Philippine Council for Advanced Science and Technology Research and Development

Human Resources and Institution Development Division Scholar's Thesis/Dissertation Progress Report for the 1st 2nd 3rd 4th Quarter / Year 2010

THESIS/DISSERTATION TITLE: ASSESSMENT ON THE HYPERACCUMULATION AND PROTEIN PROFILING OF WATER SPINACH (*IPOMEA AQUATICA*) WHEN GROWN UNDER HEAVY METAL (LEAD) STRESS

GRANTEE: IRISH A. TEJANO

DEGREE PROGRAM:

MS CHEMISTRY

DURATION: 10 MOS.

DATE STARTED: MAY 2010 **EXPECTED DATE OF COMPLETION:** MAR 2011

OBJECTIVES:

This study aims to examine the ability of water spinach to accumulate heavy metal when treated with lead and to detect the distinct protein profiles of the treated and untreated plant sample.

Specifically, this research deals to:

1. test and identify the plant organ (sorted roots, leaves and stems of water spinach) that accumulates the greatest amount of lead through Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) technique ;
2. evaluate the effect of Pb toxicity on chlorophyll contents,hydrogen peroxide formation and on some growth parameters (biomass and length of roots)
3. fractionate proteins using SDS-PAGE technique
4. identify the amino acid sequence of some proteins distinctly expressed by the plant consequent to heavy metal stress condition.

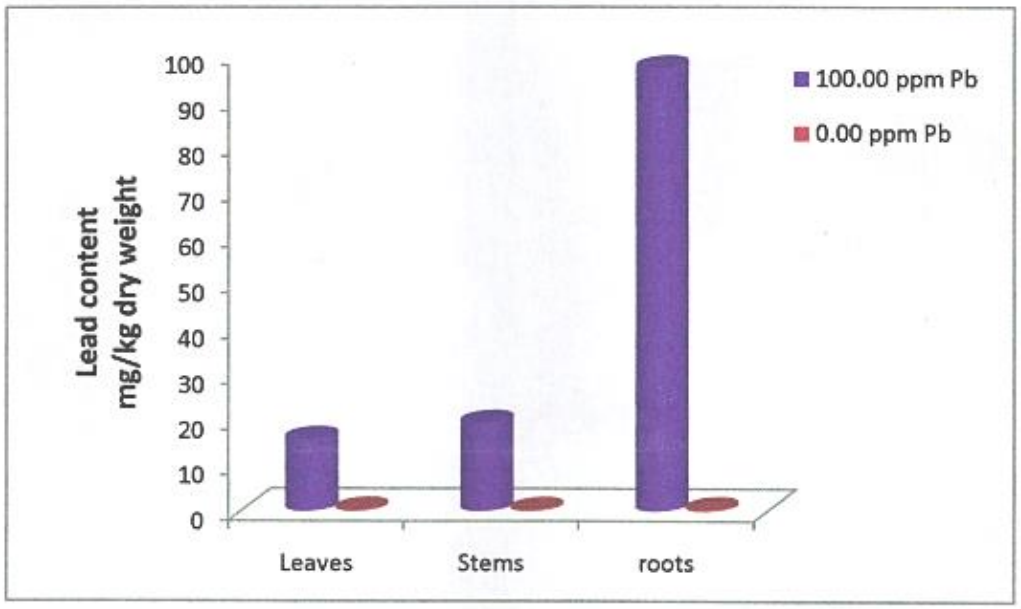


Figure 1. Lead accumulation in different parts of Water Spinach exposed to different concentrations of lead

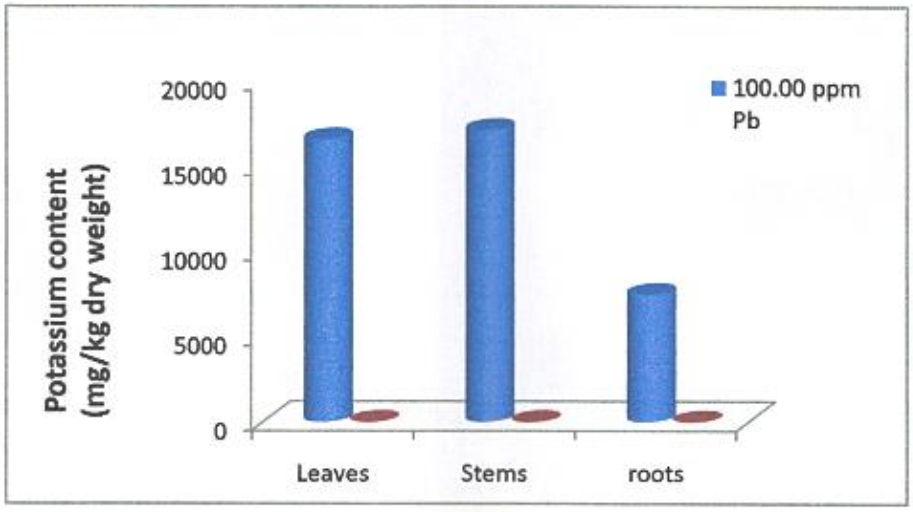
The lead contents in the leaves, stems and roots of the water spinach increased significantly under the Pb stress when compared to untreated plant. The results show that there is a significant difference in the accumulation of lead in different parts of the plant sample. The absorbed Pb was localized to a greater extent in roots than in leaves and stems for the treated plant. At highly toxic Pb concentration, 100 ppm, an 18 folds increase in leaves and 21 folds increase in stems and 102 folds increase in roots were observed for lead content when compared with their corresponding controls.

