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Preliminary anticancer activity of *Carpobrotus edulis* (L.) Bolus exposed to low and high temperature conditions against cervical cancer

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ABSTRACT

Introduction: Environmental temperature conditions might influence the composition of secondary metabolites in certain medicinal plants. This study aimed to examine how some of the phytochemicals and anticancer properties of *Carpobrotus edulis* may be impacted by temperature conditions.

Methods: The plant specimens were kept in growth chambers at 15/10 ˚C and 45/35 ˚C (day/night), and harvested at 48-hour intervals (48, 96, and 144 hours). Control conditions were maintained at 25/15 ˚C. Standard phytochemical colour tests were used to determine the presence of eight phytochemicals. The anticancer activity against cervical cancer cell lines was determined using cell viability assay, cell morphology, Hoechst staining, and wound healing assays.

Results: The phytochemical screening showed the presence of all tested phytochemicals (phenolics, flavonoids, terpenoids, saponins, tannins, steroids, and cardiac glycosides) in all treatments except for saponins in the 96- and 144-hour temperature treatments. A noteworthy IC_{50} value of 284.9 µg/mL was determined from the methanolic leaf extract exposed to high temperatures for 144 hours against cervical cancer. Treatment with this extract suggested changes in cancer cell morphology, signs of apoptosis, and cell migration inhibition.

Conclusion: The preliminary results obtained suggest that the *C. edulis* methanolic extract has potential anticancer properties against cervical cancer. These observations may have implications for the indigenous plant use in treating various ailments and broaden the type of anticancer research involving this plant.

Implication for health policy/practice/research/medical education:
The methanolic extract of *Carpobrotus edulis* from samples exposed to high-temperature conditions for 144 hours demonstrated potential anticancer activity against cervical cancer. This will have implications for the ethnomedical use of the plant, anticancer research, and climate change research on this plant.

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Introduction

More than 10 million people are diagnosed with cancer every year (1). Non-melanoma skin cancers are the most common types, often attributed to cumulative sun exposure (2). In contrast, melanoma displays more aggressive behaviour, capable of spreading to distant organs (2). The increasing prevalence of skin cancer is attributed to factors such as depletion of the ozone layer, changing lifestyles, and increased outdoor activities (3). South Africa, with its high levels of solar radiation averaging 220 W/m^2 per day due to its geographic location, experiences a substantial burden of skin cancer (4-6). The prevalence is particularly concerning in fair-skinned populations of European descent and people with albinism (7). Additionally, the country's diverse ethnic makeup and cultural practices contribute to its differing

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proneness to skin cancer (7).

Cervical cancer also results in over 300 000 deaths worldwide, with more than half a million women diagnosed with the disease (8). It is largely linked to ongoing infection with high-risk human papillomavirus (HPV) strains (8). It disproportionately affects women in low-resource settings, where access to screening, early detection, and treatment are limited (8). South Africa bears a significant burden of cervical cancer, often affecting women in their prime reproductive years (9). Socioeconomic disparities, limited access to healthcare services, and high HIV prevalence contribute to the heightened vulnerability of South African women to cervical cancer (9,10).

Cancer treatments such as chemotherapy often result in side effects and drug resistance, creating the necessity to develop alternative therapeutic approaches (11) such as herbal medicine. Medicinal plants are a source of different phytochemical compounds, which are now, due to climate change-related conditions, subjected to a variety of environmental stresses such as temperature events that persist for several days (i.e., cold fronts and heat waves) during their growth and development (12). In line with global trends, South Africa has experienced cold fronts and heat waves. Plants' survival relies heavily on their ability to adapt to these environmental conditions, and it has been indicated that climate change can affect the composition of their secondary metabolites (13,14), which can alter their medicinal properties, including anticancer activities. Nonetheless, several studies have validated the use of various medicinal plants as anticancer or antiproliferative agents (15-17).

Anticancer activity is an important aspect of cancer research and treatment, as the main goal is to slow down, inhibit, or prevent the growth and proliferation of cancer cells (18). This can be achieved in various ways, such as disrupting cellular processes that allow cancer cells to grow and multiply (i.e., cell cycle regulation and DNA replication), inducing programmed cell death (apoptosis), stimulating the body's immune system, and inhibiting the growth of blood vessels (angiogenesis) (19). Angiogenesis can potentially lead to metastasis; therefore, the anti-migratory or anti-invasive properties of a plant extract are of interest for their potential to slow down or prevent the migration and invasion of cancer cells to nearby tissues and, eventually, distant organs (20). *Carpobrotus edulis* (L.) Bolus, also known as sour fig, is one of the traditionally used medicinal plants with potential anticancer properties.

The species is native to South Africa and commonly invasive in coastal environments in many parts of the world (21). *C. edulis* belongs to the Aizoaceae family, and the *Carpobrotus* genus has 13 species, seven of which occur in Southern Africa (22). The medicinal plant is used by traditional healers, herbalists, and local people in South Africa for the treatment of various ailments such as mouth infections, sore throats, stomach aches, diabetes mellitus, tuberculosis, and HIV-related infections (23). Scientific evidence demonstrates that extracts from *C. edulis* possess anticancer properties, contributing to the inhibition of abnormal cell growth across various cell lines induced by oncogenic factors (24,25). *C. edulis* leaf extracts in methanol at non-toxic concentrations have been reported to prevent the verapamil-sensitive pump of the L5178 mouse T-cell lymphoma cell line (26). The available evidence suggests its potential in complementary and alternative cancer therapies. In light of the ethnomedicinal uses of *C. edulis* and the prolonged temperature extremes often observed in South Africa, it is essential to conduct studies on this subject. Hence, this study aimed to examine how some of the phytochemicals and anticancer properties of *C. edulis* may be impacted by low (15/10 °C) and high temperature (45/35 °C) conditions for up to six days. This is a novelty because climate change-related effects on *C. edulis* phytochemical composition and anticancer activity are limited.

Materials and Methods

Plant collection and preparation

The aerial parts (fresh cuttings) of *C. edulis* plant were collected in the city of Tshwane (Pretoria North: 25.6776° S, 28.1755° E), Gauteng, South Africa, in January 2021, planted in five-litre plastic pots in commercial potting soil, and allowed to grow for three months in the greenhouse at the University of the Witwatersrand, Johannesburg, South Africa. The prepared taxonomic identification of the voucher specimen was done and stored at the University of the Witwatersrand, Braamfontein East Campus (IMR 0009522).

Control plants (five pots with three cuttings each) were watered daily with 100 mL of water and kept under 25/15 ˚C (day and night) in the greenhouse. Two sets of fifteen experimental pot plants (three cuttings in each pot) were transferred into separate plant growth chambers (Conviron CMP6010) and kept at either 15/10 ˚C or 45/35 ˚C (day and night), respectively, watering them once daily for a period of up to six days. The control and temperature treatment samples were harvested episodically at 48-hour intervals (48, 96, and 144 hours), dried using a freeze dryer (VaCo 2, Zirbus Technology GmbH Hilfe Gottes 1, 37539 Bad Grund), and pulverised.

Plant material extraction

The pulverised leaves of *C. edulis* from the control and the two sets of temperature treatments were extracted with analytical grades of methanol and hexane, respectively. Methanol and hexane extracted different compounds depending on their polarity index; methanol is a polar solvent with an index of 5.1, and hexane is a non-polar solvent with an index of 0.1 (27). Later, the extracts were collected by filtration using a Whatman filter paper.

