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Characterization and genotoxicity evaluation of effluent from a pharmacy industry

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ABSTRACT

The pharmaceutical, textile and food industry bear much of the responsibility for environmental pollution. In order to appropriately treat and mitigate the effects of pharmaceutical effluent, it is necessary to study it in order to determine its physical and chemical composition. In this work, the physicochemical characteristics of a pharmaceutical effluent were studied, to include the concentration of phenolic compounds, heavy metals, total phosphorus, nitrate, chemical oxygen demand (COD), and dissolved oxygen (DO). The *in vivo* micronucleus test was performed in mice, for investigation and possible genotoxicity and mutagenicity of the effluent from the pharmaceutical hub in Anápolis - Goiás. In all samples, only the phenolics showed concentrations above the values established by CONAMA Resolution 430/2011. The high concentrations of total phenols and synergy between metals found in wastewater can be linked to mutagenicity and genotoxicity found in the effluent, since the results of the micronucleus test indicated higher micronucleus formation when the mice were exposed to the effluent. The results of the study highlighted the necessity of characterizing these effluents in order to determine an appropriate treatment.

Keywords: pharmaceutical wastewater, phenolic compounds, metals, micronucleus test.

Caracterização e avaliação da genotoxicidade de um efluente de indústria farmacêutica

RESUMO

Indústrias farmacêuticas, têxteis e alimentícias são umas das responsáveis por grande parte da carga de poluentes lançados no meio ambiente. Pesquisa de substâncias possivelmente presentes no efluente da indústria farmacêutica é justificada pela possibilidade de considerar um tratamento adequado para seus efluentes por meio de sua caracterização físico-química. Neste trabalho, as características físico-químicas de um efluente da indústria farmacêutica foram estudadas: concentrações de compostos fenólicos, metais pesados, fósforo total, nitrato, demanda química de oxigênio (DQO), oxigênio dissolvido (OD). O teste do micronúcleo *in vivo* foi realizado em camundongos *Swiss*, para a averiguação e possível genotoxicidade e mutagenicidade de um efluente farmacêutico do pólo farmacêutico de Anápolis - Goiás. Em todas as amostras coletadas, apenas os compostos fenólicos

apresentaram concentrações acima dos valores estabelecidos pela Resolução CONAMA 430/2011. As altas concentrações de fenóis totais e a sinergia entre metais encontrados nos efluentes podem estar ligadas à mutagenicidade e genotoxicidade encontrada no efluente, visto que os resultados do teste de micronúcleo indicaram maior formação de micronúcleos quando os camundongos foram expostos ao efluente. Através dos resultados obtidos observou-se a necessidade da caracterização desses efluentes para a indicação do tratamento mais adequado.

Palavras-chave: efluente farmacêutico, compostos fenólicos, metais, teste de micronúcleo.

1. INTRODUCTION

The pharmaceutical industry is of great economic importance to the State of Goiás due to the large growth of the sector and the consolidation of the pharmaceutical hub located in the Anápolis-Goiânia axis. The presence of drugs, metabolites, synthesis reagents and solvents, and in particular halogenated compounds (New et al., 2000), in environmental waste from the pharmaceutical industry has attracted great interest. A variety of drugs have been detected in many environmental samples (Fick et al., 2009), such as natural surface waters (Idris et al. 2013), effluents from sewage treatment plants (Halling-Sorensen et al., 1998; Bueno et al., 2012), soil and fish (White, 1983).

Historically, urban and industrial development occurred along the rivers due to the availability of water supply and the ability to use the river for waste disposal. The increase in population as the industry grows and the increasing discharge of municipal and industrial wastes into the rivers are both causes of concern. It is therefore of fundamental importance to analyze and treat the effluent. Besides the direct effects of pharmaceutical residues, secondary effects such as penetration or bioaccumulation in the food chain of aquatic systems have also been detected, and have been found to disrupt the function of the reproductive endocrine systems of impacted species, thus influencing the species' propagation (Bertoletti, 1989). It is suspected that many of these toxic compounds are potent carcinogens (Bertoletti, 1990; Bredhult et al., 2007).

Moreover, as the wastewater typically includes more than one organic compound, one should take into consideration the synergic effect of these compounds (Vanegas et al., 1997; Bervoets et al., 1996; Kopinke et al., 1995; Madureira et al., 2010).

