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Research Article

Cytotoxicity, genotoxicity, and mutagenicity of the active pharmaceutical ingredient nevirapine and a nevirapine-based drug on the plant species *Allium cepa*

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Abstract

The toxicity of the active pharmaceutical ingredient and nevirapine-based drug at analytical concentrations was evaluated under laboratory conditions, using *Allium cepa* seeds as a model. The germination index of the negative control was 86.8 ± 2.1. The concentrations of 6.42 and 9.54 mg/L of the active pharmaceutical ingredient and 11.20 mg/L of the nevirapine-based drug showed a statistically higher germination index than the negative control. We found that the root growth of the negative control was 1.7 ± 0.6 mm and that the root growth was statistically lower than the negative control at concentrations of 9.54 and 17.73 mg/L of active pharmaceutical ingredient and 5.48, 11.20, and 17.68 mg/L of the drug. The mitotic index of negative control and methyl methanesulfonate were 7.4 ± 2.7 and 12.8 ± 4.5, respectively. At a concentration of 17.68 mg/L of the nevirapine-based drug, the mitotic index of 12.7 ± 2.7 was statistically higher than the negative control and like the methyl methanesulfonate, which indicated that nevirapine was cytotoxic at this concentration. At all concentrations tested the chromosome abnormality indices were statistically higher than negative control, suggesting that nevirapine was genotoxic. The mutagenicity index of the negative control was 0.2 ± 0.3. At concentrations of 6.42, 9.54, and 17.73 mg/L of the active pharmaceutical ingredient and 17.68 mg/L of the nevirapine-based drug, the mutagenicity index was statistically higher than the negative control, indicating that nevirapine was mutagenic for *A. cespa* at these concentrations. The chromosomal adhesion was the most frequent chromosomal aberration in the groups exposed to nevirapine, suggesting that it has an aneugenic effect on the *A. cepa* species.

Introduction

Nevirapine is an antiretroviral from the non-nucleoside reverse transcriptase inhibitor class used in combination therapy for the treatment of Human Immunodeficiency Virus (HIV) infection. It is an active pharmaceutical ingredient used in the manufacture of medicines used by about 35% of the world's population, especially in low-income countries, as one of the therapeutic options available at low cost [1]. The nevirapine acts on the metabolic pathway responsible for DNA transcription, inhibiting reverse transcriptase and preventing the virus from multiplying.

Nevirapine is a dipyridodiazepine with a dipyridyl chemical structure, also found in some herbicides such as Diquat® (9,10-dihydro-8a,10a-diazoniaphenanthrene) [2]. This structure is a precursor of radical species that operate as electron acceptors in oxidative metabolism [3], inducing

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oxidative metabolism [4]. Radical species can generally induce chromosomal alterations, break the Deoxyribonucleic Acid (DNA) double-strand, and oxidize sulfhydryl groups without catalysts [5]. The cytotoxic, genotoxic, and mutagenic potential of nevirapine deserves to be evaluated through ecotoxicological tests [6], which can trigger the production of reactive oxygen species when observing the chemical structure in the nevirapine molecule [7].

Although nevirapine is a poorly water-soluble (90 mg/L), its high stability against light and heat [2], and low environmental biodegradability [8], contribute to its persistence in water, soil, and sediments [9,10]. In countries with a high prevalence of people living with HIV (PLHIV), environmental studies have identified the presence of nevirapine residue in surface water, at concentrations of 0.5 to 1.5 µg/L [9,11] and 1480 ng/L and in sediments, with concentrations of 9.5 µg/L in the dissolved fraction and 3214 µg/Kg in the undissolved fraction [12].

Contamination with micropollutants such as active pharmaceutical ingredients is a concern of the World Health Organization (WHO) regarding the human use of medicinal plants worldwide. Based on this information, Akenga, et al. [13] studied the plant absorption of molecules, including nevirapine, using hydroponic lettuce seeds (*Lactuca sativa*). Given the lack of knowledge of the effects on future generations and based on the numerous adverse effects described in the literature in children exposed to antiretrovirals in the perinatal period [14]. Therefore, they chose the plant species *Allium cepa* [15,16] as a study model, an alternative to the methodological difficulties and bioethical conflicts for conducting cytogenotoxicity assays in animal models.

Indeed, the assay with *A. cepa* to evaluate the genotoxicity of chemical compounds is consolidated in the [17,18], medicinal plants [19-25] and pharmaceuticals [26,27], showing sensitivity and good correlation compared to other systems, such as mammals [28]. This model has been widely used for monitoring basins [29], environmental pollutants [30-33], and industrial [34-36].

The presence and impacts of nevirapine residues in the environment justify carrying out studies like this one, since they imbalance numerous species and harm human health. In this sense, the present study aimed to investigate the potential cytotoxic, genotoxic, and mutagenic effect of the active pharmaceutical ingredient nevirapine and a nevirapine-based drug on the experimental model *A. cepa* to contribute with different responses to the impact of this residue in the environment.

