




ISCOM-Matrices Nanoformulation Using the Raw Aqueous Extract of *Quillaja lancifolia* (*Q. brasiliensis*)

Samuel Cibulski¹ · Thalisson Amorim de Souza² · Joanda Paola Raimundo² · Yuri Manguiera Nascimento² · Lucas Silva Abreu³ · Norma Suarez⁴ · Iris Miraballes⁵ · Paulo Michael Roehe⁶ · Demetrius Antonio Machado de Araújo¹ · Josean Fechine Tavares² · Marcelo Sobral da Silva² · Fernando Silveira⁴ 

Accepted: 29 July 2022 / Published online: 6 August 2022
© The Author(s), under exclusive licence to Springer Science+Business Media, LLC, part of Springer Nature 2022

Abstract

Quillaja saponins have an intrinsic capacity to interact with membrane lipids that self-assembles in nanoparticles (immunostimulating complexes or ISCOM-matrices) with outstanding immunoadjuvant activity and low toxicity profile. However, the expensive and laborious purification processes applied to purify *Quillaja* saponins used to assemble ISCOM-matrices show an important drawback in the large-scale use of this vaccine adjuvant. Thus, in this study, we describe a protocol to appropriately formulate ISCOM-matrices using the raw aqueous extract (AE) of *Quillaja lancifolia* leaves. In the presence of lipids, AE was able to self-assemble in nanostructures that resembles immunostimulating complexes (ISCOM). These negatively charged nanoparticles of approximately 40 nm were characterized by transmission electron microscopy and dynamic light scattering. In addition, well-known saponins with remarkable immunoadjuvant activity, as QS-21, were detected into nanoparticles. Thus, the easier, robust, cheaper, and environmentally friendly method developed here may be an alternative to the classical methods for ISCOM-matrices production that use high-purified saponins.

Keywords Quillajaceae · Saponin-based adjuvants · Vaccine adjuvants · ISCOM · DLS · HRMS

Samuel Cibulski and Fernando Silveira contributed equally to this work.

✉ Fernando Silveira
fsilveira@higiene.edu.uy

¹ Centro de Biotecnologia – CBIotec, Laboratório de Biotecnologia Celular E Molecular, Universidade Federal da Paraíba, João Pessoa, Paraíba, Brazil

² Institute for Research in Pharmaceutical and Medications – IPEFARM, Universidade Federal da Paraíba, João Pessoa, Paraíba, Brazil

³ Departamento de Química Orgânica, Instituto de Química, Universidade Federal Fluminense, Niterói, RJ, Brazil

⁴ Departamento de Desarrollo Biotecnológico, Facultad de Medicina, Instituto de Higiene, Universidad de La República (UdelAR), Montevideo, Uruguay

⁵ Cátedra de Inmunología Clínica – Departamento BIOCLIN, Laboratorio de Biotecnología Facultad de Química, Instituto Polo Tecnológico de Pando, Universidad de La República (UdelAR), Montevideo, Uruguay

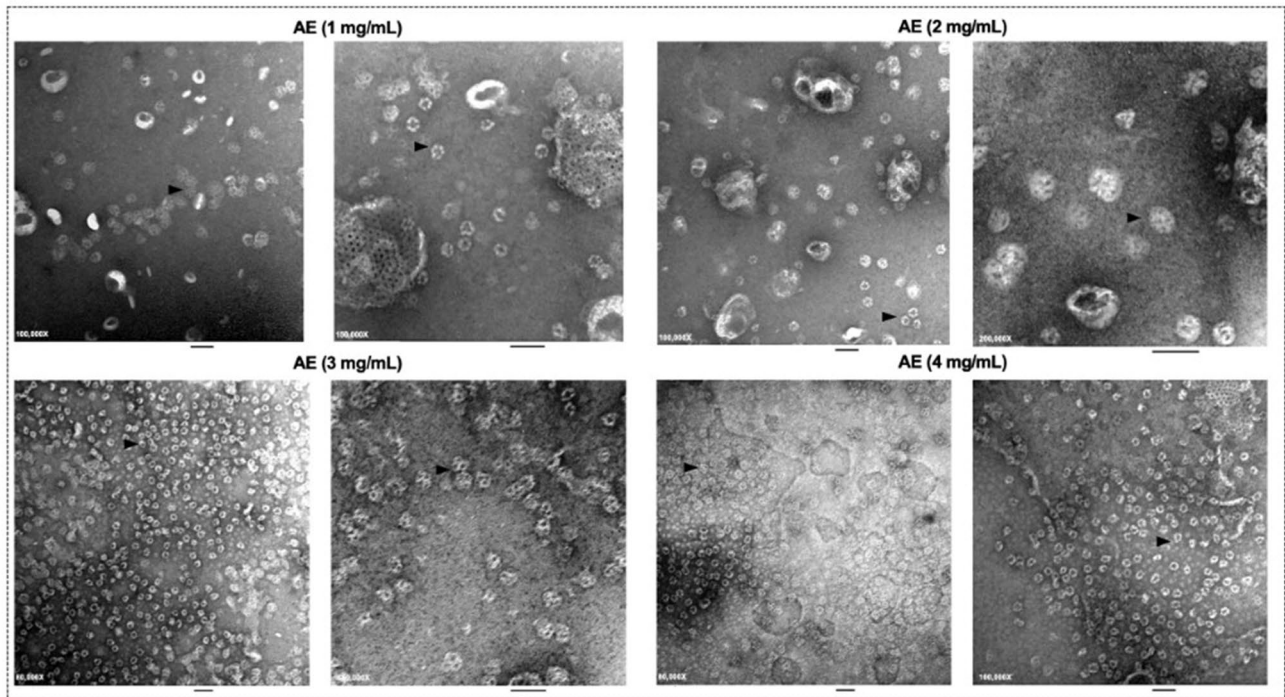
⁶ Departamento de Microbiologia Imunologia E Parasitologia, Laboratório de Virologia, UFRGS, Porto Alegre, Brazil

