after a post-operation time of 6 h, mice were sacrificed. Liver, lung and kidney tissues were collected for histological analysis. Blood was collected for hematological and biochemical parameters evaluation.

1.7. Hematological assays

Blood was collected from the abdominal vena cava. Total leukocytes and mononuclear (MN) and polymorphonuclear (PMN) leukocytes were counted by an automated system, according to the manufacturer's instructions (Mindray BC-2800 vet, São Paulo, SP, Brazil).

1.8. Biochemical assays

The amount of TNF-α, IL-1β and IL-10 cytokines in the blood plasma were determined by enzyme-linked immunosorbent assay kits according to the manufacturer's instructions. Three replicates were carried out for each different treatment. The absorbance of each group was measured by a microplate reader at the wavelength of 450 nm. To determine the total concentration of nitric oxide (NO) in the blood plasma, Griess reagent was added to 40 mL of plasma and the absorbance at 545 nm was evaluated with an ELISA kit (BD Biosciences, San Jose, CA, USA) following the manufacturer´s manual.

Bilirubin, creatinine (CREA) and urea, as well as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels and activities were analyzed from plasma by an automated system, according to the manufacturer's instructions (Mindray BS-200, São Paulo, SP, Brazil).

1.9. Histopathological analysis

Lungs, liver and kidney tissue sections were fixed in ethanol at room temperature. After fixation, samples were dehydrated in a graded series of increasing ethanol before paraffin embedding. Thin sections (4 mm) from paraffin blocks were processed for histology, and the tissue was stained with hematoxylin eosin. The slides were then analyzed by light microscopy.

1.10. Statistical analysis

The results were expressed as mean ±SEM and were statistically analyzed by one-way analysis of variance (ANOVA) and Tukey's test as a post hoc test ($P < 0.001$). GraphPad Prism 5.0 (La Jolla, CA) program was used for both statistical analysis and figure design. Histological analysis was performed by qualitative observations.

2. Results and Discussion

2.1. Analysis of the polysaccharides of Cabernet Sauvignon wine

Approximately 750 mL from commercial wine was concentrated and precipitated with excess ethanol. The polysaccharides were recovered by ethanol precipitation and then centrifugated and dialysed against water. The solution was then freeze-dried, generating the WCS polysaccharide fraction with yield of 0.05% in the harvests of the three years evaluated and the table 2 show total polysaccharide yield in mass to 750 mL of wine.

Previous studies carried out by our group on polysaccharide structure demonstrated that fraction WCS from Cabernet Sauvignon is composed by a mixture of mannan, type II arabinogalactan, type I and II rhamnogalacturonans (Bezerra et al., 2018).

^{1}H NMR spectra analyses of the crude polysaccharide fractions from Cabernet Sauvignon wine from 2014 (WCS-2014), 2015 (WCS-2105) and 2016 (WCS-2016) were performed (Fig. 1). ^{1}H-NMR data of WCS of the three vintages showed characteristic polysaccharide NMR signals, showing a complex profile at the anomeric region (4.0~6.0 ppm). The ^{1}H NMR signals shown in figure 1 the anomeric region and the binding region were similar for all three fractions. This experiment demonstrated that the wines have similar polysaccharide patterns even though they were produced in different years.

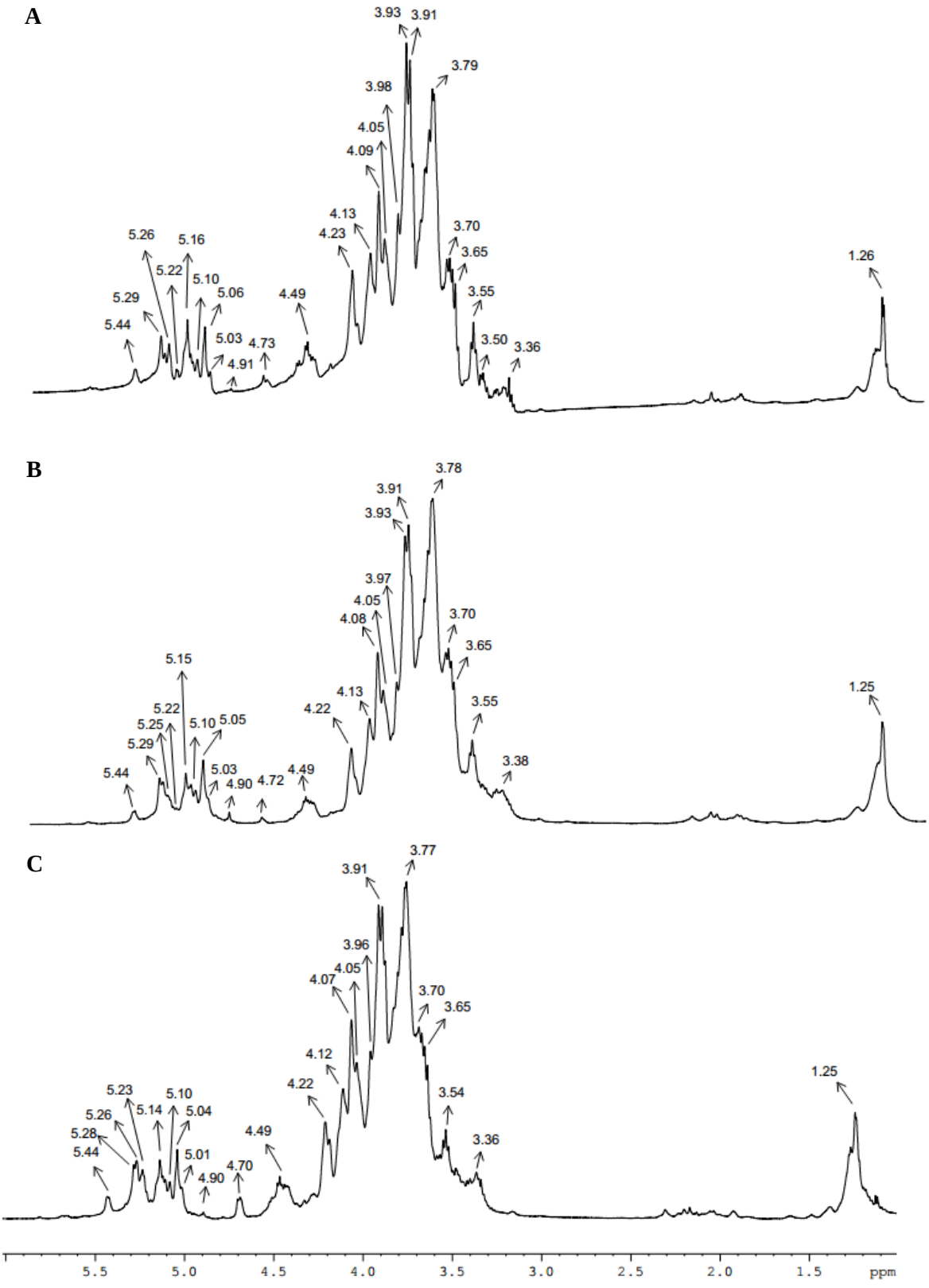

Figure 1: ^{1}H-NMR spectra of (A) WCS-2014, (B) WCS-2015 and (C) WCS-2016 in D_2O at 30 ˚C (chemical shifts are expressed in δ ppm).

The monosaccharide composition was performed for the crude fractions extracted from wine bottles belonged to three vintages (table 1). All the fractions are composed by galactose, mannose, glucose, rhamnose, galacturonic acid, arabinose, 2-*O*-methyl-fucose and

2-*O*-methyl-xylose and it was observed that there was a minimum variation among the fractions analyzed, showing that all the fractions presented the same polysaccharides in different vintages.

