SCIENTIFIC CULTURE

www.sci-cult.com

SCIENTIFIC CULTURE, Vol. 11, No. 4, (2025), pp. 2418-2424
Open Access. Online & Print

DOI: 10.5281/zenodo.11425285

IN VITRO GENOTOXICITY SCREENING OF SEARSIA RHEMANNIANA EXTRACTS AGAINST VERO CELLS

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Received: 11/11/2025
Accepted: 18/12/2025

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ABSTRACT

Medicinal plants offer a valuable therapeutic potential, but their safety is not guaranteed. Genotoxicity assessment is an essential part of the framework for the development of herbal products and risk assessment. As the use of traditional medicinal products increases and regulatory control increases, the integration of genotoxic endpoints with standard toxicology is becoming a key requirement for public health. Cells were seeded in 96-well plates at a density of 3000 cells/well and incubated overnight for attachment. Treatments were applied in complete medium and included a positive control, Griseofulvin. After 48 hours of incubation at 37°C and 5% CO2, cells were fixed with 4% formaldehyde, washed with PBS, and stained with Hoechst 33342. Fluorescence micrographs were captured at nine sites per well using a widefield microscope and analysed with MetaXpress software to determine the total cell count and percentage of micro-nucleated cells per well. Bark water extract produced a significant increase in micronucleus formation across all tested concentrations indicating that it may contain constituents capable of interacting directly with DNA or interfering with the mitotic spindle apparatus. Also, bark DCM extract induced a significant genotoxic response despite exhibiting cytotoxicity at higher concentrations. Leaves MeoH also demonstrated a statistically significant increase in the proportion of micro-nucleated cells within the concentration. The micronucleus assay confirmed that bark water, bark DCM, and leaves MeoH, possess genotoxic potential, as evidenced by a significant increase in the frequency of micro-nucleated cells.

KEYWORDS: Genotoxicity, Micronucleus, Searsia Rhemaniana, DNA.

1. INTRODUCTION

The global reliance on medicinal plants for health care continues to be significant (1), with estimates indicating that around 70% to 80% of the population worldwide depends on herbal remedies either fully or partially for their primary health needs (2). In many traditional medical systems, plants are utilized to address various physiological and pathological conditions due to their diverse phytochemical profiles and perceived safety (3). However, the belief that natural equates to safe is increasingly questioned by toxicological studies, which demonstrate that some plant-derived extracts may present mutagenic or genotoxic risks (4). Consequently, rigorous evaluation of genotoxic potential is now a crucial aspect of phytopharmacological research and safety assessment (5). Genotoxicity is defined as the ability of chemical or biological agents to damage cellular genetic material, including DNA strand breaks, chromosomal aberrations, gene mutations, and micronucleus formation (6). If unrepaired, such damage can result in mutagenesis, carcinogenesis, or hereditary defects (7). Medicinal plant extracts may contain a variety of secondary metabolites, such as alkaloids, flavonoids, phenolics, diterpenes, or pyrrolizidine alkaloids (8), some of which are inherently genotoxic or may become genotoxic following metabolic activation (9). For instance, pyrrolizidine alkaloids found in certain herbal medicines mainly the *Boraginaceae, Compositae and Leguminosa*e families are known to induce DNA adducts and chromosomal damage (10).

