Journal of Experimental Biology and Agricultural Sciences, February - 2024; Volume – 12(1) page 76 – 92

Journal of Experimental Biology and Agricultural Sciences

http://www.jebas.org

ISSN No. 2320 – 8694

Phytotoxicity and genotoxicity assessment of organic and inorganic contaminants detected in pharmaceutical industrial wastewaters using *Vigna radiata* and *Allium cepa*

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Received – September 04, 2023; Revision – December 24, 2023; Accepted – February 25, 2024
Available Online – March 15, 2024

DOI: http://dx.doi.org/10.18006/2024.12(1).76.92

KEYWORDS

Pharmaceutical industrial wastewater

Phytotoxicity

Genotoxicity

Chromosomal aberration

Mitotic index

GC-MS

ABSTRACT

The discharged effluent of pharmaceutical industrial wastewater treatment plants (PIWWTPs) exhibits substantial environmental toxicity due to the intricate combination of organic and inorganic pollutants. This study assessed the phytotoxicity, genotoxicity, and cytotoxicity of untreated and treated pharmaceutical industrial wastewater (PIWW). Most of the physicochemical parameters viz. COD, BOD, EC, sulfide, sulfate, nitrate, phosphate, grease, phenols, and metal concentrations viz. B, Cr, Ca, Cd, Cu, Zn, Pb, Hg, and As in untreated wastewater (UTW) were noted beyond the permissible limit and remained higher in treated wastewater (TW). The findings revealed that the performance of PIWWTP was woefully inadequate. The GC-MS spectra of UTW and TW revealed the presence of various organic contaminants. The toxicological studies showed that the UTW had a high degree of phytotoxicity, which persisted even after the treatment as it inhibited the seed germination in *Vigna radiata*. The seed germination was inhibited up to 70% and 50% tested at 50% concentration of UTW and TW respectively. Genotoxicity was measured by determining mitotic index and chromosomal aberrations in *Allium cepa* root apex grown in untreated and treated PIWW. Compared to the negative control, the mitotic index dropped to 85% and 75% at the 50% concentrations of UTW and TW, respectively. Chromosomal aberrations were also found in the cellular mass of root apex growing in both UTW and TW. According to the findings, it is unsafe for the environment to release PIWW that has not been properly treated, as this could pose serious risks to environmental health.

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Peer review under responsibility of Journal of Experimental Biology and Agricultural Sciences.

Production and Hosting by Horizon Publisher India [HPI] (http://www.horizonpublisherindia.in/).

1 Introduction

Wastewater production due to anthropogenic activities is an inevitable practice. Numerous sources, like households, industries, hospitals, agricultural runoff, etc, can generate such wastewater. Among these sources, the pharmaceutical industry is the most significant because its effluent consists of numerous residual pharmaceutical compounds and metabolites. Over the past few years, the pharmaceutical industries have expanded rapidly, contributing to substantial global economic growth (Gros et al. 2019; Ramirez-Morales et al. 2021). Approximately three thousand compounds are used as pharmaceuticals, with hundreds of tonnes produced yearly to meet global demand (Grenni et al. 2018). India is among the world's leading manufacturers of pharmaceuticals, with over 10,500 pharmaceutical industries (Ray et al. 2019). The pharmaceutical industry's expansion coincided with an increase in wastewater output. Large-cap pharmaceutical industries have effluent treatment plants (ETPs) to treat wastewater, whereas small-sized industries use a common effluent treatment plant (CETP). The CETP in the pharmaceutical industrial area collects wastewater from a cluster of industries that manufacture different pharmaceutical products. As a result, the effluent from this CETP contains a range of water-soluble, non-biodegradable pharmaceutical residues. Among the various types of wastewater, the equity of effluent generated by the PIWWTP is very low, but its composition is quite enough to upset the equilibrium of receiving environmental bodies. The effluent comprises a substantial quantity of recalcitrant organic pollutants discharged from the pharmaceutical industry, mainly drug residues, animal and plant steroids, personal care products and heavy metals (Babaahmadi et al. 2017; Patel et al. 2019).

The introduction of pharmaceutically active chemicals in the environment has sparked worldwide concern. The pharmaceutical industry uses various treatment strategies to treat and repurpose wastewater to reduce environmental damage. The majority of these treatment plants are incapable of properly treating PIWW and are unable to meet the acceptable range of physicochemical characteristics and metal concentrations suggested by the World Health Organization (WHO) and Central Pollution Control Board, India (CPCB) (Rana et al. 2017). As a result, the effluent of the PIWWTP contains an abundance of pollutants. The amalgamation of such wastewater into the environment may have hazardous implications for human and environmental health. The antibiotics and metals present in the effluent can generate a selective pressure for antibiotic and metal resistance (Soni et al. 2022). Additionally, it could promote the growth of specific kinds of biotic communities and adversely affect the biodiversity index.

The pharmaceutical metabolites found in the PIWW can disrupt biogeochemical cycles and cause a variety of toxicities, including phytotoxicity, genotoxicity, cytotoxicity, and mutagenicity (Pashaei et al. 2022; Samal et al. 2022). Kumari and Tripathi (2019) investigated the genotoxic effects of PIWW using the growing root apex of *A. cepa*. The mitotic index (MI) and chromosomal aberrations (CAs) were used to analyze the toxicity of PIWW, which demonstrated that it can cause a drop in the MI and can induce various CAs such as chromosome loss, c-mitosis, fragile chromosomes, and micro-nucleated conditions (Kumari and Tripathi 2019). Sharif et al. (2016) used a bacterial reverse mutation test and an Ames test to determine the mutagenic and genotoxic impact of PIWW. The mutagenic index of both examined *Salmonella typhimurium* strains (TA-100 and TA-102) decreased dose-dependent. The Ames test of wastewater revealed a drop-in frequency of chromosomal damage as wastewater concentration decreased. These findings suggested that PIWW, an intricated mixture of various organic-metallic contaminants, may have potent mutagenic and genotoxic implications on the exposed biotic community. Therefore, immediate action is needed to halt the spread of this global menace. Government regulatory bodies must act quickly to prevent the spread of PIWW without proper treatment. There is also a need to develop novel treatment strategies for adequately treating pharmaceutical metabolites.

Previous studies have looked into the occurrences of phytotoxicity, genotoxicity, and cytotoxicity caused by effluent from various treatment plants (Roy et al. 2015; Yadav et al. 2019; Haq and Kalamdhad 2021). To the best of our knowledge, limited studies have looked into the toxicity of the PIWW treatment plant's influent and effluent, and the topic is data-deficient. No study has evaluated the performance of PIWWTP based on PIWW toxicity. The novelty of this study was the performance evaluation of the PIWWTP by comparing data of physicochemical parameters, metal concentrations, and GC-MS analysis of influent and effluent, as well as assessment of its phytotoxic, genotoxic, and cytotoxic consequences.

2 Materials and methods

2.1 Sample collection

The wastewater samples were collected from a CETP located at the Integrated Industrial Estate (IIE), State Infrastructure Development Corporation Uttarakhand Limited (SIDCUL), Haridwar, Uttarakhand, India (29.9671° N, 78.0596° E). This CETP collected the PIWW from a cluster of industries. CETP has a treatment capacity of 4.5 MLD and uses a fully biological process, particularly a moving bed biofilm reactor (MBBR) (Ali et al. 2021). The untreated (UTW) and treated (TW) wastewater samples of PIWWTP were collected at the end of March 2023. For the assessment of physicochemical parameters, GC-MS, FTIR, genotoxicity and phytoxicity the samples of wastewater were collected in a sterilized 2L glass container. For metal analysis, the samples were collected in a 500 ml food-grade plastic container preadded with 4-5 ml

concentrated HCl to avoid saltation of metals. Samples were brought to the lab in the icebox, stored at 4°C, and then filtered with Whatman filter paper for further analysis.

