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RESEARCH ARTICLE

Health impact of heavy metals stressed hydroponically grown garden lettuce (*Lactuca sativa* L.) on rat models

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Abstract

In this study, *in vivo* pharmacological effects of plant extracts of hydroponically grown lettuce (HyL), compared with heavy metals (HMs) stressed hydroponically grown plants (HyCd, HyCr and HyPb) were studied. *Lactuca sativa* L. (lettuce) was grown hydroponically. The highest salt tolerance was estimated against 0.256 mM of Cd, Cr and Pb, respectively. So, we selected plants from 0.256 mM salt stress and extracts were prepared for *in vivo* pharmacological studies and data was analyzed statistically using ANOVA. Heavy metal stressed plants of HyCd, HyCr and HyPb showed elevated levels of glucose on the 21st day of the experiment, being as low as 355 mg/dl, 364 mg/dl and 210 mg/dl, respectively. In the case of the liver, kidney and serum biomarkers; it was seen that the levels were restored to normal in HyL, in comparison to heavy metal stress groups ($p<0.05$). The histopathological studies showed normal distribution and morphology of cells, while in metal-induced plant extracts deformed, submerged and distorted morphology was noted, representing toxicity of heavy metals. We evaluated the antioxidant markers of different organs, which showed variation from normal control values, indicating a less protective effect of metal on antioxidant profiles. The administration of HyL treatment resulted in increased levels of serotonin (0.127 µg/mg of tissue) and dopamine (0.089 µg/mg of tissue) in HPLC quantification. Among heavy metals, HyPb showed minor levels of neurotransmitters (Dopamine; 0.024 and Serotonin; 0.236 µg/mg of tissue) while these neurotransmitters were not detected in the case of HyCd, HyCr. Overall, HyPb showed comparatively higher activity than HyCd and HyCr ($p<0.05$). *Lactuca sativa* possesses good HMs accumulation potential; still, HMs had significant effects on the histology and antioxidant profiles in rat models.

Keywords: antioxidant; Alzheimer's disease; diabetes; elevated plus maze; Morris water maze test

Introduction

Lettuce (*Lactuca sativa*) is a leafy green vegetable. 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 (1). 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 (2). Various research has documented the scientific evidence about the biological activities of antibacterial, antioxidant and neuroprotective properties (2).

Food quality as an aspect of nutrition and food safety is most important for consumer health as well as food production and availability. Food must be free from any chemicals or other substances that can be harmful to people for it to be deemed safe. The ability of organic farming to produce healthy food and lessen the environmental harm caused by conventional agricultural techniques has risen over the past few years (3). Concerns about health, environment and most crucially the food safety have boosted people's desire to purchase organic goods. Concentrated pesticides and highly soluble fertilizers must not be used by farmers who practice organic farming (4). Organic systems encourage conservation, which not only minimizes environmental harm but also produces high-quality goods. According to the Food and Agriculture Organization report of 2017, lettuce was produced in 106 countries in 2017 (5). In 2020, 27.7 m tons of lettuce were produced globally, with China, India and USA leading the global lettuce markets (6).

In the soil environment and nutrient medium, heavy metals can accumulate and move around. Due to industrialization, rapid population expansion and urbanization modifies the soil. Urban and semi-urban soils and water are frequently contaminated with metals including lead (Pb), cadmium (Cd), arsenic (As) and zinc (Zn) and chromium (Cr) (7). Long-term cumulative impacts may endanger the ecology, 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 (8). 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 (9). WHO/FAO permitted levels of HMs, such as Cu, Cd, Cr, Pb and Zn in vegetables are 73 mg/kg, 0.1 mg/kg, 0.25 mg/kg, 0.3 mg/kg and 100 mg/kg, respectively (10).

Plants have a natural tendency to screen out 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 (11). When these HMs stressed plant parts are consumed by human or animals for long time, these accumulate in their bodies due to long half-life. Hence, this starts stressing the human and animal normal healthy life cycles. Heavy metals present in food crops can cause significant threat to food safety, leading to potential health hazards, including neurodegenerative diseases, damage to renal function and increased susceptibility to certain cancers (12). Therefore, understanding the impact of heavy metal stress on commonly consumed vegetables like lettuce is crucial for ensuring public health. By exploring the toxicity of heavy metal-stressed lettuce, our study directly addresses the need for safer agricultural practices and improved food quality standards (13).

Here, in this research, we investigated the effects of extracts of HMs stressed plants to study their anti-diabetic, antioxidant and anti-Alzheimer's disease in rat model. These extracts were also tested for their behavioral tests including elevated plus maze test and Morris-water maze test. The serum biomarkers and antioxidant enzymes and neurotransmitters in the brain region of rats were also evaluated.

Although earlier research has investigated the heavy metal accumulation in hydroponic lettuce and their possible health implications, less is understood regarding the particular *in vivo* impacts of these heavy metal-stressed plants on neurological and behavioral parameters, especially about diabetes, Alzheimer's and anxiety-related behaviors. This research seeks to fill this gap by examining the impact of HM-stressed hydroponically cultivated lettuce extracts on blood glucose, liver and kidney function, behavior, antioxidant status and neurotransmitter levels in a rat model (14-17).

Material and Methods

Selection of lettuce cultivar variant

The lettuce cultivar, Grand Rapids, *Lactuca sativa L.* was selected because of its high market demand and suitability for hydroponic culture. Seeds were sown in different ways, i.e., on soil and filter paper soaked in HM solution. Seeds were purchased from Awan Seed Store, Rawalpindi, Pakistan and voucher number (128085) was submitted in the Herbarium of Medicinal Plants of Pakistan. Seeds were germinated on 14 September 2019 in a mixture of agricultural soil and sand (3:1) and watered using half-strength Hoagland's Solution at 24 °C and maintained at 8/16 night/ day photoperiod.

Seed germination experiments

Seeds were germinated on filter paper moistened with heavy metal solutions of different concentrations (0.002 mM - 1.064 mM) in sterilized plastic boxes. After 24 hr in darkness, the boxes were subjected to natural light (21-22 °C daytime, 18-19 °C nighttime) (18).

Heavy metal stress

Following a germination period of 15 days, twelve polystyrene boxes having dimensions L x W x H = 45 x 30 x 20 cm, with six plants each, were used for each HM. 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. For each heavy metal concentration, the HM solution was added just after preparing the nutrient media and all boxes were labelled for date of transfer and HM concentration. The plants were collected for analysis on the 45th day following transplantation. In our previously published article, various morphological characteristics were studied and it was found that at 0.256 mM of Cd, Cr and Pb the plants showed the highest salt tolerance respectively (18). Hence for animal model plants from this concentration were selected and extracts were prepared using analytical grade methanol (97 %).

Animal grouping

At the primate facility of University of Gujarat, Gujarat, Pakistan, Sprague Dawley male rats weighing 180-200 g were kept in hygienic circumstances with a regular feed, water and good sanitary conditions in aluminum cages. With reference number 321, the Institutional Ethics and Biosafety Committee (IBC) accepted the study and minimal animal safety was guaranteed in accordance with National Institutes of Health (NIH) standards. In all animal studies, efforts were made to adhere to the 3Rs-principles of replacement, reduction and refinement. Replacement was assured by considering alternative *in vitro* methods. All animals were subjected to daily checks for any signs of distress or adverse effects and veterinary care was provided during the entire study. Different groups of seven rats each were randomly assigned. The first group received no treatment (disease control), the second group took glibenclamide (a positive control for diabetes), the third group took rivastigmine (a positive control for Alzheimer's disease), the rest of the groups took three different doses of hydroponically grown lettuce (HyL) and stress induced lettuce including HyCd, HyCr and HyPb, each at 200, 100 and 50 mg/kg, respectively, The last group was referred to as the normal healthy group and received no treatment. The choice of doses was based on previous studies that demonstrated pharmacological effects of plant extracts at similar concentrations (19, 20).