1 Introduction

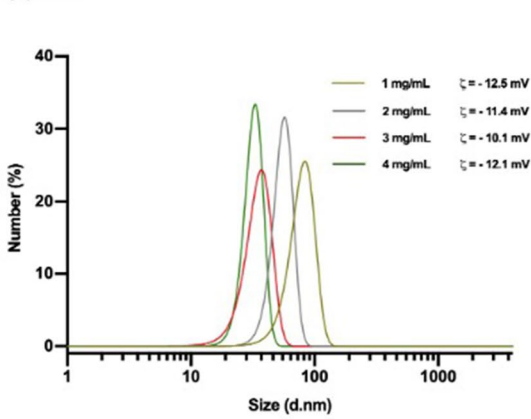
The monotypic genus *Quillaja* (from Quillajaceae) consists of two temperate evergreen tree species. The geographic distribution of *Quillaja* is quite intriguing, with the Andes Mountains being a geographical barrier isolating the two species. *Quillaja saponaria* Molina is found in Chile, while *Quillaja lancifolia* D. Don (previously known as *Q. brasiliensis*) is a representative tree of Araucaria forests in southern Brazil, northern Uruguay, northeastern Argentina, and eastern Paraguay [1, 2]. *Quillaja* saponins have a wide range of industrial applications—as adjuvants in vaccines, food additives, and cosmetic industry. According to Central Bank of Chile, quillaja extracts brought US\$ 52.6 million to the Chilean economy in 2019, as 515 tons were exported.

Several *Q. saponaria* saponins fractions were utilized as vaccine adjuvants. The most studied, Quil-A®, a crude saponin fraction extracted from *Q. saponaria* barks [3], is widely used in veterinary vaccines. However, *Quillaja* saponins has inherent toxicity. Therefore, two main approaches were applied to circumvent this matter. The first is isolating molecules with less toxicity from the saponin pool. A

