Anticancer Activity of Beetroot (*Beta vulgaris L.*) Extracts (Human Colon Carcinoma Cell Line)

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ABSTRACT

Beta Vulgaris Linnaeus ranks among the 10 most powerful vegetables as excellent sources of phytochemicals, which showed potent antioxidant and anticancer activities. Aim of study: To determine the cytotoxicity and activity of *B. Vulgaris* root extracts against colon carcinoma cells. Methods: The Beetroot extracted into ethanol/water (80/20 v/v) solvent at a solid/liquid ratio of 1/3 (g/mL) for 1 hr, HCT-116 cells (human colon carcinoma cell line) were obtained from VACSERA Tissue Culture Unit. The effect of extract was performed using MTT assay and the evaluation of Apoptotic mechanism of cytotoxicity against HCT-116 cell line also Cell Cycle was analyzed using Flow Cytometry. The results: The MTT cytotoxicity assay revealed that the beet-root ethanol extract has an obvious cytotoxicity on HCT-116 cancer cell lines at low significant IC50 value as104±4. 7µg/ml. Inhibitory activity against colon carcinoma cells was detected under these experimental conditions in vitro. The total percentages of early, late apoptosis and Necrosis ratio after treatment with a beet-root extract and a control group (non-treated cells) for 48 hours in the HCT-116 cell lines were found to be 2.72%, 17.85%, 7.52%, 0.55%, 0.18% and 1.24% respectively. On these findings, the apoptosis-inducing effect of the beet-root extract is true. However, the apoptotic effects of beet-root extract on HCT-116 cell lines were significant. In conclusion: the present study indicates that treatment of cancer cell lines (Hct-116) with red beet-root extract inhibited the cell proliferation and significantly induced apoptosis at the level of the used extracts. However, the exact proliferative and apoptotic mechanisms of beet-root extract of different cancer cells is still unknown and further studies are needed in this regard.

Keywords: Beetroot, Bulgari's root extracts, cancer, natural product.

INTRODUCTION

Human diseases such as cancer could result from oxidative stress produced by continual and excess production of reactive oxygen and nitrogen species (RONS) that induce long-term cellular disruption. Therefore, many antioxidant food sources have been evaluated for their ability to scavenge RONS and prevent oxidative stress (Lobo *et al.*,2010)

Cancer is a complex disease that shows abnormal cell growth with the invasion of surrounding cells and tissues. Cancer is caused by a progressive accumulation of multiple genetic mutations which are evoked due to environmental stress, microbial infection, food adulteration, smoke, tobacco, ionizing radiation, heavy metal exposure, and multiple genetic reasons. Approximately 5–10% of cancers are due to inherited genetic defects inherited from parents.

Since ancient times, pigment extract of various cultivated forms of red beet (*Beta vulgaris L.*, of family Chenopodiaceae) has been widely used as a natural colorant in food, cosmetics, decorative art, paintings, as a medicinal product in the management of blood, heart, liver, pancreas, digestive, neurological, and other common diseases (Nottingham, 2004).

Red beet (*Beta vulgaris var. rubra L.*; BVr) and green beet (*B. vulgaris var. cicla L.*; BVc) belong to the same plant family (Amaranthaceous– Chenopodiaceae). *Beta vulgaris var. rubra L.*; BVr) is valued for its root, while BVc is grown for its leaves. The most important bioactive phytochemicals in BVr are battalions; pigments derived from betalamic acid and grouped into yellow betaxanthins (BX) and red betacyanins (BC) with its powerful antioxidant and anti-inflammatory effect (Ninfali and Angelino, 2013)

Beetroots contain both red (betacyanin) and yellow pigments (betaxanthins) known collectively as battalions, which constitute a class of highly bioavailable natural antioxidant pigments (Kanner *et al.*, 2001and Tesoriere *et al.*, 2004, 2013)

The antioxidant, anti-inflammatory and vascular-protective effects offered by beet-root and its constituents have been investigated by several in vitro and in vivo human and animal studies; hence it might be considered for treatment of several pathological disorders (Vulić *et al.*,2014)

Beetroot (*B. vulgaris var. rubra L.;* BVr), which has been a part of the traditional western diet, the powder or extract form of betanin, a natural pigment, is an antioxidant used in the food industry. The antioxidant activity of betanin in biologic lipid environments has been indicated in human macromolecules such as membranes low-density lipoproteins (LDL) and whole cells. (Oroian and Escriche, 2015)

Moreover, betanin exerts have anti-inflammatory effects and protect hepatic functions in human cells. The compound regulates redox signalling pathways mediated by the inflammatory response in cultured endothelial cells and exerts anti proliferative effects on human tumour cell lines (Rahman *et al.,*2006)

Betanin, the original nutritional betacyanin, shows significant inhibition to the growth of tumour cells of the stomach, breast, lung, colon, and central nervous system (Rodríguez-Ramiro *et al.,* 2012); induce apoptosis in K562 human myeloid leukaemia cells; and weakly exhibit epigenome-regulated gene expression in MCF-7 breast cancer cells. However, the potential ant proliferative, chemo preventive and epigenetic activities of betaxanthins are yet to be investigated.

