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# Evaluation of Apoptotic Effect of Betanin Nanoparticles Against Squamous Cell Carcinoma Cell Line Compared to Doxorubicin

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ABSTRACT

***Aim:*** to evaluate the anticancer effect of nano sized betanine particles (betanine NP) on squamous cell carcinoma cell line compared to doxorubicin (DOX) by measuring apoptosis through caspase 3. ***Material and Methods:*** Three groups of the human tongue squamous cell carcinoma cell line (SCC 25) were created, and two of them were treated with DOX and betanine NP. The third group served as a negative control and was not given any treatment. To evaluate the concentration of caspase 3 using the EIISA technology, different quantities of betanin NP and DOX were administered to SCC25 at 48- and 72-hour intervals. ***Results:*** Caspase 3 levels in the current investigation were 501.69.93, 543.86.71 pg/ml for DOX and 336.913.1, 405.53.5, and respectively for betanine NP in 48h and 72h intervals. Additionally, morphological analysis was performed to demonstrate the cells' apoptotic alterations. There was a highly statistically significant difference between samples of various materials, as revealed by the ANOVA test for Comparison between groups using the ELISA technique for caspase 3 detection at 48h and 72h (p0.001). These findings suggest that DOX has a stronger apoptotic effect on SCC25 cells than betanin NP. ***Conclusion:*** Our findings explained that DOX and betanine NP can induce cancer cell death against SCC 25 cell lines by increasing the concentration of caspase 3. DOX has higher apoptotic effect on SCC25 cells than betanin NP according to ELISA technique.

## 1. INTRODUCTION

The rate of growth of cancer is alarming. It is regarded as one of the major global causes of death [(1)]. One of the most prevalent malignant tumours in the world is oral cancer. The type of oral cancer that affects the tongue frequently is oral squamous cell carcinoma (SCC)[(2,3)].Chemotherapy has been the preferred method of treating cancer for a long time [(4)]. Doxorubicin (DOX), a potent anti-cancer drug, is effective against OSCC and other cancer forms [(5)]. Both the cancer cell's mitochondrial DNA and nuclear DNA can intercalate with DOX,by attaching to plasma proteins, it also directly disrupts the cell membrane. The cytotoxic effect of Dox on normal cells is its primary drawback, hence it is crucial to seek for additional medications that lessen its negative effects [(6)].

Cancer cells can be effectively treated by phytochemicals without harming healthy cells. They have anticancer qualities, including actions that promote apoptosis and have anti-proliferative and anti-angiogenic effects[(7)].

Due to its significant antioxidant and anti-inflammatory effects, red beetroot is regarded as one of the most powerful phytochemicals. Vegetable red beetroot contains inorganic nitrate, lipids, micronutrients, and other elements with biological properties[(8)].Consuming beetroot juice can assist cancer patients have better outcomes since it may keep their lean body mass after chemotherapy. Previous research demonstrated that betanins or beetroot juice enhance the cytotoxic effects of chemotherapy on cancer cells [(9)].

The primary active phytochemical in beetroot is betanin, a water-soluble nitrogenous molecule that accounts for between 75% and 95% of the red beet colours. It is a strong anti-inflammatory, antioxidant, and chemopreventive agent. Different processes, including stabilising free radicals (ROS), triggering apoptosis, reducing cell proliferation, angiogenesis, and inflammation of cancer cells, are thought to be responsible for betanine's anticancer properties[(10)].

Nanotechnology a rapidly expanding field related to nanoparticles it is one of the most important topics for the development of novel medical applications. Due to their special qualities, such as their ability to target cancer cells with barely detectable side effects, nanoparticles with sizes ranging from 1 to 100 nm are very important. NPS can be divided into a variety of groups based on their shapes, sizes, and characteristics. Due to its ease of production, the spherical form of nanoparticles has recently been used most frequently. There are several different types of nanoparticles, including polymeric, metal, and ceramic NPs [(11,12)].

