

Inhibition of Proliferation in Ovarian Cancer Cell Line (PA-1) By The Action of Green Compound 'Betanin'

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Abstract

Ovarian carcinoma has a cure rate of 30% which makes it deadlier than any other disease. There are a number of genetic and epigenetic changes that lead to ovarian carcinoma cell transformation. Chemoprevention of cancer through application of natural compounds is the need of present generation as other methods are rigorous and have many side effects. Betanin, a compound from *Beta vulgaris* extract is used in present study to check its potential for inhibition of (PA-1) cancer cell proliferation. Determination of IC50 values through MTT assay was carried out, in addition measurement of Mitochondrial Membrane Potential (MMP), effect of Reactive Oxygen Species (ROS) generation and induction of Apoptosis in ovarian cancer cells through betanin was also observed. Results have shown betanin as a potential candidate for inhibition of ovarian cancer cell proliferation and it can be taken up as a serious compound for further studies for its application in cancer cure.

Introduction

Most death among women is caused by ovarian cancer. Early diagnosis of Ovarian Cancer (OC) is very difficult since symptoms are seen only at the later stages. Women from age 20 to 55 are largely affected. More than 60% of patients with OC have shown symptoms of recurrence even after surgery. With the progression of cancer's various stages, the 5 years rate of survival decreases eventually.

Major treatments for cancer include radiotherapy, chemotherapy and surgery. At various levels, the cancer cells are affected by chemical agents and through apoptotic program induction the cell is destroyed. Rapidly growing cancer cells as well as normal cells are targeted by classic chemotherapeutic agents that cause side effects in the patients [1]. Worldwide with the increase in cancer cases, development of novel, potent and safe anticancer agent is still a serious challenge.

Because of the serious side effects of chemotherapy, high medicinal values containing natural products are gaining greater attention [2-4]. In recent times, dietary phytochemicals have allured attention for their human health benefits. Natural pigments have also gained considerable attention due to their antioxidant properties which has beneficial effects on disease prevention and human health.

Anticancer activities are exhibited by certain plant derived compounds that have the ability to prevent and treat cancers and at present, about 60% of the anticancer compounds which are in use are extracted from natural sources [5-6]. Flavonoids are well known and most studied naturally occurring secondary metabolite found in plants that has anti-inflammatory and antioxidant properties. Betanin is one of the flavonoids which is isolated from fruits and vegetables exhibits anti-proliferative activity in OC cells. The compound betanin is obtained from *Beta vulgaris*. Both yellow (betaxanthins) and red pigments (betacyanins) are contained in beetroot and these are collectively called betalains, which is one of the highly, naturally available antioxidants [7-9]. Betanin is the predominant betacyanin found in the red beetroot species and it constitutes about 75% to 90% of the red beetroot's total pigment [10] and isobetanin is its C15 stereoisomer. Studies have proven that betalains have an inhibitory role against

cancers besides their powerful antioxidant properties [11-13]. Cytotoxicity assays and betalain's antitumoral activities were evaluated in various cell lines, but the results obtained were disparate [3, 14-15]. Compounds with antitumor activity are selected through *in vitro* analyses during preclinical testing prior to *in vivo* studies are conducted in animal models. And to screen highly important molecules, cell culture approach is hugely suggested. Results from the cell lines that were cultured conventionally as monolayers showed that concentrate of betanin induced inhibition of cancer cell growth which is accompanied by cell death through apoptosis and an increase in MCF-7 cell's autophagic activity and no deleterious effect over normal cells [16]. In recent years, researchers have come out with various studies to find out the effect of betanin in inducing extrinsic and intrinsic apoptosis pathways in cells [12, 16-18]. Fig.1 have shown a detailed diagram on how betanin induce apoptosis pathways.

Keeping this in mind, the present study used Betanin to check its efficacy in inhibiting Ovarian cancer cells (PA-1). Various tests like MTT assay, measurement of Mitochondrial Membrane Potential (MMP), Reactive Oxygen Species (ROS) generation and induction of Apoptosis in ovarian cancer cells through betanin was also observed.

Materials And Methods:

Source of reagents and chemicals:

Betanin (Red Beet extract diluted with Dextrin) purchased from TCI Chemicals, streptomycin, penicillin-G, Dulbecco's Modified Eagle's Medium (DMEM), L-glutamine, 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide, acridine orange (AO), phosphate buffered saline, bovine serum albumin, ethidium bromide (EB), 2',7'-diacetyl dichloro fluorescein, ethanol, trypan blue, triton X-100, Rhodamine 123 (Rh-123), dimethyl sulfoxide (DMSO), Dichloro-Dihydro-Fluorescein Diacetate (DCFH-DA) and trypsin-EDTA were bought from Sigma Aldrich chemicals Pvt. Ltd (India). All other chemicals used were of analytical grade, purchased from Hi media laboratories Pvt. Ltd., India.

