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POTENTIAL GENOTOXICITY OF *ZINNIA PERUVIANA* EXTRACT

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Abstract

Cytogenetic and DNA damaging effects of 30% ethyl acetate/n-hexane extract obtained from *Z. peruviana*, were examined through chromosome aberrations on the root meristem cells of *Allium cepa* and comet assay. *A. cepa* seeds were exposed to extract (0.1, 1, 5 and 20 mg/mL) for 72 h. Exposure revealed significant effect inhibition ($p<0.05$) of mitotic index, induction of chromosome aberrations, mitotic aberrations, and micronucleus formation at 1, 5 and 20 mg/mL. Similarly, for comet assay at all concentrations tested significant increase in the damage index (312.5 ± 1.3, 314.5 ± 1.2 and 345.0 ± 1.1) was observed respectively, in comparison to control (106.6 ± 1.3). Findings of this study confirm the genotoxic activity of extract of *Z. peruviana*.

Key words: *Zinnia peruviana*, genotoxicity, *Allium cepa* assay, chromosome aberrations, comet assay, DNA damage

Introduction

The consumption of plants, plant extracts, or plant-derived phytochemicals to treat various diseases is a normal therapeutic activity that has been practiced since time immemorial. The World Health Organization estimates that up to 80 % of the world's population relies on the traditional medicinal system for some aspects of primary health care [1, 2]. However, it is also essential to note that most of the traditional herbal products have never been the subject of comprehensive toxicological investigations, such as is required for modern pharmaceutical products [3]. Based on their traditional use for long periods of time, they are often assumed to be safe. However, many researchers have exposed that numerous herbal products, which are used as food ingredients or in traditional medicine, have *in vitro* mutagenic or toxic properties [4, 5]. Toxicity is an expression of being poisonous, indicating the state of adverse effects led by the interaction between plant extract and cells. This interaction may vary depending on the active ingredient presence in the extract, as it may occur on the cell surface, within the cell body, DNA, or in the tissues beneath as well as at the extracellular matrix. Hence, evaluation of toxic properties of a plant extract is crucial when considering in public health protection because exposure to chemicals can be hazardous and results adverse effects on human beings. Studies have revealed that some plants frequently used in folk medicine are potentially genotoxic [6, 7, 8, 9, 10].

In practice, the evaluation typically includes acute, subchronic, chronic, carcinogenic, genotoxic, and reproductive effects [11]. *Zinnia peruviana* (L.) (Asteraceae) is a native plant of center and northern Argentina [12, 13]. This species is widely used in folk medicine for the treatment of malaria, for stomach pain, as hepato protective and antiparasitic, antifungal and antibacterial agent [14, 15, 16, 17, 18]. Its antimicrobial properties have been investigated *in vitro* in previous studies [19, 20], but no toxic effects have been tested yet. Therefore, the aim of this study was to determine the possible genotoxicity of *Z. peruviana* extract using *Allium cepa* test and the comet assay, single-cell gel electrophoresis.

Methods

Plant material

Zinnia peruviana (L.) (Asteraceae) aerial parts were collected in Río Grande, San Luis, Argentina in February 2008. Voucher specimens were deposited in the herbarium of the Botany Department, San Luis National University (UNSL).

Preparation of extract

Extract of *Z. peruviana* was prepared using ethyl acetate and mixtures of n-hexane and ethyl acetate of increasing polarity on flash chromatography as described in Satorres et al, 2012 [19]. The extract tested in this work was 30% ethyl-acetate/n-hexane and was disolved in dimethyl sulfoxide (DMSO) to the highest concentration to be tested (20 mg/mL) and, then, dilutions were made in concentration ranges from 20 mg/mL to 0.1 mg/mL.

Genotoxicity assay

Allium cepa Seeds Test.

