ORIGINAL PAPER

JMS Journal of Medical Science

Mutagenic and antimutagenic evaluation of *Asparagus laricinus* Burch., *Senecio asperulus* DC., and *Gunnera perpensa* L. to hepatic cells

Polo-Ma-Abiele Hildah Mfengwana

Department of Health Sciences, Central University of Technology, Free State, Bloemfontein, South Africa

https://orcid.org/0000-0002-4208-8026

Corresponding author: pntsoeli@cut.ac.za

DOI: https://doi.org/10.20883/medical.e745

Keywords: neutral red uptake, VITOTOX, Alkaline Comet assay, VITOTOX , Alkaline Comet assay, DNA mutation toxicity

Published: 2022-12-27

How to Cite: Mfengwana P-M-AH. Mutagenic and antimutagenic evaluation of Asparagus laricinus Burch., Senecio asperulus DC., and Gunnera perpensa L. to hepatic cells. Journal of Medical Science. 2022;91(4);e745. doi:10.20883/medical.e745

ABSTRACT

Introduction. The use of traditional medicinal plant concoctions to cure or treat different diseases daily in African folk medicine. However, the effects of most medicinal plants on human health or genetic material remain unknown. This study thus aimed to evaluate the mutagenic and antimutagenic potentials of *Asparagus laricinus Burch.* cladodes, *Senecio asperulus* DC., and *Gunnera perpensa* L. roots extract *in vitro*.

Material and methods. Neutral red uptake assay, alkaline comet assay, and the VITOTOX test was used with plant extract dilutions of 4, 20, 50, and 100 µg/ml, respectively, on hepatic (C3A) cells and *Salmonella Typhimurium* TA104 strains. Ethyl methane-sulfonate and 4-nitroquinoline oxide were used as positive controls for the comet and VITOTOX assays, respectively.

Results. *In vitro* cytotoxicity and genotoxicity were not observed from all tested extracts, except for the two dichloromethane (DCM) extracts of *S. asperulus* and *G. perpensa,* which appeared to be cytotoxic with S9 metabolic activation, but not genotoxic or mutagenic. From the VITOTOX test results, none of the extracts appeared to have antimutagenic properties after treating S. Typhimurium strains with a known mutagen.

Conclusions. These results confirm that previously reported anticarcinogenic properties of *A. laricinus, S. asperulus,* and *G. perpensa* did not result from the protective mechanism against genotoxicity but from other ones. Moreover, the negative mutagenic and cytotoxic activities of the tested plants highlighted the safe use of these medicinal plants in vitro. Therefore, S. asperulus and G. perpensa DCM extracts require further investigation for their possible in vivo cytotoxic effects on humans.

Introduction

Medicinal plants play an essential role in African communities' folk medicine as they are used as remedies for minor ailments and even severe and significant ailments such as cancer [1–3]. Nevertheless, medicinal plants with mutagenic activity can induce deoxyribonucleic acid (DNA) damage in human body cells. Plants' chemical agents may directly or indirectly damage the cell's genetic information in the DNA, cause mutations, or

even lead to cancer when not repaired [4]. When mutations occur in the cell division genes, oncogenes are formed, and a cell may begin to proliferate abnormally [5]. The process occurs when cells with mutations escape repairs by a regular cellular repair system, then uncontrollably divide when they were not supposed to, thus ultimately becoming cancerous. The affected cancerous cell will either undergo programmed cell death or the damage to its oncogene will be passed on to descendant cancer cells as they divide. Moreover, gene mutations can result from chromosomal abnormalities and re-arrangements through deletion, translocation, and inversion. However, cells can protect themselves from a genotoxic mutation by triggering DNA repair or a programmed cell death process (apoptosis); failure to commit to one of these two options leads to mutagenicity. Medicinal plants can sometimes have genotoxic effects that are mutagenic or could even have antimutagenic effects that reverse and prevent or reverse or prevent mutation, which usually leads to oncogene formation [6]. Medicinal plants can be helpful in the development of new anticancer drugs.

Africa is blessed with rich flora. This abundance, especially in medicinal plants, contributes to the growing number of people using traditional medicinal plants. Other factors for the increased dependence on medicinal plants are easy accessibility even at local markets, affordability, and a belief that they have few or no side effects [7]. Local people use the medicinal plants selected for this study to treat different ailments. However, their mutagenicity has not been reported. *Asparagus laricinus Burch.* is native to the Southern African region and is used to treat cancer, tuberculosis, sores, red water, uterine infection, general alignments, and umbilical cord inflammation, and it serves as a diuretic [8–9]. Previous studies on the leaves of *Asparagus laricinus* showed the presence of tannins, saponins, terpenes, steroids, flavonoids, glycosides, steroids, and carbohydrates [10–11]. However, this plant showed the absence of alkaloids. The leaves of Asparagus laricinus also demonstrated antioxidant, antibacterial, and anticarcinogenic activities [11–12].

Senecio asperulus is ethnomedicinally used in Lesotho to treat back pain, swollen feet [13], colic, flu, colds, sore throat, mouth ulcers, and sore joints and to improve blood circulation [14]. Moreover, Kose et al., in their ethnobotanical study [15], further reported on the use of this plant for the treatment of tuberculosis, herpes, syphilis, and itchy feet by the Lesotho community. *Senecio asperulus* infusion has also been used as a remedy for internal poisoning [16]. Mugomeri et al. [13] identified phytochemicals, such as flavonoids, glycosides, and phytosterols, with beneficial medicinal properties from Senecio asperulus *Gunnera perpensa* is widely known for its high medicinal importance in several traditional medicine systems. The plant treats cancer, regulates the menstrual cycle, treats impotence, induces labour, treats stomachache, eases period pains, and relieves colic during pregnancy [17–18]. Some phytochemicals, including saponins, phenols, alkaloids, tannins, steroids, cardiac glycosides, flavonoids, and proanthocyanins and flavonols, have been identified from Gunnera perpensa [19–20]. Khan et al. [21] further isolated and identified Z-venusol as a significant component of *Gunnera perpensa.*

Many active compounds used to develop drugs come from medicinal plants. Even pharmaceutical companies show an increased interest in plant-derived drugs mainly because of the current widespread belief that 'green medicine' is safe, with fewer side effects [22–23]. However, there are still challenging tasks for drug research scientists, including investigating the safety of herbal medicine [23]. To distinguish favourable from adverse effects and to ban poisonous plants or contaminations from herbal mixtures, the assessment of the effect of plant-derived products' toxicity on the tissue or organs of mammalian recipients is still required. Most plants used in traditional medicine have *in vitro* mutagenic properties [24–25]; therefore, it is important to evaluate their mutagenic potency. Plants with mutagenic properties must thus be considered potentially unsafe and require further testing before their continued use is recommended. To evaluate the potential of tested sample to cause mutations to the DNA of cells, the use of high throughput assays such as Neutral red uptake assay, alkaline comet assay, and the VITOTOX tests are valuable as they provide information on the genotoxicity and cytotoxicity of the sample. The study aimed to investigate the mutagenic and antimutagenic properties of *Asparagus laricinus Burch., Senecio asperulus DC.,* and *Gunnera perpensa L*.

