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Molecular Mechanism of the Effects of Violacein on Cancer Cells

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1. Abstract

1.1.Background: Several effective microbiological and natural treatments of breast, lung, and prostate cancer can prevent surgery and remove damaged tissues. Chromobacterium violaceum produces a violet-colored pigment known as Violacein, was investigated in the present study for its anti-cancer properties in cancer cell lines. About the crystal violet proliferation assay, the exposure of tumor cells to serially diluted Violacein inhibits cancer cell lines' proliferative capacity.

1.2.Methods: The anti-cancer activity of 14N23 strain violacein extract from Chromobacterium violaceum was evaluated in vitro on BT549, BT20, and PC3 cancer cell lines. The crystal violet assay determines cell viability, and Violacein inhibits the proliferation of BT20 and PC3 cancer cell lines. In addition, transwell cell migration and invasion assay that measures the capacity of cell motility and invasiveness toward a chemo-attractant gradient was determined after exposure to violacein extract. Also, the peroxidation of lipid assays was used to determine the antioxidant activity of Violacein extracted in BT20 and PC3 cancer cell lines.

1.3. Results: The crude extract of 14N23 strain violacein from Chromobacterium violaceum stimulated cell proliferation against BT20 cells. Also, a crude extract of Violacein did not affect the viability of PC3 cells. Violacein had a significant effect on the migration of BT549 cells at a low concentration; however, it had no significant impact on BT20 breast cancer cells. As well as PC3 cell was inconclusive with the cell migration assay. Finally, violacein extract did not affect reactive oxygen species (ROS) on BT20 and PC3 cancer cell lines.

1.4. Conclusion: This study supports the research articles showing the anti-cancer effect of violacein extract from Chromobacterium violaceum on breast cancer BT549, BT20, and prostate cancer PC3 cell lines. Further studies in vitro with Violacein are needed to determine specific proteins and receptors that affect the migration of those cancer cell lines.

2. Keywords: Violacein Extract; Cytotoxic; Anti-cancer; Apoptosis

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3. Background

According to the American Cancer Society, cancer is the second leading cause of death in the United States. Several effective microbiological and natural treatments of breast, lung, and prostate cancer can prevent surgery and remove damaged tissues. Chromobacterium violaceum (CV) produces a violet colored pigment known as Violacein. We extracted Violacein from Chromobacterium violaceum 14N23 strain isolated from Copper Basin, Tennessee, to study its anti-cancer activities in cancer cell lines. The previous studies showed Violacein isolated from Chromobacterium violaceum affected the growth of some types of breast and lung cancer cell lines. However, the sole purpose of this study is to find and evaluate the anti-cancer properties of violacein extract against human breast cancer BT549, BT20, and prostate cancer PC3 cell lines. The exposure of cancer cells to serially diluted concentrations of Violacein for 24 hours demonstrated anti-cancer effects. The crystal violet assay determines cell viability, and Violacein inhibits proliferation of BT20 and PC3 cancer cell lines. The results indicated that violacein extract stimulated cell proliferation at lower concentrations of 1, 0.5, and 0.25 µg/ml in BT20 breast cancer cell line. However, violacein extract did not affect the

viability in the PC3 prostate cancer cell line. Because migration is a crucial estate of live cells and essential for immune response and disease processes such as cancer metastasis, cell migration is another efficacious method for cancer research [1-5].

Transwell cell migration and invasion assay that measure the capacity of cell motility and invasiveness toward a chemo-attractant gradient are one of the procedures that we used in these studies.

In the cell migration experiment, different doses of Violacein (1,0.5, 0.25, 0.125 µg/ml) were examined in breast and prostate cancer cell lines to see if Violacein affects those cell lines' cell migrations. We found that Violacein had a significant effect on the migration of BT549 breast cancer cells at a concentration of 0.25 µg/ml; however, it had no significant effect on BT20 breast cancer cells [6-8]. As well as PC3 cancer cell line was inconclusive with the cell migration assay.

This result provides us mechanistic information that Violacein has an inhibitory effect on BT549 cell migration. Further, in the present study, the peroxidation of lipid assays was used to determine the antioxidant activity of Violacein extracted from Chromobacterium violaceum in BT20 and PC3 cancer cell lines. The results indicate that violacein extract did not affect reactive oxygen species (ROS) compared to the control [9,10].