SCHEDULE OF ACTIVITIES:

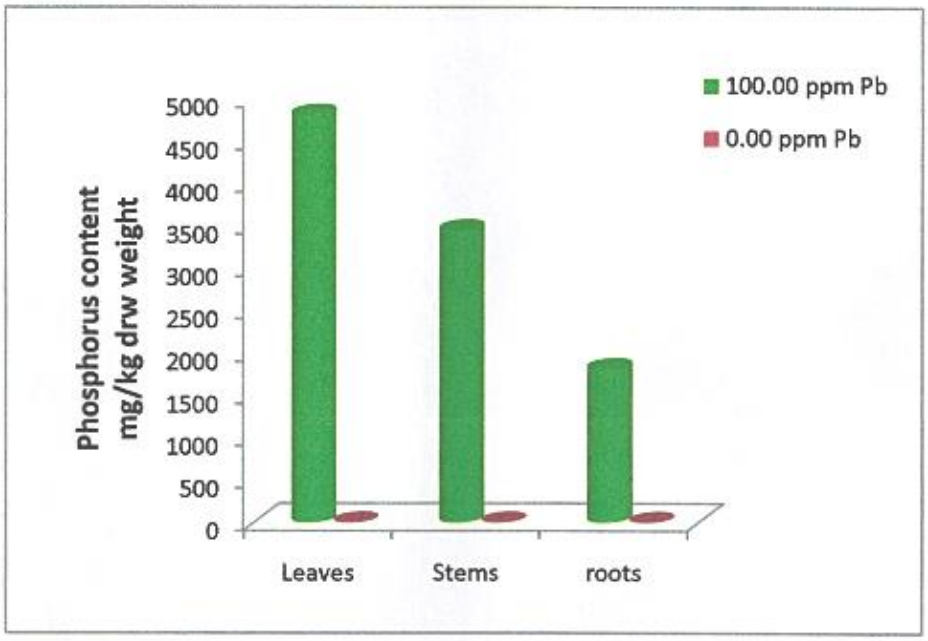
Month	Scheduled Work
• 4th week of March	• Plant Propagation
• April	• Hydroponic cultivation of Plant
	• Lead treatment
	• Harvest
• May	• Preparation of sample for ICP- OES
• June- August	• Protein Extraction for SDS-PAGE and Amino Acid Sequencing
• Sept.- Oct.	• Waiting for the ICP- OES Results
• Nov- Dec.	• Sending of sample/ Waiting for the SDS-PAGE and Amino acid sequencing results
• Jan.- Feb.	• Analysis and Interpretation of the Data and Results
• March	• Manuscript Writing

RESULTS AND DISCUSSION:

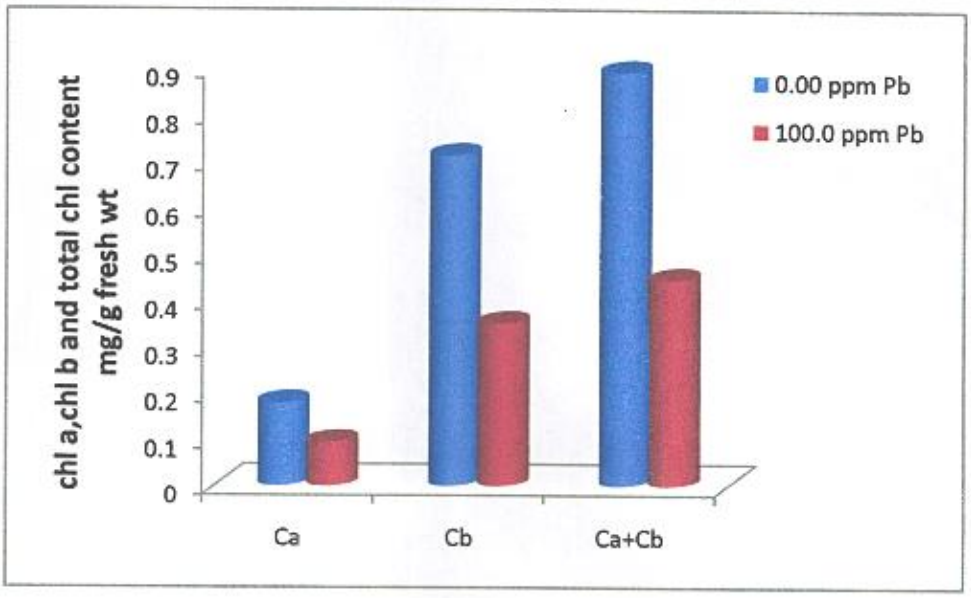
The following graphical illustrations were the preliminary results on the determination of the elements: phosphorus, potassium and lead present on water spinach samples. These will served as the results for the screening on the hyperaccumulation of water spinach when induced to heavy metal stress.



