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Impact of Faisalabad industrial and domestic wastewater on stress morphophysiological and biochemical responses of lettuce (*Lactuca sativa* L.)

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ABSTRACT

Irrigation with wastewater imperils human health by introducing toxic metals, harmful pathogens, and organic and inorganic pollutants into crops that potentially contaminate the entire food chain. This pot experiment investigated the impact of Faisalabad wastewater (Madhuana drain, Pharang drain, University of Agriculture) and canal water (control) on lettuce (*Lactuca sativa* L.), focusing on morphophysiological parameters, which were assessed alongside biochemical stress markers such as photosynthetic pigments, enzymatic antioxidants, oxidative and secondary metabolites. Water physiochemical analysis showed that the Madhuana drain wastewater contained the maximum heavy metal concentration, including lead (1.5 mg/L), cadmium (0.13 mg/L), chromium (0.83 mg/L), and zinc (5.46 mg/L), then other irrigation treatments but all wastewater sources beyond the recommended permissible limit. The growth results indicated that Madhuana drain wastewater irrigation significantly altered and decreased plant growth patterns (plant fresh weight (19%) and plant dry biomass (22%), leaves per plant (26%), leaf area (40%), and plant height (45%), and also increased metal accumulation, including Pb (3.75 mg/kg), Zn (12.92 mg/kg), Cd (1.21 mg/kg), and Cr (2.05 mg/kg), while photosynthetic pigments revealed a significant reduction, with chlorophyll content declining by 40%, 31%, and 60% than all other irrigation treatments. Oxidative stress markers including MDA, EL, and H_2O_2 were elevated, indicating cellular damage by 64%, 62%, and 73%. Enzymatic antioxidant assays (peroxidase, superoxide dismutase, and catalase) and secondary metabolites (flavonoids, alkaloids, phenolics, and anthocyanin) elevated by 50% and 40%, respectively, indicating a response against heavy metal stress. These findings underscore that untreated wastewater adversely influences plant metabolic mechanisms that ultimately reduce growth and physiological processes. To protect food security and yield, strict wastewater treatment procedures must be put in place to reduce environmental contamination.

Keywords: wastewater, lettuce, morphology, oxidative stress markers, antioxidants, metal accumulation.

INTRODUCTION

The productivity and cultivation of crops have been impacted by various environmental factors like high wind, temperatures, soil salinity, flood, and drought, but one of the worst ecological stresses is water scarcity, which significantly decreases agricultural productivity. Because the critical demand of crop husbandry is water. Leafy vegetables need plenty of water, but inadequate and insufficient irrigation significantly reduce their different attributes such as natural growth, development, quality, and poor leaf yield. Similarly, qualitative, and time-scale irrigation significantly affects the quality and production of the crop. Furthermore, different sources of irrigation like canals and tubewells are also limited due to geographic limitations resulting from population and civic infrastructure (Naz et al., 2020). Rapid population expansion, rising urbanization, and improved living standards have contributed to increased industrial activity, which has produced massive amounts of industrial and domestic wastewater. However, the readily available wastewater from domestic, sewage, and industrial is a reasonable option and attracts urban and peri-urban farmers to fulfill the irrigation demand for crops, especially vegetables (Pratap et al., 2023).

The sewerage infrastructure in Faisalabad is broadly categorized into two distinct zones- the Western and the Eastern zones. Both zones are subdivided into two other zones. The western zone has a wastewater treatment plant (WWTP) but the Eastern zone does not. So, in the eastern zone, all untreated sewerage water is released directly into the Madhuana drain by dumping stations that result in discharge into river Ravi. However, in the western zone, wastewater of about 20 million gallons per day (MGD) is processed and purified at the Uchkera treatment plant but the remaining untreated sewerage water directly discharges into the Pharang drain and ultimately flows into river Chenab (Noreen and Younes, 2023). So, the farmers used these drains water for irrigation purposes because wastewater is the biggest source of micro and macro-nutrients making it a cost-effective alternative to exogenous plant growth regulators and different fertilizers, but many heavy metals (zinc, mercury, chromium cadmium, lead, iron, etc.) are uptake and translocated into the edible part of vegetables that are deleterious to environment, human beings, and ecosystem (Shahzad et al., 2024). Long-term wastewater contaminates agricultural soils with heavy metals and salts, rendering them unsuitable for microbes and crops (Musa et al., 2011). According to past studies, applying sewage water led to a rise in the deposition of Cr, Pb, and Cd, which decreased the quality of the crops (Iqbal et al., 2016).

In small amounts, some heavy metals such as copper, zinc, and nickel are important for plant growth and development; however, an excess of these metals may be harmful because they generate reactive oxygen species (ROS), which may interact with the cell's vital biomolecules, including DNA and protein. As a result, the plant body has severe morphological, physiological, and metabolic problems, including protein breakdown, lipid peroxidation, and chlorosis of the leaves. Plants use many antioxidant enzymes to limit the production of ROS and safeguard themselves against the harmful effects of environmental stressors. Ascorbate peroxidase, SOD, POX, and CAT are the main antioxidant enzyme systems found in plants (Moreira et al., 2020).

Vegetables hold a key position in supporting human health and nutrition because they contain many nutrient molecules i.e., dietary fibers, minerals, different vitamins (A, E, C), folic acid, zinc, magnesium, phytochemicals, and phosphorus. Both nutritive and non-nutritive molecules contribute to preventing chronic health issues. There are approximately 10000 species of plants that are used as vegetables throughout the world. Leafy vegetables are excellent sources of vitamin C, iron, vitamin A, calcium, and riboflavin (Ulger et al., 2018). Lettuce, a green leafy vegetable, belongs to the Asteraceae family and is globally cultivated. It has organoleptic qualities and significant source of health-boosting metabolites like antioxidants, vitamins, phenolics, macronutrients, micronutrients, carotenoids, etc (Du et al., 2020). Lettuce is a hyperaccumulator of heavy metals, so it is considered an important source of harmful metal dietary absorption (Wu et al., 2022). So, considering the aforementioned factors, the objectives of the current research are, to (1) evaluate the influence of domestic and industrial wastewater on lettuce biomass, photosynthetic pigments, and relative water content, (2) estimate the acceleration of lipid peroxidation, antioxidant defense system and cell membrane permeability under different metal contaminated irrigation, (3) elucidate the accumulation of heavy metals in edible part of lettuce.

METHODOLOGY

Experimental setup

This pot research was performed at the University of Agriculture in Faisalabad (UAF) old botanical garden under natural temperature and light (20–35 °C and a mean photoperiod of 16–18 hours). The wastewater used in this research was collected in 5L polystyrene bottles from the University of Agriculture, Madhuana drain, Pharang drain, and canal water (Faisalabad, Pakistan). The bottles were rinsed and washed properly with tap and distilled water for wastewater collection. Nitic acid (2 ml/L) was used in bottles and stored at 4 °C for just 2 days hours before physiochemical and heavy metal (HMs) analysis. This analysis was performed in the Water and Sanitation Agency Faisalabad (WASA).

