Original Article
Hesperidin-triggered necrosis-like cell death in skin cancer cell line A431 might be prompted by ROS mediated alterations in mitochondrial membrane potential

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Abstract: Skin cancer is one of the lethal causes of cancer related deaths around the globe and with limited drug options and the side effects associated with the currently used drugs; there is pressing need for identification of novel anticancer lead molecules. The aim of the present study was to evaluate the anticancer activity of hesperidin against skin carcinoma cell line A431 and to investigate the underlying mechanism. IC_{50} was determined by MTT assay. Fluorescent probes DCFH-DA, Indo 1/AM, $DiOC_6$ were used to determine ROS, Ca^{2+}, mitochondrial membrane potential ($\Delta\Psi_m$). ATP levels were determined by using ATP liteTM kit. DNA damage was investigated by DAPI and comet assays. Protien expression was investigated by western blotting. Hesperidin exhibited lowest IC_{50} of 25 μM against skin A431 cell line. Moreover, hesperidin reduced the cell viability of A431 cells concentration and time-dependently. It also augmented the discharge of ROS and Ca^{2+} and lessened the mitochondrial membrane potential ($\Delta\Psi_m$) and ATP levels in A431 cells. Additionally, hesperidin also prompted DNA damage in A431 cell line. Notably, hesperidin stimulated the cytochrome c release only and exhibited no effect on the expression of apoptosis-related protein levels such as caspase-3, caspase-8, and Apaf-1. Taken together, hesperidin induced A431 cells death displayed a cellular pattern characteristic of necrotic cell death but not of apoptosis.

Keywords: Hesperidin, necrosis, skin carcinoma A431 cells, ROS

Introduction

Skin cancer is one of the main causes of cancer related mortality around the globe According to U.S.A. estimates approximately 1 in 5 Americans will develop skin cancer. The increasing frequency of skin cancer has pressed the need for exploration of multiple treatment options. Despite preliminary responses to chemotherapy and /or surgical interventions, the tumors consistently relapse. Moreover, the side effects associated with the synthetic drugs severely affects the quality of life of patients [1]. Consistent with this drugs from natural have gained considerable attention in the recent past. Among the natural products flavonoids form a large group of compounds ubiquitously found across plant kingdom [2]. Flavonoids represent an important part of the human diet and in United States the estimated regular dietary intake of mixed flavonoids ranges from 500 to 1000 mg. The actual figure may be even higher for people improving their diets with flavonoid rich herbal preparations [3]. With advancements in medical research, flavonoids are being evaluated for diversity of bioactivities. So far they have been reported to exhibit wide range of activities which include, but are not limited to, anti-inflammatory, estrogenic, enzyme inhibition, antimicrobial anti-antiallergic, antioxidant and antitumor [2, 3]. Owing to their fairly consistent structure, flavonoids impede the activity of a wide range of eukaryotic enzymes and therefore exhibit diversity of activities. The different parts of flavonoid molecules have been considered critical for their bioactivities [4]. Moreover, flavonoids are ubiquitously present in edible plants and beverages, they are therefore expected to have minimal toxicity [5]. Against this backdrop, the current study investigated the antitumor potential of a natural flavonoid, hesperidin against a panel of cancer cell

lines using MTT assay. Our results revealed that hesperidin exhibited lowest IC$_{50}$ against skin carcinoma A431 cancer cell line which was then selected for further study. Flow cytometry and caspase activity, ROS and protien expression studies revealed that hesperidin exerts its antitumor activity through scavenging of ROS and alterations in mitochondria membrane potential and then caused cell necrosis.

Materials and methods

Cytotoxicity of hesperidin against different cancer cell lines

The cytotoxic effect of Hesperidin was measured against colorectal adenocarcinoma LS-180, cervical cancer HeLa, human brain glioma Hs 683. Skin cancer A431 and human promyelocytic leukemia cells HL-60 using the MTT assay. The cytotoxic effect of hesperidin against all the cancer cell lines were expressed as IC$_{50}$ values.

Assessment of cell viability and colony formation of A431 cells

Cancer A431 cells were seeded at the density of 2×10^5 cells/well (2 ml) were plated in 6 well plates and treated with hesperidin for 12, 24 and 48 h and treated with 10, 25 and 50 μM hesperidin or only with vehicle (DMSO, 1% in culture media). The cells were investigated cell viability was estimated from each treatment by PI exclusion method and flow cytometry as described previously [6].

