# Use of *Artemia franciscana* in Evaluating the Toxicity of Plants Used to Treat Infectious Disease in Northern Peru

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## ABSTRACT

**Background:** The *curanderos* of Northern Peru have a long history of treating diseases with plants. This study focuses on plants and plant mixtures used to treat conditions believed to be caused by bacterial infection.

**Hypothesis:** Combined with bacterial inhibition data, toxicology data can be used to help select plants for further study.

**Methods:** Ethanol extracts made from dried, ground plants obtained in local markets were dried by rotary evaporation. This material was resuspended in boiling water and filtered. *A. franciscana* were incubated in serial dilations for 24 hours. Surviving brine shrimp were counted. Counts were converted to percent survival. $LC_{50}$ values, the concentration of extract lethal to 50% of shrimp, were calculated from dose-response curves (overall range from 0.81 to 5.67 mg/mL).

**Results:** Examination of data collected from various extracts demonstrated reproducibility. The effect of expanding the assay's scale around the area with the greatest change in percent survival was tested. Relative activity indices (RAI), the ratio of $LC_{50}$ for brine shrimp to $IC_{50}$ for *Staphylococcus aureus*, were determined.

**Conclusions:** $LC_{50}$ values for extracts of plant mixtures were significantly different from $LC_{50}$ values of corresponding single plant extracts. RAI values revealed that two sets of plants/mixtures had potential for further study.

## BACKGROUND

Northern Peru is at the center of an ecologically diverse region of the old Central Andean "Health Axis", an area stretching from Ecuador to Bolivia. The practice of traditional healing here dates at least back to the Moche culture, 100 CE [1]. Although the rainforests of Peru (Fig. 1) are given the majority of scientific attention, the biological diversity (Fig. 2)[4] present in other Peruvian ecosystems also merits investigation.

Figure 1: Research area and ecosystems of Peru.

Bussmann et al. classified 510 plant species as medicinal plants used in Peru. These plants were used to prepare 974 mixtures employed in the treatment of 164 different conditions [2]. This study focuses on the sixteen two-plant mixtures being used to treat conditions thought – from an allopathic perspective – to be infectious diseases. This project involved six of those two-plant mixtures.

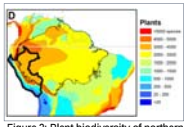

Figure 2: Plant biodiversity of northern South America. Bass et al. (2010).

While about half of the plants used during the colonial period have fallen out of popular use, the overall number of plant species used in Northern Peru has increased since that time [1]. This suggests that the medicine practiced by Peru's *curanderos* (native healers) is a dynamic, evolving art (Fig. 3) focusing on modern health concerns.

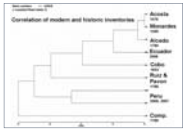

Figure 3: Dendrogram of medicinal plant use and knowledge in the "Health Axis".

Today, medicinal plants remain an accessible and affordable form of healthcare in Peru, despite the rise of pharmaceutical medicine [3, 2].

## METHODS

### Plant Collection:

Plants used in this study were purchased at a local market (Fig. 4) and identified by botanists. Voucher specimens were prepared and deposited at the Universidad Privada Antenor Orrego (UPAO) and the Missouri Botanical Garden.

Figure 4: A plant vender's stall in Chiclayo, Peru.

Figure 5: A hand-crank maize grinder was used to homogenize dried plants.

### Extract Preparation:

Plants gathered from the market were dried, ground (Fig. 5), and soaked in ethanol for seven days to extract medicinal compounds. Extracts made from single plants contained 50g of plant material in 1L of ethanol, while those made from plant mixtures contained 25g of each plant. Extracts were then filtered through Whatman #2 filter paper (to remove plant debris), dried by rotary evaporation, and stored at -30°C. As needed, frozen extracts were thawed and resuspended in boiling water. Extract dry weights were used to determine concentration.

### Toxicity Assay:

Brine shrimp (*A. franciscana*), shown in Figure 6, were used as a model to evaluate plant extract toxicity. Shrimp were hatched in a 3.5% solution of Instant Ocean with a 40W light and aeration for 48 hours. Approximately 20 shrimp were counted into each of six tubes containing resuspended extract. Resuspensions were serially diluted by a ratio of 1:4.

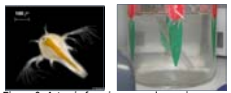

Figure 6: *Artemia franciscana* under a microscope (left) and jar with aerator used to hatch brine shrimp (right). Shrimp are visible without magnification.

