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Anti-Proliferative and Genotoxic Activities of the *Helichrysum petiolare* Hilliard & B.L. Burtt

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Abstract: *Helichrysum petiolare* (Asteraceae family) is part of the *Helichrysum* genus which comprises of an estimated 600 species. Several parts of the plant have been used traditionally for the treatment of various ailments, such as cough, infection, asthma, chest problems, diabetes and wounds. Given its various chemical constituents with anticancer properties, there has been no scientific evidence of its usage for the treatment of cancer. This study aims to investigate the anti-proliferative and genotoxic activities of *H. petiolare* methanol extract. The cytotoxic effect and cell cycle analysis of mouse melanoma cells (B16F10) and human melanoma cells (MeWo) were assessed using the ImageXpress Micro XLS Widefield High-Content Analysis System. The genotoxic potential of the extract towards Vero cells was also assessed using the micronucleus assay. The extract displayed cytotoxicity towards B16F10 and MeWo skin melanoma cells, thereby showing a dose-dependent decrease in cell density. This was preceded by cell cycle arrest in B16F10 cells at the S phase and MeWo cell arrest at the early M phase with a significant increase in apoptosis in both cells. Furthermore, the extract displayed genotoxic potential at the tested concentrations (12.5–200 µg/mL). Overall, the results revealed that *H. petiolare* extract may have the potential to eradicate skin cancer.

Keywords: cytotoxicity; genotoxic; proliferation; skin cancer; *Helicrhysum petiolare*

1. Introduction

Cancer is one of the most dreadful diseases globally and it is described by abnormal cellular proliferation. It develops due to the cellular accumulation of various epigenetic and genetic events [1,2]. Some factors such as lifestyle, environment and nutrition have also been reported to play a crucial role in the pathogenesis of cancer. In a normal cell cycle, the growth and division of cells occur in a well-organized manner, but in cancerous cells, defective caspase-mediated cell death (apoptosis) leads to increased cell proliferation [3]. Skin cancer is among the most prevalent types of cancer globally and its incidence is increasing at an alarming rate. One report showed that there were more than two million new cases of skin cancer diagnosed in 2018 [4]. This number has been growing, likely due to the earlier detection of the disease, increased sun exposure, and longer lifespans. However, the treatment of skin cancer is commonly overwhelmed by the fact that most synthetic chemotherapeutic agents are frequently associated with a variety of toxic effects, mainly due to their non-selectivity in killing cancerous cells [5,6]. These limitations and costs of modern treatments for skin cancer pose many shortfalls. Many patients plagued with this disease do not seek proper treatment, resulting in increased morbidity and mortality.

Recently, several medicinal herbs have attracted numerous researchers' attention for cancer treatment. A number of medicinal plants and their bioactive components have been revealed to possess anti-carcinogenic and anti-proliferative effects against cancerous skin cells with fewer side effects [7–9]. Hence, medicinal plants may serve as potential sources for developing new anticancer drugs.

Helichrysum is a genus consist of an estimated 600 species of flowering plants in the family of Asteraceae. Out of the 600 species, 244 species including *Helichrysum petiolare* are used for various traditional medicine treatments in South Africa. Some of the observed pharmacological activities include antioxidant, antimicrobial, anticancer, antidiabetic and anti-inflammatory effects [10,11]. Several parts of *H. petiolare* have been used traditionally for the treatment of various ailments such as cough, cold fever, infection, asthma, chest problems, diabetes, hypertension and wounds [12]. Some of the biological activity of *H. petiolare* previously reported include anti-tyrosinase, anti-inflammatory, antifungal and antioxidant effects [13].

Although *H. petiolare* is not used traditionally for the treatment of cancer, reports have shown that this plant has some chemical constituents that are responsible for its anti-cancer activity, such as flavonoids, polyphenolics and saponins [12,14]. Based on this reason, in this study, we examine the anti-proliferative activities of *H. petiolare* methanol extract against two cancerous skin cells: mouse melanoma cells (B16F10) and human melanoma cells (MeWo). In addition, an attempt is also made to examine the genotoxic potential of the extract towards Vero cells using the micronucleus assay.

