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Preliminary investigation of in *vitro* anti-proliferative effects of ethanolic plant extract of *hypoxis argentea* in colon carcinoma cell lines

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Abstract

Colorectal cancer (Caco-2) is the third most common cancer with a high mortality rate worldwide. The current therapeutic approaches, such as radiotherapy and chemotherapy, are characterized by several limitations. The search for alternatives has led to the use of medicinal plants such as *Hypoxis argentea*. This study aimed to investigate the anti-proliferative effects of ethanolic plant extract of *Hypoxis argentea* in colon carcinoma cell lines. Ethanolic extract of *Hypoxis argentea*, Caco-2 cell lines, Vero cell lines, and melphalan (positive control) were used for this study. The antioxidant potential of the extract was evaluated using the DPPH assay. The MTT assay and total cell count were performed to determine cell metabolic activity, viability, and cell proliferation in both cell lines with varying concentrations of *H. argentea* extract (15.6 to 500 µg/mL) and a reference chemotherapeutic agent, melphalan (6.25 to 100 µM). Treatment with *H. argentea* revealed a significant ($P<0.01$) dose-dependent decrease in cell viability in Caco2 cancer and Vero cells at concentrations 15.6-62.5 µg/mL. Also, the total cell count of Vero and Caco-2 cells was significantly reduced ($p<0.01$) compared to the control at every dose concentration of *Hypoxis argentea*. Likewise, the total cell count of Vero and Caco-2 cells was significantly reduced ($p<0.01$) compared to the control at every dose concentration of melphalan. These findings indicate that *H. argentea* has a promising selective antiproliferation effect on cancer cells while maintaining a safe profile for normal cells.

Keywords: Cell lines, Colon carcinoma, Hypoxis argentea, in vitro.

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1. Introduction

Despite advancements in oncology, cancer is a life-threatening disease throughout the world [1]. Estimates show that in 2020, approximately 19.3 million cases were reported, resulting in nearly 10 million deaths [2]. Furthermore, the global cancer burden is anticipated to increase to 28.4 million cases by 2040, representing a 47% rise from the case load of 2020; hence, cancer remains a leading cause of morbidity and mortality affecting both men and women [3].

Cancer is a disease characterized by uncontrolled proliferation of cells by an activation of growth-promoting oncogenes and inactivation of tumor suppressor genes that invade surrounding tissues through the bloodstream or lymphatic system- a process called metastasis [4]. It is caused by genetic mutations that can be inherited in genes affecting DNA repair, cell growth, and apoptosis, or exposure to environmental carcinogens including tobacco, consumption of high fatty foods, unsafe sexual practices, exposure to high levels of ultraviolet (UV) radiation, air pollution and comorbidities such as tuberculosis and human immunideficiency virus [5, 6].

Common cancers include breast, colorectal, lung, prostate, skin, cervical, liver, oesophageal, and pancreatic cancers [1]. Of these cancers, colorectal cancer (CRC) ranks as the third most common cancer and the second most common cause of cancer mortality worldwide [7]. In South Africa, CRC is the fourth most common cancer, both in men (7.17/100,000/year) and women (5.80/100,000/year) [8, 9].

Cancer mortality in South African rural areas is likely caused by the disease being detected at advanced stages, limited access to treatment services, high cost of treatment, and the presence of comorbid disease, including HIV and TB [10, 11]. For instance, patients from the Eastern Cape's Nelson Mandela Academic Hospital (NMAH) are compelled to travel more than 200 km to East London to access therapeutic cancer care as they cannot afford a private healthcare centre [12].

Chemotherapy, radiotherapy, surgery, gene therapy, and immunotherapy are known current treatments for cancer [13]. However, adverse effects have been observed in patients, causing a lot of strain, psychosocial problems, premature mortality, increased risk of second cancers, and disability [14, 15]. The complexity associated with current treatments, their limitations, and high costs necessitate the need to explore less toxic, affordable, and efficacious therapeutic drugs. One such alternative is the use of phytotherapeutics.