Results show that there is a significant difference in the uptake of potassium by water spinach when exposed to heavy metal stress. Stems show the highest concentration of potassium compared to leaves and roots.



Results show that there is significant difference in the uptake of phosphorus of the water spinach when treated with lead compared to the untreated plant. Leaves have the highest content compared to stems and roots.



A significant effect of lead treatment on the plant sample was observed. A decrease of chlorophyll was recorded in the plants treated with 100 ppm of lead compared to the untreated plant.

PROBLEMS ENCOUNTERED/PROPOSED SOLUTIONS:

There were lacking results in the determination of elements: lead, phosphorus and potassium in the plant sample. The researcher decided to repeat the set-up for plant propagation to enhance and to acquire reliable results. The SDS-PAGE on the sample did not show good results due to the incapacity of the electrophoresis set-up used. The researcher made some modifications on the set-up being used since it was designed for vertical electrophoresis and not for horizontal purpose but still it did not work properly. Hence good and reliable results were not obtained at some specific conditions desired by the researcher. The researcher had contacted for collaborations in the SDS-PAGE and amino acid sequence analyses of the plant sample.

The following pictures show the new set-up for plant propagation at the growth chamber.

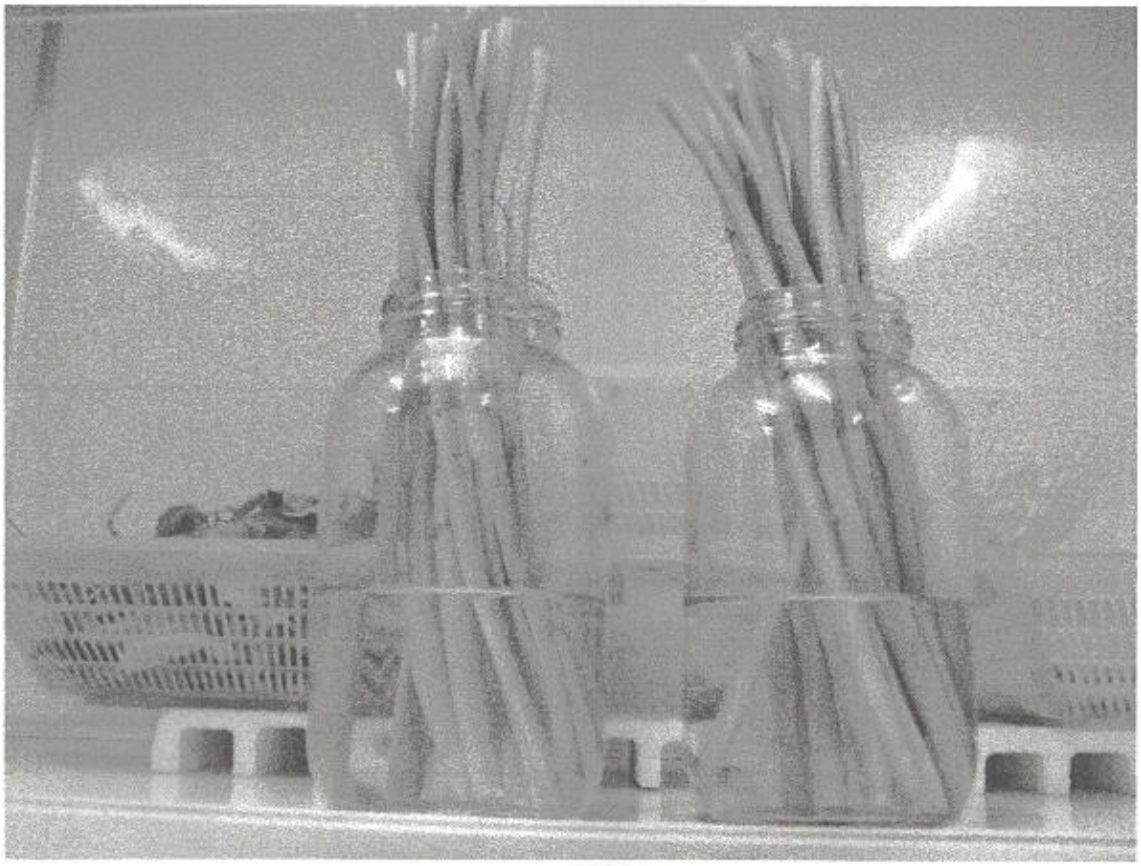


Figure 1. Plant sample after being cleaned and exposed to distilled water for 24 hours.

