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ORIGINAL ARTICLE

Hospital Waste:
Impact on *Allium Cepa* Cells

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Highlights

1. Hospital waste contains substances that can be metabolized and stored in eukaryotic organisms. 2. Propoxur, one of the products found in the waste, offers clastogenic potential as it is a synthetic carbamate and acetylcholinesterase inhibitor. 3. Hospital waste has cytotoxic and genotoxic potential.

ABSTRACT

The dumping of chemical substances, from urban, industrial and hospital waste, is the main responsible for the high concentrations of toxic substances in the environment. The adverse effects and risks that these substances may cause to the ecosystem have become the target of research in the scientific environment. The chemical substances present in tailings can be metabolized, stored as well as interact directly with the DNA of eukaryotic cells promoting pre-oncogenic events. To assess the impact of so-called emerging contaminants (EDC's) on the genome of eukaryotes, it is possible to use micronucleus (MN) and nuclear abnormalities (NA) tests through onion root (*Allium cepa)* meristematic cells. The objective of this research was to investigate the cellular, genotoxic and clastogenic alterations in onion root meristematic cells, through exposure in three hospital waste sampling efforts at three different times. The collected samples were analyzed in a gas chromatograph coupled to a mass spectrometer (GC-MS QP 2010) in order to qualify the constituents present in the tailings. By analyzing semi-permanent slides stained by the Shiff method, the nuclear and micronucleus abnormalities were computed in 3000 *Allium cepa* meristematic cells exposed to hospital waste for 96 hours. The results obtained show that the calculated MI (Mitotic Index) of the cells submitted to the waste were significantly lower when compared to the negative control group. There is also an important correlation between mitotic index and MN appearance. In the GC-MS analysis, several main peaks were observed and different retention times were identified. In treatment 1 T (3.44) and treatment 2, T (3.47), Propoxur was one of the products found that offers clastogenic potential as it is a synthetic carbamate, composed of aromatic ether and acetylcholinesterase inhibitor, widely used as a pesticide. The results show that the waste from the hospital under study has cytotoxic and genotoxic potential.

Keywords: *Allium cepa;* emerging contaminants; micronucleus.

REJEITO HOSPITALAR: IMPACTO EM CÉLULAS DE *ALLIUM CEPA*

RESUMO

O despejo de substâncias químicas, tanto do lixo urbano, industrial ou hospitalar, é o principal responsável pelas altas concentrações de substâncias tóxicas no ambiente. Os efeitos adversos e os riscos que essas substâncias podem causar ao ecossistema têm se tornado alvo de pesquisa no meio científico. As substâncias químicas, presentes em rejeitos, podem ser metabolizadas, armazenadas bem como interagir diretamente com o DNA das células eucarióticas, promovendo eventos pré-oncogênicos e epimutações. Para avaliar o impacto dos chamados emergentes contaminantes (EDCs) sobre o genoma de eucariotos, é possível utilizar testes de micronúcleo (MN) e anormalidades nucleares (AN) por meio das células meristemáticas da raiz de cebola (*Allium cepa*). O objetivo desta pesquisa foi investigar as alterações celulares, genotóxicas e clastogênicas em células meristemáticas da raiz de cebola pela exposição em três esforços amostrais de rejeito hospitalar em três momentos diferentes. As amostras coletadas foram analisadas em cromatógrafo de gás acoplado a espectrômetro de massa (GC-MS QP 2010), a fim de qualificar os constituintes presentes nos rejeitos. Por meio de análise de lâminas semipermanentes coradas pelo método de Shiff, foram computadas as anormalidades nucleares e micronúcleo em 3.000 células meristemáticas de *Allium cepa* expostas ao rejeito hospitalar por 96 horas. Os resultados obtidos mostram que o Índice Mitótico (IM), calculado das células submetidas aos rejeitos, foram significativamente menores quando comparados com o grupo controle negativo. Há também uma correlação importante entre índice mitótico e aparecimento de MN. Na análise de GC-MS vários picos principais foram observados e identificados diferentes tempos de retenção. No tratamento 1 T (3,44) e tratamento 2, T (3,47), o Propoxur foi um dos produtos encontrados que oferece potencial clastogênico por ser um carbamato sintético, composto de éter aromático e inibidor da acetilcolinesterase, muito usado como pesticida. Os resultados evidenciam que o rejeito do hospital em estudo tem potencial citotóxico e genotóxico.