Recently, the interest has shifted to the use of natural products to improve human health as a mean of disease prevention. Thus, the number of studies on the application of battalions in medical sciences are increasing. Therefore, a narrative review of the therapeutic uses of battalions and the genes involved in battling metabolism may help future investigations regarding the advantages of natural products. Because of the importance of the issue, few interesting reviews, articles have been published very recently (Rahimi *et al.,*2019).

Aim of study: To determine the cytotoxicity and activity of *B. Vulgaris* root extracts against colon carcinoma cells.

MATERIALS AND METHODS

Beetroot extraction:

The fresh red beet roots were brought from local market at El-Gharbia Governorate, the roots were carefully washed under tap water, mechanically chopped into small pieces using magic mix, then ground. and extracted into ethanol/water at a concentration of (80/20 v/v) as solvent using a solid/liquid ratio of (beetroot)/(solvent) (1g/3ml) for 1hr. under continuous mechanical stirring. The solid material was separated from the macerate by centrifugation at 12 000 (R.P.M) for 15 min. at 4 °C followed by filtration on a membrane filter, then was concentrated by ethanol evaporation under vacuum at 30 °C and freeze dried. (Stintzing *et al*., 2002)

The extraction method was performed at the National Research Centre laboratory.

HPLC conditions

HPLC analysis was carried out using an Agilent 1260 series. The separation was carried out using Eclipse C18 column (4.6 mm x 250 mm i.d., 5 μm). The mobile phase consisted of water (A) and 0.05% trifluoroacetic acid in acetonitrile (B) at a flow rate 1 ml/min. The mobile phase was programmed consecutively in a linear gradient as follows: 0 min (82% A); 0–5 min (80% A); 5-8 min (60% A); 8-12 min (60% A); 12-15 min (85% A) and 15-16 min (82% A). The multi-wavelength detector was monitored at 280 nm. The injection volume was 10 μl for each of the sample solutions. The column temperature was maintained at 35 °C.

Mammalian cell lines: HCT-116 cells:

Cells of Human colon carcinoma cell line were obtained from the VACSERA Tissue Culture Unit. Dimethyl sulfoxide (DMSO), crystal violet and trypan blue dye were purchased from Sigma (St. Louis, Mo., USA). FBS, DMEM, RPMI-1640, HEPES buffer solution, L-glutamine, gentamycin and 0.25% Trypsin-EDTA were purchased from Lonza. Crystal violet stain (1%): It's composed of 0.5% (w/v) crystal violet and 50% methanol then made up to volume with ddH_2O and filtered through a Whatmann No.1 filter paper. The cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, HEPES buffer and 50μg/ml gentamycin. All cells were maintained at 37ºC in a humidified atmosphere with 5% CO_2 and were sub-cultured two times a week (Mosmann, 1983).

Cytotoxicity evaluation using viability assay:

The MTT method of monitoring in vitro cytotoxicity is a mean to measure the activity of living cells by mitochondrial dehydrogenases. The key component is (3-[4,5dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) or MTT. Solutions of MTT, dissolved in medium or balanced salt solutions without phenol red are yellowish in colour. Mitochondrial dehydrogenases of viable cells cleave, the tetrazolium ring,

yielding purple formazan crystals are insoluble in aqueous solutions. The crystals are dissolved in acidified isopropanol, the resulting purple solution is spectrophotometric ally measured (Mosmann, 1983). The degree of cytotoxicity caused by the test material using the MTT assay was done according to (Gomha *et al*.,2015)

Mechanistic study on the antitumor activity:

Apoptosis analysis (Annexin V-FITC assay)

Apoptotic cells were further analysed by Annexin V-FITC assay. Briefly, HCT-116 (colorectal carcinoma) cells were cultured to a confluent monolayer then treated with the tested sample at the IC50 concentration (104 µg/ml) as have been described earlier. The produced cells were analysed using the flow cytometer BD FACS Calibur (BD Biosciences, San Jose, CA**)** (Wagdy *et al.,*2018).

Cell cycle analysis using flow cytometry:

To determine the effect of the tested sample on the cell cycle distribution HCT-116 cell line; cell cycle analysis was performed using the CycleTEST™ PLUS DNA Reagent Kit (Becton Dickinson Immunocytometry Systems, San Jose, CA). The HCT-116 cells (treated with the tested sample or nontreated) were stained with propodium iodide stain following the procedure provided in the kit and then run on the cytometer. Cell-cycle distribution was calculated using Cell Quest software (Becton Dickinson Immunocytometry Systems, San Jose, CA) (Eldehna *et al.,*2017).