Table 1

Composition Monosaccharide composition of the red wine polysaccharides of different vintages

Fraction	Monosaccharide %							
	Gal	Ara	Rha	Man	Glc	Xyl[1]	Fuc[2]	GalA*
WCS-2014	36.5	11.2	8.1	23.9	12.9	0.3	0.2	6,9
WCS-2015	38.1	13.2	6.5	25.8	10.2	0.2	0.2	5,8
WCS-2016	35.3	10.0	7.0	28.4	12.7	0.3	0.2	6.1
PCS	nd	nd	nd	100	nd	nd	nd	nd
FCS	61.8	21.5	9.7	nd	nd	0.6	0.4	6.0

1) Xylose found is 2-*O*-methyl-xylose. (2) Fucose found is 2-*O*-methyl-fucose. *Galacturonic acid was determined by the spectrophotometric method of Filisetti-Cozzi and Carpita (1991). Not detected (nd).

Through of the ^{1}H NMR and NMR-HSQC analysis (Fig. S1 – supporting information), using the values obtained by the integrals of the monosaccharide signals found in the anomeric region of the spectra of the crude fraction of each wine and the retention time of the monosaccharides 2-*O*-methyl xylose and 2-*O*-methyl-fucose in GC-MS (Fig. S2 – supporting information) it was possible to quantify each polysaccharide in relation to the total amount obtained in each wine (table 2). In this analysis the reproductively of wine produced in different years was evaluated, the table 2 shows that the percentage of each polysaccharide and also show that the mass of each polysaccharide recovery remained practically the same in Cabernet Sauvignon wines of different vintages. The similar percentage in the monosaccharide composition, as well as the amount of polysaccharides may suggest maintenance of quality of wine, indicating that the winery hold the same standard in the wine production process along the years.

Table 2

Quantification of each polysaccharide in relation to the total yield

Fraction	Polysaccharides								
	Total Yield	AG-II		RG-I/RG-II		Mannan		Dextrin	
	mg	%	mg	%	mg	%	mg	%	mg
WCS-2014	375.0	54.9	205.9	7.7	28.9	34.8	130.5	2.6	9.7
WCS-2015	390.0	55.1	214.9	6.8	26.5	35.7	139.2	2.4	9.4
WCS-2016	381.0	52.6	200.4	6.4	24.4	39.1	148.9	1.9	7.3

AG-II: type II arabinogalactan; RG-I/RG-II: type I and II rhamnogalacturonans.

The PCS and FCS fractions were studied by Bezerra et al (2018) through NMR, monosaccharide composition and methylation. The PCS fraction is a mannan, it is composed only by mannose (Table 1). The FCS polysaccharide is composed by galactose, rhamnose, galacturonic acid, arabinose, 2-*O*-Me-Fucose and 2-*O*-Me-Xylose (Table 1). This fraction presents a type II arabinogalactan, a type I rhamnogalacturonan and type II rhamnogalacturonan. These fractions (PCS and FCS) were used for the biological tests shown below in this work.

2.2. Biological experiments

Sepsis is one of the most prevalent diseases and one of the main causes of death among hospitalized patients. Uncontrolled severe infection often leads to sepsis, a systemic inflammatory response associated with fever, leukocytosis and multiple organ failure (Stearns-Kurosawa, 2011). This organ dysfunction can be represented by in the sequential organ failure assessment an increase including lung, kidney, and liver (Abraham et al., 1997; Bone et al., 1995; Singer et al., 2016).

In this work, we investigated the effect of Cabernet Sauvignon wine polysaccharides on survival of septic mice *in vivo*, as well as important hematological, biochemical and histopathological parameters related to organ dysfunction in sepsis. Here, it is suggested that polysaccharides may exert anti-inflammatory action that may contribute to the benefits of wine consumption.

Polymicrobial sepsis was induced by CLP in mice to investigate WCS effects. This model mimics the human sepsis caused by pathogens derived from the intestinal tract and

simulates a clinical situation (Otero-Anton et al., 2001). Many polysaccharides isolated from a variety of sources have shown immune responses *in vivo* and *in vitro*. Polysaccharides such as pectins and hemicelluloses had their anti-septicemic effect determined by being tested subcutaneously (Lim et al., 2002; Ruthes, Rattmann, Carbonero, Gorin & Iacomini, 2012; Tzianabos, Kasper, Cisneiros, Smith & Onderdonk, 1995) and orally (Dartora et al., 2013; Scoparo et al., 2013).

In this work, we treated orally the animals with the Cabernet Sauvignon wine polysaccharide mixture (CSO) to evaluate the protective effect of wine consumption and subcutaneously with the polysaccharide mixture (CSS) and the isolated fractions (FCS and PCS) to achieve very low doses and still be able to show the efficiency of the polysaccharides as anti-inflammatory and protective in an aggressive model of sepsis by CLP.

Lethality was found to be markedly reduced in mice receiving CSO, CSS, FCS and PCS treatments (Fig. 2). It was demonstrated that the CSO fraction prevents lethality caused by polymicrobial sepsis in mice. Lethality was markedly delayed in mice treated orally with CSO. The corresponding areas under the lethality curve increased to 9.120, 11.400 and 13.500 (arbitrary units) after 3, 7 and 10 mg/kg administration, respectively (Fig. 2A). The overall survival in the CSO group was 30%, 50% and 70%, increasing with the dose. The 10 mg/kg CSO dose was established for the analysis of this fraction effects in CLP.

The CSS fraction prevents lethality, obtaining a survival rate of 40% (0.1 mg/kg), 80% (1 mg/kg) and 78% (10 mg/kg) (Fig. 2B). Lethality was markedly delayed in subcutaneously treated mice. The corresponding areas under the lethality curve were 10.680, 14.160 and 14.040 (arbitrary units) after administrating 0.1, 1 and 10 mg/kg, respectively. The 1 and 10 mg/kg doses presented similar effects and obtained better results when compared to dexamethasone (0.5 mg/kg), which promoted a 50% survival rate. Therefore, the 1 mg/kg CSS dose was established for the analysis of the CLP fraction effects.

Mice treated with FCS had a survival rate of 45% at the 0.1 mg/kg dose and 80% at 1 and 10 mg/kg doses (Fig. 2C). The 1 and 10 mg/kg doses presented similar effects. The corresponding areas under the lethality curve were 12.300, 14.860 and 15.120 (0.1, 1, 10 mg/kg, respectively). Dexamethasone, used as control, had an area under curve of 12.000, with an overall survival rate of 50% at the end of the observation period. As the doses of 1 and 10 mg/kg presented similar results, the 1 mg/kg FCS dose was established for the analysis of the effects of this fraction in CLP.

Lethality was markedly delayed in mice subcutaneously treated with PCS and their areas under the curve were 10.560, 13.020 and 12.900 after administrating 0.1, 1 and 10 mg/kg doses, respectively (Fig. 2D). At the end, the corresponding overall survival rates were 40%, 65% and 63%. The 1 and 10 mg/kg doses presented similar effects. Therefore, the 1 mg/kg PCS dose was established for the analysis of the effects of this fraction in CLP. In summary, considering survival rates, the following doses were chosen for following analyses in sepsis: 10 mg/kg for CSO and 1 mg/kg for CSS, FCS and PCS.

