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Evaluation of Cytotoxic Effect of Betanin Nanoparticles against Squamous Cell Carcinoma Cell Line Compared to Doxorubicin

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ABSTRACT

Purpose: This study was aimed to evaluate the anticancer effect of nano sized betanin particles (betanin NP) on tongue squamous cell carcinoma cell line compared to doxorubicin (DOX) using cytotoxicity assay. **Materials and Methods:** Human tongue squamous cell carcinoma cell line (SCC 25) were cultured to obtain 3 groups, the first one was subjected to DOX and the second group was subjected betanin NP. The third group was not subjected to treatment and used as a control. Different concentrations of betanin NP and DOX were applied on SCC25 to choose the doses with high cytotoxic effects according to their IC50 using the methyl thiazolyl tetrazolium (MTT) viability assay at 48h and 72h intervals. **Results:** In the current study, IC50 doses for SCC 25 cell line was determined to be 0.91 ± 0.02 $\mu\text{g/ml}$, 0.37 ± 0.03 $\mu\text{g/ml}$ for DOX and 4.30 ± 0.08 $\mu\text{g/ml}$, 1.39 ± 0.07 $\mu\text{g/ml}$ for betanin NP at 48h and 72h intervals, respectively. T-test for comparison between IC50 level in different groups showed that there was highly statistically significant difference between samples of different materials ($p < 0.001$). These results indicate that higher doses of betanin are required to kill 50 % of the SCC25 cells in comparison to DOX doses. **Conclusion:** Our findings explained that DOX and betanin NP can induce cancer cell death against SCC 25 cell lines. DOX has higher cytotoxic effect than betanin NP according to MTT viability assay.

INTRODUCTION

Cancer is developing at a dangerous pace and its metastatic potential which is the main reason of death and remains the major challenge in treating cancer patient, accentuated the need for urgent efficient therapeutic approaches⁽¹⁾. Oral cancer is the sixth most common malignancy

KEYWORDS

Betanine NP; DOX; Cytotoxicity.

- Paper extracted from Master thesis titled "Evaluation of Anticancer Effect of Beet Root Nanoparticles against Squamous Cell Carcinoma Cell Line Compared to Doxorubicin"

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in the world and more than 90% of them are oral squamous cell carcinoma (SCC). Oral cancer may derive from any tissue in the mouth, but frequently it affects the tongue. Tongue squamous cell carcinoma (TSCC) is one of the most fatal cancers all over the world. It is relatively silent, and it can develop from a premalignant condition to reach to invasive carcinoma without any frightening symptoms. This causes delay in diagnosis and eventually leading to poor prognosis. In spite of the fact that the medical care has been extremely improved, the survival rate is still decreased with a disappointing cure percentage. At present, commonly used clinical treatment options for TSCC are surgery, chemotherapy, radiotherapy, and comprehensive treatment ^(2,3).

Chemotherapy has been the first line of treatment in many types of cancer for several years. One of the most effective of the Food and Drug Administration (FDA)-approved chemotherapeutic drugs is doxorubicin (DOX). DOX has been widely used in the last few decades for its usefulness in the management of different types of cancers, limited by its organotoxic potential (cardio-, hepato- and nephrotoxicity). Anticancer activity of DOX has been accredited to its potential to intercalate with both nuclear and mitochondrial DNA causing its inhibition of synthesis and damage ⁽⁴⁻⁶⁾. As a result, the use of DOX as a cytotoxic drug has some restrictions. These drawbacks made it very crucial to search for other agents that can decline the DOX induced side effects without influencing its antitumor effect ⁽⁷⁾.

Many medicinal plants have been used in prevention and treatment of cancer as an alternative way of treatment. These plants have less toxic effects on normal cells than chemotherapeutic drugs. Natural compounds isolated from these plants can act effectively on cancer cells. They are capable of inducing apoptosis or suppressing the proliferation of tumor cells. In addition, the combination therapy decreases side effects of traditional chemotherapy ^(8,9).

