Screening for antiviral activity of two purified saponin fractions of *Quillaja* spp. against *Yellow Fever Virus* and *Chikungunya Virus*

Eduardo Artur Troian[1a], Karoline Schallenberger[1a], Francini Pereira da Silva[a], Gabriela Klein Dietrich[b], Fernando Ferreira Chiesa[c], Cristina Olivaro[e], Federico Wallace[e], Juliane Fleck[d], Simone Gasparin Verza[a, d*]

[a] *Graduate Program in Virology, Feevale University, Novo Hamburgo, Rio Grande do Sul, Brazil*

[b] *Institute of Health Sciences, Feevale University, Novo Hamburgo, Rio Grande do Sul, Brazil*

[c] *Organic Chemistry Department, Carbohydrates and Glycoconjugates Laboratory, Udelar, Montevideo, Uruguay*

[d] *Graduate Program in Toxicology and Analytical Toxicology, Feevale University, Novo Hamburgo, Rio Grande do Sul, Brazil*

[e] *Espacio de Ciencia y Tecnología Química, Centro Universitario de Tacuarembó, Cenur Noreste, Udelar, Tacuarembó, Uruguay*

[1] The authors worked together and contributed equally

***Corresponding author**

Simone G. Verza, Phd*

Feevale University, Campus II - 2755, RS 239

Postal code 93525-075 Novo Hamburgo, Rio Grande do Sul, Brazil

Abstract

Yellow Fever Virus (YFV) and *Chikungunya Virus* (CHIV) are neglected reemerging pathogens that cause comorbidities worldwide. Since no antiviral drug is prescribed for those infections, there is a demand on researching compounds that inhibit viral replication. Saponins are amphiphilic compounds that already demonstrated *in vitro* activity against enveloped virus. Therefore, two purified saponin fractions from *Quillaja* spp. were evaluated regarding their antiviral potential against YFV and CHIKV. The cell line used in this study was VERO (African green monkey kidney cells) since it is permissive to the replication of both viruses. The antiviral activity of both saponins fractions was screened using the plaque reduction assay protocol. Although saponins did not inhibited YFV replication, they strongly inhibited CHIKV. To confirm the absence of antiviral activity of *Quillaja* saponins against YFV, the cytopathic effect inhibition assay was performed also. Further studies are required to determine the antiviral mechanisms involved in the CHIKV inhibition.

Keywords: Antiviral; *Chikungunya virus*; Quil-A®; *Quillaja brasiliensis*; saponins; *Yellow Fever virus*

1. Introduction

Blood-feeding arthropods, like mosquitoes and ticks usually carry viral agents (commonly called arboviruses) and remain as an important concern worldwide especially in tropical regions due to their prevalence [1]. *Yellow Fever Virus* and *Chikungunya* virus are remerging neglected pathogens that have the potential to cause epidemies [2]. Although these infections tend to be self-limited, they often leave sequelae that can last for years [2].

The *Yellow Fever Virus* (YFV) was the first viral disease described for humans in 1927. It is a 40 nanometers single-stranded RNA enveloped virus belonging to the *Flaviviridae* family genus *Flavivirus* [3–7]. Clinical manifestations are variable, and the infection can occur asymptomatic or in severe forms, as individuals can undergo spontaneous cure or hemorrhagic shock [8–10]. Even with the immunization, yellow fever (YF) causes recurring outbreaks with devastating outcomes specially in South America and Africa [5,11].

The Chikungunya Virus (CHIKV) is a member of the *Togaviridae* family genus *Alphavirus* first described in 1952. It is an enveloped virus, and its genome consists of a single-strand positive RNA [12]. A typical clinical symptom is the immobilizing arthralgia that usually have long-term sequelae, mostly because the virus can create a reservoir and evade the immune response. There is no immunization for this infection and the treatment focus on alleviating the symptoms [13,14].