2. Materials and Methods

2.1. Reagents

Skin melanoma cell lines (B16F10 and MeWo) were acquired from Highveld Biological, Johannesburg, South Africa, while the Vero cells were purchased from ATCC (Manassas, VA, USA). Roswell Park Memorial Institute (RPMI) 1640, Dulbecco's phosphate-buffered saline (DPBS) with Ca^{2+} and Mg^{2+} and DPBS without Ca^{2+} and Mg^{2+}, trypsin-EDTA and Dulbecco's modified Eagle's medium (DMEM) low-glucose cell culture medium were purchased from HyClone, Longa, UT, USA. Fetal bovine serum (FBS) was purchased from Biowest (Logan, UT, USA). BisBenzamide H 33342 trihydrochloride (Hoechst 33342), propidium iodide, griseofulvin and etoposide were purchased from Sigma-Aldrich (St. Louis, MO, USA). An annexin V-FITC kit was obtained from MACS Miltenyi Biotec, Cologne, Germany. NucRed was purchased from Molecular Probes®, Life Technologies—Thermo Fisher Scientific (Logan, UT, USA).

2.2. Plant Material and Extract Preparation

Fresh, mature leaves of *H. petiolare* were collected in December 2019 from the town of Alice, Amatole District, Eastern Cape, South Africa. The voucher specimen (Voucher no. HEL-1340) was authenticated by a botanist (Prof. Chris Cupido) and preserved in the Giffen Herbarium of the University of Fort Hare. For the extraction process, the milled plant (60 g) was mixed using methanol (1000 mL) at room temperature for 24 h. The resulting mixture was filtered and then concentrated to dryness by means of a rotary evaporator (RVO 004; Ignos, Prague, Czech Republic) to obtain a dried extract (14.43% dry extract).

2.3. Cell Culture Conditions

MeWo and B16F10 skin melanoma cells were separately maintained in 10-cm culture dishes in complete DMEM (low-glucose culture medium) supplemented with FBS (10%) and incubated at 37 °C in a humidified incubator with 5% CO_2 [15].

2.4. Imaging and Analysis

The ImageXpress Micro XLS Widefield High-Content Analysis System (Molecular Devices®, San Jose, CA, USA) with the MetaXpress® High-Content Image Acquisition and Analysis Software (Molecular Devices®, San Jose, CA, USA) was used to acquire and analyze all images [15]. Nine image sites per well were acquired for all imaging experiments.

2.5. Cytotoxicity Assay and Cell Cycle Analysis

Briefly, B16F10 and MeWo skin melanoma cells were seeded separately in 96-well plates at densities of 3000 cells/well using 100 µL aliquots and left to attach overnight. For treatment, cells were treated by adding 100 µL of *H. petiolare* (0–100 µg/mL) and then incubated for 48 h at 37 °C in a humidified 5% CO_2 incubator. Melphalan was used as a positive control. The treatment medium was removed and replaced with a 50 µL staining solution (5 mL binding buffer containing 50 µL Annexin V-FITC reagent (Milteny Biotec Annexin V Kit: Cat no 130-092-052) and a 2 µL Hoechst 33342 solution (10 mg/mL in DMSO)) and then incubated for 15 min at 37 °C. Thereafter, 50 µL of propidium iodide (PI) (2 µg/mL in binding buffer) was added and then incubated for an additional 5 min, after which the images were acquired. The cell cycle analysis was then carried out using a cell cycle module.