The lettuce variety (grand rapids) was collected from the Green Gold Pvt. Ltd. Faisalabad. The manure, compost, and loam (1:1:3 w/w) soil were filled in 5 kg plastic pots. Seeds were treated with 5% NaOH solution for 1 to 5 minutes, then washed 3 to 5 times with distilled water carefully before seed sowing. Seeds were planted at a depth of a half-inch. The pots were arranged in a CRD (completely randomized design) with three repetitions. The pots were irrigated with two types of water: one canal water (control group) and the other group treated with wastewater (UAF sewage water, Madhuana and Pharang drain wastewater) after 2 days intervals approximately 1 to 2 litter. At the maturation stage, samples were taken from treated and nontreated plants. Inductively coupled plasma optical emission spectrometry (ICP-OES) was used to analyze the samples for heavy metal contents. After one month of treatment, the soil samples were collected from each treatment using standard soil analysis protocols to determine their physiochemical and nutritional properties. This analysis was performed in the soil and water testing laboratory, soil chemistry Ayub Agriculture Research Institute, Faisalabad.

Growth attributes

The plants were assessed for important morphological features. Plant height and root-shoot length (cm) were measured by measuring tape. The leaf area (cm^2) was calculated by taking the width (W), length (L), and then a formula was applied with constant specific value (c).

$$Leaf\ area\ (\mathrm{cm}^2) = cLW \quad (1)$$

While number of leaves/plants was counted manually. For dry weight, half plants were stored in paper envelopes and dried in an oven (Memert, Germany) for 5 to 6 days until the weight constant at 70 °C.

Photosynthetic attributes

The estimation of chlorophyll contents was done by Davis (1979) procedure and the carotenoid was estimated by Arnon (1949) method. From each sample, one gram of leave was chopped and homogenized in 10 ml of 80% ethanol and acetone solution. After that, the mixture was incubated for four hours at 4 °C. Following incubation, the sample was filtered and centrifuged for five minutes at 5000 rpm. To determine a baseline absorbance, a control sample, containing the same solution without a leaf, was made and handled in the same way. The control sample was used as a reference for spectrophotometrically measuring the absorbance of the supernatant that resulted from the samples at wavelengths of 645, 663 and 480 nm.

Determination of relative water content (RWC) and electrolyte leakage (EL)

A fresh leaf was saturated with water for 4 hours under illuminated conditions to achieve turgid weight, then dried at 80 °C for 24 hours to calculate dry weight. The given formula was used to calculate the Relative water content (Turner, 1986).

$$RWC\ \% = \frac{Fresh\ weight - Dry\ weight}{Turgid\ weight - Dry\ weight} \times 100 \quad (2)$$

The conductivity meter (professional bench type conductivity (EC), TDS-TEMP Bench Meter, USA) was used to determine the electrolyte leakage (EL) by Lutts et al. (1996). After 2–3 weeks of stress injury, the leaves were collected from each replicate and cut into 1 cm sections. The samples were cleaned with deionized water to eliminate surface-adhered impurities and electrolytes. After that, leaves were stored in stoppered tubes having distilled water (10 ml) and put into a rotary shaker at 100 rpm. After 3 hours, the initial EL was determined. Then, samples were autoclaved at specific conditions (120 °C, 20 minutes); then cooled, and measured the final reading of EL by applying the following formula.

$$EL\ \% = \frac{L1}{L2} \times 100 \quad (3)$$

Determination of oxidative stress attributes

Malondialdehyde (MDA) content was determined by thiobarbituric acid (TBA) test (Heath and Packer, 1968). 0.1 g leaf was ground in 0.5 ml TCA (01%). The homogenate was centrifuged at 15.000 rpm for 10 min. The resulting supernatant (0.5 ml) in 1.5 ml TCA (20%) and TBA (0.5%). The extract was incubated in a 95 °C water bath for 15 minutes, then cooled on ice. The formation of a red-colored complex was measured spectrophotometrically at 532 nm and 600 nm. The optical density taken at 600 nm is used to correct by deducting the non-specific absorbance. The given formula calculated the MDA content.

$$MDA(\mathrm{nmol/mL}) = \frac{(A532 - A600)}{155000} \quad (4)$$

On the other hand, fresh (0.5 g) leaves were crushed in 5 ml TBA (0.1%) solution using a chilled mortar and pestle. The ground material was centrifuged at 12.000 rpm for 15 min. 0.5 ml supernatant, 1 ml KI solution, and phosphate buffer were mixed. The extract was vortexed, and the OD was determined at 390 nm (Velikova et al., 2000). 0.1% TCA was used as blank.

Determination of secondary metabolites

For the estimation of secondary metabolites, leaf tissue (1 g) was extracted in 5 mL of 80% acetone. After the mixture was centrifuged at 15000 rpm for 10 minutes. The supernatant was stored in 500 µl aliquots for phenolics and flavonoid contents. Phenolic was measured by Julkunen Tiitto (1985) method. 1 ml H_2O_2, 0.5 mL Folin-Ciocalteu reagent was poured in 100 µL extract and vigorously shaken. Then the extract was vortexed by adding 2.5 mL of 20% sodium carbonate and left the mixture for 20 minutes before measuring the optical density at 750 nm. Flavonoids were estimated by Zhishen et al. (1999) protocol. In the test tubes, 4 ml of deionized water was added to 1 mL of extracted solution. Kept the solution for 5 minutes then poured 0.5 mL of 5% sodium nitrite, 0.5 mL of 10% aluminum chloride and 2 mL of sodium hydroxide in each tube. The optical density was measured at 510 nm. Using 80% acetone as a blank for flavonoids and phenolics. Alkaloid was measured using the Singh and Sahu (2006) method. 0.1 g fresh leaves were homogenized in methanol (1 ml); then, the distilled water was used for dilution. A 0.01 M and SP1 (sodium meta periodate) were mixed in 1ml extract. The homogenized samples were boiled, and 0.01M MBIT (3-metyle-2-benzothiazol) solution was added. The homogenized samples were put in a water bath for 20 min and then shifted into a microfuge tube. After cooling the samples at room temperature, the optical density was determined at 470 nm. Anthocyanin was measured using the Stark and Wray protocol (1989). Acidified methanol (2.5 ml) was used to grind 0.1 g fresh leaves. After that, the extract was heated at 50 °C for 1 hour; then, samples were filtered. The filtered material was used to measure the absorbance at 535 nm.

Determination of enzymatic antioxidants

For the enzymatic antioxidant assays, leaf tissue (0.25 g) was extracted in 5 mL cold potassium phosphate buffer (pH 7.8) in a pre-chilled pestle and mortar. At 4 °C for 20 minutes, the mixture was centrifuged at 15000 rpm. The supernatant was stored in 500 µl aliquots for enzyme estimation. Superoxide dismutase (SOD) was determined using the methodology of Ries and Giannopolitis (1977). 13 mM methionine, NBT (50 µM), EDTA (75 mM), riboflavin (1.3 µM), and phosphate buffer (pH 7.8) 50 mM was mixed in 50 µL enzyme extract. For 20 minutes, the homogenized samples were illuminated in white fluorescent light. The optical density was determined at 560 nm using a spectrophotometer. Peroxidase (POD) and catalase (CAT) were measured using the procedure of Chance and Maehly (1955). For POD a homogenized mixture contained 20 mM guaiacol and 50 mM potassium phosphate buffer (pH 7.8). Similarly, the CAT mixture contained 5.9 mM hydrogen peroxide, 50 mM potassium phosphate buffer (pH 7.0), and an extract of the enzyme (0.1 µL). The absorbance was taken in 60-second intervals for 3 minutes at 470 and 240 nm respectively.