For clonogenic assay, skin cancer cell line A431 cells at the exponential growth phase were harvested and counted with a hemocytometer. Seeding of the cells was done at 200 cells per well, incubated for a time period of 48 h to allow the cells to attach and then to the cell culture different doses (0, 10, 25 and 50 μM) of hesperidin were added. After treatment, the cells were again kept for incubation for 6 days, washing was done with PBS, methanol was used to fix colonies and then stained with crystal violet for about 30 min before being counted under light microscope.

Estimation of cell cycle dissemination of A431 cells

The cells were seeded in 6 well plates at a density of 2×10^5 cells/well and hesperidin was administrated to the cells at the concentrations of 0, 10, 25 and 50 μM followed by 24 h of incubation. DMSO was used as a control. For estimation of DNA content, PBS was used to wash the cells followed by fixation in ethanol at 0°C. This was followed by re-suspension in PBS holding 40 μg/ml PI and, RNase A (0.1 mg/ml) and Triton X-100 (0.1%) for 30 min in a dark room at 37°C. Afterwards, analysis was carried out by flow cytometry as described previously [6].

Determination of ROS, Ca^{2+} release and ΔΨ$_m$

A431 cells were seeded at a density of 2×10^5 cells/well in a 6-well plate and kept for 24 h and treated with 25 μM hesperidin for 6-72 h at 37°C in 5% CO$_2$ and 95% air. Thereafter cells from all treatment were collected, washed 2 times by PBS and re-suspended in 500 μl of DCF-DA (10 μM) for ROS estimation, Indo 1/AM (3 μg/ml) for Ca^{2+} generation and DOC$_6$ (1 μmol/l) for ΔΨ$_m$ at 37°C in dark room for 30 min. The samples were then analyzed instantly using flow cytometry as previously described in literature [7].

Evaluation of ATP level

A431 skin carcinoma cells (density 1×10^5 cells/well) were plated in 100 μl phenol red-free medium at varied concentrations (0, 10, 25 and 50 μM) of hesperidin for 6 h in 96-well microplate and the intracellular ATP content was estimated by Luminescence ATP Detection Assay by ATP liteTM kit (PerkinElmer) as described in literature [8].

Comet assay and DAPI staining

A431 cells/well at a density of 2×10^5 cells/well were seeded in 6-well plates were administrated with 10 to 50 μM hesperidin for 48 h. The cells were then separated into two shares for comet staining by PI and as well as DAPI staining. Afterwards, the cell sample was studied and photographs taken under fluorescence microscopy as previously described [9].

Western blotting analysis

The hesperidin administrated cells were harvested and lysed. The protein concentrations of the lysates were quantified by BCA assay using specific antibodies. β-actin was used as a loading control. From each sample equal amounts

Table 1. IC$_{50}$ of hesperidin against different cancer cell lines as determined by MTT assay

Cell line	IC$_{50}$ (µM)
Colorectal adenocarcinoma LS-180	20
Cervical cancer HeLa	50
Human brain glioma Hs 683	30
Skin cancer A431	25
Human promyelocytic leukemia cells HL-60	50
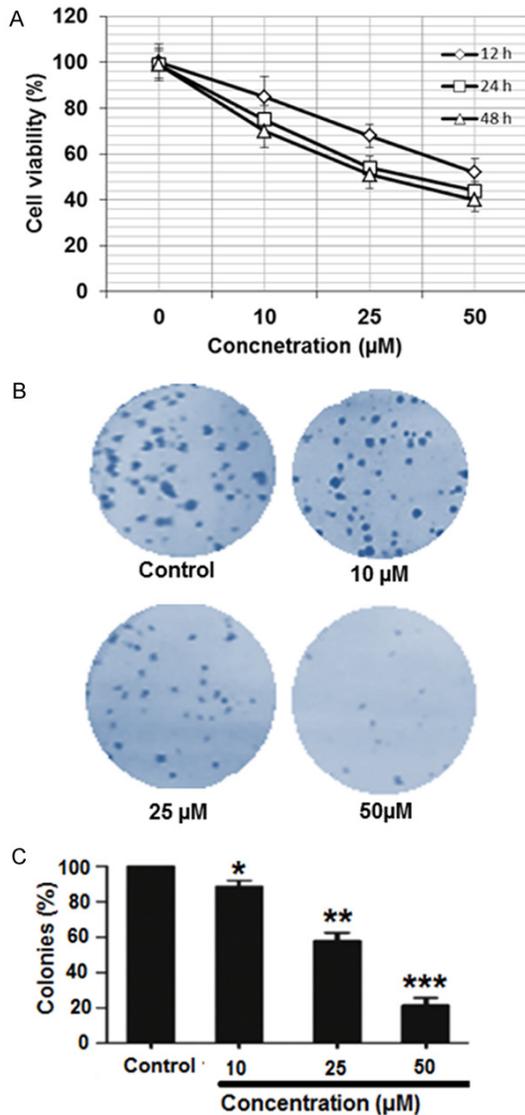
Figure 1. Hesperidin induced alterations in (A) cell viability. (B) Colony formation (C) Quantification of effect of hesperidin on colony (%) formation. Each value is mean of three replicates ± S.D. The values were considered significant at *P<0.01, **P<0.001, ***P<0.0001.

