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In vitro efficacy of *Quillaja saponaria* extracts on the infective life-stage of ectoparasite *Caligus rogercresseyi*

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Abstract

The effect of two different commercial products of *Quillaja saponaria* saponin extract on mortality of the free-living copepodite stage sea-lice *Caligus rogercresseyi* were assessed in vitro. Parasites were exposed for 24 hr to different concentrations of *Quillaja* extracts and then observed until 30 hr in non-*Quillaja* media. The EC_{50} and EC_{90} were evaluated at 30 hr. High mortalities of copepodites were obtained at higher doses (500 ppm) for both extracts. Higher mortalities were obtained using the product with higher saponin content at 24 hr (23.3% vs. 86.6%) and 30 hr (63.3% vs. 93.3%) compared with the product containing less saponins. In addition, mortalities increased even after removing the extracts suggesting a residual and prolonged

effect on the survival of the parasite. Our results indicated that the free-living stage of the parasite is sensitive to saponins from *Q. saponaria* extracts and may be used to control or prevent the infestation of *C. rogercresseyi* in salmonids in Chile.

KEYWORDS
Caligus rogercresseyi, copepodid, *Quillaja saponaria*, salmon, sea lice

1 | INTRODUCTION

Sea lice infestations in farmed salmon are considered one of the most important fish health issues in countries such as Norway, the United Kingdom, and Chile (McNair, 2015; Torrissen et al., 2013). In the northern hemisphere, *Lepeophtheirus salmonis* is the main louse species affecting wild and farmed salmon, whereas *Caligus rogercresseyi* affects salmon farming in the southern hemisphere (Torrissen et al., 2013). The global negative economic impact of these fish parasites in 2006 was estimated in US$ 480 million, affecting the health and welfare of approximately 500 million of animals (Torrissen et al., 2013; Yatabe, Arriagada, Hamilton-West, & Urcelay, 2011).

Governmental initiatives such as the National Active Surveillance Program for Sea lice in combination with the Chilean salmon industry stakeholders have proved useful to decrease the negative effects on fish health and welfare. The National Program is based on the early detection and control of highly prevalent farms with consecutive compulsory treatment. However, the main tool to control infestation has been the use of antiparasitic drugs given orally or by bath, which are not entirely successful as the parasite has adapted quickly (SERNAPESCA, 2015). For example, in Chile approved oral antiparasitic drugs such as emamectin benzoate, azamethiphos, and dichlorvos are administered by bath, diflubenzuron were successfully used in recent years but parasite resistance generated quickly making these therapies currently almost ineffective (Agusti et al., 2016; Bravo, Sevatdal, & Horsberg, 2010; Bravo, Silva, Agusti, Sambra, & Horsberg, 2015; Bravo, Silva, & Monti, 2012). Also, immune-stimulating substances or prebiotics (i.e., β-glucans) are used to maintain or increase the resistance of fish to parasite infestation (Jensen, Provan, Larssen, Bron, & Obach, 2014) and classical preventive measures such as vaccines have been used with very limited positive effect as immune mechanisms (i.e., antibodies and lymphocytes) do not reach the skin and mucus efficiently (Carpio et al., 2011).

Plant extracts have been increasingly used as alternatives to synthetic drugs as a way to increase the sustainability of farmed animals (Makkar, Francis, & Becker, 2007). Plant extracts have been used to increase nutrient uptake and growth (Chakraborty, Horn, & Hancz, 2013), enhance the fish immune system as prebiotics (Harikrishnan, Balasundaram, & Heo, 2011) and for bacterial (Reverter, Bontemps, Lecchini, Banaigs, & Sasal, 2014) and viral (Harikrishnan et al., 2011) infections in fish. However, only a few plant extracts have been tested for external parasite infestations.

