Effect of heavy metals stress on the hydroponic growth of lettuce and evaluation of stressed induced extracts for their potential antioxidant and enzyme inhibition potential

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Abstract- *Lactuca sativa* (lettuce) is an edible plant commonly used by local communities for the management of diabetes and stomach problems. This study focuses on the optimization of hydroponic conditions to investigate the effects of heavy metal stress on the lettuce. In the second part, in vitro pharmacological effects of plant extracts of hydroponically grown lettuce (HyL), in comparison with heavy metals stress induced (HyCd, HyCr and HyPb) were studied. Among three heavy metals, including Cd, Cr and Pb; Pb showed comparatively positive effects on morphological and physiological changes in lettuce, including leaf length, plant height, number of leaves, stem girth, root fresh weight, shoot dry weight, shoot fresh weight, and root dry weight of plants. The highest salt tolerance was estimated at 0.256 mM of Cd, Cr and Pb respectively. So, we selected plants from 0.256 mM salt stress and extracts were prepared in DMSO for in vitro pharmacological studies. Results showed that the highest levels of TPC, TFC, TAC and TRP contents were quantified in HyL, while among the stress induced plants the contents were highest in HyPb. In lipid peroxidation, DPPH and DNA damage assay HyPb showed comparatively better activity than HyCd and HyCr. The results of α-glucosidase inhibition assays represented good inhibition potential by HyL (IC_{50} 1.09 mg/ml). In stress plants, HyPb showed comparatively better activity than HyCd (IC_{50} 1.30 mg/ml) and HyCr (IC_{50} 1.28 mg/ml), having IC_{50} value 1.25 mg/ml. In α-amylase, acetylcholinesterase and butyrylcholinesterase assays, the lowest IC_{50} value was shown by HyPb, following HyCd and HyCr. Overall, the study showed that *Lactuca sativa* is enriched with medicinal effects and these effects can be further enhanced using controlled conditions like hydroponics.

Index Terms- Antioxidant, Enzyme inhibition, Hydroponics, Heavy metal stress,

I. INTRODUCTION

Lettuce (*Lactuca sativa*) is a leafy green vegetable. It belongs to the family Asteraceae. It features vibrant red and crisp green foliage. Around the world, lettuce is widely eaten as a leafy vegetable. It has fewer calories, fat, and salt. Consuming lettuce has several advantages for human health, including being a high source of iron, fiber, and vitamin C. It also contains a lot of other health-promoting bioactive substances such as phenolics, lutein, folate, and alpha-carotene (1). It is widely grown and appropriate for hydroponic growing (2). It is a quickly grown vegetable with a short lifespan. Due to its excellent suitability for fresh cutting and eating, it is mostly farmed for salads. It requires easy cultivation methods and low-cost upkeep. The crop may be harvested in 30 to 40 days, making it ideal for artificial indoor farming and research that needs quick results and straightforward replication (3). Numerous scholarly investigations have been dedicated to the examination of cultivated lettuce (4). Lettuce serves as a significant provider of a wide range of vitamins and minerals, encompassing calcium, phosphorus, iodine, iron, copper, and arsenic. Lettuce is recognized for its robust immune system support and its potential to combat anemia, owing to its substantial vitamin C concentration. The phytochemicals present in *Lactuca sativa* predominantly consist of secondary metabolites, which are formed as a part of the plant's normal growth process or in reaction to various environmental stimuli. Plants have been utilized in traditional therapy for several decades to address various health conditions, such as inflammation, pain, gastrointestinal issues (e.g., indigestion and lack of appetite), bronchitis, and urinary tract infections (5). Research has documented the scientific evidence pertaining to the biological activities of antibacterial, antioxidant, and neuroprotective properties (5, 6).

The ability of organic farming to produce healthy food and lessen the environmental harm caused by conventional agricultural techniques has risen over the past many years. Concerns about health, the environment, and most crucially, food safety have boosted people's desire to purchase organic goods. Pesticides and extremely concentrated and highly soluble fertilizers must not be used by farmers that practice organic farming. Organic systems encourage conservation, which not only minimizes environmental harm but also produces high-quality goods. Consumers choose foods like fruits and vegetables that have certain functional qualities. (7). According to the Food and Agriculture Organization report of 2017, lettuce was produced in 106 countries in 2017. In 2020, 27.7 m tons of lettuce were produced globally, with China, India and the USA leading the global lettuce market. China leads with more than 50% of global lettuce production. (8, 9). A 2023 report by Global news wire places the global hydroponic systems market at a value of USD

12.1 Billion, which is further projected to be USD 25.1 Billion by the end of 2027.

In the soil environment and nutrient medium, heavy metals can accumulate and move around. Due to industrialization and rapid population expansion, urbanization modifies the soil. Urban and semi-urban soils and water are frequently contaminated with metals, including lead, cadmium, arsenic, zinc and chromium (10). Long-term cumulative impacts may endanger the ecology and flora and fauna. Even diseases can result from heavy metals entering the human body through the food chain. Fruits and vegetables are a staple of the daily diet for humans. The main dietary sources of heavy metal exposure for humans are fruits and vegetables. For instance, oral ingestion accounted for nearly 70% of the Cd intake. WHO/FAO (2016) permitted levels of HM such as Cu, Cd, Cr, Pb, and Zn in vegetables are 73 mg/kg, 0.1 mg/kg, 0.25mg/kg, 0.3mg/kg, and 100 mg/kg respectively (11). Roots are the first organ where HMs are absorbed and eventually translocated from roots to shoots and leaves. Spectrophotometric analysis of these plant tissues is conducted for quantification of HMs. This analysis is used to determine the Bio-Concentration Factor (BCF = HM in Roots/ HM in media) and Translocation Factor (TF= HM in Aerial Parts/ HM in Roots) (12). Bioconcentration factor can be used to assess the concentration of heavy metals in plants absorbed from nutrient media or soil and the Translocation factor may be used to calculate the quantity of heavy metals that are moved from one organ to another. This data is compared with WHO/FAO allowed HM standards for leafy vegetable crops to assess their consumption and marketability. Studies have shown that increases in HMs like cadmium, chromium and lead in the nutrient solution result in increasing concentrations of these HMs in roots and aerial parts of the plant. One study found that a 10-fold rise of Cd concentration in solution enhanced the Cd content in leaves about 10-fold and about 8-fold in roots (13). A rise in bioavailability causes a rise in root to shoot uptake, which has a detrimental impact on plant biomass. Pb accumulation in roots is greater than that in shoots, indicating that Pb has a stronger propensity for building complexes with plant enzymes than Cd. Metal persistence and gradual degradation are demonstrated by the three-month spike in post-harvest availability of metals. Similarly, Chromium also proves to be toxic, at higher concentrations it affects plant morphology, its growth and hence biomass (14).

When plants face heavy metal (HM) stress, they cope with this by activating their natural antioxidant or chelation mechanisms. But, when HM stress crosses their tolerance index (TI), their growth pattern is disturbed, resulting in stunted or slow growth or it may even result in death of the plants. Plants have a natural tendency to screen out these HMs but an increased concentration beyond tolerance limits compels them to translocate these from underground roots to their edible portions like stems and leaves, thus making an entry to the natural food web and food chain. When these HM stressed plant parts are consumed by humans or animals for a long time, these accumulate in their bodies due to their long half-lives. Hence, this starts stressing the human and animal normal healthy life cycles. In this research, we investigated the effects of extracts of HM stressed plants to study their antioxidant and enzyme inhibition potential.

II. METHODOLOGY

Seed germination in soil

Seeds of *Lactuca sativa* L. cv. Grand Rapids was purchased from Awan Seed Store, Rawalpindi, Pakistan. Seeds were germinated on 14 September 2019 in a mixture of agricultural soil and sand (3:1) and watered using half-strength Hogland's Solution at 24℃ and maintained at 8/16 night/ day photoperiod. 2 x weeks of seedlings were shifted to optimized nutrient media after initial survival trials. To test different seed germination dynamics after applying HMs, a filter paper disc method was employed. Filter paper discs cut to the size of plastic boxes having a diameter of 20 cm were soaked in increasing HM solutions in a two-fold concentration manner from 0.002mM, 0.04mM, and 0.08mM to reach 1.064 mM. This was repeated for Pb ($PbCl_2$ Soln.), Cr ($K2Cr_2O_7$ Soln.) and Cd ($CdCl_2$ Soln.). The boxes were previously disinfected with a hydro alcoholic solution (ethanol/distilled water 80/20). Each experiment was replicated 3 times and each was replicated at a total of 10 concentration levels, counting 100 seeds (15).

Experimental design for screening optimum Hoagland solution

The nutritional solutions were made utilizing the full-strength modified Hoagland solution recipes, employing standard ingredients sourced from reputable suppliers: Merck and Sigma Ltd. The concentrations of nitrogen (N), potassium (K), and calcium (Ca) employed in the experiment were as follows: N1 - 150 ppm N, 100 ppm K, and 150 ppm Ca; N2 - 210 ppm N, 235 ppm K, and 200 ppm Ca; N3 - 250 ppm N, 300 ppm K, and 250 ppm Ca; and N4 - 300 ppm N, 350 ppm K, and 350 ppm Ca. The nutrient solution labeled as N2 exhibited comparable quantities of nitrogen (N), calcium (Ca), and potassium (K) to the Hoagland solution. However, it did not contain sulfur (S), sodium (Na), chlorine (Cl), copper (Cu), and molybdenum (Mo). The N2 solution was designated as the control treatment (12).

Transferring seedlings from soil to nutrient media

The germination process of the seedlings commenced 24 hours after sowing. Starting on the seventh day, the seedlings underwent a daily double application of a Hoagland's nutrient solution (diluted to one-fourth of its original strength) for a period of six days. Following a germination period of 15 days, the seedlings were subsequently transferred to a hydroponic system. Twelve polystyrene boxes having dimensions L x W x H = 45 x 30 x 20cm, with six plants each, were used for each heavy metal (HM) i.e., one for Hydroponically grown lettuce as control, and ten for different concentrations of HM from 0.002mM to 1.064 mM. Each polystyrene box was aerated using a 3W 5.6x5.2x4.7cm ANSELF air pump with an adjustable timer facility. Small pores d=3-5mm were introduced in the ROCO polystyrene sheets: plain, Styrofoam board, white having 1.5inch thickness and cut into circular shapes (r= 6.5 cm). Glass tubs having 5 L Nutrient media capacity were used. 1L Nutrient media was used in each tub and seedlings were shifted from soil to these sheets such that their stems were outside of nutrient media and roots were emersed inside nutrient media while the polystyrene sheets were floating on the nutrient media surface.

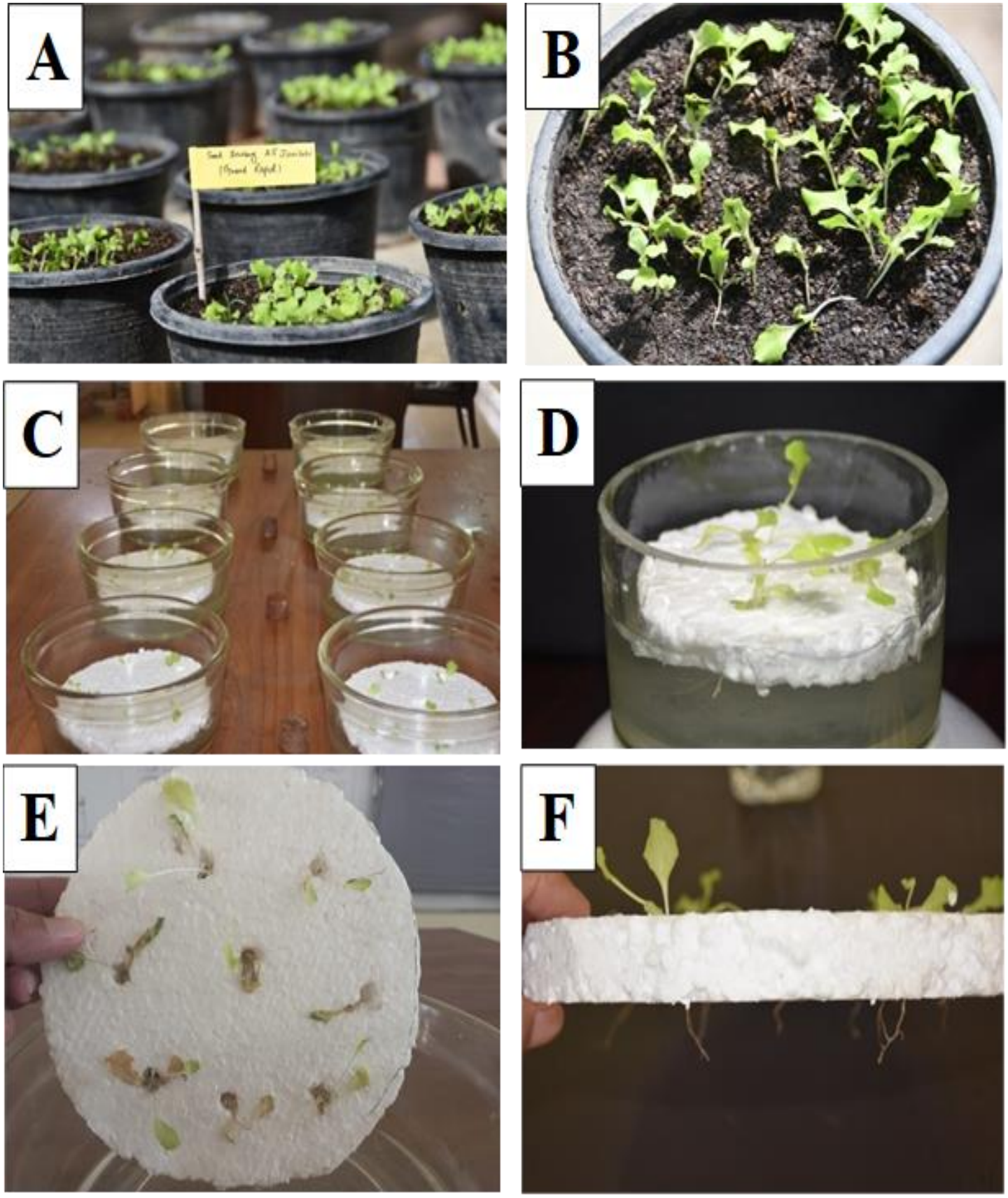

Figure 1 (A) Seed sown in Soil & Sand Mixture (3:1) (B) Two weeks seedlings (C) Initial Nutrient Media Trails (D) 15 Days Seedlings shifted to nutrient media for Heavy metal trials (E) Plants death at higher concentrations in initial trails (F) Healthy plants developing leaves and roots in initial trials.

After shifting the seedling from soil to nutrient media, a 16/8-hour photoperiod was maintained throughout the experiment (Figure 1). Once the plants were acquainted (minimal 4 days) with the new microclimate, they were shifted to polystyrene boxes with aeration pumps. The nutritional solution was maintained at a pH range of 5.5 - 6.5 and an electrical conductivity (EC) range of 0.8 - 1.2 mS/cm. The levels of electrical conductivity (EC) and pH were monitored daily and modified as necessary. Old nutrient mediums were replaced with freshly made nutrient media every 7 days. For each heavy metal concentration, the HM solution was added just after preparing the nutrient media and all boxes were labeled for the date of transfer and HM concentration (Figure 1). The plants were collected for analysis on the 45th day following transplantation. Various morphological characteristics, including plant height (centimeters), stem girth (centimeters), number of leaves, leaf length (centimeters), leaf breadth (centimeters), root length (centimeters), shoot fresh weight (grams), and root fresh weight (grams), were recorded for each heavy metal (HM) sample (Figure 2). A four-digit electronic digital caliper (Brand-UNUNS) with an LCD display having a minimum measurement range of 0.1mm and a 30 cm Scale (Brand-Prima) were used for measuring scale/ heights of plant parts (Figure 2).

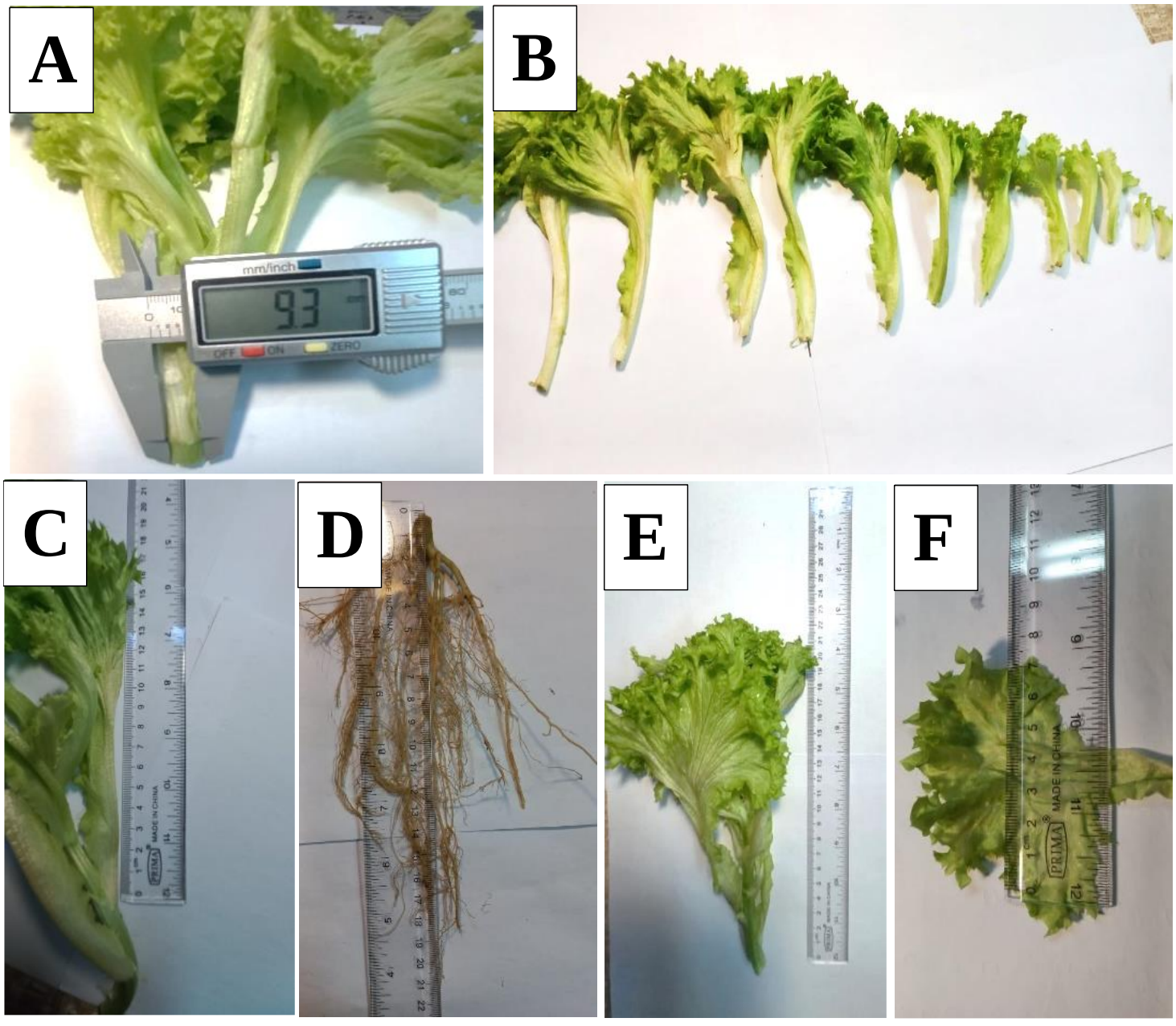

Figure 2 Measurement of different Plant parameters (A) Stem Girth (B) Total number of leaves (C) Plant Height (E) Leaf Length (F) Leaf Width

Sample preparation using acid digestion method

Plant tissue samples were washed with regular potable water, diluted HCl solutions, and distilled water. The plant parts were then sliced into tiny pieces. These portions (20 g each of roots, shoot, and leaves) were dried at 60℃ for five to seven days to obtain a permanent dry weight. After labeling the dry weights (16) of distinct plant parts of all HMs, i.e., DW-HyCd, DW-HyCr & DW-HyPb, the dried samples were powdered using a mechanical blender and carefully sieved (2 mm) before being stored at room temperature. Then, 1 g of each sample was dissolved in 15 mL of solvent (a mixture of HNO_3 (70%), $HClO_4$ (65%), and H_2SO_4 (70%), all from (Sigma-Aldrich) and homogenized. This melange was heated to the boiling point at 80°C on a hot plate. Continued digestion produced approximately 3 mL of clear liquid. In a 50-mL volumetric flask, the final mixture was filtered with Whatman filter paper (No. 41) and diluted with 50 mL of deionized water (17, 18).