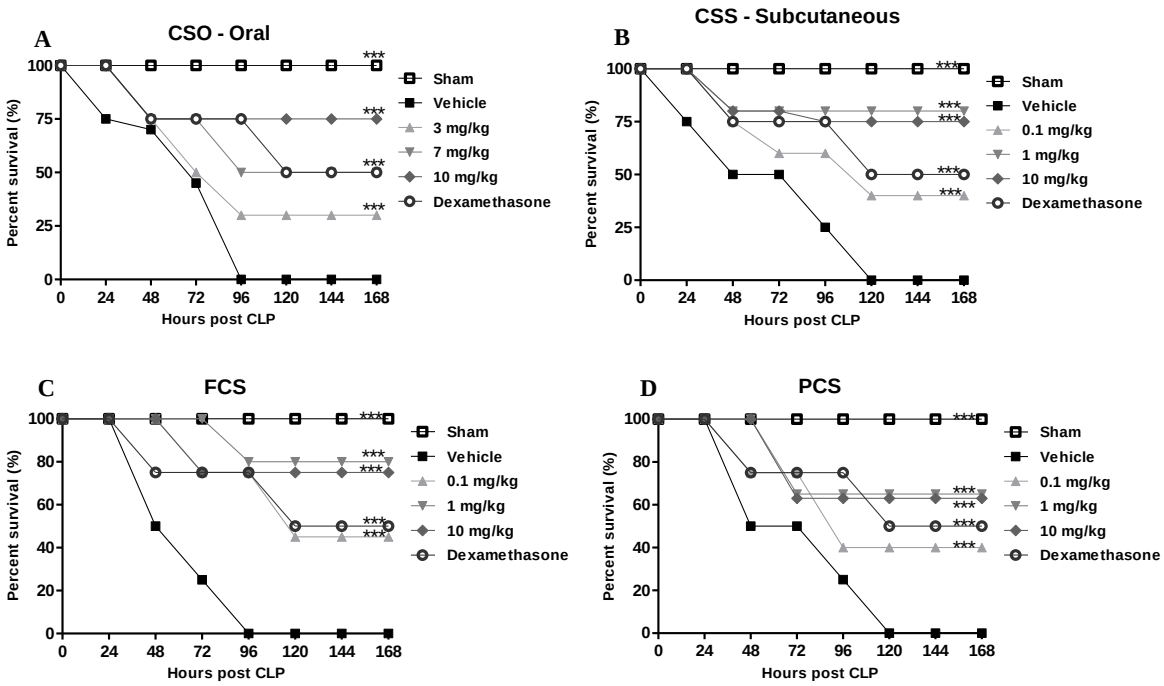

Fig. 2: Effects of CSO (A), CSS (B), FCS (C) and PCS (D) fractions on survival rate. Mice (10 animals/group) were orally administered various doses of CSO (3, 7 or 10 mg/kg) or subcutaneous administered various doses of CSS, FCS and PCS (0.1, 1, 10 mg/kg), vehicle (saline s.c.) and dexamethasone (0.5 mg/kg s.c.). Values represent means ± SEM. ***p < 0.001 when compared with CLP plus vehicle group; ANOVA followed by the Tukey's test.

In sepsis model, the acute immune response is organized and executed predominantly by innate immunity. Sepsis by CLP promotes migration of leukocytes through the bloodstream to the site of infection, peritoneal cavity and affected organs such as lung, liver and kidneys (Bone et al., 1992). Results showed that CSS, CSO, FCS, PCS and dexamethasone were able to reduce by 60%, 59%, 59%, 51% and 55% respectively, white blood cell (leukocyte count), when compared to the vehicle group (Table 3). Differential leukocyte counts demonstrated a decrease in the mononuclear leukocyte (MN) subset and the

PMN subset in the bloodstream (Table 3). Mononuclear leukocyte subset reduction consisted in 62%, 59%, 59%, 58% and 60% for CSS, CSO, FCS, PCS and dexamethasone, respectively. For de PMN subset, these reductions were 51%, 53%, 50%, 46% and 47% (CSS, CSO, FCS PCS, dexamethasone, respectively), when compared to the vehicle group. This exacerbated inflammatory response was observed on the vehicle group, in which there was an increased number of leukocytes, mononuclear leukocytes and PMN, whereas all fractions tested were able to reduce these effects significantly, reducing the amplification of the inflammatory response.

Neutrophils are the first cells to migrate to the site of infection, where they produce both reactive oxygen and nitrogen species that act to kill the infectious agents. These neutrophils and tissue resident cells also produce inflammatory cytokines, that can recruit and activate other inflammatory cells that, in turn, amplify and extend the initial systemic inflammatory response, as happened in the vehicle group. However, treatment with wine polysaccharides seems to reduce this effect (Yamashiro et al., 2001).

Table 3

Hematological parameters evaluated during sepsis in mice

Treatment	WBC (U/L)	PMN (U/L)	MN (U/L)
Sham	3316.7 ± 365.6	1450.0 ± 216.8	1850.0 ± 104.9
Vehicle	9317.9 ± 248.3***	3850.0 ± 225.8***	5283.3 ± 297.1***
CSS	3766.0 ± 266.5$^{\#}$	1900.0 ± 322.5	2016.7 ± 271.4
CSO	3850.0 ± 176.1$^{\#}$	1890.0 ± 206.6	2183.3 ± 183.3
FCS	3816.7 ± 343.0$^{\#\#}$	1916.7 ± 292.7	2186.7 ± 116.0
PCS	4600.0 ± 346.4	2133.3 ± 311.6	2200.0 ± 282.8
Dex	4216.7 ± 242.7	2050.0 ± 242.9	2116.7 ± 216.1

***$p < 0.001$ when vehicle was compared with all groups; $^{\#}$ $p < 0.05$ and $^{\#\#}$ $p < 0.01$ when compared with PCS; ANOVA followed by the Tukey's test. WBC:White blood cell count; PMN: polymorphonuclear leukocytes; MN: mononuclear leukocytes; Dex: dexamethasone.

During sepsis, the immune response starts sensing of danger by pattern-recognition receptors on the immune competent cells, such as macrophages. The sensed danger signals, through specific signaling pathways, activate transcription factors and gene regulatory systems, which up-regulate the expression of proinflammatory mediators (Akira & Takeda,

2004). Inflammatory mediators, including proinflammatory cytokines TNF-α and IL-1β, and anti-inflammatory cytokines, such as IL-10, generated by macrophages, induce the immune cells recruitment, mainly neutrophils (Overhaus et al., 2004; Pan, Lin-Shiau & Lin, 2000). Neutrophils damage tissue directly by releasing proinflammatory mediators, such as NO, which amplificate the systemic inflammatory response and contribute to multiple organ failure (Landry & Oliver, 2001; Nieuwenhuijzen et al., 1996). This study shows that CSS, CSO, FCS, PCS and dexamethasone significantly decreased the production of the pro-inflammatory cytokines TNF-α (57%, 42%, 57%, 57% and 53%, respectively) and IL-1β (54%, 52%, 53%, 53%, 53% and 52%, respectively) and increased the level of the anti-inflammatory cytokine IL-10 (3x for all fractions) when compared to the vehicle group (Fig. 3 A-C). In addition, production of excessive NO has been associated with a range of inflammatory diseases, including septic shock (Pacher, Beckman & Liaudet, 2007). As shown in figure 3D, treatments with CSS, CSO, FCS, PCS fractions and dexamethasone induced a significant inhibitory effect in NO production (60%, 57%, 59%, 57% and 54%, respectively), indicating anti-inflammatory properties. Reduction of the effects of pro-inflammatory mediators and increased levels of the anti-inflammatory mediator suggests that the all fractions exerted anti-inflammatory effect in sepsis. We performed assays regarding the phenol content of the polysaccharides sample using the Folin Ciocalteau method, and found that on the 1, 10, 100 and 1000 µg/mL polysaccharides solutions, the polyphenols content, measured as galic acid equivalents, were of ~0, 0.8, 3 and 7 µg/mL respectively. Thus the data in the literature regarding macrophage activation by plant/fruit extract containing polyphenols (Hooshmand et al, 2015) suggest that only at concentrations between 100 and 1000 µg/mL these compounds could significantly affect anti-inflammatory assays. Thus we can conclude that is unlikely that the observed biological activities demonstrated by the polysaccharide treatments could be somewhat influenced by their contents of polyphenols.