Statistical analysis.

All statistical evaluations were performed for three replicates Cell Vitality (n=3). The results were statistically analysed by analysis of variances as described by (SPSS,1997). Significant differences among individual means were analysed by Duncan´s multiple range test at $p<0.05$ as the level of the significance (Duncan,1955)

Continuous variables are expressed as means ± standard deviation (SD)

RESULTS AND DISCUSSION

Data presented in Table (1) and Figure (1) showed that eleven different phenolic compounds could be identified in red beet-root extract by using HPLC fractionation. These compounds involved: Chlorogenic acid, 872.69, µg/g), Gallic acid, 823.32, µg/g), (Ellagic acid, 54.77 µg/g), (Syringic acid, 32.01 µg/g), Catechin,13.93 µg/g), Caffeic acid, 7.73 µg/g), Methyl gallate, 7.51, µg/g), (Taxifolin, 6.51µg/g), (Ferulic acid, 5.84 µg/g), (Vanillin, 4.56 µg/g), and Naringenin, 3.04 µg/g) respectively. On the other hand, Pyro catechol, Rutin, Coumaric acid, Cinnamic acid, and Kaempferol were not detectable.

From the tabulated data in the same table, it could be also noticed that Chlorogenic acid, Gallic acid, Ellagic acid, Syringic acid and Catechin were the major phenolic compounds, meanwhile, Caffeic acid, Methyl gallate, Taxifolin, Ferulic acid, Vanillin, and Naringenin represented the minor compounds found in the studied beet roots where the lowest values of these compounds were recorded as previously revealed.

Our results obtained by HPLC analysis of beet root extracts are relatively similar to those reported by Shalaby and Hassenin (2020)who mentioned that the identified phenolic compounds of the red beet root powder using HPLC into RBP recorded levels ranged from 0.0562 to 325.0395 ppm. These compounds were Coumaric acid, Quercetin, Kaempferol, Naphthaline and Resorcinol acid. The results reported by (Steel and Torrie 1980) and (Fernández-García and McGregor 1997) revealed that Beet root powder has important bioactive agents (betaine and polyphenols). The work performed by (Young, 2001) included a variety of physiologic factors. Meanwhile (Steel and Torrie 1980) found that phenolic compounds as antioxidants have a lot of possible pathways, for example free radical-scavenging, and oxygen radical absorbance.

Evaluation of Cytotoxicity Against HCT-116 Cell Line

Table. (2) and Fig (2&3) showed the inhibitory and viability effect of different concentrations against HCT-116 cell line. Seven concentrations of Beetroot extract were prepared to experiment the inhibitory effect. Recorded numbers showed that the inhibited cell lines increased with increasing of Beetroot concentration. The highest Bet.Ex. concentration (500 µg/ml) inhibited 90.17% of HCT-116 cell line. Meanwhile, the concentration (7.8 µg/ml) inhibited 1.07% of the cell line as the lowest effected ratio of HCT-116, The relationship between viable cells and Bet. Ex. concentration was very important to detect. The curve of Fig. (2) also illustrated the reduction of cell viability with increasing Bet.Ext. concentration. The fifty percent inhibitory concentration that causes toxic effects in 50% of the HCT-116 cell which called (IC50) was = 104 ± 4.7 µg/ml.as shown in (fig.3)

Generally, the using of Beet root extract was able to inhibit HCT-116 cells completely at concentrations ranged between 500 and 100 μg/ml. The inhibition effect of beetroot extracts may be related to the highly Bet./IsoBet.-enriched concentrate produced from red beetroots that was capable to inhibit cancer cell proliferation. Betanin and isobetanin are the most predominant betalains in red beetroot (80% of Bet. /IsoBet. mixture (of which betanin accounts for 64% and isobetanin for 36%), as indicated by Nowacki, *et al*. (2015). Moreover, he reported that the induces MCF-7 cell death has no obvious effect towards normal cells.

These results relatively agreed with the observation of Kapadia *et al*. (2011& 2013), they have previously evaluated the cytotoxic effect of a red beet-root extract in MCF-7 cell line and the IC50 value was 600 μM (after 72 hr. of exposure). Reddy *et al*. (2005) also observed that the growth inhibition of MCF-7 cells treated with a betanin concentrate for 48 hr, (IC50 value was 294 μM). The betanin purification processwas applied to the crude beet-root extract and allowed to obtain a significant MCF-7 growth inhibition associated with cell death for very low concentrations (below 40 μM). Saber *et al*., (2020) found that the beet-root- extract inhibited cell proliferation in the HT-29 cells by a dose of 92 μg/ml, as well in the Caco-2 cells in a dose of 107 μg/ml at 48 h time-point. Thus, it was obvious that our data clarify these previous studies.