Current literature has identified a number of medicinal herbs with potential anticancer properties. Among these are medicinal plants, such as *Hypoxis argentea* [16]. Africa has the largest diversity of the genus *Hypoxis*, accounting for 61% of the current globally accepted taxa within the genus, including some endemic species [17]. *Hypoxis argentea* belongs to the family *Hypoxidaceae* and is a perennial herb with leaves enclosed in a sheath arising directly from an underground adventitious rootstock (corm), in what appears to be a false stem, while a slender stalk from the middle of the leaves bears an inflorescence of bright yellow, often star-shaped flowers [18]. It grows in the Eastern Cape province of South Africa on rocky outcrops and grassy areas [19]. The fresh corms of the plant are stamped, boiled in water, and then administered orally to treat cancer in the Eastern Cape [20].

Communities in South Africa rely on traditional plant-based medicines as their main source of healthcare. However, there has been no previous work published on the *in vitro* anti-proliferative effect of *H. argentea* in colorectal cancer cells, and there is no literature report with regard to its anticancer properties. Thus, it is critical to validate herbal remedies while preserving indigenous medicinal plants for the benefit of these communities [21].

Hence, this study investigates the anticancer effect of the ethanolic plant extract of *Hypoxis argentea*, highlighting its benefits in therapeutic applications as a treatment of cancers and the mechanisms through which it will inhibit growth and initiate apoptosis in colorectal cancer cells.

2. Materials and Methods

2.1. Study Design

This laboratory-based *in vitro* experimental study was done at Walter Sisulu University's chemistry laboratory, cell culture laboratory, and Nelson Mandela University's Microbiology laboratory.

2.2. Materials

Corms of *Hypoxis argentea*, Colorectal cancer (Caco-2) cell lines, Vero (noncancerous kidney cells) cell lines, Melphalan (positive control), Ethanol, Methanol, Distilled water, Analytical balance, Rotary evaporator, Filter paper (Whatman 1), Funnel, Glass Beakers, Aluminium foil, MTT (3-(4,5-dimethyl- thiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent, Dimethyl sulfoxide (DMSO), Culture medium e.g., DMEM (Dulbecco's Modified Eagle Medium with 10% Fetal bovine serum (FBS) or RPMI), DPPH (2, 2-Diphenyl-1-Picrylhydrazyi) powder, Ascorbic acid, Plastic pipettes and micropipettes with sterile tips, Volumetric flask, Test tubes, Vortex mixer, Incubator, UV-vis spectrophotometer set at 517 nm and Microplate reader.

3. Methods

3.1. Plant Collection and Identification

H. argentea was collected in August 2024, at Mthatha, Eastern Cape, South Africa. The taxonomic identity of the plant was authenticated at the Faculty of Natural Sciences, Department of Botany at Walter Sisulu University.

3.2. Preparation of Plant Extract

Corms of *Hypoxis argentea* were washed free of soil attachment with tap water and air-dried at room temperature for a few hours. The fresh corms were peeled and sliced into small pieces to increase surface area and allow the solvent to penetrate the plant material more thoroughly. About 261 g of plant material was soaked in 95% ethanol in the ratio 1:5 (weight/volume), sealed the flask with aluminum foil to prevent evaporation, and then allowed the mixture to macerate at room temperature for 1 week while occasionally stirring it. Samples were filtered using Whatman No. 1 filter paper. The corms were then remacerated two times. The solvents obtained were evaporated using a rotary evaporator (Buchi R-215, Switzerland) at 40- 60°C dryness under reduced pressure to concentrate the ethanolic extract by removing excess ethanol. The crude extract produced was collected and stored at room temperature to further air-dry.

3.3. Preparation of Cell Culture Lines

Caco-2 and Vero cell lines were cultured and seeded in Dulbecco's modified Eagle's medium (DMEM) with 1% penicillin-streptomycin and 10% fetal bovine serum (Sigma, USA). All cell cultures were incubated at 37°C in a humidified atmosphere with 5% CO_2.