2.6. Genotoxicity (Micronucleus Assay)

The micronucleus assay was performed as described previously [16]. Vero cells were seeded in a 96-well plate at a density of 3000 cells/well using 100 µL aliquots and left overnight to attach. For the treatment, an additional 100 µL of the plant extract at different concentrations (12.5–200 µg/mL) or positive controls, griseofulvin, (35 µg/mL) and etoposide (29 µg/mL), was added. The treated cells were incubated for 48 h at 37 °C in a humidified 5% CO_2 incubator. The spent culture medium was removed by aspiration and cells were fixed using 4% formaldehyde for 15 min at room temperature, then 100 µL of NucRed™ Live 647 stain dye (50 µL NucRed in 10 mL of PBS, according to the manufacturer's instructions) was added. Thereafter, cells were further incubated for an additional 15 min at room temperature prior to imaging.

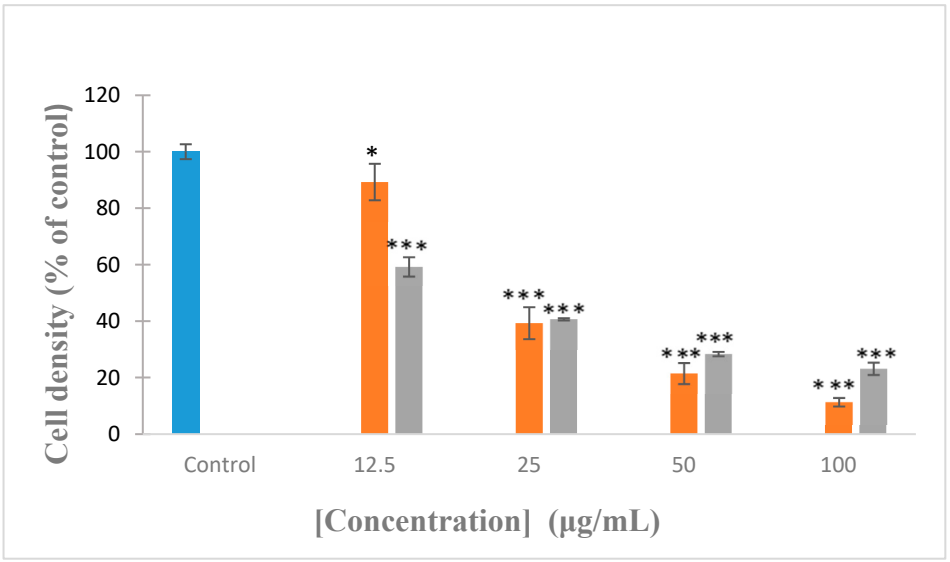
2.7. Statistical Analysis

Three replicates of each test sample (methanol extract) were used. The data were statistically analyzed by Student's *t*-test (two-tailed paired).