Cell Culture Maintenance:

Human ovarian (PA-1) cancer cells were obtained from the cell repository, National Centre for Cell Sciences (NCCS), Pune, India. For cell line maintenance DMEM was used and 10% of FBS (Fetal Bovine Serum) was used as a supplement. To prevent any bacterial contamination in the medium, streptomycin (100µg/ml) and penicillin (100 U/ml) were added. Cell lines were then incubated with 5% CO₂ in a humidified condition at 37°C.

MTT assay:

10 mL of PBS was used to dissolve 50 mg of MTT dye to make stock solution. Using 0.45-micron filter, it was filtered after 1 minute of vortexing. To prevent the exposure of light aluminium foil was used to wrap the apparatus as MTT is light sensitive. Until further use, it was stored at 4°C. To determine betanin cytotoxicity on PA-1 cells, Mosmann method (1983) [19] was used. Firstly, Haemocytometer was used to

count the harvested viable PA-1 cells and DMEM medium was used further. In 96 well plates 1×10^4 cells/ml of density was seeded in each well and to allow attachment it was incubated for 24 hours. After treating the PA-1 cells with control, each well was applied with different concentrations of Betanin (10 - 60 $\mu\text{g/ml}$). At 37°C PA-1 cells were incubated for 24h in a humidified CO_2 (5%) and air (95%) incubator. After the incubation period is over the drug containing cells were washed with fresh medium and each well was added with MTT dye (5 mg/ml in PBS) followed by another 4h of incubation at 37°C . The concentrated DMSO (100 μl) was used to dissolve the precipitated purple formazan and using multi-well plate reader the viability of cell was measured at absorbance 540 nm. The results were represented as percentage stable cells in comparison to the control. The values for half maximal inhibitory concentration (IC_{50}) was calculated and the optimum doses were analysed at different time period. From the dose responsive curve of betanin, the values of IC_{50} were determined where 50% cytotoxicity inhibition was compared to control cells. All experiments were performed in triplicates to minimize errors.

Statistical Analysis:

The values were obtained using mean \pm SD. One Way Analysis of Variance (ANOVA) and Duncan's Multiple Range Test (DMRT) were used for statistical comparisons on Statistical Product and Service Solutions (SPSS) software (version 12.0, SPSS Inc. Chicago; <http://www.spss.com>). The values were taken as statistically significant when p values come under 0.05.

ROS generation:

Using the method given by Pereira et al. (1999) [20] ROS values were generated. PA-1 cells were added in 6 well plates (2×10^6 cells/well) for 24 h before exposing them to different concentrations of the betanin (40 cells/well) for 24 h prior to exposure and different concentrations of the betanin (40 $\mu\text{g/ml}$) and untreated cells were maintained at 37°C (5% CO_2). Overnight grown PA-1 cells were transferred to 24 well plates for 24 hours. After being exposed to 40 $\mu\text{g/ml}$ of betanin, PBS (1%) is used to wash PA-1 cells and loaded with DCFH-DA (25 μM) in DMEM at 37°C for 30 minutes. DMEM was then used to wash the treated cells and fluorescence was recorded for every 5 minutes for the next 30 minutes (emission 535nm, excitation 485nm) through spectrofluorometry at 37°C . Increase in ROS was calculated by mean slope per min and normalized to the control which is unexposed to betanin.

Measurement of Mitochondrial Membrane Potential (MMP):

Method given by Bhosle, et al. (2005) [21] was used to measure MMP. For the assay, stock solution was made using 10mg Rh-123dye dissolved in 1ml PBS buffer while working solution was made by taking 4 μL of the stock solution and dissolved into 0.96ml PBS. PA-1 cells were exposed to betanin of different concentrations (40 $\mu\text{g/ml}$) and were placed in a six well plate with a cover slip. The cells are kept under incubation for 15 minutes once they are stained with Rh-123. PBS was used to wash and fix the cells. At 535nm wavelength, the intensity of fluorescence was measured and the percentage of PA-1 cells reflecting pathological changes were calculated.