3.4. Antioxidant activities using 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) assay

The DPPH assay was performed according to the method by Sagbo and Otang-Mbeng [22] and Sharifi-Rad, et al. [23]. DPPH (0.01 g) powder was weighed and dissolved in 500 mL of methanol to prepare a 0.1 mM DPPH solution. The reaction mix was wrapped with aluminum foil and kept in the dark for 30 min, and its absorbance was measured at 517 nm. A stock solution prepared with 0,1 g of ascorbic acid dissolved in 100 ml of distilled water was used as a positive control. Serial dilutions of 160, 80, 40, and 20 µg/ml were done, creating working comparison standards. A crude extract stock solution was prepared with 0,257 g dissolved in 4 ml of Dimethyl Sulfoxide (DMSO). Initially, 1 ml of methanol was added into the test tubes, and 1 ml of crude extract stock solution was added to each test tube for serial dilution. A total volume of 1 ml of 0,1 mM DPPH free radical in methanol was added to 1 ml of serially diluted extract at 16, 8, 4, 2, 1, 0.5, 0.25, and 0.125 µg/ml concentration range in three replicates. The reaction mixture was then kept in the dark for 30 minutes. The absorbance was measured at 517 nm on a microplate reader against a blank (DPPH + methanol). Ascorbic acid was used as a standard. The percentage inhibition of the radical was calculated using the formula below.

$$\%\text{DPPH inhibition} = \frac{\text{ABS control} - \text{ABS sample}}{\text{ABS control}} \times 100$$

Where ABS control is the mean absorbance of the control (DPPH + methanol), while ABS sample is the mean absorbance of the sample (DPPH + crude extract).

3.5. Determination of Cytotoxicity Activity Using 3, 4, 5-Dimethylthiazol-2-yl-2, 5-Diphenyltetrazolium Bromide (MTT) Assay

Colorectal cancer cells and Vero noncancerous kidney cells were stored at -196 °C, thawed at room temperature in a laminar flow hood (Labtech™, Ortenberg, Germany) at 23°C with a windspeed of 0.53 m/s, to ensure sterility for subsequent culturing in 17 mL of Eagle's essential minimal medium (EMEM) that was pre-warmed to 37 °C and supplemented with non-essential amino acids, sodium pyruvate (1 mM), foetal bovine serum (FBS) (10%), L-glutamine (1%) and glucose (19.5 mM). Inoculation of the cell suspension into a 15 cm cell culture petri dish was performed. The dish was then incubated at 37 °C and humidified with 5% CO_2 overnight. Passage numbers were recorded (restricted to below 30 passages) to avoid phenotypic drift from the number of times the cells were trypsinized and reseeded. The medium was refreshed (every second day) until the cells reached the targeted 80 % confluency to resume the experimentation.

The cells were sub-cultured using the standardized protocol until they reached 75-80% confluence to be used for further experimentation. The spent media was aspirated, and the remaining cells were washed with 8 mL of [1x] phosphate-buffered saline (PBS). To retrieve the cells, 3 mL of trypsin was added to each 15 cm petri dish, and the cells incubated, as above, for 3-7 min. Standardized cell culture growth medium (7 mL) was added to the cells to inactivate trypsin, and a single cell suspension was obtained by pipetting the cell suspension with a 10 mL serological pipette. A 15 mL centrifuge tube was then used to centrifuge the transferred cell suspension at 800 x g using the SL16R centrifuge (ThermoFischer Scientific™, Massachusetts, USA) for 5 min. Depending on the size of the cell pellet, the pellet was then re-suspended in 5-

10 mL cell culture growth medium, and 0.5 mL of the suspension was transferred into a centrifuge tube (2 mL) for cell counting.