Red beetroot is a vegetable contains carbohydrates, fat, micro-nutrients, inorganic nitrate and

components that has bioactive properties. Beetroot has antihypertensive and anti-platelet aggregation effect due to the high inorganic nitrate content which has a fundamental role in conserving normal cardiovascular homeostasis with cytoprotective action and immunological defense. It is also showing high antioxidant and anti-inflammatory activities ⁽¹⁰⁾. Betalains which is the main component of red beets is used as natural coloring agent in lots of food industries as in dairy products, cattle products (cooked, smoked), beverages, and desserts. Betalains contain both red (betacyanins) and yellow pigments (betaxanthins) which are a class of natural antioxidant pigments that are highly bioavailable. The predominant betacyanins is betanin which represents between 75% and 90% of the total pigments of red beetroot ^(11,12).

Betanin as the main active phytochemical of beetroot is water-soluble nitrogenous compound which has several good biological effects including antioxidant, anti-inflammatory, hepatoprotective, and antitumor activities due to its aromatic amino compound moieties that are brilliant electron donors which can stabilize free radicals. It also affect the molecular mechanisms of transformed cells, by declining their growth level and augmenting apoptosis through increasing some apoptotic proteins and encourage alteration in the mitochondrial membrane potential, including both intrinsic and extrinsic apoptotic pathways ⁽¹³⁻¹⁵⁾.

Nanotechnology is one of the most essential fields for the progress of new applications in medicine. Nano sized particles that are extracted from a plant are materials which have one dimension less than 100 nm at least. Nanoparticles are very important as they enhance many characteristics of the material such as bioavailability, decreasing toxicity and side effects as it influences the circulation time, cellular uptake, bio distribution, and cancer drug delivery of nanoparticles ^(16,17).

Cell culture is the procedure by which human, animal, or insect cells are full-grown in a promising

artificial atmosphere. The original culture which is taken directly from an individual is called the primary culture and when diluted and transported into additional containers (a procedure which called subculture or passage), it becomes a cell line. Cell lines attained from vitro transformed cell lines or cancerous cells are undefined cell lines and can be grown in many forms monolayer or suspension. These cells split rapidly with a generation time of 12-14 hours and have the capacity to be sub cultured for an indefinite period. Unlimited cell lines are easy to operate and preserve. But, these cell lines have affinity to change over a period of time. Cell lines are very important because they are a renewable basis of cell material for replications many studies. Cancer cell lines can be obtained from many tumor tissues; they are very important materials which is used in cancer research. Oral SCC cell lines such as SCC4, SCC9, and SCC25 are commonly used to study tumorigenesis, numerous signal pathways intricate in the development and progression of oral cancer and in cell reaction to chemotherapy^(18, 19). So, the present study was performed to evaluate the anticancer effect of betanin NPs on squamous cell carcinoma cell line (SCC25) compared to DOX by measuring their cytotoxicity.

MATERIAL AND METHODS

Research Ethics Committee approval for Faculty of Dental Medicine for Girls Al-Azhar University was obtained (REC-PA-21-02).

Materials used in this study

1. Cell Line: cells of human tongue squamous cell carcinoma (SCC 25) were obtained from American Type Culture Collection (ATCC) through innovation lab, VACSERA, Cairo, Egypt.
2. Betanin: was purchased from Best Nutrition Product Inc. (California).
3. Doxorubicin: was purchased from Sigma Aldrich (USA).
4. Reagents for cell culture: Dulbecco's Modified Eagle Medium (DMEM), 10% Fetal Bovine Serum (FBS Hyclone), 10 ug/ml of insulin. 1% penicillin-streptomycin. 0.25% Trypsin EDTA solution and 100µl complete growth medium. Chemicals and reagents were obtained from Sigma Aldrich (USA).
5. Reagents for Methyl Thiazolyl Tetrazolium (MTT) viability assay: 15 mg/ml serum vial of methyl thiazolyl tetrazolium (MTT) (product # M-5655) and 125 ml MTT Solubilization Solution (10% Triton X-100 + 0.1 N HCl in anhydrous isopropanol). All of the reagents for MTT Cytotoxicity assay were obtained from Sigma Aldrich (USA).

Study design

SCC 25 cell line was cultured to obtain 3 groups, the first one was subjected to DOX and the second group was subjected betanin NP. Last group was not subjected to treatment and used as negative control. Doses of both DOX and betanin NP were determined using the MTT viability assay to calculate their IC50 value

Preparation of betanin NPs

Nanoparticles were prepared at National research center, Egypt. The plant extract was utilized to cultivate nano-suspension with calcium chloride and poly acrylic acid (PAA). PAA solution was used at a concentration of 0.05% (in water) with NaOH to pH 8 and 0.1% calcium chloride solution in water. The stability of nanoparticles was noticed for 5 days in term of color, turbidity and sediment. Evaluation of nanoparticle included particles size (400-500nm) using a Malvern Particle sizer and examination of the zeta potential uses a Malvern zeta potential measuring device.