Materials and methods

We adopted the methodology of the study by Caritá, et al. [34] adapted for laboratory conditions to conduct this study, standardizing the experimental variables such as the (i) use of seeds, (ii) preparation of solutions, (iii) exposure method, (iv) assay time, (v) radicle fixation, (vi) preparation and staining of slides with histological sections, and (vii) preparation of permanent slides.

Chemical substances

Anhydrous form active pharmaceutical ingredient nevirapine (batch C5028-12009M), raw material with a purity content of 100.49%, manufactured by Zhejiang Huahai Pharmaceutical Co. Ltd (Linhai, China) was employed to prepare an active pharmaceutical ingredient nevirapine stock solution. Tablets of the product Nevirax® 200 mg (batch 14030011) with a purity content of 100.20%, manufactured by Fundação Ezequiel Dias (FUNED) (Belo Horizonte, Brazil), were pulverized to prepare a stock suspension of the drug nevirapine. The three concentrations of the active pharmaceutical ingredient working solutions and the three-drug working suspensions were analyzed by a validated analytical method using the United States Pharmacopeia (USP) anhydrous chemical reference substance (CRS) NVP (batch G0M270). Methyl methanesulfonate reagents (Sigma-Aldrich, 66-27-3), trifluralin (Sigma-Aldrich, 1582-09-8), Carnoy reagent solutions, 1% acetic carmine and Schiff reagent were provided by the Public Health/Water Laboratory of the Faculty of Pharmacy of the Federal University of Minas Gerais. The reagents and solvents used in this study were analytical grades and concentrations approved by FUNED's quality control for all preparation steps.

Solutions, suspensions, and reagents

A standard stock solution was prepared with 15.82 mg of anhydrous chemical reference substance nevirapine, solubilized in a 25 mL volumetric flask containing 10 mL of acetonitrile and 15 mL of purified water (Millipak® 20 millipore Direct – Q® 3UV). From this solution, three other working standard nevirapine solutions were prepared at concentrations of 12.66, 31.64, and 63.28 mg/L for the calibration curve.

For the tests, a solution and a stock suspension, respectively, of the active pharmaceutical ingredient and the drug were prepared to obtain, after dilution, working solutions and suspensions at concentrations of 5, 10, and 20 mg/L of nevirapine in purified water, having its pH adjusted to 6.9 ± 0.1. Such concentrations are close to 10 mg/L, similar to the plasma concentration of nevirapine found in humans [37]. An aqueous suspension of trifluralin (0.019 mg/L) [38,39], an aqueous solution of methyl methanesulfonate (4×10^{-4} mol/L) [40] were prepared for the positive controls, and purified water with adjusted pH of 6.9 ± 0.1 was used for the negative control.

Stains for preparing permanent slides

Following the protocol by Caritá, et al. [34] with adaptations, Carnoy's fixative was prepared with ethanol and glacial acetic acid at a ratio of 3:1 (v/v) for immediate use for the fixation and conservation of radicles. Acetic carmine 1% was prepared by solubilizing 1 g of carmine in 100 mL of 45% acetic acid, followed by a boiling process for two to three hours and filtration.

For the Schiff reagent, 1.5 g of basic fuchsin and 4.5 g of sodium metabisulfite were solubilized under stirring in 300 mL of heated water (50 °C) and 45 mL of 1 mol/L hydrochloric acid.

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Then, the solution was heated and stirred for fifteen minutes and stored in the dark for 24 hours. After standing, 1 g of activated carbon was added to the solution and filtered, separating 10 mL aliquots in a dropper flask. Finally, the aliquots were stored in the refrigerator in a dark bottle, wrapped in aluminum foil, and kept from light. A new aliquot was used for each working day.

Analytical determination of nevirapine

To determine the analytical concentration of nevirapine, at the initial time of the test, an aliquot of three mL of each working solution/suspension sample (active pharmaceutical ingredient and nevirapine-based drug) was filtered through a 0.45 µm filter and transferred to Falcon tubes for further quantification by ultra-performance liquid chromatography (UHPLC) (Shimadzu Nexera-Prominence®) coupled to a photodiode detector (model SPD-M20A), both Shimadzu (Columbia, USA), using the analytical method under the chromatographic conditions described by Diniz, et al. [41] [Separation was done using a 2.0 × 100 mm, 2.2 µm particle diameter C18 column (ShimPack XR® UHPLC). The nevirapine peak was monitored at 214 nm and occurred 11 minutes after the start of the run.

Test organism

Allium cepa (Baia Periforme variety) seeds, batch 42011-52, from ISLA PAK, germination index of 89% and purity of 100% acquired in the retail trade of Belo Horizonte, Southeast of Brazil.

