Effect of Beta Vulgaris Extract on the Michigan Cancer Foundation-7 (MCF-7) and Rat Embryo Fibroblast (REF)

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Beta Vulgaris, Cancer, Breast Carcinoma (MCF-7), REF

ABSTRACT
The study aimed to study the cytotoxic effect of the aqueous extract of Beta vulgaris on the breast carcinoma cell MCF-7 and the normal cell REF. Six diluted concentrations of the aqueous extract of the plant roots including (7.8, 31.25, 62.5, 125, 250, 500) µg/ml, with three exposure periods of 24, 48, and 72 hrs. The quantitative estimation of the active compounds of the aqueous extract was calculated. It was found that the aqueous extract of plant contains many active compounds such as (tannins, phenols, flavonoids and glycosides). The effect of the extract on normal REF cells after an exposure period of 72 hrs, which is represented by the concentration that causes inhibition of the proliferation of normal REF cells by 50%, is (IC50) with the aqueous extract of B. vulgaris was (6.35) mg/ml. The aqueous extract of plant was tested to detect the cytotoxic effect of the aqueous extract and all concentrations used on the MCF-7 breast cancer cell. The results showed that increase in the percentage rate of inhibition of cancer cell growth was observed during the three exposure periods of 24, 48, and 72 hrs. There is significant differences at ($p \leq 0.05$) and compared with the control. The rate of inhibition ranges at the lowest concentration 500 µg/ml were (92.204±1.455) (93.616 ±1.376) and (95.325±1.407) respectively.

1. Introduction

Plant extracts are a source of many effective and important compounds that used as anti-cancer cell growth (Stefkó et al., 2020). Kusz et al. (2021) and Ma et al. (2016) have been showed that the compounds dihydrophenanthrenes, flavonoids, apigenin, and phenols have a toxic effect on the cells of some human cancer lines. In addition, it is anti-inflammatory effect and anti-bacterial growth. It also used as antioxidants (Petrovska, 2012). Keskin et al. (2018) and Roy and Bharadvaja, (2017) have been worked on medicinal plants and the active substances derived from them indicated an increase in interest in these plants in recent years. In general, it showed the importance of using these plant products by sick people who suffer from cancer, chronic diseases, liver diseases, rheumatic disorders, and the circulatory system.

Beta vulgaris L., known locally as beet that contains powerful antioxidant phenolic compounds, ascorbic acid, carotenoids and betalains (Clifford et al., 2015). High-pressure liquid chromatographic (HPLC) analysis of beet extract also confirmed the presence of bioactive polyphenols such as quercetin, sinapic acid, pumaric acid, syringic acid, gallic acid, coumarin, caffeic acid, chlorogenic acid and catechin (Indu et al., 2017). Current results also indicate that *B. vulgaris* (root) extracts have antihypertensive, hypoglycemic, antioxidant (El Gamal et al., 2014), anti-inflammatory, and hepatoprotective activities (Charde, 2011; Singh et al., 2011).

Cancer is widely known as a silent killer and it is still among the leading causes of death worldwide, claiming millions of lives every year. It is very common throughout the world. In both developing and developed countries, the risk associated with cancer awareness is still weak in our society. Preventing and mitigating risk factors is the best way to combat cancer (Tezerjani et al. 2015). Thus, the study aimed to study the effect of aqueous extract of beet on breast cancer cells MCF-7 and the normal REF.

2. Methodolgy

Plant collection and extraction

The experiment was applied at Biotechnology and Research Center laboratories, Al-Nahrain University, Iraq. The roots of the plant were collected from the markets of Al-Muthanna Province. The plant was classified by Assistant Professor Dr. Azhar Abdel-Amir in the Department of Life

Sciences, College of Education, Al-Qadisiyah University. The roots were cleaned of dust. After drying the plant roots at room temperature, they were ground in an electric device. Then, the Sachselite equipment was used by placing 20 g of plant powder with 300 ml of distilled water at a temperature of 100°C for three hrs. A Lypholyzer equipment was used to force the conversion of the liquid extract into powder to determine the concentrations used in the study.

