Saponins from leaves of *Quillaja brasiliensis*, a new source of immunoadjuvants with low toxicity: biotechnological strategies for improving yields

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Biotechnology World Convention, São Paulo, August 15-17, 2016

OUTLINE OF THIS PRESENTATION:

- A glimpse at the vaccine market and the need for new adjuvants
- Saponins as effective and less toxic vaccine adjuvants
- *Quillaja brasiliensis* as a renewable source of useful saponins
- Immunoadjuvant activity of *Q. brasiliensis* in antiviral vaccines
- Production of *Q. brasiliensis* biomass for saponin supply
- Stimulating saponin production by environmental and hormonal signals
- Future directions

GLOBAL VACCINE MARKET: RAPID GROWTH and CHANGING STATUS

- Tripled in value from USD 5B in 2000 to almost USD 24 B in 2013
  - Influenza vaccine market: estimated at $2.9 billion in 2011 to $3.8 billion by 2018
  - US : $1.6 billion in 2011 to $2.2 billion in 2018
- Global market projected to rise to USD 100 B by 2025
- More than 120 new products in the development pipeline
- 60 are of importance for developing countries
- Vaccines: becoming an engine for the pharmaceutical industry
- Changing status of the vaccines within the pharmaceutical industry
- New business model for vaccines is emerging?

World Health Organization

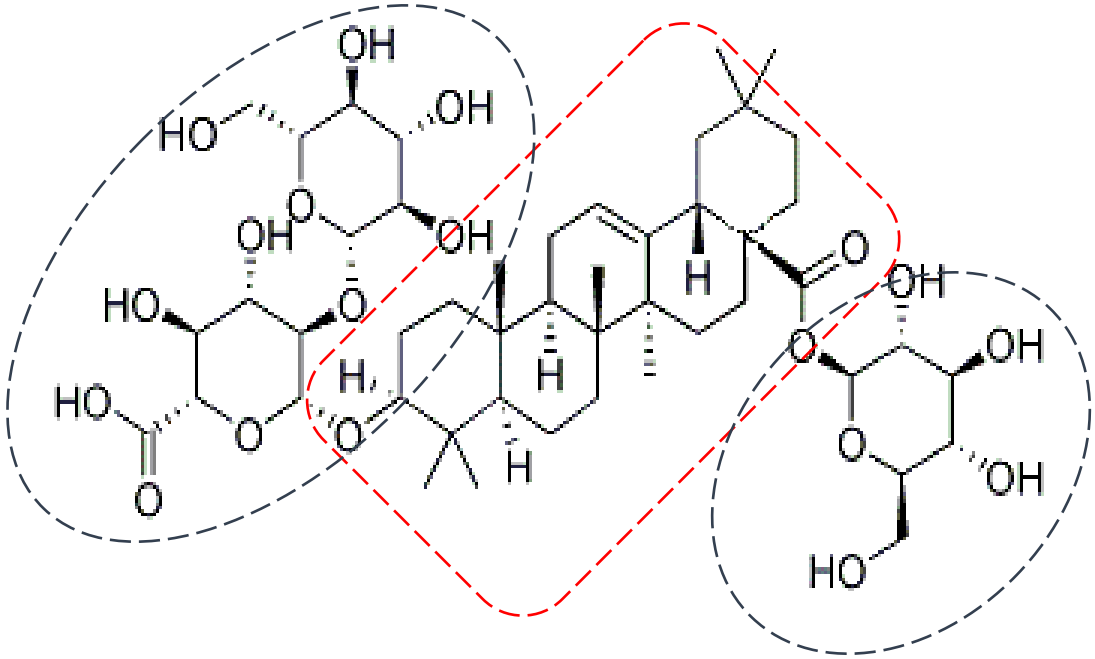
Source: slide presentation by Miloud Kaddar, Senior adviser heath economist – WHO, IVB, Geneva

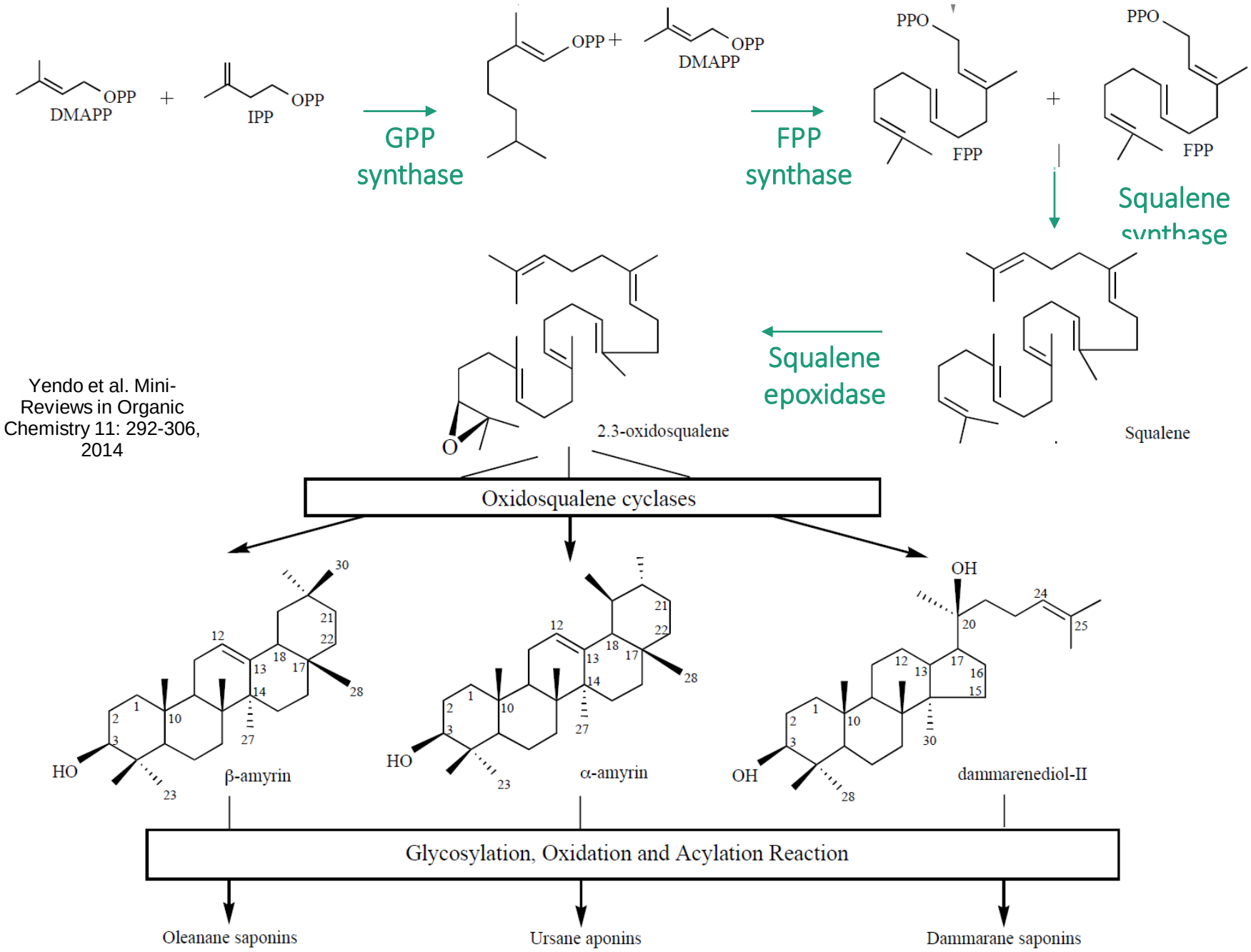
Saponins

Secondary metabolites widely distributed in plants

Lipophilic portions = triterpene or steroidal

Hydrophilic portions = sugars





Yendo et al. Mini-Reviews in Organic Chemistry 11: 292-306, 2014

High Industrial Relevance

- **Food industry**:

  emulsifier and foaming agents;

- **Textile**: fine fabric cleaning;

- **Photographic** industry;

- Pharmaceutical:

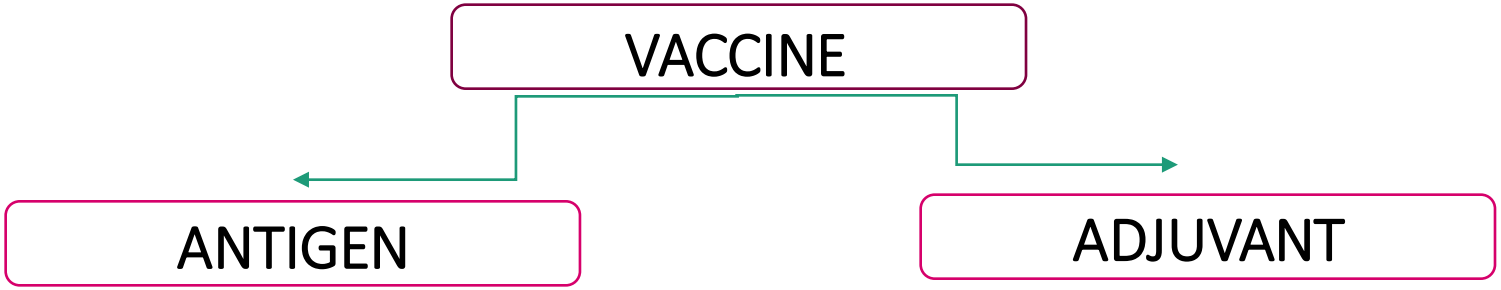
  medicines, vaccines, cosmetics e clinic lab reagents.

High value chemicals of complex and expensive organic synthesis

Immunoadjuvant activity in vaccines → *Panax ginseng*, *P. notoginseng*, *Platycodon grandiflorum*, *Polygala senega* e *Quillaja saponaria*.



De Costa et al., *Mini-Reviews in Medicinal Chemistry*, v. 11, p. 857-880, 2011.



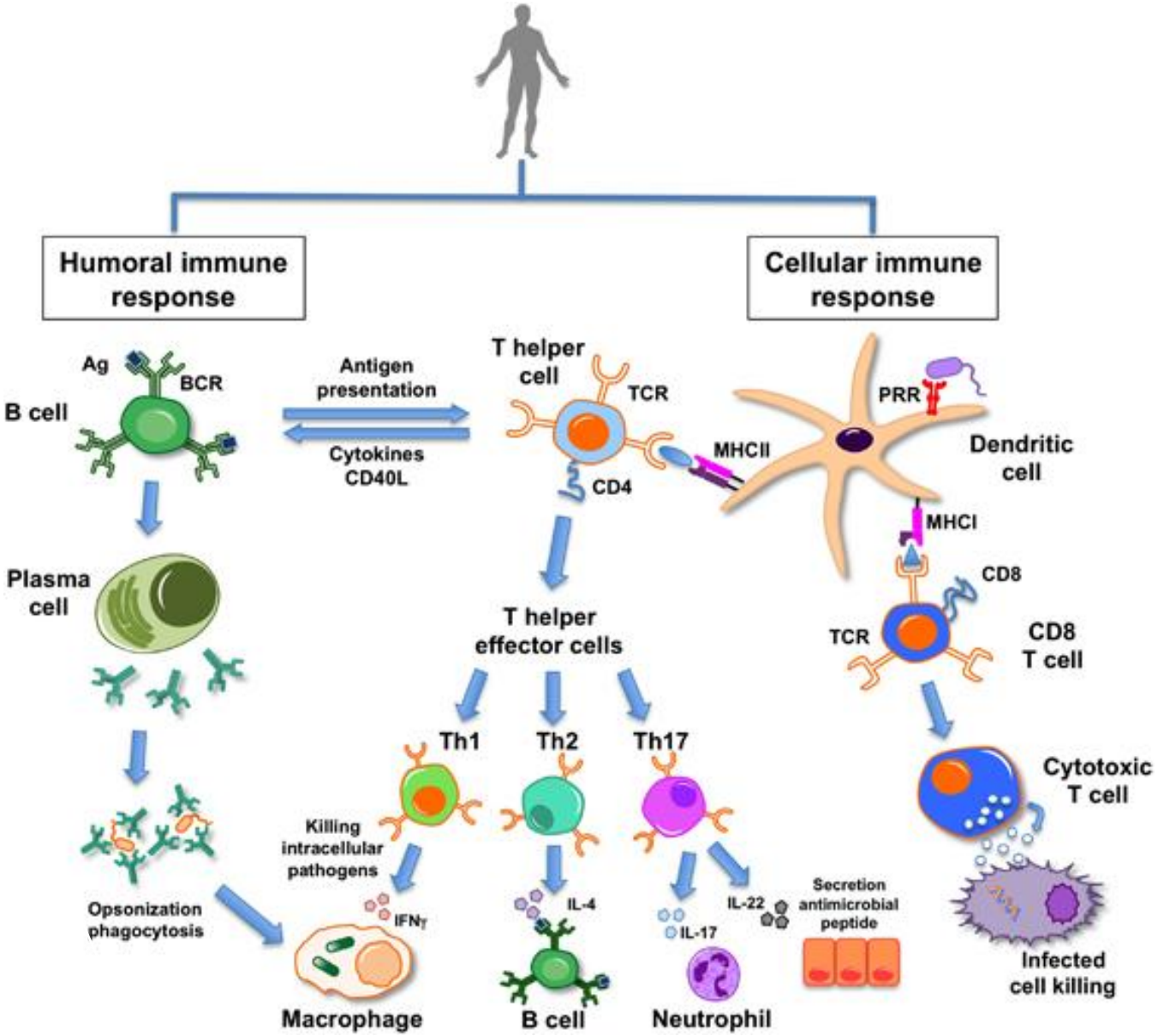
Specificity of immune response

Intensify and prolong immune response

Saves at least 25% of antigen

Very important to boost inactivated virus vaccines

Aguillar e Rodríguez, *Vaccine*, v. 25, p. 3752-3762, 2007.



Mortelaro e Ricciardi-Castagnoli, Immunol. Cell. Biol. 89: 332-339, 2011.

Quillaja saponaria Molina (Quillajaceae)

Bark is one of the main industrial sources of saponins, mostly produced in Chile.

