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PRODUÇÃO DE SAPONINAS TRITERPÊNICAS BIOATIVAS EM CULTIVOS CELULARES DE *Quillaja brasiliensis*.

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PRODUÇÃO DE SAPONINAS TRITERPÊNICAS BIOATIVAS EM CULTIVOS CELULARES DE *Quillaja brasiliensis*.

Tese apresentada como requisito parcial para obtenção do título de Doutor em Botânica na Universidade Federal do Rio Grande do Sul.

Orientador: Prof. Dr. Arthur Germano Fett-Neto.

Porto Alegre
2022

“Some people will never learn anything, for this reason, because they understand everything too soon.” — Alexander Pope, Miscellanies in Verse and Prose.

“A pessoa sábia está sempre pronta para aprender.” — Provérbios 18.15.

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Nestes dois anos da pandemia da COVID-19, estivemos preocupados com a segurança das nossas vidas, e de nossos queridos. Ao mesmo tempo, seguimos pensando no futuro, sabendo da importância de continuar lutando por nossa educação e pelo nosso trabalho como cientistas. Estou concretizando esta etapa de defesa da tese, que foi construída metade em um mundo pré-pandemia e metade em um mundo pandêmico. Muitas pessoas foram importantes neste processo, e espero poder contemplá-las nestes agradecimentos.

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Esta tese é dedicada ao meu querido Adilson e a nossa filha.

SUMÁRIO

RESUMO

Quillaja brasiliensis é uma espécie arbórea conhecida popularmente como pau-sabão, que produz saponinas triterpênicas em suas folhas. As saponinas de *Q. brasiliensis* apresentam pronunciada atividade adjuvante em vacinas veterinárias experimentais, de forma comparável ao produto comercial Quil-A®, obtido de cascas de *Quillaja saponaria*. Adjuvantes são adicionados às vacinas inativadas para aumentar a potência e duração da resposta imune, garantindo a segurança e eficiência dessas formulações. Tendo em vista a possibilidade da utilização de *Q. brasiliensis* para a obtenção de saponinas com potencial uso comercial e considerando a necessidade de maior conhecimento sobre a produção e acúmulo sustentável de metabólitos ativos em geral, esta tese visou definir procedimentos para estabelecer culturas celulares de *Q. brasiliensis* e avaliar sua competência e viabilidade para produção de saponinas. Extensa análise da literatura de metabolismo especializado de plantas resultou em dois artigos de revisão, um voltado a saponinas imunoadjuvantes de *Quillaja* sp. e outro, à biotecnologia e biossíntese de saponinas triterpênicas. Cultivos celulares foram estabelecidos, sendo que o ciclo de cultivo de suspensões celulares foi definido como sendo de 21 dias. A produção de saponinas acompanhou o acúmulo da massa seca celular, e conteúdo máximo de saponinas coincide com o final da fase exponencial de crescimento. A caracterização química inicial das saponinas produzidas por suspensões celulares foi obtida. Sete compostos foram tentativamente identificados por LC-MS, e os padrões de fragmentação indicam que ácido quiláico foi a principal aglicona presente. As saponinas purificadas de suspensões celulares foram testadas em um ensaio com vacinas adjuvantadas contra o vírus da gripe. Houve um aumento de anticorpos em indivíduos vacinados com vacinas adjuvantadas com extratos celulares, principalmente IgG total e IgG2, contudo em um nível menor que as formulações adjuvantadas com compostos purificados de folhas de *Q. brasiliensis*. Além disso, protocolos de quantificação de saponinas e esteróis por GC-MS foram desenvolvidos. Estes protocolos irão subsidiar estudos de fluxo metabólico e avaliação do investimento de carbono nos pools desses compostos em suspensões celulares. Em suma, cultivos celulares de *Q. brasiliensis* constituem uma fonte de saponinas bioativas e representam uma excelente plataforma para o estudo da biossíntese destes produtos naturais.
PALAVRAS-CHAVE: terpenos, saponinas, cultura de células, vacinas, adjuvante, *Quillaja*.

ABSTRACT

Quillaja brasiliensis is a tree species popularly known as soap-tree, which produces triterpenoid saponins in its leaves. Saponins purified from leaves of *Q. brasiliensis* show pronounced adjuvant activity in experimental veterinary vaccines, comparable to the commercial product Quil-A®. Quil-A is purified from barks and woods of *Quillaja saponaria.* Adjuvants are added to inactivated vaccines to increase potency and extent of the immune response, ensuring safety, and increasing formulation efficacy. Considering the possible use of *Q. brasiliensis* for production of bioactive saponins and considering the need for advancing the knowledge about the sustainable supply of active metabolites in general, this thesis aimed at defining procedures to establish cell cultures of *Q. brasiliensis* as a potential source of saponins. Extensive analyses of the literature on plant specialized metabolism resulted in two review articles, one examining immunoadjuvant saponins of *Quillaja* sp. and the other focusing on plant triterpene saponin biotechnology and biosynthesis. *Q. brasiliensis* cell cultures were successfully established. Cell suspension culture cycle lasted 21 days, and the production of saponins followed the accumulation of cell dry mass. Maximum saponin content was seen at end of exponential phase of the growth cycle. Chemical characterization of saponins produced by cell suspensions showed seven compounds tentatively identified by LC-MS. Fragmentation patterns indicated quillaic acid as the main aglycone present in cell extracts. Saponins purified from cell suspensions were tested in an assay for adjuvant activity, using formulations against the influenza virus. There was an increase in antibodies in groups that received vaccines prepared with cell extracts, mainly total IgG and IgG2, even though to a lesser extent than those seen for formulations that received saponins fractions purified from leaves of *Q. brasiliensis*. In addition, protocols for quantification of saponins and sterols by GC-MS were developed. These methods will allow studies of metabolic flux analyses and the evaluation of carbon investment in the pools of these compounds in cell suspensions. To sum up, *Q. brasiliensis* cell cultures constitute a source of bioactive saponins and can provide a platform for the study of the biosynthesis of these natural products.

KEYWORDS: terpenoids, saponins, plant cell cultures, vaccines, adjuvants, *Quillaja*

Introdução

Saponinas compõem um grupo complexo de metabólitos especializados, sintetizados principalmente por plantas. A estrutura básica central das saponinas deriva do óxido de esqualeno e, portanto, estas moléculas são usualmente classificadas pela natureza de suas agliconas: triterpenoides (C-30) ou esteroidais (C-18 a C-29). Este núcleo central é decorado com uma ou mais porções de oligossacarídeos, além de outros tipos de resíduos, como cadeias de ácidos graxos. A combinação entre um esqueleto hidrofóbico (aglicona) e resíduos hidrofílicos torna as saponinas altamente anfipáticas, o que confere a essas moléculas a propriedade de formar espuma persistente em água (Figura 1).

Pode-se perceber a relevância desses compostos por seu apelo comercial, pois as saponinas são utilizadas em diversos ramos da indústria, como os setores alimentício, têxtil e cosmético. Também apresentam importantes atividades farmacológicas, sendo registradas na literatura atividades antiplaquetária, hipocolesterolêmica, antitumoral, antiviral, imunoadjuvante, anti-inflamatória, antibacteriana, inseticida e fungicida (Costa, de *et al.*, 2011; Reichert, Salminen e Weiss, 2019; Yendo *et al.*, 2010).

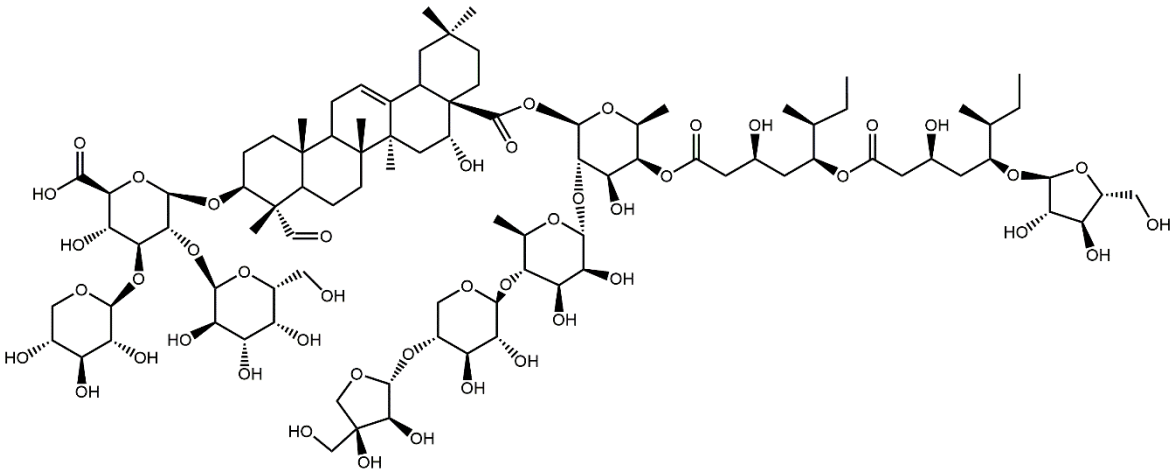

Figura 1. Estrutura típica de saponinas em *Quillaja saponaria*. O núcleo central triterpênico é conhecido como aglicona, e neste caso é o ácido quiláico. Decorações de oligossacarídeos e cadeia acil ocorrem tipicamente nestas estruturas.

Nesta tese, são apresentados dois capítulos de revisão sobre saponinas sintetizadas por plantas. No capítulo 1, revisamos as saponinas no gênero *Quillaja*: sua obtenção,

características químicas, atividade adjuvante e síntese química. Mais informações sobre o papel dos adjuvantes em vacinas podem ser encontradas a seguir nesta introdução, e nos capítulos 1 e 3.

O capítulo 2 é mais abrangente e trata do metabolismo de saponinas triterpênicas em espécies de interesse econômico, como as espécies de *Avena* sp. e *Panax* sp. São também discutidas estratégias para obtenção de saponinas: aplicação de tratamentos elicitores, identificação de fatores de transcrição que controlam a expressão gênica nas vias de biossíntese, transformação genética de plantas/culturas e expressão heteróloga da via metabólica de saponinas.

Na América do Sul, as cascas e a madeira de *Quillaja saponaria* Molina (Quillajaceae) são exploradas para a produção de adjuvantes. Esta espécie arbórea pode ser encontrada na região central Chile, principalmente (Luebert, 2013). As frações de saponinas extraídas desta espécie apresentam atividade imunoadjuvante acentuada, produzindo resposta humoral e mediada por células, frente a uma grande diversidade de antígenos e em diferentes espécies animais (Campbell e Peerbaye, 1992).

Em 2020, o comércio de extratos de plantas (especialmente extratos de *Quillaja*) mobilizou um montante de US$ 26 milhões, sendo exportadas 674 toneladas pelo Chile para Estados Unidos, Europa, Austrália, México, Coréia do Sul e Japão (IEC, 2021). A fração comercial Quil-A®, purificada a partir das cascas de *Q. saponaria*, é utilizada, por exemplo, em vacinas comerciais veterinárias contra leucemia felina, influenza equina e vírus da diarreia bovina (Sun, Xie e Ye, 2009). Já a fração QS-21 foi aprovada para o uso em vacinas para seres humanos, e está presente nas formulações contra Malária e Herpes Zoster, produzidas pela GlaxoSmithKline. Tendo em vista essa demanda, e o modelo econômico adotado pelo Chile, há uma exploração bastante importante dos recursos naturais no país. Estima-se uma redução de 237.126 hectares de área florestal nativa entre 1999-2015. No caso de *Q. saponaria*, o anelamento do floema para obtenção das cascas resulta na morte dos indivíduos. Por isso, a exploração da madeira, a partir do corte de galhos, é considerada importante para a manutenção de florestas nativas e comerciais, uma vez que preserva o indivíduo adulto (San Martin, 2000).

Nosso grupo tem estudado a produção e atividade de saponinas na espécie *Quillaja brasiliensis* (A.St.-Hil. & Tul.) Mart., que é sinônimo de *Quillaja lancifolia* D.Don (Quillajaceae). Este último é o nome aceito atualmente, mas, por consistência histórica com os trabalhos anteriores, optamos por usar o nome original. Popularmente, *Q. brasiliensis* é conhecida como sabão-de-soldado ou pau-sabão. Indivíduos adultos são hermafroditas, apresentam casca escura e folhas perenes. No Brasil, a distribuição da espécie é irregular e descontínua, sendo considerada ecologicamente uma espécie pioneira. Atualmente, *Q. brasiliensis* ocorre nos estados da região Sul do Brasil (Carvalho, 2003). As folhas são lanceoladas, alternas, de cor verde escura na face adaxial e verde amarelada na face abaxial. As flores estão dispostas em corimbos axilares de aproximadamente 12 cm de diâmetro, pediceladas, com cálice tomentoso. O fruto é formado por 5 folículos dispostos em forma de estrela, tomentosos. As sementes são aladas e sua dispersão é anemocórica (Figura 2) (CNCFlora, 2022). Esta espécie pode ocorrer em tipos diferentes de solo, podendo atingir 6 a 10 m de altura e DAP (diâmetro a altura do peio) entre 20 e 40 cm (Carvalho, 2003). Em uma avaliação realizada em 2012, *Q. brasiliensis* foi classificada como espécie "em perigo" de extinção no Brasil (CNCFlora, 2022).

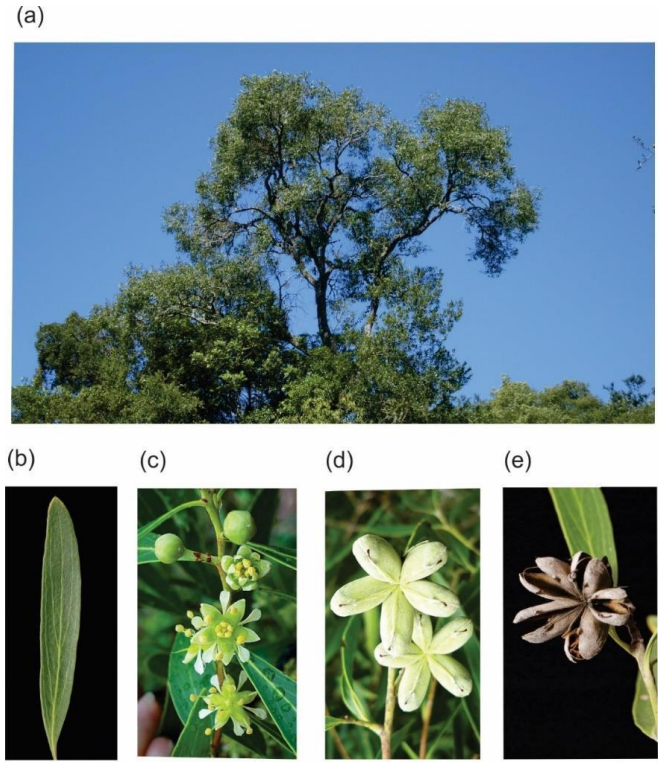

Figura 2. *Quillaja brasiliensis*. a) indivíduo adulto; b) folha; c) flores; d), frutos verdes; e) fruto maduro. Reproduzido com autorização do autor (De Costa, 2014).

Atividade adjuvante também foi demonstrada para saponinas de *Q. brasiliensis*. O extrato aquoso de folhas de *Q. brasiliensis* e a fração enriquecida em saponinas, QB-90, apresentam atividade adjuvante em vacinas contra *herpesvirus* bovino tipo 1 e tipo 5, poliovirus, vírus da diarreia viral bovina, vírus da raiva e da influenza em modelo murino. Os extratos brutos e as frações purificadas de *Q. brasiliensis*, nas doses empregadas, foram capazes de potencializar a resposta imune aos antígenos de forma comparável à fração comercial chilena Quil-A®, apresentando menor toxicidade (Cibulski *et al.*, 2016; Fleck *et al.*, 2006; Kauffmann *et al.*, 2004; Rivera-Patron *et al.* 2021; Yendo *et al.*, 2016).

Saponinas de *Quillaja brasiliensis* também apresentam um papel protetor na planta. Ação deterrente foi observada em testes com herbívoros invertebrados, assim como a inibição do crescimento de culturas de alguns fungos fitopatogênicos (Yendo, 2016). Além disso, o acúmulo de saponinas em folhas de *Q. brasiliensis* pode ser induzido por estresse osmótico, dano mecânico, hormônios vegetais, radiação ultravioleta e aumento da intensidade luminosa, indicando um possível papel das saponinas nos mecanismos de resposta ao estresse abiótico e biótico (Costa, de *et al.*, 2013; Yendo *et al.*, 2015).

Caracterização química

Saponinas de *Quillaja* são substâncias complexas que ocorrem naturalmente em isômeros. Isso torna a caracterização química e a quantificação destes compostos bastante difícil. Por exemplo, QS-21 é uma fração natural de 4 isômeros, dois isômeros de posição e dois isômeros que se diferenciam pelo açúcar terminal do oligossacarídeo ligado à aglicona na posição C-28. É também comum encontrar saponinas que ocorrem aos pares, havendo substituição no trissacarídeo ligado ao C-3 da aglicona (Kite, Howes e Simmonds, 2004). A Tabela 1 resume as principais frações de saponinas purificadas a partir de *Quillaja* spp.

Tabela 1. Frações de saponinas purificadas em *Quillaja* spp.

Nome	Espécie	Órgão	Composição	Uso
Quil-A	*Q. saponaria*	Cascas e madeira	Cerca de 25 saponinas diferentes	Vacinas veterinárias para uso comercial
QS-21	*Q. saponaria*	Cascas e madeira	4 isômeros	Vacinas para seres humanos
Fração B	*Q. brasiliensis*	Cascas e folhas	Cerca de 54 saponinas diferentes	Atividade adjuvante em modelo experimental
QB-90/QB-80	*Q. brasiliensis*	Folhas	-	Atividade adjuvante em modelo experimental

Atividade adjuvante

Em mamíferos, a imunidade compreende um sistema de defesa contra toxinas e infecções, que também auxilia no controle do crescimento de células anormais (câncer). De maneira bastante simplificada, é possível dizer que o sistema imune atua em duas frentes (Figura 3). A imunidade inata compreende a primeira linha de defesa, que se estabelece no momento da infecção ou na aplicação de vacinas. A imunidade inata conta com barreiras epiteliais e uma série de células de defesa que são capazes de destruir invasores ou células doentes. As células do sistema imune inato também são responsáveis por apresentar antígenos aos linfócitos T, fazendo uma ponte entre a imunidade inata e adaptativa. Na imunidade adaptativa, diversas respostas de defesa mais específicas são ativadas, e estas respostas são mediadas por linfócitos T e linfócitos B.

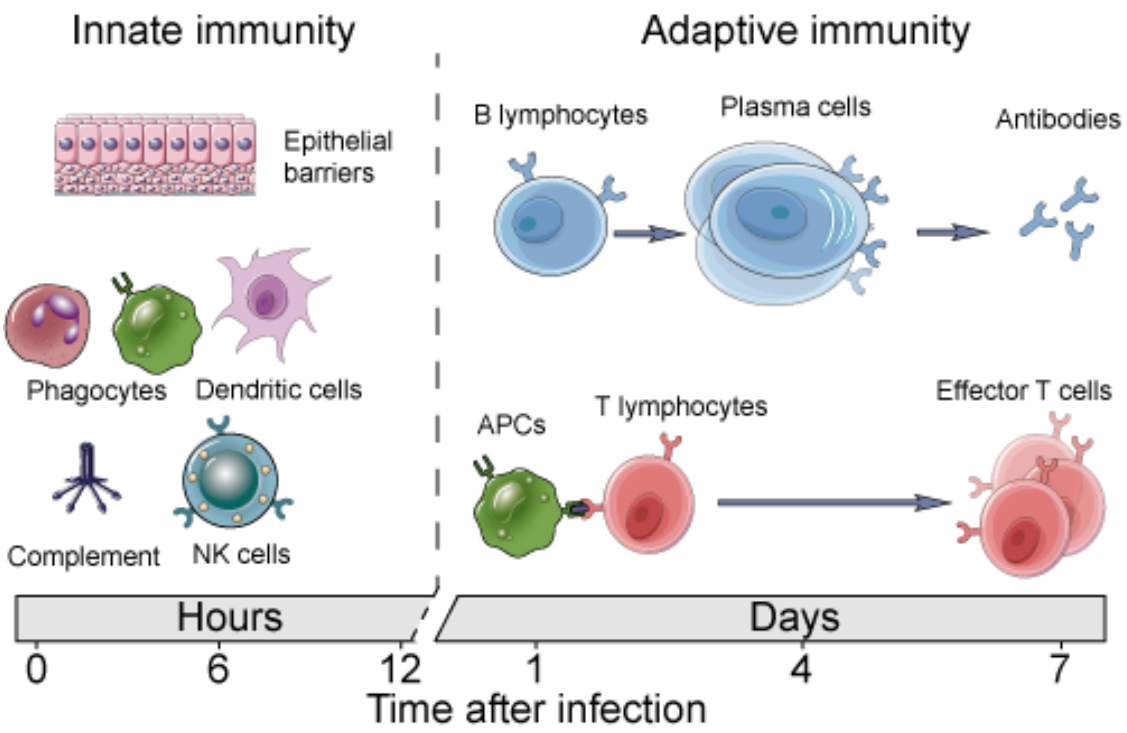

Figura 3. Células de defesa do sistema imune em mamíferos. Fonte: https://www.creative-diagnostics.com/innate-and-adaptive-immunity.html

A imunidade adaptativa depende da ativação e amadurecimento dos linfócitos T após a apresentação de antígenos (Figura 4). Linfócitos T do tipo Th1 produzem IFN-γ, entre outras substâncias, que estimulam a atividade de macrófagos e a síntese de anticorpos opsonizantes pelos linfócitos B. Essa resposta é caracterizada, portanto, por imunidade mediada por células (resposta celular), que é efetiva em destruir patógenos intracelulares e células cancerosas. Por sua vez, linfócitos do tipo Th2 secretam citocinas, como IL-4, o que leva a ativação de linfócitos B para produção de anticorpos neutralizantes. Isto é importante para resposta humoral contra patógenos extracelulares, além da formação de células de memória (Figura 4) (Bonilla e Oettgen, 2010; Schijns *et al.*, 2021).

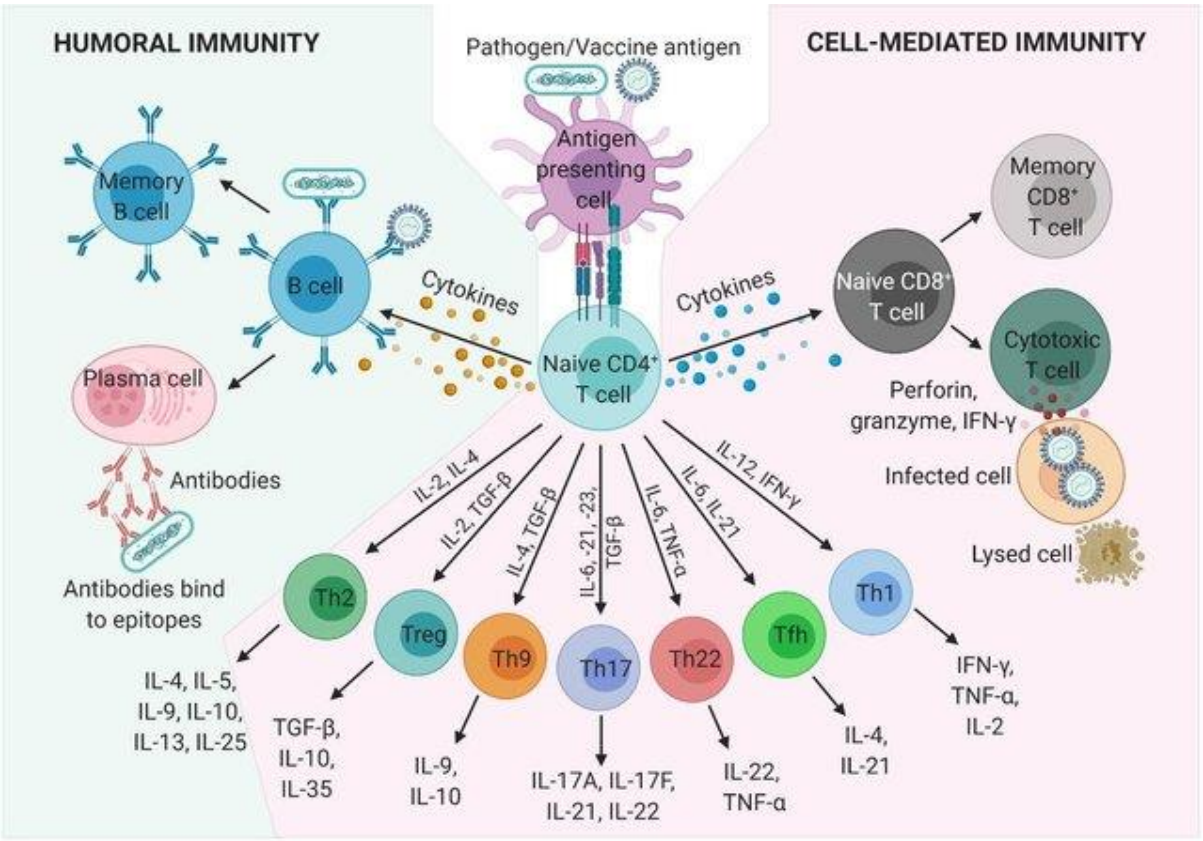

Figura 4. Imunidade adaptativa. A apresentação de antígenos ativa a maturação de linfócitos T CD4$^+$, que é mediada pela produção de interleucinas e outras moléculas. Reproduzido de Schijns *et al.*, 2021.

As vacinas são formulações capazes de estimular o sistema imune ao expô-lo ao patógeno, ou partes deste, protegendo o indivíduo vacinado. Tipicamente, vacinas podem ser vivas ou inativadas. Vacinas vivas recebem o patógeno vivo atenuado em sua formulação, sendo que este não é capaz de estabelecer uma infecção. Já as vacinas inativadas podem ser formuladas com patógenos mortos, algum de seus componentes, ou antígenos. Em geral, vacinas inativadas são mais seguras, mas menos potentes. Por isso, essas vacinas recebem um adjuvante para aumentar sua imunogenicidade. Isto é particularmente importante para a formulação de vacinas seguras e efetivas para pessoas que têm um sistema imune imaturo ou frágil, como crianças e idosos (Marciani, 2018). Mais recentemente, vacinas de mRNA foram desenvolvidas para prevenir a infecção por SARS-CoV-2. Estas formulações contêm fragmentos de RNA mensageiro que codificam para uma proteína spike do vírus. As células do indivíduo vacinado irão produzir esta proteína, que é reconhecida como estranha, o que ativa a resposta imune (Hogan e Pardi, 2022). Vacinas de mRNA não recebem adjuvantes

em sua formulação pois são capazes de estimular uma resposta imune bastante potente.

Os adjuvantes são substâncias que estimulam as respostas imunes adaptativas, aumentando sua duração e potência. Adjuvantes são capazes de:

- Aumentar títulos de anticorpos em indivíduos vacinados;
- Facilitar o uso de doses menores de antígeno, o que diminui o custo da produção de vacinas;
- Permitir a imunização completa com menos doses de uma vacina;
- Modular a natureza da resposta imune adaptativa.

Adjuvantes são capazes de definir a natureza da resposta imune adaptativa: inflamatória (Th1) ou anti-inflamatória (Th2) (Figura 4). Tipicamente, as saponinas de *Quillaja* sp. são efetivas em induzir respostas do tipo Th1 (Figura 5). O mecanismo de ação de QS-21 ainda está sendo estabelecido (Marciani, 2018). Esta saponina é internalizada por células dendríticas, ainda na fase inicial da infecção (imunidade inata). Acredita-se que QS-21 antecipe a apresentação de antígenos ao promover o rompimento das membranas dos endossomos. QS-21 pode também interagir com receptores CD2 na membrana do linfócito T, iniciando uma cascata de sinalização que resulta na ativação da resposta do tipo Th1 (Figura 5). Esta resposta Th1, induzida por saponinas, sempre é seguida por uma resposta imune reparadora do tipo Th2 (Marciani, 2018). O adjuvante comercial alum (sais de alumínio) geralmente promove um viés direcionado a resposta do tipo Th2 (Bonilla e Oettgen, 2010). Dessa forma, o uso combinado de adjuvantes, além da descoberta de nova substâncias imunoestimulantes, são fundamentais para o desenvolvimento de vacinas, aumentando seu espectro de ação na imunidade adaptativa.

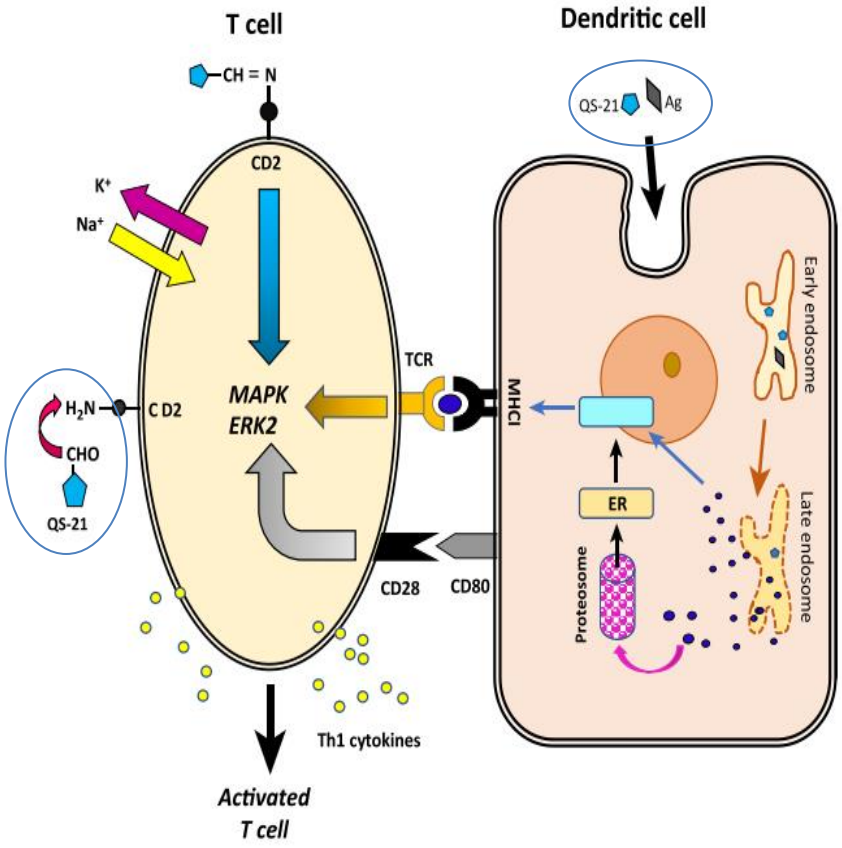

Figura 5. Mecanismo de ação de QS-21. Reproduzido de Marciani, 2018.

A formulação de adjuvantes é um processo historicamente moroso. Em 1920, as primeiras vacinas para seres humanos formuladas com adjuvantes receberam alum. Somente na década de 90, novas formulações adjuvantes foram aprovadas para uso em seres humanos, como AS01, que contém saponinas de *Quillaja saponaria* (Didierlaurent *et al.*, 2017; Pulendran, S. Arunachalam e O'Hagan, 2021). As vacinas que recebem AS01 incluem a formulação pioneira na prevenção da malária (Mosquirix®), recomendada pela OMS, e a vacina para herpes zoster (Shingrix®), aprovada pela agência Food and Drug Administration nos Estados Unidos. Da mesma forma, sistemas adjuvantes têm se mostrado bastante eficazes e seguros em vacinas para uso veterinário (Sun, Xie e Ye, 2009).

Produção biotecnológica de saponinas

A cultura de tecidos vegetais compreende um conjunto de técnicas que permite a obtenção de grande quantidade de material vegetal, em ambiente controlado e uniforme, livre de

contaminação. A produção de produtos naturais a partir dessas culturas tem sido amplamente estudada, com particular interesse em suspensões celulares. O potencial desse sistema de cultivo está em permitir a obtenção de metabólitos em condições padronizadas, e possibilitar a manipulação do metabolismo, seja pela aplicação de tratamentos de estresse, seja por vias de transformação ou edição gênica (Biswas e Dwivedi, 2019; Espinosa-Leal, Puente-Garza e García- Lara, 2018). O capítulo 3 desta tese trata do estabelecimento de cultivos celulares de *Quillaja brasiliensis* para a produção de saponinas triterpênicas bioativas.

Suspensões celulares de plantas são utilizadas para a obtenção de produtos naturais (Tabela 2). Este tipo de cultivo pode ser escalonado para biorreatores, similar ao que ocorre com cultivos de microrganismos. Comparados a células animais, o custo com a manutenção de cultivos de células vegetais é menor, com a vantagem que não há contaminantes de origem animal, o que é vantajoso para a produção de proteínas recombinantes, por exemplo. A droga anticâncer paclitaxel é o caso mais proeminente de produção comercial de um bioativo vegetal em suspensões celulares de plantas (Espinosa-Leal, Puente-Garza e García- Lara, 2018).

Tabela 2. Produtos naturais obtidos em suspensões celulares de plantas.