After 24 hours, expired shrimp were counted using a dissecting microscope. Surviving shrimp were exposed to 0.1% (w/v) lemon essential oil solution. After five minutes, surviving shrimp slow down enough to be counted. All shrimp present are then counted. Dead shrimp are subtracted from this total to calculate the number of surviving shrimp. Each duplicate (n=4) was tested with a positive control (shrimp in Instant Ocean) and a negative control (shrimp in 400ppm solution of potassium chromate, a toxicology industry standard for lethality).

### Bacterial Growth Assay (BGA):

Resuspended extracts were serially diluted (n=4). Bacteria (*Escherichia coli* or *Staphylococcus aureus*) were directly added to serial dilution tubes. After 24 hours, tubes were centrifuged, decanted, and resuspended in 0.9% saline solution. Absorbance was read on a Spec20 spectrophotometer at 660nm. Controls included full growth, antibiotic, and extract tubes (to ensure sterility).

### Data Analysis:

Data were normalized to percent survival or percent growth, in comparison to negative controls (without extract). Data were then plotted as dose-response curves and the area of the curves showing maximum change was fit to a line (y=mx+b). The concentration which caused 50% inhibition of bacteria ($IC_{50}$) and killed 50% of shrimp ($LC_{50}$) were calculated where y=50. Two-tailed unpaired t-tests were used when comparing two $LC_{50}$ values. A p < 0.05 was considered significant. Data are presented as the mean ± standard deviation.

## RESULTS and DISCUSSION

### Reproducibility of Toxicology Screening Assay:

Toxicology assays were repeated to test whether our results could be reproduced day-after-day from a given extract resuspension. Repeated experiments for *Plantago linearis* L. "small variety" were performed on several, separate days. The results of two of these experiments are shown in Figure 7. $LC_{50}$ values were calculated as described in Methods. (Note that replicates (n=4) are sometimes superimposed.) Three of the $LC_{50}$ values were within the range of 1.15 to 1.68 mg/mL with a relative standard deviation (RSD) less than 5% (Table 1). The $LC_{50}$ value calculated from Experiment 2, however, varied from the average $LC_{50}$ value of the other three experiments (1.49 mg/mL) by over a factor of three and had a RSD of 23%.

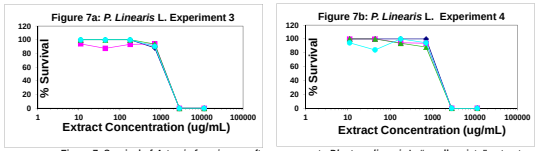


Figure 7: Survival of *Artemia franciscana* after exposure to *Plantago linearis* L. "small variety" extract.

Table 1: Summary of *Plantago linearis* L. "small variety" toxicology experiments.

| Experiment | $LC_{50}$ (mg/mL) |
|---|---|
| Exp. 1 (113S) | 1.15 ± 0.06 |
| Exp. 2 (131S) | 4.84 ± 1.13 |
| Exp. 3 (136S) | 1.65 ± 0.04 |
| Exp. 4 (138S) | 1.68 ± 0.06 |
| Exp. Average | 2.33 ± 1.59 |

The average $LC_{50}$ values for each *Plantago linearis* L. "small variety" experiment are shown in Table 1. The average $LC_{50}$ value for all four *P. linearis* L. "small variety" extract toxicology experiments is also shown. Numbers in parentheses refer to a specific lab experiment number.

The data from some plant extracts were more variable. The results from two experiments using *Salix chilensis* Molina extracts are shown in Figure 8. Note that the $LC_{50}$ values for these two experiments varied by over a factor of five, although their RSD values were both approximately 6%. The results from five *S. chilensis* extract toxicology experiments are shown in Table 2. Experiments 2, 4, and 5 had RSD less than 8%, while Experiments 1 and 3 had RSD values of 35% and 40%, respectively. The average $LC_{50}$ value for the *S. chilensis* Molina extract experiments was 2.99 ± 1.47 mg/mL, which has a RSD value of almost 50%.

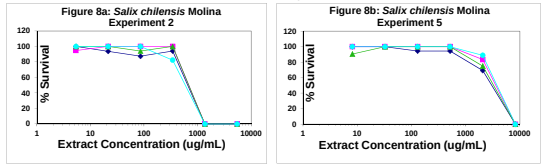


Figure 8: Survival of *Artemia franciscana* after exposure to *Salix chilensis* Molina extract.

