MERIT RESEARCH JOURNALS
www.meritresearchjournals.org

Merit Research Journal of Microbiology and Biological Sciences (ISSN: 2408-7076) Vol. 11(1) pp. 001-010, July, 2024
Available online http://www.meritresearchjournals.org/mbs/index.htm

DOI: 10.5281/zenodo.12685692

Original Research Article

Evaluation of the Toxic Activity of *Zinnia Peruviana* Products

Ana Mariel Mohamed[1*], Franco Escobar[2], Cristina Torres[2], Sara Elena Satorres[1], Diego Alberto Cifuente[3], Claudia Maricel Mattana[1]

[1]Microbiology and Immunology Area, Faculty Chemistry, Biochemistry and Pharmacy, National University of San Luis, San Luis, Argentina.

[2]Virology Area, Faculty of Exact, Physical-Chemical and Natural Sciences, National University of Río Cuarto, Córdoba, Argentina.

[3]Organic Chemistry Area, Faculty Chemistry, Biochemistry and Pharmacy, National University of San Luis, San Luis, Argentina.

[*]**Corresponding Author's E-mail: marielmohamed@gmail.com**

Abstract

Although plants have been used in popular medicine for ages, their safety is often not well studied, and many can be toxic. In this study, the toxicity of *Zinnia peruviana* products was evaluated through cytotoxicity and genotoxicity assays. Neutral Red (NR) uptake and MTT reduction were used to measure cell growth inhibition by these products. Genetic damage was evaluated using the comet assay. Acetonic extracts of roots, leaves and flowers and ziniolide (major metabolite of the roots extract), were evaluated. The flowers and roots extracts, as well as the ziniolide did not present significant cytotoxic effects on Vero cells using neutral red and MTT assays (CC_{50}> 20 µg/mL). These products also did not cause DNA damage at the lowest concentration tested (0.156 mg/mL). Significant genotoxic damage was observed at concentrations equal to or greater than 1.25 mg/mL, following a dose-dependent behavior. However, the leaves extract showed cytotoxic activity with NR and MTT (CC_{50} = 18 µg/mL and CC_{50}= 15 µg/mL respectively) and also genotoxicity at low concentration evaluated in previous studies. The results of this research are promising for advancing studies in search of the bioapplicability of these natural compounds as antimicrobial agents.

Keywords: Cytotoxicity, Genotoxicity, *Zinnia peruviana,* Ziniolide

INTRODUCTION

Although plants have been used since ancient times in popular medicine, there are few studies that determine their safety and many are toxic to the human body. Toxicity is the ability of a chemical compound to produce adverse effects in a living being when it comes into contact with it (Sánchez-Bayo *et al*., 2011). In this sense, given the growing trend to integrate traditional medicine with primary health care, ensuring the safety and understanding the toxicology of commonly used medicinal plants has become a significant challenge.

Cytotoxicity tests, like Neutral Red uptake (NR) and MTT reduction assays, are crucial for assessing cellular damage caused by plant extracts. These tests indirectly measure cell growth inhibition (Liu *et al*., 2018; Riss *et al*., 2004). The NR assay measures cell viability by dye accumulation in lysosomes, while MTT assesses mitochondrial activity. The 50% cytotoxic concentration (CC_{50}) is the most common way to determine the concentration that causes toxicity in 50% of the cells in a culture (Grela *et al*., 2018; Repetto *et al*., 2008; Indrayanto *et al*., 2021). Genotoxicity assays, such as the comet assay, detect compounds causing genetic damage, crucial for evaluating mutagenicity. The comet assay quantifies DNA strand breaks in cells, showing the extent of damage (Repetto Jiménez and Repetto Kuhn, 2009; Cordelli *et al*., 2021; Singh *et al*., 1988; Speit *et al*., 2009). These *in vitro* tests are essential for ensuring the safe use of natural products in clinical pharmacology.

Zinnia peruviana (L.) (*Asteraceae*) is a native plant that grows in central and northern Argentina (Goma *et al*.,

2019). The aerial parts of species from the genus *Zinnia* have various ethnopharmacological uses and have been evaluated for different biological properties (Barboza *et al*., 2009; Mohamed *et al*., 2017; Satorres *et al*., 2012). Likewise, this genus exhibits a diversity of secondary metabolites from various classes (Cavalcante *et al*., 2020). Among them, sesquiterpene lactones represent one of the main isolated constituents, being responsible for various biological activities, such as anticancer, anti-inflammatory and immunomodulatory effects, antiulcer, antifungal and antiviral activities (Lent *et al*., 1977; Subramaniam *et al*., 2014).

The antimicrobial and antibiofilm activity of *Z. peruviana* extracts, as well as the major metabolite isolated from roots, were evaluated in previous studies by our research group, with promising results (Mohamed *et al*., 2021). However, when evaluating the biological activities of natural products with potential use in clinical pharmacology, it is essential to analyze their toxicity through different methodologies, thus allowing their safe use to be substantiated. The aim of this research was to evaluate the toxicity of these extracts *in vitro* through cytotoxicity and genotoxicity assays.

MATERIALS AND METHODS

Zinnia peruviana extracts and ziniolide

The acetone extracts of roots, leaves and flowers of *Z. peruviana* were prepared as described in Mohamed *et al.* (2021). In that study, a major metabolite in the root extract, a sesquiterpene lactone called ziniolide, was isolated and identified. This metabolite was included in the analysis of toxic activity.

Cytotoxic effect of plant products on eukaryotic cells

The effect of extracts from flowers, leaves, and roots of *Z. peruviana*, as well as ziniolide, on eukaryotic cells was evaluated using Vero cells (C76 ATCC TL 1459) seeded in 96-well microplates (ThermoScientifi Nun MicroWel 96-Well, Nunclon Delta-Treated, Flat-Bottom Microplate). Each assay was performed in quadruplicate, and cellular controls (untreated cultures) were included. All cultures were incubated at 37ºC. Cellular toxicity was evaluated at 48 hours post-treatment by microscopic observation of cellular alterations, uptake of neutral red and reduction of MTT.

