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# Natural Products as Potential Inhibitors of Cruzain

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# Natural Products as Potential Inhibitors of Cruzain

**Honors Senior Project by Ashley F. Penton**

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## Honors Senior Project
## Approval

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**Abstract:**

The purpose of this research is to identify biologically active compounds from natural product extracts collected in Monteverde, Costa Rica with respect to the enzyme cruzain. The extracts are procured from plant samples collected in the Costa Rican cloud forest. These extracts are then made into solution for screening purposes. A biochemical fluorescence assay is used to screen natural product extracts for inhibition activity towards cruzain. The data from this assay is then analyzed, generating the percent inhibition and standard deviation. These measurements were taken for extracts collected from 2000 to 2005 in Monteverde, Costa Rica.

**Introduction:**

Cruzain is a key enzyme in the replication of the parasitic protozoan *Trypanosoma cruzi*[1,2] which is the causative agent of Chagas Disease. Chagas disease, which is also known as *American trypanosomiasis*, is primarily found in South and Central America as well as Mexico. *T. cruzi* is spread by Triatomine bugs (or "kissing" bugs) through their infected feces entering the human body by way of the eyes, mouth, or open wounds. Symptoms include fever, fatigue, swelling, heart and brain damage, often causing death. This disease kills approximately 50,000 people each year.[3]

For the past fifteen years, the Natural Products Drug Discovery Group has been collecting plant samples from the cloud forest in Monteverde, Costa Rica. Cloud forests offer a great diversity of plants to collect from. Also, with the necessity for protection from other plants, insects, and animals, these plants have a vast arsenal of chemical defenses. An assortment of over 500 extracts has already been accumulated.

**Methods and Materials:**

After the plant materials (leaves or bark) have been collected and logged, the plants are cleaned, macerated, and extracted by percolating with organic solvents for 24 hours. The solvents used include acetone, chloroform, dichloromethane, ethanol, methanol, and sometimes combinations of these solvents. This results in the crude extract which is then made into a 1% by weight DMSO solution for testing.

Activity of natural product extracts against cruzain (prepared as described in Eakin et al., 1992)[4] is measured by a fluorescence assay using Z-Phe-Arg-AMC·HCl. The cruzain solution (4nM) is prepared with 20μL of cruzain per liter of 100mM sodium acetate buffer with 5mM DTT and a pH of 5.5. The substrate solution (40μM) is prepared with 26mg Z-Phe-Arg-AMC·HCl, first dissolved in DMSO, per liter of 100mM sodium acetate buffer with 5mM DTT and a pH of 5.5. For each four wells of a 96 well plate 475μL of the cruzain solution is mixed with 25μL of the extract solution to be tested. 100μL of this mixture is then pipetted into each of the four wells. Each extract is tested in quadruplicate with DMSO negative controls and a 1% DMSO solution of TLCK for the positive controls. After approximately 10-20 minutes incubation at room temperature, 100μL of the substrate solution is pipetted into each well. The plate is then immediately read by the SpectraMax M2 plate reader. After an initial mixing period of 5 seconds the fluorescence is measured 9 times over a period of 5 minutes with an excitation wavelength of 355nm and an emission wavelength of 460nm. The slope given by the change in fluorescence is then exported into an Excel Spreadsheet for the calculations of percent inhibition and standard deviation.

**Results:**

The results of the screening for cruzain inhibition on the Costa Rican 2000-2005 extracts are listed in the table below. In addition to the year of collection and the scientific names of the plants, the table also provides information about what part of the plant was sampled and what solvent was used. The percent inhibition is listed along with the standard deviation of that calculation.

