Hindawi
Adsorption Science & Technology
Volume 2023, Article ID 9861072, 1 page
https://doi.org/10.1155/2023/9861072

Retraction

Retracted: *In Vitro* Evaluation of the Cytotoxic Potential of Environmental Contaminant Mixtures Present in Water for Human Use

Adsorption Science and Technology

Received 3 October 2023; Accepted 3 October 2023; Published 4 October 2023

This article has been retracted by Hindawi following an investigation undertaken by the publisher [1]. This investigation has uncovered evidence of one or more of the following indicators of systematic manipulation of the publication process:

(1) Discrepancies in scope

(2) Discrepancies in the description of the research reported

(3) Discrepancies between the availability of data and the research described

(4) Inappropriate citations

(5) Incoherent, meaningless and/or irrelevant content included in the article

(6) Peer-review manipulation

The presence of these indicators undermines our confidence in the integrity of the article's content and we cannot, therefore, vouch for its reliability. Please note that this notice is intended solely to alert readers that the content of this article is unreliable. We have not investigated whether authors were aware of or involved in the systematic manipulation of the publication process.

Wiley and Hindawi regrets that the usual quality checks did not identify these issues before publication and have since put additional measures in place to safeguard research integrity.

We wish to credit our own Research Integrity and Research Publishing teams and anonymous and named external researchers and research integrity experts for contributing to this investigation.

The corresponding author, as the representative of all authors, has been given the opportunity to register their agreement or disagreement to this retraction. We have kept a record of any response received.

References

[1] I. J. Hakeem and N. Amare, "*In Vitro* Evaluation of the Cytotoxic Potential of Environmental Contaminant Mixtures Present in Water for Human Use," *Adsorption Science & Technology*, vol. 2022, Article ID 3102007, 14 pages, 2022.

Hindawi
Adsorption Science & Technology
Volume 2022, Article ID 3102007, 14 pages
https://doi.org/10.1155/2022/3102007

Research Article

In Vitro Evaluation of the Cytotoxic Potential of Environmental Contaminant Mixtures Present in Water for Human Use

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Received 1 August 2022; Accepted 11 September 2022; Published 3 October 2022

Academic Editor: Rabia Rehman

Chemicals from Pharmaceuticals and Personal Care Products (PPCPs) have become much more prevalent in the environment in recent years. The effects of these substances on human health and the environment are frequently debatable because they typically have poorly understood mechanisms of toxic action. For this reason, we set out to evaluate a binary mixture consisting of butylhydroxyanisole (BHA) and propylparaben (PPB), two approved additives that have been found in the environment and have a range of health-related effects. Based on our prior research, we chose an experimental model called Vero cells, kidney fibroblasts from the African green monkey (*Chlorocebus aethiops*), with high sensitivity in toxicology studies and ideal characteristics for the analysis of chemical compounds' mechanisms of action. The experimental design includes a battery of tests using many complimentary biochemical and morphological biomarkers, the usefulness of which we have previously established. The outcomes demonstrated that the mixtures of BHA and PPB cause significant functional alterations brought on by osmotic imbalance, which are connected to irregularities in cell cycle progression, increases in ploidy, which included cell cycle imbalances as well as increases in proliferation. On the other hand, we have been able to show that the quantitative estimate of the anticipated cellular responses generally does not adjust precisely to the observed effect through the analysis of the prediction of the combined effect using mathematical models. However, once the individual compounds' respective mechanisms of action have been established, the toxicity caused by the mixtures can be qualitatively predicted.

1. Introduction

Living organisms are exposed to more than 100,000 chemical compounds due to the rise of the chemical industry in the second half of the 20th century. Two thousand new xenobiotics are introduced annually in the EU, and between 30,000 and 70,000 are used daily, with more than 20% lacking control and evaluation. Bioaccumulation of xenobiotics is concerning because it is linked to health problems [1]. Currently, it is known that environmental pollution causes bronchitis, severe immunosuppression, allergies, and other diseases with bleak prognoses. Pesticide and metal exposure has been connected to Parkinson's and Alzheimer's disease. Chronic fatigue and hypersensitivity reactions to chemical substances have been described after continuous or punctual exposure to everyday substances: cleaning products, pesticides, fresh paint, construction materials, perfumes, plastic products, etc. [2]. Most xenobiotic-induced changes result from nonspecific interactions with subcellular structures, resulting in basal cytotoxicity [3]. The relevance of basal cytotoxicity assays depends on the experimental system and techniques [4]. In this sense, our previous experience determining the basal cytotoxicity of environmental contaminants from various chemical groups has shown that the Vero cell line (*Chlorocebus aethiops*) is more sensitive than HeLa (*Homo sapiens*) and 3T3 (*Mus musculus*). Vero cells were chosen to test the in vitro toxicity of BHA (E320) binary mixtures and PPB (E216) [5].

Since the mid-1920s, parabens have been used. These compounds (methyl-, ethyl-, propyl-, isopropyl-, butyl-, and isobutyl-paraben) are used in more than 22,000 cosmetic formulations and 98% of marketed products [5]. In the last 50 years, propylparaben has become the most popular cosmetic, pharmaceutical, and food preservative. Recent publications [6], the chemical analytical data of the presence of the residues of pharmaceutical, have highlighted the possible role of parabens in breast cancer induction. Its ability to enter the bloodstream and accumulate in human tissues and other organisms remains controversial. According to some studies, parabens are endocrine disruptors in vivo and in vitro. Others have described their ability to increase animal tissue proliferation through diet. Propylparaben is linked to tumour promotion but not induction [6–8].

Since the 1950s, butylhydroxyanisole has been widely used in food and cosmetics. BHA has been linked to tumour promotion but not induction and is possibly carcinogenic to humans. Its use is authorized because no health risks have been shown. Authors say its antioxidant properties are beneficial [9].

The large number of substances to which living organisms are exposed has sparked a growing interest in the toxic effects of chemical combinations. Multiple experimental approaches, including complex mathematical models for prediction, have not always produced convincing results. We wanted to know if an assay strategy based on a sensitive cellular model could be used to evaluate and predict the toxicity of chemical mixtures [10].

This study assessed the toxicity of binary chemical mixtures made of *tert*-butylhydroxyanisole and propyl p-hydroxybenzoate in vitro. Analyse BHA, PPB, and their combinations on Vero cells. Check if mathematical models can predict BHA-PPB binary outcomes. Explain the toxic action mechanism of both industrial additives, separately and together. Genotoxic chemicals induce DNA damage and mutations and chronic exposure to low doses of these chemicals may affect biodiversity, to predict mixture effects based on individual substances' mechanisms of action.

2. Materials and Methods

2.1. Compounds Selected for Study. 3-*tert*-Butyl-4-hydroxyanisole, better known as butylhydroxyanisole (BHA), and propyl-4-hydroxybenzoate, known as propylparaben (PPB), were purchased from Sigma-Aldrich with 98% purity AR grade quality. BHA has a molecular weight of 180.25 g/mol and appears as a white or slightly yellow waxy solid with a faint characteristic odor. It is insoluble in water, but dissolves in 50% (*v*/*v*) ethanol and other nonpolar solvents, with a molecular weight of 180.21 g/mol. It appears as a white crystalline powder, without odor or taste. In general, parabens are soluble in organic solvents such as alcohols, ether, glycerin, and propylene glycol. The esters of p-hydroxybenzoic acid, including PPB, are very stable over a wide range of pH and temperature. A 0.1 M solution of PPB in dimethyl sulfoxide was prepared for our experiments. BHA and PPB solutions were prepared fresh before each experiment and kept protected from light and moisture at room temperature.

2.2. Cell Cultures

2.2.1. Cell Line. The established mammalian cell line Vero (ATCC CCL-81) has been selected to carry out our study. They have been widely used in virology studies, and their renal origin has also made them a good recommended model for toxicological studies.