Assays were carried out using of *Allium cepa* L. variety Angaco seeds (98% expected germination), provided by National Agricultural Technology Institute, San Luis. Seeds were placed on filter paper in Petri dishes and different concentrations of *Z. peruviana* extract (0.1, 1, 5 and 20 mg/mL) were added to the dishes followed by incubation for 72 h at 25 °C. Control tests were performed using distilled water as the negative control (NC) and copper sulphate (1%) as the positive control (PC). Copper sulphate is a compound with known effects on *A. cepa* root cells, presenting both cytotoxicity and genotoxicity [21]. Dimethylsulfoxide (DMSO, 1 % v/v) was also added as diluent control. After exposure, the number of seeds germinated was counted, and 10 root tips were randomly removed and placed in a solution of ethanol (99 %) and glacial acetic acid (3:1) for 24 h and stained in chloridric alcoholic carmin. Then, the meristematic regions were cut and squashed in 2 drops of 45 % acetic acid solution on the slide. The cover slip was carefully lowered on to avoid air bubbles and the sides were sealed with Euparal. Ten roots tips were made for each concentrations of *Z. peruviana* extract, along with NC and PC. The mitotic index (MI), micronucleus (MN) in the interphase, and chromosome aberrations (CA) in the dividing cells were evaluated in 500 cells per slide, totaling 5000 cells per sample. In each slide, chromosomal aberrations, considered being the presence of C-metaphase cells, chromosome losses, chromosome breaks, anaphases with spindle abnormalities, vagrant chromosomes, anaphase/telophase fragments and bridges were quantified. The MI was obtained as follows: MI= Number of cells in mitosis / Total number of cells.

The slides were observed under the An Olympus CH2 light microscope at 400X magnification and Panasonic DMC-FZ8, 12 X optical zoom digital cameras was used in order to get the clear image of

the chromosome aberrations.

Comet assay, single-cell gel electrophoresis

A pool of human blood was obtained by venous puncture from healthy, adult, young and nonsmoking volunteers (with prior consent). Briefly, 50 µL of heparinised whole blood was mixed with 950 µL of RPMI-1640 medium (Sigma) and 50 µL extract of *Z. peruviana* at 1, 5 and 20 mg/mL concentrations. Then, the cell suspensions were incubated at 37 ºC during 2 h and centrifuged at 2000g for 5 min at room temperature. Negative (whole blood and RPMI-1640) and positive controls (whole blood, 50µM H_2O_2 and RPMI-1640), were included. Cellular viability was determined by exclusion method with Trypan Blue. Briefly, after the incubation, 40 µL of negative control and positive control were mixed with 30 µL of phosphate buffered solution (PBS) and 50 µL of Trypan Blue solution 0.4% for 8 min. Cell viability was determined microscopically (400X magnification) and two categories of cells were scored: (1) live cells, which appeared uncolored or light blue, (2) dead cells, with a blue color.

Comet assay was essentially performed as described Singh et al, 1988 [22] with a few modifications: the cell suspensions were embedded in 100 µL of 1% low melting point agarose (LMPA) and they were spread on a slide pre-coated with a film of 1% normal melting point agarose. Two slides were prepared for each sample in which agarose cell suspensions were allowed to solidify at 4 ºC. After the slides were transferred to lysis solution (ClNa 2.5 M, Na_2EDTA 100 mM, Tris buffer 10 mM, DMSO 10%, triton X-100 0.8%, pH 10 at 4 º C1h) and protected from light until the neutralization. Slides were placed in an electrophoresis chamber exposed to alkali for 20 min by unwinding of DNA. Then, electrophoresis was performed for 20 min at 25 V/300 mA, electrophoresis slides were neutralized (three times), fixed in ethanol for 5 min, air-dried and stained with gel red (Biotium). Analysis of comets was carried out employing fluorescence microscope and images were taken using the camera attached to the microscope. The comet images were visualized and captured at 400X magnification. Comets were analysed on the basis of four categories according to the average queue length (comet) ± standard deviation, as follows: Category 0 (no damage): 0 to 27 µm; Category 1 (low damage): 28 to 31 µm; Category 2 (medium damage): 32 to 35 µm; Category 3 (high damage): greater than 36 µm [23]. The rate of DNA damage for each sample was calculated using the following formula: damage index, DI = n_1 +2n_2 +3n_3 +4n_4, where n_1 are cells included in Category 1, n_2 in Category 2, n_3 in Category 3 and n_4 in Category 4. Bioassays were performed in duplicate and 200 cells were analyzed per treatment: negative control, positive control and cells treated with *Z. peruviana* extract at 1, 5 and 20 mg/mL.