For both viral infections there is no specific antiviral treatment approved [7]. Therefore, the increase in the number of cases associated with the comorbidities caused by these infections suggests the search for molecules with specific activity against these viruses [15]. In this context, saponins are being studied regarding their antiviral properties against enveloped and non-enveloped virus [16]. They consist of a natural occurring glycosides found in a diversity of plants, small crustaceans and some bacteria [17,18]. Because of its amphiphilic structure, they easily interact with cholesterol and phospholipids, leading to morphological changes in cell membranes, triggering different biological activities, including antimicrobial [18]. Quil-A® is a commercially available purified fraction obtained from *Quillaja saponaria* and is widely studied, to which many antimicrobial activities are reported, including antiviral [16,19]. A concern regarding the use of *Q. saponaria* saponins refers to plant depredation since the barks are rich in saponins. In this context, alternative renewable sources are of major importance. Special attention has been paid to the congener specie *Quillaja brasiliensis* since the highest content of saponins are found in the leaves [20,21], which decreases depredation. A purified fraction named Fraction B (FB) demonstrated similar constitution to *Q. saponaria* [22], and it can be inferred that they share similar biological properties. It is well established the obtaining method for Quil-A® as it is a commercial product. On the other hand, FB is obtained by solid phase extraction of the aqueous extracts of *Q. brasiliensis* and characterized as previously described [22]. Although previous reports demonstrated antiviral activity for *Q. saponaria* [19,23], no data is described regarding its activity against arboviruses. Therefore, for the first time, we report the *in vitro* antiviral activity of a Quil-A® and FB against YFV and CHIKV.

2. Material and Methods

2.1 Cells and Viruses

VERO cells were grown on Eagle's Minimum Essential Medium (MEM, Sigma-Aldrich, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Cultilab, Brazil) and maintained at 37ºC in humid atmosphere with 5% CO_2. *Yellow fever virus* 17D and CHIKV were kindly given by Dr. Laura Helena (Fiocruz, Brazil), propagated and titrated in VERO cells.

2.2 Saponin Fractions

Quil-A® was purchased from Breentag Biosector (Frederikssund, Denmark). Fraction B (FB) was kindly given by Dr. Fernando Chiesa (Universidad de la República de Uruguay, Uruguay) and obtained as previously described [22]. Both saponin fractions were resuspended in MEM and filtered using 0.22 µm polyethersulfone membrane syringe filter (Millipore, USA). Once filtered, saponin fractions were stored in -80ºC ultrafreezer.

2.3 Cell Viability Assay

Cell viability was measured by mitochondrial activity using MTT (Thiazolytetrazolium bromide, Sigma-Aldrich, USA) assay as described by Mosmann [24] and Fotakis and Timbrell [25] Briefly, $1.5x10^5$ VERO cells were plated and incubated for 24 hours without FBS. Then, cells were treated with solutions of Quil-A® or FB starting at 25 µg/mL and incubated for 24, 48 and 120 hours, referring to acute and prolonged exposition, respectively. After incubation, compounds were removed and an MTT solution (1 mg/mL) was added and left incubating for 30 minutes. Then, the overlay was removed and Dimethyl sulfoxide (DMSO- Sigma-Aldrich, USA) added. The plate was read using SpectraMax M3 microplate reader (Molecular Devices, USA) at 570 nm and the results expressed as percentage of viable cells compared to untreated control. The concentrations that reduced 50% of cell viability (CC_{50}) were determined using non-linear logistic regression model. The percentages refer to the average of three independent experiments.

2.4 Plaque reduction assay

The plaque assays were performed as previously described [26] with some modifications. Briefly, VERO cells were plated and incubated for 24 hours. Then, the plates were infected with 100 PFU/mL at 37ºC with humid atmosphere with 5% CO_2. For YFV, after 2 hours of viral adsorption, a 1:1 (v/v) solution of 1.5% carboxymethylcellulose (CMC) diluted in high glucose Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 2% penicillin/streptomycin and 20 % FBS, was added. Two wells were reserved as negative control (without virus or saponins), two as viral control (without saponins) and two as cytotoxicity control, which received supplemented CMC with the highest saponins concentration (5µg/mL) tested in the experiment. The other wells were treated with serial solutions of FB or Quil-A® in duplicate. For CHIKV, the adsorption time was 1 hour, and the CMC solution consisted of a 1:1 (v/v) solution of 3% CMC with MEM without FBS and antibiotics.