2.2 Analysis of physicochemical parameters and heavy metal

The methodologies applied to assess the physicochemical characteristics and metal concentration were based on the APHA 23rd edition, 2017 (Rice 2012). The physicochemical characteristics of UTW and TW studied in this study were pH, temperature, TDS, TSS, COD, BOD, EC, sulfide, sulfate, nitrate, phosphate, chloride, oil, grease, and phenols. The metal concentration was evaluated using an Inductively Coupled Plasma Mass Spectrometry (ICP-MS) instrument (ThermoFisher Scientific- ICAP Qnova Series). In this investigation, the concentrations of eleven metals were determined, viz., B, Cr, Ca, Cd, Cu, Ni, Zn, Fe, Pb, Hg, and As.

2.3 Estimation of organic contaminant

2.3.1 Preparation of samples and extraction of organic pollutant

Organic contaminants were extracted using ethyl acetate at pH 2.0 from untreated wastewater (UTW) and treated wastewater (TW) samples of effluent treatment plant (ETP) (Singh et al. 2023). A 500 ml separating funnel was filled with the 50 ml acidified sample. An equal quantity of ethyl acetate was added to the sample and agitated thoroughly for four to five hours. After proper shaking, the ethyl acetate extract was isolated and dried at 40°C using a rotatory evaporator (Rotavapor® R-300, BUCHI). The dried extracts of each sample were separately dissolved in 5 ml of ethyl acetate and passed through a syringe filter (0.22 µm; Sigma-Aldrich, USA). For further FTIR spectroscopy and GC-MS analysis, the filtrate of both samples was kept at 4°C.

2.3.2 FTIR spectroscopy analyses

The extract of wastewater samples was initially utilized to determine the functional groups of organic contaminants using Fourier Transform Infrared (FTIR) spectroscopy (Nicolet 6700, Thermo Scientific, USA). The method of FTIR analysis was based on a previous study by Kumar and Chandra (2020). Using an infrared spectrophotometer, infrared spectra of samples were obtained in the scanning range of 4000 to 500 cm^{-1} (wave number) at 25ºC and a resolution of 0.15 cm^{-1}.

2.3.3 Derivatization of extracted samples and GC-MS analysis

For silylation (Derivatization), 250 µl of extracted samples were put in GC vials and evaporated in the presence of N_2 gas. After adding 40 µl of C_5H_5N (pyridine) to the dried samples, silylation was conducted at 70°C for half an hour in the presence of N, O-trimethylchlorosilane (TMCS) and N, O-bis (trimethylsilyl) trifluoroacetamide (BSTFA). Derivatization of organic compounds was performed in full scan mode with low mass (m/z): 50 amu to high mass (m/z): 800 amu.

The 1.5 µl silylated solution was introduced into a GC-MS column and segregated in a capillary tube (Thermo Fisher Scientific GC Ultra XLS Mass Spectrometer, America). The analytical GC capillary tube was filled with an inert carrier gas (Helium gas) at 300°C temperature and a flow rate of 1 $ml\text{-}min^{-1}$. The mass spectrum of electron ionization (EI) was recorded at 40 to 70 eV, covering a range of 50 to 800 m/z ratio. The resulting GC-MS spectra contain peaks representing distinct chemical compounds at certain retention times (RTs). Such compounds were identified by accessing the National Institute of Standards and Technology (NIST) library, USA (Babushok et al. 2007; Koo et al. 2016).

2.4 Toxicological implications of UTW and TW for environmental safety estimation

The toxicological impacts of untreated and treated PIWW were estimated using mung bean (*V. radiata*) seeds and onion bulbs (*A. cepa*). *V. radiata* was used to test the phytotoxicity, while *A. cepa* was used to test the cytotoxicity and genotoxicity.

2.4.1 Phytotoxicity assessment using mung bean *(V. radiata)* seeds

The method of seed germination experiments on mung bean (*V. radiata*) was based on a previous study by Salian et al. (2018). Mung bean seeds were obtained from a verified seed-selling outlet and superficially sterilized with 0.3% ($^{w}/_{v}$) $HgCl_2$ solution. By adding the required amount of distilled water, five test solutions of untreated and treated PIWW were prepared with concentrations ($^{v}/_{v}$) of (15%, 25%, 50%, 75%, and 100%). Petri plates (90 mm × 15 mm) containing 10 seeds were watered with 8 ml of test solutions, while tap water-irrigated seeds served as negative controls. The petri plates were incubated in a BOD incubator (Bionics Scientific, India) at 30 ±1°C. After 48 hours, the number of germinated seeds was noted and displayed as a percentage (%) of germination. After 5 days of treatment, seedling development variables (root and shoot length) were evaluated (Salian et al. 2018; Kumari and Tripathi 2019). The experiments were carried out in triplicate.

2.4.2 Genotoxicity estimation

Chromosomal aberrations in the root apex cells of *A. cepa* were used to determine the genotoxicity of both UTW and TW. Three test solutions ($^{v}/_{v}$) of 15%, 25%, and 50% of UTW and TW were used in this experiment. Above this concentration, there was almost no visible development in the root apex. To complete the cell cycle, the dividing cells of the root apex of onion bulbs were first rooted in tap water for 48 hours or until the length of the roots reached 1-2 cm. After that, the bulbs were transferred to test solutions for 24 hours. To determine the genotoxicity, the root apex was fixed in a CH_3COOH (glacial acetic acid) and alcohol

(1:3) for 12 hours at 65°C. The root apex was cleaned with distilled water and then hydrolyzed for five minutes at 65°C using 1N HCl. Following appropriate washing, hematoxylin was used as the stain during the processing of the root apex for slide preparation (Haq et al. 2017). About 4000 cells (400–1200 cells per slide) were scored to determine the MI. The CAs and MI were analyzed by using a phase contrast microscope (Nikon Eclipse Ci, Japan) and calculated by using the given formula:

$$\text{Mitotic Index (MI)}(\%) = \frac{\text{Number of dividing cells}}{\text{Number of total cells}} \times 100$$

$$\text{Chromosomal Aberrations (CA)}(\%) = \frac{\text{Number of aberrant cells}}{\text{Number of total cell}} \times 100$$

2.5 Statistical analysis

The standard deviation of triplicate data of physicochemical parameters, metal concentrations, phytotoxicity, and genotoxicity was determined on Microsoft Excel 2019.

