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Molecular Approach for the Identification of Stress Response Proteins and Improvement of Phytoremediation of Heavy Metals by Plants

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5-9-194, Chirag-Ali-lane, IV Floor, A.P.S.F.C. Building,
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Submitted By

Dr.T.Selvankumar- Principal Investigator
Mrs. Rathika Rajiniganth- Co-Investigator

PG & RESEARCH DEPARTMENT OF BIOTECHNOLOGY
MAHENDRA ARTS & SCIENCE COLLEGE
(AUTONOMOUS)
(*Affiliated to Periyar University, Salem-11*)
Accredited by NAAC with "A" Grade & Recognized with 2(f) and 12(B) u/s UGC Act 1956.
90th All INDIA-NIRF Ranking 2020
Attayampatti (Via), Kalippatti (Po), Tiruchengode (Tk),
Namakkal (Dt), Tamil Nadu, India.

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CERTIFICATE

This is to certify that the UGC sponsored minor project entitled **"Molecular Approach for the Identification of Stress Response Proteins and Improvement of Phytoremediation of Heavy Metals by Plants"** is a bonafide research work carried out by **Dr.T.Selvankumar-Principal Investigator and Mrs. Rathika Rajiniganth -Co-Investigator.** The research report has been submitted to UGC-Sponsored Minor Project Report to the **University Grants Commission (UGC), South Eastern Regional Office (SERO), Hyderabad.**

Co-Investigator **Principal Investigator** **Principal**

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Content

I. Introduction

Soil contamination has been a serious problem worldwide, due to population explosion, rapid industrial expansion and lack of funds for pollution control and renewal of the environment (Kim et al., 2014; Patil et al., 2016; Yılmaz, 2016). Heavy metals are among the most important sorts of contaminant in the environment. Several methods already used to clean up the environment from these kinds of contaminants, but most of them are costly and difficult to get optimum results. Heavy metals, the most hazardous environmental contaminants, are natural elements that could be supplied to soils by atmospheric deposition, agricultural practices such as application of fertilizer, pesticide and municipal wastes such as composts and sewage sludge (Zaimoglu et al., 2009; Abou-Elwafa et al., 2019). Heavy metals such as lead (Pb), and nickel (Ni) causes morphological, physiological or biochemical harmful effects to live organisms. In plants, it decreases seedling development, root elongation, transpiration, chlorophyll production, cell division and eventually plant growth (Maestri et al., 2010; Pourrut et al., 2011; Krzeslowska et al., 2016). Although low concentrations of nickel (Ni) has several roles in enhancing plant growth and metabolism, higher concentrations pose harmful effects (Parlak, 2016; Gautam et al., 2017). Small quantities of these metals are essential for human health; though in higher concentrations they become toxic or dangerous, affecting brain, kidney, lungs, liver and other important organs. Moreover, long-term exposure to them can cause physical, muscular and neurological degenerative processes, and even cancer (Jarup, 2003; Álvarez-Mateos et al., 2019).

Naturally, plants are exposed to many adverse environmental conditions like biotic and abiotic stress. Despite all others stresses heavy metal stress is one of great importance which has a notable adverse effect on crop productivity and growth. Heavy metal stress triggers different responses in plants, ranging from biochemical responses to crop yield. Stress proteins are a

diverse group of proteins that are synthesized at increased levels by cells exposed to a variety of stressful stimuli and which have a protective effect against the stress.

Bioremediation of heavy metals has been emerging, which is growing up as the attention in researchers in the present decade to clean up and maintain the pollution-free environment. Several studies reported the physicochemical methods of remediation and its disadvantages. Bioremediation, particularly phytoremediation using plants, is an eco-friendly and efficient method for the removal of metals (Li et al., 2019). Among phytoremediation, phytoextraction of heavy metals from contaminated soils has been receiving great importance. Phytoextraction to reduce the heavy metal levels in the soil by hyperaccumulator plants. The hyperaccumulator plant shoots accumulate the heavy metals and produce a considerable amount of biomass. The hyperaccumulator plants usually accumulate only a specific metal in a small amount. Several studies reported the Pb hyperaccumulator plants and their Pb uptake abilities. With advances in stress biology, some of the high biomass crops such as *Zea mays, Pisum sativum, Avena sativa,* and *Brassica juncea* plants were used as the promising alternative to hyper accumulating plants.

Production of reactive oxygen species (ROS) is increased by stress conditions. The ROS are highly cytotoxic can disrupt cell metabolism via oxidative damage to cellular components (Halliwell, 1982). Plants have evolved protective mechanisms to reduce the risk of ROS, which are effective at different levels of stress-induced deterioration (Sinhal et al., 2010). Among the ROS protective mechanisms of plants (*V.radiata, S. officinarum,* and *S. bicolor),* antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD) and catalases (CAT) received much attention against ROS.

Phytoremediation refers to the use of green plants and their related microbiota, soil amendments, and agronomic techniques to eliminate, comprise, or reduce harmless

environmental contaminants (Cunningham and Ow, 1996; Li et al., 2019). Plants remove pollutants through several bioactive processes; several factors such as growth rate of plants, reduced biomass, phytotoxicity of metals, geochemistry of the contaminated soil, biotic and abiotic stress in plants, and limited uptake of metals are known to hinder these processes (Loganathan et al., 2015).

Recently, the use of specific chelating agents and PGPB to increase the solubility and consequently stimulate the potential of metal accumulation in plants (assisted phytoextraction) is receiving significant attention (Meers et al., 2008). Two phytoextraction methods are commonly applied (1) use of hyper-accumulator plants, and (2) addition of chemicals (synthetic/organic) to enhance the growth of high-biomass producing plants to accelerate metal uptake in their aerial parts (Evangelou et al., 2007; Murakami et al., 2007; Shakoor et al., 2014). Heavy metals mobility and solubility in the soil are most important for their bioavailability. For this purpose, different chelating agents have been used to enhance metal solubility in the soil (Ehsan et al., 2014). Also, the use of chelating agents increases the resistance of a plant to drought stress and accumulation of metals in the plant (Farid et al., 2013). Numerous studies suggested that the addition of chelating agents, such as diethylene triamine penta acetic acid (DTPA) and ethylene-diamine tetraacetic acid (EDTA) and N-(2-hydro- xyethyl)-ethylene diamine tri acetic acid (HEDTA) and organic chelates such as citric acid may enhance metal mobility and bioavailability (Sinhal et al., 2010; Szczygłowska et al., 2011; Bareen, 2012; Chigbo and Batty, 2013). Synthetic chelators are capable of phytoextraction but cause groundwater contamination and are non-biodegradable (Anwer et al., 2012; Bareen, 2012). Also, the negative effects of synthetic chelators have been observed, and include increased toxicity of metals to plants and soil microorganisms (Tandy et al., 2006; Evangelou et al., 2007). It has been reported synthetic

chelating agents should be replaced by biodegradable low molecular weight citric acid (CA) to alter the chemical nature of heavy metals, bioavailability and to improve the phytoremediation of heavy metals (Al Mahmud et al., 2018). Due to the eco-friendly nature, easily biodegradable, non-toxic properties of the CA, has been applied as an amendment for the enhanced phytoextraction of heavy metals (Anwer et al., 2012).