Seed germination test

The adapted test by Christofoletti, et al. [42] was used, using 25 seeds of *A. cepa* continuously exposed to four mL of the working solution/suspension of the active pharmaceutical ingredient or nevirapine-based drug in each Petri dish autoclaved with filter paper. The positive (trifluralin and methyl methanesulfonate) seeds and negative controls (purified water) received four mL of the respective component. Then, the plates were closed, coated with polyvinyl chloride (PVC) film, and incubated in an oven (Fanem - Biochemical Oxygen Demand) for 120 hours in the absence of light at a temperature of 22 ± 2 °C. Four plates were prepared, totaling at least 100 seeds for each concentration in each group. After incubation, the germination index was calculated for each concentration of each group per the percentage of germinated seeds against the total number of exposed seeds.

Root growth

The Onwuamah, et al. [14] root growth assessment method was adapted and used in this study. After 120 hours, the radicles emerging from the seeds were evaluated for phenotypic aspects, such as (i) color, (ii) texture, and (iii) growth alterations (folds, swollen mass, and bifid radicles). Then, with a caliper (with a scale of 0.05 mm – 1/128") (Disma), the radicle length in millimeters (mm) was measured for each seed that developed in each group, except methyl methanesulfonate (for biological safety reasons, the radicles were not manipulated for measurement).

Slide preparation: staining and fixation

The methodology described by Caritá, et al. [34] was adopted. After incubation for 120 hours, ten rootlets from each plate were collected, sectioned, and fixed with Carnoy (3:1) one hour before the start of the slide preparation. Before the preparation, the radicles were washed with purified water and dried. The Feulgen reaction [43] was performed in a beaker containing 5 mL of 1 mol/L Hydrochloric Acid (HCl) and heated in a water bath at 60 °C for 11 minutes. Then, the radicles were quickly washed to stop hydrolysis, dried, and placed inside an amber glass bottle containing the Schiff reagent. After two hours of incubation in the dark, the excess reagent was removed with a paper towel.

The meristematic region (region of cell division) of the rootlets and the F1 region (region of root elongation or growth, located immediately after the meristematic region and preceding the region of cell maturation or differentiation) of each group were separated and cut with a scalpel. Then each region was covered with a coverslip, and 1% acetic carmine was dripped onto the sample. The excess solution was removed, and the slide was exposed to the flame rapidly for two seconds. In the end, the prepared slides were immersed in liquid nitrogen for fixation and conservation of the samples for later evaluation in an optical microscope (Eclipse E200) in the 40 times objective.

Assessment of the mitotic index

At least 500 cells from the meristematic region of each slide were analyzed, making a total of 5,000 cells analyzed from each group. Following the criteria of Caritá and Marin-Morales [40], the stage of cell division was identified by the phase of the nucleus of each cell (such as interphase or phases of mitosis - prophase, metaphase, anaphase, and telophase). The mitotic index was calculated through the percentage of dividing cells (nucleus in mitotic phases) against the total number of analyzed cells (nucleus in mitotic and interphase phases).

Evaluation of chromosome aberrations and nuclear alterations in the meristematic region

The test was adapted from the Grant [44,45] and Caritá, et al. [34] protocol. At least 5,000 cells from each group were analyzed in the meristematic region for the presence of binuclei, polyploid cells, C-metaphase, micronuclei, microcells, cell sprouts, breakage, loss, chromosomal adhesion, multipolar anaphases, bridges, and anaphase or telophase delays. Following the criteria of Caritá and Marin-Morales [40], the stage of cell division was identified by the phase of the nucleus of each cell (such as interphase or phases of mitosis - prophase, metaphase, anaphase, and telophase). The mitotic index was calculated through the percentage of dividing cells (nucleus in mitotic phases) against the total number of analyzed cells (nucleus in mitotic and interphase phases).

The chromosome aberration index was obtained by the percentage of cells with chromosomal alterations against the total number of cells analyzed for each group. The nuclear

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alteration index was obtained by the percentage of cells with alterations in the nucleus (minicells, micronuclei, and sprouts) against the total number of cells analyzed for each group.

Assessment of the mutagenicity index

At least 3,000 cells were analyzed for each group in the F1 region for micronuclei and sprouts. Briefly, the mutagenicity index was obtained by the percentage of cells with micronuclei and sprouts against the total number of analyzed cells of each concentration in each group [34,46].

Statistical analysis

Statistical analysis was performed using the free software R, version 4.0.3. Initially, a Shapiro-Wilk test was conducted to verify data normality. Then, from the results obtained in each group, a two-by-two Mann-Whitney comparison was implemented for non-parametric data, as it was more sensitive. Values of $p < 0.05$ (i.e., 95% confidence) were considered a statistically significant difference.