Induction of Apoptosis by betanin:

The method proposed by Baskić et al. (2006) [22] was used to analyse the apoptotic cell death using fluorescence microscopy. For staining, 200µL of dye mixture was prepared by using 100µL/mg EB and 100µL/mg AO and dissolving it in 1% PBS. A density of 5×10^4 cells/well of PA-1 cells were added in a 6 well plate and given 24hrs of incubation. The cells were detached after 24hr treatment with betanin (40 µg/ml), and then washed using cold PBS. Further, staining was performed using the mixture of AO (100 µg/ml)/EB (100µg/ml) at a ratio 1:1 for 5 min at room temperature. At a magnification of 40x the stained cells were observed using fluorescence microscope. After the treatment finished, the cells were collected and washed with PBS up to three times.

The number of cells showing feature of apoptosis was counted as a function of the total number of cells present in the field.

Results And Discussion:

While strategies for early OC diagnosis have yet to be developed, dietary compound chemoprevention might be an advisable strategy for preventing ovarian cancer. Previous studies have reported that betanin inhibited the cell growth in ovarian cancer (Huang et al., 2015). In this research, betanin have more cytotoxicity to ovarian cancer cell line PA-1 cells.

MTT assay:

The PA-1 cancer cells lines were treated with various concentration of Betanin (10-60µg/ml) for 24hr and the results are expressed as a % of the control value in presenting as a cell cytotoxicity ratio for PA-1 cells using MTT assay (**Fig. 2**). The mitochondrial dehydrogenase enzyme of viable cell reduces MTT (3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazoliumbromide) and a visible colour change from yellow to purple was observed in the product formed. A NAD(P)H-dependent reductase was present in viable cells that reduces the reagent MTT to Formazon thus giving a deep purple colour. Solubilizing solution was then used to dissolve Formazon crystals and by plate reader 500-600 nm of absorbance was measured. Data were presented as mean \pm SD asterisks indicate statistically different experiments compared to control. For PA-1, IC₅₀ was found to be about 40 µg/ml. Result have shown that when betanin dose was increased the cell viability was decreased gradually.

The results of MTT assays can be further correlated with the photomicrographic representation of changes in morphology of PA-1 cells like detachment, shrinkage, membrane blebbing, and distortion in shape induced by Betanin treatment (40 µg/ml for 24hr) as compared with control (**Fig. 3**). Normal intact cell morphology was seen in control cells whereas morphological changes attributed to non-viability of cells was observed in betanin treated cells

ROS generation:

PA-1 cells were exposed to Betanin (40 µg/ml) for 24hr and DCFH-DA was used for staining.. Through Fluorescence microscope (Labomed, USA) the digital images were obtained. DCFH-DA assay was used to measure intracellular ROS level. DCFH-DA is cell permeable, lipophilic compound. DCFH-DA is deacetylated to DCF in the cytoplasm by cellular esterases (Pereira et al.,1999). A fluorescent molecule is obtained by oxidation of DCF by various radicals such as hydroxyl, alkoxy, peroxy, carbonate and nitrate (emission 485nm, excitation 530nm). One thing to note is that DCF was not oxidized by superoxide radical or hydrogen peroxide. Control cells (green fluorescence) and a bright DCF fluorescence was observed in PA-1 cells treated with betanin (40 µg/ml) (**Fig. 4**), which shows intracellular ROS production induced by betanin in PA-1 cells.

Measurement of MMP:

For MMP characterization, 'Rh-123' a laser dye was used as a specific probe because of its specificity for localizing mitochondria in living cell. Photo luminescent quenching occurs when Rh123 enters the mitochondrial matrix and MMP depletion accompanied by fluorescence emission can be observed when Rh123 exits mitochondria. MMP description is indicated by high value of fluorescence. Decrease in MMP indicated by a gradual decrease of green fluorescence in cells treated with Betanin (40 µg/ml) which was analysed by fluorescent microscope (Labomed, USA) at 40x magnification (**Fig. 5**). The image shows Rh accumulated control cells and Betanin (40 µg/ml) treated cells had no Rh accumulation.

Induction of Apoptosis by betanin:

To check the apoptosis in PA-1 cells induced by betanin, dual staining AO/EB method was used. AO is used to stain PA-1 because of its permeability to viable/nonviable cells. It emits green fluorescence if intercalated with double stranded nucleic acid (DNA) or red fluorescence if bound to single stranded nucleic acid (RNA). When treated with betanin, PA-1 cells lost membrane integrity and the nonviable cells have taken up EB (non-permeable to cells) because of which red fluorescence was emitted due to intercalation into DNA. So, because of the stained nuclei, the chromatin condensation and fluorescence emission, cells can be distinguished into four different Viable cells are those with well-organized nuclear structure emitting green fluorescence. In early apoptotic cells, the membrane is still intact but the DNA starts to cleave which is in a state of perinuclear chromatin condensation, these can be distinguished by bright green fragments or patches. Late apoptotic cells have red or orange fluorescence with fragmented or condensed chromatin. Nuclei of necrotic cells can be identified by uniform distribution of red or orange fluorescence with no chromatin condensation. PA-1 control cells, and Betanin (40µg/ml) treated cells for 24hr were stained with dual dye AO/EB and then analysed by fluorescence microscopy (**Fig. 6**). In the microscopy images, living cells have shown clear green nucleus, late apoptotic cells have shown chromatin fragmentation or condensation orange-stained nucleus, while early apoptotic cells have shown fragmented or condensed form of yellow color nucleus with chromatin and necrotic cells have shown uniform red stained cell nuclei.

Thus, these findings of betanin's anticancer effects in ovarian cancer, add further value to the nutritional characteristics of various other fruits of *Beta vulgaris* and further studies in human trials and animal

models are required to determine the effectiveness of this natural compound as an agent to treat and prevent ovarian cancer.

Conclusion And Future Prospects:

Cancer chemoprevention can be defined as the use of natural and pharmacologic agents to prevent the activation of various procarcinogens thereby inhibiting the initiation, progression and promotion of tumor. Two mechanisms are involved in the execution of anti-proliferative effects: (i) inducing alterations in the pattern of differentiation of cells, (ii) intervening the metabolic activation of various carcinogens by ROS scavenging. Effects of betanin on PA-1 cell line have been analysed in this study. Betanins a natural pigment is widely used as food colorants which are water soluble and a good source of nitrogenous chromo alkaloids whose pharmacokinetic effects in human cell lines were demonstrated elsewhere [17–18, 23]. Besides being a part of food colorant, a nutraceutical role is also played by natural pigments like betanins thus avoiding the hazards posed by chemical and synthetic colorants. Unlike other natural pigments the pharmacological properties as well as the mechanism of action of betanins are still been studied on various cancer cells. In this study, the betanin's anti proliferative effects on ovarian cancer PA-1 cell line have been studied. From the results, it was found that a time and dose dependent inhibition in growth of PA-1 cells is induced betanin with IC₅₀ of 40µM. The use of betanin in the current study has showed typical characteristics of apoptosis like laddering of DNA, chromatin condensation membrane blebbing and decrease in membrane potential. But further studies are required to understand the mechanisms that are in involved in altering the integrity of the mitochondrial membrane under betanin treatment in PA-1 cells. This research manifests the importance of betanins against ovarian cancer in women. Biochemical alterations exhibited by betanins are reflected in structural changes of the cell that is undergoing apoptosis.

Thus, these findings of betanin's anticancer effects in ovarian cancer, add further value to the nutritional characteristics of various other fruits of *Beta vulgaris* and further studies in human trials and animal models are required to determine the effectiveness of this natural compound as an agent to treat and prevent ovarian cancer.

Declarations

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Author contributions

R performed experiments and written initial manuscript, MS did editing and made figure, NK designed the structure and did formatting of the manuscript. MS and NK critically read and finalized the manuscript. All the authors contributed to the article and approved the submitted version.

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Conflicts of interest/Competing interests - Authors have no conflict of interests.

Ethics approval – OC cell line PA-1 used, so no need of ethical approval.