Apoptosis is characterised as a precise form of programmed cell death that eliminates damaged cells via precisely controlled genes. Any slight flaw in this system has the potential to cause autoimmune disorders or cancer.

Suppression of apoptosis in the process of carcinogenesis plays a crucial role in the formation and progression of several forms of cancer. Therefore, in malignant cells, this specific mechanism is stimulated by either the mitochondrial intrinsic or extrinsic routes controlled by caspase[13,14] .

There will be certain morphological changes in the cell as a result of stimulation of the major caspases in extrinsic and intrinsic pathways, including membrane blebbing, cell shrinkage, chromatin condensation, and chromosomal DNA fragmentation. The activation of several useful genes and pro/anti-apoptotic proteins, which are activated by naturally occurring anticancer chemicals collected from medicinal plants, regulates cell survival and apoptosis [14,15].

Therefore, the present study was performed to evaluate the anticancer effect of betanine NPs on tongue squamous cell carcinoma cell line (SCC25) compared to DOX by measuring apoptosis.

## 2. MATERIAL AND METHODS

### 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, Egypt.
2. Betanine: was purchased from Best Nutrition Product Inc. (California).
3. Doxorubicin: was purchased from Sigma Aldrich (USA).
4. Caspase 3: ( 96 Test Kit) were obtained from VACSERA, Cairo, Egypt.

### Experimental groups:

**Group 1:** Control group without treatment.

**Group 2:** Doxorubicin.

**Group 3:** Betanin NP.

**Table 1:**

Summary of study design

| Study group | Control | DOX | Betanin NP |
|---|---|---|---|
| SCC 25 | Without treatment | Exposed to DOX | Exposed to betanin NP |
| | | All concentrations done at 48h,72h | All concentrations done at 48h,72h |
| Apoptotic analysis | Quantitative Sandwich immunoassay- ELISA technique for detection of caspase 3 | | |

### Preparation of betanin NPs

The National Research Center in Egypt developed nanoparticles.. To create nano-suspension with calcium chloride and polyacrylic acid (PAA). Plant extract was used with NaOH to a pH of 8 and 0.1 with calcium chloride solution in water, PAA solution at a concentration of 0.05 percent was utilised. In terms of colour, turbidity, and sediment, the stability of nanoparticles was observed over 5 days. Malvern Particle Sizer was used to measure the size of the nanoparticles (400–500 nm), and Malvern Zeta Potential Measurement Device was used to examine the zeta potential.

### ELISA technique for detection of caspase 3:

Enzyme linked immunosorbent assay (ELISA) is a technique used to detect and quantify the level of active caspase-3 protein.

### Principle of the Method

- The wells of the microtiter strips have been coated with a monoclonal antibody that is specifically employed for caspase-3. A rabbit antibody that is specific for caspase-3 is then added to the wells of microtiter strips together with samples such as a standard that contains human active caspase-3, control samples, and unknowns. The immobilised (capture) antibody binds to the caspase-3 protein during the first incubation, and the specific caspase-3 antibody acts as a detection antibody by attaching to the immobilised caspase-3 protein.
- A horseradish peroxidase-labeled Anti-Rabbit IgG (Anti-Rabbit IgG HRP) is added after the initial incubation phase and washing to get rid of extra protein and detecting antibody. To complete the four-member sandwich, this binds to the detecting antibody.
- A substrate solution is added after the third incubation and washing to get rid of all the extra Anti-Rabbit IgG HRP, and the attached enzyme reacts with it to produce colour. The amount of caspase-3 present in the original specimen directly correlates with the intensity of this coloured product.
- Betanin NP and DOX were individually added to the wells with the previously estimated IC50 concentration and incubated for 48 and 72 hours, respectively. The microtiter strips were then injected into the ELISA reader to provide readings [16].