Results and discussion

In this study, the active pharmaceutical ingredient and the nevirapine-based drug showed similar analytical concentrations (Table 1), which facilitated the comparison between them regarding the possible interference of the excipients in the observed results. The germination index of the seed lot used in the tests met expectations based on the negative control germination index (86.8 ± 2.1). The germination index of the seeds evaluated the nevirapine cytotoxicity on *A. cepa*

The germination of *A. cepa* seeds exposed to a trifluralin of 48.2 ± 6.2 was statistically lower than the negative control ($p = 0.0294$). Usually, herbicides inhibit the protein activity of cells, inhibiting development and causing injury or death of the organism [47]. Trifluralin belongs to the dinitroaniline group and acts selectively as a mitosis disruptor, inhibiting cell division in meristematic tissues, with aneugenic effects [38,39].

As for the seeds exposed to methyl methanesulfonate, a germination index of 91.8 ± 1.9 was observed, therefore statistically higher than the negative control ($p = 0.0396$). methyl methanesulfonate acts on the guanine and adenine nitrogenous bases of DNA by adding or replacing alkyl groups, which causes incorrect base pairing, blocking cell replication, and permanent changes in the genetic material [48]. This macroscopic result agrees with the expected for methyl methanesulfonate as a positive control because, at the concentrations used, it did not prevent seed germination and allowed the elongation of the radicles to observe genetic alterations in *A. cepa* and its use in the comparison of genotoxicity and mutagenicity.

At concentrations of 6.42 ± 0.58 and 9.54 ± 0.87 mg/L of active pharmaceutical ingredient and 11.20 ± 1.13 mg/L of the nevirapine-based drug, germination index was statistically higher than the negative control (germination index 95.2 ± 2.5; $p = 0.0284$; 95.2 ± 2.5; $p = 0.0294$ and 96.8 ± 2.6; $p = 0.0284$, respectively) and similar to methyl methanesulfonate ($p > 0.05$). We observed that about 10% inhibition of germination (germination index 79.8 ± 2.1; $p = 0.0284$) at a concentration of 5.48 ± 0.44 mg/L of the nevirapine-based drug was evidenced when compared to the negative control. The concentrations of 17.73 ± 1.31 mg/L of the active pharmaceutical ingredient and 17.68 ± 1.2 mg/L of the drug showed a germination index similar to the negative control (germination index 84.2 ± 2.5 and 86.8 ± 4.5; $p > 0.05$), which allows us to state that nevirapine did not inhibit germination at these concentrations, but we cannot say about the feasibility of the organism and its generations.

Concerning growth, the negative control radicles showed homogeneous mass and color and without morphological abnormalities, with a mean growth of 1.7 ± 0.6 mm in 120 test hours, while bifid, twisted, brittle roots and presence of tumors (bulging roots) when exposed to concentrations of active pharmaceutical ingredient and nevirapine-based drug.

Table 2 shows the root growth of the groups. At concentrations 9.54 ± 0.87 and 17.73 ± 1.31 mg/L of active pharmaceutical ingredient and 5.48 ± 0.44; 11.20 ± 1.13 and 17.68 ± 1.29 mg/L of the drug, the root growth was statistically lower than the negative control, reaching a 25% reduction in the concentration 5.48 ± 0.44 mg/L of the drug (root growth 1.5 ± 0.6 mm; $p = 0.0108$; 1.4 ± 0.6 mm; $p = 0.0180$; 1.3 ± 0.6 mm; $p = 0.0002$; 1.4 ± 0.5 mm; $p = 0.0017$ and 1.4 ± 0.6 mm; $p = 0.0000$, respectively). The root growth of 1.7 ± 0.5 was similar to the negative control ($p > 0.05$) only at the concentration of 6.42 ± 0.58 mg/L of API.

These results are corroborated by Onwuamah, et al. [14]. These authors used the syrup pharmaceutical form, but active pharmaceutical ingredient results that would allow comparing the possible interferences of syrup and excipients

Table 1: Analytical concentrations of Nevirapine (NVP) in Active Pharmaceutical Ingredient (API) and drug used in experiments with *Allium cepa*.

Solution	NVP concentration (mg/L)	
	API	Drug
A	6.42 ± 0.58	5.48 ± 0.44
B	9.54 ± 0.87	11.20 ± 1.13
C	17.73 ± 1.31	17.68 ± 1.29

Legend: Nevirapine concentrations were expressed as mean and standard deviation.

Table 2: Root growth (mm) between groups of *A. cepa* seeds exposed to three concentrations of active pharmaceutical ingredient (API) and three concentrations of nevirapine-based drug.