Disease induction

To induce diabetes in the rats, 0.2 mL of 30 mg/kg streptozotocin (STZ) produced in 0.1 M citrate buffer solution was injected intraperitoneally for three consecutive days (21). With the aid of a VitaTM test meter (Life scan), blood samples were taken from each rat's tail each day to measure blood sugar levels. Aluminum chloride ($AlCl_3$) was given orally every day for three weeks after hyperglycemia was induced to induce Alzheimer's disease symptoms (22). After 14 days of therapy, behavior assays were conducted and all dosages were administered orally for the remaining 21 days.

Acute oral toxicity

Studies of the acute toxicity of the lettuce extracts were carried

out by OECD guidelines 420 (23) . To achieve this objective, a single dose of 400 mg/kg of each extract separately, including seven rats, was administered orally. In the control, the seven rats were administered orally with a simple saline solution. Up to one week's worth of vital statistics, toxicity levels and changes in behavior were tracked.

Elevated plus maze test (EPM)

The elevated plus maze test was used to examine the rat's anxiety-related behavior (24). It was an equipment in the shape of a plus sign, with two sides enclosed to form the closed arm end and two sides uncovered to form the open arm end. Each rat was initially placed in the maze for a while so that it could become accustomed to the equipment. The rats were then slowly put into the labyrinth, each one facing the end with the open arm. A camera positioned on the stand captured the movements of each rat in the maze over the course of five minutes. The videos were analyzed using ANY-maze video tracking software to determine the duration of time spent in the open and closed arms, the number of entries made into each arm and the distance traversed inside both arms of the maze.

Morris water maze test (MWM)

The MWM test was used to assess the memory patterns (25). A platform was positioned in a 90 cm in diameter circular pool that had been filled with water. The platform was located in the north-west quadrant of the pool, which was divided into four quadrants. Each rat underwent one visible trial and one hidden trial. The quadrant that formerly housed the platform was where the time spent there was recorded. The 60 sec probe trial was completed. Based on this premise, an assessment was made to ascertain the extent to which the rats had retained memory in their ability to locate the platform, taking into account their previous trials. The motion was recorded by a camera that was positioned on a stable stand and the ANY-maze video tracking program was employed for its analysis.

Dissection of rats

The rats were given isoflurane anesthesia on the twenty-first day (26). The rats were then carefully dissected and blood was drawn using a 3 mL syringe and heart puncture to fill BD vacutainer® containers. The skull bone was properly removed and the brain was removed and placed in small containers. These containers contained 0.9 % saline solution and kept at -20 °C until needed. These brain tissues underwent histology and homogenization, the blood was centrifuged.

Serum preparation and serum analysis

The blood was centrifuged at 3500 rpm for 10 min. Prior to usage, the serum was kept at a temperature of -20 °C. After centrifugation, the serum was analyzed using Cobas® kits in accordance with the supplier's specified kit level. Serum blood urea nitrogen (BUN), total bilirubin, ALT, creatinine and AST levels were examined.

Histology

Slides were prepared from the formalin-fixed tissues of different organs including, (brain, pancreas, liver, kidney) using hematoxylin and eosin (H & E) staining and examined under a light microscope.

Tissue homogenate preparation

Homogenization was applied to the brain tissues that had been preserved in saline solution. To create the tissue homogenate, 100 mg of tissue was added to 1 mL of a pH 7.4 solution containing 100 mM phosphate buffer and 1 mM EDTA. After that, the tissue homogenate was centrifuged for 30 min at 4 °C at 1000 rpm. The supernatant was taken and kept at -20 °C for HPLC, neurotransmitter and antioxidant analyses.

Determination of antioxidant enzymes

The different organs tissues were subjected to antioxidant analysis. The activity of antioxidant enzymes was studied along with total protein estimation of brain samples. The antioxidant enzymes included peroxidase (POD) (27), reduced glutathione (GSH) (28), catalase (CAT) (27), thiobarbituric acid reactive substances (TBARS) (29) and superoxide dismutase (SOD) (30, 31) was performed using previously reported methods.

Quantification of dopamine and serotonin by HPLC

Dopamine and serotonin (neurotransmitters) levels in brain were quantified using HPLC method (32). The supernatant was filtered using 0.2 µm syringe filter before being injected into the apparatus injector at a volume of 20 µL. The mobile phase employed with the C-18 column was 50 mM phosphate buffer prepared in 3 % methanol. Dopamine and serotonin levels were quantified utilizing a quaternary pump and a UV/visible detector. The flow rate utilized in the experiment was recorded as 1.5 mL/min, while the duration of the experiment was set to 15 min. The measurements were taken at a wavelength of 240 nm. The dopamine and serotonin standard curves were generated by repeated dilutions at concentrations of 0.0125, 0.025, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 µg/µL. For quantification, the linear regression equation (y=1536.6x-07846) and serotonin (y=2155.8x-0.1567) were employed.

Statistical analysis

All values were represented as mean ± standard deviation. ANOVA followed by Tukey's post-hoc test was used to compare multiple groups. Statistical significance was considered at a p-value < 0.05.

Results and Discussion

Acute toxicity

No instances of mortality or observable alterations in behavior were documented among the experimental rats within the study duration. Consequently, it was determined that the extract utilized in the research posed no significant risks.

Elevated plus maze test (EPM)

The EPM test was employed to assess anxiety-like behavior in the experimental rats. The distance travelled was compared for close arm and open arms as shown in Fig. 1. In normal control group, the total distance was greater in closed arms than open arms. The distance travelled by HyL was 88.6 cm for closed arm while in open arms it was recorded as 64.2 cm