3 Results

3.1 Physicochemical parameters and metal concentration

The value of all the physicochemical parameters, the concentration of metals, the permissible limit of CPCB (2020) and the treatment efficiencies of PIWWTP are listed in Table 1. The pH and temperature of the UTW and TW were within an acceptable range of CPCB (2020). The values of TDS and TSS in UTW were

Table 1 Physicochemical characteristics and metal content in the untreated and treated wastewater of WWTP of pharmaceutical industrial area

Physicochemical Analysis	UTW	TW	Permissible limits (CPCB 2020)	Treatment Efficiency (%)
pH	6.68 ±0.28	7.29 ±0.26	6.0-8.5	-
Temperature (ºC)	33.08 ±0.04	30.45 ±0.06	34	-
TDS (mg/L)	2822.56 ±26.22	1543.34 ±18.16	2100	45.32
TSS (mg/L)	2080.06 ±18.28	483.48 ±7.42	1500	76.75
COD (mg/L)	3819.86 ±15.70	3196.62 ±18.16	100	16.31
BOD (mg/L)	1190.72 ±8.18	664.67 ±7.12	250	44.17
EC (mS/L)	380.0 ±4.42	183 ±4.22	ND	51.84
Sulfide(mg/L)	124.60 ±2.80	28.06 ±0.92	2	77.47
Sulfate(mg/L)	400.81 ±3.62	100.82 ±1.58	NS	74.84
Nitrate (mg/L)	44.07 ±1.86	30.04 ±0.68	NS	31.83
Phosphate (mg/L)	600.65 ±9.46	240.54 ±4.95	5	59.95
Chloride (mg/L)	280.73 ±4.12	210.04 ±3.45	15	25.18
Oil and grease (mg/L)	709.66 ±11.46	412.60 ±8.78	10	41.85
Phenols (mg/L)	10.03 ±0.64	5.02 ±0.02	1.0	49.95
Metals Analyses (mg/L)	**UTW**	**TW**	**Permissible limits (CPCB, 2020)**	**Treatment Efficiency (%)**
B	14.56 ±1.45	9.19 ±0.62	2.0	36.88
Cr	5.09 ±0.23	3.23 ±0.01	2	36.54
Ca	120.07 ±0.02	105.03 ±0.07	ND	12.52
Cd	9.09 ±0.01	4.05 ±0.001	1.0	55.44
Cu	7.83 ±0.78	3.85 ±0.10	3	50.83
Ni	3.73 ±0.20	2.27 ±0.05	3.0	39.14
Zn	8.98 ±17.12	5.76 ±0.91	5	35.85
Fe	15.57 ±1.02	12.82 ±1.04	15	17.66
Pb	1.44 ±0.08	0.9 ±0.009	0.1	37.50
Hg	0.43 ±0.07	0.04 ±0.02	0.01	90.69
As	7.49 ±0.16	2.47 ±0.04	0.2	67.02

UTW- Untreated wastewater; TW- Treated wastewater; CPCB- Central Pollution Control Board; COD- Chemical oxygen demand; BOD- Biological oxygen demand; TSS- Total suspended solids; TDS- Total dissolved solids; EC- Electrical conductivity; ND- Not determined; ± - Standard deviation

2822.56 ±26.22 mg/L and 2080.06 ±18.28 mg/L, respectively, which was substantially higher than the permissible limit of CPCB (2020). Although both parameters were remediated after treatment (TW), the values of TDS and TSS fell below the threshold. The values of COD, BOD, EC, sulfide, sulfate, nitrate, phosphate, chloride, oil, grease and phenols remained higher than the CPCB (2020) permitted limit in the UTW and TW (Table 1). The PIWWTP's best treatment efficacy (77.47%) was observed for sulfide, whilst the lowest efficiency (16.31%) was noted for COD.

The UTW and TW samples included eleven metals and heavy metals viz. B, Cr, Ca, Cd, Cu, Ni, Zn, Fe, Pb, Hg, and As. All metals and heavy metals in the UTW sample were considerably higher than the CPCB (2020) acceptable range (Table 1). Following treatment, the levels of Ni and Fe dropped below the CPCB (2020) permitted limit, with respective treatment efficiencies of 17.66% and 39.14%. The highest and lowest treatment efficiency was shown for Hg (90.69%) and Ca (12.52%), respectively.

3.2 FTIR spectroscopy

The FTIR spectra of the UTW and TW samples revealed several peaks corresponding to various wave numbers. These peaks indicated the presence of multiple contaminants with distinct functional groups. The UTW and TW have infrared wave numbers ranging from 4000 to 500 cm^{-1}. Figure 1 shows the infrared spectra of UTW and TW, which are interpreted in Table 2. The FTIR analysis was employed to compare the change in bond pattern and functional groups in the influent and effluent during the wastewater treatment process for the supporting data of compound characterization (Yang et al. 2015).

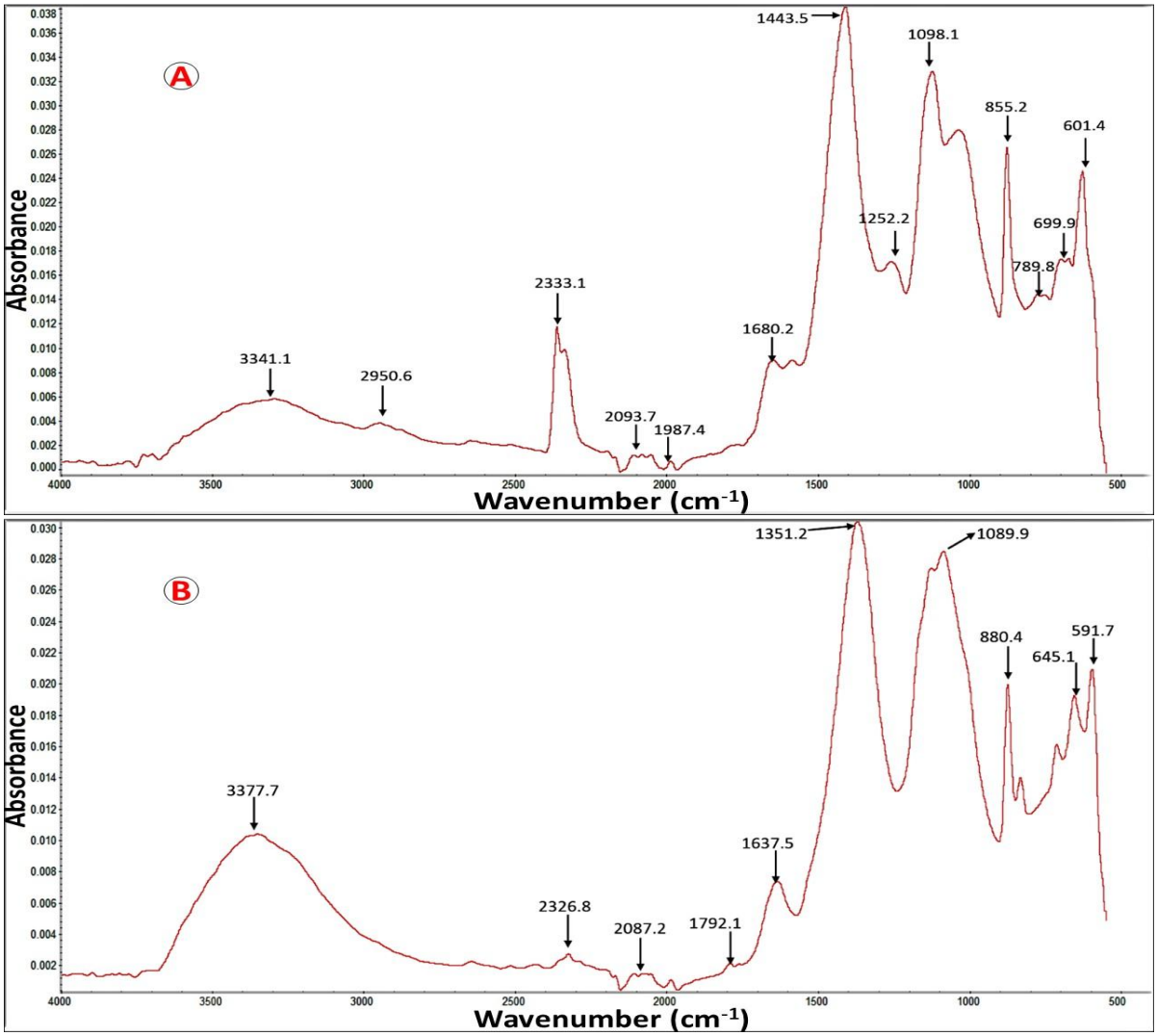


Figure 1 Fourier transform infrared (FTIR) spectroscopy analysis of ethyl acetate extract obtained from wastewater samples, A= UTW (Untreated wastewater); B= TW (Treated wastewater)