Counting of the cells was done by pipetting the cell suspension (10 µL) into a centrifuge tube (2 mL), which contained 10 µL of trypan blue solution at 0.4% and mixed by pipetting up and down. The mixture (10 µL) was then pipetted onto a LUNA slide and inserted into the LUNA machine for Caco-2 and Vero cell counting protocol.

Briefly, cells (50 µL) (1x10¬2 cells/mL) as well as 50 µL DMEM were seeded in 96-well flat-bottom plates and incubated for 24 h at 37°C in a humidified incubator under 5% CO2. Cells were then treated with 50 µL of sample extract prepared in 5% DMSO (7.8-1000 µg/mL) and incubated for 24 h. Melphalan was used as the positive control. MTT reagent (20 µL, 5 mg/mL) was added to the cells and incubated (3 h at 37°C). Finally, 100 µL of DMSO was added to each well in order to solubilize the formazan salt formed. The absorbance was read at 570 nm on a microplate spectrophotometer (Multiscan Go, Thermo Scientific).

3.6. Statistical Analysis

Results were analyzed using ANOVA (GraphPad Prism software, San Diego, CA, USA). All analyses were done in quadruplicate except for the DPPH assay (n = 3). The results were presented as mean ± Standard error of mean (SEM). Data was considered significant when $p < 0.05$.

4. Results

4.1. Antioxidant Activity Using DPPH Assay

The IC_{50} value of 1.0 µg/ml for *Hypoxis argentea* extract was calculated using the linear regression equation y = 76.062x + 22.713, where $R^2 = 0.89$ [Figure 1A], while the IC_{50} value of 45 µg/ml for ascorbic acid was calculated using the linear regression equation y = 1.2026x + 4.22, where $R^2 = 0.83$ [Figure 1B].

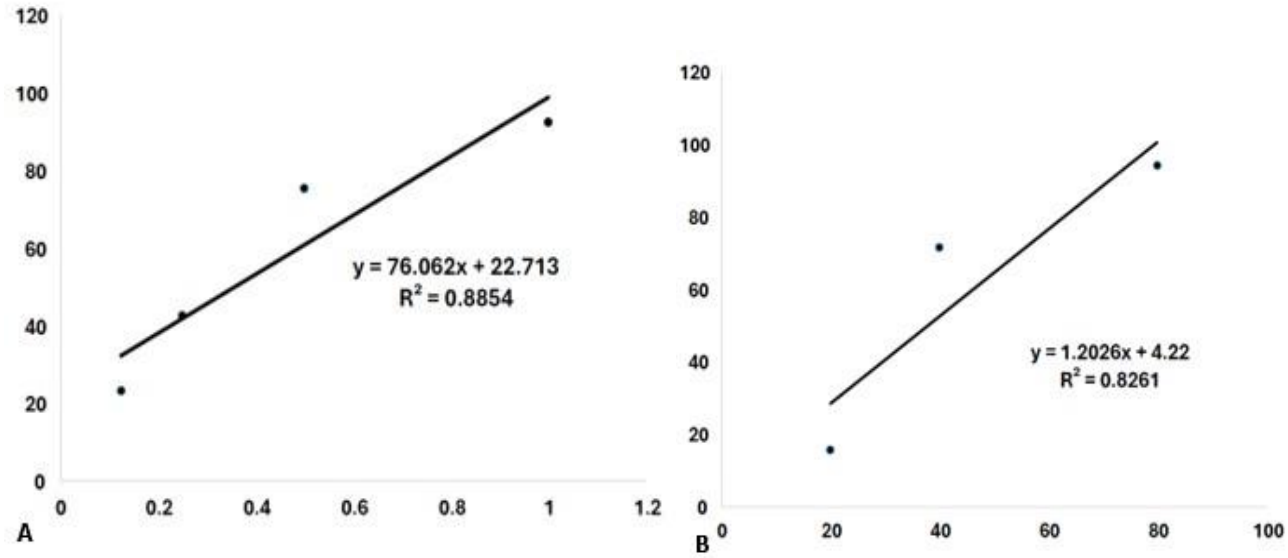

Figure 1.
Showing the linear regression graph of *Hypoxis argentea* extract (A) and ascorbic acid (B) on DPPH inhibition. Each point is a mean of three repetitions.