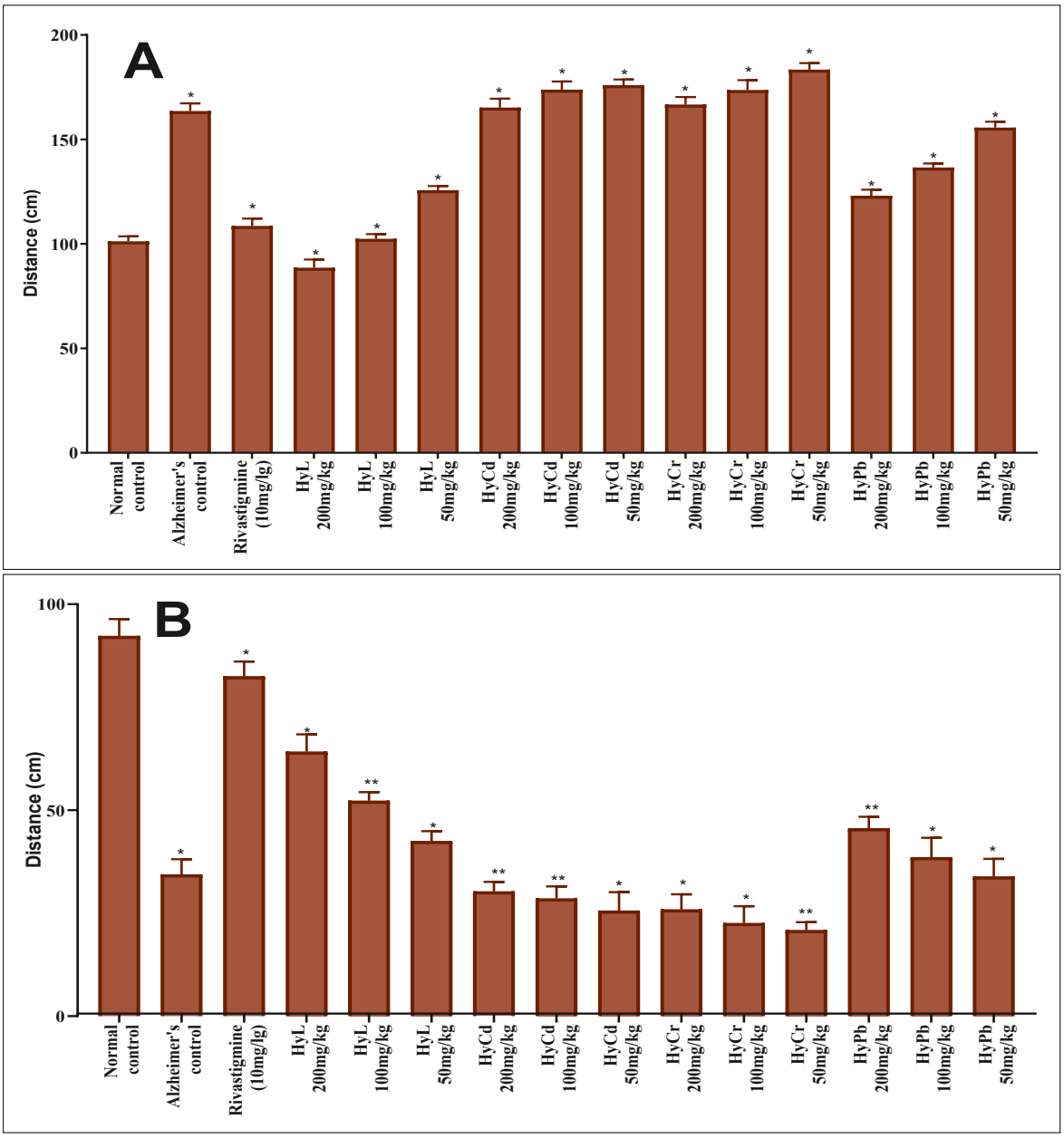

Fig. 1. Results of elevated plus maze test. (A) distance in close arm; (B) distance in open arm. Here * p<0.05 and ** p<0.01 as compared with normal control.

respectively (p<0.05). In contrast, the disease control group exhibited a mean distance of 163.6 cm for the close arm condition, whereas the open arm condition resulted in a mean distance of 34.4 cm in statistically significant manner (p<0.05). In a similar way, the normal control group exhibited measurements of 101.2 cm for the closed arm and 92.3 cm for the open arm (p<0.05). In case of metal stress, the results were comparatively low as compared with HyL, but the highest distance travelled in open arm was recorded for HyPb (45.6 cm) followed by extracts of HyCd (30.3 cm) and HyCr (25.9 cm) at 200 mg/kg concentration (p < 0.05). EPM is used to estimate anxiety behavioral indices which have strong ability to calculate and validate the selection of plant extracts (33). Anxiety and depression may arise as a result of fluctuations in the functioning of neurotransmitters, including serotonin, noradrenaline and dopamine (34). Monoamine oxidase inhibitors (MAOIs) have been recognized for their ability to augment certain naturally occurring amines, such as serotonin and catecholamine (35). Nevertheless, there are a number of plant species that have been seen to enhance the exploration of open arms in the elevated plus-maze test, a commonly used method to assess anxiety levels. Notably, *Trichilia catigua* and *Plumeria rubra* are among the plant species that have been traditionally employed in folk medicine to alleviate anxiety (36).

Morris water maze test (MWM)

The MWM test was employed to assess the memory performance of rats. The results are given in Fig. 2. In the 1st entry trial, rats in normal group reached quadrant platform in 8.3 sec as compared to the rats in Alzheimer's group which have value of 31.3 sec and when the treatment of rivastigmine was administered rats showed improved memory patterns with platform reaching time as 10.5 sec (p<0.05). HyL group showed value of 12.7 sec, representing better response then Alzheimer's control after treatment. Among the heavy metals for HyPb, HyCd and HyCr the platform reaching time was 22.6 sec, 28.9 sec and 25.8 sec respectively at 200 mg/kg concentration (p<0.05). In the second parameter as total time spent in platform quadrant