Chemical detection of the active compounds in the aqueous extract of beet roots

Chemical tests were applied on the aqueous extract that was previously prepared in the Biotechnology and Research Center laboratories, Al-Nahrain University for the purpose of identifying the chemical components of it.

1- Detection of tannins

Detection was applied according to Naing, (2018).

2- Detection of flavonoids

Detection was applied according to Naing, (2018).

3- Detection of alkaloids

Detection was applied according to Hussein and Ameer (2017).

4- Detection of Poly phenols

Detection was applied according to Hussein and Ameer, (2017).

5- Detection of Saponins

Saponins were detected by shaking the aqueous extract of the plant well, and when foam formed at the top, an indication of saponins (+).

6- Detection of glycosides

Detection was applied according to Hussein and Ameer, (2017).

Solutions for tissue culture

Solutions for tissue culture were prepared according to (Freshney, 2000).

1- Sodium bicarbonate (NaHCO3)

It was prepared by adding 4.4 g of sodium bicarbonate (NaHCO3) to 100 ml of distilled water. Then, it was sterilized in an incubator at a temperature of 121°C for 15 mins and then stored at a low temperature (4°C).

2- Antibiotics

It was prepared by dissolving the components of Benzyl penicillin with a capacity of 1,000,000 IU in a volume of 5 ml of distilled water. Then, 0.5 ml of it was taken, and 1 L of culture medium was added and stored at a low temperature (-20).

3- Fetal Bovine Serum

It was used from the supplier (Sigma) and was incubated at a temperature of (56°C) for half an hour and then added to the culture medium.

4- Trypsin

It was prepared by dissolving 1 g of trypsin powder in 100 ml of PBS. Then, sterilizing it with a filter with holes with a diameter of 0.22 um and placing it at a temperature (-20 °C).

5- Medium Rosswell Park Memorial Insitute- 1640 (RPMI- 1640)

Items	Quantity/value
RPMI-1640 with hepes buffer and L-glutamine	10.4 g
4.4 % NaHCO3	14 ml
Penicillin	0.5 ml
Streptomycin	0.5 ml
Fetal Bovine Serum	100 ml

800 ml of distilled water (ion-free water) was added to 10.4 g of culture medium (RPM-1640). Then, solutions of antibiotics and sodium bicarbonate were added, adjusting the pH to 7.2, and then fetal bovine blood serum was added. Then, filtered the culture medium and the volume reaches 1 L by adding distilled water, then it is sterilized using a filter with 0.22um holes, then placed in clean sterile glass bottles and kept in the incubator at a temperature of 37°C for three days for the purpose of verification. Ensure that it is not contaminated, and then it is stored at a temperature of (4°C) until use (Yaseen, 1990).

6- Serum Free Media (SFM)

It was prepared in the same way as mentioned in (5), but without adding the serum to the culture medium.

7- Phosphate buffer saline solution (PBS)

It was prepared by dissolving 0.20 g of KCl, 8 g of Nacl, 0.20 g of KH2PO4, and 0.92 g of Na2HPO4, then supplementing with 100 m of distilled water and sterilizing it in an autoclave at a temperature of 121°C for 15 mins. Then, stored it at a temperature of 4°C until use.

8- Preparation of Methyl Thiazolyl Tetrazolium Stain (MTT)

It was prepared by dissolving 0.005 g of dye powder in 1 ml of PBS solution in a glass beaker. The flask is placed on a vibrating magnetic plate, then the mixture is filtered with a filter (0.22 um) in order to remove the blue crystals that form from it. The dye was prepared immediately and when used. It was stored in a dark place and in sterile conditions so as not to oxidize (Betancur-Galvis et al .,1999).

Cell lines that used in the study

They were obtained from the Center for Research and Biotechnology, Al-Nahrain University, Iraq. They are the human breast cancer cell line Michigan Cancer Foundation-7 (MCF-7) , and the natural cell line Rat Embryo Fibroblast (REF). Then, it conducted a cytotoxicity test for the aqueous extract of beet roots on line cells according to (Khashan et al., 2020).