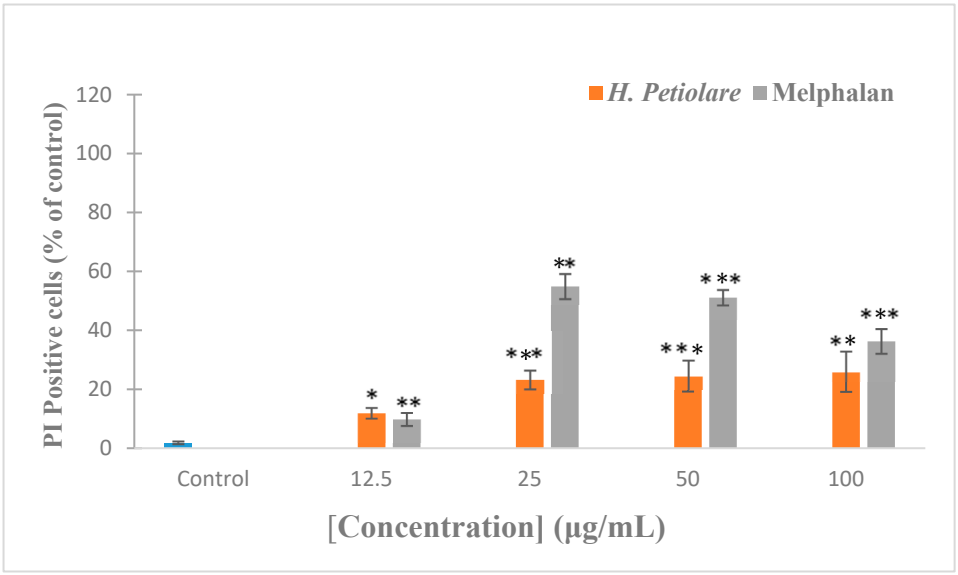
3. Results and Discussion

3.1. Cytotoxicity towards B16F10 Melanoma (Hoechst/Propidium Iodide)

The dose-dependent anti-proliferative effect of *H. petiolare* extract and the positive control (Melphalan) against B16F10 cells is shown in Figure 1. The extract exhibited a strong significant ($p < 0.001$) decrease in cell density at the tested concentrations of 25 µg/mL and higher (50–100 µg/mL). This reduction was less marked compared to the trend seen by the control cells and the positive control, Melphalan. However, despite the sharp decline in cell density by the extract, cell death, as determined using propidium iodide (PI) staining, does not show a reciprocal response and cell death appears to remain relatively constant above 25 µg/mL. This indicates that cell death is not the only contributory mechanism responsible for the decline in cell density and suggests that the inhibition of cell proliferation is an additional mechanism. It is interesting to note that the cytotoxic effect of *H. petiolare* extract against B16F10 melanoma cells has never been reported in the literature. However, some studies have been conducted on the anticancer properties of *Helichrysum* genus. For example, *Helichrysum plicatum*, a prominent member of the *Helicrysum* genus, was found to be active against K562 and PC3 cancer cells [15]. Those results support the findings observed in this study, although the cancerous cells used in that study are quite different from the ones reported in the present study. Taken together with the cytotoxic results, it could be said that *H. petiolare* extract is therefore a promising candidate for the treatment of cancer.



(**A**)



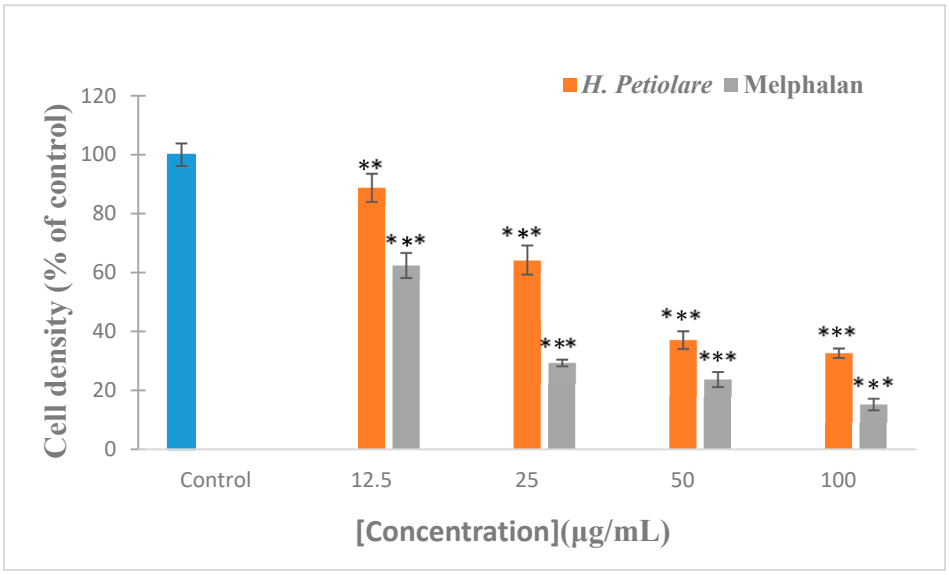
(**B**)

Figure 1. Cytotoxicity of *H. petiolare* methanol extract towards mouse melanoma cells (B16F10). (**A**) Cell density represents the number of nuclei stained with Hoechst and is expressed as a percentage of the untreated control. (**B**) Cell death is quantified as the percentage cells which stain positive for propidium iodide (PI). Melphalan was included as a positive control. Data are expressed as the mean ± SD, $n = 3$. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to control.