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Figures

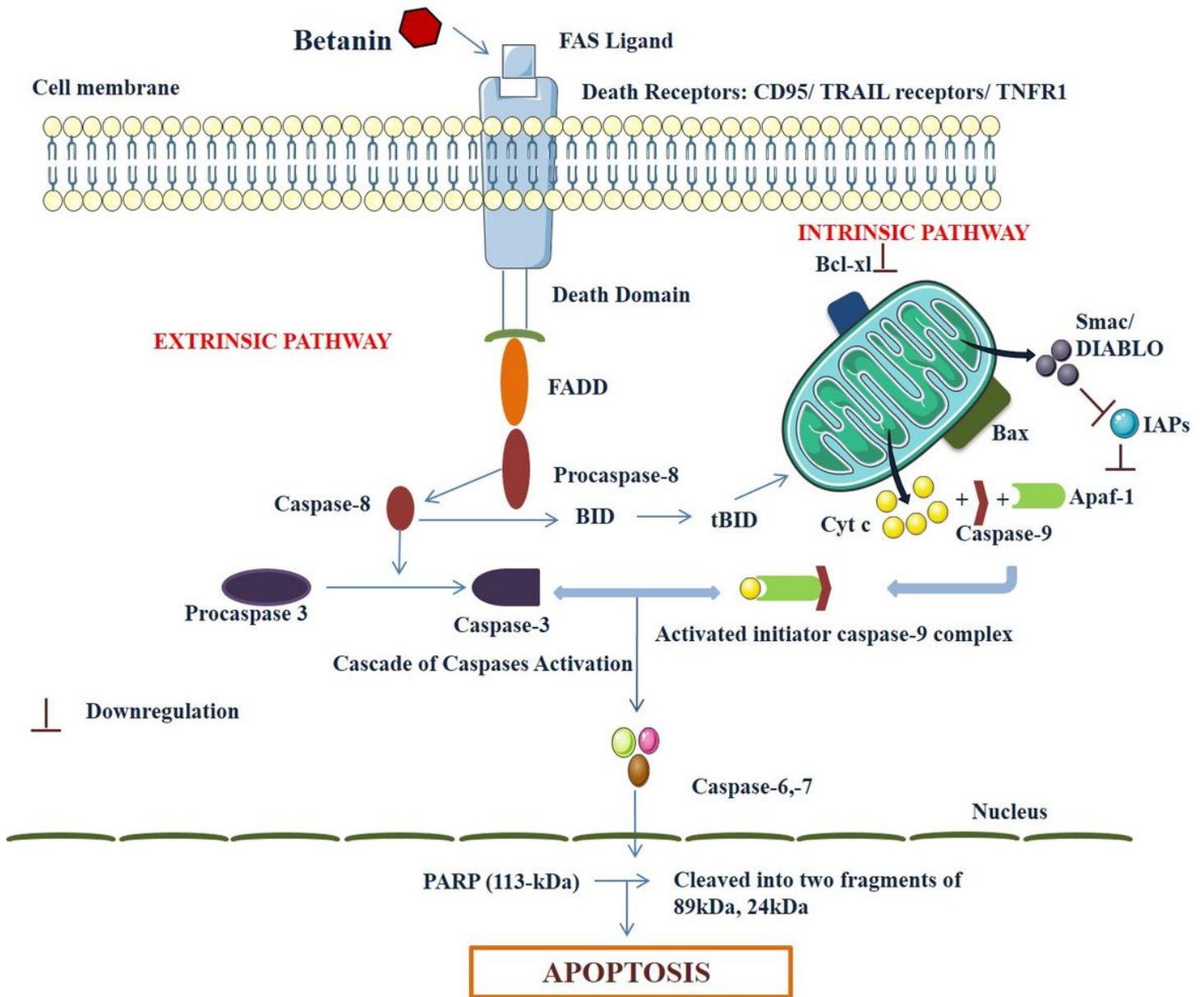


Figure 1

Effect of betanin on induction of extrinsic and intrinsic apoptosis pathways in cells