Vermiwash (VW) vermicompost extract, a brown-coloured liquid obtained by washing the vermicompost system. It contains amino acids, vitamins, plant growth-promoting hormones, nitrogen, potassium, zinc, calcium (Aghamohammadi et al., 2016). Several studies applied VW as a potential bio-fertilizer, pesticide to enhance the growth and productivity of crops.

The structure of a protein determines its biological function and its interactions with other factors; the binding regions tend to be conserved in sequence and structure, and the interacting residues involved are usually in close 3D space. The Protein Data Bank currently contains more than 110000 protein structures, approximately one-third of which contain metal ions. Identifying and characterizing metal ion-binding sites is thus essential for investigating a protein function and interactions.

Aim and Objectives

This present study aims to assess the "Molecular approach the Identification stress on proteins and improvement of Phytoremediation of heavy metals in plants" with the following objectives:

- To isolate and identify the major heavy metals soil and plant tissues.
- To identify the stress related protein evaluation and study the property of adaptation of the plants.
- To identify the impacts of heavy metals influence and their regulation of stress proteins.
- To assess the role of enzymes/protein in stress regulatory mechanism for stress tolerance.
- To evaluate the binding of various receptor and heavy metals by docking study.
- To predict the inhibitor for the enhancement of stress response in plants.

II. Materials and Methods

Soil sample

Garden soil (0-30 cm topsoil) used in the experiments was collected from, Namakkal and Erode Districts of Tamil Nadu, India.

Determination of soil physicochemical properties

Soil samples were analyzed for particle size by the international pipette method, bulk density by metal-core sampler method and porosity and moisture content according to Maiti (2003). The different physicochemical characteristics such as pH, Electrical conductivity (EC), organic carbon (OC), water holding capacity (WHC) Nitrogen (N), phosphorus (P), sodium (Na), potassium (P) of the soil sample was analyzed according to standard methods. The soil sample was air-dried and sieved through a 2 mm metal sieve to remove the stones, seeds and other components of the soil and autoclaved for 20 min at 121°C for 15 min. The sterilized soil was artificially contaminated with 100 mg/kg of Pb and Ni individually, whereas in control soil also prepared without any metal contamination. After the appropriate mixing of the metal solutions, the soil samples were incubated for 3-4 weeks. The moisture content of the soil samples were maintained at 60-70% using sterilized distilled water.

Pot experiments

V. radiata, S. officinarum, and *S. bicolor* seeds and seed cane were purchased from the agricultural training centre at Mallasamudram, Namakkal (Dt), Tamil Nadu, India. The seeds and seed cane were surface sterilized with 2% NaCl solution followed by sterile distilled water with 2-3 times and the seeds and seed cane were soaked in distilled water for 24-48 h at room temperature for germination. After the germination, the seedlings were transplanted into the

plastic pot (8 cm in diameter with 10 cm height) filled with 500 g of soil contaminated with Pb and Ni respectively.

X-ray diffraction investigation of soil

The soil samples were analyzed by XRD to identify the metal crystals produced by heavy metals.

Characterization of plant biomass using FT-IR

At the end of bioremediation experiments, the plant biomass was collected, washed several times with sterile distilled water, and dried for 24–48 h in a freeze dryer. The dried plant biomass was analyzed by using FT-IR, and the spectrum was obtained using the KBr method on a Perkin-Elmer FT-IR spectrophotometer in the region of 4000–400 cm.

SEM analysis

Heavy synthesis was carried out as described in Govarthanan et al. (2015). Briefly, the plant extract was mixed with1 mM heavy metal until the color changed indicated a successful synthesis. After centrifugation, the pellet was dispersed and dried. An aliquot of 4 ml of the prepared extract was added to 96 ml of heavy metals (1 mM) and mixed with vigorous magnetic stirring for 30 min until the color change colour indicated the synthesis. The mixture was centrifuged at 3000 rpm for 15 min, the pellet was dispersed in double-distilled water and dried in lyophilizer.

Vermiwash and Citric acid on *V. radiata, S. officinarum*, and *S. bicolor* plants

The pot experiments were maintained under fixed incubation conditions of 10 h photoperiod, 26°C with 60-65% of humidity. After 5-7 days of adaptation in Pb and Ni contaminated soil with seedlings were treated with 10 ml of vermiwash and 10 ml of (10 mM) citric acid respectively. Whereas, the control pot was treated with 10 ml of distilled water. After

30 days, the plants were harvested and the root system was cleaned with tap water followed by sterile distilled water. The shoot and root lengths were measured recorded. The Pb and Ni concentration in the samples were determined using atomic absorption spectroscopy (AAS) (Thermo Scientific™ iCE™ 3500).

Microscopic observation of shoot structure of *V. radiata, S. officinarum* and *S. bicolor*

After a month treatment with citric acid and vermiwash, the shoot thin-section of the *V. radiata, S. officinarum*, and *S. bicolor* was prepared and stained with basic strain safranin and fast green. After appropriate strain washing, the sectioning was observed under an inverted phase contrast microscope and photographed to check the accumulation of Pb and Ni on the shoots of *V. radiata, S. officinarum*, and *S. bicolor*.

Biochemical assays

Non- enzymatic antioxidant assays

Total Chlorophyll

0.2 g leaves were ground in cold pestle and motor with 10 ml of 80% acetone. The extract was centrifuged at 3000 g for 5 min. The upper phase was transferred into a new tube and its absorbance was measured at 663, 646 and 470 nm, respectively, for chlorophyll a, b and carotenoid with acetone 80% as a blank.

