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UNIVERSIDAD PERUANA CAYETANO HEREDIA  
INSTITUTO DE MEDICINA TROPICAL  
"ALEXANDER VON HUMBOLDT"

AP. 5045 - LIMA 100, PERU - ☎ 815111

March 30, 1988

ACTION COPY	
ACTION TAKEN:	<i>letter</i>
DATE:	<i>4/12/88</i>
INITIALS:	<i>[Signature]</i>

*du*  
*dat*  
*4/13*  
RECEIVED  
MAY 1988  
USAID/LIMA

Miss  
Joan La Rosa  
Chief, Division of Nutrition Health  
Agency for International Development  
A. I. D.

Dear Miss La Rosa :

The present letter is to report all what we have been doing in our project on "natural remedies, diarrhea and parasites".

First, I am going to refer to several difficulties and drawbacks we have had and, afterwards, to interesting findings and developments.

The difficulties :

- . We haven't gotten yet helecho macho to test its effect on parasites; apparently it is not easy to get it.
- . Also, it has been hard to get enough mice for the experiments. To solve this problem we are now breeding these animals at the University.
- . Very high mortality among mice immunosupressed with corticoids to produce the Trichocephalus dispar-like model. This problem has been almost totally solved putting the mice isolated in small plastic cages. The cause of mortality was infections occurring in the animals when living in crowded spaces.
- . Great difficulty to get and keep mice free of Enterobius vermicularis-like (Aspicularus) parasites to produce the Trichocephalus dispar-like model. This problem has also been solved isolating the mice in small plastic cages.
- . The first trials to produce diarrhea with two strains of aeromona have been unsuccessful; we are going to try with other strains.
- . We have tested the effect of shigella on rabbit bowel and the resultant lesion is severely ulcerative and

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hemorrhagic; with this type of lesion it is going to be very difficult to determine the effect of any treatment on the evolution of the lesion.

Now, the findings and developments :

We have assayed the toxicity of various "antiparasitic" plants. It was very interesting to see that while drugs with proven antiparasitic effect (like praziquantel and mebendazole) can be given in doses much higher than the usual ones without producing toxicity, the natural remedies showed a markedly narrower margin of dose safety. Persons using those natural remedies should be informed about their potential adverse effect.

We have not tested yet the effect of helecho macho. But, with the other "antiparasitic" plants we have found that most of them are very effective in vitro but not in vivo. Some of the plants, like leche de oje, are used in the Jungle in the form of enemas with apparently good results. Consequently, we have had the idea of testing the effect of some of the plants on Trichocephalus dispar-like parasites when given in enemas. In anesthetized mice we have been able to fill all the colon, including the cecum, with 0.8ml of water given in enema without causing any harm to the animals. I think that after testing toxicity of the plant extracts enemas, we will be able to find out their effect on colonic parasitoses. It is very probable that the results obtained with the enema model could approximate more to the ones obtained with the in vitro model.

Using the ELISA-like method to investigate possible enterotoxin binding effects of "antidiarrheic" plants, we have had positive results with 5 of the plants: ratania, chili-chili, granada, chuchuhuasi and tara. Of those plants, tara, granada and chili-chili also exert a definite effect in vivo with the rabbit intestinal loop model. Chuchuhuasi and chili-chili were not originally considered in our protocol. Both plants showed marked effect with the ELISA, and of the two Chili-chili has been found to produce also a definite effect in vivo with the rabbit intestinal loop model. And, what is most interesting, the effect is greater than with tara and granada. the ELISA-like method seems thus to have great potentiality as a simple, rapid and inexpensive way of screening plant extracts for enterotoxin binding effect. Here I would like to remark the important role played by Mrs. Dori Finch in the course of our investigations; she had the brilliant idea and initiative of using the ELISA method and of assaying the effect of chuchuhuasi and chili-chili.

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Considering the time we have lost solving the problems which appeared in the investigation on parasites, would it be possible to get an extension of the project for at least 3 additional months?

I include reports of our investigators on their observations.

Once more, I want to express our gratitude for all your help and my sincere appreciation,

Dr. Raúl León-Barúa  
Professor of Medicine  
Co-Principal Investigator, Project  
"Diarrhea and Nutrition".