4. Methods

4.1. Bacterial Strains & Growth of the Chromobacterium violaceum

Strains of Chromobacterium violaceum were collected from the Tennessee Copper Basin, which is a suitable environment for studying the ability of microbial organisms to adapt to consistent changes in their environment and biodiversity. Different strains of Chromobacterium violaceum were isolated by Drs. Johnson, Ejiofor, and Gaston (2010) from water and soil samples collected from Tennessee Copper Basin. 14N1, 14N23, and ATCC 12472 could produce Violacein. In this study, Violacein was extracted and purified from CV strain 14N23 [11].

Chromobacterium violaceum (14N23) was inoculated from pure culture stocks into 50 ml LB broth media, and cells were grown in a rotary shaker set at 175 rpm and 30C° for 48 hours. Then a loop full of this growth was streaked on nutrient agar plates, and colonies were allowed to develop overnight. Next, single colonies were selected, and a second LB agar plate was streaked for isolation. Again, this was done to obtain single colonies and ensure purity of the organisms [12].

4.2. Extraction and purification of violacein

The Violacein was extracted from the 50ml growth medium, which was mixed with 50ml ethanol. The purpose of adding the ethanol was to solubilize Violacein, which is not soluble in water. This mixture was centrifuged for 10 minutes at 10,000 rpm and 40C°, and centrifugation pelleted the cells. Next, the supernatant (mixture of ethanol and broth culture) from the centrifuge tube was poured, so that the pelleted cells were not disturbed, into three separate funnels along with 50 ml chloroform. The funnel was shaken to mix the liquid, and the three funnels were closed after releasing any internal pressure and were allowed to sit for 1 hour. When the layers were separated, the nose of the funnel was opened, and the purple color pigment in the chloroform layer was collected into 100 ml beakers that were pre-weighted. The samples were allowed to air dry, and then the exact weights of crude extract were taken from the dried samples [13-15].

Purification of Violacein was done using filter paper. The filter paper was placed in a glass funnel where extracted chloroform portion was poured directly on to the filter paper where purple colored pigment was trapped inside the filter paper; Violacein was collected. In the next step, the filter paper was air dried. After drying, methanol was used to get purple pigment from the filter paper and was then collected in the beaker. Lastly, the beaker was placed under the hood to dry the purple pigment, and then the weight of the purple pigment was obtained. After dissolving it in solvent or DMSO, the analysis was carried out. Finally, the pure Violacein in DMSO was then stored in a refrigerator until required [16].

4.3. Cell Culture and Tissue Samples

All cancer cell lines were obtained from the American Type Culture collection. In addition, BT549, BT20 (breast cancer), and PC3 (Prostate cancer) cells were purchased from the American Type Culture Collection Center. All the cell lines were routinely cultured and maintained in Dulbecco's Modified Eagles Medium- DMEM(1X) + GlutaMAXI (Gibco by Life Technologies, Carlsbad, CA-USA). The media containing 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA-USA) and 1% penicillin-streptomycin (Gibco by Life Technologies, Carlsbad, CA-USA). Cells were grown in a monolayer at 37°C in a humidified atmosphere containing 5% CO_2. In all the experiments, cells were plated in 24 healthy plates until they reached 50-80% confluency, and then they were treated with different concentrations of Violacein. DMSO (Ameresco, Solon, OH-USA) was used as a control [17-20].

4.4. Cell proliferation assay

The crystal violet assay was used for cell viability. BT20 and PC3 cells were plated in 6 well plates and allowed to grow for 24-48 hours. After the cells were at least 60-80% confluent, they were treated with 0.5 µg/ml, 0.25 µg/ml, and 0.125 µg/ml of Violacein and allowed to incubate for 48 hours. DMSO

(Dimethyl sulfoxide) was used as the vehicle control. After that, the tissue culture medium was removed from the plate, and the cells were washed with 1X PBS and later fixed with 100% methanol for 5 min; the cells were then stained with 0.5% crystal violet in 25% methanol for 10 min. Each well was then gently washed 4-5 times with distilled water in order to remove excess dye and allowed to dry overnight at room temperature. The incorporated dye was then solubilized in 0.1 M sodium citrate in 50% ethanol, and 200 µL of 0.1 M sodium citrate was added to each well and incubated for 10 minutes at room temperature. Next, the cells were examined under a microscope in order to study the cell viability. Finally, we found that 0.5 µg/ml, 0.25 µg/ml, and 0.125 µg/ml of Violacein were the best concentrations to be used in our experiments [21,22].