Composto	**Espécies**	**Uso**
Antocianinas	*Euphorbia* sp., *C. roseus*	Corantes na indústria têxtil e alimentícia.
Berberine	*Coptis japonica*	Anticâncer
Geraniol	*Geraminea* spp.	Essential oil, anti-inflammatory
Saponinas de Ginseng	*Panax ginseng*	Suplemento dietético
Paclitaxel	*Taxus* spp.	Anticâncer
Proteínas recombinantes	Tobacco cells (BY2), Rice cells	Antígenos (Hep. B), anticorpos, imunossupressores

Estudos que utilizam isótopos para marcação de compostos em plantas são empregados na avaliação dos mecanismos básicos de controle do metabolismo (Bergman *et al.*, 2020;

Zamboni, 2011), frequentemente aplicados em cultivos celulares. O capítulo 4 desta tese trata de protocolos desenvolvidos para avaliação de saponinas e esteróis em cultivos celulares de *Q. brasiliensis* por GC-MS. Nosso objetivo com estes protocolos é poder avaliar o aporte de carbono nos *pools* de saponinas e esteróis em suspensões. Esteróis têm um papel central no metabolismo primário das plantas, uma vez que fazem parte da estrutura de membranas celulares, por exemplo. Já as saponinas são um componente do metabolismo especializado e são importantes nas interações ecológicas das espécies que acumulam estes compostos. Para avaliar o incremento de carbono 13 nas suspensões celulares, será realizado o cultivo destas com glicose marcada com este isótopo natural. Ao comparar diferentes condições de cultivo e de indução do conteúdo de saponinas (com adição ou não da glicose marcada), o incremento de carbono 13 no esqueleto central das saponinas (aglicona) e de esteróis livres poderá ser examinado. Dessa forma, será possível avaliar o nível de competição pelo aporte de carbono entre a síntese de saponinas e esteróis. É importante ressaltar que isso é possível porque terpenos e esteróis compartilham precursores metabólicos, sendo o último deles o 2,3- óxido esqualeno. Com esta futura investigação, esperamos subsidiar os estudos sobre o metabolismo e biossíntese de saponinas em *Quillaja brasiliensis.*

Esta tese visa contribuir, em um panorama mais global, para a utilização racional, rentável e sustentada de *Quillaja brasiliensis* em cultivos celulares, preservando sua diversidade genética e ocorrência natural. Além disso, almeja-se consolidar as bases de conhecimento necessárias a estudos futuros do metabolismo de saponinas triterpênicas bioativas e a produção heteróloga das vias de biossíntese em microrganismos, em linha com as tendências mais recentes de investigação do metabolismo especializado de plantas.

Objetivo Geral

Definir procedimentos para estabelecer culturas celulares de *Q. brasiliensis* e avaliar sua competência e viabilidade para produção de saponinas.

Referências

BERGMAN, M. E. *et al.* Distinct Metabolic Pathways Drive Monoterpenoid Biosynthesis in a Natural Population of *Pelargonium graveolens* (Rose Scented Geranium). **Journal of Experimental Botany**, v. 71, n. 1, p. 258–271, 2020.

BISWAS, T.; DWIVEDI, U. N. Plant Triterpenoid Saponins: Biosynthesis, *In Vitro* Production, And Pharmacological Relevance. **Protoplasma**, 2019.

BONILLA, F. A.; OETTGEN, H. C. Adaptive Immunity. **Journal of Allergy and Clinical Immunology**, v. 125, n. 2 SUPPL. 2, p. S33–S40, 2010.

CAMPBELL, J. B.; PEERBAYE, Y. A. Saponin. **Research in Immunology**, v. 143, n. 5, p. 526–530, 1992.

CARVALHO, P.E.R Espécies Arbóreas Brasileiras. Saboneteira. Disponível em: https://www.embrapa.br/florestas/busca-de-publicacoes/-/publicacao/1140114/saboneteira-quillaja-brasiliensis. 2003.

CIBULSKI, S. P. *et al. Quillaja brasiliensis* Saponins Induce Robust Humoral and Cellular Responses in a Bovine Viral Diarrhea Virus Vaccine in Mice. **Comparative Immunology, Microbiology and Infectious Diseases**, v. 45, p. 1–8, 2016.

CNCFLORA. ***Quillaja brasiliensis* (A.St.-Hil. & Tul.) Mart.** Disponível em: <http://www.cncflora.jbrj.gov.br/>.

COSTA, F. DE *et al.* Immunoadjuvant and Anti-Inflammatory Plant Saponins: Characteristics and Biotechnological Approaches Towards Sustainable Production. **Mini Reviews in Medicinal Chemistry**, v. 11, n. 10, p. 857–880, 2011.

COSTA, F. DE *et al.* Accumulation of a Bioactive Triterpene Saponin Fraction of *Quillaja brasiliensis* Leaves is Associated with Abiotic and Biotic Stresses. **Plant Physiology and Biochemistry**, v. 66, p. 56–62, 2013.

COSTA, F. DE. **Saponinas Triterpênicas: Biossíntese e Atividade Biológica**. Federal University of Rio Grande do Sul, 2014.

DIDIERLAURENT, A. M. *et al.* Adjuvant System AS01: Helping to Overcome the Challenges of Modern Vaccines. **Expert Review of Vaccines**, v. 16, n. 1, p. 55–63, 2017.

ESPINOSA-LEAL, C. A.; PUENTE-GARZA, C. A.; GARCÍA-LARA, S. *In vitro* Plant Tissue Culture: Means for Production of Biological Active Compounds. **Planta**, v. 248, n. 1, 2018.

FLECK, J. D. *et al.* Adjuvant Activity of *Quillaja brasiliensis* Saponins on the Immune Responses to Bovine Herpesvirus Type 1 In Mice. **Vaccine**, v. 24, n. 49–50, p. 7129–7134, 2006.

FLECK, J. D. *et al.* Saponins From *Quillaja saponaria* and *Quillaja brasiliensis*: particular Chemical Characteristics and Biological Activities. **Molecules**, 2019.

HOGAN, M. J.; PARDI, N. mRNA Vaccines in the COVID-19 Pandemic and Beyond. **Annual Review of Medicine**, v. 73, p. 17–39, 2022.

IEC - Indicadores de Comercio Exterior 2021. Banco Central de Chile. Disponível em: https://www.bcentral.cl/documents/33528/3409132/ICE_cuarto_trimestre_2021.pdf/7da75032-35b3-94e3-5fc2-e7872ea63e91?t=1645724619553. Acesso em 20/04/2022.

KAUFFMANN, C. *et al.* Constituents From Leaves of *Quillaja brasiliensis*. **Natural Product Research**, v. 18, n. 2, p. 153–157, 2004.

KITE, G. C.; HOWES, M. J. R.; SIMMONDS, M. S. J. Metabolomic Analysis of Saponins in Crude Extracts of *Quillaja saponaria* by Liquid Chromatography/Mass Spectrometry for Product Authentication. **Rapid Communications in Mass Spectrometry**, v. 18, n. 23, p. 2859–2870, 2004.

LUEBERT, F. Taxonomy and Distribution of the Genus *Quillaja* Molina (Quillajaceae). **Feddes Repertorium**, v. 124, n. 4, p. 157–162, 2013.

MARCIANI, D. J. Elucidating the Mechanisms of Action of Saponin-Derived Adjuvants. **Trends in Pharmacological Sciences**, v. 39, n. 6, p. 573–585, 2018.

PULENDRAN, B.; S. ARUNACHALAM, P.; O'HAGAN, D. T. Emerging Concepts in the Science of Vaccine Adjuvants. **Nature Reviews Drug Discovery**, v. 20, n. 6, p. 454–475, 2021.

REICHERT, C. L.; SALMINEN, H.; WEISS, J. *Quillaja* Saponins Characteristics and Functional Properties. **Annual Review of Food Science and Technology**, v. 10, n. 1, p. 43–73, 2019.

RIVERA-PATRON, M. *et al.* Iscom-like Nanoparticles Formulated with *Quillaja brasiliensis* Saponins Are Promising Adjuvants for Seasonal Influenza Vaccines. **Vaccines**, 9 (11), 1–18, 2021.

SAN MARTIN, R. Sustainable Production of *Quillaja saponaria* Mol. Saponins. *In*: OLESZEK, W.; MARSTON, A. (Eds.). . **Saponins in Food, Feedstuffs and Medicinal Plants**. [s.l.] Kluwer Academics Publishers, 2000. p. 271–279.

SCHIJNS, V. *et al.* Rational Vaccine Design in Times Of Emerging Diseases: the Critical Choices of Immunological Correlates of Protection, Vaccine Antigen and Immunomodulation. **Pharmaceutics**, v. 13, n. 4, 2021.

SUN, H. X.; XIE, Y.; YE, Y. P. Advances in Saponin-Based Adjuvants. **Vaccine**, v. 27, n. 12, p. 1787–1796, 2009.

YENDO, A. C. A. *et al.* Production of Plant Bioactive Triterpenoid Saponins: Elicitation

Strategies and Target Genes to Improve Yields. **Molecular Biotechnology**, v. 46, n. 1, p. 94–104, 2010.

YENDO, A. C. A. *et al.* Irradiance-based Treatments of *Quillaja brasiliensis* Leaves (A. St.- Hil. & Tul.) Mart. as Means to Improve Immunoadjuvant Saponin Yield. **Industrial Crops and Products**, v. 74, p. 228–233, 2015.

YENDO, A. C. A. *et al.* A Rabies Vaccine Adjuvanted With Saponins From Leaves of The Soap Tree (*Quillaja brasiliensis*) Induces Specific Immune Responses And Protects Against Lethal Challenge. **Vaccine**, v. 34, n. 20, p. 2305–2311, 2016.

YENDO, A. C. A. **Saponinas de *Quillaja brasiliensis*: Purificação e Atividade Biológica**. [s.l.] Federal University of Rio Grande do Sul, 2016.

ZAMBONI, N. 13C Metabolic Flux Analysis in Complex Systems. **Current Opinion in Biotechnology**, v. 22, n. 1, p. 103–108, 2011.

Capítulo 1

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Review

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Future Medicinal Chemistry

Foamy matters: an update on *Quillaja* saponins and their use as immunoadjuvants

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Immunoadjuvant *Quillaja* spp. tree saponins stimulate both cellular and humoral responses, significantly widening vaccine target pathogen spectra. Host toxicity of specific saponins, fractions and extracts may be rather low and further reduced using lipid-based delivery systems. Saponins contain a hydrophobic central aglycone decorated with several sugar residues, posing a challenge for viable chemical synthesis. These, however, may provide simpler analogs. Saponin chemistry affords characteristic interactions with cell membranes, which are essential for its mechanism of action. Natural sources include *Quillaja saponaria* barks and, more recently, *Quillaja brasiliensis* leaves. Sustainable large-scale supply can use young plants grown in clonal gardens and elicitation treatments. *Quillaja* genomic studies will most likely buttress future synthetic biology-based saponin production efforts.

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The genus *Quillaja* consists of two temperate evergreen tree species. *Quillaja saponaria* Molina is mainly found in central Chile and was reported once in Andean Bolivia. *Quillaja brasiliensis* (A. St.-Hil. & Tul.) Mart. is a typical representative of Araucaria forests and occurs in southern Brazil, northern Uruguay, northeastern Argentina and eastern Paraguay [1]. *Quillaja* spp. is commonly known as 'soap tree' because its saponins produce persistent foam in water.

Saponins are molecules derived from plant specialized metabolism that are characterized by an amphipathic nature, due to their hydrophobic central aglycone decorated with several hydrophilic sugar residues. Barks and wood of *Q. saponaria* and leaves of *Q. brasiliensis* are the main sources of *Quillaja* saponins. The exploration of different sources has important implications for sustainable production of bioactive metabolites. Saponin-rich extracts of *Q. saponaria* have been extensively produced to be used in food and beverages, cosmetics, photography and in the pharmaceutical industry, as adjuvants in vaccine formulations.

Over the last decades, it has been shown that saponins from *Quillaja* spp. and other plant species have adjuvant activity [2]. Adjuvants are added in vaccines aiming at increasing and directing adaptive immune responses to antigens, which comprise mainly lymphocytes B and T activities. Antigens generally activate B cells by using T helper cells (Th1 or Th2). Th1 cells produce IFN-γ, which stimulates macrophages activation and the synthesis of B cells' opsonizing antibodies. This kind of response is characterized mostly by cell-mediated immunity (cellular response), which is effective in destroying pathogens and cancer cells. Cytotoxic T lymphocytes are the subgroup of T cells responsible for infected cell death. In viral infections and cancer, natural killer cells are important in apoptosis induction. Th1-mediated immunity induced by saponin adjuvants is always followed by Th2 response, which is a repairing type of immunity response. Also, Th2 cells secrete cytokines, such as IL-4, leading to the activation of B cells to produce neutralizing antibodies, critical for humoral responses against extracellular pathogens (e.g., extracellular microbes and helminths) [3].

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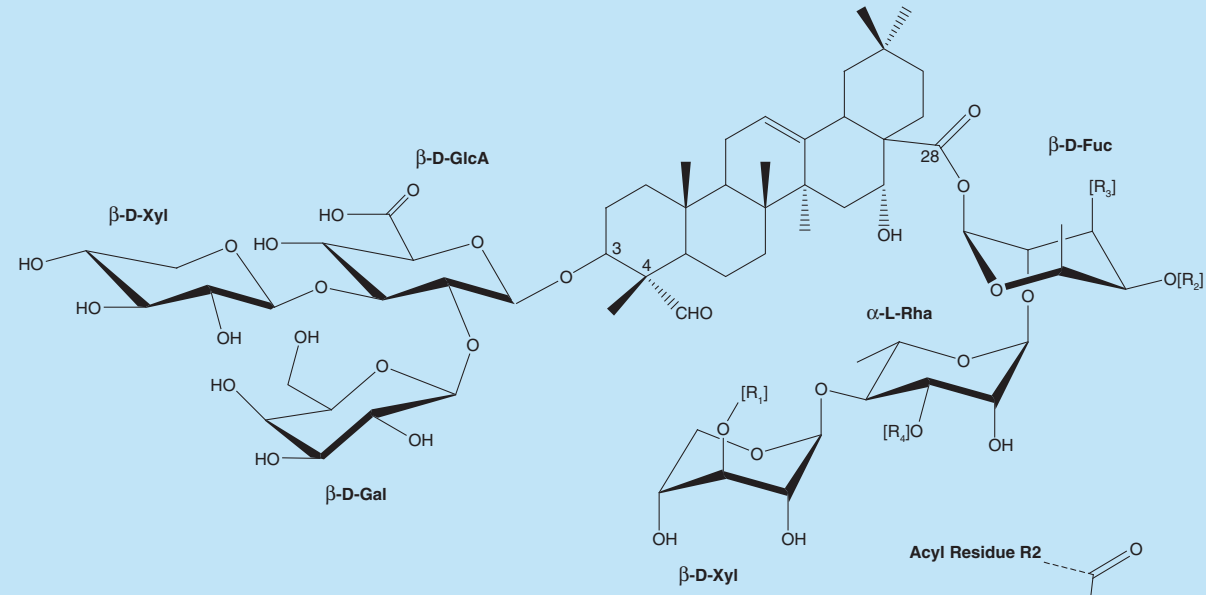


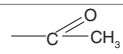
Saponin	R1	R2	R3	R4	R5
QS-7	-β-D-Api		-β-D-Glc	-β-D-Glc	
QS-17	-β-D-Api	-Acyl	-OH	-β-D-Glc	-α-L-Rha
QS-18	-β-D-Api	-Acyl	-OH	-β-D-Glc	-H
QS-21-Api	-β-D-Api	-Acyl	-OH	-H	-H
QS-21-Xyl	-β-D-Xyl	-Acyl	-OH	-H	-H

Figure 1. Chemical features of bioactive saponins identified after purification of the commercial fraction Quil-A.

Quillaja saponins are capable of stimulating both cellular and humoral immune responses, widening the spectrum of vaccine targets. In contrast, some commonly used adjuvants promote essentially Th2 responses, and one of the best studied of this kind is alum [4]. In this article, we review the main features of *Quillaja* saponins regarding chemical structure and analysis, immunoadjuvant activity and considerations on sustainable sources for commercial saponin production.

Chemical features of *Quillaja saponaria* saponins

The first study of a saponin-rich extract from *Q. saponaria*, named Quil-A, was published in the mid seventies [5]. The crude mixture of saponins is extracted with water from the milled inner bark [6] (traditional method) or the whole wood of trees, often obtained from harvest operations carried out in natural forests. After extraction, the bark extract is purified by dialysis, ion-exchange and gel filtration chromatography to result in a mixture up to 25 different saponins enriched in adjuvant and hemolytic activity [7]. The heterogeneity of the product and its toxicity hindered the use in human vaccines, but it has been widely applied in the veterinary sector [8–11].

A breakthrough toward the isolation of saponins with less hemolytic activity was achieved in the early nineties [7]. The enriched fraction Quil-A was used as the starting material that was submitted to reversed-phase HPLC, resulting in the separation of four different saponins (QS-7, QS-17, QS-18 and QS-21), which were elucidated and characterized (Figure 1). In spite of being one of the most promising molecules, QS-7 was present in significantly lower amount in the mixture and, as a result, was poorly explored.

Structure elucidation of *Quillaja* spp. saponins started in the late eighties [12]. Most of the isolated saponins are amphiphilic glycosides composed of a quillaic acid (3β, 16α-dihydroxy-23-oxolean-12-en-28-oic acid) aglycone substituted with saccharide chains residues at C-28 and C-3. The main aglycones identified are quillaic acid, phytolaccagenic acid, 22-β-hydroxy-quillaic acid and echinocystic acid [13]. The C-28 oligosaccharide substitution

is mainly conserved, with an α-L-Rha*p*-(1→2) β-D-Fuc*p* disaccharide ester attached to the aglycone. In the majority of *Q. saponaria* saponins, this saccharide chain is extended by a (1→4)-linked β-D-Xyl*p* to which could be linked a (1→3) β-D-Api*f* or other β-D-Xyl*p*. This latter difference is responsible for the immunoadjuvant saponin QS-21 not being a homogeneous substance, but rather a mixture including either apiose (QS21-Api) or xylose (QS21-Xyl) as the terminal sugar residue within the tetrasaccharide segment, in a proportion of 65 and 35%, respectively (Figure 1) [14,15].

The Rha*p* unit of the disaccharide may have a (1→3) β-D-Glc*p*, whereas the β-D-Fuc*p* unit can be linked to a (1→3) β-D-Glc*p* (sometimes acetylated) or a (1→3) α-L-Rha*p*. The oligosaccharide is acyl substituted in the O-4 fucosyl residue by an acetyl substituent. Alternatively, it may have an (1→5) ester substituent comprising a pair of 3,5-dihydroxy-6-methyloctanoic acid molecules terminated by (1→5) α-L-Ara*f*. The fatty acid domain can also migrate to fucose O-3, leading to an equilibrium mixture of both isomers in basic solution. For QS-21, each isomer (QS-21Api and QS-21Xyl) has two regioisomers named QS-21A and QS-21B, depending on the point of attachment of the acyl group on the O-4 or O-3 fucosyl residue, respectively [16].

The C-3 di- or trisaccharide substitution also appears to be conserved, being composed of an ether-linked glucuronic acid (β-D-Glc*p*A) bearing a galactose (β-D-Gal*p*) at O-2 and xylose or rhamnose (β-D-Xyl*p* or α-L-Rha*p*) at O-3. Saponins with quillaic acid have been often isolated in pairs, differing only in whether the C-3 trisaccharide contains Xyl*p* or Rha*p* [13,16,17].

Since saponins have an amphiphilic nature, they display surface activity and are able to adsorb on aqueous interfaces. Once the interface becomes saturated with saponin molecules, micelles are formed and can incorporate water-insoluble compounds [18,19]. Each micelle contains a nonpolar aglycone facing the core of the structure with anionic hydrophilic sugar residues directly in contact with the aqueous solution. The defined concentration above which micelles are formed is termed 'critical micelle concentration' [20], which is strongly affected by aqueous phase conditions such as pH, temperature and ionic strength [21]. For instance, formation of *Quillaja* micelles with Na-caseinate, pea protein and different lecithins was drastically affected by pH, temperature and saponin concentration [22–24].

In a study involving Q-Naturale 200 emulsifier® (Ingredion, IL, USA), a natural food-grade surfactant based on *Q. saponaria* saponin, interfacial properties comparable to those of Tween 80, a synthetic nonionic surfactant, were reported. *Quillaja* saponins were able to form oil-in-water emulsions with relatively small droplet sizes (<200 nm in diameter), which were stable in a wide range of pH (3–8), temperature (30–90°C) and salt concentration (≤300 mM NaCl) [25]. However, origin and composition of *Quillaja* saponins can result in completely different surface properties [26].

In a study using *Quillaja* bark saponin (QBS; Sigma), the combination of QBS and the active biopolymer β-casein, a protein from bovine milk, was analyzed [27]. The best result in terms of interfacial tension decay was obtained in a mixture of 1:1 of both surfactants, showing a synergistic effect. It has also been shown that for each saponin molecule (QBS) 60 water molecules are necessary for hydration of the sugar moieties [28].

The effective solubilization of the oil-soluble carotenoid lutein ester, which is used as a natural food colorant, was demonstrated using *Quillaja* saponins (Ingredion) as emulsifier [29]. The particle size of each micelle increased from 7.5 nm (unloaded micelle) to 130 nm (lutein ester loaded). This poorly water-soluble molecule can be degraded (oxidized) during processing and storage, resulting in color change. *Quillaja* saponins stabilized the color in micelles and nanoemulsions during the processes of atomization, spray drying and storage [30].

The structure and conformation of QS-21 in micelles have been described using molecular modeling and dynamics and small-angle x-ray scattering [31]. When QS-21 forms micelles in aqueous solutions, the ester linkage between the fucose residue and the acyl chain is less solvated and, consequently, more protected against degradation by spontaneous hydrolysis, which increases storage stability. Besides, it was shown that increased number of QS-21 molecules in a micelle (up to 20 molecules were tested), yielded lower accessibility of the aqueous solvent to the ester linkage.

Methods for chemical analysis

Recently, significant research efforts have been made to establish new high resolution methods of saponin structural characterization. In addition, improvements on available analytical methods have also been sought [32]. Saponins are typically separated in fractions using reversed-phase HPLC or high-performance thin-layer chromatography before being analyzed by mass spectrometry (MS) and/or nuclear magnetic resonance (NMR) [33–35]. The introduction of new pulse sequences, such as heteronuclear 2-bond correlation, constant time inverse-detected gradient accordion

rescaled long-range–heteronuclear multiple bond correlation and heteronuclear single quantum correlation–total correlation spectroscopy has been extremely helpful in NMR elucidation studies [36].

Regarding MS, MALDI–TOF–MS is most useful to generate molecular ions $[M+H]^+$ [30,37]. On the other hand, ESI–MS and ESI–MS^n have been increasingly explored as they allow obtaining peculiar fragmentation patterns, which help to provide missing information regarding the structural data of unknown saponins [36,38].

Various saponin derivatives have been found in *Q. saponaria* saponin extracts, ranging from a MW of 1500 to >2200 g/mol [30,34]. In order to authenticate *Q. saponaria* extracts in some commercial products and perform quality control analysis, a combination of HPLC and subsequent ESI–quadrupole ion trap–LC–MS successfully separated >100 saponins [34]. Qualitative analysis of *Quillaja* saponin extract as a surfactant was performed using high-performance thin-layer chromatography coupled with MALDI–TOF–MS [30].

Chemical features of *Quillaja brasiliensis* saponins

Chemistry elucidation of *Q. brasiliensis* saponins is not completed, but it is already well know that these triterpenes are similar to those reported for *Q. saponaria* [37,38]. The prosapogenin 3-O-β-D-glucuronopyranosyl-quillaic acid was isolated after acidic hydrolysis of the aqueous extract (AE) from leaves of *Q. brasiliensis*, resembling the aglycone found in the congener species [39]. Subsequently, a methodology for purifying *Q. brasiliensis* saponins from dried leaves was developed. Extraction of powdered leaves was carried out in distilled water. An AE was obtained after filtering, partitioning with ethyl acetate and lyophilizing the first extract. AE was further purified through reverse-phase chromatography, yielding fractions QB-80 and QB-90 [40–42].

AE and the saponin-enriched fractions QB-80 and QB-90 proved to be effective immunoadjuvants used with a wide range of antigens [37,43–46]. The saponin fraction QB-80 and AE were compared with Quil-A using MALDI–TOF MS. Fractions showed similar spectral patterns and characteristic peaks of cleavages, supporting the close chemical similarities between saponins present in the two species of Quillajaceae [37].

Another approach starting with an AE of *Q. brasiliensis* leaves, followed by SPE on reversed phase C-18 and elution with a step-wise gradient of methanol and water, resulted in a purified fraction named Fraction B [46]. Recently, this fraction was used for further chemical studies, which identified 27 saponins in *Q. brasiliensis* using C-18 reverse-phase ultra HPLC followed by LC–ESI–IT–MS^n (ESI ion trap multiple-stage MS) [38]. All of the structures were characterized as having quillaic acid, gypsogenin, phytolaccinic acid and its acetate as aglycones, as well as saccharide substitutions in C-3 and C-28 (Table 1). The immunoadjuvant saponin QS-21 was also found in *Q. brasiliensis*, including its isomers and regioisomers, although the yield of the compound in *planta* has not been determined [38].

Regarding biosurfactant properties of *Q. brasiliensis*, a single study showed that the method of extract preparation (dried versus fresh plants) affected the foam index [47], being higher when using dried plants. *Q. brasiliensis* extract could reduce superficial tension of aqueous solutions, showing a potential application for this species that needs to be further explored.

Immunoadjuvant activities

Adjuvant activity of *Quillaja saponaria* saponins

Saponins from *Q. saponaria* have been widely studied due their strong immunoadjuvant potential. The saponin fraction Quil-A showed high capacity of stimulating both humoral and cellular immunity, leading to several pre-clinical studies, including vaccines against measles, foot-and-mouth disease, *Neisseria meningitidis*, *Mycobacterium tuberculosis*, *Taenia solium*, *Echinococcus granulosus* and *Fasciola hepatica* [48–52], as well as commercial use in veterinary vaccines, including equine influenza virus, canine parvovirus and feline leukemia virus [53]. Quil-A may be used in formulations together with other adjuvants, such as oil-in-water emulsions, aluminum salts and liposomes, increasing its immunostimulatory capacity. However, its heterogeneous composition and toxicity do not allow it to be used in human vaccines.

Fractions QS-7 and QS-21, due to their lower toxicity relative to QS-18, have been the subject of more in-depth studies. QS-21 has been the main focus of vaccine development research, having applicability in human vaccines. QS-21 stimulates a strong Th1 response through the production of cytokines IL-2 and IFN-γ, and antigen-specific antibodies responses of both IgG1 and IgG2a, which makes it ideal for use in vaccines against intracellular pathogens [4]. In fact, QS-21 has been already used commercially as an effective adjuvant with a recombinant retroviral subunit vaccine against feline leukemia virus, and together with deacylated saponins in Leishmune® formulation against *Leishmania donovani* [54,55].

Table 1. *Quillaja brasiliensis* saponins (Fraction B).

Saponins n°	A ion (*m*/*z*)¶	Aglycone	R_0	R_1	R_3	R_4
1–5, 7	895.5	P-Ac	H	H	Glc‡	–
6	853.5	P	H	H	Glc‡	–
8, 10	955.5	Q	Xyl	Xyl	Glc	–
9	955.5	Q	Xyl	Xyl	H	–
11	969.5	Q	Rha	Xyl	Glc	–
12	955.5	Q	Xyl	Xyl-Api	Glc	–
13	939.5	G	Xyl	Pent-Pent	Hex	–
14	955.5	Q	Xyl	Xyl-Xyl or Xyl-Api	H	Fa+Ara
15	955.5	Q	Xyl	Xyl-Xyl or Xyl-Api	H	Fa+Ara
16	955.5	Q	Xyl	Xyl-Xyl or Xyl-Api	H	Fa+Ara
17	955.5	Q	Xyl	Xyl-Xyl or Xyl-Api	H	Fa+Ara
18	939.5	G	Xyl	Xyl-Xyl or Xyl-Api	H	Fa+Ara
19	939.5	G	Xyl	Xyl-Xyl or Xyl-Api	H	Fa+Ara
20	955.5	Q	Xyl	Xyl-Xyl or Xyl-Api	H	Fa+OH
21	955.5	Q	Xyl	Xyl-Xyl or Xyl-Api	H	Fa+OH
22	955.5	Q	Xyl	Xyl-Xyl or Xyl-Api	H	Fa
23	955.5	Q	Xyl	Xyl†	H	Fa+Ara†
24	955.5	Q	Xyl	Xyl†	H	Fa+Ara†
25	955.5	Q	Xyl	Xyl†	H	Fa+Ara†
26	955.5	Q	Xyl	Xyl†	H	Fa+Ara†
27	955.5	Q	Xyl	Xyl	H	Fa

†Could be other pentose.
‡Glucose could be on R1 or R4 positions.
Summary of data obtained from LC-ESI-IT-MS2 analysis reproduced with permission from [38] © Elsevier (2017).
¶The main characteristic fragment ion observed in the MS^2 spectra of the known *Q. saponaria* saponins when $[M-H]^-$ was selected as precursor ion is a product ion in the region *m*/*z* 800–1000 designated as the 'A ion'
G: Gypsogenin; P: Phytolaccinic acid; P-Ac: Acetylated phytolaccinic acid; Q: Quillaic acid.

However, the toxicity and the undesirable hemolytic effect of QS-21 are a disadvantage for widespread use in humans, limiting doses to approximately 50 μg/application for most patients, with the exception of cancer treatment vaccines (melanoma, prostate and breast cancers), for which the limit dose is 100 μg [56,57]. To overcome this obstacle, methods have been developed to increase immunogenicity of small amounts of QS-21. For example, a synergistic effect is obtained by combining with adjuvant systems (AS) stimulants, eliciting a more robust immune response with reduced adverse events. AS AS01, containing QS-21 and 3-O-desacyl-4′-monophosphoryl lipid A formulated in a liposome, significantly decreases the adverse effects in rat skeletal muscle and enhances antibody titer with an improved $CD8^+$ T-cell response [58]. AS01 is currently being tested in human clinical trials for several vaccine candidates, which include malaria, tuberculosis, HIV and chronic obstructive pulmonary disease exacerbations associated with nontypeable *Haemophilus influenzae* and *Moraxella catarrhalis* [59].

Two vaccines in a more advanced stage of development are an example of the potential use of this system. AS01 is used as an adjuvant in RTS,S vaccine (Mosquirix™) against *Plasmodium falciparum*. This vaccine was developed jointly by the Malaria Vaccine Initiative and GlaxoSmithKline, and it is the only recombinant malaria vaccine to reach Phase III clinical trials. Although efficacy of RTS,S/AS01 is rather modest (39%), protection is relatively short term, and a series of four vaccinations is required [60], there are pilot implementation projects with start of vaccinations in 2018 to consider its quality, safety and efficacy [61]. AS01 was also used in Shingrix, a recombinant vaccine approved for herpes zoster prevention of adults over 50 years old. Shingrix was more effective than the commercially available option, with 97% of efficacy in adults aged 50–69 years and 91% efficacy in older adults. This efficacy remains high even after 4 years of vaccination, being at least 85% in patients aged 70 and older [62].

Another strategy adopted to overcome toxicity of saponins is combining them with cholesterol and phospholipids to form a 40 nm spherical structure named ISCOM. Hydrophobic interactions are established with amphipathic molecules derived from cell walls and membranes of the antigens, so that the latter are exposed on the surface. These have been used as antigen delivery systems that proved to trigger powerful immune-stimulating activities, yet

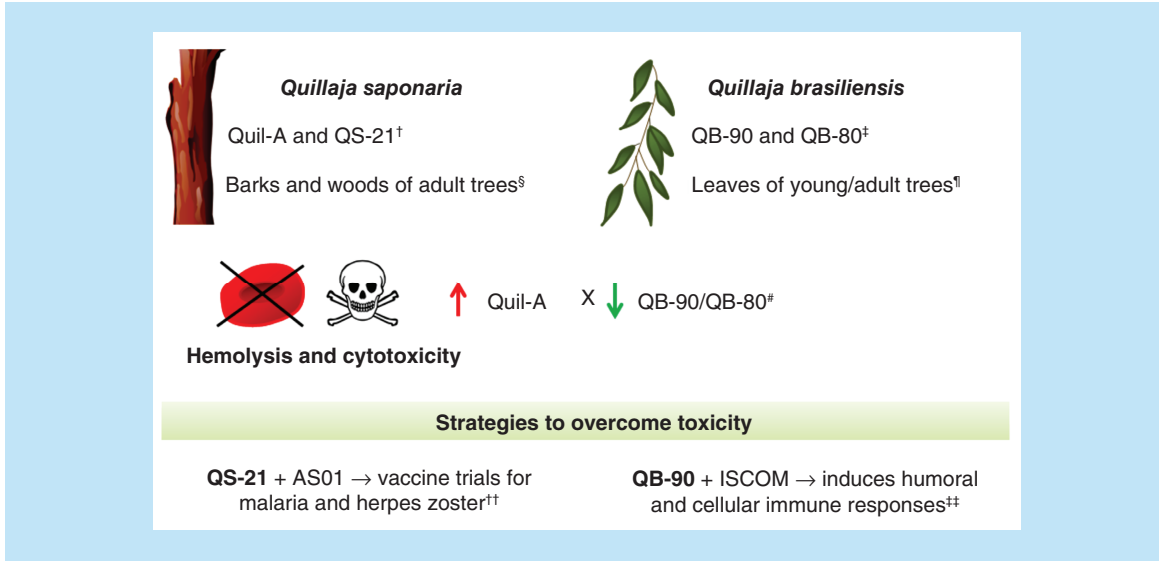


Figure 2. ***Quillaja* species provide biomass for immunoadjuvant saponins production.** Toxicity of saponins toward different human and animal models has been evaluated. Assembling saponins with lipid matrixes has been used to overcome toxicity.
†Data taken from [7].
‡Data taken from [40,42].
§Data taken from [80].
¶Data taken from [95].
#Data taken from [37,43,45,70].
††Data taken from [61,62].
‡‡Data taken from [70].

displaying reduced toxicity in several animal models [63,64]. There are commercial vaccine options on the market using ISCOMs for diseases such as equine influenza [65]. If the structure comprises only cholesterol, phospholipid and saponin – without antigen – it is named ISCOMATRIX [66]. Both systems can produce more immunogenic and less toxic responses, since saponins are trapped inside the structure [11]. QS-21 has been extensively investigated in human vaccine formulations, either alone in purified form or as a major component of adjuvant mixtures (ISCOMs, ISCOMATRIX, AS01 and AS02) [67–69].