Study of the effect of aqueous extract of beet on the growth of cell lines

1- Preparation of culture medium and cell lines

The previously prepared culture medium was distributed and placed inside tightly covered glass bottles with a capacity of 200 ml. The bottles were kept at a temperature of -20°C until use. The cancer cell line and the normal cell line for the study were prepared (MCF-7, REF). The steps for tissue culture were performed under sterile conditions (Freshney, 1994) and according to the following steps:

2 ml of trypsin solution was added to the 50 cm tissue culture bottle containing the cells after removing the culture medium and washing it with the solution (PBS). The bottle was gently moved and incubated in the incubator at a temperature of 37 °C for 5 mins in order to break up the adherent cells and disrupt their adhesion to the wall to obtain single monocytes. Then, added 15 ml of new growth medium (RPMI-1640) to the bottle containing the disassembled cells. The bottle was stirred well and then the contents of the bottle were distributed equally with another new bottle so that the

amount of cells with the culture medium was equal between the two bottles. This process is called secondary culture (Subsculpturing Process). Finally, the bottles were then incubated at a temperature of 37°C for a day, after complete information was written on them, the type of cells, and the date of the culture procedure. The bottles were followed up in order to ensure that they were free of contamination and that the cultured cells were in good condition, by examining them with an inverted phase microscope.

Testing the toxicity of the aqueous extract of the plant on the growth of cancer cell lines

The 0.5 g of the extract powder was dissolved in 10 ml of serum-free medium, and the extract was sterilized using a filter with holes with a diameter of 0.22 µm. Then, six concentrations (half dilutions) were prepared using the serum-free medium including (125, 250, 500, 7.8, 31.25, 62.5) ml/ug and under sterile conditions. All the concentrations that were prepared were used immediately after the preparation process was completed.

The cell suspension was prepared by adding a 50 cm^2 trypsin solution to the layer of cells grown in the tissue culture bottle. Then, 20 ml of culture medium containing 10% serum was added. After each mixing, 0.2 ml is transferred to the microtiter plates with 96-fat bottom well by using a micropipette. The dishes were placed in the incubator at a temperature of 37°C for a period of 24 hrs until the cells adhered inside the well hole, after which the old culture medium in the hole was disposed of and 0.2 ml of the extract concentrations that had been prepared previously were added in eight replicates for each concentration.

Eight replicates were used as a control unit. The dishes were incubated at 37°C for 24 hrs. Then took the dishes out of the incubator and removed the culture medium. Then, wash the cells with a solution of (PBS). After that, add 0.1 ml of MTT stain to each hole and left it for three hrs, after which the hole was washed with a solution of (PBS) in order to remove the excess dye, as the cells attached to the bottom of the hole were stained yellow. Then, add 0.1 ml of dimethyl sulfoxide solution (DMSO), followed by an incubation period at 37 °C for 15 mins, and after the plates dried, the results were read using the Eliza plate reader at a wavelength of 620 nm. These steps were performed on cell lines (MCF-7, REF) by using aqueous extract and with three exposure periods (24), 48, and 72 hrs. The rate of inhibition of cell growth (percentage of cytotoxicity) was calculated with the following equation.

Inhibition rate (IR) = (A-B)/A *100

A indicates the optical density of the control, and B indicates the optical density of the samples (Abdullah et al., 2020).

3. Result and Discussion

Chemical detection of the active compounds of the aqueous extract of *B. vulgaris*

The results showed that when conducting the detection of secondary metabolites and active compounds present in the aqueous extract of the *B. vulgaris* plant showed the presence of flavonoids, phenols, alkaloids, saponins, tannins and glycosides (Table 2).