Figure 2. Plant sample being placed in the growth chamber prior to lead treatment.

REMARKS:

The researcher had screened the plant sample on its ability to hyperaccumulate heavy metal. Plant sample prior to the analysis of SDS-PAGE and Amino acid sequencing will be send to Taiwan University upon collaboration with Conmar Malmis, a PhD student in biochemistry.

Prepared by:

IRISH A. TEJANO
Grantee's name/signature

This portion is to be filled up by adviser and returned to PCASTRD in a sealed envelope together with the scholar's report:

PERCENTAGE OF TOTAL WORK COMPLETED:

Roughly about 50% of the Thesis work was done.

COMMENTS:

She encountered difficulty in running the horizontal SDS-PAGE set-up for her protein analysis. SDS-PAGE and amino acid sequence analyses will be done at Taiwan University by Mr Conmar Malmis, our collaborator.

ADVISER'S NAME/SIGNATURE:

LYDIA M. BAJO, Ph.D.

11/8/10
DATE

Philippine Council for Advanced Science and Technology
Research and Development
Human Resources and Institution Development Division

Scholar's Thesis/Dissertation Progress Report

for the ☑ 1st ☐ 2nd ☐ 3rd ☐ 4th Quarter / Year 2010

THESIS/DISSERTATION TITLE:

ASSESSMENT ON THE ACCUMULATION AND PHYTOCHELATIN PEPTIDE PROFILING OF THE WATER SPINACH [*Ipomoea aquatica* Forsk] GROWN UNDER HEAVY METAL (LEAD) STRESS

GRANTEE:
IRISH A. TEJANO

DEGREE PROGRAM:
MS CHEMISTRY

DURATION:
6 MOS.

DATE STARTED:
MAY 2010

EXPECTED DATE OF COMPLETION:
OCT. 2010

OBJECTIVES:

This study aims to examine the ability of water spinach to hyperaccumulate heavy metal when treated with different level of lead and to detect and quantify metal-binding peptides (phytochelatins) of the plant extract.

Specifically, this research aims to:

1. test and identify the plant organ (sorted roots, leaves and stems of water spinach) that accumulates the greatest amount of lead through Inductively Coupled Plasma- Optical Emission Spectrometry (ICP-OES) technique ;
2. isolate and analyze phytochelatins peptides chromatograms found in plant organ with greatest amount of lead using HPLC analysis; and

3. identify the amino acid sequence of some proteins distinctly expressed by the plant consequent to heavy metal stress condition employing the LC-MS/MS technique.

SCHEDULE OF ACTIVITIES:

Month	Scheduled Work	Duration
• May	• Seed Propagation	• one mo.
• June- July	• Hydroponic cultivation of Plant • Lead treatment • Harvest • Preparation of sample for ICP-OES • Protein Extraction for HPLC • Protein Extraction for SDS-PAGE and LC-MS/MS Analysis	• two mos.
• August	• Waiting for the Results • Analysis and Interpretation of the Data and Results	• one mo.
• Sept.- Oct.	• Manuscript Writing	• two mos.

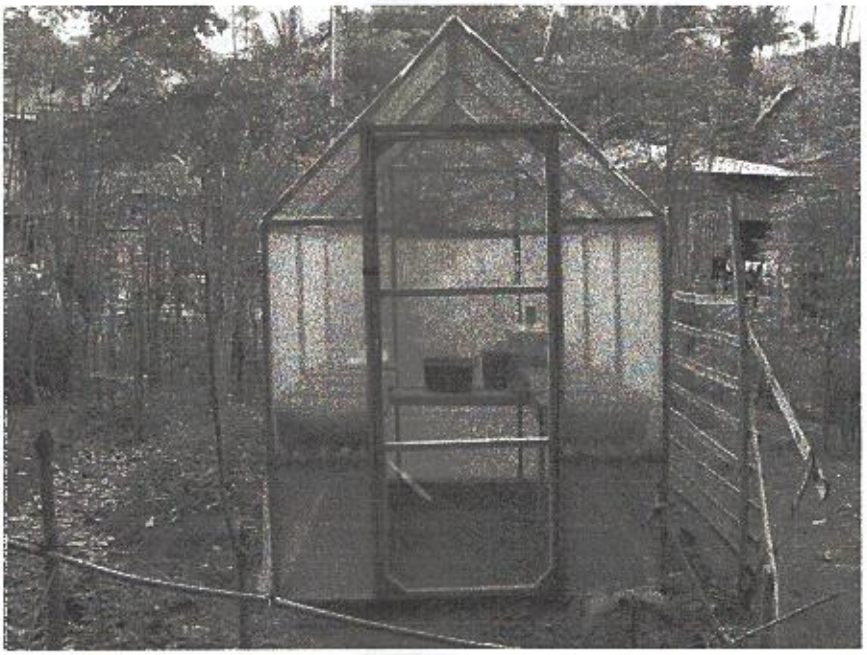
RESULTS AND DISCUSSION:

The plant samples (water spinach) were successfully cultivated using hydroponic system of planting inside a makeshift greenhouse. Plant samples were treated with 100 ppm Pb for one week. The observable characteristics of the plants were monitored and photographed.