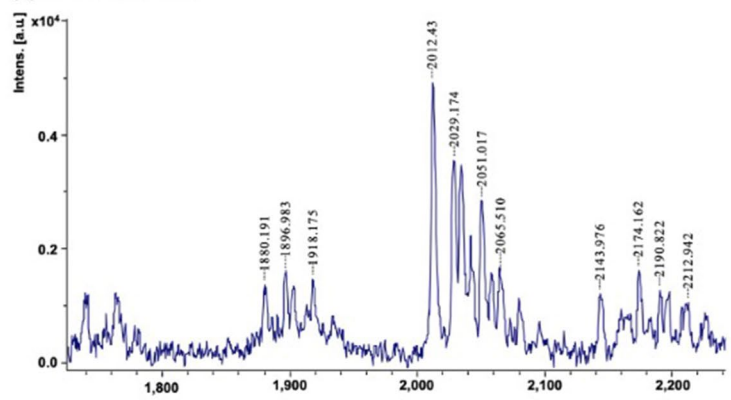
(a) TEM



(b) DLS



(c) MALDI-ToF-MS



(d) DI-ESI-ToF-MS

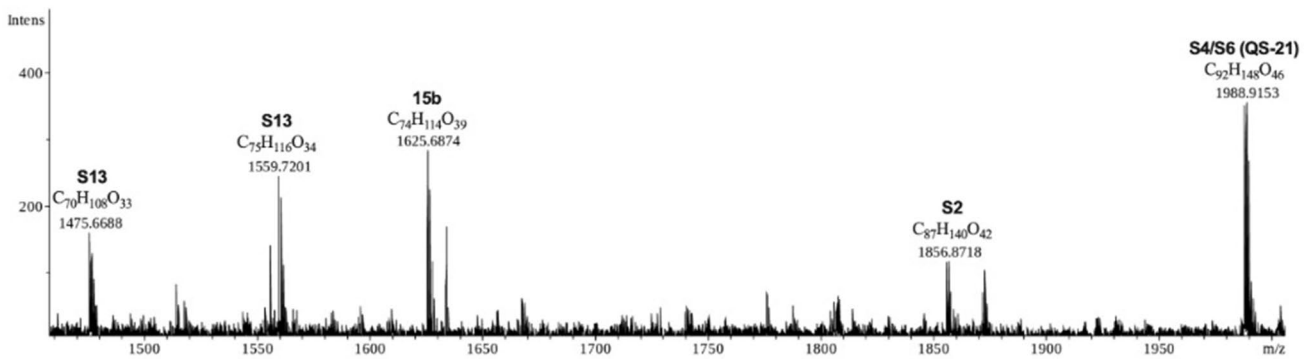


Fig. 1 ISCOM-like nanoparticles assembled with *Q. lancifolia* AE. **a** TEM were obtained after negative staining with uranyl acetate. The arrowhead shows the assembly of cage-like particles. The scale bar bellows each figure represents 100 nm. **b** DLS measurements of average hydrodynamic diameter and ζ -potential of nanoformulations. Size distribution were stated as an average of ten measurements. All measurements were carried out at 25 ± 0.1 °C. **c** MALDI-ToF mass spectra of the nanoformulation containing 3 mg/mL of AE showed characteristic peaks of saponins between m/z 1900 and 2200. The products were detected as $[M+Na]^+$ and/or $[M+K]^+$ ions. **d** DI-ESI-ToF-MS data from *Q. lancifolia* AE. The most frequent ions were assigned, based on its homologous, well characterized, saponins from *Q. lancifolia* and *Q. saponaria*

successful example, QS-21, was purified from Quil-A® and it has been demonstrated to have less toxicity than its parent saponin fraction. Today, QS-21 has been extensively studied in the form of a sole or combination adjuvant in several promising human vaccine formulations and it was recently approved to malaria and shingles vaccines [4]. The second approach is based on the inherent capacity of *Quillaja* saponins to interact with membrane lipids. Thus, in the presence of cholesterol and phosphatidylcholine, *Quillaja* saponins self-assembles in nanoparticles (immunostimulating complexes or ISCOM-matrices) with outstanding immunoadjuvant activity [5]. ISCOM-matrices included in human vaccines were found to be safe and well tolerated, with no serious adverse events [6]. Recently, the recombinant spike protein from SARS-CoV-2 was formulated with a *Q. saponaria* saponin-based nanoadjuvant (Matrix-M®). This vaccine demonstrate strong efficacy in phase 3 clinical trials demonstrating and is a possible candidate for fighting the SARS-CoV-2 pandemic [7].

The congener species *Q. lancifolia* (“pau-de-sabão”) has saponins that are chemically similar to those of *Q. saponaria* barks [8–10], showing a remarkable immunoadjuvant activity. The immunoadjuvant activity of purified *Q. lancifolia* saponin fractions (denominated QB-90 and QB-80) [11–14], as well as their poorly purified AE [15, 16], is equivalent to Quil-A®, being already demonstrated in experimental vaccines. Regarding AE, MALDI-ToF-MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) revealed peaks that showed similar patterns between Quil-A® in spectra regions of m/z 750–1000 and 1850–2200, which are characteristic of *Quillaja* saponins [15].

Purified *Quillaja* saponins fractions such as Quil-A® [17], QB-90, and QB-80 [13, 18–20] are recognized to self-assemble in cage-like nanoparticles (ISCOM) in the presence of lipids. However, the processes utilized to purify these saponin-rich fractions are expensive and time-consuming, involving silica, gel filtration, and reversed-phase chromatography. Therefore, processes that minimize and or eliminate steps in formulation of ISCOM-like nanoparticles are still desired. Here, we presented for the first time, ISCOM-matrices nanoparticles assembled using raw AE of *Q. lancifolia*.