Table 2: Summary of *Salix chilensis* Molina toxicology experiments.

| Experiment | $LC_{50}$ (mg/mL) |
|---|---|
| Exp. 1 (118S) | 2.49 ± 0.88 |
| Exp. 2 (128S) | 0.82 ± 0.05 |
| Exp. 3 (132S) | 3.11 ± 1.27 |
| Exp. 4 (134S) | 4.22 ± 0.35 |
| Exp. 5 (135S) | 4.34 ± 0.26 |
| Exp. Average | 2.99 ± 1.47 |

The average $LC_{50}$ values for each *Salix chilensis* Molina experiment are shown in Table 2. The average $LC_{50}$ value for all five *S. chilensis* Molina extract toxicology experiments is also shown. Numbers in parentheses refer to a specific lab experiment number.

### Expansion of Toxicology Screening Range:

Expansion experiments were done to more accurately determine the $LC_{50}$ value being measured by having more data points in the "changing region" of the dose-response curves. Typically, the broad-range Screening assay would provide an $LC_{50}$ value between two concentrations – one that was lethal to 100% of brine shrimp and one that didn't kill any after a 24 hour incubation. The higher concentration was the "starting point" for our Expansion. $LC_{50}$ values were calculated, as described in the Methods, from several different graphical fits. Depending on how the data was fit to a curve (Fig. 9), the calculated average of our *Spartium* junceum L. screening $LC_{50}$ values could be statistically significantly different from the $LC_{50}$ value of the expanded scale experiment (Table 3). However, these results did not differ appreciably from one another. Considering that "reproducibility experiments" can vary by over a factor of three, variance of $LC_{50}$ value due to curve fitting is insignificant. Additionally, this work was being done with crude ethanol extracts (not isolated compounds) and there is limited time to work in Peru. Due to these factors, and the observed variability of the data obtained, the time involved in performing assays to expand the toxicology assay range might be better spent elsewhere.

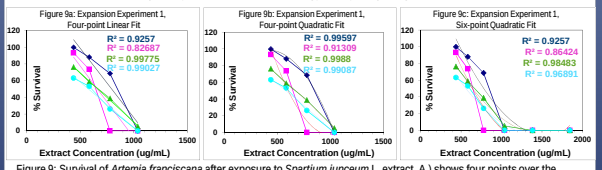


Figure 9: Survival of *Artemia franciscana* after exposure to *Spartium junceum* L. extract. A.) shows four points over the "region of change" fit to a line (where y=mx+b). B.) shows four points fit to a quadratic curve (where $y=ax^2+bx+c$). C.) shows six points fit to a quadratic curve. All three experiments involve six extract serial dilutions.

## RESULTS and DISCUSSION CONTINUED

Table 3: Summary of *Spartium* junceum L. toxicology Expansion Experiment.

| Experiment | $LC_{50}$ (mg/mL) |
|---|---|
| Average of Toxicology Screenings[a] | 1.28 ± 0.25 |
| Expansion Exp. 1 (112E) | **0.67 ± 0.09** (Linear fit, 4 points) |
| | **0.95 ± 0.12** (Quadratic fit, 4 points) |
| | **1.16 ± 0.22** (Quadratic fit, 6 points) |

[a] Refers to the calculated average of *S. junceum* L. toxicology screening experiments (104S), (108S), (109S), (110S),and (114S).

The effect on $LC_{50}$ value of different graphical representations of an Expansion Experiment are shown in Table 3. Numbers in parentheses refer to a specific lab experiment number.

### Ratio of toxicity to antimicrobial activity - the Relative Activity Index (RAI):

The toxicology data collected from the *Artemia franciscana* assay were compared to extract antimicrobial activity to obtain a more empirical measure of therapeutic potential – a Relative Activity Index (RAI) (Table 4). The RAI is defined as $LC_{50}$ divided by $IC_{50}$. While a "relative activity index" is analogous to a therapeutic index (TI), RAI values are more applicable to the study of the effects of crude ethanol extracts on bacterial growth and brine shrimp toxicity.