Immunoadjuvant activity: **humoral and celular responses**

Human vaccines in phase III clinical trials:

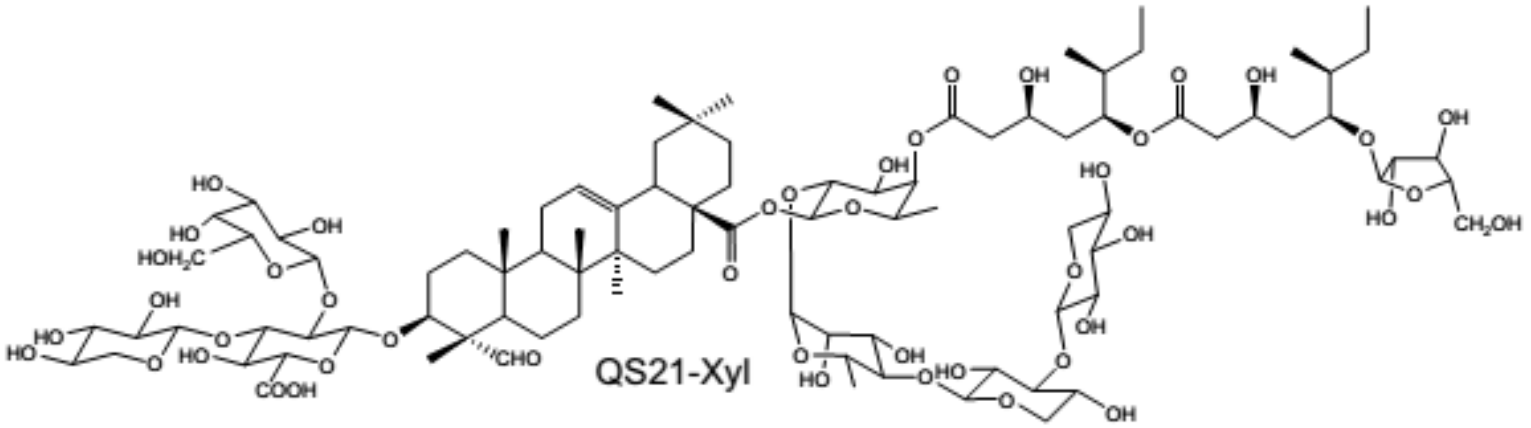
Malaria, melanoma, lung cancer and herpes zoster with over 40,000 patients

Used commercially in veterinary: feline leukemia, equine influenza, BVDV vaccines



Sun et al., Vaccine 27:1787-96, 2009; GlaxoSmithKline, 2013.

Chemical components



Triterpene and bidesmosidic saponins

Aglicones:
- quillaic acid,
- equinocystic acid,
- phytollacagenic acid,
- 22-β-hydroxyquillaic acid

Nord and Kenne, *Carbohydrate Research* 320: 70-81, 1999.

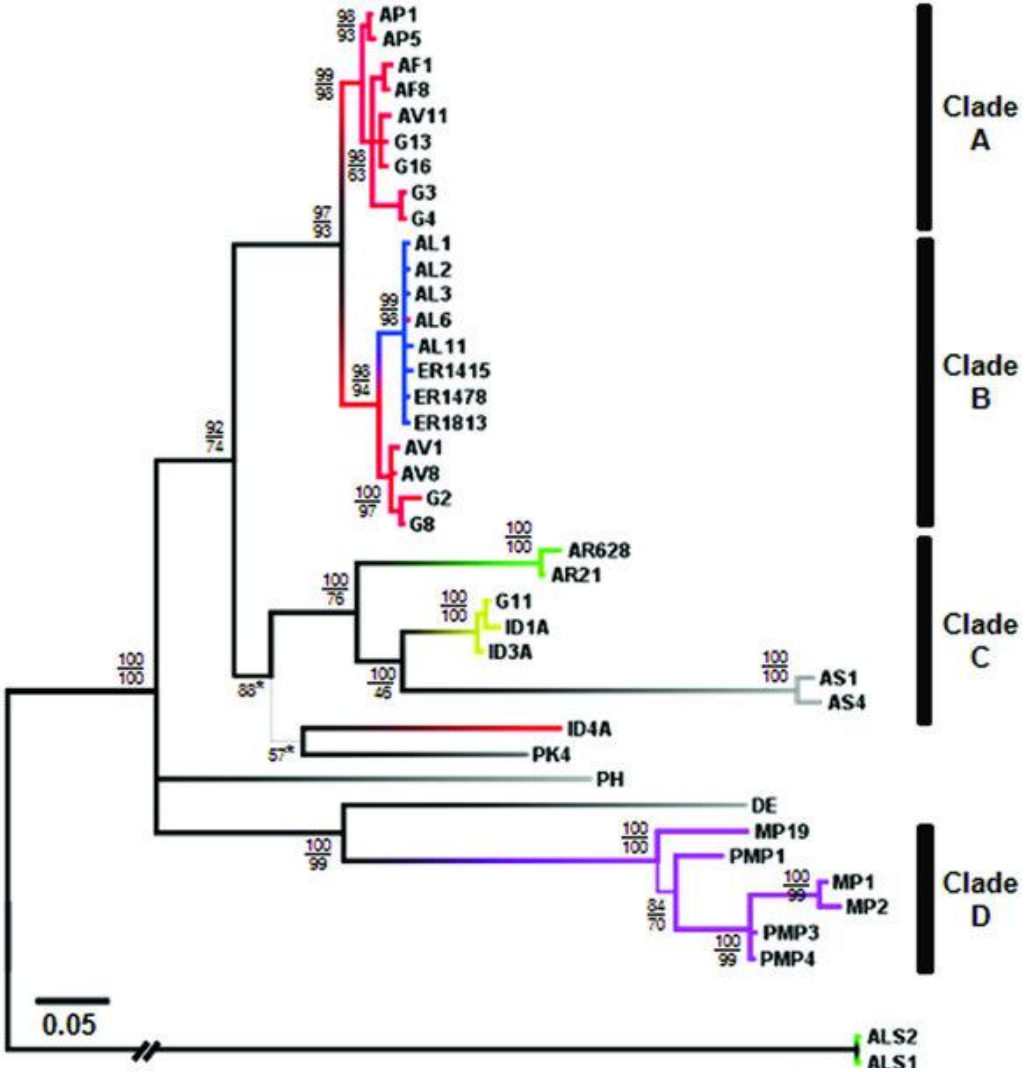
Supply of current demand

- 2015: US$ 13.4 million in *Quillaja* bark raw extract exports from Chile;
- ~ 700 tons per year to USA, Japan and Europe;
- Felling of 28,000 trees / year: each ~ 30 to 50 years old (2012);
  - WHERE CAN WE LOOK FOR VIABLE ALTERNATIVE SOURCES?



Pro-Chile 2013; IEC 2015.

ALTERNATIVE NEW SOURCES? CHEMOTAXONOMY AS A LEAD FOR SEEKING BIOACTIVE PLANTS, i.e. BET ON SIMILAR CHEMISTRY



Quillaja brasiliensis (A. St.-Hill. & Tul.) Mart.

SOAP TREE - Southern Brazil, Uruguay and Northeast Argentina.



Leaves and barks are rich in saponins.





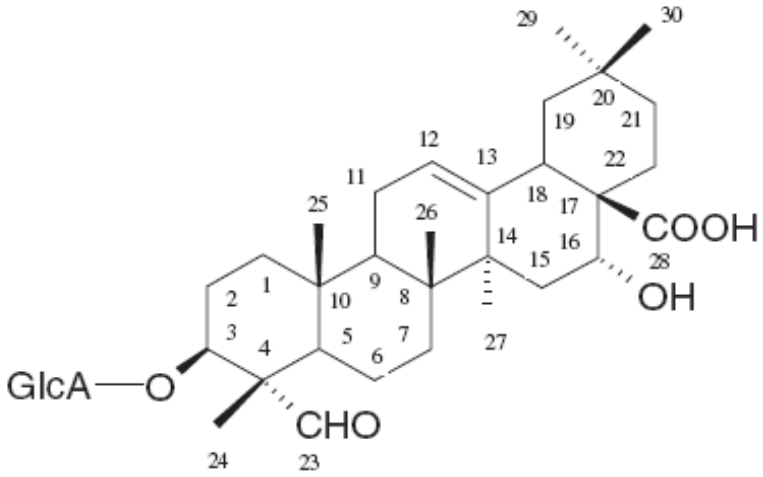




Sobral et al. 2006 - http://www.ufrgs.br/fitoecologia/florars/open_sp.php?img=15002

Saponins found in *Q. brasiliensis* are similar to those of *Q. saponaria*

AE-leaves — Acid hydrolysis — QB-H1



Pro-sapogenin of
3-O-β-D-glucuronopyranosyl-
quillaic acid

AE - leaves

QB-80 QB-90

Saponins with similar chromatographic profile

Kauffmann et al., Nat Prod Res 18: 153-157, 2004.

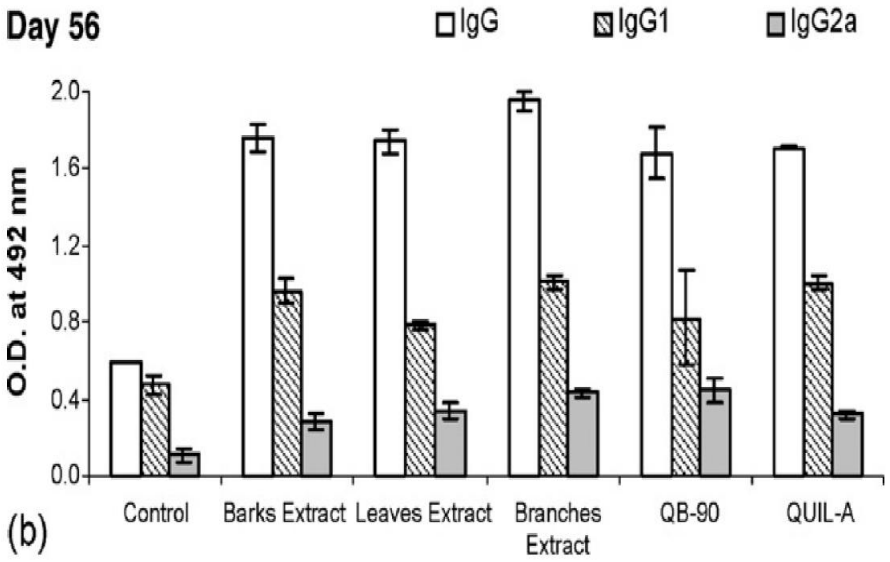
ENCOURAGING, BUT...

ARE THESE SAPONINS ALSO IMMUNOADJUVANT?

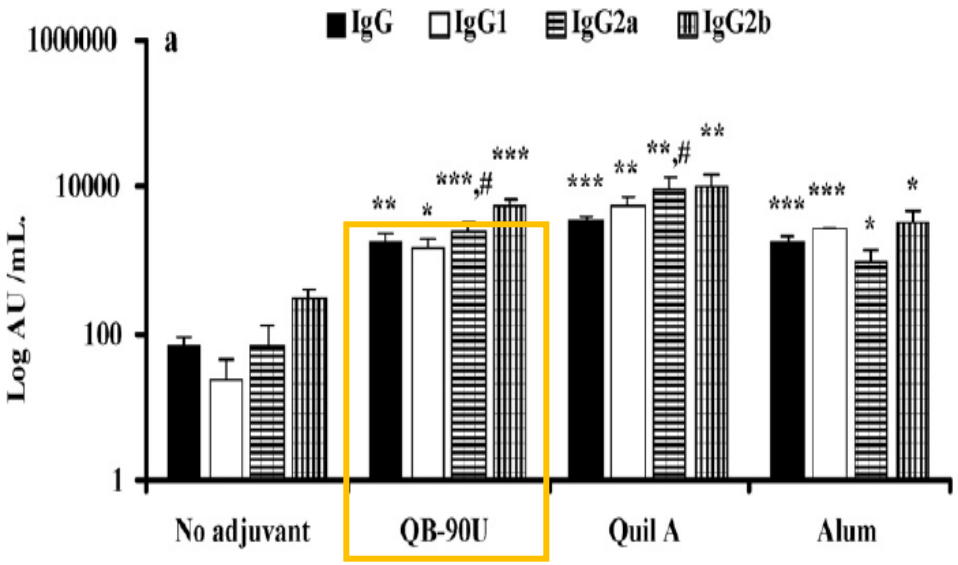
AND IF SO, HOW DO THEY FARE AGAINST Quill A AND OTHER STANDARD ADJUVANTS?

Immunoadjuvant activity on BHV

BHV type I



BHV type V



AE of barks – 100 µg
leaves – 400 µg
stems – 400 µg
Fract QB-90 – 100 µg

→ Comparable to Quil-A of *Q. saponaria* (100 µg)

Fleck et al., Vaccine 24: 7129-7134, 2006; Silveira et al. Vaccine 29: 9177-9182, 2011.

Immunoadjuvant activity on poliovirus

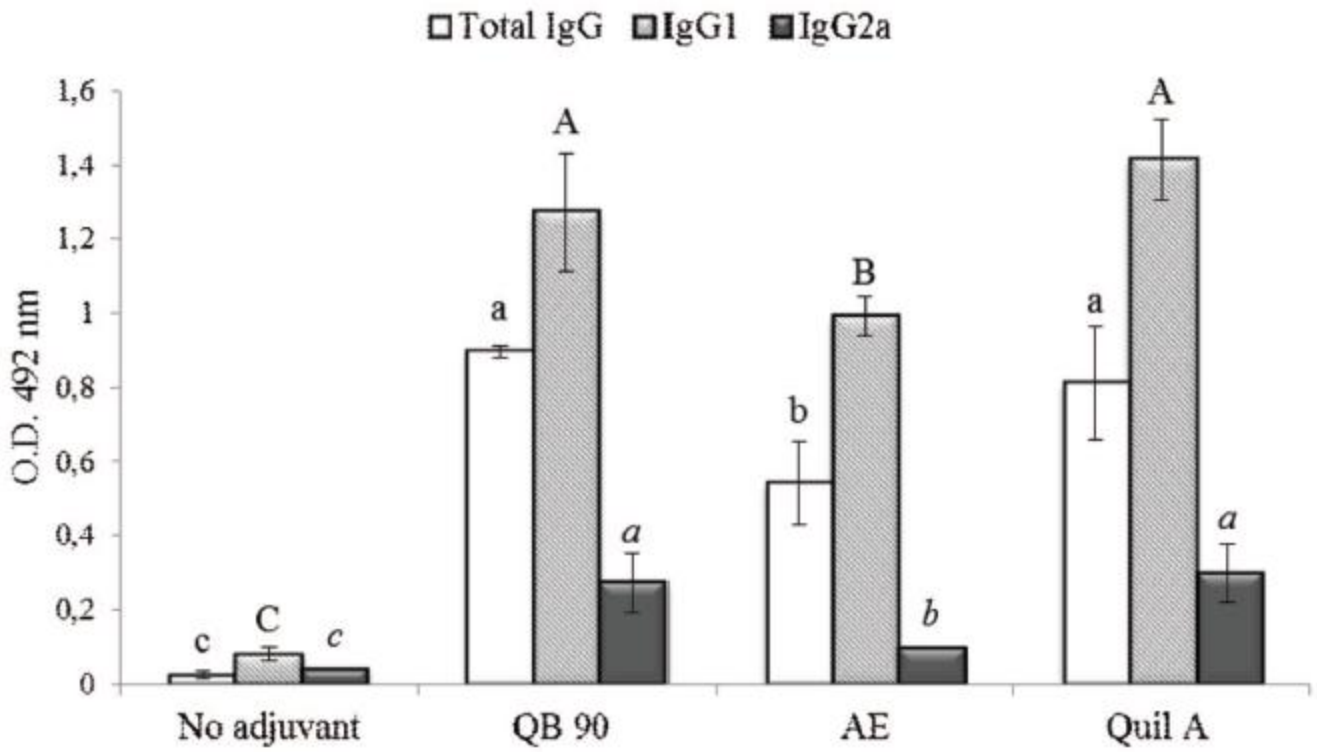
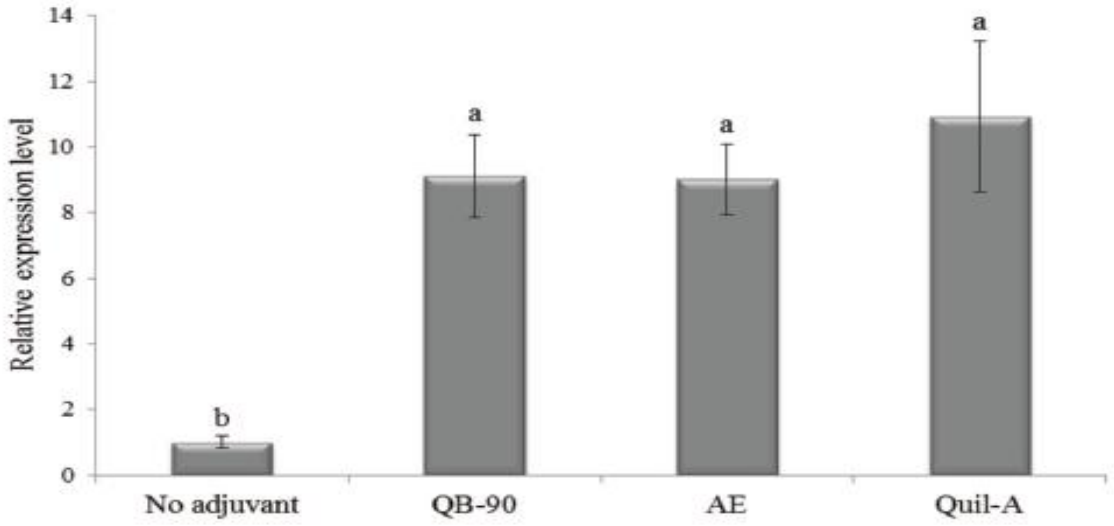