2 Material and Methods

2.1 Plant Material and Aqueous Extract Preparation

Q. lancifolia D. Don leaves were collected from a 5-year-old specimen in Eldorado do Sul, RS, Brazil ($29^{\circ} 59' 26''$ S– $51^{\circ} 19' 39''$ W). A herbarium specimen is on deposit at Herbarium of the Department of Systematics and Ecology of Universidade Federal da Paraíba, João Pessoa, Brazil. AE preparation were carried out as previously described [21]. Briefly, leaves were dried in a circulating air oven at 37 °C for 5 days and submitted to a knife mill. The powered leaves (100 g) were transferred to a flat bottom balloon with 800 mL of distilled water and maintained for 8 h under slight agitation. After, the AE was filtered, and tannins were precipitated with gelatin. The AE was partitioned with ethyl acetate and the organic phase is discarded. The aqueous phase is concentrated in a rotary evaporator at 40 °C.

2.2 ISCOM-Matrices Nanoparticle Preparation

Concisely, a lipid-mix (2 mL) containing chloroform-dissolved cholesterol (Sigma-Aldrich, USA) and di-palmitoyl phosphatidylcholine (Avanti Polar Lipids, USA) (both at 100 mg/mL) was mixed with 10 mL of 20% octyl β -D-glucopyranoside (OGP, Sigma-Aldrich, USA). Five hundred microliters of the lipid-mix were added to 5, 10, 15, or 20 mg of AE dissolved in 4.5 mL of water. The mixture was dialyzed in a 12–14 kDa pre-hydrated dialysis membrane at 22 °C in 50 mM Tris-Cl (pH 8.5) containing 0.001% of thimerosal for 5 days against 20 L of buffer, changing the dialysis buffer every 24 h.

2.3 Transmission Electron Microscopy (TEM)

For TEM analysis, 10 μ L of the nanoformulation was placed on a copper grid covered with a formvar and carbon film for 2 min. After, the remaining liquid was drained, and the sample was negatively stained with uranyl acetate (2% w/v) for 2 min. The analysis was performed in a Jeol JEM 1010 transmission electron microscope (Jeol, Japan) at an accelerating voltage of 90 kV and at magnifications between 30,000 and 150,000X.

2.4 Dynamic Light Scattering (DLS) and ζ -Potential Parameters

Standard operating procedure (SOP) for the dynamic light scattering (DLS): material RI = 1.59, dispersant RI (water) = 1.33, T = 25 °C, viscosity (water) = 0.887 cP, measurement angle = 173° backscatter, measurement position = seek for optimum position, automatic attenuation. Additionally, for ζ -potential, automatic attenuation and voltage selection.

2.5 Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-ToF-MS)

MALDI-ToF-MS measurements were conducted on a Microflex LR MALDI-ToF-MS instrument (Bruker Daltonics, USA) with a 337-nm nitrogen laser operated in positive ion lineal mode with delayed extraction and optimized in the m/z range of 0–20 kDa. One microliter of each nanoparticle preparation was mixed with 1 μL of matrix solution (2,5-dihydroxybenzoic acid, 10 mg/mL in H_2O with 1% of TFA). The sample and matrix mixes were spotted onto a 96-well stainless-steel plate and allowed to air dry for 15 min at room temperature before measurements. Calibrations were performed with a peptide calibration standard mix (Bruker Daltonics, USA). The laser was fired 100 times at each of ten locations for each sample well on a 96-well plate for a cumulative 1000 shots per sample well taken at 30% intensity.

2.6 AE Saponin Profile by Direct Injection Electro spray Ionization (ESI) and Time-of-Flight Detection (DI-ESI-ToF-MS)

For saponin identification, DI-ESI-ToF-MS were applied on a micrOTOF II high-resolution mass spectrometer (Bruker Daltonics, USA). Spectrometer analysis parameters were as follows: 4.5 kV capillary, ESI in negative ion mode, a 500 V end plate offset, a 40.6 psi nebulizer, a dry gas (N_2) flow rate of 8.0 L/h, and a temperature of 200 °C. Spectra (m/z 1400–2400) were recorded every 2.0 s. The ESI Tuning mix® (Sigma Aldrich, USA) was used as the internal calibrator.