Table 2 Fourier-transform infrared (FTIR) spectroscopy analyses of ethyl acetate extract of untreated and treated wastewater samples

S. No.	Wavenumber (cm^{-1})	Functional group	UTW	TW
1.	3341.1	OH carbohydrates proteins and polyphenols	+	-
2.	3377.7	OH carbohydrates proteins and polyphenols	-	+
3.	2950.6	CH and CH_2	+	-
4.	2333.1	C=C conjugated and C≡C	+	-
5.	2326.8	C=C conjugated and C≡C	-	+
6.	2093.7	CH and CH_2	+	-
7.	2087.2	CH and CH_2	-	+
8.	1987.4	CH and CH_2	+	-
9.	1792.8	C=O ester fatty acid group	-	+
10.	1680.2	C=O group of quinone compounds	+	-
11.	1443.5	Stretching –C=O inorganic carbonate	+	-
12.	1351.2	C–N amide III band	-	+
13.	1252.2	C–N amide III band	+	-
14.	1098.9	C–O carbohydrate	+	-
15.	880.4	Bending –C=O inorganic carbonate	-	+
16.	855.2	Bending –C=O inorganic carbonate	+	-
17.	789.8	CH out of plane aromatic band	+	-
18.	699.9	CH out of plane aromatic band	+	-
19.	645.1	CH out of plane aromatic band	-	+
20.	601.4	CH out of plane aromatic band	+	-
21.	591.7	CH out of plane aromatic band	-	+

UTW: Untreated wastewater; TW: Treated wastewater; + : Present; - : Absent

3.3 GC-MS data analysis

The GC-MS chromatogram of ethyl acetate extract of UTW and TW samples displayed many major and minor peaks at different RTs (Figure 2; Table 3). The major peaks in the UTW extract were recorded at RT 7.49, 10.37, 12.35, 13.23, 16.04, 19.49, 22.03, 26.30, 27.95, 28.55, 37.31, and 37.56 minutes. These peaks corresponded to 2-Methyl-1,3-propanediol 2TMS; Silane, trimethyl (phenylmethoxy); 1-tert-Butyl-2,5-dimethoxybenzene; Ethyl isopropylaminooximinoacetate; Pentanedioic acid, bis(trimethylsilyl) ester, 4-Bromo-5-methyl-2-phenylthiazole; 2-(5-acetyl-2-thienyl)-1,4-naphthoquinone; 5-Bromo-2-hydroxy-N'-(3-pyridinylmethylene) benzohydrazideditms; 6-methoxy-7-methy-2,3-diphenyl-5,8-quinoxalinedione; Garciniaxanthone F; n-Hexadecyloxy(triethyl)silane; (2R,3R)-2,3-Diacetoxy-a,a-carotene; and 2-methyl-3-acetyl-1a,3a,5,6-tetra(trimethylsiloxy)-benzo-furan-4,7-dione, respectively.

The primary peaks in TW were found at RTs of 8.05, 14.04, 16.04, 24.62, 26.48, 27.60, 30.47, 33.64, 39.80 and 42.80 minutes. These peaks were interpreted as 2-Trimethylsilylmethylcyclopentanone; Disiloxane, hexamethyl; Pentanedioic acid, bis(trimethylsilyl) ester, 4-Bromo-5-methyl-2-phenylthiazole; 2-Bromoethyl 3-bromotetrahydrofuron-2yl ether; 1,2-Benzenedicarboxylic acid, dibutyl ester; Hexadecanoic acid, trimethylsilyl ester; octadecanoic acid, trimethylsilyl ester; 1,2-Benzenedicarboxylic acid, dioctyl ester; Silane, [[(3a,5a)-cholestan-3-yl]oxy]triethyl; and 3,5-di-tert-butylstilbenyl phenyl ketone, respectively. Peaks at RT 16.04 minutes were detected in the chromatograms of both samples.

3.4 Phytotoxicity of pharmaceutical industrial wastewater

Table 4 shows the impact of UTW and TW on the early phase of seedling growth (5-seedling) after five days of incubation. On increasing the wastewater concentrations, seed germination efficiency (%), shoot growth, root growth, and biomass of germinating seed all reduced (Table 4; Figure 3). Maximum seed germination rate (%), shoot and root length growth, and increase in biomass were observed when the negative control (tap water) was used.

Table 3 Organic compounds detected by GC-MS analysis of untreated and treated wastewater samples collected from WWTP of pharmaceutical industrial area

RT	Compound Name	UTW	TW	Toxicity
6.43	1,2-Bis(trimethylsiloxy)ethane	-	+	Dermal and ophthalmic irritation
7.49	2-Methyl-1,3-propanediol 2TMS	+	-	Diarrhea, yellow nasal discharge
8.05	2-Trimethylsilylmethylcyclopentanone	-	+	Neurotoxicity
8.26	Docosane	+	-	ND
10.37	Silane, trimethyl(phenylmethoxy)	+	-	Dermal and ophthalmic irritation
11.05	Ethanol, 2-(2-ethoxyethoxy)	-	+	Neurotoxin - Acute solvent syndrome
12.35	1-tert-Butyl-2,5-dimethoxybenzene	+	-	Acute toxicity (Oral), Carcinogenicity
13.23	Ethyl isopropylaminooximinoacetate	+	-	Gastrointestinal irritation, rashes, and photosensitization
14.04	Disiloxane, hexamethyl	-	+	Low acute inhalation toxicity
14.43	bis(trimethylsilyl) methylmalonate	-	+	Respiratory tract and eye irritation
15.02	Sym-tetramethyl(diisopropyl)disiloxane	+	-	ND
16.04	Pentanedioic acid, bis(trimethylsilyl) ester, 4-Bromo-5-methyl-2-phenylthiazole	+	+	Endocrine disrupting chemical
16.93	(4-(Ethynyl-2,3 dinitrophenylethynyl)trimethylsilane	+	-	Dermal and ophthalmic irritation
18.32	1-(Trimethylsilylmethyl)dimethylsilyloxypentane	+	-	ND
19.49	2-(5-acetyl-2-thienyl)-1,4-naphthoquinone	+	-	Cellular toxicity
20.38	4-Acetyl-2-methoxy-trimethylsiloxy-benzene	+	-	ND
22.03	5-Bromo-2-hydroxy-N'-(3-pyridinylmethylene) benzohydrazideditms	+	-	Dermal and ophthalmic irritation
24.22	1-Pentamethyldisilyloxy-2-phenylethane	+	-	Affects the gastrointestinal and respiratory tract
24.62	2-Bromoethyl 3-bromotetrahydrofuron-2yl ether	-	+	ND