Palavras-chave: *Allium cepa;* emergentes contaminantes; micronúcleo.

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INTRODUCTION

Anthropogenic action has caused significant changes in water quality, leading to human health harm as a result. Several researches are carried out in order to measure the damage caused by anthropization, as well as to propose mitigation methods. Strategies, such as physical-chemical and biological analyzes of surface waters, have been reported in order to determine the contaminants present and assess possible toxicity index[1,2].

Living organisms can produce a set of information on the bioavailability of contaminant substances in tailings, which makes biosensors great references for toxicity assessment. Toxicological tests are carried out with the objective of determining the effect of physical, chemical, and biological agents that may offer a risk to human health. Toxicological tests used in studies of liquid effluents have been an important tool for public and private institutions in order to identify problems with the disposal of toxic substances, as well as establish control priorities in critical regions, enabling appropriate corrective actions[3].

According to Montagner et al. (2017)[4], the issue of emerging contaminants has been addressed in different aspects by researchers around the world. These are numerous compounds detected in soil, water and air, those of human origin, present in domestic, industrial, hospital effluents and those from agricultural and livestock activities, as well as naturally occurring ones. These compounds may offer a risk to the ecosystem and are not included in routine monitoring programs, that is, they are not legislated. Thus, they will be likely for future regulation depending on the results obtained in ecotoxicity studies when the effects on human health are determined, potential for bioaccumulation, transport, and destination in different environmental compartments, in addition to the amount in which they are released and the concentration in the environment.

Several substances have been considered emerging contaminants, such as: drugs, compounds used in personal hygiene products (sunscreens), hormones, alkylphenols and their derivatives, illicit drugs, sucralose and other artificial sweeteners, pesticides; by-products from water disinfection processes, brominated flame retardants; perfluorinated compounds; siloxanes; benzotriazoles; naphthenic acids; perchlorates; dioxins; nanomaterials; ionic liquids and microplastics[4-6].

Genotoxic substances have as a major problem their ability to chemically interact with the genetic material of all types of organisms, inducing breaks in the DNA molecule. These lesions are usually repairable by activating specific ways. In case of error in the repair ways, mutations are established, perpetuating in the daughter cells during the cell division process[7].

Higher plants are excellent genetic models to detect environmental mutagens being used in monitoring studies[8]. The *Allium cepa* species is indicated for its high sensitivity and excellent correlation with other test systems, mainly with mammals. These factors are relevant for the detailed assessment of environmental risks, as well as for the analysis of other target organisms, such as man[9]. Tests with this species have shown a correlation of 82% with carcinogenicity in rodents due to its high sensitivity[10].

The goal of this work was to analyze the cytotoxicity of hospital waste in *Allium cepa* meristematic cells by evaluating the Mitotic Index (MI) and genotoxicity by counting nuclear abnormalities (AN) and micronuclei (MN). In addition, the tailings were qualitatively evaluated through gas chromatography coupled to a mass spectrometer.

MATERIAL AND METHODS

Study area and collection protocol

Hospital Vida & Saúde, in Santa Rosa (RS), provides services to the community in the northwestern region of the state. It covers 22 municipalities (240 thousand people), receiving patients for the most diverse types of care and performs an average of 1,200 hospitalizations a month.

The three waste collections were carried out at three different times out in August 2020, December 2020, and April 2021. All liquid samples were placed in glass bottles and stored under refrigeration. The physical-chemical parameters of all samples, such as temperature (ºC), pH, dissolved oxygen (DO) and conductivity (COD) were evaluated in the Genetics laboratory using the Asko SX836 Multiparameter Meter and stored in refrigeration until the moment of use.