Figure 1. Serum titers of poliovirus total IgG, IgG1 and IgG2a isotypes. Sera were collected one month after the second antigen dose (day 56), administered either with no adjuvant or formulated with QB-90, AE or Quil-A. Results are expressed as the mean value ± S.D. (n = 7). Different lowercase, uppercase and italic letters indicate significant difference among the treatment samples of total IgG, IgG1 and IgG2a, respectively, by a Tukey test ($\rho \leq 0.01$).
doi:10.1371/journal.pone.0105374.g001

Cytokine gene expression with poliovirus vaccines

INF - Y



IL-2

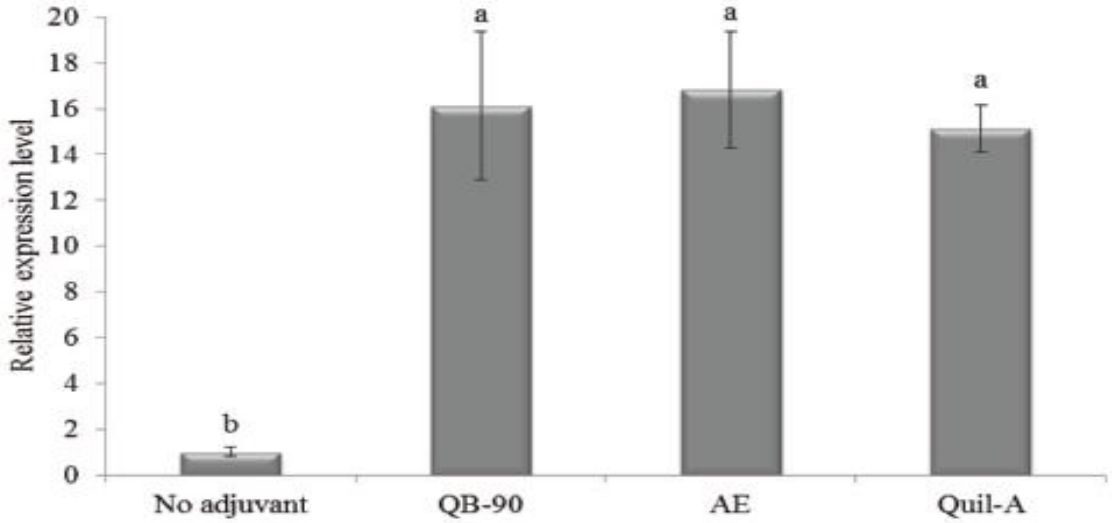


Figure 3. Relative expression profile of IFN-γ (a) and IL-2 (b) in poliovirus-reestimulated splenocytes from mice immunized with poliovirus vaccine containing either no adjuvant or formulated with QB-90, AE or Quil-A, one month after the second immunization. β-actin gene was used as reference to normalize mRNA expression and results were expressed as a ratio relative to the group that received non-adjuvanted vaccine, which was arbitrarily set to 1. The vertical bars represent the S.D. of four biological replicates. Different letters indicate significant difference by a Tukey test ($p \leq 0.01$).
doi:10.1371/journal.pone.0105374.g003

De Costa et al. PLoS One e105374, 2014

Poliovirus vaccines induce mucosal and systemic antibody production

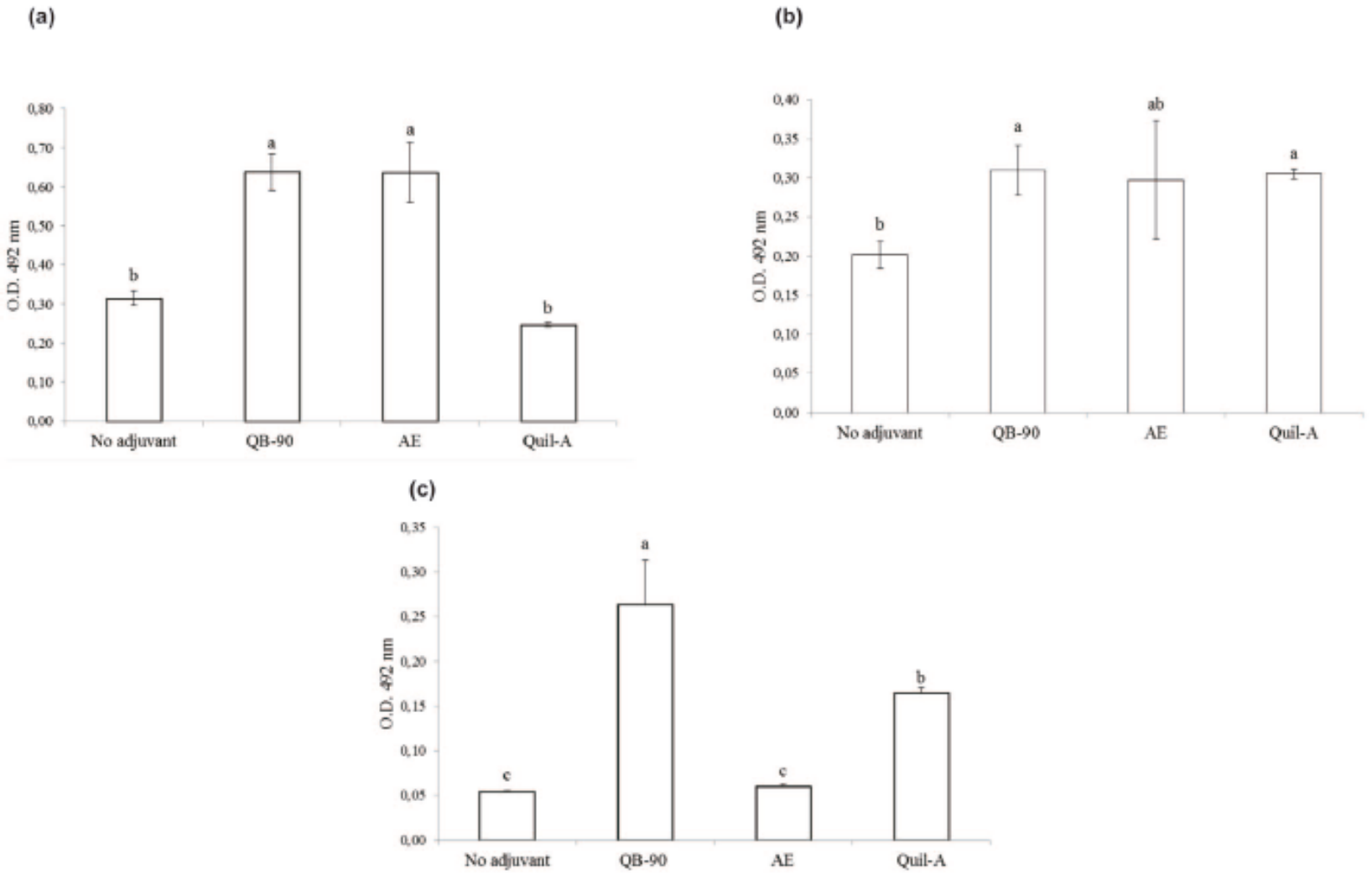
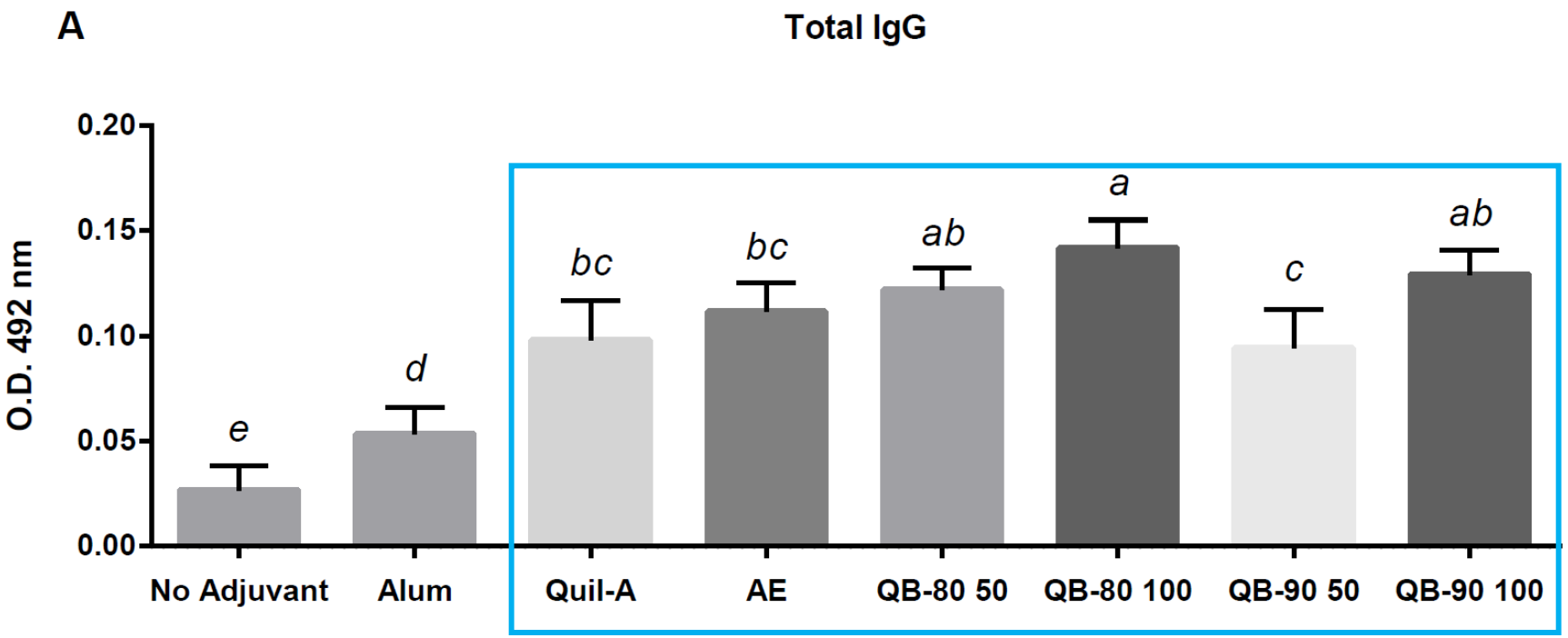


Figure 4. Poliovirus-specific IgA antibody responses in mice vaccinated with non-adjuvanted vaccine or QB-90-, AE-, or Quil-A-adjuvanted preparations in bile (a), fecal (b) and vaginal washings (c). Mice were euthanized at day 56 and bile, feces and vaginal washing samples were collected, processed and assayed for poliovirus-specific IgA antibodies. Results are expressed as the mean value ± S.D. (n = 4). Different letters indicate significant difference by a Tukey test ($\rho \leq 0.05$).
doi:10.1371/journal.pone.0105374.g004

Immunoadjuvant activity rabies virus



ESSENTIALLY SAME RESULTS FOR IgG1 AND IgG2

Figure 1a. Total IgG isotypes in serum. Sera were collected four weeks after the second antigen dose (day 42). Results are expressed as the mean value ± S.D. (n=6). Bars not sharing a letter have significant differences by Tukey test ($p \leq 0.05$).