The researcher already prepared the needed reagents and solutions for the analysis of lead in the plant sample. The samples were harvested and prepared for dry ashing. Apparatus and needed materials for plant digestion were ready prior to further

analyses. Plant samples were ready to undergo analysis in the Inductively Coupled-Plasma (ICP) apparatus for the determination of lead content.

The above accomplishments of the researcher in regards to the study are supported by the following pictures taken during the experimental period.



Makeshift greenhouse located near the residential place of the researcher.

The researcher preparing the hydroponic set-up and planting of sample.

Hydroponic set-up treated with 100 ppm of heavy metal lead.

The researcher is currently optimizing the experimental procedures for the protein extraction of the plant material for further analyses: HPLC, SDS-PAGE and Amino acid sequencing.

PROBLEMS ENCOUNTERED/PROPOSED SOLUTIONS:

The first hydroponic propagation of water spinach was unsuccessful. Leaves were turning yellow. This might be due to too much sunlight that was trap in the makeshift greenhouse. The proposed solutions to address the problem were to wrap the roof of the greenhouse with a net so that less sunlight could pass through. The nutrient level in the hydroponic set-up was also increased to compensate the transpired or evaporated liquid/water which could upsurge the yellowing of the leaves.

REMARKS

Thesis plan was realized. Optimization of experimental methods was done to maximize reliable results.

Prepared by:

IRISH A. TEJANO

Grantee

This portion is to be filled up by adviser and returned to PCASTRD in a sealed envelope together with the scholar's report:

PERCENTAGE OF TOTAL WORK COMPLETED:

Ms. Luisa A. Tejano has completed 50% of her total thesis work. She is now on the isolation of peptides found in the plant sample.

COMMENTS:

Hopefully, Ms. Tejano will be finishing the experimental portion by the end of October.

ADVISER'S NAME/SIGNATURE:

LYDIA M. BAJO, Ph.D.

July 23, 2010
DATE

SCHEDULE OF ACTIV[redacted]

Month	Scheduled Work
◦ October	◦ Plant Propagation ◦ Hydroponic cultivation of Plant
◦ November	◦ Lead treatment ◦ Harvest ◦ Preparation of sample for ICP- OES
◦ Dec.	◦ Protein Extraction for SDS-PAGE and Amino Acid Sequencing
◦ January	◦ Waiting for the ICP- OES Results ◦ Sending of sample/ Waiting for the SDS-PAGE and Amino acid sequencing results
◦ February	◦ Analysis and Interpretation of the Data and Results
◦ March	◦ Manuscript Writing

Prepared by:

Irish A. Tejano
Scholar/Advisee

Noted and endorsed by:

Dr.Lydia M. Bajo
Adviser

LINE ITEM BUDGET

ITEM	QUANTITY	PRICE
CHEMICALS:		
• Hydrochloric acid; approx. 6 M	4L	1,000
• Nitric acid, concentrated	4L	2,000
• chelating agent EDTA (1 M)	1L	3,000
• 96% acetic acid	4L	800
• 60% Perchloric acid		
• Laemmli-β-mercaptoethanol lyses buffer	1L	1,200
• Lead Nitrate	4L	3,000
• Nutrient Solution	50L	4,000
• Synthetic PCs	10 pcs.	5,000
		20,000
APPARATUS:		
• Hydroponic tank	8 pcs.	1,000
• Epperdorf Tubes & cryovials	30 pcs.	2,000
• Porcelein crucible	30 pcs.	3,000
• Greenhouse	1 unit	5,000
		11,000
ANALYSES FEE:		
• ICP-OES		10,000
• HPLC		5,000
• SDS-PAGE		10,000
• Amino acid Sequencing		15,000
		40,000
Manuscript Expenses/ Documentation:		10,000
TOTAL COST		**81,000**

Prepared by: **IRISH A. TEJANO**
Advisee

NOTED BY: **LYDIA M. BAJO Ph.D**
Adviser

preparation; limits of detectable contamination depend on the sequences of the analysed proteins.

The most important prerequisite for a protein to be sequenced by Edman degradation is that the N-terminal residue is not blocked, but free to react in the first step of the sequencing procedure. Proteins can be blocked either naturally e.g. by acetylation, or during purification by cyclisation of glutamine. It is estimated that >50% of all proteins are blocked. In these cases no sequence is obtained.