Culturing and sub culturing procedure for cell line

Cells were cultured using DMEM supplemented with 10% FBS (Hyclone.), 10 ug/ml of insulin

and 1% penicillin-streptomycin at Tissue Culture Laboratory of the Research and Development (R&D) sector, VACSERA, Cairo, Egypt. Before the MTT assay procedure, plate cells for 24 hours in a volume of 100 μ l complete growth medium + 100 μ l of the tested material per well in a 96-well plate. The cultures were incubated at 37°C for 24h. After treatment of SCC-25 cells with the serial concentrations of the DOX, betanin NP drug (100, 25, 6.25, 1.56 and 0.39 μ g/ml). Incubation is carried out for 48h and 72h at 37°C, then the plates are to be examined under the inverted microscope and proceed for the MTT assay.

Measurement of the cell viability by MTT viability assay

By definition, MTT assay is a colorimetric assay that uses reduction of a yellow tetrazolium salt (or MTT reagent) to measure cellular metabolic activity, in order to reduce the MTT reagent to formazan (an insoluble crystalline product with a profound purple color) cells should be energetic and embrace NADPH- dependent oxidoreductase enzymes. Then solubilizing solution was used to dissolve formazan crystals. The absorbance is measured at 500-600 nanometers with a plate-reader. As the solution gets darker it indicates a high number of metabolically active cells ⁽²⁰⁾.

Experimental media were removed, and cells were washed in PBS. After treatment of cells with antibiotic and antifungal for 24-72h. DOX and betanin NP were separately added for cell line with serial dilution (100 mg/ml, 25 mg/ml, 6.25 mg/ml, 1.56mg/ml, and 0.39 mg/ml) incubated for 48h then 72h, respectively and then washed by PBS twice. Cells were continuously examined under the inverted phase microscope. The medium was aspirated, and the formazan product was solubilized with MTT Solubilization Solution (**Fig.1**). Absorbance at 570 nm was measured for each well using (ROBONIK P2000 Eia reader). The results were translated and the half maximal inhibitory concentration (IC₅₀) which is defined as the concentration of drug at which 50% of your target is inhibited was calculated.

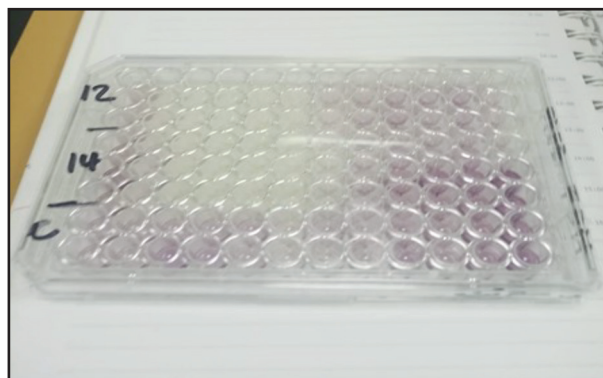


Figure (1) MTT plates showing variations in color intensity according to cell viability.

Statistical analysis

All data were investigated using the statistical package for social sciences, version 20.0 (SPSS Inc., Chicago, Illinois, USA). A one-way analysis of variance (ANOVA) test was used when comparing between more than two means. For multiple comparisons between different variables Tukey's test (t-test) was used. The P value, if less than or equal to 0.05 ($P \leq 0.05$), was considered significant.

RESULTS

I- MTT cytotoxicity assay (Cell viability percentage)

Different concentrations of betanin NP and DOX were applied on SCC25 to choose the doses with high cytotoxic effects according to their IC₅₀ values. IC₅₀ values that were found in betanin NP samples are (4.30 \pm 0.08) and (1.39 \pm 0.07) and those of DOX samples are (0.91 \pm 0.02) and (0.37 \pm 0.03) at 48h and 72h respectively (**Fig.2**).