Yendo et al. Vaccine 34: 2305-2311, 2016

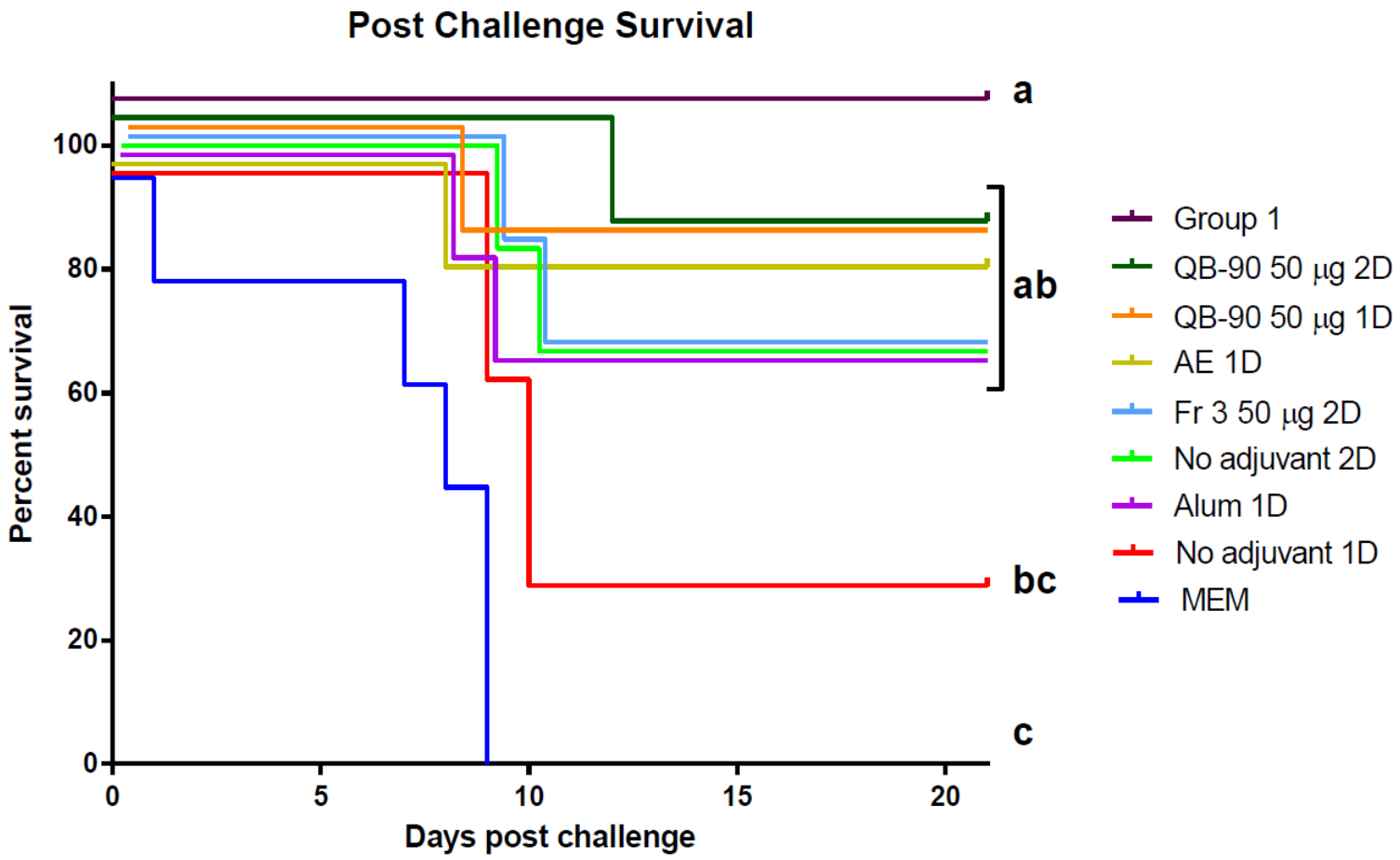


Figure 4. Percent survival of rabies infected mice after challenge by CVS strain. Group 1 represents treatments that protected 100% of challenged mice: AE 2D, QB-90 100 µg 1D, QB-90 100 µg 2D, QB-80 50 µg 1D, QB-80 50 2D, QB-80 100 µg 1D, QB-80 100 µg 2D, Fr B 50 µg 2D, Quil-A 50 µg 1 D, Quil-A 50 µg 2 D, Alum 2 D.

Yendo et al. Vaccine 34: 2305-2311, 2016

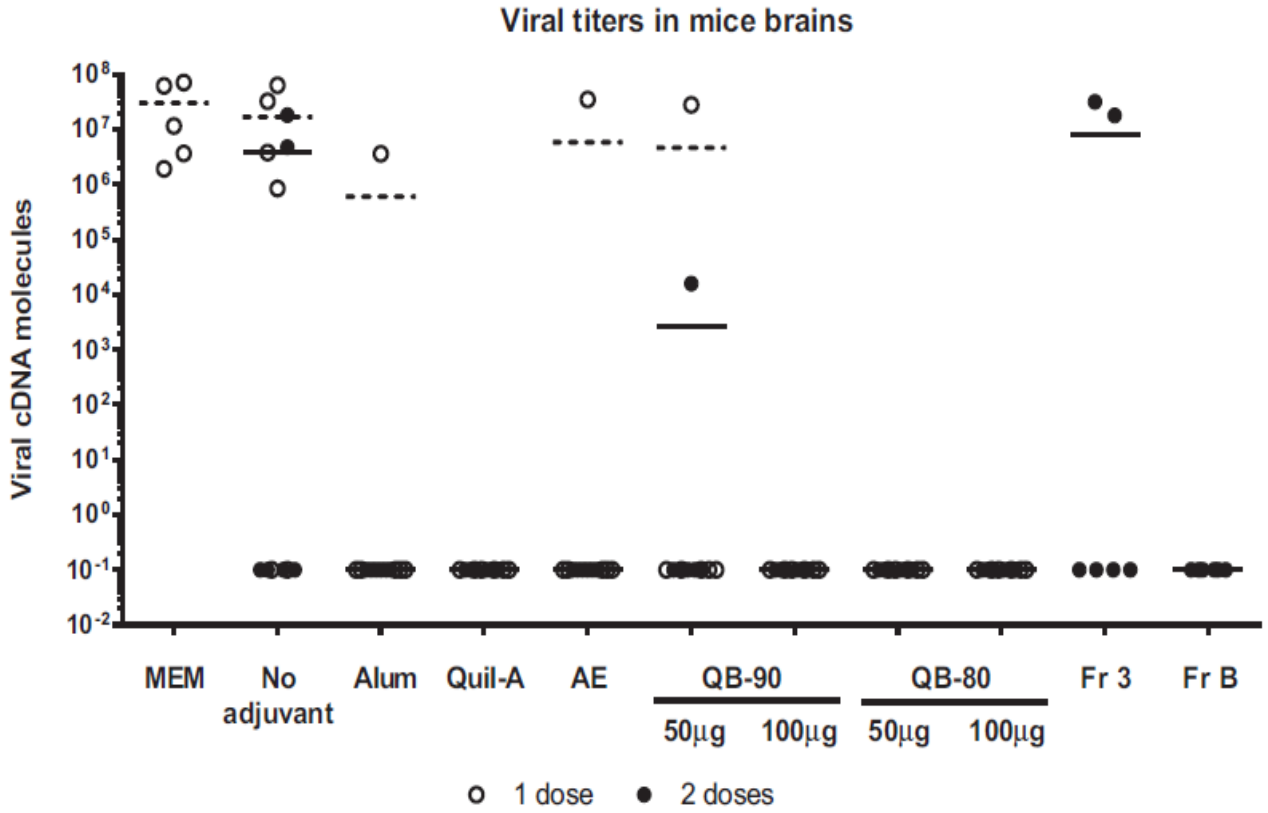


Fig. 5. Rabies virus RNA recovered from mice challenged with a lethal dose of CVS 35 days after the first immunization. Viral RNA was detected in the brain of mice that died of rabies within ten days post challenge (day 45). Mice that did not develop rabies showed no detectable viral RNA. Black lines indicate the geometric mean. Groups received one (1D) or two (2D) immunizations on day 0 or 0 and 14, respectively, of rabies vaccine preparation either without adjuvant or formulated with QB-80 (50 µg or 100 µg), QB-90 (50 µg or 100 µg), AE (400 µg), Fraction 3 (50 µg), Fraction B (50 µg), Alum (200 µg) or Quil-A (50 µg). Fraction 3 (50 µg) and Fraction B (50 µg) groups received only two immunizations. A control group was carried out administering only MEM ($n = 6$).

Yendo et al. Vaccine 34: 2305-2311, 2016

Immunogenicity Fr 3 e Fr B

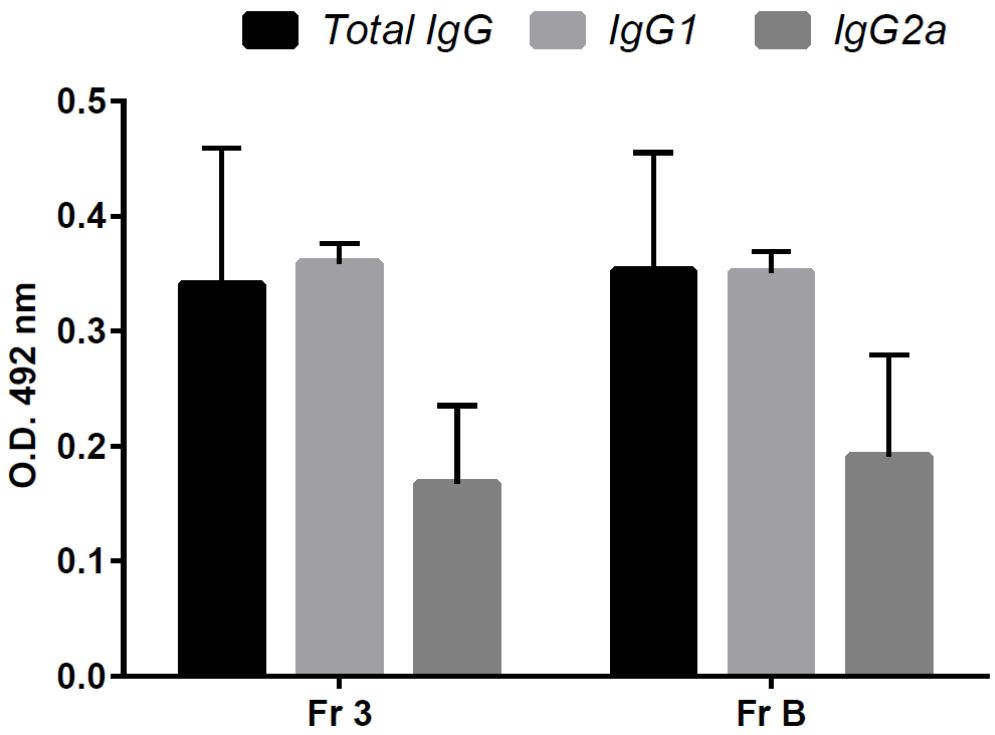
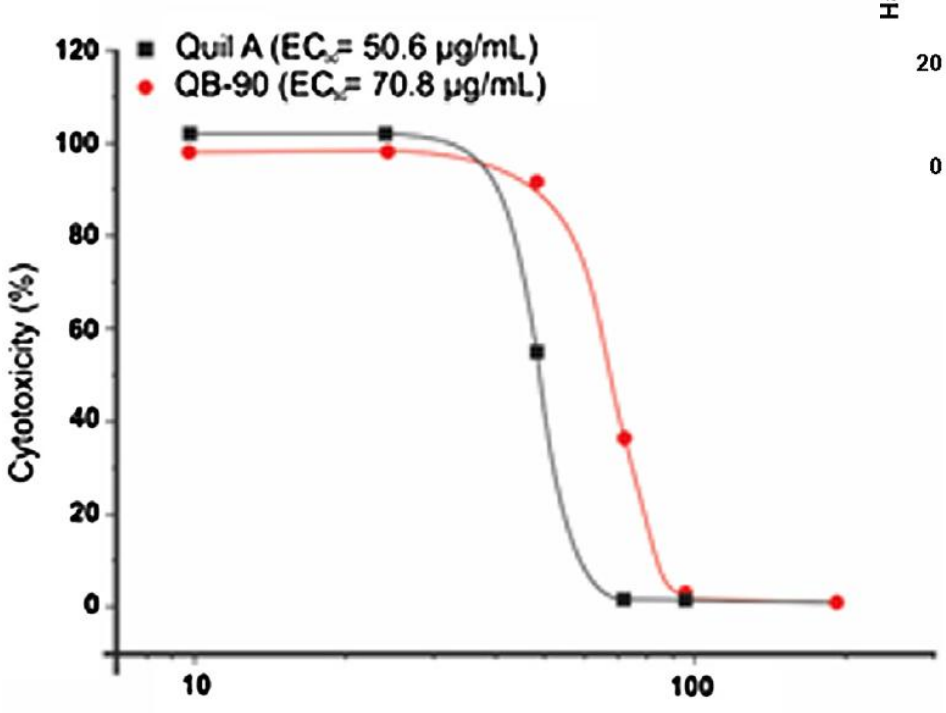
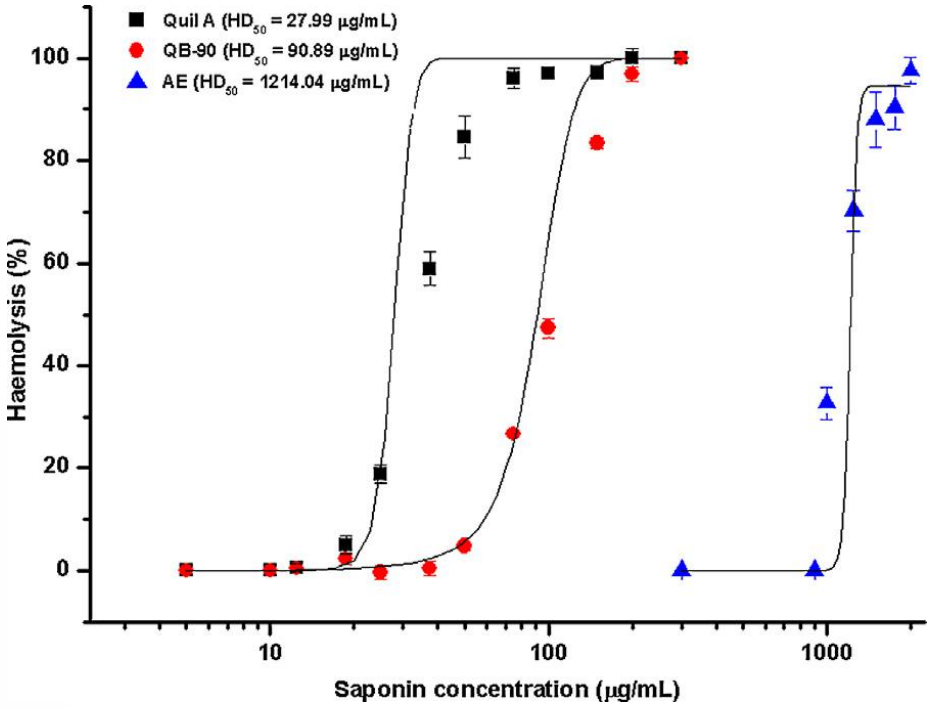


Figure 2. Total IgG, IgG1 and IgG2a isotypes in serum. Sera were collected two weeks after the second antigen dose (Day 28) formulated with 50 µg of Fraction 3 or 50 µg of Fraction B. Results are expressed as the mean value ± S.D. (n=6). No significant differences in total IgG, IgG1 and IgG2a were found between fractions by Tukey test ($p \leq 0.05$).

Yendo et al. Vaccine 34: 2305-2311, 2016

Lower toxicity: ↓ hemolytic and cytotoxic activity compared Quil-A®.





Cibulski et al., Vaccine 34: 1162–1171, 2016; Cibulski et al., Comp Imm Microb Infec Dis 45: 1-8, 2016.

MAIN DATA FROM VACCINE STUDIES

Increased antibodies – **humoral** (IgG total, IgG1) and **cellular** (IgG2a) responses, as well as DTH, equal or superior to those of Quil-A;

Induced systemic and mucosal immunity – polio and BVDV

Homogeneous and consistent biological activity – Fr B, Fr 3, QB-80 and QB-90: geographical origin and purification method do not affect activity;

Protection in challenge with RV – one dose yielded moderate protection and two doses full protection;

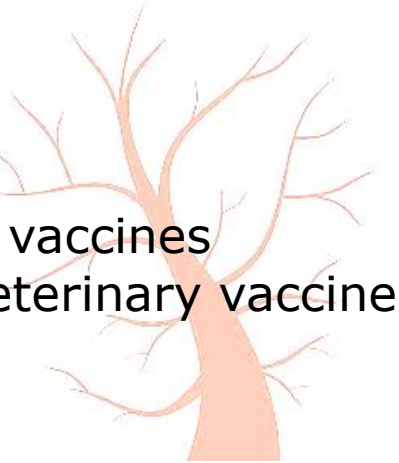
Effective in inactivated virus vaccines - several cases.

