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Evaluation of cytotoxicity and genotoxicity of *Pappea capensis* extracts

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ABSTRACT

The cytotoxicity and genotoxicity of the ethnomedicinal plant *Pappea capensis* used mostly at the Northern parts of South Africa and the neighbouring countries and found at the Limpopo province boarders was tested for cytotoxicity against the three cell lines Vero cells, MCF7 and PC. The known chemotherapeutic drug melphalan was used as the positive control for all cell lines. Cytotoxicity was determined using the dual staining procedure with a nuclear dye, Hoechst 33342 and propidium iodide (PI). All live cells will stain positive with Hoechst 33342 but only dead/dying cells will stain positive with PI. The results of the cytotoxicity screening against Vero, MCF7 and PC3 cells showed no significant change for most of the extracts concentration. Both the ethanoic extract 6 and aqueous extract 7 showed no cytotoxic effect against any of the three cell lines. Methanolic extract 8 showed slight cytotoxicity at a high concentration of 250 µg/mL against Vero and MCF7 cells. This concentration of 250 µg/mL is considered physiologically irrelevant and for this reason, all extracts are considered non-cytotoxic. All the extracts are not active (cytotoxic) enough to be used for the treatment of cancer cells. The extracts were further screened for genotoxicity. Two extracts showed no genotoxic effect to the cells and were considered non-genotoxic and the ethanoic extract could be considered genotoxic. Only 30% micronucleated cells were observed at the high concentration (200 ug/ml) of ethanoic extract. The plant was found to be partially cytotoxic and moderately genotoxic for methanolic extract and ethanoic extract at 250 µg and 200 ug/ml, respectively. The active concentration of the plants extracts for both cytotoxicity and genotoxicity testing are considered physiologically irrelevant and for this reason, the plant is considered safe. It's worth noting that the assertion made by the traditional healers that cancer can be cured using aqueous extraction method was proven otherwise in this study; however their claims on *Pappea capensis* warrant further investigations by GCMS and antimicrobial testing as it was shown to possess a little toxicity in this study.

KEYWORDS: *Pappea capensis*, genotoxicity, cytotoxicity, plant extract, medicinal plant.

INTRODUCTION

World Health Organization (WHO) has reported that 80% of the population in African and Asian countries mostly rely on traditional medicine for survival [1]. The herbal medicine products gained interest globally because of growing costs related to primary health care treatment. However, there is fewer scientific evaluation evidence to the safety of the plants consumed for the different diseases. As part of the growing efforts to establish the safety and efficacy of the different preferred medicinal plants by the people of South Africa, in this study three extracts of *Pappea capensis* which is mostly used in the Northern part of South Africa were prepared. The plant is commonly used by the

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traditional healers for the treatment of several illnesses including cancer.

Cancer continues to deteriorate the lives of both males and females globally. This calls for the need for the discovery of new anticancer drugs with fewer side effects [1]. The scientific interest in the use of natural products as potential anti-cancer agents can be dated to 1550 BC during the Ebers papyrus time [2]. Nevertheless, during the early nineteen-sixty's scientific interest focused more on the application of podophyllotoxin and its derivatives as possible anti-cancer agents [3]. There has been a growing scientific and commercial interest in the discovery of new drugs from medicinal plants over the last three decades due to high economic demands, increased side effects and drug resistance [4, 5]. Natural products play a vital role in the discovery of new drugs, with more than 60% of those approved derived from natural plants [6]. More than 49% of the drugs tested in the clinical trials as potential anti-cancer agents are derived from natural products or are purely natural products [7]. The scientific literature supports the rationale behind scientific research done using medicinal plants for the discovery of novel drugs [8].

In this study three *Papea capensis* plant extracts were used to evaluate cytotoxicity against three cell lines. Cytotoxicity is defined as the ability of an agent to produce a toxic effect on a cell. Cytotoxicity assays were used to test the ability of cells to continue proliferating in the presence of a test compound or substance over a specific time period. Cytotoxicity testing is a popular method used for the screening of pharmaceutical products and synthetic organic compounds. This is an important initial step when investigating possible new therapies or developing new compounds for the treatment of an ailment, which is the determination of their cytotoxic potential and the determination of their harmful effects.