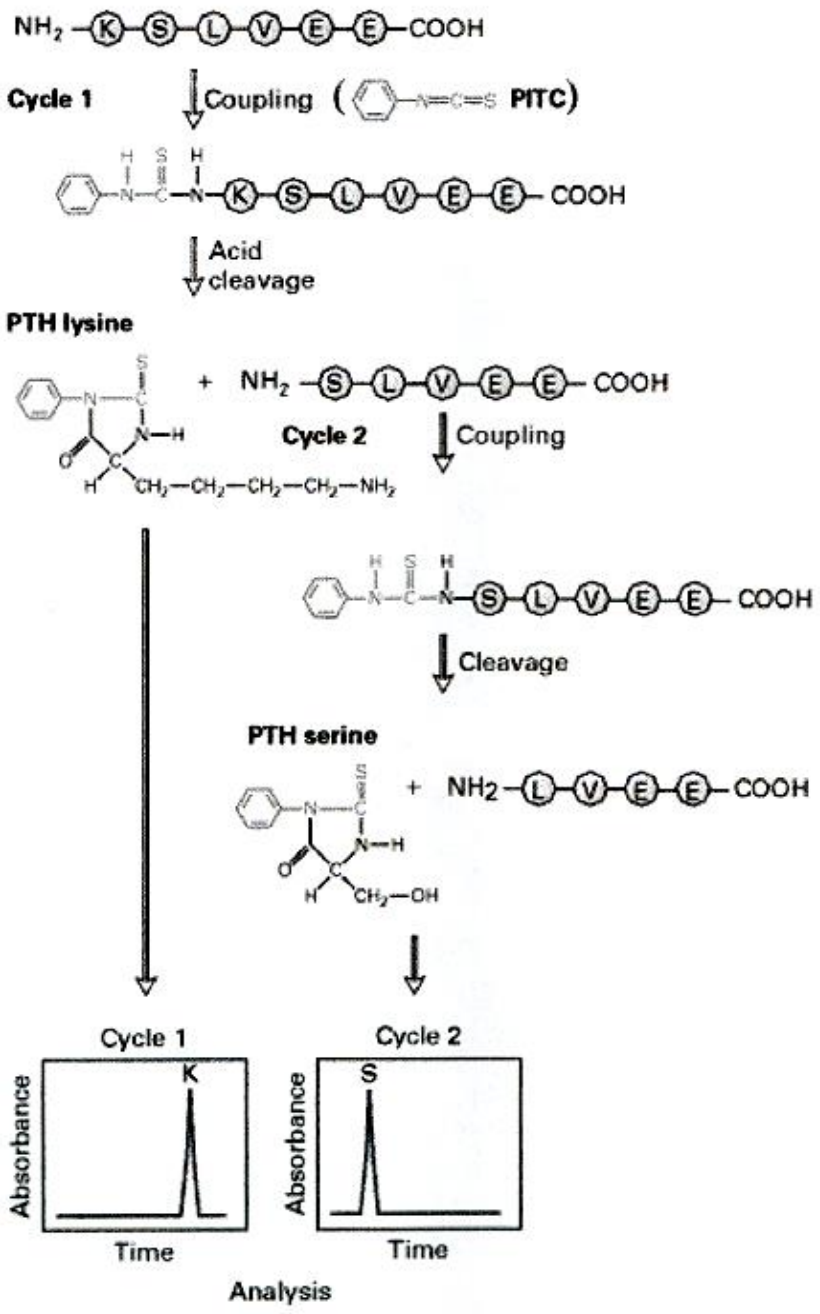


Figure 4 Overview of the Chemical determination of the sequence of a protein by Edman degradation.

In the first step, the polypeptide N-terminus is reacted with phenylisothiocyanate (PITC). In the second step, the N-terminal amino acid is cleaved from the polypeptide by acid hydrolysis, yielding the cyclic phenylthiohydantoin (PTH) derivative and a polypeptide that is shorter at its N-terminus by one residue. These two steps are then repeated with the shortened polypeptide. The PTH derivative formed in each cycle is identified by liquid chromatography (W. H. Freeman and Company, 2000).

CHAPTER 3

METHODOLOGY

3.1 Preparation of Plant Material

- **Materials Needed:**
  - Nutrient Solution
  - Lead nitrate
  - Deionized water
  - Greenhouse
  - Hydroponic Tank

Stem cuttings will be employed for propagating the plant. Water spinach will be grown under hydroponic conditions, using Hoagland nutrient solutions (to be made with deionized water), with varying lead concentration and with sand to be used as a mechanical support. The plants will be grown under greenhouse at the vicinity of MSU-IIT, Iligan City. Three pots per treatment of heavy metal with three replicates (plants) per pot will be used. Seedlings will be grown for 5 weeks. Each pot would be spiked with 0, 50, 125, 375, 750 and 1,000 μM $Pb(NO_3)_2$ concentrations for once a week.