(i) Infrastructure and culture material: the maintenance of in vitro cell cultures requires a high degree of sterility; FLVA150 vertical laminar flow cabinet suitable for biosafety level 2 [11]; Heracell 150 CO_2 incubator; CH-BI45-2 Olympus (Japan) inverted binocular microscope with 4x, 10x, and 20x objectives; Dulbecco's minimal essential medium (DMEM) with 4.5 g/L glucose and phenol red; L–Glutamine 200 mM, 0,85% NaCl; antibiotic mix: penicillin–streptomycin (10,000 U/mL); bovine fetal serum, 1x phosphate-buffered saline (PBS), pH = 7.2 – 7.4; trypsin 2.5%, trypsin–EDTA (200 mg/L Versene EDTA); culture media, complete medium without serum (MCSS); DMEM mixture with 1% L-glutamine (*v*/*v*) and 1% antibiotics (*v*/*v*)

(ii) Complete medium with serum (MCCS): this is the medium in which the culture is kept in passage; it is MCSS to which 5% bovine fetal serum (*v*/*v*) is added—freezing medium. It is composed of a mixture of dimethyl sulfoxide (Serva, Germany) at 10% (*v*/*v*) in bovine fetal serum. Disposable material: sterile polystyrene plastic material for cell culture (flasks and multiwell plates) was obtained from BD Falcon™ (BD Biosciences, USA). The 1.2 mL cryotubes for freezing are from Cultek (Spain), while the 15×15 and $20 \times 20\,mm^2$ glass coverslips are from Menzel–Glaser (Germany)

2.2.2. Crop Maintenance. The culture begins with frozen cells in a vial. After thawing in a 37°C bath, cells are seeded in 25 cm^2 flasks with a complete medium containing 5% (*v*/*v*) serum and kept at 37°C in an incubator with 5% carbon dioxide. After 4-5 hours, the culture medium is removed, the cells are washed with PBS 1x, and 5 mL of clean culture medium is added to remove DMSO and nonviable cells.

First, using dilution factors, count the cells in the cultures. From these values, cell suspension seeding volume is estimated. The subculture process must be done 2 or 3 times a week, with 1 PBS wash and a medium change every 3 days. The experiments always used 3-10 passage cells.

2.2.3. Treatment Conditions

(1) Continuous Exposure. Sowing of 8×10^4 cells/mL was carried out for experiments lasting no more than 24 hours. Between 20 and 24 hours after the exact seeding and always within the period of exponential growth of the culture, the cells were exposed to the different concentrations of the

compounds. The BHA and PPB solutions were sterilized using 0.22 μm filters (Millipore®, USA) once dissolved in culture medium in the so-called test solution.

(2) Delayed Toxicity. After seeding the culture (2.5×10^4 cells/mL), we exposed the cells to the toxicant to be evaluated for the estimated time (24 h). After this incubation, we removed the medium with drug, washed abundantly with PBS 1x, and replaced it with a complete culture medium at 5% (v/v) without the drug for the chosen time (24 and 48 hours). At the end of this period, the effect produced was quantified using different parameters.

2.2.4. Design of the Experimental Protocol. The experimental design, biological model, and evaluation parameters affect the quality and specificity of in vitro data. Our approach to mixture studies requires a step-by-step protocol. Our proposal is based on the toxic effects of the chemical substances to be evaluated in the experimental model chosen for the study and others to which we have bibliographic information.

First, the individual compounds that make up the mixture were tested for viability, proliferation, and cell morphology. Microscopic analysis of cell morphology reveals interferences with essential structures not always detectable with quantitative basal cytotoxicity assays. General cellular morphology was studied using relevant concentrations of each compound and simple techniques to define toxic effects.

Individual toxicity studies used 24-hour exposures, which exceeds a crop division cycle. This approach lets us determine the baseline toxicity range of each compound's toxicological profile.

Literature review [12, 13] allowed the construction of a list of potential alterations or targets on which the compounds can interact individually or in combination.

In the second stage, we determine the concentrations that will be used to analyse the mixture's toxicity using a complete factorial design. We can estimate the combined effect in our model system with individual toxicity data, assuming the model's mathematical assumptions are met.

This study's second phase used the same methodology as the first (viability, proliferation, and general cell morphology). In all experiments, the individual treatments were repeated to ensure that their effects did not differ. With the second-stage results, we analysed which combinations produced the most toxic effects to study their mode and/or mechanism of action.

In the third stage, damaged pathways involved in the observed toxic process were defined to determine if the proposed mode of action is correct.

Our experimental design includes a wide variety of tests that cover a wide range of toxic action mechanisms, without being a closed set. One of the great advantages of the system used is the possibility of adding techniques as they arise. Needs for evaluation in our research, we have employed tests to assess DNA damage, cytoskeleton condition, mitochondrial physiology, cell adhesion, cell membrane stability, gene and protein expression, oxidative stress, and cell cycle progression.

2.2.5. Cell Viability

(1) MTT Reduction.

(i) A 5 mg/mL MTT stock solution was prepared in 1x phosphate-buffered saline. Once dissolved, it was sterilized by filtration through a Millipore® filter

(ii) 0.22 μm and stored in an opaque glass bottle at 4°C

(iii) This stock solution was diluted before the assay in complete medium without serum in the proportion 1/11 and kept at 37°C

(iv) Under sterile conditions and once the treatment was finished, the culture medium was emptied from the wells by aspiration and they were washed twice with 1x saline phosphate buffer at 37°C

(v) 1 mL of culture medium with the MTT already incorporated was added to each well, and the plates were incubated for 2 hours at 37°C and with a 5% CO_2 atmosphere

(vi) After incubation and outside the cabinet, the medium was removed by aspiration, draining the wells well

(vii) 1 mL of DMSO was added to each well and to facilitate the dissolution of the formazan; an ultrasound bath was used in which 3 pulses of 5 seconds were performed

(viii) After 10 minutes at room temperature, the color developed was read

(ix) Data reading and processing

(x) The SPECTRAFluor plate reader (Tecan, Austria) was adjusted; mode: photometric (absorbance). $\lambda = 570$ nm

(xi) The previously measured blank was conveniently subtracted from the results obtained

(2) Neutral Red Capture.

(i) The stock solution of neutral red (4 mg/mL) in bidistilled water sterilized by filtration through a Millipore® 0.22 μm filter was stored in an opaque glass bottle at 4°C

(ii) With it, a solution was prepared in complete culture medium without serum (50 μg/mL) that was left overnight in an oven at 37°C and centrifuged before using it for 10 minutes at 1500 rpm to remove crystalline precipitates

(iii) After removing the medium by aspiration once the desired treatment had been completed and under sterile conditions, the wells were washed with PBS 1x at 37°C

(iv) Subsequently, 1 mL of culture medium with neutral red already incorporated was added to each well and the plates were incubated for 3 hours at 37°C and with a 5% CO_2 atmosphere

(v) After this time, the medium was removed by aspiration, and for washing the wells, a buffer containing 1% (*v*/*v*) formaldehyde and 1% (*p*/*v*) $CaCl_2$ in distilled water was used

(vi) Once the wells were washed, the buffer was removed by aspiration, draining the wells well. Dye extraction buffer was added which was a mixture of 1% glacial acetic acid (*v*/*v*) and 50% absolute ethanol (*v*/*v*) in distilled water. To facilitate the extraction of the incorporated dye, an ultrasonic bath (Branson 2200) was used, in which 3 pulses of 5 seconds were performed

(vii) After 10 minutes at room temperature, the color developed was read

(3) Trypan Blue Exclusion Assay.

(i) After treatment, cells from the supernatant and those that remained anchored to the substrate were collected separately following the steps described above for trypsinization

(ii) A mixture of cell suspension and trypan blue was prepared as explained for cell number counts

2.2.6. Proliferation Analysis

(i) The absorbance of the dye shows linearity starting from a stock solution of BSA (100 μg/mL) in distilled water, just before the titration

2.2.7. Study Samples

(i) Once the treatment was finished, the wells were washed twice with 1x saline phosphate buffer at 37°C. Then, 0.5 mL of 1x PBS was added to each well and the cells were lysed from the plate using a scraper

(ii) In another empty plate, the samples were processed: 120 μL of cell lysate from each well was added to 680 μL of distilled water, and subsequently, 200 μL of concentrated Bradford reagent was added. The plate was shaken and read at 595 nm. When the absorbance values were outside the range of values of the standard line, it was necessary to dilute starting from the cell lysate

2.2.8. Split Rate Analysis

(i) After treatment, cells were collected from the supernatant and those that remained anchored to the substrate following the steps described above for trypsinization

(ii) They were centrifuged for 10 minutes at 1500 rpm, and the medium was removed. The pellet was resuspended in 1 mL of PBS 1x and centrifuged again for another 10 minutes at 1500 rpm

(iii) The 1x phosphate-buffered saline was removed, and 1 mL of cold (-20°C) 70% ethanol was added dropwise while slowly vortexing the cells. Thus, they were stored at 4°C for at least 18 hours

(iv) After resuspending the pellet, the cells were centrifuged for 10 minutes at 1500 rpm. The supernatant was removed, and 1 mL of cycling buffer (RNase A 50 μg/mL and propidium iodide 50 μg/mL in 0.1% (*w*/*v*) tribasic sodium citrate dihydrate in bidistilled water) was added

(v) It was mixed well, and the cells were incubated for 30 minutes in the dark

(vi) After this time, the DNA load of the cells was quantified by flow cytometry

2.2.9. Cell Morphology

(1) Toluidine Blue Staining.