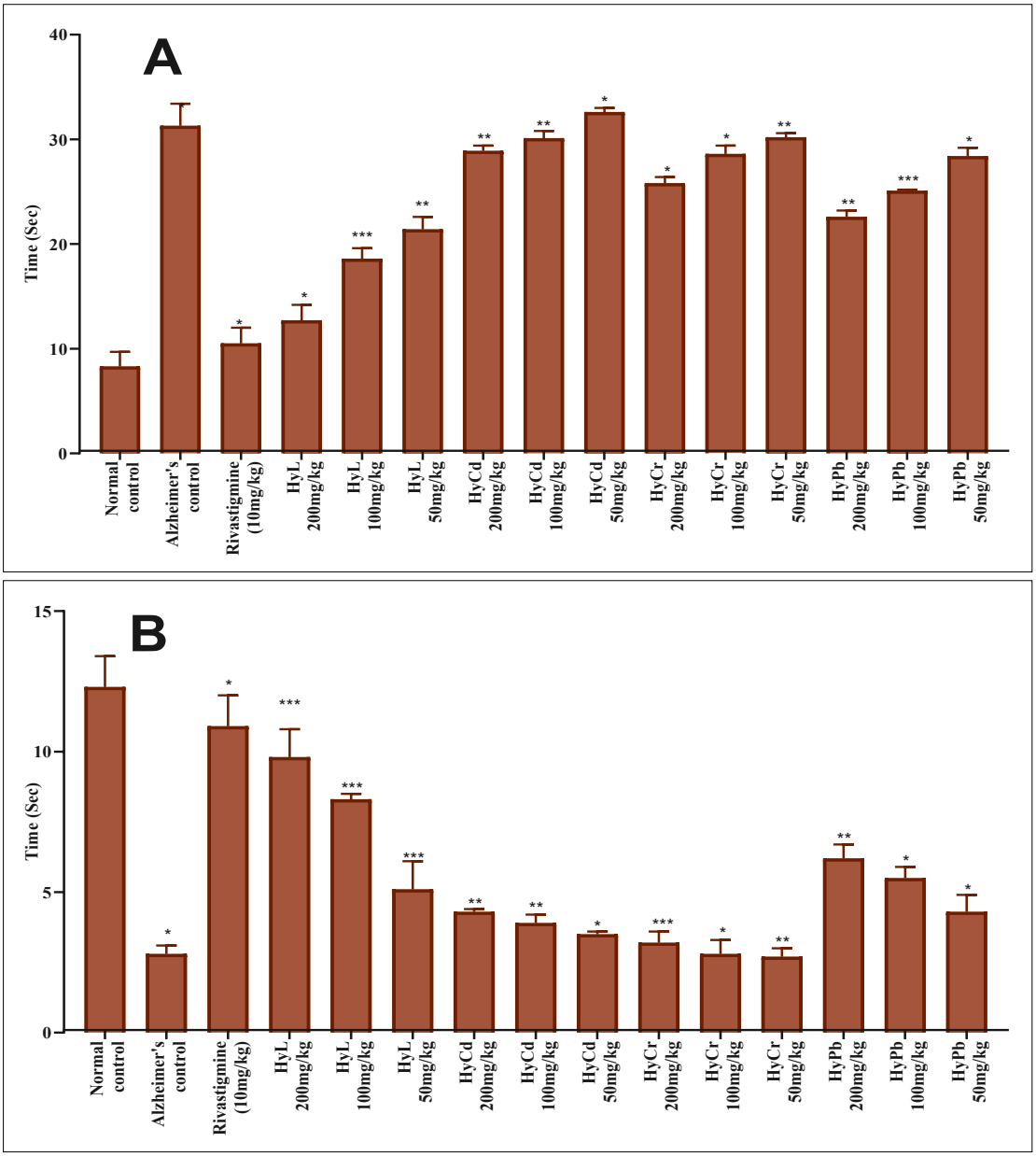

Fig. 2. Results of Morris water maze test. (A) 1st entry in platform quadrant; (B) total time in platform quadrant. Here * p<0.05, ** p<0.01, *** p<0.001 as compared with normal control.

normal group rats spent 12.3 sec in platform as compared to Alzheimer's group, which have spent only 2.8 sec while rivastigmine treated rats, spent 10.9 sec in platform quadrant (p<0.05). HyL group spent 9.8 sec representing improved orientation and memory pattern. In case of metal stress, the results were representing minimum time spent in platform quadrant. HyPb, HyCd and HyCr spent total 6.2 sec, 4.3 sec and 3.2 sec respectively, at 200 mg/kg concentration in platform quadrant (p<0.01).

The MWM test is a significant method utilized to assess memory impairments in individuals with Alzheimer's disease, specifically by examining the functioning of the hippocampus in the brain (37). The administration of aluminum chloride ($AlCl_3$) has been found to result in a decline in both spatial and contextual memory. However, it has been observed that the effects of $AlCl_3$-induced memory impairment can be mitigated by the administration of Asiatic acid, which aligns with the findings of our investigation (38). The present study employed the Morris Water Maze (MWM) test as a means to assess the enduring cognitive capacities about spatial learning and reference memory (39). It can serve as an indicator of spatial learning, as animals are required to acquire knowledge of the precise location of a concealed platform and create appropriate strategies to access there (40). The assessment of reference memory in the absence of the platform is mostly determined by the virtual-platform crossing numbers seen during the probe test (40). Consistent with prior research findings, extracts demonstrated a significant increase in the time it took for subjects to escape in the acquisition phase, indicating a longer latency.

Blood glucose level

Streptozotocin exerts an impact on the endogenous secretion of insulin, leading to a rise in the concentration of glucose in the bloodstream (41). The impact of lettuce extracts on blood glucose levels was assessed using the Life Scan One Touch Vita™ test meter at the time of injecting the rats with streptozotocin and then after every 3-day intervals and results are presented in Table 1. The diabetic control group, consisting of rats with diabetes, showed a glucose range of 401-525 mg/dl, but the glucose level of the Glibenclamide remained within the range of above 95-

133mg/dl during the 21st day experiment ($p<0.05$). The HyL group exhibited a substantial reduction in blood glucose levels, decreasing from an initial measurement of 414 mg/dl at 0 hr to 127 mg/dl on the 21st day ($p<0.05$). Compared with all the above controls, heavy metal stress induced plant extract of HyCd, HyCr and HyPb showed an elevated level of glucose on the 21st day of the experiment, being 355 mg/dl, 364 mg/dl and 210 mg/dl respectively in statistically significant way ($p<0.05$). However, HyPb group showed a moderate reduction in blood glucose levels, decreasing from an initial measurement of 385 mg/dl at 0 hr to 210 mg/dl on the 21st day ($p<0.001$). Streptozotocin, functioning as an alkylating agent, induces DNA fragmentation (42). DNA damage triggers the initiation of the DNA repair mechanism, which subsequently facilitates an increase in ATP dephosphorylation. This process provides a substrate for xanthine oxidase, ultimately leading to the generation of ROS (43). The DNA damage assay provided further confirmation of the protective effects of HyL.

Serum analysis

The serum biomarkers of kidney and liver were assessed in rats with STZ-induced diabetes and the findings are presented in Table 2. It was seen that the levels of all these biomarkers were found restored in the HyL extracts, in comparison to the diabetic group in a statistically significant manner ($p<0.05$). The serum electrolytes are described as liver prime functioning enzymes that controls the metabolism of whole body and are an indicator of hepatic efficiency (44). In a study, when 300mg/kg/day plant extract was given to Pb-acetate toxic rats for fourteen days, the antioxidant depletion was prevented (45). Comparable results were seen with CCl_4 induced toxicity in rats. These findings align with the values of the control group, indicating that HyL treatments have beneficial effects on kidney and liver biomarkers. In case of heavy metal stress, including HyCd, HyCr and HyPb group had values close to the disease group, representing negative effect of heavy metals on the liver and kidney biomarkers. It can be assumed from this study that heavy metals like Pb, Cd and Cr adversely affect the liver and kidney functions. If we compare the toxicity, then Pb can been seen slightly less toxic than Cd and Cr, or we can say lettuce can accommodate more Pb as compared to Cr and Cd.

Organ histology

Fig. 3a depicts microscopic images of pancreas samples observed under a light microscope at a magnification of 10X. In the normal group, distinct Islets of Langerhans (ILs), ducts and a visible pattern were observed. In contrast, no visible ILs, visible ducts, or visible patterns were observed in the diabetes group. The glibenclamide treated group exhibited the presence of distinct ILs, accompanied by visible ducts and a visible pattern. The group treated with HyL had a distinct cellular arrangement characterized by the presence of ILs and observable ducts. Within the HyPb group, we found the

Table 1. Effect of lettuce extracts on blood glucose level (mg/dl)