**% Inhibition of Costa Rican Natural Product Extracts**

| Year | Family | Species | Sample | Solvent | % Inhibition | Std. Dev. |
|---|---|---|---|---|---|---|
| 2005 | Solanaceae | *Acnistus arborescens* | Bark | Chloroform | 97.45721 | 1.107365 |
| 2005 | Myrcinaceae | *Ardisia compressa* | Bark | Dichloromethane | 72.90628 | 3.85244 |
| 2005 | Lauraceae | Beilschmiedia aloiophyllum | Plant | Essential Oil | 75.51713 | 0.955346 |
| 2005 | Lauraceae | Beilschmiedia brenesii | Bark | Chloroform | >100 | 1.5137386 |
| 2005 | Lauraceae | Beilschmiedia brenesii | Bark | Methanol | 94.6409 | 0.094004 |
| 2005 | Lauraceae | Beilschmiedia brenesii | Plant | Essential Oil | 92.44849 | 1.14632 |
| 2005 | Lauraceae | Beilschmiedia brenesii | Leaves | Chloroform | 99.14568 | 1.389946 |
| 2005 | Lauraceae | Beilschmiedia brenesii | Leaves | Methanol | 98.45917 | 0.637602 |
| 2005 | Lauraceae | Beilschmiedia tilaranensis | Plant | Essential Oil | 98.12251 | 1.907792 |
| 2005 | Solanaceae | *Cestrum megalophyllum* | Bark | Dichloromethane | 93.2279 | 2.157949 |
| 2005 | Melastomataceae | *Conostegia xalapensis* | Bark | Chloroform | 88.89639 | 1.185243 |
| 2005 | Fabaceae/Papilionoideae | *Erythrina lanceolata* | Leaves | Acetone | 84.25419 | 1.780673 |
| 2005 | Euphorbiaceae | *Euphorbia elata* | Leaves | Acetone | >100 | 1.005999 |
| 2005 | Myrtaceae | Eugenia octopleura | Plant | Essential Oil | 28.311 | 2.381221 |
| 2005 | Myrtaceae | *Eugenia salamensis* | Plant | Essential Oil | 94.0891 | 2.096434 |
| 2005 | Sapindaceae | *Exothea paniculata* | Bark | Chloroform | 66.57407 | 6.277488 |
| 2005 | Sapindaceae | *Exothea paniculata* | Bark | Methanol | 75.21077 | 2.447541 |
| 2005 | Apocynaceae | Mandevilla veraguasensis | Bark | Chloroform | 95.49698 | 2.595761 |
| 2005 | Apocynaceae | Mandevilla veraguasensis | Bark | Methanol | 70.97175 | 0.635552 |
| 2005 | Apocynaceae | Mandevilla veraguasensis | Leaves | Chloroform | 96.7428 | 1.453121 |
| 2005 | Apocynaceae | Mandevilla veraguasensis | Leaves | Methanol | 93.91037 | 2.337301 |
| 2005 | Myrtaceae | Myrcia sp. "fuzzy leaf" | Bark | Acetone | 88.68056 | 2.90282 |
| 2005 | Myrtaceae | Myrcia sp. "fuzzy leaf" | Leaves | Acetone | 98.67479 | 1.323782 |
| 2005 | Myrtaceae | *Myrcia splendens* | Plant | Essential Oil | 67.58187 | 2.973577 |
| 2005 | Nyctaginaceae | *Neea psychotrioides* | Bark | Chloroform | 73.53057 | 3.534767 |
| 2005 | Nyctaginaceae | *Neea psychotrioides* | Bark | Methanol | 72.72326 | 0.871009 |
| 2005 | Lauraceae | Ocotea valeriana | Plant | Essential Oil | 93.653938 | 1.3419433 |
| 2005 | Lauraceae | Persea caerulea | Plant | Essential Oil | 93.11926 | 0.926134 |