Determination of heavy metal in plants and soil

Oven-dried and ground samples (plant/soil) 0.5 g were added to nitric acid (5 ml) in a 250 ml digestion flask. The flasks were put on the hot plate for 10 min at 95 °C. After cooling the solution, more conc. nitric acid (2.5 ml) was added and placed on the hot plate again for 30 min at 95 °C. Then 2 ml distilled water and 3 ml H_2O_2 (30%) were mixed. After that, the mixture was heated to the level of transparency. Then the solution was filtered when reached room temperature and made up the volume to 50 ml with deionized water. The filtrate was measured for heavy metal concentration by atomic absorption spectrophotometer.

Statistical analysis

An analysis of the variance of a CRD with three repetitions was calculated by statistical software Statistix 8.1. OriginPro 2025 software was used for box plot, correlation, chord and heatmap. The mean of all pairwise comparisons was used for lettering, and significant differences were found among the treatments with HSD $p \leq 0.05$.

RESULTS

Physiochemical analysis of Faisalabad wastewater

The results of the physical analysis revealed that all wastewater has an alkaline pH ranging from 6.8 to 7.9 but the Madhuana drain wastewater has the highest alkaline pH as compared to others. The electrical conductivity (EC) ranged from 83.8 to 543 dS/m. The highest values (543 dS/m) were observed in wastewater and then canal water (83.8 dS/m, indicating an increased level of salinity. Furthermore, turbidity and hardness were ranged from 0.42–460 NTU and 290–560 ppm, respectively. In contrast, the maximum turbidity was recorded in the Pharang drain (460 NTU) and hardness in the Madhuana drain (560 ppm) as compared to canal water. The total dissolved and suspended solids (TDS and TSS) were significantly higher in wastewater than in control, ranging from 419–2724 mgL^{-1} and 14–1012 mgL^{-1}, respectively. The maximum TDS was noted in the Madhuana drain wastewater, and the minimum TSS was noted in the Pharang drain. The biological and chemical oxygen demand (BOD and COD) ranged from 37–103 mgL^{-1} and 80–192 mgL^{-1}. The maximum BOD and COD were noted in the Madhuana drain wastewater (103 mgL^{-1} and 192 mgL^{-1}, respectively) indicating the maximum decaying of organic pollutants in wastewater. The nutritional analysis of wastewater, such as NPK was recorded in the range of 19–37 mgL^{-1}, 10–30 mgL^{-1}, and 40–60 mgL^{-1}, respectively. The highest NPK value was recorded in UAF wastewater, followed by Pharang and Madhuana drains wastewater. These nutrients come from detergents, human urine, feces, food waste, garbage, etc. The major noxious heavy metals (HMs) including lead (Pb), zinc (Zn), copper (Cu), mercury (Hg), cadmium (Cd), nickel (Ni), chromium (Cr), and iron (Fe) were in wastewater. The Cr (0.8 mgL^{-1}), Pb (1.5 mgL^{-1}), Zn (5.46 mgL^{-1}), and Cd (0.13 mgL^{-1}) were significantly higher in wastewater. However, Ni, Fe, and Hg (0.001 mgL^{-1}) were observed as the least common HM in all sources. The highest value of HMs was observed in the Madhuana drain wastewater, while UAF wastewater has the highest plant nutrients (NPK). It also contained heavy metals, but these were within permissible limits (Table 1)

Physiochemical analysis of soil

The physical analysis of soil has an alkaline pH ranging from 6.8 to 8.1, so it is considered that pH directly affects several nutritive and toxic chemicals. The highest values (pH 8.1) were observed in the Madhuana drain wastewater irrigated

Table 1. Quality parameters of wastewater for all irrigation treatments

Parameters	Unit	Treatments			
		CW	UAFWW	MDWW	PDWW
pH	–	6.8	7.3	7.9	7.5
EC	µS/cm	838	1130	5430	2209
Turbidity	NTU	0.42	59	166	460
Hardness	ppm	290	350	560	390
BOD	mg L^{-1}	37	61	103	68
COD	mg L^{-1}	80	96	192	148
TDS	mg L^{-1}	419	565	2724	1104
TSS	mg L^{-1}	14	110	1002	1012
Cadmium (Cd)	mg L^{-1}	0.02	0.05	0.13	0.11
Zinc (Zn)	mg L^{-1}	0.07	2.34	5.46	4.66
Chromium (Cr)	mg L^{-1}	0.47	0.73	0.83	0.78
Mercury (Hg)	mg L^{-1}	nd	0.002	0.015	0.009
Nickel (Ni)	mg L^{-1}	nd	nd	nd	nd
Iron (Fe)	mg L^{-1}	0.2	1.9	2.3	2.1
Copper (Cu)	mg L^{-1}	nd	nd	nd	nd
Lead (Pb)	mg L^{-1}	0.5	0.9	1.5	1.25
Total nitrogen (N)	mg L^{-1}	7	37	19	28
Total phosphorous (P)	mg L^{-1}	4	30	10	20
Potassium (K)	mg L^{-1}	13	60	40	54

Note: CW – canal water, UAFWW – sewage water, MDWW – Madhuana drain, PDWW – Pharang drain, nd – not detectable.

soil then canal water wastewater irrigated soil (pH 6.8). The electrical conductivity (EC) ranged from 83.8 to 176 dS/m but wastewater irrigated soil has higher EC then canal water irrigated soil. The nutritional analysis of wastewater irrigated soil, such as percent organic matter, total phosphorous (P) and potassium (K), was recorded in the range 0.9–1.37%, 3.49–23.5 mg/kg and 6.66–57 mg/kg, respectively. The highest NPK value was recorded in UAF wastewater irrigated soil, followed by Pharang and Madhuana drains wastewater irrigated soil. The highest organic matter was found in Madhana drain wastewater irrigated soil (1.37%) because these nutrients come from detergents, human urine, feces, food waste, garbage, etc. The major noxious HMs (Cr, Pb, Cd, Ni, Zn, Cu, Fe, and Hg) were present in soil irrigated with wastewater. The highest HM concentration ranges from Zn>Fe>Pb>Cr>Cd>Hg. However, the least common heavy metals, Ni and Cu, are available in ≤ 0.001 mg/L; both are within a safe limit. The maximum HM was observed in soil irrigated with a Madhuana drain wastewater (Table 2).

Growth attributes

Statistically, differences were observed for growth attributes in three wastewater and canal water treatments (Fig. 1). Untreated wastewater adversely affects the morphology of lettuce, and as a result, stunted growth, leaf and root damage, and reduced productivity were observed. The results indicated that both plant fresh and dry weight reduced (19% and 22%) in the Madhuana drain wastewater (MDWW) than canal water (CW). Moreover, the results indicated that the wastewater has a significant influence on plant height. All wastewater treatments (UAF wastewater – UAFWW, Madhuana drain wastewater – MDWW, Pharang drain wastewater – PDWW) caused a significant decrease (25%, 45%, 31%) in plant height compared to CW. Moreover, leaf area was reduced in MDWW (40%) than in PDWW (34%), while a slight decrease (23%) was observed in UAFWW than in canal water. Additionally, number of leaves was reduced (26%) in MDWW compared to canal water. Overall, all growth parameters were reduced in wastewater treatment particularly in Madhuana drain wastewater as compared to canal water.