Hypoxis argentea extract showed a strong IC_{50} value of 45 µg/ml for ascorbic acid using a linear regression equation y=1.2026x + 4.22 as a reference standard. Also, ascorbic acid showed a weak antioxidant activity compared to the IC_{50} value of 1.0 µg/ml for *Hypoxis argentea* extract, which was calculated using a linear regression equation y=76.062x + 222.713.

4.2. Cell Viability and Proliferation Assay (MTT Assay)

As shown in Figures 2 and 3, MTT assay data show a significant reduction ($P<0.001$) in cell mitochondrial activity with the melphalan control for Vero noncancerous and colorectal cancer cells. This reduction was also seen with the various treatments at low concentrations, significantly at P-value less than 0.01 and 0.001 compared to the untreated control, except for high concentrations of 125, 250, and 500 µg/ml of *H. argentea* treatment.

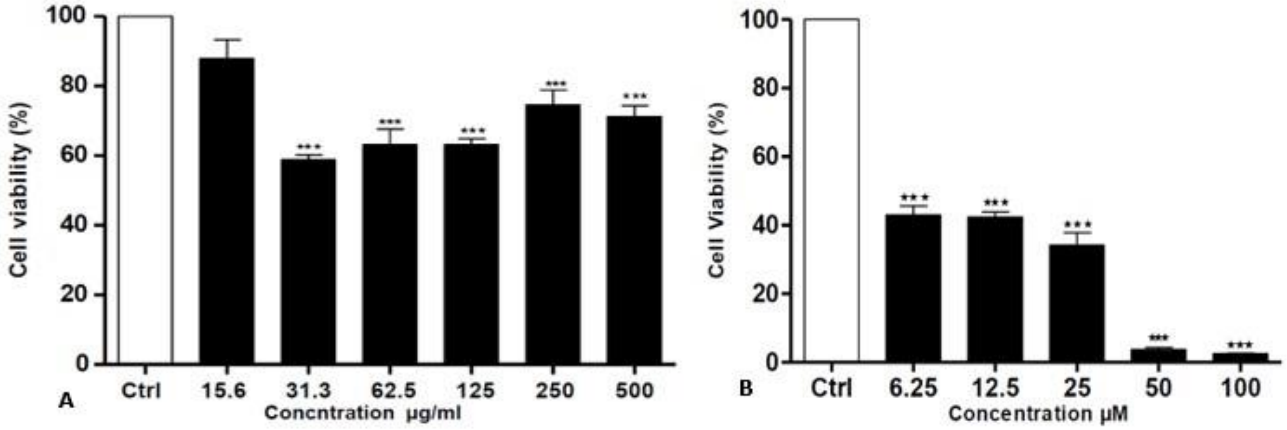

Figure 2.
Showing the results of the MTT assays on the viability of Vero cells treated with different dose concentrations of *Hypoxis argentea* **(A)** and melphalan **(B)** (***p < 0.001).

Hypoxis argentea significantly reduced the cell viability of Vero cells dose dependently with doses 31.3, 62.5, 125, 250, and 500 µg/ml, reducing viability to 59 ± 1.3, 63 ± 5, 63 ± 1.8, 75 ± 4, and 71 ± 3 % respectively [Figure 2A]. Melphalan significantly reduced the cell viability of Vero cells dose dependently with doses of 6.25, 12.5, 25, 50, and 100 µM, reducing viability to 43 ± 2.3, 42 ± 1.5, 34 ± 4, 4± 0.6, and 3 ± 0.2 % respectively [Figure 2B].