Toxic substances which directly interfere with cell viability and produce genetic knockout by damaging the genome and cause mutation are referred to as genotoxic [9]. Genotoxicity refers to the destructive effect that a compound or extract has on the nuclear material of the cell causing mutations in the cell [10]. The molecular genotoxic assay such as end-point assays have been used to study and detect DNA damage, can also be employed as indicators for primary damage or analysing the genotoxicity of new compounds [11]. Micronucleus test, chromosomal test and many other tests are used for evaluating antimutagenic action of any drug [12]. With regard to hereditary qualities, genotoxicity represents the property of a mixture of operators that damage the hereditary data [13]. In an attempt to survive, the cell prevents enunciation of the genotoxic transformation by either DNA repair mechanisms or programmed cell death (apoptosis); as a result, the harm may not generally settle, prompting mutagenesis [13].

The purpose of genotoxicity test is to decide whether a substrate will impact genetic material or may cause growth [14]. Genotoxicity tests can be characterized as *in-vivo* and *in-vitro* test intended to recognize compounds which incite genetic damage directly or indirectly by different mechanisms [14]. A feature of a genotoxic compound is the resulting formation of micronuclei after treatment of cells with the genotoxin [15]. Micronuclei are extra-nuclear bodies that contain damaged chromosome fragments and/or whole chromosomes that have not been incorporated into the nucleus after cell division. The formation of micronuclei can lead to cell death, genotoxic instability and cancer development [15]. Previous studies also indicated that different plants and different extraction methods may present different patterns when coming to micronuclei determination [12, 16, 17].

MATERIALS AND METHODS

Plant material

The plant material (*Pappea capensis*) was authenticated by Dr. Zietsmann, Bloemfontein museum and scientists at the National Botanical Gardens in Pretoria, South Africa. The collected bark and wood materials were dried at room temperature and pulverised by a mechanical mill and weighed. It was then stored at room temperature until analysis.

Three 360 g wood samples were weighed out for extraction with 1080 ml acetone, water or methanol respectively. Volumes were adapted according to the consistency of the sample. Then the rest of the solvent was added, and solutions allowed to seep out for 24 hours.

Filtering was performed after 24 hours, then the solids were removed from each solution by filtration with a Millipore funnel with medium filter paper (Bright sign nr102) connected to a Millipore vacuum pump. Where needed, samples were centrifuged in 50 ml conical tubes. Removing Solvents: Most extracts contained both aqueous and organic solvents and we employed both Freeze-drying steps with a Virtis Freeze drier to remove aqueous solvent as well as a Rotary evaporate (55°C) to remove organic solvents. After repeated steps of both freeze-drying and vacuum evaporation the samples were moved to pre-weighed containers and the yield determined.

Cytotoxicity evaluation of three plant extracts against three different cell lines

Sample preparation

Test compounds were reconstituted in dimethyl sulphoxide (DMSO) to give a final concentration of 100 mg/mL. Samples were sonicated if solubility was a problem. Samples were stored at 4 °C until required [18].

In vitro anti-cancer screening

The human prostate cancer cell line, PC3; human breast cancer cell line, MCF7 and the African green monkey kidney cell line and Vero were used for cytotoxicity screening. Cells were seeded into 96 well microtiter plates at a density of 4000 cells/well using a volume of 100 µl in each well. The microtiter plates were incubated at 37 °C, 5% CO_2, and 100% relative humidity for 24 hours prior to the addition of test compounds to allow for cell attachment.

Cells were treated with 12.5 µg/mL-250 µg/mL of each extract. One hundred microliters aliquots of the diluted compound in fresh medium were used to treat cells. Cell lines were incubated at 37 °C in a humidified 5% CO_2 for 48 hours.

Treatment medium was aspirated from all wells and replaced with 100 µL of Hoechst 33342 nuclear dye (5 µg/mL) and incubated for 10 minutes at room temperature. Thereafter, cells were stained with propidium iodide (PI) at 100 µg/mL in order to enumerate the proportion of dead cells within the population. Cells were imaged immediately after addition of PI using the ImageXpress Micro XLS Widefield Microscope (Molecular Devices).