Phytochemical screening

Standard phytochemical screening procedures were

adapted from Roghini and Vijayalakshmi (28) to determine the presence or absence of phenolics, flavonoids, terpenoids, saponins, tannins, steroids, and cardiac glycosides in *C. edulis* leaves.

Anticancer activity

Sample preparation

The extracts were finally concentrated by evaporating the solvent using a fume hood and kept at 4°C until use. The methanol and hexane extracts' concentrates of each *C. edulis* treatment were dissolved in dimethyl sulfoxide (DMSO) to form a final 100 mg/mL stock solution and stored at −20 °C until use.

Cell culture

Skin cancer (A375), cervical cancer (ME-180), and normal human lung tissue (MRC-5) cell lines were obtained from the University of Johannesburg, South Africa, and cultured in Dulbecco's Modified Eagle's Medium (DMEM). Cells were incubated in a humidified incubator (HERAcell 150i CO2 incubator (Thermo Fisher scientific, South Africa)) at 37 °C with 5% CO_2.

Cell viability assay

The cell viability assay was adapted from Kumar et al (29) with slight modifications. Cells were grown to approximately 70% confluence in a 94-well plate along with the vehicle/DMSO control for about 24 hours. Following that, the media was aspirated to wash the cells with phosphate-buffered saline (PBS) and treated with 500 µg/mL (screening purposes) of each extract, then incubated at 37 °C for 22 hours. Ten microliters of alamarBlue™ was added to each well in the dark, and the plates were incubated at 37 °C for 2 hours. The fluorescence was measured at 590 nm using a plate reader (Synergy HT microplate reader; BioTek, United States). The extract that exhibited the highest cytotoxicity was further treated with a range of concentrations (62.5-500 µg/mL) of extract. The treatment's DMSO final concentration was kept below 0.1%. The experiments were repeated at least three times to acquire the data. The cell viability was calculated using the following formula:

$$Cell\ viability\ (\%) = \frac{(Fluorescence\ of\ treated\ cells - Fluorescence\ of\ blank)}{(Fluorescence\ of\ untreated\ cells - Fluorescence\ of\ blank)} \times 100$$

The selectivity index (SI) for normal cells to that for cancer cells was determined using the following formula:

$$Selectivity\ index = \frac{IC50\ of\ non\text{-}cancerous\ cell}{IC50\ of\ cancerous\ cell}$$

Cell morphology

Cells were seeded in 4-well plates and treated with DMSO or extract (at the IC_{50} concentration determined using the standard curve line equation: y = mx + c) for 24 hours. After the treatment period, images were captured using phase contrast microscopy with an HBO 50/AC Axiovert microscope (Zeiss, SA).

Hoechst staining assay

The Hoechst staining assay was adapted from Sutherland and Durand (30) with slight modifications. Cells were grown to 70%-80% confluency and then washed with sterile PBS to remove the debris. Trypsin was added to detach the cells and incubated (HERAcell 150i CO2 incubator (Thermo Fisher scientific, South Africa)) for 2 minutes. After that, cells were centrifuged (Heraeus Biofuge Primo R centrifuge; Thermofisher Scientific, SA) to get a pellet of cells, which were later stained with 1 mL of Hoechst 33342. The stained cells were then plated on a slide, and a cover slip was mounted for viewing under a microscope (HBO 50/AC Axiovert microscope; Zeiss, SA).

Wound healing assay

The wound healing assay was adapted from Yarrow et al (31) with slight modifications. Cells were cultured in 4-well plates and allowed to reach 80%–90% confluency. Subsequently, a consistent scratch or wound was created in each well using a 10 µL pipette tip. Following the scratch, cells were washed with sterile PBS to remove the debris before treatment with the extract at IC_{50} concentration or vehicle for 24 hours, following which the images of the cells were captured using an inverted microscope (Zeiss Axioplan 2 compound microscope coupled with Axiocam HRC digital camera and software; Spectrum, UJ).

Data analysis

The obtained study results were analysed using Microsoft® Excel and GraphPad Prism 10.1.0 software. A one-way analysis of variance (ANOVA) was used to determine a significant difference among various treatments of cell viability at a significance level of $P \leq 0.05$. Where a significant difference was observed, a two-sample *t* test was used to identify the difference; an asterisk (*) represents the number of zeros after the decimal point. All analyses were accomplished in triplicate, and cell viability percentages were expressed as the mean and standard deviation.

Results

Phytochemical screening

The phytochemical screening of *C. edulis* methanolic leaf extract under control conditions (25/15 °C day and night) showed a high presence of phenolics and tannins, a moderate presence of terpenoids, steroids, and cardiac glycosides, and a low presence of flavonoids and saponins (Table 1). Volatile oils were absent. The hexane extract showed a high presence of saponins and volatile oils, a moderate presence of cardiac glycosides and high volatile oils, and a low presence of flavonoids (Table 1).

Table 1. Phytochemical screening of *Carpobrotus edulis* in control and temperature conditions

	Control (25/15°C)		Low temperature (15/10°C) treatments		High temperature (45/35°C) treatments		
	Methanol	Hexane	Methanol	Hexane	Methanol	Hexane	
48 hours							Phenolics
96 hours							
144 hours							
48 hours							Flavonoids
96 hours							
144 hours							
48 hours							Terpenoids
96 hours							
144 hours							
48 hours							Saponins
96 hours							
144 hours							
48 hours							Tannins
96 hours							
144 hours							
48 hours							Steroids
96 hours							
144 hours							
48 hours							Cardiac glycosides
96 hours							
144 hours							
48 hours							Volatile oils
96 hours							
144 hours							

Note: Dark green, green, light green and white cells indicate high, moderate, low and absent respectively.

The phytochemical screening of *C. edulis* methanolic leaf extract from samples that were harvested after 48 hours under low-temperature conditions showed a high presence of phenolics, terpenoids, and tannins, while steroids and cardiac glycosides were moderately present (Table 1). Interestingly, the presence of terpenoids in the control methanolic extract of the plant was moderate; however, after exposure to low-temperature conditions for 48 hours, the presence of terpenoids increased (high presence). The low presence of flavonoids and saponins as well as the absence of volatile oils remained the same even after 48 hours of low-temperature conditions. The hexane extract showed a high presence of saponins and volatile oils, a moderate presence of cardiac glycosides, and a low presence of flavonoids.

The *C. edulis* methanolic leaf extract from the samples that were harvested after 96 hours of low temperature conditions showed a high presence of phenolics, terpenoids, and tannins, a moderate presence of steroids and cardiac glycosides, and a low presence of flavonoids and saponins (Table 1). Volatile oils were absent. The same sa the methanol extract exposed to low-temperature conditions for 48 hours, the presence of terpenoids after 96 hours increased compared to the control group. The hexane extract showed a high presence of saponins and volatile oils, a moderate presence of cardiac glycosides, and a low presence of flavonoids.

The *C. edulis* methanolic leaf extract from the samples that were harvested after 144 hours of low-temperature conditions showed a high presence of phenolics and terpenoids, a moderate presence of steroids and cardiac glycosides, and a low presence of saponins and tannins (Table 1). Terpenoids increased compared to the control, and the presence of tannins, which was high in the control, was low after exposure to 144 hours of low-temperature conditions. The hexane extract showed a high presence of saponins and volatile oils, a moderate presence of cardiac glycosides, and a low presence of flavonoids. Phenolics, terpenoids, tannins, and steroids were absent.