Materials and methods

Plant material

The study received plant collection and export permits from the Ministry of Tourism Environment and Culture, Lesotho, and from the Department of Economic Development, Tourism and Environmental Affairs, South Africa (NC.553/2017), for import approval. The plant materials were collected from the mountains of Lesotho (Mohale's Hoek district, Mpharane). Then, they were authenticated by scientists at the National Botanical Gardens in Bloemfontein, South Africa. Their voucher specimens were deposited at the National Botanical Gardens with herbarium numbers MAS001 for Asparagus laricinus, PHM01 for Senecio asperulus, and PHM02 for Gunnera perpensa. Roots of *Senecio asperulus DC.* and *Gunnera perpensa L.,* and *Asparagus laricinus Burch.* cladodes were washed, air-dried at room temperature (22°C), then grounded into a fine powder using an electric blender and weighed. They were then stored in a cool place until analysis. The crude extracts were used in this study as lay people frequently use these plants to treat different ailments and use concoctions prepared from the whole part of the plant, not the isolated compound.

Extraction method

The extraction was done using maceration [6]. Plant material (10 g of the dried powdered roots, and cladodes, respectively) were weighed, pulverised, and soaked in distilled water (DH_2O), methanol (MeOH), and dichloromethane (DCM), for 72 hours with occasional stirring using a mechanical shaker. The extracts were filtered, and new solvents were added again, respectively, for more extraction until the solvents remained clear (this process was repeated three times). The extracts were then filtered, and aqueous extracts were concentrated with a freeze-drier and organic solvents with a rocket evaporator. Percentage yields were calculated.

C3A cell culture

The toxicology of the studied plant extracts was determined using mycoplasma-free human hepatocyte cells (C3A). C3A cells are a sensitive model for *in vitro* predictive of human genotoxic exposure. Cell suspensions of human C3A cells in Dulbecco's modified Eagle's culture medium supplemented with 10% fetal calf serum were seeded into each well of a 96-well microtiter plate, so the cell density was 40,000 cells/well. Plates were incubated overnight (24 hours) at 37°C and 5% carbon dioxide (CO_2). Humidity was maintained using a water bath containing distilled water inside the incubator.

The neutral red uptake (NRU) test

The NRU test estimates the dose of medicinal extracts that is not cytotoxic to human hepatic cells (C3A). The NRU test is based on the ability of live cells to take up and bind the 3-amino-7-dimethyl-2-methylphenazine hydrochloride (NR) dye. This dye is known to accumulate in the lysosomes of the viable cell after penetrating the cell membranes through non-ionic diffusion. Increased unabsorbed NR dye shows increased cell death. Therefore, viable cells can be distinguished from dead or dying cells based on their NRU, and quantitative measurement of the number of viable cells can be undertaken. In this study, the neutral red uptake (NRU) test was performed using the method described by Repetto et al. [26]. After overnight incubation, the cells were then treated with dilutions of the extracts, which were 4 µg/ml, 20 µg/ml, 50 µg/ml, and 100 µg/ml. Cells were further incubated for another 24 hours to allow the extracts to work. Then cells were washed in phosphate-buffered saline (PBS), after which 200 ml of a 0.625 mg/ml neutral red (NR)-solution was added. Cells were rewashed with PBS after being incubated for three hours to remove excess dye. Two hundred millilitres of a 50:1 ethanol-acetic acid solution were then added, and cells could mix with this solution for 1.5 hours on the shaker to remove the dye from the cells. The absorbances were measured with a microplate spectrophotometer at 540 nm wavelength. The absorption of non-treated cells (negative control) was given a 100% value to which data from treated cells were compared. Sodium dodecyl sulfate was used as a positive control.

The comet assay

The protocol by Olive and Banáth [27], was followed to evaluate the DNA damaging and protective effects of the three-plant species. Microscope slides were pre-coated by spreading 300 µl 1% standard melting point agarose in water evenly over the slides and allowing the agarose to

harden. Hepatic (C3A) cells at a density of 200000 cells/ml were treated with different concentrations of the test sample in 24-well plates and incubated for 24 hours at 37°C in a 5% CO_2 incubator. The plant extracts were tested at 250, 100, 50, and 4 µg/ml concentrations. Ethyl methanesulfonate (EMS) at 1 mM was used as a positive control/mutagen. The cells were exposed to plant extracts alone for mutagenicity testing, and for antimutagenicity testing, the cells were exposed to a combination of the plant extracts and 1 mM EMS. After incubation, cells were trypsinized, and 10 µl of a 10 000-cell suspension was added to 300 µl of 0.8% low melting point agarose at 37°C.

The mixture was spread on the pre-coated slides and hardened under a coverslip on ice. Once the agarose had been prepared, the coverslips were removed. The microscope slides were placed in a lysis buffer overnight. First, denaturation was conducted using the electrophoresis buffer at 17°C for 40 minutes. Next, electrophoresis was conducted using the same solution at 25V, and the current was adjusted to 300 mA for 20 minutes. After electrophoresis, the microscope slides were neutralised in a Tris buffer (pH 7.5), and dried. The slides were then placed in ice-cold ethanol for 10 minutes and dried at room temperature. Finally, the gels were stained with 100 µl of 20 µg/ml ethidium bromide, left for 10 minutes, and rinsed in distilled water. The slides were analyzed using a fluorescence microscope supplied with a camera. The tail length, percentage DNA in the tail, and tail moment were determined using the computer image-analysis program Tri-Tek CometScore™. This program allows the measurement of tail length, percentage of DNA in the tail, and tail moment as parameters to measure DNA damage in the comet assay.

Moreover, for mutagenicity testing, differences in parameters used to measure DNA damage (i.e. tail length, percentage DNA in tail and tail moment) were compared between sample concentration and solvent blank (negative control). For antimutagenicity testing, the same parameters used for genotoxicity testing were used. In this case, the measurements in the test samples were compared to the positive control (EMS).