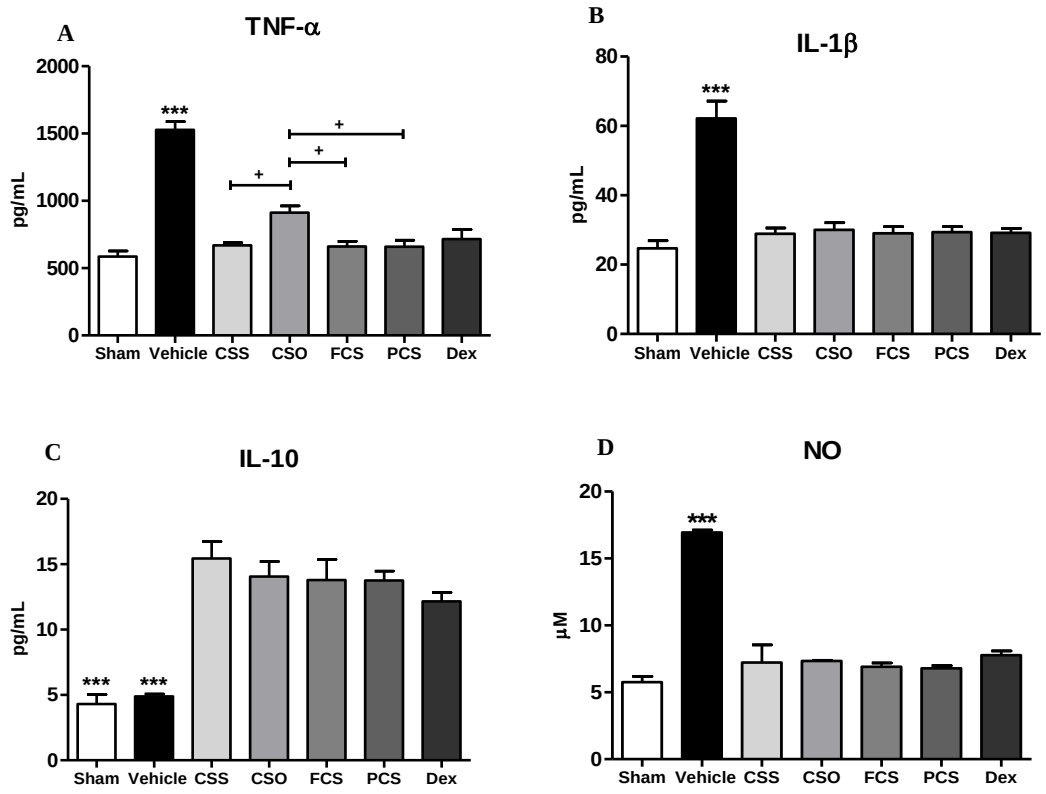

Fig. 3: Expression levels of TNF-α. (A), IL-1β (B), IL-10 (C), and NO (D) production levels. Dex (dexamethasone). ***p < 0.001 when vehicle was compared with all groups; $^{+}$ p <0,05 comparison between fractions. ANOVA followed by the Tukey's test.

Sepsis is characterized by a systemic inflammatory response to infection resulting in acute organ dysfunction and associated with a high mortality rate (Hotchkiss & Karl, 2003). There is progressive deterioration of various organs function, starting mostly with lung and followed by failure of liver and kidneys (Bau, Durhan & Faist, 1998). During sepsis, acute lung injury can be caused directly by bacterial damage or secondarily by the systemic inflammatory response of sepsis (Chen et al., 2016). The activated polymorphonuclear leukocytes play an important role in mediating microvascular damage and lung tissue injury. PMN leukocytes infiltrate the lung tissue and their persistence in the lungs is an important determinant of poor survival (Bhatia & Moochhala, 2004). Histopathological analysis showed the lungs of the treated and untreated mice after CLP (Fig. 4 H-N). Vehicle group showed characteristics of acute alveolar damage and inflammation, such as congestion, accumulation of neutrophils (Fig. 4I) and markedly thickened alveolar walls. CSS, CSO, FCS, PCS and dexamethasone groups showed reduced sepsis-induced damage and even normal lung histologic feature (Fig. 4 J-N). This study showed that the wine polysaccharides significantly

reduced the PMN number in blood and this may have reduced leukocyte infiltration in tissues and protected against lung injury.

Liver plays a major function in host defense in sepsis. Kupffer cells scavenge bacteria as well as clear cytokines from the liver. Hepatocytes modify metabolic pathways, increase gluconeogenesis, activate cellular repair and enzymes such as transaminases (AST and ALT) and alter bilirubin levels (Dhainaut, Marin, Mignon & Vinsonneau, 2001). ALT and AST transaminases are markers of hepatocyte injuries, so they were evaluated in this study. It was observed an increase in plasma AST and ALT activity in the septic group (Table 4). Treatment with CSS, CSO, FCS, PCS and dexamethasone showed a significant reduction of ALT in the blood in 59%, 64%, 57%, 58% and 58%, respectively, compared to the vehicle group (Table 4). Although ALT is an enzyme present in the hepatocyte cytoplasm, AST is present in mitochondria and at the cytoplasm. Moreover, AST has activity in the liver, heart, kidneys, pancreas, muscles and erythrocytes. Therefore, increased levels of plasma AST indicate the occurrence of major damage to the hepatocyte mitochondria or significant damage in a different organ. Thus, AST levels are higher than ALT in the vehicle group, but were not affected in all fractions treated groups and dexamethasone, which were able to maintain the low levels of the two enzymes. When compared to the vehicle group, it was observed the reduction in AST levels by 65% (CSS), 69% (CSO), 64% (FCS), 63% (PCS) and 63% (dexamethasone) (Table 4).

This study also evaluated liver function by measuring bilirubin levels. Bilirubin is recognized as an indicator of liver dysfunction, so severe elevations can cause damage to brain and erythrocytes (Brito, Silva & Brites, 2006; Fevery, 2008). Increased serum level of bilirubin was observed in the septic group, which was in accordance with other results already shown (Table 4). All treatments with fractions and dexamethasone showed a significant reduction of bilirubin serum in relation to septic group, consisting in 67% (CSS), 61% (CSO), 61% (FCS), 58% (PCS) and 58% (dexamethasone) (Table 4). Histopathological analyses demonstrated hepatic alterations in the vehicle group (Fig. 4B). Lesions were characterized by a reduction in sinusoids within the lumen, indicating tumefaction, and capsular infiltration of inflammatory cells. These lesions were found in septic group and could be prevented by treatments with each of all fractions, showing that the wine polysaccharides were able to preserve the liver, similar to dexamethasone (Fig. 4C-G) and sham group (Fig. 4A). These results showed that the wine polysaccharides were effective in protecting the liver and maintaining the organ functions during sepsis.