(i) Bright-field optical microscopy studies were carried out on coverslips. After exposure to xenobiotics, the medium was removed and the cells were fixed with absolute methanol at −20°C for 7 minutes and left to dry

(ii) Later, staining with toluidine blue 0.025% (*p*/*v*) in distilled water was carried out for 20 seconds

(iii) Finally, a wash was carried out in distilled water, and the preparations were mounted in EuKitt®, after letting the stained coverslips dry and performing a brief immersion in xylene

2.2.10. Subcellular Damage Markers

(1) Supravital Labeling with Acridine Orange.

(i) Once the desired treatment was completed and the cells grown on coverslips were washed with 1x saline phosphate buffer, a mixture of 10 μM acridine orange in serum-free culture medium was added to each well

(ii) After an 8-minute incubation at 37°C, the mixture was removed, the cells were abundantly washed with 1x phosphate-buffered saline, and observation was quickly carried out by optical fluorescence microscopy using a blue excitation filter ($\lambda = 470 - 490$ nm), a 520 nm barrier filter

2.3. Microscopy. The digital images were captured with the Olympus DP Controller 1.1.1.65, 2002 (Olympus) program, which controlled the Olympus DP–70 CCD digital camera. The image processing was carried out using ImageJ 1.41o

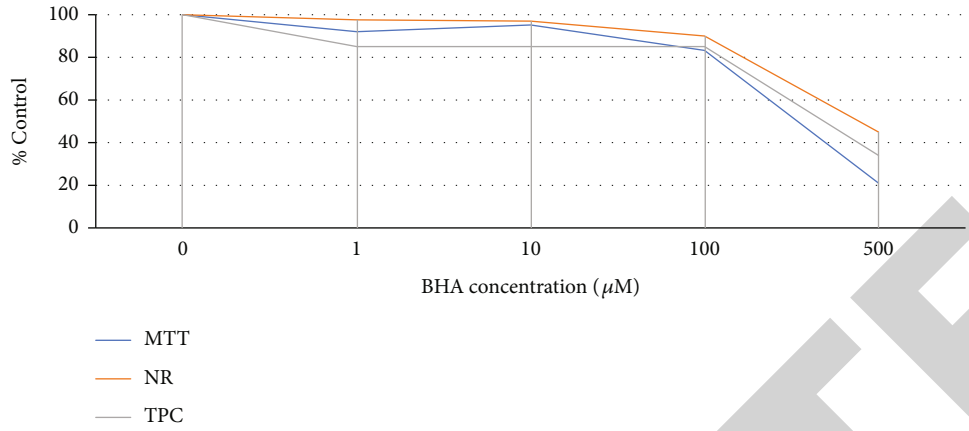


Figure 1: Results were obtained with the three basal cytotoxicity assays after 24 hours of exposure to BHA (ANOVA, $p = 0.063$).

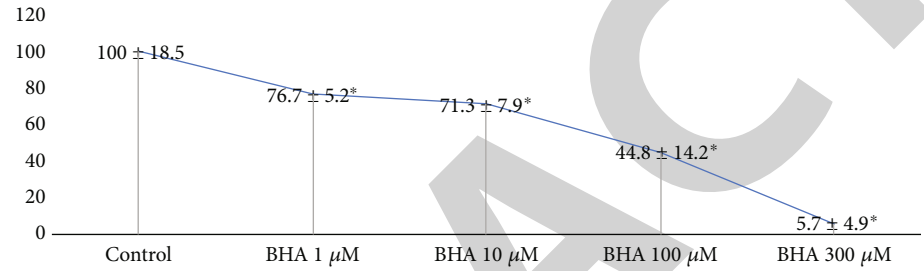


Figure 2: Cell division rate of Vero cell cultures exposed for 24 hours to BHA. Asterisks indicate statistically significant values concerning the control (ANOVA, $p = 0.067$).

(National Institutes of Health, USA), depending on the requirements of each analysis.

2.4. Statistical Analysis. Basal cytotoxicity assays (MTT reduction, neutral red incorporation) were performed in multiwell plates, obtaining at least 10 mean values per dose group. In experiments analysing division rate, phase index, metaphase types, nuclear morphology, and DNA double-strand break frequency, a minimum of 6 samples per treatment were obtained from at least three independent experiments. Three independent experiments using the trypan blue exclusion assay showed $n \geq 9$. Base oxidation image analyses analysed 10 photos from at least 3 experiments for each treatment ($n \geq 30$). The microarray studies used samples from at least three independent experiments for each treatment group to obtain the biochip's mean values. In western blot, each dose group's experiment was repeated at least three times, so $n \geq 6$ proteins were analysed.

3. Results

3.1. Individual Cytotoxicity Assessment. Quantitative tests were performed after 24 hours of exposure to butylhydroxyanisole in the concentration range of 1 to 300 μM and propylparaben from 50 to 500 μM. These studies include viability and proliferation assays. Qualitative tests were performed at different doses to study the general morphological changes induced by each compound by staining with toluidine blue.

Butylhydroxyanisole showed a dose-dependent cytotoxic response. All biochemical tests showed an identical trend (Figure 1). The integrity of the cell membranes and the content of total cellular protein (TPC) were only altered concerning the control after exposure to the highest dose (300 μM). However, oxidative metabolism (MTT) was affected with treatments ≥ 100 μM.

The most sensitive parameter of those tested was the rate of division of the culture, based on mitotic index counts (Figure 2). The percentage of cells in mitosis showed a notable decrease from the concentration of 1 μM, a dose for which no other tests indicated significant alterations. The cleavage rate was reduced to half the control value (EC_{50}) at 63.8 μM. On the other hand, the proliferation rate was practically null in the treatments with 300 μM BHA after 24 hours of exposure.

Taking into account that the viability tests presented a statistically significant correlation with the quantification of the total cellular protein (MTT-TPC, $r = 0.86$; NR-TPC, $r = 0.72$; $p < 0.0001$) and that the most sensitive test was the mitotic index count, we estimate that butylhydroxyanisole produces an apparent antiproliferative effect on Vero cells and has a particular preference in its toxic action on mitochondria, except after exposure to 300 μM, where a severe cytoplasmic vacuolization was observed.

The same battery of tests was used to evaluate the basal cytotoxicity of propylparaben. The observed effects also showed a clear dose-dependent response. Propylparaben caused changes concerning the control in incorporating

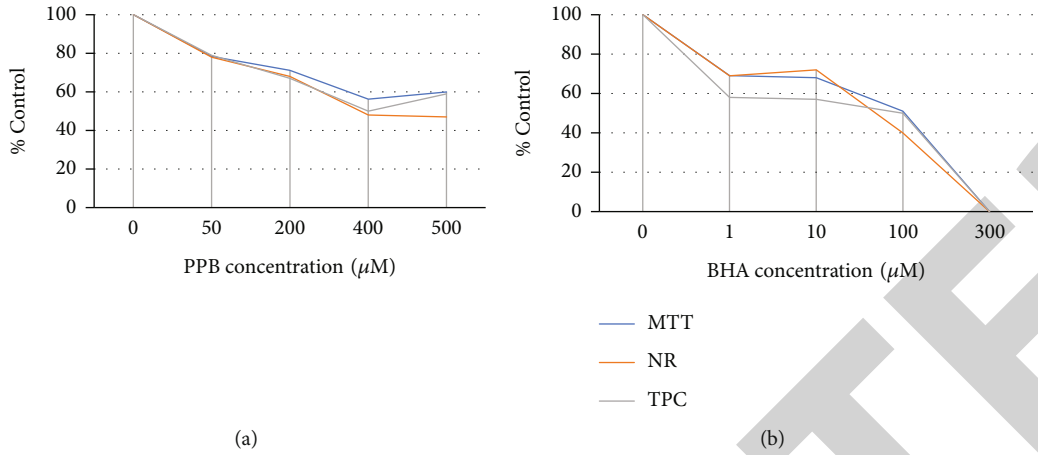


Figure 3: MTT reduction tests, neutral red capture, and TPC after 24 hours of exposure to binary mixtures of BHA and PPB. (a) Binary pools are composed of 100 μM BHA and a range of PPB concentrations (50-500 μM). (b) Binary pools are composed of 500 μM PPB and a range of BHA concentrations (1-300 μM) (ANOVA, $p = 0.065$).

neutral red from 200 mM. At this point, compared to the untreated cultures, the total protein content of the cells was substantially lower. However, no significant differences were observed with respect to the control in the reduction of MTT up to the concentration of 400 μM. As had happened with butylhydroxyanisole, the mitotic index counts were the most sensitive parameter of all those analysed with an EC_{50} of 160.0 μM, obtaining significant decreases in proliferation from the lowest dose evaluated.

