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Evaluating the Toxicity and Genotoxicity of Naproxen and Ketoprofen Using Factorial Design: A Study with *Artemia salina* and *Allium cepa*

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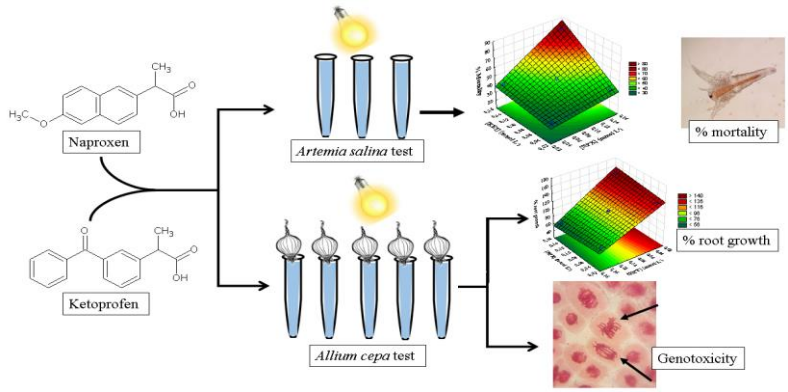
The nonsteroidal anti-inflammatory drugs (NSAID) are excreted unchanged in the environment that can have toxic effects on living organisms. Among these drugs, naproxen and ketoprone are widely used. Thus, a study was proposed to evaluate the interaction between different concentrations (mmol L^{-1}) of the variables naproxen [NPX] and ketoprone [KET] against the acute toxicity of the *Artemia salina* (*A. salina*) and *Allium cepa* (*A. cepa*) applying the 2^2 factorial design. Responses were used: percent *A. salina* mortality (% mortality) and *A. cepa* root growth (% root growth). For *A. salina*, after 72 h of exposure with ([NPX] and [KET] = 0.15 mmol L^{-1}) caused an 80% mortality. While *A. cepa* root growth was higher with ([NPX] and [KET] = 0.03 mmol L^{-1}) exhibiting 133.58% root growth. However, genotoxicity was shown by the highest frequency of the values of chromosomal alterations (CA) with 46.8% CA±9.16, when compared with the negative control equal 12.6% CA±6.39. Thus, from the test ($p<0.05$) with the p-values of 0.0302. The lower concentrations showed necrosis and micronuclei with 1.82%±1.66 apoptotic index and 5.4%±1.40 micronuclei for 5025 cells counted. Therefore, drugs demonstrated high *A. salina* acute toxicity and potential genotoxic and mutagenic effect for *A. cepa* based.

Graphical abstract



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1. Introduction

Drugs are substances designed to cause a specific and beneficial biological effect both in animal and human health care. However, some drugs used in human and veterinary medicine are not completely metabolized and, thus, can be

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excreted unchanged or with metabolites that exhibit biological activity. These substances are resistant to degradation in the environment or in sewage treatment plants (STPs) because they are considered persistent compounds. Thus, when these compounds enter the environment, they can cause harmful effects on aquatic organisms and humans, due to the biological activity that they still exhibit [1-3].

Among drug classes, non-steroidal anti-inflammatory drugs (NSAIDs) are pharmaceutical compounds with anti-inflammatory, analgesic, and antipyretic effects, attracts the attention of the scientific community, because substances used indiscriminately and are considered persistent compounds in the range of millions of tons back into the environment. Some NSAIDs have become one of the most prescribed and consumed pharmacological substances in modern medicine, such as naproxen (NPX), ibuprofen (IBU), diclofenac (DCF), ketoprofen (KET), and phenazone (PHE) [1, 2, 4, 5].

The most commonly detected and quantified drugs in the aquatic environment and among the most used NSAIDs worldwide are naproxen (NPX) and ketoprofen (KET) (Fig 1). Thus, were chosen as the pharmaceuticals for our study, due to the continuous release of these drugs into the environment [6]. Fig. 1a shows the structure of NPX (drug) with molecular formula $C_{14}H_{14}O_3$, molar mass 230.3 g mol^{-1} and log Kow 3.18. From this the drugs naproxen (NPX) was quantified in surface waters in the concentration range of 1.0-32.8 µg L^{-1} in the countries of Canada, China and France [5, 7]. KET, which has the molecular formula $C_{16}H_{14}O_3$, molar mass 254.3 g mol^{-1}, log Kow 3.12 and molecular structure (Fig. 1b) has already been quantified in surface waters in the concentration range of 1.0-190 µg L^{-1} in studies carried out in China, USA, Spain [5, 7].

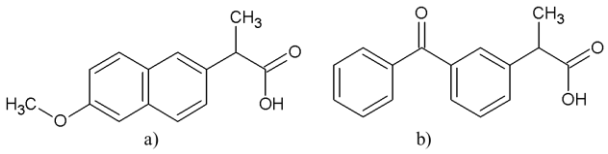

Fig. 1. Molecular structure a) naproxen and b) ketoprofen.