/cm

P.D. Within a few days, I will be sending a complete report, prepared by Dr. Elba Miranda, on parasitologic findings.

D. Finch  
24-3-88

PROGRESS REPORT ON THE PROJECT "THE USEFULNESS OF TRADITIONAL  
MEDICINE IN THE TREATMENT OF DIARRHEA AND PARASITES"  
MICROBIOLOGY SECTION

I. In Vitro Assay : GM1 Elisa Method

To date we have tested 14 medicinal plants against the crude cholera toxin NIH001 in the rabbit ileal loop assay, an animal model of toxigenic diarrhea. Using between 4 and 8 rabbits per test plant, we have found two plants, Tara and Granada, that reduce loop fluid accumulation by 50% and 80% respectively. This effect rather than being antisecretory as previously reported for berberine (Sack, R.B. 1982) appears to be either binding of the toxin or competitive binding of gut epithelial gm1 receptors by a yet unknown factor in the medicinal plant infusion. More recently, a third plant, Chili-Chili, was also found to reduce loop fluid accumulation in the rabbit ileal loop by 35%. This plant has yet to be tested to determine if it has an anti-secretory effect.

In this assay, more than 100 rabbits have already been used to find three medicinal plants out of 14 that significantly reduce loop fluid ~~loop~~ accumulation. The amount of time involved in obtaining and maintaining the rabbits, and to set up the animal model as an assay is considerable. To reduce the time and expenses in carrying out a study of traditional remedies against enterotoxins, we devised a screening method using the GM1 Elisa blocking or neutralization assay described below to detect which of the traditional remedies being tested will likely show an effect in the rabbit ileal animal model. This method is simple and reduces the time spent screening each plant by about 90%.

A. Materials and Methods

The crude cholera toxin NIH001 was supplied by Dr. Bradley Sack (The Johns Hopkins University, School of Public Health). Polyvinyl microtiter "U" plates (Immulon) were used for all ELISA procedures. The GM1 ganglioside (Sigma) was used in a concentration of 1 ug/ml in phosphate buffered saline. The antiserum against crude cholera toxin NIH001 was produced by immunizing 2 adult rabbits (about 2 kg. each). The rabbits were pre-bled and then injected intramuscularly with 20 ug of purified cholera toxin (Schwarz-Mann) in Freund complete adjuvant on day 0. On day 17, the injection was repeated subcutaneously with 50 ug of pure cholera toxin in Freund incomplete adjuvant. Sera from the 2 rabbits were ~~were~~ collected on day 31. Goat anti-rabbit globulin (Sigma) conjugated with peroxidase was used as the second antiserum. p-Orthophenylenediamine was used as a substrate in the reaction.

All medicinal plants tested in the GM1 ELISA neutralization assay were prepared fresh as infusions in a concentration of 0.4 g/4 ml and then filtered using Whatman filter paper #3.

## The Neutralization Assay :

Microtiter plates were pre-coated with GM1 ganglioside (1 ug/ml) 100 ul per well and incubated overnight at room temperature. Thereafter, each of the following steps was followed by 3 washings of PBS-Tween, 200 ul for 3 minutes, each at room temperature. Crude cholera toxin (1000 ug/ml) in PBS-Tween + 0.1% bovine serum albumin was added in each of the positive control wells, 100 ul per well. Each of the test plants 50 ul/ml were added to each test well and mixed with 50 ul of the cholera toxin (2000 ug/ml). After an overnight incubation, 100 ul of the rabbit anti-cholera toxin diluted 1:1000 in PBS-Tween+0.1% BSA was added to each of the test wells and then incubated for one hour at 37C. The peroxidase labelled goat anti-rabbit globulin, diluted 1:1000 in PBS-Tween+0.1% BSA was added to each of the test wells, 100 ul per well. After one hour incubation at 37C the substrate, p-orthophenylene diamine was added, 100 ul per well. The reaction was stopped with diluted H2SO4 after 15 minutes. Optical density was read at 490 nm in a Dynatech ELISA reader.