4.5. Dimethyl sulfoxide (DMSO)

DMSO is Dimethyl Sulfoxide that was purchased from Sigma Aldrich. DMSO is an important polar aprotic solvent. DMSO is a sulfur-containing organic compound that is readily soluble in a wide variety of compounds. It is a colorless, clear hygroscopic liquid with a molecular formula C_2H_6OS. It is also capable of being entirely dissolved in water. DMSO was used as a positive control in the experiments [23,24].

4.6. Violacein effects on cell migration assay

Cell migration was tested using a 24-well transwell plate that has 8 micrometer, pore polycarbonate membrane inserts, according to the manufacturer's protocol (Corning, NY, USA).

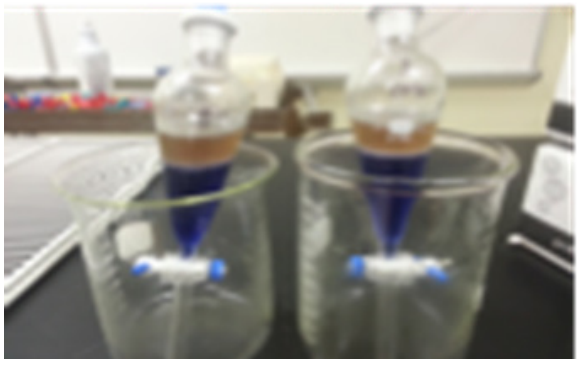

Figure 2: Extraction of Violacein from the ethanol-water layer with chloroform

BT549, BT20, and PC3 cells were plated and allowed to grow in six-well plates for 24 hrs. Cell migration assays were conducted according to the manufacturer's instructions (Cell Biolabs Inc., San Diego, CA, USA) (Figure 2). First, a cell suspension containing 0.5- $1.0X10^6$ cells/ml was prepared in serum-free media along with DMSO (vehicle) and 1 µM Violacein. Next, 500 µl of media containing 10% fetal bovine serum (which is the most used growth factor in cell culture due to its high level of nutrients) was added to the lower chamber of the migration plate. Three hundred microliters of the cell suspension containing vehicle or 1 µM Violacein were then added to the inside of each insert and allowed to incubate for 24 hours at 37 °C and 5% CO_2.

Then, the media was removed from the insert. Next, we transferred the insert to a clean well containing 400 µL of cell stain solution and incubated it for 10 minutes at room temperature. The stained inserts were washed several times in a beaker of water; then the insert was allowed to air dry [25-28].

Finally, each insert was transferred to an empty well, and 200 µL of extraction solution was added per well, then incubated for 10 minutes at room temperature.

The migratory cells were counterstained, solubilized, and optical density was read at 560nm using an X-mark microplate absorbance spectrophotometer (Bio-Rad).

4.7. Lipid peroxidation assay

Lipid peroxidation assay was used to determine the levels of MDA, which is malondialdehyde. The formation of lipid peroxidation products led to the spread of free radical reactions, leading to cell damage. PC3 and BT20 cancer cell lines were treated with the violacein extract and incubated for 24 hours at 37 °C. Then the media was removed, DMSO was added to the first two wells as the control, and concentrations 1,2, 3, and 4 of the violacein extract were added to the other wells in serum-free media. After 24 hours of incubation, the media was removed, and the wells were rinsed in 1ml of 1x PBS. Then 600 µl of trypsin was added, and the plate was incubated for 3 minutes. Next, the plate was shaken for 5 minutes until all the cells were floating. Finally, the cell lines were transferred to the microcentrifuge tubes, and 600 µl of media was added and centrifuged for 10 minutes. The supernatant was discarded, and the pellet was stored in the -80 freezer until use. The pellets were removed from the freezer and put in a bucket of ice. 110 µl of 1x PBS was added to the pellets, and the pellets were sonicated by a sonicator tool. The following modifications were used for the MDA assay kit. The R1 solution was added to each sample, and HCL (Chromogenic reagent) was added to each tube and mixed. The samples were incubated for 5 minutes. Finally, the samples were transferred to 96 well plates, and the absorbance was measured at 500 nm in a Microplate Reader. The MDA levels were calculated from a standard curve [29-31].