Atomic absorption spectrophotometer (ASS) analysis

AAS analysis was used to ascertain the heavy metal concentrations in the selected vegetable samples. By modifying the slit width to 0.7 nm, the metals were measured at wavelengths of 283.3 nm for Pb, 228.8 nm for Cd, and 357.9 nm for Cr. To verify the instrument's sensitivity, standard solutions were repeatedly analyzed. The concentrations of Pb, Cd, and Cr were measured (19) (using a Perkin Elmer atomic absorption spectrophotometer Model 2380, USA). The results were reported as the mean standard deviation, and each experiment was conducted three times. Finally, a series of standard solutions of $CdCl_2$, $K_2Cr_2O_7$, and $PbCl_2$ were prepared: 0, 1, 2, 4, 8, 16, 32, 64, 126, 256, and 512 mg/L for Cd, Cr, and Pb. To create calibration curves, their absorbances were measured at 283.3 nm for Pb, 228.8 nm for Cd, and 359.9 nm for Cr. The slope and intercept of the line provided the relationship between absorbance and concentrations of these heavy metals (20).

In-vitro study

Extract preparation

For the experiment, aerial portions of lettuce were harvested by removing vegetation above the perlite growing medium. The harvested parts separately from normal hydroponically grown lettuce (HyL), Cd-induced hydroponically grown lettuce (HyCd), Cr-induced hydroponically grown lettuce (HyCr) and Pb-induced hydroponically grown lettuce (HyPb) were washed with distilled water and shade dried, ground to make fine powder and then were macerated into methanol (99% from Sigma-Aldrich). After five days of vigorous stirring and mixing, the extracts were properly filtered through filter paper (Whatman-1) and all filtrates were dried under vacuum at 40°C temperature. The resulting extracts were named as HyL, HyCd, HyCr and HyPb respectively. The isolates were stored at -20 ℃ for pharmaceutical evaluation.

The total phenolic contents (TPC)

TPC components were ascertained by the technique suggested by Kim, Jeong (18) utilizing Folin and Ciocalteu's reagent, with some alterations. The crude extracts of 5 µl (1mg/ml) are taken into the 96-well plate then added 98µl of Folin & Ciocalteu's phenol mixture (10-fold dilute with water) and mixed. The mixture was retained for 5 min and then 7% Na_2CO_3 solution (98 µl) was included and mixed well. The final mixture was maintained at 26°C for a total of 90 min and absorbance was estimated at a wavelength of 630 nm with the micro titer (Elx 800) reader plate. The TPC can be stated as Quercetin (21) equivalents. The Graph-Pad Prism software is used to calculate the percentage change in TPC.

The total flavonoid contents (TFC)

TPC components were estimated via the aluminum-chloride calorimetric method with some modifications to the technique suggested by Park, Jung (22). The crude extract of 5 µl (1mg/ml) was taken, separately mixed with 20 µl of $AlCl_3.6H_2O$ (10%), 20µl K-acetate (1 M) and 155µl of H_2O into the 96-well plate. The final admixture was incubated for approximately 30 min (at 37 °C). Then absorbance was assessed at 405 nm wavelength with the micro titer Elx 800 reader. The TFC was denoted as Gallic acid (GAE) equivalents.

The total antioxidant capacity (TAC)

The TAC was ascertained via the phosphomolybdenum method with some modifications to the technique suggested by Phatak and Hendre (23). The 4 µl of crude extract (1mg/ml) independently coalesced with chemical reagent solutions of 196 µl consisting of 28 mM of Na3PO4, 0.6 mM of H_2SO_4 and 4 mM of $(NH_4)_6Mo_7O_{24}$, respectively. The final admixture was incubated for 90 minutes in water (at 95°C). The assessment of absorbance was done by UV-spectrophotometer (Agilent) at wavelength 765 nm. The TAC performed in triplicate was expressed as Ascorbic acid equivalents. In order to compute the percentage change in TAC by the software system Graph-Pad Prism was applied.

The reducing power assay (TRP)

The plant sample has the power of reduction determined by previously reported modified protocol (24). In a 200 µl plant sample (1mg/ml), 500 µl of 6.6 pH 200mM phosphate buffer and 1% of 500µl of $K_3[Fe(CN)_6]$ were added and mixed up accompanied by incubation at 50°C (just for 20 min). Then 500 µL of approx. 10 % TCA was included and mixed properly. Then samples were put into centrifugation at 3500rpm just for 10min. The upper most layer of admixture (500 µL) was separated and put 100 µL of approximately 0.1% $FeCl_3$ in it. All samples were mixed well and after 10 minutes, absorbance was recorded by UV/V spectrophotometer (Agilent) at 630nm. 100 µl of DMSO was used as blank instead of crude extract. The TRP performed in triplicate was denoted as Ascorbic acid (ASB) equivalents. The computer software Graph-Pad Prism was employed for the computation of percentage changes in TRP.

Lipid peroxidation assay

LPA was estimated using a protocol reported by Kalpana, Devipriya (25). The addition of 490 µl of 0.4M $FeCl_3$ and 490µl of 0.2M ascorbic acid induced lipid peroxidation. 20 µl of extract was added to this mixture. The extracts were then incubated for 60 minutes at 37°C. To cease the reaction, 1 ml of a solution mixture consisting of TCA (1.5%), TBA (0.375%), and 250mM HCl was added to the final mixture. After 15 minutes of boiling, these reaction mixtures were chilled and centrifuged. Now, 200µl

of the mixture was transferred to the microplate and absorbance at 532nm was recorded using Elx-800 microplate reader. IC_{50} was computed by computer software Graph-Pad prism V8.

DPPH scavenging assay

Through DPPH scavenger abilities, one can estimate free radical species' harmful impressions suggested via the technique reported earlier (26). DPPH activity was determined by preparing 0.24 g of DPPH solution in methanol (100 ml), and the resulting bulk solution was held on at a temperature of 20°C. The absorbance of DPPH was aligned to (1.108± 0.02) at the wavelength of 515 nm. Then 900 µl of diluted DPPH was added to 100 µl of crude extract. The final solutions were mixed and incubation was performed at 37°C in the dark for 30 minutes using ascorbic acid as a positive control. Experimentation was done thrice and the change in color from dark purple to yellow was determined by measuring the absorbance with the help of a UV-Vis spectrophotometer (Agilent) at 515nm. The percent inhibition and IC_{50} were computed by computer software Graph-Pad prism V8.

DNA damage assay

According to the procedure outlined by Shabbir, Ahmad (27), lettuce extracts were evaluated for their DNA-protective properties. A 36% H_2O_2 solution was diluted with water to produce a 30% H_2O_2 solution. In 100 milliliters of distilled water, 0.18 grams of NaH_2PO_4 and 0.55 grams of Na_2HPO_4 were dissolved to produce a buffer having a pH of 7.4. $FeSO_4$ was produced by dissolving H_2O in 100 milliliters of distilled water. The plasmid DNA for pBR322 was obtained from the German company Fermentas. The concentration of pBR322 plasmid DNA was reduced threefold with a 50mM buffer. The DNA of a plasmid was attenuated to create the positive control (P). In order to examine the pro-oxidant effect, a control (X) was created by combining 3µl of diluted plasmid, 5µl of extract (1 mg/ml), and 7 µl buffer in a PCR tube. The experiment reaction was conducted on 15 µl of final volume in PCR tubes. Each PCR tube contained 3 µl of plasmid dilution, 5 µl of extract, 3 µl of 2mM $FeSO_4$, and 4 µl of 30% H_2O_2. In order to determine the DNA damaging effect, the reactions were incubated in the dark at 37°C for one hour before being run on an agarose gel containing 0.9% agarose. In a 250 ml flask, 1 g of analytical agarose was melted in 110 ml of 1X TBE buffer. The emulsion was gently blended with 5 µl of ethidium bromide. After pouring the gel into the gel tray, it solidified for 30 minutes. After gelation, 1X TBE was added to immerse both electrodes. Each reaction mixture was loaded with 3 µl of bromophenol blue and placed on 0.9% agarose gel wells following a run at 100 volts and 90 amps for 1 hour on a Biometra electrophoresis device. Gel-Doc (BioRad) visualized gels.

Assay for α-glucosidase inhibition

In a 96-well microtiter plate, the α-glucosidase inhibition action of lettuce was determined using the protocol described previously (28). The α-glucosidase enzyme solution (2 µl), PNPG substrate solution (10 µl), 5µl extract, and 68µl buffer were combined in a 96-well microtiter plate. After obtaining the initial values at 405 nm, the reaction mixtures were incubated for 30 minutes at 37ºC. Following incubation, 100µl of 0.5 mM $NaHCO_3$ was mixed to terminate the reaction. The increase in absorbance (Abs) caused by the hydrolysis of PNPG by α-glucosidase was determined by the Elx-800 microplate reader at 405 nm. Acarbose served as a standard drug. IC_{50} was computed by computer software Prism V8.

Assay for α-amylase inhibition

This test was run on a microplate using the technique reported previously (29) with a few minor modifications. Each well in the experiment included 40 µl of 0.5 mg/ml starch, 30 µl of buffer having pH 6.8 and 20 µl of extract. Then, each well received 20 µl (0.1 U) of the enzyme in the 100mM buffer, and the plates were incubated for 30 minutes at 50°C. Then the mixture was given 20 µl (100mM) HCl as a stopping reagent. In this mixture, 0.1ml of Lugol's iodine solution was added and an Elx-800 plate reader was used to record the absorbance at 540 nm. A dark blue hue in the reaction mixture denotes the existence of starch, a yellow hue the lack of starch, and a brownish hue the partial degradation of starch. Acarbose served as a standard drug and IC_{50} was calculated using the Graph-Pad prism V8 program.

Assay for acetylcholinesterase inhibitory activity

The inhibition of acetylcholinesterase (30) was estimated using Ellman's protocol (31). The evaluation was conducted on 96-well plates utilizing the colorimetric technique. The experiment's components were 25 µl of the AuCh enzyme solution, prepared in 100mM phosphate buffer pH 8, 25 µl of 15 mM AuCh, 50 µl of 100mM buffer, 125 µl of DTNB, prepared in 100mM phosphate buffer pH 8, and 25 µl of the test sample, dissolved in DMSO, with various concentrations between 1, 0.5, and 0.25 mg/ml. After combining, the reactants were incubated at 37 °C for 30 minutes. Galantamine hydrobromide (Sigma) and DMSO were employed as the experiment's negative and positive controls, respectively. Changes in absorbance at 405 nm were recorded using an Elx-800 reader. The percent inhibition and IC_{50} were computed by computer software Graph-Pad prism V8.

Assay for butyrylcholinesterase inhibitory activity

Butyrylcholinesterase (BuCh) inhibition was estimated using Ellman's protocol (32). The evaluation was conducted on 96-well plates utilizing the colorimetric technique. Experiment components included 25 µl of BuCh enzyme solution having 0.08 U/ml; 25 µl of 15 mM BuCh prepared in 100mM buffer having pH 8; 50 µl of 100mM buffer pH 8; 125 µl of DTNB (3mM) and 25 µl of extracts at concentrations ranging from 1, 0.5 and 0.25 mg/ml. The reactants were combined and incubated at 37 °C for 30 minutes. In the experiment, galantamine hydrobromide (Sigma) served as a positive control. Changes in absorbance at 415 nm were recorded using an Elx 800 reader. The percent inhibition and IC_{50} were computed by computer software Graph-Pad prism V8.

Statistical analysis

All recorded data was evaluated by ANOVA, and then Tukey's test for multiple comparisons was executed. $P < 0.05$ is considered to be the threshold for statistical significance, and the findings are summarized using mean and standard deviation. The IC_{50} value was determined with the assistance of table curve analysis software.

III. Results and Discussion

The toxicity of heavy metals like cadmium, chromium and lead in plant organisms can impair photosynthetic pigments and reduce dry matter production, which is crucial in agronomy. The research presented here aims to evaluate morphological and pharmacological variation in metal stress induced lettuce plants and results are presented sequentially.

Seed germination

A comparison of seed germination of hydroponically grown lettuce and three heavy metals i.e., Cd, Cr and Pb-induced stressed seeds showed that HMs caused toxicity and seed germination was proportionally affected by increasing HM concentrations (Figure 3). This toxic effect was less prominent from 0.002mM to 0.0032mM and more visible beyond 0.032mM. HM concentrations 0.256mM & 0.0512 mM were the most toxic. Mutually comparing these three HMs, it was found that Cr was most toxic at higher concentrations while Pb was least toxic. Maximum inhibition of seed germination by cadmium was caused between 0.128 mM and 1.056 mM (Figure 3). The germination process started with almost 48 hours of water spray on wet filter paper and continued till the 7^{th} and 8^{th} days. It is observable from figure 4 that the germination percentage increased as the number of days increased. The germination peak reached at the peak on the 5^{th} to 6^{th} day in almost all the experimental seed growth boxes. On the 6^{th} day of the experiment, the seed germination was inversely proportional to the HM concentrations. Maximum seed germination was significant between 0.002 to 0.008 mM concentrations on the 5^{th} to 6^{th} day of seed plantation, where germination for Cd was 80% (C1=0.002 mM) and for Cr and Pb was 83 % (C3= 0.008 mM). The maximum germination value is defined as the final germination percentage (FGP) divided by the "x" days required to reach that FGP (Figure 5). Out of all HM stressed seeds, the maximum germination value has been shown by Cr indicating minimum stress, while Cd-stressed seeds showed the least germination percentages at 0.512mM and 1.056mM (Figure 5).

Effect of HMs on the height of the lettuce plants

Heavy metal-induced abiotic stress affects the dynamic stages of plant growth and development, hence, polluting our agriproducts and entering at this level in the human food chain (14). In the Cadmium, Chromium and Lead stressed lettuce groups of hydroponic plants i.e., HyCd, HyCr, and HyPb the HM uptake was positively related to the concentration level of these HMs in the optimized nutrient medium. The differences in plant heights could be assumed as an indicator of HM toxicity, which affects plant metabolism and disrupts normal metabolite production pathways. Stunted or low growth is observable at high HM concentration levels, i.e., from 0.128 mM to 0.512 mM in all the HM stressed plant experiments. It is evident from figure 6 that higher concentrations of HMs resulted in low growth of lettuce plants. Therefore, plant heights in high concentration groups i.e., at 0.128mM, 0.256 mM and 0.512mM were found to be lower in comparison to the plant heights at lower concentrations, i.e., 0.002mM and 0.0040 and 0.008 mM. Overall, the plant height linearly decreased from 0.002 mM to 0.064 mM but at 0.128mM a sharp decline was observed in average plant heights. It indicates increasing toxicity levels, which were tolerated well till 0.064 mM of HM concentrations. But beyond 0.064mM, the heavy metal toxicity was non-tolerable and plant heights were compromised. At concentration levels of 0.512 mM and 1.024 mM, the growth was either ceased or not detected and plants could not survive. Growth of lettuce plants at cadmium, chromium and lead concentrations of 0.128mM, 0.256mM, 0.512mM and 1.024 mM was severely decreased. Out of these three HMs, this toxicity range affected the lead stressed plants to the maximum and the cadmium stressed plants to the minimum. The studies showed that plants exposed to heavy metal toxicity of cadmium showed signs of nutritional imbalances in their leaves and roots. These studies related this heavy metal toxicity to decreased photosynthetic activity and increased lipid peroxidation (33).

Effect of HM on root length of the lettuce plants

Cadmium metal toxicity in roots showed a different trend than the one in plant heights. A slight increase was observed in average plant root lengths, i.e., from $HyCd_{0.002}$ (11.9cm) to $HyCd_{0.064}$ (11.1cm), beyond which the change was abrupt (HyCd0.128 and beyond). These variations in plant height might be interpreted as a sign of cadmium poisoning, which affects plant metabolism and interferes with the typical routes for metabolite formation. At concentrations $HyCd_{0.512}$ and $HyCd_{1.024}$, the seedling ceases growing in toxic environments (Figure 7). Chromium also showed similar effects and results are in agreement with (19, 34) who observed that the root system was the most susceptible part of the plant to chromium toxicity. These inhibitory changes in root length and root count prove to be the most important indexes to measure the effect of chromium on plants. Some reports (35, 36) also showed a similar root inhibitory effect of chromium. In the case of HM lead, the average root length of lettuce plants decreased as the concentration of the lead increased except at 0.002mM concentration ($HyPb_{0.002}$), where it showed a hermetic effect on the average root length of the grand rapids. This stimulatory effect was also observed by John (37) in the Great Lakes 428 cultivar of HyL with 10ppm $CdCl_2$. Also, he reported a significant increase in root growth of New York 12 and Imperial 847 varieties when exposed to 2ppm of $PbCl_2$ in nutrient media for two weeks. He further connected these differences to genetic differences among different cultivars, time of exposure and applied lead concentration nutrient media. Similarly, Capelo, Santos (38) also noted that roots rapidly respond to Pb exposure, through a reduction in growth length and modification of the branching pattern.

Effect of HM on stem girth of the lettuce plants

In cadmium induced plants, the stem girth changed little from concentration $HyCd_{0.002}$ to $HyCd_{0.004}$ until a sharp drop at $HyCd_{0.128}$ was observed (Figure 8), which indicates that the lettuce plant could tolerate toxicity until $HyCd_{0.064.}$ After this limit, the growth declines gradually ($HyCd_{0.512}$ & $HyCd_{1.024}$). For Chromium, the stem girth changed little from concentration $HyCr_{0.002}$ to $HyCr_{0.032}$ to a threshold at $HyCr_{0.064}$ was observed. It indicates that the lettuce plants could tolerate toxicity till $HyCr_{0.064.}$ After this beyond which the growth was either compromised or ceased at all ($HyCr_{1.024}$). For lead, stem girth

was inversely proportional to the increasing concentration of applied lead in nutrient media. This effect was most prominent on $HyPb_{0.256}$ and $HyPb_{0.512}$ while the decrease in stem girth diameter from low concentration ($HyPb_{0.002}$) towards increased concentration ($HyPb_{0.128}$) was gradual.