Groups	0 hour	3rd hour	6th hour	12th hour	2nd day	6th day	10th day	14th day	18th day	21st day
Diabetic control	401±4.0**	525±2.1**	512±4.4*	513±3.7*	502±3.2*	503±2.2*	491±3.4*	491±4.3*	491±2.3*	488±3.3*
Normal control	98±2.4	96±3.0	99±2.6	97±4.2	94±3.5	96±3.3	93±2.0	99±3.5	98±1.2	96±4.1
Glibenclamide	411±1.8**	133±1.4**	106±4.3*	104±2.9*	131±3.5*	102±3.4*	96±2.7*	95±2.3*	96±2.9*	96±1.2**
HyL 200 mg/kg	414±4.4*	252±3.3*	188±2.7*	161±1.8**	146±3.3*	136±1.9*	136±1.7**	134±3.3*	133±4.3*	127±4.2*
HyL 100 mg/kg	413±5.3*	245±4.4*	227±3.4*	199±3.5*	196±4.5*	191±5.2*	187±6.1*	185±5.5*	185±4.7*	181±3.7*
HyL 50 mg/kg	406±3.5*	253±5.3*	236±5.3*	211±4.7*	206±4.4*	205±4.1*	208±4.4*	209±5.0*	201±6.0*	204±4.1*
HyCd 200 mg/kg	390±4.9**	410±5.1*	398±5.8*	401±2.5***	398±4.4*	396±3.8*	375±3.8*	372±2.3***	365±5.2*	355±2.9***
HyCd 100 mg/kg	392±5.6*	402±3.8*	395±5.2*	402±3.6*	385±4.9*	391±3.2*	398±5.0*	386±3.8*	369±4.4*	356±3.4**
HyCd 50 mg/kg	401±4.2*	395±3.9*	402±4.2*	398±4.6*	388±5.1*	391±5.1*	402±5.6*	377±2.9**	366±3.8*	368±5.4*
HyCr 200 mg/kg	403±2.3***	398±4.6*	398±3.8*	395±5.1*	392±6.1*	386±4.9*	383±1.9***	381±4.5*	364±3.0*	369±2.9***
HyCr 100 mg/kg	399±3.9*	402±4.5*	402±2.9***	396±2.9***	384±5.2*	382±3.8*	391±5.3*	372±5.3*	360±4.9*	351±3.9*
HyCr 50 mg/kg	394±4.1*	405±3.1*	401±3.8*	399±3.8*	386±4.5*	379±4.1*	382±4.8*	376±4.9*	371±5.6*	359±3.3*
HyPb 200 mg/kg	385±3.7*	350±3.9*	325±4.5*	310±5.4*	295±3.7*	281±4.3*	265±4.6*	241±3.8*	233±3.4*	210±2.7***
HyPb 100 mg/kg	387±4.8*	375±4.2*	365±6.1*	345±4.8*	325±3.4*	310±4.4*	292±3.3*	276±4.3*	264±2.8***	255±3.8*
HyPb 50 mg/kg	406±5.2*	395±4.1*	385±3.7**	375±3.7*	355±3.8*	342±2.9***	332±3.8*	311±4.4*	299±1.9***	285±5.1*

Values are means ± SD with *p<0.05, **p<0.01 and ***p<0.001 statistically significance compared to normal group

Table 2. Effects of lettuce extracts on serum biomarkers

Groups	Liver			Kidney	
	AST (U/L)	ALT (U/L)	Total bilirubin (mg/dl)	Creatinine (mg/dl)	BUN (mg/dl)
Diabetic control	78.3±3.3*	53.0±3.6*	1.21±0.05*	1.49±0.04*	37.0±2.1*
Normal control	22.7±1.2	22.4±1.3	0.29±0.01	0.34±0.01	19.2±0.6
Glibenclamide	27.3±2.1*	23.4±2.8*	0.39±0.02*	0.61±0.01*	21.2±2.2*
HyL 200 mg/kg	37.4±2.5*	27.3±2.1*	0.58±0.04*	0.73±0.04*	27.4±2.4*
HyL 100 mg/kg	45.2±3.5*	34.2±3.2*	0.84±0.05*	0.92±0.07*	32.5±3.1*
HyL 50 mg/kg	56.1±3.3*	44.1±4.1*	0.95±0.03*	1.21±0.06*	29.3±2.6*
HyCd 200 mg/kg	75.6±3.8*	52.1±2.3*	1.31±0.06*	1.43±0.42*	35.6±3.5*
HyCd 100 mg/kg	76.9±4.2*	53.6±3.6*	1.38±0.07*	1.49±0.48*	37.9±3.9*
HyCd 50 mg/kg	79.3±4.9*	55.6±1.8**	1.42±0.05*	1.56±0.53*	40.3±4.3*
HyCr 200 mg/kg	80.6±4.7*	56.7±2.7*	1.29±0.03**	1.5±0.45*	39.4±4.0*
HyCr 100 mg/kg	82.4±3.8*	58.1±2.1*	1.35±0.08*	1.59±0.49*	42.5±4.5*
HyCr 50 mg/kg	85.9±2.5**	60.9±3.7*	1.39±0.01***	1.63±0.56*	45.6±4.9*
HyPb 200 mg/kg	70.6±2.9*	40.6±1.8*	1.12±0.02**	1.35±0.35*	30.2±3.1*
HyPb 100 mg/kg	72.8±4.6*	42.6±1.9*	1.22±0.04*	1.39±0.39*	35.9±3.5*
HyPb 50 mg/kg	70.4±2.7*	45.9±1.2***	1.28±.07*	1.51±0.43*	39.7±3.9*

Values are means ± SD with *p<0.05, **p<0.01 and ***p<0.001 statistically significance compared to normal group

presence of deteriorated ILs, as well as the very low visibility of ducts and no proper pattern while in the HyCr and HyCd group, observations were made about the degradation of the Islets of Langerhans, the presence of no visible ducts and no pattern.

The provided Fig. 3b displays microscopic pictures of liver samples acquired from rats belonging to each respective group. No instances of overlapping were detected in the normal control. A visible pattern was discovered, characterized by distinct sinusoidal shapes and peripheral cells. In the context of diabetes control, an absence of visible pattern, a high density of cells and indistinct sinusoids, accompanied by a notable presence of immune cells, were observed. The glibenclamide treated group had highly populated cells lacking any discernible pattern, as well as sinusoids and prominently present immune cells. A distinct pattern was observed within the HyL groups, along with sinusoids, immune cells and peripheral cells apparent. No discernible pattern was identified within the HyPb, HyCd and HyCr groups and no clear structures that included sinusoids, immune cells and peripheral cells were observed.

The Fig. 3c displays microscopic views of kidney samples from several groups. During the examination of the normal group, the Bowman's capsule and glomerulus were observed to be clear and distinct, with no observed overlapping of tubules. The examination of diabetes control revealed the presence of tubules that overlapped, accompanied by the nonexistence of a distinct Bowman's capsule. Additionally, a distinct glomerulus was observed. During the glibenclamide experiment, we noticed the presence of disordered Bowman's capsule accompanied with a distinct overlapping tubules and glomerulus. In the group treated with HyL, the presence of a well-defined Bowman capsule and glomerulus was seen, with no observed overlap of tubules. Within stress induced HyPb, HyCd and HyCr groups, it was seen that no clear Bowman's capsule and glomerulus were present, alongside the presence of overlapping tubules.

The Fig. 3d displays brain pictures acquired by microscopic analysis of slides that were made from distinct groups. In a normal group, the presence of homogeneous cells and discernible cell borders was observed. The study of Alzheimer's disease revealed the presence of aberrant cellular morphology characterized by structural deformities and a loosely arranged cellular architecture. The rivastigmine group revealed the presence of cells that exhibited no deformities and were densely arranged. Within the group treated with HyL, the presence of homogeneous, non-damaged and closely arranged cells with visible cell borders was observed. In HyPb, HyCd and HyCr groups, deformed and loosely packed cells were observed additionally cells were however not uniform as in the normal control. In conclusion, in all histopathological analysis in HyL groups, visible cell boundaries and uniform cells are noted. In heavy metal stress induced plant including HyPb, HyCd and HyCr groups, distorted and loosely packed cells were observed representing the toxic effects of heavy metals on different tissues.