Allium cepa Test

To carry out the study, *Allium cepa* bulbs were purchased at the local market and placed in 50 mL beakers in contact with distilled water for 96 hours for rooting. After the rooting period, the experiment was carried out as follows[11]: i. three bulbs were exposed for 96 hours to hospital waste samples, undiluted (collections 1, 2 and 3), ii. another group with three bulbs, negative control, was kept in distilled water for the same period iii. and another group, positive control, with three bulbs was submitted to lead nitrate ($Pb(NO_3)_2$).

After the exposure time, the root meristems were collected and conditioned for a period of 24 hours in microtubes, type Ependorf®, containing Carnoy's fixative (3: ethanol / 1: acetic acid). Subsequently, the roots were washed in distilled water and stored in 70% alcohol under refrigeration until the slides were made. Three slides were prepared for each sample collected, including a negative control and a positive control with lead nitrate (0.5 mg/L).

The staining of the slides followed the Feulgen protocol with modifications, where the roots were subjected to acid hydrolysis with 1N HCl for 15 minutes at 60 ºC, the hydrolysis allows good staining due to its action on the purine bases of DNA, releasing aldehyde groups and carbohydrates. Then, they were immersed in Schiff's reagent for 60 minutes in the dark and at room temperature. Root tips were sectioned to extract their meristematic regions and macerated on a slide, added a drop of 2% acetic orcein for cytosol contrast and closed with a coverslip.

In Olympus® CX31 Optical Microscopy, 1000 cells were observed per slide, that is, 3000 cells per sample, counting Micronucleated cells (MN) and Nuclear Anomalies (AN) of the types: Binucleation, "Lobbed", Broken-egg, Anaphasic Bridge, Stikness, C-Mitosis, Apoptosis, Chromosome Loss and Dragging.

The mitotic index was obtained by following the recommendations of Oliveira et al. (1996)[12], dividing the number of cells in mitosis (prophase + metaphase + anaphase + telophase) by the total number of cells (interphase + mitosis) multiplied by 100. The data extracted from the total Micronucleus (MN) and Nuclear Abnormalities (NA) by subjects were subsequently subjected to statistical analysis.

Qualification of hospital waste through Gas Chromatography Coupled to Mass Spectrometer (GC-MS)

By-product identification was performed by using a gas chromatograph coupled to a mass spectrometer (GCMS-QP2010, SHIMADZU, Japan) using an NST 05 MS column (30 mx 0.25 mm x 0.25 µm coating thickness) by 95% dimethyl polysiloxene and 5% diphenyl. Initially, an extraction procedure based on 3 x 20 mL of CH_2Cl_2 was performed on 40 mL of the SMX samples. Then, the combined

organic layer was dried with anhydrous $MgSO_4$ and concentrated by rotary evaporation (Hei-VAP Precision, HEIDOLPH) at 40°C.

The initial temperature was 55 °C, which was held constant for 3 min followed by an increase of 25 °C min-1 to 300 °C, then held for 6 min. The inlet and detector temperature was 260 °C, with an interface temperature of 240 °C. The temperature of the ionization source was maintained at 250 °C for the selection and quantification of organic compounds. The equipment was adjusted to a voltage of 0.88 kV, generating an impact ionization of 70 eV for molecular fragmentation and ion production in a mass / electric charge (m/z) field of 20 to 600 to identify the structure and determine the concentrations of by-products in the samples. The structural configuration of the by-products is based on the NIST 08 library of GCMS-QP2010. This methodology is described by Borba et al. (2018) and Bueno et al. (2018)[13-14].

Statistical treatment of data

For the statistical analysis of the total AN and MN counts, the sampling efforts were submitted to the Shapiro-Wilk normality test. Not finding normal values, the Kruskal-Wallis test was chosen. For all tests, p≤0.05 was considered significant. The BioEstat 5.3 program was used.