RT	Compound Name	UTW	TW	Toxicity
26.30	6-methoxy-7-methyl-2,3-diphenyl-5,8-quinoxalinedione	+	-	Chronic health effects- acute bronchospasm, hives (urticaria), deep dermal wheals (angioneurotic edema), running nose (rhinitis) and blurred vision
26.48	1,2-Benzenedicarboxylic acid, dibutyl ester	-	+	Skin and eye irritation
27.60	Hexadecanoic acid, trimethylsilyl ester	-	+	Endocrine disrupting chemical
27.95	Garciniaxanthone F	+	-	Neurotoxic and phenylketonuria
28.55	n-Hexadecyloxy(triethyl)silane	+	-	ND
30.47	Octadecanoic acid, trimethylsilyl ester	-	+	Endocrine-disrupting chemical, reproductive toxicity
31.20	Octadecanoic acid, trimethylsilyl ester	+	-	Endocrine-disrupting chemical, reproductive toxicity
32.16	1,1-Diisobutoxy-isobutane	-	+	Carcinogenic
32.36	Octadecanoic acid, butyl ester	+	-	Inflammation and skin irritation
33.64	1,2-Benzenedicarboxylic acid, dioctyl ester	-	+	Gastrointestinal irritation, rashes, and photosensitization
33.80	n-Hexadecyloxy(triethyl)silane	+	-	Skin and eye irritation
34.33	3-(4-nitrophenyl)quinazolin-4(3h)-one	+		Ulcerative colitis
34.52	Dirithromycin	-	+	Overdose causes nausea, vomiting, epigastric distress, and diarrhea.
37.31	(2R,3R)-2,3-Diacetoxy-a,a-carotene	+		Chronic vitamin A toxicity
37.56	2-methyl-3-acetyl-1a,3a,5,6-tetra(trimethylsiloxy)-benzo-furan-4,7-dione	+	-	ND
38.12	(4S)-2,2,4-tribenzyl-5,5-dimethyl-3-[(5R)-3-phenyl-2-isoxazoline-5-carbonyl]0xazolidine	-	+	LD50-Oral-Rat, LC50-Inhalation-Rat and carcinogen
39.80	Silane, [[(3a,5a)-cholestan-3-yl]oxy]triethyl	-	+	ND
41.23	4-ethly-1,2,3,9,10-pentamethoxybenzo[a]heptalene	-	+	Eye and skin irritation
42.80	3,5-di-tert-butylstilbenyl phenyl Ketone		+	Burning sensation in the throat and chest. Abdominal pain
45.32	Dibutyl 2-bromovinylphosphonate	-	+	Irritation

RT- Retention time; UTW- Untreated wastewater; TW- Treated wastewater; + Present; - Absent; ND- Not determined

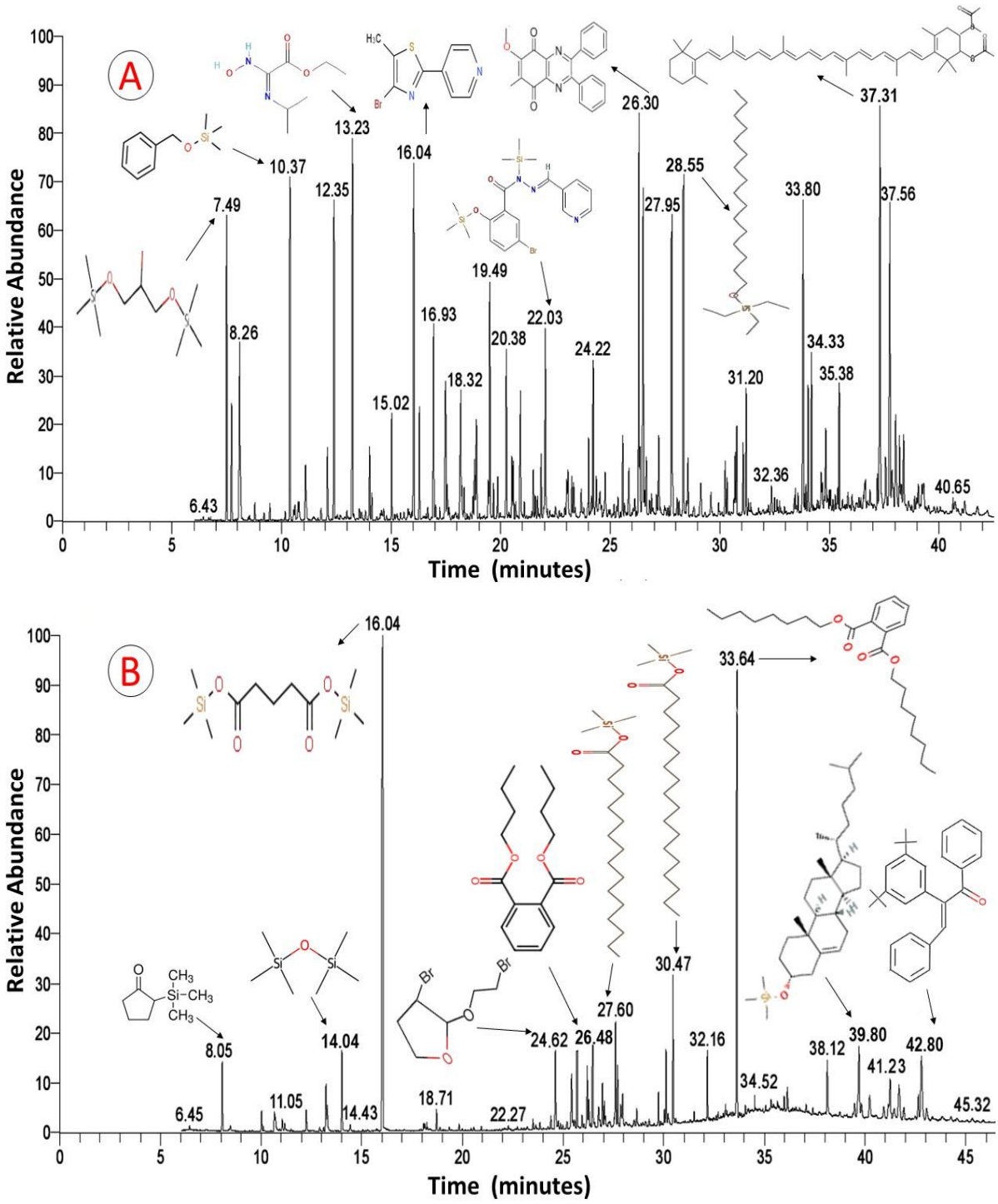


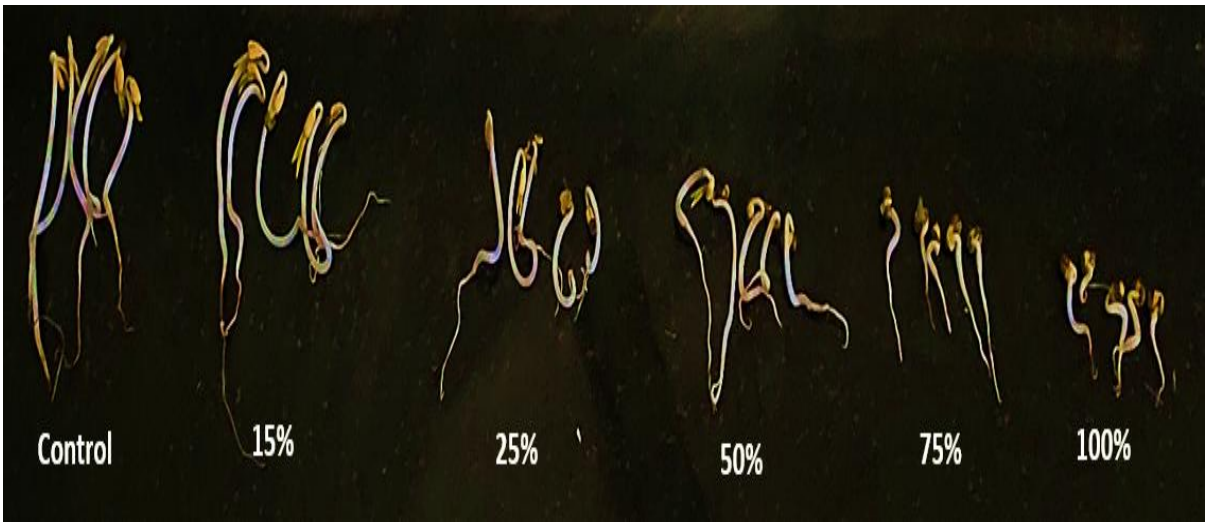
Figure 2 Chromatogram obtained during GC-MS analysis of ethyl acetate extract of wastewater samples showing the presence of various residual organic pollutants (ROPs), A= UTW (Untreated wastewater); B= TW (Treated wastewater).