Estimation of total flavonoids

The antioxidative properties of flavonoids are due to several different mechanisms, such as scavenging of free radicals, chelation of metal ions, and inhibition of enzymes responsible for a free radical generation. Depending on their structure, flavonoids can scavenge practically all known ROS. The amount of total flavonoid content can be determined by aluminium chloride method. The reaction mixture (3.0 ml) comprised of 1.0 ml of extract, 0.5 ml of aluminium

chloride (1.2%) and 0.5 ml of potassium acetate (120 mM) is incubated at room temperature for 30 min and absorbance measured at 415 nm. Quercetin can be used as a positive control. The flavonoid content is expressed in terms of standard equivalent (mg/g of the extracted compound).

Total phenolic content Assay

The amount of total phenolic content can be determined by Folin-Ciocalteu reagent (FCR) method. Commonly 0.5 ml of extract and 0.1 ml of Folin-Ciocalteu reagent (0.5 N) are mixed and incubated at room temperature for 15 min. Then 2.5 ml of saturated sodium carbonate is added and further incubated for 30 min at room temperature and absorbance measured at 760 nm. Guaiacol can be used as a positive control. The total phenolic content is expressed in terms of standard equivalent (mg/g of the extracted compound).

Antioxidant enzymes activity analysis

The sample preparation and analysis of antioxidant enzyme activity of the plant samples were followed according to Singh et al., (2009) with minor modifications. Briefly, 2 g of plant material was ground with 10 mL of phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 1% polyvinylpyrrolidone. The homogenate was centrifuged at 15,000 rpm for 15 min at 4°C, and the supernatant was collected for the determination of SOD, POD, CAT activities.

SOD activity

SOD activity of the plant sample was estimated according to Dhindsa et al., (1981). The complete reaction mixture containing 1.5 ml of 0.1 M potassium phosphate buffer (pH 7.0), 0.2 ml of 200 mM methionine, 0.1 ml of nitro-blue tetrazolium chloride (NBT), 0.1 ml of EDTA, 0.1 ml of 1.5 M sodium carbonate, 0.1 ml of enzyme solution and 0.8 ml of distilled water. The reaction is based on the formation of blue-coloured formazone by nitro-blue tetrazolium chloride and O_2 radical. The absorbance was read at 560 nm using a UV-Vis spectrophotometer (UV-

1800, Shimadzu, Japan). Tubes containing reaction mixture in the absence of enzyme extract was used as a control. One unit of enzyme activity was defined as the quantity of SOD required to produce a 50 % inhibition of the reduction of NBT.

POX activity

POX of the plant extract was estimated based on the method described by Dhindsa et al., (1981). The 0.1 ml of the reaction solution was mixed with 1 mL of 100 mM phosphate buffer, 0.5 mL of 96 mM guaiacol, 0.5 mL of H_2O_2, 0.4 mL of double-distilled water. Addition of H_2O_2 converted guaiacol into the tetraguaiacol due to the oxidation reaction. The changes in the absorbance were determined at 470 nm. One unit of POX activity was determined as an absorbance change of 0.001 U/min.

CAT activity

Plant catalase was determined according to the method described by Dhindsa et al., (1981) with minor modifications. Briefly, the enzyme mixture was mixed with 10 mL of 30% H_2O_2. The reaction was initiated by adding H_2O_2 into the enzyme mixture. The resultant product absorbance was analyzed by UV-spectrophotometer at 240 nm. The catalase activity was defined as the amount of enzyme catalyzing the decomposition of 1mmol of H_2O_2/min.

AOX activity

Ascorbate oxidase activity can be measured with the method of Dhindsa et al., (1981). 1.0 ml of reaction mixture contained 20 mM potassium phosphate buffer (pH 7.0) and 2.5 mM ascorbic acid. The reaction was initiated with the addition of 10 µl enzyme extract. The decrease in absorbance was observed for 3 min at 265 nm due to ascorbate oxidation and calculated using the extinction coefficient, $mM^{-1}cm^{-1}$.

Extraction and estimation of protein

The stem/leaf tissues were cut into pieces and immediately plunged in 0.5M phosphate buffer (pH 7) or water. 10.0 to 15.0 ml of buffer for every gram of tissue was used. The tissues were thoroughly macerated in a mortar with a pestle for 5 to 10 minutes. This was then passed through two layers of muslin cloth and re-extracted the ground tissues by second grinding. This second extraction ensures complete removal of buffer soluble substances. This second extract passed through a muslin cloth. Both the extracts were pooled, filtered through what man No. 41 filter paper and the volume was raised with buffer. Protein estimation in this extract was carried out by Lowry's method (1951).

Protein analysis by SDS PAGE

SDS PAGE (Sodium dodecyl sulfate Polyacrylamide Gel Electrophoresis) gel was used for visualizing the proteins on the gel method according to Kumar Sahu et al., (2017).

Estimation of Rubisco protein

Rubisco protein was isolated from the leaves of *V. radiata*, *S. officinarum*, and *S. bicolor* grown in Pb and Ni contaminated soils according to Wang et al., (1992). The activity of the isolated Rubisco was spectrophotometrically determined at 340 nm according to Racker (1962). The enzyme unit was calculated as one unit of the enzyme was defined as the amount of enzyme-producing 1 M of RuBP per min.

Metal-Stress docking study

MIB is a binding site prediction and docking server (http://bioinfo.cmu.edu.tw/MIB/.) for metal ions, and this server provides an accurate, integrated approach to search the residues in metal ion-binding sites using the fragment transformation method.18 Predictions of residues that bind 2 types of metal ions are supported. The query protein structure is compared with each

metal-binding template in the database to locate the metal-binding residues. Each residue of the query protein is assigned a binding score, which is composed of sequence and structure conservation measures.

III. Result and Discussion

Soil testing

The soil used for the growth of plants were analyzed for its physiological properties (Table 1) (initial and final day) such as pH, conductivity, bulk density, phosphates, nitrates, organic matter, organic carbon and moisture content were decreased during the final day (60^{th} day) compared to the initial day (0^{th} day).In contrast the sulphates and nickel concentration increases during the final day (60^{th} day) than the initial day (0^{th} day).