Apoptosis Analysis Using Flow Cytometry

Apoptosis, as an accurate programmed cell death, removes damaged cells via precisely regulated genes and plays an important role in the development and homeostasis of normal tissues (Hassan *et al*.,2014) and (Fuchs and Steller.,2011).

As HCT-116 cell viabilities were strongly decreased during Bet. /Ex. treatment, the nature of cell death induced was analysed by the pigments. First, the apoptosis induction was checked and quantitated using Annexin-V-FITC assay labelling and cell analysis by flow cytometry as indicated by the results in Table (3), Fig. (4) which revealed that Bet. /Ex. Treated samples with the IC_{50} concentration (104μg/ml) as earlier described has significantly increased the percentage of Annexin-V-positive/PI-negative HCT-116 cells, positive control (non-treated cells)

HCT-116 cell culture promoted Phosphatidylserine externalization; the percentage of Annexin-V-positive cells were found to be increased with Beet root extract.

Finally, it was found that the total percentages of early, late apoptosis and Necrosis ratio after treatment with a beet-root extract and a control group (non-treated cells) for 48 hours in the HCT-116 cell lines were 2.72%, 17.85%, 7.52 and 0.55%, 0. 18%1. 24 respectively. However, the apoptosis-inducing effect of beet-root extract in the control group was higher increased on HCT-116 cell lines, since HCT-116 treated cells, the expressions of apoptosis-related proteins were strongly increased, and the mitochondrial membrane potential was altered, demonstrating the involvement of both intrinsic and extrinsic apoptotic pathways. The results were also in relative agreement with the results of (Nowacki *et al*.,2015) who reported that treatment of MCF-7 cells with betanin-enriched red beet-root (Beta vulgaris L.) extract increased the expression level of apoptosis-related proteins (Bad, TRAILR4, FAS, p53) and altered the mitochondrial membrane potential. These alterations confirm the involvement of both intrinsic and extrinsic apoptosis pathways due to red beet-root extract treatment.

Saber *et al*., (2020) investigated and demonstrated that red beet-root hydro-alcoholic extract and bitumen can inhibit cell proliferation and can induce apoptosis in treating HT-29 and Caco-2 cancer cell lines.

Cell Cycle Analysis Using Flow Cytometry

Table (4)and Figure (6&7)reveal Bet. /Ex. concentrate effect throughout focusing on treatment and no treatment human cell (HCT-116) lines that represent two different and specific phenotypes. The cell cycle progression was studied after 48 hr. post-seeding without (control) or with 104 μg/ml (IC_{50}) Bet. /Ex. For each cell line, using the Cycle TEST™ PLUS DNA Reagent Kit were significantly different: there were fewer cells in G1 phase (42.91%) and in the S phase was (26.74%) and G2/M phase (30.35%), as a result of the effect related to 104 μg/ml (IC_{50}) Bet. /Ex which decreased the G1 cell number and promoted S phase increase as previously described in MCF-7 cells treated with resveratrol or riproximin in the observation of Joe *et al*., (2002) and Pervaiz *et al*., (2015). On the other hand, a fewer effect was observed with the untreated human HCL116 cell lines and registered the ratios (51.88%) during the G1 phase analysis, (36.17%) for S phase and (11.95%) with the phase G2 -M.

Plati *et al.*, (2008) and Fulda, (2009) reported that during the process of preventing cancer cell formation, the DNA damage in precancerous lesions triggers apoptosis pathways with the purpose of removing potentially harmful cells and blocking tumor growth. Nevertheless, deregulation of this exact death process by different carcinogenic factors resulted in uncontrolled cell proliferation, progress, and development of cancerous cells and predisposed to resistance against drug therapies.

However, the significant role of pro/anti-apoptotic proteins and over/down expression of their effective genes by natural anticancer compounds is very important in the cell survival and apoptosis (Saber *et al*.,2017) (Newman& Cragg,2010).

CONCLUSION

In conclusion, the present study indicates that treatment of cancer cell lines (HCT-116) with red beet-root extract inhibited the cell proliferation and significantly induced apoptosis. However, the exact proliferative and apoptotic mechanisms of beet-root extract and betanin of different cancer cells is still unknown and further studies are needed in this regard.