4.8. Statistical analysis

Values are expressed as the mean ± standard error. One-way ANOVA analysis was applied to determine the statistical differences. $P>0.05$ was considered to indicate a statistically not significant difference between values [32-35].

5. Results

14N23 strain of Chromobacterium violaceum was inoculated from pure culture stock into 50 ml broth and grown on a rotary shaker for 48 hours. After 48 hours' exposure, dark purple color in the broth and the growth of bacteria were seen. Nutrient agar plates were streaked with loopful of single colonies. The colony morphology, small, round, smooth, convex, non-gelatinous, and dark purple colonies, was observed for 14N23 (Figure 1). Therefore, Violacein was extracted and purified from Chromobacterium violaceum 14N23 strain for studying and testing its effects on different cancer cell lines' growth [36-38].

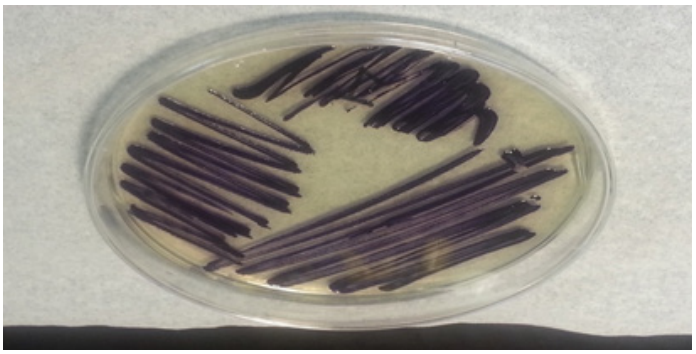

Figure 1: Growth of CV on LB agar showing isolated colonies and the purple pigment produced.

It was noted that the addition of ethanol to the growth medium effectively solubilized the purple pigment so that the bacterial cells were easily removed by centrifugation at 10,000 RPM. The purple pigment was then effectively extracted from the supernatant, which contained water and ethanol primarily, using chloroform. It was noted that nearly 100% of the purple pigment was extracted into the chloroform layer. Once the chloroform was evaporated, the dry residue was re-suspended using DMSO.

We established the cytotoxicity of Violacein on cancer cells; two cell lines- PC3 (prostate) and BT20 (breast) cancer cell lines were tested, and cell viability was assessed using cell proliferation assay. Prostate and breast cancer are two of the most common cancers impacting humans and exhibit a high incidence of causing tumor metastasis.

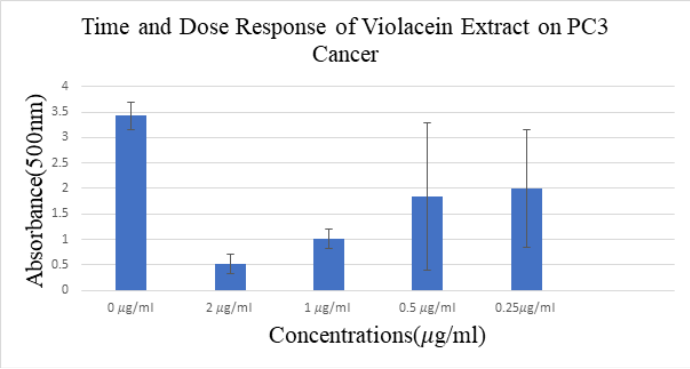

Figure 3: Time and dose response of violacein extract on PC3 cancer.

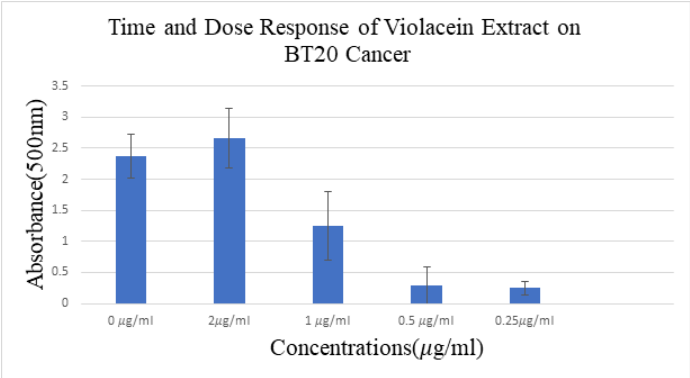

Figure 4: Time and dose response of violacein extract on BT20 Cancer.