Adjuvant activity of *Q. brasiliensis* saponins

Saponins isolated from *Q. brasiliensis* are a promising alternative source for the development of new vaccines, with studies certifying their immunoadjuvant activity against several viral types in murine model. The crude AE from leaves, barks and branches, as well as the purified fraction QB-90, were able to potentiate the immune response against bovine *herpesvirus* type 1 antigen in a manner comparable to Quil-A, not having a dose-dependent profile and presenting low *in vivo* toxicity [41]. In fact, the induction of IgG1 and IgG2a levels indicates potential stimulation of both humoral and cellular responses, pointing to viable use of these saponins against a wide range of pathogens [41]. Subsequent studies revealed that QB-90 and AE are safe adjuvants based on hemolytic activity and cell cytotoxicity assays, with significantly less toxicity compared with Quil-A (Figure 2) [37,43,45,70]. Performance of QB-90 compared with Quil-A and aluminum hydroxide against bovine *herpesvirus* type 5 antigen was evaluated. Responses of IgG antibodies and delayed type hypersensitivity (DTH) were monitored showing that these saponins stimulate the production of high antibody titers and a strong DTH response, the latter being an indicative of cell-mediated immune responses associated with Th1 reactivity. Moreover, QB-90 is capable of inducing Th2 and Th1 $CD4^+$ cells in antibody responses, by induction of high levels of IgG1 and IgG2a - IgG3, respectively. The Th1 $CD4^+$ cells generation was confirmed by assessing the expression levels of Th1 cytokines IL-2 and IFN-γ in splenocytes from immunized mice [45].

The strong DTH response and high Th1 and Th2 stimulation by QB-90 have been further corroborated in studies against other viral types, such as poliovirus, rabies and bovine viral diarrhea virus (BVDV) [37,43,44,46]. Indeed,

vaccine formulations with QB-90 or AE stimulated humoral and cellular immune responses and enhanced IL-2 and IFN-γ cytokine mRNA expression in mice splenocytes similarly to Quil-A [43,44]. AE-adjuvanted formulations have also been able to induce long-term specific humoral immune response against BVDV antigen and provide dose-sparing effect, causing the amount of antigen required for the formulation to decrease fivefold, without affecting the quality of the immune response [37].

QB-90 fraction and AE immune responses induce cytokine profiles comparable to those of *Q. saponaria* saponins [43]. When immunized with BVDV vaccine adjuvanted with QB-90, mice enhanced BVDV-specific $CD8^+$ T-cell proliferation, which produces IFN-γ, IL-2 and lymphotoxins, playing a central role against intracellular pathogens by the cell-mediated immune response. Memory $CD8^+$ T-cells induction by external antigen indicates that QB-90 enhances antigen cross-presentation presumably by a chemotactic effect recruiting dendritic cells (DCs) to the site of immunization. Thus, viral antigen recognition by $CD8^+$ T cells occurs in the form of short-length peptides presented by MHC-I molecules on the exterior of virus-infected cells leading to their lysis, or noncytopathic intracellular inactivation of the pathogen involving cytokines IFN-γ and TNF-α. Besides, QB-90 enhanced the production of IL-10 and IL-17, but did not increase IL-4 and IL-6, suggesting a Th1 bias. The vaccine containing QB-90 as adjuvant promoted significant increases in IFN-γ^+ $CD4^+$ and IFN-γ^+ $CD8^+$ T cells when compared with mice vaccinated with adjuvant-free vaccine. This profile shows that the vaccine with QB-90 was able to cause antigen-specific activation of $CD8^+$and $CD4^+$ T cells [43].

QB-90 is also able to form ISCOMs (IQB-90) akin to *Q. saponaria* saponins. Subcutaneously applied IQB-90 induced intense humoral and cellular immune responses, whereas intranasal delivery significantly increased ovalbumin immunogenicity without resulting in systemic IgG2a responses or DTH effects [70]. The capacity of *Q. brasiliensis* saponins to elicit mucosal responses after subcutaneous immunization is in agreement with previous assays demonstrating high levels of IgA antibodies in bile, feces and vaginal washings [44]. IQB-90 displays no hemolysis and enhances the antigen internalization efficiency, as demonstrated by bone marrow-derived DCs ovalbumin-fluorescein conjugate uptake assay [70]. Both QB-90 and its ISCOMATRIX IMQB-90 (formulated without antigen) promoted recruitment of neutrophils, DCs, natural killer, B and T cells, and may have activated inflammasomes by differential production and secretion of IL-1β by bone marrow-derived DCs. However, whereas QB-90 promoted an immunocompetent environment inducing changes in the expression of 41 genes, IMQB-90 altered transcript levels of only 23 genes, suggesting that inflammation induced by QB-90 alone is stronger than that by IMQB-90. Although 19 genes encoding cytokines and chemokines were upregulated by both treatments, the fact that IMQB-90 did not activate as many proinflammatory and activation genes as QB-90 may partly explain this nanoparticulated formulation's lower toxicity and hemolytic activity [71].

Another fraction of saponins isolated from leaves, known as QB-80, has also proven highly effective in inducing both humoral and cellular immune responses in experimental vaccines. Formulations containing purified fractions of QB-80 and QB-90 saponins or AE of *Q. brasiliensis* leaves stimulate humoral and cellular immune responses comparable to those induced by Quil-A fraction. The protective efficacy of the saponin-adjuvanted vaccines (QB-80, QB-90 and AE) could be demonstrated even with the administration of only one dose during challenge against the lethal rabies virus strain, which allows reducing the cost of using a commercial vaccine and proves the relevant bioactivity of these compounds [46].

QB-80 was less toxic than Quil-A in *in vitro* hemolytic activity and in *in vivo* toxicity assays. Mice injected with QB-80 kept healthy, displaying no signs of local toxicity, with any of the solutions tested (up to 150 μg/ds), until the end of the assay, which did not occur with animals inoculated with 150 and 75 μg of Quil A. QB-80 promoted both humoral and cellular long-term immune responses in murine model BVDV vaccines, significantly enhancing specific IgM, IgG, IgG1 and IgG2a antibodies titers against BVDV antigen. Furthermore, the positive DTH reaction caused by QB-80 indicated that this fraction used as BVDV vaccine adjuvant promoted $CD8^+$ T cells, resulting in a Th1-type response. This is further suggested by flow cytometry analysis because mice immunized with QB-80-adjuvanted formulations had higher intracellular IFN-γ production by $CD4^+$ and $CD8^+$ T cells when compared with nonadjuvanted formulation [37]. The favorable results obtained with QB-80, QB-90 and AE reinforce the use of saponins of the Brazilian species as an effective substitute or complement to Quil-A.

Mechanism of action

QS-21 and similar saponins, such as QS-18, seem to interact with both DCs and T cells. QS-21 induced cytotoxic T-lymphocytes proliferation and production of Th1 cytokines, IFN-γ and IL-2. Moreover, these saponins promoted the release of antibodies IgG2a isotype to protein antigens [72]. A review of its probable mechanisms of action was

recently published [4]. Briefly, these saponins bind to the T-cell surface receptors, like CD2, and generate a T-cell costimulatory signal that results in changes in Na^+/K^+ transport. In addition, there is a T-cell receptor-mediated signaling, which occurs by kinase ERK2 phosphorylation and mobilization of Ca^{2+}. These events promote T-cell activation and boost the production of Th1 cytokines by T cells [4].

QS-21 and other *Quillaja* spp. saponins have an aldehyde group attached to C-4 (Figure 1), which is capable of forming imine carbonyl groups with T-cells surface ligands [73]. It has also been reported that QS-21 lost adjuvant activity after modification of its C-4 aldehyde [74]. Therefore, this feature may be important for Th1/Th2-induced immunity response. Plant saponins lacking aldehyde residues seem to have other imine-forming carbonyl groups, such as ketones and acyl chains [4,75].

Saponin adjuvants facilitate the endosomal escape of antigen necessary for cross-presentation in MHC-I by intracellular lipid body formation in DCs. There is probably an endocytosis of antigen/QS-21 dependent on cholesterol that leads to the lysosomes, where the processing of the antigen by lysosomal proteases and QS-21 destabilization occur, allowing membrane disruption and rapid antigen translocation to the cytosol for proteasome-independent cross-presentation of antigen–MHC-I complexes. This cross-presentation induction ability leads to the stimulation of potent $CD8^+$ T-cell responses [4,76]. Triterpenic cores of saponins have high affinity for cholesterol and may disrupt cell membranes by intercalating into its cholesterol-rich areas [4,77].

QS-21 was further tested through deacylation of the fucosyl pyranose at C-28, resulting in QT-0101. This product was capable of inducing only Th2 responses [78]. This apparent paradox was reviewed in [4] and may be explained by the fucopyranosyl residue attached to QS-21 aglycone at C-28. This residue seems to bind to a C-type lectin receptor of DCs inducing preferentially the Th2 type response [79].

ISCOMs and other particulated saponin formulations also enhance antigen uptake, prolong retention by DCs in draining lymph nodes, induce activation of DCs, lead to potent Th1- and Th2-like immune responses, besides activating cytotoxic T cell, specific antibody responses and innate immune cells [80–84]. The key role of innate immunity in the mode of action of adjuvants has been well established by several studies [83,84]. On the other hand, AS01 adjuvant system response is the result of the synergistic action of monophosphoryllipid A, an immunoadjuvant, and QS-21, which promotes a fast and transient innate immune response at injection site and the draining lymph node, also leading to the activation of a broad range of DCs and the modulation of protective immune response [58].

Sustainable biological sources

The sources of bioactive *Quillaja* spp. saponins are a matter of considerable relevance, particularly taking into account the major technical challenges of the chemical synthesis of these complex triterpenes (see next section). Hence, strategies toward sustainable biomass production as a way to preserve native trees and to obtain high-quality plant material for industrial applications are needed.

Quillaja saponaria

Q. saponaria bioactive saponins were first identified from bark extracts (Figure 2), and Quil-A is a recognized commercial saponin fraction. Plant extracts (mostly *Quillaja* extracts) brought US$ 13.3 million to the Chilean economy in 2017, as 613 tons were exported to the USA, Europe, Australia and Eastern Asia [85].

Only barks and woods of adult trees over 25 years old are currently explored for saponin extraction. Reports present different total saponin content from bark/wood extractions in water, ranging from 1.2% (w/w) yield in branches to 20% (w/w dry mass) in extracted solids from barks [86,87]. Saponin content can vary among different genotypes and field conditions [88].

Quillaja forests cover about 320,000 hectares of Chilean land, with an estimated 4.5 tons of biomass production per acre [89]. Chilean government regulates *Quillaja* commercial and native forests exploitation, and authorizes producers to harvest 35% biomass of each tree every 5–10 years, so as to allow plant regeneration [80]. *Q. saponaria* grows slowly, approximately 30 cm/year in height and 0.6–0.8 cm/year in diameter [90]. Also, despite governmental regulation, it has been estimated a 237,000 ha area reduction of native Chilean forest between 1999 and 2015 [91]. Therefore, one limiting aspect to consider *Q. saponaria* barks/wood as sustainable sources of saponin is production rate of plant biomass.

Quillaja spp. sustainability was first addressed in 1996, when Catholic University of Chile improved saponin extraction from barks and wood. Traditional bark peeling was reducing *Q. saponaria* native forests very fast due to phloem removal, an essential vascular tissue. The new method allowed producers to harvest shoots and branches from degraded forests, which usually present bush-like trees, alleviating the pressure on older native trees [92].

Pursuing new sources like leaves, which are relatively fast growing organs, could be an interesting alternative. Leaves from 3-year-old trees of *Q. saponaria* were harvested along 1 year, and average content of saponins was 2.58% (w/w), displaying seasonal variation. Therefore, leaf saponins content in young trees was comparable to that obtained from bark/wood extracts, as described above. On the other hand, industrial extraction protocols may not be suitable to leaves of the species [93].

Improving extraction methods, as a means to provide higher saponin yields, is a relevant strategy to increase whole process sustainability. For example, it was shown that ultrasound could enhance saponin extraction from bark and wood of *Q. saponaria.* Including ultrasound reduces extraction time from hours to minutes and temperature from 60 to 20°C [94]. However, further investigation is necessary regarding ultrasound frequency and raw material/solvent ratio for industrial treatment chambers.

Quillaja brasiliensis

Q. brasiliensis field-grown trees were used to obtain purified saponin fraction QB-90 (Figure 2). Detached leaves from adult trees and barks showed an average QB-90 content of 0.085 and 0.022 (% dry weight) [95]. Interestingly, QB-90 content in leaves of laboratory cultivated seedlings was four-times higher compared with that of adult plant leaves [40].

Studies concerning elicitation of QB-90 in *Q. brasiliensis* leaves were carried out. Bioactive saponin fractions could be induced by osmotic stress, mechanical damage, plant hormones (e.g., jasmonic acid, salicylic acid), ultraviolet radiation and increased light intensity, indicating a possible role of saponins in plant defense mechanisms [96]. Postharvest treatment with red light or UV-C radiation was able to increase QB-90 content in leaves harvested from young and adult plants. Saponin content of harvested leaves remained stable after 10 days of dark storage at room temperature. It was also observed that young leaves had higher basal and elicited saponin content [95].

Taken together, data suggests saponins can be purified from leaves of both adult and young *Quillaja* trees. In *Q. brasiliensis*, saponin content could be artificially elicited. Leaves grow relatively faster than branches, and are available all year long in *Quillaja* species. Also, harvesting leaves does not compromise tree viability, unlike bark and wood removal that could result in death. Some authors suggest that harvesting whole plants of *Q. saponaria* in commercial farms, especially young-aged trees, would be interesting to optimize biomass production and preserve native trees [88,93]. Preparing for both of these scenarios of sustainable biomass production, selected high saponin-yielding genotypes of *Q. brasiliensis* have been grown and studied in miniclonal gardens [97].

In vitro propagation could also provide sustainable sources of plant material, since it allows rapid growth of individuals or tissues in controlled environment. These individuals can be selected for high production of secondary metabolites. Since native arboreal species usually are not subjected to breeding programs, biotechnological approaches are an interesting way of obtaining specific plant materials for basic and applied studies (Figure 3). A micropropagation protocol was described for *Q. brasiliensis* [98]. *Q. brasiliensis* seeds were aseptically germinated, reaching up to 95% of germinability. From *in vitro* seedlings, shoot explants were grown in semisolid culture medium supplemented with the cytokinin phytohormone 6-benzylaminopurine. Derived shoots were cultivated for 4 months. Elongated shoots rooted promptly, even without exogenous auxin hormonal supplementation. Overall, however, best rooting responses were observed in medium having 10 mgl^{-1} of the auxin indole-3-acetic acid. Rooted explants transferred to soil had 95% survival rate, after 10 months of experiment. QB-90 content in leaves of 6-month micropropagated plants was comparable to those found in field-grown individuals. Therefore, it would be possible to have high-quality plant material in approximately 1 year using micropropagation.

In vitro propagation also includes plant cell cultures. In 1993, a patent was deposited on methods of plant cell cultures of *Quillaja* species [99]. This patent included cell cultures parameters, saponin extraction methods and bioactivity characterization. Cell cultures allow homogeneous biomass production under controlled conditions, being a potential source of specialized metabolites of interest [100].

Chemical synthesis

Considering technical and logistic difficulties in obtaining natural *Q. saponaria* saponins in adequate purity and homogeneity to meet vaccine market demands, chemical syntheses of saponins could be an alternative to obtain these compounds. Nevertheless, chemical syntheses require huge efforts due the complexity of these molecules. Natural *Q. saponaria* saponins can be divided into four domains: a quillaic acid triterpene core, a branched trisaccharide attached at the terpene C-3 hydroxyl, a linear oligosaccharide linked to the triterpene C-28 carboxyl group and a dimeric acyl chain. Their isomeric components differ mainly in the constitution of sugar residues [101].

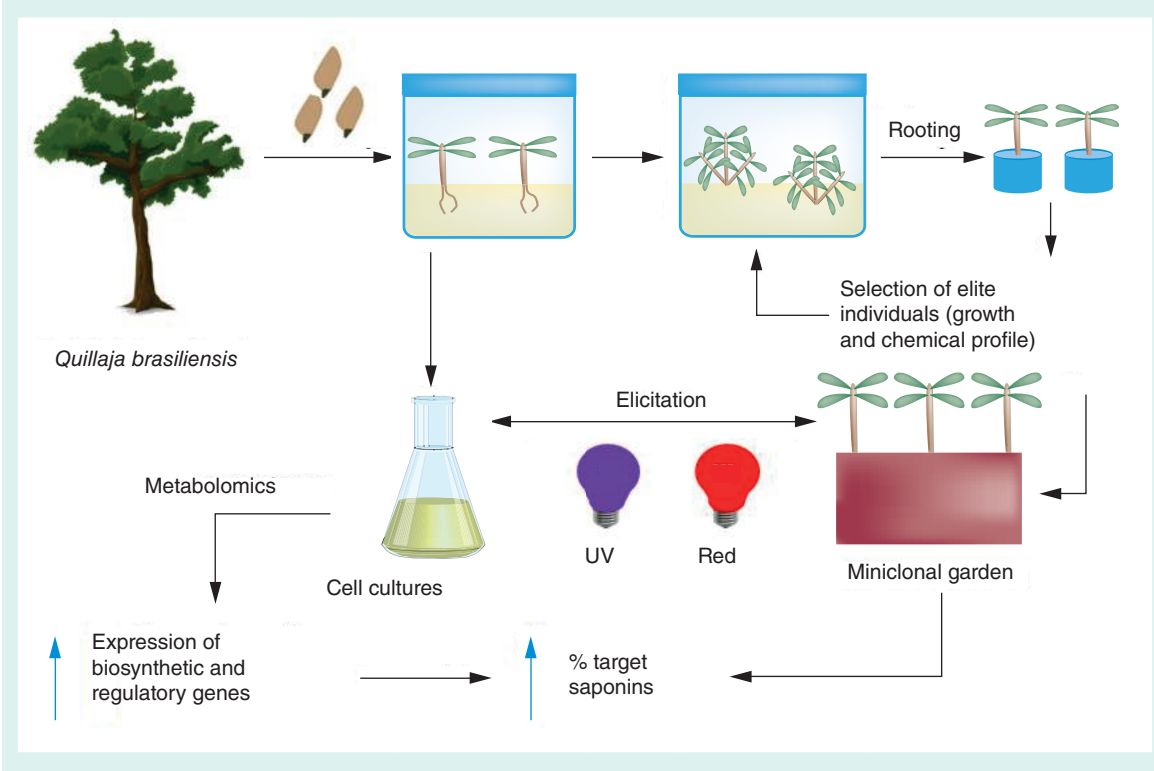


Figure 3. Biotechnological approaches for obtaining plant material targeting enhanced saponin yields in *Quillaja brasiliensis*. Seeds germinated *in vitro* are used to obtain shoot cultures; after rooting, young high saponin producing clones are selected and transferred to miniclonal gardens to serve as ministumps (cutting donor plants). Also, *in vitro* seedlings can be used to generate cell cultures. Saponin content can be elicited with UV and red light, enhancing target saponin content. Cell cultures are useful for metabolomic analyses aiming at upregulating biosynthetic and regulatory genes.

After the year 2000, some key papers addressed the saccharide chain syntheses, as well as the synthesis and absolute stereochemistry of the acyl moiety of *Quillaja* saponins [102–104]. Further, the first chemical synthesis of one isomer of QS-21, namely QS-21-Api, was achieved with major effort by Gin and colleagues in 2005 [105]. In 2006, Schmidt *et al.* achieved the synthesis of branched cholesteryl, cholestanyl and friedelanyl trisaccharide saponin glycosides using glucose, galactose and xylose as sugar residues in an ingenious route [106]. Later in 2013, Michalek and colleagues published the synthesis of QS-21-based immunoadjuvants [107]. All these advances provided valuable leads to further obtain the synthesis of analogs of saponins in order to enhance adjuvant activity, attenuate toxicity and develop this potentially important source of saponins.

The group of Gin and colleagues continued studying QS-21 chemical synthesis, considering its clinical potential as a vaccine adjuvant. They achieved its chemical synthesis along with many analogs in order to overcome hydrolytic degradation and toxicity associated with QS-21. Moreover, a pilot clinical trial demonstrated that synthetic QS-21 (SQS-21) had mild toxicity and potent immune responsiveness in comparison with the vaccine using natural QS-21 [15,108,109]. Also, these authors were the pioneers in revealing the first probe variants to evaluate the mechanism of action using *in vitro* and *in vivo* assays together with molecular dynamic simulations of *Q. saponaria* saponins analogs [101,110].

To the best of our knowledge, the detailed immune response profile of the synthetic saponins is still unknown; however, the structure–activity relationships established so far have shed light on the importance between saponin conformation and adjuvant effect. The synthetic efforts to obtain novel saponins will provide new insights on the mechanism of action and aid in the rational discovery of new adjuvants for future vaccines [101].

Conclusion

Data to date show that the use of *Quillaja* spp. saponins as immunoadjuvants is expanding at a fast pace. The chemical features of Quillajaceae saponins, notably their interactions with membranes, result in potent immune responses, useful for boosting defenses against both intra- and extracellular pathogens. Saponins of *Q. saponaria* and *Q. brasiliensis* are chemically similar, displaying different degrees of toxicity, which can be modulated by strategies such as use in combination with other adjuvants or incorporation to nanoparticles. Sustainable production of *Quillaja* biomass is critical for advances in the large scale use of these molecules and has been shown to be viable, as exemplified by leaves of *Q. brasiliensis* as a source of saponins. Improvement of saponin yield is also a reality, mostly based on the manipulation of plant defense responses using abiotic factors and signaling molecules. Chemical syntheses of immunoadjuvant saponins have advanced considerably, providing a platform for the development of novel saponin analogs. The complementary and concerted efforts of developing improved sustainable biological sources, pharmacological preparations, and chemical syntheses strategies of *Quillaja* immunoadjuvant saponins are key elements in the consolidation and expansion of the industrial use of these unique compounds.

Note added in proof: during the editorial production of this paper, a review article has been published on chemical characteristics and biological activities of *Quillaja* saponins [111], and new data on structures of saponins of *Q. brasiliensis* were also reported [112].

Future perspective

The use of isolated saponins in human vaccines and total saponins or purified fractions in their veterinary counterparts should dominate the coming years. Detailed studies on the mechanisms of action are required for guiding the design of saponin analogs and the definition of useful combinations of immunoadjuvants. Chemical synthesis studies will likely focus on more direct reaction series and structure–activity studies to achieve simpler and more stable structures retaining bioactivity. Toxicity issues, dose sparing and the application of lipid-based delivery systems are other topics to watch in coming years.

The development of alternative and more sustainable sources of saponins from both species of *Quillaja* spp. will experience an expansion, with new scenarios for the market of these triterpenes, including uses beyond vaccines. Increasing focus on molecular studies involving genome sequencing and transcriptome of *Quillaja* spp. will pave the road toward synthetic biology for production of its saponins, most likely in engineered yeast.

Executive summary

- *Quillaja* spp. triterpene saponins are powerful immunoadjuvants.
- Active saponins have complex structure and biosyntheses.
- Chemical synthesis of saponins may lead to new bioactive analogs based on structure–activity studies.
- The *in planta* defense functions of saponins provide a means to increase production.
- *Quillaja brasiliensis* is consolidating itself as a viable sustainable source of highly active immunoadjuvants.
- Future genomic and transcriptomic studies may launch the bases for synthetic biology production systems of *Quillaja* spp. saponins.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

References

Papers of special note have been highlighted as: • of interest; •• of considerable interest

1. Luebert F. Taxonomy and distribution of the genus *Quillaja* Molina (Quillajaceae). *Feddes Repert.* 124(4), 157–162 (2014).
2. de Costa F, Yendo AC, Fleck JD, Gosmann G, Fett-Neto AG. Immunoadjuvant and anti-inflammatory plant saponins: characteristics and biotechnological approaches towards sustainable production. *Mini Rev. Med. Chem.* 11(10), 857–880 (2011).
3. InvivoGen. Vaccine adjuvants review. www.invivogen.com/review-vaccine-adjuvants

4. Marciani DJ. Elucidating the mechanisms of action of saponin-derived adjuvants. *Trends Pharmacol. Sci.* 39(6), 573–585 (2018).

•• **A comprehensive up-to-date analysis of the mechanisms of action of saponins focused on adjuvant activity and recent developments.**

5. Dalsgaard K. Saponin adjuvants. 3. Isolation of a substance from *Quillaja saponaria* Molina with adjuvant activity in food-and-mouth disease vaccines. *Arch. Gesamte Virusforsch.* 44(3), 243–254 (1974).

6. van Setten DC, van de Werken G. Molecular structures of saponins from *Quillaja saponaria* Molina. In: *Saponins Used in Traditional and Modern Medicine.* Waller GR, Yamasaki K (Eds), Springer, MA, USA, 185–193 (1996).

7. Kensil CR, Patel U, Lennick M, Marciani D. Separation and characterization of saponins with adjuvant activity from *Quillaja saponaria* Molina cortex. *J. Immunol.* 146(2), 431–437 (1991).

•• **Breakthrough article, which paved the way for saponin isolation and elucidation.**

8. Hodgins DC, Barjesteh N, St. Paul M, Ma Z, Monteiro MA, Sharif S. Evaluation of a polysaccharide conjugate vaccine to reduce colonization by *Campylobacter jejuni* in broiler chickens. *BMC Res. Notes* 8(1), 204 (2015).

9. Lampe K, Gottstein B, Becker T, Stahl-Hennig C, Kaup FJ, Matz-Rensing K. Immunization of rhesus macaques with *Echinococcus multilocularis* recombinant 14-3-3 antigen leads to specific antibody response. *Parasitol. Res.* 116(1), 435–439 (2017).

10. Tahoun A, Jensen K, Corripio-Miyar Y *et al.* Functional analysis of bovine TLR5 and association with IgA responses of cattle following systemic immunisation with H7 flagella. *Vet. Res.* 46(9), 1–15 (2015).

11. Sun HX, Xie Y, Ye YP. Advances in saponin-based adjuvants. *Vaccine* 27(12), 1787–1796 (2009).

12. Higuchi R, Tokimitsu Y, Komori T. An acylated triterpenoid saponin from *Quillaja saponaria*. *Phytochem.* 27, 1165–1168 (1988).

13. Guo S, Kenne L. Structural studies of triterpenoid saponins with new acyl components from *Quillaja saponaria* Molina. *Phytochemistry* 55(5), 419–428 (2000).

14. Cleland JL, Kensil CR, Lim A *et al.* Isomerization and formulation stability of the vaccine adjuvant QS-21. *J. Pharm. Sci.* 85(1), 22–28 (1996).

15. Deng K, Adams MM, Damani P, Livingston PO, Ragupathi G, Gin DY. Synthesis of QS-21-xylose: establishment of the immunopotentiating activity of synthetic QS-21 adjuvant with a melanoma vaccine. *Angew. Chem. Int. Ed. Engl.* 47(34), 6395–6398 (2008).

16. Jacobsen NE, Fairbrother WJ, Kensil CR, Lim A, Wheeler DA, Powell MF. Structure of the saponin adjuvant QS-21 and its base-catalyzed isomerization product by ^{1}H and natural abundance ^{13}C NMR spectroscopy. *Carbohydr. Res.* 280(1), 1–14 (1996).

17. Nord LI, Kenne L. Novel acetylated triterpenoid saponins in a chromatographic fraction from *Quillaja saponaria* Molina. *Carbohydr. Res.* 329(4), 817–829 (2000).

18. Bottcher S, Drusch S. Saponins – self-assembly and behavior at aqueous interfaces. *Adv. Colloid Interface Sci.* 243, 105–113 (2017).

19. Stanimirova R, Marinova K, Tcholakova S, Denkov ND, Stoyanov S, Pelan E. Surface rheology of saponin adsorption layers. *Langmuir* 27(20), 12486–12498 (2011).

20. Zdziennicka A, Szymczyk K, Krawczyk J, Jańczuk B. Critical micelle concentration of some surfactants and thermodynamic parameters of their micellization. *Fluid Phase Equilib.* 322, 126–134 (2012).

21. Mitra S, Dungan SR. Micellar properties of *Quillaja* saponin. 1. Effects of temperature, salt, and pH on solution properties. *J. Agric. Food Chem.* 45(5), 1587–1595 (1997).

22. Reichert CL, Salminen H, Badolato Bönisch G, Schäfer C, Weiss J. Concentration effect of *Quillaja* saponin – co-surfactant mixtures on emulsifying properties. *J. Colloid Interface Sci.* 519, 71–80 (2018).

23. Reichert CL, Salminen H, Leuenberger BH, Hinrichs J, Weiss J. Miscibility of *Quillaja* saponins with other co-surfactants under different pH values. *J. Food Sci.* 80(11), 2495–2503 (2015).

24. Reichert CL, Salminen H, Leuenberger BH, Weiss J. Influence of heat on miscibility of *Quillaja* saponins in mixtures with a co-surfactant. *Food Res. Int.* 88(A), 16–23 (2016).

25. Yang Y, Leser ME, Sher AA, McClements DJ. Formation and stability of emulsions using a natural small molecule surfactant: *Quillaja* saponin (Q-Naturale®). *Food Hydrocoll.* 30(2), 589–596 (2013).

26. Wojciechowski K. Surface activity of saponin from *Quillaja* bark at the air/water and oil/water interfaces. *Colloids Surf. B Biointerfaces* 108, 95–102 (2013).

27. Wojciechowski K, Kezwon A, Lewandowska J, Marcinkowski K. Effect of β-casein on surface activity of *Quillaja* bark saponin at fluid/fluid interfaces. *Food Hydrocoll.* 34, 208–216 (2014).

28. Wojciechowski K, Orczyk M, Marcinkowski K *et al.* Effect of hydration of sugar groups on adsorption of *Quillaja* bark saponin at air/water and Si/water interfaces. *Colloids Surf. B Biointerfaces* 117, 60–67 (2014).

29. Tippel J, Lehmann M, von Klitzing R, Drusch S. Interfacial properties of *Quillaja* saponins and its use for micellisation of lutein esters. *Food Chem.* 212, 35–42 (2016).

30. Tippel J, Reim V, Rohn S, Drusch S. Colour stability of lutein esters in liquid and spray dried delivery systems based on *Quillaja* saponins. *Food Res. Int.* 87, 68–75 (2016).
31. Pedebos C, Pol-Fachin L, Pons R, Teixeira CV, Verli H. Atomic model and micelle dynamics of QS-21 saponin. *Molecules* 19(3), 3744–3760 (2014).
32. Lacaille-Dubois M, Melzig MF. Saponins: current progress and perspectives. *Planta Med.* 82(18), 1495 (2016).
33. Cheok CY, Salman HAK, Sulaiman R. Extraction and quantification of saponins: a review. *Food Res. Int.* 59, 16–40 (2014).
34. Kite GC, Howes MJ, Simmonds MS. Metabolomic analysis of saponins in crude extracts of *Quillaja saponaria* by liquid chromatography/mass spectrometry for product authentication. *Rapid. Commun. Mass Spectrom.* 18(23), 2859–2870 (2004).
35. San Martín R, Briones R. Quality control of commercial quillaja (*Quillaja saponaria* Molina) extracts by reverse phase HPLC. *J. Sci. Food Agric.* 80(14), 2063–2068 (2000).
36. Scognamiglio M, Severino V, D'Abrosca B, Chambery A, Fiorentino A. Structural elucidation of saponins: a combined approach based on high-resolution spectroscopic techniques. In: *Studies in Natural Products Chemistry (Volume 45).* Atta-ur-Rahman (Ed.). Elsevier, Amsterdam, The Netherlands, 85–120 (2015).

•• **Thorough review showing the most advanced techniques for saponin elucidation.**

37. Cibulski S, Rivera-Patron M, Suarez N *et al.* Leaf saponins of *Quillaja brasiliensis* enhance long-term specific immune responses and promote dose-sparing effect in BVDV experimental vaccines. *Vaccine* 36(1), 55–65 (2018).
38. Wallace F, Bennadji Z, Ferreira F, Olivaro C. Analysis of an immunoadjuvant saponin fraction from *Quillaja brasiliensis* leaves by electrospray ionization ion trap multiple-stage mass spectrometry. *Phytochem. Lett.* 20, 228–233 (2017).

• **First article reporting the isolation and elucidation of *Quillaja brasiliensis* saponins.**

39. Kauffmann C, Machado AM, Fleck JD *et al.* Constituents from leaves of *Quillaja brasiliensis*. *Nat. Prod. Res.* 18(2), 153–157 (2004).
40. Fleck JD, de Costa F, Yendo AC *et al.* Determination of new immunoadjuvant saponin named QB-90, and analysis of its organ-specific distribution in *Quillaja brasiliensis* by HPLC. *Nat. Prod. Res.* 27(10), 907–910 (2013).
41. Fleck JD, Kauffmann C, Spilki F, Lencina CL, Roehe PM, Gosmann G. Adjuvant activity of *Quillaja brasiliensis* saponins on the immune responses to bovine herpesvirus type 1 in mice. *Vaccine* 24(49-50), 7129–7134 (2006).
42. Yendo AC, de Costa F, Kauffmann C, Fleck JD, Gosmann G, Fett-Neto AG. Purification of an immunoadjuvant saponin fraction from *Quillaja brasiliensis* leaves by reversed-phase silica gel chromatography. *Methods Mol. Biol.* 1494, 87–93 (2017).
43. Cibulski SP, Silveira F, Mourglia-Ettlind G *et al. Quillaja brasiliensis* saponins induce robust humoral and cellular responses in a bovine viral diarrhea virus vaccine in mice. *Comp. Immunol. Microbiol. Infec. Dis.* 45, 1–8 (2016).
44. de Costa F, Yendo AC, Cibulski SP *et al.* Alternative inactivated poliovirus vaccines adjuvanted with *Quillaja brasiliensis* or Quil-A saponins are equally effective in inducing specific immune responses. *PLoS ONE* 9(8), e105374 (2014).
45. Silveira F, Cibulski SP, Varela AP *et al. Quillaja brasiliensis* saponins are less toxic than Quil A and have similar properties when used as an adjuvant for a viral antigen preparation. *Vaccine* 29(49), 9177–9182 (2011).
46. Yendo AC, de Costa F, Cibulski SP *et al.* A rabies vaccine adjuvanted with saponins from leaves of the soap tree (*Quillaja brasiliensis*) induces specific immune responses and protects against lethal challenge. *Vaccine* 34(20), 2305–2311 (2016).
47. Muller LE, Schiedeck G. Physical properties of botanical surfactants. *Sci. Total Environ.* 610, 1133–1137 (2018).
48. Stittelaar KJ, Boes J, Kersten GF *et al. In vivo* antibody response and *in vitro* CTL activation induced by selected measles vaccine candidates, prepared with purified Quil A components. *Vaccine* 18(23), 2482–2493 (2000).
49. Xiao C, Rajput ZI, Hu S. Improvement of a commercial foot-and-mouth disease vaccine by supplement of Quil A. *Vaccine* 25(25), 4795–4800 (2007).
50. da Fonseca DP, Frerichs J, Singh M, Snippe H, Verheul AF. Induction of antibody and T-cell responses by immunization with ISCOMS containing the 38-kilodalton protein of *Mycobacterium tuberculosis*. *Vaccine* 19(1), 122–131 (2000).
51. Lightowlers MW, Heath DD. Immunity and vaccine control of *Echinococcus granulosus* infection in animal intermediate hosts. *Parassitologia* 46(1–2), 27–31 (2004).
52. Haçariz O, Sayers G, McCullough M, Garrett M, O'Donovan J, Mulcahy G. The effect of Quil A adjuvant on the course of experimental *Fasciola hepatica* infection in sheep. *Vaccine* 27(1), 45–50 (2009).
53. Spickler AR, Roth JA. Adjuvants in veterinary vaccines: modes of action and adverse effects. *J. Vet. Intern. Med.* 17(3), 273–281 (2003).
54. Marciani DJ, Kensil CR, Beltz GA, Hung CH, Cronier J, Aubert A. Genetically-engineered subunit vaccine against feline leukaemia virus: protective immune response in cats. *Vaccine* 9(2), 89–96 (1991).
55. Santos FN, Borja-Cabrera GP, Miyashiro LM *et al.* Immunotherapy against experimental canine visceral leishmaniasis with the saponin enriched-Leishmune® vaccine. *Vaccine* 25(33), 6176–6190 (2007).
56. Kensil CR, Kammer R. QS-21: a water-soluble triterpene glycoside adjuvant. *Expert Opin. Investig. Drugs* 7(9), 1475–1482 (1998).
57. Ragupathi G, Gardner JR, Livingston PO, Gin DY. Natural and synthetic saponin adjuvant QS-21 for vaccines against cancer. *Expert Rev. Vaccines* 10(4), 463–470 (2011).