At 48h and 72h, There was highly statistically significant difference between samples of different materials ($p < 0.001$). The highest IC₅₀ values were found in betanin NP samples (4.30 \pm 0.08) and (1.39 \pm 0.07) μ g/ml followed by DOX samples (0.91 \pm 0.02) and (0.37 \pm 0.03) μ g/ml at 48h and 72h respectively. IC₅₀ of betanin NP is higher than DOX at 48 h which means higher dose of betanin

is required to kill 50 % of the SCC25 cells, while DOX requires fewer doses to kill 50% of SCC25 cells. As time passing, at 72h, the dose required for both materials become less but still betanin NP is higher than DOX samples (Table1).

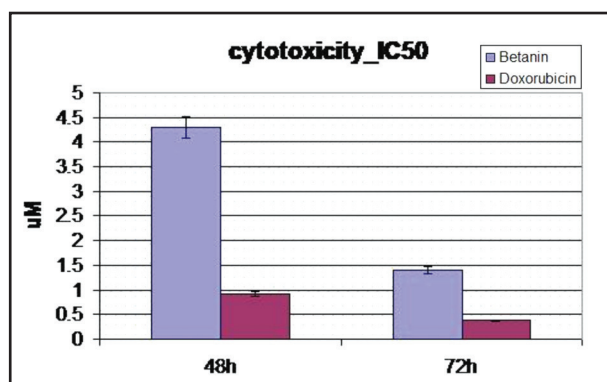


Figure (2) Bar chart showing the different IC₅₀ values of betanin NP and DOX at 48h and 72h intervals.

Table (1) Statistical results using *t*-test for comparison between IC₅₀ level in different groups at 48h and 72h intervals.

	Sample code	M.W g/mol	Cytotoxicity		
			SCC25		
			IC50 µg/ml		
			48h	72h	<i>p-value</i>
1	Betanin NP	550.47	4.30 ±0.08	1.39 ±0.07	<0.001**
2	Doxorubicin	543.52	0.91 ±0.02	0.37 ±0.03	<0.001**
p-value			<0.001**	<0.001**	

***p*-value <0.001 highly significant

Comparison between control and different concentrations of betanin NP and DOX at 48h:

Regarding the cell viability of all studied concentrations after 48h, ANOVA test revealed that there was highly statistically significant difference between different concentrations of betanin NP

and DOX ($P < 0.001$). 100 μm DOX concentration showed the statistically significant least mean cell viability (0.12), while the highest mean cell viability was noted in the control group (0.60) followed by 0.39 μm betanin NP concentration with (0.38) mean cell viability. While, there was no statistically significant difference noted between some concentrations of DOX and betanin NP such as (25 μm) of DOX and (100 μm) betanin NP, (1.56 μm) of DOX and (6.25 μm) betanin NP, (0.39 μm) of DOX and (1.56 μm) betanin NP (Fig.3).

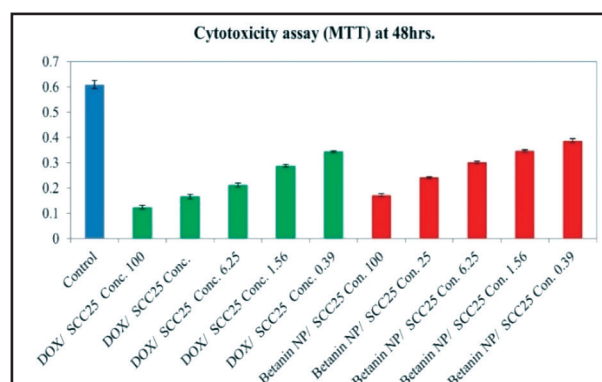


Figure (3) Bar chart compare between control and different concentrations of betanin NP and DOX according to MTT cytotoxicity assay at 48h.

Comparison between control and different concentrations of betanin NP and DOX at 72h:

Regarding mean cell viability of all studied concentrations after 72h, ANOVA test revealed that there was highly statistically significant differences between different concentrations of betanin NP and DOX ($P < 0.001$). 100 μm DOX concentration showed the statistically significant least mean cell viability (0.073). While, the highest mean viability was noted in the control group (0.61) followed by 0.39 μm betanin NP concentration with (0.36) mean cell viability. While, there was no statistically significant difference noted between some concentrations of DOX and betanin NP such as (25 μm) of DOX and (100 μm) betanin NP, (1.56 μm) of DOX and (6.25 μm) betanin NP, (0.39 μm) of DOX and (1.56 μm) betanin NP (Fig.4).