3.2. Cytotoxicity towards MeWo Melanoma (Hoechst/PI)

A further assessment of cytotoxicity was also carried out in MeWo cells. The result (Figure 2) showed that treatment of MeWo cells with the extract caused a dose-dependent decrease in the cell density compared to the control cells at all the tested concentrations (12.5–100 µg/mL). In addition, cell death, as indicated by PI staining, was more prominent in MeWo cells with increasing concentrations (12.5–50 µg/mL) of the extract. On the other hand, the positive control, Mephalan, induced cell density and cell death better than the trend seen with the plant extract and the control cells. Indeed, the decline in cell density, as exhibited by the extract, appears to involve both cell death and the inhibition of cell proliferation. However, several constituents of the *H. petiolare* extract, as described by

Maroyi [12], have been described in the literature for their cytotoxic activity on different cancer cell lines [16,17]. These plant constituents have been reported to affect different intracellular signaling pathways implicated in the initiation, promotion and progression of cancer [18,19]. Then, the observed cytotoxicity activity of the plant extract against MeWo melanoma cells might be attributed to the synergic effects of some of its chemical constituents that function on the cells, thereby leading to growth inhibition. The findings from this study suggest that the extract suppressed the growth of MeWo melanoma cells, therefore showing anticancer properties. However, it is imperative to perform a cell cycle analysis to assess population cell death and to examine whether the extract can induce cell cycle arrest.



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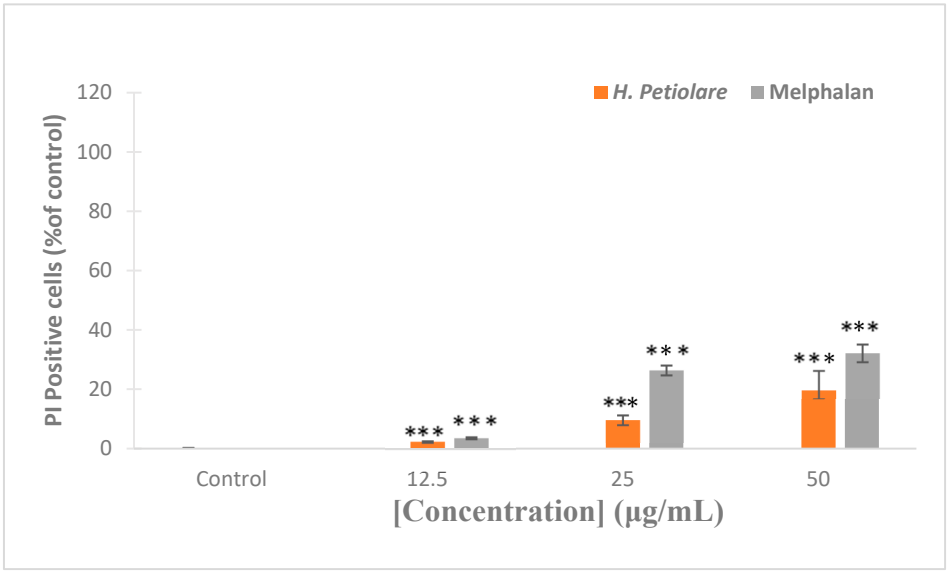

(**B**)

Figure 2. Cytotoxicity of *H. petiolare* methanol extract towards human melanoma cells (MeWo). (**A**) Cell density represents the number of nuclei stained with Hoechst and expressed as a percentage of the untreated control. (**B**) Cell death is quantified as the percentage cells which stain positive for PI. Melphalan included as a positive control. Data are expressed as the mean ± SD, $n = 3$. ** $p < 0.01$ and *** $p < 0.001$ compared to control.