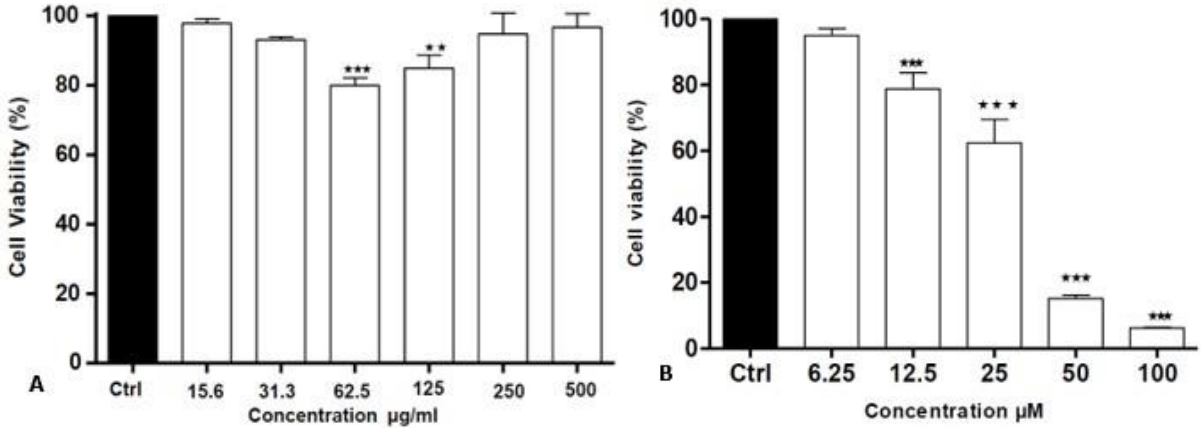

Figure 3.
Showing the results of the MTT assays on the viability of Caco-2 cells treated with different dose concentrations of *Hypoxis argentea* (A) and melphalan (B).
Note: (**p < 0.01; ***p < 0.001).

Hypoxis argentea reduced the cell viability of Caco-2 cells dose dependently, with all doses, but significantly with doses of 62.5 and 125 µg/ml, reducing viability to 80 ± 2 and 85 ± 4%, respectively [Figure 3A]. While melphalan significantly reduced the cell viability of Caco-2 cells dose dependently with doses of 12.5, 25, 50, and 100 µM, reducing viability to 79 ± 5, 62 ± 7, 15 ± 1.0, and 6 ± 0.3 % respectively [Figure 2B].

4.3. Total Cell Count of Vero and Caco2 Cells

As observed in Figures 4A and 5A, the total cell count of Vero and Caco-2 cells was significantly reduced ($p < 0.001$) compared to the control at every dose concentration of *Hypoxis argentea*. Likewise, in Figures 4b and 5b, the total cell count of Vero cells and Caco-2 cancer cells was significantly reduced ($p < 0.001$) when compared to the control at every dose concentration of melphalan.