In recent decades, several studies have assessed plant extracts for genotoxic effects using a variety of in vitro and in vivo tests (11). A quantitative systematic review and found that 28.4 percent of 458 studies with medicinal plant extracts reported a positive genotoxic outcome (12). The most frequently used tests were those recommended by the regulatory agencies, including bacterial mutation assays, micronucleus or chromosomal aberrations in mammalian cells (13), comet (gel electrophoresis of single cells) and in vivo micronucleus or bone marrow tests (14). These observations highlight that genotoxicity is not uncommon in herbal preparations (15) and highlight the importance of full testing prior to use in medicinal products or in the nutraceutical sector (16). Regional studies further demonstrate the prevalence of potentially genotoxic herbal materials (17). In South Africa, a study of 51 herbal medicinal products used in traditional practices evaluated dichloromethane and 90 percent of the methanol extract against Ames and other parameters and showed a variable but significant response (18). More recently, aqueous extracts of Tulbaghia violacea was reported to induce significant chromosomal aberrations and micronuclei in the test on the root tip of Allium cepa, with more pronounced effects in extracts than in leaves or roots (19). The families Fabaceae, Asteraceae, Euphorbiaceae, Rosaceae, Lamiaceae and Apocynaceae were identified as common genotoxic positive families (20). This underlines the need for genotoxicity testing of herbal medicinal products from both a regulatory (21) and a pharmacological point of view, as genotoxicity risk cannot be presumed to be absent simply because a plant belongs to a traditionally used or safe group of medicinal products (22). These frameworks aim to ensure that products distributed for human use do not present an unacceptable risk of DNA damage or mutagenicity (23). However, several key issues remain, genotoxicity, cytotoxicity, pharmacological efficacy and mechanistic studies, are still uncertain in many cases (24). Although medicinal plants offer valuable therapeutic potential, their safety cannot be taken for granted. This research, addresses micronucleus endpoints in *Searsia rhemanniana* extracts, addresses this need and contributes to the pharmacological safety profile of the candidate medicinal products.

2. MATERIALS AND METHODS

2.1. Plant Material

The plant material was authenticated as *S. rhemanniana* by botanists from the botanical garden in Pietermatzburg, South Africa. Following verification, the purchased plant material was thoroughly cleaned with distilled water to remove soil and debris. The plant was then separated into its major anatomical parts: roots, bulbs, and leaves. Each plant component was dried in a ventilated oven at a temperature range of 30–60°C for five days to ensure gradual dehydration and preservation of phytochemicals. Once fully dried, the material was coarsely ground using a hammer mill and stored at room temperature in airtight containers until required for extraction.

2.2. Extract Preparation

Plant material was ground into a fine powder using an IKA grinder (IKA Labortechnik, Germany). Extraction was performed using methanol (MeOH), dichloromethane (DCM), and

water (H_2O) at a ratio of approximately 1:4 (w/v). The maceration was placed on a shaker (Labcon, Lab Design Engineering, Maraisburg, South Africa) for 72 hours. Following extraction, the mixture was filtered through Whatman No. 1 filter paper (Merck Chemicals (Pty) Ltd, Wadeville, South Africa) using a vacuum filtration system (Merck Chemicals (Pty) Ltd, Wadeville, South Africa). This process was repeated until the filtrate was clear. The organic solvents (MeOH and DCM) were removed under reduced pressure using a BÜCHI Rotovapor (Labotec (Pty) Ltd, Halfway House, South Africa), and the resulting extracts were dried at room temperature under a fume hood and stored at 4°C. The aqueous extract was frozen at -80°C and subsequently freeze-dried to a powder, then stored at 4°C.

In Vitro Genotoxicity Determination of S. Rhemanniana Extracts Against Vero Cells

Dulbecco's Modified Eagle Medium (DMEM), Foetal Bovine Serum (FBS) and PBS with and without Ca^{2+} and Mg^{2+} were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Bis-benzamide H 33342 trihydrochloride (Hoechst) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Cell Line Maintenance

The African green monkey kidney cell line, Vero cells, were purchased from Cellonex (South Africa). Cells were maintained in 10 cm culture dishes in complete medium (DMEM, 10% FBS) and incubated at 37°C in a humidified atmosphere with 5% CO_2.

2.4. Sample Preparation

Test samples were reconstituted in dimethyl sulfoxide (DMSO) to a stock concentration of 100 mg/mL and sonicated if solubility was a problem. Samples were stored at 4°C until required.