## II. RESULTS.

As can be seen from the graph, Neutralization of cholera toxin NTH001 by 10 medicinal plants, five out of ten plants showed neutralization of more than or equal to 60%. Out of the five that showed neutralization, Tara, Granada, and more recently, Chili-Chili, have been shown to reduce loop fluid accumulation significantly in the rabbit ileal loop assay. Ratania and Chuchussi did not reduce loop fluid accumulation significantly. The rest of the five plants that showed neutralization less the 60% all were tested negative in the rabbit ileal loop assay. We propose therefore that the GM1 ELISA assay could be used as a screening tool for many medicinal plants to determine their effects against cholera toxin and it could be adapted to testing effects against other enterotoxins.

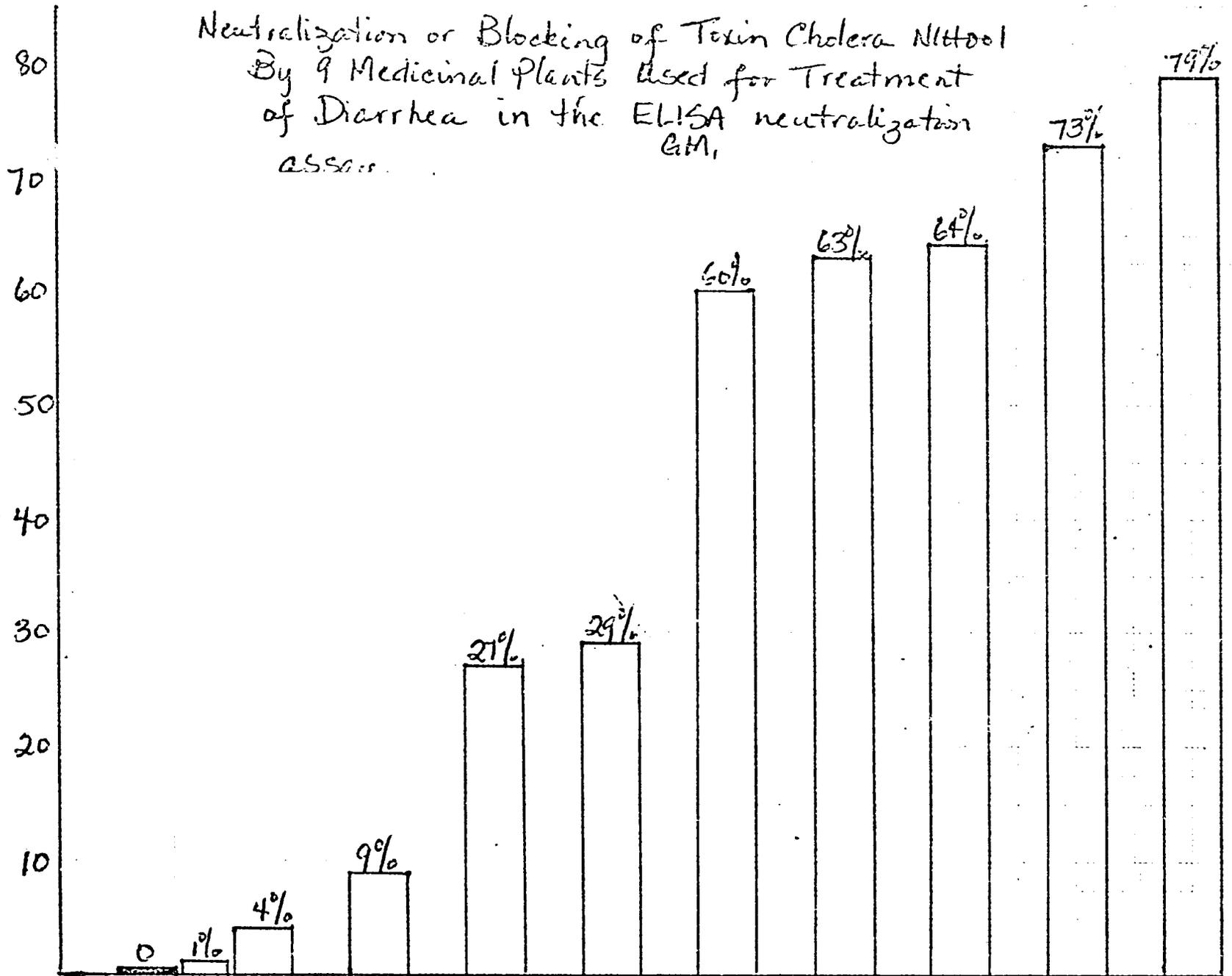
## Reference:

1. Sack, R. B. et.al. 1982. Berberine inhibits intestinal secretory response of *V.cholerae* and *E.coli* enterotoxins. Infect. Immun. 35:471-475.
2. Sack, R. B. et.al. Microtiter ganglioside enzyme-linked immunosorbent assay for *Vibrio* and *E.coli* heat-labile and enteric and enterotoxin. J.Clin. Microbiol. 1982.

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$$\% \text{ Neutralization} = \left( 1 - \frac{\text{O.D. of unknown sample} - \text{B.G.}}{\text{O.D. of positive sample} - \text{B.G.}} \right) \times 100$$

Neutralization or Blocking of Toxin Cholera Nitrool  
 By 9 Medicinal Plants Used for Treatment  
 of Diarrhea in the ELISA neutralization  
 assay.



Concentration of each test plant.

Tox. Cont.  
 1000000  
 1/5th Arayagan Lanchuli  
 1000000  
 Berberine Sulfate  
 1000000  
 Paico  
 0.4g/ml  
 Yeso  
 0.4g/ml  
 Arayagan  
 0.4g/ml  
 Retania  
 0.4g/ml  
 Chili-chili  
 0.4g/ml  
 Granada  
 0.4g/ml  
 Chuchuesi  
 0.4g/ml  
 Tara  
 0.4g/ml

Chili-chili + Tox NIH881

- 1.) Press RETURN twice to continue data entry.
  - 2.) Press RETURN, then F2 if no data for that cell.
  - 3.) Press RETURN, then F10 after last data entry.
- Sample 3
- 1.) Press RETURN twice to continue data entry.
  - 2.) Press RETURN, then F2 if no data for that cell.
  - 3.) Press RETURN, then F10 after last data entry.

only enterotoxin

Enterotoxin + Chili-chili added at 0, 5 and 15 minutes after the enterotoxin

	Sample 1 ToxCont	Sample 2 Tox+CHO	Sample 3 Tox+CH5	Sample 4 Tox+Ch15
1:	2.5	0.5	2.3	2
2:	2.5	0.25	0.5	1.15
3:	1.5	0.86	1.98	1.27
4:	2.0	0.78	0.86	0.90
5:	1.41	1.21	1.57	2.14
6:	1.25	1.17	0.9	1.22
7:	3.0	0.8	2.4	1.6
8:	3.2	0.5	0.86	1.4
9:				

NO.	8	8	8	8
MEAN	2.160000	0.768875	1.305000	1.422500
NED	2.250000	0.790000	0.990000	1.345000
SDEV	0.798024	0.348517	0.711698	0.426505

Press space bar to return to menu:

#####  
: STUDENT'S T-TEST (two-tailed) :  
#####

What is the name of the Database you wish to analyze? C:none  
(Press RETURN if you wish to skip directly to T evaluation)

What are the SAMPLE NUMBERS of the 2 groups you want to compare?  
Sample #: 1 'ToxCont'                      Sample #: 2: 'Tox+CHO'

Means =                      2.16    .768875

Are these INDEPENDENT or PAIRED samples? (1 or 2)                      1

T = 4.716139    df = 14

p = 3.30058E-04

The MEANS of these 2 samples are significantly different.