Table (2) detection of active compounds in the aqueous extract of *B. vulgaris*

Active compounds	Name of reagent	reagent guide	reagent result
Phenols	Ferric chloride reagent 1%	Greenish blue	+
Flavonoids	aqueous lead acetate C4H6O4Pb	Bright yellow	+
Tannins	lead acetate C4H6O4Pb	Gelatinous	+
Glycosides	Benedict reagent	Orange-red	+
Alkaloids	Dragendrov reagent	Brown	-

Saponins	shaking the solution	Bubbles	+

(+) indicates a positive test, while the sign (-) indicates a negative test

The cytotoxic effect of the aqueous extract of *B. vulgaris* on normal cell line REF

The results indicated that the effect of the extract on normal cells represented by the concentration that causes inhibition of the proliferation of normal cells (REF) by 50% (IC50), using the aqueous extract of beetroot beta vulgaris was 6.35 mg/ml as (Figure 2). The results also showed change in the percentage of cell viability at the different concentrations used (Table 3).

Table (3) Vitality rate of normal cell lines exposed to the extract at different concentrations

Con. mg/ml	Mean± SD
1	96.329±2.310
2	88.080 ±1.920
4	56.103±1.103
6	53.473±1.383
8	35.144±1.372
10	19.128±3.014
12	13.289±2.130

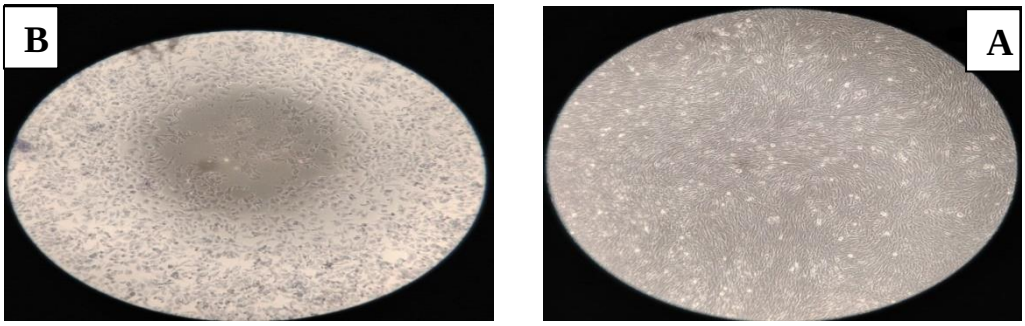

Figure (1) A- REF normal line cells not exposed to extract; B- REF normal line cells exposed to extract

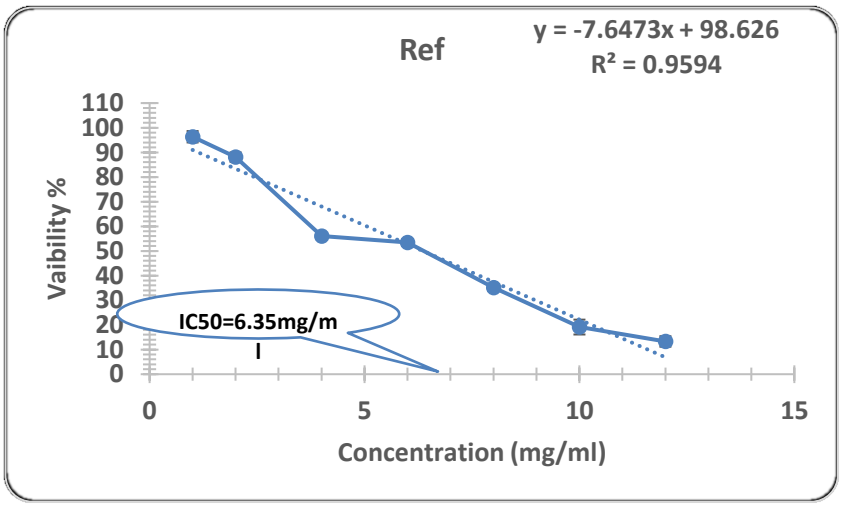

Figure (2) the concentration causing inhibition of normal cell proliferation (IC50) of the extract of *B .vulgaris*

The effect of aqueous extract of *B. vulgaris* on the MCF-7 breast cancer cell line

The results showed that the effect of the aqueous extract began 24 hours after exposure. There is a significant differences at ($p \leq 0.05$) compared to the control. The average of cancer cell inhibition for

the lowest concentrations of 7.8 µg/ml was 5.612±2.776, and the toxic effect on cells increased at the highest concentrations of 500 µg/ml, reaching 92.204±1.455 at the same level of probability and exposure period. However, when the 48 hrs period of exposure to the extract, it is noted that the percentage of inhibition increased for almost all concentrations, reaching 7.539±3.030 at the concentration of 7.8 µg/ml, while the percentage of inhibition at the highest concentration of 500 µg/ml reached 93.616±1.376 (Table 4).