The *in vivo* micronucleus test has been widely used and accepted by regulatory agencies and the scientific community (Mateuca et al., 2006) as a test to investigate genotoxicity (Papis et al., 2011). The test detects genomic alterations and / or damage to the mitotic apparatus where micronuclei indicate the irreversible loss of DNA (deoxyribonucleic acid). While genetic toxicity is not a measure of carcinogenicity, it is often used as an indicator for cancer due to the associations between high positive responses in genetic toxicity tests and carcinogenicity in rodents and humans (Azevedo et al., 2003; Rezende et al., 2006; Monteiro and Boxall, 2010).

Many dyes in use possess mutagenic or carcinogenic properties. The disposal of dyes in river sand lakes diminishes the absorption of light energy by changing aquatic ecosystems (Ferreira et al., 1999; Carneiro et al., 2010). Azos dyes are commonly used in the textile, paper, food, cosmetics and pharmaceutical products (Knapp and Newby, 1995).

Phenolic compounds are used in disinfectants, in phenolic resins and in other materials, such as solvents. Phenol is toxic, but it has negative effects on waters even before reaching levels harmful to the human health, because its chemical reactions form chlorophenol compounds that affect the water's appearance, taste and cause an unpleasant smell (Imhoff and Imhoff, 1986). It therefore requires treatment with chlorine. According to Tziotzios et al.

(2005), phenol contaminants have toxic effects capable of penetrating cell membranes and cause coagulation of cytoplasmic membranes, thereby damaging the cells and possibly causing severe damage to human health and to the environment. In activated sludge systems (systems used for wastewater treatment of pharmaceutical waste), concentrations of phenols in the range of 40 to 200 mg L^{-1} were able to inhibit nitrification, and concentrations of 40 mg L^{-1} are sufficient to cause such inhibition.

The State of Goiás establishes limits only for the organic load in relation to the Biochemical Oxygen Demand (BOD), establishing the maximum concentration of 60 mg O_2 L^{-1} or its reduction by 80%. CONAMA Resolution Nº 430 of May 13th, 2011, establishes conditions and standards for effluent discharge directly into a water body. The primary quality standards for the discharge of effluents into water bodies are shown in Table 1, except for special cases where there is a history of cyano-bacterial bloom and sewage wastewater treatment systems.

Ecotoxicological data have been collected by researchers to identify drugs that are potentially dangerous to the environment; however, the data available in the literature are insufficient. The occurrence of these drugs in surface and subsurface waste waters demonstrates a need for studies to determine the toxic effects of these drugs on the environment. In this context, research has been done to analyze potential risks for some drugs in the environment (Henschel et al., 1997; Gros et al., 2010).

The objectives of this study were to characterize the physico-chemical effluent of a pharmaceutical industry, to include inorganic compounds such as nitrogen, phosphorus, heavy metals(iron, zinc, lead and copper), phenolic compounds of a specific pharmaceutical industry and to investigate the genotoxic potential of that wastewater.

2. MATERIAL AND METHODS

2.1. Pharmaceutical effluent

The effluent samples were collected quarterly over a period of one year at a pharmaceutical industry near Anápolis, Goiás, Brazil. The effluent pH was measured immediately after collection by a digital potentiometer. The effluent was stored under refrigeration at approximately 4°C (Santiago, 1999). All effluent samples were characterized chemically and evaluated for genotoxic potential. Table 1 shows the methodology used in the characterization of the effluent, according to the Standard Methods of Water and Wastewater-APHA/ AWWA (APHA et al., 1995).

Table 1. Standard methods for water and wastewater of APHA/AWWA for characterization of effluents (APHA et al., 1995).