Statistical Data Analysis

The results represent the mean ± standard error of the mean values and statistical comparisons were analysed using Analysis of Variance Technique (ANOVA) at significant level of $p < 0.05$.

Results and Discussion

***Allium cepa* assay**

Higher plants such as *A. cepa* are accepted as admirable genetic models to evaluate genotoxic effects such as chromosome aberrations and disturbances in the mitotic cycle and frequently used to evaluate the genotoxicity of herbal extracts [24]. *A. cepa* assay enabled the assessment of different genetic endpoints, which are mitotic index (MI) and chromosome aberration (CA). Mitotic index was characterized by the total number of dividing cells in cell cycle. MI is used as an indicator of cell proliferation biomarkers which measures the proportion of cells in the M-phase of the cell cycle. Hence, the decrease in the MI of *A. cepa* meristematic cells could be interpreted as cellular death. The Table 1 show the cytological effects of *Z. peruviana* extract and control tests on the *A. cepa* root tip cells. The cells of *A. cepa* root tips after treatment with extract of *Z. peruviana* showed decreased mitotic index with increasing concentration, from 1.0 mg/mL to 20 mg/mL. The MI decreased significantly ($p < 0.05$) when compared to the NC. According to Hoshina *et al.* [25], MI significantly lower than the negative control can indicate alterations, deriving from the chemical action in the growth and development of exposed cells. The mitotic indexes in treated cells were lower compared to the NC which was 11.20. By way of exception, the MI was not significantly different in the 0.1 mg/mL concentration of *Z. peruviana* extract, when compared to the NC and the mitotic activity of *Z. peruviana* extract was significantly decreased at concentration of 20 mg/mL (MI= 2.20). Copper sulphate (1%) was used as positive control in this study. The mitodepressive effect suggests that *Z. peruviana* extract had some effects on cell division of *A. cepa*. This may be due to abnormal conditions of the cells after induced by the treatments. The abnormalities of chromosomes could be due to the

blockage of DNA synthesis or inhibition of spindle formation. They may not even allow the initiation of their biosynthesis [26]. The reduction of the mitotic index might be explained as being due to the obstruction of the onset of prophase, the arrest of one or more mitotic phases, or the slowing of the rate of cell progression through mitosis [27]. As shown in Table 1, the MI decreased at the same rate as the concentration increased from 1.0 mg/mL to 20 mg/mL. Chromosomal aberrations (CA) are characterized by changes in either chromosomal structure or in the total number of chromosomes, which can occur both spontaneously and as a result from exposure to physical or chemical agents [28]. Structural chromosomal alterations may be induced by several factors, such as DNA breaks, inhibition of DNA synthesis and replication of altered DNA. The CA, such as chromosome bridges and breaks, are indicator of clastogenic action, whereas chromosomes losses, adherence, multipolarity and C-mitosis, result from aneugenic effects [29]. According to Rank *et al*. [30], chromosome aberrations analysis not only allowed to estimate of genotoxic effects, but also enabled evaluation of their clastogenic and aneugenic actions.