Soil pH generally plays an important role in metal bioavailability, toxicity and leaching capability to surrounding areas. Heavy metals are mostly soluble and leached out in acidic pH. Soil pH of 6.5 indicates that heavy metals may remain in the soil for long time exposed to plants that come into contact with them.

Structural studies

The seed germination was observed in (*Vigna radiata*, *Sorghum bicolor* & *Saccharum officinarum*) plants up to 0-14 days under green house condition. After 60 days of interval the following parameters were calculated. The length of shoots and roots (Fig 1a,b and 5) were decreased in respective to the concentration of metal salts (100,300,500 mg/kg) added to the soil compared to control plant shoots and roots. The plant leaves (Fig 2a, b) showed reduced surface area at both the intervals in contrast to control leaf surface area. The fresh and dry weight of whole plant also showed the similar loss of weight in the respective intervals (Table 2).

The decrease in shoot length, root length, fresh weight, dry weights and total leaf area and yield gets affected by the presence of toxic pollutants in the effluent. That kind of pollutants mainly affects the respiration of the root. Respiration of root and soil organism tends to reduce

the oxygen and increase the CO_2 concentration. The soil becomes harder and closed the pores of the soil are closed causing less aeration and retarding the growth of plant.

Seed stress

The rate of germination of metal stressed seeds (*Vigna radiata*, *Sorghum bicolor* & *Saccharum officinarum*) showed decreased germination rate at different time intervals (24, 48, 72 hrs) with respect to the metal concentrations (100,300,500 mg/l). Every 24 hrs the physical deformities such as radical development were observed (Fig 3). Le (2012) investigated lead pollution near the road side soil. The results indicate that concentration of lead in road side soils range from 23-90 mg/kg with an average value of 37.11mg/kg, exceeded environmental background value.

XRD analysis

The result from sequential metal extractions might not be sufficient enough to confirm the role of carbonate precipitation and mineral formation. Thus, detailed XRD patterns of the bioremediated soils were investigated. Accordingly, the XRD results showed the formation of various minerals such as, calcite, aragonite, halite, and quartz. Many calcite peaks could be observed in the bioremediated soils (Fig 8).

FT-IR analysis

The FT-IR results revealed that the carboxyl, alkanes and amide groups served as the preliminary molecules for the subsequent precipitation of metals.

SEM analysis

The SEM image of metals obtained from plant extract exhibit spherical and triangle shapes and particles size was found in the average range of 10–70 nm.

Enzymic antioxidant assays

The spectrometric analysis using the plant extract showed gradual increase of the following enzyme activities. Catalase (CAT), Ascorbate-Oxidase (AOX), Guaicol peroxidase (GPX), Super Oxide Dismutalase (SOD) with control and increasing concentration of metal salts(100,300,500 mg/l) at both the intervals (Table 3).

Similar results have been reported in different plant species, where oxidative stress-induced by heavy metals was alleviated by increasing antioxidant enzyme activities. Increased oxidative defense systems have been reported to be correlated with tolerance to different stress conditions, thus plants perform normally under the adverse environment.

Non- enzymic antioxidant assays

The spectrometric analysis using the plant extract showed gradual increase of the following non enzyme activities. Total phenolic content, radical inhibition activity of DPPH with control and increasing concentration of metal salts (100,300,500 mg/kg) at both the intervals. In contrast, the photosynthetic pigments such as total chlorophyll and total flavonoids showed gradual decreased in their content (Table 4,5).

The increase in phenolic content may be due to protective function of these compounds against heavy metal stress by metal chelation and ROS scavenging.

The increase in pigment contents at low doses of lead and nickel have also been reported earlier. Inhibitory effect of high level of nickel was more marked on chlorophyll a than chlorophyll b. It is indicative of more lead and nickel sensitivity of chlorophyll a. Chlorophyll a/b ratio also decreased with increase in nickel concentrations.

Estimation of protein

The protein concentration of plant extracts decreases with increasing metal concentration, time intervals and control. Lipids and proteins are important constituents of the cell that easily damage in environmental stress condition. Hence, any change in these compounds can be considered as an important indicator of oxidative stress in plants (Table 6). The results of this study showed variable changes in soluble protein content in different metal treatments that reflect different levels of antioxidant defense. It is thought that decrease in total soluble protein content under heavy metals stress may be due to increase in protease activity.

SDS-PAGE analysis

The SDS-PAGE analysis of the protein in profile plants (Fig 6) leaf sample of Pb and Ni treated plants revealed major changes than control.

Vermiwash and Citric acid on *V. radiata, S. officinarum*, and *S. bicolor* plants

The pots were maintained under fixed incubation conditions of 10 h photoperiod, 26°C with 60-65% of humidity. After 5-7 days of seedlings adaptation, the pots were treated with 10 ml of VW and 10 ml of (10 mM) CA respectively. However, the control pot was treated with 10 ml of distilled water. After 30 days, the plants were harvested and the root system was cleaned with tap water followed by distilled water. The shoot and root lengths were recorded. The dry weights of shoot and roots are recorded as the biomass of the plant. The Pb and Ni concentration in the samples were determined (Table 7) using atomic absorption spectroscopy (AAS).

Morphological changes of *V. radiata, S. officinarum* and *S. bicolor*

Effects of VW and CA on the morphology of *V.radiata, S. officinarum* and *S.bicolor* cultivated in the Pb and Ni contaminated soils is represented. The biomass of *V.radiata, S.*

officinarum and *S.bicolor* grown with CA and VW treated plants were significantly increased with the control plants. Compared to control, CA treated plants showed 24, and 26% of increased biomass cultivated with Pb and Ni contaminated soils, whereas, VW treated plants showed 11 and 9% of increased biomass compared to CA cultivated with Pb and Ni contaminated soil. The chelators (CA and VW) treatment enhanced the growth of *V. radiata, S. officinarum* and *S. bicolor* resulting shoot and root lengths compared to control plants (Fig 4 a,b, c). Compared to control, CA treated plants showed 3, and 5 cm of the increased shoot and root length cultivated Pb and Ni contaminated soils, whereas, VW treated plants showed 6 and 8 cm of increased shoot and length compared to CA cultivated with Pb and Ni contaminated soil. Statistical analysis showed significant differences ($P < 0.05$) were observed for biomass, shoot length and root length in CA and VW treatments. The biomass and plant growth results indicated that the addition of VW improved the growth characteristics as well as amelioration of Pb and Ni toxicity symptoms in of *V. radiata, S. officinarum* and *S. bicolor*.