The VITOTOX® test

The VITOTOX® test is performed following the method described by Verschaeve [28], with *Salmonella Typhimurium* bacteria that lack the necessary oxidative enzyme systems for the metabolization of foreign compounds as they can react with DNA. As for most other in vitro assays, the bacteria were treated with the test compound in the presence and absence of a post-mitochondrial supernatant ('S9'). Micro-organisms were incubated overnight and then a dilution of the bacterial suspension was incubated for an hour on a rotative shaker. Multiwell plates were used to contain the solvent, different concentrations of the test compound, or the positive control for genotoxicity testing (4-Nitroquinoline 1-oxide (4-NQO) with S9 or 4-NQO without S9). Genotoxicity and toxicity measurements were performed using a microplate luminometer that enabled online measurements of emitted light (e.g., every five minutes over four hours). After completion of the measurements, the data were transferred into a Microsoft Excel macro sheet, and the signal-to-noise (S/N) ratio, i.e., the light production of exposed bacteria divided by the light production of non-exposed bacteria, was calculated for each measurement. The S/N ratio was calculated for the recN2–4 and pr1 strains separately. So was the ratio between the maximum S/N values of the recN2–4 and pr1 strains. All calculations occurred automatically and were based on measurements between 60 and 240 minutes of incubation. Based on experimental grounds, a compound was considered genotoxic when the following criteria were met:

- max S/N (*rec*N2–4) / max S/N (*pr1*) (to be indicated as: *rec*/pr1) was greater than 1.5,
- max S/N in *rec*N2–4 must show a good dose-dependent effect.

Statistical analysis

All experiments per sample were repeated in triplicates. The Mann-Whitney U test was used for the statistical analysis, and $p < 0.05$ was considered significant.

Results

The results from this study showed that the viability of cells treated with different concentrations (4 µg/ml, 20 µg/ml, 50 µg/ml, and 100 µg/ml) of *Asparagus laricinus, Senecio asperulus,* and *Gunnera perpensa* plant extracts to be dose-dependent as shown in **Figure 1** (**A-I**). DCM extracts of *Aspar-*

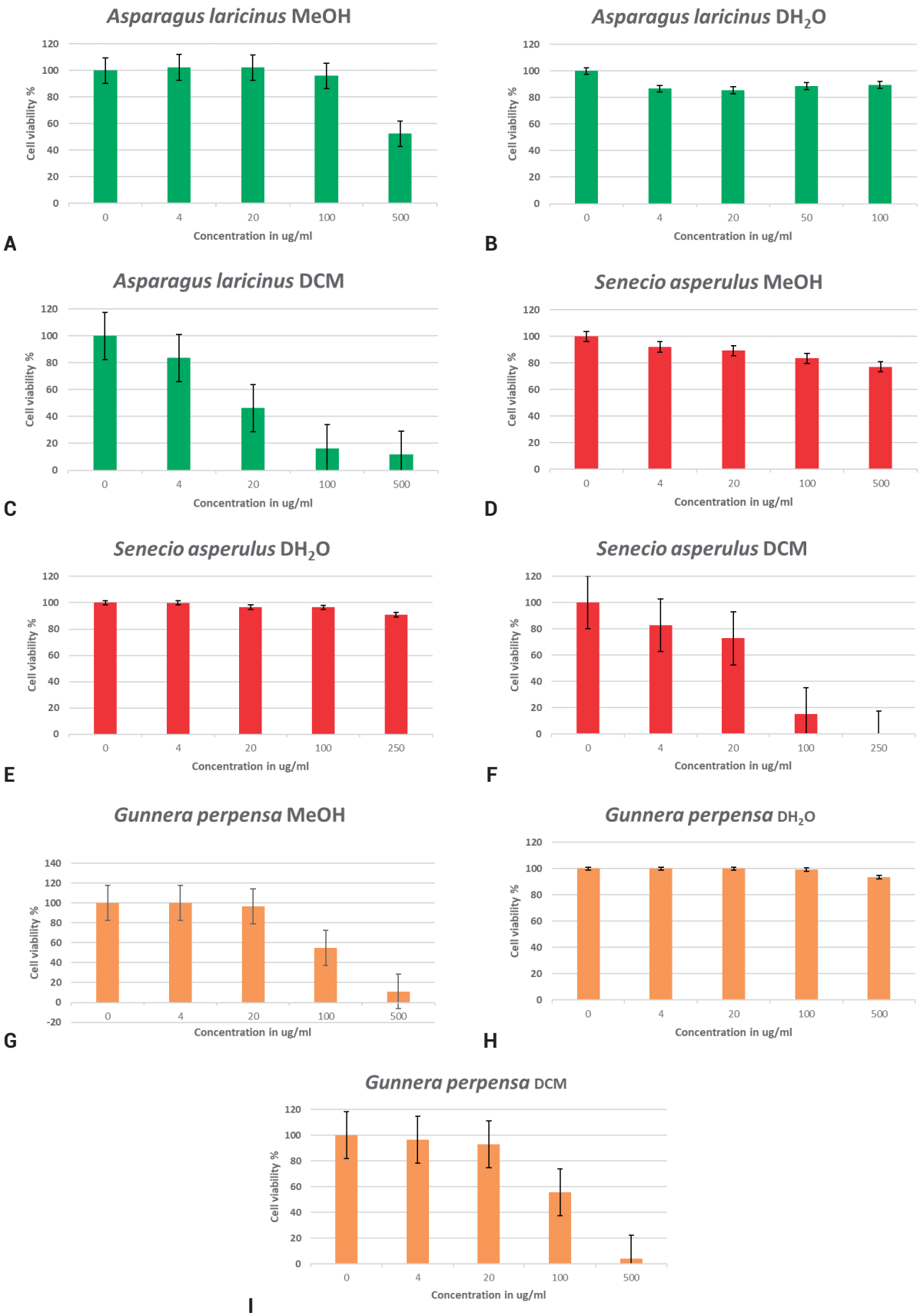

Figure 1. Neutral Red Uptake test results of *Asparagus laricinus* (**A**: methanol, **B**: water, and **C**: dichloromethane), *Senecio asperulus* (**D**: methanol, **E**: water, and **F**: dichloromethane) and *Gunnera perpensa* (**G**: methanol, **H**: water and **I**: dichloromethane) plant extracts. On the x-axis, 0 represents the negative control. Cells were treated with different concentrations of the extracts between 4 µg/ml and 500 µg/ml, then treated with a dye to differentiate between live and dead cells. Live cells took up the dye, and their viability was measured and presented by red bars

agus laricinus and *Senecio asperulus* showed proliferation inhibition to C3A cells in a dose-dependent manner from the 50 µg/ml concentrations. *Gunnera perpensa* MeOH and DCM extracts showed cytotoxic effects from the 100 µg/ml concentrations. The remaining extracts had no significant cell proliferation inhibition effects even at the higher concentration of 100 µg/ml compared to the untreated (negative control) C3A cells.

The concentration of the tail formed (due to damaged/broken pieces of DNA) relative to the head (intact DNA), reflecting the number of DNA breaks and the extent of DNA damage, was calculated automatically by the use of imaging software. **Figure 2** (**A-I**) shows the results. Deviations that were statistically significant for Asparagus laricinus were found at as low as 4 µg/ml for aqueous extracts, 20 µg/ml for methanolic extracts, and 250 µg/ml for DCM extracts. After that, concentration showed increased DNA damage due to the observed percentage of the tail (**Figure 2**: **A-I**). Nonetheless, these effects were low compared to DNA damage caused by the well-known mutagen EMS. *Senecio asperulus* extracts did not show any formation of statistically significant comets nor *Gunnera perpensa* aqueous extracts. However, Gunnera perpensa organic extracts showed statistically significant DNA damage at the highest tested concentration of 500 µg/ml for MeOH extracts and 100 µg/ml for DCM extracts. However, there was no significant DNA damage increase for the latter as the concentrations increased.