Effect of HM on the number of leaves of the lettuce plants

Increasing concentrations of cadmium, chromium and lead have an increasing cytotoxic effect on the number of leaves. With increasing concentration, the number of leaves decreases gradually. This cytotoxic effect was less prominent in lower HMs concentrations, i.e., from 0.002 mM to 0.032mM and most prominent in higher HM concentrations i.e., from 0.512 mM to 1.024mM. Overall, chromium was more toxic and cadmium was least in terms of number of leaves (Figure 9). For cadmium, our findings are similar to the findings of Loi, Sanzharova (39) who grew lettuce at 5mg/kg of cadmium concentration in soil and noted a decrease in the number of leaves by 16.4–61.3% of control plants and beyond 5mg/Kg this effect was proportionally increasing. A similar trend was observed for chromium and lead and findings for HM chromium were similar to the findings of Mallick, Sinam (40) in Zea mays which was exposed for 7 days to a hydroponic solution containing 173 micromolar concentration of Cr (VI).

Effect of HMs on leave length of the lettuce plants

Out of all three HMs, cadmium proved to be more cytotoxic in comparison to chromium and lead (Figure 10). These observations of the cytotoxic effect of Cadmium are in agreement with Monteiro, Santos (33) who found the same results and related his observations with the induction of some enzymes over a 14-day exposure to Cadmium. He further suggested that a complex of antioxidative enzymes (predominantly Peroxidase (POX) and Superoxide Dismutase (39)) act in combination to decrease the effect of Cd toxicity, especially in young leaves. However, plants do appear to have a limited capacity to boost antioxidant defense in order to resist the harmful effects of oxidative stress brought by heavy metals. Other studies also revealed the same cytotoxic behaviour of Cadmium in other plants where exposure to high concentrations of Cd resulted in a decreased antioxidant capacity (41, 42). Chromium also showed similar trends, while for lead the lower concentrations show a stimulatory effect or hormetic effect on leave length and its higher concentrations prove to be toxic as at higher concentration levels, i.e., at $HyPb_{1.064}$ the growth stops.

Effect of HM on leave breadth of the lettuce plants

The leave breadth of HyCd, HyCr and HyPb decreased with an increase in the applied concentration of HM from 0.004 mM to 1.024 mM as shown in figure 11. These observations are in agreement with literature (20) on two varieties of lettuce named Divina and Melina. According to them, an exposure to 15 µM concentration of $CdCl_2$, resulted in decreased leaf expansion (i.e., total leaf area per plant and individual leaf area). This harmful effect was more noticeable in 'Divina' (–57% and –50%, respectively) than in 'Melina' (–30% and –32%) respectively. The effect of chromium toxicity on the overall morphology of plants has been studied and similar trends are reported, for example: *Zea mays*, *Oryza sativa*, *Hordeum vulgare* and *Brassica juncea*, have all been reported with a decreased biomass in hydroponic growth media (40, 43-45) but literature on toxic effects of chromium, on leave breadth of *Lactuca sativa* generally and its grand Rapid cv specifically, is scarce. Similarly, a stimulatory effect of Cr can be observed at $HyCr_{0.002}$. This hormetic effect has already been reported by many researchers for different crops where HM at trace levels has a positive effect on plant growth and development. With different HMs concentrations of Pb the average breadth of lettuce leaves decreases down the group. Only $HyPb_{0.004}$ has shown to have hermetic effect on leaf breadth of lettuce plants. Our findings were similar to Capelo, Santos (38) who reported a significant decrease in leaf area with an increase in Pb concentration at 125 mg/L of Pb in comparison to control. Ikkonen and Kaznina (10) also reported a decrease in leaf area at 250 mg/L.

Effect of HM on shoot fresh weight of the lettuce plants

Shoot fresh weight of HyCd, HyCr and HyPb decreased with the increasing concentration of HMs (Figure 12). These observations are in agreement with Zorrig, El Khouni (20) who observed the same results while studying two varieties of lettuce named Divian and Melina. At 15 µM concentration of $CdCl_2$, the shoot biomass dropped in comparison to the control plants. Our observations (Figure 12) are in agreement with Manzocco, Foschia (46) who reported that hydroponically grown lamb's lettuce showed higher yield compared to the soil-grown lettuce, (Hydroponically grown=1585 g/m2, soil =1203 g/m2, respectively). This outcome can be explained by the genetic factor by which these cultivars could have variable hydroponic adaptations. Similarly, the anomalous behaviour of $HyCd_{0.004}$ & $HyCr_{0.004}$ is notable, which can be attributed to the stimulatory behaviour of small metal concentrations, as also noticed by Zorrig, El Khouni (20) at 0.1 micromolar concentration of $CdCl_2$ where a maximum 52% increase in shoot size was reported or there may be some environmental or genetic factor involved but exact reason needs to be investigated for this anomaly. Literature for grand rapids cv is scarce, though other plant species have been reported with a reduced shoot growth. For example, an exposure of *Arabidopsis thaliana* at 800 micromolar Cr (VI) resulted in 50% fresh weight reduction in comparison to the control after an exposure of only two days. A maximum of fresh weight reduction in our researched literature (45) has been reported for *Brassica juncea* (Mustard) where exposure of brassica plants to nutrient media containing 300 micromolar Cr(VI) for 15 days resulted in 89.1% shoot weight reduction. HM lead also proves to be toxic. The same was observed by John (37) who reported that a 50ppm $CdCl_2$ concentration, after 4 weeks' exposure, reduced the average shoot weight by 19%. Also Capelo, Santos (38) reported similar observations for shoot fresh weight reduction at 125 mg/L concentration.

Effect of HM on root fresh weight of the lettuce plants

Roots are the first organ of all plants that come across pollutants either in the soil or in the nutrient medium. Out of three tested HMs, Cadmium was less stressful while Chromium and lead were more towards fresh weight reduction (Figure 13). When comparing the fresh root weight of HyL (2.11g), we found that HyL has more overall root weight (0.10g). These findings are comparable to the findings of Lei and Engeseth (47) who found

that the fresh root weight of HyL=6.72g of cultivar Giant Caesar lettuce was more than the soil grown lettuce. For HyPb we observed that beyond $HyCd_{0.032}$ i.e., $HyCd_{0.004}$ to $HyCd_{1.024}$, the weight loss was much higher than the weight loss at lower heavy metal concentrations i.e., $HyCd_{0.002}$ till $HyCd_{0.032}$. Similar observations i.e., a 13% decrease in fresh root weight were noted by Ahmed, Ahmed (47) in soil grown lettuce irrigated with drainage waste water in comparison to the soil grown control. For HyCd, the average root weight reduction from $HyCr_{0.032}$ to $HyCr_{0.512}$ was higher than the weight loss at lower Cr concentrations, i.e., $HyCr_{0.02}$ to $HyCr_{0.016}$. When comparing Chromium-induced reduction in root growth as compared to control of various plant species, we found similar results. For example, Wakeel, Ali (48) reported a 92.8% root growth reduction in *Arabidopsis thaliana* species after treatment with 200 micromoles of Cr(VI) for only one day. Similarly, Chen, Zhang (42) reported a 78% root growth reduction in *Oryza sativa* after exposure to 80 micromolar Cr (VI) for 7 days. For HyPb Capelo, Santos (37) also reported a similar root fresh weight decrease at 125mg/L of PbCl2. In addition, he also reported a change in the development of sub-root patterns.

Effect of HM on shoot dry weight of the lettuce plants

In our results, $HyCd_{0.002}$ to $HyCd_{1.024}$ a sharp drop in average shoot dry weight was observed at $HyCd_{0.064}$ which is due to increased cytotoxic concentrations of heavy metal cadmium (Figure 14). These trends are the same as reported by Fontana, Rossi (49), Manzocco, Foschia (46) and Siomos, Beis (50) who found that the shoot dry weight of hydroponically grown lettuce was lower than the shoot dry weight of soil grown lettuce. From figure 14, it is evident that Cr (VI) at lower concentrations of $HyCr_{0.002}$ and $HyCr_{0.004}$ does not affect the root dry weight of the lettuce. After this, however, there is a decline in the dry root weight which decreases with increasing Cr concentration. Till $HyCr_{0.032}$ the tolerance limit of plants for Cr (VI) is maximum. The average dry shoot weight reduction is maximum at $HyCr_{0.512.}$ Though literature is scarce for lettuce cultivars under chromium stress in hydroponic growth cultures but numerous other plant species in hydroponic environment have been reported with reduced shoot dry weight reduction in comparison to their control plants. For example, UdDin, Bano (51) reported a decrease in dry shoot weight of *Parthenium hysterophorus* and *Solanum nigrum* by 64% and 106% respectively after exposure to 500 micromole Cr(VI) for 21 days. Ikkonen and Kaznina (10), and Capelo, Santos (38) also witnessed similar results in their independent findings.

Effect of HM on root dry weight of the lettuce plants

The average dry root weight values of our HyL are slightly more than reported for soil grown lettuce. Similar results were found by Li, Li (52) who reported that not only the root length but average root diameter, root area, root volume, and the maximum number of roots of hydroponically grown 'Nenglv naiyou' lettuce were significantly greater than conventionally grown lettuce. Also, a drainage-fed lettuce plant versus control comparison was made by Ahmed, Ahmed (53) who found heavy metal toxicity caused a reduction in dry root mass by 56%. Cr toxicity is more prominent in higher concentrations. This is the result of cytotoxic effect of Cr ions on root growth. Similar results were reported by Wakeel, Ali (48) who attributed these findings of root growth inhibition mediated by Cr(VI), to the inhibition of cell division or reduction in the cell size of the elongation zone, which resulted in decreased fresh root weight and subsequently reduced dry root weight. A similar trend was followed by the root dry weight of HyPb. These observations were also reported by Capelo, Santos (38), Michalska and Asp (13) in different lettuce cultivars (Figure 15).

Atomic absorption spectrophotometer (ASS) analysis

Edible portions of plants may collect heavy metals, providing a route into the human food chain. These bioaccumulation and translocation factors are unique to each crop species and even specific for specific cultivars of the same species. Thus, it provides the basis for cultivar selection for employing against contaminated soils (phytoremediation) and even studying the physiological effects of HM pollutants on plant growth. A spectrophotometric analysis of plant parts at different growth stages of plants gives an estimate of the HM bioaccumulation in roots and then its translocation to shoots and leaves. Figure 16 shows the accumulation of HM Cadmium, Chromium and Lead in lettuce plant roots, shoots and leaves. Generally, the maximum of HM accumulation takes place in lettuce roots. Then translocation from roots to the shoots and leaves is different for all groups. Out of Cadmium, Chromium and Lead, the maximum root accumulation was observed in roots of HyCd (150mg/kg), followed by HyPb (144.84mg/kg) and HyCr (126mg/kg) respectively (Figure 16). Maximum translocation of HMs was observed for Cadmium in HyCd stressed plants (47.26 mg/kg), followed by HyCr (24.6mg/kg) and HyPb (8.2mg/kg). Figure 17 shows Bioconcentration Factors (BCF) for roots and translocation factors (TF) for shoots and leaves. Out of Cadmium, Chromium and Lead stressed plants, the maximum translocation factor is observed for Cadmium (0.80) showing maximum absorption of Cadmium by lettuce roots followed by Chromium (0.50) and Lead (0.41). Translocations from roots to shoots and leaves are maximum for Cadmium (0.06), followed by Lead (0.02) and Chromium (0.01).

Antioxidant assays

Total phenolic contents (TPC)

Phenolic contents are secondary metabolites that commonly spread throughout the plant kingdom. Phenolic contents are most important for many functions of plants like pigmentation, reproduction, growth and defense against pathogens, etc. (54). The "Folin-Ciocalteu" method as reported previously was carried out to quantify the phenolic content of plants. In our study, TPC was calculated from the standard curve (R2=0. 91) of Quercitin and results are given in figure 18. Results showed that the highest level of TPC was quantified in HyL (3.83 mg/g) extract as compared with stress induced lettuce (HyCd, HyCr and HyPb). Among the stress-induced plants, the TPC was highest in HyPb (2.91 mg/g) while in HyCd and HyCr the TPC was 2.46 mg/g and 2.55 mg/g. The results of the current study indicate that lettuce might contain different phenolic constituents like aglycone or glycosides and phenolic acids, especially rosmarinic acid and quercetin glycosides, which makes it an efficient radical scavenger and potential antioxidant might be correlated and ascertained by prior data search (55-57).

Total flavonoid contents (TFC)

Flavonoids are the major class of phenolic components of plants. It has been demonstrated through a great number of research that flavonoids also possess antioxidant action (21). TFC was determined from the standard curve (R2=0.91) and was presented as mg of GAC per gram of dry plant extract (figure 19). Results showed that the highest levels of TFC were calculated in HyL (12.22 mg/g) while the stress-induced lettuce (HyCd, HyCr and HyPb) expressed comparatively low TFC. Among the stress-induced plants, the TPC was highest in HyPb (11.67 mg/g) while in HyCd and HyCr the TPC were 9.73 mg/g and 10.54 mg/g. The existence of phenolic and flavonoid compounds makes lettuce rich in hydroxyl moieties, which are capable of donating hydrogen or electron atoms in order to oxidize the unstable radical species as supported by previous studies (58-60). The plant derived extracts containing total phenolic and total flavonoid components (TPC and TFC) show significant radical scavenger properties as endorsed by earlier investigations (55, 61-64).

Total antioxidant capacity (TAC)

The TAC assay was performed on the principle of Phospho-molybdenum assay which converts Mo-VI into Mo-V by utilizing plant extract containing antioxidant activity and, in the end, a green compound is formed. The TAC of the sample was determined at 700 nm wavelength in a spectrophotometer (65). The results of TAC are shown in figure 20. Results showed that the highest level of TAC was calculated in HyL (5.48 mg/g), while the stress induced lettuce (HyCd, HyCr and HyPb) expressed comparatively low TAC. Among the stress induced plants, the TAC was highest, in HyPb (5.15 mg/g) while in HyCd and HyCr the TAC was 4.13 mg/g and 4.54 mg/g. The results suggested that hydroponic lettuce has a good total antioxidant capacity (TAC) index, indicating its electron/hydrogen donating capacity, so it can show significant antioxidant potential against free radicals by transforming them into stable products (non-reactive) and thus act as chain terminator (59, 60, 64).

Total reducing power assay (TRP)

TRP was estimated by the standard curve of ascorbic acid and results are shown in figure 21. Results showed that the highest level of TRP was calculated in HyL (3.54 mg/g), while the stress induced lettuce (HyCd, HyCr and HyPb) expressed comparatively low TRP. Among the stress induced plants, the TRP was highest in HyPb (3.13 mg/g) while in HyCd and HyCr the TRP was 2.94 mg/g and 2.92 mg/g. Our results correlate to prior investigations that the plant derived extracts containing total reduction potential (TRP) show significant radical scavenger properties that might be due to their redox potential via different mechanisms like 1) free radical scavenges, 2) chemical chelating of metals, such as Fe and Cu and 3) prevent lipid peroxidation (58, 61, 66, 67).

Assay for lipid peroxidation activity

The thiobarbituric acid assay was used to determine whether lettuce extracts inhibited lipid peroxidation (25). As shown in figure 22, all samples demonstrated significant (p0.05) antioxidant activity. The highest activity was reported by Vitamin E (88%) followed by HyL (77%) at 1mg/ml. In the case of stress-induced plants, the activity was comparatively low. Among three different metal stresses, the highest activity was calculated for HyPb (66%) extracts while HyCd and HyCr exhibited comparatively low percentage activity 60% and 59% respectively at 1mg/ml concentration. The results are also presented in the form of IC_{50} in figure 22. The results of IC_{50} represented that the lowest IC_{50} value was shown by vitamin E (0.03 mg/ml) and HyL (0.55 mg/ml) respectively. In stress induced plant extracts, HyPb showed comparatively better activity than HyCd (0.82 mg/ml) and HyCr (0.86 mg/ml) having an IC_{50} value of 0.63 mg/ml. Moreover, all the plant extracts showed activity in a concentration dependent manner. Overall, stress induced plant extracts presented moderate lipid peroxidation activity. The formation of free radicals is what causes lipid peroxidation, which can be induced by multiple factors, including organic hydro peroxides, iron-containing compounds, and redox cycling compounds. Investigations into the phytochemistry of this plant have previously uncovered the fact that it possesses a variety of flavonoids (68). Antioxidant flavonoids are known to have an effect of lipid peroxidation scavenging as well as the inhibition of radicals (69).

DPPH scavenging activity

The DPPH method is one of quick, reliable and commercial technique for the approximation of the antioxidant capability of extracts (59). The outcomes depicted that the antioxidant potency of lettuce extract might be due to such phytochemical constituents that have a hydrogen-donating ability to scavenge or inhibit DPPH stable free radicals with a colour change from dark violet to yellow. In the DPPH assay, all extract samples showed significant activity as shown in figure 23. The highest activity was reported with Vitamin E was used as positive control (95%) followed by HyL (87%) at 1mg/ml. In the case of stress-induced plants, the activity was comparatively low. In the case of different metal stresses, the highest activity was calculated for HyPb (69%) extracts while HyCd and HyCr exhibited comparatively low percentage activity being 66% and 63% respectively, at 1mg/ml concentration. For more clarification, IC_{50} was calculated and results of IC_{50} values are presented in Table 1. The results of IC_{50} represented that the lowest IC_{50} value was shown by vitamin E (0.08 mg/ml) followed by HyL (0.42 mg/ml). In stress induced plant extracts, HyPb showed comparatively better activity than HyCd (0.63 mg/ml) and HyCr (0.70 mg/ml) having an IC_{50} value of 0.56 mg/ml. Moreover, all the plant extracts showed activity in a concentration-dependent manner. It is observed that the intensity level of colour is directly proportional to the antioxidant scavenging capacity of extracts (70). Our results have been supported by other studies that plants have antioxidant capacity to scavenge DPPH radicals (55, 59, 64, 70, 71). Overall, the results indicate that lettuce is abundant in radical scavenger molecules, such as flavonoids, phenolic acids, and their derivatives; therefore, the DPPH radical scavenging potential of the Grand Rapids may be associated with the presence of these phytochemicals (72).

DNA damage assay

The plasmid pBR322 break system, triggered by free radicals, is employed for the assessment of a compound's antioxidant properties on DNA. The foundation of this bioassay is rooted in

the fundamental principle of the Fenton reaction (73). When hydroxyl radicals (•OH) initiate an attack on DNA, the occurrence of single-stranded nicking leads to the relaxation of the supercoiled structure, resulting in the formation of an open circular form that exhibits slower mobility. If double-stranded nicking occurs, it will result in the production of a linear shape that transitions between an open circular form and a supercoiled form (16). An in vitro assay was performed to estimate the antioxidant and prooxidant potential of plant extracts obtained from both normal and stress-induced plants. The intensity of bands generated on a 0.9% agarose gel was analyzed, and the resulting data was used to calculate the percentage protection activity, as shown in figure 24. The substances were evaluated at three distinct concentrations. HyL demonstrated effective protection against •OH radicals at concentrations of 1 and 0.5mg/ml while exhibiting modest protection at a dose of 0.25 mg/ml. The results of the experiment indicated that HyPb exhibited a modest level of protection at a dose of 0.25 mg/ml while demonstrating detrimental effects at 1 and 0.5 mg/ml. On the contrary, both HyCd and HyCr exhibited detrimental effects across all three concentrations. Consequently, it was determined that these extracts exhibited concentration-dependent DNA protection, whereby the degree of DNA protection increased proportionally with the concentration of the tested chemical. The findings align with Kalpana, Devipriya (23) who documented the protective effects of hesperidin against cellular damage generated by free radicals and was identified. Both HyCd and HyCr effectively transformed the supercoiled pBR322 plasmid DNA into linear and open circular DNA forms across all three doses. From the results it is clear that test compounds caused more damage to plasmid pBR322 DNA at their higher concentration. These findings are supported by Lima, Pereira (74) that heavy metals have shown catalytic activity in the oxidation of carbohydrates and in oxidative damaging to 2-deoxy-D-ribose (75).