Data quantification

Quantification of live and dead cells for the screening assay was performed using the ImageXpress Micro XLS Widefield Microscope (Molecular Devices) and the acquired images were analysed using the MetaXpress software and Multi-Wavelength Cell Scoring Application Module. Acquired data was transferred to an EXCEL spreadsheet and data was analysed and processed.

Genotoxicity evaluation of three plant extracts against Vero cells using micronucleus assay

Sample preparation

The NucRed Live 647 Probe (ThermoFischer Scientific) which is a far-red, cell-permeant nuclear stain for live and dead cells was used in the study to determine the genotoxicity of three *Pappea capensis* extracts. The assay was used to determine any existence or the formation of micronuclei.

The Vero cells (African green monkey kidney cells) were seeded at 4000 cells/well and left overnight to attach. Then the cells were treated with the different concentrations of each extract (200, 100, 50, 25 and 12.5) µg/mL. The cells were then treated for 48 hours (incubated at 37 °C). The medium and treatments were aspirated and stained with NucRed solution. The NucRed working solution was prepared by adding two drops of NucRed per mL PBS (+Ca +Mg) in both the aspirate medium and the treatments. This was followed by adding 100 µL NucRed working solution to each well. Then the plate was incubated for 15-30 minutes at 37 °C. Then the plates were acquired using the ImageXpress XLS microscope.

RESULTS

Cytotoxic evaluation of plant extracts (ethanol 6, water 7 and methanol 8)

All extracts were screened for cytotoxicity against all three cell lines. Figures 1-3 indicate the results of the dual staining cytotoxicity assay.

Genotoxicity

The formation of micronuclei was detected using NucRed nuclear stain and the ImageXpress microscope. Results are shown in Figure 4 which clearly shows the number of micronuclei formed