58. Didierlaurent AM, Laupèze B, Di Pasquale A, Hergli N, Collignon C, Garçon N. Adjuvant system AS01: helping to overcome the challenges of modern vaccines. *Expert Rev. Vaccines* 16(1), 55–63 (2017).
59. Garçon N, Di Pasquale A. From discovery to licensure, the Adjuvant System story. *Hum. Vaccin. Immunother.* 13(1), 19–33 (2017).
60. The RTS,S Clinical Trials Partnership. Efficacy and Safety of the RTS,S/AS01 malaria vaccine during 18 months after vaccination: a Phase III randomized, controlled trial in children and young infants at 11 African sites. *PLoS Med.* 11(7), e1001685 (2014).
61. European Vaccine Initiative. Annual Report 2016. Universitätsklinikum Heidelberg, Heidelberg, Germany (2017). www.euvaccine.eu/sites/default/files/uploads/Files/annual_reports/EVI_Annual%20Report%202016_Final_171031.pdf
62. James SF, Chahine EB, Sucher AJ, Hanna C. Shingrix: the new adjuvanted recombinant herpes zoster vaccine. *Ann. Pharmacother.* 52(7), 673–680 (2018).
63. Lovgren Bengtsson K, Morein B, Osterhaus AD. ISCOM technology-based matrix M™ adjuvant: success in future vaccines relies on formulation. *Expert Rev. Vaccines* 10(4), 401–403 (2011).
64. Zaman M, Chandrudu S, Toth I. Strategies for intranasal delivery of vaccines. *Drug Deliv. Transl. Res.* 3(1), 100–109 (2013).
65. Paillot R. A systematic review of recent advances in equine influenza vaccination. *Vaccines (Basel)* 2(4), 797–831 (2014).
66. Morein B, Bengtsson KL. Functional aspects of iscoms. *Immunol. Cell Biol.* 76(4), 295 (1998).
67. Alving CR, Peachman KK, Rao M, Reed SG. Adjuvants for human vaccines. *Curr. Opin. Immunol.* 24(3), 310–315 (2012).
68. Didierlaurent AM, Collignon C, Bourguignon P *et al.* Enhancement of adaptive immunity by the human vaccine adjuvant AS01 depends on activated dendritic cells. *J. Immunol.* 193(4), 1920–1930 (2014).
69. Garçon N, Van Mechelen M. Recent clinical experience with vaccines using MPL – and QS-21 – containing adjuvant systems. *Expert Rev. Vaccines* 10(4), 471–486 (2011).
70. Cibulski SP, Mourglia-Ettlin G, Teixeira TF *et al.* Novel ISCOMs from *Quillaja brasiliensis* saponins induce mucosal and systemic antibody production, T-cell responses and improved antigen uptake. *Vaccine* 34(9), 1162–1171 (2016).
71. Cibulski SP, Rivera-Patron M, Mourglia-Ettlind G *et al. Quillaja brasiliensis* saponin-based nanoparticulate adjuvants are capable of triggering early immune responses. *Sci. Rep.* 8(1), 13582 (2018).
72. Kensil CR. Saponins as vaccine adjuvants. *Crit. Rev. Ther. Drug Carrier Syst.* 13(1–2), 1–55 (1996).
73. Press JB, Reynolds RC, May RD, Marciani DJ. Structure/function relationships of immunostimulating saponins. *Stud. Nat. Prod. Chem.* 24(Part E), 131–174 (2000).
74. Soltysik S, Wu J, Recchia J *et al.* Structure/function studies of QS-21 adjuvant: assessment of triterpene aldehyde and glucuronic acid roles in adjuvant function. *Vaccine* 13(15), 1403–1410 (1995).
75. Oda K, Matsuda H, Murakami T, Katayama S, Ohgitani T, Yoshikawa M. Adjuvant and haemolytic activities of 47 saponins derived from medicinal and food plants. *Biol. Chem.* 381(1), 67–74 (2000).
76. en Brok MH, Büll C, Wassink M *et al.* Saponin-based adjuvants induce cross-presentation in dendritic cells by intracellular lipid body formation. *Nat. Commun.* 7, 13324 (2016).
77. Lorent JH, Quentin-Leclercq J, Mingeot-Leclercq MP. The amphiphilic nature of saponins and their effects on artificial and biological membranes and potential consequences for red blood and cancer cells. *Org. Biomol. Chem.* 12(44), 8803–8822 (2014).
78. Marciani DJ. New Th2 adjuvants for preventive and active immunotherapy of neurodegenerative proteinopathies. *Drug Discov. Today* 19(7), 912–920 (2014).
79. Svajger U, Anderluh M, Jeras M, Obermajer N. C-type lectin DC-SIGN: an adhesion, signaling and antigen-uptake molecule that guides dendritic cells in immunity. *Cell Signal.* 22(10), 1397–1405 (2010).
80. Maraskovsky E, Schnurr M, Wilson NS, Robson NC, Boyle J, Drane D. Development of prophylactic and therapeutic vaccines using the ISCOMATRIX adjuvant. *Immunol. Cell Biol.* 87(5), 371–376 (2009).
81. Sun HX, Xie Y, Ye YP. ISCOMs and ISCOMATRIX™. *Vaccine* 27(33), 4388–4401 (2009).
82. Zhao L, Seth A, Wibowo N *et al.* Nanoparticle vaccines. *Vaccine* 32(3), 327–337 (2014).
83. Coffman RL, Sher A, Seder RA. Vaccine adjuvants: putting innate immunity to work. *Immunity* 33(4), 492–503 (2010).
84. Duthie MS, Windish HP, Fox CB, Reed SG. Use of defined TLR ligands as adjuvants within human vaccines. *Immunol. Rev.* 239(1), 178–196 (2011).
85. Banco Central de Chile. Indicadores de comercio exterior: cuarto trimestre de 2017, Chile (2017). https://si3.bcentral.cl/estadisticas/Principal1/Informes/SE/COMEX/Indicadores.html
86. San Martín R, Briones R. Industrial uses and sustainable supply of *Quillaja saponaria* (Rosaceae) saponins. *Econ. Bot.* 53(3), 302–311 (1999).
87. San Martín R. Sustainable production of *Quillaja saponaria* Mol. saponins. In: *Saponins in Food, Feedstuffs and Medicinal Plants.* Oleszek W, Marston A (Eds), Springer, Dordrecht, The Netherlands, 271–279 (2000).

88. Szakiel A, Pączkowski C, Henry M. Influence of environmental abiotic factors on the content of saponins in plants. *Phytochem. Rev.* 10(4), 471–491 (2011).

89. ChileBotanics®. https://chilebotanics.cl/en/

90. Correa C, Martínez A. Información tecnológica de productos forestales no madereros del bosque nativo en Chile. *Productos Forestales No Madereros em Chile* (2013). www.pfnm.cl/paqtecnologicos/paqtecpfnm.htm

91. Ministry of Environment of Chile. Tecer reporte del estado del medio ambiente. Governo de Chile (2017). http://sinia.mma.gob.cl/wp-content/uploads/2017/09/REMA-2017.pdf

92. Walther RU, Padilla L, González J, Otero R. *Quillaja saponaria* wood extract: refined processing and forestry management guarantee sustainability and ecological benefits. *Compendium on Detergency* 2, 3–4 (2011).

93. Schlotterbeck T, Castillo-Ruiz M, Cañon-Jones H, San Martín R. The use of leaves from young trees of *Quillaja saponaria* (Molina) plantations as a new source of saponins. *Econ. Bot.* 69(3), 262–272 (2015).

94. Gaete-Garretón L, Vargas-Hernández Y, Cares-Pacheco MG, Sainz J, Alarcón J. Ultrasonically enhanced extraction of bioactive principles from *Quillaja saponaria* Molina. *Ultrasonics* 51(5), 581–585 (2011).

95. Yendo ACA, de Costa F, Fleck JD, Gosmann G, Fett-Neto AG. Irradiance-based treatments of *Quillaja* brasiliensis leaves (A. St.-Hil. & Tul.) Mart. as means to improve immunoadjuvant saponin yield. *Ind. Crops Prod.* 74, 228–233 (2015).

96. de Costa F, Yendo AC, Fleck JD, Gosmann G, Fett-Neto AG. Accumulation of a bioactive triterpene saponin fraction of *Quillaja brasiliensis* leaves is associated with abiotic and biotic stresses. *Plant Physiol. Biochem.* 66, 56–62 (2013).

- **First report on factors able to induce accumulation of bioactive *Quillaja saponins* in leaves and seedlings.**

97. Arborea Biotech®. www.arboreabiotech.com/

98. Fleck JD, Schwambach J, Almeida ME *et al.* Immunoadjuvant saponin production in seedlings and micropropagated plants of *Quillaja brasiliensis*. *Vitr. Cell Dev. Biol. Plant.* 45(6), 715–720 (2009).

99. Dalsgard K, Henry M. US5716848 (1992).

100. Matsuura HN, Malik S, de Costa F *et al.* Specialized plant metabolism characteristics and impact on target molecule biotechnological production. *Mol. Biotechnol.* 60(2), 169–183 (2018).

101. Fernández-Tejada A, Tan DS, Gin DY. Development of improved vaccine adjuvants based on the saponin natural product QS-21 through chemical synthesis. *Acc. Chem. Res.* 49(9), 1741–1756 (2016).

- **Provides information on chemical synthesis of QS-21 and discusses it as model for investigating action mechanisms of saponins.**

102. Zhu X, Yu B, Hui Y, Higuchi R, Kusano T, Miyamoto T. Synthesis and absolute stereochemistry of the acyl moiety of *Quillaja* saponins. *Tetrahedron Lett.* 41(5), 717–719 (2000).

103. Zhu X, Yu B, Hui Y, Schmidt RR. Synthesis of the trisaccharide and tetrasaccharide moieties of the potent immunoadjuvant QS-21. *Eur. J. Org. Chem.* 2004 (5), 965–973 (2004).

104. Yu B, Zhang Y, Tang P. Carbohydrate chemistry in the total synthesis of saponins. *Eur. J. Org. Chem.* 2007(31), 5145–5161 (2007).

105. Wang P, Kim YJ, Navarro-Villalobos M, Rohde BD, Gin DY. Synthesis of the potent immunostimulatory adjuvant QS-21A. *J. Am. Chem. Soc.* 127(10), 3256–3257 (2005).

106. Schimmel J, Eleutério MIP, Ritter G, Schmidt RR. Synthesis of saponins with cholestanol, cholesterol, and friedelanol as aglycones. *Eur. J. Org. Chem.* 2006(7), 1701–1721 (2006).

107. Wang P, Dai Q, Thogaripally P, Zhang P, Michalek SM. Synthesis of QS-21-based immunoadjuvants. *J. Org. Chem.* 78(22), 11525–11534 (2013).

108. Kim YJ, Wang P, Navarro-Villalobos M, Rohde BD, Derryberry J, Gin DY. Synthetic studies of complex immunostimulants from *Quillaja saponaria*: synthesis of the potent clinical immunoadjuvant QS-21Aapi. *J. Am. Chem. Soc.* 128(36), 11906–11915 (2006).

109. Adams MM, Damani P, Perl NR *et al.* Design and synthesis of potent *Quillaja* saponin vaccine adjuvants. *J. Am. Chem. Soc.* 132(6), 1939–1945 (2010).

110. Walkowicz WE, Fernández-Tejada A, George C *et al. Quillaja* saponin variants with central glycosidic linkage modifications exhibit distinct conformations and adjuvant activities. *Chem. Sci.* 7(3), 2371–2380 (2016).

111. Fleck JD, Betti AH, da Silva FP *et al.* Saponins from *Quillaja saponaria* and *Quillaja brasiliensis*: particular chemical characteristics and biologicalactivities. *Molecules*24(171), 1–29 (2019).

112. Wallace F, Bennadji Z, Ferreira F, Olivaro C. Structural characterization of new immunoadjuvant saponins from leaves and the first study of saponins from the bark of *Quillaja brasiliensis* by liquid chromatography electrospray ionization ion trap mass spectrometry. *Phytochem Analysis* https://doi.org/10.1002/pca.2837 (2019) (Epub ahead of print).

Capítulo 2

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Production of plant bioactive triterpenoid saponins: from metabolites to genes and back

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Abstract Saponins are specialized plant terpenoids derived from the mevalonic acid pathway. Triterpenoid saponin cores are decorated with sugar residues, conferring a highly amphipathic nature to these molecules, which show hypocholesterolemic, immunoadjuvant and anti-inflammatory activities, among others. Natural sources of bioactive saponins are relevant in light of the technical challenges of chemical synthesis of these compounds. Current supply of high quality, homogeneous and renewable plant material falls short of industrial demand. Research regarding molecular regulation of saponin metabolism has advanced considerably in recent years. Recent studies have focused on transcriptome analysis and identification of key transcription factors regulating gene expression patterns related to saponin biosynthesis. Biotechnological approaches to engineer saponin production in plants, organ and cell cultures, as well as development of heterologous expression systems, are being actively pursued as alternative sources of these high value plant terpenoids. It is expected that these efforts will impact industrial scale sustainable production systems in the coming years.

Keywords Saponin · Biosynthesis · Transcription factors · Triterpenes · Natural products

Abbreviations

AcCoA	Acetyl-CoA
BAS	Beta amyrin synthase
DMADP	Dimethylallyl diphosphate
DS	Dammarenediol synthase
FDP	Farnesyl diphosphate
FDS	Farnesyl diphosphate synthase
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
HMGR	3-hydroxy-3-methylglutaryl-CoA reductase
HMGS	3-hydroxy-3-methylglutaryl-CoA synthase
IDP	Isopentenyl diphosphate
MEP	2*C*-methyl-D-erythritol-4-phosphate
MVA	Mevalonic acid
SE	Squalene epoxidase

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SS	Squalene synthase
OSC	Oxidosqualene cyclase
SCPL	Serine-carboxypeptidase-like acyltransferase

Introduction

Saponins are specialized plant terpenoids mainly derived from the mevalonic acid pathway (Bach et al. 1999). Their name comes from the Latin word *sapo*, meaning soap, a term which reflects detergent qualities due to their amphipathic chemical nature. Saponins from dicotyledonous lineages feature a C_{30} triterpenoid hydrophobic core (sapogenin) that is composed of 6 five carbon isoprene units, whereas the corresponding sapogenin in monocotyledonous plants is typically a C_{27} steroidal nortriterpene structure (Netala et al. 2015), having lost several carbon atoms from the original C_{30} core following cyclization. In limited cases, both types of saponins are found in the same species (Osbourn 2003). The amphipathic quality of both types of saponins is due to hydrophilic substituents on the terpenoid skeleton, which consist mainly of sugar residues but also, to a lesser extent, small organic acids and polyketide fragments.

Natural sources of bioactive saponins are relevant in light of the technical challenges of chemical synthesis of these compounds. Currently, cultivated plants are the main commercial sources of bioactive saponins, whereas cell and tissue cultures are mostly important as tools for research on saponin biosynthesis (Biswas and Dwivedi 2019).

The synthesis and accumulation of specialized plant metabolites are strictly controlled in a spatial and temporal manner. This regulation can be affected by biotic and abiotic factors. Transcription factors (TFs) play a crucial role in the induction of specialized metabolite biosynthesis in response to stress (Xie et al. 2019). Transcription factors are capable of interacting with specific DNA sequences (cis-like) in the promoter region of target genes. This interaction activates or represses the target gene expression in response to internal or external cues. There are some TFs that only interact with other proteins to form regulatory complexes, and these complexes will bind to the promoter region of target genes (Patra et al. 2013). The current state of knowledge surrounding TFs related to saponin biosynthesis is summarized in the section "Transcription factors regulating saponin biosynthesis".

Transcriptomic and genomic analyses of plant species that produce bioactive saponins are increasingly available. These data are useful in the elucidation of biosynthetic pathways and regulatory mechanisms governing them. Over the last five years, new information obtained from transcriptomic and genomic analyses of saponin producing plants has been published (Mertens et al. 2016a; Chu et al. 2018; Shang and Huang 2019). Herein, we provide a critical overview of saponin biosynthesis, natural sources, and metabolism-related transcription factors involved in regulating saponin biosynthesis, emphasizing species of pharmaceutical interest. In addition, we will discuss strategies for improving yields of bioactive saponins and future advances towards this goal.

Saponins are versatile molecules with several activities and applications

Saponins are highly versatile molecules finding applications in a diverse array of industrial activities. In the pharmaceutical sector, saponins are relevant for their activities as immunoadjuvants (promoting immune responses in both veterinary and human vaccine formulations), anti-inflammatory, antiallergic, anti-hypercholesterolemic, antihyperlipidemic, antioxidant, antifungal, antibacterial, antiviral, antiprotozoan, anti-tumoral, anti-diabetic, cardioprotective, angiogenic, hemolytic, anticoagulant, antihypertensive, vasodilatory, adaptogenic against stresses, memory stimulant, cardiac modulator, skin cleanser, hair dyeing, hair color preparation, shampoos, and cosmetics manufacture (Table 1) (Barbosa 2014; Singh and Chaudhuri 2018; Reichert et al. 2019).

Saponins are also relevant products in the food industry. Some of its main uses are as foaming agents in beverages and emulsifiers in processed foods, including encapsulation and stabilization of bioactive compounds. One particular case is glycyrrhizin, a saponin synthesized by *Glycyrrhiza* spp. that is 50 times sweeter than sucrose, being used as sweetener in the food industry (Hayashi and Sudo 2009; Kao et al. 2014). Other applications in this economic sector include aeration of food products, bread dough fermentation improvement, solubilizing agent, egg

replacer, removal of cholesterol and protein-caused turbidity (Reichert et al. 2019).

In agriculture, livestock, and pet industries, saponins are used as natural biocontrol agents and in animal nutrition. These terpenes have shown a number of properties that make them interesting environmentally-friendly alternatives to conventional pesticides and natural adjuvants for animal food improvement. In the biocontrol forefront, saponins have proven useful as molluscicidal agents, insecticides (both deterrent and antifeedant), and antimicrobials to treat and prevent a variety of plant diseases (reviewed in Faizal and Geelen 2013; Singh and Kaur 2018; Hussain et al. 2019).

Avenacins have an important role in resistance of *Avena* spp. against the root infecting fungus *Gaeumannomyces graminis* var. tritici, which is known for causing "take-all" disease in cereals (Osbourn et al. 1994). The characterization of the *sad* (saponin-deficient) mutants from *Avena strigosa* Schreb. and the oat-infecting *G. graminis* var. avenae, which synthetizes avenacinase, a saponin detoxifying enzyme that enables infection, were crucial for the establishment of avenacin antifungal activity (Osbourn et al. 1994; Papadopoulou et al. 1999).

Most bioactivities and uses of saponins depend on their unique chemical properties. The capacity to interact with several membrane components and access these in various cell compartments as a result of their amphipathic properties seems to be at the center of triterpene saponins mechanism of action. The triterpene cores of saponins have high affinity for cholesterol-rich zones in membranes, being able to intercalate with this sterol, thereby causing membrane disruption (Lorent et al. 2014). Some examples of how these properties affect saponin bioactivities and industrial applications are discussed below.

Immunoadjuvant activity of QS-21, a triterpene saponin from *Quillaja saponaria* Molina, probably results from its interaction with dendritic and T-cells, both by receptor-mediated and receptor independent pathways. QS-21 and related *Quillaja* saponins can modulate proliferation of T-cells, as well as promote release of IgG2a antibodies. In dendritic cells, action of QS-21 involves lipid body formation and endosomal antigen release facilitation required for cross-presentation in MHC-1 and establishment of complexes (Marciani 2018). Among the structural features of *Quillaja* saponins relevant for their immunoadjuvant effects is the presence of an aldehyde group in its C4 (Soltysik et al. 1995). Saccharides of saponins are important for their immunoadjuvant action since they impact on the triggering of Th1 or Th2 immune responses (Soltysik et al. 1995; Gringhuis et al. 2014).

The toxic effects of triterpene saponins on herbivores and pathogens have also been associated with membrane disruption. In lepidopteran insects, cell membrane permeation was correlated with entomotoxic activity (De Geyter et al. 2012). Saponins are also able to interact with proteins, which may also contribute to their effects on herbivores, including changes in proteinases and other digestive enzymes (Potter et al. 1993; Hussain et al. 2019).

The roles of saponins in the cardiovascular system are related to their interaction with membrane components, such as cholesterol (Enomoto et al. 1986). Sodium permeability may be increased as a result of the interaction with the membrane sterol. The blocking of calcium channels by ginsenosides Rb1, Rb2 and Rb3 is another possible mode of action, as these saponins have been able to inhibit contractility and action potential in cardiac cells. Re-establishment of membrane potentials in rat cardiomyocytes partly damaged by oxidative stress was observed after treatment with ginsenosides, indicating antioxidant activity. The myocardium necrosis inducer isoproterenol had its damaging effects in rats reduced by Rb3 ginsenoside. The protective role of *Panax* saponins against heart disease, including their anti-inflammatory activity, have supported the use of remedies based on these plants in traditional Chinese medicine for many years (Yang et al. 2014; Singh and Chaudhuri 2018; Shi et al. 2019a).

The diverse properties and useful biological activities of triterpene saponins make them valuable chemicals in industry. The number of known saponins will surely increase, particularly as a result of phytochemical studies in plants of tropical and subtropical biomes. As individual molecules are isolated and characterized, new applications are likely to unfold.

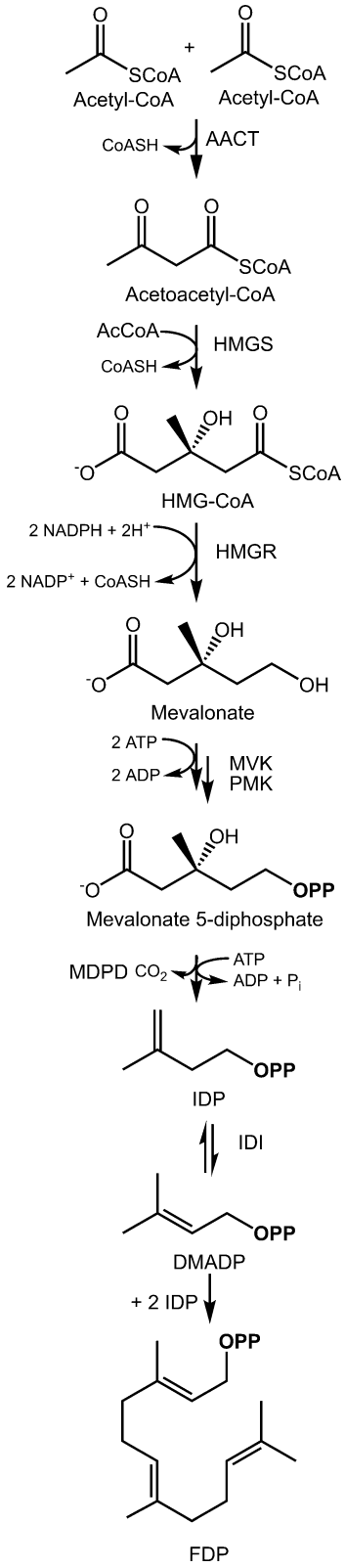
Table 1 Examples of biological activities described for triterpene saponins identified in plants

Activities	Saponins	Plant species	References
Adaptogenic against plant stresses	*Quillaja* saponins	*Quillaja brasiliensis*	de Costa et al. (2013)
Angiogenic	Panaxtriol	*Panax notoginseng*	Hui et al. (2017)
Antibacterial	Hederagenin and its derivatives	*Medicago arabica* (L.) Huds.	Avato et al. (2006)
Anticoagulant and Anti-diabetic	Notoginsenosides	*Panax notoginseng*	Yao et al. (2008) and Uzayisenga et al. (2014)
Antifungal	Avenacins	*Avena sativa* L.	Osbourn et al. (1994)
Anti-herbivory/hemolytic	Medicagenic acid and hederagenin glucosides	*Medicago spp.*	Szczepanik et al. (2001) and Mazahery-Laghab et al. (2011)
Anti-hypercholesterolemic	*Quillaja*/*Yucca* saponins	*Quillaja saponaria* and *Yucca schidigera* Roezl ex Ortgies	Kim et al. (2003)
Antihypertensive/vasodilatory	Notoginsenosides	*Panax notoginseng*	Loh et al. (2019)
Anti-inflammatory/ antiallergic	Rb1, Rb2	*Panax ginseng*	Cho et al. (2001) and Matsuda et al. (1990)
Antioxidant	Asiaticoside	*Centella asiatica* (L.) Urb.	Xu et al. (2012)
Anti-protozoan	*Maesa* saponins	*Maesa balansae* Mez.	Vermeersch et al. (2009)
Anti-tumoral	Raddeanin A	*Anemone raddeana* Regel	Li et al. (2017)
Antiviral	*Quillaja* saponins	*Quillaja saponaria*	Tam and Roner (2011)
Cardioprotective	Ginsenosides	*Panax* spp.	Scott et al. (2001) and Yang et al. (2014)
Immunoadjuvant	Quil-A, QS-21, QB-90	*Quillaja* spp.	Santos et al. (2007), de Costa et al. (2011), Yendo et al. (2016) and Marciani (2018)
Memory stimulant	Asiaticoside	*Hydrocotyle sibthorpioides* Lam.	Lin et al. (2013)

Biosynthesis of β-amyrin from central metabolic intermediates: the central precursor to triterpene saponins

Saponins, like all terpenes, are synthesized from the universal intermediates of terpenoid metabolism, isopentenyl and dimethylallyl diphosphate (IDP and DMADP). These prenylated diphosphate precursors are produced by two distinct routes in the plant cell, the plastid localized 2*C*-methyl-D-erythritol-4-phosphate (MEP) pathway and the cytosolic mevalonic acid (MVA) pathway (Fig. 1) (Rodriguez-Concepción 2014). The MVA pathway is generally understood to supply the synthesis of C_{15} sesquiterpenes as well as $C_{27\text{-}29}$ sterols and C_{30} triterpenes (Bach et al. 1999). Cytosolic acetyl-CoA (AcCoA) is the sole carbon source for the MVA pathway, and in the initial enzymatic steps, two molecules of AcCoA are condensed by acetoacetyl CoA thiolase to yield acetoacetyl-CoA (Ahumada et al. 2008). This intermediate is then condensed with a third AcCoA unit to yield 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) (Liao et al. 2014). HMG-CoA is the substrate for the main regulatory step of the MVA pathway, HMG-CoA reductase (HMGR). Two subsequent phosphorylation steps and a decarboxylation yield IDP (Cordier et al. 1999; Lluch et al. 2000), which can be isomerized to its double bond congener DMADP via IDP isomerase (Phillips et al. 2008). One molecule of DMADP and two molecules of IDP are condensed by the prenyltransferase farnesyl diphosphate (FDP) synthase (FDS) to yield FDP in a head-to-tail-fashion (Cunillera et al. 1996).

FDP is a common precursor to sesquiterpenes, sterols, and triterpenes alike. For sterol and triterpene biosynthesis, two units of FDP are condensed to form squalene by squalene synthase (SS) (Nakashima et al. 1995), an NADPH and Mg^{2+} dependent reaction



◀**Fig. 1** Synthesis of farnesyl diphosphate (FDP) from acetyl-CoA (AcCoA) through the mevalonate (MVA) pathway. FDP is a central intermediate in the synthesis of triterpene saponins. MVA pathway enzymes include acetoacetyl-CoA thiolase (AACT), 3-hydroxy-3-methylglutaryl-Coa synthase (HMGS), HMG-Coa reductase (HMGR), mevalonate kinase (MVK), phosphomevalonate kinase (PMK), mevalonate diphosphate decarboxylase (MDPD), and isopentenyl diphosphate isomerase (IDI)

which features a head-to-head condensation yielding a pure olefinic hydrocarbon product (Fig. 2). Squalene is then oxidized to 2,3-oxidosqualene by squalene epoxidase (SE) (Thimmappa et al. 2014), the branch point intermediate leading alternately to the sterol pathway (usually via cycloartenol) or to triterpene saponins, mostly through the intermediate β-amyrin (Haralampidis et al. 2001; Hayashi et al. 2001; Shibuya et al. 2009), lupeol (Shibuya et al. 2009) or, in the case of dammarane type triterpenes, through a related intermediate such as dammarenediol-II (Tansakul et al. 2006). Collectively, enzymes acting on 2,3-oxidosqualene are known as oxidosqualene cyclases (OSCs). A phylogenetic analysis of the various subclades of plant OSCs has been reviewed (Thimmappa et al. 2014). Combined metabolomics, transcriptomics and biochemical evaluation in recombinant yeast have been expanding the collection of OSCs (Srivastava et al. 2020).

The functionalization of the triterpene skeleton is preceded by the introduction of oxygen functional groups via cytochrome P450 monooxygenases, a large family of modifying enzymes responsible for significant chemical diversity in the terpenoid natural products family (Pateraki et al. 2015; Ghosh 2017). Cytochrome P450s introduce alcohol, epoxide, and carboxylic acid groups into the triterpene core which later provide attachment points for carbohydrate and acyl groups. β-Amyrin is the best characterized substrate for triterpene monooxygenation, and cytochrome P450s adding a hydroxyl group at C16 (Geisler et al. 2013; Tamura et al. 2017a, b), C21 (Leveau et al. 2019), C23 (Liu et al. 2019a), and C30 (Seki et al. 2011) are known. At least one of these modifying enzymes is multifunctional; CYP51, responsible for C16 hydroxylation, also introduces an epoxide at the C12-13 alkene (Geisler et al. 2013). Double oxidations introducing either a ketone at C11 (Seki et al. 2008) or an aldehyde function at C23 have also been characterized. In the latter case, an initial

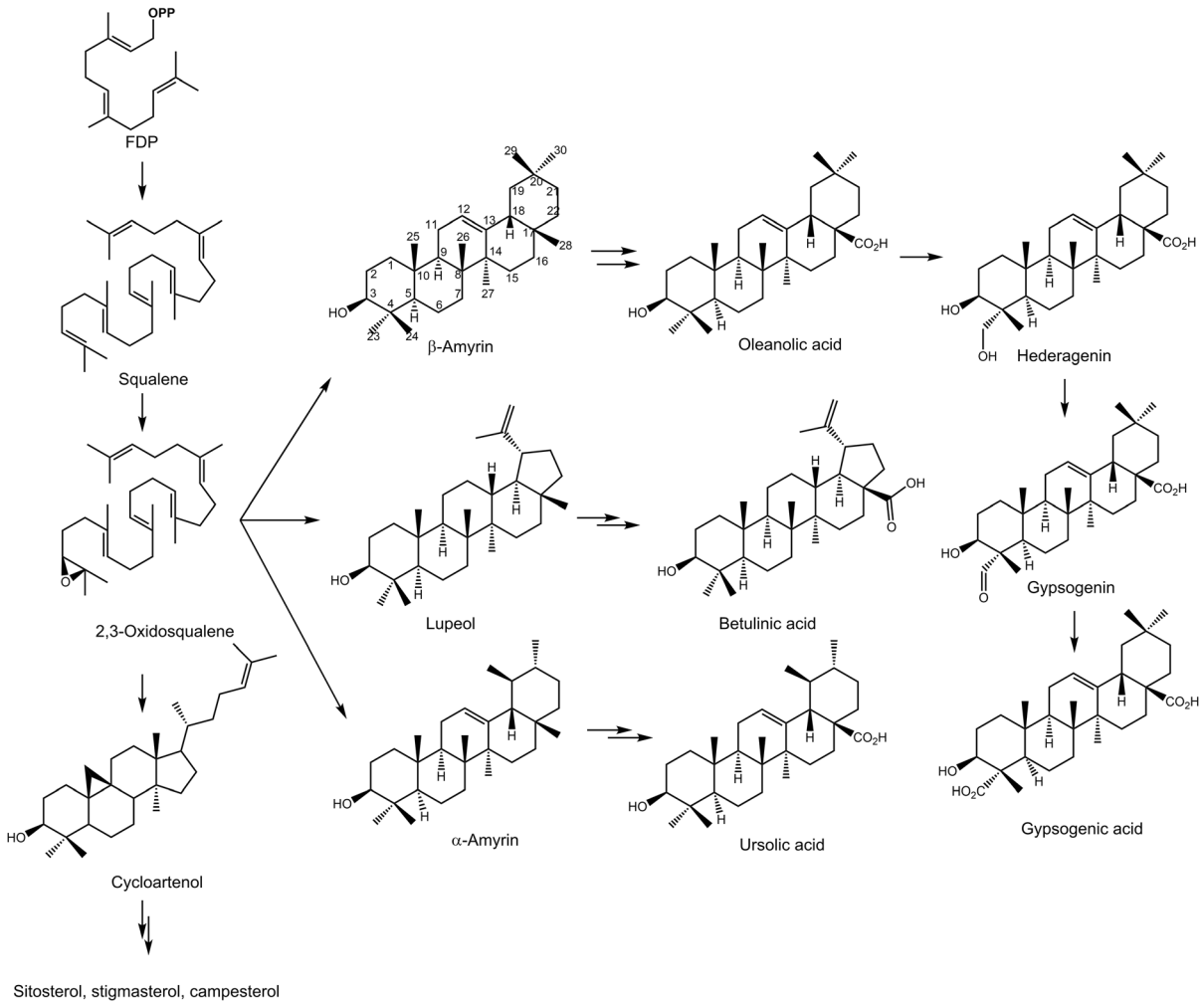


Fig. 2 Cyclization of 2,3-oxidosqualene leads to the major triterpene carbon skeletons. The intermediates β-amyrin, lupeol and α-amyrin receive oxygen functional groups via cytochrome P450 monooxygenases. An initial hydroxylation of oleanolic acid at C23 yields hederagenin, which can be further oxidized to yield gypsogenin, and then gypsogenic acid in *Medicago truncatula*. Sterol biosynthesis shares the same precursor (2,3-oxidosqualene) with triterpene carbon skeletons

hydroxylation of oleanolic acid at C23 yields hederagenin, which can be further oxidized by the same cytochrome P450 to yield gypsogenin (Fig. 2) and then gypsogenic acid in *Medicago truncatula* Gaertn. (Tzin et al. 2019). However, the most common P450 modifying the β-amyrin skeleton carries out a triple oxidation at the C28 methyl to produce a carboxylic acid, as in the case of oleanolic acid (Carelli et al. 2011; Jo et al. 2017), and multi-substrate enzymes carrying out a similar modification to the equivalent methyl group of β-amyrin or lupeol to yield ursolic and betulinic acids, respectively, have also been described (Huang et al. 2012).