3.2 Analysis of Lead Content (Dry Ashing)

- **Materials needed:**
  - crucible,15 mL,porcelain,tall form
  - muffle furnace
  - water bath
  - filter paper,Whatman no.541
  - Hydrochloric acid; approx. 6 M
  - Nitric acid, concentrated

After five weeks the water spinach will be harvested and sorted into different parts: roots, stems and leaves. Sorted plant parts will be dried in an oven at 70 °C. A 2.5 g of dried and ground plant material will be placed into an acid- washed porcelain crucible. The crucibles with samples will then be put in a muffle furnace with the temperature slowly raised over 2 h to reach 500 °C and ashed for at least 4 h. Thereafter, crucible will be removed and allowed to cool. Ten milliliter of 6 M HCl will be added then covered and will be heated on a steam bath for 15 min. One milliliter of concentrated nitric acid will then be added and evaporated to dryness. Heating will be continued for 1 h to dehydrate silica. One milliliter of 6 M HCl will be added. The solution will be consequently swirled and will be added also with 10 mL of deionized water. It will be heated again on a

steam bath to complete dissolution. Cool and filter through a Whatman filter paper into a 25 mL volumetric flask and make up to the mark with water. A blank will then be prepared by repeating procedure but omitting the plant sample.

3.3 Phytochelatin Analyses

3.3.1 Protein Extraction prior for HPLC Analysis

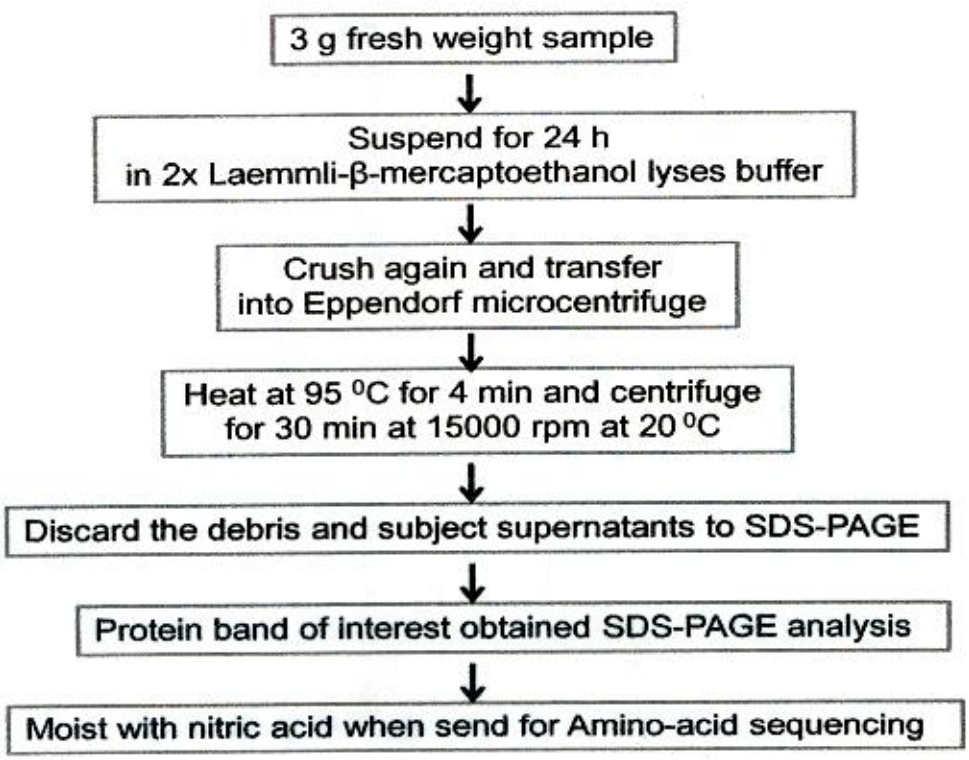
- **Materials needed:**
  - plastic bag
  - blender
  - ice bath/ liquid nitrogen
  - centrifuge instrument
  - chelating agent EDTA (1 M)
  - 96% acetic acid
  - 60% Perchloric acid
  - cryovials

Plant's organ (roots, stems or leaves) of high concentration of lead level as detected by ICP-OES will undergo PC analysis. Plants that are mature and fully developed will be harvested. Each part will be weighed, cut into pieces and ground using a blender. Two grams of fresh weight will

be taken and will be added with 0.8 mL of chelating agent EDTA. Homogenates will then be vortexed for 10 min and added with 0.8 mL of 96% acetic acid. Vortex again for 5 min and add 2.4 mL of 60% perchloric acid. Homogenates will then be transferred to centrifuge tubes and centrifuge at 9000 g for 10 min at 4°C. Supernatants will be transferred in an eppendorf tube and ready for HPLC analysis. A synthetic PC will be purchased for further detection of PCs in the experimental sample and will also be subjected for HPLC analysis.