Determination of antioxidant enzymes

The quantification of antioxidant enzymes in the pancreas (Table 3), liver (Table 4), kidney (Table 5) and brain (Table 6) was performed using a spectrophotometric method. In general, the findings indicated a rise in the levels of CAT, POD, SOD and GSH across all examined organs in comparison to the diabetes control group. Conversely, reduction in TBARS activity was detected as presented in Tables 3-6. HyCd, HyCr and HyPb group showed the toxic effect of metal on plant extracts which in turn is responsible for the reduction of antioxidant enzymes in different organs. If we compare the toxicity, then Pb can be seen slightly less toxic than Cd and Cr. Oxidative stress arises as a result of the action of free radicals, necessitating the consideration of antioxidant enzymes that function as the primary protective mechanism against these radicals ($p<0.05$). Catalase, glutathione and lipid peroxidation enzymes all account for neuron protection against oxidative

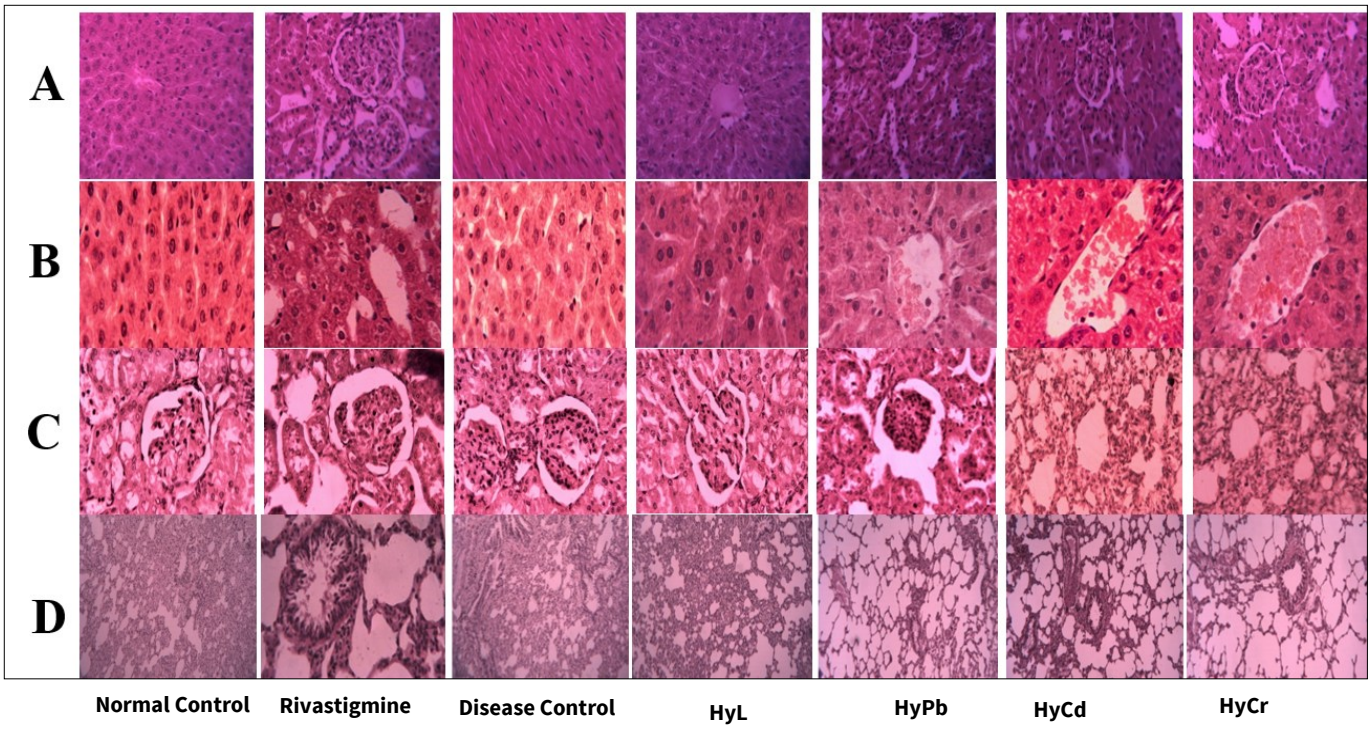

Fig. 3. Histology of different rat tissues. (A) pancreas; (B) liver; (C) kidney and (D) brain.

Table 3. Effects of lettuce extracts on pancreas biomarkers

Treatment	TBARS (nM/min/ mg)	SOD (U/mg)	GSH (mM/g)	POD (U/min)	CAT (U/min)
Diabetic control	42.1±2.3*	1.6±0.07**	4.9±0.5*	2.8±0.2**	1.6±0.3**
Normal control	34.8±1.0	2.5±0.5	8.9±0.4	5.4±0.6	5.5±0.2
Glibenclamide	32.9±1.8***	2.3±0.03**	8.5±0.2***	4.8±0.2***	5.0±0.4*
HyL 200mg/kg	35.8±2.7*	2.2±0.04**	7.8±0.6*	4.4±0.7*	4.5±0.5**
HyL 100mg/kg	36.4±3.8*	1.7±0.03*	6.8±0.7*	3.5±0.5*	3.2±0.4*
HyL 50mg/kg	37.2±2.5*	1.4±0.07*	6.1±0.4*	3.0±0.3*	2.4±0.5*
HyCd 200mg/kg	39.3±2.1*	1.8±0.03*	4.9±0.6*	3.1±0.4*	1.9±0.02**
HyCd 100mg/kg	42.8±2.6*	1.5±0.02*	4.5±0.4*	2.9±0.3*	1.5±0.03*
HyCd 50mg/kg	44.2±3.5*	1.3±0.03*	4.2±0.2**	2.5±0.5*	1.2±0.04*
HyCr 200mg/kg	40.1±3.4*	1.7±0.04*	5.2±0.5*	3.0±0.6*	1.8±0.03*
HyCr 100mg/kg	42.7±4.6*	1.6±0.04*	4.0±0.3*	2.8±0.1***	1.4±0.01***
HyCr 50mg/kg	44.6±4.0*	1.3±0.01**	4.8±0.1***	2.5±0.2**	1.1±0.04*
HyPb 200mg/kg	35.7±2.6*	2.5±0.03*	6.3±0.2**	3.8±0.1***	2.3±0.05*
HyPb 100mg/kg	37.4±2.8*	2.2±0.02*	5.8±0.1***	3.2±0.3*	2.1±0.01***
HyPb 50mg/kg	39.2±2.7*	1.9±0.04*	5.2±0.1***	2.8±0.3*	1.8±0.03*

Values are means ± SD with *p<0.05, **p<0.01 and ***p<0.001 statistically significance compared to normal group

Table 4. Effects of lettuce extracts on liver biomarkers

Treatment	TBARS (nM/min/ mg)	SOD (U/mg)	GSH (mM/g)	POD (U/min)	CAT (U/min)
Diabetic control	54.5±2.4*	3.5±0.1**	5.5±0.4*	4.2±0.1**	10.5±1.3*
Normal control	36.9±3.0	4.9±0.2	10.0±0.4	5.0±0.3	14.5±1.5
Glibenclamide	38.5±3.5*	4.5±0.3***	9.8±0.3**	4.7±0.2**	13.6±1.3*
HyL 200mg/kg	44.8±3.4*	3.8±0.7*	9.4±0.6*	4.3±0.5**	12.0±1.2*
HyL 100mg/kg	46.8±4.4*	3.2±0.5*	7.9±0.2*	3.9±0.4*	11.0±1.3*
HyL 50mg/kg	49.2±3.8*	4.3±0.2*	7.0±0.7*	3.5±0.1*	10.2±1.5*
HyCd 200mg/kg	54.5±4.2*	3.5±0.6*	6.2±0.5*	3.9±0.03*	9.5±1.2*
HyCd 100mg/kg	56.0±4.1*	3.2±0.5*	6.0±0.5*	3.5±0.02*	9.2±1.3*
HyCd 50mg/kg	57.8±4.3*	2.9±0.5*	5.5±0.3*	3.1±0.05*	8.7±1.5*
HyCr 200mg/kg	55.1±3.6*	3.6±0.4*	5.8±0.3*	3.8±0.04*	9.2±1.7*
HyCr 100mg/kg	56.7±3.5*	3.4±0.4*	5.2±0.2*	3.3±0.04*	8.5±2.2*
HyCr 50mg/kg	58.3±2.5**	3.1±0.1**	4.5±0.3*	2.9±0.06*	9.1±1.3*
HyPb 200mg/kg	45.6±2.1**	4.1±0.3*	7.5±0.4*	4.3±0.05*	11.2±2.3*
HyPb 100mg/kg	49.3±2.3**	3.6±0.3*	7.0±0.1**	4.0±0.02*	10.5±2.5*
HyPb 50mg/kg	51.3±2.7*	3.0±0.1**	6.3±0.1**	3.8±0.01**	9.5±1.4*