Modification of triterpene skeletons by acylation or glycosylation

Triterpenes by themselves are highly non-polar molecules. The amphipathic nature of saponins implied by their name results from the presence of highly polar substituents which are added following the cyclization and oxidation of the central core. The most common modification involves transfer of one or more sugar residues, often several, through the action of UDP-glycosyl transferases (Bowles et al. 2006). Functional specialization of these enzymes by changes in specificity has been associated with saponin

diversification (Nomura et al. 2019). The most frequent sugar resides are glucose, galactose, rhamnose, arabinose, and glucuronic acid activated with a nucleotide diphosphate such as UDP. Multiple sugar residues may be added, although this appears to occur sequentially, rather than via pre-assembly of a bloc of residues and transferred as a group (Sawai and Saito 2011). Sugar attachment occurs most frequently at the C3 alcohol (relative to β-amyrin numbering) (Augustin et al. 2012; Louveau et al. 2018) or C28 acids groups (Meesapyodsuk et al. 2007; de Costa et al. 2017; Kim et al. 2017) to generate an acetal or ester linkage, respectively. However, glycosylation at C4, 16, 20, 21, 22, and 23 positions has also been reported (Thimmappa et al. 2014). Most of these reactions take place in the cytosol. However, vacuolar glycosyl transferases have also been described (Orme et al. 2019).

The second type of modification involves transfer of acyl groups. Unlike BAHD acyltransferases, which utilize thioesterified high-energy acyl donors, saponin acylation relies on serine carboxypeptidase-like acyltransferases (SCPLs) which first conjugate an acyl group to glucose through an ester linkage (Shirley and Chapple 2003; Mugford and Milkowski 2012). SCPLs reported to date are evidently all vacuolar proteins. The best example of SCPL acylation comes from avenacin biosynthesis. There the SAD7 protein transfers benzoate or N-methylanthranilate to C21 of desacyl avenacin via their O-glucosides (Mugford et al. 2009). This necessarily requires an N-methyltransferase to methylate anthranilic acid and a UDP-glucosyl transferase to form the sugar-acyl conjugate in addition to SCPL activity to transfer the acyl group onto the triterpene backbone (Mugford et al. 2013). The most significant variation on the acyl and glycoside transfer reactions described above can be found with the triterpene saponins of *Quillaja saponaria*, which features branched chain acyl groups conjugated to sugar residues (Guo and Kenne 2000a). However, the biochemical details of this process are currently unknown.

Transport and storage of triterpene saponins

After final modification of triterpene skeletons, saponins are most likely stored in the vacuole (Szakiel et al. 2005; Mylona et al. 2008). This is a known strategy for storage of water soluble toxic compounds in plants. The active form of avenacin A-1, for example, is stored in the vacuole (Mylona et al. 2008). Upon infection, some fungal pathogens release enzymes (*e.g.* avenacinase) that remove one or more sugar residues from avenacin A-1, rendering it less toxic (Bowyer et al. 1995).

Even though several transporter proteins are described for moving plant specialized metabolites (Lv et al. 2016), few were identified for triterpene saponin transport, with evidence pointing to ATP-binding cassette (ABC) and multidrug and toxic compound extrusion (MATE) families as candidates (De Brito Francisco and Martinoia 2018). Two ABC cell membrane transporters involved in ginsenoside transport were cloned and characterized in *Panax ginseng* (Zhang et al. 2013a, b). Two vacuolar transporter proteins were tentatively detected in leaf protoplasts of *Calendula officinalis* L. (Szakiel and Janiszowska 2002). The mining of candidate genes from transcriptome data is still limited regarding the identification of saponin transporters (Ramilowski et al. 2013; Cao et al. 2015; Zhang et al. 2015).

Triterpene saponin biosynthesis, transport and storage can also involve different organs and tissues. Generally, this distribution dynamics follows a defense rationale, with accumulation directed towards organs and tissues more prone to face specific biotic stress challenges. Examples of this regulatory distribution profile are presented and briefly discussed in the next section.

Natural sources of bioactive saponins

Triterpene saponins are relatively widespread among eudicot groups (Fig. 3). A brief survey on published reviews yielded a summarized list of plant species that accumulate triterpene saponins (Supplementary Table 1) (Vincken et al. 2007; de Costa et al. 2011; Biswas and Dwivedi 2019). Fabales and Apiales include the largest number of species producing triterpene saponins, but Fabales is known for having the most diverse occurrence of different types of sapogenins (Vincken et al. 2007). In our survey, the number of species producing triterpenoid saponins was evenly distributed between Asterids (n = 100) and Rosids (n = 99) groups (Fig. 3). Nonetheless, triterpenoid sapogenins show convergent evolution in

Eudicots

Ranunculales - 13 spp
Santalales - 2 spp
Caryophylalles - 26 spp

Rosids

Zygophyllales - 1 sp
Celastrales - 9 spp

Rosid I
Fabales - 48 spp
Rosales - 8 spp
Cucurbitales - 5 spp

Rosid II
Myrtales - 6 spp
Crossosomatales - 1 sp
Sapindales - 22 spp

Asterids

Ericales - 19 spp

Asterid I
Gentianales - 12 spp
Lamiales - 12 spp

Asterid II
Asterales -17 spp
Apiales - 31 spp
Dipsacales - 8 spp

Fig. 3 Distribution of plant species that produce triterpene saponins in orders of Eudicots (compiled from data by Vincken et al. 2007; de Costa et al. 2011; Biswas and Dwivedi 2019)

Angiosperm phylogeny (Cardenas et al. 2019). In monocots, it is known that *Avena* species and the closely related *Arrhenatherum elatius* (L.) P.Beauv. ex J.Presl & C.Presl (Poaceae) can produce triterpene saponins, being an exception to this group of plants (Crombie and Crombie 1986).

Natural sources of bioactive saponins are a relevant matter, especially considering the technical challenges of the chemical synthesis of these complex triterpenoid glycosides. Strategies to obtain high quality plant material for industrial applications and developments towards sustainable production to reduce the pressure on native landscapes are still a challenge in several cases. Next, some of the main natural sources of bioactive saponins are described.

Panax spp. (Araliaceae)

Roots from *Panax ginseng* C.A. Meyer and *Panax notoginseng* (Burk.) F.H. Chen are traditionally used as medicinal herbs in China (Guo et al. 2010). These particular *Panax* species are commercially cultivated in geographically restricted areas of China and South Korea due to climate specificities required by the different cultivars (Baeg and So 2013; Wang et al. 2016). In vitro cell cultures (*e.g.* roots, callus, and cell suspensions) are well-established for saponin metabolism-related research (Deng et al. 2017b; Zhang et al. 2019).

Hundreds of saponins have been isolated and identified mainly from roots, but also from leaves and flower buds of *Panax* spp. These saponins are mostly dammarane derived with 20(S)-protopanaxadiol (PPD) or 20(S)-protopanaxatriol (PPT) sapogenins (Fig. 4). Hydroxyl groups replace the C3 and C12 of the dammarane core. A methyl group is placed at C8, the configuration at C13 is β-H and an S configuration is found at C20. The 20(S)-protopanaxadiol sapogenin has a hydroxyl at C6 that is not present in the 20(S)-protopanaxatriol. Unlike *P. ginseng*, *P. notoginseng* does not produce saponins derived from oleanolic acid (Shin et al. 2015; Wang et al. 2016; Yao et al. 2019).

P. notoginseng roots produce five main saponin constituents: the notoginsenoside R1 and the ginsenosides Rb1, Rg1, Rd, and Re. Among these, Rb1, Rg1 and R1 are usually used as standard compounds in quality evaluation of *P. notoginseng* (Wang et al. 2016). In *P. ginseng*, the most abundant PPT-type saponins are the ginsenosides Re and Rg1, while the typical PPD-type ginsenosides are Rb1, Rb2, Rc, and Rd (Shin et al. 2015).

Quillaja spp. (Quillajaceae)

Quillaja comprises two temperate evergreen tree species. *Quillaja saponaria* occurs mainly in the central area of Chile, whereas *Quillaja brasiliensis* (A. St.-Hil. & Tul.) Mart. can be found in southern Brazil, northeastern Argentina, eastern Paraguay and northern Uruguay (Luebert 2014). *Quillaja saponaria* bioactive saponins are extracted from bark and wood of adult trees, and Quil-A has long been recognized as a commercially useful immunoadjuvant purified from bark extracts (Dalsgaard 1974; Magedans et al. 2019). Leaves from *Quillaja brasiliensis* trees can be used to obtain bioactive saponin fractions with similar properties (Fleck et al. 2013). Saponin content can vary among different genotypes and environmental conditions (Szakiel et al. 2011; de Costa et al. 2013).

Saponins from *Quillaja* are marked by an unusually high degree of substituent complexity, which consists of multiple glycoside residues and branched lipid chains attached to a C_{30} triterpene sapogenin (Fig. 4). So far, the main known sapogenins are quillaic acid, phytolaccagenic acid, and echinocystic acid (Guo and Kenne 2000a, b; Wallace et al. 2019).

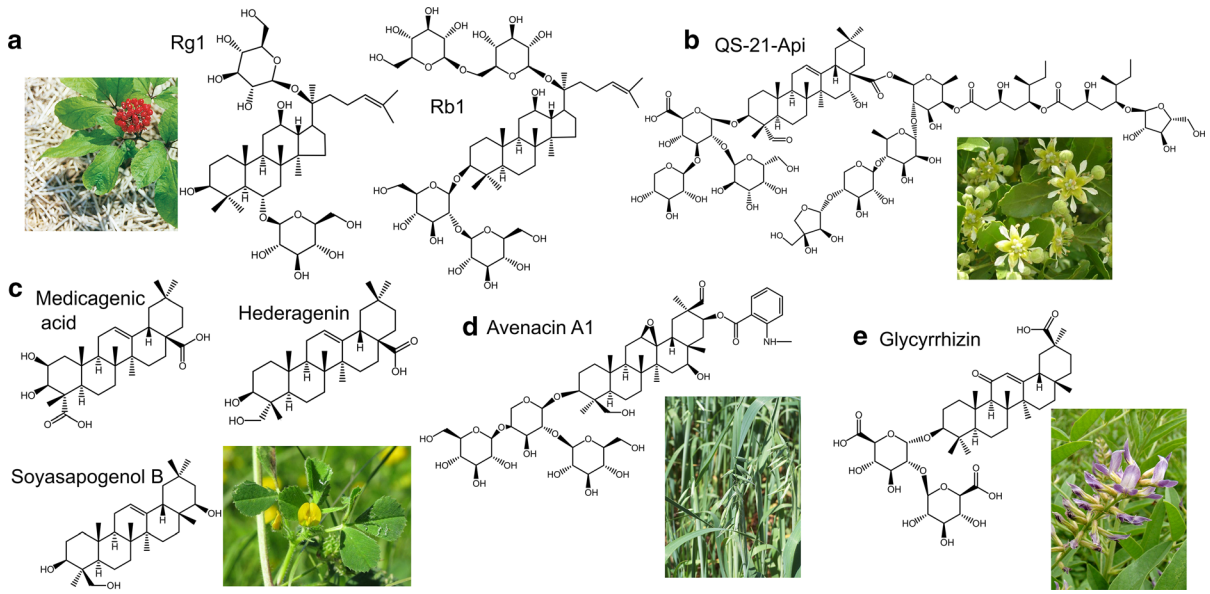


Fig. 4 Natural sources of triterpenoid saponins. **a** *Panax ginseng*. The sapogenin typically divides ginsenosides in two groups. Rg1 has protopanaxatriol (PPT) core and Rb1 has the protopanaxadiol (PPD) sapogenin. **b** *Quillaja saponaria.* QS-21-Api has an apiose as the terminal sugar residue within the tetrasaccharide attached to C28. The sapogenin is quillaic acid, which is a typical aglycone found in *Quillaja* saponins. **c** *Medicago truncatula.* Two classes of saponins can be found in *M. truncatula:* the hemolytic class are compounds modified from medicagenic acid and hederagenin, for example. Non-hemolytic saponins are the soyasaponins, modified from triterpenoids like soyasapogenol B. **d** *Avena sativa*. Avenancin A-1 is the major compound found in root extracts of *Avena* spp. It has an esterified N-methyl anthranilic acid and emits fluorescence under UV light. **e** *Glycyrrhiza glabra.* The main saponin found in *Glycyrrhiza* spp. roots is the monodesmoside glycyrrhizyn. Credits: *Panax ginseng*. Wikimedia Commons. FloraFarm GmbH/Katharina Lohrie. *Quillaja saponaria* – Juan Pablo Gabella (Reproduced with permission. http://www.chilebosque.cl/). *Medicago truncatula* - Stephen Mifsud (Reproduced with permission. http://www.maltawildplants.com/). *Avena sativa* – Wikimedia Commons. Henrik Sendelbach

The challenges of providing biomass to the market of *Quillaja* extracts, while preventing deforestation, were reviewed by Magedans et al. 2019. Commercial forests of *Quillaja saponaria*, government regulation on tree harvesting, and extraction protocols for branches and wood account for decreasing the pressure on older native forests. For *Quillaja brasiliensis*, micro-propagation and clonal garden cultivation are some of the techniques established towards sustainable biomass production for the purification of saponins (Fleck et al. 2009).

Medicago truncatula (Fabaceae)

Triterpene saponin biosynthesis has been extensively studied in the model species *Medicago truncatula*. In fact, many *Medicago* species accumulate pentacyclic oleanane-derived saponins.

Typically, *Medicago* saponins are divided in two classes based on sapogenin modifications. The first group of compounds has a carboxyl group at C28 and might present diverse oxidized states at C23 position. In *Medicago* spp., the sapogenins that belong to this class are hederagenin, oleanolic acid, queretaroic acid, medicagenic acid, and zanhic acid, among others. The second class of saponins has soyasapogenols as sapogenins, which have a hydroxyl residue at C24 position and no modification at C28 (Tava et al. 2011; Gholami et al. 2014) (Fig. 4). Hemolytic activity further distinguishes these two classes of saponins. This feature is probably affected by the sapogenin to sugar residue ratio, as well as sugar substitutions. Soyasaponins do not have hemolytic activity, unlike the other class of *Medicago* saponins (Oleszek 1996).

Differential spatial accumulation of saponins is found in *M. truncatula*. The soyasapogenol glycosides are synthesized in the border cells of roots (Watson

et al. 2015). These saponins play a role in plant defense, inhibiting nematode attack (Pollier et al. 2011). The saponins zanhic acid and medicagenic acid can only be found in the aerial parts of *M. truncatula* and show potential toxicity against herbivores (Kapusta et al. 2005; Gholami et al. 2014).

Glycyrrhiza spp. (Fabaceae)

Roots and stolons of *Glycyrrhiza* spp. (licorice) accumulate oleanane-type saponins, soyasaponins, betulinic acid, among other compounds, such as flavonoids. Glycyrrhizin and licorice saponin extracts can be found in over-the-counter drugs commercialized in Japan, including anti-inflammatory and anti-allergic drugs (Hayashi and Sudo 2009). The main source of *Glycyrrhiza* saponins are plants harvested from wild forests or commercial farming (WHO 1999).

Glycyrrhizin has been described as the main saponin produced in the roots of *Glycyrrhiza* spp. (Yamamoto and Tani 2005; Kojoma et al. 2010). Glycyrrhizin is a monodesmoside saponin derived from glycyrrhetinic acid, a sapogenin modified from β-amyrin that has a ketone at C11 and a carboxyl group at C30 (Fig. 4). The yield of glycyrrhizin in methanolic extracts obtained from *Glycyrrhiza glabra* L. roots has been estimated at 9% (w/v) (Gantait et al. 2010). Protocols for in vitro plant regeneration and cell propagation have been established for licorice species (Hayashi et al. 1988; Kojoma et al. 2010). Glycyrrhizin can be purified from root cultures of *G. glabra* and callus cultures of *Glycyrrhiza* spp. (Wongwicha et al. 2008; Shabani et al. 2009; Srivastava et al. 2019).

Avena spp. (Poaceae)

Avena spp. produce both steroidal and triterpenoid saponins, unlike other monocotyledonous species that only accumulate steroidal saponins (Osbourn 2003). Avenacins are a class of triterpene saponins synthesized in roots of *Avena* spp. Avenacin A-1 was identified as the major triterpene saponin produced by *Avena* spp. (Fig. 4). Young roots of *Avena sativa* L. proportionally accumulate more avenacin A-1 than A-2 (73% and 14% of saponin extract, respectively, with the remainder composed of minor isomers), but this profile shifts to a more even distribution as the plant ages (Crombie and Crombie 1986). Avenacins are preferentially accumulated in the epidermal cells of root meristems, which is consistent with the protective activity of these terpenes against pathogens (Osbourn et al. 1994; Kemen et al. 2014).

Avenacin A-1 has an esterified N-methylanthranilic acid group (Fig. 4) and emits fluorescence under UV light (Crombie et al. 1984), which is a useful phenotype for mutant isolation and subsequent biochemical characterization (Papadopoulou et al. 1999; Osbourn 2003; Mylona et al. 2008). Therefore, *Avena* spp. have been used as model organisms for the study of saponin biosynthesis.

Transcription factors regulating saponin biosynthesis

Research on the molecular regulation of saponin biosynthesis has focused on mining transcriptome data for transcription factor-like sequences that are up regulated by jasmonic acid (JA). JA is a well characterized plant defense hormone which activates the synthesis of a wide variety of specialized plant defense compounds (Wasternack and Hause 2013; Zhou and Memelink 2016; Shoji 2019).

One family of JA-responsive TFs is the APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) family (De Boer et al. 2011). AP2/ERF TFs are only found in plants (Phukan et al. 2017). The DNA biding domain has up to 60 amino acids forming a three-stranded anti-parallel β-sheet followed by an α-helix that is arranged in parallel to the β-sheet (Allen et al. 1998). The AP2/ERF TFs recognize a GCC-box element promoter sequence (5′-AGCCGCC-3′) of target genes.

A member of AP2/ERF TF family was identified in cell cultures of *P. notoginseng* treated with methyl jasmonate (MeJA) (Deng et al. 2017a). The conserved domain of an ORCA sequence identified in *Catharanthus roseus* (L.) G.Don was used as template for primer design. Sequence of *PnERF1* was amplified from a RACE-Ready cDNA library with a length of 1113 bp. Alignment of PnERF1 amino acid sequence with known plant ERFs and phylogenetic analysis supported PnERF1 as a member of AP2/ERF family. Also, electrophoretic mobility shift assays (EMSA)

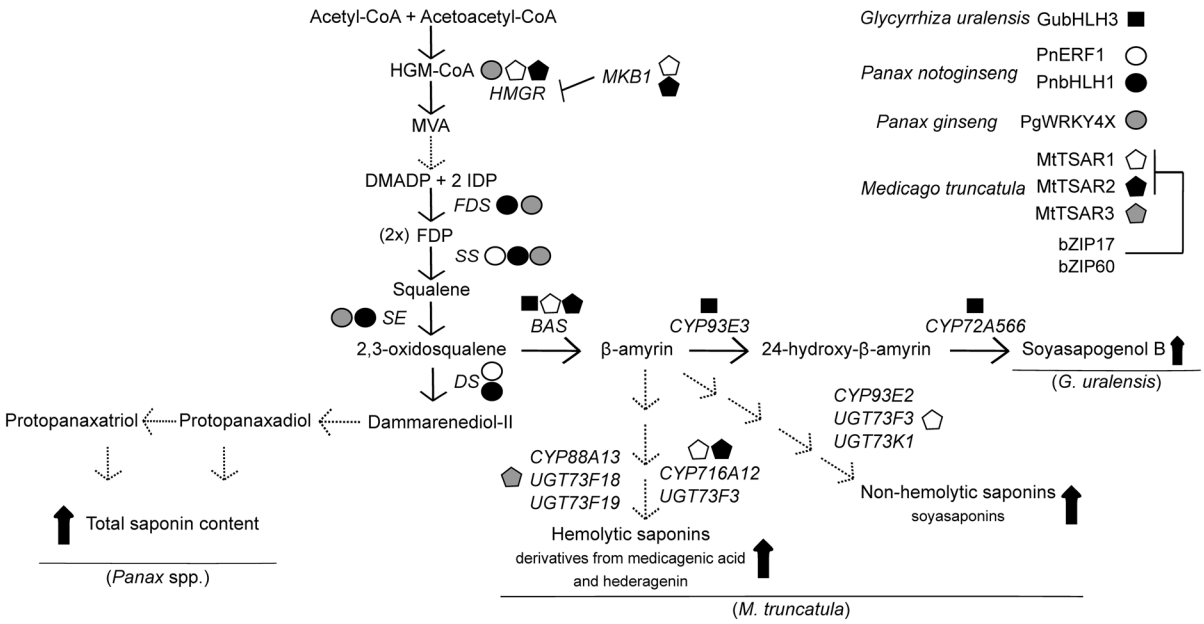


Fig. 5 Transcription factors involved in regulating saponin biosynthesis in *Glycyrrhiza uralensis* (GubHLH3: black square), *Panax notoginseng* (PnERF1: white circle; PnbHLH1: black circle), *Panax ginseng* (PgWRKY4X: grey circle), and *Medicago truncatula* (MtTSAR1: white polygon; MtTSAR2: black polygon; MtTSAR3: grey polygon). Two bZIP proteins (bZIP17 and bZIP60) may act as negative regulators of MtTSAR1 and MtTSAR2. For each species, modulation of the different transcription factor levels changed the relative expression of biosynthetic genes and saponin yield accordingly. Black arrow: one biosynthetic step. Dashed arrow: multiple biosynthetic steps. See text for further details. Abbreviations: BAS: *β-AMYRIN SYNTHASE*. DMADP: dimethylallyl diphosphate. DS: *DAMMARENEDIOL-II SYNTHASE*. FDP: farnesyl diphosphate. FDS: *FARNESYL DIPHOSPHATE SYNTHASE*. HGM-Coa: 3-hydroxy-3-methylglutaryl-Coa. HMGR: *3-HYDROXY-3-METHYLGLUTARYL SYNTHASE*. IDP: isopentenyl diphosphate. MKB1: *MAKIBISHI1*. MVA: mevalonic acid. SE: *SQUALENE EPOXIDASE*; SS: *SQUALENE SYNTHASE*

showed that recombinant PnERF1 could interact specifically with GCC-box cis-like elements (Deng et al. 2017a).

Cell suspension lines of *P. notoginseng* overexpressing *PnERF1* successfully produced higher levels of ERF1 transcripts. Expression of *SS* and *DAMMARENEDIOL SYNTHASE* (*DS*) genes were also upregulated in these cell lines (Fig. 5). Concentrations of total saponins and bioactive saponin monomers (*e.g.* Re and Rg1) were increased by overexpression of *PnERF1* (Deng et al. 2017a).

A MeJA-inducible member of the WRKY TF gene family was identified in *Panax quinquefolius* L. (*PqWRKY1*), presenting a WRKY domain and a Zinc-finger motif (Sun et al. 2013a). In *Arabidopsis* plants overexpressing this TF, transcripts of genes related to triterpene biosynthesis increased up to fivefold; in addition, transgenic plants were more tolerant to osmotic stress. Based on these results it was suggested that *PqWRKY1* acts as a positive regulator of triterpene ginsenoside accumulation and osmotic stress responses in *P. quinquefolius*.

Ginsenoside biosynthesis in *P. ginseng* adventitious roots was significantly stimulated by the endophyte fungus *Chaetomium globosum*, and differential transcriptomic analysis of control and elicited roots allowed the identification of the pathogen-related TF PgWRKY4X (Yao et al. 2020). Overexpression and RNA interference experiments targeting this TF in transgenic cell lines of *P. ginseng* indicated that PgWRKY4X was able to positively regulate the expression of several saponin biosynthesis-related genes, such as *HMGR*, *FDS, SS*, and *SE*. Increased expression of *PgWRKY4X* correlated with higher levels of ginsenosides. Moreover, binding of PgWRKY4X to the *SE* promoter was shown by E-MSA. Curiously, a putative direct interaction of PgWRKY4X and SE proteins was identified, although its meaning is unknown (Yao et al. 2020).

The Basic Helix-Loop-Helix (bHLH) TFs comprise another family of TFs capable of responding to JA signaling. In the bHLH TF family, the domain responsible for protein dimerization has a size of 50 amino acids and consists of two amphipathic α-helices separated by a loop of variable length. The second domain is an N-terminal DNA biding region enriched in basic residues (Atchley and Fitch 1997). Transcription factors of the bHLH family are capable of forming homodimers or heterodimers that interact with E-box (5′-CANNTG-3′) sequences present in the promoter regions of their target genes, like the N-box (5′-CACGAG-3′) and G-box (5′-CACGTG-3′) elements. The bHLH-type TFs are very common in eukaryotes, and phylogenetic analyses suggest distinct evolutionary events among clades (Goossens et al. 2017).

PnbHLH1 was characterized in *P. notoginseng*. This TF has a bHLH motif with high homology to the same motif present in other bHLH TFs, and EMSA assays showed that PnbHLH1 could successfully interact with E-box (CANNTG) cis-like elements. Transgenic cell lines overexpressing *PnbHLH1* had a significant increase in gene expression of *DAMMARENEDIOL-II SYNTHASE*, *SS, SE*, and *FDS*. Unexpectedly, *HMGR* expression was barely upregulated. As recorded for *PnERF1* overexpression, production of total saponins and saponin monomers was induced in transgenic cell lines (Zhang et al. 2017) (Fig. 5).

Genomic sequencing analysis of *P. ginseng* revealed 169 candidate genes encoding bHLH TFs. Twenty-one RNA-Seq data sets were evaluated for expression patterns of bHLH TF in different organs and under MeJA treatment. Six PgbHLH genes were potentially related to the regulation of ginsenoside biosynthesis. For example, *PG40693* and *PG07173* showed stronger correlation with PPD-type ginsenosides than with PPT-type ginsenosides. In addition, *PG40693* levels had a strong positive correlation with expression of multiple genes in the MVA and MEP pathways. These results suggested that *PG40693* is a candidate regulator of ginsenoside biosynthesis deserving further analysis (Chu et al. 2018).

GubHLH3 could modulate soyasaponin biosynthetic related genes of *Glycyrrhiza uralensis* Fisch. GubHLH3 has high identity with Medtr0246s0020.1 (65% amino acid sequence) and AtbHLH018 (42%). The TF sequence was successfully cloned from a cDNA library of *G. uralensis* to produce transgenic root cultures (Tamura et al. 2018). Transgenic hairy roots of *G. uralensis* overexpressing *GubHLH3* had higher expression of *BETA-AMYRIN SYNTHASE (BAS)*, *CYP93E3*, and *CYP72A566*, which are necessary to produce soyasapogenol B from 2,3-oxidosqualene (Fig. 5). In addition, two-fold increase in the content of soyasapogenol B and its intermediate, 22β-hydroxy-β-amyrin, was detectable in transgenic lines. An increment of oleanolic acid level in transgenic lines was not observable, which suggested a competition between oleanolic acid biosynthesis and soyasaponin biosynthesis (Tamura et al. 2018).

Two bHLH TFs were identified in *M. truncatula* and named TRITERPENE SAPONIN ACTIVATION REGULATOR1 (MtTSAR1) and MtTSAR2. These are encoded by genes that are homologous to *Medtr7g080780* and *Medtr4g066460*, respectively. MtTSAR1 and MtTSAR2 preferably bind to the cis-like G-box sequence (CACGTG), and also show affinity for N-box element (CACGAG). *MtTSARs* transcription levels are upregulated by MeJA treatment (Mertens et al. 2016a).

Hairy root lines overexpressing *MtTSARs* had higher expression levels of *3-HYDROXY-3-METHYL-GLUTARYL-CoA SYNTHASE 1 (HMGS)* and *MAKIBISHI 1* (*MKB1*), which are usually co-expressed. *MKB1* encodes a protein that targets HMGS to the proteasome, preventing excessive allocation of AcCoA to the MVA pathway. Also, MtTSARs upregulated the expression of *BAS*, *P450s* and *UDP-DEPENDENT GLUCOSYLTRANSFERASE* genes (Fig. 5) (Mertens et al. 2016a). Cell lines overexpressing *MtTSAR1* accumulated both non-hemolytic saponins and hemolytic saponins. However, overexpression of *MtTSAR2* exclusively upregulated the content of hemolytic saponins. Accordingly, knockout mutants exhibited lower content of saponins and downregulation of related biosynthetic genes (Mertens et al. 2016a).

The participation of endoplasmic reticulum (ER) stress-related TFs bZIP17 and bZIP60 (basic leucine zipper type) has been proposed as negative regulators of MeJA-induced triterpene saponin biosynthesis in *M. truncatula*. These TFs would act by interfering with MtTSAR1 and MtTSAR2 as part of a mechanism to coordinate defense, growth, and metabolism under specific stress conditions that lead to changes in the ER network structure (Ribeiro et al. 2020a).

TRITERPENE SAPONIN ACTIVATION REGULATOR3 (TSAR3) is an *M. truncatula* seed-specific

TF involved in regulating hemolytic saponin accumulation. Co-expression analyses of *TSAR3* led to the identification of genes encoding relevant late biosynthetic enzymes of the metabolic branch of hemolytic saponins in the legume species. These included a cytochrome P450 oxidase, CYP88A13, as well as two uridine diphosphate glycosyltransferases, UGT73F18 and UGT73F19 (Ribeiro et al. 2020b).

The bHLH TF IRIDOID SYNTHESIS1 (BIS1) was shown to regulate the terpenoid branch of the monoterpene indole alkaloid pathway in *Catharanthus roseus.* CrBIS1 and MtTSARs are orthologues that belong to same subclade IVa of the bHLH family (Heim et al. 2003). When expressed in *C. roseus*, *MtTSARs* are capable of inducing iridoid biosynthesis genes. Likewise, *M. truncatula* transgenic cell lines expressing *CrBIS1* showed increased transcripts of genes from the triterpene saponins pathway and had an increase in saponin amount (Mertens et al. 2016b).

Another large family of plant TFs is MYB (v-myb avian myeloblastosis viral oncogene homolog), characterized by variable numbers of MYB DNA binding domain repeats. These TFs, of which the most frequent type in plants is R2R3-MYB, are part of complex regulatory systems involved in stress responses, development, and metabolism (Dubos et al. 2010). In adventitious roots of *P. ginseng*, *PgMYB2*, an R2R3-MYB gene was isolated and its product shown to localize to the nucleus. It binds to the promoter of the *DS* gene, possibly causing its transactivation in a MeJA responsive fashion (Liu et al. 2019b). However, the correlation of this activity with saponin accumulation remains to be established.

Strategies for improving yields of bioactive saponins

Like most plant specialized metabolites, saponins have essentially evolved to act as defense molecules, protecting against both biotic and abiotic stresses. In addition, it has been well established that responses to stress conditions generally involve transduction signals based on ROS, Ca^{2+}, NADPH oxidases, redox regulation, MAP kinases, and phytohormones, such as JA (and MeJA), salicylic acid (SA), ethylene (ETH), abscisic acid (ABA), and nitric oxide (NO) (Sewelam et al. 2016). Not surprisingly, the application of moderate intensity stresses, preserving plant cell integrity and functionality, and/or treatment with some of the signaling molecules involved in stress response transduction at different concentrations and times of exposure, have long been successfully used to increase saponin production (Yendo et al. 2010).

Production of specialized plant metabolites, including triterpene saponins, can be done in whole plants, organ, tissue, or cell cultures, as well as in heterologous systems, such as yeast, bacteria, or other plant species, all of which can be used as hosts for expressing biochemical pathways leading to metabolites of interest. Each one of these approaches has its own advantages and limitations, the choice of which is best for increasing metabolite yields varying on a case by case basis (reviewed by Matsuura et al. 2018). Some examples of improving yields of triterpene saponins produced by the main types of biological sources are discussed below.