Table 4

Plasmatic parameters evaluate during sepsis in mice

Treatment	AST (U/L)	ALT (U/L)	Bilirubin (mg/dL)	Urea (mg/dL)	Creatinine (mg/dL)
Sham	138.4 ± 28.1	73.9 ± 5.8	1.0 ± 0.1	60.7 ± 9.4	0.4 ± 0.1
Vehicle	459.8 ± 25.5***	248.7 ± 19.1***	3.6 ± 0.4***	209.1 ± 14.0***	1.3 ± 0.1***
CSS	161.2 ± 14.5	101.8 ± 5.7	1.2 ± 0.3	86.2 ± 9.6	0.4 ± 0.1
CSO	140.5 ± 14.9	90.7 ± 6.9	1.4 ± 0.2	89.9 ± 6.8	0.5 ± 0.1
FCS	166.0 ± 21.5	106.9 ± 9.1	1.2 ± 0.1	97.5 ± 10.6	0.4 ± 0.1
PCS	169.5 ± 24.6	104.6 ± 14.5	1.5 ± 0.3	101.8 ± 5.4	0.5 ± 0.1
Dex	168.7 ± 10.9	105.0 ± 2.8	1.5 ± 0.2	99.9 ± 4.1	0.5 ± 0.1

***$p < 0.001$ when vehicle was compared with all groups; There was no statistical difference between the treated groups. ANOVA followed by the Tukey's test. WBC:White blood cell count; Dex: dexamethasone.

Sepsis is a systemic inflammatory response syndrome caused by bacteria, viruses or fungi. It can cause organ damage far from the primary site of infection, leading to multiple organ failure and even death. Acute kidney injury (AKI) is one of the complications of sepsis that can cause increased mortality rate in patients (Rewa & Bagshaw, 2014; Yamada et al., 2013). Kidney infiltration by neutrophils and subsequent release of free radicals is one major mechanism leading to AKI (Huang et al., 2015). The ability of CSS, CSO, FCS and PCS to have a protective effect on the kidneys was evaluated through the analysis of creatinine and urea (Table 4), which are markers of renal function (Fig. 4 O-U and Fig. S3 - supporting information). There was a significant increase in serum urea and creatinine levels in mice in vehicle group, while all fractions showed a significant reduction of serum levels of these markers (Table 4). The observed reductions were 59% (CSS), 57% (CSO), 53% (FCS), 51% (PCS) and 52% (dexamethasone), in relation to the vehicle group for urea levels. For creatinine level, the reductions were of 69% (CSS), 62% (CSO), 69% (FCS), 62% (PCS) and 62% (dexamethasone). Histological analysis of kidney reveled a renal tubular dilatation, bleeding, leukocyte infiltrate and tubular epithelial cell vacuolar degeneration with presence of necrosis, Bowman's capsule and proximal convoluted tubule retraction with degeneration were observed in the vehicle group (Fig. 4P and Fig. S3B - supporting information). Pre-treatment with each of all fractions effectively improved these kidney histopathological changes in mice with sepsis, as well as dexamethasone (Fig. 4Q-U and Fig. S3 C-G -

supporting information) and sham group (Fig. 4O and Fig. S3A - supporting information). Wine polysaccharides were able to preserve the kidneys during sepsis and seem to have helped in maintaining kidney function.

Due to the results presented, we were able to show that pre-treatment with Cabernet Sauvignon wine polysaccharides prevented lethality caused by polymicrobial sepsis in mice, by both subcutaneous and oral administration. Polysaccharides demonstrated a potent anti-inflammatory effect in the sepsis model, what may indicate beneficial effects of wine moderate consumption for human health. The results were very satisfactory and encouraging, in future work we aim to include polysaccharides from commercial suppliers as controls. The anti-inflammatory effect of polysaccharides obtained from Caberrnet Sauvignon wine and pure polysaccharide preparations (AGs, RGs and mannans) from commercial suppliers will be compared in the biological experiments, we will evaluate if the same polysaccharide obtained from different sources in which it presents small variations in its structure and different branching points may influence the biological response studied.

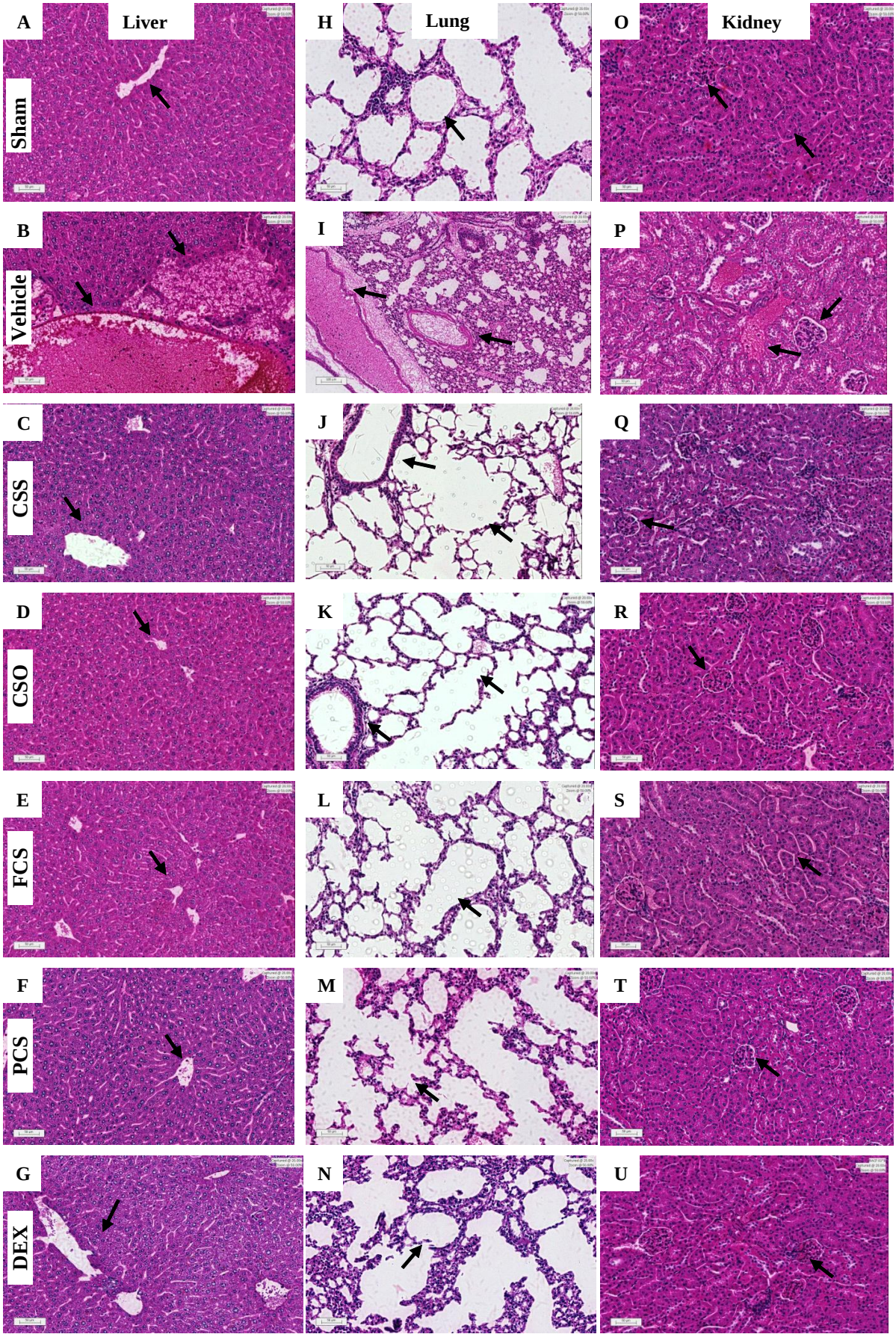
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Fig. 4: Representative histological sections from mice in groups: sham, vehicle, CSS, CSO, FCS, PCS and DEX (dexamethasone). Liver (A-G), lung (H-N) and kidney (O-U). Original magnification × 50. H&E. Scale bar = 50µm.