Table 1 reports the summarized results of the toxicological properties of *Asparagus laricinus, Senecio asperulus,* and *Gunnera perpensa* plant extracts. These plants were assessed using the VITOTOX assay by investigating both genotoxicity and cytotoxicity effects with and without S9. The tested concentrations were guided by the NRU results reported in **Figure 1** (**A-I**).

With the VITOTOX assay, the plant extracts' genotoxicity and cytotoxicity are assayed simultaneously to identify false-positive results caused by non-specific light production induced by other mechanisms besides the genotoxic effect [27]. Both the genotoxic strain with luciferase operon (TA104 recN2–4) and Cytox strain expressing the lux operon (TA104 pr1) were used with and without metabolic activation by the S9 enzyme. The light production showed the genotoxicity of the plant extract after the genotoxic extract had activated the recN promoter in the TA104 recN2–4 strain. Non-specific light production when the compound activates the pr1 in the TA104 pr1 strain was indicative of the cytotoxicity of the plant extract. The genotoxicity and cytotoxicity of each extract at concentrations of 100 µg/ml, 250 µg/ml, and 500 µg/ml were investigated and reported in **Table 1**. The positive control, 4-nitroquinoline 1-oxide, is known to have mutagenic and carcinogenic effects. For the validity of the test, 4-NQO showed genotoxicity with an S/N ratio greater than 1.5. Yet no cytotoxicity with an S/N ratio greater than 0.8 **(Table 1)**.

Table 1. The genotoxicity and cytotoxicity performed with the highest tested concentration (as guided by the neutral red uptake results) of *Asparagus laricinus (A. laricinus), Senecio asperulus (S. asperulus),* and *Gunnera perpensa (G. perpensa)* with, and without S9 activation

Extract and concentration	Genotoxicity (S/N ratio)	Genotoxicity with S9 (S/N ratio)	Cytotoxicity (S/N ratio)	Cytotoxicity with S9 (S/N ratio)
A. laricinus MeOH [500 µg/ml]	<0.6	>0.8 but <1.0	<0.8	>0.8 but <1.0
A. laricinus DH_2O [100 µg/ml]	0.0	0.0	0.0	0.0
A. laricinus DCM [500 µg/ml]	>1.5	1.0	>1.6	<1.2 but >1.0
S. asperulus MeOH [500 µg/ml]	>1.5	1.0	>0.8	1.0
S. asperulus DH_2O [250 µg/ml]	0.0	<0.6	0.0	<0.6
S. asperulus DCM [250 µg/ml]	<0.6	>0.8 but <1.5	>0.8	<0.8
G. perpensa MeOH [500 µg/ml]	>1.5	<1.0	<0.8	>0.8
G. perpensa DH_2O [500 µg/ml]	1.5	0.8	<0.8	<0.8
G. perpensa DCM [500 µg/ml]	<0.8	<0.6	<0.8	<0.8
4-NQO 100 µg/ml	>1.5	>1.5	>0.8	>0.8

Abbreviations: S9, hepatic post-mitochondrial supernatant; S/N, signal-to-noise; MeOH, methanol; DH_2O, distilled water; DCM, dichloromethane; 4-NQO, 4-Nitroquinoline 1-oxide; <, less than; >, greater than.