Values are means ± SD with *p<0.05, **p<0.01 and ***p<0.001 statistically significance compared to normal group.

Table 5. Effects of lettuce extracts on kidney biomarkers

Treatment	TBARS (nM/min/ mg)	SOD (U/mg)	GSH (mM/g)	POD (U/min)	CAT (U/min)
Diabetic control	57.8±4.2*	1.2±0.02**	3.0±0.5*	1.3±0.02**	11.2±0.7**
Normal control	30.2±3.1	3.8±0.3	7.9±0.4	4.4±0.4	13.5±0.5
Glibenclamide	34.7±3.5*	3.3±0.02***	7.5±0.3***	4.5±0.1***	13.1±0.7***
HyL 200mg/kg	39.1±4.4*	2.4±0.5*	6.9±0.5*	3.8±0.5*	12.8±0.5**
HyL 100mg/kg	44.3±4.3*	1.8±0.3*	6.0±0.3*	2.2±0.4*	12.0±1.6*
HyL 50mg/kg	51.8±5.0*	4.3±0.2*	5.3±0.4*	1.2±0.5*	11.5±1.8*
HyCd 200mg/kg	55.5±4.2*	1.6±0.2*	3.9±0.5*	1.1±0.5*	11.5±1.2*
HyCd 100mg/kg	58.6±4.3*	1.4±0.2*	3.5±0.5*	0.9±0.3*	10.6±1.6*
HyCd 50mg/kg	60.2±4.6*	1.1±0.3*	3.1±0.6*	0.8±0.4*	10.1±3.2*
HyCr 200mg/kg	58.6±4.5*	1.2±0.1**	3.7±0.3*	1.0±0.1**	11.1±2.5*
HyCr 100mg/kg	60.8±4.1*	1.0±0.2*	3.1±0.2*	0.7±0.2*	10.2±2.4*
HyCr 50mg/kg	65.3±4.3*	0.9±0.1*	2.8±0.3*	0.5±0.1*	9.5±2.1*
HyPb 200mg/kg	47.9±4.5*	1.9±0.4*	4.1±0.1**	2.5±0.3*	12.3±1.8*
HyPb 100mg/kg	50.3±3.2*	1.7±0.3*	3.5±0.1**	2.2±0.4*	11.7±1.4*
HyPb 50mg/kg	53.2±3.5*	1.3±0.2*	3.1±0.2*	1.8±0.4*	11.1±1.7*

Values are means ± SD with *p<0.05, **p<0.01 and ***p<0.001 statistically significance compared to normal group

Table 6. Effects of lettuce extracts on brain biomarkers

Treatment	TBARS (nM/min/ mg)	SOD (U/mg)	GSH (mM/g)	POD (U/min)	CAT (U/min)
Alzheimer control	29.3±3.3*	0.4±0.01*	1.8±0.7*	3.4±0.6*	3.5±0.02***
Normal control	15.5±2.3	1.2±0.04	4.5±0.5	5.7±0.2	5.0±0.3
Rivastigmine	18.7±3.5*	1.0±0.06*	4.4±0.2*	5.4±0.01***	4.0±0.03***
HyL 200mg/kg	20.0±3.3*	0.8±0.01*	4.0±0.3*	4.8±0.3*	3.8±0.8*
HyL 100mg/kg	25.4±4.4*	0.7±0.02*	3.3±0.5*	4.3±0.2*	3.5±0.5*
HyL 50mg/kg	28.3±4.2*	0.5±0.03*	2.5±0.1*	3.5±0.4*	4.3±0.7*
HyCd 200mg/kg	28.5±2.2*	0.5±0.01*	2.2±0.3*	3.9±0.3*	4.0±1.3*
HyCd 100mg/kg	30.3±2.5*	0.4±0.03*	1.9±0.3*	3.5±0.2*	3.7±1.5*
HyCd 50mg/kg	33.2±2.6*	0.3±0.05*	1.5±0.3*	3.1±0.3*	3.3±3.6*
HyCr 200mg/kg	31.6±2.8*	0.4±0.04*	1.8±0.2*	3.6±0.1**	3.9±2.4*
HyCr 100mg/kg	35.6±3.2*	0.3±0.06*	1.5±0.1*	3.2±0.4*	3.4±2.8*
HyCr 50mg/kg	38.2±3.1*	0.1±0.03*	1.2±0.1**	3.0±0.1**	3.1±2.7*
HyPb 200mg/kg	27.9±3.0*	0.7±0.01*	3.3±05*	4.3±0.1***	4.3±2.1*
HyPb 100mg/kg	29.4±3.6*	0.5±0.02*	3.1±0.4*	4.0±0.2*	4.0±2.1*
HyPb 50mg/kg	32.3±2.1*	0.3±0.02*	2.8±0.2**	3.5±0.3*	3.6±1.3*

Values are means ± SD with *p<0.05, **p<0.01 and ***p<0.001 statistically significance compared to normal group

stress (46). GSH levels are disrupted as observed in neurodegenerative diseases. In the present study, it was observed that there was an increase in the levels of GSH in the control group. However, the administration of lettuce extracts resulted in a reduction of GSH levels, bringing them closer to the levels observed in the normal control group. Generally, SOD and CAT levels are observed to be very low in Alzheimer rats (47) which was also reported in this study. In literature, methanolic plant extracts are reported to inhibit 70 % lipid peroxidation activity. These methanolic extracts also showed strong antioxidant activity equivalent to 74.19 µg/mL gallic acid (48). On the other hand, selenium is an important micronutrient involved in essential pathways as keratin integrity, coenzyme Q and antibody synthesis, along with protection of DNA and oxidative damage (49). It is reported to increase catalase and glutathione levels in $AlCl_3$ treated Alzheimer rats (50). Similarly, in our study, findings indicated that the values tended to align with those of the normal control group, suggesting a restoration of the altered enzyme levels caused by the adverse effects of diabetes.

Determination of neurotransmitters

Alzheimer group displayed a negligible amount of the neurotransmitter, while the Rivastigmine group (serving as the positive control) had considerably high levels as compared to the normal group (Fig. 4). The administration of HyL treatment resulted in a statistically significant ($p<0.001$) elevation in the concentration of serotonin neurotransmitter (0.13 µg/mg of tissue). In the context of dopamine, HyL (0.089 µg/mg of tissue) exhibited a notable and statistically significant elevation in the neurotransmitter levels as compared to the normal group. No neurotransmitters were detected in case of heavy metals induced plant extracts including HyCd and HyCr groups at all concentrations. In case of HyPb at concentration of 200 mg/kg plant extract minute concentration of dopamine (0.024 µg/mg of tissue) and slightly higher concentration of serotonin (0.236 µg/mg of tissue) was detected ($p<0.001$). So, HyL maintained the level of neurotransmitters in rat's brain that were merely detected in diseased and heavy metal stressed induced rats.