The confidence limits on the DIFFERENCE between the means of these samples can be calculated as:

$$1.391125 (-1(14) + .2949712$$

Do you want perform another T-TEST using this database? (Y or N)

What are the SAMPLE NUMBERS of the 2-groups you want to compare?  
Sample #: 1 'ToxCont'                      Sample #:                      'ToxCont5'

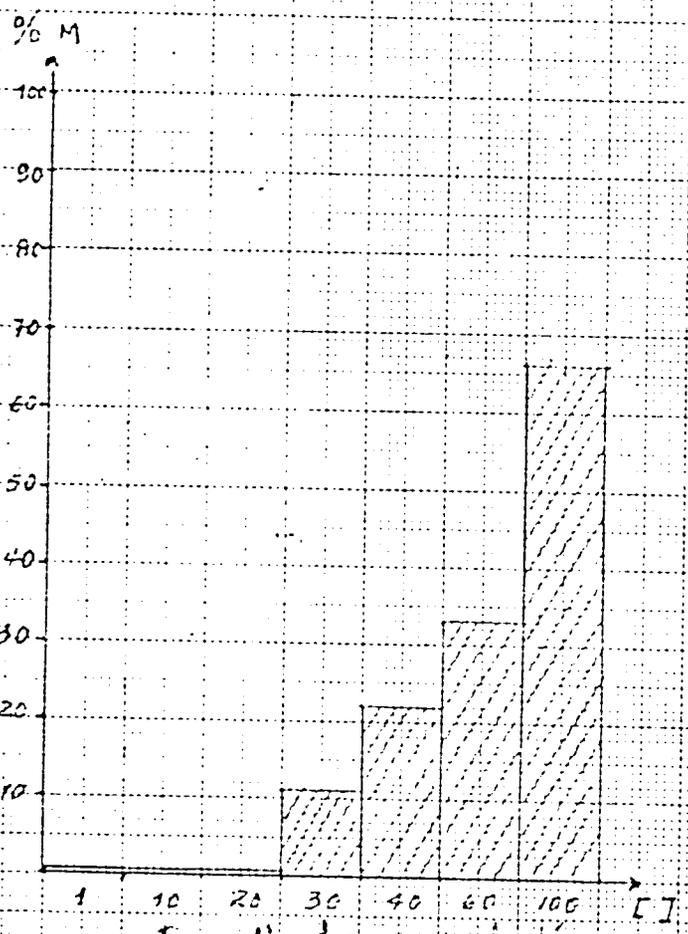
Means =                      2.16    1.305



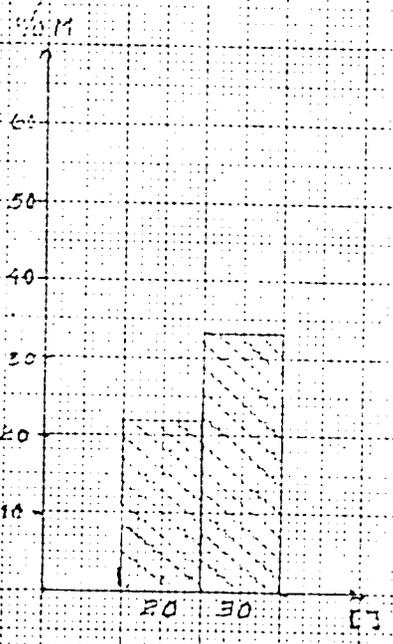


PRUEBA DE TOLERANCIA IN VIVO

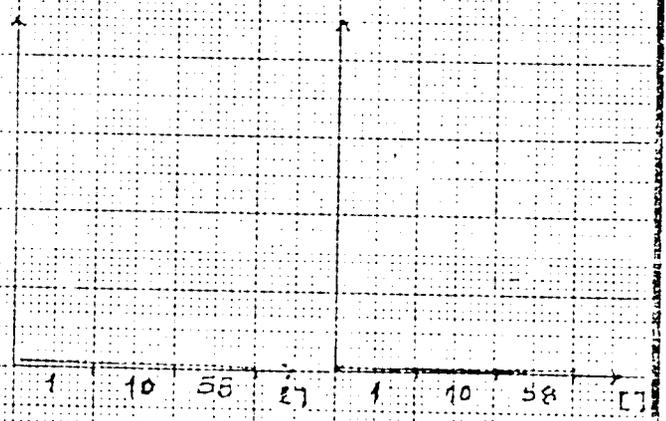
- I. LECHE DE DYE FORD
- II. LECHE DE DYE + MEDICINA
- III. PASTA DE HELICHO MACHO
- IV. HOJAS DE HELICHO MACHO