of protein samples were loaded and separated by electrophoresis on a 12% denaturing SDS

gel. Afterwards, the proteins were electroblotted on polyvinylidene difluoride membranes (0.45 m pore size).

Statistical analysis

The quantitative data was expressed as mean ± SD. Results are representative of three independent biological replicates. The statistical differences between the caffeic acid n-butyl ester -treated and control samples were calculated by Student's *t*-test with a *p*-value of P*<0.01, P**<0.001 and P***<0.0001 were considered significant.

Results

Hesperidin decreases cell viability of A431 cells

Hesperidin was evaluated against different cancer cell lines and it was found to exhibit lowest IC$_{50}$ against skin carcinoma cell line A431 (**Table 1**). Therefore, it was further evaluated against skin carcinoma cell line A431 only. After administration of several doses of hesperidin for varied time intervals, cells were studied for alterations jn viability. Our results revealed that hesperidin decreased the percent viability of cells concentration-dependently (**Figure 1A**) and decreased the colony forming potential of cancer cells in a dose dependent manner (**Figure 1B, 1C**).

Hesperidin caused S phase arrest and inhibition of related protein levels

The cancer cells were administrated with different doses of Hesperidin for 24 h and estimation of A431 cell cycle was carried out. Our results indicated that hesperidin triggered S phase arrest (**Figure 2A**). The administration of A431 cells with 10-50 µM hesperidin for 24 h caused a greater number of cells in S phase than control. Also, the decrease in the expression level of cyclin D, CDK2 and thymidylate synthase were observed in Hesperidin-administrated A431 cells at the concentrations of 0, 10, 25 and 50 µM (**Figure 2B**).

Hesperidin augmented the accretion of ROS and Ca^{2+} and reduced the ΔΨ$_m$ level