3.3.2 Protein extraction prior for SDS-PAGE and Amino-acid Sequencing Analyses

- **Materials needed:**
  - Stirring rod
  - Ice bath
  - Laemmli-β-mercaptoethanol lyses buffer
  - Eppendorf microcentrifuge tubes
  - centrifuge



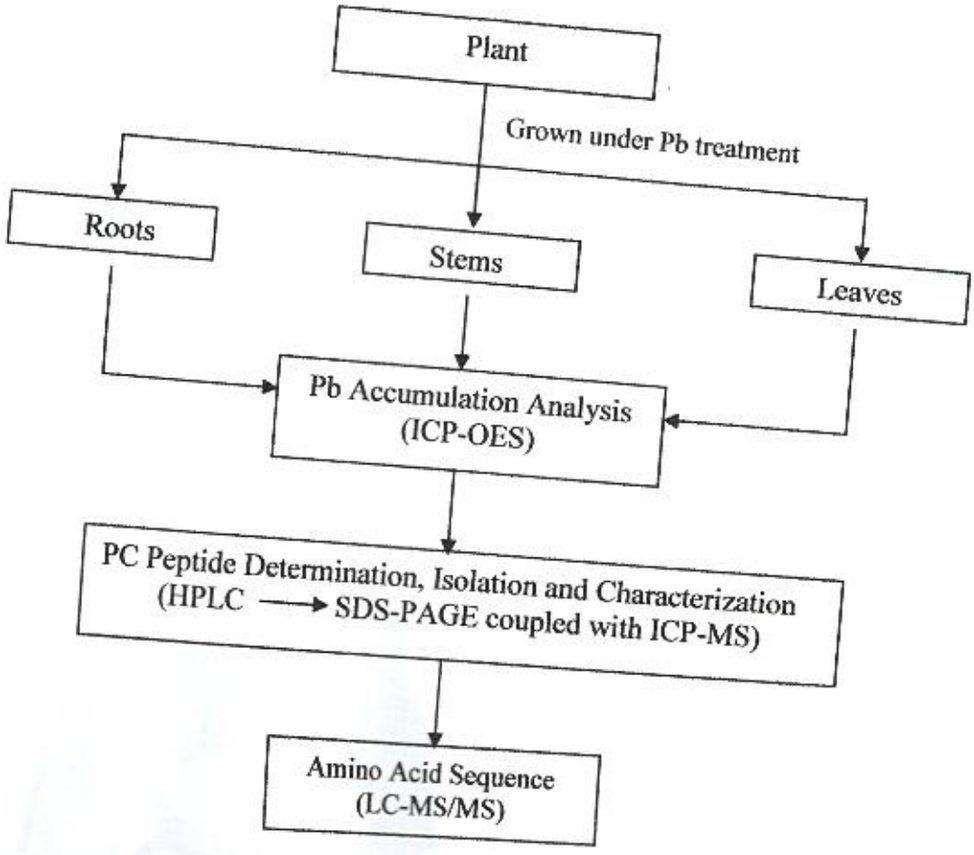


Figure 5 The experimental framework of the study.

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LINE ITEM BUDGET

ITEM	QUANTITY	PRICE
CHEMICALS:		
• Hydrochloric acid; approx. 6 M	4L	1,000
• Nitric acid, concentrated	4L	2,000
• chelating agent EDTA (1 M)	1L	3,000
• 96% acetic acid	4L	800
• 60% Perchloric acid		
• Laemmli-β-mercaptoethanol lyses buffer	1L	1,200
• Lead Nitrate	4L	3,000
• Nutrient Solution	50L	4,000
• Synthetic PCs	10 pcs.	5,000
		20,000
APPARATUS:		
• Hydroponic tank	8 pcs.	1,000
• Epperdorf Tubes & cryovials	30 pcs.	2,000
• Porcelein crucible	30 pcs.	3,000
• Greenhouse	1 unit	5,000
		11,000
ANALYSES FEE:		
• ICP-OES		10,000
• HPLC		5,000
• SDS-PAGE		10,000
• Amino acid Sequencing		15,000
		40,000
Manuscript Expenses/ Documentation:		10,000
TOTAL COST		**81,000**

TIME TABLE OF THE STUDY

Month	Scheduled Work	Duration
• last week of March	• Seed Propagation	• one week
• April	• Hydroponic cultivation of Plant • Lead treatment	• one mo.
• May	• Harvest • Preparation of sample for ICP-OES • Protein Extraction for HPLC • Protein Extraction for SDS-PAGE and LC-MS/MS Analysis	• one mo.
• June- July	• Waiting for the Results	• two mos.
• August	• Analysis and Interpretation of the Data and Results	• one mo.
• Sept.- Oct.	• Manuscript Writing	• two mos.