Assay for α-glucosidase activity

α-glucosidase inhibitors limit carbohydrate digestion with the help of competitive inhibition of α-glucosidase, which is found on the edge of enterocytes that line the intestinal walls. This enzyme inhibition results in a decline of blood glucose levels and hence helps to counter hypoglycemia. In the α-glucosidase assay, all extract samples showed significant antioxidant potential as shown in figure 25. The highest activity was reported with acarbose being used as positive control (90%) followed by HyL (46%) at 1mg/ml. In the case of stress-induced plants, the activity was comparatively low. In the case of different metal stresses, the highest activity was calculated for HyPb (41%) extracts while HyCd and HyCr exhibited comparatively low percentage activity, being 37% and 36% respectively at 1mg/ml concentration. For more clarification, IC_{50} was calculated and results of IC_{50} values are presented in Table 1. The results of IC_{50} represented that the lowest IC_{50} value was shown by acarbose (0.05 mg/ml) and then by HyL (1.09 mg/ml) respectively. In stress induced plant extracts, HyPb showed comparatively better activity than HyCd (1.30 mg/ml) and HyCr (1.28 mg/ml) having an IC_{50} value of 1.25 mg/ml. Moreover, all the plant extracts showed activity in a concentration-dependent manner. α-glucosidase inhibitors act competitively and reversibly inhibit the activity of α-glucosidase activity. This helps in the delayed digestion of carbohydrates and the slow release of sugar molecules (76). Inhibition of α-glucosidase activity is very important for diabetes type 2 patients. This phenomenon results in the maintenance of a consistent blood glucose concentration over the day, particularly following the consumption of meals. Recently, substantial focus has been on the finding of powerful -glucosidase inhibitors obtained from natural sources. This aim has been pursued with several different approaches (77). Numerous substances, including anthocyanins, alkaloids, flavonoids, glycosides, terpenoids, and phenolic compounds, have been recognized for their ability to block α-glucosidase (77). The putative α-glucosidase inhibition seen in Grand Rapids may be due to the significant occurrence of phytochemicals.

Assay for α-amylase activity

α-amylase (*α*-1, 4-glucan-4glucanohydrolases) is the enzyme that hydrolysis starch into maltose which is then converted into glucose, this glucose is readily available for the body to utilize for various functions. One of the therapeutic targets in current research is the inhibition of α-amylase, which will in return decrease glucose production, hence leading to a decreased blood glucose level. α-amylase activity is measured in lettuce samples and results are presented in figure 26. Acarbose was employed as a positive control, exhibiting a significant inhibition rate of 86% at a concentration of 1mg/ml. Among plant extracts, the highest activity was reported by HyL having 72% inhibition at 1mg/ml. In the case of stress-induced plants, the activity was comparatively low. In the case of different metal stresses, the highest activity was calculated for HyPb (66%) extracts while HyCd and HyCr exhibited comparatively low percentage activity, being 56% and 52% respectively at 1mg/ml concentration. For a more accurate estimate of activities, IC_{50} was calculated and results of IC_{50} values are presented in Table 1. IC_{50} values showed that the highest activity with the lowest IC_{50} value was shown by acarbose (0.09 mg/ml) and then by HyL (0.64 mg/ml) respectively. In stress induced plant extracts, HyPb showed comparatively better activity than HyCd (1.30 mg/ml) and HyCr (0.96 mg/ml) having an IC_{50} value of 0.71 mg/ml. Moreover, all the plant extracts showed activity in a concentration-dependent manner. Several authors have analyzed the function potential of plants as amylase inhibitors (78). Several plants are stated to own anti-amylase potential and may therefore apply to the cure of diabetes (79). Bioactivity against hyperglycemia has been demonstrated by a variety of plant-derived compounds, primarily glycosides, alkaloids, polysaccharides, galactomannan gum, guanidine, steroids, hypoglycin, peptidoglycans, terpenoids and glycopeptides, (80). Lo Piparo, Scheib (81) comprehended the molecular requirement for enzyme inhibition, the linkage between flavonoids and human amylase was investigated. They were able to establish that the degree of enzyme inhibition is inversely proportional to the quantity of hydroxyl groups of flavonoids. This finding provides evidence that the mechanism is involved.

Assay for acetylcholinesterase inhibitory activity

Extracts of hydroponically grown and stressed induced lettuce were tested for acetylcholinesterase inhibition. The experiment was performed at three concentrations consisting of 1, 0.5 and

0.25mg/ml. In the acetylcholinesterase assay, all extracted samples showed significant ($p<0.05$) antioxidant activity as shown in figure 27. Galantamine hydrobromide was used as a positive control which exhibited 88% inhibition potential at 1 mg/ml concentration. Among plant extracts highest activity was reported by HyL having 42% activity at 1mg/ml. In the case of stress-induced plants, the activity was comparatively low. In the case of different metal stresses, the highest activity was calculated for HyPb (35%) extracts while HyCd and HyCr exhibited comparatively low percentage activity, being 25% and 21% respectively, at 1mg/ml concentration. For a more accurate estimate of activities, IC_{50} was calculated and results of IC_{50} values are presented in Table 1. IC_{50} values showed that the highest activity with the lowest IC_{50} value was shown by galantamine hydrobromide (0.13 mg/ml) and then by HyL (1.13 mg/ml) respectively. In stress induced plant extracts, HyPb showed comparatively better activity than HyCd (1.80 mg/ml) and HyCr (1.69 mg/ml) having an IC_{50} value of 1.37 mg/ml. Moreover, all the plant extracts showed activity in a concentration-dependent manner. Alzheimer's disease is distinguished by the absence of acetylcholine, which is responsible for the majority of its symptoms, including the decline in cognition and memory (82). Neurodegenerative illnesses frequently exhibit the deterioration of the cholinergic system, a widely recognized phenomenon (82). This assertion can be substantiated through the utilization of muscarinic inhibitors of esterase enzymes, with the latter having the capacity to augment the levels of acetylcholine (83). The lettuce has promising enzyme inhibition capabilities, indicating the presence of active compounds that effectively hinder acetylcholinesterase activity. Further investigation is warranted to ascertain and isolate the specific active component responsible for this inhibition.

Assay for butyrylcholinesterase inhibitory activity

Extracts of hydroponically grown and stressed induced lettuce were tested for butyrylcholinesterase inhibition. In the butyrylcholinesterase assay, all extracted samples showed prominent activity as shown in figure 28. The highest activity was reported by galantamine hydrobromide being used as positive control (97%) followed HyL (68%) at 1mg/ml. In the case of stress-induced plants, the activity was comparatively low. In the case of different metal stresses, the highest activity was calculated for HyPb (61%) extracts while HyCd and HyCr exhibited comparatively low percentage activity, being 56% and 55% respectively at 1mg/ml concentration. For more clarification, IC_{50} was calculated and results of IC_{50} values are presented in Table 1. The results of IC_{50} represented that the lowest IC_{50} value was shown by galantamine hydrobromide (0.09 mg/ml) and then by HyL (0.70 mg/ml) respectively. In stress induced plant extracts, HyPb showed comparatively better activity than HyCd (0.88 mg/ml) and HyCr (0.96 mg/ml) having an IC_{50} value of 0.71 mg/ml. Moreover, all the plant extracts showed activity in a concentration-dependent manner. The enzyme characteristics of butyrylcholinesterase are distinctive, and its distribution throughout the nervous system suggests its potential role in neural function (84). Butyrylcholinesterase is an enzyme belonging to the serine hydrolase family and shares a relationship with acetylcholinesterase. Its primary function involves facilitating the breakdown of choline esters, such as acetylcholine. The findings of our study provide a comprehensive overview of the biochemical impact of lettuce on butyrylcholinesterase. These effects are supposed to be key players in cholinergic neurotransmission and potentially contribute to several activities within the nervous system, as well as the development of neurodegenerative illnesses.

IV. CONCLUSION

This study was performed to determine the effects of different morphological and pharmacological effects of hydroponically grown lettuce (HyL) and stress induces (heavy metals) plant extracts (HyCd, HyCr and HyPb) of *Lactuca sativa*. From the results, it is evident that the seed germination rate was 75-82% and the best hydroponic composition was N3. In terms of phenotypic and morphological changes, HyPb was found to be more accommodative than HyCr and HyCd. HyCr and HyCd have almost similar effects and the drastic change phenotypic and morphological characters was appeared at 0.256mM collectively in all three heavy metal cases. HyPb showed comparatively higher pharmacological effects then HyCr and HyCd. However, stress plants showed much lower activity than control plants.

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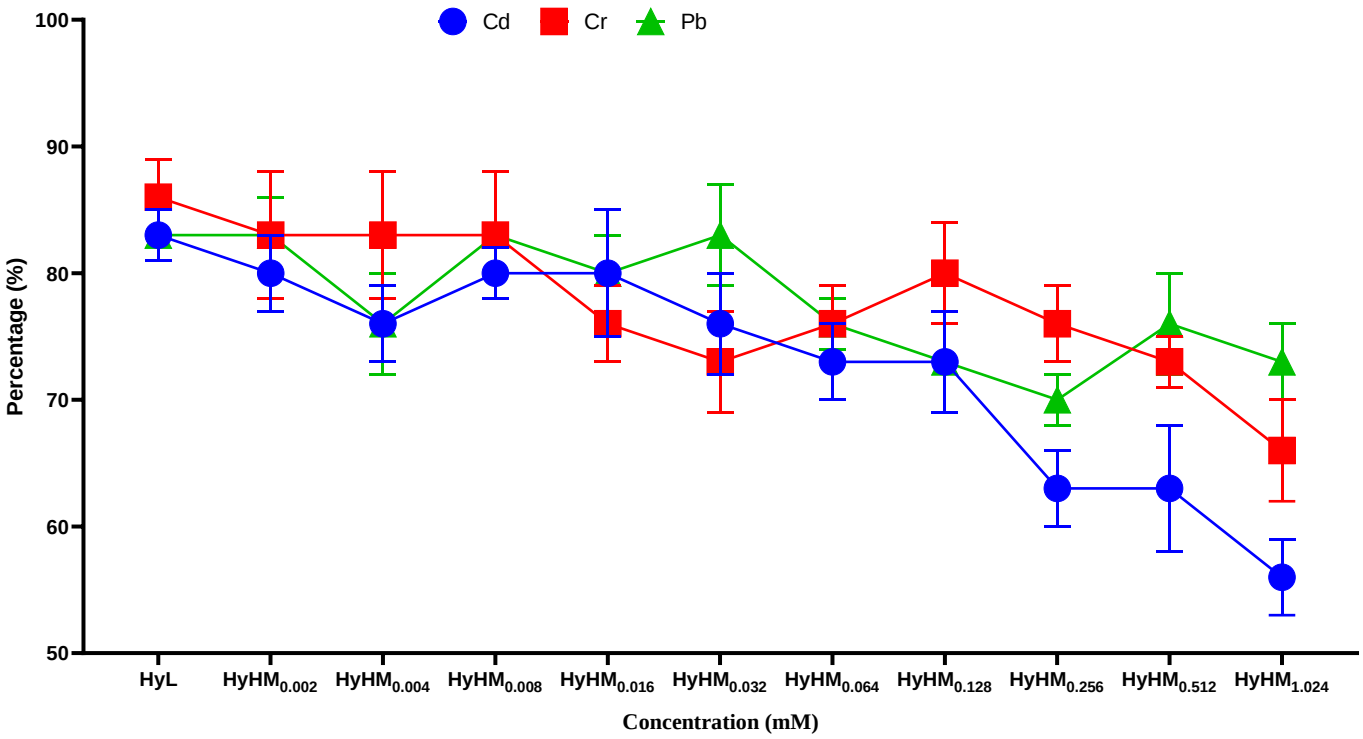

Figure 3 Germination Percentage of Grand Rapids seeds for increasing heavy metal concentrations