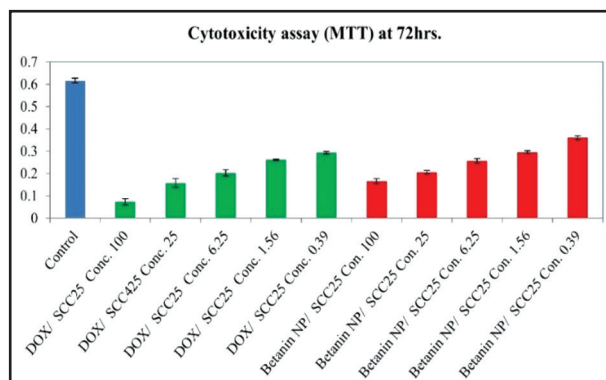


Figure (4) Bar chart compare between control and different concentrations of betanin NP and DOX according to MTT cytotoxicity assay at 72h.

DISCUSSION

Oral cancer is very frequent cancer among other types of cancers with more than 90% of them is oral squamous cell carcinoma (OSCC) ⁽²⁾. For many years, DOX has been widely used for its ability to reduce rapidly proliferating cells and suppress cancer progression, limited only by its side effects on normal cells ^(21,22). So, to overcome chemotherapeutic side effects of DOX, in this study we evaluated the effect of betanin NPs (beetroot extract) on SCC25 in trial to be used as anticancer treatment with minimal cytotoxic effect on normal cells. Three groups of SCC25 cell line were included, one control group without drug treatment and two groups were treated with different doses of betanin NP and DOX. In addition, all the previously mentioned groups were evaluated after 48 and 72 hours.

MTT cytotoxicity assay was performed to choose the doses of betanin NP and DOX with high cytotoxic effects according to their IC₅₀ values. The MTT assay technology that has been used for evaluation of the viability of the treated cells is a widely accepted technique and is very popular as a way of valuing the number of viable cells ⁽²³⁾.

In the present study, we found that there was highly statistically significant difference between samples of different materials ($p < 0.001$). The highest

IC₅₀ values were found in betanin NP samples (4.30 ± 0.08) and (1.39 ± 0.07) $\mu\text{g/ml}$ followed by DOX samples (0.91 ± 0.02) and (0.37 ± 0.03) $\mu\text{g/ml}$ at 48h and 72h respectively. IC₅₀ of betanin NP is higher than DOX at 48 h which means higher dose of betanin is required to kill 50 % of the SCC25 cells, while DOX requires fewer doses to kill 50% of SCC25 cells. As time passing, at 72h, the dose required for both materials become less but still betanin NP group is higher than DOX group. This could be explained by the simple fact that DOX is highly cytotoxic in nature by producing direct destruction effect on DNA and therefore is more effective at lower concentrations in inducing apoptosis compared to betanin NP ⁽²⁴⁾.

Our results were opposite to previous study which found that the MTT cytotoxicity assay of beet root NP and silica-beet root NP had clear cytotoxicity on SSC-090 tongue cancer cell lines at low significant IC₅₀ value as $0.73 \mu\text{g/ml}$ and $2.94 \mu\text{g/ml}$ respectively after incubation period 48h. While 5 Fluorouracil (5FU) drug showed evident cytotoxicity on SSC-090 cancer cell lines at higher value as IC₅₀ $8.83 \mu\text{g/ml}$ after the same incubation period. These results signify that low doses of beet root NP may apply notable cell death on tongue cancer cells line SCC-090 and contemplate beet root plant as pharmaceutical active agent since the US Food and Drug Administration (FDA) and USA national cancer institute considered that plant extract with IC₅₀ values $\leq 20 \mu\text{g/ml}$ after incubation between 48h and 72h are considered pharmaceutically active agent and can be used as a mark for appropriate screening cancer drugs conducted from plants and herbs ⁽²⁵⁾.