Cells were administrated with 25 µM hesperidin for various time periods (12, 24, 48 and 72) and the levels of ROS, Ca^{2+} and ΔΨ$_m$ were evalu-
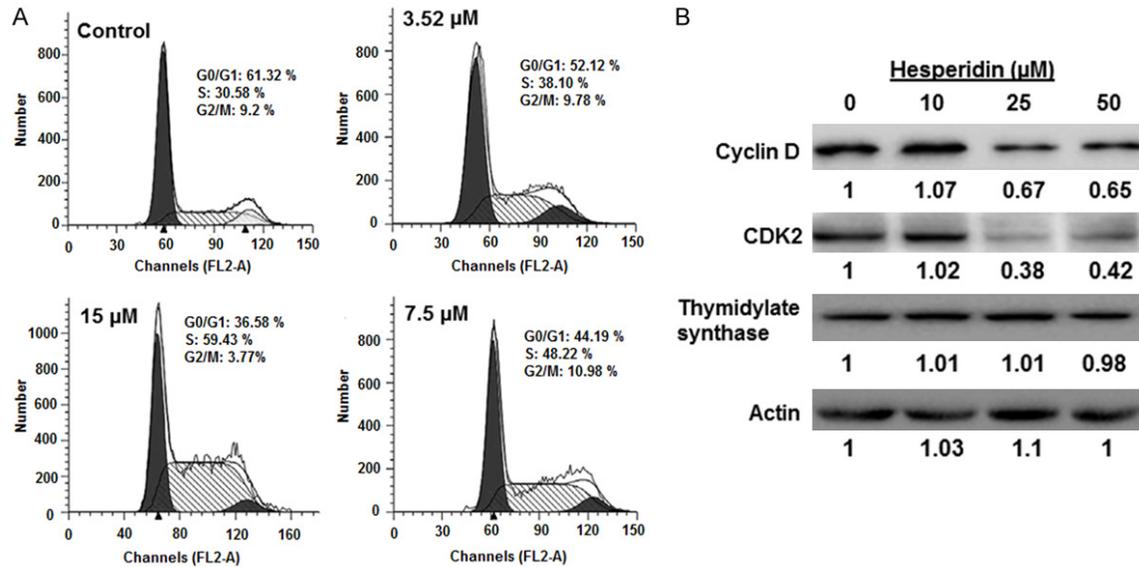
Figure 2. Hesperidin triggered S phase arrest and regulated protein expression in A431 cells. Cells were administrated with indicated concentrations of hesperidin for 24 h. (A) The cells were then collected and examined for cell cycle distribution by flow cytometry (B) Western blots showing expression of cyclin D, CDK12 and thymidylate synthase in hesperidin-treated A431 cells. Each experiment was carried out in at least three times and β-actin was used as an internal control.
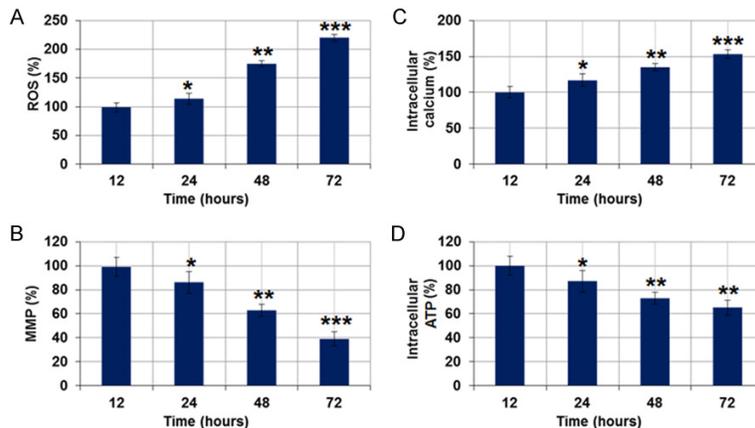
Figure 3. Hesperidin caused alterations in the levels of (A) ROS (B) $\Delta\Psi_m$ (C) Ca^{2+} production, and (D) ATP content in A431 cells. Cells were administrated with 25 µM hesperidin for 12, 24, 48 and 72 hours. Each experiment was done in triplicates and expressed as mean ± SD. The values were considered significant at *P<0.01, **P<0.001, ***P<0.0001.

ated (**Figure 3**). A considerable upsurge in intracellular ROS (**Figure 3A**) and cytosolic Ca^{2+} (**Figure 3B**), and a significant reduction of $\Delta\Psi_m$ level (**Figure 3C**) were experienced in the hesperidin-treated A431 cells as compared to the control. Moreover, it was detected that hesperidin treatment considerably augmented the ROS levels from 12 h to 72 h than the control (**Figure 3**). Similarly, hesperidin treatment (6 h) increased cytosolic Ca^{2+} level significantly till 72

h exposure (**Figure 3B**). Furthermore, hesperidin considerably reduced $\Delta\Psi_m$ level around 28 % in 72 h administration in A431 cells compared to control (**Figure 3C**).

Hesperidin induced alterations in ATP levels

A431 cells were administrated with various concentrations of hesperidin, and the levels of ATP were measured. Results indicated that hesperidin reduced ATP levels up to 40% at the 10-50 µM concentration in A431 cells (**Figure 3D**).