Parameters	Reference	Quantification Limit
Total solids	2540	0,1 mg L^{-1}
Phenolics	5530-C	0,1 mg L^{-1}
Total phosphorus	4500 P-E	0,1 mg L^{-1}
Nitrate	4500-NO_3-D	0,1 mg L^{-1}
Chemical Oxygen Demand-COD	5220-D	1,0 mg L^{-1}
Total Nitrogen	ISO 2005. Method ISO	0,1 mg L^{-1}
Iron	3120-B	0,02 mg L^{-1}
Lead		0,01 mg L^{-1}
Copper		0,002 mg L^{-1}
Zinc		0,002 mg L^{-1}
Apparent Color, True Color and Turbidity	2120-C	1,0 mg PtCo 1,0 NTU
Dissolved Oxygen - DO	4500-H^+ B	0,1 mg L^{-1}

2.2. Animals

The chemical industry of the State of Goiás (IQUEGO) provided *Swiss* mice (males aged 7 to12 weeks) for the experiments. The animals were randomly grouped in plastic cages and maintained at a constant temperature of 20° C with a light-dark cycle of 12 h. The regular diet of the mice was the classic, with standard commercial feed and water provided *ad libitum*. To achieve mutagenicity, the assay animals were divided in control and exposed to 0.2 mL of raw effluent orally for 10 days. Animal studies were conducted according to the Ethical Principles of Animal Experimentation of the Brazilian Council for Animal Experimentation.

2.3. Genotoxicity assay

In order to evaluate the potential mutagenic effluent, animals received 0.2 ml of the effluent for 10 days. Twenty-four hours after exposure, the animals were terminated by cervical dislocation and their marrow was harvested with a syringe. The control group received only saline. The experimental groups (five animals per group) were distributed as follows: Group I: negative control (received only saline), Group II: positive control (cyclophosphamide 200 mg kg^{-1}), Group III: received the raw effluent (pool of 4 samples; 0.2 mL day^{-1}).

2.4. Preparation of slides for micronucleus research

Bone marrow was collected in a test tube containing 1 mL of saline. The tube was then placed in a centrifuge twice, for 5 minutes each time, at 2000 RPM. The supernatant was then discarded. A small drop of the resulting suspension was then removed and placed on the end of the blade to be smeared on a slide. After drying, the smear slides were stained with *Leishmann* and again dried at room temperature. A small drop of Canada balsam was then applied in the center of the blade to fasten the cover slip. The slides were analyzed blind using a 1000x magnification. One thousand erythrocytes were analyzed per slide (in triplicate).

2.5. Statistical Analysis

Data were analyzed using the Analysis of Variance (ANOVA) statistical method and the Tukey post-hoc test. For all groups, results were considered statistically significant when $P < 0.05$.

3. RESULTS AND DISCUSSION

Chemical characterization was performed in parallel to assess the genotoxic potential of wastewater. Effluent sample collections were conducted at different times in order to obtain representative samples, since the manufacture of medicinal products is seasonal and the effluent varies according to the products that are being manufactured in a given period. The hydrogen potential (pH) of all samples is below 7, indicating an acidic character. The pH is an important parameter in wastewater treatment and determines process selection. It is also one of the most important factors in the treatment of water, as it affects coagulation, hardness and prevents corrosion of iron pipes in the distribution network.

The effluent was kept refrigerated in order to determine the concentration of total solids and dissolved particulate compounds such as metals, inorganic salts, oils and greases and organic matter (APHA et al., 1998). This is important in order to determine the amount of waste present in the effluents. In this study, the amount of total solids was approximately 3 g L^{-1} for all effluent samples, with the lowest in sample 4 (1.8 g L^{-1}). Figure 1 is a comparative chart of total solids and total phenols.