The presence of NSAIDs, both in aquatic ecosystems and in soil, has the capacity to alter biochemical reactions, genotoxicity, endocrine disruption, locomotive disorders, biomass composition, as well as the metabolic and enzymatic processes, can have toxic effects on living organisms based on their high bioactivity [8, 9, 10]. In addition, it is important to highlight that some authors report report a noteworthy relationship between to the synergistic or antagonistic effects on the toxicity of substances that are considered persistent [6, 9, 11, 12, 13]. In this context, studies aimed at combining substances, such as drugs, to counteract toxicity are relevant and, therefore, form the starting point of the present research project.

To evaluate the toxicity levels of several substances the *Artemia salina* (*A. salina*) microcrustacean, a popular model organism, due to being a substance-sensitive test for persistent pollutants, short generation time, ease of culture, the commercial availability of its cysts, and it is also a biological model with acceptance already established in the scientific field [11, 14, 15, 16, 17]. Furthermore, it is an organism that does not belong to the Chordata phylum and, therefore, it is not necessary to submit the project to the ethics committee based on Brazilian Federal Law Nº. 11,794/08. Moreover, another widely used organism in bioassays is *Allium cepa* (*A. cepa*) assay provides for cytotoxicity and genotoxicity assessment in the samples taken from the environment, plant extracts, and chemical substances. Because of its easy handling, low cost, greater sensitivity, and an interesting correlation with mammalian test systems *in vitro*. Therefore, the use of the *Allium cepa* bioassay is important to evaluate the chromosomal alterations that substances can produce in the test organism [18-20]. However, some NSAIDs such as ibuprofen, paracetamol, ketoprofen, naproxen can have an effect on promoting growth in the roots of plants such as *Lactuca sativa*, spring barley, rice (*Oryza sativa. L*) and *A. cepa* [9, 21, 22]. This behavior is concentration-dependent and may present chronic toxicity in the tested organism, being the starting point for our study.

Therefore, in the present study the objective was to analyze the interaction on the toxicity of naproxen and ketoprofen, applying a 2^2 factorial design with added center point, employed to bioassays for the *A. salina* microcrustacean and *A. Cepa*. In addition, cytotoxicity and genotoxicity analysis was performed using A. cepa assays (*A. cepa* root growth), with a highlight on genotoxicity in the research.

2. Material and Methods

2.1 Reagents

The bioassays were performed with naproxen (NPX) and ketoprofen (KET) (99% Galena Chemical and Pharmaceutical – Brazil). The synthetic seawater used for the test with *A. salina* was commercial. Meanwhile, reagents for the preparation of solutions were purchased from Synth: hydrochloric acid (37% P.A.), sodium hydroxide (P.A.), glacial acetic acid (100% P.A.), and ethyl alcohol (99.5% P.A.). For the study with *A. cepa*, the dye orcein P.A. was used from Dinâmica.

2.2 Acute toxicity to *Artemia salina*

Acute toxicity (*A. salina* tests) was carried out with microcrustacean larvae hatched in synthetic seawater (32 g L^{-1}), at pH 9 (±0.2), aerated for 48 h. Thus, the bioassays were performed in triplicate (10 individuals per replicate), at 20 ± 2 °C, with a 16 h light and 8 h dark photoperiod for 72 hours in a static system with 10 mL solution for each test, where dead larvae were counted for each test. The NPX and KET solutions were prepared at pH 9 (±0.2) to increase water solubility. Moreover, the experiments were carried out with synthetic seawater as the sample without dilution, where the mortality value for application in the experimental design was for the solution at 100% [11, 23, 24].

2.3 Cytotoxicity and genotoxicity evaluation of *Allium cepa*

The *A. cepa* toxicity test employed equal-sized commercial onion bulbs, cleaned, washed and acclimated in tap water for 24 hours. Equal-sized bulbs were exposed to the solutions for 48 hours with five replicates for each drug mixture. The measure the length of the three longest roots (to calculate their average length) of each onion, it was possible to compare the test with the negative control. It is worth mentioning that the response evaluated was *A. cepa* root growth [11, 25, 26].

Thus, the NPX and KET solutions were prepared at pH 9 (±0.2) to increase water solubility, and a negative control group with distilled water at a basic pH equal to that drug solutions. The *A. cepa* bioassays were performed in

quintuplicate analysis (5 bulbs per experiment), at 20 ± 2 °C, with a 16 h light and 8 h dark photoperiod for 48 hours of exposure [11, 25, 26].

Cytotoxicity and genotoxicity produced by naproxen and ketoprofen can be determined by the mitotic index (MI) and chromosome alteration (CA). Thus, roots were cut and suspended in Carnoy solution for 24 hours and thereafter preserved in 70% ethanol. Then, root tips were hydrolyzed in HCl (1 mol L^{-1}) under heating at 60 ºC for 10 min, followed by the addition of orcein (2%) and crushing against microscopy slides. The percentage mitotic index (%MI) for each bulb was calculated as the ratio of the number of dividing cells for 1,000 cells. Furthermore, 200 cells at anaphase/telophase were studied for the presence of chromosome alteration (CA), with five slides per sample were analyzed. Thus, one for each onion (5,000 cells) was analyzed for cell division (MI) and 1,000 cells for CA experimental condition [11, 25, 26].