Determination of the Maximum Non-Cytotoxic Concentration (MNCC)

The morphology of the cells was observed under an inverted optical microscope to detect alterations such as cell rounding, granulations, cytoplasmic vacuolization, and/or cell detachment. Concentrations of the tested samples that caused visible morphological alterations under the microscope were considered cytotoxic. The maximum concentration that did not alter the cell monolayer was defined as the Maximum Non-Cytotoxic Concentration (MNCC). Different concentration ranges were tested: ziniolide (10-125 µg/mL), leaves and roots extracts (5-95 µg/mL) and flowers extract (95-170 µg/mL).

Determination of CC_{50} by analysis of lysosomal activity: Neutral Red (NR) uptake assay

After the treatment period (48 h) with the samples at different concentrations, the culture medium was replaced with 0.2 mL of a NR solution (50 µg/mL Sigma Aldrich) and incubated at 37ºC for 3 h to promote the incorporation of the dye into the cells. After incubation, the medium containing NR was discarded and the cell monolayers were washed with PBS. Subsequently, 150 µL/well of a clarifying solution (49% distilled water + 50% ethyl alcohol + 1% acetic acid in water) was added, which dissolved the NR contained in the lysosomes. It was shaken for 15 min and the optical density (OD) was measured at 540 nm in an ELISA reader (Microplate Spectrophotometer Reader Multiskan GO - ThermoFisher Scientific).

The cell survival fraction (SF) was determined using the following formula:

$SF\ \% = (OD_{treated\ cells}/OD_{control\ cells}) \times 100$

These values allowed us to build a graph based on the different concentrations tested for each sample and calculate the 50% Cytotoxic Concentration (CC_{50}) by non-linear regression analysis.

Determination of CC_{50} by detection of mitochondrial activity using the colorimetric MTT reduction method

Vybrant MTT Cell Proliferation Assay Kit (Molecular Probes Invitrogen Detection Technologies, Eugene, Oregon, USA) was used. MTT is a tetrazolium salt [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide], which is cleaved by the mitochondria of living cells, giving rise to a product (formazan) of blue color, which can be detected in a spectrophotometer.

After the treatment period (48 h) 20 µl of an MTT solution (1 mg/mL) was added to each well and the plate was incubated at 37°C for 4 h. Then, the untransformed MTT was removed and the formazan blue crystals were solubilized by the addition of DMSO (150 µl/well). The OD was measured at 540 nm using an ELISA reader (Microplate Spectrophotometer Reader Multiskan GO - ThermoFisher Scientific).

Different concentration ranges were tested: ziniolide (10-150 µg/mL), roots extract (5-65 µg/mL), leaves extract (5-50 µg/mL) and flowers extract (110-170 µg/mL). The cell survival fraction was determined as detailed in the previous point.

Genotoxic effect of plant products on eukaryotic cells. Comet Assay

The comet assay is a method to measure DNA breaks in cells, based on the principle that fragmented DNA migrates faster than intact DNA during electrophoresis through an agarose matrix. The technique was performed as described by Singh *et al.* (1988) with some modifications.

The preparation of mounting the cells on slides was carried out. Each slide was degreased with alcohol and dried at 60°C for 24 h. Subsequently the slides were pre-coated with 200 µl 1% normal-melting-point agarose (NMPA) (Sigma-Aldrich) and completely dried at room temperature. The cell suspension was made up of human whole blood obtained by venipuncture from young, healthy, non-smoking volunteers with prior consent. The viability of cell suspension was determined via staining with trypan-blue 0.4% (Sigma-Aldrich).Thus, 50 µL of heparinized whole blood was mixed with 950 µL of RPMI-1640 medium (Sigma-Aldrich) and 50 µL of the extracts: flowers extract (10, 1.25 and 0.156 mg/mL), roots extract (20, 10, 1.25 and 0.156 mg/mL) and ziniolide (20; 10 and 0.156 mg/mL). Then, the cell suspensions were incubated at 37ºC for 2 h and centrifuged at 2000 rpm for 10 min at room temperature (Sigma Laboratory Centrifuges 3K30). Negative controls (whole blood and RPMI-1640) and positive controls (whole blood, 50 µM H_2O_2 and RPMI-1640) were included. A total of 200 µl of 1% low-melting-point agarose (LMPA) (Thermo Fisher Scientific Inc., Waltham, MA, USA) kept at 37°C was mixed with the cell suspension and placed on top of the agarose layer, and a lamella was placed on top. The slides thus prepared were placed on ice for 10 min for solidification, after which the lamella was removed, and the slides were subjected to a lysis step. Two slides were prepared for each sample.

Finally, the coverslips were removed and the glasses were immersed in koplins containing cold lysis solution protected from light (2.5 M of NaCl, 10 mM of Tris, and 100 mM of Na_2EDTA plus 1% sodium lauryl sarcosinate (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) at pH 10. Before use, 1% Triton X-100 (Sigma Aldrich, Merck KGaA, Darmstadt, Germany) and 10% DMSO (dimethyl sulfoxide) (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) were added, with the role of capturing the radicals generated by hemoglobin iron. The lysis time was 1h, and lysis occurred at 4°C and in the dark.

After incubation with the lysis solution the slides were placed in a horizontal electrophoresis tank (BIO-RAD), immersed in ice, oriented in the cathode-anode direction. Positive and negative controls were incorporated in each electrophoretic run. The glasses were immersed in electrophoresis buffer (NaOH 10 N, Na_2EDTA 200 mM) and left in contact with it for 20 min for unwinding of the DNA. After this time, the electrophoretic run was carried out at 25 volts and 300 mA for 20 min. At the end of the run, the glasses were carefully removed and washed three times with neutralization buffer (base tris 0.4 M 48,5 g, distilled water 1000 mL), 5 min each wash, in order to eliminate excess salts and improve their subsequent visualization under the microscope. The slides were drained and dehydrated in alcohol for 5 min. They were air dried for 1 h and stored in boxes inside the drying chamber until use.

Assessment of comets by image analysis

To carry out the assessment of comets, the prepared slides were stained with GelRed Nucleic Acid Gel Stain (Biotium). The evaluation of DNA damage was carried out using a fluorescence microscope (Epifluorescence microscope Nikon Eclipse 50i), and the images were captured with a digital camera (Nikon) attached to the microscope. The comets were analyzed by visual observation based on five categories according to the length of the tail (comet) ± standard deviation (Brugés *et al*., 2007): Category 0 (no damage), Category 1 (low damage), Category 2 (medium damage), Category 3 (high damage) and Category 4 (extremely high damage). The DNA damage rate 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. The bioassays were performed in duplicate and 200 cells were analyzed per treatment, performed by a single observer.