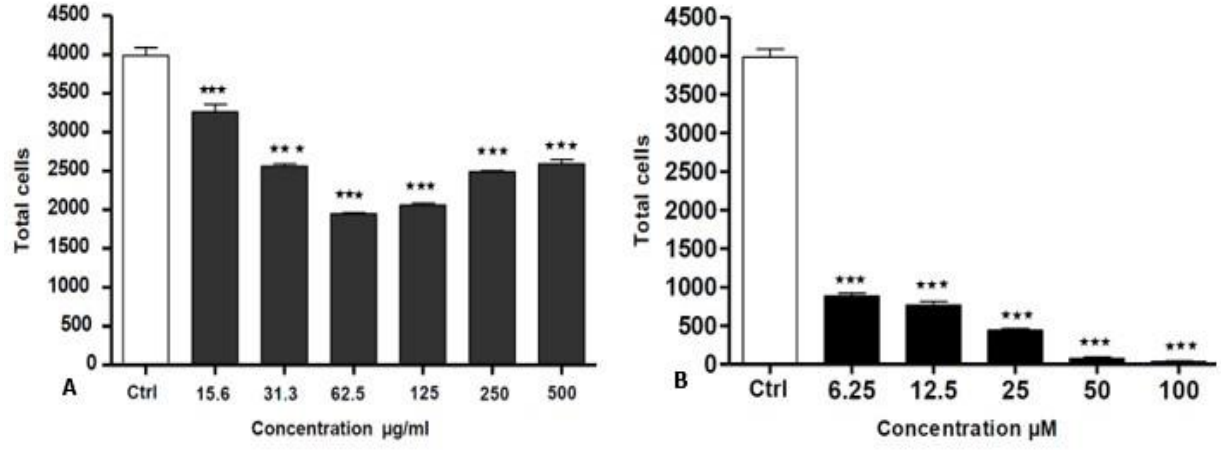

Figure 4.
Showing the effect of *Hypoxis argentea* (A) and melphalan (B) on the total cell counts of Vero cells at different dose concentrations.
Note: (***p < 0.001).

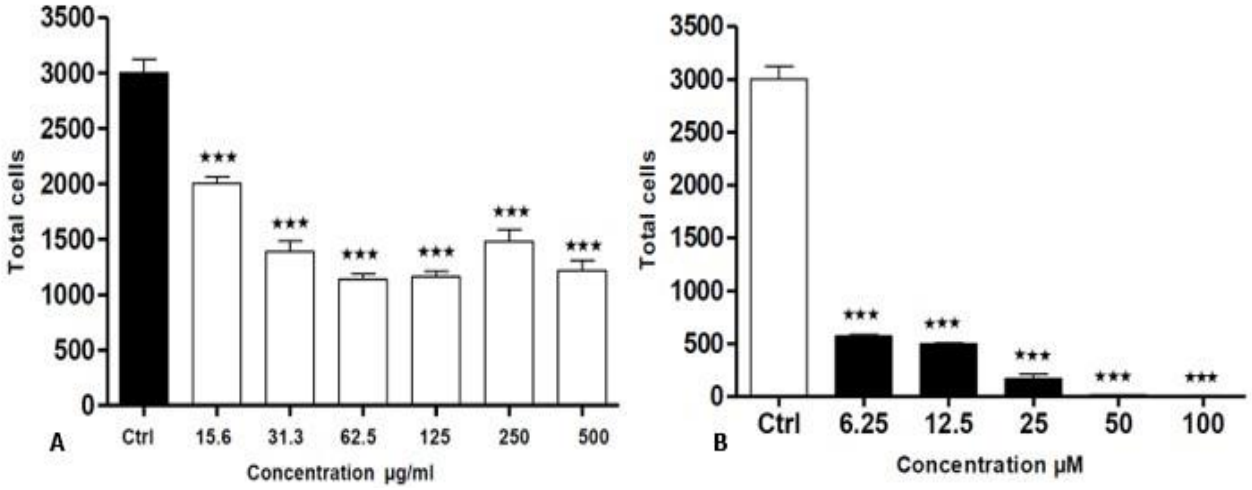

Figure 5.
Showing the effect of *Hypoxis argentea* **(A)** and melphalan **(B)** on the total cell counts of Caco-2 cells at different dose concentrations.
Note: (***p < 0.001).

5. Discussion

Despite several advances in oncological management of cancer, cancer-related morbidity and mortality are still high and devastating [3]. Patients sometimes suffer adverse effects from current treatments of cancer, such as chemotherapy, radiotherapy, gene therapy, etc [13-15]. In addition, conventional treatments for cancer are unaffordable (high cost) to populations in developing countries. Hence, exploring less toxic, affordable, and efficacious therapeutics for cancer management and treatment is necessary.