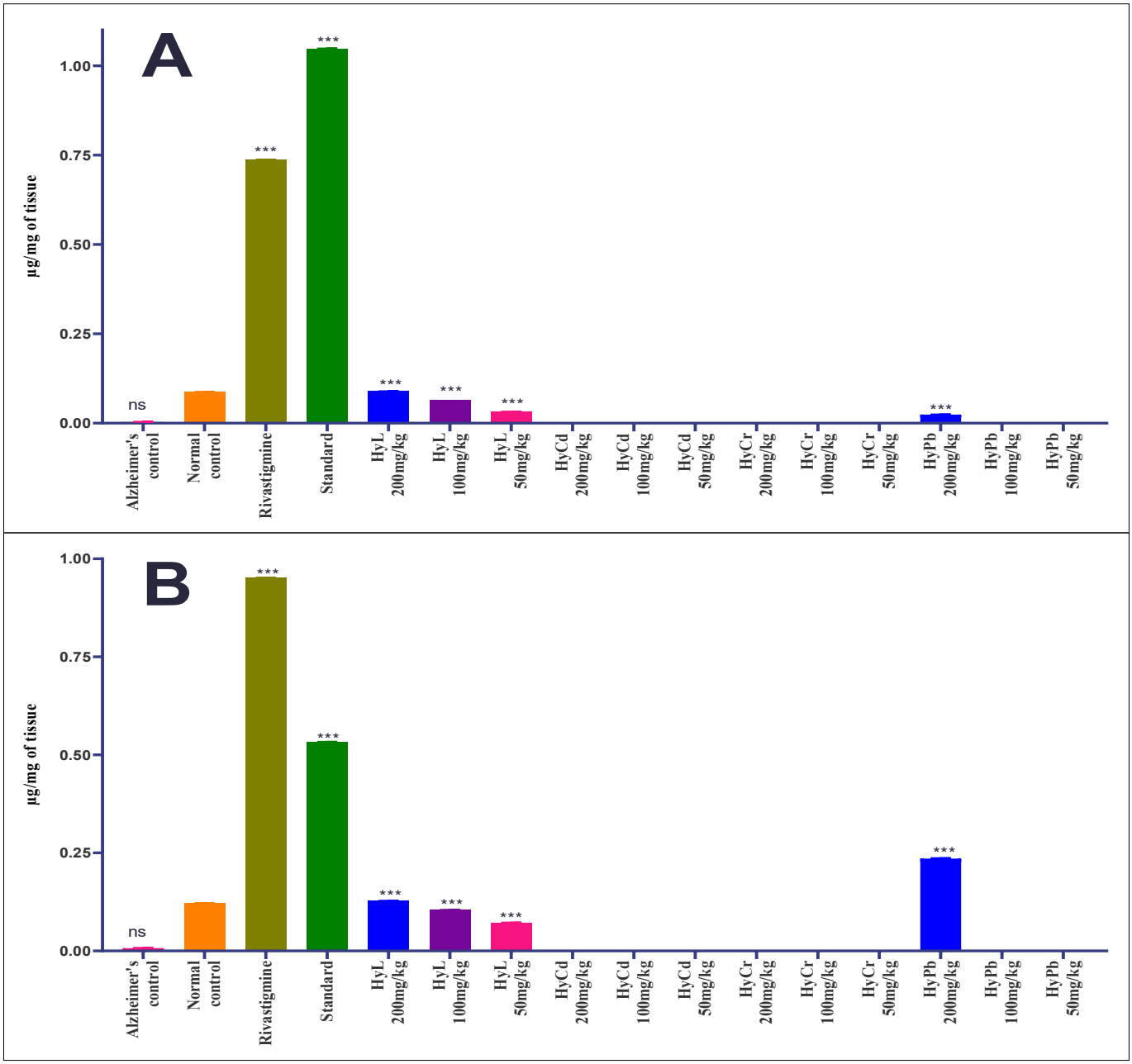

Fig. 4. Effects of lettuce extracts on brain neurotransmitters. (A) Dopamine; (B) Serotonin. ***P<0.001 as compared with normal control and ns represents non significant.

Serotonin and dopamine are catecholamines that are involved in the regulation of brain neural pathways and play a crucial role in cognitive learning and memory processes (51). A reduction in serotonin levels has been observed in individuals diagnosed with Alzheimer's disease, a finding that aligns with cognitive processes (52). According to a study, the levels of serotonin and dopamine in the brains of rats with Alzheimer's disease were found to be 0.886 ± 0.087 and 5.353 ± 1.016 ng/mg, respectively. In comparison, the levels of serotonin and dopamine in the brains of normal control rats were measured to be 1.551 ± 0.262 and 7.225 ± 0.873 ng/mg, respectively (53). In another study, the administration of *Hedera nepalensis* to rats with Alzheimer's disease resulted in an elevation of serotonin and dopamine neurotransmitter levels (54). Likewise, in our study, HyL can restore the levels of neurotransmitters, which are significantly depleted in the disease control group. Additionally, the above results also give us the idea that heavy metals are not good for the human body, especially as they decrease the levels of neurotransmitters, which ultimately affect the proper functioning of the body.

This study has several limitations. First, the study was conducted on rat models and the results may not directly translate to humans. Second, the study used controlled heavy metal exposure, which may not reflect the complex mixtures and varying concentrations found in real-world environments. The implications of our research extend to both food safety and medical applications. From a food safety perspective, our results highlight the need for careful monitoring of heavy metal levels in hydroponic systems to ensure that vegetables produced are safe for human consumption (55). From a medical application perspective, our study provides valuable insights into the potential mechanisms by which heavy metals exert their toxic effects on the body, particularly concerning serum biomarkers, the nervous system and behavior. These findings could inform the development of targeted interventions to protect individuals from heavy metal toxicity and alleviate associated symptoms.

Conclusion

These findings highlight the potential health risks associated with the consumption of heavy metal-contaminated vegetables. This opens the door for future research focusing on the investigation of the mechanisms underlying the observed effects. Overall, this study provides valuable insights into the impact of heavy metal stress on plant quality and the potential consequences for human health, thereby underscoring the need for careful monitoring and mitigation strategies in hydroponic agriculture.

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Authors' contributions

SN was responsible for preparing the initial draft of the manuscript, including the organization and writing of the primary content. HI conceived the overall study design, provided critical supervision and ensured the research was conducted according to the planned methodology. Both SN and HI were equally involved in conducting the experiments, including the hydroponic cultivation of garden lettuce (*Lactuca sativa* L.), applying heavy metal stress and carrying out the health impact studies on rat models. Together, they performed data collection, analysis and interpretation. Both authors participated in reviewing and refining the manuscript and gave their final approval for submission.

Compliance with ethical standards

Conflict of interest: The Authors do not have any conflicts of interest to declare.

Ethical issues: With reference number 321, the Institutional Ethics and Biosafety Committee (IBC) accepted the study and minimal animal safety was guaranteed by National Institutes of Health (NIH) standards.

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