Viability assays and protein quantification presented a statistically significant correlation (MTT-TPC, $r = 0.829$; NR-TPC, $r = 0.769$; $p < 0.0001$). However, the compound exhibits a preferential action on cell membranes, as indicated by the increased sensitivity of the neutral red capture assay. Toluidine blue staining in cells exposed to different propylparaben treatments did not show remarkable morphological alterations, even at the highest dose tested.

3.2. Combined Cytotoxicity Assessment. Maintaining a fixed dose of one compound while varying the other's concentration was the experimental method. Coexposure was based on each compound's toxicity. Fixed doses of butylhydroxyanisole and propylparaben were 100 and 500 μM, respectively; the maximum concentrations studied did not alter morphology and had a significant effect on some of the parameters. None of the total cellular protein content treatments exceeded a 50% effect level. Seven BHA-PPB mixtures were tested. Both tests showed similar trends in cell viability after 24 hours of exposure to binary combinations (reduction of MTT and capture of neutral red). In addition, both compounds were nearly identical in total cellular protein quantification (Figure 3).

Correlations between viability assays and TPC were statistically significant, again demonstrating the existence of an apparent antiproliferative effect. Of note, the mitotic index count was again found to be the most sensitive assay, showing that the preferred mode of action of the combinations, genotoxic biomarker alone is not sufficient for evaluating the toxicological response of pollutants, like that of the individual compounds, is inhibition of culture proliferation.

Taking all the basal cytotoxicity data as a whole, the mixtures were ordered according to the response of the four quantitative parameters used (Figure 4). Based on this table, there are three groups of BHA-PPB mixtures: the least toxic 100-50 (green), the most toxic 300-500 (red), and a set of five combinations of intermediate toxicity and with variable orders depending on each parameter (green-orange gradient).

Defining the effect as the difference between the mean viability (or proliferation) of the control (100%) and the treatment group, we can see that the least toxic mixture produced an effect of around 20% (BHA 100 μM+PPBμ50μM), with identical values and correlated with the TPC. Also, the cleavage rate decreases. These data show that the compounds produce an antiproliferative effect without altering mitochondrial function or endosomal integrity. The most toxic mixture, BHA 300 μM+PPB 500 μM, showed null viability, proliferation, and many floating cells.

The remaining mixtures had total cellular protein and viability between 75% and 35% of the control. 500 μM PPB with 1 and 10 μM BHA and 100 μM BHA with 200, 400, and 500 μM PPB showed similar responses in all three trials, confirming their antiproliferative effect. The mitotic index was affected more than the other tests, except for the 100 μM BHA+200 μM PPB mixture.

After the baseline cytotoxicity of the seven mixtures was quantitatively evaluated, the cultures were morphologically analysed with toluidine blue. Figure 5 shows that Vero cells in the control culture (Figure 5(a)) have a fibroblastic morphology, with a wide, 100 μM BHA+500 μM PPB mixture caused cytosolic vacuolization and pleiomorphism (Figure 5(b)). In cultures exposed to 300 μM BHA+500 μM PPB, the significant effect is a decrease in confluence level due to loss of adhesion and hydropic degeneration (Figure 5(c)). Toluidine blue staining showed no cytological changes with 500 M propylparaben (Figure 5(d)) or 100 μM BHA (Figure 5(e)). 300 μM BHA caused cytoplasmic vacuolization (Figure 5(f)), but not as severely as BHA

	100-50	100-200	1-500	10-500	100-400	100-500	300-500
MI	68.6	51.7	17	17	7.1	2.3	0
NR	81.4	56.4	65.9	73.7	38.2	36.5	0
MTT	77.1	63.5	67.4	67.1	50.2	54.2	0
TPC	81.9	56.8	58.4	59.9	43.7	52.1	0

Figure 4: The binary mixtures were sorted according to the response's toxicity at 24 hours according to the four quantitative parameters. The green-red color gradient indicates the magnitude of the effect from the least to most toxic.

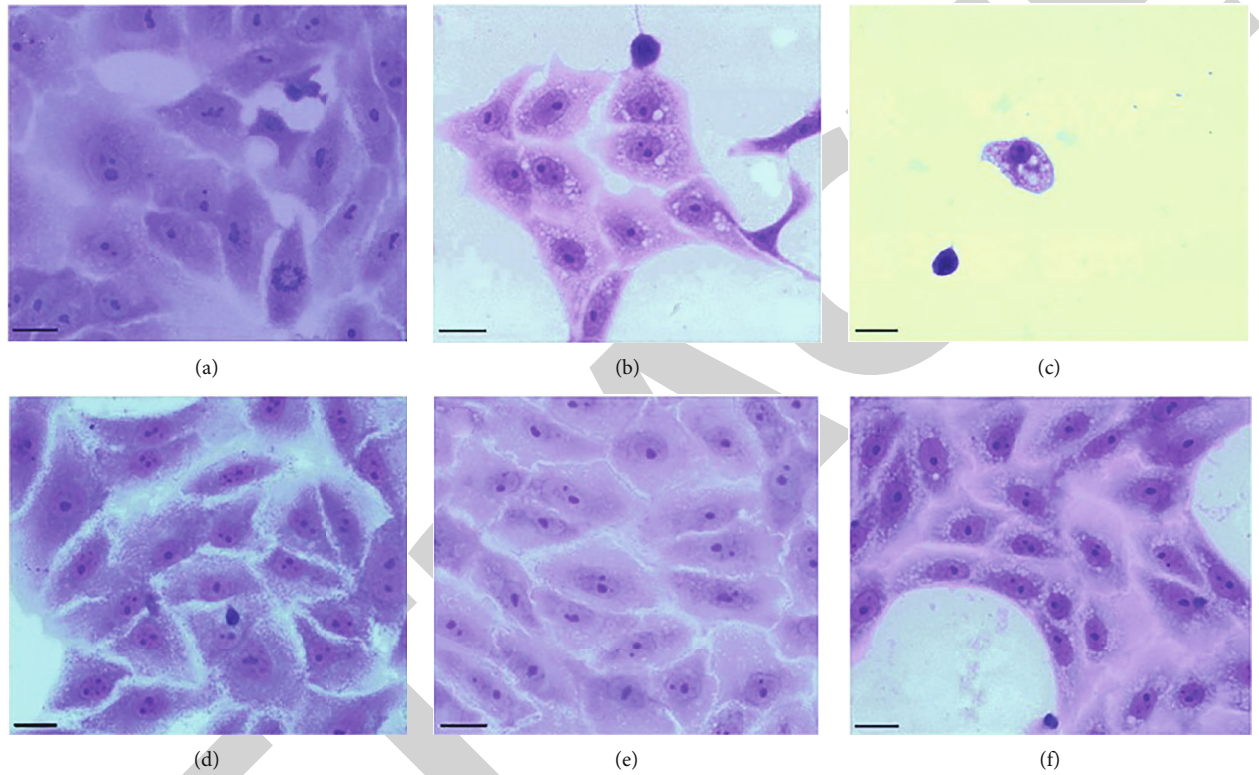


Figure 5: General morphology study by bright-field microscopy and toluidine blue staining of cultures exposed to butylhydroxyanisole and/or propylparaben for 24 h. 20 μm bar.