Cancer cells were incubated with several concentrations of violacein (0, 0.5, 0.25, and 0.125 µg/ml) to study its effects on cell viability using dose-response experiments. Cell- viability dose-response assays displayed that there were decreases in PC3 cells treated with 0.5 µg/ml, 0.25, and 0.125 µg/ml respectively, as compared to the cell viability of vehicle (DMSO) treated control cells (Figure 3). Subsequently, BT20 cells showed decreased cell viability after treatment with the same concentrations as above compared to that with vehicle-treated control cells (Figure 4). Thus, the cytotoxicity effects of Violacein are shown to be more cell-specific [39,40].

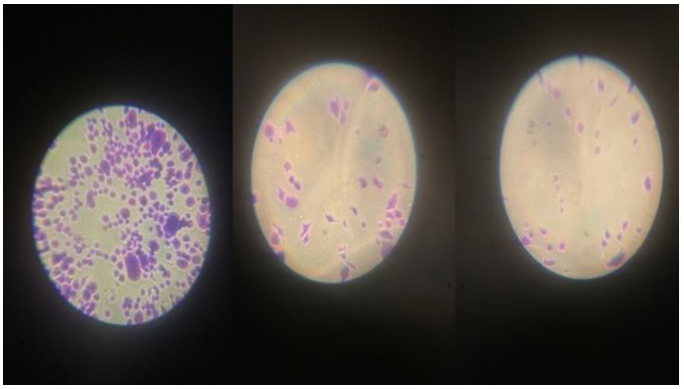

Figure 5: The effect of Violacein on cell viability in the growth of PC3 (Prostate) cancer cell lines.

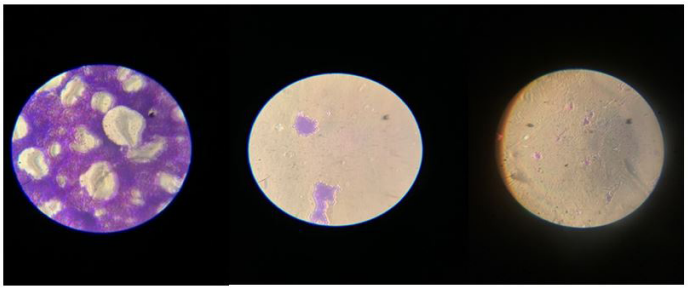

Figure 6: The effect of Violacein on cell viability in the growth of BT20 (Breast) cancer cell lines.

The results of the effect of Violacein on cell viability in the growth of PC3 (prostate) cancer cell lines was shown (Figure 5). the effect of Violacein on cell viability in PC3 prostate cancer cells for different concentrations of Violacein (0. µg/ml, 0.5 µg/ml, and o.25 µg/ml) under 40X magnification was conducted. Violacein induces morphological changes that affect cancer cell motility. PC3 prostate cells showed a decreases of cell numbers in response to treatment with 0.5 µg/ml violacein after 24 hours of exposure. Cells were treated with dimethyl sulfoxide- vehicle control, 0.5, and 0.25 µg/ml of Violacein. Also, the effect of Violacein on cell viability in BT20 breast cancer cells under 40X magnification was studied (Figure 6) [41,42]. Violacein induces morphological changes that affect cancer cell motility. BT20 breast cells showed a decreases of cell numbers in response to treatment with 0.5 µg/ml violacein after 24 hours of exposure [43].

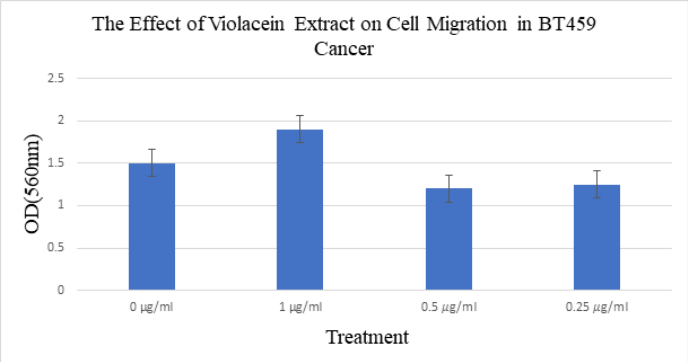

Figure 7: The effect of violacein extract on cell migration in BT549 cancer.