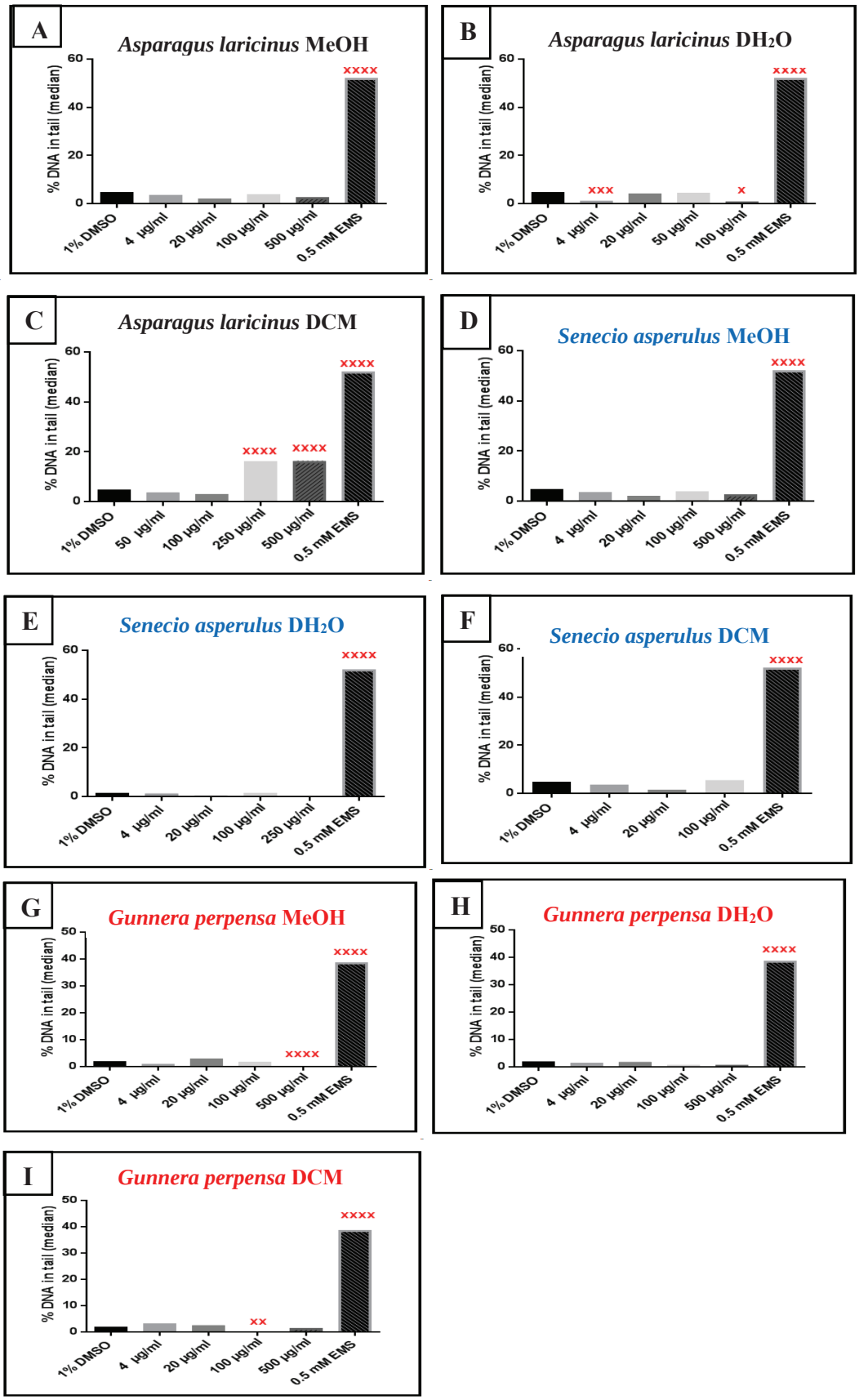

Figure 2. Comet test results of *Asparagus laricinus* (A-C), *Senecio asperulus* (D-F) and *Gunnera perpensa* (G-I) plant extracts. The statistically significant increases above background levels when compared with cells treated with 1% dimethyl sulfoxide(DMSO), and this was indicated as **x** = $P < 0.05$; **xx** = $P < 0.01$, and **xxx** = $P < 0.005$ and **xxxx**= $P < 0.001$. Abbreviations: MeOH, methanol; DH_2O, distilled water; DCM, dichloromethane; EMS, Ethyl methane-sulfonate

Discussion

For the continuous use of medicinal plants with a history of being used for the treatment of various ailments and cancer to be recommended, their safety to mammal cells must be evaluated. Although medicinal plant users believe they are safe by their users, research has proven that natural products, including medicinal plants, can be mutagenic [24–25]. Mutagenicity is the ability of a chemical agent to cause mutations to the DNA of cells, and these agents are said to be genotoxic [4, 29]. Genotoxic compounds from medicinal plants can cause mutations that are mutagenic. All mutagens are thus genotoxic, but not all genotoxic agents are mutagenic.

Moreover, mutations are known to be significant contributors to carcinogenesis; mutagens are thus most likely considered carcinogens [30]. However, plants with antimutagenic potential are considered interesting sources for new therapeutic uses [28]. The paper reports how the mutagenicity and antimutagenicity of Asparagus laricinus, Senecio asperulus, and Gunnera perpensa affect mycoplasma-free human hepatocyte (C3A) cells using two tests, namely the bacterial VITOTOX test, and the alkaline comet assay.

The VITOTOX test is a high throughput bacterial genotoxicity test that is very fast, sensitive, and uses only tiny quantities of a sample. This test uses two different *Salmonella Typhimurium* TA104 recombinant test strains that carry a luciferase operon to determine the genotoxicity and cytotoxicity of the sample. Furthermore, the assay correlates well with the Ames test or the SOS chromotest [4, 31]. SOS gene response is triggered when there is cell DNA damage in which the cell cycle is arrested, and DNA repair and mutagenesis are induced [32]. Thus genotoxic compounds are considered SOS-inducing compounds. The VITOTOX test is based on the induction of SOS function transcription by inserting an operon-less "lux" gene next to recN, resulting in light production when the bacterial DNA is damaged. Infusing luxCDABE on the multicopy plasmid to recN promoter allows genotoxicity to be detected. On the other hand, its fusion with the pr1 promoter allows cytotoxicity to be detected. According to Verschaeve et al. [4], when recN-luxCDABE fusion occurs, the sample genotoxicity at sublethal will increase light production. In contrast, this light production decreases as a function of the sample toxicity due to the infusion of Pr1-luxCDABE. Therefore, a signal-to-noise (S/N) ratio greater than 1.5 indicates genotoxicity. An S/N ratio <0.8 shows cytotoxicity.

The comet assay is a single-cell gel electrophoresis assay that is simple to perform, versatile and sensitive for single- and double-strand break measurements in damaged DNA [29, 33]. The comet assay was performed only in the absence of S9 as the cells used (liver cells, C3A) could already retain their metabolic activity. **Figure 1** (**A-I**) demonstrates concentrations used in this test guided by the results obtained from the NRU test as demonstrated, as well as the solubility of the extracts. Moreover, overly toxic concentrations will influence the percentages of DNA fragments in the tail of the formed comets. Thus, their DNA damage properties were assessed at lower concentrations for extracts that reduced the viability of cells at higher concentrations, such as Senecio asperulus dichloromethane extracts.

Furthermore, the comet assay was performed in the dark to prevent light-induced DNA damage [34]. According to Chang et al. [35], this migration results when the structural loop of the DNA break loses its supercoiling and is pulled towards the anode under the electrophoresis field. These formed comets were observed by fluorescence microscopy after the DNA was stained with DNA-specific fluorescent dyes such as ethidium bromide or propidium iodide.

Asparagus laricinus methanol and *Senecio asperulus* aqueous extracts were neither genotoxic nor cytotoxic with or without S9. Their S/N ratio in response to the Genox strain was less than 1.5 and to the Cytox strain was below 0.8 in a dose-dependent manner (**Table 1**). *Asparagus laricinus* water and *Gunnera perpensa* dichloromethane extracts were neither genotoxic nor cytotoxic with or without S9. The *Asparagus laricinus* dichloromethane extracts were cytotoxic and genotoxic, with or without S9 activation, which was in line with the NRU test and comet assay results. Furthermore, the results coincided with findings by Mfengwana et al. [12]. Asparagus laricinus dichloromethane extracts were reported to be cytotoxic after nuclear morphological changes, and their cytotoxicity was due to induced apoptosis. VITOTOX test results in this study showed Asparagus laricinus to be non-mu-

tagenic. The outcome correlated with the Ames test findings reported by Mashele and Fuku36. The test evaluated the mutagenic and antimutagenic properties of Asparagus laricinus aqueous root extracts. The Asparagus laricinus leaf aqueous extracts were reported to be cytotoxic to Vero cells at the concentration of 200 µg/ml, which contradicts the finding by Mashele and Kolesnikova [8] as the roots of the same plant showed no cytotoxic effect.However, the study by Mfengwana [37] showed the presence of nine compounds from the leaves of *Asparagus laricinus* (publication on the identification of these compounds is in progress), while only a few compounds were isolated from the roots of *Asparagus laricinus*: indole-3-carbinol, α-sitosterol, and ferulic acid [10]. Thus, certain compounds are present in the leaves that are absent from the root part of this plant, which could be why the leaf extracts were cytotoxic at higher concentrations. It indicates the importance of determining the correct dose that will not lead to cytotoxicity or genotoxicity before medicinal plant application. The in vivo toxicity of the *Asparagus laricinus* leaves has not been evaluated yet, as only the toxicological evaluation of the roots has been reported [38].