Hesperidin prompted DNA damage in A431 cells

The cells were separated from hesperidin and DNA damage was evaluated DAPI staining assay as well as by comet. Our results indicated that hesperidin caused DNA damage dose-dependently as evident from the comet assay (**Figure 4A, 4B**). Comment assay further confirmed that hesperidin induced dose-dependent DNA breakage (**Figure 4C, 4D**) as evident
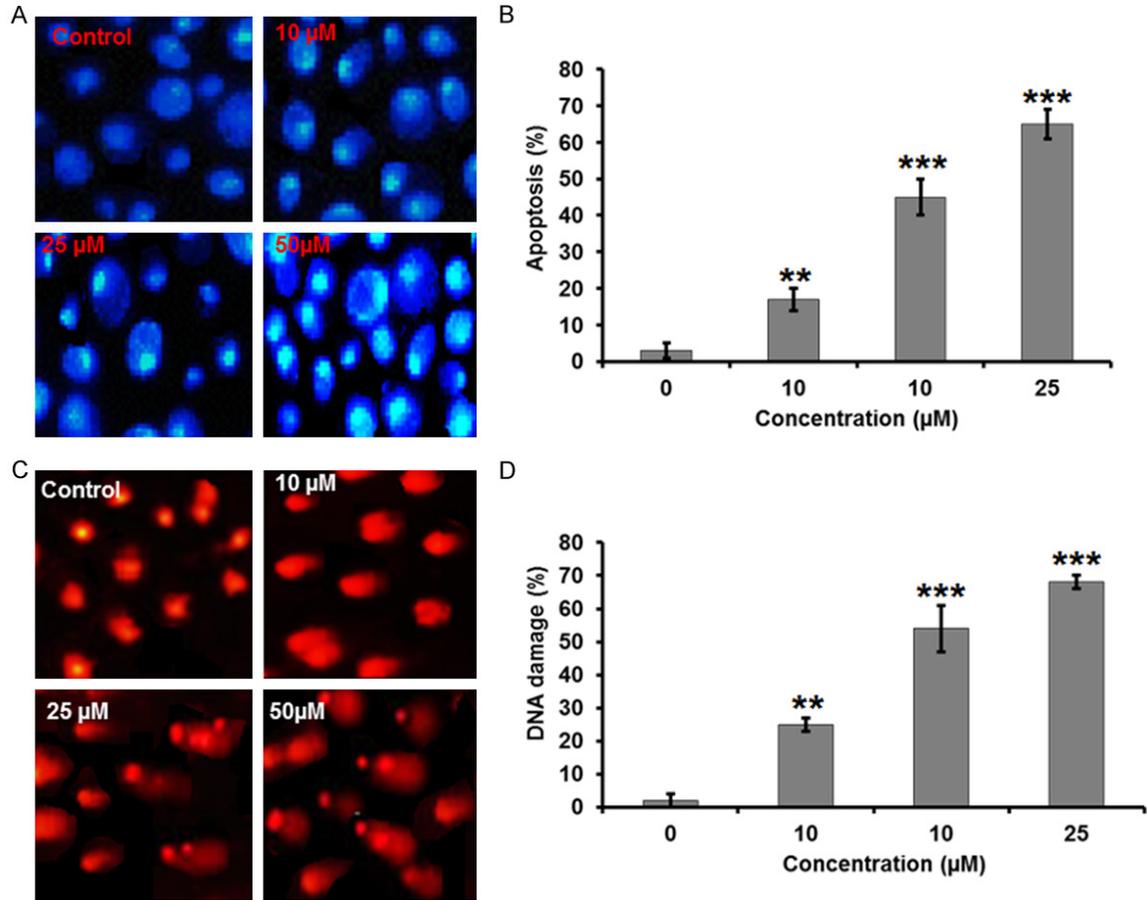
Figure 4. Cells were administrated with various concentrations of hesperidin (0, 10, 25 and 50 µM) for 48 h. The cells were then collected and assessed for DNA damage by (A) Comet assay (B) Percent DNA damage (C) DAPI staining (D) Percentage of apoptotic cells. The cell samples were observed under fluorescent microscope at 200 × magnification. The values were considered significant at *$P<0.01$, **$P<0.001$, ***$P<0.0001$.
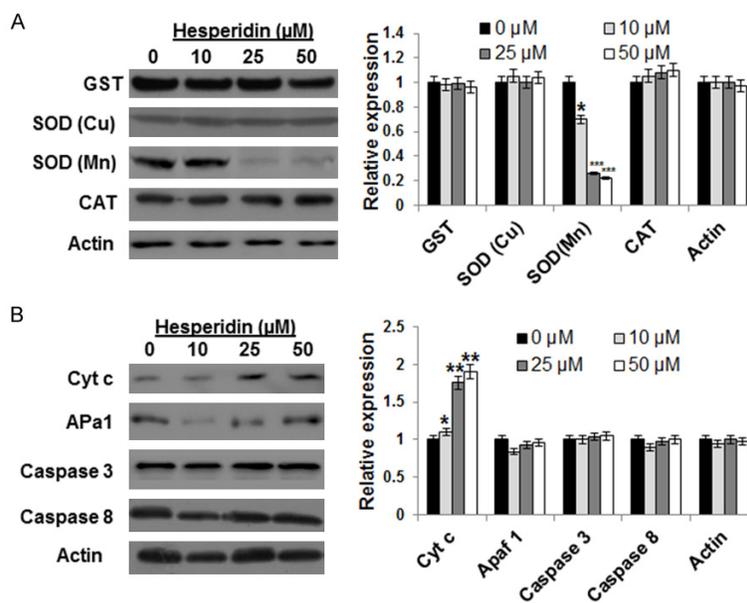
Figure 5. Western blots showing Hesperidin induced the changes in the expression levels and quantification of (A) GST, CAT, SOD (Cu), SOD and (Mn) and (B) cytochrome c; PARP, caspase-3, caspase-8, and Apaf-1. The values were considered significant at *$P<0.01$, **$P<0.001$, ***$P<0.0001$.