3.3. Cell Cycle Analysis in B16F10 Melanoma (Annexin V/Hoechst)

Loss of control of the cell cycle plays a significant role in the growth of cancer. Thus, halting the cycle of cancer cells at any stage is an important strategy in the treatment of cancer [20]. In the present study, cell cycle analysis was determined to further confirm whether the *H. petiolare* extract mediated any alteration of a specific phase in cell cycle progression. As may be expected, the majority of cells were in the G0/G1 phase, a typical feature, as this represents the longest phase of the cell cycle. Significant changes in the G0/G1 and S phases of the cell cycle are apparent after treatment with the extract, suggesting an S phase arrest (Figure 3). In contrast to the extract, the positive control, Melphalan, induced a robust increase in G2 arrested cells, which is consistent with DNA crosslinking agents. To exclude apoptotic cells from the cell cycle analysis, Annexin V staining was used to identify apoptotic cells. Treatment with the extract caused a significant but small increase in apoptosis. However, it is known that cell cycle arrest is an important cause of the inhibition of cancer cell proliferation. For example, previous studies conducted by Liu et al. [21] revealed that *Ophiorrhiza pumila* inhibited liver cancer cell proliferation by inducing G2/M arrest. Another study conducted by Swanepoel et al. [22] also showed that *Anemone nemorosa* extract inhibited HeLa cervical cancer cell proliferation by inducing cell arrest at the early M phase. Thus, in this study, the extract demonstrated growth inhibitory effects in the B16F10 skin melanoma cells by inducing cell cycle arrest at the S phase. The cell cycle arrest at the S phase observed in this study is in support of other previous studies that reported plant extracts that induced an S phase arrest [23,24]. Studies have indicated that cell cycle arrest at the S phase is controlled by cyclin and cyclin-dependent kinase (CDKs) [25]. The CDKs are upregulated by cyclins and downregulated by cyclin-dependent kinase inhibitors (CDKI) such as $p16^{INK4a}$ and $p21^{Cip1}$ [26]. However, it should be noted that gene expression analysis was not investigated in this present study. From this perspective, it is clear that the proliferation of B16F10 skin melanoma cells was halted by DNA arrest during the S phase at 50 µg/mL, therefore suggesting that *H. petiolare* may be a potential candidate in eradicating skin cancer.

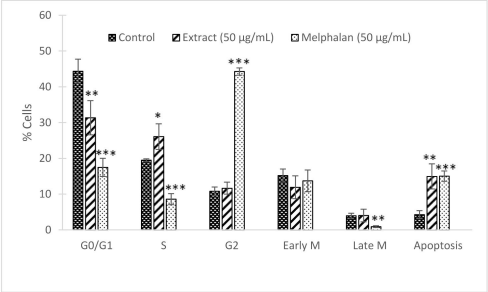

Figure 3. Cell cycle analysis in B16F10 cells after exposure of *H. petiolare* extract (50 µg/mL) and Melphalan (50 µg/mL). Cell cycle analysis was determined by the NucRed ™ Live 647 stain dye. The results are presented as the percentage of cells detected in each phase. Data are expressed as the mean ± SD, $n = 3$. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to control.

3.4. Cell Cycle Analysis in MeWo Melanoma (Annexin V/Hoechst)

Significant changes in the G0/G1 phase of the cell cycle are apparent after treatment with the extract. The extract caused a predominant cell cycle arrest at the early M phase (Figure 4). In contrast to the extract, Melphalan induced a significant increase in both the S and G2 phases, but this still remains consistent for a DNA crosslinking agent. To exclude apoptotic cells from the cell cycle analysis, Annexin V staining was used to identify apoptotic cells. Treatment with the extract caused a significant but small increase in apoptosis. One report showed that mitotic arrest is predominantly due to the disruption of the formation of the mitotic spindle, resulting in mitotic catastrophe [27]. The observed cell cycle arrest at the early M phase, as exhibited by the extract in the MeWo melanoma cells, therefore suggests that the extract may act by triggering the disruption of the formation of the mitotic spindle, thereby causing mitotic catastrophe [27]. However, several researchers have indicated

that phytochemical components play an important role in regulating the genes that control the cell cycle, proliferation and apoptosis pathway in cancerous cells [28–30]. Therefore, this effect of *H. petiolare* extract on cell cycle progression may be attributed to its secondary metabolites, which were previously reported [12,14].