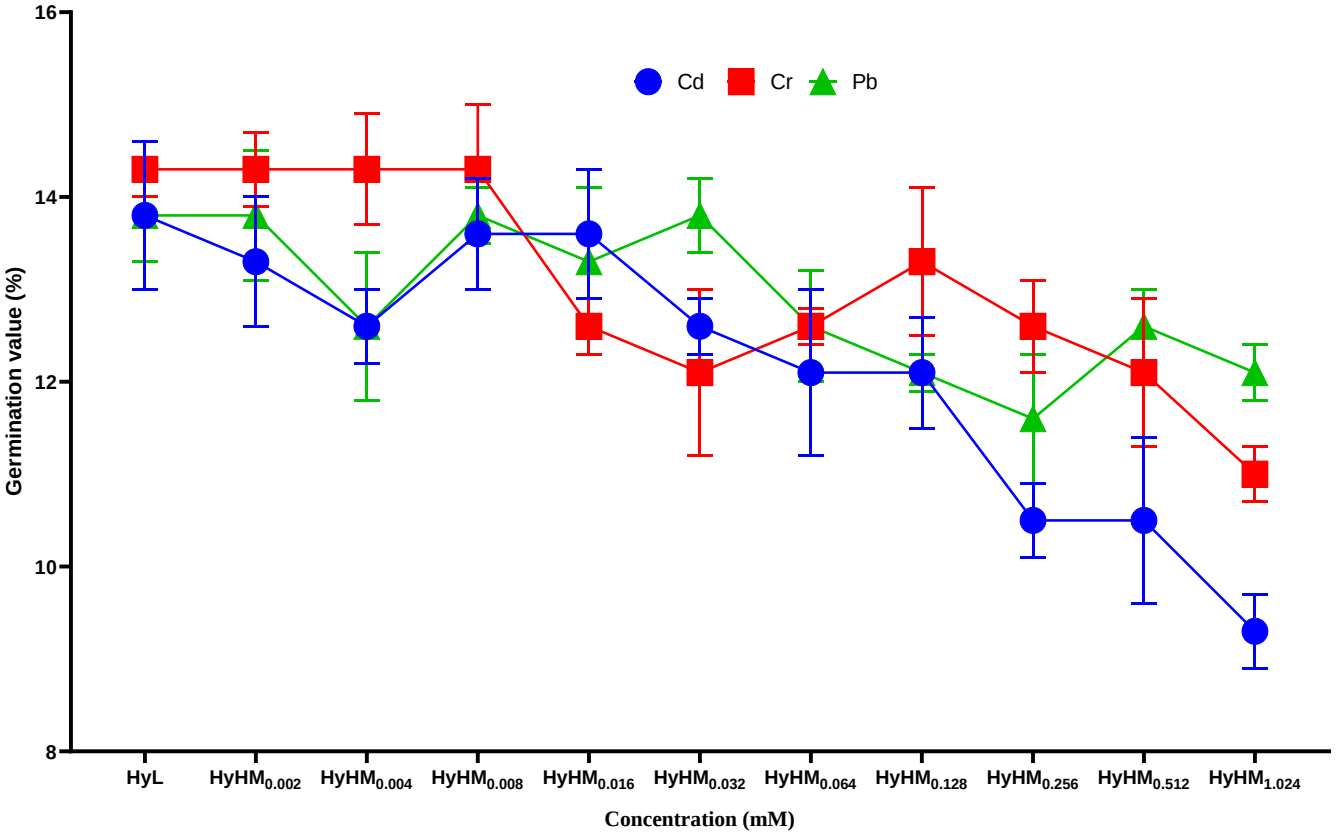

Figure 4 Maximum germination value of lettuce plants

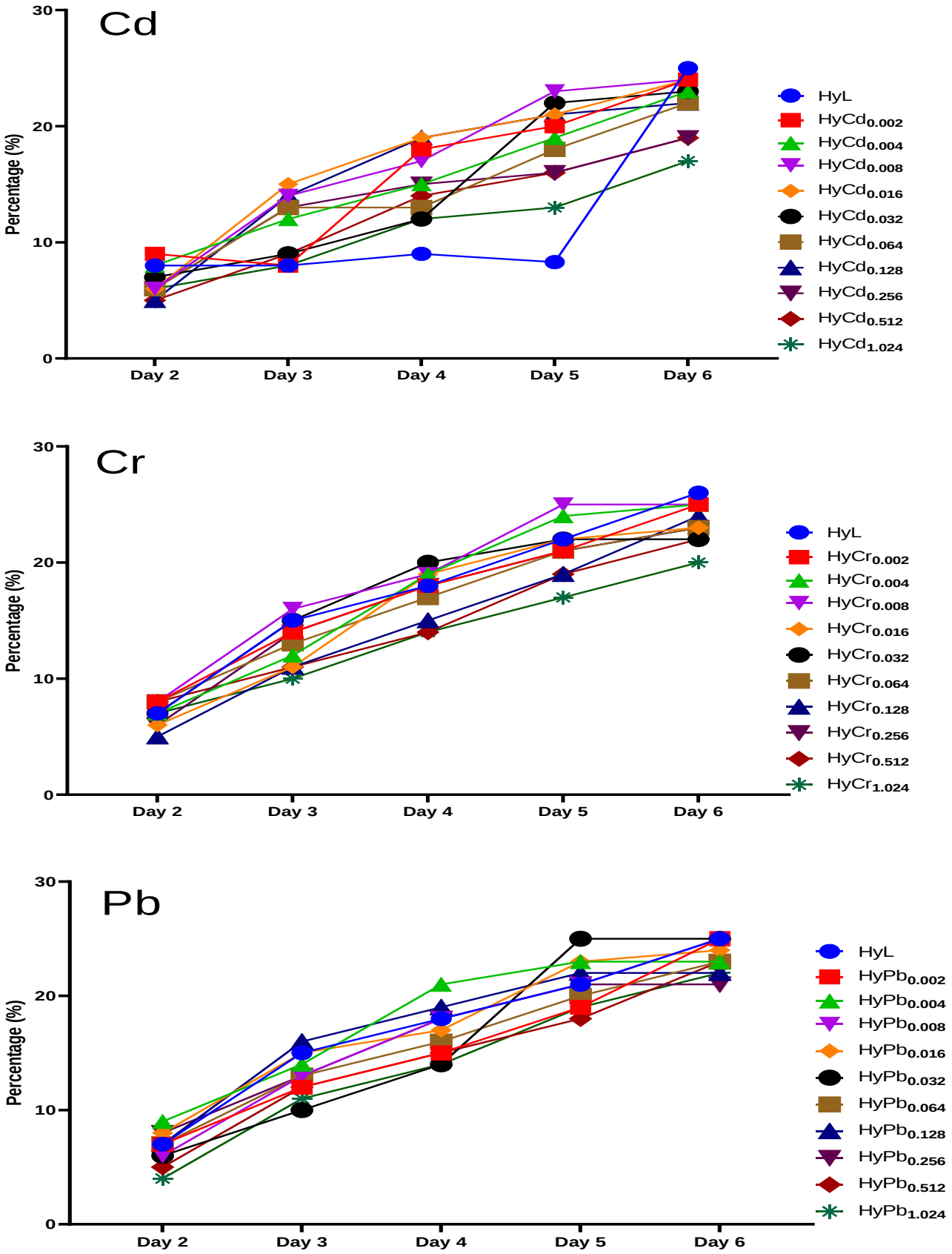

Figure 5 Final germination percentage (FGP) of lettuce seeds

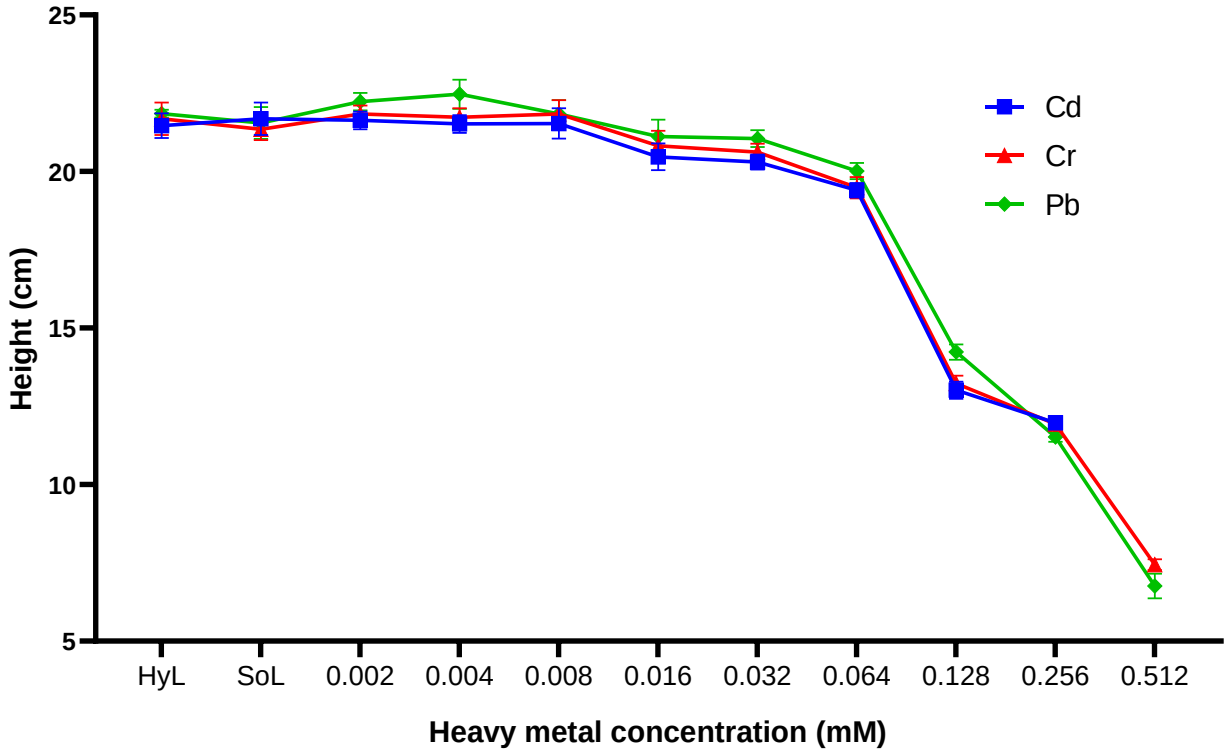

Figure 6 Effect of HM Cadmium, Chromium and Lead on Plant Heights

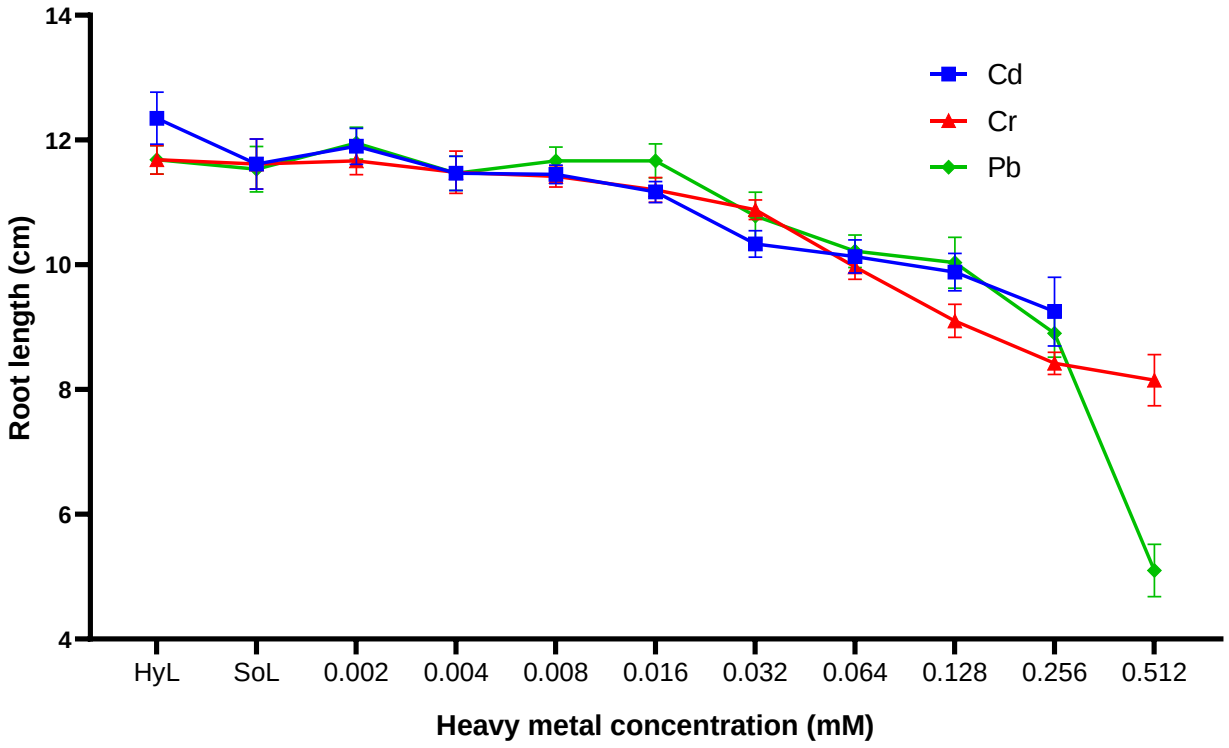

Figure 7 Effect of HMs toxicity on plant roots

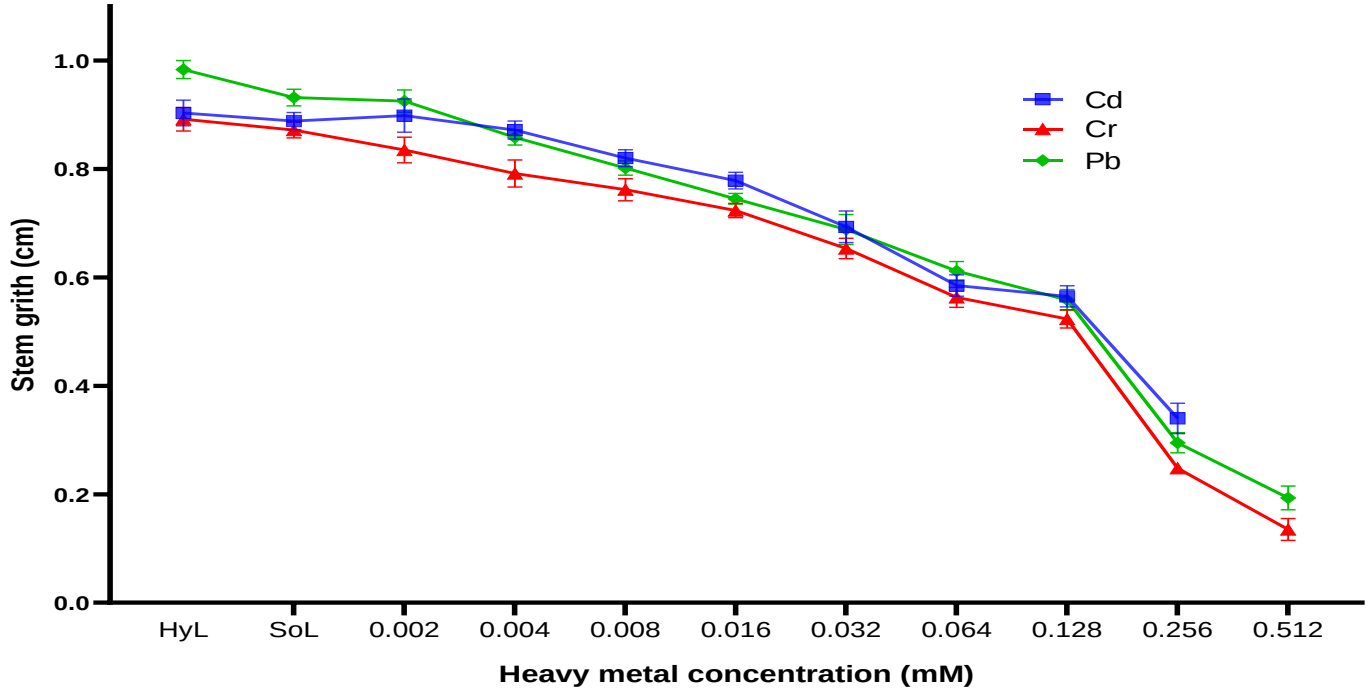

Figure 8 Effect of HM on stem girth of the lettuce plants

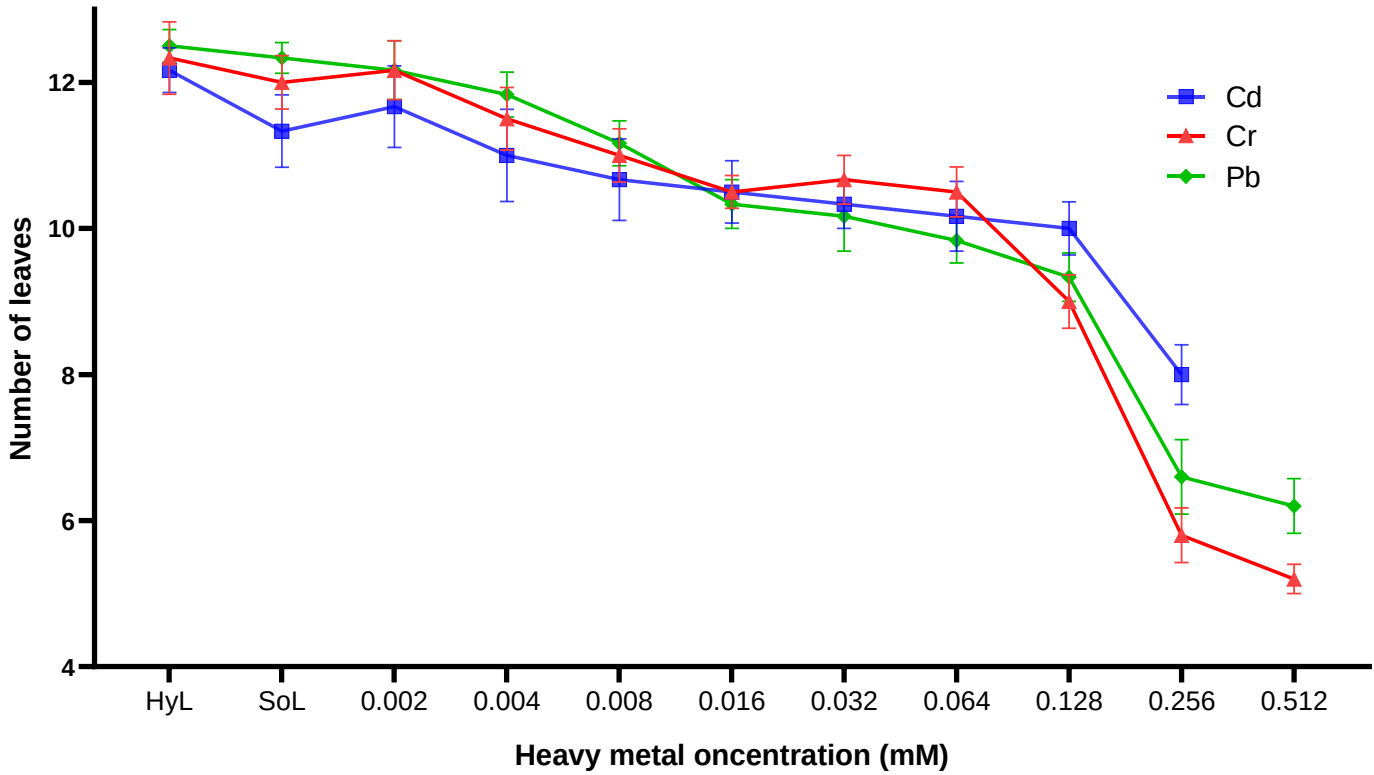

Figure 9 Effect of HMs on the numbers of leaves of the lettuce plants

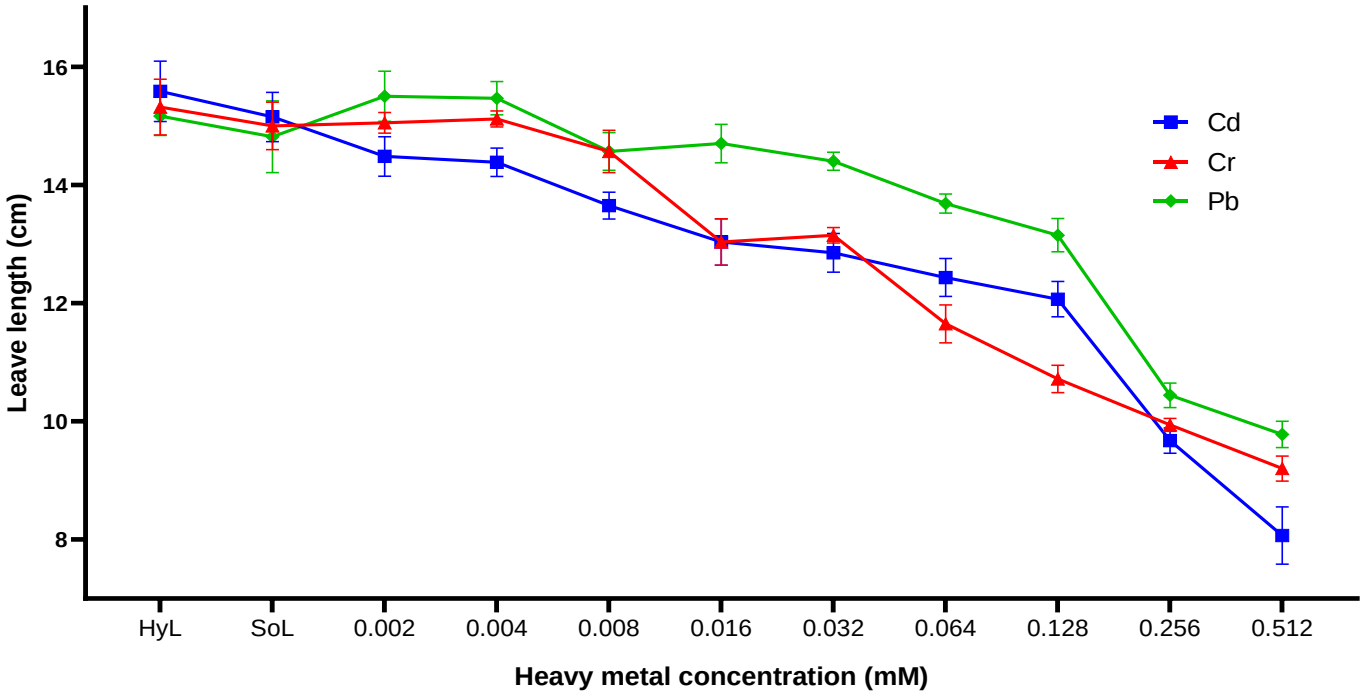

Figure 10 Effect of HM on leave length of the lettuce plants

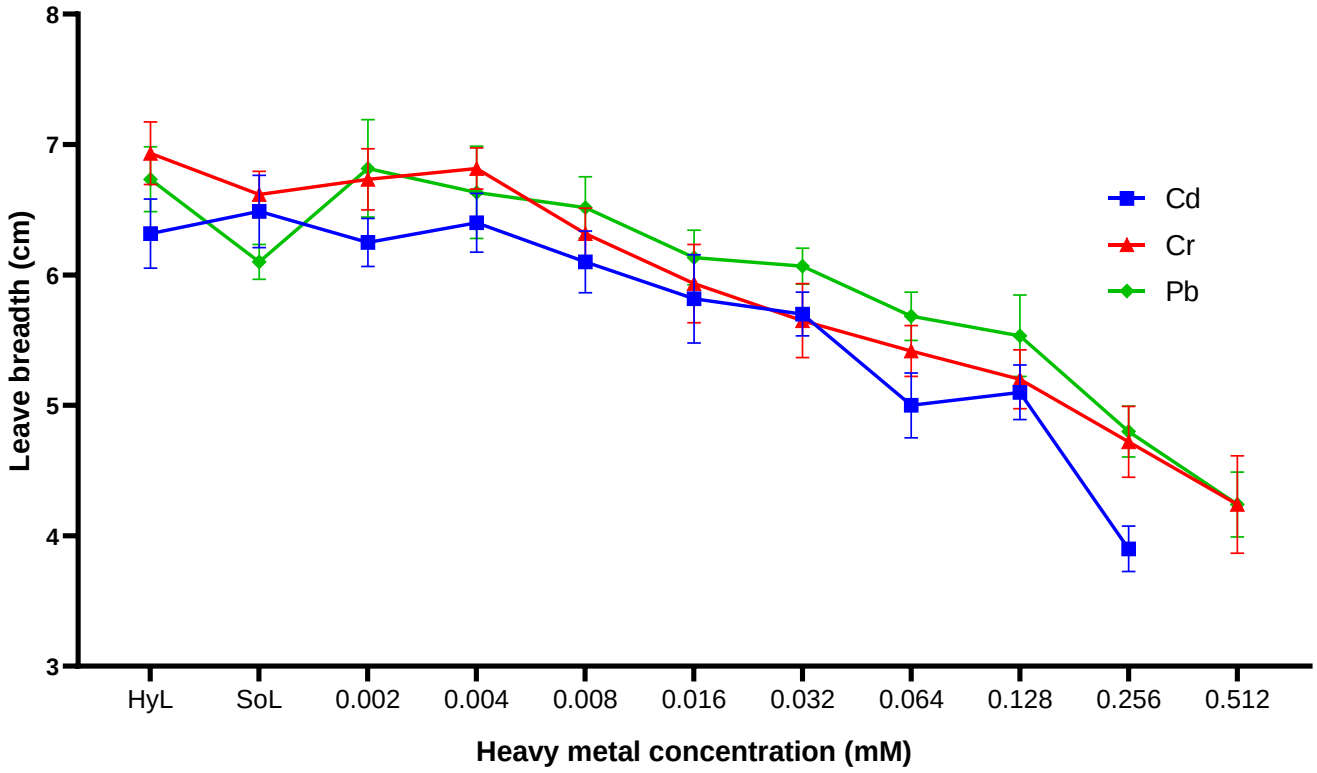