Q. brasiliensis saponins are viable alternative/complement to Quil-A.

WHAT ABOUT THE CATALYTICALLY ACTIVE BIOMASS SUPPLY ISSUE?

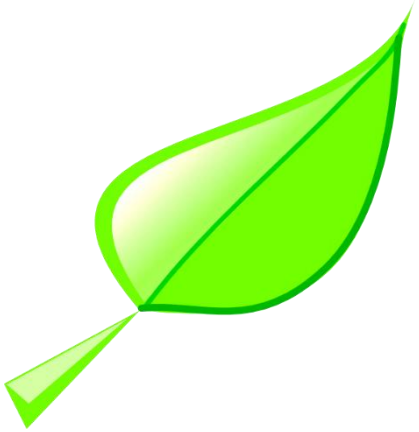
Immunoadjuvant saponins of *Quillaja*

Barks of ***Q. saponaria***



- Native tree of South America (Chile, Bolivia, Peru)
- **QS-21** – used in phase III clinical trials of human vaccines
- Saponin fraction **Quil-A** – commercially used in veterinary vaccines
- Destructive source by annealing adult trees

Leaves of ***Q. brasiliensis***



- Native species of southern Brazil
- Easily renewable source of saponins
- Non-destructive source

- **ALTERNATIVE SOURCE OF SAPONINS**

Q. brasiliensis micropropagation



Aseptic germination



Shoot tip culture in BAP 0.05 mg/L



Rooting – IAA 10mg/L



Ex vitro acclimation



Soil grown plants

Fleck et al.,In Vitro Cell Dev Biol – Plant 45: 715-720, 2009.

Miniclonal garden and minicutting system can be applied to *Quillaja brasiliensis*

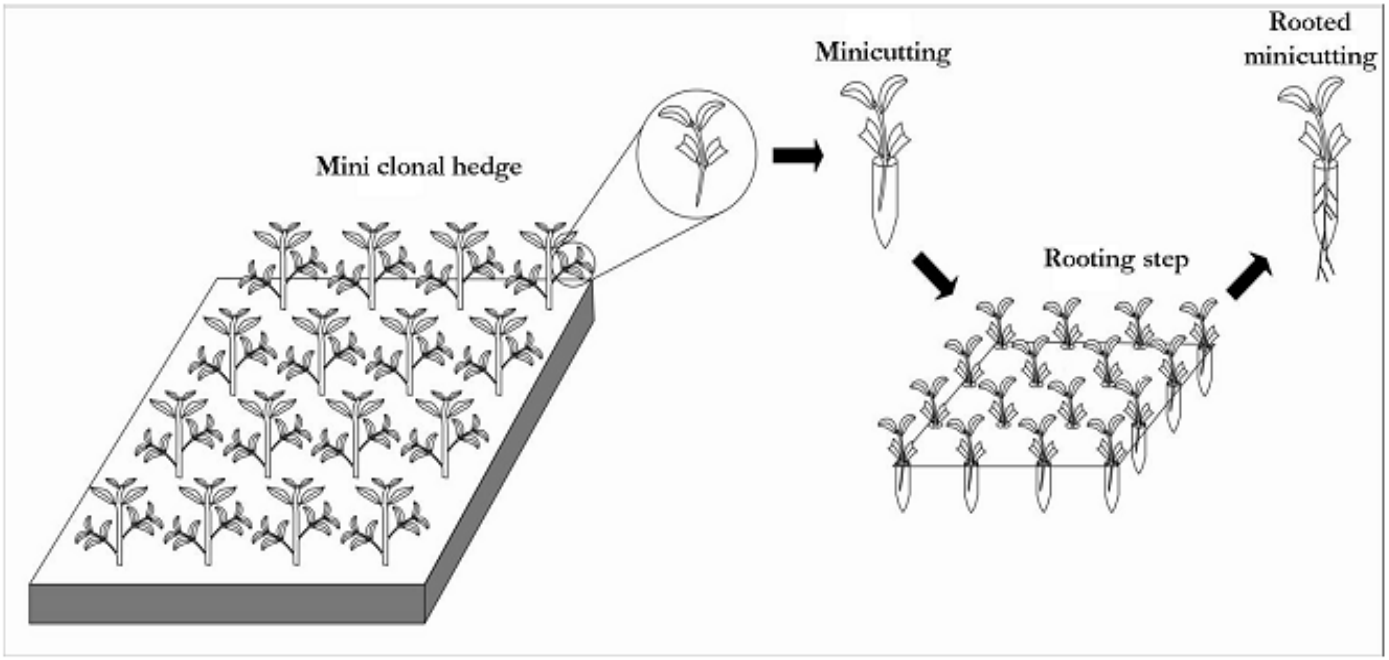


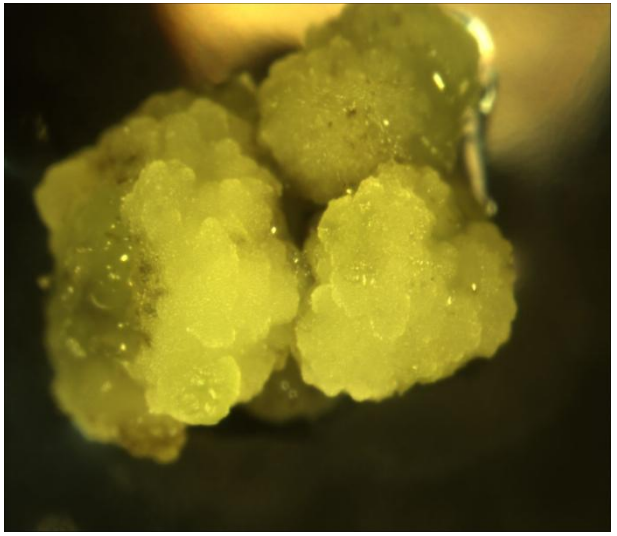
Fig. 1. A schematic diagram of miniclonal hedge–based production of eucalypts (*Eucalyptus* sp.). Ministumps grown in the hedge are usually maintained in greenhouse sandbeds and originate from *in vitro* micropropagated hybrids. Modified from [11] with the permission from New For.

Schwambach et al. New Forests, v. 36, p. 261-271, 2008

Cell cultures – successfully established

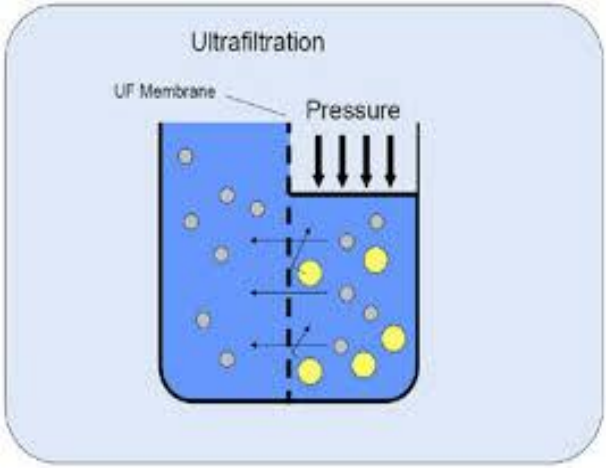
Chemical signatures of relevant saponin markers present

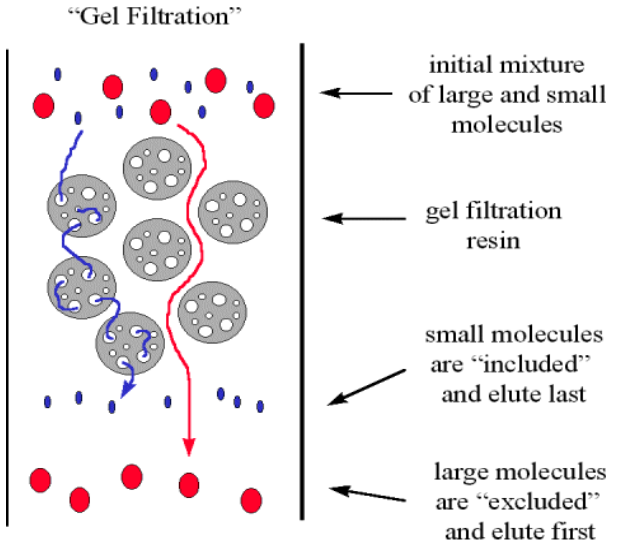
Chemical interference of polysaccharides and pectins

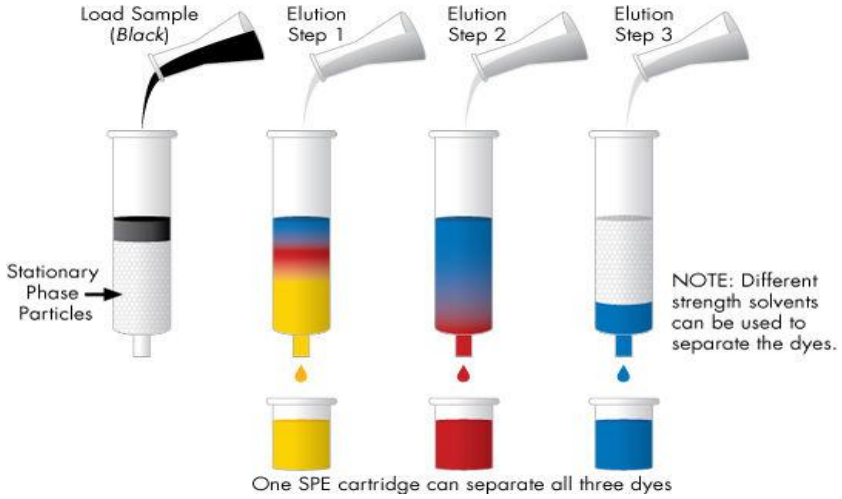


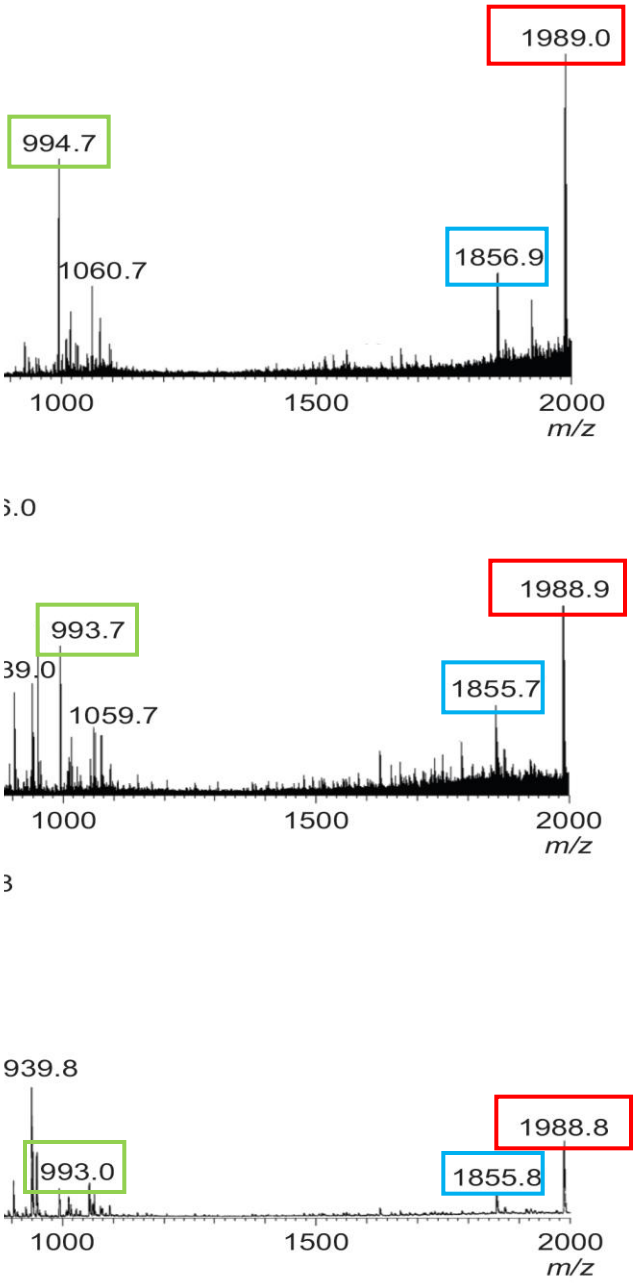
CAN BIOACTIVE SAPONINS BE OBTAINED EFFECTIVELY AND REPRODUCIBLY AND ARE THEY CONSISTENT IN COMPOSITION?

SAPONIN FRACTION PURIFICATION METHODS







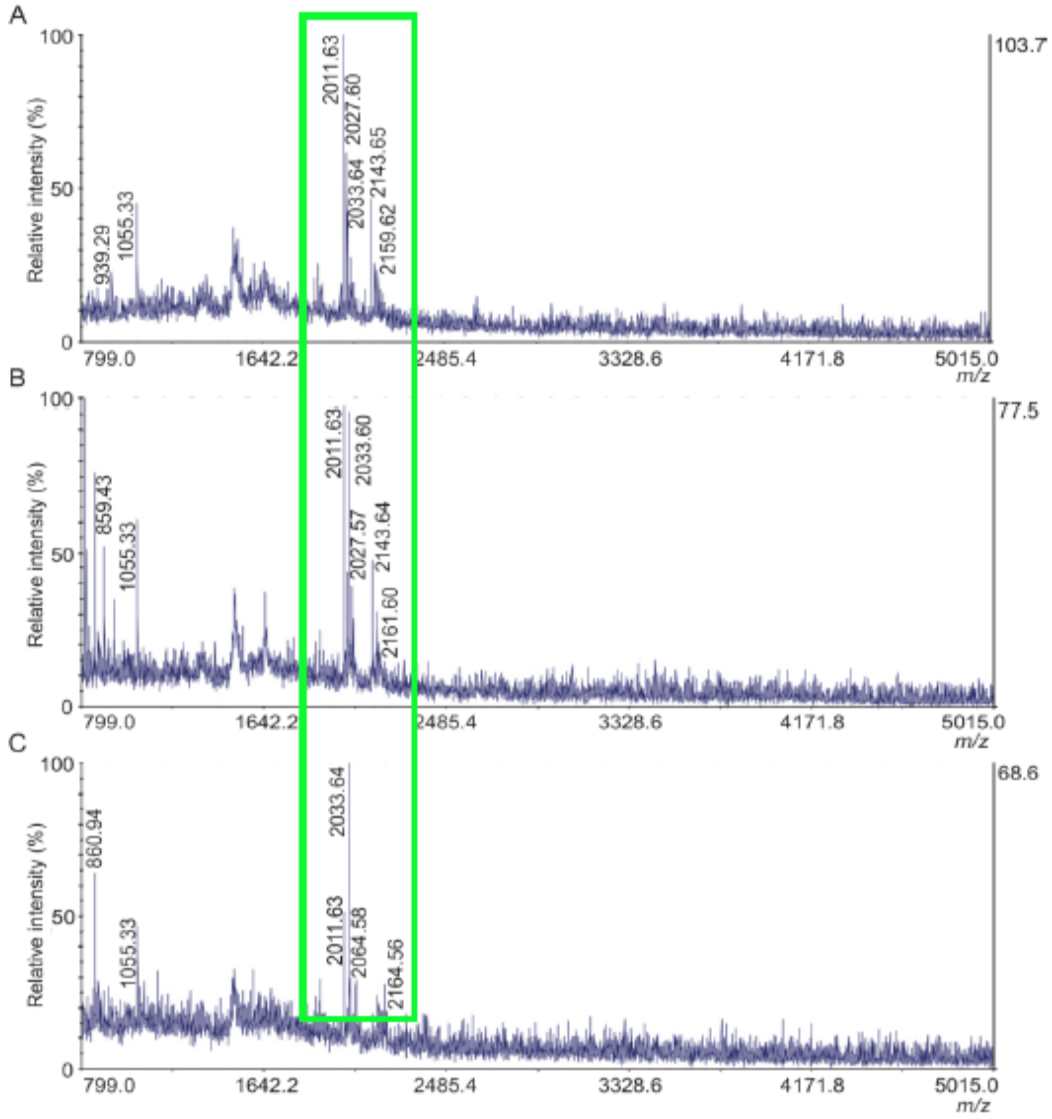


ESI MS

- Ion-trap MS (Bruker Daltonik)