Photosynthesis attributes

Statistical analysis showed that different wastewater treatments significantly influenced the lettuce photosynthetic pigments as compared to the control group (Fig. 2). Chlorophyll-*a* was significantly increased in control CW (76%) as compared to the treatment groups. MDWW (42%) was drastically reduced than in PDWW (26%) while a slight decrease (15%) was observed in UAFWW. Chlorophyll-*b* was significantly higher in CW (64%) and reduced in MDWW (46%) than in PDWW (40%) while a slight decrease (28%) was observed in UAFWW. Carotenoids were maximum in CW (80%) and lowered drastically in MDWW (60%) than in PDWW (50%) while a slight decrease (40%)

Table 2. Soil physio-chemical analysis after different sources of irrigation

Parameters	Unit	Treatments			
		CW	UAFWW	MDWW	PDWW
pH	–	6.8	7.3	8.1	7.6
EC	dS/m	83	137	176	165
Organic matter	%	0.9	1.26	1.37	1.07
Phosphorous (P)	mg kg^{-1}	3.49	23.5	9.2	9.3
Potassium (K)	mg kg^{-1}	6.66	57	35	54
Lead (Pb)	mg kg^{-1}	0.9	1.23	2.25	1.85
Cadmium (Cd)	mg kg^{-1}	0.1	0.17	1.18	1.12
Chromium (Cr)	mg kg^{-1}	0.89	1.13	1.22	1.18
Copper (Cu)	mg kg^{-1}	≤ 0.001	≤ 0.001	≤ 0.001	≤ 0.001
Zinc (Zn)	mg kg^{-1}	0.11	3.5	7.46	5.76
Nickel (Ni)	mg kg^{-1}	≤ 0.001	≤ 0.001	≤ 0.001	≤ 0.001
Iron (Fe)	mg kg^{-1}	0.2	1.9	2.3	2.1
Mercury (Hg)	mg kg^{-1}	≤ 0.001	0.001	≤ 0.015	0.009

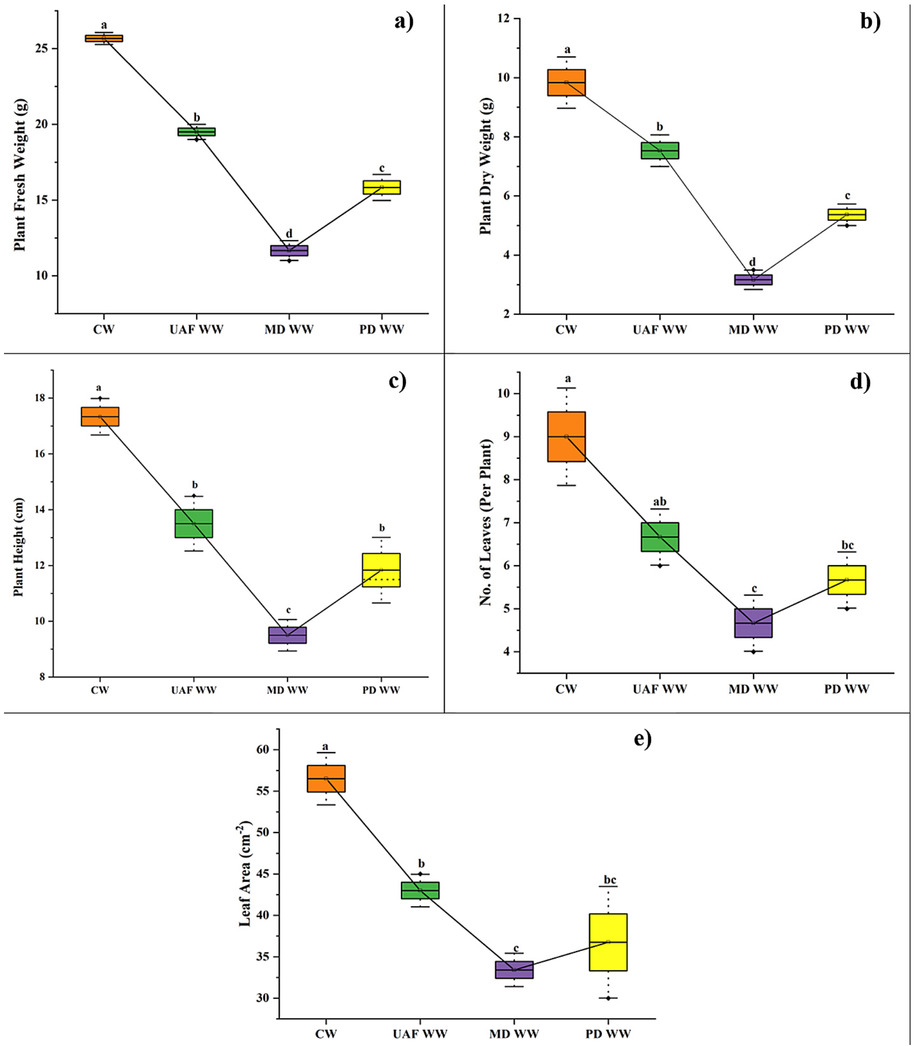

Figure 1. Growth parameters: plant fresh weight (a), plant dry weight (b), plant height (c), number of leaves (d) and leaf area (e) of lettuce, under canal water (CW) and different wastewater (UAFWW: UAF sewage water; MDWW: Madhuana drain; PDWW: Pharang drain) conditions. The standard errors are represented by error bars among replications. A significant difference ($P < 0.05$) is indicated by different lettering among the treatments computed using the HSD test

was observed in UAFWW. Total chlorophyll was increased in CW (87%) and lowered drastically in MDWW (33%) than in PDWW (31%) while a slight decrease (19%) was observed in UAFWW. UAFWW and PDWW groups had a lower reduction of photosynthetic pigments than the MDWW group, but all treatment groups were observed in still lower as compared to canal water.

Stress indicator attributes

Statistical analysis showed that different wastewater treatments significantly influenced the lettuce water content as compared to the control group (Fig. 3). All wastewater treatments caused a significant increase in EL than canal water. EL increased in MDWW (62%) than in PDWW (56%) while a slight increase (27%) was observed in UAFWW as compared to CW. However, all wastewater treatments caused a significant decrease in RWC than canal water. RWC decreased in MDWW (47%) than in PDWW (25%) while a slight decrease (19%) was observed in UAFWW as compared to CW.

Oxidative stress attributes

Statistical analysis showed that different wastewater treatments significantly influenced the lettuce oxidative stress attributes as compared to the control group (Fig. 4). Results of malondialdehyde (MDA) content showed a

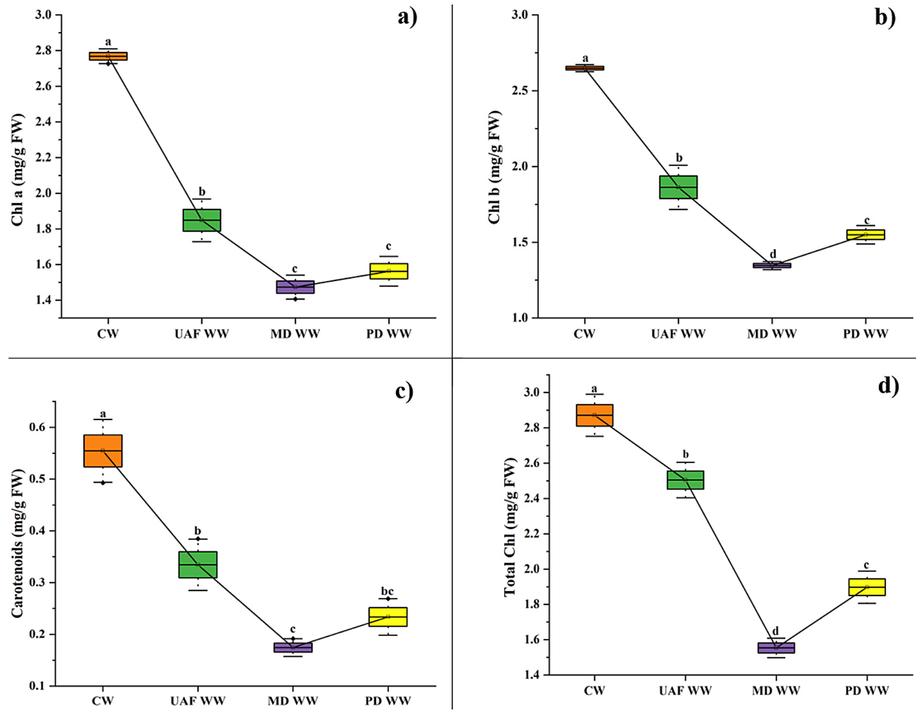

Figure 2. Photosynthetic pigments (chlorophyll a (a), chlorophyll b (b), carotenoids (c), and total chlorophyll (d)) of lettuce under canal water (CW) and different wastewater (UAFWW: UAF sewage water; MDWW: Madhuana drain; PDWW: Pharang drain) conditions. The standard errors are represented by error bars among replications. A significant difference ($P < 0.05$) is indicated by different lettering among the treatments computed using the HSD test