3 Results and Discussion

ISCOM-matrices nanoparticles assembly were verified by TEM and DLS and are shown in Fig. 1a. The self-assembled nanostructures formulated with AE (1–4 mg/mL) in the presence of lipids give a spherical-shaped cage-like particles of ~40 nm, typical of ISCOM or ISCOM-matrices nanoparticles. However, in formulations containing 3 or 4 mg/mL of AE, the number of cage-like particles is high and more homogeneous in comparison to formulations containing fewer saponins. These observations support the fact that the

experimental conditions that used a high concentration of AE (it also increases the saponin's concentration) are more adequate for the preparation of the observed micelles. Moreover, aggregates and amorphous structures were also observed, particularly in formulations containing 1 and 2 mg/mL of AE.

Concerning DLS measurements (Fig. 1b), the z-average size (size distribution by number) was consistent with TEM data. Interestingly, the size of the nanoparticles decreases when greater amounts of saponins are used in the formulation (Fig. 1b). The particle size in the formulation containing 3 or 4 mg/mL AE was similar, showing once again, that these concentrations are suitable for the formulation of ISCOM-matrices from raw *Q. lancifolia* AE. The nanoparticles assembled with AE has a negative zeta (ζ)-potential (fluctuating from –10.1 to –12.5 mV) since carrying a net negative charge due to the glucuronic acid component present in *Quillaja* saponins [22]. Entire ζ -potential data are presented in Table 1.

The saponin profile of ISCOM-matrices particles assessed by MALDI-ToF-MS is quite similar in all formulations (Fig. 1c), showing saponins with m/z of 1700–1950 and 2000–2200. This is in agreement with *Q. lancifolia* saponins MS data and shows that these saponins can form ISCOM-matrices structures [8, 9, 15]. The most frequent ion is 2012.4, which may correspond to saponin with m/z of 1988.9 (QS-21), detected as $[\text{M} + \text{Na}]^+$ ion. In order to confirm the identity of QS-21 and tentatively identify another Quillajaceae saponins, a direct injection in a high-resolution mass spectrometer followed by electro spray ionization and time-of-flight MS detection (DI-ESI-MS) was performed.

The most frequent ions were tentatively assigned, based on its homologous and well-characterized saponins from Quillajaceae, and the full scan of DI-ESI-MS is presented in Fig. 1d and in Table 2. Detailed high-resolution MS data can be obtained in Fig. 1d. Using this approach, it was possible to identify the most frequent saponins ions in the *Q. lancifolia* AE. These includes the S4/S6 (QS-21; exact mass 1988.9242, detected mass, 1987.9100, error 3.5 ppm), 15b, S2, and S13 saponins [23–25]. Some ions could not be attributed to saponins already identified in *Quillaja* by spectroscopic or spectrometric analysis, indicating probable novel molecules in *Q. lancifolia* leaves (data not shown).

Furthermore, the ISCOM-matrices saponin profile is quite similar to QB-90 saponin fraction (Fig. 1). QB-90 is

Table 1 ζ -potential data

	ζ -potential (mV)		Mob ($\mu\text{mcm/Vs}$)		Cond (mS/cm)	
	Mean	SD	Mean	SD	Mean	SD
AE 1 mg/mL	–12.5	0.624	–0.9774	0.04745	15.7	0.551
AE 2 mg/mL	–11.4	0.493	–0.8897	0.04005	16.4	0.603
AE 3 mg/mL	–10.1	0.738	–0.7901	0.05713	17.2	0.603
AE 4 mg/mL	–12.1	1.19	–0.9472	0.09329	16.7	0.755

Table 2 Most frequent saponin ions tentatively identified by high-resolution mass spectrometry (DI-ESI-ToF)

Saponin*	[M-H] ⁻	Molecular formula	Error (ppm)	References
S13 [#]	1475.6688	C ₇₀ H ₁₀₈ O ₃₃	0.8	[29]
S13	1559.7201	C ₇₅ H ₁₁₆ O ₃₄	4.8	[24]
15b	1625.6874	C ₇₄ H ₁₁₄ O ₃₉	-0.6	[25]
S2	1855.8818	C ₈₇ H ₁₄₀ O ₄₂	-3.9	[23]
S4/S6	1987.9100	C ₉₂ H ₁₄₈ O ₄₆	3.5	[23]

*The most frequent ions were assigned, based on its homologous, well characterized, saponins from Quillajaceae. [#]S13 without MeBu

a C18 reversed-phase chromatography purified *Q. lancifolia* saponin-rich fraction and extensively studied by our research group. This similar profile brings to light a possible new saponin purification/enrichment method, based on dialysis. Dialysis-based methodologies are less expensive than techniques based on reversed-phase chromatography. Furthermore, dialysis-based purifications do not use organic solvents (such as methanol, used to elute QB-1290 from the C18 column), resulting in an environmentally friendly process.