Tukey's multiple comparison test was conducted to identify significant differences. Significant differences ($p < 0.05$). The results represent the mean ± standard deviation of the mean values.

RESULTS AND DISCUSSION

The use of plants, plant extracts or phytochemicals used to treat various diseases is a therapeutic activity that has been practiced since ancient times. However, there are few studies that support the safety of medicinal plants. In this sense, several investigations have shown that numerous herbal products, which are used as food ingredients or in traditional medicine, express mutagenic or toxic properties *in vitro* (Chan, 2003; Soetan and

Table 1. Maximum Non-Cytotoxic Concentration (MNCC) values obtained for products derived from *Z. peruviana*.

Product	MNCC (µg/mL)
Flowers extract	100
Leaves extract	5
Roots extract	20
Ziniolide	25

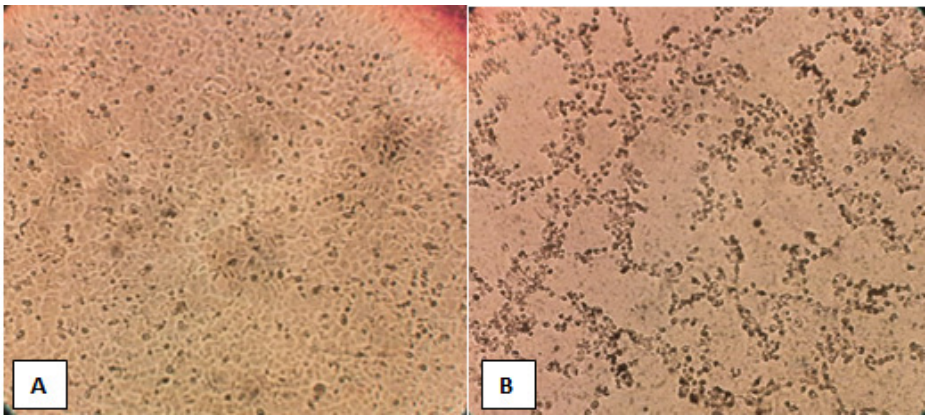

Figure 1. Cytotoxic effect on Vero cells. Cellular control, confluence of the cell monolayer is observed (A). In cells treated with *Z. peruviana* roots extract, cytotoxic effects are observed: formation of holes due to cell detachment, retraction and refraction of the cells (B). Magnification100X.

Aiyelaagbe, 2009). Toxicity is the ability of a chemical compound to produce adverse effects in a living being. Toxicological parameters include dose, exposure time, and sublethal effect. (Sánchez-Bayo *et al*., 2011). Within the battery of *in vitro* tests essential for the registration or application of clinical trials for a given substance, cytotoxicity tests play a crucial role. These tests detect adverse effects on cellular mechanisms by interfering with structures and/or properties essential for cell survival, proliferation, and functions. Cellular cytotoxicity is defined as an alteration of basic cellular functions that leads to detectable damage.

This work evaluated the cellular and genetic toxicity of extracts from the flowers, leaves, and roots of *Z. peruviana*, as well as ziniolide. Cytotoxicity was assessed in Vero cell cultures at 48 hours post-treatment. The maximum non-cytotoxic concentration was determined through microscopic observation of changes in cell morphology and the CC_{50} was established using colorimetric assays.

Determination of the Maximum Non-Cytotoxic Concentration (MNCC)

Those concentrations of the tested samples that generated morphological alterations in cells visible under the microscope were considered cytotoxic. The maximum concentration of the compound under study that did not alter the cell monolayer was defined as the Maximum Non-Cytotoxic Concentration (MNCC). Table 1 displays the MNCC values obtained for the products derived from *Z. peruviana*.

These results showed that the leaves extract produced cell damage at a lower concentration (MNCC=5 µg/mL). The root extract and ziniolide had maximum non-cytotoxic concentrations of 20 µg/mL and 25 µg/mL respectively (Table 1), suggesting that the major compound in roots (ziniolide) may be responsible for the observed cytotoxic damage. The slightly greater toxicity of the root extract compared to the ziniolide could indicate the interaction of other minor compounds that would enhance the damage. The flowers extract produced alterations in Vero cells at a higher concentration, with an MNCC of 100 µg/mL. The *in vitro* cytotoxic effect was characterized by loss of cell confluence, cell lysis, cell detachment, and cell retraction and refraction of the cells (Figure 1).

Determination of CC_{50} by analysis of lysosomal activity: Neutral Red (NR) uptake assay

The NR uptake assay is a chemosensitive method that assesses cell survival and viability by measuring the ability of viable cells to incorporate and bind NR, a supravital dye. Lysosomal alterations induced by incubation with extracts result in gradual changes that lead to a decrease in the uptake and binding of the dye.