Non- enzymatic antioxidant assays

The spectrometric analysis using the plant's extract showed a gradual increase in the following non-enzyme activities. Total phenolic content, radical inhibition activity of DPPH with control and increasing concentration of metal salts at both intervals. In contrast, the photosynthetic pigments such as total chlorophyll and total flavonoids showed a gradual decrease in their content.

Enzymatic antioxidant assays

The spectrometric analysis using the plant's extract showed a gradual increase of the following enzyme activities. Catalase (CAT), Ascorbate Oxidase (AOX), Guaiacol peroxidase

(GPX), Super Oxide Dismutase (SOD) with control and increasing concentration of metal salts at both intervals (Table 3).

Estimation of protein

The protein concentration of plants extracts decreases with increasing metal concentration, time intervals and control.

SDS-PAGE analysis

The SDS-PAGE analysis of the protein in profile plants leaf sample of Pb and Ni treated plants revealed major changes than control.

Rubisco protein activity

Rubisco protein estimation has been performed. The results indicated that the enhanced activity of Rubisco was found in the plants (grown in Pb and Ni contaminated soil) treated with both VW and CA. In particular, the plants grown in Pb contaminated soil treated with VW showed enhanced activity of plant extract, whereas, CA treatment of Rubisco. Both the treatments showed higher activity than the control (Fig 4d).

Protein-Metal docking

The web server reported here was built to predict metal ion-binding residues and to generate the predicted metal ion-bound 3D structure (Fig 7). Binding templates have been constructed for regions that bind 12 types of metal ion-binding residues have been used to construct binding templates. The templates include residues within 3.5 Å of the metal ion, and the fragment transformation method was used for structural comparison between query proteins and templates without any data training. MIB also provides the metal ions docking after prediction. The stress proteins of *V. radiata* (2FLH, 1TI5, 1SIY), *S. officinaru* (3UL6, 3UL5,

2KSK, 5WAX, 5WEG), and *S. bicolor* (5VKT, 5AOG, 5KVA, 5TQM, 5MDH, 1VO2) were studied.

IV. Summary

1. The study reveals that the presence of heavy metal causes many variations in germination, and biochemical parameters of *V. radiata, S. officinarum* and *S. bicolor* plants. The effects of heavy metals on plants resulted in growth inhibition, structure damage, a decline of physiological and biochemical activities, as well as of the function of plants. However, this is an important work to find out that toxic effect of Pb and Ni. It may be suggested that the plants are being affected by the heavy metals from the surrounding sources. So, this polluted soil should be properly treated to remove the heavy metals.

2. Environmental pollution is becoming a great threat to all forms of life. Of these, stresses on agricultural crops are increasing rapidly at present. Mainly among the abiotic stresses the toxic heavy metal stress adversely affects growth and yield of crops. In the present study the effect of heavy metals (lead and nickel) stress on the *V. radiata, S. officinarum* and *S. bicolor* was examined. Under continuous observation and various biochemical analysis in green house condition. The plants seem to be completely stressed in their structure and their continuous uptake of metal salts damaged the antioxidant enzyme activity. Activities of enzymes such as (CAT, AOX, GPX, and SOD), photosynthetic pigments (Total Chlorophyll, Total flavonoids) and protein content found to be abnormal in the studied plant in contrast to the control plant on spectrometric analysis at different periods.

3. Phytoextraction seems to have considerable potential for decontamination of soils contaminated with heavy metals (lead and nickel). However, uptake of heavy metals by plants is limited by their low solubility in soil solution especially in the case of Pb. Application of phytoremediation has been proposed to increase Pb and Ni concentration in soil solution through enhancing dissolution of sparingly soluble soil minerals. Such enhanced concentration of Pb and

Ni in soil solution occurs mostly at the expense of exchangeable, organic matter and carbonate bound fractions.

4. Effectiveness of phytoextraction to bring more Pb and Ni into soil solution depends on its rate, contamination level of Pb and Ni as well as complementary metals present in soils and method of its application. It has been proposed that the plant mediated removal onto soils enhanced translocation Pb and Ni from roots to shoots by lowering their binding with cell walls. Reduction of percolation risks by the use of more degradable alternatives to plants has been proposed over recent years. In fact, we have arrived at cross-roads at which the scientific community is distancing itself from the concept of using persistent compounds such as EDTA in the context of field application to enhance phytoremediation and is turning its attention towards more degradable alternatives. After over a decade of dedicated plant research, there is a need to clearly state that although being an interesting bench mark model for enhanced phytoextraction research, as a compound has probably a low applicability in practical field application in the context of phytoextraction due to unacceptable percolation risks associated with its environmental persistence. Discovery of and applied research towards degradable alternatives such as EDDS could help to move enhanced phytoextraction, based on past EDTA based research, to the level of practical field scale application. Although a great number of studies have been conducted during recent years to make assisted Pb and Ni phytoextraction an effective and low-risk technology, it demands extensive investigations before drawing authentic and generalized conclusions of commercial significance.

Ultimately, the scientific community needs to determine whether enhanced phytoextraction for Pb in general has a future as soil remediation technology based on observed removal efficiency and the remaining associated risks as described in literature. Regardless of the

debate whether EDTA itself should be abandoned as a potential amendment for phytoremediation due to the adverse effects associated with its use, valuable lessons still remain to be learned from the vast historic research invested in the study of this compound throughout the world, as the scientific community makes the shift towards more degradable alternatives and safer means and methods of incorporating the principle of intentional increasing metal phytoavailability for phytoextraction purposes.

5. The study reveals that the presence of heavy metal causes many variations in germination, and biochemical parameters of *V. radiata*, *S. officinarum* and *S. bicolor* plants. The effects of heavy metals on plants resulted in growth inhibition, structure damage, a decline of physiological and biochemical activities, as well as of the function of plants. However, this is an important work to find out that toxic effect of Pb and Ni. It may be suggested that the plants are being affected by the heavy metals from the surrounding sources. So, this polluted soil should be properly treated to remove the heavy metals. The results of phytoremediation with vermiwash indicated that the VW amendment showed higher biomass, shoot and root lengths, chlorophyll concentrations, antioxidant enzymes, and photosynthetic enzyme activity of plants cultivated with Pb and Ni contaminated soil compared to CA. Therefore, the present study suggested that the VW could be used as a potential chelator for phytoaccumulation of heavy metals, as well as growth-promoting formulation. Hence, the VW could be replaced the synthetic chelators like CA for the enhanced phytoremediation of heavy metals. The stress proteins of *V. radiata* (2FLH, 1TI5, 1SIY), *S. officinaru* (3UL6, 3UL5, 2KSK, 5WAX, 5WEG), and *S. bicolor* (5VKT, 5AOG, 5KVA, 5TQM, 5MDH, 1VO2) were also proved that the metal ions are the key role for the elevation of stress response proteins.