2.5. Genotoxic Evaluation Using Vero Cells – Treatment Protocol

Cells were seeded in 96 well plates at 3000 cells/well (100 µL aliquots) and left overnight to attach. Treatments were prepared in complete medium and added to cells in 100 uL aliquots. Griseofulvin (3.125 – 50 µM) was used as a positive control. Cells were incubated for 48 hours at 37°C and 5% CO_2. Spent medium was aspirated and cells were fixed using freshly prepared 4% formaldehyde for 15 minutes. Fixative was aspirated and cells were washed with 100 µL PBS with Ca^{2+} and Mg^{2+}. Hoechst 33342 (5 µg/mL) prepared in PBS with Ca^{2+} and Mg^{2+} was added to each well (100 µL) and incubated for 30 minutes. Fluorescence micrographs were captured at 9 sites per well using an ImageXpress Micro XLS Widefield Microscope (Molecular Devices) with a 10x Plan Fluor objective and DAPI filter cube. Acquired images were analysed using the MetaXpress software and Micronucleus Cell Scoring Application Module, where the total number of cells and % micro-nucleated cells per well were determined.

3. RESULTS

Genotoxicity was determined using the Hoechst 33342 nuclei dye. Cytotoxicity, as determined by the total number of cells (nuclei) is shown in Figure 1, and percentage of micro-nucleated cells is shown in Figure 2.

Table 1: Extract Numbers, Plant Parts and Solvents of S. Rhemanniana.

Extract numbers	Plant parts and Solvents
1	Barks water
2	Barks Methanol (MeoH)
3	Barks Dichloromethane (DCM)
4	Leaves Methanol
5	Leaves water
6	Leaves Dichloromethane
7	Roots Methanol
8	Roots Dichloromethane
9	Arterial Methanol

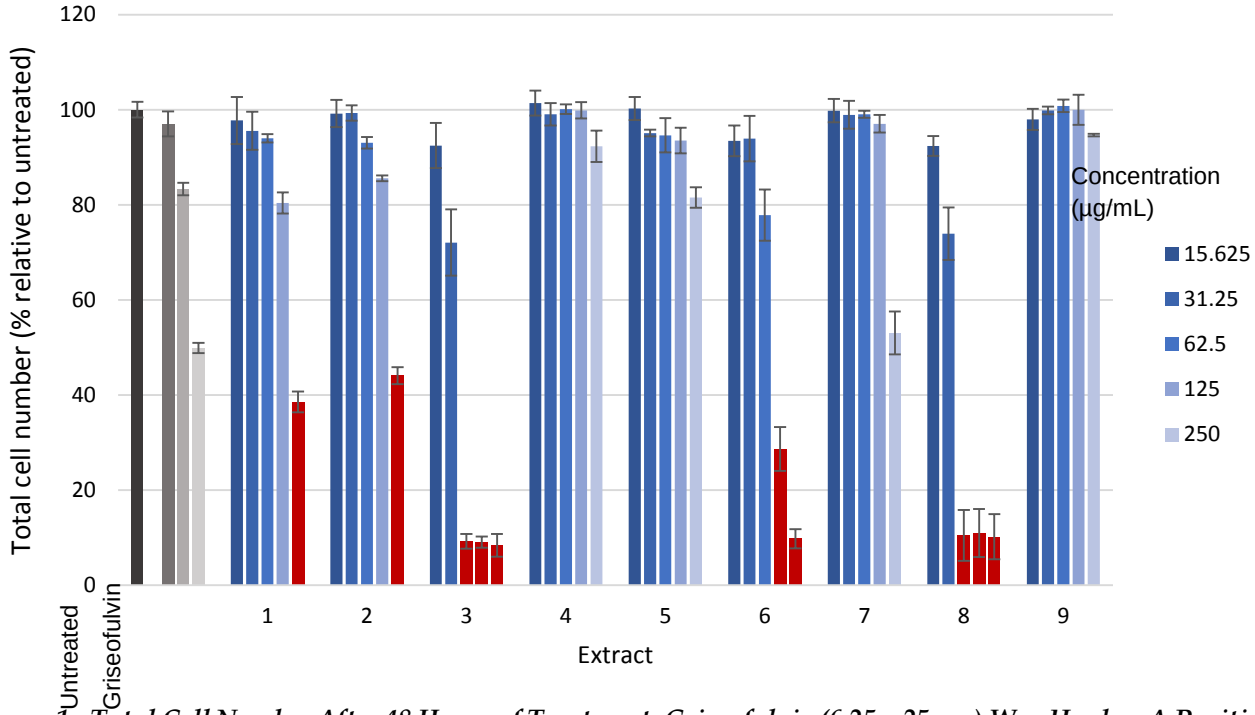

Figure 1: Total Cell Number After 48 Hours of Treatment. Griseofulvin (6.25 – 25 µm) Was Used as A Positive Control. Error Bars Indicate the Standard Deviation of The Mean of Three Separate Experiments (N=3), Each Performed in Triplicate or Quadruplicate. Red-Coloured Bars Indicate Significant Cytotoxicity.