Mutagenicity is the ability of a chemical agent to cause mutations that result in cell death. Then, those agents are genotoxic [4, 29]. *Senecio asperulus* MeOH extract was genotoxic and cytotoxic without the presence of S9. However, this extract remained cytotoxic but was not genotoxic after adding S9. Literature reports show the *in vitro* cytotoxicity of *Senecio asperulus* DCM extracts on kidney epithelial cells extracted from an African green monkey (Vero cells) The cytotoxicity of this extract was thus observed without S9 activation when the (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) MTT assay was used (11). The study findings also confirmed the cytotoxicity of the *Senecio asperulus* DCM extract as it was not genotoxic or cytotoxic without S9, then its toxicity was reversed by the presence of S9 as this extract became cytotoxic. This indicates that *Senecio asperulus* DCM extract is not a direct mutagen (inactive without S9) but becomes cytotoxic after metabolization (mutagenic in the presence of S9). It suggests that diverse assays (VITOTOX and MTT in this case) have different specificities and sensitivity against certain mutagens. Plant mutagenicity, therefore, cannot be based on one test result [39].

Both the methanol and water extract of *Gunnera perpensa* were genotoxic but could not produce an S/N ratio above 0.8 for the cytox strain. They were thus genotoxic but not cytotoxic. However, these extracts were not genotoxic but cytotoxic when S9 was present. The presence of S9 reversed the mutagenicity or even blocked the induced mutagenic activity of these extracts and made them genotoxic agents that are not mutagenic, that is, some of the compounds present in this extract are mutagens. The isolation of pure compounds and the re-analysis of those will, therefore, assist in highlighting unknown mutagens from these plant extracts. The study results indicate that the presence of the S9 enzyme (from both comet and VITOTOX assays) reduced Gunnera perpensa extract genotoxicity, which means that the safety of this plant is modified when the liver cells metabolise the plant. However, this could only be concluded after in vivo work on this plant has been completed (work in progress).

Antimutagens counteract mutagens by inactivating or preventing the Mutagen-DNA reaction or mutagenic compound transformation into mutagen. These can be natural or synthetic compounds rendering certain mechanisms, such as (i) directly interacting with mutagens, (ii) inhibiting the activation of mutagens, (iii) blocking the mutagen binding to the target, and (iv) through the generation of antioxidant mechanism [40]. These mechanisms prove that antimutagens have the potential to eliminate or reduce the mutagenic effects of potentially harmful substances. Therefore, the investigation of antimutagenic compounds provides new possibilities in anticancer drug discovery, and this quest is expanding hastily in cancer research [41–42]. DNA damage in cells exposed to the test substance was investigated to evaluate the mutagenicity and antimutagenicity of Asparagus laricinus, Senecio asperulus, and Gunnera perpensa. For satisfactory evaluation of the genotoxic potential of a compound, 3 endpoints need to be assessed: gene mutation, DNA damage, and structural and numerical chromosome aberrations [26]. Accordingly, many in vitro and in vivo toxicology test methods including the VITOTOX test, mammal cell micronucleus test, and comet assay, have been developed to

assess the potential of substances to cause mutagenicity that may lead to cancer.

Extracts were tested to assess the antimutagenic effects of the selected medicinal plants with 4-NQO and with and without S9. The same concentrations and conditions were set as in the case of the genotoxicity test. 4-nitroquinoline-N-oxide (4-NQO) is a base substitution agent that causes direct DNA damage by acting at G residues, which leads to the induction of GC to AT transitions [43]. Based on the study results, all extracts tested could not significantly decrease or improve the genotoxicity of 4-NQO. They were thus considered as not having antigenotoxic activities Hence the implication is that the observed anticarcinogenic activity of Asparagus laricinus and Senecio asperulus on breast cancer cells did not result from plant genotoxicity protection but other mechanisms, such as apoptosis [12]. *Gunnera perpensa* has been characterized before, and five compounds are identified from its methanolic extract: 3,3',4'-tri-O-methyl ellagic acid lactone, ellagic acid lactone, 1,1'-biphenyl-4,4'-diacetic acid, p-hydroxybenzaldehyde and Z-methyl lespedezate [44]. Some of these compounds are known as antihaemorrhagic, antimutagenic and anticarcinogenic agents. Contrary to the compounds reported by Brookes and Dutton [44], none of the Gunnera perpensa extracts showed antimutagenic properties. Only Gunnera perpensa DCM extracts proved anticarcinogenic properties in vitro [12].

Conclusions

Asparugus laricinus, Senecio asperulus, and *Gunnera perpensa* have been used as traditional medicines to treat several diseases, including cancer. However, the safety of these plants has yet to be investigated before with comet and VITOTOX assays, especially using liver cells to mimic how the liver will metabolise the plant. Nevertheless, *S. asperulus* and *Asparagus laricinus* water extracts are potentially safe as they demonstrate no mutagenicity or cytotoxicity. Moreover, *G. perpensa* is genotoxic but not cytotoxic; however, its genotoxic and cytotoxic effects are reversed when the S9 enzyme is present. Thus, this proves that the genotoxicity properties are lost during the metabolism of this plant. Thus, it does not cause DNA damage after being metabolized but becomes cytotoxic. This plant is, therefore, still questionable and should not be used further until its cytotoxic mechanism is well understood. Unfortunately, none of the tested extracts showed any antimutagenic effects and thus cannot be used to reverse DNA damage caused by mutagens.

Acknowledgements

Conflict of interest statement
The authors declare no conflict of interest.

Funding statement
The National Research Fund (117961), Thuthuka Grant, funded the study.

Ethical considerations
The author has observed ethical issues (including plagiarism, data fabrication, and double publication). The Faculty of Health and Environmental Sciences research committee at the Central University of Technology, Free State in South Africa (CUT-208012729) evaluated and approved the protocol.

Significance
This research emphasises the safety of Asparagus laricinus, Senecio asperulus, and Gunnera perpensa medicinal plants, which are common cancer treatment methods. This information can support further uses of these plants in human health as they are not carcinogenic.

Acknowledgements
The author would like to thank the Central University of Technology, the Free State (CUT) research office, the National Research Fund, the Department of Higher Education and Training, and the next generation of the academic program for their financial and other support. An appreciation to Luc Verschaeve and Roel Anthonissen from the Institute of Public Health, Brussels, for assisting with the data analysis. An expression of genuine gratitude to the "Ha Morena Motlatsi's" Chieftaincy, Mpharane, Mohale's Hoek district, Lesotho, for their warm welcome and permission for plant collection from their land. Special thanks to the indigenous plants' knowledge holder, Ms T.R Mochochoko, for sharing her knowledge with the CUT research team.