The *C. edulis* methanolic leaf extract from the samples that were harvested after 48 hours of high-temperature conditions showed a high presence of phenolics and terpenoids, a moderate presence of cardiac glycosides, and a low presence of flavonoids, saponins, and tannins. Volatile oils were absent. Terpenoids increased compared to the control. The hexane extract showed a high presence of saponins and volatile oils, a moderate presence of cardiac glycosides, and a low presence of flavonoids. Phenolics, terpenoids, tannins, and steroids were absent.

The *C. edulis* methanolic leaf extract from the samples that were harvested after 96 and 144 hours of high-temperature conditions showed a high presence of phenolics and terpenoids, a moderate presence of steroids and cardiac glycosides, and a low presence of tannins.

Terpenoids increased compared to the control; the detection of terpenoids was triggered by high-temperature conditions after 96 and 144 hours. Surprisingly, the saponins that were detected in the control were not present after 96 and 144 hours of high-temperature conditions. Moreover, the tannins that were present in the control decreased after exposure to high-temperature conditions for 96 and 144 hours. Volatile oils remained absent even after exposure to high-temperature conditions for up to 96 and 144 hours. The hexane extract showed a high presence of saponins and volatile oils, a moderate presence of cardiac glycosides, and a low presence of flavonoids. Phenolics, terpenoids, tannins, and steroids were absent.

Low- and high-temperature conditions versus control conditions

When looking at the overall responses under low-temperature conditions, the *C. edulis* methanolic extracts displayed an increase in the presence of terpenoids compared to the control. Phenolics, flavonoids, saponins, tannins, steroids, cardiac glycosides, and volatile oils remained the same compared to the control. In the hexane extracts, all tested compounds remained the same compared to the control. When exposed to high-temperature conditions, the methanolic extracts of *C. edulis* showed an increase in the presence of terpenoids. However, saponins and tannins decreased compared to the control. The presence of other compounds was consistent with the control. In the hexane extracts, all tested compounds remained the same compared to the control.

Anticancer activity

Presented in Figure 1 are the cell viability screening outcomes of *C. edulis* methanolic extracts under control (25/15 °C), low-temperature (15/10 °C), and high-temperature (45/35 °C) conditions against cervical and skin cancers. The cell viability screening of *C. edulis* methanolic extract after 144 hours of high-temperature conditions showed potential anticancer activity at 500 µg/mL against the cervical cancer cell line (ME-180) (Figure 1, highlighted in red colour). The cell viability screening of *C. edulis* methanolic extract after 96 hours of low-temperature conditions showed potential anticancer activity against skin cancer cell line A375 (Figure 1, highlighted in red colour). A statistically significant difference was observed in the cell viability screening between the cells treated with the majority of *C. edulis* methanolic extracts against both cervical cancer and skin cancer, respectively (Figure 1).

Presented in Figure 2 are the cell viability screening outcomes of *C. edulis* hexane extracts under control (25/15 °C), low-temperature (15/10 °C), and high-temperature (45/35 °C) conditions against cervical and skin cancers. The cell viability screening of *C. edulis* hexane extract after 144 hours of low-temperature conditions showed potential anticancer activity against the cervical cancer cell line (ME-180) (Figure 2, highlighted in red). The cell viability screening of *C. edulis* hexane extract after 96 hours of low-temperature conditions showed potential anticancer activity against skin cancer (A375) (Figure 2, highlighted in red). A statistically significant difference was observed in the cell viability screening between untreated cells and cells treated with *C. edulis* hexane extracts against both cervical cancer and skin cancer, respectively.

Subsequently, further cell viability tests were conducted using the specific extracts identified in Figures 1 and 2 to evaluate the minimum inhibitory concentration that is required to eliminate 50% of the cancer cells (IC_{50}). The *C. edulis* methanolic extract from the samples exposed to high-temperature conditions for 144 hours against cervical cancer (ME-180) showed a noteworthy IC_{50} value

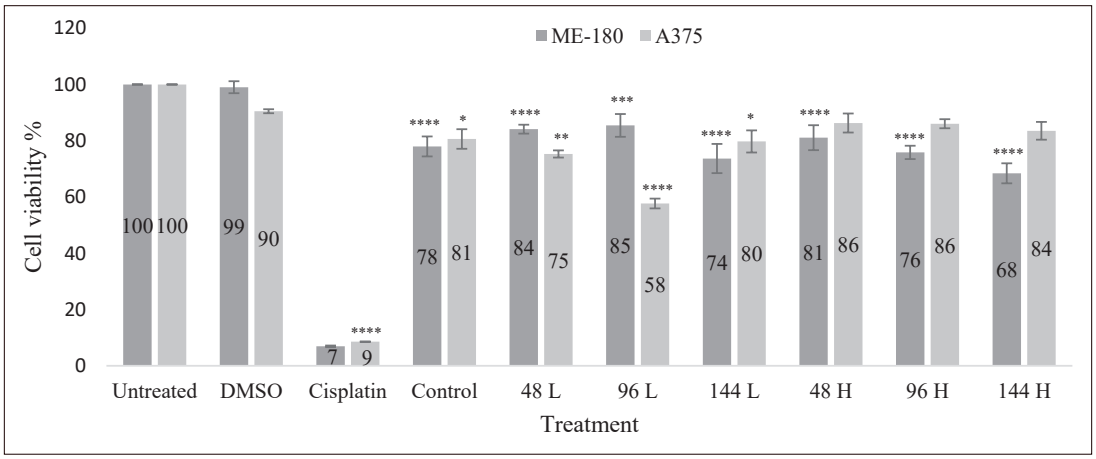


Figure 1. Cell viability screening of *Carpobrotus edulis* methanolic extracts from samples exposed to low and high temperature conditions against cervical cancer (ME-180) and skin cancer (A375) cell lines. * represents $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$, all compared to untreated cells. Untreated: Cells not treated; DMSO: Cells treated with dimethyl sulfoxide; Cisplatin: Cells treated with the positive control (cancer drug); 48 L, 96 L, and 144 L: Low-temperature conditions for 48, 96, and 144 hours; 48 H, 96 H, and 144 H: High-temperature conditions for 48, 96, and 144 hours, respectively.

of 284,9 µg/mL (Figure 3a). The *C. edulis* hexane extract from samples exposed to low-temperature conditions for 96 hours against cervical cancer (ME-180) showed an IC_{50} value of 878 µg/mL (Figure 3b). The *C. edulis* methanolic extracts from the samples exposed to low-temperature conditions for 144 hours against skin cancer (A375) showed an IC_{50} value of 480 µg/mL (Figure 3c). The *C. edulis* hexane extract from the samples exposed to low-temperature conditions for 96 hours against skin cancer (A375) showed an IC_{50} value of 3114 µg/mL (Figure 3d).