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Table 1: Identification of Phenolic Compounds in Red Beet Extract by HPLC

	Area	Conc. (µg/ml=µg/21.9mg)	Conc.(µg/g)
Gallic acid	823.32	Ellagic acid	54.77
Chlorogenic acid	872.69	Coumaric acid	ND
Catechin	13.93	Vanillin	4.56
Methyl gallate	7.51	Ferulic acid	5.84
Coffeic acid	7.73	Naringenin	3.04
Syringic acid	32.01	Taxifolin	6.51
Pyro catechol	ND	Cinnamic acid	ND
Rutin	ND	Kaempferol	ND

ND= Not detected

Table.2: The effect of different concentration of beet root extract against HCT-116 cell line.

Extract conc. (µg/ml)	Viability %	Inhibitory %	S.D. (±)
0	100	0	
3.9	100	0	
7.8	98.93	1.07	0.75
15.6	96.87	3.13	1.49
31.25	85.49	14.51	2.65
62.5	68.21	31.79	3.47
125	40.97	59.03	3.15
250	26.42	73.58	2.84
500	9.83	90.17	1.61

Table 3: Annexin-V labelling of cells HCT-116 treated with Bet. /Ex. And non-treated cells.

Sample code	Tested conc. (µg/ml)	Early Apoptosis	Late Apoptosis	Necrosis
Sample 1 (Treated cells)	104	2.72	17.85	7.52
HCT-116 cells (control)	0	0.55	0.18	1.24

Table 4: Cell cycle Analysis HCT-116 using Flow Cytometry Treated of Bet. /Ex. and non-Treated cells

Sample code	Tested conc. (µg/ml)	%G0-G1	%S	%G2-M	%Pre G1
Sample 1 (Treated cells)	104	42.91	26.74	30.35	28.09
HCT-116 cells (control)	0	51.88	36.17	11.95	1.97

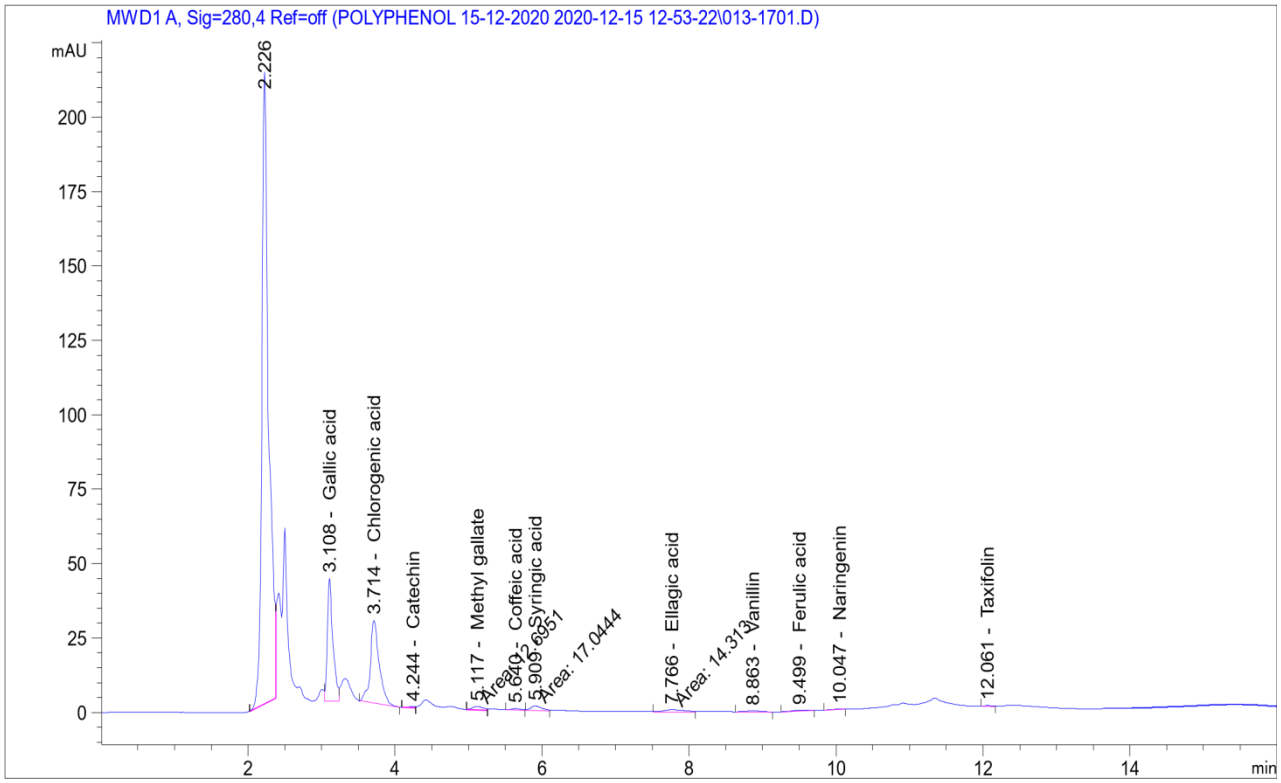
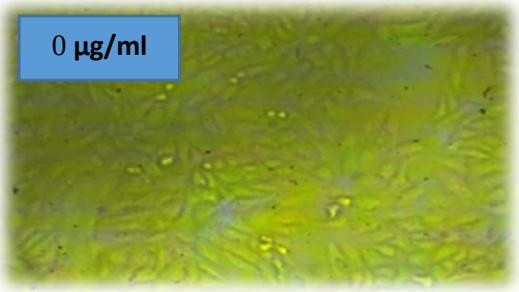