The cytotoxicity pattern detected by the NR assay was

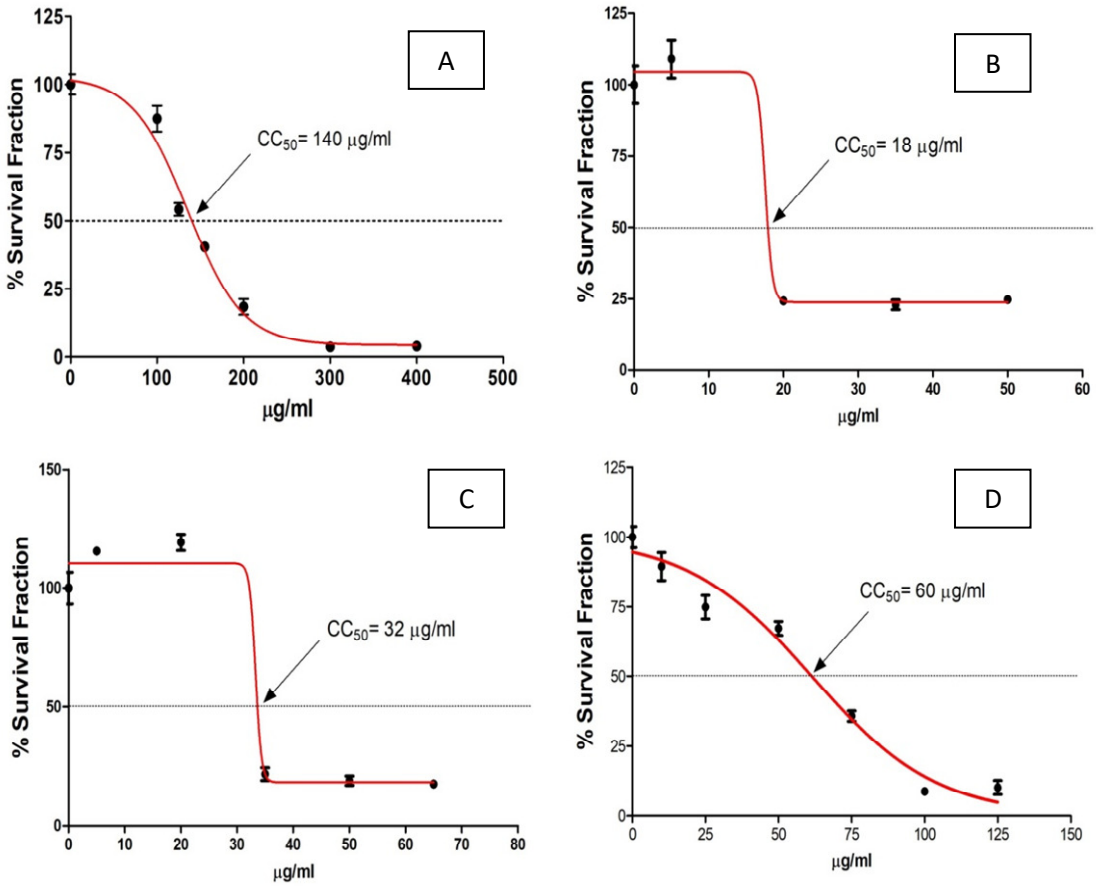

Figure 2. Percentage of viability of cultured Vero cells, incubated for 48 h in the presence of *Z. peruviana* products at different concentrations (µg/mL) determined by neutral red uptake (NR). Each point represents the mean of four independent trials. Flowers extract (A). Leaves extract (B). Roots extract (C). Ziniolide (D).

consistent with that observed by light microscopy. *Z. peruviana* flower extract exhibited the lowest cytotoxic effect, requiring a concentration of 140 µg/mL to damage 50% of the cells (Figure 2A). In contrast, the leaves extract was the most cytotoxic, with 18 µg/mL being sufficient to cause damage to 50% of the cells (Figure 2B).

Figures 2C and 2D display the CC_{50} values expressed by *Z. peruviana* roots extract and ziniolide, respectively. Comparing the cytotoxic effects of both, the greater toxicity of the roots extract is evident, as twice the concentration of ziniolide was required to produce the same effect. This observation supports the hypothesis of the participation of other compounds in lower concentrations, which not only contribute to increasing the antimicrobial activity of the roots extract but also to its greater cytotoxicity.

Determination of CC_{50} by detection of mitochondrial activity using the colorimetric MTT reduction method

The concentration that caused 50% cell death was determined using the MTT reduction method. In this method, the reagent is reduced by the mitochondria of living cells, resulting in the formation of formazan blue. After the treatment period, the cell survival fraction was determined, and the results were analyzed.

Cytotoxicity determined by the neutral red uptake assay and the MTT reduction method was consistent across all samples tested (Table 2). The highest cytotoxicity determined by the MTT assay was exhibited by the *Z. peruviana* leaves extract, while the lowest toxicity was observed with the flowers extract (Figures 3B and 3A). Figures 3C and 3D show the percentages of cell survival due to the effects of the roots extract and ziniolide respectively. The concentrations that produced metabolic alterations in 50% of the cells (CC_{50}) also induced morphological alterations in the tested cellular system.

Kuete *et al.* (2011) postulated that a CC_{50} value greater than 20 µg/mL indicates that compounds are not toxic to cells. Based on this criterion and the CC_{50} values obtained from the NR and MTT assays, only the leaves extract was cytotoxic (CC_{50} = 15-18 µg/mL). A study by Echenique *et al.* (2020) conducted *in vivo* confirmed the

Table 2. Values of Cytotoxic Concentration 50% (CC_{50}) using NR and MTT tests obtained for products derived from *Z. peruviana*.

Product	CC_{50} NR (µg/mL)	CC_{50} MTT (µg/mL)
Leaves extract	18	15
Flowers extract	140	115
Roots extract	32	26
Ziniolide	60	73