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|---|---|---|---|---|---|---|
| 2005 | Proteaceae | *Roupala montana* | Bark | Acetone | 88.7457 | 3.155446 |
| 2005 | Moraceae | Sorocea trophoides | Bark | Chloroform | 98.78866 | 1.328781 |
| 2004 | Actinidiaceae | *Saurauria montana* | Leaves | Acetone | 95.88904 | 0.461082 |
| 2004 | Campanulaceae | *Centropogon solanifolius* | Plant | Chloroform/Methanol | 94.31486 | 0.233233 |
| 2004 | Campanulaceae | *Centropogon costariceae* | Plant | Chloroform/Methanol | >100 | 0.61408 |
| 2004 | Ericaceae | *Cavendishia bracteata* | Bark | Chloroform/Methanol | 98.54456 | 0.707722 |
| 2004 | Ericaceae | *Cavendishia bracteata* | Leaves | Chloroform/Methanol | >100 | 0.708302 |
| 2004 | Euphorbiaceae | *Croton monteverdensis* | Plant | Essential Oil | 23.506122 | 0.8304487 |
| 2004 | Euphorbiaceae | *Croton niveus* | Plant | Essential Oil | 18.92971 | 3.233753 |
| 2004 | Fabaceae/Papilionoideae | *Styphnolobium monteviridis* | Bark | Chloroform | 92.63816 | 6.392692 |
| 2004 | Fabaceae/Papilionoideae | *Styphnolobium monteviridis* | Bark | Chloroform | 96.03843 | 1.349507 |
| 2004 | Fabaceae/Papilionoideae | *Styphnolobium monteviridis* | Leaves | Methanol | >100 | 0.372344 |
| 2004 | Gesneriaceae | *Monopyle maxonii* | Herb | Chloroform/Methanol | 87.4312 | 0.807911 |
| 2004 | Gesneriaceae | *Drymonia rubra* | Vine | Chloroform/Methanol | 75.82591 | 3.325258 |
| 2004 | Hippocrateaceae | *Salacia sp.* | Bark | Chloroform | 88.86545 | 1.303054 |
| 2004 | Lauraceae | *Nectandra membranacea* | Plant | Essential Oil | 9.074361 | 4.343128 |
| 2004 | Lauraceae | *Ocotea floribunda* | Bark | Chloroform | 98.2109 | 1.908005 |
| 2004 | Lauraceae | *Ocotea floribunda* | Bark | Methanol | 89.06116 | 0.74703 |
| 2004 | Lauraceae | *Ocotea floribunda* | Plant | Essential Oil | 87.91552 | 0.293303 |
| 2004 | Oleaceae | *Chionanthus panamensis* | Bark | Acetone | 85.41248 | 2.248531 |
| 2004 | Papveraceae | *Bocconia frutescens* | Bark | Methanol | 80.27133 | 1.435607 |
| 2004 | Papveraceae | *Bocconia frutescens* | Leaves | Chloroform/Methanol | 81.85142 | 2.209856 |
| 2004 | Piperaceae | *Piper umbellatum* | Plant | Essential Oil | 16.43081 | 5.891664 |
| 2004 | Rubiaceae | *Cosmibuena valerii* | Leaves | Chloroform/Methanol | 95.41643 | 2.434483 |
| 2004 | Rutaceae | *Stauranthus perforatus* | Bark | Dichloromethane | 86.33055 | 3.340383 |
| 2004 | Rutaceae | *Stauranthus perforatus* | Plant | Essential Oil | 63.59698 | 0.668614 |
| 2004 | Rutaceae | *Zanthoxylum rhoifolium* | Bark | Methanol | 92.48882 | 2.177983 |
| 2004 | Rutaceae | *Zanthoxylum rhoifolium* | Plant | Essential Oil | 36.85181 | 5.720097 |
| 2004 | Rutaceae | *Zanthoxylum rhoifolium* | Bark | Chloroform | 96.806819 | 2.5285708 |
| 2004 | Rutaceae | *Zanthoxylum setulosum* | Bark | Chloroform | 98.21828 | 1.687395 |
| 2004 | Rutaceae | *Zanthoxylum setulosum* | Bark | Methanol | 97.44228 | 2.231621 |
| 2004 | Rutaceae | *Zanthoxylum setulosum* | Plant | Essential Oil | 40.6279 | 2.3346886 |
| 2002 | Bombacaceae | *Ochroma byrmidile* | Bark | Acetone | 98.92047 | 0.919227 |
| 2002 | Boraginaceae | *Cordia eriostigma* | Bark | Acetone | >100 | 0.366273 |
| 2002 | Clusiaceae | *Vismia sp.* "red bark" | Bark | Acetone | 78.53722 | 1.21281 |
| 2002 | Ebenaceae | *Diospyros blancoi* | Bark | Acetone | 94.10869 | 3.557925 |
| 2002 | Ebenaceae | *Diospyros digyna* | Bark | Acetone | >100 | 0.619251 |
| 2002 | Lauraceae | *Cinnamomum tonduzii* | Bark | Acetone | >100 | 1.049267 |
| 2002 | Lauraceae | *Cinnamomum tonduzii* | Bark | Dichloromethane | 96.00295 | 2.999147 |
| 2002 | Myrtaceae | *Eugenia salamensis* | Bark | Acetone | 94.298389 | 0.6803956 |
| 2002 | Myrtaceae | *Eugenia sp.* "San Luis" | Bark | Acetone | 84.58524 | 1.034227 |
| 2002 | Myrtaceae | *Eugenia salamensis* | Bark | Dichloromethane | 98.17864 | 1.876217 |
| 2002 | Piperaceae | *Piper aequale* | Leaves | Acetone | 96.95756 | 1.135258 |
| 2002 | Proteaceae | *Roupala montana* | Bark | Acetone | 97.68166 | 1.145681 |
| 2002 | Sapindaceae | *Cupania glabra* | Bark | Dichloromethane | >100 | 0.737953 |
| 2002 | Sapindaceae | *Dilodendron costaricense* | Bark | Acetone | 86.49165 | 2.862386 |
| 2002 | Sapindaceae | *Paullinia costaricensis* | Stem | Acetone | 68.03124 | 1.850871 |
| 2002 | Solanaceae | *Cestrum megalophylum* | Bark | Acetone | 88.75761 | 2.239602 |
| 2002 | Sterculiaceae | *Guazuma ulmifolia* | Bark | Acetone | 98.84487 | 2.677336 |
| 2002 | Styracaceae | *Styrax argenteus* | Bark | Acetone | 52.38266 | 1.347655 |