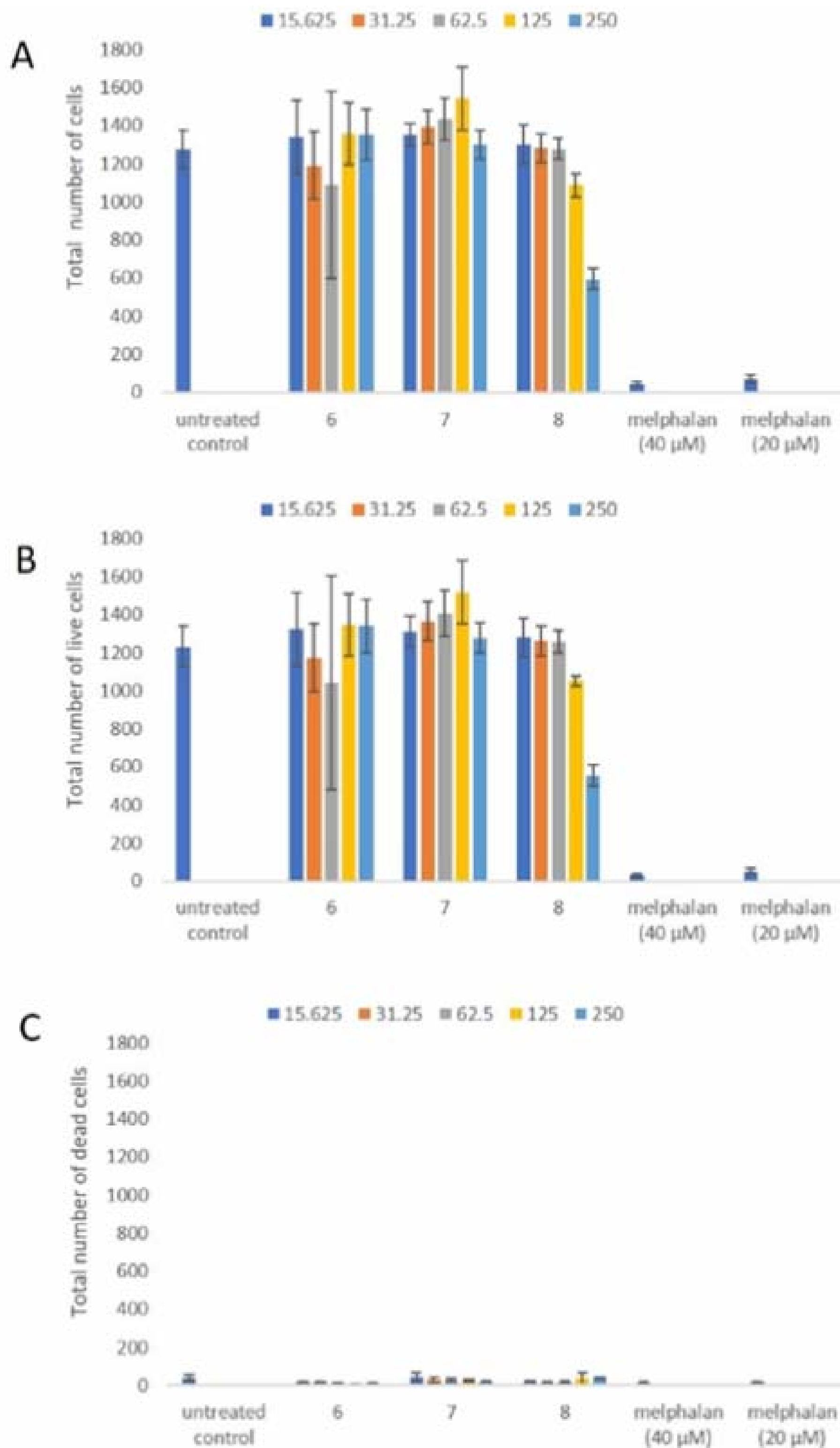

Figure 1. Cytotoxic response of Vero cells to treatments (at varying concentrations in µg/mL) with plant extracts 6, 7 and 8 as well as melphalan (positive control). A: total cell number, B: total number of live cells, C: total number of dead cells. Error bars indicate standard deviation of quadruplicate values done as a single experiment.