I Times the dose equivalent to usual accustomed dose



II



III

IV

Mortality increases markedly with increases in doses of Leche de Dye, but not with Helicho Macho

% of mortality with doses progressively greater than the one equivalent to usual accustomed dose

PRUEBA DE TOXICIDAD EN VIVO

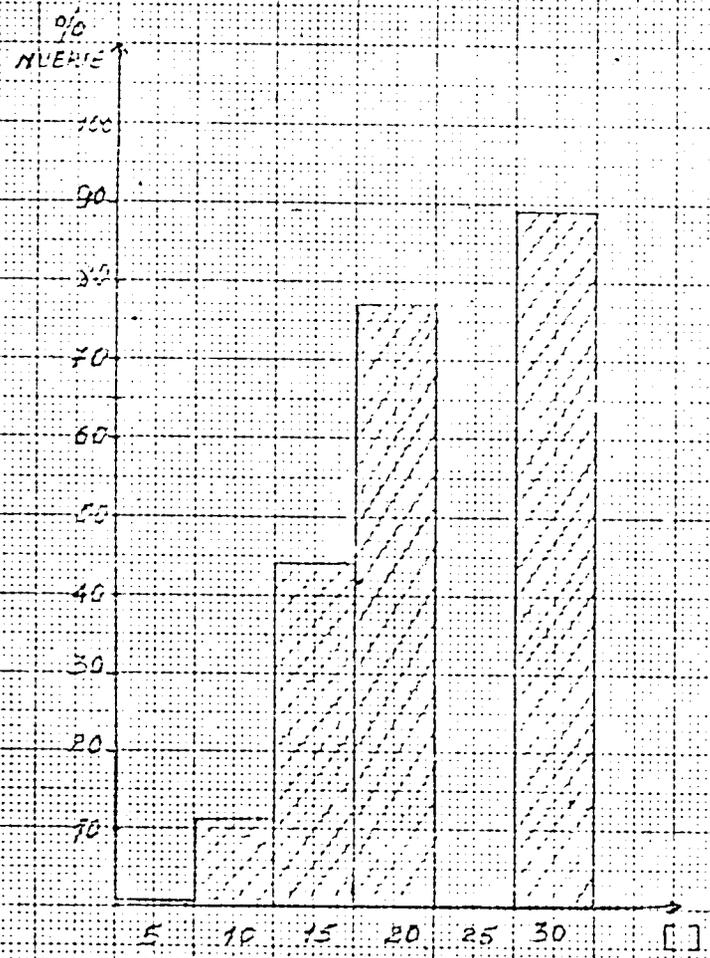
I. LATEX DE PAPAYO

CONCENTRACIONES QUE SE EQUIVALEN A 100000 UNIDADES MUEER

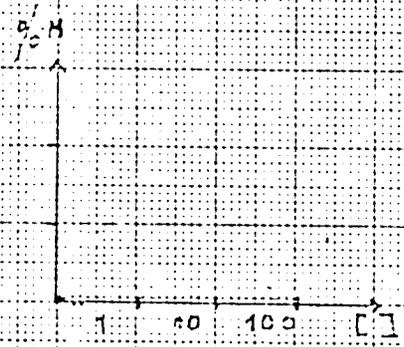
II. PEPAS DE PAPAYO

III. PEPAS DE ZAPALDO

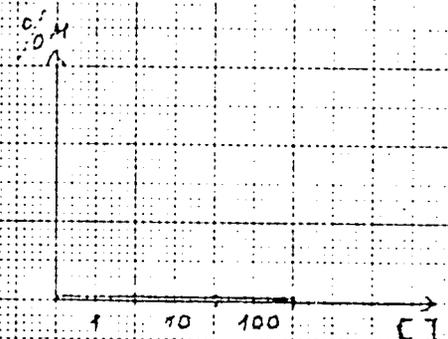
% DE MUERTE / CONCENTRACION DE LA SANGRE



I.



II.



III.

Markedly increases markedly with increase in doses of latex de papayo, but not with seeds of papayo.