As mentioned above in the transcription factor section, accumulation of triterpene saponins is often induced by MeJA and JA. This response has been shown in *P. ginseng, P. notoginseng*, *G. glabra, M. truncatula, Medicago sativa* L., and *Q. brasiliensis* in diverse systems, such as whole plants, organ (such as hairy roots and adventitious roots) and cell cultures (Shabani et al. 2009; Yendo et al. 2010; de Costa et al. 2013; Oh et al. 2014; Biswas and Dwivedi 2019). Other phytohormones have also been used to increase triterpene saponin yields, including auxins, cytokinins, SA, ETH, and ABA (Yendo et al. 2010, 2014).

Non-hormonal treatments have also proven useful in promoting triterpene saponin accumulation. Osmolytes, drought, oligosaccharides, yeast extract, chitosan, casein hydrolysate, membrane permeabilizing agents, H^{+}-ATPase inhibitors, mechanical damage, heavy metals, visible and ultraviolet irradiance, heat, and oxygen concentration have been used to that end (Yendo et al. 2010; Lambert et al. 2011; de Costa et al. 2013; Huang et al. 2013; Marsik et al. 2014; Alsoufi et al. 2019; Magedans et al. 2019). Exposure to the phenylpropene *trans*-anethole has also improved yields of triterpene saponins in hairy roots of *P. quinquefolius* (Kochan et al. 2018). As expected, several of these treatments can change the redox status of the cells and may trigger defense phytohormone synthesis and/or activation.

Nutrition can also be used to modulate saponin production; increased yield of oleanolic glycosides was recorded in hairy roots of *C. officinalis* grown in

media containing nitrate as the sole source of nitrogen (Długosz et al. 2018). Endophytic fungal filtrates combined with sucrose feeding promoted triterpenic acids (oleanolic and ursolic acid) biosynthesis in *Salvia fruticosa* Mill. cell suspensions (Kümmritz et al. 2016), which represents an example of the useful impact of combined elicitation and nutritional modulation on metabolite yield.

Metabolic engineering of both early and late pathway portions of saponin biosynthetic genes is another valuable approach to improve metabolic flow towards triterpene saponin accumulation, and this has been the topic of several reviews (Yendo et al. 2010; Lambert et al. 2011; Zhao and Li 2018). Overexpression or repression of genes involved in saponin or closely related compound metabolism have been successfully done in whole plants, organ, and cell cultures. Transformation methods used mainly *Agrobacterium* (Shirazi et al. 2018; Biswas and Dwivedi 2019), whereas in some instances RNAi has been employed (Han et al. 2010; Sun et al. 2013b; Park et al. 2016). Genes involved in biosynthesis of triterpene saponins of the crucifer *Barbarea vulgaris* R.Br. were transiently expressed in *Nicotiana benthamiana* Domin plants using a viral microsome system (Khakimov et al. 2015). Recently, a case of triterpenoid pathway engineering was reported in *Lotus japonicus* (Regel) K. Larsen hairy roots, in which genome editing technology based on clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) generated a *cyp716a51* mutant that no longer accumulated C-28 oxidized triterpenoids (Suzuki et al. 2019).

The success of metabolic engineering often depends on or can significantly benefit from the optimization of cell, tissue and organ structural features as effective metabolite sinks or storage sites. Allocation to storage structures, tissues, cells, or organelles (mainly vacuoles) can be improved by the manipulation of metabolite transporters in membranes delimiting these levels of structural organization. The various specialized metabolite transporter protein families represent another level of metabolic regulation and may be potential targets for metabolic engineering (Shitan 2016). The creation of metabolite sinks or storage sites mimics the condition found in well differentiated whole plant metabolism. There are several advantages in having sinks and storage sites of specialized metabolites, namely the alleviation of feedback inhibition of enzymes, improved expression of regulatory genes, and reduced autotoxicity (Shitan 2016; Matsuura et al. 2018).

Encouraging results on saponin production were achieved using synthetic biology approaches by transferring genes encoding parts or entire pathways of plants to bacteria or yeast (Kim et al. 2015; Takemura et al. 2017; Zhao and Li 2018). Pathway insertion in host plant species, such as *Nicotiana* spp., has proven viable (Han et al. 2014; Khakimov et al. 2015). Biosynthetic genes of the ginseng triterpene sapogenin protopanaxadiol driven by endosperm-specific α-globulin promoter have been expressed in transgenic rice grains, which accumulated this metabolite (Han et al. 2019).

Synthetic biology methods for expression of saponin-related plant genes in yeast and bacteria have advanced considerably in the last decade, partly as a result of the identification of several genes involved in saponin production based on transcriptomic and metabolomic analyses. Elicited plants or in vitro cultures, mostly by JA or MeJA, or different plant organs have been the most frequent objects of these evaluations (e.g. Ma et al. 2016; Wang et al. 2018; Zeng et al. 2018; Shi et al. 2019b). As described in the section dedicated to transcription factors, besides biosynthetic enzymes, these regulatory proteins also represent useful targets for improving triterpene saponin yields.

Plant transient expression of saponin-related genes has also been done mostly in *N. benthamiana* as host, using *Agrobacterium tumefaciens* as vector. Transient expression has often been used as tool to show accumulation of the expected triterpenes by the gene constructs. An example of this application involved new OSCs producing α-amyrin, which were discovered in *Bauhinia forficate* Link, a medicinal legume tree that accumulates high levels of this molecule. In fact, OSC genes introduced in *N. benthamiana* leaves by agroinfiltration resulted in the accumulation of α-amyrin (Srisawat et al. 2019).

The viability and significant potential of this strategy for saponin production has been shown by the development of an improved *Agrobacterium tumefaciens* infiltration method using whole plants, combined with metabolic engineering that included a feedback insensitive form of HMGR (Reed et al. 2017). Co-infiltration of *A. tumefaciens* strains carrying different genes of saponin biosynthesis using this

system yielded triterpenes in the gram range, also allowing the generation of known and novel triterpene derivatives with relevant bioactivities (Reed et al. 2017).

Given the complexity of plant cell, tissue and organ structure and its advantages to support elaborate biochemistry, transfer of metabolic pathways to relatively simpler microbial cells, either yeast or bacteria, is often not readily feasible (Kim et al. 2015; Matsuura et al. 2018). However, a number of alternatives have been developed to overcome these potential difficulties (Zhao and Li 2018). Some of these solutions include most adequate host strain selection, codon usage optimization, promoter selection and improvement, co-expression combinations using common regulated promoters, two step culture media (biomass accumulation and saponin production stage), optimized elicitation, subcellular localization (mainly in yeast), modification of cellular morphology (*e.g.* expansion of endoplasmic reticulum by disruption of phosphatidic acid phosphatase) (Arendt et al. 2017), modulation of expression of high flux control enzymes or use of feedback insensitive truncated forms (*e.g.* HMGR), precursor availability increase (*e.g.* combination of MVA and MEP pathways leading to IDP/DMADP), metabolite transporter engineering, and competing pathway inhibition (*e.g.* sterol biosynthesis after biomass accumulation) (Matsuura et al. 2018; Zhao and Li 2018; Srisawat et al. 2020).

Conclusion

The economic relevance of triterpene saponins has been driving continuous research efforts to improve yields and production systems of these valuable chemicals. Plant species that are natural sources of saponins must be continuously preserved, domesticated, and multiplied in order to support renewable and environmentally sound supply of metabolites of interest. Biotechnological approaches using conventional and genetically modified biomass, such as cell, organ, and whole plant cultures are important alternatives to meet market demands of high quantity and quality metabolites. These production methods can be particularly powerful when coupled with elicitation strategies related to defense signaling molecules and redox homeostasis associated to moderate stresses.

Biosynthesis of triterpene saponins is complex, encompassing a large number of steps and elaborate regulation. Omics studies, mainly transcriptomics and metabolomics, are providing a road map for identifying key regulator genes of metabolic flux leading to target saponins, as well as generating a more robust bioinformatics knowledge foundation to find new genes and metabolites. There are relatively few studies at the proteomic level with a focus on triterpene saponin metabolism and this should be a topic to consider in future research efforts, as it provides important information on general biochemical regulation.

Overall, genetic engineering of triterpene saponin metabolic pathways is often focused on biosynthetic genes aiming at improved yields of target metabolites. However, an attractive set of transcription factors is being made available to reinforce and improve the metabolic engineering toolbox, potentially allowing simultaneous and fine control of enzyme encoding genes in a relatively less laborious fashion. The use of more recent technologies such as genome editing is likely to increase in coming years and positively impact triterpene saponin production. Production systems of triterpene saponins in microbial hosts more amenable to industrial scale are still incipient. However, in the near future it is likely that large scale production of premium quality bioactive plant triterpene saponins will take place in stable, contained, and highly controlled industrial environments.

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Compliance with ethical standards

Conflict of interest None.

References

Ahumada I, Cairó A, Hemmerlin A et al (2008) Characterisation of the gene family encoding acetoacetyl-CoA thiolase in *Arabidopsis*. Funct Plant Biol 35:1100–1111. https://doi.org/10.1071/FP08012

Allen MD, Yamasaki K, Ohme-Takagi M et al (1998) A novel mode of DNA recognition by a β-sheet revealed by the solution structure of the GCC-box binding domain in complex with DNA. EMBO J 17:5484–5496. https://doi.org/10.1093/emboj/17.18.5484

Alsoufi ASM, Pączkowski C, Szakiel A, Długosz M (2019) Effect of jasmonic acid and chitosan on triterpenoid production in *Calendula officinalis* hairy root cultures. Phytochem Lett 31:5–11. https://doi.org/10.1016/j.phytol.2019.02.030

Arendt P, Miettinen K, Pollier J, De Rycke R (2017) An endoplasmic reticulum-engineered yeast platform for overproduction of triterpenoids. Metab Eng 40:165–175. https://doi.org/10.1016/j.ymben.2017.02.007

Atchley WR, Fitch WM (1997) A natural classification of the basic helix-loop-helix class of transcription factors. Proc Natl Acad Sci U S A 94:5172–5176. https://doi.org/10.1073/pnas.94.10.5172

Augustin JM, Drok S, Shinoda T et al (2012) UDP-glycosyltransferases from the UGT73C subfamily in *Barbarea vulgaris* catalyze sapogenin 3-O-glucosylation in saponin-mediated insect resistance. Plant Physiol 160:1881–1895. https://doi.org/10.1104/pp.112.202747

Avato P, Bucci R, Tava A, Ferrer A (2006) Antimicrobial activity of saponins from *Medicago* sp.: structure-activity relationship. Phytother Res 20:454–457. https://doi.org/10.1002/ptr.1876

Bach TJ, Boronat A, Campos N et al (1999) Mevalonate biosynthesis in plants. Crit Rev Biochem Mol Biol 34:107–122. https://doi.org/10.1080/10409239991209237

Baeg IH, So SH (2013) The world ginseng market and the ginseng (Korea). J Ginseng Res 37:1–7. https://doi.org/10.5142/jgr.2013.37.1

Barbosa AdP (2014) An overview on the biological and pharmacological activities of saponins. Int J Pharm Pharm Sci 6:47–50

Biswas T, Dwivedi UN (2019) Plant triterpenoid saponins: biosynthesis, in vitro production, and pharmacological relevance. Protoplasma 256(6):1463–1486. https://doi.org/10.1007/s00709-019-01411-0

Bowles D, Lim E-K, Poppenberger B, Vaistij FE (2006) Glycosyltransferases of lipophilic small molecules. Annu Rev Plant Biol 57:567–597. https://doi.org/10.1146/annurev.arplant.57.032905.105429

Bowyer P, Clarke BR, Lunness P et al (1995) Host range of a plant pathogenic fungus determined by a saponin detoxifying enzyme. Science 267:371–374. https://doi.org/10.1126/science.7824933

Cao H, Nuruzzaman M, Xiu H et al (2015) Transcriptome analysis of methyl jasmonate-elicited *Panax ginseng* adventitious roots to discover putative ginsenoside biosynthesis and transport genes. Int J Mol Sci 16:3035–3057. https://doi.org/10.3390/ijms16023035

Cardenas PD, Almeida A, Bak S (2019) Evolution of structural diversity of triterpenoids. Front Plant Sci 10:1–10. https://doi.org/10.3389/FPLS.2019.01523

Carelli M, Biazzi E, Panara F et al (2011) *Medicago truncatula* CYP716A12 is a multifunctional oxidase involved in the biosynthesis of hemolytic saponins. Plant Cell 23:3070–3081. https://doi.org/10.1105/tpc.111.087312

Cho JY, Yoo ES, Baik KU et al (2001) In vitro inhibitory effect of protopanaxadiol ginsenosides on tumour necrosis factor (TNF)-alpha production and its modulation by known TNF-alpha antagonists. Planta Med 67:213–218. https://doi.org/10.1055/s-2001-12005

Chu Y, Xiao S, Su H et al (2018) Genome-wide characterization and analysis of bHLH transcription factors in *Panax ginseng*. Acta Pharm Sin B 8:666–677. https://doi.org/10.1016/j.apsb.2018.04.004

Cordier H, Karst F, Bergès T (1999) Heterologous expression in *Saccharomyces cerevisiae* of an *Arabidopsis thaliana* cDNA encoding mevalonate diphosphate decarboxylase. Plant Mol Biol 39:953–967. https://doi.org/10.1023/A:1006181720100

Crombie WML, Crombie L (1986) Distribution of avenacins A-1, A-2, B-1 and B-2 in oat roots: their fungicidal activity towards "take-all" fungus. Phytochemistry 25:2069–2073. https://doi.org/10.1016/0031-9422(86)80068-1

Crombie L, Crombie WML, Whiting DA (1984) Structures of the four avenacins, oat root resistance factors to "take-all" disease. J Chem Soc Chem Commun 1:246–248. https://doi.org/10.1039/C39840000246

Cunillera N, Arró M, Delourme D et al (1996) *Arabidopsis thaliana* contains two differentially expressed farnesyl-diphosphate synthase genes. J Biol Chem 271:7774–7780. https://doi.org/10.1074/jbc.271.13.7774

Dalsgaard K (1974) Saponin adjuvants. 3. Isolation of a substance from *Quillaja saponaria* Molina with adjuvant activity in food-and-mouth disease vaccines. Arch Gesamte Virusforsch 44:243–254

De Boer K, Tilleman S, Pauwels L et al (2011) APETALA2/ETHYLENE RESPONSE FACTOR and basic helix-loop-helix tobacco transcription factors cooperatively mediate jasmonate-elicited nicotine biosynthesis. Plant J 66:1053–1065. https://doi.org/10.1111/j.1365-313X.2011.04566.x

De Brito Francisco R, Martinoia E (2018) The vacuolar transportome of plant specialized metabolites. Plant Cell Physiol 59:1326–1336. https://doi.org/10.1093/pcp/pcy039

de Costa F, Yendo ACA, Fleck JD et al (2011) Immunoadjuvant and anti-inflammatory plant saponins: characteristics and biotechnological approaches towards sustainable production. Mini Rev Med Chem 11:857–880. https://doi.org/10.2174/138955711796575470

de Costa F, Yendo ACA, Fleck JD et al (2013) Accumulation of a bioactive triterpene saponin fraction of *Quillaja brasiliensis* leaves is associated with abiotic and biotic stresses. Plant Physiol Biochem 66:56–62. https://doi.org/10.1016/j.plaphy.2013.02.003

de Costa F, Barber CJS, Kim Yb et al (2017) Molecular cloning of an ester-forming triterpenoid: UDP-glucose 28-O-glucosyltransferase involved in saponin biosynthesis from the

medicinal plant *Centella asiatica*. Plant Sci 262:9–17. https://doi.org/10.1016/j.plantsci.2017.05.009

De Geyter E, Swevers L, Caccia S et al (2012) Saponins show high entomotoxicity by cell membrane permeation in Lepidoptera. Pest Manag Sci 68:1199–1205. https://doi.org/10.1002/ps.3284

Deng B, Huang Z, Ge F et al (2017a) An AP2/ERF Family transcription factor PnERF1 raised the biosynthesis of saponins in *Panax notoginseng*. J Plant Growth Regul 36:691–701. https://doi.org/10.1007/s00344-017-9672-z

Deng B, Zhang P, Ge F et al (2017b) Enhancement of triterpenoid saponins biosynthesis in *Panax notoginseng* cells by co-overexpressions of 3-hydroxy-3-methylglutaryl CoA reductase and squalene synthase genes. Biochem Eng J 122:38–46. https://doi.org/10.1016/j.bej.2017.03.001

Długosz M, Markowski M, Pączkowski C (2018) Source of nitrogen as a factor limiting saponin production by hairy root and suspension cultures of *Calendula officinalis* L. Acta Physiol Plant 40:1–14. https://doi.org/10.1007/s11738-018-2610-2

Dubos C, Grotewold E, Weisshaar B et al (2010) MYB transcription factors in *Arabidopsis*. Trends Plant Sci 15:573–581. https://doi.org/10.1016/j.tplants.2010.06.005

Enomoto Y, Ito K, Kawagoe Y et al (1986) Positive inotropic action of saponins on isolated atrial and papillary muscles from the guinea-pig. Br J Pharmacol 88:259–267. https://doi.org/10.1111/j.1476-5381.1986.tb09494.x

Faizal A, Geelen D (2013) Saponins and their role in biological processes in plants. Phytochem Rev 12:877–893. https://doi.org/10.1007/s11101-013-9322-4

Fleck JD, Schwambach J, Almeida ME et al (2009) Immunoadjuvant saponin production in seedlings and micropropagated plants of *Quillaja brasiliensis*. Vitr Cell Dev Biol Plant 45:715–720. https://doi.org/10.1007/s11627-009-9222-x

Fleck JD, De Costa F, Yendo ACA et al (2013) Determination of new immunoadjuvant saponin named QB-90, and analysis of its organ-specific distribution in *Quillaja brasiliensis* by HPLC. Nat Prod Res 27:907–910. https://doi.org/10.1080/14786419.2012.666751

Gantait A, Pandit S, Nema NK, Mukjerjee PK (2010) Quantification of glycyrrhizin in *Glycyrrhiza glabra* extract by validated HPTLC densitometry. J AOAC Int 93:492–495

Geisler K, Hughes RK, Sainsbury F et al (2013) Biochemical analysis of a multifunctional cytochrome P450 (CYP51) enzyme required for synthesis of antimicrobial triterpenes in plants. Proc Natl Acad Sci U S A 110:E3360–E3367. https://doi.org/10.1073/pnas.1309157110

Gholami A, De Geyter N, Pollier J et al (2014) Natural product biosynthesis in *Medicago* species. Nat Prod Rep 31:356–380. https://doi.org/10.1039/c3np70104b

Ghosh S (2017) Triterpene structural diversification by plant cytochrome P450 enzymes. Front Plant Sci 8:1–15. https://doi.org/10.3389/fpls.2017.01886

Goossens J, Mertens J, Goossens A (2017) Role and functioning of bHLH transcription factors in jasmonate signalling. J Exp Bot 68:1333–1347. https://doi.org/10.1093/jxb/erw440

Gringhuis SI, Kaptein TM, Wevers BA et al (2014) Fucose-specific DC-SIGN signalling directs T helper cell type-2 responses via IKKε-and CYLD-dependent Bcl3 activation. Nat Commun 5:1–7. https://doi.org/10.1038/ncomms4898

Guo S, Kenne L (2000a) Structural studies of triterpenoid saponins with new acyl components from *Quillaja saponaria* Molina. Phytochemistry 55:419–428. https://doi.org/10.1016/S0031-9422(00)00340-X

Guo S, Kenne L (2000b) Characterization of some O-acetylated saponins from *Quillaja saponaria* Molina. Phytochemistry 54:615–623. https://doi.org/10.1016/S0031-9422(00)00161-8

Guo HB, Cui XM, An N, Cai GP (2010) Sanchi ginseng (*Panax notoginseng* (Burkill) F. H. Chen) in China: distribution, cultivation and variations. Genet Resour Crop Evol 57:453–460. https://doi.org/10.1007/s10722-010-9531-2

Han JY, In JG, Kwon YS, Choi YE (2010) Regulation of ginsenoside and phytosterol biosynthesis by RNA interferences of squalene epoxidase gene in *Panax ginseng*. Phytochemistry 71:36–46. https://doi.org/10.1016/j.phytochem.2009.09.031

Han JY, Wang HY, Choi YE (2014) Production of dammarenediol-II triterpene in a cell suspension culture of transgenic tobacco. Plant Cell Rep 33:225–233. https://doi.org/10.1007/s00299-013-1523-1

Han JY, Baek SH, Jo HJ et al (2019) Genetically modified rice produces ginsenoside aglycone (protopanaxadiol). Planta 250:1103–1110. https://doi.org/10.1007/s00425-019-03204-4

Haralampidis K, Bryan G, Qi X et al (2001) A new class of oxidosqualene cyclases directs synthesis of antimicrobial phytoprotectants in monocots. Proc Natl Acad Sci U S A 98:13431–13436. https://doi.org/10.1073/pnas.231324698

Hayashi H, Sudo H (2009) Economic importance of licorice. Plant Biotechnol 26:101–104. https://doi.org/10.5511/plantbiotechnology.26.101

Hayashi H, Fukui H, Tabata M (1988) Examination of triterpenoids produced by callus and cell suspension cultures of *Glycyrrhiza glabra*. Plant Cell Rep 7:508–511. https://doi.org/10.1007/BF00272743

Hayashi H, Huang P, Kirakosyan A et al (2001) Cloning and characterization of a cDNA encoding β-amyrin synthase involved in glycyrrhizin and soyasaponin biosyntheses in licorice. Biol Pharm Bull 24:912–916. https://doi.org/10.1248/bpb.24.912

Heim MA, Jakoby M, Werber M et al (2003) The basic helix-loop-helix transcription factor family in plants: a genome-wide study of protein structure and functional diversity. Mol Biol Evol 20:735–747. https://doi.org/10.1093/molbev/msg088

Huang L, Li J, Ye H et al (2012) Molecular characterization of the pentacyclic triterpenoid biosynthetic pathway in *Catharanthus roseus*. Planta 236:1571–1581. https://doi.org/10.1007/s00425-012-1712-0

Huang C, Qian ZG, Zhong JJ (2013) Enhancement of ginsenoside biosynthesis in cell cultures of *Panax ginseng* by N, N'-dicyclohexylcarbodiimide elicitation. J Biotechnol 165:30–36. https://doi.org/10.1016/j.jbiotec.2013.02.012

Hui Z, Sha DJ, Wang SL et al (2017) Panaxatriol saponins promotes angiogenesis and enhances cerebral perfusion after ischemic stroke in rats. BMC Complement Altern Med 17:1–10. https://doi.org/10.1186/s12906-017-1579-5

Hussain M, Debnath B, Qasim M et al (2019) Role of saponins in plant defense against specialist herbivores. Molecules 24:1–21. https://doi.org/10.3390/molecules24112067

Jo HJ, Han JY, Hwang HS, Choi YE (2017) β-Amyrin synthase (EsBAS) and β-amyrin 28-oxidase (CYP716A244) in oleanane-type triterpene saponin biosynthesis in *Eleutherococcus senticosus*. Phytochemistry 135:53–63. https://doi.org/10.1016/j.phytochem.2016.12.011

Kao TC, Wu CH, Yen GC (2014) Bioactivity and potential health benefits of licorice. J Agric Food Chem 62:542–553. https://doi.org/10.1021/jf404939f

Kapusta I, Stochmal A, Perrone A et al (2005) Triterpene saponins from barrel medic (*Medicago truncatula*) aerial parts. J Agric Food Chem 53:2164–2170. https://doi.org/10.1021/jf048178i

Kemen AC, Honkanen S, Melton RE et al (2014) Investigation of triterpene synthesis and regulation in oats reveals a role for β-amyrin in determining root epidermal cell patterning. Proc Natl Acad Sci U S A 111:8679–8684. https://doi.org/10.1073/pnas.1401553111

Khakimov B, Kuzina V, Erthmann P et al (2015) Identification and genome organization of saponin pathway genes from a wild crucifer, and their use for transient production of saponins in *Nicotiana benthamiana*. Plant J 84:478–490. https://doi.org/10.1111/tpj.13012

Kim S, Park S, Kang S, Kang H (2003) Hypocholesterolemic property of *Yucca schidigera* and *Quillaja saponaria* extracts in human body. Arch Pharm Res 26:1042–1046. https://doi.org/10.1007/BF02994756

Kim YJ, Zhang D, Yang DC (2015) Biosynthesis and biotechnological production of ginsenosides. Biotechnol Adv 33:717–735. https://doi.org/10.1016/j.biotechadv.2015.03.001

Kim OT, Jin ML, Lee DY, Jetter R (2017) Characterization of the asiatic acid glucosyltransferase, UGT73AH1, involved in asiaticoside biosynthesis in *Centella asiatica* (L.) Urban. Int J Mol Sci 18:1–11. https://doi.org/10.3390/ijms18122630

Kochan E, Szymczyk P, Kúzma Ł et al (2018) The increase of triterpene saponin production induced by trans-Anethole in hairy root cultures of *Panax quinquefolium*. Molecules 23:1–10. https://doi.org/10.3390/molecules23102674

Kojoma M, Ohyama K, Seki H et al (2010) In vitro proliferation and triterpenoid characteristics of licorice (*Glycyrrhiza uralensis* Fischer, Leguminosae) stolons. Plant Biotechnol 27:59–66. https://doi.org/10.5511/plantbiotechnology.27.59

Kümmritz S, Louis M, Haas C et al (2016) Fungal elicitors combined with a sucrose feed significantly enhance triterpene production of a *Salvia fruticosa* cell suspension. Appl Microbiol Biotechnol 100:7071–7082. https://doi.org/10.1007/s00253-016-7432-9

Lambert E, Faizal A, Geelen D (2011) Modulation of triterpene saponin production: in vitro cultures, elicitation, and metabolic engineering. Appl Biochem Biotechnol 164:220–237. https://doi.org/10.1007/s12010-010-9129-3

Leveau A, Reed J, Qiao X et al (2019) Towards take-all control: a C-21β oxidase required for acylation of triterpene defence compounds in oat. New Phytol 221:1544–1555. https://doi.org/10.1111/nph.15456

Li JN, Yu Y, Zhang YF et al (2017) Synergy of Raddeanin A and cisplatin induced therapeutic effect enhancement in human hepatocellular carcinoma. Biochem Biophys Res Commun 485:335–341. https://doi.org/10.1016/j.bbrc.2017.02.079

Liao P, Wang H, Hemmerlin A et al (2014) Past achievements, current status and future perspectives of studies on 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS) in the mevalonate (MVA) pathway. Plant Cell Rep 33:1005–1022. https://doi.org/10.1007/s00299-014-1592-9

Lin X, Huang R, Zhang S et al (2013) Beneficial effects of asiaticoside on cognitive deficits in senescence-accelerated mice. Fitoterapia 87:69–77. https://doi.org/10.1016/j.fitote.2013.03.023

Liu Q, Khakimov B, Cárdenas PD et al (2019a) The cytochrome P450 CYP72A552 is key to production of hederagenin-based saponins that mediate plant defense against herbivores. New Phytol 222:1599–1609. https://doi.org/10.1111/nph.15689

Liu T, Luo T, Guo X et al (2019b) PgMYB2, a MeJA-responsive transcription factor, positively regulates the dammarenediol synthase gene expression in *Panax ginseng*. Int J Mol Sci 20:2219. https://doi.org/10.3390/ijms20092219

Lluch MA, Masferrer A, Arró M et al (2000) Molecular cloning and expression analysis of the mevalonate kinase gene from *Arabidopsis thaliana*. Plant Mol Biol 42:365–376. https://doi.org/10.1023/A:1006325630792

Loh YC, Tan CS, Ch'ng YS, Ng CH (2019) Mechanisms of action *of Panax notoginseng* ethanolic extract for its vasodilatory effects and partial characterization of vasoactive compounds. Hypertens Res 42:182–194. https://doi.org/10.1038/s41440-018-0139-9

Lorent JH, Quetin-Leclercq J, Mingeot-Leclercq MP (2014) The amphiphilic nature of saponins and their effects on artificial and biological membranes and potential consequences for red blood and cancer cells. Org Biomol Chem 12:8803–8822. https://doi.org/10.1039/c4ob01652a

Louveau T, Orme A, Pfalzgraf H et al (2018) Analysis of two new arabinosyltransferases belonging to the carbohydrate-active enzyme (CAZY) glycosyl transferase family1 provides insights into disease resistance and sugar donor specificity. Plant Cell 30:3038–3057. https://doi.org/10.1105/tpc.18.00641

Luebert F (2014) Taxonomy and distribution of the genus *Quillaja* Molina (Quillajaceae). Feddes Repert 124:157–162. https://doi.org/10.1002/fedr.201400029

Lv H, Li J, Wu Y et al (2016) Transporter and its engineering for secondary metabolites. Appl Microbiol Biotechnol 100:6119–6130. https://doi.org/10.1007/s00253-016-7605-6

Ma CH, Gao ZJ, Zhang JJ et al (2016) Candidate genes involved in the biosynthesis of triterpenoid saponins in *Platycodon grandiflorum* identified by transcriptome analysis. Front Plant Sci 7:1–14. https://doi.org/10.3389/fpls.2016.00673

Magedans YVS, Yendo AC, de Costa F et al (2019) Foamy matters: an update on *Quillaja* saponins and their use as immunoadjuvants. Future Med Chem 11:1485–1499. https://doi.org/10.4155/fmc-2018-0438

Marciani DJ (2018) Elucidating the mechanisms of action of saponin-derived adjuvants. Trends Pharmacol Sci 39:573–585. https://doi.org/10.1016/j.tips.2018.03.005

Marsik P, Langhansova L, Dvorakova M et al (2014) Increased ginsenosides production by elicitation of in vitro cultivated *Panax ginseng* adventitious roots. Med Aromat Plants 03:1–5. https://doi.org/10.4172/2167-0412.1000147

Matsuda H, Samukawa K, Kubo M (1990) Anti-inflammatory activity of Ginsenoside Ro1. J Planta Medica 56:19–23. https://doi.org/10.1055/s-2006-960875

Matsuura HN, Malik S, de Costa F et al (2018) Specialized plant metabolism characteristics and impact on target molecule biotechnological production. Mol Biotechnol 60:169–183. https://doi.org/10.1007/s12033-017-0056-1

Mazahery-Laghab H, Yazdi-Samadi B, Bagheri M, Bagheri AR (2011) Alfalfa (*Medicago sativa* L.) shoot saponins: identification and bio-activity by the assessment of aphid feeding. Br J Nutr 105:62–70. https://doi.org/10.1017/S0007114510003120

Meesapyodsuk D, Balsevich J, Reed DW, Covello PS (2007) Saponin biosynthesis in *Saponaria vaccaria*. cDNAs encoding β-amyrin synthase and a triterpene carboxylic acid glucosyltransferase. Plant Physiol 143:959–969. https://doi.org/10.1104/pp.106.088484

Mertens J, Pollier J, Vanden Bossche R et al (2016a) The bHLH transcription factors TSAR1 and TSAR2 regulate triterpene saponin biosynthesis in *Medicago truncatula*. Plant Physiol 170:194–210. https://doi.org/10.1104/pp.15.01645

Mertens J, Van Moerkercke A, Vanden Bossche R et al (2016b) Clade IVa basic helix-loop-helix transcription factors form part of a conserved jasmonate signaling circuit for the regulation of bioactive plant terpenoid biosynthesis. Plant Cell Physiol 57:2564–2575. https://doi.org/10.1093/pcp/pcw168

Mugford ST, Milkowski C (2012) Serine carboxypeptidase-like acyltransferases from plants, 1st edn. Elsevier Inc., Amsterdam

Mugford ST, Qi X, Bakht S et al (2009) A serine carboxypeptidase-like acyltransferase is required for synthesis of antimicrobial compounds and disease resistance in oats. Plant Cell 21:2473–2484. https://doi.org/10.1105/tpc.109.065870

Mugford ST, Louveau T, Melton R et al (2013) Modularity of plant metabolic gene clusters: a trio of linked genes that are collectively required for acylation of triterpenes in oat. Plant Cell 25:1078–1092. https://doi.org/10.1105/tpc.113.110551

Mylona P, Owatworakit A, Papadopoulou K et al (2008) Sad3 and Sad4 are required for saponin biosynthesis and root development in oat. Plant Cell 20:201–212. https://doi.org/10.1105/tpc.107.056531

Nakashima T, Inoue T, Oka A et al (1995) Cloning, expression, and characterization of cDNAs encoding *Arabidopsis thaliana* squalene synthase. Proc Natl Acad Sci U S A 92:2328–2332. https://doi.org/10.1073/pnas.92.6.2328

Netala VR, Ghosh SB, Bobbu P et al (2015) Triterpenoid saponins: a review on biosynthesis, applications and mechanism of their action. Int J Pharm Pharm Sci 7:24–28

Nomura Y, Seki H, Suzuki T, Ohyama K (2019) Functional specialization of UDP-glycosyltransferase 73P12 in licorice to produce a sweet triterpenoid saponin, glycyrrhizin. Plant J 99:1127–1143. https://doi.org/10.1111/tpj.14409

Oh JY, Kim YJ, Jang MG et al (2014) Investigation of ginsenosides in different tissues after elicitor treatment in P*anax ginseng*. J Ginseng Res 38:270–277. https://doi.org/10.1016/j.jgr.2014.04.004

Oleszek W (1996) Alfalfa saponins: structure, biological activity, and chemotaxonomy. In: Waller GR, Yamasaki K (eds) Saponins used in food and agriculture. In: Advances in experimental medicine and biology (series), vol 405. Plenum Press, New York, pp 155–170

Orme A, Louveau T, Stephenson MJ et al (2019) A noncanonical vacuolar sugar transferase required for biosynthesis of antimicrobial defense compounds in oat. Proc Natl Acad Sci U S A 116:27105–27114. https://doi.org/10.1073/pnas.1914652116

Osbourn AE (2003) Saponins in cereals. Phytochemistry 62:1–4. https://doi.org/10.1016/S0031-9422(02)00393-X

Osbourn AE, Clarke BR, Lunness P et al (1994) An oat species lacking avenacin is susceptible to infection by *Gaeumannomyces graminis* var. *tritici*. Physiol Mol Plant Pathol 45:457–467. https://doi.org/10.1016/S0885-5765(05)80042-6