RESULTS AND DISCUSSION

The analyzes of the physical-chemical parameters of the tailings water are shown in Table 1. The National Council for the Environment (Conama), in the use of the powers conferred on it by item VII of art. 8 of Law No. 6.938, of August 31, 1981, regulates that effluents from any polluting source may only be discharged directly into the receiving body as long as they comply with the conditions and standards set forth in this article, subject to other applicable requirements[15].

Table 1 – Physico-chemical parameters of tailings, after collection

CARACHTERISTICS	T1	T 2	T3	Maximum values
Collection	1	2	3	
pH	4.85	8.88	6.50	5 - 9
COND. (µs)	109.2	32.82	37.82	N/C
Total Sol	9.87	2.15	8.80	minimum removal efficiency of 20%
Salinity	7.49	1.62	3.42	N/C
ODi (mg/L)	9.25	1.37	1.44	maximum 120 mg/L
Temp. H_2O (°C)	11	25.5	20	Inf. a 40

*ODi= dissolved oxigen; COND=conductivity.

Source: Elaborated by the author, 2021

The data obtained from our samples are within the acceptable limits by CONAMA Resolution No. 430 of May 13, 2011[16], which provides for the conditions and standards of effluent discharge that complements and amends Resolution No. 357, of March 17, 2005[17], of the Conama. Even observing some small variations, such as the pH of the T1 effluent, which is extremely acidic (4.85) and a dissolved oxygen value of 9.25, the values are still within the tolerable range. It is known that dissolved oxygen is responsible for oxidizing the organic material present in water, its low value is indicative of normality, while its high value may be due to the large presence of surfactants from the laundry, according to results obtained by Evaldt (2005)[18].

Table 2 presents the cytotoxic alterations in the analyzed onions. The calculated MI of the cells submitted to the waste showed significantly lower rates when compared to the negative control group.

Table 2 – Total values of the mitotic index of undiluted effluent treatment and controls in 3000 cells submitted to the Kruskal Wallis test with a 5% significance level

GROUP	MI %
NEGATIVE CONTROL	13.2[A]
POSITIVE CONTROL	4.9[B]
TREATAMENT 1	2.8[B]
TREATAMENT 2	2.76[B]
TREATAMENT 3	3.16[B]

*Different letters have statistical significance.

Source: Elaborated by the author (2021).

It is also noted that the MI of the cells that were subjected to the three tailings samples also showed lower values than the cells treated with the chosen mutagen (Lead Nitrate), although it did not present significant values. Biological events in the cell cycle that result from contact with mutagenic substances are notably important for cell integrity.

When the meristematic tissue has a low rate of mitosis, this indicates that genes that regulate the cell cycle were affected by a xenobiotic altering the programmed ways for good tissue functioning[19]. Although the statistical test does not point out the significance of the data, we see an important loss when the conditions of the cells in the prepared slides were analyzed with many cells in the process of degradation and apotosis.

Other studies show that dilutions of hospital wastewater to 50% cause a notable increase in chromosomal aberrations in *Allium cepa*, corroborating the data we obtained without dilution[20-21].

In addition to the MI, we also analyzed the incidence of Nuclear Abnormalities and Micronuclei in order to establish damage to the cells that entered mitosis and tried to complete the phase without complications. We observed the data expressed in Table 3 which compares the values of AN and MN.

Table 3 – Total values of MN and AN counts in 3000 cells submitted to the Kruskal Wallis test with a significance level of 5%

GROUP	AN	MN
NEGATIVE CONTROL	29 [A]	0 [A]
POSITIVE CONTROL	12 [A]	14 [B]
TREATAMENT 1	143 [A]	10 [A]
TREATAMENT 2	210[B]	14 [A]
TREATAMENT 3	192[B]	9 [A]

*Different letters have statistical significance.

Source: Elaborated by the author (2021).