In this study, chromosome aberrations were observed in all stages of mitosis. The *Z. peruviana* extract, excluding the 0.1 mg/mL concentration, induced a variety of chromosomal aberrations in mitotic cells of *A. cepa* root tips (Figure. 1). C-mitosis, anaphases with spindle abnormalities, vagrant chromosomes, anaphase/telophase fragments and bridges were the most frequent alterations. Exposure to *Z. peruviana* extract and positive control displayed a significantly higher chromosomal aberration than the negative control and the 0.1 mg/mL concentration of *Z. peruviana* extract. The 20 mg/mL concentration of *Z. peruviana* extract was similar to the PC in terms of caused chromosomal aberrations (Table 2). Chromosome bridges were commonly observed during anaphase (Figure 1 D). The bridges noticed in the cells were probably formed by breakage and fusion of chromatids or subchromatids [31]. An important frequency of c-mitosis was also observed at 1, 5 and 20 mg/mL of *Z. peruviana* extract (Figure 1 C and Table 2). Their presence may be attributed to the failure of the spindle apparatus to organize and function in a normal way. Similar observations have been made by other workers where C-mitosis was regarded as indicative of a weak toxic effect which may be reversible [32]. Vagrant chromosomes that were not organized to a specific stage of the mitotic division were also observed (Figure 1, E and F). This abnormality may be caused by unequal distribution of chromosomes with paired chromatids in which resulted from non disjunction of chromatids in anaphase. The outcomes of the micronuclei (MN) analysis of *A. cepa* root tips exposed to the controls and concentrations of *Z. peruviana* extract are given in Table 3 and Figure 1 A. The percentage of MN causation was dose dependent, and generally observed as significantly different in all treatments when compared with the NC ($p<0.05$), except for the 0.1 mg/mL of *Z. peruviana* application. Especially, at 20 mg/mL it was clearly higher than the other concentrations of *Z. peruviana* extract in all the treatments and the same as the PC (Table 3). The increase in the percentage of chromosomal aberrations in *A. cepa* mitotic cells 72 h after treatment with extract correlated with the inhibition of cell division. A number of factors, such as compound solubility, rate of transport and biodistribution, and concentration at the target site (which is influenced by time and cellular permeability), can modulate the time of occurrence of chemically-induced aberrations [33]. The occurrence of abnormal anaphases and C-mitoses in *A. cepa* indicated that spindle formation was adversely affected [34]. Other abnormalities such as the presence of binucleated cells were not observed.

Chromosomal aberrations provide a sensitive endpoint for assessing the genotoxicity of chemicals [35] and, as shown here, *A. cepa* may be a sensitive biosensor for screening the genotoxicity of xenobiotics.

Comet assay

The measurement of DNA damage can be used as a sensitive marker with great predictive value to detect the genotoxic properties of contaminant [24, 36]. In order to get a primary DNA damage profile characterization of the ethyl acetate/n-hexane extract obtained from *Z. peruviana* the standard comet assay was conducted. As can be observed in Figure 2, the positive control used in this work showed highly significant abnormal genetic changes: degraded nucleoids and comet formation (Figure 2C) in comparison to the negative control group (Figure 2 A and B) where, nucleoids without comet effect were observed microscopically. The results obtained in the evaluation of DNA damage for effect of *Z. peruviana* extract showed significantly genotoxic effect in the three different concentrations used. (Figure 3 A, B and C). Table 4 shows the damage index (DI) values. The DNA damage was classified in category 3 (high damage) with DI values of 312.5 ± 1.3, 316.5 ± 1.2 and 345.0 ± 1.1 for three concentrations tested

respectively. DI values calculated for positive and negative controls were 106.6 ± 1.3 and 314.5 ± 1.2 respectively. Although the damage indexes presented at all concentrations were higher than that of negative control. It is well documented in the literature that genetic mutations act by different steps on the carcinogenesis process and some tests, such as the comet assay, identify genotoxic agents and substances with potential risks to the human health. Genotoxic substances have in common chemical and physical properties that allow the interaction of nucleic acids. The most relevant consequence of these alterations in somatic cells may be the presence of benign or malignant tumors [37, 38, 39]. The above indicates higher genotoxicity for the different concentrations on human leukocyte DNA, at least in the experimental conditions tested. Genotoxic concentrations assayed in this study have antibacterial activity [19], but bioavailability *in vivo*, with these trials is not known. Future studies will be necessary in this regard. In summary, the observed chromosomal aberrations in conjunction with the comet assay results suggest that the plant extract used provide significant injury to genetic cellular apparatus and DNA fragments form the 'comet'.

No genotoxicity studies have reported on *Zinnia peruviana*, in this sense, this work represents a contribution.

Conflict of interest statement

The authors declare no conflict of interest.