Quillaja saponaria trees are rich in triterpenoids saponins (San Martín & Briones, 2000). The extracts have been successfully used as an antifungal (Chapagain, Wiesman, & Tsror, 2007), antibacterial (Arabski, Wegierek-Ciuk, Czerwonka, Lankoff, & Kaca, 2012), antiviral (Tam & Roner, 2011) agent, and as a vaccine coadjuvant (Dalsgaard, 1974) in animals and humans. Industrial uses of saponin are well documented such as food additives (Yang, Leser, Sher, & McClements, 2013), cosmetic, and mining (San Martin, Otero, & Cruz, 2005). The in-feed and bath immersion routes of administration of *Q. saponaria* extracts have shown an immunostimulatory effect in aquaculture (Ng'ambi et al., 2016; Su & Chen, 2008; Wang et al., 2016).

The use of *Q. saponaria* extracts against *C. rogercresseyi* in salmon has not been reported before and it may become a new, sustainable, and useful way to control the infestation. The objective of this study was to investigate the sensitivity of copepodids of *C. rogercresseyi* to different commercially available products of *Q. saponaria*.

2 | MATERIALS AND METHODS

2.1 | *Quillaja saponaria* products

Two liquid-based commercial *Quillaja saponaria* products from Desert King Chile S.A. were tested: QL-35 (30–32% p/v solids; with an 8.92% saponin content, p/v) and QL-1000 (43–45% p/v solids; with a 17.43% saponin content, p/v). A stock solution of 1 mg/mL of each product was prepared using filtered sterile (5 μm and UV-disinfected) seawater obtained from a nearby sea-farm and refrigerated at 12 ± 1°C until further use.

2.2 | Parasite collection

Live female ovigerous parasites (50 females) were obtained from Atlantic salmon, *Salmo salar* in two salmon farms located within 50 km of Puerto Montt (41°48″S and 73°20″W) in southern Chile. The farms had not been treated with any antiparasitic drug during the current growing phase in sea. The parasites were collected directly from the surface of the fish using tweezers and transferred to the laboratory facilities where they were kept in a thermally regulated chamber (12 ± 1°C), salinity (30‰) and constant aeration (85 ± 5% of saturation) from collection until hatching of larvae (nauplii). These environmental conditions were maintained constant during the study. Subsequently, the larvae were placed in containers for incubation using the same environmental conditions until achieving the copepodid stage (approximately 5 days).

2.3 | Sensitivity tests

The sensitivity tests were performed according to established protocols for the evaluation of resistance/susceptibility to chemical compounds in sea lice (Horsberg, Jackson, Haldorsen, Burka, & Colleran, 2006). Dilutions with concentrations of 10, 50, 100, and 500 ppm were obtained through dilutions with filtered and sterilized seawater from the stock commercial solution. Negative control groups (No *Q. saponaria* added) were maintained the same way as treated groups. Each experimental group was tested in triplicate.

For each replicate, 10 copepodids were placed in petri dishes with the dilutions described previously.

The vitality of copepods was assessed before starting the experiments by observation of their swimming activity and response to light stimuli or air bubbling, as described below.

The saponin exposure time of *C. rogercresseyi* copepodids was 24 hr, after which all copepodids were washed with filtered seawater, transferred, and maintained in untreated seawater container for a further 6 hr reaching a final 30 hr evaluation time. The latter was carried out to evaluate any potential recovery or persistence of the effect after exposure.

Evaluation of copepodid's sensitivity was carried out at 24 and 30 hr for each product using a stereoscopic magnifying glass by two experienced researchers. The researchers were blinded to experimental groups. The number of living, dying, and dead copepodites was quantified categorically as follow:

- Living: copepodid was swimming actively, with the movement of the appendages and rapid reaction to light stimuli and producing air bubbles.

- Dying: erratic swimming, little reaction to stimuli, and little movement of appendages.
- Dead: no swimming, floating in the water, no reaction to light stimuli, and no appendages movements.

2.4 | Analysis of results

A probit regression model (Ross & Finney, 1972), obtaining the effective concentration (EC_{50}, 50% dead; EC_{90}, 90% dead), was calculated as the sum of dead copepodites.