2.4 Experimental design

The experimental design applied was a factorial design with an added center point based on response surface methodology (RSM). Thus, it was employed to evaluate the interaction between naproxen and ketoprofen on acute toxicity in *A. salina* and *A. cepa*. It is important to mention that experimental design is a statistical strategy for organizing, reducing, and mainly to study the interaction of variables [11, 16, 27].

The combination of naproxen and ketoprofen induced *A. cepa* root growth. Therefore, root growth was used as the response to evaluate toxicity to *A. cepa*. The responses used to analyze toxicity in the combination of the drugs were: percent *A. salina* mortality (% mortality) and percent *A. cepa* root growth (% root growth). The independent variables were the concentrations of naproxen ([NPX] (mmol L^{-1})) and ketoprofen ([KET] (mmol L^{-1})), following the methodology applied in studies conducted by our research group Nolasco et al. (2023) [11], Svobodníková et al (2020) [28], Wang et al (2020) [21].

Furthermore, the drug concentration values used in the experimental design were based on toxicity studies performed by the authors Svobodníková et al (2020) [28], Wang et al (2020) [21], Nolasco et al. (2023) [11] and Pawłowska et al. (2023) [9]. A 2^2 factorial design was then constructed, with a total of seven experimental combinations: four cube points and a triplicate at the center point [11, 27]. The Table 1 shows the variables and levels with the concentration range of 0.03 to 0.15 mmol L^{-1} for *A. saline* and *A. cepa* bioassays. To generate the experimental matrix was employed Statistica 10 software (StatSoft, Tulsa, USA).

Table 1. Levels of the 2^2 factorial design with an added center.

Bioassay	Variables	Levels		
		-1	0	+1
Artemia salina **and** ***Allium cepa***	**[NPX] (mmol L^{-1})**	0.03	0.09	0.15
	[KET] (mmol L^{-1})	0.03	0.09	0.15

3. Results and Discussion

3.1 Factorial design with an added center point

The results obtained for the seven experiments with a factorial design with added center point are summarized in Table 2 from the independent variables (Table 1). Based on the response surface methodology, the following polynomial Equation 1 was deduced to describe the interaction between independent and dependent variables:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{1 \le i \le j}^{k} \beta_{ij} X_i X_j \quad (1)$$

where k represents the number of variables, Y is the dependent variable (percentage *A. saline* mortality and *A. cepa* root growth), and β_0, β_i, β_{ij}, denote the regression coefficients for the linear effects related to the linear X_i and X_iX_j interaction terms.

Table 2. Observed and predicted values of the percentage of mortality and *A. cepa* root growth, using different combinations factorial design with added center point.

Exp.*	[NPX] (mmol L^{-1})	[KET] (mmol L^{-1})	% Mortality		[NPX] (mmol L^{-1})	[KET] (mmol L^{-1})	% root growth	
			Obs.	Prev.			Obs.	Prev.
1	0.03	0.03	30	29.64	0.03	0.03	133.58	133.1
2	0.15	0.03	40	39.64	0.15	0.03	148.12	147.64
3	0.03	0.15	40	39.64	0.03	0.15	70.93	70.45
4	0.15	0.15	80	79.64	0.15	0.15	62.41	61.93
5	0.09	0.09	45	47.14	0.09	0.09	105.2	103,.28
6	0.09	0.09	45	47.14	0.09	0.09	100.2	103.28
7	0.09	0.09	50	47.14	0.09	0.09	102.51	103.28

*Experiments (Exp.).

3.2 Percentage *A. salina* mortality and *A. cepa* root growth response

Responses for percentage mortality and root growth the predicted by factorial design with added center point were generated as arithmetic averages with ±95% confidence limits. Figures 2a and 2b depict the relationship between predicted values (red line) and observed values (blue points). In addition, the correlation coefficients (R^2) and adjusted correlation coefficients (R^2_{adj}) were determined from the observed and predicted values, respectively. The R^2-values between 0.988 and 0.997, as well as adjusted correlation coefficients (R^2_{adj}) between 0.976 and 0.995, for % mortality and % root growth, respectively, demonstrated good agreement for the experiments [11, 27, 29]. Figures 2c and 2d show the residuals that correspond to the difference between predicted and observed results. Thus, the appropriateness of the responses was evidenced by the corresponding expected normal value (red line) that varies linearly with the residuals (blue points) [11, 27, 29].