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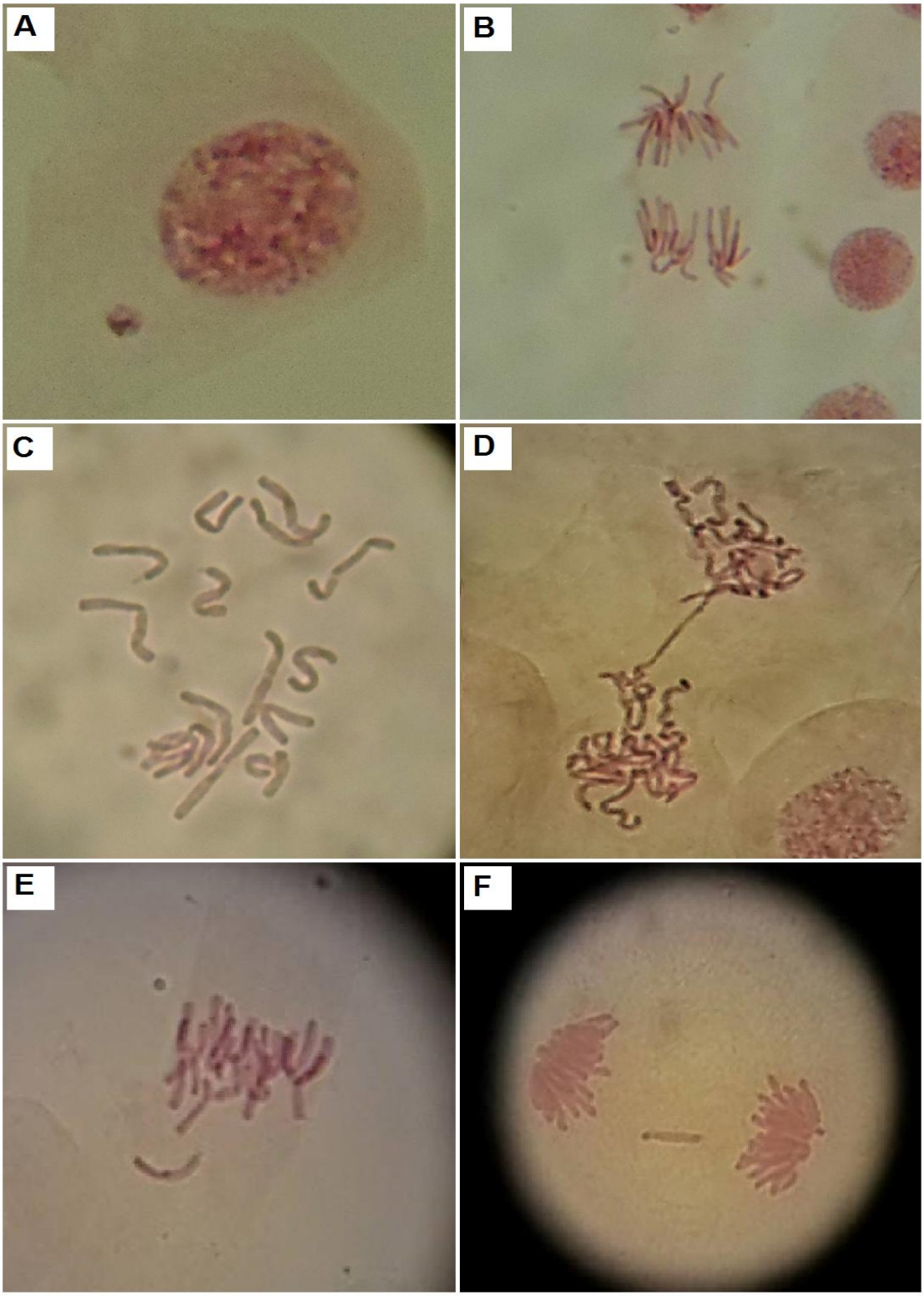

Figure 1. Chromosome aberrations induced by *Z. peruviana* extract in *A. cepa* roots tips. A: micronucleous in interphase. B: anaphase multipolar. C: C-mitosis. D: chromosome bridge. E and F: vagrant chromosome. Magnification 400X.