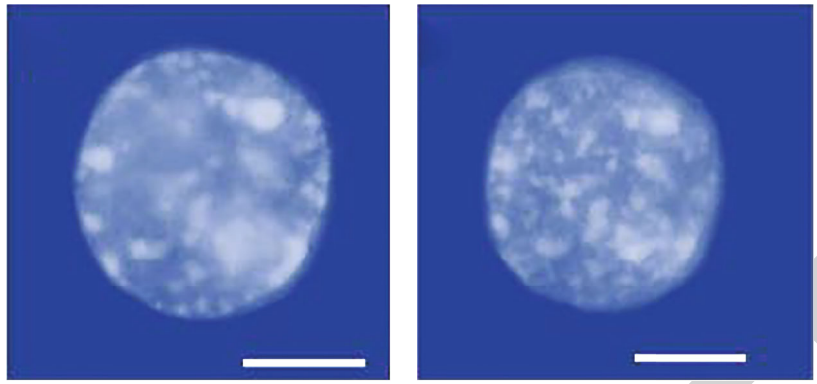
300 μM+PPB 500 μM. The remaining mixtures showed no morphological changes.

3.3. Determination of the Mechanism of Toxic Action. To determine the mechanism of toxic action by which proliferation inhibition is induced in binary mixtures, the studies were complemented with various more specific analysis techniques. In this study's second phase, the two binary combinations defined as the most toxic of the seven evaluated were analysed, corresponding to BHA 300–PPB 500 (Mix 300).

3.3.1. Study of the Mechanism of Action of Mix 300. Considering that almost all culture exposed to the mixture detached from the substrate, a hemocytometer count was made to determine the percentage of adherent cells. They were also processed using the trypan blue exclusion assay to determine if the plasma membrane was intact. 86.6 ± 9.6% of the exposed cells floated. None of the 24 h-exposed cells were permeable to trypan blue. Individual treatments also caused significant adhesion loss, but not as much (ANOVA, $p = 0.058$). 300 M BHA and 500 M PPB produced 20.1 ± 2.4% buoyant cells. Control cultures showed no adhesion loss (1.1 ± 3.3%) and no altered plasma membranes. Neither treatment had statistically significant trypan blue-positive cells.

To determine the state of the cells in suspension, optical fluorescence microscopy with Hoechst 33258 staining was used. Figure 6 shows different nuclei morphologies after Mix 300 treatment.

Counting of nuclear morphologies showed that 21.2 ± 3.2% were live cells, although no mitotic figures were observed, confirming that the culture was undergoing a strong proliferation arrest. In contrast, in the floating fraction of the individual treatments, only typically apoptotic nuclei were observed, which turned out to be 17.5 ± 3.9% of the total culture for 300 μM BHA and 2.3 ± 0.7% for 500 μM PPB.



(a) (b)

Figure 6: Different nuclear morphologies are present in the floating fraction of the cell culture after exposure to Mix 300 for 24 hours. (a, b) Interphase nucleus analysed in different focal planes showing highly positive punctate regions after labeling with Hoechst 33258. Bar 5 μm.

At this stage of the study, the interphase cell cycle blocking mechanism was delved into, for which an analysis was carried out using flow cytometry. Figure 7 shows the histogram of DNA loading frequencies of a control culture of Vero cells after 24 h (Figure 7(a)). In the cultures exposed to the compounds individually, what is most striking is the strong blockade in G0-G1 and the decreases in the S and G2-M phases (Figures 7(b) and 7(c)). However, the Mix 300 treatments show an evident change in profile (Figure 7(d)). There is a halving of the percentage of cells in the G0-G1 phase, an almost doubling of the proportion of cells in the S phase, and there are no significant variations in the G2-M period. The most striking differences were observed in the region where the cells with the highest DNA content are distributed. The treated culture increases its mean level of polyploid cells up to almost five times with respect to the control.

In light of these results, data were analysed using a diploid-tetraploid cell cycle modeling software (ModFit LTTM 3.0), which estimates the phase of the tetraploid cell cycle in which cells are found. The results indicated that the cells with a charge equal to or greater than 4c were fundamentally in the S period and their diploid cycle lacked G2-M (Figure 8).

As seen in Figure 9, longer exposures over time (48 h) showed that the histogram of the control did not vary (Figure 9(a)) while those of the individual compounds showed a greater number of cells in sub-G1 (Figures 9(b) and 9(c)). In addition, the culture exposed to the mixture for 48 h showed an increase in dead cells and cell debris, drastically changing the profile observed in the control and the same treatment 24 hours before (Figure 9(d)).

To analyse the chronological sequence of effects that lead to cell death in the culture and thus the mechanism of toxic action, we analysed the changes observed in these same treatments at shorter exposure times. After 4 hours, Mix 300's cell viability and protein content were evaluated. Targeted chemical analysis would be required to identify the genotoxic contaminants in these samples. Total cellular protein quantification, MTT reduction, and neutral red capture assays determined that the primary target of this binary combination was cell membranes. The neutral red test was the most sensitive, showing viability lower than 40% compared to the control, while the rest of the tests showed results around 75%. We conclude that Mix 300 induces a specific alteration of endosomal membranes after 4 hours of exposure because the plasmatic membrane was intact and the cells were impermeable to trypan blue. 300 M BHA affected neutral red capture and total protein content. After 4 hours of exposure to 500 μM PPB, only endosomal integrity was affected.

The quantitative results were supplemented with specific tests to evaluate endosomal and mitochondrial reticulum integrity. Vero cells have acidic endosomes (A). Butylhydroxyanisole treatments alkalinized, swelled, and changed lysosome distribution (B). PPB treatments alkalinized endosomes mildly (C). Cells exposed to the binary mix (D) could not accumulate acridine orange in their lysosomes and suffered severe damage, including cytosolic vacuolization and altered morphology.

Vero cells labelled with rhodamine 123 (E) showed reticular mitochondria around the nucleus. Butylhydroxyanisole caused mitochondrial reticulum fragmentation (F). Propylparaben caused no damage (G). Mix 300 caused severe mitochondrial reticulum fragmentation and membrane potential loss (H). Mitotic index counts finished the results. After 4 hours of exposure, neither individual treatment nor their combination affected culture division. The mitotic index values of the binary mixture were supported by an increase in metaphases, according to the phase index count. This explains some Bradford test results. Loss of culture adhesion can affect total protein quantification, and since the cells showed severe damage, actin cytoskeleton changes could not be ruled out.

We then quantified the percentage of floating and adhered cells after different Mix 300 exposure times. After 4 hours, cells exposed to the binary combination lost adhesion significantly. At 9 h, cultures treated with Mix 300 had slightly more than 40% of the culture floating, while individual treatments were around 5%. The binary combination caused 86.6 ± 9.6% of the cells to float after 24 h, and adhesion was affected at 48 h.

These quantitative studies were supplemented with specific cytoskeleton detections to analyse possible actin