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|---|---|---|---|---|---|---|
| 2002 | Styracaceae | *Styrax argenteus* | Bark | Dichloromethane | 7.077622 | 4.867669 |
| 2002 | Urticaceae | *Urera elata* | Leaves | Acetone | >100 | 1.412285 |
| 2002 | Verbenaceae | *Citharexylum costaricensis* | Bark | Acetone | 87.83052 | 2.12338 |
| 2001 | Fabaceae/Mimosoideae | *Albizia adinocephala* | Bark | Acetone | >100 | 1.10471 |
| 2001 | Apocynaceae | *Stemmadenia donnelsmithii* | Bark | Acetone | 60.543927 | 1.3282399 |
| 2001 | Asteraceae | *Baccharis pedunculata* | Bark | Acetone | 98.94172 | 2.036269 |
| 2001 | Asteraceae | *Verbesina turbacensis* | Bark | Acetone | 97.65187 | 0.790958 |
| 2001 | Clusiaceae | *Vismia baccifera* | Bark | Acetone | 99.22806 | 0.246492 |
| 2001 | Combretaceae | *Terminalia oblongata* | Bark | Acetone | 94.11305 | 0.384147 |
| 2001 | Combretaceae | *Terminalia oblongata* | Leaves | Acetone | 99.13849 | 0.380085 |
| 2001 | Ebenaceae | *Diospyros blancoi* | Bark | Acetone | 96.7289 | 1.457794 |
| 2001 | Ericaceae | *Cavendishia bracteata* | Leaves | Acetone | >100 | 0.069976 |
| 2001 | Euphorbiaceae | *Euphorbia elata* | Bark | Acetone | 71.80022 | 2.399403 |
| 2001 | Euphorbiaceae | *Euphorbia elata* | Leaves | Acetone | 99.91413 | 1.061612 |
| 2001 | Euphorbiaceae | *Sapium glandulosum* | Bark | Acetone | 97.76969 | 0.788455 |
| 2001 | Euphorbiaceae | *Sapium glandulosum* | Leaves | Acetone | >100 | 0.535447 |
| 2001 | Euphorbiaceae | *Sapium macrocarpum* | Bark | Acetone | 99.94036 | 0.794197 |
| 2001 | Euphorbiaceae | *Sapium macrocarpum* | Leaves | Acetone | 99.82039 | 1.336504 |
| 2001 | Fabaceae/Papilionoideae | *Erythrina lanceolata* | Bark | Dichloromethane | 95.55196 | 1.539901 |
| 2001 | Fabaceae/Papilionoideae | *Erythrina vertioana* | Bark | Acetone | 98.34571 | 0.0603519 |
| 2001 | Fabaceae/Papilionoideae | *Lonchocarpus costaricensis* | Bark | Acetone | 75.33674 | 1.481962 |
| 2001 | Fabaceae/Papilionoideae | *Lonchocarpus* "fuzzy leaf" | Bark | Acetone | >100 | 0.683268 |
| 2001 | Fabaceae/Papilionoideae | *Machaerium biovulatum* | Bark | Acetone | 99.49675 | 0.691475 |
| 2001 | Hernandaceae | *Gyrocarpus jatrophifolus* | Bark | Mixed | 57.50804 | 1.501903 |
| 2001 | Lauraceae | *Cinnamomum brenesii* | Bark | Mixed | 97.71099 | 0.402668 |
| 2001 | Lauraceae | *Ocotea* "los llanos" | Bark | Acetone | 81.72718 | 2.3733 |
| 2001 | Lauraceae | *Ocotea* "los llanos" | Bark | Dichloromethane | 98.51376 | 1.556532 |
| 2001 | Malpighiaceae | *Byrsonima crassifolia* | Bark | Mixed | >100 | 0.763908 |
| 2001 | Myrsinaceae | *Ardizia revoluta* | Bark | Dichloromethane | 99.42189 | 2.177159 |
| 2001 | Myrtaceae | *Eugenia salamensis* | Bark | Acetone | 99.78662 | 0.60746 |
| 2001 | Myrtaceae | *Eugenia salamensis* | Bark | Dichloromethane | 97.7267 | 1.245774 |
| 2001 | Phytolaccaceae | *Trichostigma octandrum* liana | Bark | Acetone | 54.8316 | 0.265382 |
| 2001 | Piperaceae | *Piper grabrescens* | Leaves | Acetone | >100 | 0.589792 |
| 2001 | Piperaceae | *Piper hispidum* cf | Leaves | Acetone | >100 | 1.038888 |
| 2001 | Piperaceae | *Piper lanceifolium* | Leaves | Acetone | >100 | 1.04563 |
| 2001 | Polygonaceae | *Coccoloba acapulcensis* | Bark | Acetone | 99.44935 | 0.679426 |
| 2001 | Proteaceae | *Roupala glaberrima* | Bark | Dichloromethane | >100 | 0.150472 |
| 2001 | Rutaceae | *Casimiroa edulis* | Bark | Acetone | 95.10044 | 2.718723 |
| 2001 | Sapindaceae | *Thouinidium decandrum* | Bark | Acetone | 81.05503 | 2.190924 |
| 2001 | Sapotaceae | *Sideroxylon capiri* | Bark | Acetone | 79.92685 | 1.848451 |
| 2001 | Styracaceae | *Styrax argenteus* | Bark | Acetone | 84.76495 | 1.389711 |
| 2001 | Styracaceae | *Styrax argenteus* | Bark | Dichloromethane | 52.18483 | 5.108985 |
| 2001 | Thymelaceae | *Daphnopsis americana* | Leaves | Dichloromethane | 98.18927 | 1.646619 |
| 2001 | Thymelaceae | *Daphnopsis americana* | Leaves | Ethanol | 98.39591 | 0.404916 |
| 2001 | Tiliaceae | *Luehea speciosa* | Bark | Mixed | 93.04841 | 2.480753 |
| 2000 | Actinidiaceae | *Saurauia pittieri* | Bark | Acetone | 96.18638 | 0.682075 |
| 2000 | Araliaceae | *Oreopanax xalapensis* | Leaves | Chloroform | 98.06448 | 0.737396 |
| 2000 | Asteraceae | *Verbesina turbacensis* | Bark | Acetone | 99.55561 | 0.406818 |
| 2000 | Bombaceae | *Quararibea costaricensis* | Bark | Acetone | 70.80286 | 2.510932 |
| 2000 | Celastraceae | *Perrottetia longistylis* | Bark | Acetone | 94.6178 | 1.026238 |