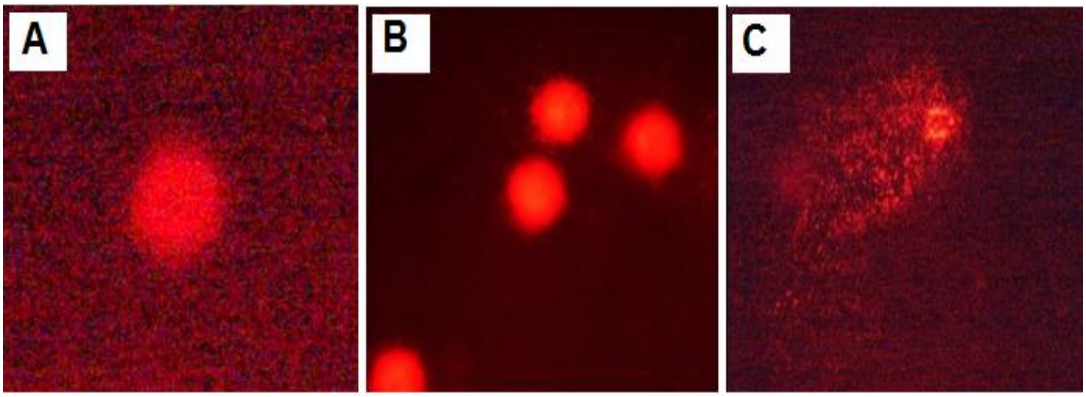

Figure 2. Nucleoids without and with genotoxic damage. A and B: Negative control: nucleoids without genotoxic damage (category 0). C: Positive control (H_2O_2, 50µM), degraded core and formation of comet (category 3).

Table 1: Cytogenetic analysis of *A. cepa* root tips exposed to different concentrations of *Z. peruvianna* extract, negative control (NC) and positive control (PC)

Concentration (mg/mL)	N° of cells	Mitotic Index*
NC	5000	11.20±0.45
PC	5000	2.00±0.54
0.1	5000	10.30±0.35
1.0	5000	6.15±0.04
5.0	5000	5.60±0.04
20.0	5000	2.20±0.24
DMSO	5000	11.00±0.30

* $p < 0.05$ versus NC (Distilled water). ±SD (Standard Deviation

Table 2: Type and frequency of chromosomal aberrations in the root tips of *A. cepa* exposed to concentrations of *Z. peruviana* extract and controls

Treatments	C. mit. %	S. abn %	Vag. C %	Frag. %	Bridges %	CA total %
NC	0.50 ±0.04	0.43±0.22	0.25±0.04	0.20±0.04	0.10±0.16	1.48
PC	6.49±0.08*	4.23±0.16*	3.15±0.20*	3.05±0.04*	2.99±0.11*	19.91
0.1 mg/mL	0.33±0.04	0.32±0.08	0.27±0.04	0.23±0.08	0.20±0.04	1.35
1 mg/mL	2.13±0.12*	2.15±0.08*	1.75±0.27*	1.85±0.26*	1.34±0.33*	9.22
5 mg/mL	2.51±0.16*	2.05±0.23*	1.60±0.33*	1.60±0.33*	0.99±0.44*	8.75
20 mg/mL	5.25±0.40	4.99±0.46*	2.15±0.04*	1.99±0.44*	2.13±0.20*	16.51

*Different from NC $p < 0.05$. **C. mit:** c. mitoses, **S. abn:** anaphases with spindle abnormalities, **Vag. C:** vagrant chromosomes, **Frag:** fragmentation, **CA:** chromosomal aberrations.

Table 3. Result from the micronucleus analysis testing of *Z. peruviana* extract and using controls in the *A. cepa* test

Concentration (mg/mL)	N° of cells	Micronucleus %
NC	5000	-
PC	5000	0.10±0.05*
0.1	5000	-
1.0	5000	0.07±0.02*
5.0	5000	0.08±0.02*
20.0	5000	0.10±0.04*
DMSO	5000	-

* $p < 0.05$ versus NC (Distilled water).-

Table 4. Values of damage index (DI) of *Z. peruviana* extract at differents concentrations and controls

Extract	Concentration (mg/mL)	Damage Index (DI)
Z. peruviana	1	312.5 ± 1.3
Z. peruviana	5	316.5 ± 1.2
Z. peruviana	20	345.0 ± 1.1
NC	0	106.6 ± 1.3
PC	50*	314.5 ± 1.2