Violacein's effect on tumor cell migration in BT549, and BT20 cancer cells was shown by using Boyden Chamber assay. The Boyden Chamber assay showed that 0.25 and 0.125 µg/ml of Violacein decreased the BT549 cell migration as compared to that of vehicle (DMSO) treated control cells (Figure 7). However, there was no significant effect on BT20 breast cancer cell migration when using different concentrations of Violacein (Figure 8). Finally, Reactive Oxygen Species (ROS) are ions, radicals, or molecules built by single and unpaired electrons. However, excessive amounts of it can lead to cell death. Malondialdehyde is the final product of ROS and the high level of MDA indicates cancer cells. Consequently, the measurement of the MDA level is used as an indicator of lipid peroxidation and to detect oxidative change. In these experiments (Figure 9 and 10), we tested the effects of violacein extract on the lipid peroxidase level which was measured by the production of cellular malondialdehyde [44,45].

6. Discussion

The first aim of the study was to determine the growth inhibitory effect of Violacein on PC3 and BT20 cancer cell lines. The second aim of the study was to test the ability of Violacein to block BT549, BT20, and PC3 cancer cell migration. The third aim of the study was to evaluate the effect of Violacein on the lipid peroxidase level in PC3 and BT20 cancer cell lines. The results of the first aim of the study indicated that violacein extract stimulated cell proliferation at lower concentrations of 1, 0.5, and 0.25 µg/ml in BT20 breast cancer cell line. However, violacein extract did not affect the viability in the PC3 prostate cancer cell line. The second aim of the study involved determining the Violacein's effect on tumor cell migration in BT549, BT20, and PC3 cancer cells. The effect of violacein extract on those cancer cell lines was shown by using Boyden Chamber assay. The Boyden Chamber assay showed that 0.25 and 0.125 µg/ml of Violacein decreased the BT549 cell migration as compared to that of vehicle (DMSO) treated control cells (Figure 7). The experiment was done in duplicate; three separate experiments showed similar results. DMSO (Vehicle control) and 0.5, 0.25, and 0.125 µg/ml concentrations of violacein were used to treat cells. Finally, by using the ANOVA test, the p-value was <0.05 which indicate that there is significant difference between values. The finding of the study suggests that the violacein extract did inhibit the cell migration in BT549 cancer cell lines [46-50].

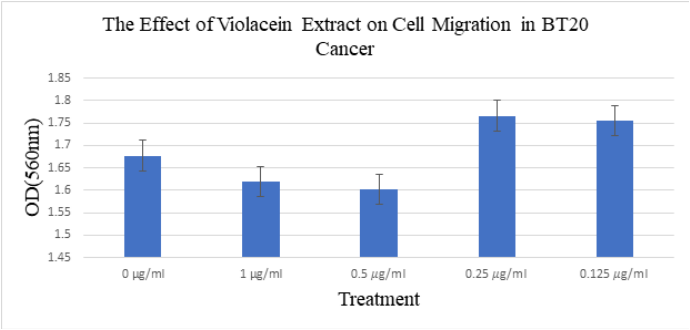

Figure 8: The effect of violacein extract on cell migration in BT20 cancer.

However, there was no significant effect on BT20 breast cancer cell migration when using different concentrations of Violacein (Figure 8). The experiment was done in duplicate; all three separate experiments showed similar results. DMSO (Vehicle control) and 0, 1, 0.5, 0.25, and 0.125 µg/ml concentrations of violacein were used to treat cells. Finally, by using the ANOVA test, the p-value was >0.05 which indicate that there is no significant difference between values. The finding of the study suggests that the violacein extract did not inhibit the cell migration in BT20 cancer cell lines. As well as PC3 cancer cell line was inconclusive with the cell migration assay. The third aim of the study involved determining the antioxidant activity of Violacein extracted from Chromobacterium violaceum in BT20 and PC3 cancer cell lines. The lipid proxied level

activates in PC3 cancer cell lines were examined in different concentrations of Violacein that were extracted from the Chromobacterium violaceum 14N23 strain. The cells were first plated at 100,000 cells per well in a 96-well plate. DMSO, and different concentrations of violacein (0, 0.125, 0.25, 0.5, and 1 µg/ml) were added to the plate.