Descriptive statistical analysis was carried out for each product according to evaluation time and concentrations used. Assessment of statistical differences in concentration and time for each *Q. saponaria* product was carried out using analysis of variance and multivariate analysis of variance. All statistical analyses were carried out using R (R Development Core Team, 2014).

3 | RESULTS

All *Quillaja* commercial products were able to kill the copepodids at 500 ppm. For QL35 experiments, there were statistically significant higher mortalities at 500 ppm at 24 hr ($F_{3,8} = 49$, $p < .001$, 23.3 ± 5.7%) compared to lower concentrations. Mortality increased further and significantly after transfer to clean seawater at 30 hr ($F_{3,8} = 6.23$, $p = .05$, 63.3 ± 5.7%), compared to lower concentrations of QL35 (Figure 1).

A similar situation occurred when using QL1000, where 500 ppm of the product produced statistically significant higher mortalities at 24 hr ($F_{3,8} = 214.6$, $p < .001$) compared to lower concentrations. However, mortality did not increase significantly ($F_{6,24} = 1.29$, $p = .29$) after transfer to clean seawater at 30 hr ($F_{3,8} = 70.6$, $p < .001$; Figure 2).

Comparatively, at 500 ppm product concentration, QL1000 had significantly higher mortalities than QL35 at 24 hr (86.6 vs. 23.3%, $F_{1,4} = 180.5$, $p < .001$) and also at 30 hr (93.3 vs. 63.3%, $F_{1,4} = 40.5$, $p = .003$).

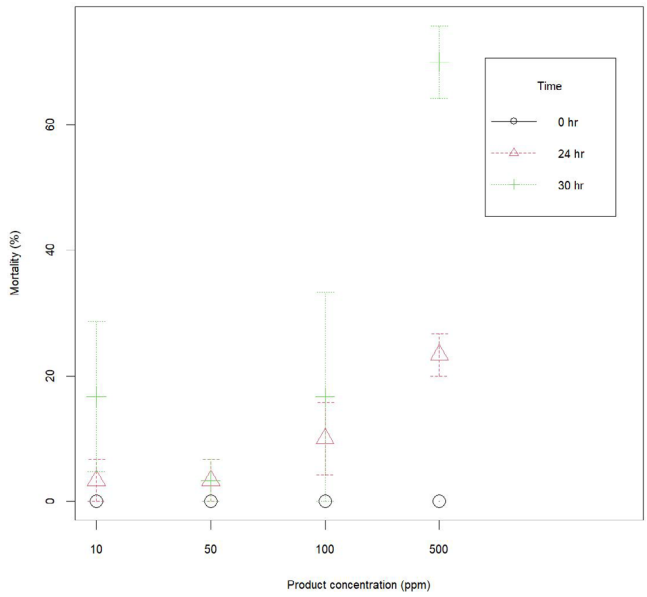

FIGURE 1 Mortality (mean ± SD) of *C. rogercresseyi* at 0, 10, 50, 100, and 500 ppm of QL35 at 24 and 30 hr