NC: Negative control. PC: Positive control (*μM)

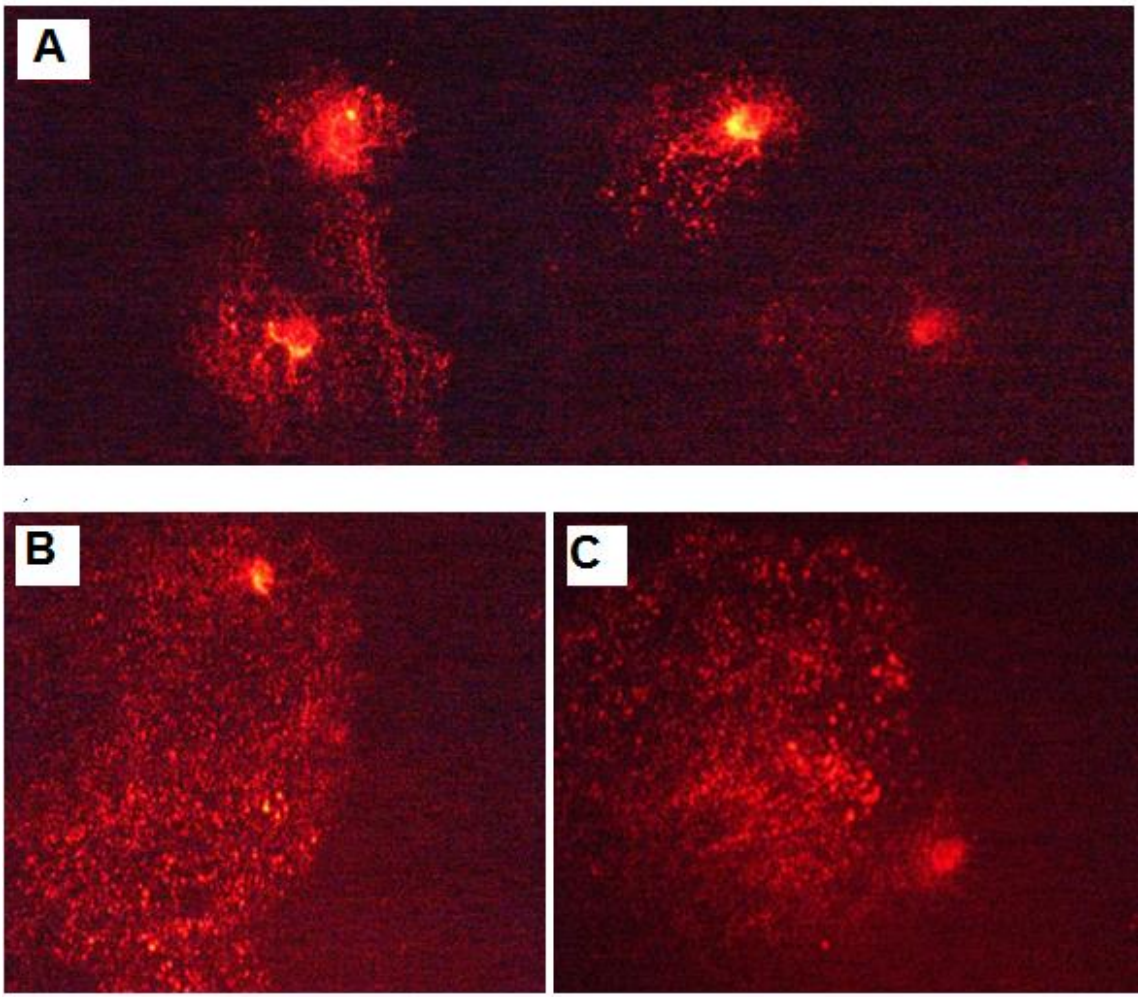

Figure 3. Evaluation of genotoxic effects at different concentrations of *Z. peruviana*. Nucleoids with genotoxic damage (Category 3) in A: *Zinnia peruviana* (1 mg/mL) B: *Zinnia peruviana* (5 mg/mL) and C: *Zinnia peruviana* (20 mg/mL).