Papadopoulou K, Melton RE, Leggett M et al (1999) Compromised disease resistance in saponin-deficient plants. Proc Natl Acad Sci U S A 96:12923–12928. https://doi.org/10.1073/pnas.96.22.12923

Park SB, Chun JH, Ban YW et al (2016) Alteration of *Panax ginseng* saponin composition by overexpression and RNA interference of the protopanaxadiol 6-hydroxylase gene (Cyp716a53v2). J Ginseng Res 40:47–54. https://doi.org/10.1016/j.jgr.2015.04.010

Pateraki I, Heskes AM, Hamberger B (2015) Cytochromes P450 for terpene functionalisation and metabolic engineering. In: Schrader J, Bohlmann J (eds) Biotechnology of isoprenoids. Springer, Switzerland, pp 107–139

Patra B, Schluttenhofer C, Wu Y et al (2013) Transcriptional regulation of secondary metabolite biosynthesis in plants. Biochim Biophys Acta Gene Regul Mech 1829:1236–1247. https://doi.org/10.1016/j.bbagrm.2013.09.006

Phillips MA, D'Auria JC, Gershenzon J, Pichersky E (2008) The *Arabidopsis thaliana* type I isopentenyl diphosphate isomerases are targeted to multiple subcellular compartments and have overlapping functions in isoprenoid biosynthesis. Plant Cell 20:677–696. https://doi.org/10.1105/tpc.107.053926

Phukan UJ, Jeena GS, Tripathi V, Shukla RK (2017) Regulation of Apetala2/Ethylene response factors in plants. Front Plant Sci 8:1–18. https://doi.org/10.3389/fpls.2017.00150

Pollier J, Morreel K, Geelen D, Goossens A (2011) Metabolite profiling of triterpene saponins in M*edicago truncatula* hairy roots by liquid chromatography fourier transform ion cyclotron resonance mass spectrometry. J Nat Prod 74:1462–1476. https://doi.org/10.1021/np200218r

Potter SM, Jimenez-Flores R, Pollack JA et al (1993) Protein-saponin interaction and its influence on blood lipids. J Agric Food Chem 41:1287–1291. https://doi.org/10.1021/jf00032a023

Ramilowski JA, Sawai S, Seki H et al (2013) *Glycyrrhiza uralensis* transcriptome landscape and study of phytochemicals. Plant Cell Physiol 54:697–710. https://doi.org/10.1093/pcp/pct057

Reed J, Stephenson MJ, Miettinen K, Brouwer B (2017) A translational synthetic biology platform for rapid access to

gram-scale quantities of novel drug-like molecules. Metab Eng 42:185–193. https://doi.org/10.1016/j.ymben.2017.06.012

Reichert CL, Salminen H, Weiss J (2019) *Quillaja* saponin characteristics and functional properties. Annu Rev Food Sci Technol 10:43–73. https://doi.org/10.1146/annurev-food-032818-122010

Ribeiro B, Erffelinck M-L, Colinas M, Williams C (2020a) ER-anchored transcription factors bZIP17 and bZIP60 regulate triterpene saponin biosynthesis in *Medicago truncatula*. bioRxiv. https://doi.org/10.1101/2020.01.17.910802

Ribeiro B, Lacchini E, Bicalho KU et al (2020b) A seed-specific regulator of triterpene saponin biosynthesis in *Medicago truncatula*. Plant Cell 32:2020–2042. https://doi.org/10.1105/tpc.19.00609

Rodriguez-Concepción M (2014) Plant isoprenoids: a general overview. In: Rodríguez-Concepción M (ed) Plant isoprenoids. Methods and protocols. Humana Press, pp 1–8

Santos FN, Borja-Cabrera GP, Miyashiro LM et al (2007) Immunotherapy against experimental canine visceral leishmaniasis with the saponin enriched-Leishmune®vaccine. Vaccine 25:6176–6190. https://doi.org/10.1016/j.vaccine.2007.06.005

Sawai S, Saito K (2011) Triterpenoid biosynthesis and engineering in plants. Front Plant Sci 2:1–8. https://doi.org/10.3389/fpls.2011.00025

Scott GI, Colligan PB, Ren BH, Ren J (2001) Ginsenosides Rb1 and Re decrease cardiac contraction in adult rat ventricular myocytes: role of nitric oxide. Br J Pharmacol 134:1159–1165. https://doi.org/10.1038/sj.bjp.0704377

Seki H, Ohyama K, Sawai S et al (2008) Licorice β-amyrin 11-oxidase, a cytochrome P450 with a key role in the biosynthesis of the triterpene sweetener glycyrrhizin. Proc Natl Acad Sci U S A 105:14204–14209. https://doi.org/10.1073/pnas.0803876105

Seki H, Sawai S, Ohyama K et al (2011) Triterpene functional genomics in licorice for identification of CYP72A154 involved in the biosynthesis of glycyrrhizin. Plant Cell 23:4112–4123. https://doi.org/10.1105/tpc.110.082685

Sewelam N, Kazan K, Schenk PM (2016) Global plant stress signaling: reactive oxygen species at the cross-road. Front Plant Sci 7:1–21. https://doi.org/10.3389/fpls.2016.00187

Shabani L, Ehsanpour AA, Asghari G, Emami J (2009) Glycyrrhizin production by in vitro cultured *Glycyrrhiza glabra* elicited by methyl jasmonate and salicylic acid. Russ J Plant Physiol 56:621–626. https://doi.org/10.1134/S1021443709050069

Shang Y, Huang S (2019) Multi-omics data-driven investigations of metabolic diversity of plant triterpenoids. Plant J 97:101–111. https://doi.org/10.1111/tpj.14132

Shi Y, Wang H, Shi F (2019a) Ginsenoside Rg3 suppresses the NLRP3 inflammasome activation through inhibition of its assembly. FASEB J 34:208–221. https://doi.org/10.1096/fj.201901537R

Shi Y, Zhang S, Peng D et al (2019b) Transcriptome analysis of *Clinopodium chinense* (Benth.) O. Kuntze and identification of genes involved in triterpenoid saponin biosynthesis. Int J Mol Sci 20:2643. https://doi.org/10.3390/ijms20112643

Shibuya M, Katsube Y, Otsuka M et al (2009) Identification of a product specific β-amyrin synthase from *Arabidopsis thaliana*. Plant Physiol Biochem 47:26–30. https://doi.org/10.1016/j.plaphy.2008.09.007

Shin BK, Kwon SW, Park JH (2015) Chemical diversity of ginseng saponins from *Panax ginseng*. J Ginseng Res 39:287–298. https://doi.org/10.1016/j.jgr.2014.12.005

Shirazi Z, Aalami A, Tohidfar M, Sohani MM (2018) Metabolic engineering of glycyrrhizin pathway by over-expression of beta-amyrin 11-oxidase in transgenic roots of *Glycyrrhiza glabra*. Mol Biotechnol 60:412–419. https://doi.org/10.1007/s12033-018-0082-7

Shirley AM, Chapple C (2003) Biochemical characterization of sinapoylglucose:choline sinapoyltransferase, a serine carboxypeptidase-like protein that functions as an acyltransferase in plant secondary metabolism. J Biol Chem 278:19870–19877. https://doi.org/10.1074/jbc.M302362200

Shitan N (2016) Secondary metabolites in plants: transport and self-tolerance mechanisms. Biosci Biotechnol Biochem 80:1283–1293. https://doi.org/10.1080/09168451.2016.1151344

Shoji T (2019) The recruitment model of metabolic evolution: jasmonate-responsive transcription factors and a conceptual model for the evolution of metabolic pathways. Front Plant Sci 10:1–12. https://doi.org/10.3389/fpls.2019.00560

Singh D, Chaudhuri PK (2018) Structural characteristics, bioavailability and cardioprotective potential of saponins. Integr Med Res 7:33–43. https://doi.org/10.1016/j.imr.2018.01.003

Singh B, Kaur A (2018) Control of insect pests in crop plants and stored food grains using plant saponins: a review. LWT Food Sci Technol 87:93–101. https://doi.org/10.1016/j.lwt.2017.08.077

Soltysik S, Wu JY, Recchia J et al (1995) Structure/function studies of QS-21 adjuvant: assessment of triterpene aldehyde and glucuronic acid roles in adjuvant function. Vaccine 13:1403–1410. https://doi.org/10.1016/0264-410X(95)00077-E

Srisawat P, Fukushima EO, Yasumoto S, Robertlee J (2019) Identification of oxidosqualene cyclases from the medicinal legume tree *Bauhinia forficata*: a step toward discovering preponderant alpha-amyrin-producing activity. New Phytol 224:352–366. https://doi.org/10.1111/nph.16013

Srisawat P, Yasumoto S, Fukushima EO et al (2020) Production of the bioactive plant-derived triterpenoid morolic acid in engineered *Saccharomyces cerevisiae*. Biotechnol Bioeng 117:2198–2208. https://doi.org/10.1002/bit.27357

Srivastava M, Singh G, Sharma S et al (2019) Elicitation enhanced the yield of glycyrrhizin and antioxidant activities in hairy root cultures of *Glycyrrhiza glabra* L. J Plant Growth Regul 38:373–384. https://doi.org/10.1007/s00344-018-9847-2

Srivastava G, Garg A, Chandra R et al (2020) Transcriptome analysis and functional characterization of oxidosqualene cyclases of the *Arjuna* triterpene saponin pathway. Plant Sci 292:110382. https://doi.org/10.1016/j.plantsci.2019.110382

Sun Y, Niu Y, Xu J et al (2013a) Discovery of WRKY transcription factors through transcriptome analysis and characterization of a novel methyl jasmonate-inducible PqWRKY1 gene from *Panax quinquefolius*. Plant Cell

Tissue Organ Cult 114:269–277. https://doi.org/10.1007/s11240-013-0323-1

Sun Y, Zhao SJ, Liang YL et al (2013b) Regulation and differential expression of protopanaxadiol synthase in Asian and American ginseng ginsenoside biosynthesis by RNA interferences. Plant Growth Regul 71:207–217. https://doi.org/10.1007/s10725-013-9821-8

Suzuki H, Fukushima EO, Shimizu Y et al (2019) *Lotus japonicus* triterpenoid profile and characterization of the CYP716A51 and LjCYP93E1 genes involved in their biosynthesis *in planta*. Plant Cell Physiol 60:2496–2509. https://doi.org/10.1093/pcp/pcz145

Szakiel A, Janiszowska W (2002) The mechanism of oleanolic acid monoglycosides transport into vacuoles isolated from *Calendula officinalis* leaf protoplasts. Plant Physiol Biochem 40:203–209. https://doi.org/10.1016/S0981-9428(02)01370-0

Szakiel A, Ruszkowski D, Janiszowska W (2005) Saponins in *Calendula officinalis* L. - Structure, biosynthesis, transport and biological activity. Phytochem Rev 4:151–158. https://doi.org/10.1007/s11101-005-4053-9

Szakiel A, Pączkowski C, Henry M (2011) Influence of environmental biotic factors on the content of saponins in plants. Phytochem Rev 10:493–502. https://doi.org/10.1007/s11101-010-9164-2

Szczepanik M, Krystokowiak K, Jurzysta M, Bialy Z (2001) Biological activity of saponins from alfalfa tops and roots against Colorado potato beetle larvae. Acta Agrobot 54:33–45. https://doi.org/10.5586/aa.2001.021

Takemura M, Tanaka R, Misawa N (2017) Pathway engineering for the production of β-amyrin and cycloartenol in *Escherichia coli*—a method to biosynthesize plant-derived triterpene skeletons in *E. coli*. Appl Microbiol Biotechnol 101:6615–6625. https://doi.org/10.1007/s00253-017-8409-z

Tam KI, Roner MR (2011) Characterization of in vivo antirotavirus activities of saponin extracts from *Quillaja saponaria* Molina. Antivir Res 90:231–241. https://doi.org/10.1016/j.antiviral.2011.04.004

Tamura K, Teranishi Y, Ueda S et al (2017a) Cytochrome P450 monooxygenase CYP716A141 is a unique β-amyrin C-16β oxidase involved in triterpenoid saponin biosynthesis in *Platycodon grandiflorus*. Plant Cell Physiol 58:874–884. https://doi.org/10.1093/pcp/pcx043

Tamura K, Teranishi Y, Ueda S, Suzuki H, Kawano N, Yoshimatsu K, Saito K, Kawahara N, Muranaka T, Seki H (2017b) Cytochrome P450 monooxygenase CYP716A141 is a unique b-amyrin C-16b oxidase involved in triterpenoid saponin biosynthesis in platycodon grandiflorus. Plant Cell Physiol 58(6):1119. https://doi.org/10.1093/pcp/pcx067

Tamura K, Yoshida K, Hiraoka Y et al (2018) The basic helix-loop-helix transcription factor GubHLH3 positively regulates soyasaponin biosynthetic genes in *Glycyrrhiza uralensis*. Plant Cell Physiol 59:778–791. https://doi.org/10.1093/pcp/pcy046

Tansakul P, Shibuya M, Kushiro T, Ebizuka Y (2006) Dammarenediol-II synthase, the first dedicated enzyme for ginsenoside biosynthesis in *Panax ginseng*. Plant Cell Physiol 580:5143–5149. https://doi.org/10.1016/j.febslet.2006.08.044

Tava A, Scotti C, Avato P (2011) Biosynthesis of saponins in the genus *Medicago*. Phytochem Rev 10:459–469. https://doi.org/10.1007/s11101-010-9169-x

Thimmappa R, Geisler K, Louveau T et al (2014) Triterpene biosynthesis in plants. Annu Rev Plant Biol 65:225–257. https://doi.org/10.1146/annurev-arplant-050312-120229

Tzin V, Snyder JH, Yang DS et al (2019) Integrated metabolomics identifies CYP72A67 and CYP72A68 oxidases in the biosynthesis of *Medicago truncatula* oleanate sapogenins. Metabolomics 15:1–20. https://doi.org/10.1007/s11306-019-1542-1

Uzayisenga R, Ayeka PA, Wang Y (2014) Anti-diabetic potential of *Panax notoginseng* saponins (PNS): a review. Phyther Res 28:510–516. https://doi.org/10.1002/ptr.5026

Vermeersch M, Foubert K, Inocêncio da Luz R et al (2009) Selective antileishmania activity of 13,28-epoxy-oleanane and related triterpene saponins from the plant families Myrsinaceae, Primulaceae, Aceraceae and Icacinaceae. Phyther Res 23:1404–1410. https://doi.org/10.1002/ptr

Vincken JP, Heng L, de Groot A, Gruppen H (2007) Saponins, classification and occurrence in the plant kingdom. Phytochemistry 68:275–297. https://doi.org/10.1016/j.phytochem.2006.10.008

Wallace F, Bennadji Z, Ferreira F, Olivaro C (2019) Structural characterisation of new immunoadjuvant saponins from leaves and the first study of saponins from the bark of *Quillaja brasiliensis* by liquid chromatography electrospray ionisation ion trap mass spectrometry. Phytochem Anal 30:644–652. https://doi.org/10.1002/pca.2837

Wang T, Guo R, Zhou G et al (2016) Traditional uses, botany, phytochemistry, pharmacology and toxicology of *Panax notoginseng* (Burk.) F.H. Chen: a review. J Ethnopharmacol 188:234–258. https://doi.org/10.1016/j.jep.2016.05.005

Wang N, Wang L, Qi L, Lu X (2018) Construct a gene-to-metabolite network to screen the key genes of triterpene saponin biosynthetic pathway in *Panax notoginseng*. Biotechnol Appl Biochem 65:119–127. https://doi.org/10.1002/bab.1580

Wasternack C, Hause B (2013) Jasmonates: biosynthesis, perception, signal transduction and action in plant stress response, growth and development. An update to the 2007 review in Annals of Botany. Ann Bot 111:1021–1058. https://doi.org/10.1093/aob/mct067

Watson BS, Bedair MF, Urbanczyk-Wochniak E et al (2015) Integrated metabolomics and transcriptomics reveal enhanced specialized metabolism *in Medicago truncatula* root border cells. Plant Physiol 167:1699–1716. https://doi.org/10.1104/pp.114.253054

WHO (1999) WHO monographs on selected medicinal plants. WHO, Malta

Wongwicha W, Tanaka H, Shoyama Y et al (2008) Production of glycyrrhizin in callus cultures of licorice. Zeitschrift fur Naturforsch - Sect C J Biosci 63c:413–417. https://doi.org/10.1515/znc-2008-5-617

Xie Z, Nolan TM, Jiang H, Yin Y (2019) AP2/ERF transcription factor regulatory networks in hormone and abiotic stress responses in *Arabidopsis*. Front Plant Sci 10:1–17. https://doi.org/10.3389/fpls.2019.00228

Xu CL, Wang QZ, Sun LM et al (2012) Asiaticoside: attenuation of neurotoxicity induced by MPTP in a rat model of

Parkinsonism via maintaining redox balance and up-regulating the ratio of Bcl-2/Bax. Pharmacol Biochem Behav 100:413–418. https://doi.org/10.1016/j.pbb.2011.09.014

Yamamoto Y, Tani T (2005) Field study and pharmaceutical evaluation of *Glycyrrhiza uralensis* roots cultivated in China. J Trad Med 22:86–97

Yang X, Xiong X, Wang H, Wang J (2014) Protective effects of *Panax notoginseng* saponins on cardiovascular diseases: a comprehensive overview of experimental studies. Evid Based Complement Altern Med 2014:1–13. https://doi.org/10.1155/2014/204840

Yao Y, Wu WY, Guan SH et al (2008) Proteomic analysis of differential protein expression in rat platelets treated with notoginsengnosides. Phytomedicine 15:800–807. https://doi.org/10.1016/j.phymed.2008.06.013

Yao L, Wang S, Liang W et al (2019) Screening and evaluation of adventitious root lines of *Panax notoginseng* by morphology, gene expression, and metabolite profiles. Appl Microbiol Biotechnol 103:4405–4415. https://doi.org/10.1007/s00253-019-09778-z

Yao L, Wang J, Sun J et al (2020) A WRKY transcription factor, PgWRKY4X, positively regulates ginsenoside biosynthesis by activating squalene epoxidase transcription in *Panax ginseng*. Ind Crops Prod 154:112671. https://doi.org/10.1016/j.indcrop.2020.112671

Yendo ACA, de Costa F, Gosmann G, Fett-Neto AG (2010) Production of plant bioactive triterpenoid saponins: elicitation strategies and target genes to improve yields. Mol Biotechnol 46:94–104. https://doi.org/10.1007/s12033-010-9257-6

Yendo ACA, de Costa F, da Costa CT et al (2014) Biosynthesis of plant triterpenoid saponins: genes, enzymes and their regulation. Mini Rev Org Chem 11:292–306. https://doi.org/10.2174/1570193x1103140915111425

Yendo ACA, de Costa F, Cibulski SP et al (2016) A rabies vaccine adjuvanted with saponins from leaves of the soap tree (*Quillaja brasiliensis*) induces specific immune responses and protects against lethal challenge. Vaccine 34:2305–2311. https://doi.org/10.1016/j.vaccine.2016.03.070

Zeng X, Luo T, Li J et al (2018) Transcriptomics-based identification and characterization of 11 CYP450 genes of *Panax ginseng* responsive to MeJA. Acta Biochim Biophys Sin (Shanghai) 50:1094–1103. https://doi.org/10.1093/abbs/gmy120

Zhang R, Huang J, Zhu J et al (2013a) Isolation and characterization of a novel PDR-type ABC transporter gene *PgPDR3* from *Panax ginseng* C.A. Meyer induced by methyl jasmonate. Mol Biol Rep 40:6195–6204. https://doi.org/10.1007/s11033-013-2731-z

Zhang R, Zhu J, Cao HZ et al (2013b) Molecular cloning and expression analysis of PDR1-like gene in ginseng subjected to salt and cold stresses or hormonal treatment. Plant Physiol Biochem 71:203–211. https://doi.org/10.1016/j.plaphy.2013.07.011

Zhang S, Wu Y, Jin J et al (2015) De novo characterization of *Panax japonicus* C. A. Mey transcriptome and genes related to triterpenoid saponin biosynthesis. Biochem Biophys Res Commun 466:450–455. https://doi.org/10.1016/j.bbrc.2015.09.048

Zhang X, Ge F, Deng B et al (2017) Molecular cloning and characterization of PnbHLH1 transcription factor in *Panax notoginseng*. Molecules 22:1–12. https://doi.org/10.3390/molecules22081268

Zhang X, Yu Y, Jiang S et al (2019) Oleanane-type saponins biosynthesis in *Panax notoginseng* via transformation of β-amyrin synthase gene from *Panax japonicus*. J Agric Food Chem 67:1982–1989. https://doi.org/10.1021/acs.jafc.8b07183

Zhao YJ, Li C (2018) Biosynthesis of plant triterpenoid saponins in microbial cell factories. J Agric Food Chem 66:12155–12165. https://doi.org/10.1021/acs.jafc.8b04657

Zhou M, Memelink J (2016) Jasmonate-responsive transcription factors regulating plant secondary metabolism. Biotechnol Adv 34:441–449. https://doi.org/10.1016/j.biotechadv.2016.02.004

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Soapbark triterpenes: *Quillaja brasiliensis* cell culture sapogenin and free sterol analysis by GCMS.

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Abstract

Triterpene saponins of the genus *Quillaja* (Quillajaceae) are known for their immunoadjuvant, hypocholesterolemic, and anti-inflammatory activity. Plant cell cultures are useful for the study of saponin metabolism and industrial production of these bioactive compounds. While structurally related phytosterols are primary metabolites essential to growth and development, saponins are responsive to pathogen and abiotic stress, fulfilling roles in plant specialized metabolism. For cell culture production of saponins, phytosterols may be considered a competing pathway which relies on a common pool of cytosolic isoprenoid precursors. Understanding the metabolic allocation of resources between these two related pathways is key to maximizing saponin production in *in vitro* production systems. Sterols and saponins naturally occur in multiple conjugated forms, which complicates separation and quantification. The acid hydrolysis of conjugated sterols and saponins to their free forms is a useful technique to simplify their analysis by

gas chromatography. Here we provide the workflow for the quantification of free sterols and sapogenins in cell cultures of *Quillaja brasiliensis*.

Keywords: triterpenes, saponins, sterols, vaccine adjuvant, vaccine development, plant cell culture, *Quillaja brasiliensis.*

1. Introduction

Saponins are C30 triterpenoid glycosides known for producing persistent foam in aqueous solutions due to their amphipathic chemical structures. Saponins have a hydrophobic triterpenoid central core decorated with hydrophilic sugar residues and branched acyl chains, which distinguishes them from the esterified fatty acyl conjugations, glycosides, and acylglycosides frequently seen in sterols (1). Bark and wood of *Quillaja saponaria* Molina and leaves of *Q. brasiliensis* A.St.-Hil. & Tul. are the main sources of *Quillaja* saponins. The triterpenoid core free of conjugations is referred to as a 'sapogenin' and is thought to constitute an important metabolic intermediate that precedes attachment of sugar and acyl groups (2, 3). Quillaic acid is a common sapogenin occurring in *Quillaja* spp. (4).

Quillaja saponins have several applications in industry, but they are best known for their vaccine potentiating adjuvant activity (5, 6). Adjuvants are added to vaccines to increase the adaptive immune responses to antigens. Quillaic acid-based saponins are currently added as adjuvants in veterinary formulations (7) and are being tested in human vaccine trials. For example, QS-21 is combined with lipids to form the less toxic adjuvant system AS01, which has been tested in vaccines for

malaria and herpes zoster (8-10). New sources of adjuvants are also strategic in a pandemic scenario like COVID-19 where shortages reduce vaccination availability. Adjuvants hold the potential to increase the number of effective doses from a limited stock without compromising efficacy. AS01 was tested in vaccine formulations against SARS- CoV-1 and produced immunogenic responses in mice (11). The potential use of *Quillaja* saponins in formulation against SARS-CoV-2 has been recently reviewed (12, 13). Forests in Chile and Southern Brazil as well as cultivated farms are the main commercial sources of *Quillaja* saponins. Given the technical challenges of chemical synthesis of these compounds, cell and tissue cultures are the only viable alternative to harvesting trees. Tissue culture also constitutes a platform for the study of saponin metabolism in plants (14).

Besides saponins, sterols constitute the other major group of co-occurring cytosolically derived triterpenes in *Quillaja* spp. These modified triterpenes (C27-C29), generally derived from cycloartenol, play structural, ecological, and developmental roles in plants (15). Sterols are associated with cell membrane components, such as glycolipids and sphingolipids, where they contribute to the maintenance of membrane permeability and fluidity (16, 17). Sterols also serve as precursors to the brassinosteroid family of phytohormones where they regulate cell elongation, division, and differentiation (18).

In contrast, saponins fulfill roles in plant defense as anti-herbivore (19) and anti-fungal compounds (20) and are responsive to pathogen and abiotic stress (14). Both groups of triterpenes are derived from the mevalonic acid pathway and share common intermediates through 2,3-oxidosqualene. Despite sharing early metabolic

intermediates, sterols and saponins play distinct roles in primary and secondary plant metabolism, respectively, with essentially no overlap. In this sense, the relative substrate commitment into sterols and saponins is indicative of the division of cytosolic isoprenoid resources between primary and secondary metabolism. Methods for the comparative quantification of these two classes of triterpenes in *Quillaja* tissues would be advantageous for understanding how the division of carbon flux between primary and secondary isoprenoid metabolism is controlled in the cytosol. The increasing role of *Quillaja* saponins in vaccine development underscores the importance of methods to evaluate saponin accumulation while also monitoring efforts to suppress competing pathways such as sterols.

Previous methods for the separation and analysis of saponins and sterols have been reported using liquid or gas chromatography coupled to mass spectrometry (LCMS and GCMS) (21-25). Sterols and sapogenins usually occur in multiple conjugated forms in the plant, which complicates their analysis by dramatically increasing the number of related end products. Sterols are frequently esterified to fatty acids or conjugated to glycoside residues, which themselves may be further esterified to fatty acids (16). While sterols are soluble in polar organic solvents such as methanol and chloroform, saponins are generally water soluble, necessitating separate extraction procedures for each group of metabolites. Here, we present a workflow for free sterol and sapogenin analysis by gas chromatography coupled to mass spectrometry (GCMS), following acid hydrolysis of the cell free crude lysate. Acid hydrolysis of the initial organic or aqueous extract to release free sterols and sapogenins, respectively, results in cleavage of glycosidic bonds, greatly simplifies their

analysis, and improves the reliability of their quantification (21, 26, 27). In contrast, alkaline hydrolysis is more effective at cleavage of steryl esters (28). Derivatization of free alcohol groups to their trimethylsilyl ethers is performed just prior to GCMS analysis. In summary, we have found that a combination of aqueous and organic extraction conditions followed by acid hydrolysis are effective for the complete comparative quantification of triterpene and sterol derivatives in *Quillaja* cell extracts. A detailed protocol outlining these procedures follows.

2. Materials

Calli cultures and cells from suspension cultures should be lyophilized to complete dryness prior to the procedure described herein. When working with suspension cell cultures, filter cells and wash the remaining media away with distilled water (29). After harvesting cells, flash freeze in liquid nitrogen, and store at -80 °C until lyophilization.

To perform this protocol, a shaking incubator for microtubes, a nitrogen evaporator device, and a heat block with temperature control are needed. We recommend a heating plate with a metal block adapted for microtubes and 12 mm glass vials (figure 1) attached to a nitrogen blow down manifold which accommodates 24 tubes. Nitrogen may be supplied by a nitrogen generator or compressed gas tank of high purity (>99.99%).

2.1 Extraction of sterols

1. Extraction solution (stock): chloroform-methanol (1:1 v/v). Store in a

glass bottle at room temperature.

2. Internal standard (IS) stock solution: weight out 5.0 mg of 5-α-cholestane (Sigma-Aldrich) in a 5 mL glass volumetric flask and dissolve in hexane to give a final concentration of 1 $mg.mL^{-1}$. Aliquot and store at -20 °C.
3. Extraction solution with internal standard (working solution): dilute the stock solution of cholestane 1:50 (v/v) into fresh chloroform-methanol to arrive a final concentration of 20 $\mu g \cdot mL^{-1}$. To prepare 1 mL of this working solution, add 20 µL of IS stock solution to 980 µL chloroform- methanol. Vortex thoroughly. Prepare 250 µL of this working solution for each sample + 1 (see Note 1).

2.2 Extraction of saponins

1. Prior to hydrolysis of saponins, an enriched saponin fraction is prepared by low pressure column and thin layer chromatography of an aqueous *Q. brasiliensis* leaf extract as described previously (30). The lyophilized aqueous extract is applied to a silica Lichroprep® (Merck, 40–63 µm particle size) column and eluted with a stepwise gradient of aqueous methanol (0–100%). The saponin enriched fraction elutes at 90% methanol. Following evaporation to dryness, this enriched fraction, denoted QB-90, is further purified by TLC prior to acid hydrolysis (30).
2. Dilute IS stock solution (prepared in item 2.1) 1:100 (v/v) in

hexane to achieve a final concentration of 100 µg.ml^{-1}.

2.3 Acid hydrolysis for free sterol analysis

1. Pasteur glass pipette (5 ¾ in) and rubber bulb for sample transfer.
2. Prepare a Pasteur pipette clean-up column as follows: pack a small plug of deactivated glass wool into the constriction of the pipette, then add ~100 mg silica gel (Sigma-Aldrich, Davisil Grade 635, pore size 60 Å, mesh 60-100) and ~50 mg $MgSO_4$ in this order (see Note 2). Prepare 1 glass Pipette column for each sample.
3. Prepare a 1 N HCl solution in methanol.
4. Hexane, GCMS grade.
5. Two 5 mL glass screw cap vials for each sample. Use caps with a PTFE coated septum.

2.4 Acid hydrolysis for sapogenin analysis

1. Pasteur glass pipette (5 ¾ in) and rubber bulb for sample transfer.
2. Prepare 1 glass Pipette column for each sample as described above using only glass wool and $MgSO_4$.
3. Prepare a 2 N HCl solution in water.
4. Ethyl acetate.
5. Ammonium hydroxide solution, 30% (v/v) (Sigma-Aldrich).
6. Two 5 mL glass screw cap vials for each sample. Use caps with a PTFE coated septum.

2.5 Derivatization for GCMS analysis

1. Glass GC vial with a 200 µL pulled point glass insert for each sample.

2. High purity pyridine (Sigma-Aldrich).
3. N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) in 1 mL sealed aliquots (Supelco).

2.6 GCMS analysis

1. Gas chromatograph coupled to a single quadrupole mass spectrometer.
2. A non-polar phase capillary GC column (for example: HP-5MS ultra inert column, 30 m length, 0.25 mm i.d., 0.25 μm film thickness; Agilent Technologies).
3. Prepare stock solutions of authentic sterols: dissolve stigmasterol, sitosterol and campesterol (Sigma-Aldrich) in hexane to reach a final concentration of 1 $mg \cdot mL^{-1}$ each. Store at – 20 °C.
4. Prepare stock solution of authentic quillaic acid (Extrasynthese) in ethyl acetate to reach a final concentration of 1 $mg \cdot mL^{-1}$. Store at – 20 °C.

3. Methods

3.1 Extraction of total sterols

1. Weigh out 5.0 mg ± 0.05 of each lyophilized sample into a 1.5 mL Safelock microtube and record the exact weight.
2. Transfer the lyophilized sample to a 2 mL glass Tenbroeck homogenizer (See Note 3).
3. Add 250 μL of working solution (methanol:$CHCl_3$ + IS) to the

glass homogenizer and grind thoroughly. After complete homogenization, transfer sample back to the Safelock microtube.

4. Extract on a shaking incubator at 60 ℃ for 20 min at maximum speed.
5. Centrifuge at 12,000 *g* for 7 min.
6. Transfer the supernatant to a 5 mL glass vial, being careful to avoid the pellet.
7. Add 250 μL of extraction solution without IS to the pellet and repeat steps 3 to 5. Pool the supernatants.
8. Retain the pellet for sapogenin extraction (See Note 4).
9. Dry the pooled organic extracts on a nitrogen blow down device at 40 ℃ until completely dry.

3.2 Acid hydrolysis for free sterols analysis

1. Add 1 mL of 1 N HCl in methanol to each sample and dissolve by briefly vortexing.
2. Incubate samples at 80 °C for 2 h.
3. Allow samples to cool down to room temperature.
4. Add 1 mL hexane. Agitate samples at room temperature for 10 min in a shaking device or large format vortex.
5. Set the glass pipette clean-up column inside a fresh 5 mL glass vial. Pass the organic hexane layer over the column. Make transfers with a glass Pasteur pipette.
6. Repeat the hexane extraction two more times, pooling the same extracts together.
7. Wash the glass Pasteur pipette column with 1.5 mL hexane.
8. Dry the hexane extracts under a nitrogen stream to dryness at 40 °C.

9. Derivatize samples for GCMS analysis (Section 3.4)

3.3 Extraction of saponins

1. Weight out 1 mg of an enriched saponin fraction (QB-90) in a microtube (See Note 4).
2. Add 50 µL of diluted IS solution (100 $\mu g.ml^{-1}$) to a 5 mL glass vial.
3. Transfer saponin sample to the 5 mL glass vial. Proceed to acid hydrolysis immediately.

3.4 Acid hydrolysis for sapogenin analysis

1. Add 1 mL 2 N HCl in water to each 5 mL glass vial and resuspend by vortexing briefly .
2. Incubate samples at 90 °C for 3 h .
3. Allow samples to cool down to room temperature.
4. Add 200 uL of fresh NH_4OH (30%) dropwise to neutralize the acid (it will give a final pH around 8). Ensure proper use personal protective equipment and a glass shield during this step.
5. Extract the sapogenins with 1 mL ethyl acetate. Agitate samples at room temperature for 10 min in a shaking device or vortex.
6. Pass the upper organic layer over a Pasteur pipette clean-up column prepared with glass wool and $MgSO_4$ only. Make transfers with a glass Pasteur pipette.
7. Repeat the extraction two more times, pooling all three extracts together into a fresh 5 mL glass vial.