Ovarian cancer PA-1 cells

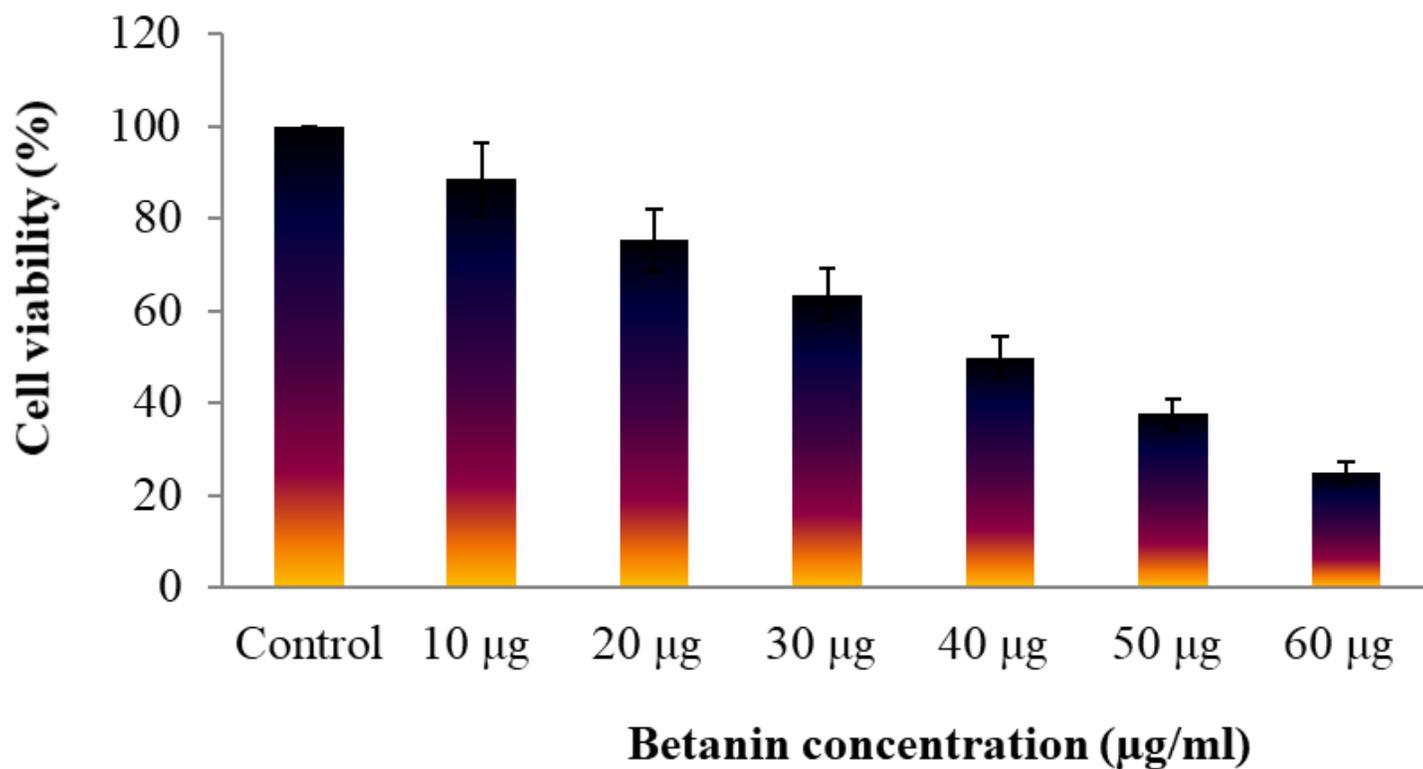


Figure 2

Anti-proliferative effects of betanin on PA-1 cells

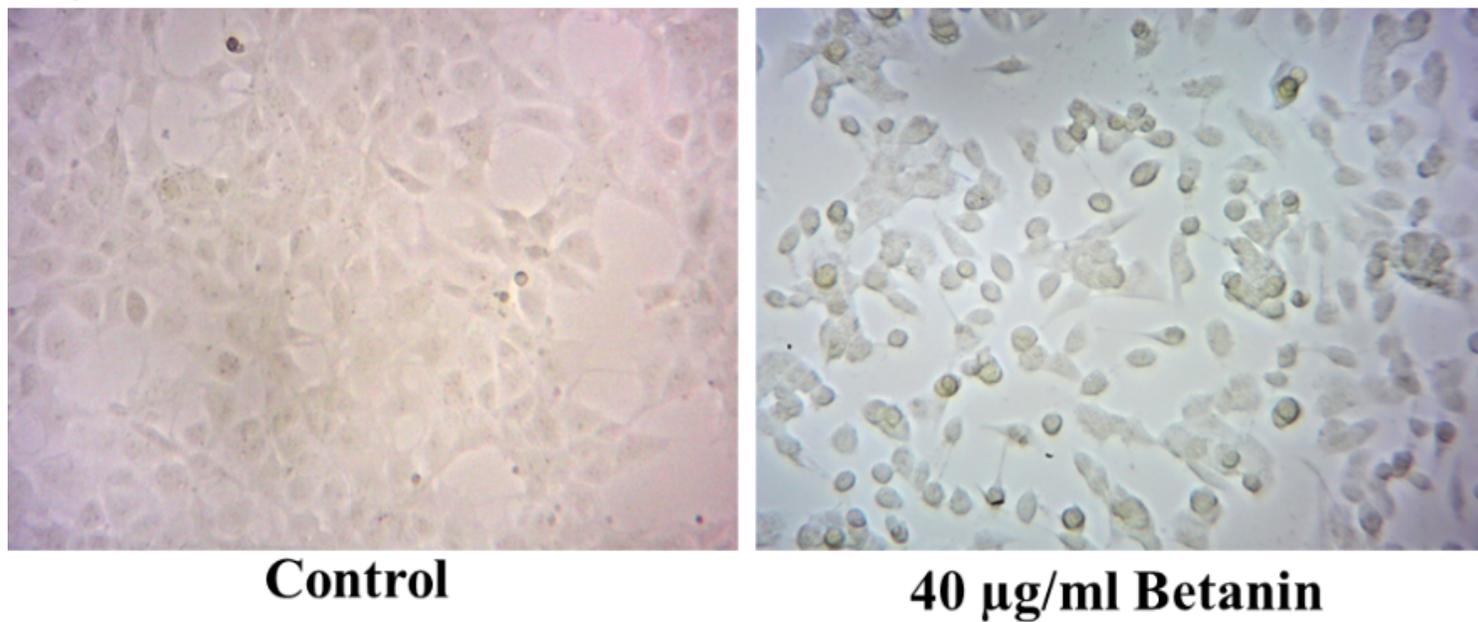