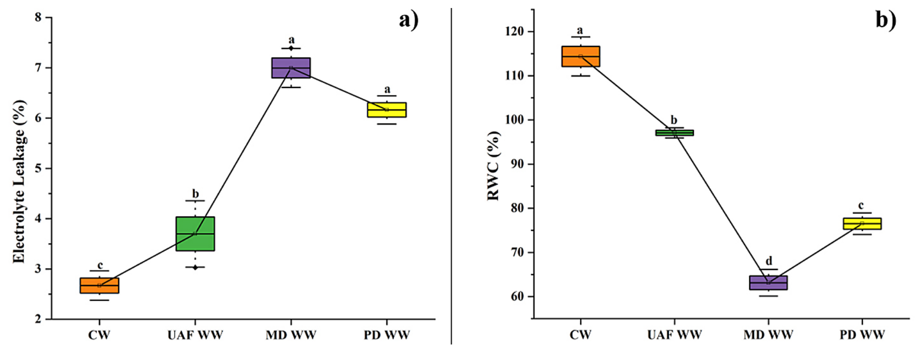

Figure 3. Stress indicator attributes (electrolyte leakage (a) and RWC (b)) of lettuce under canal water (CW) and different wastewater (UAFWW: UAF sewage water; MDWW: Madhuana drain; PDWW: Pharang drain) conditions. The standard errors are represented by error bars among replications. A significant difference ($P < 0.05$) is indicated by different lettering among the treatments computed using the HSD test

significant influence of wastewater on lettuce. All wastewater treatments caused a significant increase in MDA level than canal water. MDA increased significantly in MDWW (64%) than in PDWW (46%), while a slight decrease (29%) was observed in UAFWW as compared to CW. On the other hand, all wastewater treatments significantly enhanced the level of H_2O_2 as compared to canal water. H_2O_2 increased drastically in MDWW (73%) than in PDWW (55%), while a slight decrease (42%) was observed in UAFWW as compared to CW.

Determination of secondary metabolites

Statistical analysis showed that the different wastewater sources increased the concentrations of phenolic, anthocyanins, flavonoids and alkaloids as compared to the control group (Fig. 5). Total alkaloids increased in MDWW (35%) than

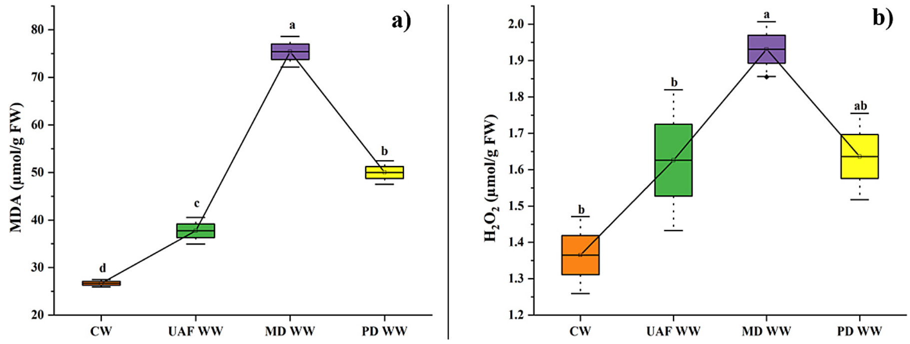

Figure 4. Oxidative stress Indicators (MDA (A) and H2O2 (B)) of Lettuce canal water (CW) and different wastewater (UAF WW: UAF sewage water; MDWW: Madhuana Drain; PDWW: Pharang Drain) conditions. The Standard errors are represented by error bars among replications. A significant difference ($P < 0.05$) is indicated by different lettering among the treatments computed using the HSD test

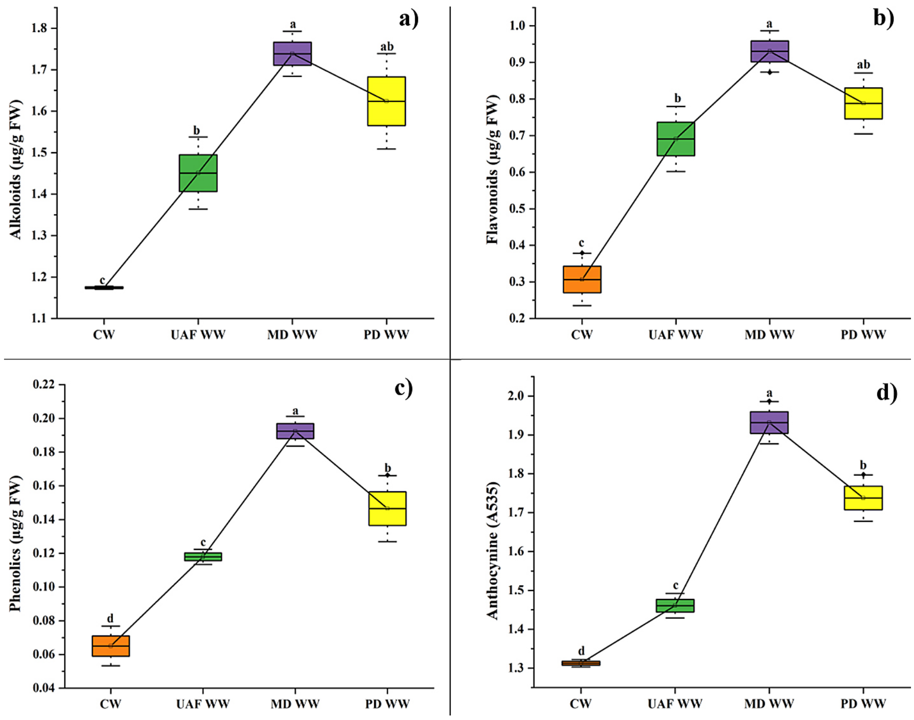

Figure 5. Secondary metabolites (total alkaloids (a), flavonoids (b), phenolics (c), and anthocyanin (d)) of lettuce under canal water (CW) and different wastewater (UAF WW: UAF sewage water; MDWW: Madhuana drain; PDWW: Pharang drain) conditions. The standard errors are represented by error bars among replications. A significant difference ($P < 0.05$) is indicated by different lettering among the treatments computed using the HSD test

in PDWW (26%). At the same time, a slight decrease (13%) was observed in UAFWW as compared to CW. Similarly, phenolic content was elevated in MDWW (68%) than in PDWW (57%). In comparison, a slight decrease (45%) was observed in UAFWW as compared to CW. Flavonoids increased drastically in MDWW (43%) than in PDWW (37%). In comparison, a slight decrease (28%) was observed in UAFW as compared to CW. Anthocyanin increased significantly in MDWW (46%) than in PDWW (30%).In comparison, a slight decrease (22%) was observed in UAFWW as compared to CW.