Lysis plaques were counted using a magnifying glass, and the percentage of inhibition of viral

replication was calculated according to the formula: (mean number of plaques counted in each concentration tested / number of plaques in viral control) x 100.

2.5 Cytopathic effect inhibition assay

The cytopathic effect inhibition assay (CEI) was conducted as previously described [27] with adaptations. A 96-well plate was prepared with VERO cells at a concentration of $1.5x10^{5}$ cells/mL and incubated for 24 hours at 37 ° C with 5% CO^2 atmosphere. Then, 1:10 serial dilutions of the viral suspension were added, and the plate incubated for two hours. After incubation, the overlay was removed and added MEM to the first four columns, reserved for viral titration. The last four columns were treated with a single concentration of the saponins (5µg/mL). The two central columns were reserved as negative control, to which the last four wells were used as cytotoxicity control and were also treated. The microplate was incubated for five days for viral replication. The reading was performed using inverted microscope and the titer determined using the Spearman & Kärber method [28].

3. Results and Discussion

Two purified saponins fractions were screened to access their cytotoxicity. For the acute exposition, the cytotoxic concentration for 50% of the cells (CC_{50}) were 18.1 and 25 µg/mL respectively. Then, the cells were exposed to 48 and 120 hours, referring to CHIKV and YFV replication times. Quil-A® was more toxic than FB at all exposure times (Table 1).

Table 1. Cytotoxicity of Quil-A® and FB.

	Quil-A® CC_{50} (µg/mL)	**FB CC_{50} (µg/mL)**
Acute (24 hs)	18.11	25.05
48 hours	10.85	14.68
120 hours	9.27	13.1

Cytotoxicity is the crucial factor that determines whether a molecule will be tested for its biological properties. Molecules which exhibit activity at toxic concentrations are not feasible for use in commercial formulations and therefore are discarded or undergo structural changes in order to reduce toxicity [29]. Because of the amphiphilic structure of the saponins, they have affinity with cell membranes. *Q. saponaria* and *Q. brasiliensis* features an aldehyde side chain at carbon 4 (Figure 1), which interacts with free amino acids and membrane proteins, causing changes in the cells which generally lead to loss of cell viability [30,31]. It is hypothesized that highly cytotoxic saponins easily interact with cholesterol and phospholipids, recruiting them from the membrane and redistributing as they are solubilized, causing membranolytic activity. On the other hand, less hemolytic saponins interact with cholesterol in order to alter their distribution without removing them from the membrane, resulting in morphological changes in cell structure, therefore triggering toxicity [32,33].

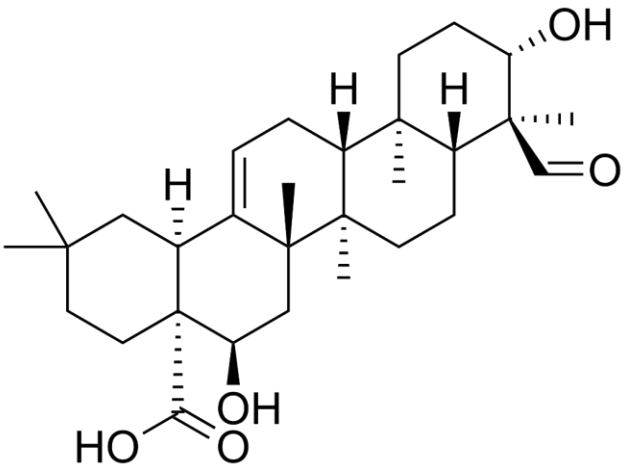

Figure 1. Quillaic acid structure, a common aglycone in both Quil-A® and FB.