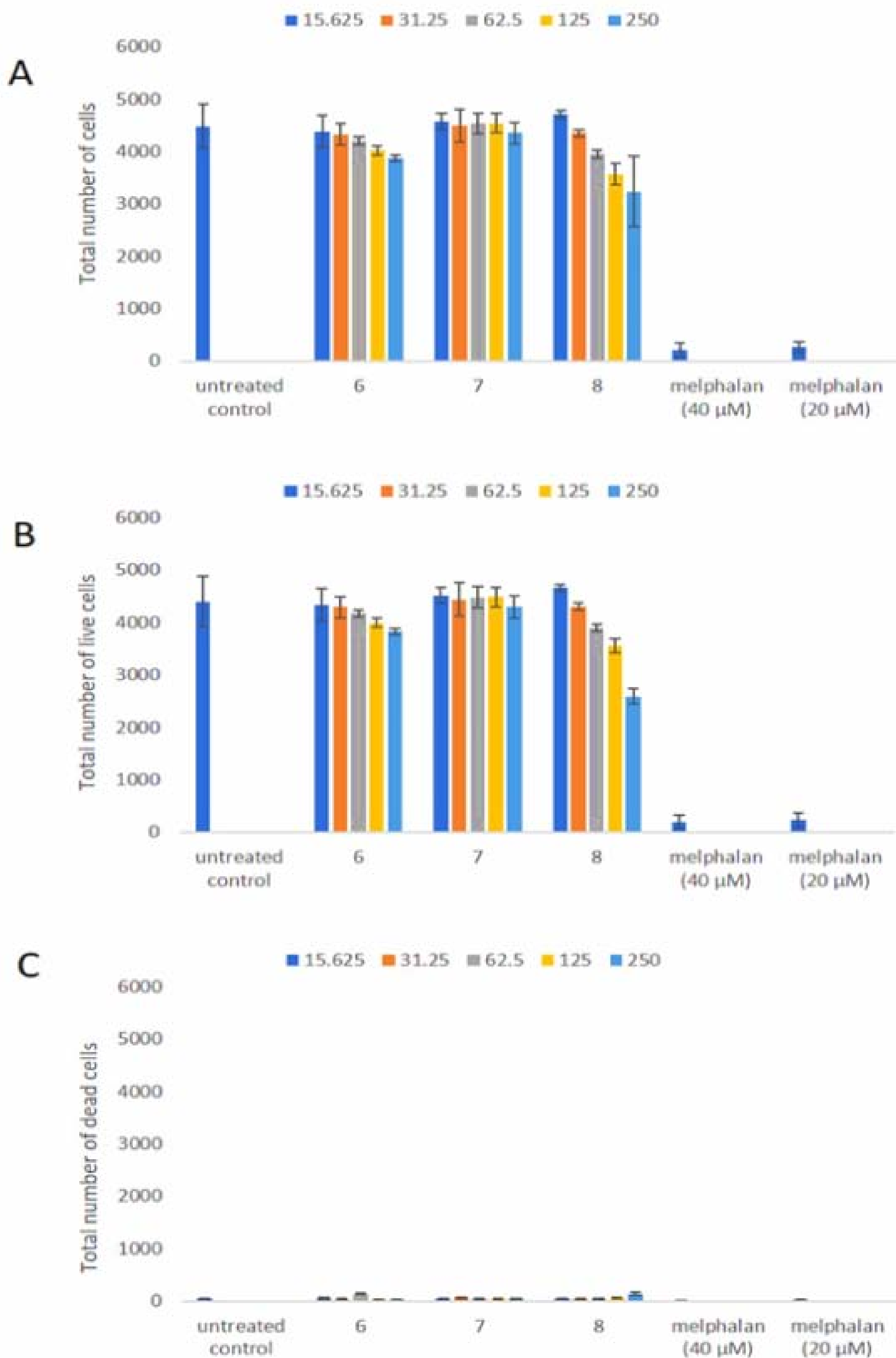

Figure 2. Cytotoxic response of MCF7 cells to treatments (at varying concentrations in µg/mL) with plant extracts 6, 7 and 8 as well as melphalan (positive control). A: total cell number, B: total number of live cells, C: total number of dead cells. Error bars indicate standard deviation of quadruplicate values done as a single experiment.