### Statistical analysis

The statistical software for social sciences, version 20.0, was used to analyse all the data (SPSS Inc., Chicago, Illinois, USA). When comparing more than two means, a one-way analysis of variance (ANOVA) test was applied. Tukey's test (t-test) was employed for multiple comparisons between various variables. The P value was deemed significant if it was less than or equal to 0.05 (P 0.05).RESULTS

### I- Morphological assessment:

The cells were checked on a regular basis under the Inverted phase microscope to ensure their viability. Cultured cells of the SCC cell line showed signs of dysplasia as nuclear and cellular pleomorphism and increased mitosis. The cancer cells displayed features of apoptosis following treatment with betanin NP and DOX these include shrinkage, DNA fragmentation ladder, cell membrane blebbing and Margination of chromatin(**Fig. 1-5**).

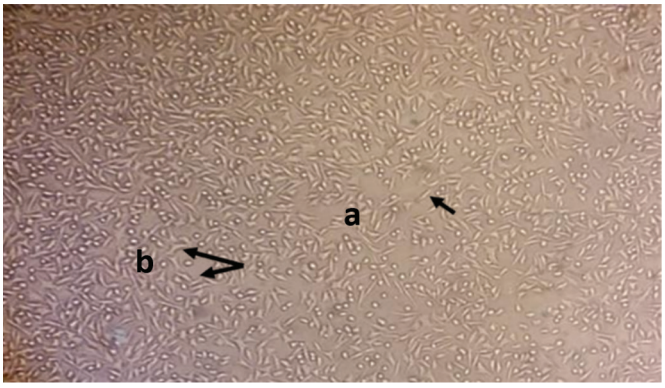


**Figure (1) —Phase contrast inverted microscope photomicrograph of SCC 25 cells of the control group showing high number of angular fusiform (a) and spindle shape cells (b) (original magnification X10).**

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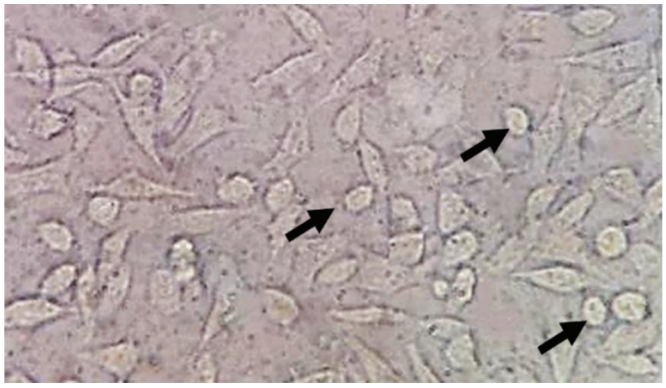

**Figure (2) —Phase contrast inverted microscope photomicrograph showing SCC 25 after addition of betanin NP at 48h with many cells undergoing apoptosis (arrows) (original magnification X40).**

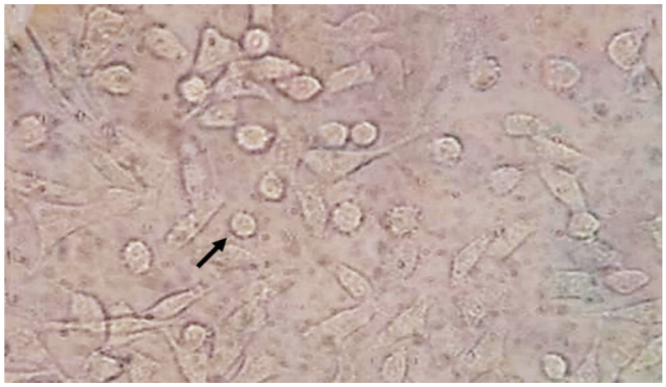

**Figure (3) — Phase contrast inverted microscope photomicrograph of SCC 25 showing many cells undergoing apoptosis with change in cell morphology like membrane blebbing (arrow) after addition of betanin NP at 72h (original magnification X40).**