The seed germination was noticed till 50% UTW test solution, but above this concentration, it was not significant (Figure 3). However, seed germination was detected in all the five tested solutions of TW, though the magnitude of germination reduced with each step. When using 100% UTW, there was no significant increase in shoot length, root length, or biomass. For 100% TW, a just-reverse trend was seen, indicating the higher toxicity of UTW (Table 4).

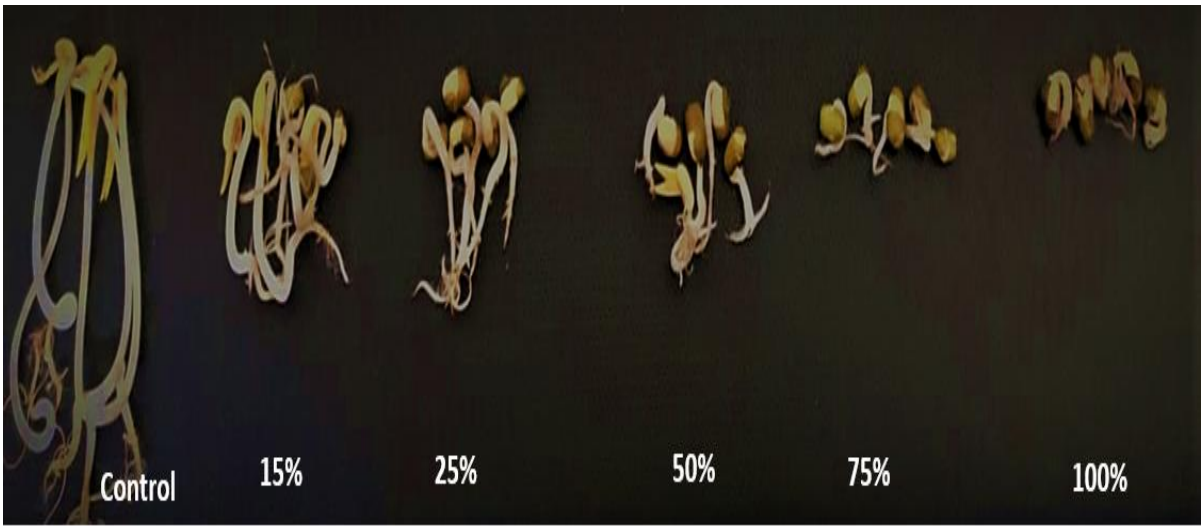
Table 4 Effect of different concentrations of pharmaceutical treated wastewater on seed germination, root length, shoot length and biomass in mung bean (*Vigna radiata*) plants

	Germination (%)		Shoot length (cm)		Root Length (cm)		Biomass (gm)	
Wastewater (%)	UTW	TW	UTW	TW	UTW	TW	UTW	TW
15	80	100	8.4 ±0.38	10.6 ± 0.41	1.2 ±0.02	1.8 ±0.05	1.0 ±0.002	1.9 ±0.004
25	60	80	5.7 ±0.23	9.6 ±0.26	1.1 ±0.9	1.6 ±0.11	0.9 ±0.001	1.4 ±0.002
50	30	50	2.5 ±0.06	7.9 ±0.03	0.7 ±0.18	1.5 ±0.02	0.4 ±0.04	0.8 ±0.17
75	0	5	0.2 ±0.02	4.3 ±0.03	0.2 ±0.009	1.4 ±0.08	0.0	0.3 ±0.02
100	0	4	0.0	2.3 ±0.04	0.0	1.1 ±0.06	0.0	0.2 ±0.04
Negative Control	100		12.2 ±0.15		2.8 ±0.05		3.23 ±0.057	

UTW- Untreated wastewater; TW- Treated wastewater; ± - Standard Deviation; Negative Control- Tap water



(A)



(B)

Figure 3 Effect of different concentrations of pharmaceutical industrial wastewater on early seedling growth of mung bean (*Vigna radiata*), A= UTW (Untreated wastewater); B= TW (Treated wastewater)

3.5 Cytotoxicity and genotoxicity of pharmaceutical industrial wastewater

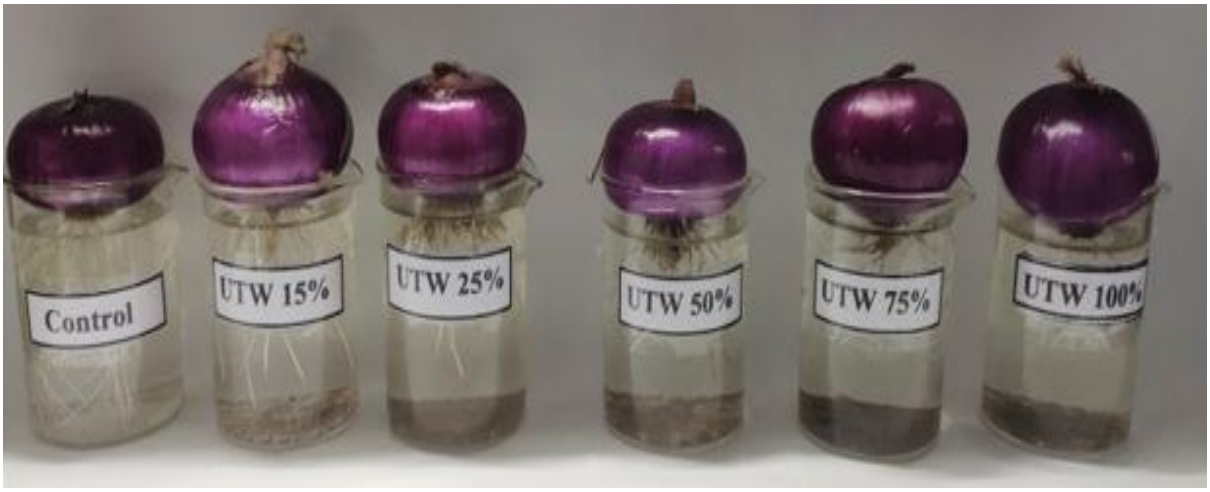
The cytotoxicity and genotoxicity of untreated and treated PIWW were assessed in growing cells of the root apex of *A. cepa* using the MI and CA.

3.5.1 Mitotic index (MI)

The MI is a useful research tool for measuring the cytotoxic effects of various pollutants on cell division. MI quantifies the fraction of cells in the division phase as a percentage of the total cell population (Mercado et al. 2020). Table 4 highlights the cytotoxic effects of untreated and treated PIWW on *A. cepa* roots. The results showed that the mitotic index (MI%) value of the plant root apex was in the order of 37.62 (UTW 15%), 30.86% (UTW 25%), and 13.20 (UTW 50%) for untreated wastewater, and 44.92 (TW 15%), 38.18 (TW 25%), and 21.34 (TW 50%) for treated wastewater. The MI% for the negative control (tap water) was 85.54%. The MI% fell significantly with increasing UTW and TW concentrations, showing the presence of numerous cytotoxic residual contaminants in untreated and treated PIWW. The magnitude of the MI% decrease of the UTW test solution was greater than that of the TW test solution, indicating the PIWWTP's treatment efficiency.