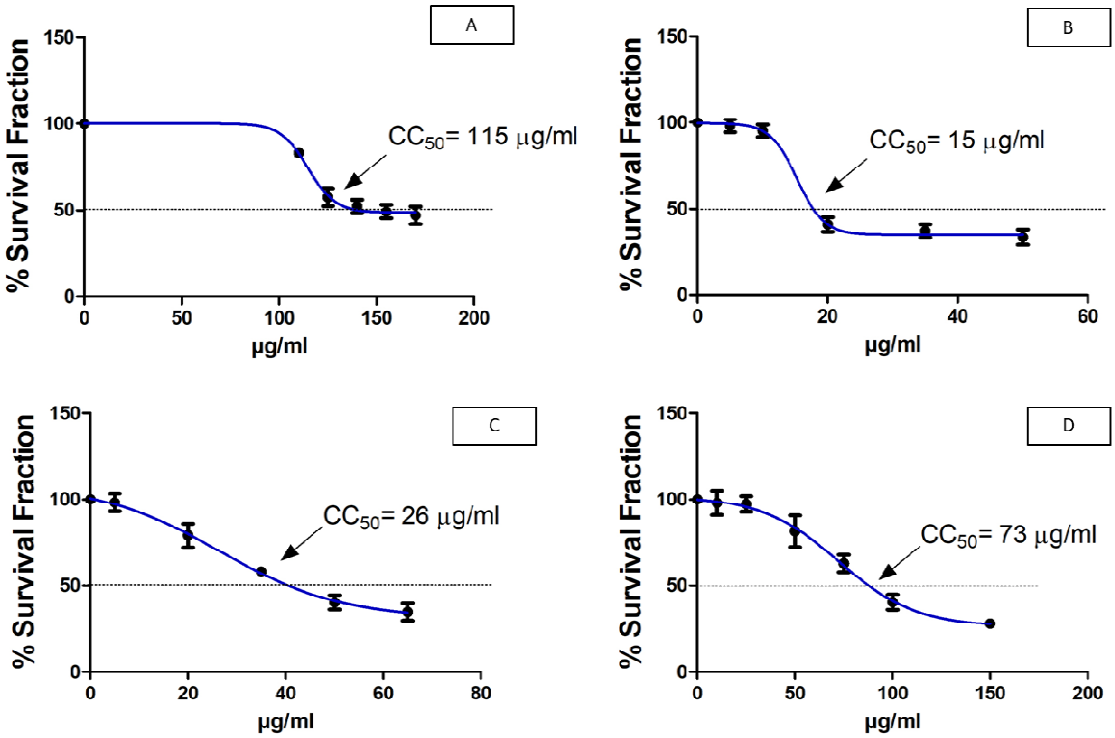

Figure 3. Percentage of viability of cultured Vero cells, incubated for 48 h in the presence of *Z. peruviana* products at different concentrations (µg/mL) determined by the MTT reduction method. Each point represents the mean of four independent trials. Flowers extract (A). Leaves extract (B). Roots extract (C). Ziniolide (D).

cytotoxicity observed *in vitro*. The researchers administered a mixture of *S. aureus* and *Z. peruviana* leaves extract (70:30 ethyl acetate:n-hexane) into the nostrils of mice. While the extract controlled the infection, preventing its spread to other organs, it was found to be cytotoxic, causing histological alterations.

The cytotoxicity of the acetonic extract of *Z. peruviana* leaves may be related to its major compounds. Khaw *et al.* (2020) studied phytosterols (campesterol, stigmasterol, and β-sitosterol) from *Carica papaya*, finding cytotoxic activity for all. Stigmasterol showed significant selectivity for cancer cells, possibly due to a double bond in its side chain (C-22). Additionally, leaves may be more toxic than roots as a defense against herbivores (War *et al*., 2012; Zaynab *et al*., 2021).

In contrast, *Z. peruviana* flowers extract did not show significant cytotoxicity (CC_{50} = 140-115 µg/mL). Although the cytotoxic activity of this fraction of the plant has not been extensively studied, it could be associated with the low toxicity of its compounds, which include carotenoids and anthocyanins (Qing *et al*., 2022; Ino and Yamaguchi, 1993).

In relation to the toxicity expressed on Vero cells by the *Z. peruviana* roots extract, a concentration range of 26 to 32 µg/mL was required to induce metabolic alterations in 50% of the cells. Several studies have investigated the cytotoxicity of sesquiterpene lactones, which are present in the roots of *Z. peruviana*. Bordignon *et al.* (2017) tested the cytotoxic activity of sesquiterpene lactones from another species belonging to the *Asteraceae* family and found cytotoxicity against mammalian cell lines with ranges between 10 and 35 µg/mL, consistent with our results (Bordignon *et al*., 2017). Sesquiterpene lactones represent a large group of

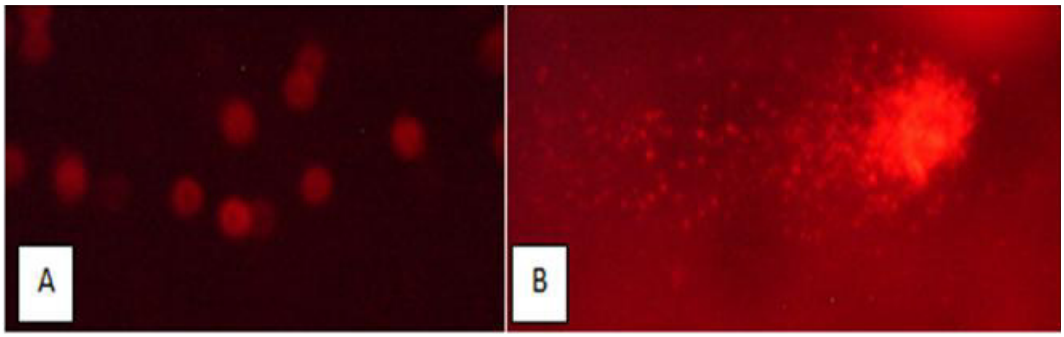

Figure 4. Comet Assay. Microscopic observation. Negative control (A): Tailless nucleoids (category 0). Positive control (B): Nucleoid with genotoxic damage (category 3). Magnification 400X.

natural products and constitute one of the largest groups of plant secondary metabolites, with more than 5000 compounds found in species of the plant kingdom, particularly in the *Asteraceae* family (Chadwick *et al*., 2013). This group has received significant attention due to its bioactivity, including cytotoxicity against cancer cells and *in vivo* antineoplastic efficacy (Wu *et al*., 2016). Li *et al*. (2020) studied sesquiterpene lactones from *Asteraceae* roots and highlighted the α-methylene-γ-butyrolactone ring as a key structure for various bioactive effects, such as anti-inflammatory, antioxidant, cytotoxic, and antimicrobial activity (Li *et al.,* 2020).

The cytotoxicity of the metabolite isolated from the roots of *Z. peruviana*, ziniolide, has not been previously reported. However, this compound was isolated by Bader *et al.* (2013) from the roots of another species belonging to the *Asteraceae* family, *Xanthium spinosum* L. In their work, they investigated the biochemical mechanisms that could justify some of the plant's popular uses as an anti-inflammatory. They concluded that one of the main anti-inflammatory mechanisms of this plant species is the rapid inhibition of pro-inflammatory eicosanoids and the increase in the production of anti-inflammatory mediators, followed over time by a longer-lasting downregulation of these enzymes through the inhibition of NFκB (nuclear factor κB), identifying ziniolide as one of the active principles responsible for these activities (Bader *et al*., 2013).