Figure 1: Phenolic Compounds in Red Beet Extract by HPLC



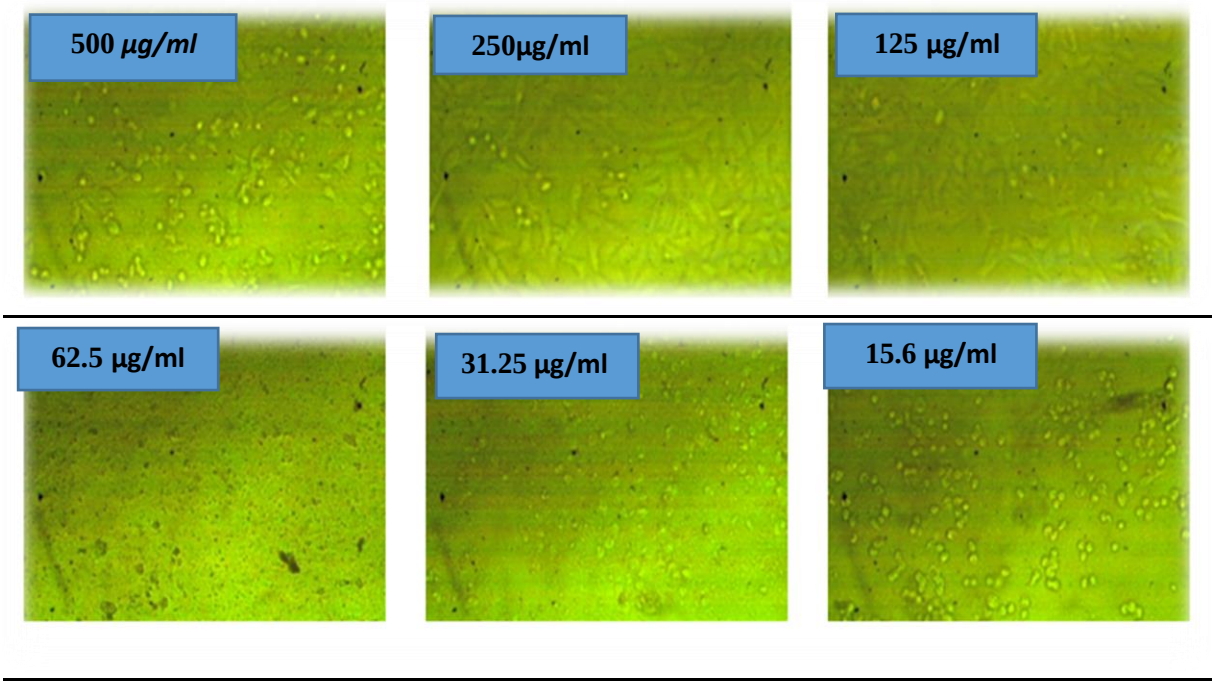


Figure 2: Effect of Tested Sample on Hct116 Cells at Different Concentrations:

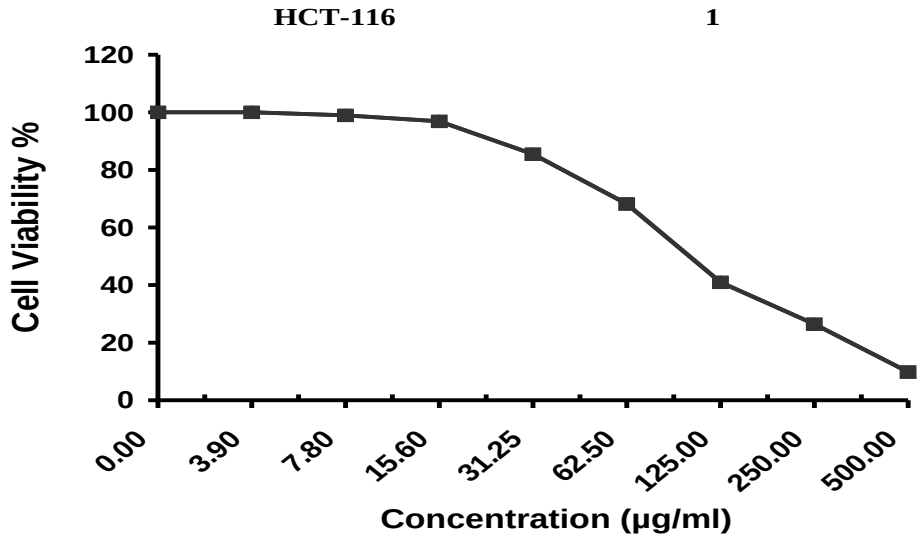
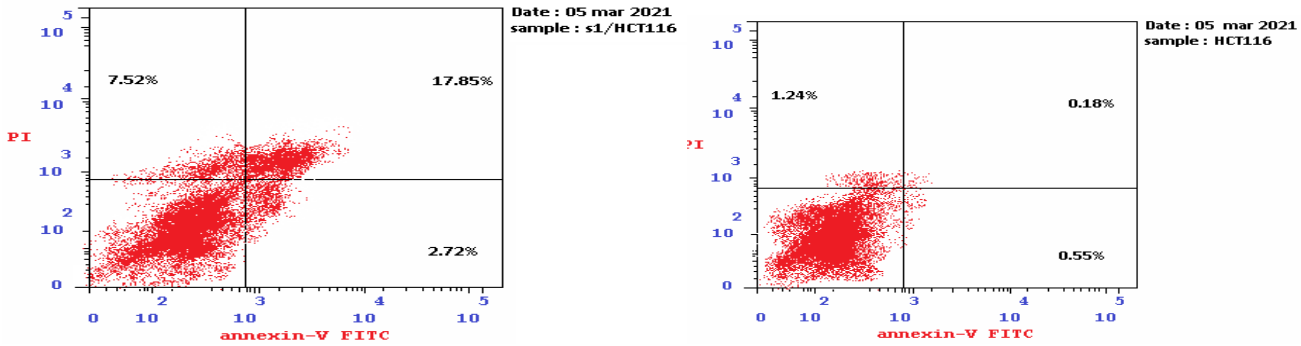


Figure 3: The relation between beet-root extract concentration and the level of HCT-116 cell viability.



HCT-116 Cells treated with extract HCT-116 Control (Non-treated cells)

Figure 4: Flowcytometric analysis of treated/untreated cancerous. Cells were treated with FITC Annexin V in combination with a PI to detect apoptosis and necrosis before being subjected to analysis by flow cytometry and early apoptosis, late apoptosis, and necrotic.

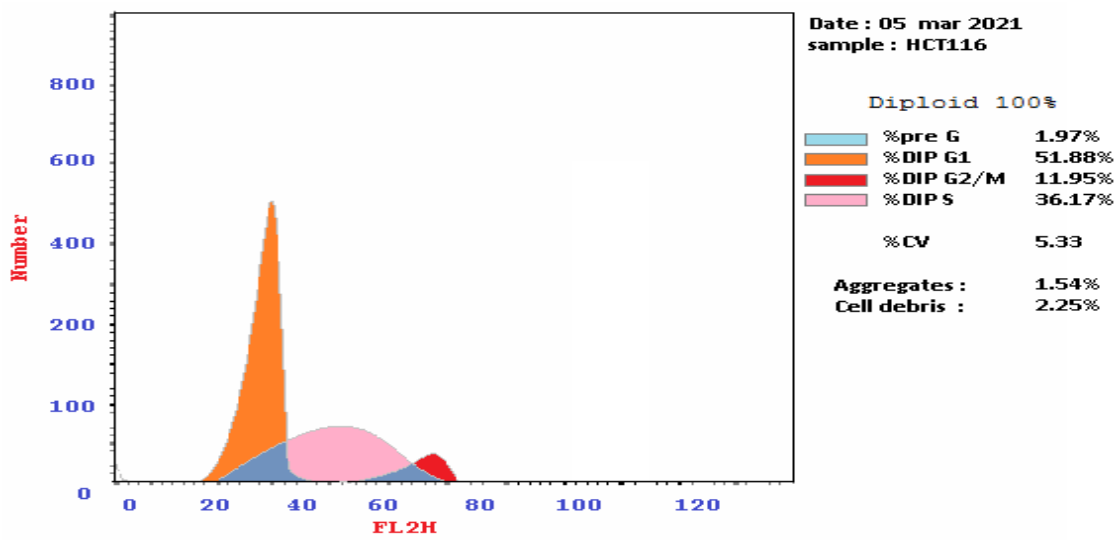


Figure 5: HCT-116 Control (Non-treated cells) showing cell cycle phase.