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1. R.Rathika, P.Srinivasan, Jawaher Alkahtani, L.A. Al-Humaid, Mona S. Alwahibi, R. Mythili, T. Selvankumar, Influence of biochar and EDTA on enhanced phytoremediation of lead contaminated soil by *Brassica juncea*. Chemosphere, 2021, 271, 129513. **(IF. 5.77)**

2. R.Rathika, Ashraf YZ Khalifa, P.Srinivasan, L. Praburaman, S.KamalaKannan, T. Selvankumar, Woong Kim, M.Govarthanan, Effect of citric acid and vermi-wash on growth and metal accumulation of *Sorghum bicolor* cultivated in lead and nickel contaminated soil. Chemosphere, 2020, 243, 125327. **(IF. 5.77)**

3. M.Govarthanan, R.Mythili, S.Kamala-Kannan, T.Selvankumar, P.Srinivasan, H. Kim, In-vitro bio-mineralization of arsenic and lead from aqueous solution and soil by wood rot fungus, *Trichoderma* sp. Ecotoxicology and Environmental Safety, 2019, 174,699-705. **(IF. 4.87)**

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Table Legends

Table: 1 Soil Physico-chemical analysis

	pH	Conductivity (millimols)	Bulk density	Sulphates (mg/lit)	Phosphates (mg/lit)	O.M (%)	O.C (%)	M.C (%)	Pb (mg/kg)	Ni (mg/kg)
Initial day (0^{th})	6.2	0.11	1.156	0.64	1.26	1.47	0.85	2.150	20.25	22.80
Final day (60^{th})	5.5	0.05	0.987	0.87	1.02	1.23	0.64	1.875	63.10	77.32

Table: 2 Structural studies

Treatments	Shoot length(cm^2)		Root length(cm^2)		Leaf surface area(cm^2)		Fresh Weight (gms)		Dry Weight (gms)	
	30^{th} day	60^{th} day	30^{th} day	60^{th} day	30^{th} day	60^{th} day	30^{th} day	60^{th} day	30^{th} day	60^{th} day
Control	30	54	6	24	28	32.13	27	43	11	15.1
100 mg/kg	26	43	4.5	13.5	17.83	24.67	18	25	7.92	9.2
300 mg/kg	23	30	3.2	9	12.88	18.34	12	17	5.48	7.43
500 mg/kg	18	23	2.4	3.5	9.76	7.16	10	13	4.16	5.17

Table: 3 Antioxidants assay

Treatments	CAT (µmol mg-1 protein min-1)		AOX (µmol mg-1 protein min-1)		GPX (µmol mg-1 protein min-1)		SOD (U/mg protein)	
	30^{th} day	60^{th} day	30^{th} day	60^{th} Day	30^{th} day	60^{th} day	30^{th} day	60^{th} Day
Control	41.54	47.31	0.97	0.77	104.42	110.27	20.356	21.656
100 mg/kg	50.38	59.78	1.05	0.97	126.54	131.75	21.212	23.382
300 mg/kg	57.85	63.55	1.47	1.16	133.71	136.43	21.885	25.585
500 mg/kg	62.94	74.04	2.53	1.89	145.67	148.51	23.485	28.653

Non-Enzymatic antioxidants assay

Table: 4 Total Phenol Content And DPPH Radical Inhibition Activity

Assays	Control		100 (mg/kg)		300 (mg/kg)		500 (mg/kg)	
	30th day	60th day	30th day	60th day	30th day	60th day	30th day	60th day
Total Phenol content (mg/g)	7.88	8.26	8.17	8.63	8.74	9.25	9.61	10.27
DPPH (%)	23.25	25.42	26.2	28.54	30.68	33.42	32.79	40.12

Table: 5 Photosynthetic pigments

Treatments	Chlorophyll a (mg/g FW)		Chlorophyll b (mg/g FW)		Total chlorophyll (mg/g FW)		Total flavonoids (mg/g FW)	
	30th day	60th day	30th day	60th day	30th day	60th day	30th day	60th day
Control	0.0212	0.14	0.081	0.062	0.021	0.024	7.23	10.48
100 (mg/kg)	0.0155	0.021	0.069	0.035	0.012	0.013	5.42	7.87
300 (mg/kg)	0.001	0.017	0.018	0.029	0.008	0.015	4.05	6.21
500 (mg/kg)	0.002	0.003	0.008	0.009	0.01	0.006	3.64	4.78

Table: 6 Estimation of Protein

		Control	100 (mg/kg)	300 (mg/kg)	500 (mg/kg)
Protein (mg/g FW)	30th day	38.8	30.3	26.7	22.9
	60th day	32.5	27.6	23.8	19.4

Table:7 Analysis of Heavy metal

		Control	100 (mg/kg)	300 (mg/kg)	500 (mg/kg)
Heavy metal (Ni) (µg/g)	30th day	20.3	62.4	69.3	74.4
	60th day	22.6	71.7	76.5	83.2

Figure Legends

Fig. 1a. Heavy metals trated plant growth after 30 days intervel (*Vigna radiata.L*)

Fig. 1b. Heavy metals trated plant growth after 60 days intervel (*Vigna radiata.L*)

Fig. 2a. Heavy metals trated root length after 30 days intervel (*Vigna radiata.L*)