For 72 hours, the percentage of inhibition rate reached its highest levels, compared to the same concentrations, as the percentage concentration in cancer cells was recorded as 8.074±3.150 at a concentration 7.8 µg/ml compared to 95.325±1.407 at a concentration 500 µg/ml.

Table (4) effect of extract of *B. vulgaris* on the breast cancer cell line MCF-7 after 24, 48 and 72 hrs at different concentrations

Time / **Concentration**	**24 hr (Mean± SD)**	**48 hr (Mean± SD)**	**72 hr (Mean± SD)**
0 µg/ml	3.077±1.600a A	4.397±1.939a A	4.498±1.971a A
7.8 µg/ml	5.612±2.776a A	7.539±3.030a A	8.074±3.150a A
31.25 µg/ml	25.542±5.142a B	31.271±5.201bc B	32.576±5.312b B
62.5 µg/ml	35.873±5.588a C	41.868±5.888b C	43.772±6.122b C
125 µg/ml	63.408±2.015a D	65.036±2.162a D	70.559±2.248b D
250 µg/ml	77.942±2.710a E	81.101±2.997b E	85.624±3.116a E
500 µg/ml	92.204±1.455a F	93.616±1.376b F	95.325±1.407c F
LSD 0.05	4.95		

Small letters indicate differences between times; capital letters indicate differences between concentration; * different letters indicate significant differences, * Similar letters indicate no significant difference The results also showed that the lowest vital percentage of cancer cells was recorded at a concentration of 500 µg/ml, which was 7.80, while the highest vital percentage of cells recorded at a concentration 7.8 µg/ml was 94.39. The results also noted that this vitality decreases with the passage of time at the same concentrations used in 48 hours. The percentage of cell vitality at a concentration 500 µg/ml was 6.38, while the highest percentage of cell vitality was at a

concentration 7.8 µg/ml was92.46. Finally, the lowest vital percentage of cancer cells was at a concentration 500 µg/ml after 72 hours, the vital percentage of cancer cells reached 4.67 (Figure 3, 4, and 5).

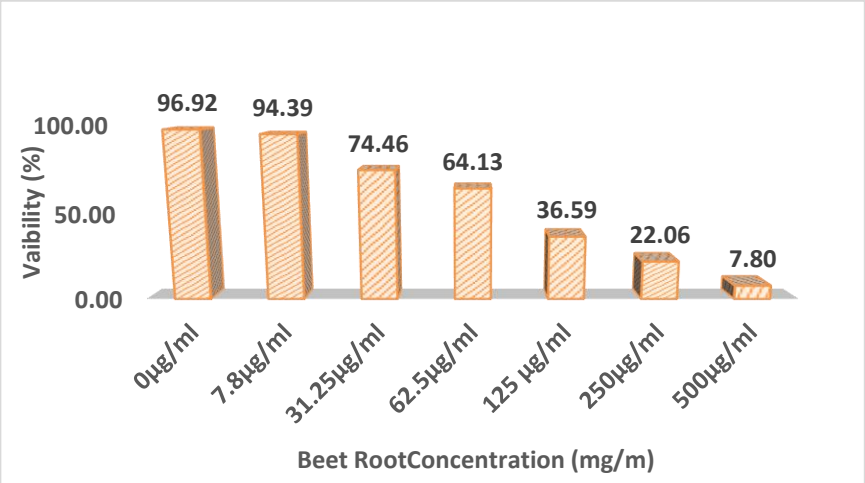

Figure (3) effect of aqueous extract of *B. vulgaris* on the vitality of breast cancer cell line MCF-7 after 24 hrs at different concentrations