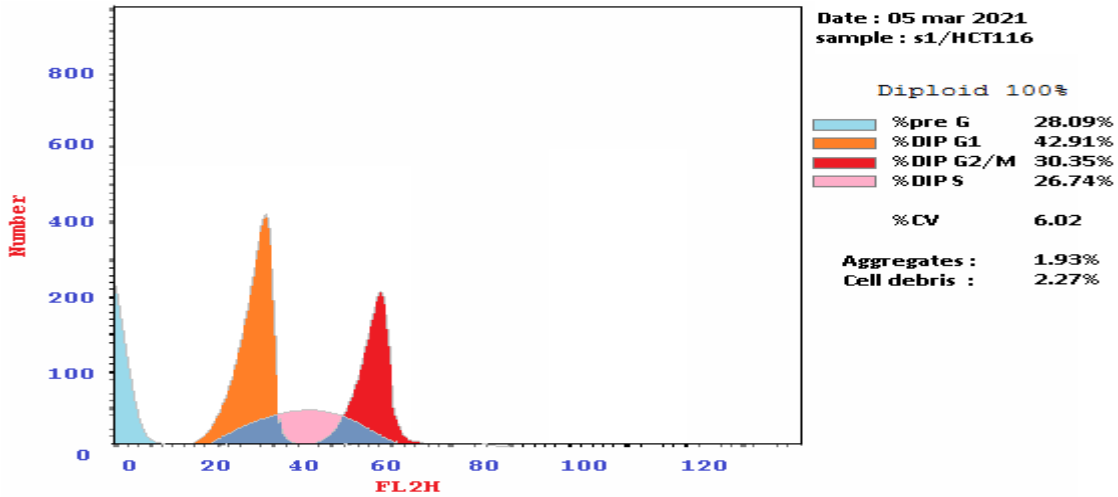


Figure 6: gHCT-116 Cells treated with sample showing changes in cell cycle phases

النشاط المضاد للخلايا السرطانية لمستخلصات درنات البنجر الأحمر (*Beta vulgaris L.*) (خلايا قولون الإنسان المسرطنة)

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الملخص العربي

يعتبر البنجر من أقوي 10 خضراوات كمصدر ممتاز للمركبات الفينتو التي لها نشاط بالغ الأهمية كمضاد للأكسدة ومضاد للسرطانالهدف من الدراسة:هو تحديد السمية الخلوية (MTT) لنشاط مستخلص درنات البنجر ضد خلايا القولون المسببة للاورام الطرق: تم الحصول علي مستخلص البنجرعن طريق الاستخلاص بالايثانول/الماء بنسبة (20/80) حجم/حجم كمذيب واستخدم البنجر / المذيب (1\ 3) وزن/حجم. كما تم الحصول علي خلايا سرطان القولون (HCT- 116) من وحدة زراعة الانسجة بمركزالفطريات. تم دراسة تأثير سمية المستخلص الإيثانولي لدرنات البنجر(الشمندر) عليخط خلايا القولون ثم تقييم آلية موت الخلايا المبرمج (Apoptotic)وتحليل دورة الخلية باستخدام جهاز التدفق الخلوي(الفلوسيتومتري Flow Cytometry). أوضحت نتائج دراسة السمية الخلوية (MTT) لمستخلص درنات البنجران له تأثير فعال ضد خلايا سرطان القولون حتى عند استخدامه بتركيزات منخفضة. وتم حساب النسبة المئوية المتوسطة لتثبيط خلايا سرطان القولون (IC50) وجدت 104±4.7 µg/ml تحت الظروف التجريبية.كما وجد أن مجموع النسب المئوية لموت الخلايا المبكر والمتاخر وأيضا كانت درجة تحلل الخلايا بعد المعالجة بمستخلص درنات الشمندر بالمقارنة بخلايا الكنترول (غير المعاملة بالمستخلص) 2.72٪ ، 17.85٪ ، 7.52 %، 0.55٪ ، 0.18٪ و 1.24٪ على التوالي بعد 48 ساعة في خط خلايا (HCT-116) تحت التجربة. وفي ضوء هذه النتائج تم التأكد من صحة وجود التأثير الفعال لمستخلص درنات البنجر على تحفيز موت الخلايا السرطانية المبرمج وأيضا كان له تأثير واضح في حث الاستماتة علي خط خلايا سرطان القولون. الخلاصة: توضح الدراسة الحالية أن معالجة خط خلايا سرطان القولون بواسطة مستخلص درنات البنجرالاحمر (الشمندر) أن له تأثير مثبط عالٍ علي تكاثر الخلايا كما أن له تأثير ملحوظ علي تنشيط موت الخلايا المبرمج و بالرغم من ذلك فان الية عمل مستخلص جذور البنجر علي مختلف خلايا السرطان مازالت غير معروفة وتحتاج لمزيد من الدراسات في هذا المجال.

الكلمات الاسترشادية: درنات البنجر، مستخلص البنجر، خلايا السرطانية، مواد طبيعة.