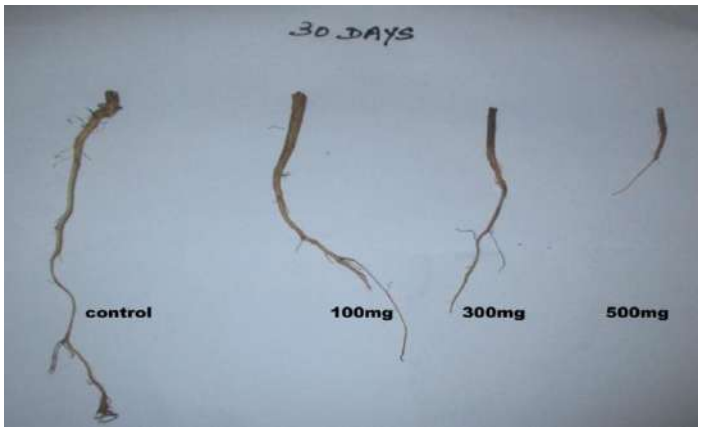

Fig. 2b. Heavy metals trated root length after 60 days intervel (*Vigna radiata.L*)

Fig. 3. Heavy metals trated Seeds Growth (*Vigna radiata.L*)

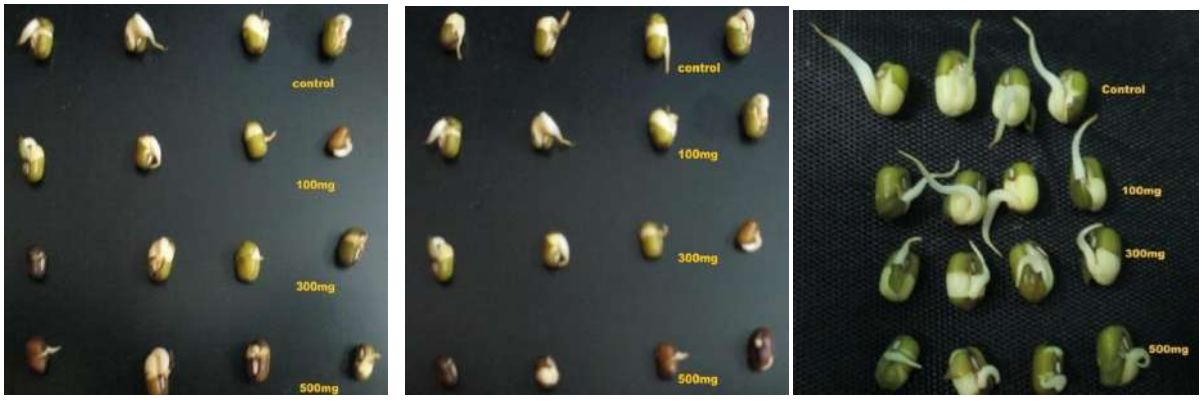

Fig.4. Effects of VW and CA on morphology and microscopically images of *S.bicolor* cultivated in the Pb and Ni contaminated soils.

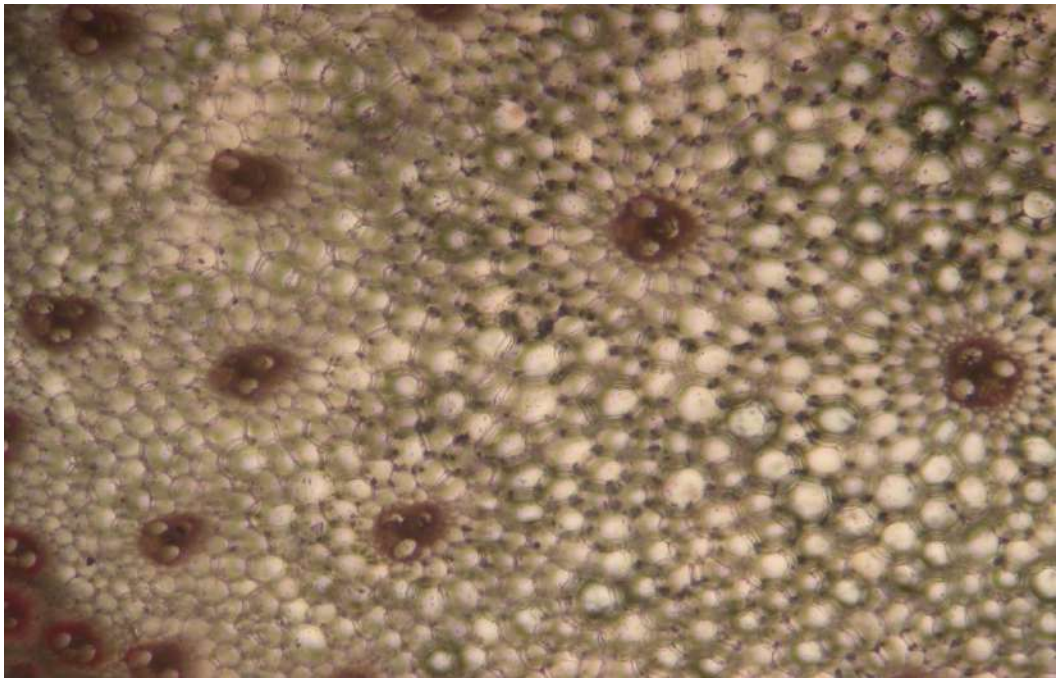

Fig.4a. Effects of VW and CA on morphology of *S.bicolor* cultivated in the Pb and Ni contaminated soils.

Fig.4b. Influence of VW and CA on biomass of *S.bicolor* cultivated and Chlorophyll concentrations in the Pb and Ni contaminated soils.

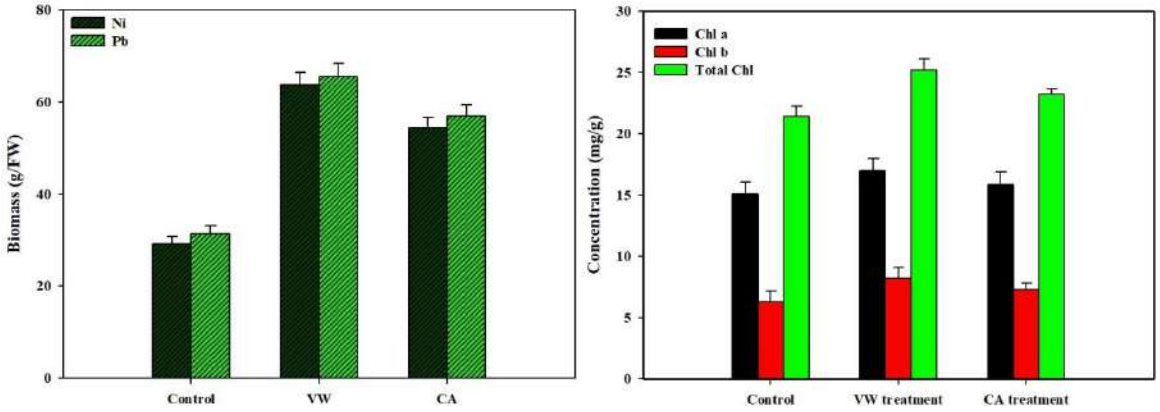

Fig. 4c. Effect of VW and CA on antioxidant enzymes of *S. bicolor* cultivated in the Pb and Ni contaminated soils.