There is an important correlation between these two events. When the cell suffers damage but is able to activate repair ways and maintain a certain nuclear integrity, MN levels decrease and AN tends to increase. Likewise, when the cell cannot repair the damage, it has two ways, organizing the nucleus even with some loss of material (leading to the formation of MN) or inducing apoptosis, which consequently decreases MI values[22]. When we look at our Table, we can see this correlation in all treatments. The positive control can be seen as an example: in 3000 cells we have 14 MN, which is

a high value, since the tolerable value would be 3 for 3000. On the other hand, the ANs are in lower value and so is the MI.

The results show that all tailings' samples induced MN values above the physiologically expected, as well as a large amount of ANs when compared to the negative control. This indicates that meristematic cells treated with the tailings, which managed to enter mitosis, suffered DNA damage activating repair ways and organizing their nuclei as they could (ANs)[23]. And the cells carrying MN are those that the repair ways could not minimize the damage and in this way the cell organized the lost DNA in the form of a small nucleus inside the cytosol. This cell, in turn, has no more chance of reversing this event[24].

Hooftman and de Raat (1982)[25] suggested that variations in the shape of micronuclei may represent an alternative approach to detecting genotoxicity. Despite the fact that there is a correlation between nuclear abnormalities and genotoxic effects has not yet been established, preliminary observations strongly suggest that such morphological changes may be a manifestation of the effects of xenobiotics[26].

Genotoxicity in hospital wastewater has already been proven by the Ames test[27] and *Allium cepa* tests[20-21], and it is believed that the combination of genotoxic substances from these wastewaters may be one of the possible causes of cancer in recent decades. Genotoxicity can be attributed to the presence of drugs, such as anticancer drugs (phosphamide, cisplatin, doxorubicin) and antimicrobial agents (ciprofloxacin); however, the correlation of genotoxic dose effect data on a given compound in wastewater is not an easy task, mainly due to the presence of limited data available in literature and the variable characteristics of wastewater, which depend on the type of hospital activity[28].

The results presented in Table 4 show the compounds identified in the gas chromatography, the data selected in the test were above the 75% reliability.

Table 4 – Residual compounds identified in Gas Chromatography Coupled to Mass Spectrometer (GC-MS)

Retention time	Molecular formula	Identified residual compound	T1	T2	T3
3.44	$C_{11}H_{15}NO_3$	Propoxur	x	x	x
3.47	C_8H_9NO	N-phenylacetamide		x	
3.47	$C_8H_{10}O$	2-phenylethanol		x	
3.47	C_7H_7I	Toluene		x	
3.47	$C_6H_5C(O)CH_3$	acetophenone		x	
3.66	$C_6H_{11}N_3O_4$	caracemide	x		
3.89	$C_{10}H_{12}O_2$	eugenol		x	x
4.84	$C_{16}H_20Br_4$	tetrabromopentacycle		x	
4.94	$C_{10}H_{12}N_2S$	N-propyl-2,1-benzothiazol-3-amine	x		
9.82	$C_5H_{10}O_2$	somaleric acid			x
9.89	$C_6H_{12}O_2$	butyl acetate		x	
10.29	$C_{10}H_{18}O_4$	sebaceous acid	x		
10.50	$C_8H_{16}O_2$	octanoic acid	x	x	
12.06	$C_6H_{11}N_3O4$	Gly-Asn			x
13.40	C_8H_6ClN	4-chlorophenylacetonitrile	x	x	
13.96	$C_5H_{12}O$	tert-butyl methyl ether			x
14.84	$C_4H_7NO_3$	N-acetylglycine			x
16.84	$C_4H_8N_4O_4$	allantoic acid	x	x	
17.50	C_2H_3NO	methyl isocyanate	x		

Source: elaborated by the author (2021).

In the GC-MS analysis, several main peaks were observed, and different retention times were identified, in treatment 1, T (3.44) and treatment 2 and treatment 3 (Figure 2), it is observed that propoxur, which is a synthetic carbamate, composed of aromatic ether and acetylcholinesterase inhibitor and used as a pesticide and the one that appears most frequently. Propoxur is reported to biodegrade very quickly in water, particularly when bacterial activity and temperature are high. Volatilization of water surfaces is not expected to be a major destination process based on the estimated Henry's Law constant of this compound.