Group ($n = 100$)	NVP (mg/L)	Root growth (mm)	P-value (compared to NC)
Negative control	-	1.7 ± 0.6	-
Positive control	-	0.7 ± 0.2	> 0.0001
API [A]	6.42 ± 0.58	1.7 ± 0.5	> 0.05
API [B]	9.54 ± 0.87	1.5 ± 0.6	0.010820
API [C]	17.73 ± 1.31	1.4 ± 0.6	0.018020
Drug [A]	5.48 ± 0.44	1.3 ± 0.6	0.000179
Drug [B]	11.20 ± 1.13	1.4 ± 0.5	0.001720
Drug [C]	17.68 ± 1.29	1.4 ± 0.6	0.000003

Legend: Nevirapine concentrations in water and root growth were expressed as mean and standard deviation. Negative control = Purified type I water with adjusted pH of 6.9 ± 0.1; Positive control = Methyl methanesulfonate in water (4×10^{-4} mol/L).

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in the determination of the effective inhibition concentration of 50% (EC50%) of *A. cepa* root growth were not presented. It is noteworthy that the concentrations of the isolated active pharmaceutical ingredient and the drug in the present study were within the range tested by Onwuamah, et al. [14]. However, they were lower than the EC50% of 24.63 mg/L of nevirapine determined by them.

However, these macroscopic results did not allow a complete inference about the cytotoxic potential of the substances tested because damage to the genetic material may have occurred even without a significant change in germination and root growth, making it necessary to assess the integrity of the proliferative process through histological sections in the meristematic cells [49]. Thus, the mitotic index was used to confirm evidence of cytotoxicity [50].

The mitotic index (MI) of negative control and methyl methanesulfonate were 7.4 ± 2.7 and 12.8 ± 4.5, respectively. At the concentration of 17.68 ± 1.29 mg/L of the nevirapine-based drug, the mitotic index of 12.7 ± 2.7 was statistically higher than the negative control (p = 0.0029) and similar to the methyl methanesulfonate (p > 0.05), which indicated that nevirapine was cytotoxic at this concentration. The mitotic index of 8.5 ± 4.0 observed at the concentration of 6.42 ± 0.58 mg/L of the active pharmaceutical ingredient was the only one similar to the negative control (p > 0.05) and that differed from the methyl methanesulfonate (p = 0.0185), which suggested that nevirapine did not cause disturbances in the proliferative process of *A. cepa* at this concentration. On the other hand, at the concentration of 17.68 ± 1.29 mg/L of the drug, the mitotic index of 12.7 ± 2.7 was statistically higher than the negative control (p = 0.0029) and similar to the methyl methanesulfonate (p > 0.05), indicating that nevirapine can be considered cytotoxic at this concentration. This result was similar to that of Onwuamah, et al. (2014), who observed a significant reduction in the mitotic index after exposure of onion bulb roots to a nevirapine syrup at a concentration of 12.32 mg/L of nevirapine; however, without the comparison with the alkylating agent.

Fiskejo [16] and Krüger [51] consider that root growth and mitotic index can be endpoints to be observed for the evaluation of cytotoxicity. We observed that, in this study, the macroscopic findings of root growth corresponded to the mitotic index at concentrations of 6.42 ± 0.58 mg/L of active pharmaceutical ingredient (non-cytotoxic) and 17.68 ± 1.29 mg/L of the drug (cytotoxic).

Genotoxicity was assessed using the chromosome aberration index. We found that the chromosome abnormality indices of the negative control, methyl methanesulfonate, and trifluralin were 0.2 ± 0.4; 0.9 ± 2.1, and 1.0 ± 3.3, respectively. At the concentrations of 6.42 ± 0.58; 9.54 ± 0.87; 17.73 ± 1.31 mg/L of active pharmaceutical ingredients and 5.48 ± 0.44; 11.20 ± 1.13 and 17.68 ± 1.29 mg/L of the nevirapine-based drug, chromosome aberration index were statistically higher than the negative control and similar to methyl methanesulfonate and trifluralin (p > 0.05), suggesting that nevirapine showed genotoxic potential at all concentrations tested chromosome aberration index 1.4 ± 0.6; p = 0.0003; 1.7 ± 1.3; p = 0.0016; 1.1 ± 1.3; p = 0.0086; 0.8 ± 1.5; p = 0.0202; 0.6 ± 0.4; p = 0.0419; 2.0 ± 1.3; p = 0.0011, respectively). It is important to note that the highest chromosome aberration index was observed at the concentration of 17.68 ± 1.29 mg/L of the nevirapine-based drug, besides the most significant inhibition of root growth and the highest mitotic index. Abnormalities such as loss, breakage, and adhesion of chromosomes were observed at this concentration. Figures 1 and 2 show examples of chromosome aberrations identified in the groups exposed to nevirapine.