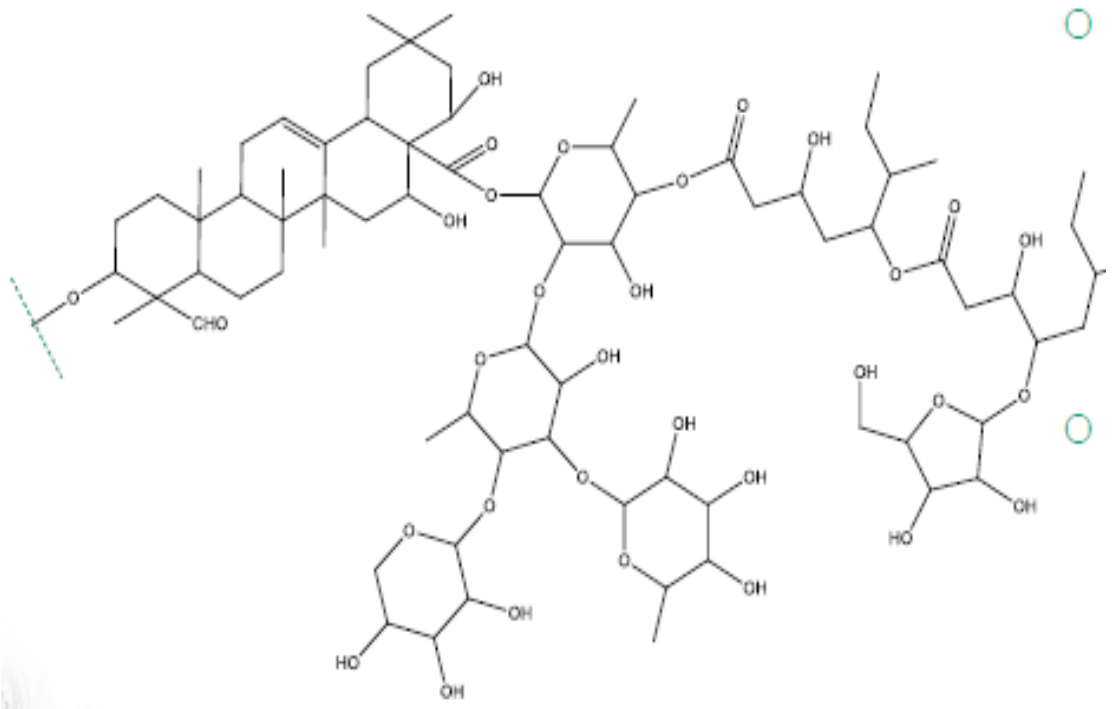
Figure 2. Negative MS spectra for (A) QB-90; (B) Fraction 3; and (C) Fraction 4 from *Q. brasiliensis* leaf AE.

MALDI-TOF

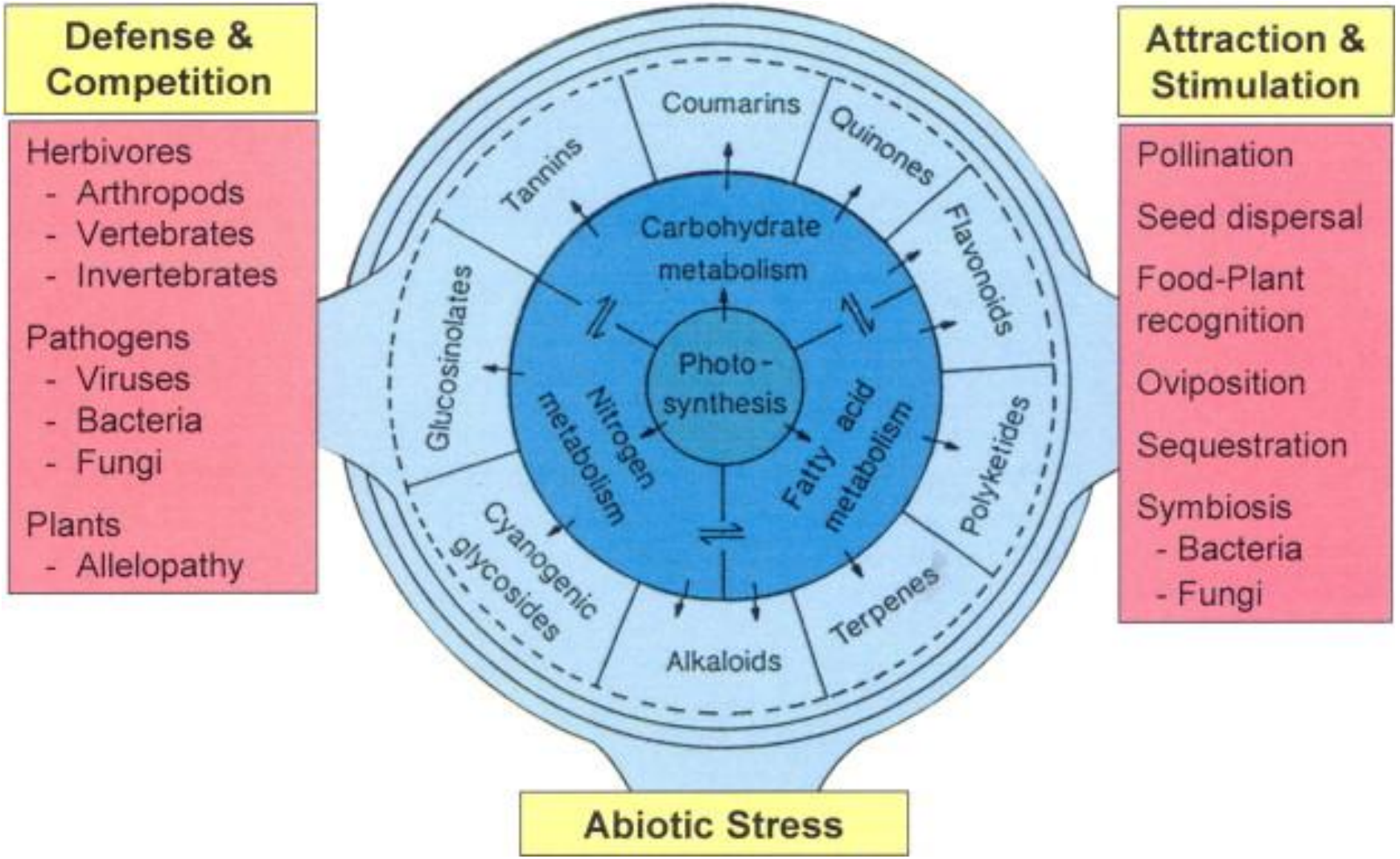


MALDI-TOF mass spectra of (A) QB-80; (B) QB-90; and (C) Fraction 3 from *Q. brasiliensis* leaf AE.

NMR INITIAL EVALUATION SHOWED CHEMICAL SIGNATURES OF AGLYCONE AND APPROPRIATE C SUBSTITUENTS SIMILAR TO THOSE OF QUILL A.

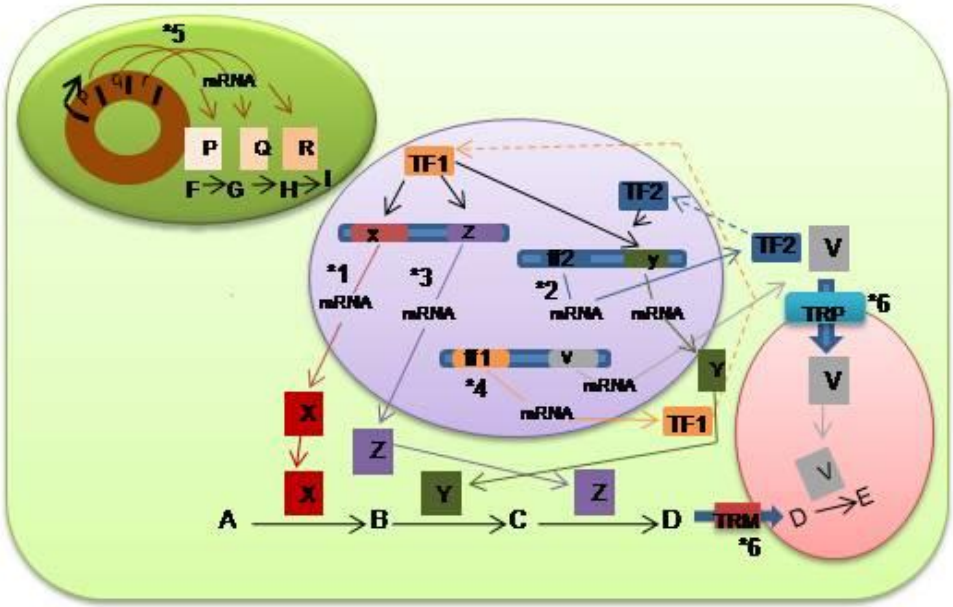


CAN WE STIMULATE PLANTS TO YIELD MORE BIOACTIVE SAPONIN PER BIOMASS UNIT?



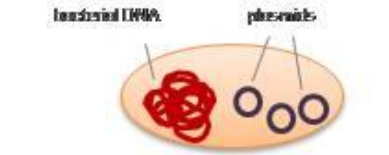
Hartmann, T. Phytochemistry 68: 2831-2846, 2007

B

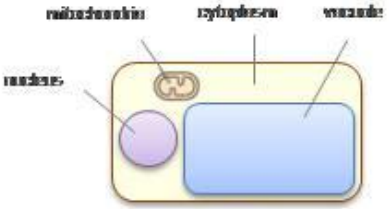


Fett-Neto A.G. (Ed.) Plant Secondary Metabolism Engineering, Springer Protocols, Methods in Molecular Biology, vol .643, Humana Press, Springer, New York, 2010

Heterologous Systems for Natural Products Expression:



bacteria



yeast



Other plant species

Key Regulatory Inputs:

Developmental control



Organ/tissue specific

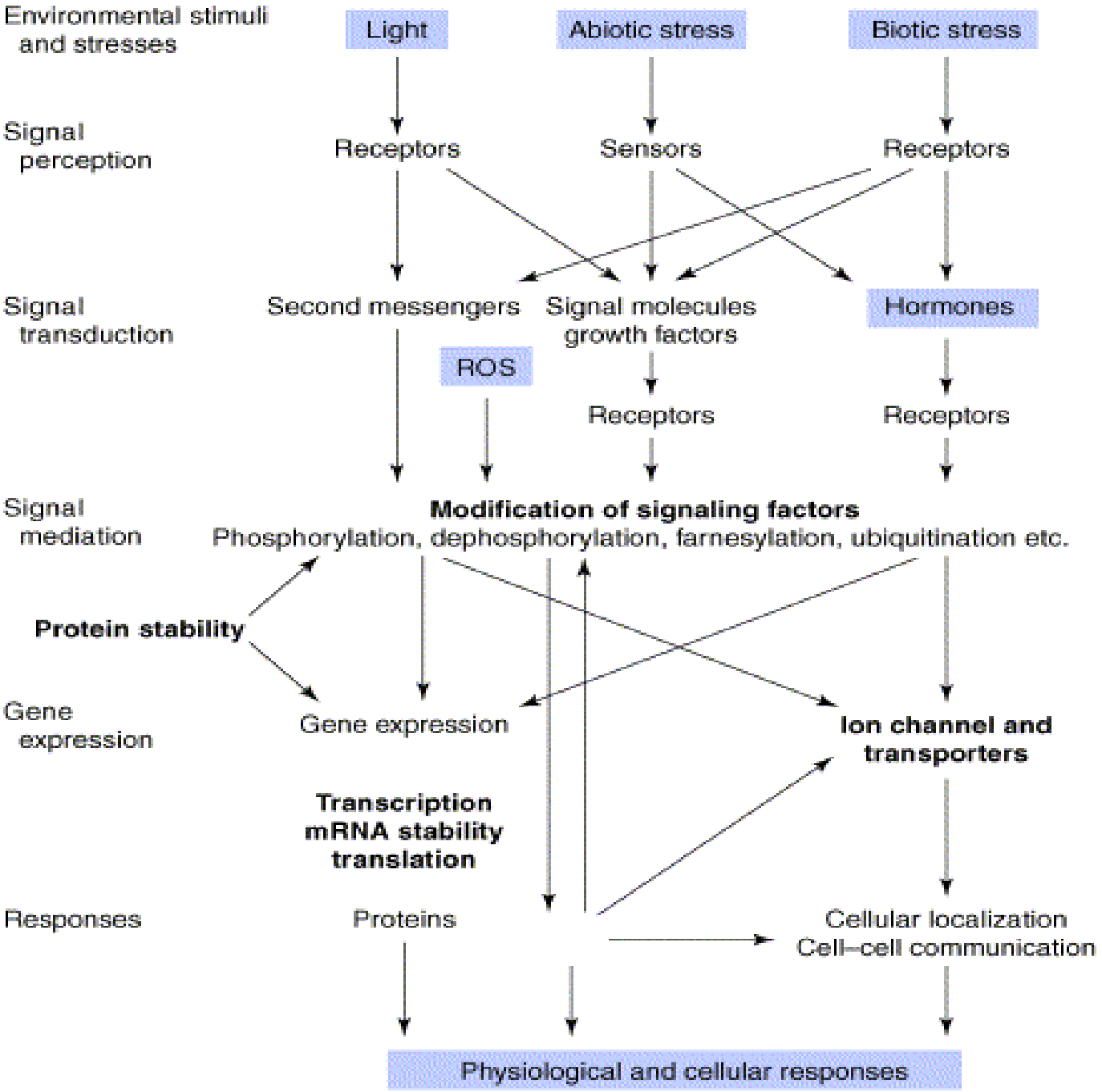


Abiotic/Biotic Stresses



Circadian rhythms





Curr Opinion Plant Biol. 6: 405-409, 2003

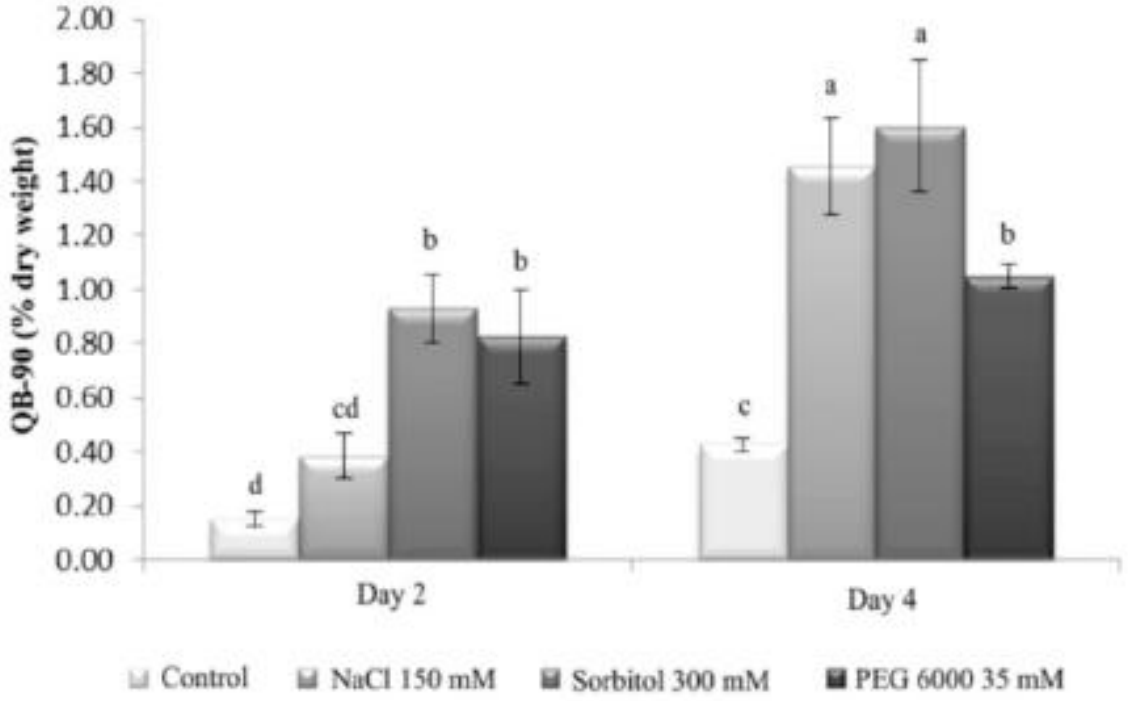


Fig. 1. QB-90 content in leaf disks of *Q. brasiliensis* after 2 and 4 days of exposure to control (0.1× MS) and treatments with NaCl 150 mM, sorbitol 300 mM and PEG 35 mM. Different letters indicate significant difference by a Duncan test ($P \leq 0.05$). Bars represent the means ± standard error.

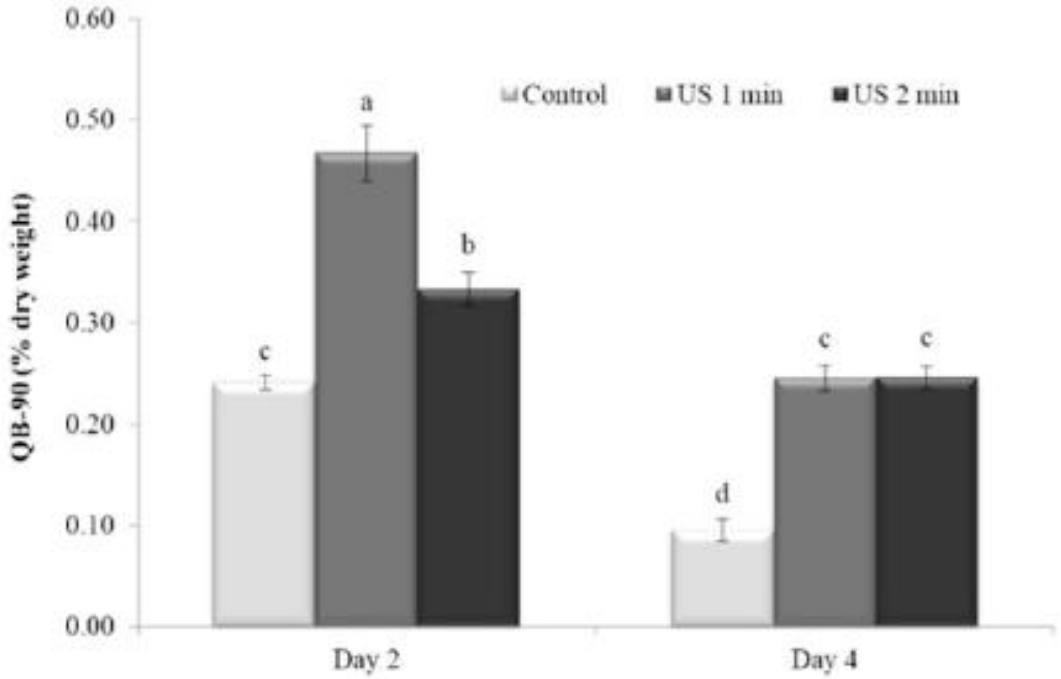
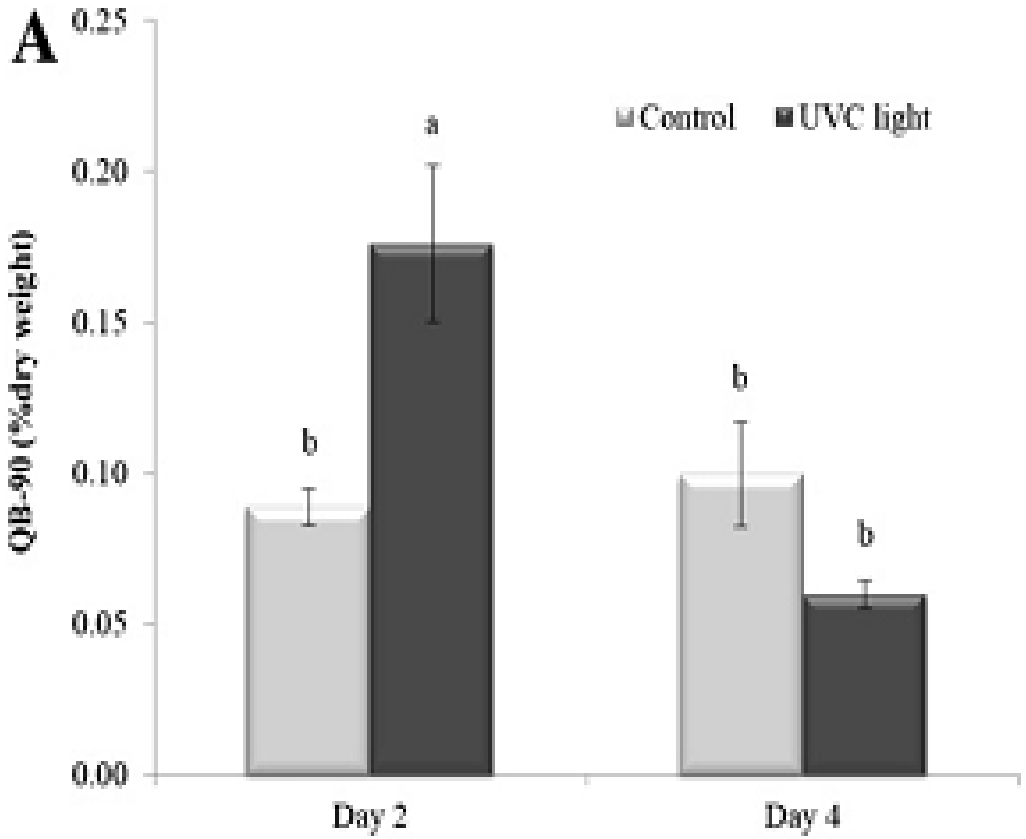


Fig. 2. QB-90 content in leaf disks of *Q. brasiliensis* submitted to ultrasound exposure. Control (untreated disks kept in 0.1× MS), US treatments applied as a single initial exposure for different lengths of time (1 and 2 min) on disks incubated in the same medium. QB-90 yield is shown for 2 and 4 days after treatment application. Different letters indicate significant difference by a Duncan test ($P \leq 0.05$). The bars represent the means ± standard error.



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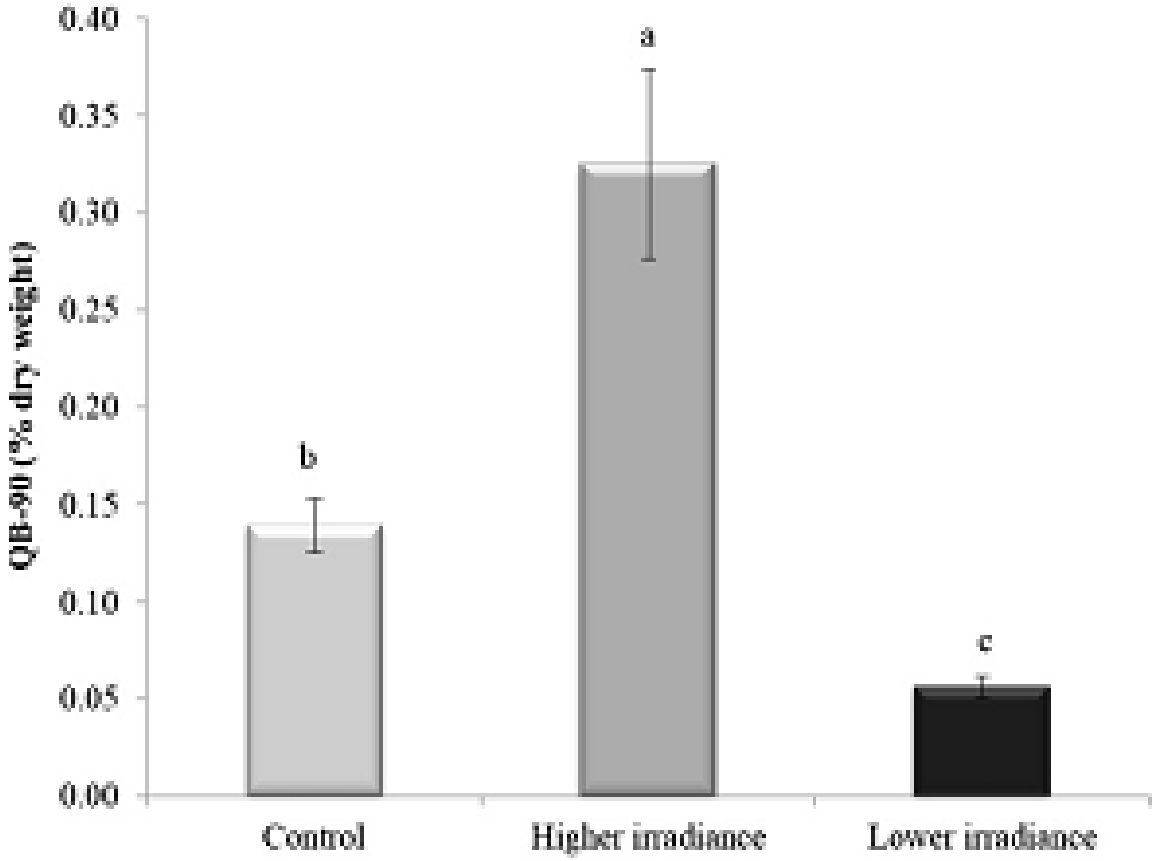


Fig. 6. Influence of light intensity on QB-90 content of *Q. brasiliensis* shoots on day 30. Different letters indicate significant difference by a Duncan test ($P \leq 0.05$). The bars represent the means ± standard error.

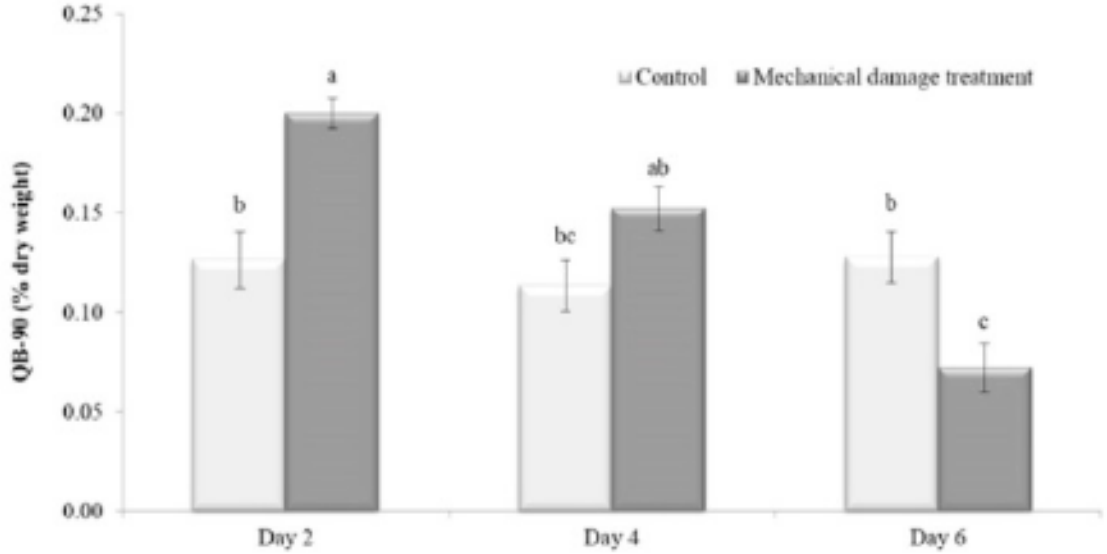


Fig. 5. Influence of mechanical damage treatment on QB-90 content of *Q. brasiliensis* shoots on days 2, 4 and 6 after damage. Different letters indicate significant difference by a Duncan test ($P \leq 0.05$). The bars represent the means ± standard error.