8. Wash the glass Pasteur pipette column with 1.5 mL ethyl acetate.
9. Dry the extracts under a nitrogen stream to dryness at 40 °C.
10. Derivatize samples for GCMS analysis (Section 3.4)

3.5 Derivatization for GCMS analysis

1. Thoroughly re-suspend saponin or sterol hydrolysate residues in 100 µL pyridine with vigorous pipetting and centrifuge at maximum speed for 5 min. Alternative aprotic solvents such as tetrahydrofuran may be substituted if high purity pyridine is unavailable.
2. Prepare a GC vial with a glass insert for each sample. Add 20 µL MSTFA taken from a septum sealed vial with a Hamilton glass syringe (See Note 5). Place a crimp cap on top of each tube to reduce air exposure during preparation.
3. Transfer the upper 20 µL of each pyridine resuspension to the 20 µL MSTFA in the glass insert and mix by pipetting.
4. Briefly direct a gentle stream of nitrogen gas into the insert to remove air and immediately seal the vial with a crimping wrench.
5. Incubate at 37 °C for 1 h.
6. Analyze by GCMS within 24 h of completing the derivatization reaction.

3.6 GCMS analysis

1. Inject 1 µL each extract in split mode (1:25) with the injection port set to 250 °C. Set oven conditions as it follows: 120 °C for 1 min then increasing at 6 °C min^{-1} to 320 °C, with a final hold time of 11.6 min.
2. Use electron impact ionization at 70 eV (positive mode) to generate spectral data.
3. Acquire data in scan mode ranging from *m/z* 50 to 550. Adjust scan speeds to reach a final cycle time of ~330 ms (~3 cycles/s).
4. Run standards curves for authentic sterols and quillaic acid ranging from 50 $\mu g \cdot mL^{-1}$ to 350 $\mu g \cdot mL^{-1}$. Add the IS at the same concentration used in samples.

3.7 Quantification and data analysis

1. Agilent MassHunter workstation (version B.07 service pack 2) is used for peak integration of total ion chromatograms and extraction of mass spectra. Identification of sterols and sapogenins can be achieved in MassHunter Qualitative Analysis based on spectral matches to the NIST2014 mass spectral library. Confirm compound identification by comparing the retention time and mass spectra to those authentic standards (figure 2).
2. Absolute compound quantification is performed by linear regression to individual sterols and quillaic acid standard curves, corrected for recovery of the internal standard (See Note 6).

4. Notes

1. Adjust the IS concentration according to your extraction yield. Here we suggest 1 µg of IS per mg dry weight sample.
2. Use a wooden stick to pack glass wool inside the Pasteur pipette. Aim for 0.5 cm length in the first layer. You can add the silica gel and $MgSO_4$ by picking them up directly with the glass pipette. Passing samples through this column will remove water and acidic components from hexane and ethyl acetate extracts. Silica gel will retain the sapogenins; it should not be added to the clean-up columns for sapogenin preparation.
3. Alternatively, cells can be ground using a mortar and pestle. We found a better extract yield when using the glass homogenizer.
4. Saponin extraction is described in the literature for different tissues, and is usually achieved using butanol, methanol, or water as extraction solvents. To establish the acid hydrolysis protocol for sapogenins, we used an enriched saponin fraction purified after *Q. brasiliensis* leaf extracts (figure 2) (31). Add Yendo et al 2017 here too, please.
5. MSTFA must be fresh (maximum 1 month since first use).
6. Two no tissue controls should be performed to calculate recovery of the internal standard. Both should include the IS but no tissue. One should

follow the complete extraction and hydrolysis protocol. The second should simply be dried and resuspended in pyridine for the MSTFA derivatization steps.

5. References

1. Lara J A, Burciaga-Monge A, Chávez A *et al.*, (2018) Identification and characterization of sterol acyltransferases responsible for steryl ester biosynthesis in tomato. Frontiers in Plant Science 9: doi.org/10.3389/fpls.2018.00588.
2. Reichert C L, Salminen H, Weiss J, (2019) *Quillaja* saponin characteristics and functional properties. Annual Review of Food Science and Technology 10: 43-73.
3. Osbourn A, Reed J, (2019) Metabolic Engineering. Patent cooperation treaty EP2018/086430, application WO 2019/122259 A1, 20 Dec 2018
4. Magedans Y V, Yendo A C, Costa F d, Gosmann G, Fett-Neto A G, (2019) Foamy matters: an update on *Quillaja* saponins and their use as immunoadjuvants. Future medicinal chemistry 11: 1485-1499.
5. Kensil C R, Patel U, Lennick M, Marciani D, (1991) Separation and characterization of saponins with adjuvant activity from *Quillaja saponaria* Molina cortex. The Journal of Immunology 146: 431-437.
6. Yendo A C A, de Costa F, Cibulski S P *et al.*, (2016) A rabies vaccine adjuvanted with saponins from leaves of the soap tree (*Quillaja brasiliensis*) induces specific immune responses and protects against lethal challenge. Vaccine 34: 2305-2311.
7. Santos F N, Borja-Cabrera G P, Miyashiro L *et al.*, (2007) Immunotherapy against experimental canine visceral leishmaniasis with the saponin enriched-Leishmune® vaccine. Vaccine 25: 6176-6190.
8. James S F, Chahine E B, Sucher A J, Hanna C, (2018) Shingrix: the new adjuvanted recombinant herpes zoster vaccine. Annals of Pharmacotherapy 52: 673-680.
9. Guerra Mendoza Y, Garric E, Leach A *et al.*, (2019) Safety profile of the RTS, S/AS01 malaria vaccine in infants and children: additional data from a phase III randomized controlled trial in sub-Saharan Africa. Human vaccines & immunotherapeutics 15: 2386-2398.
10. Arslan I, (2020) Quillaic acid–containing saponin-based immunoadjuvants trigger early immune responses. Revista Brasileira de Farmacognosia: 1-7.
11. Roberts A, Lamirande E W, Vogel L *et al.*, (2010) Immunogenicity and protective efficacy in mice and hamsters of a β-propiolactone inactivated whole virus SARS-CoV vaccine. Viral immunology 23: 509-519.
12. Gupta T, Gupta S K, (2020) Potential adjuvants for the development of a

SARS-CoV-2 vaccine based on experimental results from similar coronaviruses. International immunopharmacology: 106717.
13. Sharma R, Palanisamy A, Dhama K *et al.*, (2020) Exploring the possible use of saponin adjuvants in COVID-19 vaccine. Human vaccines & immunotherapeutics: 1-10.
14. da Silva Magedans Y V, Phillips M A, Fett-Neto A G, (2020) Production of plant bioactive triterpenoid saponins: from metabolites to genes and back. Phytochemistry Reviews.
15. Valitova J N, Sulkarnayeva A G, Minibayeva F V, (2016) Plant sterols: Diversity, biosynthesis, and physiological functions. Biochemistry (Moscow) 81: 819-834.
16. Ferrer A, Altabella T, Arró M, Boronat A, (2017) Emerging roles for conjugated sterols in plants. Prog. Lipid Res. 67: 27-37.
17. Rogowska A, Szakiel A, (2020) The role of sterols in plant response to abiotic stress. Phytochemistry Reviews 19: 1525-1538.
18. Planas-Riverola A, Gupta A, Betegón-Putze I *et al.*, (2019) Brassinosteroid signaling in plant development and adaptation to stress. Development 146: dev151894.
19. Osbourn A, Goss R J M, Field R A, (2011) The saponins – polar isoprenoids with important and diverse biological activities. Natural Product Reports 28: 1261-1268.
20. Yang C-R, Zhang Y, Jacob M R *et al.*, (2006) Antifungal activity of C-27 steroidal saponins. Antimicrobial Agents and Chemotherapy 50: 1710-1714.
21. Meesapyodsuk D, Balsevich J, Reed D W, Covello P S, (2007) Saponin biosynthesis in *Saponaria vaccaria*. cDNAs encoding β-amyrin synthase and a triterpene carboxylic acid glucosyltransferase. Plant physiology 143: 959-969.
22. Assimopoulou A, Papageorgiou V, (2005) GC-MS analysis of penta-and tetra-cyclic triterpenes from resins of *Pistacia* species. Part I. *Pistacia lentiscus* var. Chia. Biomedical Chromatography 19: 285-311.
23. Lai C, Li S, Yu H *et al.*, (2006) A rapid HPLC–ESI-MS/MS for qualitative and quantitative analysis of saponins in "XUESETONG" injection. Journal of Pharmaceutical and Biomedical Analysis 40: 669-678.
24. Christie W, Han X. (The Oily Press, Bridgwater, UK, 2010).
25. Abidi S, (2001) Chromatographic analysis of plant sterols in foods and vegetable oils. Journal of Chromatography A 935: 173-201.
26. Moreau R A, Nyström L, Whitaker B D *et al.*, (2018) Phytosterols and their derivatives: Structural diversity, distribution, metabolism, analysis, and health-promoting uses. Prog. Lipid Res. 70: 35-61.
27. Flores-Sánchez I J, Ortega-López J, Montes-Horcasitas M d C, Ramos-Valdivia A C, (2002) Biosynthesis of sterols and triterpenes in cell suspension cultures of *Uncaria tomentosa*. Plant and Cell Physiology 43: 1502-1509.
28. Moreau R A, Whitaker B D, Hicks K B, (2002) Phytosterols, phytostanols, and their conjugates in foods: structural diversity,

quantitative analysis, and health-promoting uses. Prog. Lipid Res. 41: 457-500.
29. Mustafa N R, De Winter W, Van Iren F, Verpoorte R, (2011) Initiation, growth and cryopreservation of plant cell suspension cultures. Nature Protocols 6: 715-742.
30. Yendo A C, de Costa F, Kauffmann C *et al.*, (2017) Purification of an immunoadjuvant saponin fraction from *Quillaja brasiliensis* leaves by reversed-phase silica gel chromatography. In: Fox CB (ed) Vaccine Adjuvants. Methods in Molecular Biology, vo. 1494. Springer, New York, pp 87-93.
31. Fleck J D, de Costa F, Yendo A C *et al.*, (2013) Determination of new immunoadjuvant saponin named QB-90, and analysis of its organ-specific distribution in *Quillaja brasiliensis* by HPLC. Natural Product Research 27: 907-910.
32. Tava A, Biazzi E, Mella M, Quadrelli P, Avato P, (2017) Artefact formation during acid hydrolysis of saponins from *Medicago spp*. Phytochemistry 138: 116-127.

Figures

Fig. 1. Custom made metal block adaptor for 12 mm (O.D.) glass tubes used during solvent evaporation in a heat block under a nitrogen gas stream.

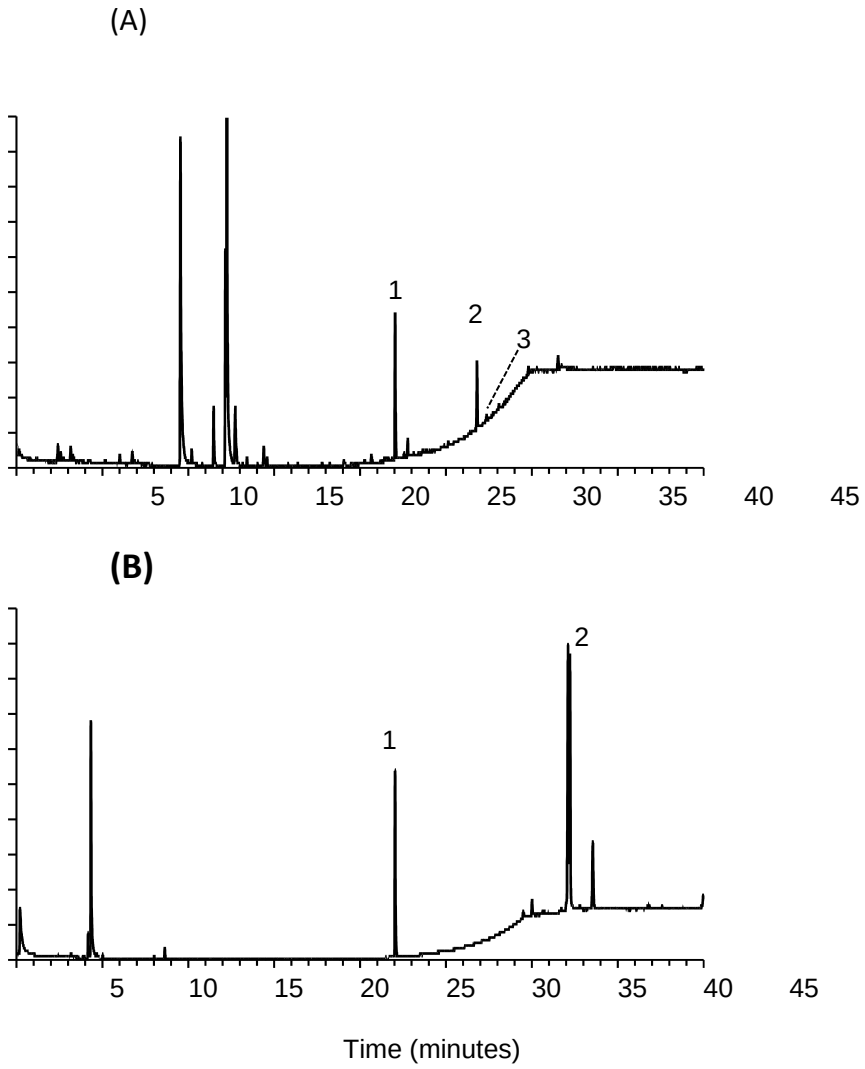

Fig. 2. Free sterol and sapogenin analysis by GCMS. A) Total ion chromatogram (TIC) of free sterols from cell cultures of *Quillaja brasiliensis* (1 – cholestane, 2 – stigmasterol, and 3 – sitosterol. B) TIC of sapogenins in an enriched saponin fraction purified from *Q. brasiliensis* leaves. 1 – cholestane; 2 – quillaic acid (the other peak in the doublet is an artefact produced during acid hydrolysis (32)). In B, cholestane was added as a surrogate.

Considerações Finais

As saponinas de *Quillaja* são amplamente exploradas comercialmente, inclusive para produção de vacinas. Conforme discutido no capítulo 1, saponinas para uso comercial são obtidas exclusivamente a partir das cascas e madeira de *Quillaja saponaria*. Avanços foram feitos quanto à sustentabilidade do processo de obtenção de saponinas nesta espécie, aliviando a pressão sobre a vegetação nativa do Chile. Contudo, a crescente demanda do mercado mundial por extratos de *Quillaja* configura um panorama em que há a possibilidade de falta de matéria-prima, particularmente de boa qualidade, para produção destes extratos. As cadeias produtivas foram ainda mais afetadas pela pandemia da COVID-19, o que ressalta a importância de novas fontes e processos para obtenção de produtos naturais com vistas ao suprimento das demandas da sociedade.

Quillaja brasiliensis vem sendo estudada como uma fonte alternativa muito promissora de saponinas bioativas. Em *Q. brasiliensis*, saponinas são purificadas a partir das folhas, que configuram uma fonte mais sustentável de produtos naturais porque são órgãos de rápido crescimento e reposição na planta. O conteúdo de saponinas nas folhas de *Q. brasiliensis* pode ser induzido por uma série de tratamentos, ligados ao estresse abiótico ou biótico, o que pode contribuir para maior rendimento de saponinas bioativas por unidade de biomassa.

A biotecnologia é grande aliada na obtenção de saponinas bioativas, conforme revisado no capítulo 2. A caracterização das vias metabólicas em espécies de interesse comercial, especialmente com ênfase na descrição de fatores de transcrição, tem avançado nos últimos anos. A identificação de genes relacionados à biossíntese de saponinas é importante para o desenvolvimento de estratégias de transformação de plantas com vistas ao aumento da produção destes compostos. Esta caracterização é também relevante para a produção heteróloga (*e.g* em culturas de leveduras) de saponinas com vistas à obtenção de fontes alternativas, aliviando a pressão sobre populações naturais, e ao escalonamento industrial.

Nesta tese, nosso objetivo foi estabelecer culturas celulares de *Quillaja brasiliensis* para subsidiar estudos de metabolismo e oferecer uma alternativa de produção sustentável de saponinas. O capítulo 3 descreve o estabelecimento de culturas de calos e suspensões

celulares de *Q. brasiliensis*, que produzem saponinas triterpênicas bioativas. Preliminarmente, foi possível observar que saponinas sintetizadas pelas suspensões celulares têm perfil químico parecido com as purificadas a partir das folhas de *Quillaja brasiliensis.* Contudo, somente uma pequena fração de compostos (sete) foram preliminarmente identificados em suspensões celulares. A produção destes compostos ativos também pôde ser modulada pela aplicação de tratamentos ligados a estresse, como observado em experimentos anteriores com folhas. Além disso, as saponinas obtidas pelos cultivos celulares foram capazes de induzir a produção de anticorpos em camundongos, em um ensaio com vacinas para gripe.

Durante o estágio de doutorado sanduíche, foram desenvolvidos dois métodos para quantificação de esteróis livres e agliconas de saponinas em cultivos celulares de *Quillaja brasiliensis*. Estes dois grupos de compostos compartilham os mesmos precursores metabólicos, contudo divergem na sua função *in planta*. Esteróis são componentes de membrana, apresentando funções ligadas ao crescimento e desenvolvimento do vegetal. Já as saponinas têm sido relacionadas às interações ecológicas entre planta e patógenos ou herbívoros. Ao tratar os cultivos celulares com açúcar marcado com C-13, a quantificação dos pools de esteróis livres e agliconas (*i.e.* ácido quiláico) permitirá avaliar o investimento de carbono no metabolismo de esteróis ou saponinas por células de *Quillaja brasiliensis*. Dessa forma, espera-se ampliar o conhecimento acerca da regulação da biossíntese de saponinas com vistas ao enriquecimento do cultivo *in vitro* com metabólitos de interesse, possivelmente aumentando seu valor agregado.

As culturas de tecido representam plataformas que possibilitam a obtenção de material vegetal de alta qualidade e renovável para a produção de produtos naturais, como é conhecido para taxol (*Taxus* sp.) e saponinas de Ginseng (*Panax* sp.). Em *Quillaja* sp., a obtenção de novas fontes de saponinas bioativas é chave para suprir as demandas por moléculas bioativas, tornando a produção destes compostos mais sustentável. Nesta tese, vimos que as culturas de tecido são uma fonte alternativa de saponinas para a produção de adjuvantes para vacinas. Além disso, estes cultivos podem ser úteis na investigação do metabolismo e vias de biossíntese de saponinas bioativas, visando subsidiar estudos de engenharia metabólica e biologia sintética.

Apêndices

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Review

Sustainable production of bioactive alkaloids in *Psychotria* L. of southern Brazil: propagation and elicitation strategies

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ABSTRACT

Psychotria is the largest genus in Rubiaceae. South American species of the genus are promising sources of natural products, mostly due to bioactive monoterpene indole alkaloids they accumulate. These alkaloids can have analgesic, antimutagenic, and antioxidant activities in different experimental models, among other pharmacological properties of interest. Propagation of genotypes with relevant pharmaceutical interest is important for obtaining natural products in a sustainable and standardized fashion. Besides the clonal propagation of elite individuals, the alkaloid content of *Psychotria* spp. can also be increased by applying moderate stressors or stress-signaling molecules. This review explores advances in research on methods for plant propagation and elicitation techniques for obtaining bioactive alkaloids from *Psychotria* spp. of the South Region of Brazil.

Keywords: abiotic stress, alkaloids, elicitation, monoterpenes, plant propagation, *Psychotria*, southern Brazil, sustainability

Introduction

Psychotria belongs to Rubiaceae, one of the major families of flowering plants having economic interest. The family includes coffee, a few significant poisonous plants to livestock, besides several important ornamental and medicinal species (Souza & Lorenzi 2012). *Psychotria* has captured researchers' attention mostly because of its medicinal properties.

Psychotria colorata is an Amazonian species that produces polyindolinic alkaloids with analgesic activity (Matsuura *et al*. 2013). The promising results obtained with *P. colorata* motivated the investigation of southern Brazilian *Psychotria* species and the discovery of new bioactive alkaloids (Porto *et al*. 2009). Moreover, leads on *in planta* alkaloid functions were also topic of experimental evaluation.

One of the key elements that needs to be addressed early on during the process of developing new bioactive molecules from plants is the capacity to generate catalytically active biomass to support extraction and steady supply. There are a number of ways through which these goals may be reached, including greenhouse rooting of cuttings (mini-cutting

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Projeto com autorização para testes de bioatividade

Porto Alegre, 17 de agosto de 2021.

FORMULÁRIO PARA SOLICITAÇÃO DE ADENDO E/OU MUDANÇA EM PROJETO

Título do Projeto: INFECÇÕES POR ZIKA VÍRUS: MODELOS EXPERIMENTAIS *IN VITRO* E *IN VIVO*

Nº de registro na CEUA: UP Nº 5402/17

Pesquisador responsável: Paulo Michel Roehe

A) Metodologia original descrita no projeto:

Local de execução

O estudo será desenvolvido no laboratório do Serviço de Medicina Experimental do IC/FUC - Centro de Cardiologia Experimental (CCE) onde está sendo montado um laboratório exclusivo para pesquisas com infecções virais. O laboratório terá três salas sendo uma delas para cultivos celulares (área limpa), outra sala (área suja) onde serão manipulados cultivos infectados, preparo de vacinas e onde serão manipulados os animais infectados e a terceira sala onde serão mantidos os animais de experimentação. Esta última sala contará com rack de ventilação intra-caixa (rack isolado) para a manutenção dos animais a serem infectados. A construção deste laboratório destinado para pesquisas exclusivas com o ZIKV é resultado de uma parceria entre o IC/FUC e UFRGS (ICBS - Departamento de Bioquímica) com financiamento do edital aprovado MCTIC/FNDCT-CNPq/MEC-CAPES/MS-Decit / N° 14/2016 e da Diretoria Científica do IC/FUC.

Amostras de vírus e linhagem celular

Amostras do vírus Zika de origem brasileira (ZIKV-BR) e africana (ZIKV-AF), serão obtidas de laboratórios colaboradores integrantes da rede Zika (http://www5.usp.br/tag/rede-zika/), incluindo o Instituto de Ciências Básicas da USP (ICB/USP) (gentileza do Prof. Edison Durigon) (ICB/USP, SP), laboratório de pesquisa em virologia da Faculdade de Medicina de São José do Rio Preto, SP (gentileza Dr. Maurício Nogueira) e o Instituto de Microbiologia Prof. Paulo de Góes (UFRJ) (gentileza Dr. Davis Ferreira). Os vírus serão multiplicados em células Vero E6 (originariamente ATCC CCL 81) seguindo métodos usuais (Caine et al., 2013). Os estoques de vírus serão quantificados seguindo métodos usuais (Cugola et al. 2016) e armazenados a -70 °C no LABVIR.

Animais

O uso de animais de experimentação é essencial para a execução deste estudo, pois não há, até o presente momento, métodos alternativos que substituam o uso dos mesmos para a metodologia proposta neste projeto (Art. 2º da resolução normativa nº18 de 24 de setembro de 2014, Conselho Nacional de Experimentação Animal - CONCEA).

O presente estudo será realizado após a aprovação pela Unidade de Pesquisa e pelo Comitê de Ética na Utilização de Animais do IC/FUC. Serão seguidos os princípios éticos de experimentação animal estabelecidos pela Lei Arouca no 11.796/08, pelo CONCEA e pela Sociedade Brasileira para Ciência de Animais de Laboratório - SBCAL.

Serão utilizados camundongos fêmeas da linhagem C57BL/6N. Os animais serão fornecidos pelo biotério do Centro de Cardiologia Experimental (CCE) do IC/FUC, Porto Alegre, Brasil. O número de camundongos que serão utilizados nos experimentos é o mínimo estatisticamente necessário para cumprir os objetivos propostos. Cada etapa apresenta um número variável de animais. Na primeira etapa, serão necessários 54 camundongos (Tabela 1), na segunda etapa, serão necessários 84 camundongos (Tabela 2) e na terceira etapa, serão necessários 54 camundongos (Tabela 3) num total de 192 animais.

Produção de vacinas experimentais

Para imunização com YFV será utilizada a vacina utilizada na rede pública de saúde, administrada por via subcutânea. A vacina para DENV será igualmente adquirida de fornecedores. Para a produção de vacina experimental para ZIKV, o vírus será multiplicado em células Vero; as suspensões de meio de cultivo contendo células infectadas serão clarificadas e inativadas com formalina. Os títulos das amostras virais antes da inativação deverão estar na ordem de pelo menos 10^6 unidades formadoras de placas (PFU)/mL. Para o preparo da vacina o imunógeno será acrescido de adjuvante. A escolha deste adjuvante será realizada na primeira etapa do experimento.

Avaliação da resposta humoral e celular produzida pela vacina inativada de zika vírus, administrada com diferentes adjuvantes (etapa 1).

Nesta etapa os grupos serão compostos por 9 camundongos fêmeas da linhagem C57BL/6N cada. Em cada grupo será avaliado a resposta induzida com o uso de diferentes adjuvantes, conforme (Tabela 1).

Tabela 1. *Descrição dos grupos para o teste dos adjuvantes*

Grupos (G)	*Adjuvante*
G1	*Salina*
G2	*Antígeno*
G3	*Al*
G4	*MF59*
G5	*QB-90*

A vacina inativada de Zika vírus (volume máximo 200uL) será administrada por via subcutânea. No dia zero será coletado sangue da veia lateral da cauda dos camundongos e, posteriormente, administrada a primeira dose vacinal. No dia 14 será coletado sangue dos animais e administrado um reforço vacinal. No dia 28 será coletado sangue dos animais. No dia 35 será coletado sangue dos animais. Após a coleta quatro camundongos serão eutanasiados para a remoção do baço e os demais (n=5) serão inoculados com vírus (desafio) em cada grupo. Após o desafio, será coletado sangue a cada 2 dias desses animais até o dia 42. Após esse período, os animais serão eutanasiados.

Após a coleta do soro, uma alíquota será utilizada para realizar teste de soroneutralização dos soros coletados nos dias 0, 14, 28 e 35 e teste de ELISA dos soros coletados nos dias 14 para IgM; 28 e 35 para IgG1 e IgG2a.Os soros coletados no período de 35 a 42 dias pós-imunização, serão utilizados para avaliar a viremia através de testes moleculares. O cultivo dos esplenócitos dos quatro camundongos eutanasiados no dia 35 será utilizado no ensaio de proliferação celular e quantificação das citocinas (Th1, Th2, Th17) no sobrenadante do cultivo celular e citometria de fluxo (CD4+ e CD8+). A vacina que induzir melhor resposta imunológica será utilizada no experimento.

Avaliação da dose letal (DL50) em diferentes vias de inoculação (Etapa 2).

Nesta etapa será avaliada a DL50 e o resultado obtido será utilizado na próxima etapa. Os animais serão inoculados com diferentes doses infectantes do vírus (diluição na base 10) por via intraperitonial e subcutânea. Cada grupo será composto por sete camundongos fêmeas da linhagem C57BL/6N.

Tabela 2. Identificação dos grupos utilizados para avaliação da dose letal por via intraperitonial e subcutânea.

Via de inoculação	***Grupos (G)***	***Concentração de vírus***
Intraperitonial	*G1*	*Salina (controle)*
	G2	10^5
	G3	10^4
	G4	10^3
	G5	10^2
	G6	10^1
Subcutânea	*G7*	*Salina (controle)*

	G8	10^5
	G9	10^4
	G10	10^3
	G11	10^2
	G12	10^1

Avaliação da resposta imunológica do zika vírus frente a diferentes vacinas para arbovírus (Etapa 3)

Os animais serão imunizados com vacinas para cada um dos agentes. A via e a dose do vírus de desafio serão definidas de acordo com os resultados da etapa anterior. Todos os grupos desta fase do experimento serão compostos por 9 camundongos fêmeas da linhagem C57BL/6N.

Tabela 3. *Descrição das vacinas utilizadas em cada grupo de animais.*

Grupos (G)	***Vacina***
G1	*Salina (controle)*
G2	*Vírus da febre amarela*
G3	*Dengue-1*
G4	*Dengue-2*
G5	*Zika vírus*
G6	*Herpesvírus*

As vacinas contra os diferentes antígenos virais serão administradas por via subcutânea. No dia zero será coletado sangue dos camundongos e, posteriormente, administrada a primeira dose vacinal. No dia 14 será coletado sangue dos animais e administrado um reforço vacinal. No dia 28 será coletado sangue dos animais. No dia 35 será coletado sangue dos animais e depois realizado o desafio, inoculando dose letal (previamente definida) de vírus nos animais previamente imunizados. Após o desafio, será coletado sangue a cada 2 dias desses animais até o dia 42.

B) Adendo ou modificação a ser executada na metodologia original

As modificações a seguir referem-se apenas a Etapa 3 do projeto. Essa Etapa do projeto não foi desenvolvida, uma vez que os dados obtidos com a Etapa 1 mostraram-se altamente promissores, uma vez que os adjuvantes baseados em saponinas de *Quillaja brasiliensis* tiveram um grande potencial adjuvante para vacinas contendo antígenos inativados de vírus envelopados, como o Zika vírus. De posse desses resultados, nosso grupo de pesquisa investiu no desenvolvimento de novas estratégias de obtenção desses adjuvantes de origem vegetal, desenvolvendo modernas técnicas de culturas celulares vegetais *in vitro*. A proposta é inovadora uma vez que visa investigar, comparativamente, o potencial imunogênico das saponinas de *Quillaja brasiliensis* produzidas *in vitro* das produzidas pelas folhas, em ambiente natural. Dessa forma, espera-se avaliar e comparar as respostas humoral e celular induzidas por tais imunizações.

Do ponto de vista virológico, espera-se que as alterações identificadas nos modelos experimentais contribuam significativamente para o avanço do conhecimento sobre vacinas virais inativadas, em especial contra influenzavírus. Nesse sentido, a proposta é de extrema relevância à saúde pública.

Animais

Serão utilizados camundongos (fêmeas) da linhagem Balb/C, com idade entre 30-45 dias e peso aproximado de 20 gramas. Os animais serão adquiridos do biotério o IC-FUC, Porto Alegre, Brasil. Serão necessários 50 camundongos fêmeas (grupos estão descritos na Tabela 1).

Produção da vacina experimental

Para a produção de vacina experimental adjuvantada para influenza, o antígeno vacinal comercial (vacina tetravalente contra influenza, produzida pelo Instituto Butantã, Brasil) será mesclado com diferentes concentrações de saponinas e/ou extratos ricos em saponinas extraídos de *Quillaja brasiliensis*. Para isso, 5 ug de antígeno vacinal será aplicado em duas doses, com intervalo de 14 dias contendo diferentes quantidades de adjuvantes, conforme descrito na Tabela 1.

Nesta etapa os grupos serão compostos por 5 camundongos (conforme descritos anteriormente), totalizando 45 camundongos fêmeas agrupadas conforme descrito na Tabela 1.

Tabela 1. Descrição dos grupos para o teste dos adjuvantes

Grupo	Adjuvante
G1	Salina (controle, não imunizado)
G2	Vacina comercial, contendo 5 ug de antígeno
G3	Vacina comercial (5 ug de antígeno) + Quil-A (10 ug)
G4	Vacina comercial (5 ug de antígeno) + QB-90cel (10 ug)
G5	Vacina comercial (5 ug de antígeno) + QB-90cel (50 ug)
G6	Vacina comercial (5 ug de antígeno) + QB-90 folha (10 ug)

G7	Vacina comercial (5 ug de antígeno) + AEcel (400 ug)
G8	Vacina comercial (5 ug de antígeno) + AEcel (200 ug)
G9	AE folha (400 ug)

O extrato aquoso (AE) será preparado a partir de folhas de *Q. brasiliensis*. Após a liofilização, o AE será purificado em uma coluna de sílica de fase reversa para obtenção da fração enriquecida em saponinas denominada QB-90. O extrato aquoso celular (derivado a partir de culturas in vitro de células de *Q. brasiliensis*) (AEcel) e a fração QB-90 purificada a partir deste extrato (QB-90cel) serão obtidos segundo metodologia ajustada, a partir de células liofilizadas de suspensões celulares de *Q. brasiliensis*, com 21 dias de cultivo.

A vacina inativada contra influenza será administrada por via subcutânea (volume 200 µL). No dia zero será administrada a primeira dose vacinal. No dia 14 será coletado sangue dos animais e administrado um reforço vacinal. No dia 28 será coletado sangue dos animais. Após a coleta, os animais serão eutanasiados para avaliação da resposta celular.

Os soros coletados nos dias 14 e 28 serão utilizados para realizar teste de ELISA e soroneutralização. Teste de ELISA será utilizado para avaliar os soros coletados nos dias 14 para IgM; e 28 para IgG1 e IgG2a. Adicionalmente, cultivo de esplenócitos de três camundongos eutanasiados no dia 28 será utilizado no ensaio de proliferação celular e quantificação das citocinas (Th1, Th2, Th17) no sobrenadante do cultivo celular e citometria de fluxo (CD4+ e CD8+).

C) Número total de animais e gênero apresentado no projeto original e número total e gênero a ser modificado:

Projeto Original						
Animal	Linhagem	Idade	Peso aprox.	Quantidade		
				M	F	Subtotal
Camundongo isogênico	C57BL/6N	4 a 6 semanas	+/- 23 g		X	192
				Total		192

Projeto Modificado						
Animal	Linhagem	Idade	Peso aprox.	Quantidade		
				M	F	Subtotal
Camundongo	Balb/C	4 a 6 semanas	+/- 20 g	0	50	50
				Total		50

D) Justificativa do pesquisador responsável para solicitação de adendo e/ou modificação no projeto:

A Etapa 3 do projeto foi substituída para a avaliação da atividade adjuvante de produtos naturais obtidos de cultivos celulares. O objetivo é comparar a capacidade adjuvante dos produtos obtidos dos cultivos *in vitro* com os adjuvantes já estabelecidos, que são purificados a partir de folhas do gênero *Quillaja*.

Referências

Yockey LJ et al. Vaginal exposure to Zika virus during pregnancy leads to fetal brain infection. Cell. 2016, 166:1247–1256.

Tang WW et al. A Mouse Model of Zika Virus Sexual Transmission and Vaginal Viral Replication. Cell Reports. 2016, 17: 3091–3098.

Scott JM et al. Cellular and Humoral Immunity Protect against Vaginal Zika Virus Infection in Mice. Journal of Virology. 2018, 92(7): 1–17.

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