from the greater density of white color nuclei.

Hesperidin caused alterations oxidative stress and apoptosis-related protein induction

Western blot analysis revealed that hesperidin inhibited the expression of GST, CAT and

SOD (Mn) but exhibited no effects on SOD (Cu) expression in A431 cells (**Figure 5A**). Moreover, hesperidin stimulated the expression level of cytochrome *c* in A431 cells. No effect was observed in PAR1P cspase-3, cspse-8, Apaf-1 protien expression levels in A431 cells (**Figure 5B**). Taken together, these results indicated that hesperidin prompted non-apoptotic cell death in A431 cells.

Discussion

Skin cancers are by far the most common malignancy of humans, particularly in the white population. The growing incidence of skin malignances has increased the need for multiple treatment options [10]. Anti-cancer drugs that induce DNA damage and apoptosis of cancer cells are considered effective in cancer treatment [11, 12]. It has been reported that extracts prepared from many plants as well as semi-synthesized compounds can induce apoptotic processes [13]. The present study first time reports that hesperidin inhibits human skin carcinoma A431 cell line growth *in vitro* through generation of ROS. Treatments with hesperidin lead to accumulation of A431 cells in S phase and underwent necrosis and not apoptosis in a concentration and time-dependently. It is well established that several agents arrest the cell cycle progression by downregulating the expression of cyclin D, CD1K2 and thymidylate synthase levels are essential players of DNA replication during S phase. Thus, we propose that hesperidin blocked A431 cells in the S phase by augmenting the expression of these proteins. Our results indicated that hesperidin reduced the $\Delta\Psi_m$ (**Figure 3C**) and ATP levels (**Figure 3D**). Moreover, western blotting revealed that Hesperidin administration lead to no significant change in the expression of Apf-1 caspase-3, caspase-8, and PARP proteins. Thus, we suggest that A431 cell death induced by hesperidin may not be mediated through apoptotic signaling.

The outcomes of this study also indicated that hesperidin augmented the accretion of ROS in A431 cells (**Figure 3A**). Previous studies have revealed that reduction in ROS induced by several anticancer drugs is positively associated inhibition of tumor growth [14] and the upsurge of ROS is associated with the apoptotic response [15]. Moreover, it is now well established that elevated ROS generation induces apoptosis by inducing mitochondria permeability transition pore opening, discharge of pro-apoptotic factors and stimulation of caspase-9 [16]. Nevertheless, necrosis is accompanied with a loss of $\Delta\Psi_m$ and ATP [17]. Consistent with this, our results revealed that hesperidin reduced both the $\Delta\Psi_m$ (**Figure 3C**) and ATP levels (**Figure 3D**) in A431 cells. Consequently, our results are in good agreement with other investigations as generation of ROS, loss of $\Delta\Psi_m$ and ATP exhaustion in mammalian cells causes necrosis due to failure to apoptosome formation [18].

Conclusion

Taken together, our findings indicate that skin carcinoma A431 cells are subtle to hesperidin and their cell death is due to a necrosis-like event triggered by ROS mediated reduction in $\Delta\Psi_m$ and exhaustion of ATP levels. The present study paves way for further evaluation of Hesperidin *in vivo*.

Disclosure of conflict of interest

None.

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