3. Conclusions

NMR and GC–MS analysis showed that the polysaccharides and their percentages were similar for Cabernet Sauvignon wine from different vintages. This has demonstrated the reproducibility of the different harvests wines from a polysaccharide perspective. The present study also suggests that wine polysaccharides exerted anti-inflammatory effect in sepsis induced by cecal ligation and puncture. It was demonstrated that polysaccharides prevent lethality caused by polymicrobial sepsis in mice. This beneficial effect seems to be due to a reduction in leukocyte infiltration, increased anti-inflammatory cytokine and inhibited production of pro-inflammatory cytokines, with consequent protection against tissue damage. The polysaccharide fractions were able to protect liver, lungs and kidney against injuries induced by CLP sepsis in mice.

Supporting Information

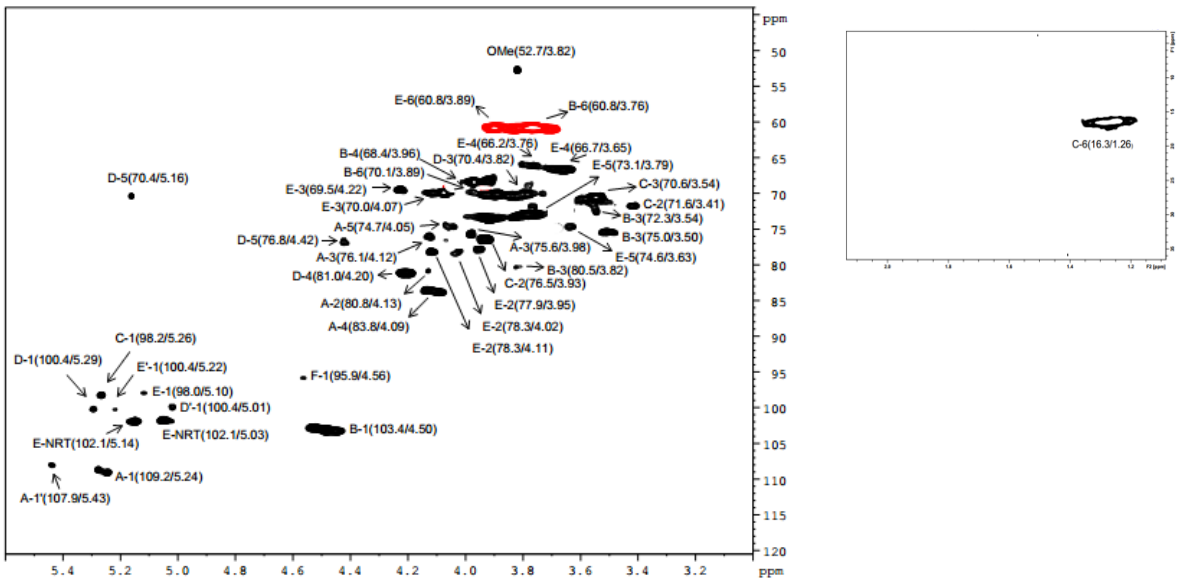

Figure S1: $^{1}H/^{13}C$ HSQC NMR spectrum of WCS. Solvent D_2O at 30 ºC; numerical values are in δ ppm. A (α-L-Ara*f*); B (β-D-Gal*p*); C (α-L-Rha*p*); D (α-D-Gal*p*A); D' (6-*O*Me-α-D-

Gal*p*A), E (Man*p)*; F (α-D-Glc). Letters are followed by the carbon number of the monosaccharide unit. Chemical shifts were determined by HSQCed experiment.

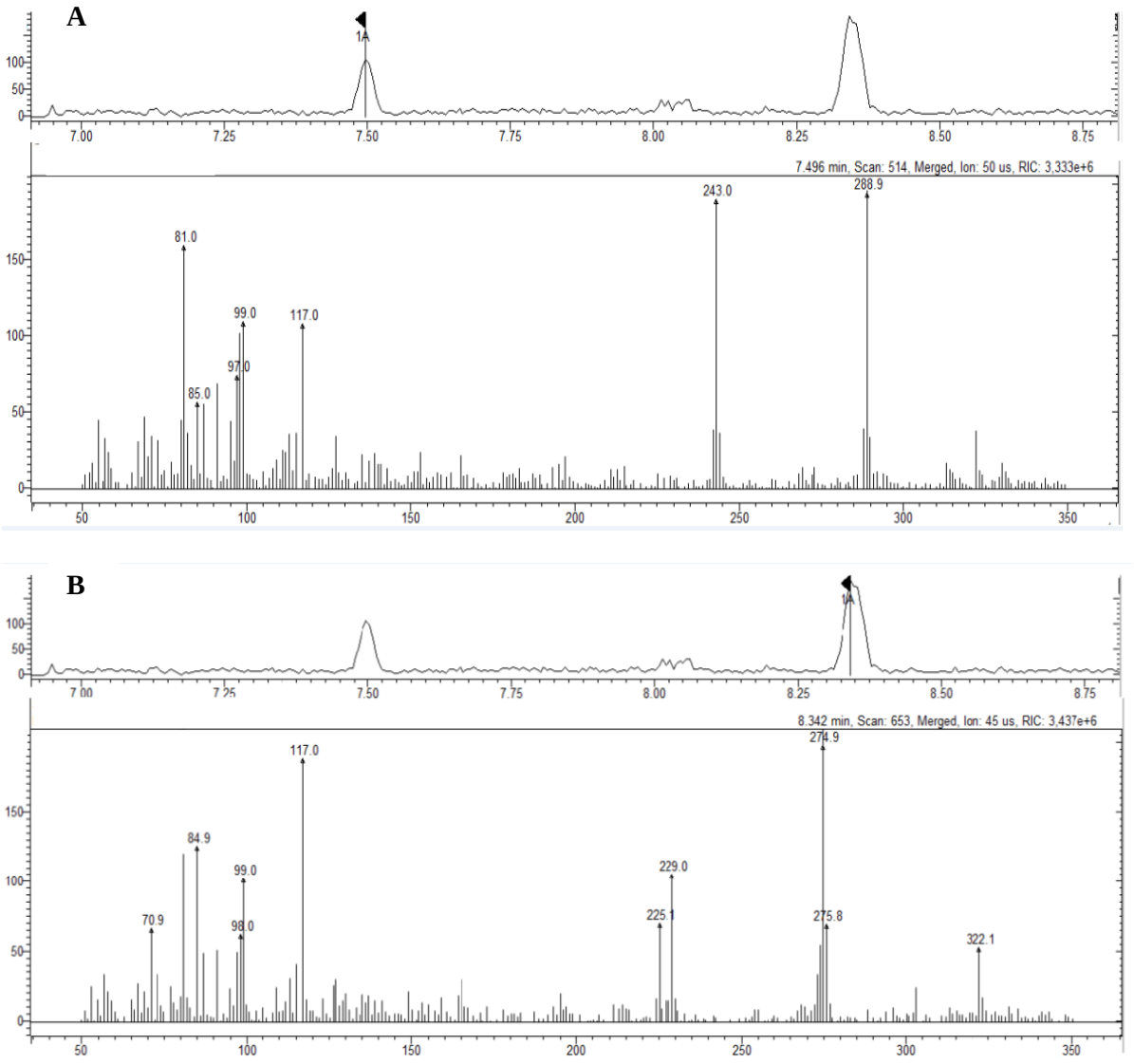

Figure S2: Chromatogram and mass spectra containing retention and fragmentation times from (A) 2-*O*-methyl-xylitol and those from (B) 2-*O*-methyl-fucitol. Identification of fragments at m/z.