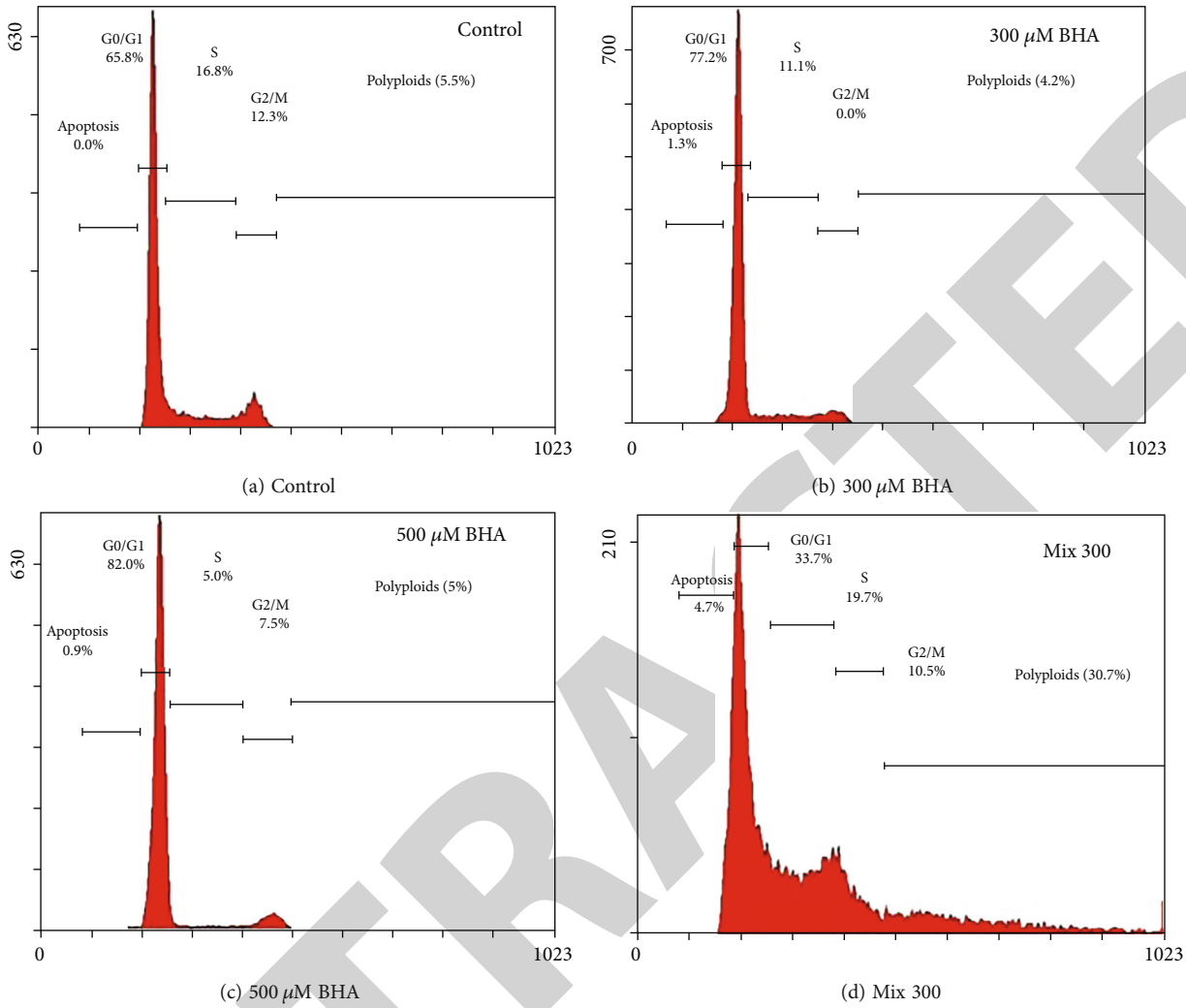


Figure 7: Representative histograms of the frequency of cells in the different phases of the cell cycle analysed after treatments with BHA and/or PPB for 24 hours ($n = 6$ per treatment).

microfilament alterations in cell adhesion. Individual treatments with BHA and PPB after 4 and 9 hours did not alter the actin cytoskeleton in control cells. Mix 300 changes are very evident after 4 hours of exposure, with dendritic appearance, loss of adhesion, and actin cytoskeleton disorganisation.

In 9-hour treatments, after 4 h, the microtubule pattern in interphase cells showed only general morphological changes from the binary mixture. As described, Mix 300-exposed cultures had a higher percentage of metaphase cells.

The detailed study of the mitotic spindles by double immunodetection against α- and γ-tubulin (Figure 10) showed that two types of metaphases could be found after treatment. In the former, the chromosomes were not arranged in the metaphase plate (Figure 10(a)) and bipolar mitotic spindles did not form (Figure 10(b)), since the two pairs of centrosomes remained together (Figures 10(c) and 10(d)). Similarly, classical metaphases were observed, with chromosomes in the equatorial plate (Figure 10(e)) and spindles with two correctly separated poles (Figures 10(f) and 10(g)) similar to those observed in control.

At this point in the study, we hypothesised that cells exposed to BHA 300 μM+PPB 500 μM could be stressed, causing premature chromatin condensation (CPC) and mitosis, unfinished DNA synthesis. CPC processes cause DNA damage, so we next tested if Mix 300 exposed cells were genotoxic. We used immunocytochemistry to detect 8-OHdG in the binary mixture and BHA- and PPB-exposed cells.

DNA bases oxidise due to intracellular reactive oxygen species (ROS), which we measure with DCFH-DA. After 4 hours of exposure to Mix 300, oxidative stress levels increased significantly, although less so than in cultures treated with 300 M BHA and 500 mM PPB. Free radicals increased after 24 h compared to 4 h in all treatments. Oxidative stress can cause base oxidation but not premature chromatin condensation. DNA double-strand breaks are associated with CPC, so we immunised γ-H2AX to verify this genotoxic damage. The percentage of culture cells with double-strand breaks (DSB) tripled after 4 hours of exposure to Mix 300. Individual treatments increased double-strand break frequencies, but not statistically significantly.

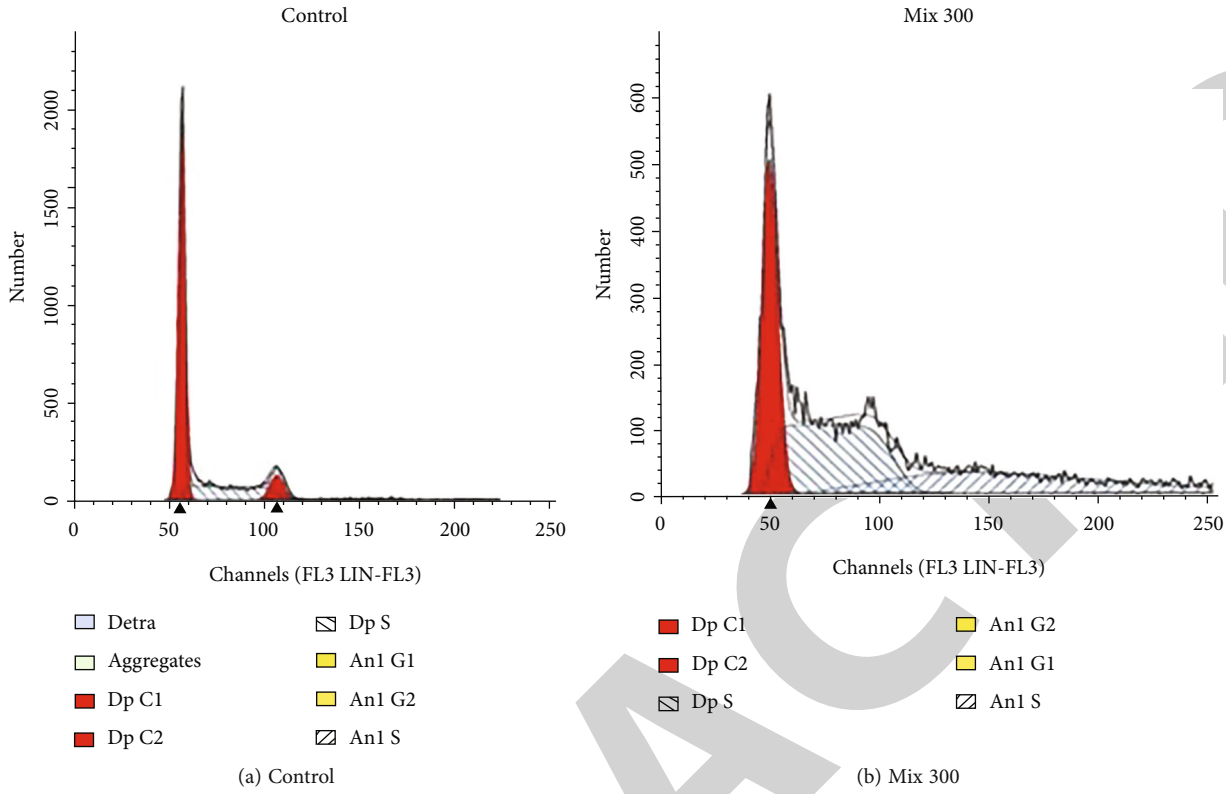


FIGURE 8: Diploid-tetraploid cycle modeling of representative cell cycle histograms of control (Dip. S: S phase of the diploid population, An1. S: S phase of the aneuploid population).