The commercial *Q. saponaria* fraction Quil-A® has been successfully used for veterinary applications, but it is known that side effects as local reactions and granulomas can be attributed to its components. *Q. lancifolia* AE have some advantages. They can induce immune responses similarly to Quil-A®, including promoting dose sparing [15, 16, 26, 27]. This could be explained by the presence QS-21, easily detected in AE of *Q. lancifolia* leaves, that may explain the strong immunoadjuvant activity of AE, including B- and T-cell immunological responses, and their low toxicity in experimental vaccines. Furthermore, AE extraction process is simple and less expensive than the laborious and costly chemical purification steps to obtain saponin-rich fractions, involving silica and reverse-phase chromatography. Regarding forest extractivism concerns, AE was derived from leaves of *Q. lancifolia* that are readily renewable alternative sources of saponins compared to *Q. saponaria* barks.

As stated previously, one of the critical issues in the use of saponins as vaccine adjuvants is the fact that they can raise safety concerns due to their toxic effects in the injection site [28]. The results obtained in our previous studies showed that AE has a low toxicity profile both in vitro and in vivo [15]. Using a hemolysis assay, a widely accepted test to predict saponins toxicity, AE has been shown to cause membrane damage at high dose concentrations (with HD₅₀ close to 1 mg/mL). This value is calculated 23-fold higher than Quil-A® (HD₅₀ 35.9 µg/mL) [15]. The nanoformulations presented here were submitted to the hemolysis test and no hemoglobin release was verified, indicating no membrane damage. This suggests that the membrane-toxic effects are abrogated when saponin formulations are complexed with lipids. Additionally, due to

the presence of the well-characterized saponin QS-21 in *Q. lancifolia* AE, this can be an enriched intermediate for obtaining/purifying this potent adjuvant for use in human vaccines.

According to previous studies involving the immunoadjuvant potential of AE and the data presented here, we propose that *Q. lancifolia* AE and their nanoparticles has a promising saponin-based vaccine adjuvant, as it presents some advantages over commercially available saponins. Foremost, AE contains known saponins with a strong immunoadjuvant activity, as QS-21. Additionally, AE is a raw extract of saponin and, as such, is easier and cheaper to produce compared to purified saponin fractions. Furthermore, in addition to its efficacy, AE was proven to be safe and well-tolerated in mice. Finally, AE proved to be able to form cage-like structures similar in shape and size to ISCOM particles and, with that, opens up new possibilities to AE in nanoformulations. These are some advantages that are carefully analyzed when a new vaccine adjuvant is considered a candidate to be escalated from experimental to industrial uses.

Acknowledgements We are grateful to Ubiratan Ribeiro and Adrielly Andrade for the help in AE isolation and Anauara Lima e Silva for the exsiccate preparation. We thank the Laboratório Multiusuário de Caracterização e Análise (LMCA-UFPA) staff for the MS data acquisition and fruitful discussions for data analyses. We are also thankful for collaborating with Rede Norte-Nordeste de Fitoprodutos (INCT-RENNOFITO). JFT, MSS and DAMA are CNPq research fellows. FS is a recipient of ANII research productivity fellowship level 1. The authors also thank the support of the Programa de Desarrollo de las Ciencias Básicas (PEDECIBA Uruguay).

Declarations

Conflict of Interest The authors declare no competing interests.

Research Involving Humans and Animal Statement None.

Informed Consent None.

References

- Magedans, Y. V. S., Yendo, A. C. A., Costa, F. De, Gosmann, G., & Arthur, G. (2019). Foamy matters: An update on Quillaja saponins and their use as immunoadjuvants. *Future Medicinal Chemistry*.
- BFG. (2021). Brazilian Flora 2020: Leveraging the power of a collaborative scientific network. *Taxon*, 00, 1–21. <https://doi.org/10.1002/tax.12640>
- Dalsgaard, K., Jensen, M. H., & Sorensen, K. J. (1977). Saponin adjuvants. IV. Evaluation of the adjuvant quil A in the vaccination of cattle against foot-and-mouth disease. *Acta veterinaria scandinavica*, 18(3), 349–360. Retrieved from <http://agris.fao.org/agris-search/search.do?recordID=US201301276478>
- Lacaille-Dubois, M. A. (2019). Updated insights into the mechanism of action and clinical profile of the immunoadjuvant QS-21: A review. *Phytomedicine*, 60, 152905. <https://doi.org/10.1016/j.phymed.2019.152905>