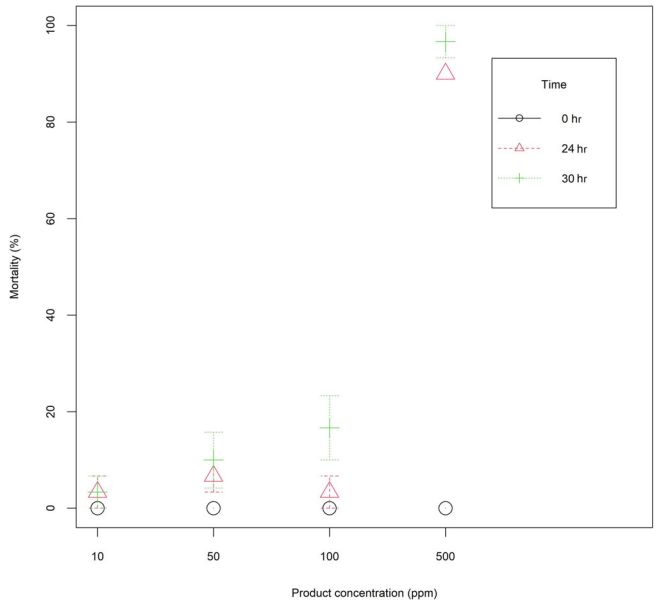

FIGURE 2 Mortality (mean ± SD) of *C. rogercresseyi* at 0, 10, 50, 100, and 500 ppm of QL1000 at 24 and 30 hr

TABLE 1 Effect concentration (EC_{50} and EC_{90}, ppm of product) of QL35 and QL1000 at 24 and 30 hr for *Caligus rogercresseyi* copepodites

Product	Saponin content (%, p/v)	EC_{50} (ppm)	EC_{90} (ppm)
QL35	8.92	274	990
QL1000	17.43	127	492
Ratio QL35/QL1000	0.511	0.46	0.49

There was almost no mortality at concentrations of 10, 50, and 100 ppm in both products. Most copepodids exposed with QL35 or QL1000 at 10, 50, or 100 ppm at 24 hr were still alive (83% of copepodids were actively swimming).

The EC_{50} and EC_{90} for QL35 were almost twice as high compared to QL1000, and similar to the relation between saponin concentration in products (Table 1).

4 | DISCUSSION

Our results demonstrated the effect of commercially available *Q. saponaria* products on the survival of the infective stage of *C. rogercresseyi*. Results showed that QL1000 was more effective than QL35, mainly at 500 ppm. Concentrations below 500 ppm did not have a clear effect on copepodids, with more than 75% of copepodids surviving within each treatment group (10, 50, and 100 ppm) for both *Q. saponaria* products.

Although QL1000 and QL35 are saponin rich extracts, QL1000 had a higher concentration of saponins. Also, *Q. saponaria* extracts contain other bioactive natural compounds apart from saponins such as tannins, polyphenols, and polysaccharides (Jiang, Hansen, Strobel, & Cedergreen, 2018; Maier et al., 2015). In fact, Jiang et al. (2018) showed that a dried quillaja bark extract consisted of 67.2% saponins and 32.8% nonsaponins. However, the ratio

saponin:nonsaponin fraction depends on the nature of extracts and purification grade. In our study, QL35 and QL1000 had 30 and 45% of soluble solids representing a very similar nonsaponin content of 23 and 27%, respectively. If these nonsaponin fractions were responsible for the effects on copepodids, then a similar EC_{50} and EC_{90} would have been achieved; a situation that did not happen in our experiments.

On the other hand, QL1000 has 1.95 times more saponin than QL35 suggesting that the effect on copepodids is both saponin and concentration-dependent, as double of concentration of QL35 is needed to achieved EC_{50} and EC_{90} as compared to QL1000. These results are in agreement with those of Jiang et al. (2018) who found saponin was responsible for the toxicity in daphnia, *Daphnia magna*, and zebrafish, *Danio rerio*, embryos.

The results show that the commercial *Q. saponaria* product from Desert King Chile had a significant negative effect on copepodids at 500 ppm and that while it is likely that the saponin component may be the active ingredient, this needs confirmation. The concentration of saponin required to have any effect on copepodids was 44 ppm (using QL35 500 ppm at 24 hr) and 88 ppm (using the QL1000 500 ppm at 30 hr). However, future studies should be conducted with more saponin-purified extracts to confirm that saponins are responsible as in this present study. Similar effects have been reported in other insects and arachnids. For example, a strong aphicidal effect was observed using *Q. saponaria* (Sigma®, 10% sapogenin) against pea aphids given orally ($LC_{50} = 0.55$ mg/mL) or by direct contact ($LC_{50} = 8.2$ mg/mL), but also had a higher deterrent effect (De Geyter, Smagghe, Rahbe, & Geelen, 2012). The LC values reported by De Geyter et al. (2012) were much higher than those reported here suggesting that terrestrial insects might be more resistant to *Quillaja* saponins than aquatic organisms. This is supported by experiments carried out using saponins against *Aedes aegyptis* and *Culex pipiens* aquatic larvae (Pelah, Abramovich, Markus, & Wiesman, 2002). In Pelah et al. (2002), *Quillaja* saponin (Sigma®, 10% sapogenin) had 100% larvicidal effect on *A. aegyptis* (800 mg/L) and *C. pipiens* (1,000 mg/L) when exposed for 5 days. Our results suggest that terrestrial insect may also be susceptible to saponins but only for the aquatic larvae life cycle.