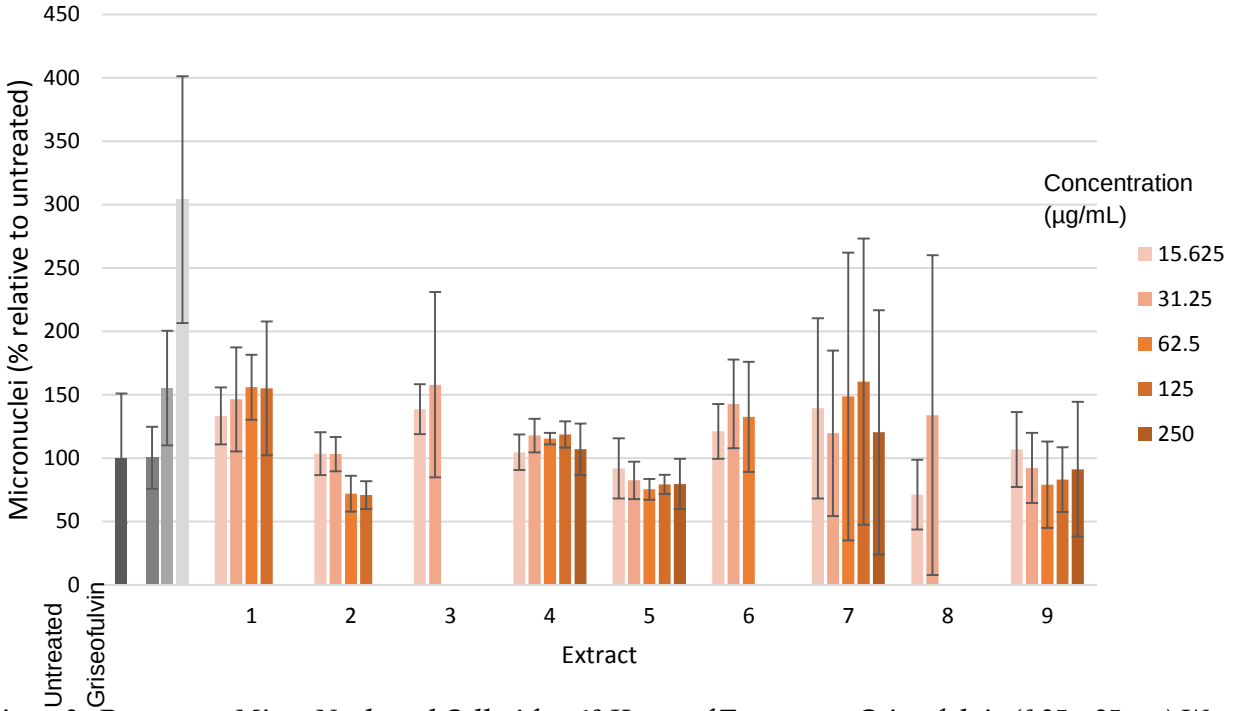

Figure 2: Percentage Micro-Nucleated Cells After 48 Hours of Treatment. Griseofulvin (6.25 – 25 µm) Was Used as A Positive Control. Error Bars Indicate the Standard Deviation of The Mean of Three Separate Experiments (N=3), Each Performed in Triplicate or Quadruplicate.

4. FINDINGS

In this study, cytotoxic effects were evaluated through a cell viability assay, and the results are summarized in Figure 1. A reduction in cell viability exceeding 50% relative to untreated control was considered indicative of significant cytotoxicity.