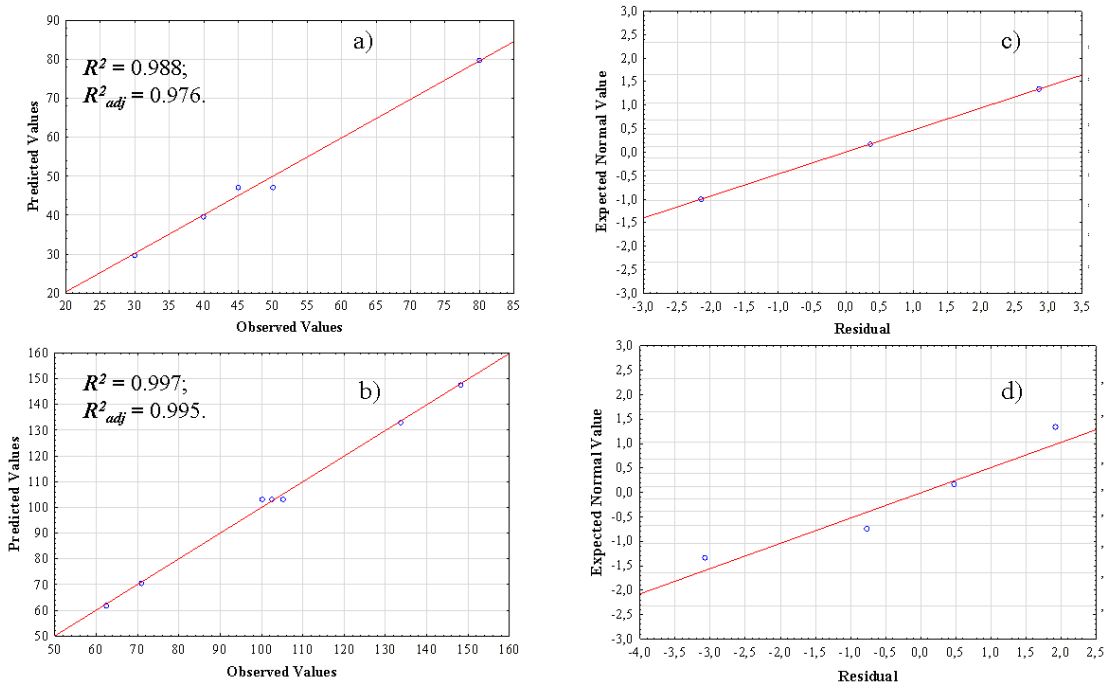

Fig. 2. The predicted and observed values for the percentage of a) % Mortality; and b) % root growth. Residual plots for the responses of the percentages of c) % Mortality; and d) % root growth.

Another analysis performed on the model was the analysis of variance (ANOVA) for the influence of each independent variable, their interactions, and curvature. Thus, the curvature analysis will make it possible to determine whether there is a possibility of using a central composite design (CCD) [30].

The ANOVA results of the linear regression model obtained for percentages of *A. salina* mortality and *A. cepa* root growth are shown in Table 3. Thus, analyzing Table 3, the sum of squares (SS), which measures the influence of the corresponding variable on the variation of the response values, the degrees of freedom (df), which corresponds to the number of columns of responses obtained, and the ratio between SS and df, which is the related mean of the squares (MS), are presented. Based on a probability level 95%, high F-values and low p-values (lower than 0.05) are evidence of the statistical significance for a model [31, 32]. From this analysis, the curvature was not statistically significant, with low F-value equal to 0.153 and 0.345 with high p-value of 0.742 and 0.616 for the percentages of mortality and root growth responses, respectively. Therefore, it was not necessary to apply the central composite design model to the study [11, 30, 33].

Table 3. ANOVA table results for factorial design 2^2 obtained for percent *A. salina* mortality and *A. cepa* root growth responses.

Response	Factor	SS	df	MS	F-value	*p*-value
%mortality	Curvatr.	1.190	1	1.1905	0.153	0.742
	[NPX] (mmol L^{-1})	625.00	1	625.00	75.00	0.013
	[KET] (mmol L^{-1})	625.00	1	625.00	75.00	0.013
	[NPX] *by* [KET]	225.00	1	225.00	27.00	0.035
	Error	16.67	2	8.33		
	Total SS	1492.86	6			
%root growth.	Curvatr.	2.163	1	2.163	0.345	0.616
	[NPX] (mmol L^{-1})	9.060	1	9.060	1.447	0.352
	[KET] (mmol L^{-1})	5502.67	1	5502.67	878.74	0.001
	[NPX] *by* [KET]	132.94	1	132.94	21.23	0.044
	Error	12.524	2	6.26		
	Total SS	5659.36	6			

SS: Sum-of-Square; df: degree of freedom; MS: Mean Square.

3.3 Percent *A. salina* mortality response

Note that in Fig. 3a, the drug concentration variables were statistically significant with a p-value less than 0.05 ($p<0.05$). In addition, the interaction was significant and, thus, the surface plot based on the dependent variable (%mortality) was generated (Fig. 3b) with 1by2 - [NPX] *by* [KET].