Determination of enzymatic antioxidant activity

Statistical analysis showed that the different wastewater sources increased the concentrations of SOD, POD and CAT as compared to the control group (Fig. 6). SOD was increased in

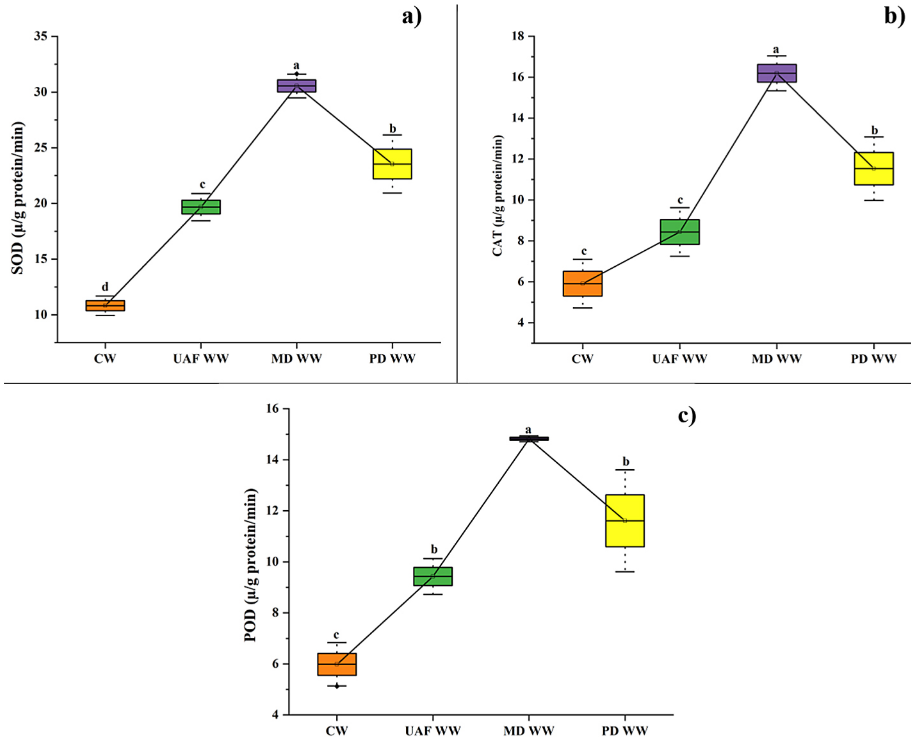

Figure 6. Antioxidant enzyme estimation (SOD (a), CAT(b) and POD (c)) of lettuce under canal water (CW) and different wastewater (UAFWW: UAF sewage water; MDWW: Madhuana drain; PDWW: Pharang drain) conditions. The Standard errors are represented by error bars among replications. A significant difference ($P < 0.05$) is indicated by different lettering among the treatments computed using the HSD test

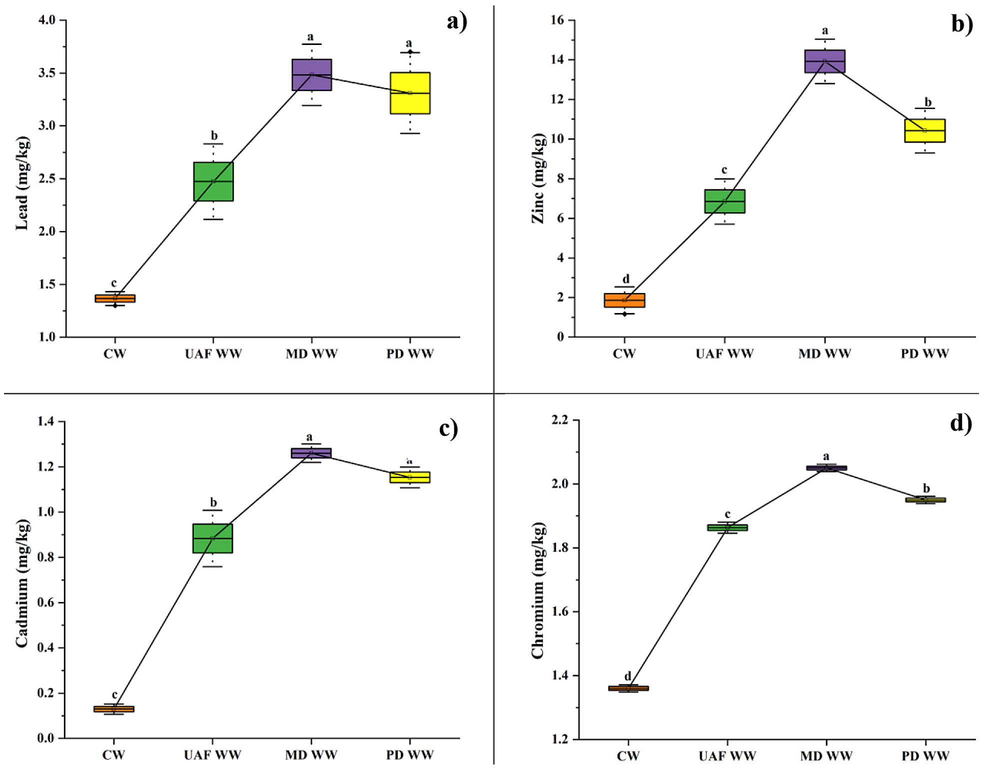

Figure 7. Most common heavy metals (lead (a), zinc (b), cadmium (c) and chromium (d)) in lettuce under canal water (CW) and different wastewater (UAFWW: UAF sewage water; MDWW: Madhuana drain; PDWW: Pharang drain) conditions. The standard errors are represented by error bars among replications. A significant difference ($P < 0.05$) is indicated by different lettering among the treatments computed using the HSD test

MDWW (81%) than in PDWW (76%), while a slight decrease (56%) was observed in UAFWW as compared to CW. CAT was greater in MDWW (36%) than in PDWW (23%), while a slight decrease (14%) was observed in UAFWW as compared to CW. POD was elevated drastically in MDWW (52%) than in PDWW (23%), while a slight decrease (16%) was observed in UAFWW as compared to CW.

Heavy metals analysis

Statistical data exhibited that different concentrations of heavy metal accumulated in lettuce due to different wastewater sources. The maximum Pb concentration (3.75 mg/kg) was recorded in lettuce using Madhuana drain wastewater, followed by 3.13 mg/kg in Pharang drain wastewater, 2.14 mg/kg in UAF wastewater and 1.36 mg/kg in canal water.

Similarly, the maximum Zn concentration accumulated in the edible part (12.92 mg/kg) irrigated with Madhuana drain wastewater, followed by Pharang drain wastewater (10.42 mg/kg), UAF wastewater (5.85 mg/kg) and canal water (1.18 mg/kg). Furthermore, the results revealed that the maximum Cd concentration (1.21 mg/kg) was recorded in Madhuana drain wastewater, followed by Pharang drain wastewater (1.17 mg/kg), UAF wastewater (0.23 mg/kg), and canal water (0.13 mg/kg). Similarly, the maximum Cr concentration (2.05 mg/kg) was recorded in Madhuana drain wastewater, followed by Pharang drain wastewater (1.95 mg/kg), UAF wastewater (1.86 mg/kg), and canal water (1.36 mg/kg) (Fig. 7).