From the outcomes in Figures 3a-d, it was evident that the methanolic extract of *C. edulis* from the samples exposed to high-temperature conditions for 144 hours against cervical cancer (ME-180) showed better anticancer activity due to the lowest IC_{50} value of 284.9 µg/mL. Before further tests were conducted using this extract, its activity

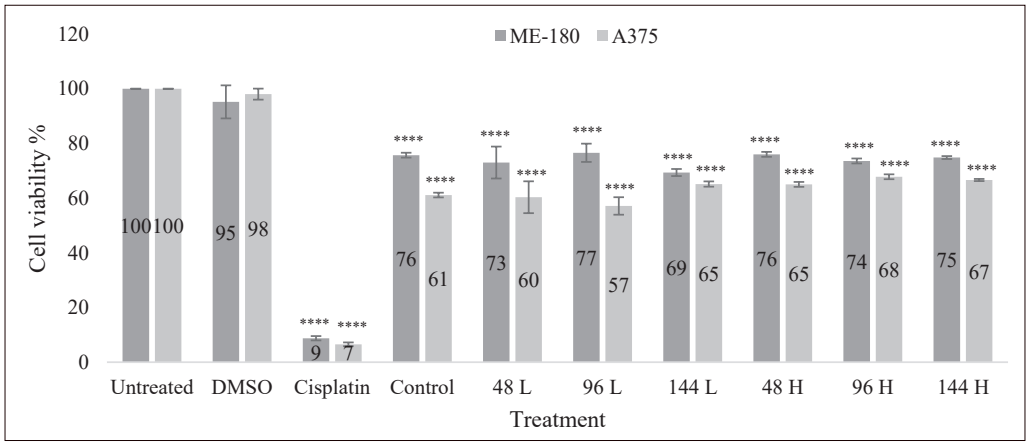


Figure 2. Cell viability screening of *Carpobrotus edulis* hexane extracts from samples exposed to low and high-temperature conditions against cervical cancer (ME-180) and skin cancer (A375) cell lines. * represents $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$, all compared to untreated cells. Untreated: Cells not treated; DMSO: Cells treated with dimethyl sulfoxide; Cisplatin: Cells treated with positive control (cancer drug); 48 L, 96 L, and 144 L: Low-temperature conditions for 48, 96, and 144 hours; 48 H, 96 H, and 144 H: High-temperature conditions for 48, 96, and 144 hours, respectively.

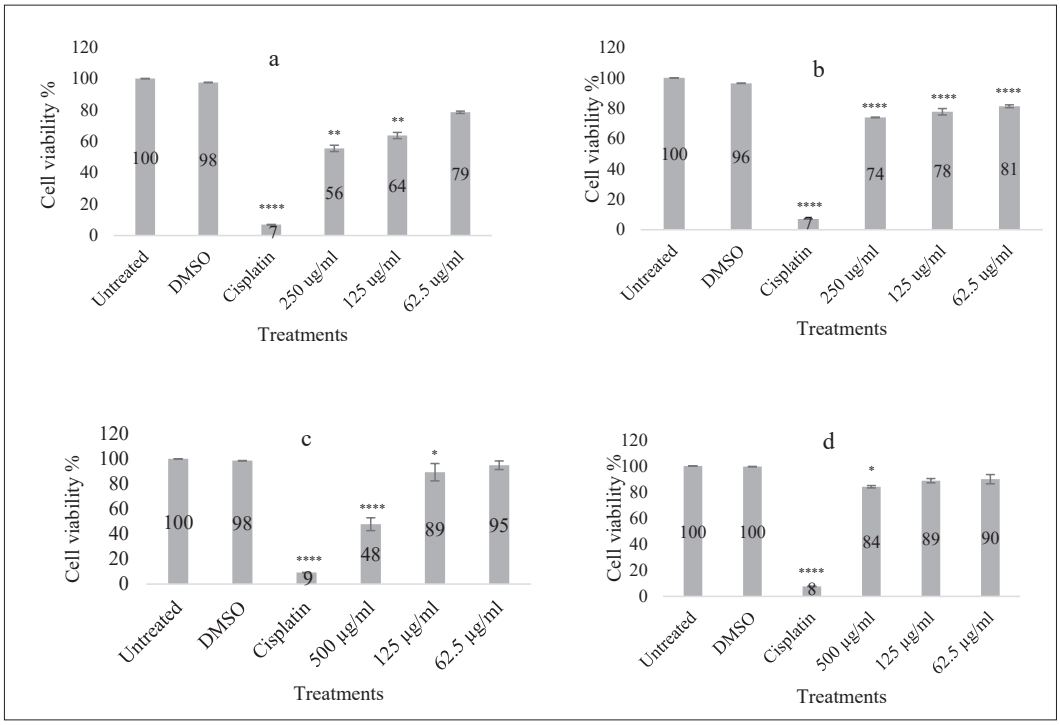


Figure 3. Anticancer effects of various concentrations of *Carpobrotus edulis* extracts from the samples exposed to low and high-temperature conditions against cervical cancer and skin cancer cell lines. * represents $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$, all compared to untreated cells. (a) The *C. edulis* methanolic extract from the samples exposed to high-temperature conditions for 144 hours against cervical cancer (ME-180). (b) The *C. edulis* hexane extract from samples exposed to low-temperature conditions for 96 hours against cervical cancer (ME-180). (c) The *C. edulis* methanolic extracts from the samples exposed to low-temperature conditions for 144 hours against skin cancer (A375). (d) The *C. edulis* hexane extract from the samples exposed to low-temperature conditions for 96 hours against skin cancer (A375). Untreated: Cells not treated; DMSO: Cells treated with dimethyl sulfoxide; Cisplatin: Cells treated with positive control (cancer drug); 500 µg/mL, 250 µg/mL, 125 µg/mL, and 62.5 µg/mL are the concentrations of the extract.

had to be tested on normal human lung tissue (MRC-5) (Figure 4). The results showed that the extract had not a negative effect on the normal cell line. The IC_{50} value was 2704 µg/mL (Figure 4). As a result, the selectivity index for normal cells (2704 µg/mL) to that for cancer cells (284,9 µg/mL), was determined to be 9.49.

The morphology of the cervical cancer cells changed after treatment with the *C. edulis* methanolic extract exposed to high temperature conditions for 144 hours (Figure 5). Several morphological signs of apoptosis were observed when comparing the treated cervical cancer cells with the untreated. The signs of karyorrhexis were observed in the cells that were treated with the *C. edulis* extract (Figure 5, red arrows). Karyorrhexis was also seen in the cells of the positive control (Figure 5, red arrow). The treated and positive control cells' membranes appeared intact, and membrane blebbing appeared to be occurring in some of the cells (Figure 5, white arrows).

The Hoechst staining results showed probable apoptotic signs such as irregular cell shapes, deep enfolding of the cells, highly condensed, and evenly distributed chromatin on the *C. edulis* treated cells, and positive control cells (Figure 6, red arrows). These changes were not observed in the untreated cells (Figure 6).

Wound healing properties of *C. edulis* methanolic leaf extract exposed to high-temperature conditions for 144 hours were observed (Figure 7). When comparing the untreated cervical cancer cells with those treated with *C. edulis* methanolic leaf extract exposed to 144 hours of high-temperature conditions, there was an observable difference in wound healing ability after 24 hours (within white boundary lines) (Figure 7). The treated cells did not exhibit complete closure of the gap created in the cell monolayer (within white boundary lines).