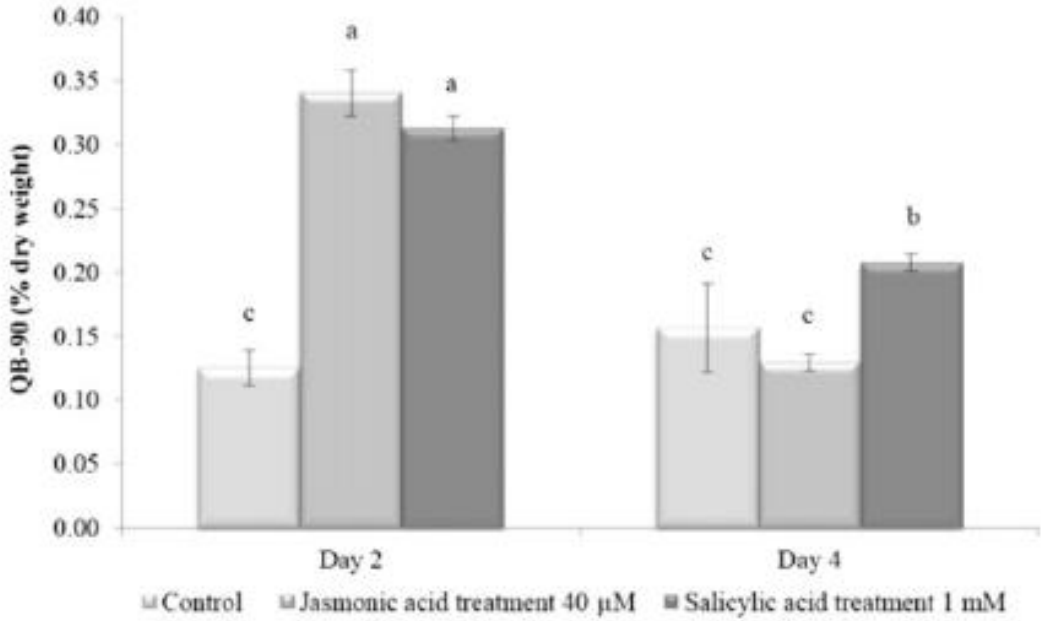
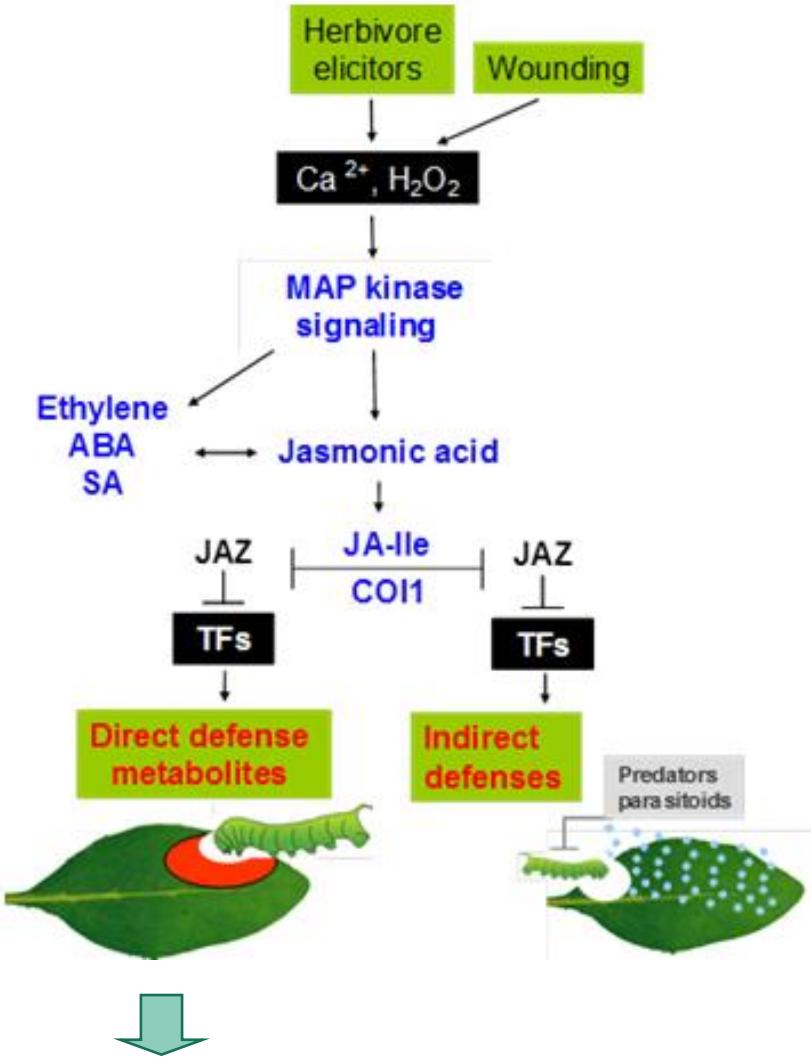
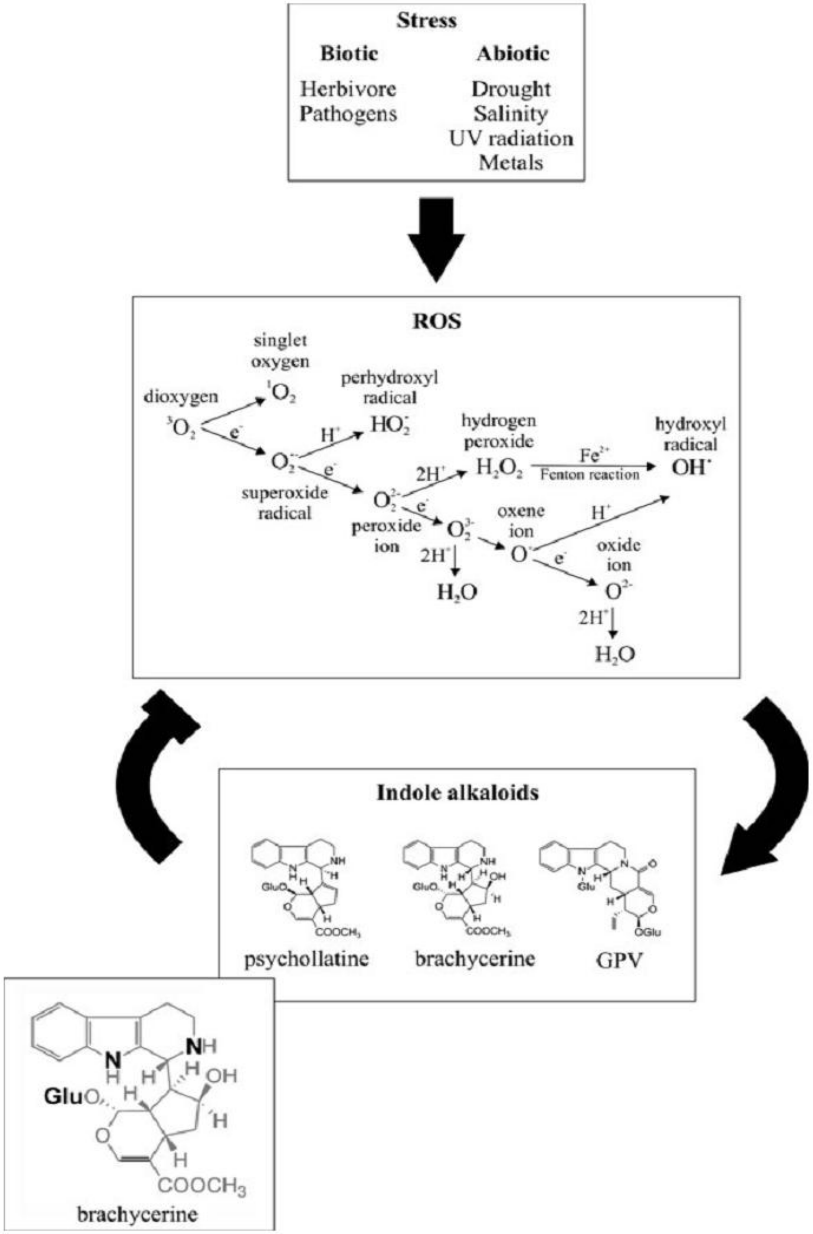


Fig. 4. QB-90 content in leaf disks of *Q. brasiliensis* after 2 and 4 days of exposure to control conditions or to treatment with JA 40 µM or SA 1 mM treatment. Different letters indicate significant difference by a Duncan test ($P \leq 0.05$). Bars represent the means ± standard error.


Herbivore elicitors
Wounding
Ca 2+, H2O2
MAP kinase signaling
Ethylene
ABA
SA
Jasmonic acid
JAZ
JA-Ile
COI1
JAZ
TFs
TFs
Direct defense metabolites
Indirect defenses
Predators
parasitoids

Saponins



Matsuura et al. Biotechnol Lett 36:191–200, 2014

Postharvest physiology and stability of bioactive saponins

Table 1

Concentration of QB-90 saponins after post-harvest incubation of leaves and barks (% dry weight). Values of day 0 represent basal content of QB-90 for all treatments of the respective organ. Values are the means of quadruplicates and respective standard errors.

	Day 0		Day 10	
	Leaves	Barks	Leaves	Barks
25 °C under vacuum	0.085 ± 0.026	0.022 ± 0.004	0.051 ± 0.011	0.017 ± 0.006
25 °C			0.058 ± 0.015	0.021 ± 0.003
40 °C			0.079 ± 0.011	0.010 ± 0.006
60 °C			0.066 ± 0.001	0.007 ± 0.002[a]
−12 °C			0.082 ± 0.007	0.026 ± 0.001

[a] Significantly different from pre-incubation content (day zero) of the respective organ ($P \leq 0.05$, Student *t*-test).

Yendo et al, Industrial Crops and Products 74: 228-233, 2015 .

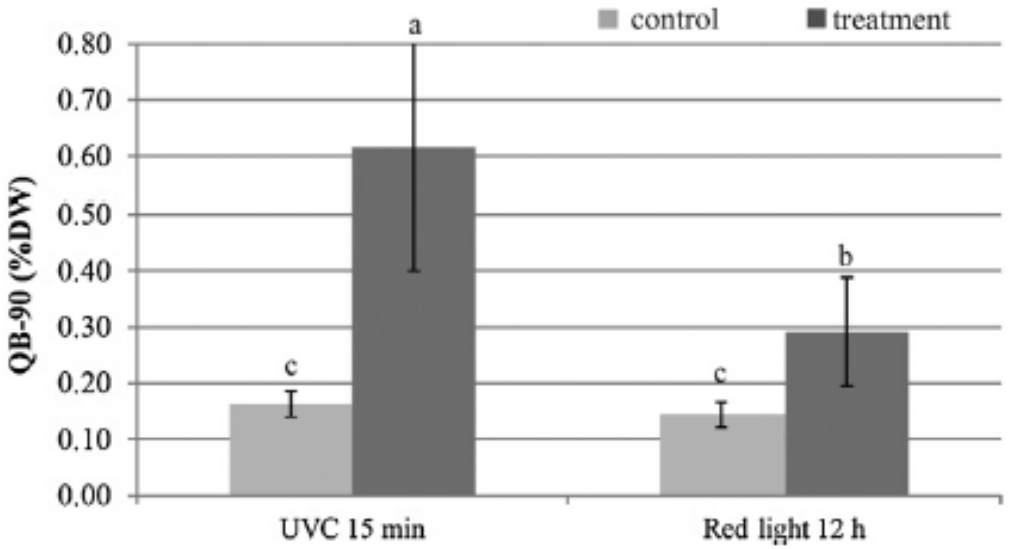


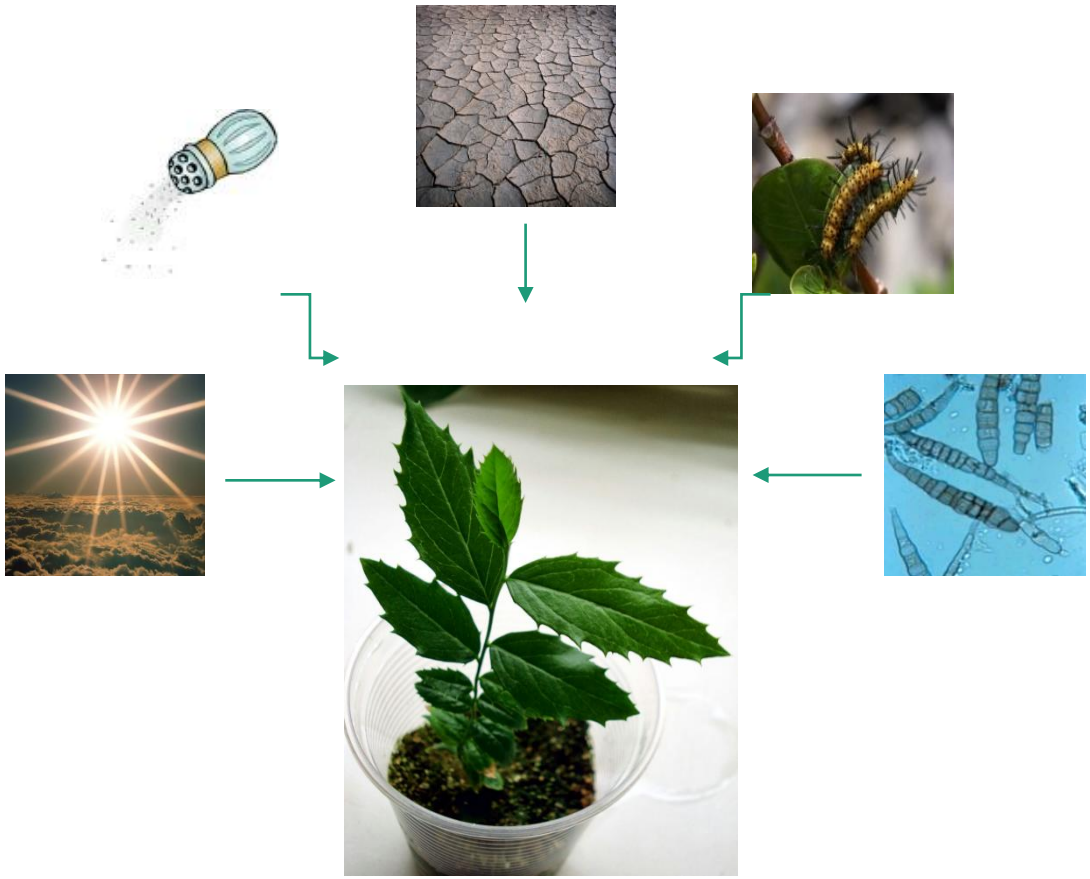
Fig. 4. Concentration of QB-90 saponins in leaves of *Quillaja brasiliensis* juvenile plants exposed to UV-C for 15 min or to red light for 12 h after harvest, and the respective untreated controls. Bars (means ± standard errors) not sharing a letter are significantly different by a Duncan test ($P \leq 0.05$).

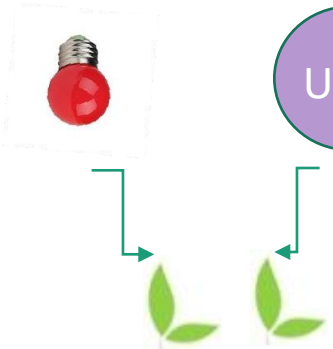
Yendo et al, Industrial Crops and Products 74: 228-233, 2015 .

Regulation of saponin production

Abiotic stress:
- osmolytes/drought
- higher irradiance
- UV-C

Biotic stress:
- salicylic acid
- jasmonic acid
- ultrasound
- wounding





Likely role in defense

Postharvest

Industrial scale application

Yendo et al, Industrial Crops and Products 74: 228-233, 2015 .

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Future steps:

-Select for high yield genotypes;

-Apply mass clonal propagation and cell cultures with 'elite' genotypes;

- ‘Saponin transcriptomics’ – RNAseq

- Isolate and structurally define single saponins as molecular scaffolds;

- Improve cheap scale up extraction of purified fractions;

- Advance tests as vaccine adjuvants in target commercial livestock.



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