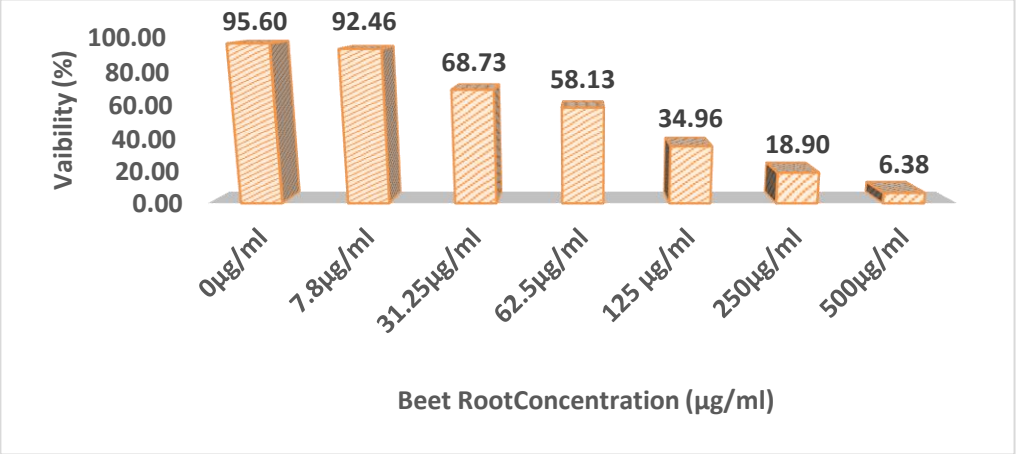

Figure (4) effect of the aqueous extract of *B. vulgaris* on the vitality of the MCF-7 breast cancer cell line after 48 hrs at different concentrations

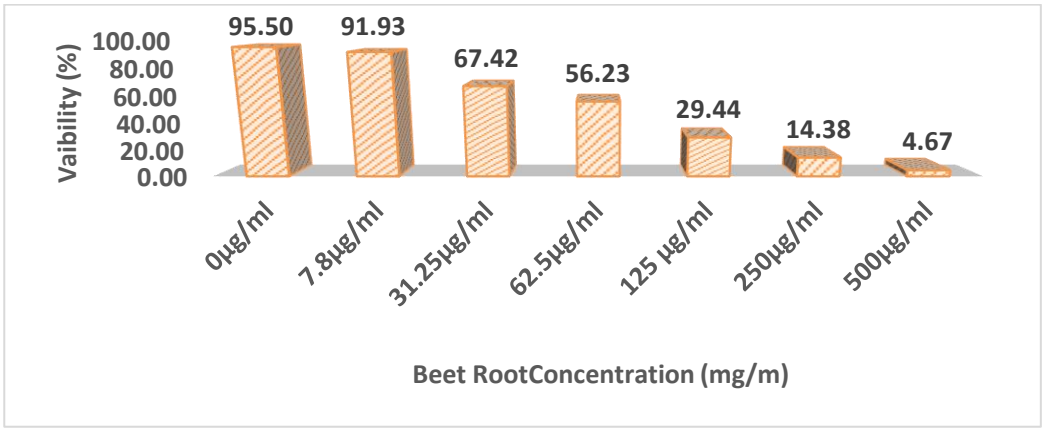

Figure (5) effect of the aqueous extract of *B. vulgaris* on the vitality of the MCF-7 breast cancer cell line after 72 hrs at different concentrations