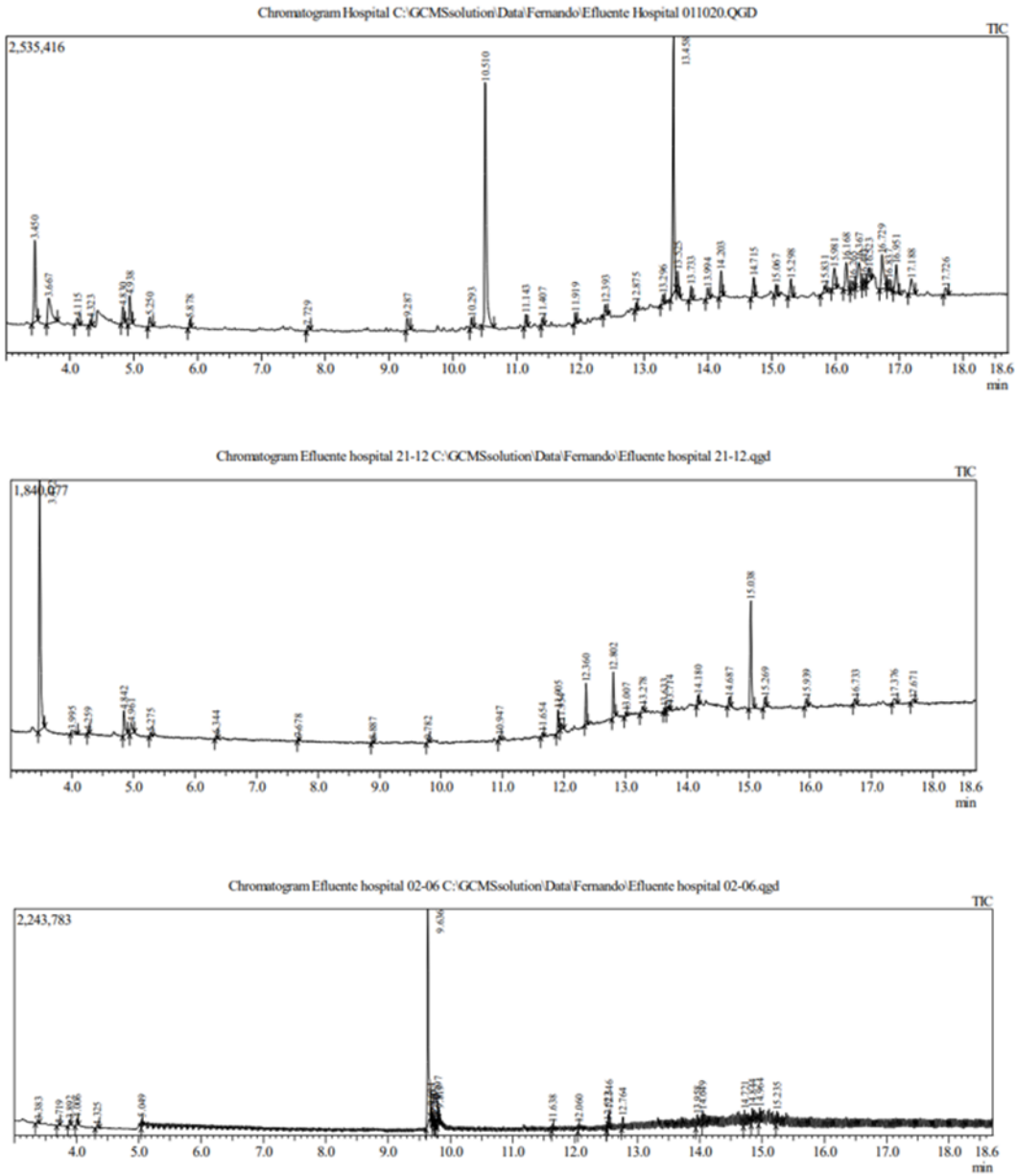

Figure 3 – Peaks observed in treatments submitted to GC-MS.