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|---|---|---|---|---|---|---|
| 2000 | Marcgraviaceae | *Ruyschia phylladenia* | Leaves | Acetone | 97.60243 | 0.212098 |
| 2000 | Marcgraviaceae | *Ruyschia phylladenia* | Bark | Chloroform | 81.4625 | 5.48145 |
| 2000 | Meliaceae | *Trichilia martiana* | Bark | Acetone | 97.5808 | 0.856273 |
| 2000 | Meliaceae | *Trichilia havanensis* | Bark | Acetone | 91.16551 | 2.118835 |
| 2000 | Meliaceae | *Trichilia glabra* | Bark | Acetone | 97.90361 | 1.092814 |
| 2000 | Monimiaceae | *Siparuna* "macra" | Bark | Acetone | 96.09643 | 1.685886 |
| 2000 | Myrsinaceae | *Parathesis glabra* | Bark | Acetone | 99.04451 | 0.903253 |
| 2000 | Piperaceae | *Piper nemorense* | Bark/Stem | Acetone | 96.75477 | 1.163593 |
| 2000 | Rubiaceae | *Palicourea padifolia* | Bark | Acetone | 87.47642 | 2.267257 |
| 2000 | Sapindaceae | *Serjania* | Stem | Acetone | >100 | 0.496371 |
| 2000 | Simaroubaceae | *Picramnea antidesma* | Bark | Acetone | 99.97953 | 0.294683 |
| 2000 | Solanaceae | *Solanum accrescens* | Bark | Acetone | 96.35857 | 0.890374 |
| 2000 | Styracaceae | *Styrax argenteus* | Bark | Acetone | 90.0491 | 0.645737 |