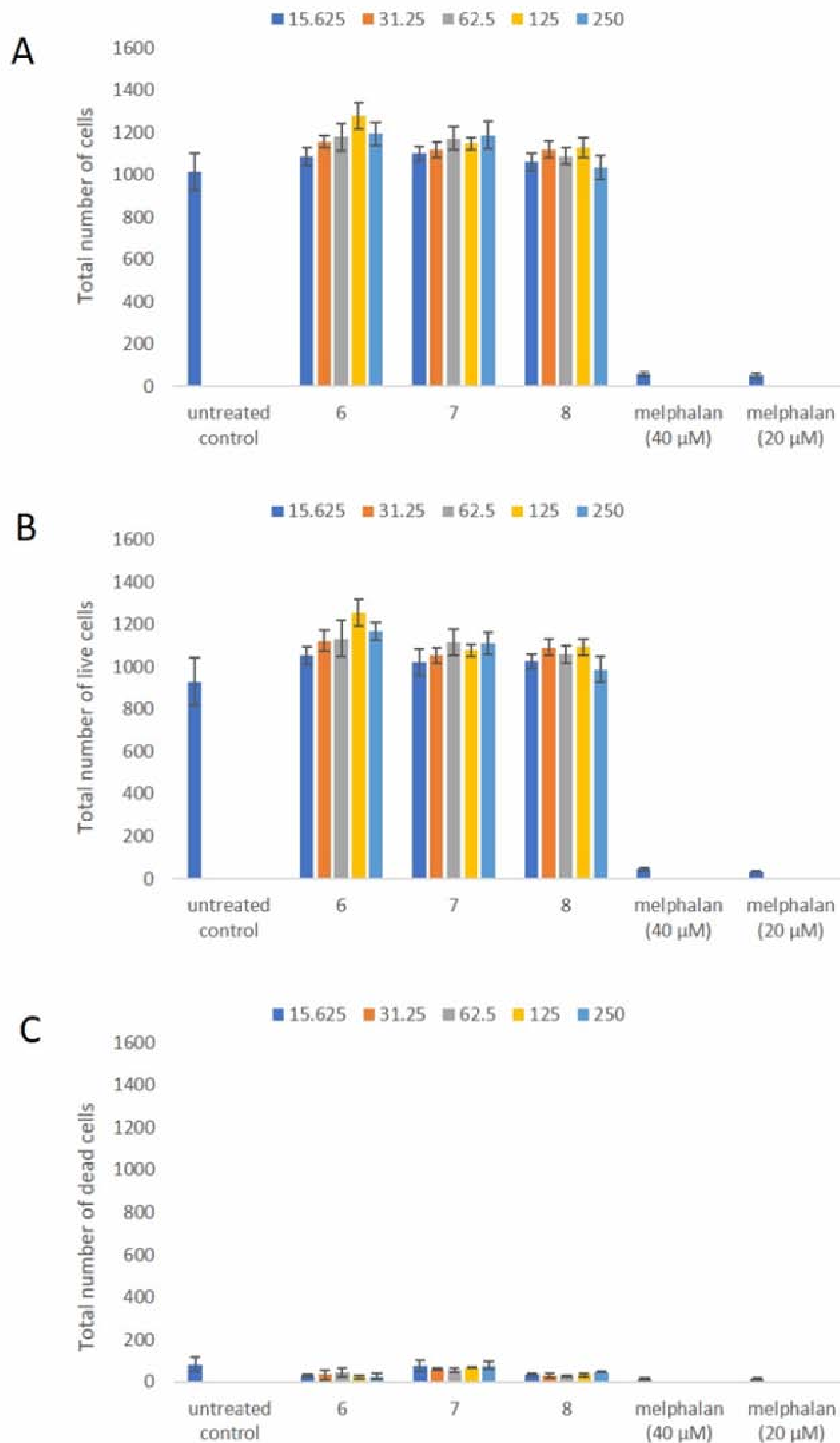

Figure 3. Cytotoxic response of PC3 cells to treatments (at varying concentrations in µg/mL) with plant extracts 6, 7 and 8 as well as melphalan (positive control). A: total cell number, B: total number of live cells, C: total number of dead cells. Error bars indicate standard deviation of quadruplicate values done as a single experiment.