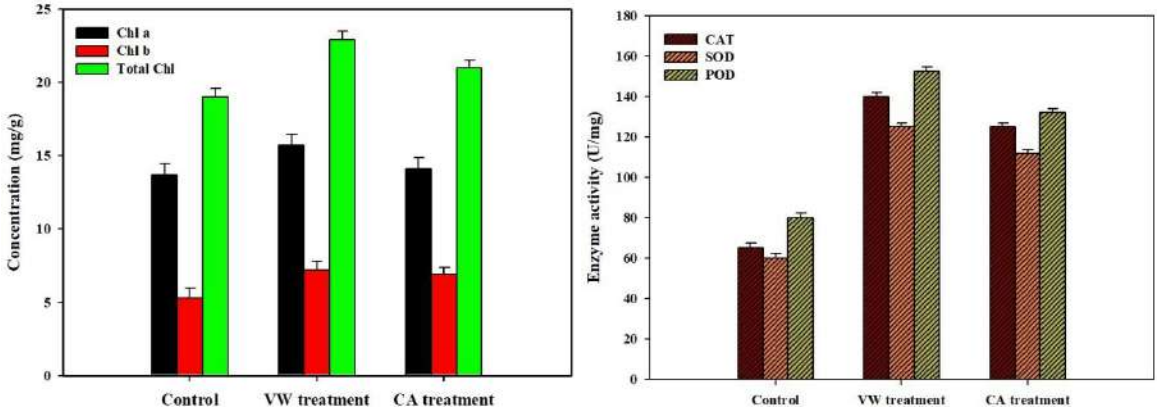

Fig. 4d. Effect of VW and CA on activity of Rubisco in leaves of *S. bicolor* cultivated in the Pb and Ni contaminated soils.

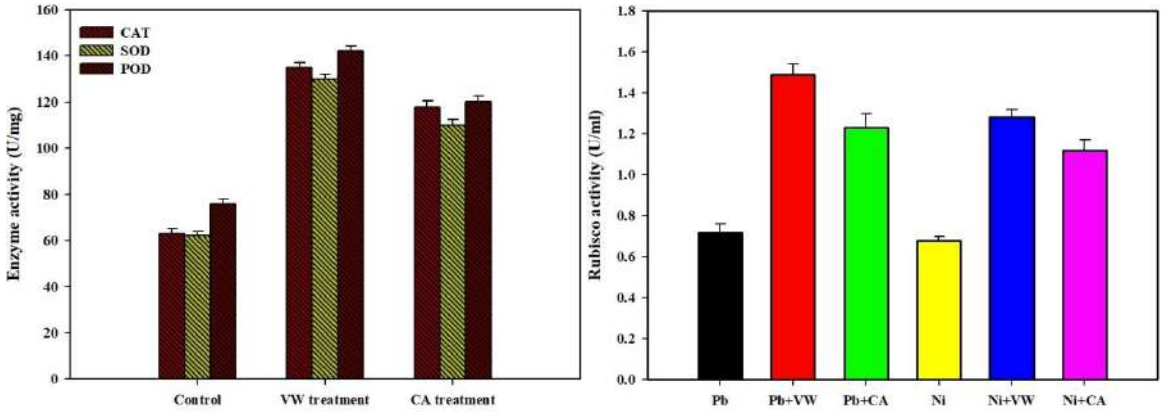

Fig. 5. Effect of lead and nickel on plant growth of *Saccharum officinarum* plant

Control

30 days

60 days

Fig. 6. SDS PAGE analysis of plant proteins

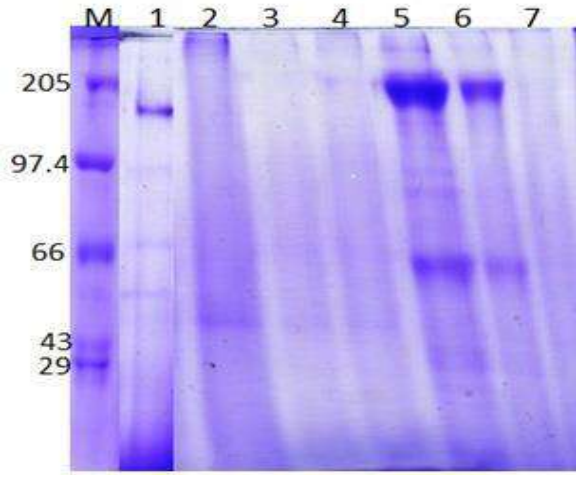

Fig.7. Characterization of heavy metals in the soil samples

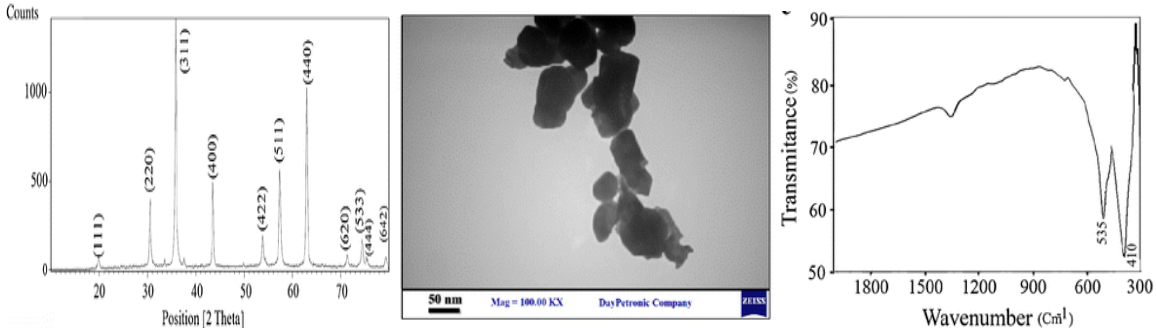

Fig.8. Docking analysis of stress proteins and heavy metals