Discussion

The phytochemical screening of plant extracts provides valuable knowledge about the bioactive compounds present in the plant material, which can have various implications for potential therapeutic, medicinal, or nutritional applications. The phytochemical screening of *C. edulis* methanolic leaf extract under control conditions

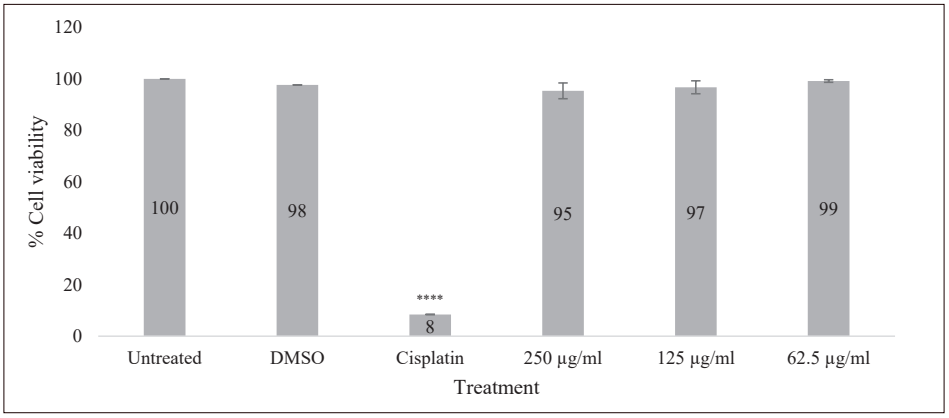


Figure 4. Various concentrations of *Carpobrotus edulis* methanolic extract from the samples exposed to high-temperature conditions for 144 hours against normal human lung tissue (MRC-5). * represents $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$, all compared to untreated cells. Untreated: Cells not treated; DMSO: Cells treated with dimethyl sulfoxide; Cisplatin: cells treated with a positive control (cancer drug); 250 µg/mL, 125 µg/mL and 62.5 µg/mL are the concentrations of the extract.

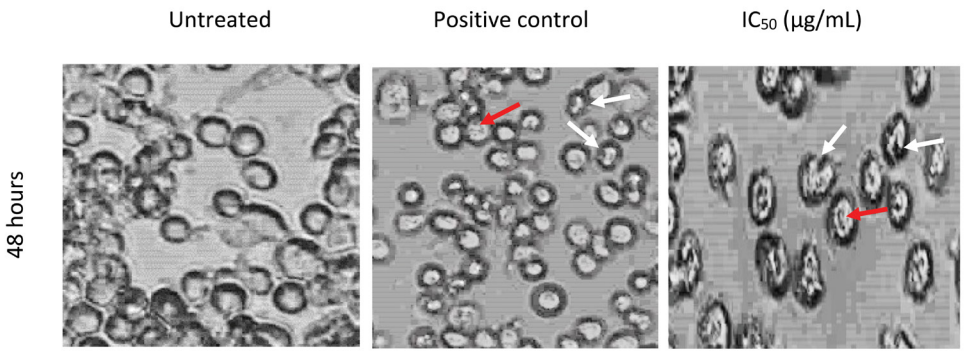


Figure 5. Morphology of cervical cancer cells after 48 hours treatment with *Carpobrotus edulis* methanolic extract exposed to high-temperature conditions for 144 hours. Red and white arrows in positive control show the effects of cisplatin (cancer drug). Red and white arrows in IC_{50} show the effects of *C. edulis* extract from samples that were exposed to high-temperature conditions for 144 hours. Untreated: Cells not treated; Positive control: Cells treated with cisplatin; IC_{50}: Cells treated with *C. edulis* extract at 284.9 µg/mL; 48 hours: Duration of treatment.

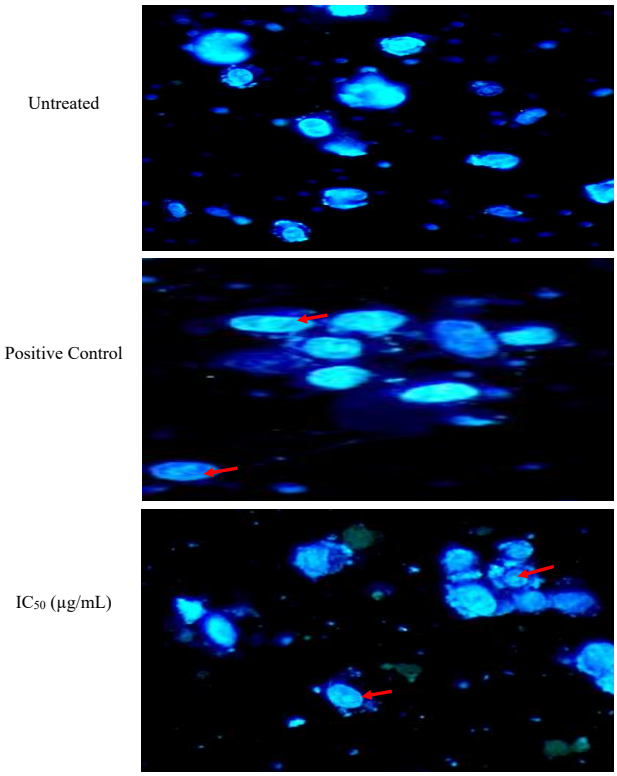


Figure 6. Hoechst-stained micrographs of cervical cancer cells after treatment with *Carpobrotus edulis* methanolic extract from samples exposed to high-temperature conditions for 144 hours. Red arrows in positive control show the effects of cisplatin (cancer drug). Red arrows in IC_{50} show the effects of *C. edulis* extract from samples that were exposed to high-temperature conditions for 144 hours. Untreated: Cells not treated; Positive control: Cells treated with cisplatin; IC_{50}: Cells treated with *C. edulis* extract at 284.9 µg/mL.

showed the presence of all tested compounds. The absence of other compounds in the hexane extract suggests that hexane might not be efficient in extracting certain classes of phytochemicals since the polarity of the two solvents is not the same. A previous study on *C. edulis* leaves in various solvent extracts found major constituents to be phenolics, proanthocyanidins, and tannins (23). The current study aligns with this, as the methanolic extract (rich in phenolics and tannins) and hexane extract (rich in saponins and volatile oils) were found to be major compounds.

The biosynthesis of phytochemicals can be influenced by a variety of factors, including temperature; however, not all compounds are equally affected. Primary metabolites are likely to be less affected by temperature fluctuations because their biosynthetic pathways have evolved to withstand changes. For example, studies have shown that the biosynthesis of essential amino acids like proline can remain stable or even increase under cold stress, helping plants cope with low temperatures (32). Just as some plants have cold-adapted enzymes, others have evolved heat-tolerant enzymes that remain functional at high temperatures (32). These enzymes are involved in various biosynthetic pathways, including those for primary metabolites, and can sustain biosynthesis under heat stress (33). Some plants employ compensation mechanisms in response to high temperatures (34). They may increase the expression of certain genes or alter metabolic pathways to maintain the biosynthesis of crucial compounds (34). For example, the biosynthesis of antioxidants like ascorbic acid and glutathione can increase in response to heat stress (34). Oh et al (35) reported on various environmental stresses in *Lactuca sativa* L. and showed that heat stress (40 °C), chilling (4 °C), or high light intensity can trigger the production of specific phenolic compounds and flavonoids, which play substantial roles in plant defence against oxidative stress caused by high temperatures. These observations are similar to those in this current study: *C. edulis* under both low and high-temperature conditions showed an increase in crucial compounds and at times employed potential compensation mechanisms in response to temperature conditions. For instance, the presence of terpenoids under control conditions in the methanolic extract of the plant was moderate; however, after exposure to low-temperature and high-temperature conditions, respectively, for up to six days (48, 96, and 144 hours, respectively), the level of terpenoids increased. Terpenoids are vital for both the growth and development of plants as well as for their defence mechanisms (36). They are particularly used in the food, cosmetics, and pharmaceutical industries because of their various medicinal properties, including antioxidant, antibacterial, antitumour, antiviral, and anti-inflammatory effects (36). Therefore, the detection of terpenoids that was triggered by 48 hours of low-temperature conditions could indicate a potential enhancement in various above-mentioned medicinal properties and possibly treat various diseases. Additionally, the saponins that were detected in the control were not present after 96 and 144 hours of high-temperature conditions. Saponins possess various pharmacological activities, including anti-inflammatory, antifungal, and anticancer properties (37). Therefore, the absence of saponins triggered by 96 and 144 hours of high-temperature conditions could indicate a potential decreased potency of the plant to treat various diseases associated with its medicinal properties.