Figure 11 Effect of HM on leave Breadth of the lettuce plants

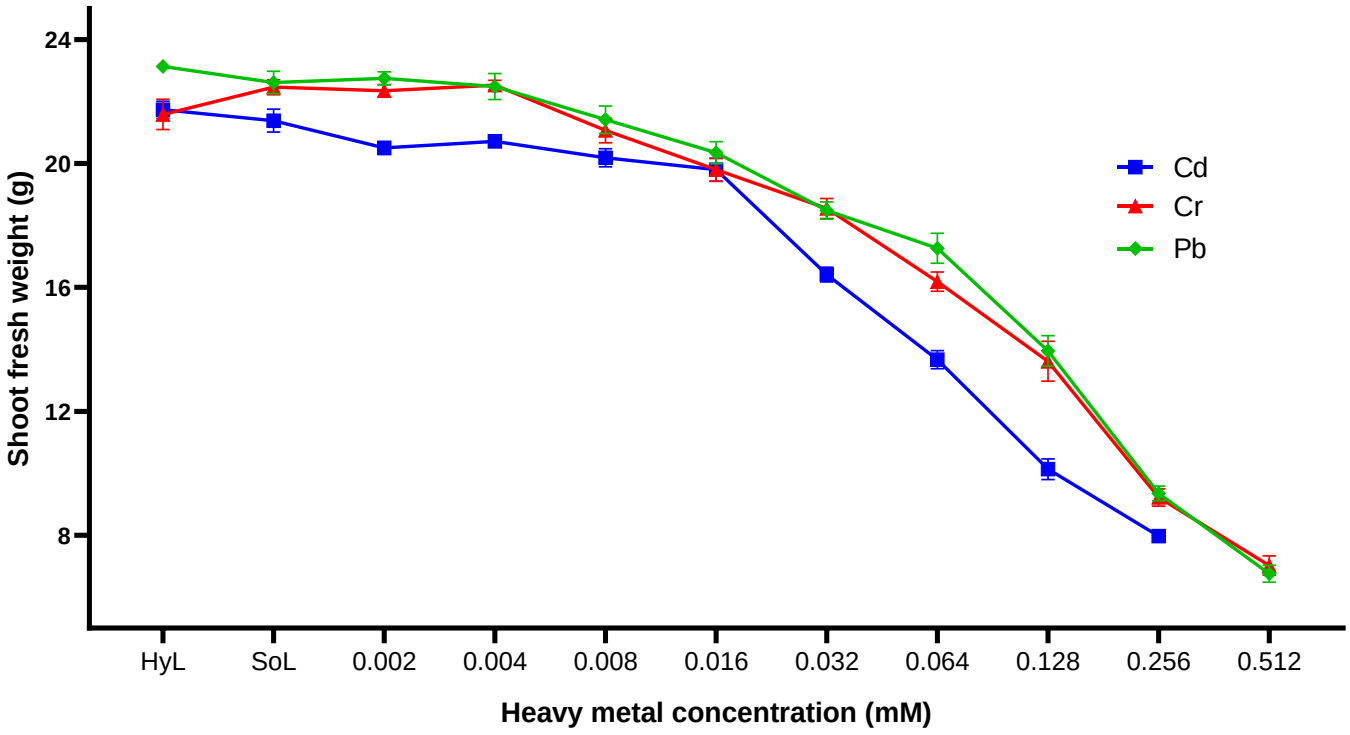

Figure 12 Effect of HM on shoot fresh weight of the lettuce plants

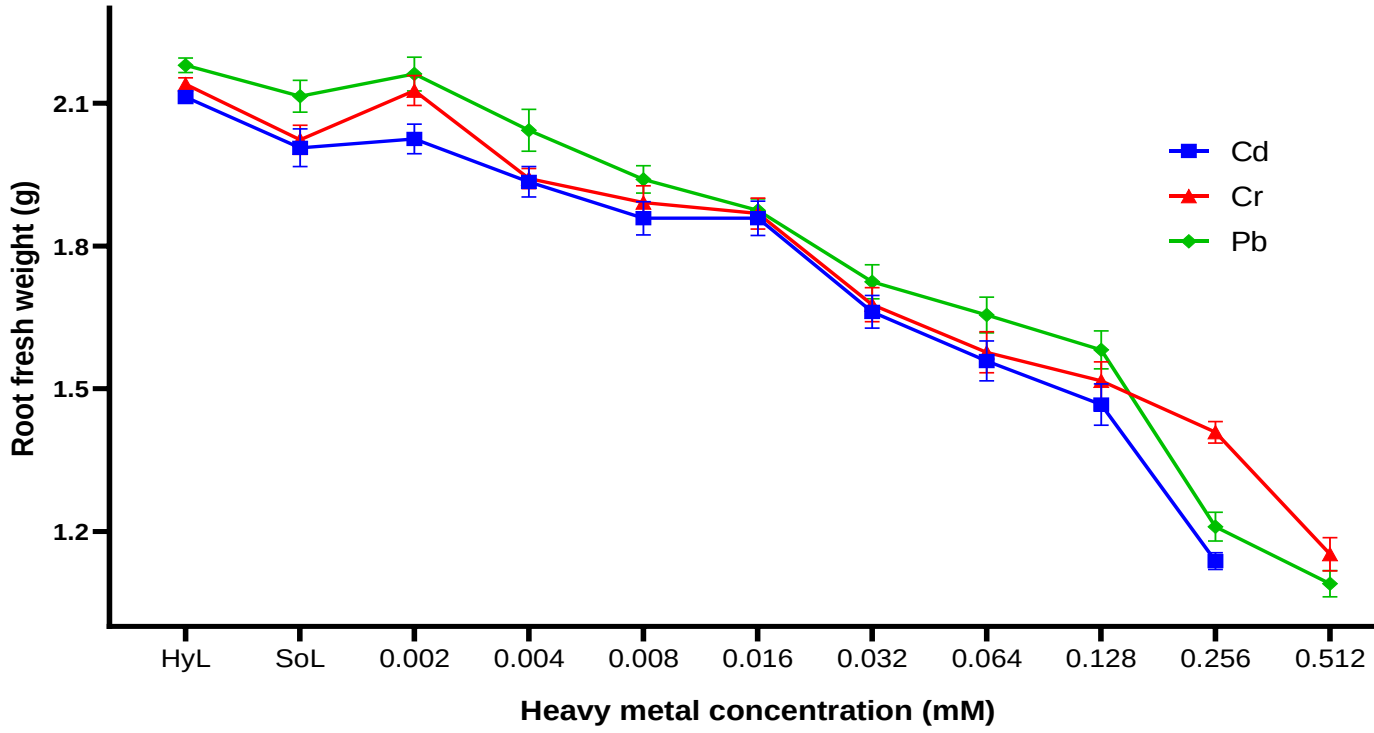

Figure 13 Effect of HM on root fresh weight of the lettuce plants

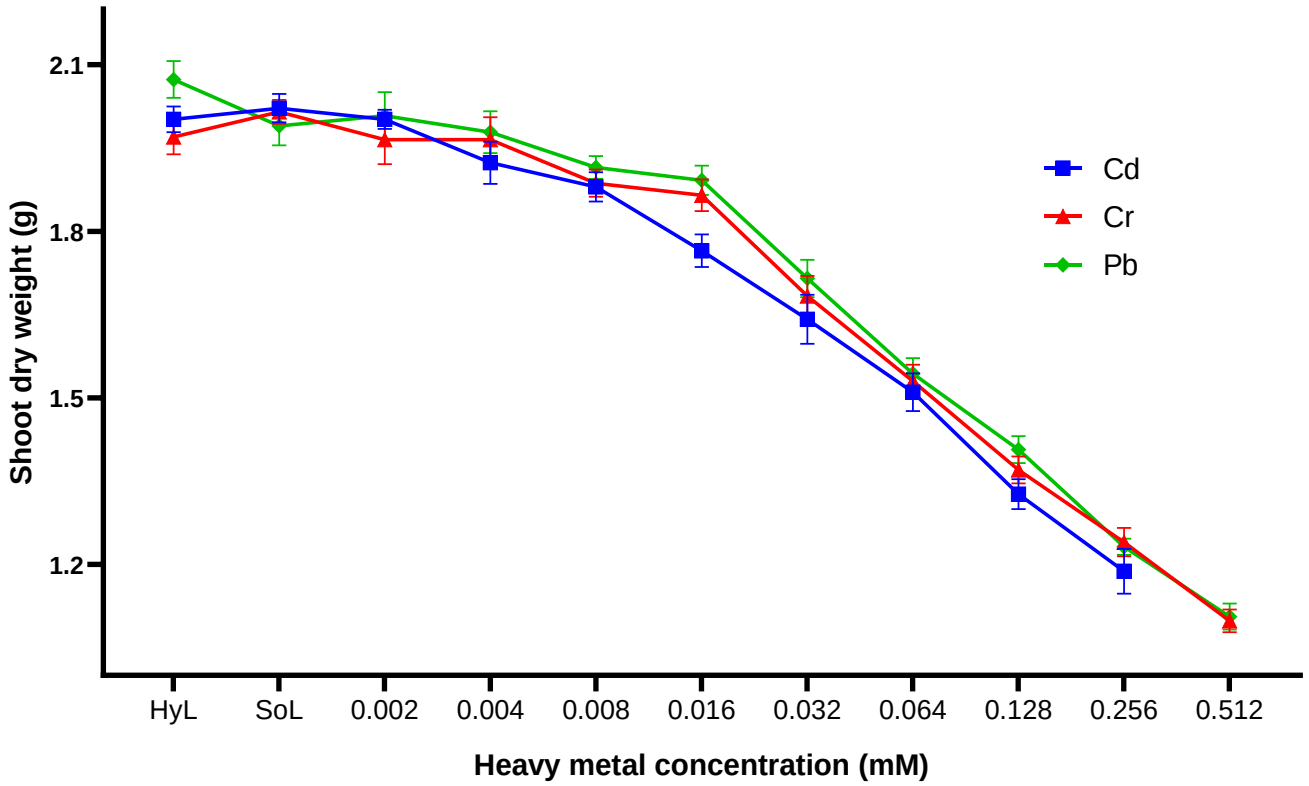

Figure 14 Effect of HM on shoot dry weight of the lettuce plants

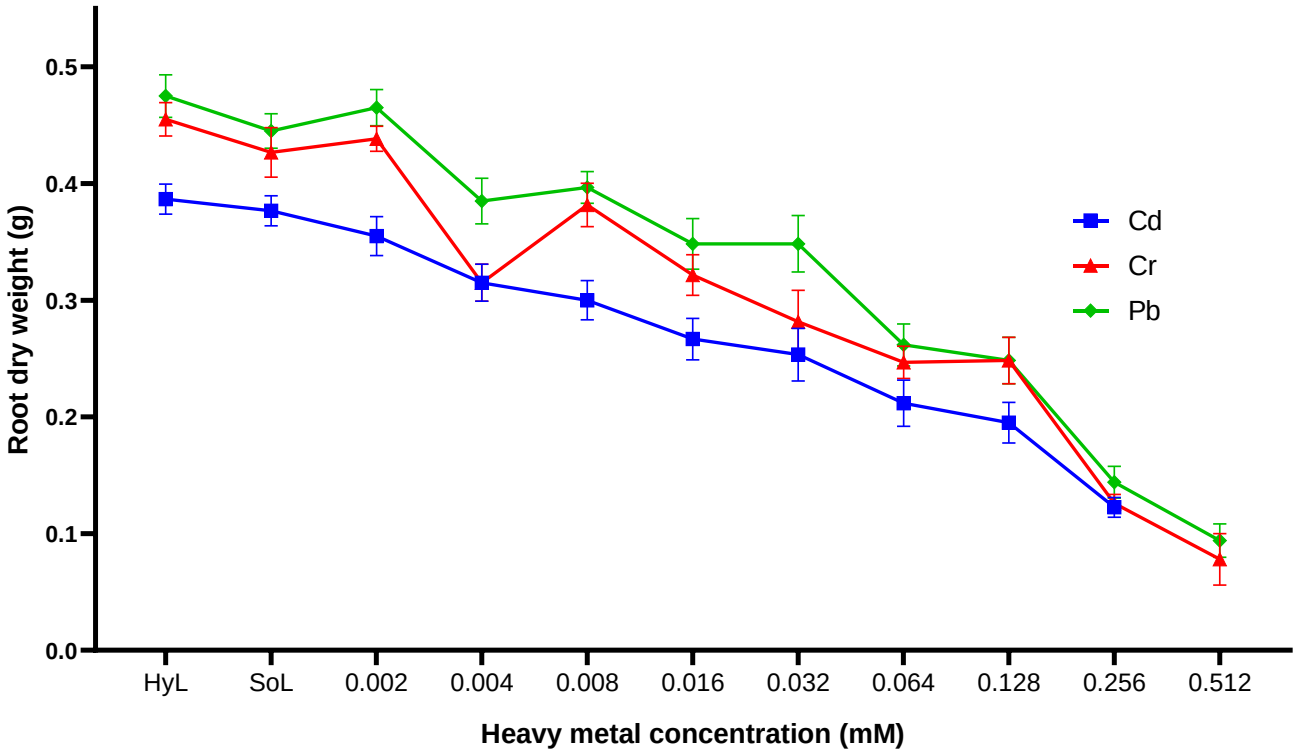

Figure 15 Effect of HM on root dry weight of the lettuce plants

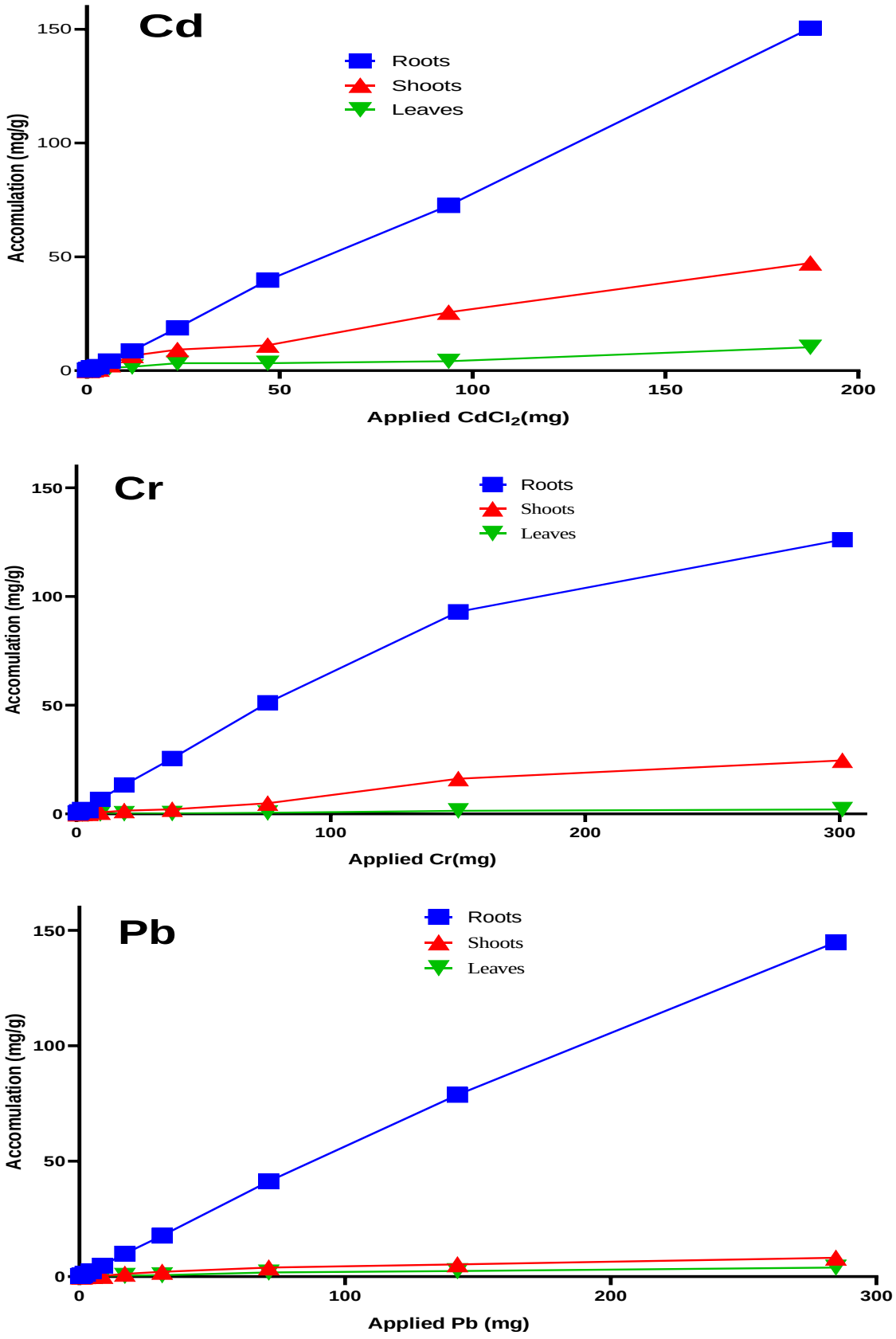

Figure 16 Concentrations of applied HMs and translocation/ Transfer to Roots, Shoots and Leaves

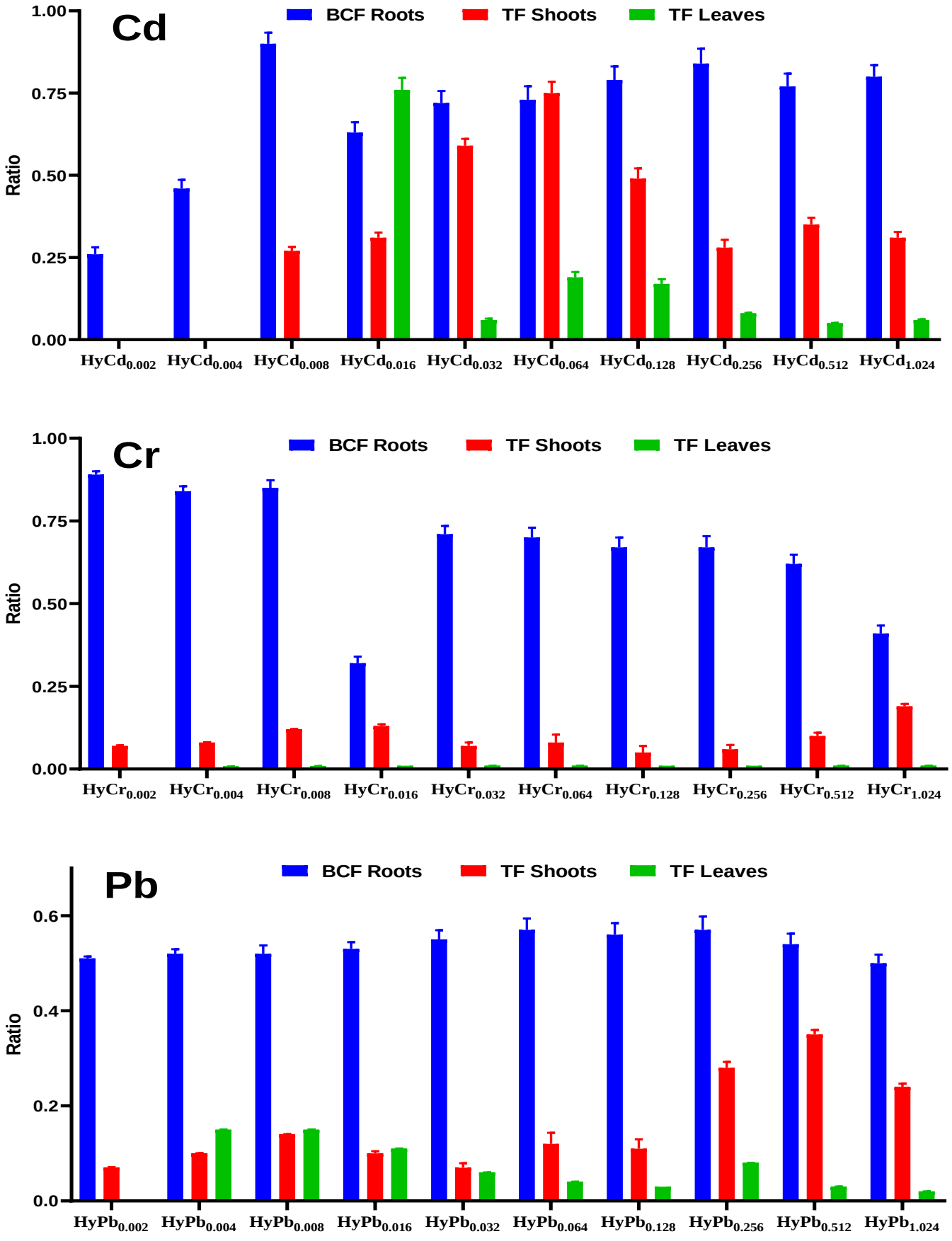

Figure 17 BCF for roots and TF for shoots and leaves of HM stressed lettuce plants