A percentage of 1.2 ± 1.7 of chromosome adhesions was verified in the negative control. At concentrations of 6.42 ±

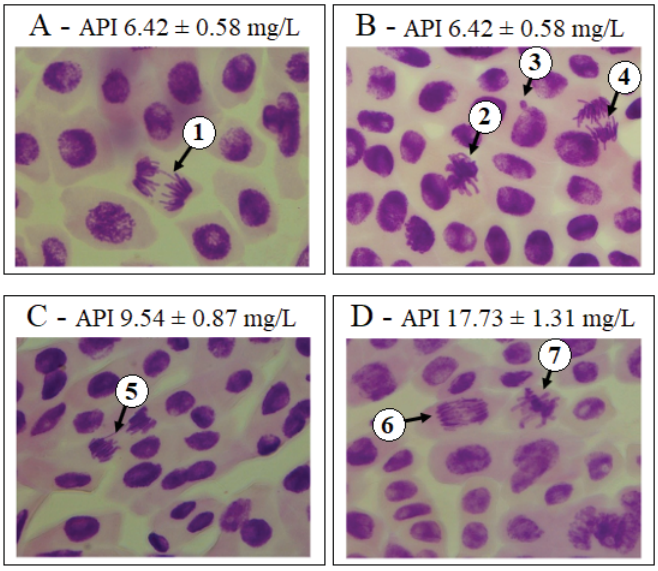

Figure 1: Chromosome aberrations in the meristematic region of *A. cepa* rootlets exposed to the active pharmaceutical ingredient nevirapine.
Legend: Arrow 1 = Anaphase with bridge; Arrow 2 = Normal anaphase, Arrow 3 = Sprout; Arrow 4 = Adherent metaphase; Arrow 5 = Anaphase with bridge; Arrow 6 = Anaphase; Arrow 7 = Adherent metaphase.

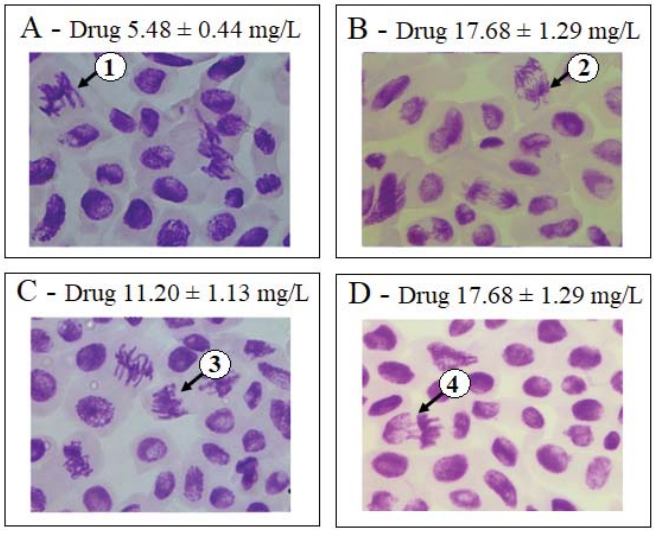

Figure 2: Chromosome aberrations in the meristematic region of *A. cepa* rootlets exposed to the nevirapine-based drug.
Legend: Arrow 1 = Adherent metaphase; Arrow 2 = Adherent anaphase; Arrow 3 = Abnormal metaphase; Arrow 4 = Adherent anaphase.

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0.58; 9.54 ± 0.87 and 17.73 ± 1.31 mg/L of active pharmaceutical ingredient and 5.48 ± 0.44; 11.20 ± 1.13 and 17.68 ± 1.29 mg/L of the nevirapine-based drug, the percentages of chromosome adhesions were higher than the negative control (chromosome adhesions 7.9 ± 3.3; $p = 0.0003$; 10.3 ± 5.5; $p = 0.0013$; 6.6 ± 5.3; $p = 0.0072$; 6.6 ± 6.0); $p = 0.0112$; 3.7 ± 2.6; $p = 0.0319$; 9.3 ± 5.2; $p = 0.0008$, respectively). This abnormality was the most frequently observed in this study. This result is corroborated by Onwuamah, et al. [14], who also observed the predominance of chromosome adhesions against other types of abnormalities when *A. cepa* was exposed to nevirapine.

According to Kurás [52], adhesions result from an alteration in the proportion between histones and other proteins responsible for the organization of nuclear chromatin that increase the adhesion, inducing atypical metaphases and anaphases, chromosomal bridges and inhibiting cytokinesis, forming binucleated cells, making them indications of aneugenic effects of the test substance [35,28].

Indeed, the percentages of adhesions in the groups exposed to nevirapine at the concentrations tested were similar ($p > 0.05$) to trifluralin (chromosome adhesions 10.5 ± 13.0). This substance acts in cell division phases, selectively inhibiting tubulin (the enzyme responsible for the formation of microtubules), inhibiting seed germination, and the formation of new cells in the rootlet and stem [46]. Thus, the predominance of chromosome adhesions observed in the groups exposed to nevirapine suggests that it has an aneugenic effect and trifluralin [53]. Based on its mechanism of action, nevirapine, like trifluralin, may act as an enzyme inhibitor in metabolic pathways responsible for synthesizing proteins in charge of mitotic spindle activities.