3.5.2 Chromosomal aberrations

The results demonstrated that chromosomal and nuclear configurations remain wild-type in cells treated with negative control (tap water). However, treatment with increasing concentrations of untreated and treated PIWW caused an array of aberrations in the chromosome (Table 5; Figure 4). The detected aberrations were sticky metaphase (chromosomal clumping in the metaphase stage),



(A)

(B)

Figure 4 Effect of different concentrations of pharmaceutical industrial wastewater on root growth of onion bulb *(Allium cepa)*; Control= Tap water; A= UTW (Untreated wastewater); B= TW (Treated wastewater)

Table 5 Assessment of different chromosomal abnormalities in *A. cepa* root apex cells. The root apex was subjected to various concentrations of untreated and treated wastewater of ETP for 24 hours

	Negative Control	UTW			TW		
	Tap Water	UTW (15%)	UTW (25%)	UTW (50%)	TW (15%)	TW (25%)	TW (50%)
Total cell	332	319	311	303	423	419	403
Dividing cell	284	120	96	40	190	160	86
Mitotic Index (%)	85.54 ±2.50	37.62 ±1.26	30.86 ±0.62	13.20 ±0.07	44.92 ±1.07	38.18 ±0.22	21.34 ±1.06
Chromosomal aberration							
Sticky metaphase	0.00	5.3 ±0.78	8.0 ±0.25	11 ±0.42	4.3 ±0.23	6.0 ±0.27	9.0 ±0.28
Vagrant chromosome	0.00	3 ±1.34	4 ±1.28	6 ±1.76	2 ±0.86	3 ±0.94	4 ±1.37
Chromosomal loss	0.00	2.6 ±.22	4.2 ±0.46	6.4 ±0.48	2.1 ±0.42	3.8 ±0.25	4.7 ±0.26
C-mitosis	0.00	4.2 ±0.98	5.2 ±1.14	7.1 ±2.01	2.8 ±0.83	3.4 ±1.04	5.5 ±1.02
Binucleated	0.00	1.9 ±0.8	7.0 ±1.02	9.0 ±1.26	1.3 ±0.48	5.0 ±0.88	8.0 ±1.00
Micronuclei	0.00	6 ±1.00	8 ±1.5	9.5 ±2.50	3 ±1.00	5 ±1.00	7.5 ±1.50
Aberrant cells (%)	0.00	13 ±1.50	18 ±2.00	22 ±2.40	7 ±1.00	12 ±1.60	14 ±1.8

UTW- Untreated wastewater; TW- Treated wastewater; ± - Standard Deviation

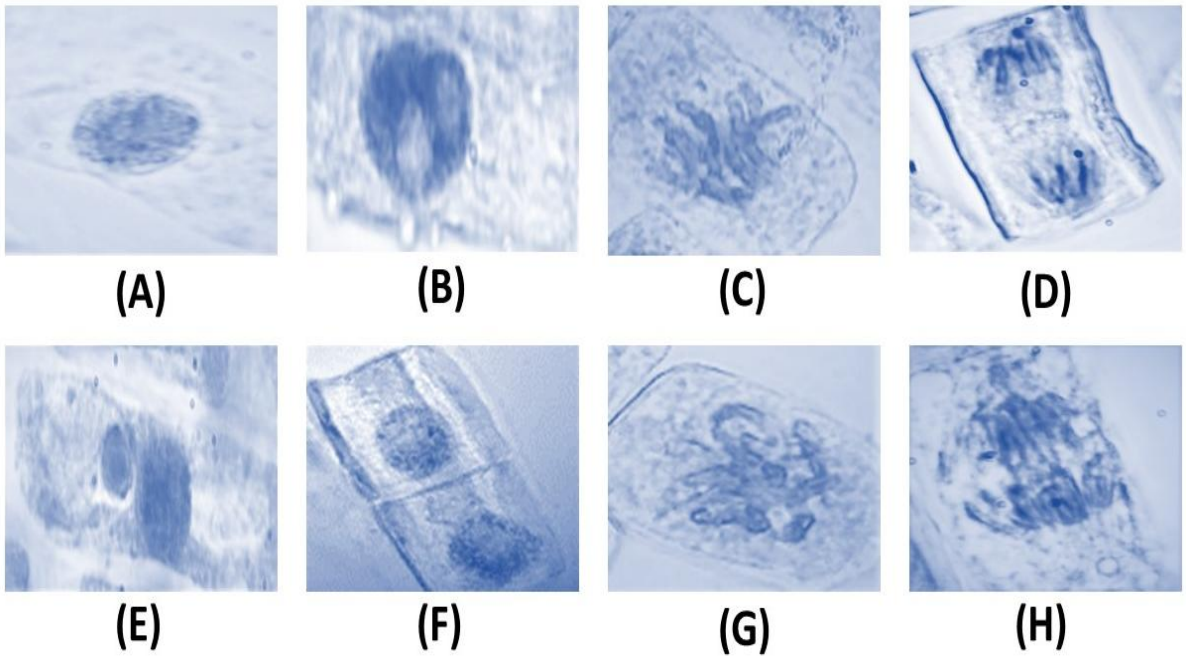


Figure 5 Chromosomal aberrations observed in root apex cells of *A. cepa* exposed to different concentrations of pharmaceutical wastewater (A) Disrupt prophase (B) C-mitosis (C) Sticky metaphase (D) lagged anaphase (E) Micronucleate (F) Binucleate (G) Chromosomal loss (H) vagrant

vagrant chromosome (unspecified wandering chromosome), chromosomal loss, c-mitosis (induced abortive division of nuclear material resulting in the doubling of chromosome), binucleated (cell with two nuclei), micronuclei (a smaller nucleus formed when a chromosome or its part fails to merge into one of the dividing nuclei during mitosis) and frequency of aberrant cells. The CAs were detectable at all concentrations of test solutions for both UTW and TW. The frequency of aberrations was higher in UTW test solutions. The most common aberrations at all the wastewater concentrations studied were micronuclei, sticky metaphase, and c-mitosis. The highest frequency of abnormal cells was reported at a 50% UTW concentration (Table 5; Figure 5).

4 Discussion

TDS and TSS values were substantially reduced after treatment, but other metrics remained high, indicating the inability of PIWWTP. Such high physicochemical parameter values can have an array of environmental hazards (Hamaidi and Brahim 2019). The high turbidity is caused by inorganic and organic substances in wastewater received at ETPs (Theoneste et al. 2020), whereas TDS represents dissolved organic and inorganic compounds and a range of minerals and salts viz., Na, Ca, Cl, bicarbonates, and sulfates (Chen et al. 2021). TDS's inorganic and organic pollutants raise the wastewater's COD and BOD value. Singh and Singh (2018) define COD and BOD as the total amount of organic compounds and their constituents in wastewater. The high levels of COD, BOD, TDS, TSS, and turbidity in wastewater indicate a significant load of inorganic and organic chemicals. Such settings may be breeding grounds for clinical bacterial strains (Soni et al. 2024).

In this study, the physicochemical parameters BOD and COD of the effluent (TW) were 664.67 ±7.12 mg/L and 3196.62 ±18.16 mg/L, respectively. Kumari and Tripathi (2019) reported that PIWW's BOD and COD values were 7253.34 mg/L and 765.67 mg/L, respectively, substantially higher than this study. Further, the BOD value was much lower, and the COD value was significantly greater in this investigation. This comparison shows that TW contained a significant concentration of inorganic components susceptible to oxidation by the oxidant. Bakare et al. (2009) determined that the COD, BOD, TDS, phosphate, chloride, and nitrate concentrations were 147.04 mg/L, 48.13 mg/L, 336 mg/L, 61.3 mg/L, 5240 mg/L, and 42 mg/L, respectively. Comparing this study (Table 1), the COD, BOD, TDS, and phosphate values revealed by Bakare et al. (2009) were much lower, whereas chloride and nitrate values were significantly higher.