5. Sun, H. X., Xie, Y., & Ye, Y. P. (2009). ISCOMs and ISCOMATRIX™. *Vaccine*, 27(33), 4388–4401. <https://doi.org/10.1016/j.vaccine.2009.05.032>
6. McKenzie, A., Watt, M., & Gittleson, C. (2010). ISCOMATRIX vaccines: Safety in human clinical studies. *Human vaccines*, 6(3), 237–246. <https://doi.org/10.4161/hv.6.3.10754>
7. Keech, C., Albert, G., Cho, I., Robertson, A., Reed, P., Neal, S., Glenn, G. M. (2020). Phase 1–2 trial of a SARS-CoV-2 recombinant spike protein nanoparticle vaccine. *New England Journal of Medicine*, NEJMoa2026920. <https://doi.org/10.1056/NEJMoa2026920>
8. 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. *Phytochemical Analysis*, (February), pca.2837. <https://doi.org/10.1002/pca.2837>
9. Wallace, F., Bennadji, Z., Ferreira, F., & Olivaro, C. (2017). Analysis of an immunoadjuvant saponin fraction from *Quillaja brasiliensis* leaves by electrospray ionization ion trap multiple-stage mass spectrometry. *Phytochemistry Letters*, 20, 228–233. <https://doi.org/10.1016/j.phytol.2017.04.020>
10. Kauffmann, C., Machado, A. M., Fleck, J. D., Provensi, G., Pires, V. S., Guillaume, D., ... Gosmann, G. (2004). Constituents from leaves of *Quillaja brasiliensis*. *Natural Product Research*, 18(2), 153–157. <https://doi.org/10.1080/14786410310001608055>
11. Silveira, F., Cibulski, S. P., Varela, A. P., Marqués, J. M., Chabalgoity, A., de Costa, F., ... Ferreira, F. (2011). *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. <https://doi.org/10.1016/j.vaccine.2011.09.137>
12. Yendo, A. C. A., de Costa, F., Cibulski, S. P., Teixeira, T. F., Colling, L. C., Mastrogiovanni, M., Fett-Neto, A. G. (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(20). <https://doi.org/10.1016/j.vaccine.2016.03.070>
13. Cibulski, S. P., Mourglia-Ettlin, G., Teixeira, T. F., Quirici, L., Roehe, P. M., Ferreira, F., & Silveira, F. (2016). Novel ISCOMs from *Quillaja brasiliensis* saponins induce mucosal and systemic antibody production, T-cell responses and improved antigen uptake. *Vaccine*, 34(9), 1162–1171. <https://doi.org/10.1016/j.vaccine.2016.01.029>
14. Cibulski, S. P., Silveira, F., Mourglia-Ettlin, G., Teixeira, T. F., dos Santos, H. F., Yendo, A. C., ... Roehe, P. M. (2016). *Quillaja brasiliensis* saponins induce robust humoral and cellular responses in a bovine viral diarrhea virus vaccine in mice. *Comparative Immunology, Microbiology and Infectious Diseases*, 45. <https://doi.org/10.1016/j.cimid.2016.01.004>
15. Cibulski, S., Rivera-Patron, M., Suárez, N., Pirez, M., Rossi, S., Yendo, A. C., ... Silveira, F. (2018). 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. <https://doi.org/10.1016/j.vaccine.2017.11.030>
16. De Costa, F., Yendo, A. C. A., Cibulski, S. P., Fleck, J. D., Roehe, P. M., Spilki, F. R., ... Fett-Neto, A. G. (2014). Alternative inactivated poliovirus vaccines adjuvanted with *Quillaja brasiliensis* or Quil-A saponins are equally effective in inducing specific immune responses. *PLoS ONE*, 9(8). <https://doi.org/10.1371/journal.pone.0105374>
17. Morein, B., Sundquist, B., Höglund, S., Dalsgaard, K., & Osterhaus, A. (1984). Iscom, a novel structure for antigenic presentation of membrane proteins from enveloped viruses. *Nature*, 308(5958), 457–460. <https://doi.org/10.1038/308457a0>
18. Rivera-Patron, M., Cibulski, S. P., Miraballes, I., & Silveira, F. (2022). Formulation of IMXQB: Nanoparticles based on *Quillaja brasiliensis* saponins to be used as vaccine adjuvants. In *Methods in molecular biology (Clifton, N.J.)* (Vol. 2469, pp. 183–191). Methods Mol Biol. https://doi.org/10.1007/978-1-0716-2185-1_15