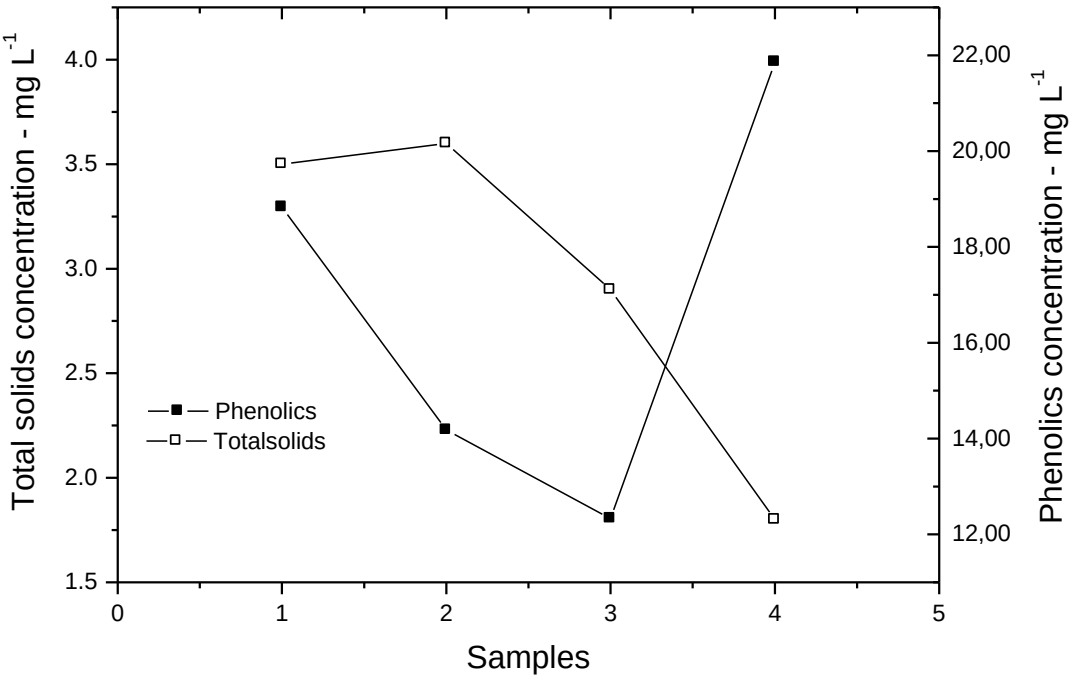

Figure1. Comparison of total solids and total phenols of samples of pharmaceutical effluent.

The high concentration of phenolic compounds found in the analyzed industrial effluent indicated that inadequate treatment of such substances can cause harm to both the environment or to human health (Tziotzios et al., 2005). Santos and Linardi (2004) have found that wastewater from industrial processes, petrochemical plants, coal conversion processes, and phenolic resins from pharmaceutical industries include phenolic compounds, and therefore require careful treatment prior to discharge into water bodies.

Phenolic compounds are among the polluting compounds of particular concern in the environment as they are readily absorbed into the skin (CETESB, 2010) and are very toxic to humans. A lethal oral dose is estimated to be about 70 mg kg^{-1} (CETESB, 2010).

Pinheiro et al. (2007) reported that such compounds may be toxic and problematic for water supplies causing odor, even at low concentrations such as 1 to 10 g L^{-1}. Moreover, they represent a great threat to the environment because of their toxicity and their acidic, mutagenic and carcinogenic characteristics. There is therefore a great need to remove these compounds from wastewater. The CONAMA 430/2011 proposes that the maximum permitted emission levels be 0.5 mg L^{-1}; the samples had concentrations up to 40 times greater than permitted.

In addition to high concentrations of phenolic compounds found in wastewater, other substances may be present, such as compounds with nitrogen in its different oxidation states: ammonia and albuminoid, nitrite and nitrate, thus increasing the risk to human health. However, these elements are not of concern pharmaceutical effluent since they are present in concentrations lower than that permitted by law.

Nitrate is one of the most frequent ions found in natural waters. It usually occurs in low concentrations in surface waters, but can reach high concentrations in deep waters. Its consumption in drinking water is associated with two adverse health effects: the occurrence of methemoglobinemia, especially in children, and the potential formation of carcinogenic nitrosamines and nitrosamines (Mato, 1996). Figure 2 compares these parameters.