Analyzing Fig 3b, the highest concentrations of naproxen and ketoprofen ([NPX] = 0.15 mmol L^{-1} and [KET] = 0.15 mmol L^{-1}) produced a higher mortality for *A. salina* neonates with 80%. While, in the experimental combination with the lowest concentrations ([NPX] = 0.033 mmol L^{-1} and [KET] = 0.033 mmol L^{-1}) showed 30% mortality. Thus, the increase in both drugs showed a higher mortality and, consequently, an effect on acute toxicity. Moreover, the combinations in experiments 2 and 3 was observed: (*i*) concentration of [NPX] = 0.03 mmol L^{-1} and [KET] = 0.15 mmol L^{-1}, produced 40% mortality; and (*ii*) concentration of [NPX] = 0.15 mmol L^{-1} and [KET] = 0.03 mmol L^{-1}, generated 40% mortality. The toxicity against the microcrustacean is correlated with the increase in the concentration of both drugs, i.e., a synergistic effect is observed.

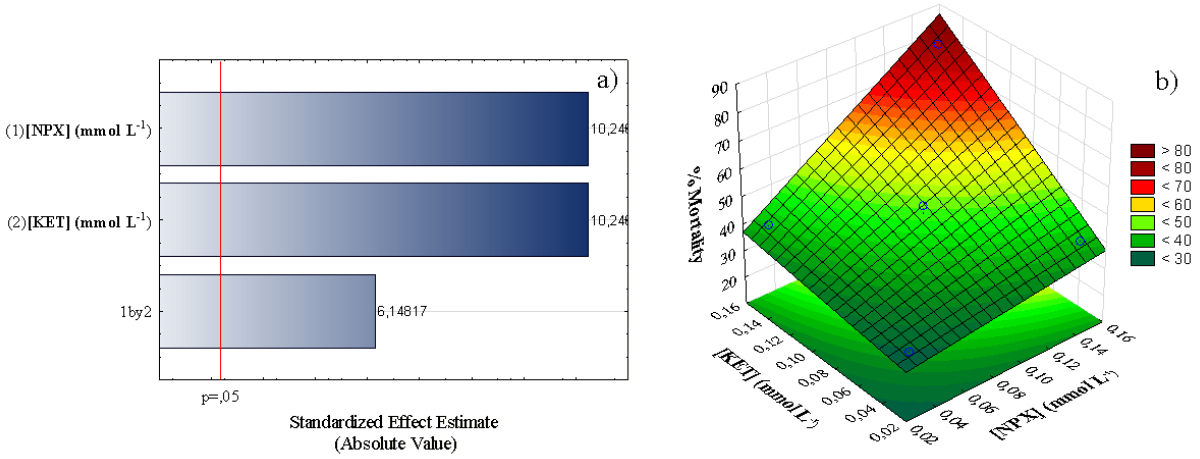

Fig. 3. a) Pareto chart; b) Response surface plot for percent *A. salina* mortality ([NPX] *vs* [KET]).

It is worth mentioning that there are some toxicity studies for aquatic organisms for naproxen drug, such as acute toxicity for *Thamnocephalus platyurus* and *Ceriodaphnia dubia* crustaceans with LC_{50} equal 62.48 and 84.09 mg L^{-1}, respectively [34]. Furthermore, the ketoprofen drug showed high acute toxicity at a concentration of 632.30 ± 10.10 mg L^{-1} for 96 h to embryonic stages of zebrafish (*Danio rerio*) [35].

From the data, Equation 2 shows the estimated regression coefficients of the generated empirical model of percent mortality, considering variables and their mutual relationships.

$$Y_{\%mortality} = 47.14 + 25X_{[NPX]} + 25X_{[KET]} + 15X_{[NPX]}X_{[KET]} \quad (2)$$

where $X_{[NPX]}$ and $X_{[KET]}$ represent naproxen and ketoprofen concentration variables, respectively, and $Y_{\%mortality}$ is the percentage of mortality response. In addition, the effect of variables can be analyzed based on the values and signs of the estimated regression coefficients.

Analyzing the signs of the coefficients (Equation 2), *A. salina* mortality was found to increase with increasing naproxen and ketoprofen concentrations, due to the positive coefficients. Based on the study Gheorghe et al (2016) [7], these the drugs have acute toxicity for microcrustaceans, for which the EC_{50} values for the *Daphnia magna* microcrustacean were 46.72 and 43.65 mg L^{-1} for naproxen and ketoprofen, respectively, in 48 hours of exposure.

The relationship between [NPX] and [KET] concentrations, was observed to have a positive coeficiente, i.e., a synergistic effect was demonstrated in the interaction between the variables with the experiments: (*i*) concentration of [NPX] = 0.03 mmol L^{-1} with [KET] = 0.03 mmol L^{-1}, produced 30% mortality; while (*ii*) concentration of [NPX] = 0.15 mmol L^{-1} with [KET] = 0.15 mmol L^{-1}, generated 80%. Comparing with the experiments in which one of the drug concentrations was increased (experiments 2 and 3, Table 2), the mortality was the same, with 40% *A. salina* mortality. Therefore, the simultaneous increase in the concentrations of both drugs affects mortality in the toxicity.