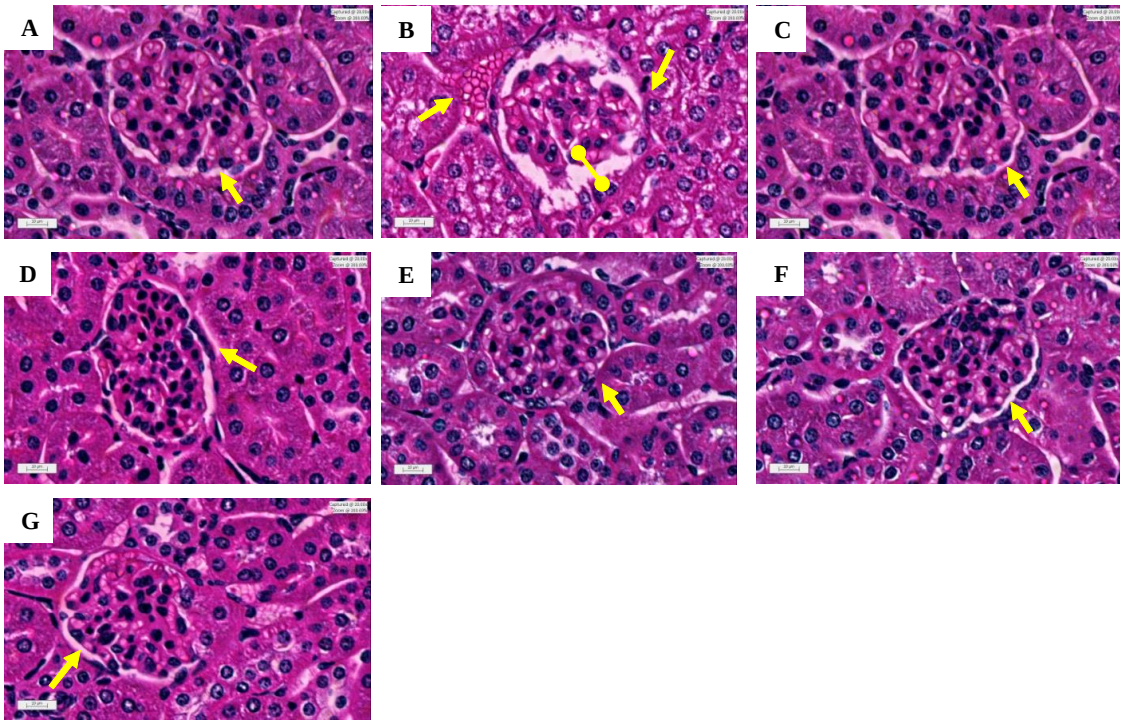

Fig. S3: Representative histological sections of kidney from mice in groups: sham (A), vehicle (B), CSS (C), CSO (D), FCS (E), PCS (F) and dexamethasone (G). Original magnification × 200. H&E. Scale bar = 20µm

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CAPITULO III

Biodistribution and anti-inflammatory activity of red wine polysaccharides: a sweet fluorescent trip in the mice body

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CAPÍTULO IV

Influence of red wine soluble polysaccharides profile on the flavanol composition and precipitation

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CAPITULO V

1. RESULTADOS COMPLEMENTARES

1.1. Análise dos pigmentos antocianos em vinhos

Para a identificação e quantificação dos pigmentos antocianos, foram adicionados 2 mL de vinho a 2 mL de HCl 0,1N. Em seguida, 2 mL desta solução foram carregados em um cartucho SPE de troca catiônica de modo misto/fase reversa Oasis MCX (60 mg, 3 mL) da Waters (Milford, MA, EUA), previamente condicionada com 2 mL de metanol seguido por 2 mL água ultrapura. Em seguida foi lavado com 4 mL de água e as antocianinas foram eluídas com 8 mL de metanol. O eluato foi rotaevaporado a 30 ºC e depois reconstituído em 500 µL de água ultrapura para obter o extrato de vinho de antocianinas. A quatificação foi realizada por HPLC - DAD - ESI / MS (García-Estévez, Alcalde-Eon & Escribano-Bailón, 2017). Para isso, 390 µL deste extrato foram misturados com 10 µL de ácido clorogênico (1 mg / mL), que foi utilizado como padrão interno, antes da injeção no sistema cromatográfico.

A análise por HPLC-MS dos compostos fenólicos nos vinhos mostrou maior concentração de antocianinas entre 141 e 364 mg/L para os vinhos Cabernet Sauvignon e 424,3 mg / L para o vinho Tempranillo (tabela 1). Os monogicosídeos foram as antocianinas mais abundantes em todos os vinhos estudados, seu conteúdo variou entre 29 e 53% (tabela 1).

Tabela 1: Antocianinas determinadas nos vinhos Cabernet Sauvignon e Tempranillo

Amostras	Pigmentos antocianos (%)					Total mg/L
	Monoglicosídeos	Acetatos	Cumaratos	F-A+	Piranoantocianinas	
CSCM	53,01^{a}	18,39^{a}	4,10^{c}	7,65^{c}	16,85^{b}	165,43
CSJA	50,92^{a}	17,55^{a}	10,73^{b}	6,30^{c}	14,50^{b}	363,58
CSVA	36,16^{b}	19,06^{a}	12,00^{ab}	9,76^{b}	23,02^{a}	141,58
CSFA	51,32^{a}	13,73^{ab}	12,00^{ab}	9,97^{b}	12,98^{c}	172,69
CSMO	29,36^{b}	18,02^{a}	15,31^{a}	12,23^{a}	25,09^{a}	206,06
TPHO	60,73^{a}	8,59^{b}	11,09^{b}	7,65^{c}	11,95^{c}	424,35

A percentagem média foi calculada a partir da percentagem individual de cada composto analisado em triplicata. F-A+: Produtos de condensação direta de flavonol antocianina

1.2. Medidas colorimétricas

Os espectros de absorção (190–770 nm) foram registrados no pH do vinho e após ajuste a pH 3,6 para todos os tipos de vinhos e em todas as pontos de amostragem em um espectrofotômetro Hewlett-Packard UV-vis HP 8453 (Agilent technologies, Waldbronn, Alemanha) com cubeta de quartzo de 2 mm de comprimento. A análise de cores foi feita a partir dos espectros visíveis (380–770 nm). A Cor CIELAB para os parâmetros (L *, a *, b *, C * ab e hab) foram calculados utilizando o software CromaLab® (Heredia, Álvarez, González-Miret e Ramirez, 2004).

A tabela 2 mostra os principais parâmetros do CIELAB em todos os vinhos analisados. No parâmentro de luminosidade (L) é avaliada a claridade do vinho. Vinhos com valor alto de luminosidade são mais escuros e possuem grande quantidade de flavonoides. TPHO apresentou valor de luminosidade mais alto (64,63). O parâmetro cromaticidade está relacionado com a unificação das cores, vinhos com padrão de cor unificado apresentam C* elevado, na tabela 1 é possivel observar que todos os vinhos analisados apresentaram C* entre 40 e 54. O parâmento Hab está relacionado a cor do vinho, valores entre 0 e 90 são correspondente as cores vermelho, laranja e amarelo, que são cores as cores encontradas nos vinhos. TPHO apresentou valor 7,02 que corresponde a cor vermelha e os outros vinhos que possuem valores mais altos correspondem as cores vermelho-alaranjadas. O vinho TPHO apresentou maiores valores dos parâmetros CIELAB, isso pode estar relacionado ao maior conteúdo de antocianos nesse vinho (424,35 mg/L) como mostrado na tabela 1 (Alcalde-Leon et al, 2019).