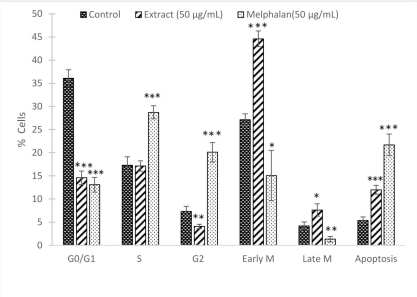

Figure 4. Cell cycle analysis in MeWo cells after exposure to *H. petiolare* extract (50 µg/mL) and Melphalan (50 µg/mL). NucRed ™ Live 647 stain dye. The results are presented as the percentage of cells detected in each phase. Data are expressed as the mean ± SD, $n = 3$. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to control.

3.5. Genotoxicity (Micronucleus Assay)

Micronuclei refer to small nuclei that are formed whenever a chromosome or a fragment of a chromosome is not incorporated into one of the daughter nuclei during cell division [31]. This is commonly a sign of genotoxic events and chromosomal instability (DNA strand breaks and chromosome loss or damage). In this study, a significant cytotoxicity towards Vero cells at the highest three concentrations tested (50–200 µg/mL) was seen following exposure compared to the trend seen in control cells (Figure 5). In addition, a significant increase in the formation of micronuclei cells was also observed with increasing concentrations (50–200 µg/mL) of the extract. Furthermore, the extract caused a dose-dependent decrease in the ratio of 2n to 4n and a dose-dependent increase in the nuclear size of the cells at the highest concentrations. On the other hand, etoposide, used as a positive control, exhibited a better increase in nuclear size compared to the trend seen in extract and control cells. It is clear that all the parameters observed in this study support the meaningful genotoxic effect of the plant extract. This is the first study to investigate the genotoxic potential of *H. petiolare* using a micronucleus assay. However, the genotoxic effect of plants from the *Helichrysum* genus has been reported. The study conducted by Eroglu et al. [32] revealed that methanol extracts of *H. pamphylicum* and *H. sanguineum* induced the formation of micronuclei in human lymphocyte cultures, thereby suggesting their genotoxic potential. This previous report is in agreement with the findings of this present study. It can be deduced from this study that *H. petiolare* extract exhibits genotoxic potential, therefore supporting its ability as an anticancer agent.