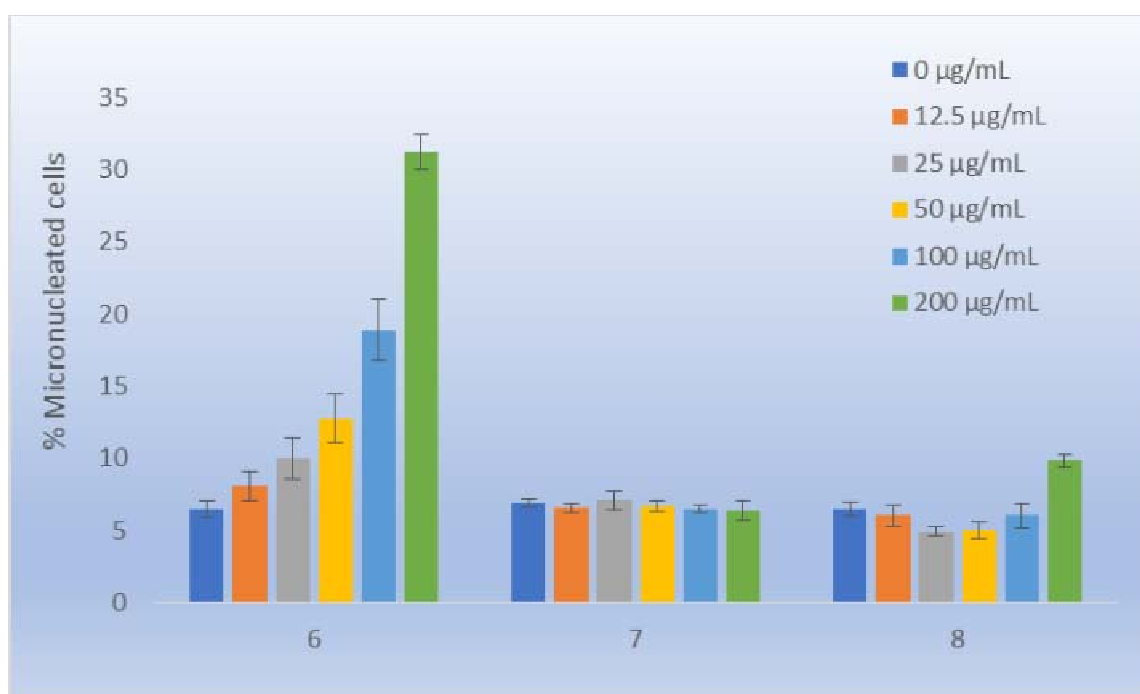

Figure 4. Formation of micronuclei in response to treatment of Vero cells with varying concentration (as indicated) of extract. Error bars indicate standard deviation of quadruplicate values.

in the presence of each extract. It is clear from this result that ethanoic extract (no6) induced the formation of micronuclei while the other two extract shows less formation of micronuclei.

DISCUSSION

Cytotoxicity was determined using the dual staining procedure with a nuclear dye, Hoechst 33342 and propidium iodide (PI). All live cells will stain positive with Hoechst 33342 but only dead/dying cells will stain positive with PI. Figures 1, 2 and 3 show the results of the cytotoxicity screening against Vero, MCF7 and PC3 cells, respectively. The known chemotherapeutic drug melphalan was used as the positive control for all cell lines. Both the ethanoic extract 6 and aqueous extract 7 showed no cytotoxic activity against any of the three cell lines. Methanolic extract 8 showed slight cytotoxicity at a high concentration of 250 µg/mL against Vero and MCF7 cells. This concentration of 250 µg/mL is considered physiologically irrelevant and for this reason, all extracts are considered non-cytotoxic [18]. Melphalan proved to be an appropriate positive control. Although Panel C in Figures 1, 2 and 3 show a small number of dead cells for melphalan treatment, this corresponds to the very low number of total live cells (Panel B) for all cell lines.

The NucRed Live 647 probe assay was used determine the formation of micronuclei in Vero cells responding to treatment with varying concentrations of the three *Pappea capensis* extracts. The ethanoic extract no6 could be considered genotoxic as it was found to have induced micronucleated cells by 30%. In a previous study the aqueous and hydro-alcoholic extracts of *Ecium amoenum* showed to have induced micronuclei [17], and chloroform root extract of *P. stellatum* correlated to the micronuclei response of the Vero cells exposed to ethanoic extract of *Pappea capensis* [16]. This ethanoic extract of *Pappea capensis* could be considered genotoxic; however other two couldn't induce micronucleated cells except for methanolic extract that shows partially micronucleated cell activity of 10% at 200 ug/ml.

CONCLUSION

Through comparing the used control and the only extract showing slight toxicity, it's conclusive to say that a high dose of methanolic extract is

physiologically inappropriate. All extracts are not active (cytotoxic) enough to suggest that *Pappea capensis* can be evaluated further for anticancer testing. Nonetheless, it's scientifically important to mention that isolation and identification of bioactive compounds in their pure state may indicate otherwise. Further testing using GC-MS may possibly indicate volatiles present, and that their individual biological effect be tested.

Genotoxins are agents that can possibly interact with the DNA, thus damage its structure, and cause mutation which may lead to cancer. Two extracts were considered non-genotoxic, and the ethanoic extract could be considered moderately genotoxic. It must also be notified that only 30% of the cells were micronucleated at the high concentration (200 ug/ml). Further tests should be done to predict what mechanism is affected by compounds in the ethanoic extract. The results indicate the potential use of this plant against cancer; however this moderate genotoxicity at 200 ug/ml is worrisome. This further supports the need for the identification of bioactive compounds using GC-MS chromatography as the *Pappea capensis* extracts show less to no genotoxicity.

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CONFLICT OF INTEREST STATEMENT

The authors state that there are no conflicts of interest in this work.

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