Even the tannins that were present in the control decreased after exposure to high-temperature conditions for 96 and 144 hours. Tannins are known for numerous biological and pharmacological properties such as antioxidant, anti-inflammatory, and antimicrobial effects (38,39). They can bind to proteins, which can be applied in leather manufacturing, and they are responsible for the astringent taste or dry, puckering sensation in drinks or foods such as tea and unripe fruits (38-40). Therefore, the decrease of tannins triggered by 96 and 144 hours of high-temperature conditions could indicate a potential decreased potency of the plant to treat various diseases or binding to proteins associated with its biological and pharmacological properties, and potential decreased

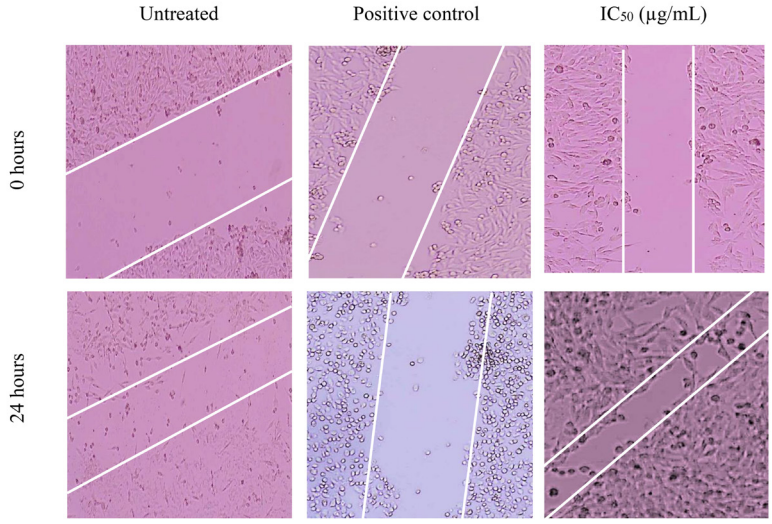


Figure 7. Wound healing comparative analysis of cervical cancer cells before (0 hours) and after (24 hours) treatment with *Carpobrotus edulis* methanolic extract from the samples exposed to high-temperature conditions for 144 hours. Untreated: Cells not treated; Positive control: Cells treated with cisplatin (cancer drug); IC_{50}: Cells treated with *C. edulis* extract at 284.9 µg/mL. The white boundary lines show the 'wound' that the cancer cells are closing.

astringency.

Similarly, the fluctuation in the appearance of compounds under environmental factors has been observed in several studies (41, 42). Ljubej et al (42) showed that cold temperatures (8 °C) in *Brassica oleracea* var. *acephala* led to a rise in phytochemical levels of phenolic acids by 3%, flavonoids by 5%, carotenoids by 15%, and glucosinolates by 21%. Olennikov et al (41) revealed that extended exposure (20 days) of *Dracocephalum palmatum* seedlings to low temperatures (1 °C) harmed organ integrity, leading to increased electrolyte leakage, reactive oxygen species imbalance, and higher levels of malondialdehyde and antioxidant enzymes. Nevertheless, this condition also led to the accumulation of various beneficial compounds in *D. palmatum*, including photosynthetic pigments, unsaturated fatty acids, essential oils, volatile monoterpenes, phenolic compounds, and soluble sugars.

The outcomes based on the cell viability IC_{50} values suggested that the methanolic extract from samples exposed to high-temperature conditions exhibited notable potency against ME-180 cervical cancer cells without any apparent negative effect on the normal cell line. This selective effect against cancer cells, while sparing normal cells, is a desirable trait in potential anticancer agents (43). The observed selectivity index highlights the extract's preferential targeting of cervical cancer cells over normal cells. A selective index that is greater than one is generally acceptable; the higher the selectivity index, the better. This selectivity is crucial for minimizing potential side effects in normal tissues (43). It suggests that the extract's phytochemical profile may target pathways or features that are more pronounced in cervical cancer cells compared to normal lung tissue cells (43). This finding aligns with existing literature suggesting that heat stress can enhance the release of bioactive compounds from plants (13, 14), which may contribute to their increased anticancer potential. Netshimbupfe et al (44) exposed African *Amaranthus caudatus, A. hypochondriacus, A. cruentus, and A. spinosus* plants to heat stress (40°C) and evaluated the accumulation of phenolic and flavonoid compounds and antioxidant capacity. The results indicated that heat stress-induced the accumulation of caffeic acid and rutin. It also showed a direct relationship between total phenolic content and antioxidant activity. The results of the present study are similar to the results obtained by Netshimbupfe et al (44) in which compounds such as phenolics, terpenoids, steroids, and cardiac glycosides known for their anticancer properties (45-48) were present in the methanolic extract. This shows a possible relationship between the phytochemical profile of the plant under high-temperature conditions and potential anticancer activity.

The change in the cervical cancer cells' morphology after treatment with the *C. edulis* methanolic extract exposed to high-temperature conditions for 144 hours was a potential sign of apoptosis. Apoptotic cells lose their attachment to the extracellular matrix and neighbouring cells. Loss of adhesion was observed in the treated cells as well as in the positive control; this could be due to alterations in the adhesion molecules of these cells. Hoechst staining further revealed specific morphological changes that could indicate apoptotic cell death in the *C. edulis-treated* cervical cancer cells. Necrotic cells often display large, irregular clumps of chromatin, while apoptotic cells typically lack such clumping and instead exhibit highly condensed, evenly distributed chromatin. Furthermore, the *C. edulis* methanolic extract-treated cells did not exhibit complete closure of the gap created in the cell monolayer, implying an inhibitory effect on cell migration and wound healing. This phenomenon can be

interpreted in the context of the potential anti-migratory and anti-invasive properties of the extract, both of which are hallmark processes in cancer metastasis (49). The anti-migratory property relates to the inhibition of cancer cells' ability to travel from the primary tumour site to other distant locations, while the anti-invasive property relates to the reduction in cancer cells' ability to permeate neighbouring tissues and initiate secondary tumour foci (49). The observation that the cervical cancer cells treated with *C. edulis* methanolic extract (from samples exposed to high-temperature conditions for 144 hours) did not completely close the wound gap suggests that the extract may possess anti-migratory or anti-invasive properties. These properties against cervical cancer cells may be attributed to the secondary metabolites found in the extract. Martins et al (50), Hafsa et al (24), and Omuruyi et al (25) have shown various mechanisms of action against different cancer cell lines using *C. edulis* extracts (in normal temperature conditions), including the inhibition of the p-glycoprotein, inhibition of protein glycation, and induction of apoptosis via the mitochondrial apoptotic pathway, respectively.