Tabela 2: Parâmetros CIELAB (L, Cab e Hab) dos vinhos Cabernet Sauvignon e Tempranillo

Amostras	L	Cab	Hab
CSCM	51,26	40,07	18,56
CSJA	57,67	47,25	25,10
CSVA	57,21	43,47	27,32
CSFA	59,48	41,02	21,55
CSMO	57,38	43,54	27,53
TPHO	¨64,63	53,71	7,02

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CONCLUSÃO GERAL

Após a análise dos resultados obtidos nesta pesquisa, pode-se concluir que:

- Os polissacarídeos encontrados nos vinhos Cabernet Franc, Cabernet Sauvignon e Sauvignon Blanc foram: arabinogalactana do tipo II, ramnogalaturonana do tipo I e II, manana e dextrina. O polissacarídeo majoritário presente nos três vinhos é uma arabinogalactana tipo II. Ela é formada por uma cadeia principal de unidades de β-D-Gal*p* (1→3)-ligadas, substituída em *O*-6 por cadeias laterais de β-D-Gal*p* (1→6)-ligadas. As cadeias laterais são substituídas em *O*-3 por terminais não redutores de α-L-Ara*f*. A ramnogalacturonana do tipo I encontrada é formada pela repetição da unidade dissacarídica [→4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→]. A manana presente nos vinhos estudados é um polissacarídeo constituído por uma cadeia principal de unidades α-D-Man*p* (1→6) ligadas, substituída em *O*-2 por cadeias laterais curtas de α-D-Man*p* (1→2) ligadas.

- O estudo realizado com vinhos Cabernet Sauvignon da mesma vinícola em diferentes safras mostrou que a porcentagem de cada polissacarídeo e também o rendimento total de polissacarídeos permaneceram praticamente inalterados nas diferentes safras. A porcentagem semelhante na composição de monossacarídeos, bem como a quantidade de polissacarídeos, pode sugerir manutenção da qualidade do vinho, indicando que a vinícola mantém o mesmo padrão no processo de produção de vinho ao longo dos anos.

- O efeito anti-inflamatório das frações polissacarídicas extraídas de vinhos foi avaliada em células RAW 264.7. As frações reduziram marcadamente a produção de óxido nítrico (NO) e citocinas pró-inflamatórias (TNF-α e IL-1β) em células estimuladas com LPS. Além disso, as análises realizadas *in vivo* em camundongos submetidos a CLP mostraram que a administração oral e subcutânea dos polissacarídeos apresentaram um aumento na sobrevivência dos camundongos com sepse, quando comparados com o controle. Levando também a diminuição dos níveis de citocinas pró-inflamatórias, bem como os níveis séricos de AST, ALT, bilirrubina, uréia e creatinina, com consequente proteção contra dano tissular. Além disso, foi observada a migração dos polissacarídeos marcados nos tecidos e órgãos após administração subcutânea em camundongos com sepse por CLP e a biodistribuição desse polímero pelo organismo aparentemente acontece através do sistema imunológico. Os polissacarídeos demonstraram potencial anti-inflamatório *in vitro* e *in vivo*, o que pode indicar efeitos benéficos do consumo moderado de vinho para a saúde humana.

- As análises realizadas mostraram que os vinhos Cabernet Sauvignon e Tempranillo têm um padrão semelhante em sua composição flavânica, embora haja diferenças marcantes entre as porcentagens de cada família de flavanóis entre os diferentes vinhos. As análises estatísticas indicaram que uma maior afinidade de certas famílias de polissacarídeos, como mananas, manoprotreínas, xilanas e dextrinas, em relação aos flavanóis, causando maior precipitação destes quando eles interagem. Isso pode explicar a relação inversa observada entre a concentração desses polissacarídeos e a dos flavanóis nos vinhos estudados, o que indica que uma quantidade maior de determinadas famílias de polissacarídeos em um vinho pode afetar o conteúdo dos flavanóis, o que teria consequências importantes para propriedades organolépticas do vinho. Além disso, a análise da composição pigmentos dos vinhos também mostrou uma variabilidade importante entre os vinhos estudados, o que se traduz em diferenças importantes nos parâmetros colorimétricos dos vinhos (principalmente clareza e tom) e, portanto, na cor destes.

CONCLUSIONES

Después de analizar los resultados obtenidos en esta investigación, se puede concluir que:

- Los polisacáridos encontrados em los vino Cabernet Fran, Cabernet Sauvignon y Sauvignon Blanc fueron: arabinogalactanos de tipo II, ramnogalacturonanos de tipo I y II, manano y dextrina. La familia de polisacáridos mayoritaria encontrada en los tres vinos fueron los arabinogalactanos de tipo II, los cuales están formados por una cadena principal de unidades de β-(1→3)-D-Gal*p* que presenta ramificaciones en las posiciones *O*-6 formadas por cadenas laterales de β-(1→6)-D-Gal*p*. A su vez, en las cadenas laterales aparecen sustituciones en las posiciones *O*-3 de monosacáridos no reductores de α-L-Ara*f*. Los ramnogalacturonanos de tipo I encontrados estaban formados por la repetición de la unidad disacarídica [→4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→]. Por último, los mananos presentes en los vinos estudiados son polisacáridos constituídos por una cadena principal de unidades α-(1→6)- D-Man*p* que presenta ramificaciones en las posiciones *O*-2 formadas por pequeñas cadenas laterales de α-(1→2)-D-Man*p*.

- El estudio realizado con los vinos Cabernet Sauvignon elaborados por la misma bodega en diferentes vendimias mostró que el porcentaje de cada polisacárido, así como el contenido total de estos compuestos en el vino, no se ven modificados en las diferentes vendimias. La estabilidad en la composición de polisacáridos (tanto en su contenido total como en su composición porcentual), podría indicar una gran regularidad en la calidad del vino, mostrando así que la bodega mantiene el mismo patrón en el proceso de producción de los vinos a lo largo de los diferentes años.

- El efecto antinflamatorio de las fracciones de polisacáridos extraídas de los vinos se estudió en células RAW 264.7. Los polisacáridos ensayados redujeron de forma importante la producción de óxido nítrico (NO) y citocinas proinflamatorias (TNF-α e IL-1β) en las células estimuladas con LPS. Además, los análisis realizados *in vivo* con ratones sometidos a CLP mostraron que la administración oral y subcutánea de los polisacáridos obtenidos no solo generaba un aumento en la supervivencia de los ratones con sepsis en comparación con la población control, sino que también provocaba la disminución de los niveles de citocinas proinflamatorias, así como los niveles séricos de AST, ALT, bilirrubina, urea y creatinina, lo que puede asociarse a una protección contra el daño celular. Por otro parte, se pudo observar

una migración de los polisacáridos marcados en los tejidos y órganos después de la administración subcutánea en los ratones con sepsis por CLP. Esta biodistribución de los polisacáridos por los órganos ocurre, aparentemente, a través del sistema inmunológico. Los polisacáridos demostraton, por tanto, un potencial anti-inflamatorio *in vitro* e *in vivo*, lo que puede indicar un posible efecto beneficioso del consumo moderado de vino para la salud humana.

- Los análisis realizados mostraton que los vinos de Cabernet Sauvignon y Tempranillo tienen un patrón similar en su composición flavánica, aunque existen diferencias marcadas entre los porcentajes de cada familia de flavanoles entre los diferentes vinos. Los análisis estadísticos señalaron que una mayor afinidad de ciertas familias de polisacáridos, como mananos, manoprotreínas, xilanos y dextrinas, hacia los flavanoles, provocando una mayor precipitación de éstos cuando interaccionan. Esto puede explicar la relación inversa observada entre la concentración de estos polisacáridos y la de flavanoles en los vinos estudiados, lo que indica que una mayor cantidad de ciertas familias de polisacáridos en un vino puede afectar al contenido de flavanoles, lo que tendría consecuencias importantes en las propiedades organolépticas del vino. Además, el análisis de la composición de pigmentos de los vinos mostró también una importante variabilidad entre los vinos estudiados, lo que se traduce en importantes diferencias en los parámetros colorimétricos de los vinos (principalmente claridad y tono) y, por tanto, en el color de éstos.