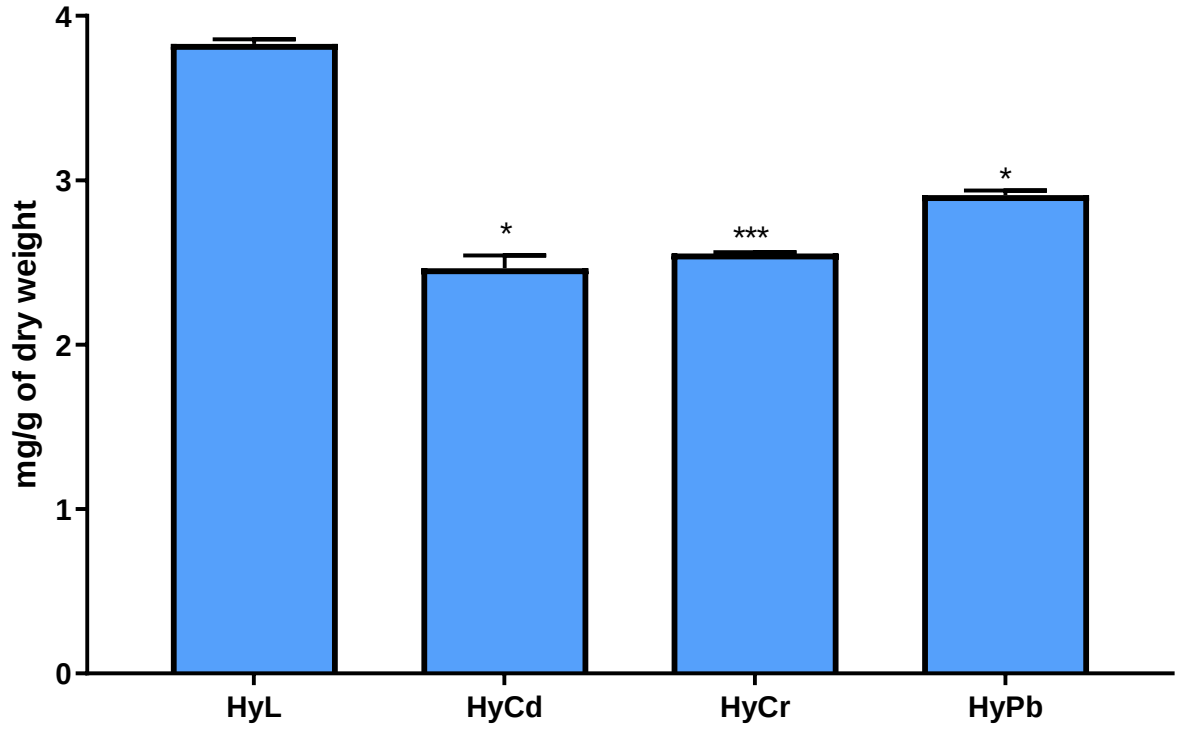

Figure 18 Total phenolic content of hydroponically grown and stressed induced plant extracts of lettuce. Graph represents mean of three ± S.D. *** $p < 0.001$ and * $p < 0.05$

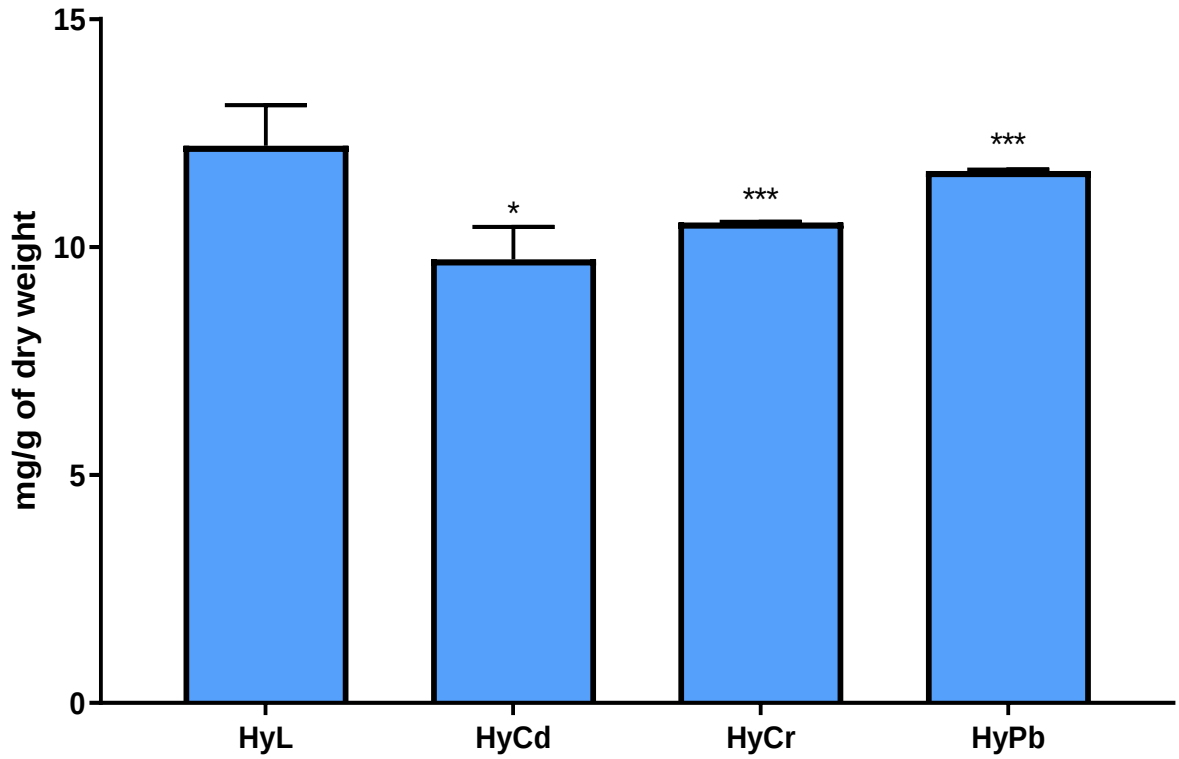

Figure 19 Total flavonoid content of soil hydroponically grown and stressed induced plant extracts of lettuce. Graph represents mean of three ± S.D. *** $p < 0.001$ and * $p < 0.05$

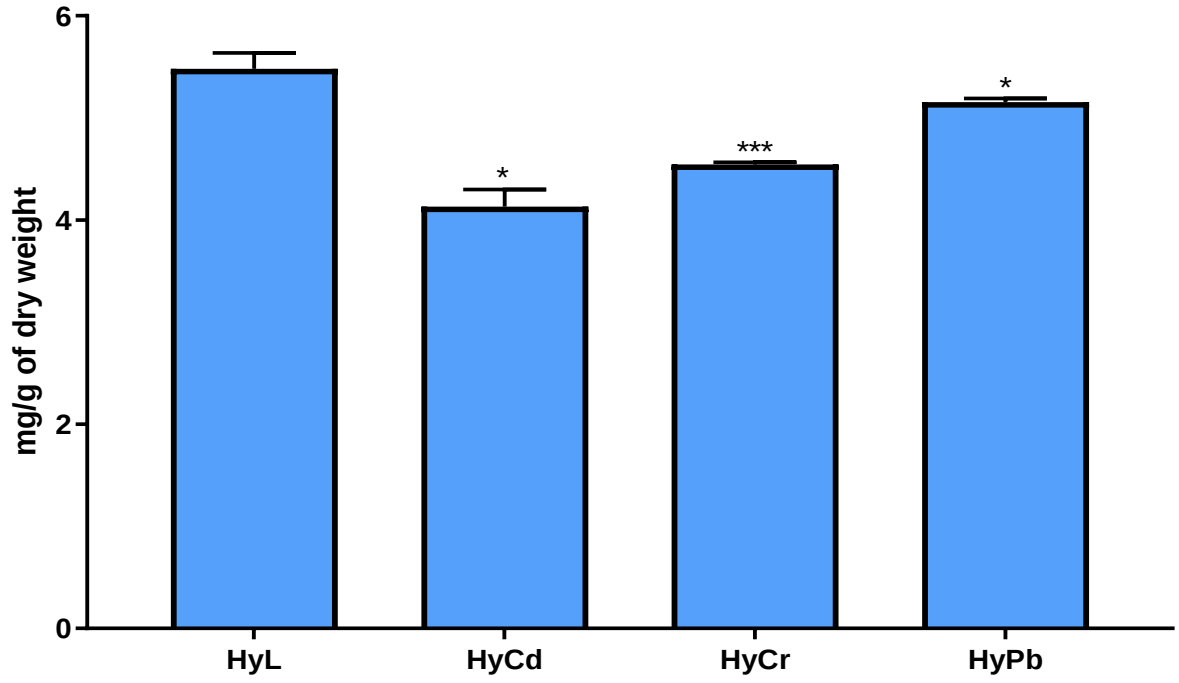

Figure 20 Total antioxidant capacity of hydroponically grown and stressed induced plant extracts of lettuce. Graph represents mean of three ± S.D. *** $p < 0.001$ and * $p < 0.05$

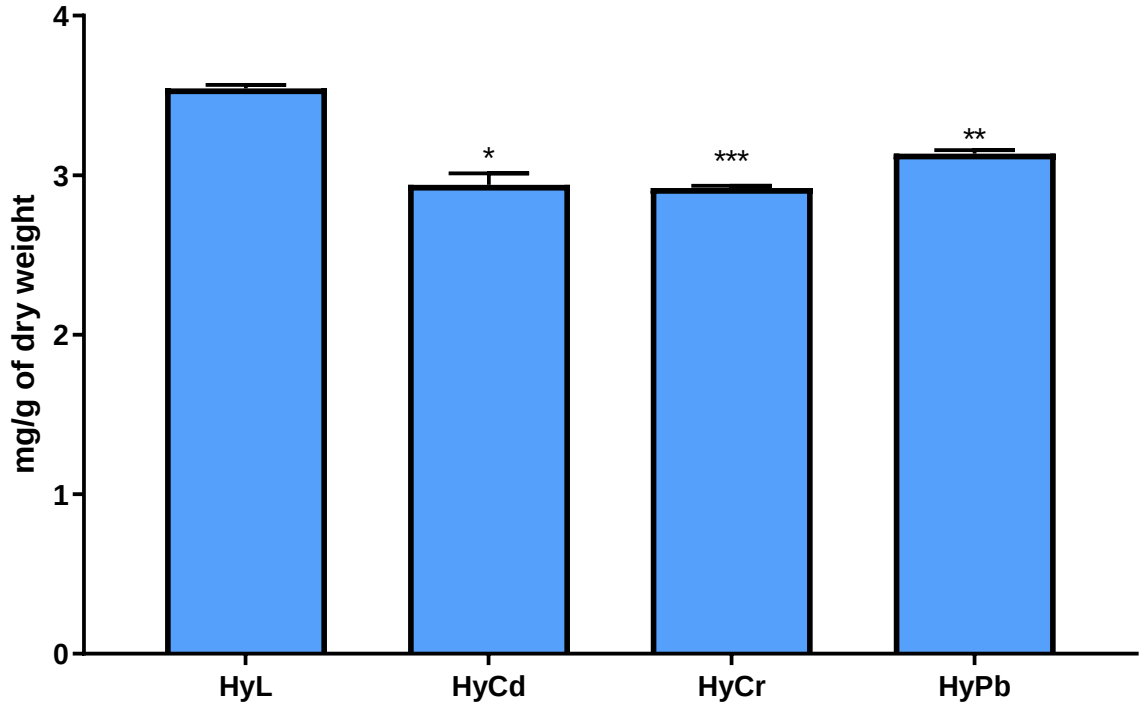

Figure 21 Total reducing the power of hydroponically grown and stressed induced plant extracts of lettuce. Graph represents mean of three ± S.D. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$

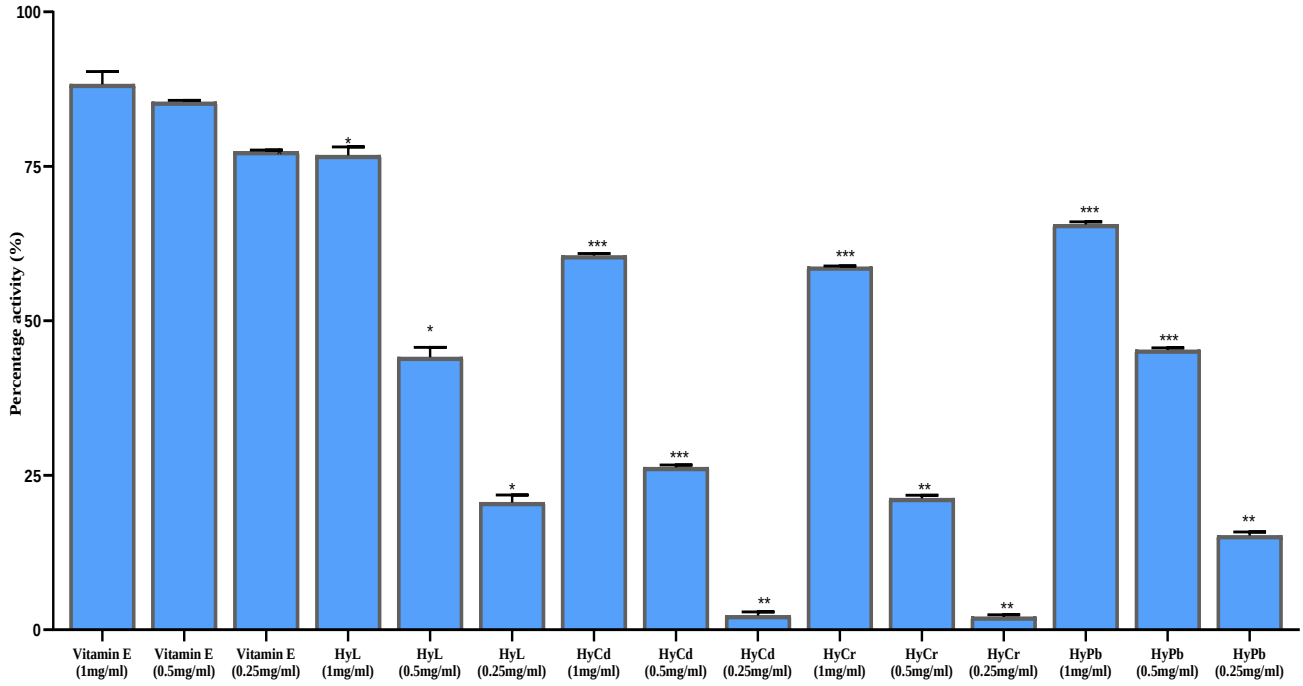

Figure 22 Lipid peroxidation activity of hydroponically grown and stressed induced plant extracts of lettuce. Graph represents mean of three ± S.D. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$.

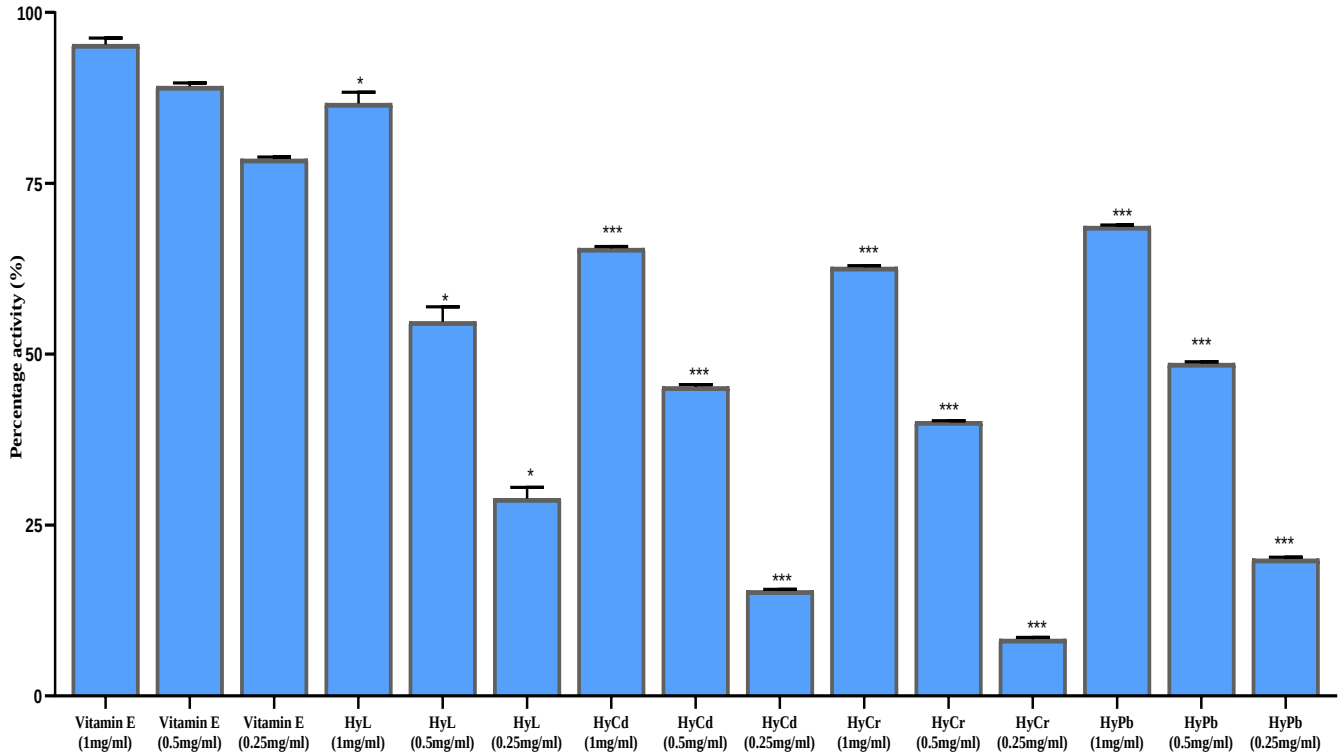

Figure 23 DPPH activity of hydroponically grown and stressed induced plant extracts of lettuce. Graph represents the mean of three ± S.D. *** $p < 0.001$ and * $p < 0.05$.