A

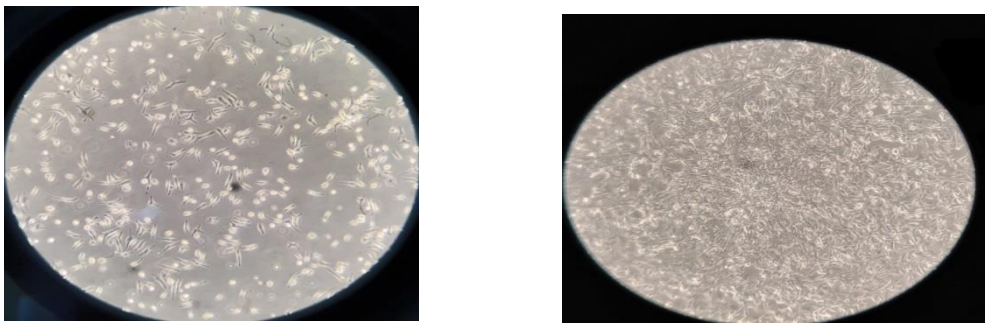

Figure (6) A- MCF-7 breast cancer cells not exposed to extract; B- MCF-7 breast cancer cells exposed to extract

Chemical detection of the active compounds of the aqueous extract of beet root *B. vulgaris*

The results of the current study agreed with (Rehman et al., 2021), which used the Soxhlet extraction method to prepare crude leaf and root extracts and reported that phytochemical analysis of root and leaf extracts showed the presence of flavonoids, phenols, alkaloids, saponins, and glycosides. Furthermore the results agreed with (Arjeh et al., 2022) that the roots of *Beta vulgaris* were harvested from West Azerbaijan Province, Iran. The peel extract of the roots gave higher values for the compounds in all the experiments conducted compared to the fresh parts in ethanol and methanol solvent, which agreed with a different study (Kujala et al., 2000). It was found that phenolic compounds are mostly distributed in the outer parts of the root. The amount of phenolic compounds in the peel (19.7 ± 1.1 g/mg db.) was more than twice the amount in the fresh part (8.3 ± 0.0 g/mg db.)

Moreover, solvents also had a significant effect on the amount of phenolic compounds, and methanol was more effective than ethanol in extracting phenolic compounds (Barreca et al., 2016). In addition, El-Beltagi et al. (2022) reported these results when total polyphenols were determined by the Folin-Ciocalteu test. They are the predominant bioactive compounds (832 mg/100 g) in the root extract. Furthermore, flavonoids account for approximately 25% (234 mg/100 g) of the total polyphenols found in the root extract. Meanwhile, the betalains content was 535 mg/100 g. These results obtained were higher than those evaluated by Tumbas Šaponjac et al. (2016) which showed the contents of total polyphenols was (326.51 mg GAE/100 g), flavonoids (10.23 mg RE/100 g) and betalains (60.52 mg betanin/100 g and 61.33 mg E) in aqueous ethanol extract of dried beetroot pomace. In addition, The results obtained were lower than those evaluated by Lazăr et al (2020) and Vulić et al., (2012) who showed that aqueous beet root peel powder extract had a high polyphenolic content.

The cytotoxic effect of the aqueous extract of B. *vulgaris* on normal cell line REF

The cytotoxicity results showed that the concentration causing inhibition of normal cell proliferation (REF) by 50% (IC50) by the aqueous extract of *B. vulgaris* was 6.35mg/ml (Figure 2). The results also showed a change in the percentage of cell vitality at the different concentrations used (Table 2). The results obtained were agrred with (Saber et al., 2022) who studied the effects of red beets and betanin on human colorectal cancer cell lines after treated with different doses (20 to 140 µg/ml) of beetroot extract and betanin during (24 and 48 hrs). IC50s were determined as 92 µg/ml, 107 µg/ml and 64 µg/ml, 90 µg/ml in HT-29 and Caco-2 cell lines at 48 hrs, respectively. Also, there was no effect on normal KDR/293 cell lines (control group) at the highest specific concentration (140 µg/ml) at 24 hrs and 48 hrs. Beet root extract and betanin significantly inhibited the growth of HT-29 and Caco-2 cell lines in a time and dose dependent (with concentrations increasing from 40 to 100 µg/mL for betanin and 60 to 100 µg/mL for beet root extract).