References

1. Abd Razak, M, F., K.E. Aidoo and A.G. Candlish, 2007. Mutagenic and cytotoxic properties of three herbal plants from Southeast Asia. Trop Biomed, 24(2): 49-59. https://europepmc.org/article/med/18209708
2. Roy, A., T. Attre and N. Bharadvaja, 2017. Anticancer agent from medicinal plants: a review. In book: New aspects in medicinal plants and pharmacognosy, 1st edition.;1(3): 54-73. https://www.researchgate.net/

profile/Arpita-Roy-3/publication/318721809_Anti-cancer_agent_from_medicinal_plants_a_review/links/597ace074585151e35aea568/Anticancer-agent-from-medicinal-plants-a-review.pdf
3. Verschaeve, L., H. Edziri, R. Anthonissen, D. Boujnah, F. Skhiri and H Chehab, 2017. In vitro toxicity and genotoxic activity of aqueous leaf extracts from four varieties of Olea europea (L). Pharmacog Magazine, 13(S1): S63-S80. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5407118/
4. Verschaeve, L., J. Van Gompel, L. Thilemans, L. Regniers, P. Vanparys and D. Van der Lelie, 1999. VITOTOX® bacterial genotoxicity and toxicity test for the rapid screening of chemicals. Environ and Mol Mutagen, 33(3): 240-8. https://onlinelibrary.wiley.com/doi/abs/10.1002/(SICI)1098-2280-(1999)33:3%3C240::AID-EM8%3E3.0.CO;2-R
5. Griffiths, A.J., J.H. Miller, D.T. Suzuki, R.C. Lewontin and W.M. Gelbart, 2000. Quantifying heritability. In Freeman WH, ed. An Introduction to Genetic Analysis, 7th edition.. http://lgb.rc.unesp.br/biomol/literatura/Griffiths_8th.pdf
6. Bouguellid, G., C. Russo, M. Lavorgna, C. Piscitelli, K. Ayouni, E. Wilson, H.K. Kim, R. Verpoorte, Y.H. Choi, D. Kilani-Atmani, D. Atmani, M. Isidori, 2020. Antimutagenic, antigenotoxic and antiproliferative activities of Fraxinus angustifolia Vahl. leaves and stem bark extracts and their phytochemical composition. PLoS One, 16;15(4):e0230690. https://pubmed.ncbi.nlm.nih.gov/32298276/
7. Mustapha, A.A, 2014. Medicinal plants with possible anti-HIV activities: A review. Int J Med Plants, 106: 439-53 https://clinphytoscience.springeropen.com/articles/10.1186/s40816-015-0004-1
8. Mashele, S.S. and N. Kolesnikova, 2010. In vitro anticancer screening of Asparagus laricinus extracts. Pharmacologyonline, 2(1): 246–252. https://pharmacologyonline.silae.it/files/archives/2010/vol2/023.Mashele.pdf
9. Mfengwana, P.H. and S.S Mashele, 2019. Medicinal Properties of Selected Asparagus Species: A Review. In: Roa V, Mans D, Roa L, eds. Phytochemicals in Human Health. London. Intechopen. https://www.intechopen.com/chapters/67855
10. Fuku, S., A.M. Al-Azzawi, I.T. Madamombe-Manduna and S. Mashele, 2013. Phytochemistry and free radical scavenging activity of Asparagus laricinus. Int J Pharmacol.9(5): 312-17. https://scialert.net/fulltext/?doi=ijp.2013.312.317
11. Ntsoelinyane, P.H. and S.S Mashele, 2014. Phytochemical screening, antibacterial and antioxidant activities of Asparagus laricinus leaf and stem extracts. Bangladesh J Pharmacol, 9: 10-4. https://www.semanticscholar.org/paper/Phytochemical-screening%2C-antibacterial-and-of-leaf-Ntsoelinyane-Mashele/72c699d9275e4802f2b92e998e2c9222ca29db8d
12. Mfengwana, P.H., S.S. Mashele and I.T. Manduna, 2019. Cytotoxicity and cell cycle analysis of Asparagus laricinus Burch. and Senecio asperulus DC. on breast and prostate cancer cell lines. Heliyon, 5(5):e01666. https://www.sciencedirect.com/science/article/pii/S2405844019324715
13. Mugomeri, E., P. Chatanga, S. Hlapisi, L. Rahlao, 2014. Phytochemical characterization of selected herbal products in Lesotho. Lesotho Med Asso J, 12: 38–47. https://www.ajol.info/index.php/ajtcam/article/view/130715
14. Moteetee, A. and B. Van Wyk, 2011. The medical ethnobotany of Lesotho: a review. Bothalia, 41(1): 209-228. http://www.ethnopharmacologia.org/prelude2020/pdf/biblio-hm-44-moteetee.pdf
15. Kose, L.S., A. Moteetee, S. Van Vuuren, 2015. Ethnobotanical survey of medicinal plants used in the Maseru district of Lesotho. J Ethnopharmacol, 170: 184–200. https://pubmed.ncbi.nlm.nih.gov/25957810/
16. Quattrocchi, U, 2016. CRC World Dictionary of Medicinal and Poisonous Plants. Common Names, Scientific Names, Eponyms, Synonyms, and Etymology. Boca Raton: Taylor & Francis Group. https://doi.org/10.1201/b16504
17. Mammo, F., V. Mohanlall and F. Shode, 2017. Gunnera perpensa L.: a multi-use ethnomedicinal plant species in South Africa. Afr J Sci Technol Innov Dev. DOI: 10.1080/20421338.2016.1269458
18. Maroyi, A, 2016. From Traditional Usage to Pharmacological Evidence: Systematic Review of Gunnera perpensa L. J Evid Based Complement Altern Med, 17: 14-25. https://www.researchgate.net/publication/311501497_From_Traditional_Usage_to_Pharmacological_Evidence_Systematic_Review_of_Gunnera_perpensa_L
19. Simelane, M., O.A. Lawal, T.G. Djarova, C.T. Musabayane, M. Singh, and A.R. Opoku, 2012. Lactogenic activity of rats stimulated by Gunnera perpensa L. (Gunneraceae) from South Africa. Afr J Tradit, Complement Altern Med, 9(4): 561–573. https://www.researchgate.net/publication/256189785_Lactogenic_Activity_of_Rats_Stimulated_by_Gunnera_Perpensa_L_Gunneraceae_from_South_Africa
20. Chigor, C.B, 2014. Development of conservation methods for Gunnera perpensa L.: an overexploited medicinal plant in the Eastern Cape, South Africa. PhD Thesis, University of Fort Hare.. https://core.ac.uk/download/pdf/145034661.pdf
21. Khan, F., X.K. Peter, R.M. Mackenzie, L. Katsoulis, R. Gehring, O.Q. Munro, R. van Heerden and S.E. Drewes, 2004. "Venusol From Gunnera perpensa: Structural and Activity Studies." Phytochemistry, 65(8): 1117–21. https://www.sciencedirect.com/science/article/abs/pii/S0031942204000986
22. Ndhlala, A.R., J.F. Finnie and J. Van Staden, 2011. Plant composition, pharmacological properties and mutagenic evaluation of a commercial Zulu herbal mixture: Imbiza ephuzwato. J Ethnopharmacol, 133(2): 663-74. https://www.sciencedirect.com/science/article/pii/S0378874110007634
23. Efferth, T. and B. Kaina, 2011. Toxicities by herbal medicines with emphasis to traditional Chinese medicine. Curr Drug Metab, 12(10): 989-96. https://www.ingentaconnect.com/content/ben/cdm/2011/00000012/00000010/art00009