Most of the metal concentrations in the TW were many folds greater than the CPCB (2020) permissible limit (Table 1). When such effluent is exposed to the environment, it can cause phytotoxicity, cytotoxicity, and genotoxicity (Zhu et al. 2024). Mehrarad et al. (2016) reported that the effluent of PIWWTP had extremely high concentrations of heavy metals, including Pb (25.89 mg/L), Cd (12.34 mg/L), Zn (26.12 mg/L), Cr (17.46 mg/L), Mn (10.21 mg/L), Cu (18.34 mg/L), and Fe (43.22 mg/L). All of the metal concentrations in this investigation were substantially lower than those reported by Mehrarad et al. (2016) (Table 1). A just-reverse trend for metal concentration was seen when comparing this study's findings to those of Kumari and Tripathi (2019).

The FTIR spectra of the UTW extract showed more peaks than TW throughout a range of wave numbers (Figure 1). These peaks imply that UTW absorbed more FTIR energy due to its high concentration of functional groups (Nandiyanto et al. 2019). According to the FTIR study results (Figure 1), the peaks at 3341.1 cm^{-1} in the UTW extract and 3377.7 cm^{-1} in the TW extract correspond to OH carbohydrates and polyphenols. Mecozzi et al. (2012) revealed peaks in a similar range in the FTIR spectra of PIWWTP's effluent. Peaks 789.8 to 591.7 cm^{-1} correspond to C-H outside the aromatic band plane (Table 2).

Organic pharmaceutical compounds in wastewater can have many phytotoxic, genotoxic, and cytotoxic effects (Chowdhary et al. 2022). The GC-MS spectra of UTW and TW revealed various peaks at different RTs, corresponding to different pharmaceutical components. The peak at RT 16.04 minutes corresponded to Pentanedioic acid, bis(trimethylsilyl) ester, 4-Bromo-5-methyl-2-phenylthiazole showed in both samples. The number of peaks in the UTW was more than TW which indicates that there was vigorous treatment of wastewater was facilitated. Several pharmacological and therapeutic chemical metabolites were found in the UTW and TW, including 2-Trimethylsilylmethylcyclopentanone (RT- 8.05), Disiloxane, hexamethyl (RT- 14.04), Pentanedioic acid, bis(trimethylsilyl) ester, 4-Bromo-5-methyl-2-phenylthiazole (RT- 16.04), 2-(5-acetyl-2-thienyl)-1,4-naphthoquinone (RT- 19.49), 5-Bromo-2-hydroxy-N'-(3-pyridinyl methylene) benzohydrazideditms (RT- 22.03), Garciniaxanthone F (RT- 27.95), 3-(4-nitrophenyl)quinazolin-4(3h)-one (RT- 34.33) and Dirithromycin (RT- 34.52).

2-Trimethylsilylmethylcyclopentanone is a cyclopentanone derivative used to manufacture rubber products, medications, and insecticides (Li et al. 2019). Disiloxane hexamethyl, an ingredient in liquid bandages (sprayers on plasters) such as pavilion spray, is meant to protect wounded skin irritation occurs due to other bodily fluids (Gowtham et al. 2022). 2-(5-acetyl-2-thienyl)-1,4-naphthoquinone, a naphthoquinone derivative, has anti-tumor, antimicrobial, and antiproliferative activity. Discharging it into the environment can potentially disrupt the microflora and microfauna (Umar et al. 2023). 5-Bromo-2-hydroxy-N'-(3-pyridinyl methylene) benzo hydrazide ditms inhibit the development of hepatic cancer by reducing the activity of TGF-β (transforming growth factor-β) (Tseng et al. 2021). Garciniaxanthone F is a bioactive Xanthone isolated from Garcinia mangostana and used to treat injuries, dermal infections, cystitis, dysentery, and gonorrhoea. It induces quinone reductase (QR) and inhibits P^{450} activity (Ong et al. 2020; Shan et al. 2011). 3-(4-nitrophenyl) quinazolin-4(3h)-one a derivative of quinozolin, while Dirithromycin is a derivative of erythromycylamine and both these are antimicrobial (Adel et al. 2021; Patel and Patel 2010). Its free dissemination into the environment may promote the evolution of antibiotic-resistant bacterial populations (Soni et al. 2022).

The determined physicochemical parameters, metal concentration, and organic pollutants revealed that the collected samples were highly contaminated (Table 1; Table 2). These pollutants may have various phytotoxic implications, including suppressing seed germination, shoot growth, root growth, and biomass development (Wadaan et al. 2023). In this investigation, the seed germination frequency was 100% when a negative control (tap water) was used, but it decreased substantially when the concentration of wastewater was increased (Table 4). The UTW had less potential to promote seed germination and growth than the TW, indicating that the UTW was more hazardous (Figure 3). Antibiotics are present in PIWW (Dias et al. 2023). These antibiotics can potentially impair nitrogen metabolism in nitrogen-fixing organisms, resulting in phytotoxicity (Fiaz et al. 2023).

To investigate the harmful effect on the root growth of onion bulbs, test solutions of different concentrations of UTW and TW were utilized. Significant differences were found when the root length data were compared to the negative control (tap water). Root development was not substantial in onions grown at 50% UTW concentrations (Figure 4). The MI for the negative control, UTW (50%) and TW (50%) observed in this investigation was 85.54 ±2.50, 13.20 ±0.07, and 21.34 ±1.06 respectively, indicating a potent cytotoxic impact of PIWW. The MI for 25% of TW observed in this study was 38.18, greater than the value (9.6) detected in Kumari and Tripathi (2019) study at the same concentration of test solution. This comparative study shows that the cytotoxic impact of effluent calculated in this study is lower than in the study of Kumari and Tripathi (2019). The study of Drzymaa and Kalka (2023) revealed a similar trend. They calculated the toxicity of diclofenac and sulfamethoxazole on *Vicia faba* and observed that both pharmaceuticals reduced the MI by 46% and 22% in soil cultures and 45% and 47% in hydroponic cultures, respectively. The cellular mass of the root apex was treated with negative control (tap water) and revealed no chromosomal abnormalities, but in the test solutions CAs were observed (Figure 5; Table 5). Moreover, the magnitude of the aberration increased with increasing wastewater contents. Other researchers have also reported many CAs in their studies (Kumari and Tripathi 2019; James et al. 2015). The maximum CA obtained in this investigation was aberrant cells, 22 ±2.40 at a 50% UTW concentration. The presence of CA in the cellular mass of the root apex of A. cepa may be due to the cumulative impact of many contaminants like heavy metals, therapeutic compounds, phenols and other organic contaminants in the PIWW. These studies demonstrated that heavy metals, therapeutic compounds, phenols and other organic contaminants in the pharmaceutical wastewater are primarily responsible for the cytotoxic and genotoxic consequences.

Conclusions

WWTPs, responsible for promising everyone access to clean water, sanitary conditions, and good health, are essential to achieving the UN Sustainable Development Goals (SDGs), particularly SDGs 3 and 6. In comparison to effluents of other WWTP, the PIWWTP's effluent contains high levels of BOD, COD, nitrate, phosphate, metal concentration, pharmaceutical metabolites, and so on. The inability of PIWWTPs to completely remove pharmaceutical metabolites has been widely overlooked. The amalgamation of this effluent with the recipient environmental bodies may have several detrimental effects. It can potentially cause phytotoxicity and chromosomal aberrations and disrupt MI and other cytological processes. Due to these grave implications, immediate action is required to limit the propagation of this worldwide hazard. Public regulatory agencies must act quickly to prevent PIWWTPs from discharging hazardous effluent. The scientific community must develop more efficient, cost-effective methods for treating the PIWW.

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