Quil-A® and another purified saponins fraction from *Q. brasiliensis* named QB-90 were previously evaluated regarding their toxicity *in vitro* using VERO cells. It was found that 50 µg/mL of Quil-A® resulted in less than 20% of cell viability after 48 hours of exposure, while QB-90 resulted in more than 90%. The same was observed concerning the hemolytic activity, since QB-90 was significantly less hemolytic [34]. The higher toxicity of FB compared to QB-90 may be related to differences in their composition of saponins. Previous studies demonstrated that FB have similar composition to Quil-A®, and therefore similar toxicity properties can be observed between these two fractions [22]. Because of this affinity with cell membrane, saponins are interesting antiviral candidates, since they can interact with viral envelope or capsid, or with an infected cell [23,35].

Using a nontoxic concentration of *Quillaja* saponins, no antiviral activity was observed for the YFV (Table 2). Even with inhibition percentiles of 75.2% and 36.8% for Quil-A® and FB, respectively, these data suggest that both fractions are not antiviral, since a good candidate should inhibit about 95% of replication [36].

Table 2. Antiviral properties of both saponins' fractions, expressed as the percentage of inhibition of viral replication.

	Percentage of YFV inhibition		**Percentage of CHIKV inhibition**	
	Quil-A®	FB	Quil-A®	FB
5 µg/mL	75.26	36.8	100	93
2.5 µg/mL	66	50	32	24.9
1.25 µg/mL	27.8	40.8	7.9	19.3

To confirm the lack of antiviral activity, we used CEI assay to determine whether a single concentration of the tested compounds could inhibit viral replication. At a concentration of 5 µg/mL, the viral titer (number of viral particles) did not change (Table 3), indicating that both FB and Quil-A® are not antiviral agents for YFV.

Table3: YFV viral titer during the Cytopathic effect inhibition assay in VERO cells

	With Antiviral treatment	Without Antiviral treatment
FB (5µg/mL)	$2.47x10^6$	$4.39x10^6$
Quil-A® (5µg/mL)	$7.83x10^6$	$1.65x10^6$

Both saponins fractions were evaluated against CHIKV, another emerging arbovirus that caused an outbreak in Latin America back in 2014 [37]. In contrast to YFV results, *Quillaja* saponins have been shown to be quite effective in blocking viral replication. Quil-A® reduced the number of lysis plaques by 100%, while FB reduced 93% (Table 2).

Previous reports demonstrated antiviral activity for *Q. saponaria* aqueous extracts against non-enveloped [19,35] and enveloped viruses [7,38,39]. It is known that saponins can inhibit replication by degrading enzymes or lysing replicating sites, by directly degrading viral envelope or capsid and by causing modifications in cell morphology, blocking the infection [23,35,40].

Flavivirus replication is similar to *Alphavirus*, as the biggest difference resides in the fact that *Flavivirus* viral assembly occurs in the endoplasmic reticulum and maturation in the Golgi apparatus, while for *Alphavirus* it occurs in cytoplasm and it matures as the virion buds to plasma membrane [41–44]. These differences could be related with not inhibition YFV by *Quillaja* saponins.

It is known that the *Flaviviridae* family have some miRNA transcripts responsible 'helping' transcription process. Interestingly, there is evidence that when an exogenous signal that may impair the replication process is recognized, some miRNA interfere, inducing the virus to evade the antiviral response [45–47]. Although we did not investigate the molecular mechanisms, this might be one of the reasons why saponins could not inhibit viral replication of YFV.

4. Conclusion

This study reports the antiviral properties of two *Quillaja* spp. purified fractions against the YFV and CHIKV. Although no antiviral activity was observed for YFV, Quil-A® and FB strongly inhibited CHIKV replication, *in vitro*. The antiviral activity of *Quillaja* saponins against CHIKV was first reported in this work and reinforce the current data about antiviral properties *Quillaja* saponins against enveloped and non-enveloped viruses. Further investigations are required to better understand the antiviral mechanism.

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