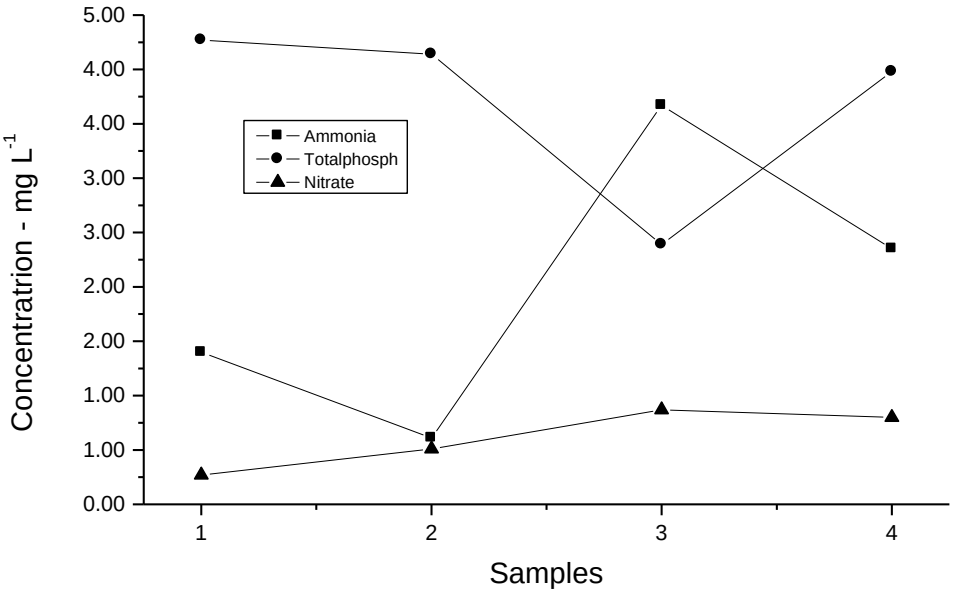

Figure 2. Comparison of the concentrations of total nitrogen, total phosphorus and nitrate samples of the effluent from a pharmaceutical industry.

Copper ions usually occur in surface waters at concentrations below 20 μgL^{-1}. However, the presence of copper was not detected in the effluent due to the sensitivity of the method used. Lead was not detected by atomic absorption spectrophotometry. Iron gives a dark color and bitter taste when present at high concentrations. Iron concentration found in the effluent is lower than those permitted by law (seven times lower on average) and therefore pose no risk. Lead concentrations (Pb) were not detected by the analytical method used. Figure 3 compares concentrations of the analyzed metals.

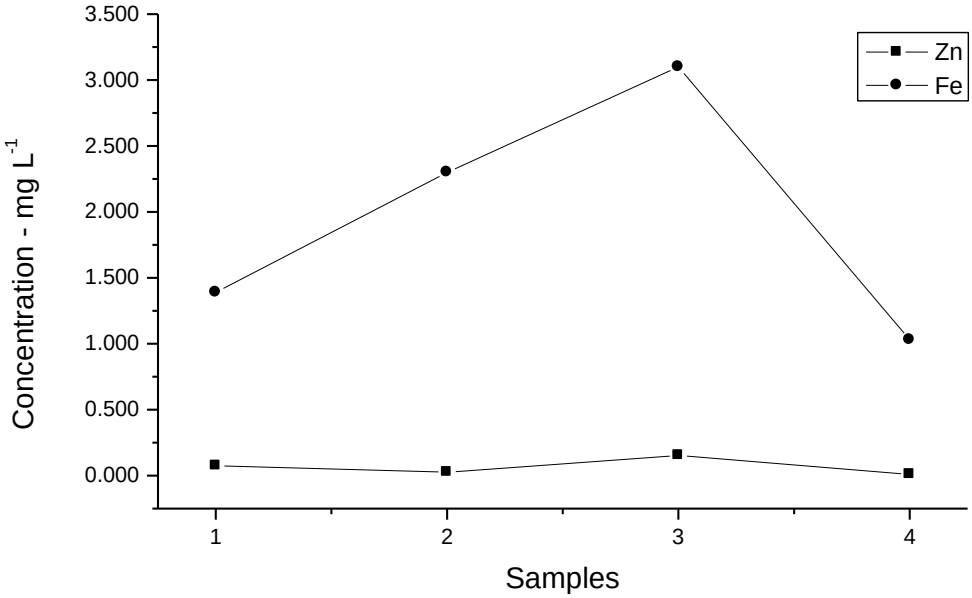

Figure 3.Comparison of metal concentrations: zinc (Zn) and iron (Fe) samples of pharmaceutical effluent.

Data regarding the color of the effluent are shown in Figure 4. The results showed great variability.