The exact mechanisms by which the *C. edulis* methanolic extract exposed to high-temperature conditions for 144 hours exerted its anti-migratory or anti-invasive effects are not known yet; however, they may include the disruption of signalling pathways involved in cell migration and invasion, the inhibition of enzymes that breakdown the extracellular matrix called matrix metalloproteinases, and the reduction in the expression of cell adhesion molecules, which allows them to attach to other cells and tissues, thereby hindering their movement (51).

Conclusion

In this study, the results showed that the methanolic extract from samples exposed to 144 hours of high-temperature conditions had potential anticancer activity against cervical cancer and did not have a negative effect on normal lung tissue. This could be a result of the phytochemicals present in the extract. The potential initiation of apoptosis and inhibition of wound healing in the context of cancer cell migration was also observed, which further highlights the potential of *C. edulis* methanolic extract in combating cancer progression.

There is a limited discussion on the underlying mechanisms of how high-temperature conditions for up to 144 hours alter the phytochemical composition, leading to the potential anticancer activity against cervical cancer. This can be achieved by conducting real-time PCR and Western blots to compare apoptosis-related gene expression in treated and untreated cervical cancer cells. Therefore, a comprehensive understanding of *C. edulis*' anticancer properties could be achieved through a multifaceted approach combining in vitro and in vivo studies with phytochemical analyses. Long-term studies assessing the effects of prolonged exposure to varying temperature conditions can provide a deeper insight into how medicinal plants adapt to climate change over time. Future research could also explore the impact of other environmental stressors on the medicinal properties of *C. edulis*.

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Conflict of interests

The authors declare that they have no known conflict of interest that could have appeared to influence the work reported in this paper.

Ethical considerations

This research does not contain any animal or human participants.

Authors' contributions

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Formal analysis: Mpho Mashigo, Ida Risenga, and Mpho Choene.
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Methodology: Mpho Mashigo, Ida Risenga, and Mpho Choene.
Project administration: Ida Risenga, and Mpho Choene.
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Writing–review & editing: Mpho Mashigo, Ida Risenga, Mpho Choene, and Kennedy Ngwira.

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References

1. Moten A, Schafer D, Farmer P, Kim J, Ferrari M. Redefining global health priorities: improving cancer care in developing settings. J Glob Health. 2014;4(1):010304. doi: 10.7189/

jogh.04.010304.
2. Leiter U, Keim U, Garbe C. Epidemiology of skin cancer: update 2019. Adv Exp Med Biol. 2020;1268:123-39. doi: 10.1007/978-3-030-46227-7_6.
3. Diffey B. Climate change, ozone depletion and the impact on ultraviolet exposure of human skin. Phys Med Biol. 2004;49(1):R1-11. doi: 10.1088/0031-9155/49/1/r01.
4. Modenese A, Korpinen L, Gobba F. Solar radiation exposure and outdoor work: an underestimated occupational risk. Int J Environ Res Public Health. 2018;15(10):2063. doi: 10.3390/ijerph15102063.
5. World Health Organization, World Meteorological Organization, United Nations Environment Programme, International Commission on Non-Ionizing Radiation Protection. Global Solar UV Index: A Practical Guide. WHO; 2002.
6. Wright CY, Norval M. Health risks associated with excessive exposure to solar ultraviolet radiation among outdoor workers in South Africa: an overview. Front Public Health. 2021;9:678680. doi: 10.3389/fpubh.2021.678680.
7. Gordon LG, Elliott TM, Wright CY, Deghaye N, Visser W. Modelling the healthcare costs of skin cancer in South Africa. BMC Health Serv Res. 2016;16:113. doi: 10.1186/s12913-016-1364-z.
8. Cohen PA, Jhingran A, Oaknin A, Denny L. Cervical cancer. Lancet. 2019;393(10167):169-82. doi: 10.1016/s0140-6736(18)32470-x.
9. Jordaan S, Michelow P, Richter K, Simoens C, Bogers J. A review of cervical cancer in South Africa: previous, current and future. Health Care Curr Rev. 2016;4(4):180. doi: 10.4172/2375-4273.1000180.
10. Botha MH, Dreyer G. Guidelines for cervical cancer screening in South Africa. South Afr J Gynaecol Oncol. 2017;9(1):8-12. doi: 10.10520/EJC-9584c6bc7.
11. Khaliq NU, Lee J, Kim J, Kim Y, Yu S, Kim J, et al. Mesoporous silica nanoparticles as a gene delivery platform for cancer therapy. Pharmaceutics. 2023;15(5). doi: 10.3390/pharmaceutics15051432.
12. Pant P, Pandey S, Dall'Acqua S. The influence of environmental conditions on secondary metabolites in medicinal plants: a literature review. Chem Biodivers. 2021;18(11):e2100345. doi: 10.1002/cbdv.202100345.
13. Figueiredo AC, Barroso JG, Pedro LG, Scheffer JJ. Factors affecting secondary metabolite production in plants: volatile components and essential oils. Flavour Fragr J. 2008;23(4):213-26. doi: 10.1002/ffj.1875.
14. Cavaliere C. The effects of climate change on medicinal and aromatic plants. HerbalGram. 2009;81:44-57.
15. Baharum Z, Akim AM, Taufiq-Yap YH, Hamid RA, Kasran R. In vitro antioxidant and antiproliferative activities of methanolic plant part extracts of *Theobroma cacao*. Molecules. 2014;19(11):18317-31. doi: 10.3390/molecules191118317.
16. Chanda S, Nagani K. In vitro and in vivo methods for anticancer activity evaluation and some Indian medicinal plants possessing anticancer properties: an overview. J Pharmacogn Phytochem. 2013;2(2):140-52.
17. Talib WH, Mahasneh AM. Antiproliferative activity of plant extracts used against cancer in traditional medicine. Sci Pharm. 2010;78(1):33-45. doi: 10.3797/scipharm.0912-11.
18. Constantinescu T, Lungu CN. Anticancer activity of natural and synthetic chalcones. Int J Mol Sci. 2021;22(21):11306. doi: 10.3390/ijms222111306.
19. Shukla S, Mehta A. Anticancer potential of medicinal plants and their phytochemicals: a review. Braz J Bot. 2015;38(2):199-210. doi: 10.1007/s40415-015-0135-0.
20. Senft C, Polacin M, Priester M, Seifert V, Kögel D, Weissenberger J. The nontoxic natural compound Curcumin exerts anti-proliferative, anti-migratory, and anti-invasive properties against malignant gliomas. BMC Cancer. 2010;10:491. doi: 10.1186/1471-2407-10-491.
21. D'Antonio CM, Mahall BE. Root profiles and competition between the invasive, exotic perennial, *Carpobrotus edulis*, and two native shrub species in California coastal scrub. Am J Bot. 1991;78(7):885-94. doi: 10.1002/j.1537-2197.1991.tb14492.x.
22. Malan C, Notten A. *Carpobrotus edulis* (L.) L. Bolus. South Africa: South African National Biodiversity Institute; 2006. Available from: https://pza.sanbi.org/references/malan-c-notten-al-2006-carpobrotus-edulis-l-lbolus-aizoaceae-plantzafrica-online.
23. Omoruyi BE, Bradley G, Afolayan AJ. Antioxidant and phytochemical properties of *Carpobrotus edulis* (L.) bolus leaf used for the management of common infections in HIV/AIDS patients in Eastern Cape Province. BMC Complement Altern Med. 2012;12:215. doi: 10.1186/1472-6882-12-215.
24. Hafsa J, Hammi KM, Khedher MR, Smach MA, Charfeddine B, Limem K, et al. Inhibition of protein glycation, antioxidant and antiproliferative activities of *Carpobrotus edulis* extracts. Biomed Pharmacother. 2016;84:1496-503. doi: 10.1016/j.biopha.2016.11.046.
25. Omoruyi SI, Enogieru AB, Ekpo OE. In vitro evaluation of the antiproliferative activity of *Carpobrotus edulis* on human neuroblastoma cells. J Herb Med. 2021;30:100519. doi: 10.1016/j.hermed.2021.100519.
26. Ordway D, Hohmann J, Viveiros M, Viveiros A, Molnar J, Leandro C, et al. *Carpobrotus edulis* methanol extract inhibits the MDR efflux pumps, enhances killing of phagocytosed *S. aureus* and promotes immune modulation. Phytother Res. 2003;17(5):512-9. doi: 10.1002/ptr.1314.
27. Abubakar AR, Haque M. Preparation of medicinal plants: basic extraction and fractionation procedures for experimental purposes. J Pharm Bioallied Sci. 2020;12(1):1-10. doi: 10.4103/jpbs.JPBS_175_19.
28. Roghini R, Vijayalakshmi K. Phytochemical screening, quantitative analysis of flavonoids and minerals in ethanolic extract of *Citrus paradisi*. Int J Pharm Sci Res. 2018;9(11):4859-64. doi: 10.13040/ijpsr.0975-8232.9(11).4859-64.
29. Kumar P, Nagarajan A, Uchil PD. Analysis of cell viability by the alamarBlue assay. Cold Spring Harb Protoc. 2018;2018(6):pdb-rot095489. doi: 10.1101/pdb.prot095489.
30. Sutherland RM, Durand RE. Radiation response of multicell spheroids--an in vitro tumour model. Curr Top Radiat Res Q. 1976;11(1):87-139.
31. Yarrow JC, Perlman ZE, Westwood NJ, Mitchison TJ. A high-throughput cell migration assay using scratch wound healing, a comparison of image-based readout methods. BMC Biotechnol. 2004;4:21. doi: 10.1186/1472-6750-4-21.
32. Szabados L, Savouré A. Proline: a multifunctional amino acid. Trends Plant Sci. 2010;15(2):89-97. doi: 10.1016/j.