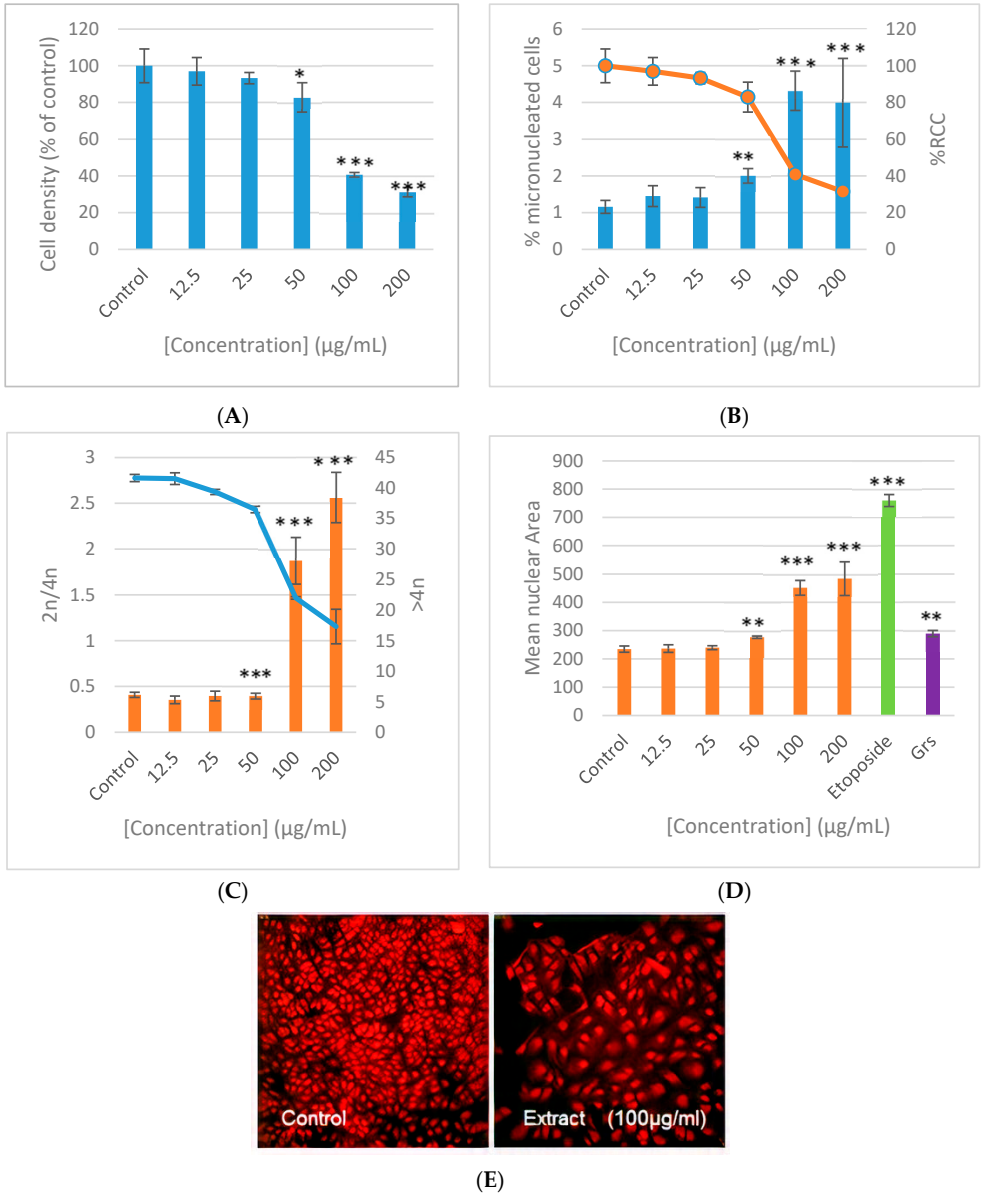

Figure 5. Micronucleus formation and nuclear parameters of Vero cells treated with different concentrations of *H. petiolare* methanol extract. Cytotoxicity is measured as decrease in the number of cells relative to the untreated control (also referred to as % Relative Cell Count). Data are expressed as the mean ± SD, $n = 3$. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to control. Griseofulvin (Grs). (**A**) Cytotoxicity; (**B**) micronucleus; (**C**) cell ploidy; (**D**) nuclear size; (**E**) micrographs (10× magnification) illustrating the nuclear size after treatment compared to the control.

4. Conclusions

In conclusion, the treatment of melanoma cells with this plant extract is characterized by a strong decline in cell density; however, cell death does not appear to fully explain the decline in cell density and suggests that proliferation is strongly impeded by the extract. To make a more definitive conclusion, additional experiments to confirm the mechanism of cell death is recommended. Data from the cell cycle analysis, however, provide some evidence that the mechanism involves aberrant cell division. In B16F10 cells, the extract caused cell arrest in the S phase, while in MeWo cells, extract treatment was characterized by arrest in the early M phase. This dissimilarity between the B16F10 and MeWo cells probably represents species differences. Data derived from all the parameters of the micronucleus assay in Vero cells support the meaningful genotoxic effect of the *H. petiolare* extract. Furthermore, a dose-dependent decrease in the

ratio of 2n to 4n observed in the micronucleus assay also supports a mechanism that involves aberrant cell division. Overall, the results of this study show that methanol extract from *H. petiolare* may have the ability to eradicate skin cancer.

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Conflicts of Interest: The authors declare that there are no conflict of interest regarding the publication of this paper.

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