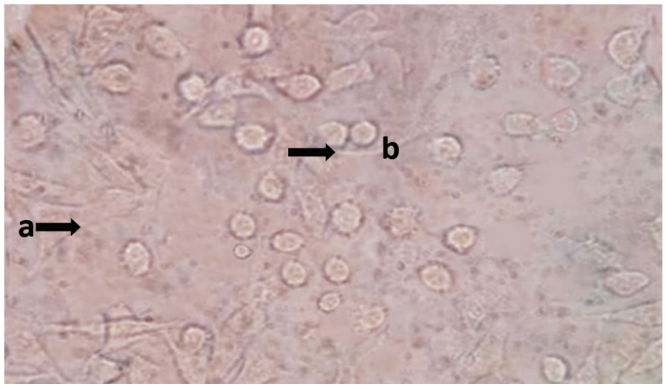


**Figure (4) — Phase contrast inverted microscope photomicrograph of SCC 25 showing marked decrease in number of viable cells (a) after addition of DOX at 48h with many cells undergoing apoptosis (b) (original magnification X40)**

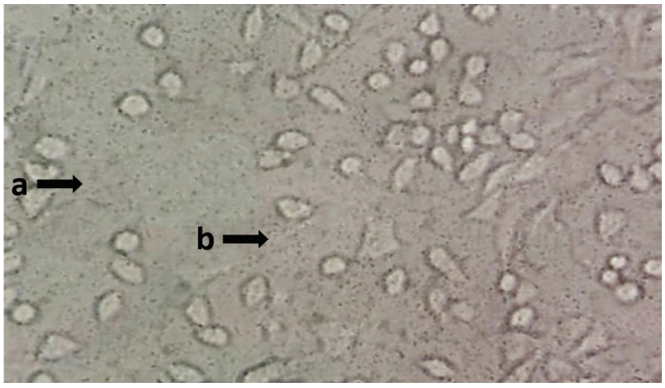


**Figure (5) —Phase contrast inverted microscope photomicrograph of SCC 25 showing marked decrease in number of viable cells (a) and change in cell morphology after addition of DOX at 72h with many cells undergoing apoptosis (b) (original magnification X40).**

### II- Results of ELISA technique for detection of caspase 3

***Comparison of caspase3 concentration between control, DOX and betanine NP groups at 48h:***

ANOVA test revealed that there was a highly statistically significant differences among the studied groups (P<0.001). The statistically significant highest concentration was noted in the DOX group (501.5) followed by betanine NP (336.9) while the statistically significant least concentration was noted in the control group (41.9) **(Table. 2) (Fig.6).**

**Table (2):**

Comparison between groups according to ELISA technique for detection of caspase 3 at 48h using ANOVA test.

| Caspase 3 at 48hrs. | Concentration | |
|---|---|---|
| | **Mean +/- SD** | **Range** |
| **Control/ SCC25** | 41.960**C**±10.663 | 30.630-51.800 |
| **Doxorubicin/ SCC25** | 501.533**A**±9.945 | 493.100-512.500 |
| **Betanin NP/ SCC25** | 336.867**B**±13.123 | 324.900-350.900 |
| **ANOVA** | 1267.99 | |
| **p-value** | <0.001** | |

***Highly significant p-value, Different letters indicate significant difference at (p<0.05).*

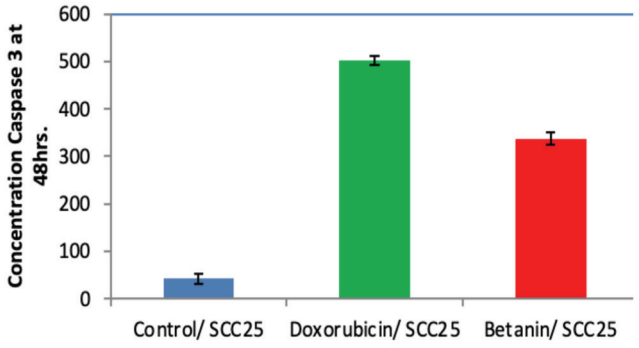


**Figure (6) — Bar chart between groups according to ELISA technique for detection of caspase 3 at 48h.**

***Comparison of caspase3 concentration between control, DOX and betanine NP groups at 72h:***

ANOVA test revealed that there was a highly statistically significant differences among the studied groups (P<0.001). The statistically significant highest concentration was noted in the DOX group (543.8) followed by betanine NP (405.5) while the statistically significant least concentration was noted in the control group (25.1) **(Table 3) (Fig.7).**