24. Cardoso, C.R., I.M. de Syllos Cólus, C.C. Bernardi, M. Sannomiya, W. Vilegas, E.A. Varanda, 2006. Mutagenic activity promoted by amentoflavone and methanolic extract of Byrsonima crassa Niedenzu. Toxicol, 225(1): 55-63. https://www.sciencedirect.com/science/article/pii/S0300483X06002812
25. Déciga-Campos, M., I. Rivero-Cruz, M. Arriaga-Alba, G. Castañeda-Corral, G.E. Angeles-López, A. Navarrete. And R. Mata, 2007. Acute toxicity and mutagenic activity of Mexican plants used in traditional medicine. J Ethnopharmacol, 110(2): 334-42. https://www.sciencedirect.com/science/article/pii/S0378874106005034
26. Repetto, G., A. Del Peso. and J.L. Zurita, 2008. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. Nat Protoc, 3(7): 1125-31. https://www.nature.com/articles/nprot.2008.75
27. Olive, P.L. and J.P. Banáth, 2006. The comet assay: a method to measure DNA damage in individual cells. Nat Protoc, 1: 23–29 https://www.researchgate.net/publication/6416166_Olive_PL_Banath_JP_The_comet_assay_a_method_to_measure_DNA_damage_in_individual_cells_Nat_Protoc_1_23-29
28. Verschaeve, L., 2005. The VITOTOX® genotoxicity test. In Pandalai SG, ed. Recent research developments in applied microbiology and biotechnology. India: Research Signpost;.pp. 33-49. https://pascal-francis.inist.fr/vibad/index.php?action=getRecordDetail&idt=17893276
29. Collins, A.R., 2011. The use of bacterial repair endonucleases in the comet assay. In Gautier JC, ed. Drug Safety Evaluation. Methods in Molecular Biology, Vol 691.New Jersey: Humana Press; pp. 137-47. https://link.springer.com/protocol/10.1007/978-1-60761-849-2_8
30. Makhuvele, R., K. Foubert, S. Apers, L. Pieters, L. Verschaeve and E. Elgorashi, 2018. Antimutagenic constituents from Monanthotaxis caffra (Sond.) Verdc. J Pharm Pharmacol, 70(7): 976-84. https://academic.oup.com/jpp/article/70/7/976/6121861?login=true
31. Vaghasiya, Y., R. Dave and S. Chanda, 2011. Phytochemical analysis of some medicinal plants from western region of India. Res J Medicinal Plant, 5(5): 567-76. https://www.cabdirect.org/globalhealth/abstract/20123177659
32. Biran, A., H.B. Yoav, S. Yagur-Kroll, R. Pedahzur, S. Buchinger, Y. Shacham-Diamand, G. Reifferscheid and S. Belkin, 2011. Microbial genotoxicity bioreporters based on sulA activation. Anal Bioanal Chem, 400(9): 3013-24. https://link.springer.com/article/10.1007/s00216-011-5007-2
33. Słoczyńska, K., B. Powroźnik, E. Pękala and A.M. Waszkielewicz, 2014. Antimutagenic compounds and their possible mechanisms of action. J Appl Genet, 55(2): 273-85. https://link.springer.com/article/10.1007/s13353-014-0198-9
34. Azqueta, A. and A.R. Collins, 2013. The essential comet assay: a comprehensive guide to measuring DNA damage and repair. Arch Toxicol, 87(6): 949-68. https://link.springer.com/article/10.1007%2Fs00204-013-1070-0
35. Chang, J.B., M.F. Wu, H.F. Lu, J, Chou, M.K. Au, N.C. Liao, C.H. Chang, Y.P. Huang, C.T. Wu, and J.G. Chung, 2013. Toxicological evaluation of Antrodia cinnamomea in BALB/c mice. In Vivo, 27(6): 739-45. https://iv.iiarjournals.org/content/27/6/739.short
36. Mashele, S.S. and S. Fuku, 2011. Evaluation of the antimutagenic and mutagenic properties of Asparagus laricinus. J Med Technol, 2: 33-36. https://www.semanticscholar.org/paper/Evaluation-of-the-antimutagenic-and-mutagenic-of-Mashele-Fuku/a9e076d98f8993f79a0dac125ad4669986adf268
37. Mfengwana, P, 2019. Evaluation of Pharmacological Properties of Traditional Medicinal Plants Used For The Treatment Of Cancer By South African And Lesotho Communities. http://hdl.handle.net/11462/2032
38. Mokgawa, S.D, 2016. Toxicology of Asparagus laricinus in rats. http://ir.cut.ac.za/bitstream/handle/11462/1332/Mokgawa%2C%20Sekobane%20Daniel.pdf?sequence=1&isAllowed=y
39. Edziri, H., M. Mastouri, A. Mahjoub, R. Anthonissen, B. Mertens, S. Cammaerts, L. Gevaert and L. Verschaeve, 2011. Toxic and mutagenic properties of extracts from Tunisian traditional medicinal plants investigated by the neutral red uptake, VITOTOX and alkaline comet assays. S Afr J Bot, 77: 703–710. https://www.sciencedirect.com/science/article/pii/S0254629911000214
40. Gautam, S., S. Saxena and S. Kumar, 2016. Fruits and vegetables as dietary sources of antimutagens. J Food Chem Nanotechnol, 2(3): 97-114. https://foodchemistryjournal.com/jfcn/articles/v2n3/jfcn-018-satyendra-gautam.pdf
41. El-Sayed, W.M. and W.A. Hussin, 2013. Antimutagenic and antioxidant activity of novel 4-substituted phenyl-2, 2'-bichalcophenes and aza-analogs. Drug Des Dev Ther, 7: 73-81. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3573810/
42. Ferguson, L.R. and M. Philpott, 2008. Nutrition and mutagenesis. Annu. Rev. Nutr, 28:313-29. https://www.annualreviews.org/doi/abs/10.1146/annurev.nutr.28.061807.155449
43. Fronza, G., P. Campomenosi, R. Iannone, and A. Abbondandolo, 1992. The 4-nitroquinoline 1-oxide mutational spectrum in single-stranded DNA is characterized by guanine to pyrimidine transversions. Nucleic Acids Res, 20(6): 1283-7. https://academic.oup.com/nar/article-abstract/20/6/1283/2386751
44. Brookes, K.B. and M.F. Dutton, 2007. Bioactive Components of the Uteroactive Medicinal Plant, Gunnera Perpensa (or Ugobo). S Afr J Sci, 103 (5): 187–189. http://www.scielo.org.za/pdf/sajs/v103n5-6/07.pdf