Other sources of saponins such as tea saponins have shown to be efficient in controlling insects (Chaieb, 2010), but this is the first evidence of the use of commercial and standardized formulations of saponins for the use against *C. rogercresseyi*.

The proposed mechanism of action of *Quillaja* saponins on arthropods and insect cultured cell lines is over cell membrane permeability forming vacuoles and pores (De Geyter et al., 2012), thus decreasing the internal insect homeostasis. This mechanism is due to physical and chemical affinity and not receptor-mediated, thus no resistance should be developed. Future studies should aim to verify this, in which case the use of *Quillaja* saponin would be of great interest for the aquaculture industry as *C. rogercresseyi* has developed resistance to almost all currently used synthetic drugs such as emamectin benzoate (Bravo et al., 2010, 2012), organophosphorus compounds and pyrethroids (Bravo et al., 2015).

Environmental conditions (pH, salinity, oxygen concentration, and saturation) did not change throughout the experiment suggesting that *Q. saponaria* products have no effect on water quality increasing the potential safety of the product. This is supported further as saponins from quinoa, *Chenopodium quinoa*, have been proved effective to control apple snail, *Pomacea canaliculata*, infestations without any damage to fish or crustaceans (Joshi et al., 2008; San Martín, Ndjoko, & Hostettmann, 2008), which make the potential use of *Quillaja* saponins as an ecological and sustainable alternative and friendly method for the control of the ectoparasite.

Our results suggest that *Q. saponaria* products could be used as a new tool to control the infestive phase of *C. rogercresseyi* in salmon farming. Currently, the most common strategies for the control of *C. rogercresseyi* in salmon have been focused on the elimination of the chalimi, pre-adults, and adult stages of the parasite (Mancilla-Schulz, Marín, & Molinet, 2019); life stages that are on the fish. These results showed the effect on the free or nonattached stage to the fish, making it possible to use it for water treatment, and increasing the antiparasitic arsenal against Chilean sea lice. Toxicity of saponins in fish is known; but not all saponins are equally toxic (Ashour, El Aziz, & Gomha Melad, 2019). Current evidence shows that *Quillaja* saponins have lower toxicity compared to other saponins both in vitro and in vivo (Cabrera-Orozco, Jimenez-Martinez, & Davila-Ortiz, 2013; Fleck et al., 2019), related to chemistry composition (triterpenic vs. stereoidal or alkaloidic). However, future studies must evaluate the toxicity of the

products at 500 ppm in farmed salmon and determine the practicability and efficacy of the products for 24 to 30 hr in commercial settings.

More importantly, future studies should be aimed to elucidate the mechanism of action of saponin in *C. rogercresseyi* and safety studies on salmonids exposed or infested with the parasite.

5 | CONCLUSIONS

Quillaja saponins products have shown to be potentially useful for the control of the infestive stage of *C. rogercresseyi* ectoparasite. The results showed a persistent effect of the saponins for 6 hr after removing the products. Environmental conditions did not change when *Q. saponaria* products were applied, which suggest that these have no negative effect on water quality increasing the potential safety of the product. More studies are needed to understand the underlying mechanism of action of *Quillaja* saponins on *C. rogercresseyi*.

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CONFLICT OF INTEREST

The authors declared no potential conflicts of interest.

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