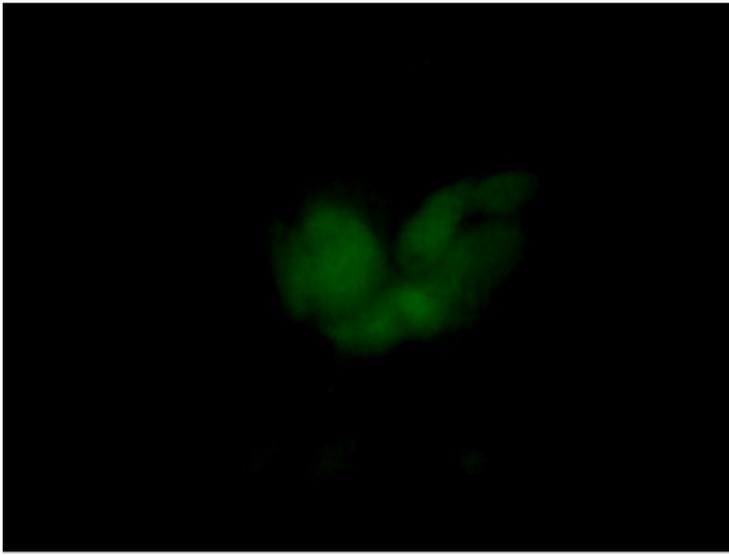
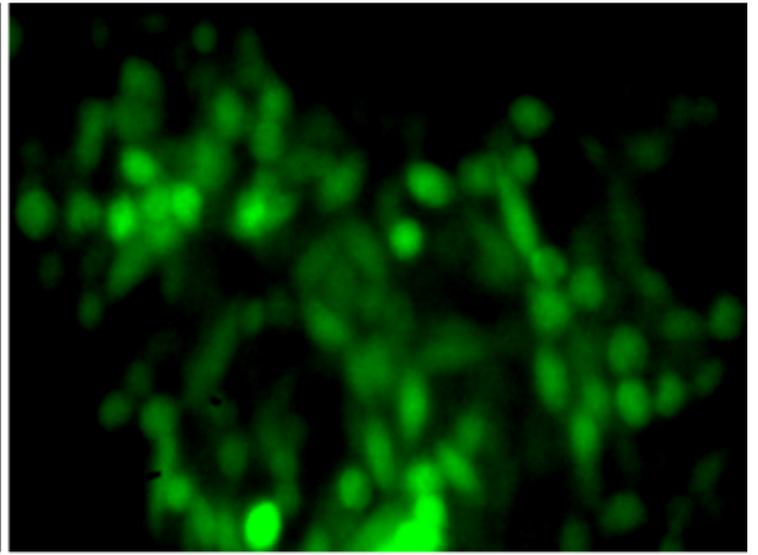


Figure 3

Betanin treated PA-1 cells in comparison to Control-A photomicrographic representation