Marked cytotoxicity was observed for several of the tested extracts at the defined treatment concentrations. Specifically, bark water and bark MeoH extracts exhibited pronounced cytotoxic effects at 250 µg/mL, while bark DCM and roots DCM extracts demonstrated a dose-dependent decrease in cell viability across the concentration range of 62.5 to 250 µg/mL. Similarly, leaves DCM displayed substantial cytotoxicity between 125 and 250 µg/mL, suggesting that the threshold for toxicity in this sample occurs within this concentration window. These findings imply that certain extracts or compounds possess potent cell-inhibitory properties, likely mediated by the disruption of essential biochemical pathways such as mitochondrial activity or membrane stability.

The observation of more than 50% cell loss reflects not only decreased proliferation but also possible induction of apoptosis or necrosis at higher exposure levels. In contrast, extracts exhibiting minimal cytotoxicity at comparable concentrations may contain components with limited interaction with vital cellular targets, or they may act through mechanisms that do not immediately compromise cell viability.

Such differential effects highlight the importance of assessing a concentration range to distinguish between cytostatic and cytotoxic responses. It is important to note that due to excessive cell loss, micro-nucleus assay results could not be reliably interpreted for bark water, bark MeoH, bark DCM, leaves DCM, and roots DCM extracts at their respective cytotoxic concentrations. The significant reduction in viable cells and the resulting morphological changes hindered accurate scoring of micronuclei formation. Consequently, these data points were excluded from Figure 2 to ensure the integrity of the genotoxicity analysis.

The percentage of micro-nucleated cells relative to the total number of cells serves as a quantitative indicator of genotoxic stress. As shown in Figure 2, several extracts exhibited statistically significant increases in the frequency of micro-nucleated cells compared with the untreated control group.

These findings reflect the genotoxic potential of specific test compounds at concentration ranges. Notably, bark water extract induced a significant increase in micronucleus formation across all tested concentrations (15.625 – 250 µg/mL), indicating that it may contain constituents capable of interacting directly with DNA or interfering with the mitotic spindle apparatus.

Similarly, bark DCM extract produced a significant genotoxic response at 15.625 µg/mL, despite exhibiting cytotoxicity at higher concentrations. This suggests that even low doses of certain compounds within this sample may be sufficient to cause DNA damage, potentially through oxidative stress or the formation of reactive intermediates. Leaves MeoH extract also demonstrated a statistically significant increase in the proportion of micro-nucleated cells within the concentration range of 62.5 – 250 µg/mL, further supporting its genotoxic potential. The absence of a dose-dependent trend in some extracts may reflect differences in compound stability, bioavailability, or cellular defense responses such as DNA repair activation. Nevertheless, the occurrence of micronucleus formation at multiple concentrations supports the conclusion that certain extracts contain genotoxic agents capable of inducing chromosomal breakage or mis-segregation events.

5. CONCLUSION

The present study demonstrated that several of the tested extracts exerted notable cytotoxic and genotoxic effects in human cell models. Cytotoxicity assays revealed that bark water, bark MeoH, bark DCM, leaves DCM, and roots DCM significantly reduced cell viability at higher concentrations, indicating potent inhibition of cellular proliferation and possible induction of apoptotic or necrotic pathways. In contrast, samples showing limited cytotoxicity may contain bioactive constituents with mild or selective cellular effects, underscoring the concentration-dependent nature of their toxicity profiles.

The micronucleus assay further confirmed that some extracts, particularly bark water, bark DCM, and leaves MeoH, possess genotoxic potential, as evidenced by a significant increase in the frequency of micro-nucleated cells.

This finding suggests that these extracts or their active components may interact with genetic material or interfere with chromosomal segregation mechanisms, leading to DNA damage. The occurrence of genotoxicity at low concentrations especially in samples that were also cytotoxic at higher doses highlights the complexity of their biological activity and the importance of concurrent evaluation of both endpoints.

Overall, these results indicate that while certain samples contain compounds with strong cytotoxic and genotoxic properties, others may have safer biological profiles suitable for further investigation. Further studies are warranted to isolate and characterize the active constituents responsible for these effects, elucidate their mechanisms of action,

and determine their potential therapeutic or toxicological relevance in vivo.

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