Source: data produced by GC-MS (2021).

In a study developed by Eraslan (2009)[29] the authors show that propoxur caused negative changes in most of the biochemical parameters investigated in Wistar rats. The observed increase in tissue and plasma oxidative marker levels in the present study in the group that received propoxur alone (control group) demonstrated that lipid peroxidation was developed. This fact is indicative of damage caused

to the examined tissues as a result of free radicals generated by propoxur. Excessive free radicals can interact with DNA causing damage that plays an important role in mutagenesis and carcinogenesis processes[30]. During mitosis of *Allium cepa* meristematic cells, the presence of propoxur may have induced high levels of peroxide, inducing errors in the processes of division and appearance of MN.

The caracemide that is present in treatment 1, T (3.66) is an acetohydroxamic acid-derived agent with potential antineoplastic activity. It is a metabolite present in drugs used in cancer treatment. Caracemide inhibits ribonuclease reductase, resulting in decreased DNA synthesis and tumor growth; it also inhibits acetylcholinesterase. In vivo, caracemide contributes to the formation of methyl isocyanate, a neurotoxine.

This effect, together with the acetylcholinesterase activity of the agent, may account for the severe central nervous system toxicity seen in clinical trials. Antineoplastic drugs have the function of interrupting the cell cycle, preventing cell divisions, an intrinsic characteristic of tumor cells, and endless mitosis. Present in the samples collected may have prevented cells in the mitotic phase of the exposed roots.

Other compounds found in the treatments may have played an important role in the results found. Acetophenone in treatment 2, T (3.47) is an organic compound found in the composition of medicines. In addition, GC-MS analysis also showed the presence of fatty acids and carboxylic acids (treatment 1), T (10.29) sebaceous and T (16.84) allantoic (treatment 1 and 2) which may be metabolites of drugs used in neurological treatments. The octanoic acid found in treatment 1, T (10.50) is a fatty acid and may be from laundry products. N-phenylacetamide (treatment 2) T (3.47) is a member of the class of acetamides in which one of the hydrogens attached to the nitrogen is replaced by a phenyl group. It has an analgesic function. It is derived from an acetic acid[31].

The discharge of phthalates causes water pollution and serious toxicological effects on aquatic organisms[32]. Benli et al. (2016)[33] also reported that the bioaccumulation of phthalates leads to genotoxic effects, endocrine disruption, dysregulation of the antioxidant defense system in plants and humans.

Phenolic compounds and phthalates are reported as potential endocrine disruptors (EDCs) Benzoic acid, a well-known EDCs, was detected in tannery wastewater by GC-MS. It has been classified as a probable human carcinogen and highly toxic to aquatic organisms[34-35].

Benzene is known to be a carcinogen and its presence in tanneries effluents may be associated with the use of phthalate and phenolic compounds in the leather industries[36-37]. Furthermore, recently some EDCs such as resorcinol, hexadecanoic acid and octadecanoic acid have also been detected in wastewater from tanneries[37].

In Spain, Gracia-Lor et al. (2012)[38] monitored the presence of various drugs (analgesics and anti--inflammatory drugs, disruptors, antibiotics) in urban effluents and found that conventional treatment processes do not completely remove these persistent micropollutants. Moreover, in wastewater from Spanish hospitals, different compounds (analgesics and anti-inflammatories, antibiotics, b-blockers, diuretics, iodinated contrast media) were found (ng-mg L1) that offer a risk to aquatic organisms belonging to different trophic levels[39]. Our results show that the waste collected presents the same risks for the environment and for living organisms.

CONCLUSION

In the ecotoxicological analyzes that we performed, we see that hospital waste demonstrates cytotoxic and genotoxic activity in *Allium cepa* cells. Substances found in hospital waste can interact with DNA promoting cell cycle damage as well as changes in the genetic material of exposed cells.

The waste from the hospital under study presents a risk to the rivers in the region, as well as to the organisms present in this ecosystem.

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