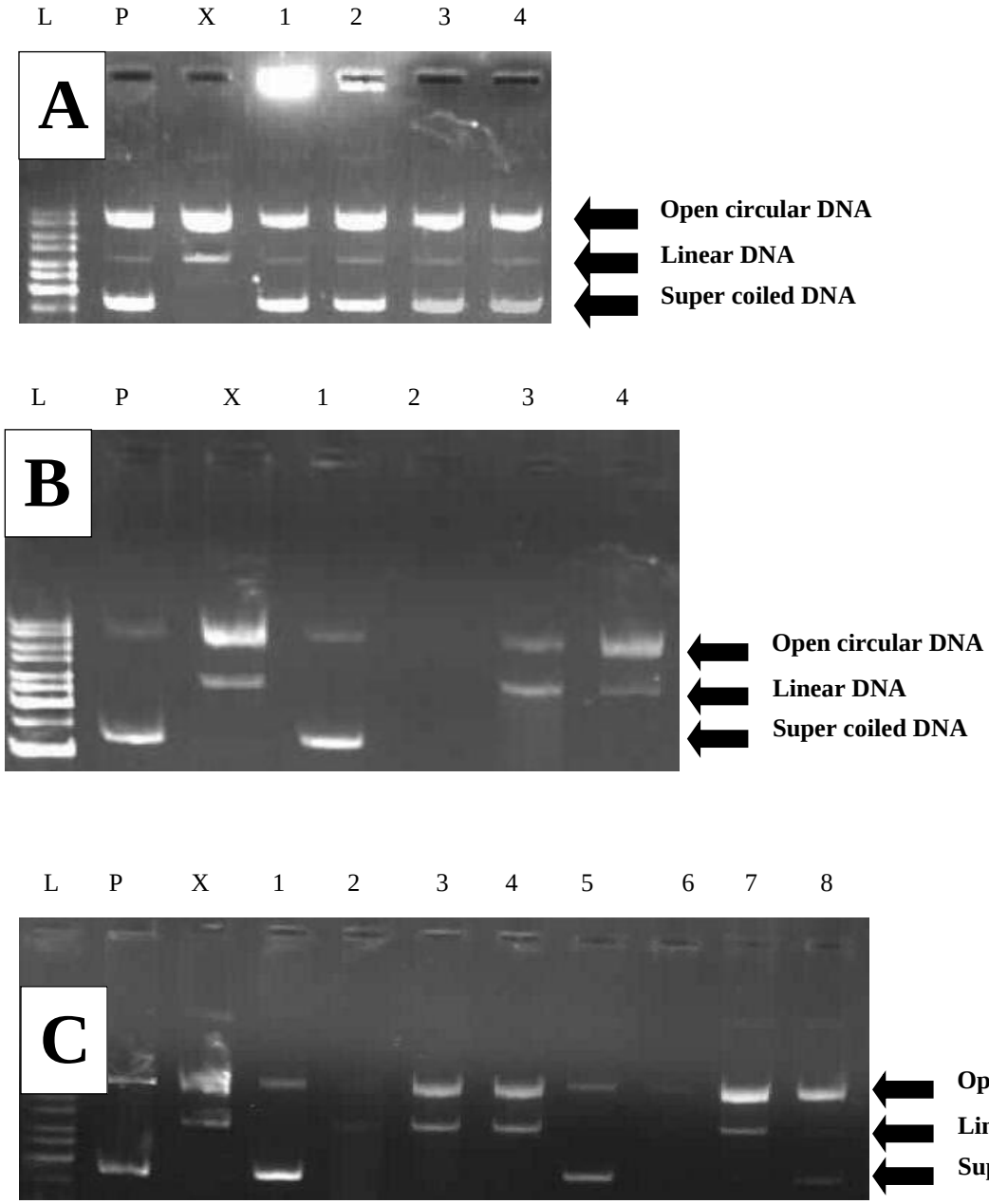

Figure 24 DNA protection activity (A) HyL (B) HyCd (C) HyCr (1-4) and HyPb (5-8). Where P=pBR322, L=1Kb ladder, X=pBR322+H2O2+FeSO4, 1=pBR322+1mg/ml prooxidant, 2=pBR322+1mg/ml concentration +H_2O_2+$FeSO_4$, 3=pBR322+0.5mg/ml concentration +H_2O_2+$FeSO_4$, 4=pBR322+0.25mg/ml concentration +H_2O_2+$FeSO_4$

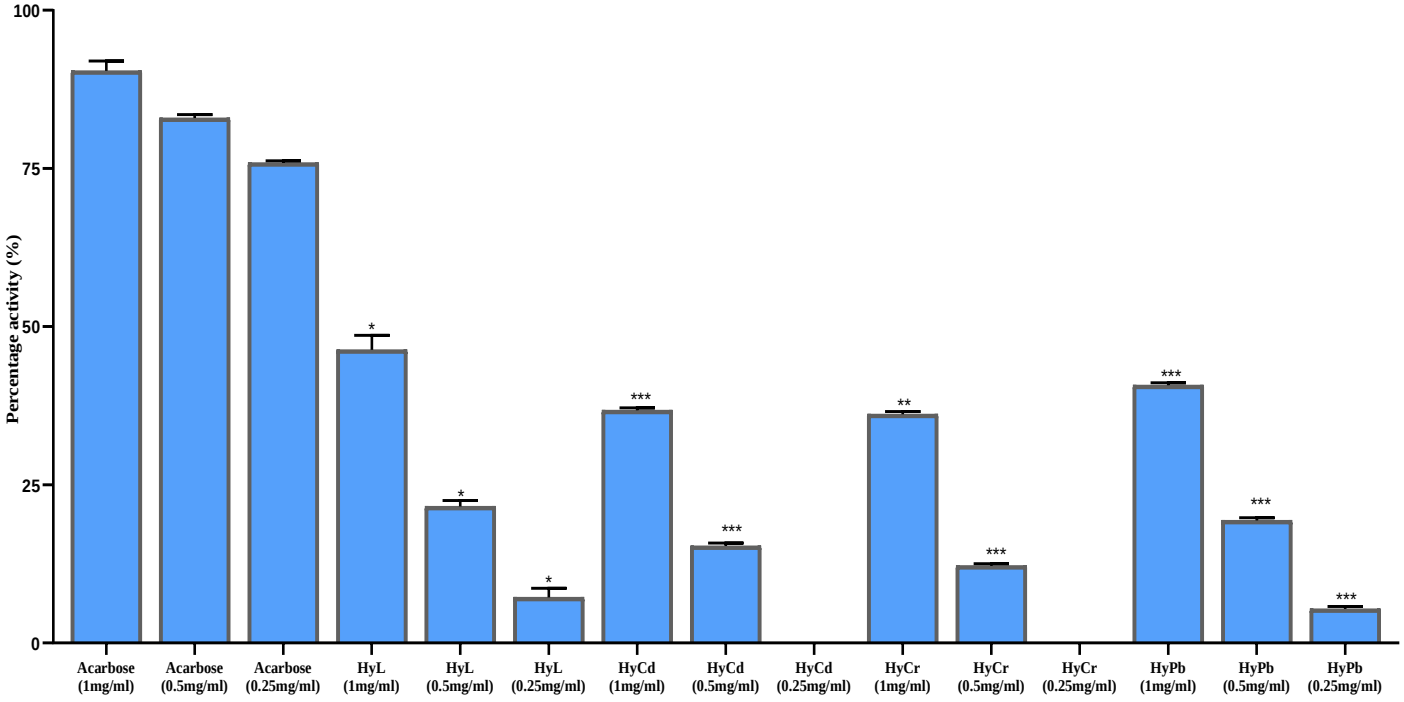

Figure 25 α-glucosidase activity of hydroponically grown and stressed induced plant extracts of lettuce. Graph represents mean of three ± S.D. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$.

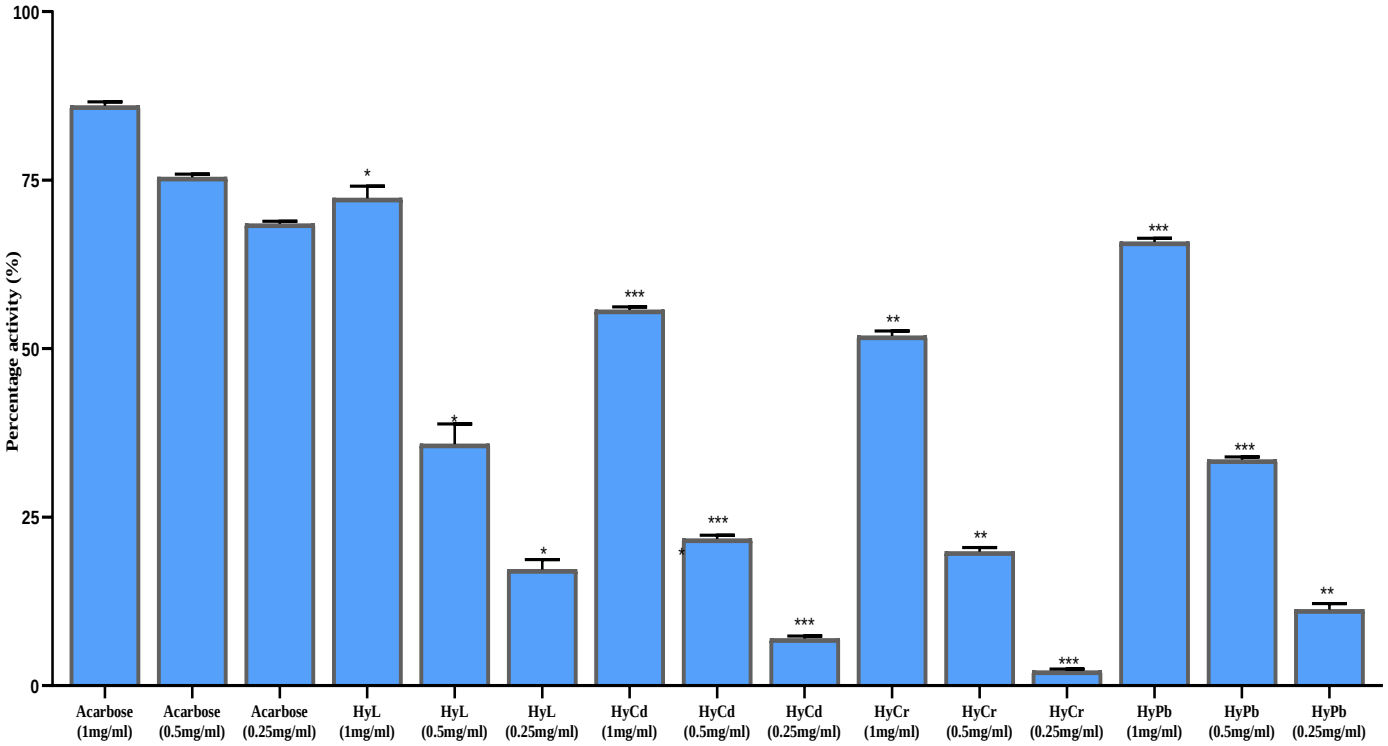

Figure 26 α-amylase activity of hydroponically grown and stressed induced plant extracts of lettuce. Graph represents mean of three ± S.D. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$

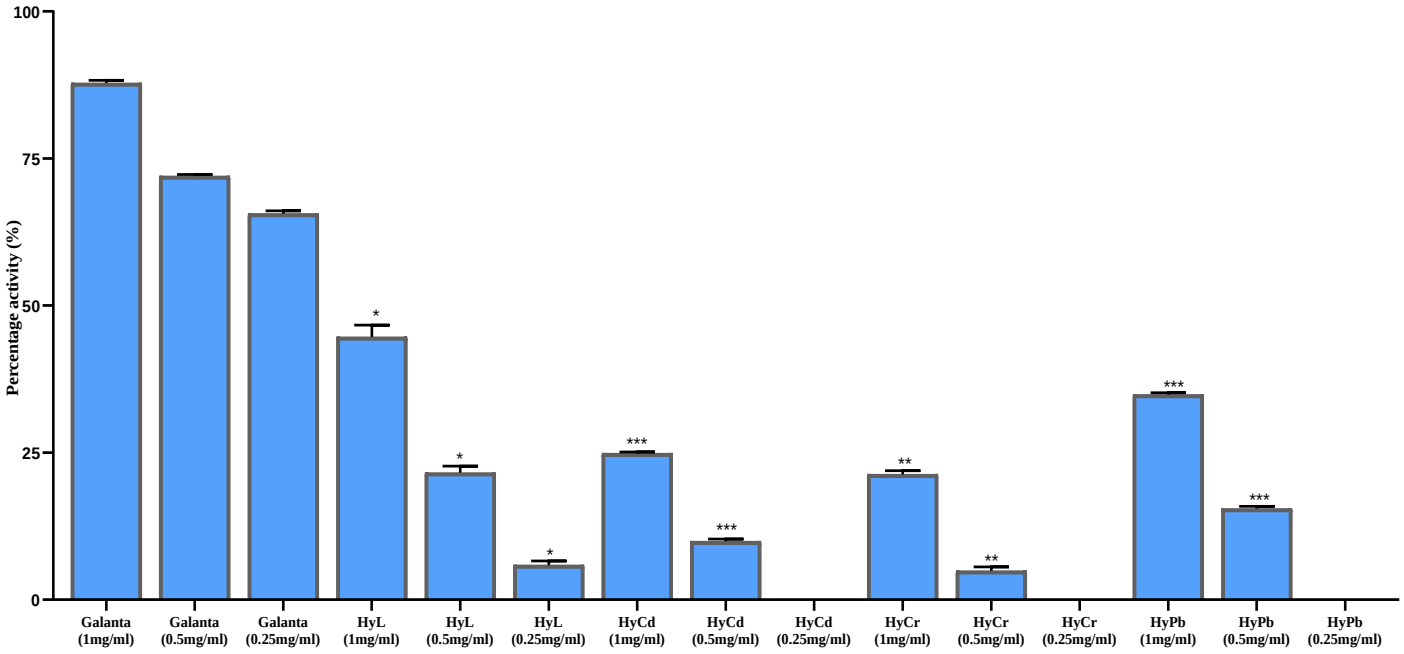

Figure 27 Acetylcholinesterase activity of hydroponically grown and stressed induced plant extracts of lettuce. Graph represents mean of three ± S.D. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$

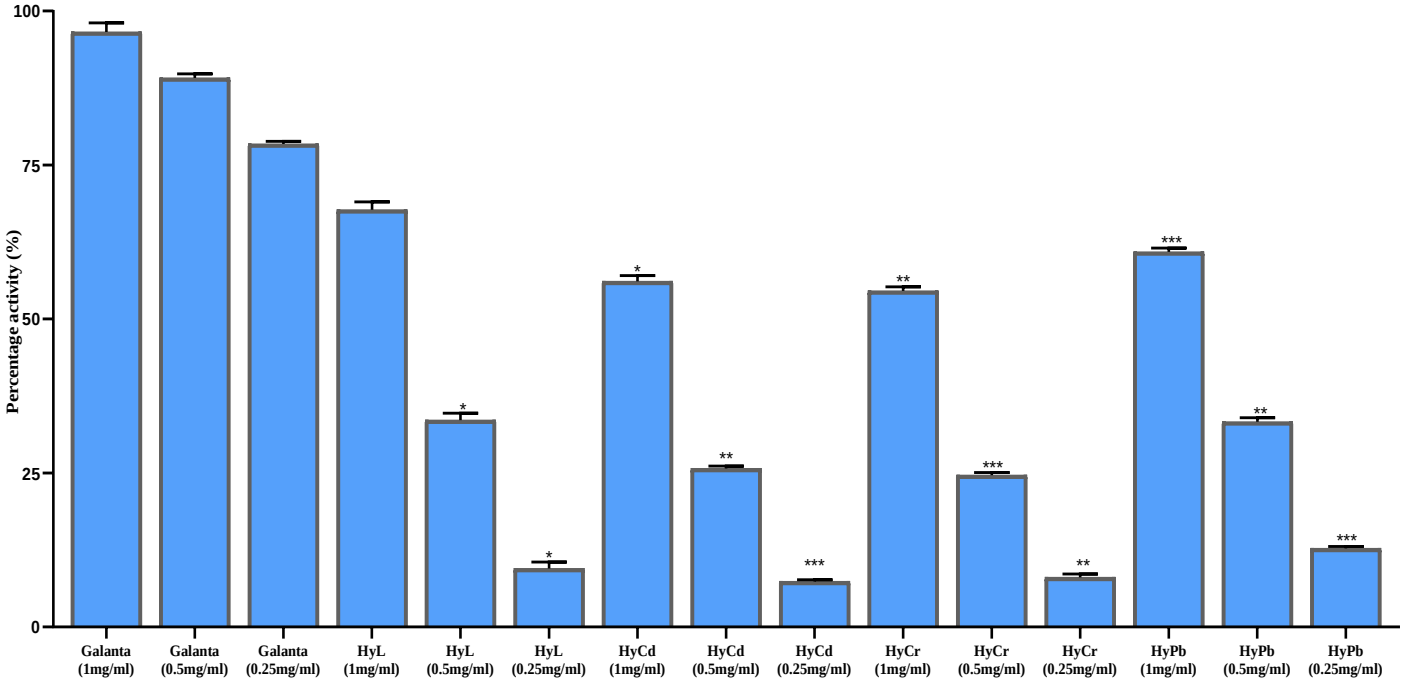

Figure 28 Butyrylcholinesterase activity of hydroponically grown and stressed induced plant extracts of lettuce. Graph represents mean of three ± S.D. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$

Table 1 IC_{50} values of in vitro activities of hydroponic and stress induced extracts

Sr.	Activity	PC (mg/ml±SD)	HyL (mg/ml±SD)	HyCd (mg/ml±SD)	HyCr (mg/ml±SD)	HyPb (mg/ml±SD)
1	Lipid peroxidation	0.03±0.01	0.55±0.17	0.82±0.04	0.86±0.03	0.63±0.06
2	DPPH	0.08±0.09	0.42±0.01	0.63±0.02	0.70±0.01	0.56±0.03
3	DNA Protection	0.06±0.01	0.48±0.01	Nil	Nil	1.20±0.01
4	α-glucosidase	0.05±0.01	1.09±0.06	1.30±0.02	1.28±0.02	1.25±0.01
5	α-amylase	0.09±0.01	0.64±0.06	1.30±0.01	0.96±0.01	0.71±0.01
6	Acetylcholinesterase	0.13±0.02	1.13±0.05	1.80±0.03	1.69±0.02	1.37±0.02
7	Butyrylcholinesterase	0.09±0.01	0.70±0.01	0.88±0.01	0.91±0.01	0.76±0.01

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