The histological analysis illustrates the presence of micronuclei in the meristematic region (Figure 3) as a possible aneugenic effect related to the induction of radicular species related to the dipyridyl structure of nevirapine, which may have contributed to damage to the nuclear genetic material that tends to be expelled from inside the cell in as sprouts or micronuclei. An essential contribution of this study was the nevirapine mutagenicity index (Table 3), which was investigated in the meristematic and the F1 regions.

The negative control mutagenicity index was 0.2 ± 0.3. At concentrations of 6.42 ± 0.58; 9.54 ± 0.87 and 17.73 ± 1.31 mg/L of the active pharmaceutical ingredient and 17.68 ± 1.29 mg/L of the nevirapine-based drug, the mutagenicity index were statistically higher than the negative control (mutagenicity index 1.8 ± 1.4; $p = 0.0013$; 1.3 ± 0.9; $p = 0.0017$; 1.0 ± 0.8; $p = 0.0003$; 0.5 ± 0.3; $p = 0.0237$, respectively), indicating that nevirapine was mutagenic for *A. cepa* at these concentrations. At concentrations of 5.48 ± 0.44 and 11.20 ± 1.13 mg/L of the drug, there was no difference regarding the negative control mutagenicity index. Thus, nevirapine was not mutagenic in these cases. Sprouts and micronuclei were observed in the F1 region. However, the most frequently observed abnormality was the presence of micronuclei at all nevirapine concentrations. We could not identify any minicell in any of the slides.

Micronuclei are generally located peripherally in cells and originate from chromosomal breaks and losses and polyploidy not repaired by the cell [54]. Sprouts and micronuclei are formed by expelling excess genetic material, which may originate in the meristematic region. When not repaired by the cell, the genotoxic effects spread and can be observed in the first generation of daughter cells in the F1 region of the radicles, modifying the rate of nuclear alterations [38,39,54].

Indeed, in Table 3, we observed that the nuclear abnormality index (sprouts, micronuclei) in the meristematic region of the negative control was 0.4 ± 0.3 and that, at concentrations of 6.42 ± 0.58; 9.54 ± 0.87, and 17.73 ± 1.31 mg/L of active pharmaceutical ingredient and 5.48 ± 0.44; 11.20 ± 1.13, and 17.68 ± 1.29 mg/L of the nevirapine-based drug, the nuclear abnormality index were statistically higher than the negative control (nuclear abnormality index of 1.4 ± 0.9; $p = 0.0003$; 1.0 ± 0.4; $p = 0.0015$; 1.3 ± 0.9; $p = 0.0094$; 1.0 ± 0.7; $p = 0.0199$; 0.8 ± 1.0; $p = 0.0417$, and 1.3 ± 0.7; $p = 0.0011$, respectively), replicating for all mutagenicity indices calculated for the F1 region, except for concentrations 5.48 ± 0.44 and 11.20 ± 1.13 mg/L of the nevirapine-based drug.

Although in the Ames test with *Salmonella sp.* and *Escherichia coli*, in the mammalian cell mutation test (Chinese hamster ovary - CHO / HGPRT cells), and in the mouse bone marrow micronucleus assay, nevirapine was not considered mutagenic or clastogenic by the International Agency of Research on Cancer (IRTC) [55], the present study showed that the plant species *A. cepa* was sensitive to exposure to this drug.

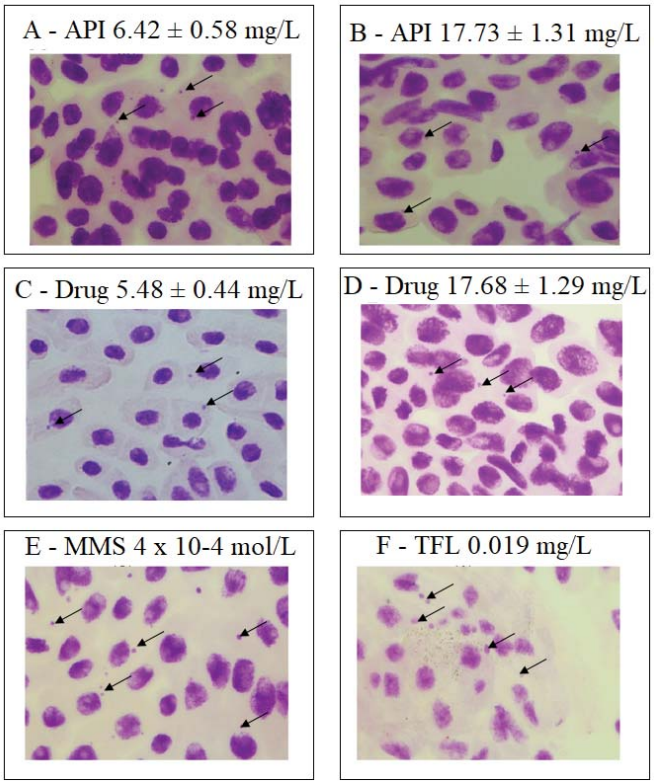

Figure 3: Micronuclei originating in the meristematic region of *A. cepa* rootlets exposed to different concentrations of nevirapine.
Legend: Black arrow = Micronuclei.