Based on established toxicity criteria (Kuete *et al*., 2011), the bioassays carried out with neutral red and MTT indicated that the acetone extracts of flowers and roots of *Z. peruviana*, as well as the ziniolide compound, do not significantly affect lysosomal and mitochondrial functions. This is an interesting finding, given that these products showed significant antibacterial, antifungal, and antibiofilm activity (Mohamed *et al*., 2021).

Genotoxic effect of plant products on eukaryotic cells. Comet Assay

Genotoxicity assays detect compounds that damage genetic material, which is crucial for evaluating mutagenicity and toxicological characterization (Repetto Jiménez and Repetto Kuhn, 2009). The comet assay is a method used to detect genotoxic effects induced by xenobiotics by quantifying DNA damage in lymphocytes. In this assay, cells with greater DNA damage exhibit more significant DNA migration toward the anode in an agarose matrix (Alvarez *et al*., 2013; Singh *et al*., 1988).

The potential *in vitro* genotoxic effect of extracts from flowers and roots of *Z. peruviana*, as well as ziniolide, considered the most promising natural products based on their antimicrobial and antibiofilm activity, was evaluated using the comet assay. Genotoxic cellular damage was determined by visually counting nucleoids observed under the microscope, which were classified into five categories according to the level of damage. As shown in Figure 4, H_2O_2, used as a positive control, exhibited a significant genotoxic effect, with degraded nucleoids and class 3 comet formation (Figure 4B) compared to the negative control, where nucleoid heads without comet formation were observed (Figure 4A).

The tested concentrations of extracts from flowers and roots of *Z. peruviana*, along with the major root metabolite ziniolide, induced varying degrees of genotoxic damage in human lymphocytes, as evidenced by the length of the comet tail. Figure 5A shows that the percentages of nucleoids with medium and high damage induced by the flowers extract increased with higher concentrations. Conversely, there was a decrease in the percentage of nucleoids without damage as concentrations increased, indicating a dose-dependent behavior. A similar pattern was observed with the roots extract and ziniolide (Figures 5B and 5C).

Based on the classification of the nucleoids observed in the different categories according to the level of damage, the damage index (DI) was calculated for each product and the results are presented in Table 3.

As shown in Table 3, flowers and roots extracts at concentrations of 1.25 mg/mL and higher were genotoxic. The toxic effects on DNA displayed a dose-dependent behavior, with higher DI values observed at higher concentrations. At 10 mg/mL, the *Z. peruviana* flowers

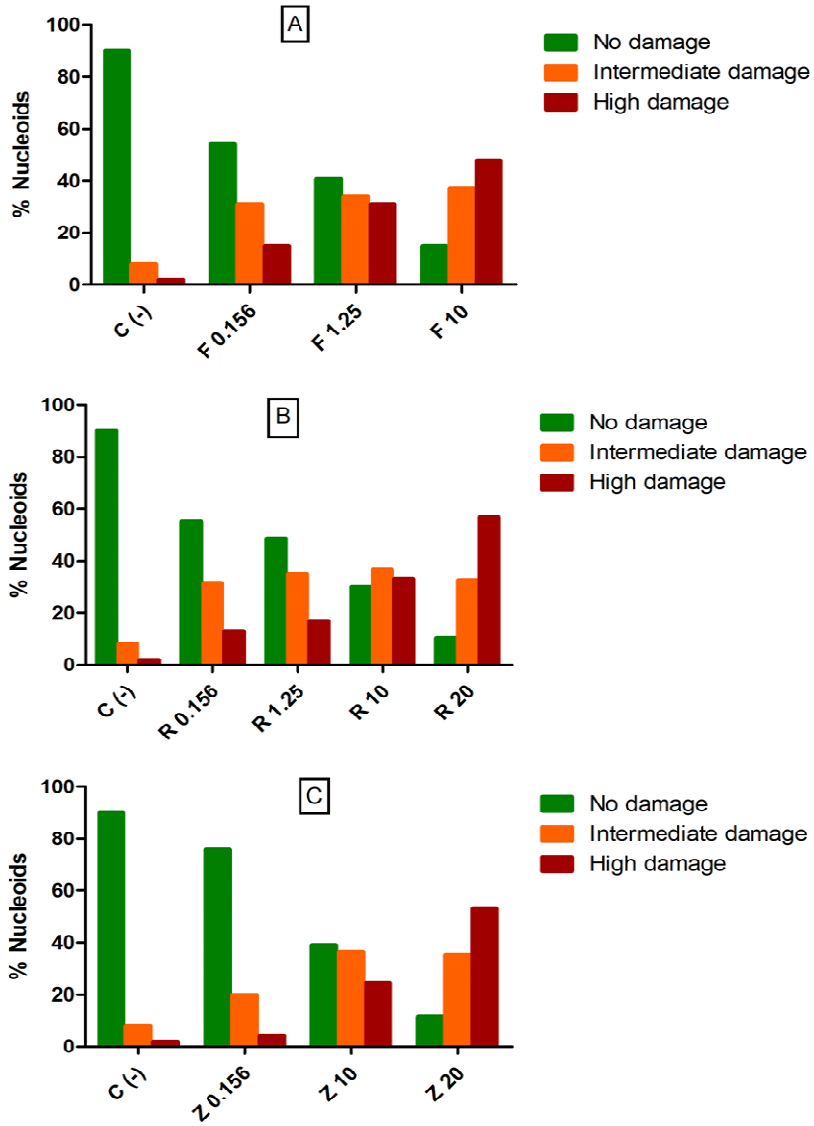

Figure 5. Percentages of nucleoids grouped into three damage categories: no damage, intermediate damage, and high damage. C (-): negative control. Concentrations expressed in mg/mL. Treatment with *Z. peruviana* flowers extract (A). *Z. peruviana* roots extract (B) and ziniolide (C).

Table 3. Damage Index (DI) values of *Z. peruviana* extracts, ziniolide and controls.

Product	Concentration (mg/mL)	Damage Index (DI)
Flowers extract	10	436 ± 1.0
Flowers extract	1.25	286 ± 1.1
Flowers extract	0.156	214 ± 1.2
Roots extract	20	464 ± 1.2
Roots extract	10	374 ± 1.1
Roots extract	1.25	272 ± 1.2
Roots extract	0.156	180 ± 1.0
Ziniolide	20	455 ± 1.0
Ziniolide	10	284 ± 1.1
Ziniolide	0.156	114 ± 1.0
Negative control	0	108.3 ± 1.3
Positive control	50*	302.2 ± 1.2

*Concentration expressed in µM.