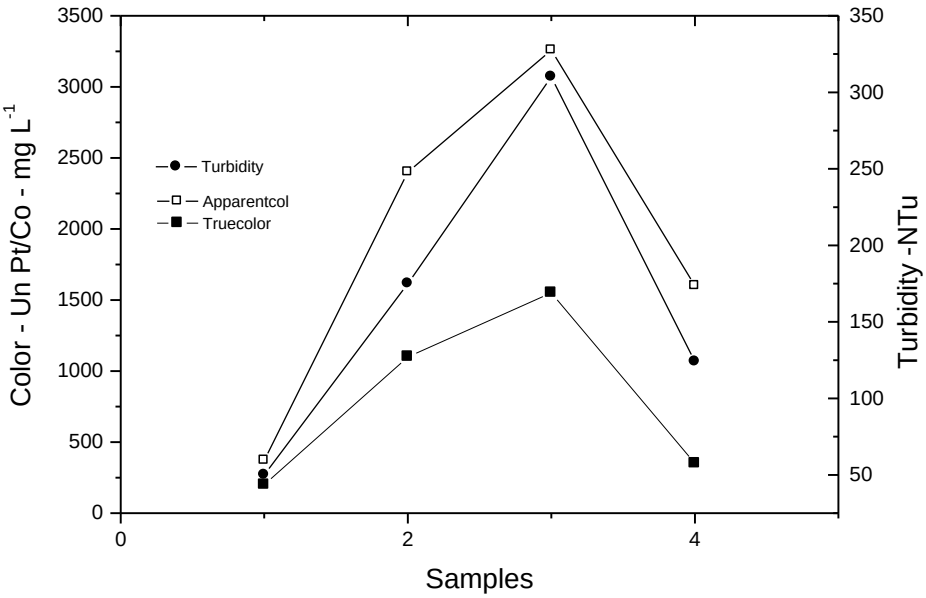

Figure 4.Comparison of apparent color variations and true color of the samples of pharmaceutical effluent.

The effluent's apparent color consists of dissolved substances (natural or artificial colorants). In all samples, the true color was approximately 50% less than the apparent color, showing a high quantity of particulate material, as evidenced by observed turbidity values (Figure 4).

The high COD values found in samples, particularly in Sample 2 (6155 $mgL^{-1}$of O_2), indicated a high load of organic compounds, suggesting that the effluent is actually composed of a mixture of substances in different stages of decomposition.

DO concentrations were observed in low and high BOD, showing that the effluent from the pharmaceutical industry has a high biologically degradable organic load. Values for the COD and the BOD found in the industry effluent can be viewed and compared in Figure 5.

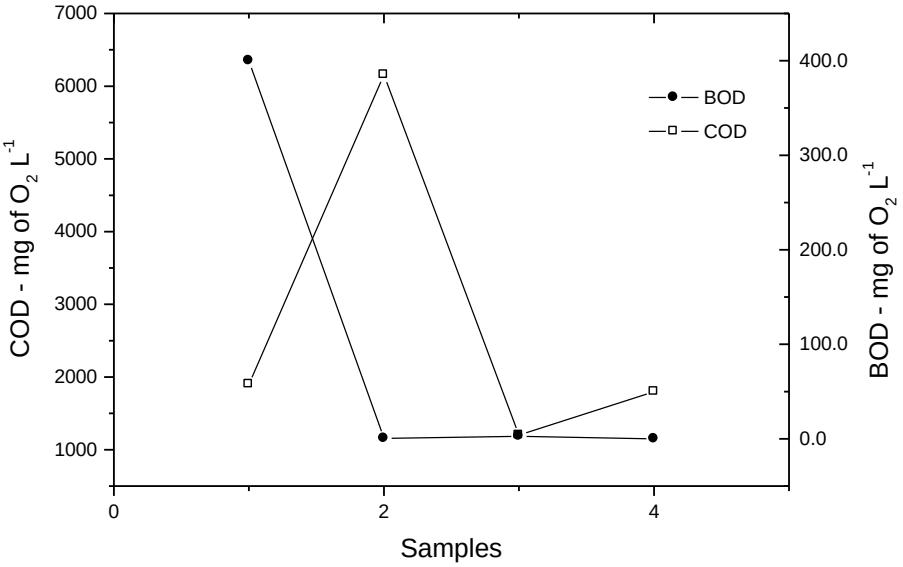

Figure 5. Variation of COD and BOD in samples of effluent from a pharmaceutical industry.

Certainly, in addition to the substances mentioned above, other organic compounds, biodegradable or not, are included in the effluents of these industries.