Another study in which they used 2 colorectal cancer cell lines (HT-29 and Caco-2) with the IC₅₀s of betanin were determined after treatment with different doses (20 to 140 $\mu\text{g/ml}$) in two time points at 24 and 48 h, they found that IC₅₀ doses of betanin were $64 \mu\text{g/ml}$ and $90 \mu\text{g/ml}$ at 48h, respectively. Their outcome showed that betanin suggestively can impede the growth of cancerous cells by stimulation of apoptosis with time and dose-

dependently (with increasing concentrations from 40 to 100 $\mu\text{g/ml}$) without apparent unfavorable effects on KDR/293 normal cells as a control group. Also, constructed on some preceding studies it can be hypothesized that the betacyanins and betanin produce chemotherapeutic and antiproliferative activities via their antioxidative activity and lower the level of reactive oxygen species to minimum level that can't stimulate proliferation by inappropriate signal transduction in that levels ⁽¹⁴⁾.

Actually, various in-vitro and in-vivo researchs suggested that betacyanins, isobetanin and betanin as the main component of red beetroot, reduce cancer cell proliferation with different IC50s and retain anti-inflammatory, hepatoprotective, radioprotective, neuroprotective, diuretic, hypolipidemic, osteoarthritis pain reliever and anti-diabetic effects in diverse doses and time-points ^(14,26).

Regarding the cell viability of all studied concentrations at 48h and 72h in the current study, ANOVA test revealed that there was highly statistically significant difference between different concentrations of betanin NP and DOX ($P < 0.001$). 100 μm DOX concentration showed the statistically significant least mean cell viability (0.12 and 0.073) $\mu\text{g/ml}$, while the highest mean cell viability was noted in the control group (0.60 and 0.61) $\mu\text{g/ml}$ followed by 0.39 μm betanin NP concentration with (0.38 and 0.36) $\mu\text{g/ml}$ mean cell viability, respectively. The least cell viability mean that was noted with 100 μm concentration of DOX at 48h and 72h is recognized to the strong cytotoxic effect of DOX. Where DOX intercalates into the DNA helix and attach covalently to proteins involved in DNA replication and transcription thus contributing to its increased apoptosis and then cytotoxicity ⁽²⁷⁾.

Although DOX was usually exhibited statistically significant difference than betanin NP in our study which may be due to the strong cytotoxic effect of DOX as mentioned above, there was no statistically significant difference noted between some concentrations of DOX and betanin NP such as (25

μm) of DOX and (100 μm) betanin NP, (1.56 μm) of DOX and (6.25 μm) betanin NP, (0.39 μm) of DOX and (1.56 μm) betanin NP at both 48h and 72h. The non-significant difference between DOX and betanin NP may be explained by study showed that the role of betanin as anticancer agent was through inhibition of reactive oxygen species (ROS) formation as the chemical structure of betanin comprises phenolic and cyclic amine groups, which delivers suitable electron donors with a high free radical scavenging property. Betanin is also a potent inducer of Glutathione which proposes an intense antioxidant property to counter act the oxidative hazards ⁽²⁸⁾.

An exciting observation showed by the betanin red beetroot extract is that there is a remarkable similarity in the chemical structure and configuration with the chemotherapeutic agent doxorubicin, both have a planar aromatic chromophore and a six-membered sugar molecule which produce DNA intercalation in cancer cells lead to cancer cell death. This proposes that betanin may play a main role in the observed cytotoxic effect of the red beetroot extract through a possible mechanism of action common with doxorubicin and correlated anthracycline chemotherapeutic drugs ⁽²⁵⁾.

Also, using betanin as NP in the current study augments its characterization as betanin is highly degradable material so will be more stable if used as nanoparticles. Converting the material into NP has been used in a wide range nowadays especially in vivo in order to limit the access of the material to a selective site and to deliver it into the site of action in a sustained release and a controlled way ⁽²⁹⁾.

CONCLUSION

Based on the results of the present study, it could be concluded that: Betanin NP and DOX have a cytotoxic effect on SCC cell line. DOX has higher cytotoxic effect than betanin NP according to MTT viability assay. They could be capable of inducing cancer cell death against oral tongue carcinoma SCC 25 cell lines in dose and time dependent.

RECOMMENDATIONS

1. Further studies should be performed to work against more cancer cell lines for the possible use of betanin as chemotherapy in the future.
2. Further investigations should be done to use low dose of DOX instead of high dose to avoid the toxic side effects of DOX.
3. Further investigations should be done to use combination of DOX and natural phytochemical which can reduce the toxicity of DOX.

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