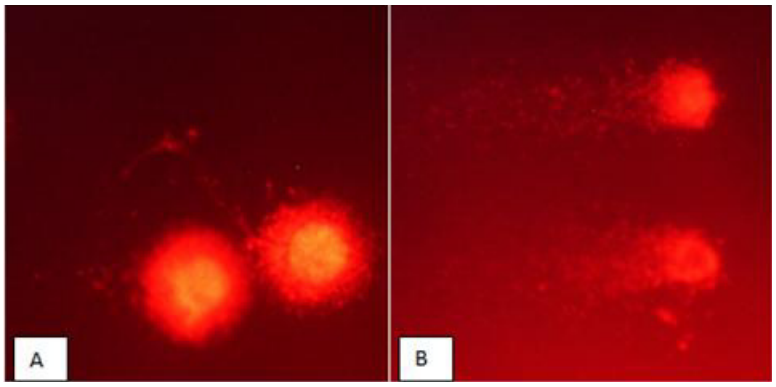

Figure 6. Comet assay. Evaluation of the genotoxic effect with the treatment of *Z. peruviana* flowers extract at 0.156 mg/mL (A) and 10 mg/mL (B). Magnification 400X.

extract caused significant DNA damage (Figure 6B), with extensive comet formation where nearly all the genetic material migrated to form a tail. This damage was greater than that induced by the positive control, with DI values = 436 ± 1.0. At a low concentration (0.156 mg/mL), the extract caused less genotoxic damage, with an DI lower than the positive control (DI = 214 ± 1.2) but twice that of the negative control, indicating a tendency towards genotoxic damage.

When testing the roots extract at different concentrations, significant DNA damage, similar to the flowers extract, was observed at high concentrations (20, 10, and 1.25 mg/mL). However, at 0.156 mg/mL, the roots extract showed a clear non-genotoxic effect (DI = 180 ± 1.0). Interestingly, cells treated with ziniolide at the same concentration (0.156 mg/mL) mostly exhibited undamaged DNA, resulting in a non-genotoxic index (DI = 114 ± 1.0). Notably, the roots extract concentration without DNA damage (0.156 mg/mL) exceeded the MIC required to inhibit microbial growth of both Gram-positive and Gram-negative bacteria and *C. albicans*. Similarly, ziniolide, the main metabolite of the roots extract, was not genotoxic at low concentrations. In contrast, the flowers extract's MICs ranged from 1.25 to 5 mg/mL for different microorganisms, and the lowest MIC (1.25 mg/mL) induced a genotoxic effect (Mohamed *et al*., 2021).

The genotoxicity of the *Z. peruviana* leaves extract was previously studied by Mattana *et al.* (2016). The authors reported chromosomal aberrations in the *Allium cepa* test and significant genotoxicity in the comet assay at concentrations of 1, 5, and 20 mg/mL. This aligns with our findings, which observed genotoxic effects of *Z. peruviana* flowers and roots extracts at concentrations of 1.25 mg/mL or higher.

No previous studies have reported *in vitro* genotoxicity of *Z. peruviana* flowers or roots extracts, nor of the ziniolide metabolite, using the comet assay. Therefore, our work represents a significant contribution to the field.

CONCLUSIONS

The present work determined the potential toxic effect of *Z. peruviana* products. This effect was demonstrated *in vitro* in eukaryotic cells at the cellular and genetic levels. All the tested bioindicators demonstrated the existence of cytotoxicity and genotoxicity with varying degrees of sensitivity.It is highlighted that flowers and roots extracts and ziniolide did not have a significant cytotoxic effect (CC_{50} > 20 µg/mL). These products also did not cause DNA damage at the lowest concentration tested (0.156 mg/mL). These results are promising for advancing studies in search of the bioapplicability of these natural compounds as antimicrobial agents.

ACKNOWLEDGMENTS

This study was supported by CONICET and National University of San Luis (PROICO 02-0218 and 02-2516).

AUTHOR CONTRIBUTIONS

AM Mohamed carried out the experimental part and wrote the manuscript. CM Mattana and DA Cifuente participated in the experimental design of the microbiological/genotoxic and chemical assays respectively. They also collaborated in the critical review of the manuscript and data content. C Torres and F Escobar supervised and guided the cytotoxicity assays. SE Satorres contributed to the revision of the manuscript. All authors read and approved the final manuscripts.

CONFLICT OF INTEREST

Authors declared that there are no conflict of interest.

REFERENCES

Alvarez MC, S. Ochoa Hernández, N. Ayala Chávez, M. E. Andrade Martínez. (2013). Prueba Cometa. Universidad de Guadalajara, México.

Bader A., Giner RM, Martini, F., Schinella, G. R., Ríos, J. L., Braca, A., & Prieto, J. M. (2013). Modulation of COX, LOX and NFκB activities by *Xanthium spinosum* L. root extract and ziniolide. Fitoterapia, (91), 284-289.

Barboza GE, Cantero JJ, Núñez C, Pacciaroni A, Ariza Espinar L (2009). Medicinal plants: A general review and a phytochemical and ethnopharmacological screening of the native Argentine Flora. Kurtziana, 34(1-2), 7-365.

Bordignon A, Frédérich M, Ledoux A, Campos PE, Clerc P, Hermann T, Quetin-Leclercq J, Cieckiewicz E (2018). *In vitro*antiplasmodial and cytotoxic activities of sesquiterpene lactones from *Vernoniafimbrillifera Less. (Asteraceae).* Natural Product Research, 32(12), 1463-1466.

Cavalcante NB, Diego da Conceição Santos A, Guedes da Silva Almeida JR (2020). The genus *Jatropha* (*Euphorbiaceae*): A review on secondary chemical metabolites and biological aspects. Chemico-Biological Interactions, 318, 108976.

Chadwick M, Trewin H, Gawthrop F, Wagstaff C (2013). Sesquiterpenoids lactones: benefits to plants and people. Int. J. Molecular Sci., 14(6), 12780-12805.