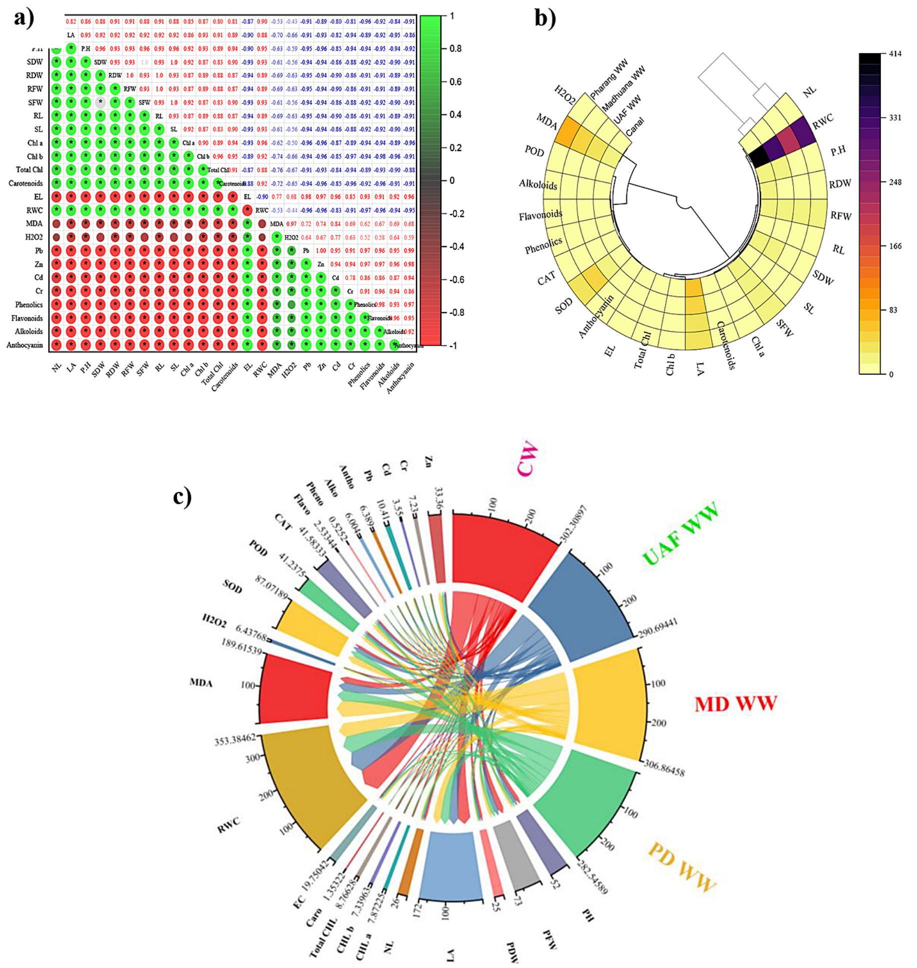

Figure 8. The correlation matrix (a) and polar heatmap with dendrogram (b) and chord diagram (c) depicted the influence of different Faisalabad wastewater (canal water, University of Agriculture wastewater (UAF WW), Madhuana drain wastewater (MDWW), Pharang drain wastewater (PDWW)) on various growth, physiological and oxidative and antioxidant attributes of lettuce

Heatmap, correlation and chord analysis

Matrix correlation of antioxidants, secondary metabolites, heavy metal and oxidative traits with lettuce morphological and photosynthetic parameters was studied for the different wastewater sources (Fig. 8-a). In lettuce, a highly positive correlation was noted between photosynthetic pigments (chlorophyll *a*, *b*, total chlorophyll, and carotenoids) with growth attributes (PFW, PDW, PH, LA and NL). A strong negative correlation was found between heavy metals (Pb, Cd, Cr, and Zn), oxidative stress (H_2O_2, EL, MDA), enzymatic antioxidants (CAT, SOD, POD), and secondary metabolites (anthocyanin, phenolics, alkaloids and flavonoids) with lettuce growth and photosynthetic pigments attributes. The polar heatmap with a dendrogram represented the effect of different water sources on photosynthetic pigments (chlorophyll *a*, *b*, total chlorophyll, and carotenoids), growth attributes (SL, RL, SFW, SDW, RFW, RDW, PH, LA and NL), heavy metals accumulation (Pb, Cd, Cr, and Zn), oxidative stress (H_2O_2, EL, MDA), enzymatic antioxidants (CAT, SOD, POD), and secondary metabolites (anthocyanin, phenolics, alkaloids and flavonoids) (Fig 8-b). Canal water and UAFWW are clustered closely together in the dendrogram, they showed similar patterns in terms of growth, oxidative stress, and antioxidant levels in the heatmap. Similarly, Madhuana drain wastewater and Pharang drain wastewater clustered together, so they showed similar patterns of high oxidative stress, heavy metals, and low growth. Similarly, canal water showed high growth values (SL, RL, SFW, etc.) and photosynthetic with low oxidative stress and antioxidant value. UAFWW, MDWW and PDWW showed low growth and high oxidative stress, heavy metal concentrations and increased production of secondary metabolites and antioxidants, this indicated that canal water promotes better growth conditions while wastewater induced stress. The chord diagram represented the effect of different water sources on photosynthetic pigments (chlorophyll *a*, *b*, total chlorophyll, and carotenoids), growth attributes (SL, RL, SFW, SDW, RFW, RDW, PH, LA and NL), heavy metals accumulation (Pb, Cd, Cr, and Zn), oxidative stress (H_2O_2, EL, MDA), enzymatic antioxidants (CAT, SOD, POD), and secondary metabolites (anthocyanin, phenolics, alkaloids and flavonoids) (Fig 8-c). The canal water represented a control treatment and had fewer or weaker connections to stress-related factors. University of Agriculture wastewater linked to positive physiological responses, such as improved chlorophyll content or antioxidant enzyme activity. Madhuana drain wastewater showed a stronger connection to oxidative stress markers like MDA (malondialdehyde) and H_2O_2 (hydrogen peroxide) and increased all enzymatic antioxidants (CAT, POD, SOD), indicating possible stress-induced damage. While Pharang drain wastewater showed a mixed influence on different biochemical responses.

DISCUSSION

Lettuce is one of the green vegetables that is widely grown around the world due to its organoleptic qualities and is regarded as a significant source of health-boosting metabolites like antioxidants, vitamins, phenolics, macronutrients, micronutrients, carotenoids, low-fat and high-water content (Du et al., 2020). Leafy vegetables need plenty of water, but inadequate and insufficient irrigation significantly reduce their different attributes, such as natural growth, development, quality, and poor leaf yield (Naz et al., 2020). However, large quantities of domestic, sewage, and industrial wastewater attract farmers to fulfill the irrigation demand for vegetables. Due to its cost-effectiveness, abundant supply of plant nutrients, and continuous availability. Apart from these positive qualities, wastewater irrigation has numerous negative consequences on humans, animals, and crops (Shahzad et al., 2023). Contaminants, both organic and inorganic, are widely distributed in wastewater. Heavy metals are particularly concerning as a class of inorganic contaminants due to their toxicity, persistence, and lack of biodegradability (Khan et al., 2008). Nearly 70% of harmful metals are intake in humans by consumption of contaminated food crops (Murtaza et al., 2010). Vegetables that are irrigated with wastewater are the main cause of heavy metal consumption in food crops. These hazardous metals induce many illnesses in humans, including kidney, neurological, and cardiovascular issues (Turkdogan et al., 2003).