tplants.2009.11.009.
33. Bita CE, Gerats T. Plant tolerance to high temperature in a changing environment: scientific fundamentals and production of heat stress-tolerant crops. Front Plant Sci. 2013;4:273. doi: 10.3389/fpls.2013.00273.
34. Foyer CH, Shigeoka S. Understanding oxidative stress and antioxidant functions to enhance photosynthesis. Plant Physiol. 2011;155(1):93-100. doi: 10.1104/pp.110.166181.
35. Oh MM, Carey EE, Rajashekar CB. Environmental stresses induce health-promoting phytochemicals in lettuce. Plant Physiol Biochem. 2009;47(7):578-83. doi: 10.1016/j.plaphy.2009.02.008.
36. Yang W, Chen X, Li Y, Guo S, Wang Z, Yu X. Advances in pharmacological activities of terpenoids. Nat Prod Commun. 2020;15(3):1934578X20903555. doi: 10.1177/1934578x20903555.
37. Juang YP, Liang PH. Biological and pharmacological effects of synthetic saponins. Molecules. 2020;25(21):4974. doi: 10.3390/molecules25214974.
38. Das AK, Islam MN, Faruk MO, Ashaduzzaman M, Dungani R. Review on tannins: extraction processes, applications and possibilities. S Afr J Bot. 2020;135:58-70. doi: 10.1016/j.sajb.2020.08.008.
39. Sharma K, Kumar V, Kaur J, Tanwar B, Goyal A, Sharma R, et al. Health effects, sources, utilization and safety of tannins: a critical review. Toxin Rev. 2021;40(4):432-44. doi: 10.1080/15569543.2019.1662813.
40. Ashok PK, Upadhyaya K. Tannins are astringent. J Pharmacogn Phytochem. 2012;1(3):45-50.
41. Olennikov DN, Chirikova NK, Kashchenko NI, Gornostai TG, Selyutina IY, Zilfikarov IN. Effect of low temperature cultivation on the phytochemical profile and bioactivity of Arctic plants: a case of *Dracocephalum palmatum*. Int J Mol Sci. 2017;18(12):2579. doi: 10.3390/ijms18122579.
42. Ljubej V, Karalija E, Salopek-Sondi B, Šamec D. Effects of short-term exposure to low temperatures on proline, pigments, and phytochemicals level in kale (*Brassica oleracea* var. *acephala*). Horticulturae. 2021;7(10):341. doi: 10.3390/horticulturae7100341.
43. Cheok CF. Protecting normal cells from the cytotoxicity of chemotherapy. Cell Cycle. 2012;11(12):2227-8. doi: 10.4161/cc.20961.
44. Netshimbupfe MH, Berner J, Van Der Kooy F, Oladimeji O, Gouws C. Influence of drought and heat stress on mineral content, antioxidant activity and bioactive compound accumulation in four African *Amaranthus* species. Plants (Basel). 2023;12(4):953. doi: 10.3390/plants12040953.
45. Yadav M, Chatterji S, Gupta SK, Watal G. Preliminary phytochemical screening of six medicinal plants used in traditional medicine. Int J Pharm Pharm Sci. 2014;6(5):539-42.
46. Rabi T, Bishayee A. Terpenoids and breast cancer chemoprevention. Breast Cancer Res Treat. 2009;115(2):223-39. doi: 10.1007/s10549-008-0118-y.
47. Wagner KH, Elmadfa I. Biological relevance of terpenoids. Overview focusing on mono-, di- and tetraterpenes. Ann Nutr Metab. 2003;47(3-4):95-106. doi: 10.1159/000070030.
48. Calderón-Montaño JM, Burgos-Morón E, Orta ML, Maldonado-Navas D, García-Domínguez I, López-Lázaro M. Evaluating the cancer therapeutic potential of cardiac glycosides. Biomed Res Int. 2014;2014:794930. doi: 10.1155/2014/794930.
49. Fares J, Fares MY, Khachfe HH, Salhab HA, Fares Y. Molecular principles of metastasis: a hallmark of cancer revisited. Signal Transduct Target Ther. 2020;5(1):28. doi: 10.1038/s41392-020-0134-x.
50. Martins A, Vasas A, Schelz Z, Viveiros M, Molnár J, Hohmann J, et al. Constituents of *Carpobrotus edulis* inhibit P-glycoprotein of MDR1-transfected mouse lymphoma cells. Anticancer Res. 2010;30(3):829-35.
51. Iqbal J, Abbasi BA, Mahmood T, Kanwal S, Ali B, Shah SA, et al. Plant-derived anticancer agents: a green anticancer approach. Asian Pac J Trop Biomed. 2017;7(12):1129-50. doi: 10.1016/j.apjtb.2017.10.016.

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