However, Bouchmaa et al (2022) found that the methanolic extract of *B. vulgaris* subsp has no

cytotoxic effect against normal cells (PBMCs) (IC50 > 50 µg/ml. On the contrary, the extract was found to have highly selective killing capacity against triple negative breast cancer (TNBC) and positive breast cancer cell lines (MDA-MB-468) and MCF-7. Rehman et al., (2021) also found that the root extract had an IC50 value of 2.32 ± 0.72. µg/ml compared to leaf extract (2.20±0.72 µg/ml). The reason behind the lower IC50 value of the leaf extract may be that it has a higher phenolic content compared to the root extract. The lower IC50 indicates the greater therapeutic activity of the bioactive components present in the extracts. This is because to the fact that betanin, which constitutes more than 95% of the total betacyanin is non-toxic at different concentrations to human umbilical vein endothelial cells and normal human fibroblasts. It also inhibits the production of reactive oxygen species and reduces the level of reactive oxygen species within the cells (Loo et al., 2003).

The effect of aqueous extract of *B. vulgaris* on breast cancer cell line MCF-7

The results that presented in Table (3) agreed with (Piasna-Słupecka et al., 2023), who used beet root on breast cancer cell lines. It was reported in the study that adding digested juice from beet root sprouts to a culture of MCF-7 cancer cells, cell proliferation was inhibited statistically significantly ($p \geq 0.05$) by 27.38% after incubation for 24 hrs; Additional incubation for 48 hrs and 72 hrs caused successive inhibition compared to the control sample by 33.03% and 58.83%, respectively. In contrast, addition of digested root juice to MCF-7 cancer cell culture resulted in a statistically significant ($p \geq 0.05$) decrease in cell proliferation by 22.97% after incubation for 24 hrs.

Continuation of the experiment for the next two days resulted in a significant progressive inhibition ($p \geq 0.05$) of the proliferation of the studied cell line, i.e. approximately 30.58% and 63.45% inhibition after 48 hrs and 72 hrs of incubation, respectively. In addition, the results also agreed with (Bouchmaa et al., 2022) on treating two cancer cell lines with different concentrations of methanol extract of beet root (1.56, 3.12, 6.25, 12.5, 25, 50, and 100 µg/ml) for 48 hrs. The results showed a dose-dependent cytotoxic effect against both breast cancer cell lines. Furthermore, methanolic beetroot extract had moderate activity against both MCF-7 and MDA-MB-468 breast cancer cell lines.

In similar studies, Reddy et al (2005) compared the effects of natural pigments, including betanin, as well as their mutual interactions, on their potential to inhibit cancer cell growth. In a 48 hrs trial, the observed inhibition of breast cancer cell growth in the MCF-7 line treated with betanin. A study conducted by Kapadia et al (2011) showed that beet root extract strongly inhibits the proliferation of cancer cells, including breast cells (MCF-7). Another study showed an anti-proliferative effect of beetroot extract on breast and prostate cancer cell lines (Kapadia et al., 2013). A study also showed complete inhibition of breast cancer cell lines MCF-7 and MD-MB-231 under the influence of a mixture of betanin, isobetanin, with no significant effect on the normal cell line HUV-EC-C (Nowacki et al., 2015). *Beta vulgaris* isolated from beets was found to suppress growth, migration, colony formation, and mammosphere formation in breast cancer cell lines. This compound also reduced the CD44+/CD24- subpopulation ratio and the expression of self-renewal-related genes such as c-Myc, Nanog, and Oct4. *Beta vulgaris* inhibits Stat3/Sox2 signaling and causes death of breast cancer stem cells (Liu et al., 2020).

4. Conclusion

The study concludes that the aqueous extract of plant contains many active compounds such as (tannins, phenols, flavonoids and glycosides). The effect of the extract on normal REF cells after an exposure period of 72 hrs, which is represented by the concentration that causes inhibition of the proliferation of normal REF cells by 50%, is (IC50) with the aqueous extract of B. vulgaris was (6.35) mg/ml. The aqueous extract of plant was tested to detect the cytotoxic effect of the aqueous extract and all concentrations used on the MCF-7 breast cancer cell. The results showed that increase in the percentage rate of inhibition of cancer cell growth was observed during the three exposure

periods of 24, 48, and 72 hrs.

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