The present study evaluated the *in vitro* anti-proliferative effects of the ethanolic extract of *Hypoxis argentea* on colorectal cancer cell lines by assessing cell viability using the MTT assay and total cell count. The antioxidant properties of *Hypoxis argentea* have been reported in previous studies following antioxidant assays [24, 25]. This was also observed in the present study as the antioxidant activity of the extract (*Hypoxis argentea*) was determined using the DPPH assay by comparing its radical scavenging ability to that of a known standard, ascorbic acid. The results showed that *H. argentea* exhibits highly significant dose-dependent antioxidant potential and has cytotoxic and genotoxic effects on cancer cells.

The findings of this study indicate a significant reduction in cell viability on Caco2 cells treated with low concentrations of *H. argentea* extract in terms of the plant's cytotoxic effect on cancer cells, causing cell death. This finding aligns with a study conducted by Siddiqui, et al. [24] on phytochemical composition, cytotoxicity screening, and antioxidant activities of extracts and essential oils of *Hypoxis argentea* corms, where the ethanolic extract demonstrated its potency towards cancer. However, at high doses, the extract shows that it promotes cell proliferation, which could be a result of a phytochemical that dominates at high concentrations or that the extract has different mechanisms of toxicity in different cell types. Furthermore, Siddiqui, et al. [24] observed that up to 50 µg/ml, the aqueous extract appeared to stimulate cell division, a property that can be utilized as a cytoprotective therapeutic target.

Concentrations of the *H. argentea* extract examined showed a significant difference ($p<0.01$) when compared to the control, as observed for melphalan (positive control). In a cytotoxicity screening of African medicinal plants, Statistics South Africa (Stats SA) [26] found that the ethanolic extract of *H. argentea* corms stimulated cell growth of DU-145 (prostate carcinoma cells), MCF-12A (non-malignant breast cancer cells), and inhibited the growth of MCF-7 cells.

Steenkamp and Gouws [27] evaluated the cytotoxicity of *H. argentea*, *H. stellipilis*, and *H. sobolifera chloroform* corm extracts in HeLa (cervical), HT-29 (colorectal), and MCF-7 (breast) cancer cell lines using the MTT assay. Findings suggest that *H. sobolifera* has the best overall cytotoxic effect against the cancerous cell lines screened, with *H. argentea* effectively inhibiting HT-29 and *H. stellipilis* having stimulated the growth of HeLa as well as HT- 29 cells.

This implies that while *Hypoxis argentea* extract does affect both cell lines, it demonstrates a greater inhibitory effect on the cancerous Caco2 cells compared to the normal Vero cells at low concentrations. The analysis outlines that *H. argentea* treatment has some cytotoxic activity and proliferation. The significant changes were observed when comparing the various treatments to the melphalan treatments. Hence, an effort must be devoted to isolating and purifying these anticancer bioactive components.

The cell count analysis revealed a significant decrease ($p<0.001$) in total Caco2 cell numbers exposed to *H. argentea*, as also observed for melphalan (positive control). By measuring the absolute number of cells after treatment, we gain a better understanding of the anti-proliferative and potentially cytotoxic effects of each compound, as well as the selectivity of *Hypoxis argentea* for cancer cells over normal cells. This analysis complements the MTT assay, offering a more comprehensive picture of how each treatment affects cell survival and proliferation. The pathogenesis of cancer involves oxidative stress mechanisms. In oxidative stress, the production of ROS exceeds the capacity of exogenous antioxidants to detoxify them, leading to cellular or tissue damage due to lipid peroxidation and other mechanisms [28].

6. Conclusion

The present study evaluated the *in vitro* anti-proliferative effects of ethanolic extract of *Hypoxis argentea* on colorectal cancer cell lines. Ethanol extract of *H. argentea* significantly inhibited cancerous cell lines while remaining nontoxic to noncancerous cell lines. This study then concludes that the ethanolic extract of *H. argentea* possesses significant antioxidant activities, attributes which may prove useful for their effectiveness in the treatment of diseases such as cancer. This study also suggests further studies to be carried out in vivo to validate the observed in vitro effects of *Hypoxis argentea* extract. In addition, testing on a wider variety of cancer and normal cell lines would enhance the generalizability of these findings.

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