19. Cibulski, S., Teixeira, T. F., Varela, A. P. M., de Lima, M. F., Casanova, G., Nascimento, Y. M., ... Silveira, F. (2020). IMXQB-80: A *Quillaja brasiliensis* saponin-based nanoadjuvant enhances Zika virus specific immune responses in mice. *Vaccine*, 39(3), 571–579. <https://doi.org/10.1016/j.vaccine.2020.12.004>
20. Cibulski, S., Varela, A. P. M., Teixeira, T. F., Cancela, M. P., Sesterheim, P., Souza, D. O., Silveira, F. (2021). Zika virus envelope domain III recombinant protein delivered with saponin-based nanoadjuvant from *Quillaja brasiliensis* enhances anti-Zika immune responses, including neutralizing antibodies and splenocyte proliferation. *Frontiers in Immunology*, 12. <https://doi.org/10.3389/fimmu.2021.632714>
21. Yendo, A., de Costa, F., Kauffmann, C., Fleck, J., Gosmann, G., & Fett-Neto, A. (2017). Purification of an immunoadjuvant saponin fraction from *Quillaja brasiliensis* leaves by reversed-phase silica gel chromatography. In C. B. Fox (Ed.), *Methods in Molecular Biology* (Vol. 1494, pp. 87–93). Springer New York. https://doi.org/10.1007/978-1-4939-6445-1_6
22. Pedersen, J. S., Oliveira, C. L. P., Hübschmann, H. B., Arleth, L., Manniche, S., Kirkby, N., & Nielsen, H. M. (2012). Structure of immune stimulating complex matrices and immune stimulating complexes in suspension determined by small-angle X-ray scattering. *Biophysical Journal*, 102(10), 2372–2380. <https://doi.org/10.1016/j.bpj.2012.03.071>
23. Nord, L. I., & Kenne, L. (1999). Separation and structural analysis of saponins in a bark extract from *Quillaja saponaria* Molina. *Carbohydrate Research*, 320, 70–81. [https://doi.org/10.1016/S0008-6215\(99\)00227-X](https://doi.org/10.1016/S0008-6215(99)00227-X)
24. Nord, L. I., & Kenne, L. (2000). Novel acetylated triterpenoid saponins in a chromatographic fraction from *Quillaja saponaria* Molina. *Carbohydrate Research*, 329(4), 817–829. [https://doi.org/10.1016/S0008-6215\(00\)00248-2](https://doi.org/10.1016/S0008-6215(00)00248-2)
25. Guo, S., & Kenne, L. (2000). Characterization of some O-acetylated saponins from *Quillaja saponaria* Molina. *Phytochemistry*, 54(6), 615–623. [https://doi.org/10.1016/S0031-9422\(00\)00161-8](https://doi.org/10.1016/S0031-9422(00)00161-8)
26. Fleck, J. D., Kauffmann, C., Spilki, F., Lencina, C. L., Roehe, P. M., & Gosmann, G. (2006). Adjuvant activity of *Quillaja brasiliensis* saponins on the immune responses to bovine herpesvirus type 1 in mice. *Vaccine*, 24(49–50), 7129–7134. <https://doi.org/10.1016/j.vaccine.2006.06.059>
27. Yendo, A. C. A., de Costa, F., Cibulski, S. P., Teixeira, T. F., Colling, L. C., Mastrogiovanni, M., & Fett-Neto, A. G. (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(20), 2305–2311. <https://doi.org/10.1016/j.vaccine.2016.03.070>
28. Petrovsky, N. (2015). Comparative safety of vaccine adjuvants: A summary of current evidence and future needs. *Drug Safety*, 38(11), 1059–1074. <https://doi.org/10.1007/s40264-015-0350-4>
29. Kite, G. C., Howes, M. J. R., & Simmonds, M. S. J. (2004). Metabolomic analysis of saponins in crude extracts of *Quillaja saponaria* by liquid chromatography/mass spectrometry for product authentication. *Rapid Communications in Mass Spectrometry*, 18(23), 2859–2870. <https://doi.org/10.1002/rcm.1698>

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Springer Nature or its licensor holds exclusive rights to this article under a publishing agreement with the author(s) or other rightsholder(s); author self-archiving of the accepted manuscript version of this article is solely governed by the terms of such publishing agreement and applicable law.