**Discussion:**

When the extract sample is mixed with the cruzain solution the compounds within the sample are given the opportunity to bind with the enzyme. If one of more compounds from the sample does bind with the enzyme, the enzyme will bind less or not at all with the substrate depending on the extent of the inhibition. The fluorescence read by the plate reader is a result of the binding of the enzyme and the substrate. Z-Phe-Arg-AMC·HCl is a fluorogenic substrate that alone does not fluoresce. This substrate does, however, fluoresce when it binds with the enzyme. The amount it fluoresces is based on how much is bound to the enzyme.

The plate reader reads the amount of fluorescence of each well in the plate nine times over five minutes. These data points are then plotted and the slope of the line is given. The slope will increase as the substrate continues to bind to the enzyme. The positive and negative controls give the upper and lower parameters of the potential binding. The DMSO control represents a situation where the substrate binds with the enzyme as much as it possibly could in any of the samples. The TLCK control represents a situation where the substrate binds with the enzyme as little as it possibly could.

The percent binding is calculated by the following equation:

$$(1-\frac{S-P}{N-P})\times 100\%$$ S=slope of sample, P=slope of TLCK, N=slope of DMSO

This calculation is done for each of the four slopes given for each sample. In some cases, only three percent inhibitions are calculated if there is one slope that is wildly different from the others. These percent inhibitions are then averaged for the final percent inhibition of the sample.

The standard deviation is a measure of how precise a calculation is. It is derived from how close the original numbers are to each other. It is given by the following equation:

$$\sqrt{\frac{\sum(x-\bar{x})^2}{n-1}}$$ $x$ =slope, $\bar{x}$ =average of all slopes, n =number of slopes averaged

This gives an accurate idea of how varied the percent inhibitions were prior to being averaged.

**Conclusions:**

The percent inhibition of a given extract represents how much the enzyme is prevented from binding with the substrate. As all of these measurements are taken from extracts in 1% DMSO solutions, this number is a comparison of those extracts. Those with higher percent inhibition numbers are more equipped to disable the enzyme from binding with the substrate.

These extracts contain many different compounds within them. Each of the highly active extracts may contain one or more compounds that actually cause the inhibition of the enzyme. In many cases, as several of these extracts are from the same families, these compounds may actually be the same.

With regards to those extracts whose percent inhibition is greater than 100%, there are possibly other factors effecting these reactions. There is the possibility that one or more of the compounds within the extract have destabilized the enzyme altogether. In order to determine exactly what causes this abnormal inhibition of cruzain binding studies would need to be conducted.

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