Younas et al. (2017) described that in Pakistan, different industries discharge their wastewater without any treatments. As a result, they contain a lot of heavy metals including chromium and lead beyond the safe limits, adversely affecting the crop, aquatic life, and food chain. Similarly, Yamin et al. (2015) studied the quality of Faisalabad wastewater and exposed that no industry follows the standards of NEQs (National Environmental Quality) to discharge their wastes. As untreated wastewater from the Madhuana and Paharang drains is discharged into the Ravi and Chenab rivers, it gradually pollutes the water, making it unsafe for irrigation and human consumption, causing many health problems in the bodies because these rivers flow through Faisalabad. Furthermore, Al-Musharafi et al. (2013) investigated Cd and Pb as highly harmful worldwide for living things. This research focused on observing the influence of different Faisalabad wastewater sources, i.e., UAF wastewater, Madhuana drain wastewater, and Pharang drain wastewater, on lettuce growth, physiology, and antioxidant parameters.

Physiochemical analyses of wastewater such as pH, EC, turbidity, hardness, TSS, TDS, BOD, COD, plant nutrients (NPK) and toxic heavy metals contents vary among industries discharging their wastes (Table 2). Physical and chemical composition and heavy metal contents were higher in Madhuana and Pharang drains wastewater while the plant nutrients NPK were rich in UAF sewage water but also contained heavy metal to some extent. Similar results were recorded by Batool et al. (2024) the maximum concentration of pollutants in wastewater compared to canal water. Chhonkar et al. (2000) measured the industrial wastewater and observed higher TDS, COD, and BOD levels. In addition, soil physiochemical analyses including pH, EC, organic matter, and HM concentration increased in wastewater-irrigated soil as compared to canal water (Table 2). In contrast, the plant nutrients (NPK) increased in UAF sewage water. A similar report from the literature shows that wastewater irrigation changes soil properties including EC, water holding capacity, pH, organic carbon ratio, and bulk density (Ali et al., 2022). In a similar report, organic matter, electrical conductivity, N, Na, and K contents were increased in soil irrigated with wastewater than in canal water (Jahan et al., 2020). Mostly, wastewater increased toxic metals such as Mn, Pb, Ni, Cr, and Zn in soil compared to permissible limits investigated by Fatoba et al. (2012).

Various plant growth attributes including shoot and root length, plant biomass, plant height, no. of leaves, and leaf area significantly reduced in wastewater irrigation as compared to canal water (Fig. 1). According to the results, the maximum reduction in growth parameter was noted in the Madhuana drain wastewater because it has a wide variety of organic and inorganic pollutants, followed by Pharang drain wastewater and UAF wastewater compared to canal water. A similar study was conducted by Rehman et al. (2013), who investigated wastewater significantly decreased vegetable growth by reducing the root length due to root damage and leaf area. Another study revealed that wastewater containing heavy metal reduced the uptake of nutrients, increasing ROS generation and producing oxidative stress to plant organelles; as a result, the overall growth of lettuce and turnip decreased (Hassanein et al., 2013). Heavy metals produce ROS and chelation with essential elements that inactivate the enzyme activity and damage the protein structure, adversely affecting plant growth (Ahmad et al., 2014). Huma et al. (2012) observed that wastewater decreased the growth of crops because of the highest concentration of salt and organic and inorganic pollutant stress.

To further investigate, a series of experiments were performed in our laboratory to calculate photosynthetic pigments. The maximum reduction of photosynthetic pigments or leaf pigments was observed in wastewater than in canal water. It was noticed that Madhuana drain wastewater harmed all photosynthetic pigments (Fig. 2). Our data, as per Alhashimi et al. (2024) studies, photosynthetic pigments are decreased in carrot plants irrigated with untreated wastewater. Similarly, Hajihashemi et al. (2020) presented that untreated wastewater decreased the chlorophyll contents by reducing the carbohydrate content in wheat cultivars.

Furthermore, in another report described by Ahmad et al. (2022), it was stated that untreated wastewater containing a wide variety of heavy metals reduces the fixation of carbon dioxide in okra leaves, and, ultimately, it reduces the photosynthetic pigments. In addition, EL, a measure of membrane stability, increased, and RWC decreased in plants irrigated with different wastewater sources compared to canal water (Fig. 3). Similarly, Khalilzadeh et al. (2020) described that wastewater containing heavy metals reduced the water content and decreased the membrane stability by increasing the electrolyte leakage. In another report, Plaza et al. (2021) investigated that wastewater reduced the water content. Therefore, our physiological parameters studies conclude that the different wastewater sources harmed lettuce photosynthesis efficiency, water content, and plasma membrane stability.

In oxidative stress, lipid peroxidation is considered an indicator of ROS mediated cell membrane damage, which is usually measured by an accumulation of malondialdehyde content, an end product of lipid peroxidation in membranes (Liu et al., 2009). So, wastewater-irrigated plants displayed an increased amount of MDA. H_2O_2 is a ROS as it hinders the C-3 cycle and reduces carbon fixation; ultimately, it is toxic for numerous metabolic functions in cells. Lettuce leaves accumulated maximum levels of H_2O_2 under wastewater stress (Fig. 4). According to Hussain et al. (2021), pharmaceutical wastewater increased MDA and H_2O_2 in carrots.

Interestingly, the plants have adopted many mechanisms for providing stability in their growth one of them is producing secondary metabolites that help in stress. This study measured the most beneficial secondary metabolites, such as alkaloids, total phenolics, anthocyanins, and flavonoids. Our data showed that heavy metal wastewater enhanced the accumulation of all these secondary metabolites (Fig. 5). Similar data showed that the total flavonoid level increased in rice by increasing the effluent level (Gupta, 2010). Furthermore, Michalak (2006) described that the level of phenolics is enhanced under stress conditions because they play a role in metal and scavenging ROS. Similarly, Hussain et al. (2021) reported that the level of alkaloids and anthocyanin increased under pharmaceutical effluent irrigation.

In addition, Plants have a defensive mechanism called antioxidants such as POD, SOD and CAT that scavenge the excess production of ROS in chloroplasts. In this experiment, it is noted that all antioxidants increased at a higher level of metal-containing wastewater (Fig. 6). Our findings correlate with the results of Yasmeen et al. (2009). In another study, Youcef et al. (2020) described that untreated wastewater increased the level of antioxidants in radish to combat ROS.

A higher accumulation of heavy metals (Zn, Cd, Cr and Pb) was reported in lettuce leaves when irrigated with wastewater than in canal water when wastewater was used for irrigation (Fig. 7). Our results, as per John et al. (2012), investigated that the concentration of HM increased from the permissible limit in vegetables irrigated with wastewater. Leafy vegetables indicated the highest accumulation of toxic metals compared to other vegetables (Tomas, 2012).

CONCLUSIONS

In conclusion, this present research demonstrated that wastewater irrigation significantly impacts lettuce morphology, metal accumulation, physiology, oxidative stress markers and antioxidant defense system highlighting potential risks to food safety and human health. The finding revealed that lettuce growth and photosynthetic pigments were reduced by the accumulation of toxic metal and elevated oxidative stress markers, antioxidants and phytochemicals as compared to canal water. The results emphasize the importance of implementing effective wastewater treatment technologies, monitoring metal accumulation, and adopting sustainable agricultural practices to mitigate environmental and health risks.

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