TABLE 4: Summary of Extract $IC_{50}$ values, $LC_{50}$ values, and calculated RAIs

| Plant | *S. aureus* $IC_{50}$ (mg/mL) | $LC_{50}$ (mg/mL) | $RAI_{S.\ aureus}$ |
|---|---|---|---|
| ***Piper aduncum* L.** | **10.86 ± 10.22** | **1.89 ± 0.39** | **0.17** |
| ***Plantago linearis* L., "small variety"** | **1.45 ± 0.38** | **2.33 ± 1.59** | **1.61** |
| ***P. aduncum/P. linearis* "small variety" mix** | **1.13 ± 0.05** | **4.76 ± 0.30** | **4.21** |
| ***Piper aduncum* L.**[1] | **10.86 ± 10.22** | **1.89 ± 0.39** | **0.17** |
| ***Plantago linearis* L. "large variety"** | **11.46 ± 0.29** | **NT: 0.0041 to 4.20** | **ND** |
| ***P. aduncum/ P. linearis* L., "large variety" mix** | **NI: 0.015 to 3.92** | **NT: 0.0011 to 1.18** | **ND** |
| ***Salix chilensis* Molina** | **7.15 ± 1.79** | **2.99 ± 1.47** | **0.42** |
| ***Scirpus californicus* Steub. Subsp. *totora*** | **14.62 ± 2.05** | **NT: 0.0044 to 4.53** | **ND** |
| ***S. chilensis* Molina/ *S. californicus* mix** | **16.34 ± 7.95** | **3.81 ± 0.07** | **0.23** |
| ***Salix chilensis* Molina**[1] | **7.15 ± 1.79** | **2.99 ± 1.47** | **0.42** |
| ***Prunus serotina* Ehrh. Subsp. *capuli* (Cav.) McVaugh** | **3.05 ± 0.35** | **0.88 ± 0.01** | **0.29** |
| ***S. chilensis* Molina/ *P. serotina* mix** | **2.47 ± 0.27** | **1.35 ± 0.00** | **0.55** |
| ***Sambucus peruviana* Kunth** | **5.58 ± 4.90** | **1.20 ± 0.23** | **0.22** |
| ***Ricinus communis* L.** | **10.51 ± 0.22** | **0.79 ± 0.03** | **0.08** |
| ***S. peruviana* Kunth/ *R. communis* L. mix** | **4.48 ± 0.69** | **1.41 ± 0.39** | **0.32** |
| ***Tiquilia paronychioides* (Phil.) A.T. Richardson** | **2.87 ± 1.30** | **NT: 0.003 to 3.07** | **ND** |
| ***Mirabilis jalapa* L.** | **2.12 ± 3.04** | **1.57 ± 0.23** | **0.74** |
| ***T. paronychioides*/ *M. jalapa* mix** | **0.82 ± 0.01** | **5.67 ± 0.67** | **6.92** |

[1] Some plant extracts are repeated on this table for clarity.
(Note: Non-Toxic plants marked as "NT"; extracts that did not inhibit growth (non-inhibitory) are marked as "NI". "ND" denotes that, as the result of NT/NI extracts, RAI values were not determined. Shading is used to separate groups of extracts which share plants in common.)

Although RAI values were calculated for experiments done with both *E. coli* and *S. aureus*, only *S. aureus* RAI values are shown in Table 4. In 10 of the 18 tested extracts, *E coli* growth was not inhibited, while in 7 of the 8 remaining extracts, $IC_{50}$ values were too high to be therapeutically relevant ($IC_{50} \geq 10$ mg/mL). Ideally, $IC_{50}$ values are minimized (i.e., a lower concentration is needed to kill bacteria) while $LC_{50}$ values are maximized (a larger concentration is required to cause adverse effects). Larger RAI values are more favorable. In 4 of the 6 plant sets studied, the RAI value of the mixture exceeded the RAI value of either single plant in the set. Two mixtures (*P. aduncum*/*P. linearis* L. "small variety" and *T. paronychioides* (Phil.) A.T. Richardson/*M. jalapa* L.) are potential candidates for further study.

The calculation of RAI values gives a holistic view of the traditional use of medicinal plants. The study of plant extract toxicology complements antimicrobial data and suggests that medicinal plants may be used in specific combinations that attenuate toxicity.

## CONCLUSIONS

When working with crude ethanol extracts of medicinal plants:

- Toxicology screening assays are mostly reproducible, but exhibited some unexplained variability.
- Expanding the scale of toxicology screening assays is not worthwhile – especially considering the variability of the screening assay.
- Relative Activity Indices, the ratio of $LC_{50}$ to $IC_{50}$, can be obtained and will help guide the natural products scientist in further studies.

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