3.4 Percent *A. cepa* root growth response

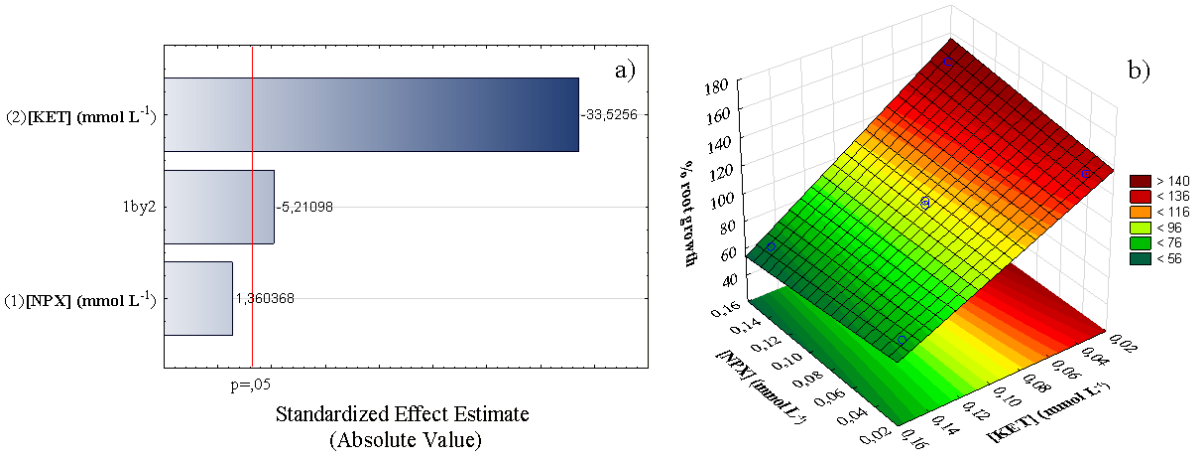

Fig. 4. a) Pareto chart; b) Response surface plot for percent *A. cepa* root growth ([NPX] *vs* [KET]).

Fig. 4a, the variable [KET] concentration and the relationship between [NPX] (mmol L^{-1}) with [KET] (mmol L^{-1}) were statistically significant with $p<0.05$. Thus, the surface plot based on the dependent variable (% root growth) was generated (Fig. 4b) with 1by2 - [NPX] *by* [KET].

Based on Fig. 4b, the lowest concentration levels ([NPX] = 0.03 mmol L^{-1} and [KET] = 0.03 mmol L^{-1}) produced 133.58 % root growth. In contrast, increasing the highest levels concentration ([NPX] = 0.15 mmol L^{-1} and [KET] = 0.15 mmol L^{-1}) provided an inhibition in *A. cepa* root growth, exhibiting

62.41 % root growth. Thus, demonstrating that by increasing the concentration of both drugs, a cytotoxicity was evidenced, i.e., hormesis effect was observed for both drugs. The phenomenon of hormesis is that at a higher concentration, an inhibition effect occurs; on the other hand, at a lower concentration, radicular growth in the plant is observed. Thus, the promotion of enzyme activity can be induced with low concentrations that promote growth of the plant [36, 37].

Analyzing the experiments 2 and 3 (Table 2) with the following combinations: (*i*) concentration of [NPX] = 0.15 mmol L^{-1} with [KET] = 0.03 mmol L^{-1}, obtained 148.12% root growth; and (*ii*) concentration of [NPX] = 0.03 mmol L^{-1} with [KET] = 0.15 mmol L^{-1}, exhibited 70.93% root growth. The hormesis effect on *A. cepa* root growth was most observed with naproxen than ketoprofen. Nonetheless, in experiment 4 ([NPX] = 0.15 mmol L^{-1} and [KET] = 0.15 mmol L^{-1}) a smaller growth with 62.41 % root growth, i.e, the increase in both concentrations results in increased inhibition. This behavior in low concentrations was observed by other authors. Svobodníková et al (2020) [28] presented in their studies that NPX affected the length of roots in pea plants, in which the concentration of 0.5 mg L^{-1} increased the root length by 30% compared to the control. Another work carried out by Wang et al (2020) [21] for the ketoprofen drug showed that low concentrations (0.5 mg L^{-1}) stimulated the growth of rice seedlings. While high concentrations (20 mg L^{-1}) significantly inhibited root growth.

The generated empirical model for $Y_{\%irootgrowth}$ expressed as a function of the concentration variable and their mutual relationship defined above, was given by Equation 3:

$$Y_{\%irootgrowth} = 103.3 - 74.2.7X_{[KET]} - 11.5X_{[NPX]}X_{[KET]} \quad (3)$$

where $X_{[NPX]}$ and $X_{[KET]}$ represent naproxen and ketoprofen concentration variables, respectively. While $Y_{\%irootgrowth}$ is the percentage *A. cepa* root growth response.