3.3.2. Delayed Toxic Effect Study. Proliferation and DNA damage assays were used to study delayed toxicity. After 24 h, the drug-containing medium was replaced for 24 and 48 h. Total cellular protein was measured then. Mix 100 binary mixture results were surprising, as TPC decreased to 39.2 ± 15.1% after 24 hours. 48 hours after drug withdrawal, it was 82.6 ± 6.6%. 100 μM BHA did not decrease TPC after 24 hours of continuous treatment but increased protein by more than 20% compared to the control 48 hours later. In cultures exposed for 24 h to 500 μM PPB, the TPC decreased to 52.4 ± 10.3% and returned to control levels after 48 h. After 72 hours of continuous treatment, the protein was only detected in control cultures, 100 M BHA (71.7 ± 10.4%), and 500 μM PPB (8.1 ± 2.6%) cultures. The growth rate of cultures pretreated for 24 hours was much higher than the controls. This variable describes a growth graph where the slope of the curves is a reasonable approximation of the crop's growth rate. In this case, we included all treatments evaluated throughout the study except Mix 300, which was lost after 24 hours. Both models performed poorly in our experiments. MTT reduction test was the most predictable of the three evaluated parameters. The neutral red capture assay was the hardest to predict, with only the full negative correlation model coming close for Mix 300.

3.4. Isobolographic Analysis. Binary isobols showed different results depending on study parameters and BHA and PPB combinations. Mix 300 synergizes all tests. In the case of 100 M BHA and 400 or 500 μM PPB, the effect fits the isobol value and can be defined as zero additivity or interaction for all basal cytotoxicity colorimetric assays. Except for BHA 100 μM+PPB 200 μ M, which showed noninteractive effects for the neutral red capture assay and synergistic effects for TPC and MTT reduction, the other mixtures reacted antagonistically for all the parameters tested.

4. Discussion

A complex network of signals controls cell physiology by communicating between compartments. Changes in the cell's exterior trigger autoregulatory responses that maintain intracellular conditions. Cellular homeostasis involves detection, response, and adaptation. Changes in cell homeostasis do not necessarily mean cell death because cells have complex control systems [14]. Altering cellular homeostasis leads to an adaptation process in which cells maintain viability but modulate function. When adaptive response limits are exceeded, cells undergo a series of events that temporarily or permanently alter cell structure or function. We currently believe that cell injury involves metabolic and morphological changes. Type, duration, and severity of injury determine cellular response to noxious stimuli. The "point of no return" that leads to cell death is determined by the cell's damage tolerance and ability to recover after stimulus withdrawal [15].

Toxic chemical compounds usually damage different cellular targets. Therefore, toxicological evaluations must be

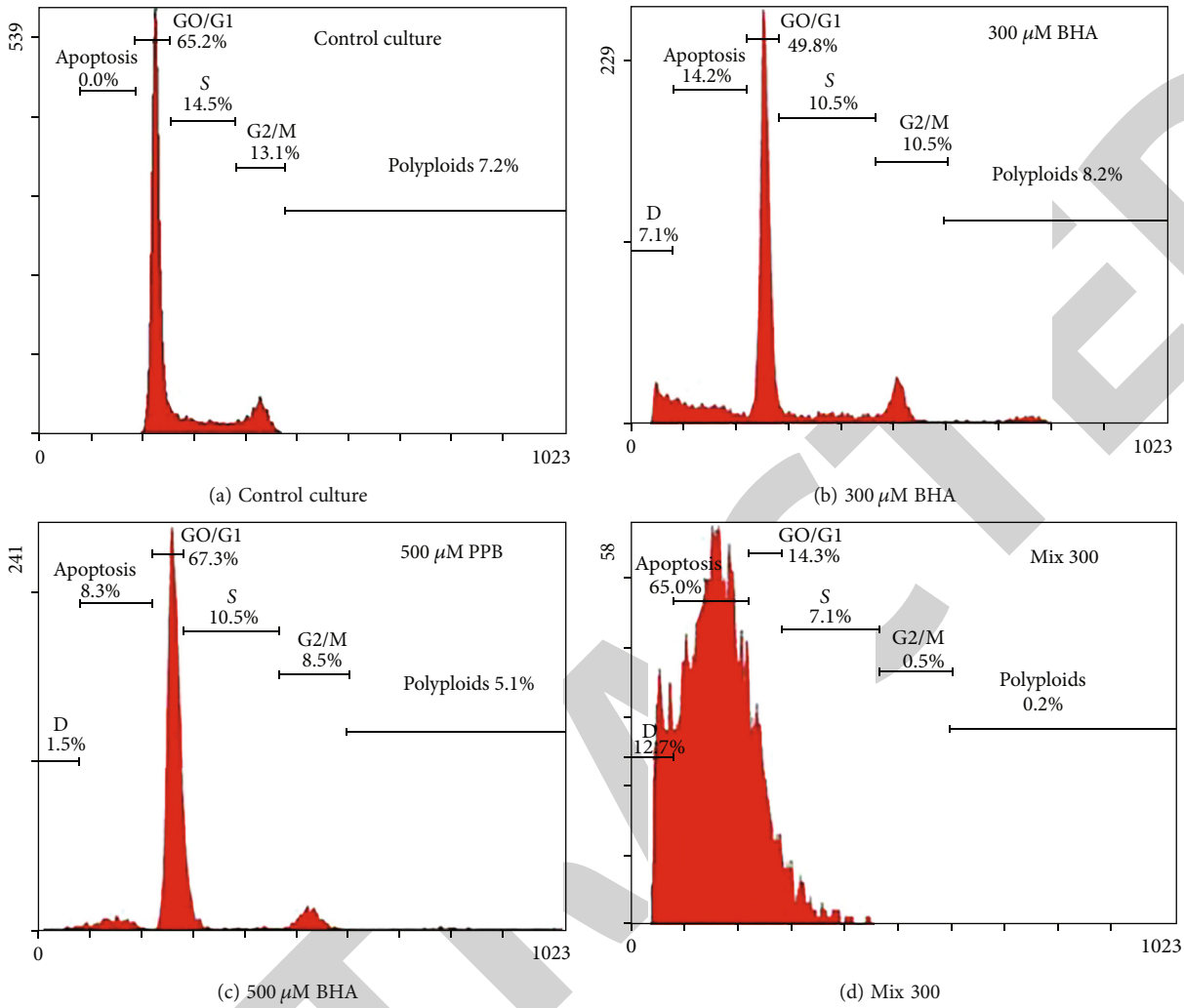


Figure 9: Representative histograms of cell frequencies in each cell cycle phase analysing DNA loading by flow cytometry after 48 hours of continuous exposure to BHA and/or PPB ($n = 6$ per treatment).

based on cell physiology parameters. Mammalian cell lines are effective in vitro model for determining a compound's basal cytotoxicity by analysing metabolic activity, viability, and cellular and subcellular morphological integrity [16].

Most studies on the toxic effects of chemical mixtures are descriptive. Mathematical models that reliably predict toxic effects lack consensus. Clearly, testing strategies are needed to develop complete toxicological profiles of chemical mixtures of social interest.

In this line, the main goal of this work was to use an in vitro experimental protocol to analyse the possible toxicity of a mixture of *tert*-butyl-4-hydroxyanisole (BHA) and propyl p-hydroxybenzoate (PPB), two additives used in the food, pharmaceutical, and cosmetic industries whose effects on cells and organisms are controversial. The study's starting hypothesis was to determine whether an assay strategy based on a cellular model, which we have used successfully to determine the mechanism of action of different chemical agents individually, could predict relevant toxic effects in a binary mixture, to intensive toxicological investigation and risk assessment and the appropriate remedial actions. The assay strategy integrates the analysis of multiple cell physiology parameters and uses different mathematical estimation models to avoid imprecise results.