**Table (3):**

Comparison between groups according to ELISA technique for detection of caspase 3 at 72husing ANOVA test.

| Caspase 3 at 72 hrs. | Concentration | |
|---|---|---|
| | Mean +/- SD | Range |
| **Control/ SCC25** | 25.133**C**±1.372 | 23.880-26.600 |
| **Doxorubicin/ SCC25** | 543.800**A**±6.720 | 536.800-550.200 |
| **Betanin NP/ SCC25** | 405.533**B**±3.485 | 401.800-408.700 |
| **ANOVA** | 10969.55 | |
| **p-value** | **<0.001**** | |

***Highly significant p-value, Different letters indicate significant difference at (p<0.05).*

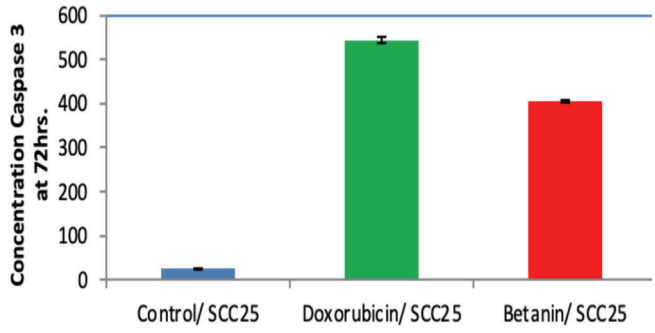


**Figure (7) — Bar chart between groups according to ELISA technique for detection of caspase 3 at 72h.**

## 5. DISCUSSION

The majority of tongue cancers, which account for 30 to 50 percent of all oral cancers, are one of the most common types of cancer. The most typical form of tongue cancer is thought to be squamous cell carcinoma of the tongue (TSCC). Unfortunately, the prognosis for (TSCC) is poor due to early metastases [17].Due to its potent cytotoxic impact in treating various cancers, DOX has been used extensively as a chemotherapeutic drug for many years. Its sole drawback is that it is poisonous to healthy tissues [18].

Thus we tried in this study to evaluate the apoptotic effect of btanin NPs (beetroot extract) on SCC25 in a trial to be used as anticancer treatment with minimal cytotoxic effect on normal cells. Three groups of SCC25 cell line were included, two groups were treated with different doses of betanin NP and DOX. One control group without drug treatment. In addition, all the previously mentioned groups were evaluated after 48 and 72 hours.

**In our study** we measure the concentration of caspase 3 to evaluate the apoptotic effect of both DOX and betanin NP in comparison to the control group. Considering that the caspase 3 enzyme belongs to the endoprotease family, which regulates the signalling networks that cause inflammation and apoptosis. Through mediating the breakdown of cellular components like DNA fragmentation or cytoskeletal protein degradation, caspase 3 plays a crucial role in apoptosis. Due to the function it plays during apoptosis, it is also known as an executioner caspase [19,20].

**Regarding the concentration of caspase 3 in this study** measured by ELISA technique in different groups treated with betanin NP and DOX as well as control group at 48h and 72h, the highest caspase 3 expression level was present with DOX group (501.6±9.93 and 543.8±6.71) followed by betanin NP group (336.9±13.1 and 405.5±3.5) while the least caspase 3 concentration was observed with the control group (41.96±10.7 and 25.1±3.7) at 48h and 72h respectively. While, the betanin NP revealed lower value of caspase 3 compared to DOX group. However, betanin NP produced also a significant apoptotic effect in comparison to nontreated cancer cells (control group) at both 48h and 72h intervals.