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Table 3: Nuclear abnormalities in the meristematic region and the F1 region and Mutagenicity Index among groups of A. cepa seeds exposed to three concentrations of Active Pharmaceutical Ingredient (API) and three concentrations of nevirapine-based drug.

Group	NVP (mg/L)	Nuclear abnormalities (%)			
		Meristematic region (n = 5,000)		F1 Region (n = 3,000)	
		Nuclear abnormality index	p-value	Mutagenicity index	p-value
Negative control	-	0.4 ± 0.3	-	0.2 ± 0.3	-
Positive control (TFL)	-	4.3 ± 4.6	0.0010	8.2 ± 11.6	0.0013
Positive control (MMS)	-	6.9 ± 5.3	0.0001	5.5 ± 6.8	0.0002
API [A]	6.42 ± 0.58	1.4 ± 0.9	0.0003	1.8 ± 1.4	0.0013
API [B]	9.54 ± 0.87	1.0 ± 0.4	0.0015	1.3 ± 0.9	0.0017
API [C]	17.73 ± 1.31	1.3 ± 0.9	0.0094	1.0 ± 0.8	0.0003
Drug [A]	5.48 ± 0.44	1.0 ± 0.7	0.0199	0.3 ± 0.3	> 0.05
Drug [B]	11.20 ± 1.13	0.8 ± 1.0	0.0417	0.5 ± 0.6	> 0.05
Drug [C]	17.68 ± 1.29	1.3 ± 0.7	0.0011	0.5 ± 0.3	0.0237

Legend: Nevirapine concentrations in water, nuclear abnormality index, and mutagenicity index were expressed as mean and standard deviation. Negative control = Purified type I water with adjusted pH of 6.9 ± 0.1; Positive control (TFL) = Trifluralin in water (0.019 mg/L); Positive control (MMS) = Methyl Methanesulfonate in water (4 x 10^{-4} mol/L).

The results showed cytotoxic, genotoxic, and mutagenic effects that can be reproduced in other species, especially when considering the nevirapine bioconcentration shown for the lettuce (*Lactuca sativa*) plant species [13]. In this sense, one should be concerned about the possible environmental residues of nevirapine contaminating medicinal plants and other vegetables, which deserve attention when controlling their release and mitigation in water and effluent treatment plants, which may affect directly and indirectly human health.

Nevirapine solubility was one of the principal challenges for the study. However, the validity of the results obtained is in the design of the study certifying the actual concentration of nevirapine to which the seeds were exposed for germination and root growth, besides the application of protocols internationally accepted that allowed confirming the results obtained. Although only one nevirapine-based drug was evaluated, this study showed that the concentration of 9.54 ± 0.87 mg/L of the active pharmaceutical ingredient promoted a similar response to the concentration of 11.20 ± 1.13 mg/L of the nevirapine-based drug, indicating that the excipients did not interfere with the toxicity observed for nevirapine only at this concentration.

The concentration of 6.42 ± 0.58 mg/L of active pharmaceutical ingredient was not considered cytotoxic based on germination index, root growth, and mitotic index but was genotoxic and mutagenic based on chromosomal abnormality and mutagenicity indices. Genotoxicity was observed at all concentrations tested, although only at concentrations of 6.42 ± 0.58; 9.54 ± 0.87, and 17.73 ± 1.31 mg/L of the active pharmaceutical ingredient and the concentration of 17.68 ± 1.29 mg/L of the nevirapine-based drug, the mutagenic potential was observed. nevirapine at a concentration of 17.68 ± 1.29 mg/L of the drug was considered cytotoxic, genotoxic, and mutagenic.

Conclusion

The drugs have therapeutic effects, but they can also cause dose-dependent or not toxic effects, such as cytotoxicity, genotoxicity, and mutagenicity demonstrated for *A. cepa* and that can threaten other species. The continuous exposure of the environment to micropollutants such as nevirapine can be mitigated with investments in sanitary sewage, effluent treatment plants, awareness, and health education for the proper disposal of drug residues. Besides nevirapine's genotoxic and mutagenic effects shown in this work, several other micropollutants can contribute to measuring the environmental risk resulting from industrial production and human environmental consumption of medicines.

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