First, we compiled all available information on the compounds in question, including physical-chemical properties and toxicological tests, especially in combination. We have no background on the toxicity of binary BHA-PPB mixtures, so we cannot compare ours directly. Both compounds interact well with other chemical groups, however. BHA can increase in vitro the toxicity of green tea antioxidants [17], tetrahydrocannabinol, and tioconazole [18]. Due to its antioxidant capacity, the anticarcinogenic properties of this compound have also been described in animal models. Some studies show BHA promotes tumours induced by other agents. In these cases, pretreatment is required and coexposure never occurs. Most toxicological evaluations of PPB have been done in combination with other esters of p-hydroxybenzoic acid, with which it shows additive or synergistic effects. Both compounds were individually toxicologically analysed in the second stage to determine the ideal mixture concentrations. The toxic responses of binary

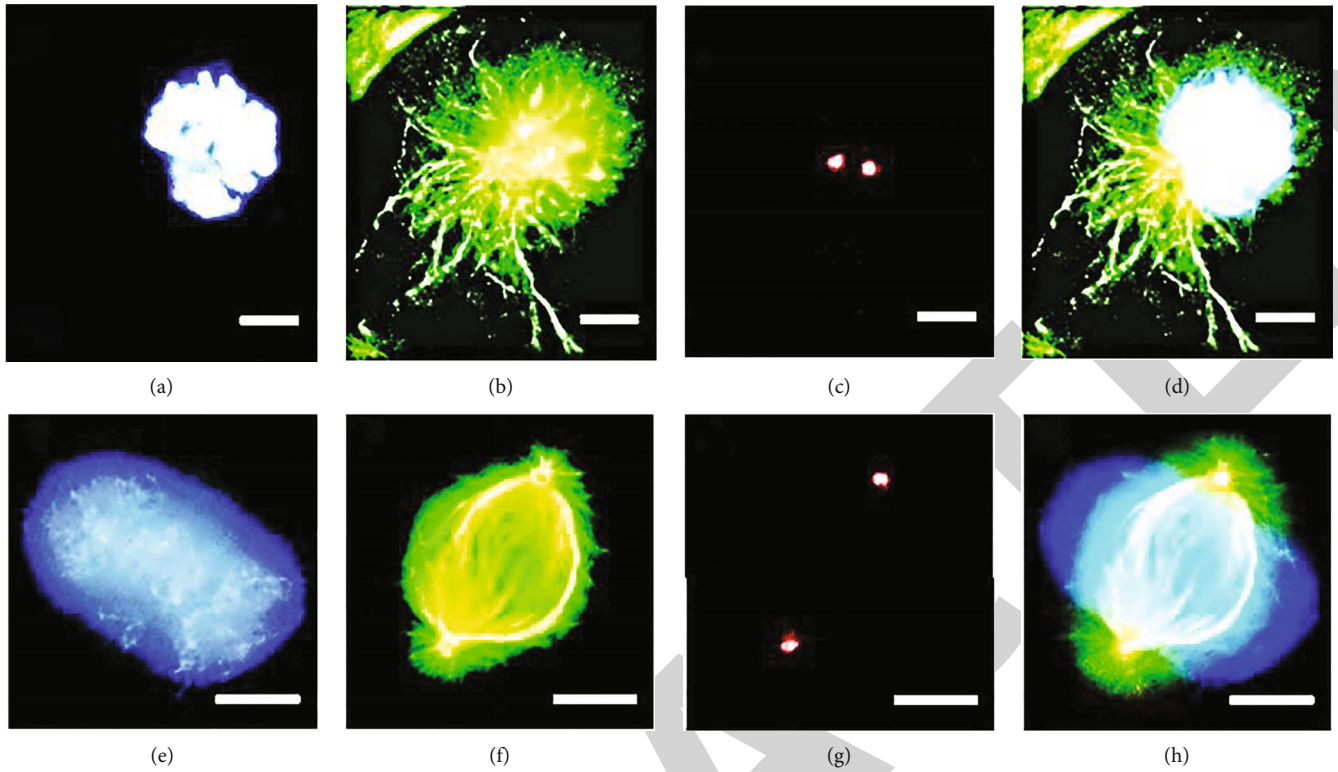


Figure 10: Vero cell metaphase analysis after 4 hours of treatment with Mix 300. (a–d) Aberrant metaphases and (e–h) control metaphase. Specific chromatin labeling with Hoechst 33258 (a, e). Immunodetection of mitotic spindle microtubules with anti-α-tubulin (b, f). Immunodetection of centrosomes with anti-γ-tubulin (c, g). Superimposed images of the three channels (d, h). 5 μm bar.

mixtures of BHA and PPB at different doses were evaluated to facilitate the choice of estimation models.

First, cell viability, a key factor in analysing chemical products' toxic effects, was evaluated. Normalized to control values, all treatments showed similar effect values. The dose-response curves for neutral red incorporation and MTT reduction are practically indistinguishable in the combined exposure.

Total cellular protein content, an indicator of cell number, followed the same trend as viability assays. Correlation studies showed that basal cytotoxicity assays were related to the number of anchored cells in each well. Mitotic index counts confirmed that fewer dividing cells explained the decrease in culture protein. In addition, the division rate was the most sensitive test of those performed, as with both compounds individually. Our results showed that exposure to Mix 300 reduced cell adhesion, which explains the basal cytotoxicity results. Flow cytometry shows that 100 M BHA increases the G0-G1 period. Statistically significant changes were only seen in the G2-M phase, while Mix 100 and 300 M BHA and 500 M PPB arrested the cell cycle in the G0-G1 interphase.

Other authors have described the cytostatic activity of BHA and PPB in different cell lines, but the stage at which proliferation is blocked has never been specified. Mix 300 reduced the number of G0-G1 cells and increased S without affecting G2-M. Both mixtures increased the percentage of cells with loads above 4c.

Our toxicity predictions have not been accurately adjusted to mixture study effects. Graph analysis of Sühnel slopes fits better than other mathematical models. The applied combination of genotoxicity and cytotoxicity testing proved be a useful combination of simple tests. Success percentages depend on the parameters used, with MTT reduction test responses being the most predictable, unlike the neutral red capture test, a relevant methodology test. ECVAM [7, 19]-validated, EU-recommended 3erres. Flow cytometry results showed that some effects could be predicted. All models correctly predicted the increase in G0-G1 cells from Mix 100 treatments. These detected effects are unpredictable because individual treatments do not show them. These are emergent effects.

Vero cells exposed to Mix 300 for 24 hours showed hydropic degeneration after 300 M BHA, but not PPB. Other authors observed the same phenomenon in vivo and in vitro but did not explain its cause. Mitochondria and lysosomes changed more after mixture treatment than after individual treatment. In mitochondria, all individual treatments except 500 M PPB for 4 h induced fragmentation of the mitochondrial reticulum, which was also observed in the combined treatments evaluated (Mix 100 24 h and Mix 300 4 h). Changes in oxidative phosphorylation and inner mitochondrial membrane potential can occur in response to cellular pathologies. After 4 hours of Mix 300 exposure, the mitochondrial membrane potential decreased, suggesting this organelle was damaged.

BHA and PPB mixtures inhibited Vero cell growth. Faced with stress, cells pause in G1 or G2-M to repair damage and avoid injury. Hydropic degeneration, associated with cycle arrest in Mix 100 and Mix 300 cultures, may be a first attempt to preserve cellular structure and function in the face of massive water influx. Vacuolization, organelle swelling, and actin cytoskeleton disorganisation suggest Vero cells were under hyperosmotic stress.

Vero cells treated with Mix 100 and Mix 300 showed cytotoxic effects, indicating the DRV regulatory mechanism was ineffective. Identifying osmotic regulation failures is difficult because molecular mechanisms are interrelated and cell type-specific. Other authors have described the key events in Vero cells, allowing us to infer possible interference from our experiment.

We evaluated western blot results similarly. Our prediction for p21 and NECC was always higher than what we could quantify, indicating that both responses are antagonistic. Taking all our results together, we can conclude that exposure to binary combinations of BHA and PPB additives triggers multiple physiological alterations, mainly from an osmotic imbalance, which can cause death. Despite the variety of mathematical models used, estimation of results does not generally match observed cellular responses. Our data show that the toxicity of a binary chemical combination can be predicted once its individual components' mechanisms of action are known. According to our results, reversibility or delayed toxicity tests can provide valuable additional information. This study shows that the toxic effects of exposing Vero cells to Mix 300 are similar, albeit of different intensity, and correspond to a mechanism of action. In this way, analogies (read across) are useful for analysing potentially toxic chemical mixtures.

5. Conclusion

(i) A proper in vitro assessment of the toxicity of chemical products and mixtures requires quantitative and qualitative parameters

(ii) BHA and PPB mixtures in Vero cells cause hypotonic stress dependent on intracellular redox potential

(iii) The experimental treatments cause an osmotic imbalance, which leads to functional changes like cell cycle imbalances, ploidy increases, DNA damage from oxidative damage, and cell death

(iv) BHA, PPB, and their binary combinations change Vero cell physiology, jeopardising organelles and cytoskeleton

(v) Withholding experimental treatments causes cell cycle imbalances and Vero cell proliferation, a delayed toxicity response

(vi) The experimental strategy has been useful for determining the toxic effects of additives and mixtures

(vii) Once the components' modes of action are known, the toxic effect of mixtures on Vero cells can be predicted

Data Availability

The data underlying the results presented in the study are available within the manuscript.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this paper.

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