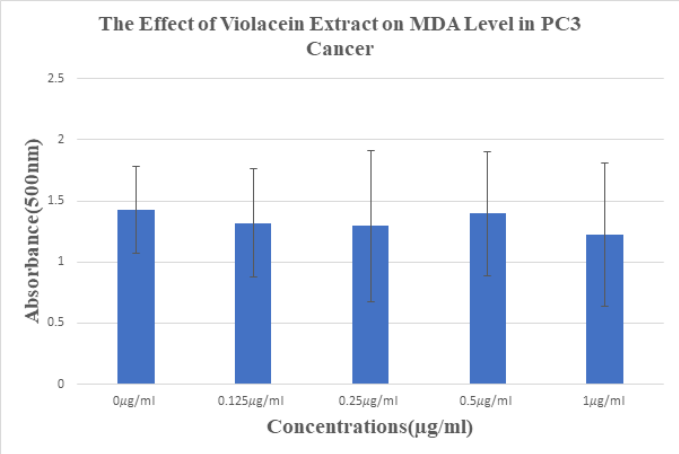

Figure 9: The effect of violacein extract on MDA Level in PC3 cancer.

The malondialdehyde levels were generally found to decrease with increase in violacein extract concentration (Figure 9). The control sample (DMSO) that had no violacein had MDA levels of 1.42 µg/ml [51-53]. However, the levels were reduced to 1,31 µg/ml in samples that were treated with 0.125 µg/ml. The value was further reduced to 1.11(g/ml in samples treated with 0.25 µg/ml. At 0.5 µg/ml, value was reduced to 0.69 µg/ml. Finally, by using the ANOVA test, the p-value for all different concentrations was >0.05, indicating no significant difference between values. The study's finding suggests that an increase in the concentration of Violacein reduces the MDA levels in the PC3 cancer cell line. Also, the lipid proxied level activates in BT0 cancer cell lines were examined in different concentrations of Violacein that were extracted from the Chromobacterium violaceum 14N23 strain. The cells were first plated at 100,000 cells per well in a 96- well plate. The plates were then treated with different concentrations of violacein (0, 0.125, 0.25, 0.5, and1µg/ml).

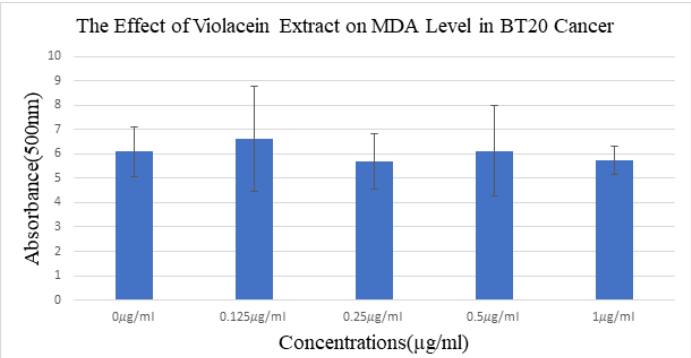

Figure 10: The effect of violacein extract on MDA Level in BT20 cancer.

The malondialdehyde levels were generally found to decrease in the presence of violacein extract (Figure 10). The control sample (DMSO) that had no violacein had MDA levels of 6.296 µg/ml; the levels were reduced to in samples treated with 0.125 µg/ml. Finally, by using ANOVA test, the p-value for all different concentrations was >0.05, indicating no significant difference between values. The study's finding suggests that the violacein extract reduced the MDA levels in BT20 cancer cell lines [54-57].

7. Conclusion

In conclusion, Violacein has potential as a therapeutic agent to treat cancer cell lines due to its versatility to cause cell death in several types of cancer and prohibit metastatic invasion. As given by the observations of this study, the violacein extracts did not decrease cancer cell growth in certain cancer cells.The extract from the CV 14N23 strain was not effective against the PC3 prostate cancer cell line. Interestingly, the CV 14N23 strain's extract was significantly effective against the BT20 cancer cell line. However, the results show that violacein extract can be an effective treatment of BT549 breast cancer by inhibiting that cell migration. Also, violacein extract did not increase the lipid peroxidase level on BT20 and PC3 cancer cells.

8. Acknowledgements

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