The negative coefficients for the ketoprofen concentration variable (Equation 3) indicate that *A. cepa* root growth is improved at lower concentrations this variable, because ketorpofen produced a greater inhibitory effect against the plant organism. Compared with the literature, ketoprofen inhibited root growth in rice seedlings at a concentration of 20 mg L^{-1} Wang et al 2020 [21]. These authors evaluated the biomarker Malondialdehyde (MDA) generated from oxidative damage to lipid membranes by lipid peroxidation in cells. Thus, increase of 3.25 times was observed in relation to the control at a concentration of 20 mg L^{-1} ketoprofen exposure; this increased oxidative stress in plants leads to increased cytotoxicity. Another study carried out by authors Pawłowska et al (2023) [9] observed that the ketoprofen and the mixture of ketoprofen with ibuprofen encouraged the germination of spring barley seeds at a concentration of 50 mg L^{-1}. However, growth inhibition began at 100 mg L^{-1}.

In relation the interaction between [NPX] and [KET] variables, an antagonistic effect (negative coefficient) was demonstrated, as can be observed with the following combinations: (*i*) concentration of [NPX] = 0.15 mmol L^{-1} with [KET] = 0.03 mmol L^{-1}, obtained 148.12% root growth; and (*ii*) concentration of [NPX] = 0.03 mmol L^{-1} with [KET] = 0.15 mmol L^{-1}, exhibited 70.93% root growth. Thus, when comparing the two drugs in terms of root growth, ketoprofen caused more root inhibition, resulting in a reduction in *A. cepa* root growth. This behavior may be correlated with the increased oxidative stress that ketoprofen produced in the target organism, as compared to the study carried out by the authors Wang et al (2020) [21]. In this sense, naproxen and ketoprofen encouraged the *A. cepa* root growth, which was concentration-dependent. However, an analysis at the cellular level is extremely relevant to assess chronic toxicity to the organism.

3.5 Cytotoxicity and genotoxicity analysis with *A. cepa*

Cytotoxic effects were estimated based on the ratio between the number of dividing cells and the total number of cells, the mitotic index (MI) for 1,000 cells. While, genotoxicity defined as damage to genetic material produced by a chemical, was determined based on the frequency of chromosomal alterations (CA) in the mitotic *A. cepa* anaphase-telophase stages. The calculation was obtained by dividing the number of CA by 200 cells in anaphase/telophase counted per slide. Thus, the frequency of CA was compared with the negative control in order to assess the increase in chromosomal alterations [19, 38, 39].

Based on cell divisions, an analysis of the mitotic index (% MI) can be carried, as shown in Table 4. Therefore, when comparing the mitotic index of experiment 1 (value equal to 5.52 %MI) with experiment 4 (value of 3.32 %MI), a statistically significant difference was observed with a p-value of 0.0008 ($p<0.05$), i.e., demonstrating that the increase in both drugs produced *A. cepa* cytotoxicity by decreasing cell divisions in mitosis.

From this, an analysis was carried out on experiments 1 and 4 to evaluate chromosomal alterations. Table 4 shows the results regarding the frequency of mitotic alterations for the following experiments: (*i*) negative control distilled water at basic pH (pH 8-9); (*ii*) experimente 1: with [NPX] = 0.03 mmol L^{-1} and [KET] = 0.03 mmol L^{-1} produced 133.58 % root growth; and (*iii*) experimente 4: with [NPX] = 0.15 mmol L^{-1} and [KET] = 0.15 mmol L^{-1} produced 62.41 % root growth.

Table 4. Mitotic index (MI) and chromosomal alterations (CA) of *A. cepa* in experiments.

Experiment	*A. cepa* Root length (cm)[a]	(%) MI[b]	CA[c]	(%)CA[c]
Negative Control	1.33± 0.06	4.84 ± 0.15	0.126± 0.06	12.6± 6.39
Experiment 1	3.11± 0.09	5.52±0.25	0.468± 0.09	46.8± 9.16
Experiment 4	2.16± 0.08	3.32±0.36	0.264± 0.06	26.4± 5.94

a. Root length: data expressed as mean ± standard deviation for five replicates. b. Mitotic Index: the mean ± standard deviation obtained from 1000 cells for five replicates. c. Chromosomal Alterations: data obtained from 200 cells and expressed as mean ± standard deviation for five replicates.

Table 4 shows that genotoxicity was exhibited by the highest frequency of chromosomal alterations, where experiment 1 showed 46.8 % CA and experiment 4 equal the 26.4 %CA, when compared with the negative control (12.6% CA). Thus, the significance was tested ($p < 0.05$) with p-values of 0.0302 and 0.0077 for experiments 1 and 4, respectively.

Another important piece of information is that the slides obtained in experiment 1 showed necrosis and micronuclei, with a mean and standard deviation 1.82% ± 1.66 for apoptotic index and 5.4 ± 1.40 for micronuclei (for 5025 cells counted), which were not observed in the negative control. Micronucleus analysis provides information on the mutagenic potential, where the combination of drugs enabled chromosomal alterations through the breakdown of genetic material (clastogenic) and/or caused a disturbance in the mitotic process (aneugenic), thus resulting in the formation of micronuclei [36]. Therefore, it was evident that although the combination of drugs encouraged *A. cepa* root growth, chronic toxic effects such as chromosomal alterations, apoptosis, and micronuclei were increased.