Chan K (2003). Some aspects of toxic contaminants in herbal medicines. Chemosphere, 52(9), 1361-1371.

Cordelli E, Bignami M, Pacchierotti F (2021). Comet assay: a versatile but complex tool in genotoxicity testing. Toxicology Research, 10(1), 68-78.

Echenique DR, Aguilera Merlo C, Cruceño AM, Mattana CM, Satorres SE (2020). Effect of the administration of *Zinnia peruviana* on nasal colonization and cutaneous infection by methicillin-resistant *Staphylococcus aureus* in a model mouse. Environments, 1(4), 5.

Gomaa AA, Samy MN, Desoukey SY, Kamel MS (2019). A comprehensive review of phytoconstituents and biological activities of genus *Zinnia*. J. Adv. Biomed. Pharm. Sci. 2(1), 9-37.

Grela E, Kozłowska J, Grabowiecka A (2018). Current methodology of MTT assay in bacteria - A review. ActaHistochemica, 120(4), 303-311.

Indrayanto G, Putra GS, Suhud F (2021). Validation of *in vitro* bioassay methods: Application in herbal drug research. Profiles of drug substances, excipients, and related methodology, 46, 273-307.

Ino I, Yamaguchi MA (1993). Acetyl-coenzyme A: Anthocyanidin 3-glucoside acetyltransferase from flowers of *Zinnia elegans*. Phytochemistry, 33(6), 1415-1417.

Khaw KY, Shaw PN, Parat MO, Pandey S, Falconer JR (2020). Compound identification and *in vitro* cytotoxicity of the supercritical carbon dioxide extract of papaya freeze-dried leaf juice. Processes, 8(5), 610.

Kuete V, Krusche B, Youns M, Voukeng I, Fankam AG, Tankeo S, Lacmata S, Efferth T (2011). Cytotoxicity of some Cameroonian spices and selected medicinal plant extracts. J. Ethnopharmacol. 134(3), 803-812.

Lent KH, Ibuka T, Wu RY, Geiman TA (1977) Structure-antimicrobial activity relationships among the sesquiterpene lactones and related compounds. Phytochemistry, 16, 1177–1181.

Li Q, Wang Z, Xie Y, Hu H (2020). Antitumor activity and mechanism of costunolide and dehydrocostus lactone: Two natural sesquiterpene lactones from the *Asteraceae* family. Biomedicine & Pharmacotherapy, 125, 1-12.

Liu X, Rodeheaver DP, White JC, Wright AM, Walker LM, Zhang F, Shannon S (2018). A comparison of *in vitro* cytotoxicity assays in medical device regulatory studies. Regulatory Pharmacology: RTP, 97, 24-32.

Mattana CM, Cangiano MA, Satorres SE, Alcaráz LE, Laciar AL (2016). Potential genotoxicity of *Zinnia peruviana* extract. Pharmacology Online, 2, 72-80.

Mohamed AM, Cifuente DA, Mattana CM (2018). Anti-*Listeria* activity of natural products single and mixed obtained from native plants of Argentina. Pharmacology Online, 3, 150-160.

Mohamed AM, Salinas A, Mattana C, Satorres S, Ortega C, Favier, L., & Cifuente, D.A. (2017) Improving in the antibacterial activity and the antioxidant properties of chemically modified extracts of *Zinnia peruviana*. Pharmacology Online, 2(1), 162-168.

Qing HS, Qian JY, Chen JH, Jiang LL, Fu JX, Huang XQ, Zhang C (2022). Carotenoid analysis and functional characterization of lycopene cyclases in *Zinnia elegans* L. Industrial Crops and Products, 188, 115724.

Repetto Jiménez M, Repetto KG (2009). Toxicología fundamental. Ediciones Díaz de Santos. 4ª ed.

Repetto G, del Peso A, Zurita JL (2008). Neutral red uptake assay for the estimation of cell viability/cytotoxicity. Nature Protocols, 3(7), 1125-1131.

Riss TL, Moravec RA, Niles AL, Duellman S, Benink HA, Worzella TJ, Minor L (2016). Cell viability assays. Assay Guidance Manual [Internet].

Sánchez-Bayo F, van den Brink PJ, Mann RM (2011). Ecological impacts of toxic chemicals. Sources and Toxicity of Pollulants. Bentham Science Publishers, 3-12.

Satorres SE, Chiaramello AI, Tonn CE, Laciar AL (2012). Antibacterial activity of organic extracts from *Zinnia peruviana* (L.) against gram-positive and gram-negative bacteria. Emirates Journal of Food and Agriculture, 24(4), 344-347.

Singh NP, McCoy MT, Tice RR, Schneider EL (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. Experimental Research, 175(1), 184-191.

Soetan KO, Aiyelaagbe OO (2009). The need for bioactivity-safety evaluation and conservation of medicinal plants-A review. J. Med. Plants Res, 3(5), 324-328.

Speit G, Vasquez M, Hartmann A (2009). The comet assay as an indicator test for germ cell genotoxicity. Mutation Research, 681(1), 3-12.

Subramaniam, S., Keerthiraja, M. & Sivasubramanian, A. (2014). Synergistic antibacterial action of β-sitosterol-D-glucopyranoside isolated from *Desmostachyabipinnata* leaves with antibiotics against common human pathogens. Revista Brasileira de Farmacognosia, 24(1), 44-50.

War AR, Paulraj MG, Ahmad T, Buhroo AA, Hussain B, Ignacimuthu S, Sharma HC (2012). Mechanisms of plant defense against insect herbivores. Plant Signaling &Behavior, 7(10), 1306-1320.

Wu Y, Wang D, Gao Y, Feng J, Zhang X (2016). New α-methylene-γ-butyrolactone derivatives as potential fungicidal agents: design, synthesis and antifungal activities. Molecules, 21(2), 130.

Zaynab, M., Sharif, Y., Abbas, S., Afzal, M. Z., Qasim, M., Khalofah, A., Ansari, M. J., Khan, K. A., Tao, L., & Li, S. (2021). Saponin toxicity as key player in plant defense against pathogens. Toxicon: official journal of the International Society on Toxicology, 193, 21-27.