Our results is consistent with the study that concluded the entire percentages of early and late apoptosis ratio after treatment of colorectal adenocarcinoma cell lines with beetroot hydro-alcoholic extract and betanin for 48 hours were found to be 81.7%, 91%, and 68.2%, 72.1% respectively. While in control group the apoptosis ratio was about 21.5% and 38% respectively[21].

An earlier work on lung cancer cell lines describes the apoptotic effect of betanin NP. This study revealed significant activation of caspase 9 and effectors (caspases 3 and 7) in lung cancer cells treated with 0.4 g/ml betanin for 48 hours**.** [22].

It may also be explained by a different study in which betanin/isobetanin mixture treatment significantly reduced the viability of the (MCF-7) breast cancer cell line. The effect of this mixture in apoptosis was addressed in detail in the same study since it significantly enhanced apoptotic-related proteins like Bad, TRAILR4, FAS, phosphorylated p53, and altered mitochondrial membrane potential. Therefore, based on the facts previously discussed, they propose that the betanin/isobetanin concentrate treatment of MCF-7 cells resulted in a p53-dependent response and the participation of both the mitochondrial and death-receptor pathways [23].

Betanin is a highly degradable substance, thus employing it as NP in the current work enhances its characterisation because it will be more stable. In order to restrict the content's access to a specific site and deliver it into the site of action in a sustained release and regulated manner, the material has increasingly been converted into NP [24].

**Regarding the control group in measuring the concentration of caspase 3 in the present study,** we noticed that concentration of caspase 3 at 48 h was 41.9 while it decreased at 72 h to become 25.1. This may be attributed to the fact that cancer cells can produce extreme amounts of anti-apoptotic proteins such as Bcl-2, Bcl-XL which shorten the extrinsic death receptor apoptotic pathway[25]. **Additionally,** when the expression of caspase 3 was evaluated in another study, it was shown that breast cancer cells had lost their ability to express caspase 3, which may indicate a crucial mechanism for cell survival and chemoresistance. In over 75% of breast cancer samples, there was no caspase 3 transcript and little caspase 3 protein expression [26].

**In the current study,** ANOVA test revealed that there was a highly statistically significant differences among the studied groups (P<0.001) at both 48h and 72h. The statistically significant highest concentration was noted in the DOX group (501.5 and 543.8) followed by betanin NP (336.9 and 405.5) while the statistically significant least concentration was noted in the control group (41.9 and 25.1). DOX revealed significant higher apoptotic effect than betanine NP at lower significant IC50 value. This is in line with a previous study that discovered DOX causes apoptosis by activating caspases and disrupting the potential of the mitochondrial membrane. Another study

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revealed that the apoptotic effects of DOX are thought to be caused by the production of free radicals and the inhibition of topoisomerase II since DOX binds to both topoisomerase enzymes I and II, causing DNA damage. When attempts to repair DNA breaks have been unsuccessful and cellular growth is impeded, the apoptotic pathway is initiated at phases G1 and G2, inhibiting DNA synthesis and resulting in a DNA double-strand break [27,28].

The chemotherapeutic medicine doxorubicin and betanin (red beetroot extract) both feature a planar aromatic chromophore and a six-membered sugar molecule that cause DNA intercalation in cancer cells, which ultimately results in cancer cell death. This remarkable finding is interesting. This suggests that betanin may have a major impact on the red beetroot cytotoxic effect through a potential mechanism of action similar to that of doxorubicin and other related anthracycline anticancer medicines [29] .

## 6. CONCLUSION

Based on the results of the present study, it could be concluded that: Betanin NP and DOX have an apoptotic effect on SCC cell line. DOX has higher apoptotic effect than betanin NP according to concentration of caspase 3 measured by ELISA. They could be capable of inducing cancer cell death against oral tongue carcinoma SCC 25 cell lines in dose and time dependent.

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