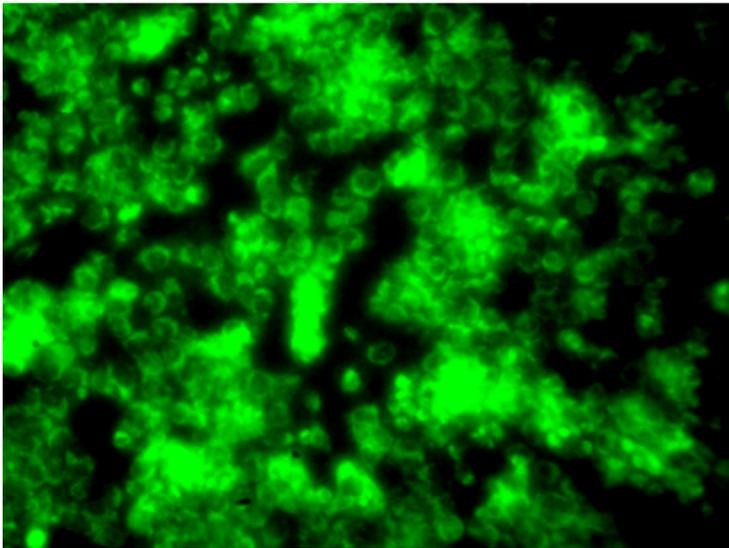
Control



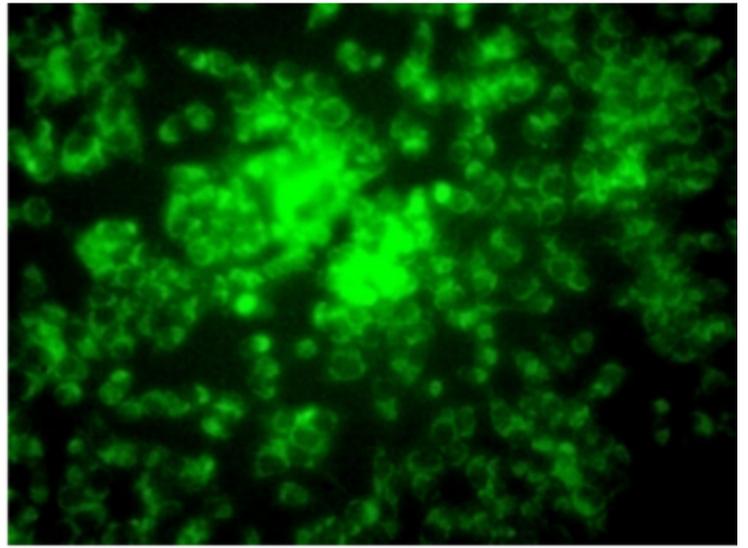
40 µg/ml Betanin

Figure 4

Intracellular production of ROS induced by Betanin



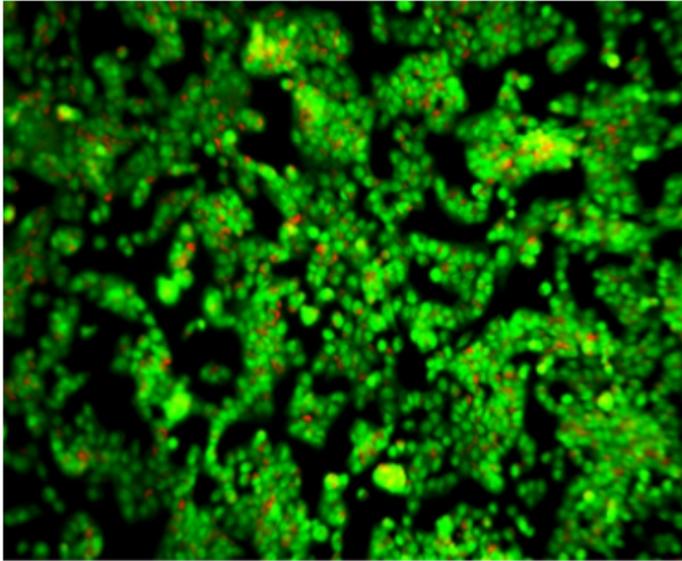
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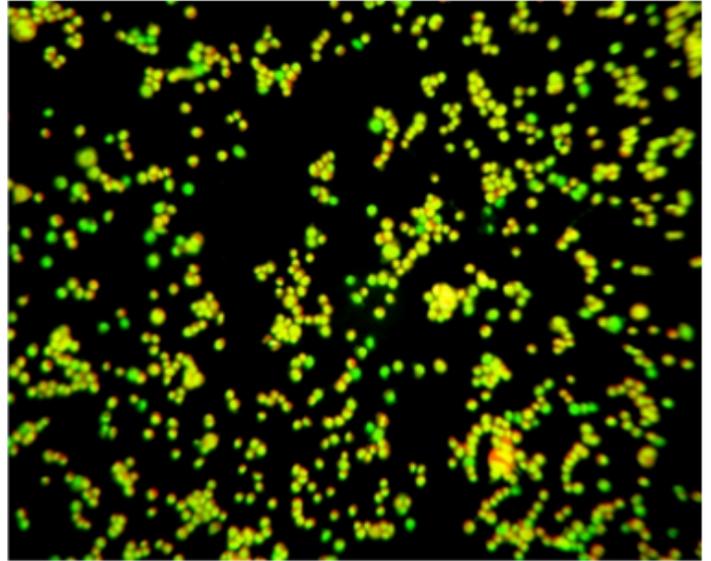
40 µg/ml Betanin

Figure 5

Betanin effects on the mitochondrial membrane potential of PA-1 cells



Control



40 µg/ml Betanin

Figure 6

Effects of Betanin induces apoptotic incidence in PA-1 cells