There is currently a need to develop analytical methods sufficiently sensitive to detect and quantify residual pharmaceuticals in aquatic environments, with detection limits in the order of μgL^{-1} and ngL^{-1} and (Sacher et al., 2001).

In regard to the genotoxic potential of the effluent, Figure 6 shows the frequency of micronuclei in erythrocytes in the bone marrow of animals exposed or 10 days to raw effluent in the pharmaceutical industry in sample 3, since samples 1, 2 and 4 did not show potential toxicity.

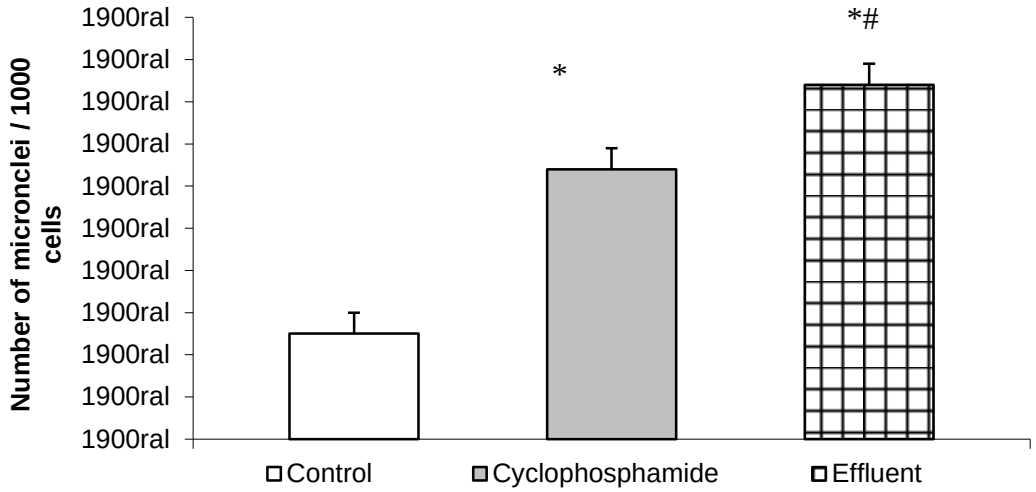

Figure 6.Assessment of micronucleus frequency in erythrocytes (1000/blade) bone marrow of animals exposed (n=5) for 10 days (0.2 mL day^{-1}) to the raw effluent of pharmaceutical industries. The animals were terminated 24 h after exposure. Cyclophosphamide (single dose, 200 mg kg^{-1}) was used as a positive control for induction of micronucleus formation. ANOVA, Tukey test.*P<0.05 compared to control group. # P<0.05 compared to the group that received cyclophosphamide.

The results clearly demonstrated the genotoxic potential of effluent from the pharmaceutical industry since we observed a significant increase (P <0.05, ANOVA, Tukey test) in the number of micronucleated erythrocytes in bone marrow of mice exposed to this effluent compared to control group that only received saline. Moreover, the frequency of micronuclei in animals exposed to the effluent was higher than the frequency of micronuclei found in animals exposed to cyclophosphamide, an agent for positive control of clastogenicity. Bakare et al. (2009) and Brausch et al. (2012) reported that in their experiments the mixture of heavy metals have a more detrimental effect than the performance of these separate elements.

4. CONCLUSIONS

The analyzes of the physico-chemical parameters of the effluent from the pharmaceutical industry has a wide variety of chemical compounds, to include a high amount of total phenols and inorganic species such as zinc, iron, nitrogen and phosphorus in smaller quantities. With regard to compliance with environmental regulations, the samples were in accordance with the established emission standards, except in the category of phenols, which was far above the permitted levels and, therefore, can be related to the toxicity of this effluent.

In addition, there was variation in the amount of certain substances in different samples. This highlights the importance of collecting representative samples of what is being discarded by the industry. In addition, the effluent has to be well analyzed in order to determine the most effective treatment methods for the effluent type and also to determine compliance with environmental regulations.

The genotoxicity and mutagenicity of the effluent in question, as shown in micronucleus tests in mice, may be due to the high concentrations of phenolic compounds which readily penetrate the skin of animal and cell membranes, causing a wide spectrum of genotoxicity and mutagenicity. It may also be due to the synergistic action of metal ions found in the effluent.

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