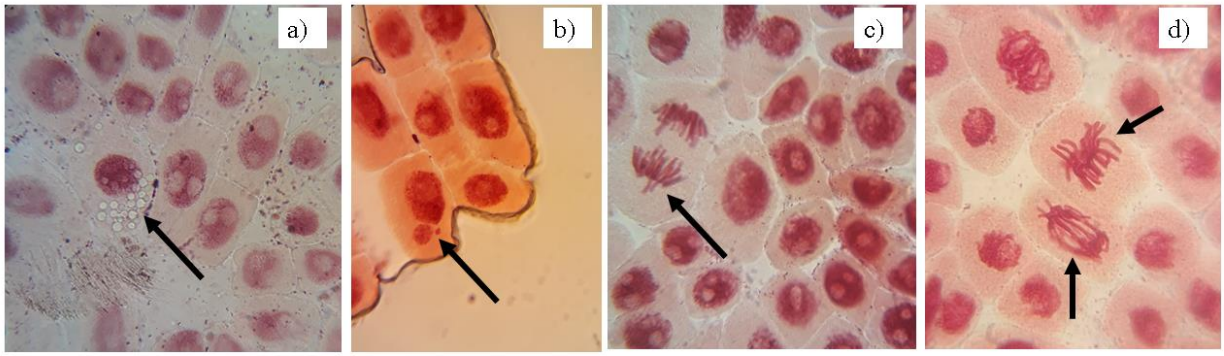

Fig. 5. Chromosomal alterations in *Allium cepa* meristem cells treated with experimente 1 with [NPX] = 0.03 mmol L^{-1} and [KET] = 0.03 mmol L^{-1}: a) necrotic cells; b) micronucleus; c) vagrant chromosomes; d) naphase bridge and sticky chromosomes.

Genotoxic effects were observed (Fig. 5), such as necrotic cells, micronuclei, sticky chromosomes, vagrant chromosomes, naphase bridge, and sticky chromosomes by the combination of drugs NPX and KET, mainly in the experiment with the lowest concentrations ([NPX] = 0.03 mmol L^{-1} and [KET] = 0.03 mmol L^{-1}). It is known that DNA damage can occur in two ways: complex DNA damage and/or simple DNA damage. Complex DNA damage is much more difficult to repair due to the lesions in the DNA, which can directly or indirectly induce double-strand breaks (DSBs). In addition, an increase in reactive species can affect different biomolecules and genetic material [39].

In this sense, oxidative stress and membrane damage are a possible mechanism of phytotoxicity caused by xenobiotics. Because the test plant produces enzymatic and non-enzymatic defense mechanisms to reduce the effects of ROS [21]. From this, the authors Wang et al (2020) [21] observed that ketoprofen triggers excessive reactive oxygen species (ROS) formation, resulting in cell structure damage and an increase in oxidative stress in the root growth in rice seedlings. These authors observed an increase in the activity of the superoxide dismutase enzyme (SOD) that was 10.99 times greater than the negative control at a concentration of 10 mg L^{-1} KET. It is worth mentioning that the concentration in experiment 1 ([NPX] = 0.03 mmol L^{-1} and [KET] = 0.03 mmol L^{-1}) of our study was 7.63 mg L^{-1} KET.

In addition, Pawłowska et al. (2023) [9] conclude that the ketoprofen drug and the mixture of ketoprofen with ibuprofen caused oxidative stress in the spring barley, with na increase in the content of H_2O_2 and increase in the activity of the catalase enzyme. Other authors, Svobodníková et al. 2020 [28], evidenced an increase in production of ROS, in our case hydrogen peroxide (by 33%) and superoxide (by up to 62% as against control) under 10 mg L^{-1} NPX in pea plant roots. Thus, the increase in *A. cepa* root growth may be indicative of an increase in chromosomal alterations resulting from damage to the genetic material, which for the combination of drugs led to an increase in the genotoxic and mutagenic effects against the target organism.

4. Conclusions

In this context, it was possible to observe that the experimental design with a central point evaluated the interaction between the concentrations of the naproxen and ketoprofen, against the mortality of the *A. salina* microcrustacean, and cytotoxicity and genotoxicity for *A. cepa* as dependent on the concentration. The concentration of [NPX] = 0.15 mmol L^{-1} with [KET] = 0.15 mmol L^{-1} generated 80% *A. salina* mortality. Furthermore, naproxen encouraged *A. cepa* root growth more and exhibited the hormesis effect only at the highest ketoprofen concentration. Another important point was that the *A. cepa* root growth became a relevant response for the evaluation of genotoxicity with an increase in chromosomal alterations. Thus, the combination with the lowest concentrations ([NPX] = 0.03 mmol L^{-1} and [KET] = 0.03 mmol L^{-1}) provided greater *A. cepa* genotoxicity and mutagenic potential. In addition, the study demonstrated the importance of evaluating the combination of substances in order to understand the interaction of drugs and contribute to the risk assessment and management of pharmaceutical products that enter the environment.

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Author Contributions

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Conceptualization, Methodology, Resources, Writing -Review & Editing. Amilcar Machulek Jr: Conceptualization, Methodology, Writing -Review & Editing.

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