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Role of autophagy induced by anthocyanins in protection of DNA damages caused by UVB irradiation in HepG2 cells

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ABSTRACT

DNA-protein cross-links and thymidine dimer are special types of DNA damages that formed when DNA exposed to many inducers as UV irradiations. Anthocyanins are members of phytochemicals found in many plants including *Hibiscus sabdariffa.* It was reported that anthocyanins protect DNA from UV-induced damages. Here we investigate the role of autophagy induced by anthocyanins in protection of UVB-induced DNA-protein cross-links and thymidine dimer in HepG2 cells. Cells were treated either with anthocyanins, Rapamycin, or combination of anthocyanins and Bafilomycin A1 or anthocyanins and 3-methyladenine for 24 h and then subjected to 0.36 J/cm^2 UVB irradiation. The results revealed that anthocyanins induce autophagy formation as detected by acridine orange pH dependent green to red (G/R) color shifting. To investigate the role of anthocyanins-induced autophagy on DNA-protein cross-links and thymidine dimer formation after UVB exposure, electrophoresis of protein covalently linked to DNA or thymidine dimer dot plot analysis of DNA were carried out. It was found that anthocyanins treated cells have reduced DNA-protein cross-links and thymidine dimer compared with the anthocyanins untreated cells. Treatment of cells with autophagy inhibitors Bafilomycin A1 or 3-

methyladenine together with anthocyanins significantly increased the DNA-protein cross-links and thymidine dimer formation compared with anthocyanins treated cells. Also, Rapamycin treatment alone protects cells from DNA-protein cross-links and thymidine dimer formation which confirm the role of autophagy in protection of cells from UVB-induced DNA-protein cross-links and thymidine dimer formation.

INTRODUCTION

DNA stability is a corner stone for the proper functioning and existence of all living organisms. Genotoxic agents in general have a negative impact on genome stability. DNA damage, which can come from a variety of external and internal causes such as reactive oxygen species, atmospheric radiation, environmental toxins, and chemotherapeutics, is a persistent danger to genomic integrity and stability. Mutations, genetic instability, premature ageing, mental cognitive retardation, and other developmental diseases, as well as cancer, can result from a failure to repair DNA damage [1]. Irradiation of DNA by UV (ultraviolet light specially UVB: 280–315nm) is an operator that can make many changes of life by inducing a variety of DNA lesions including 6-4 photoproducts (6-4PPs), cyclobutane-pyrimidine dimers (CPDs), DNA-protein cross-links (DPCs) and their Dewar valence isomers in addition to DNA strand breaks [2]. Thymine dimers, thymine-cytosine dimers and cytosine-cytosine dimers are the most occurring covalently linked pyrimidines. These dimers usually prevent both replication and transcription of that region of the genome. These types of DNA lesions are known as permutational lesions because they are fatal if left uncorrected [3]. After exposure to a physical or chemical crosslinker, DPCs are the common resulted lesions [4]. DNA-protein cross-links occur when a protein becomes covalently linked to DNA after exposure to any cross-linkers, such as UV light (physical) or aldehydes (chemical) which collectively known as (non-enzymatic DPCs). Enzymatic DPCs occur because of faulty DNA-enzymatic reactions. The formation of DPCs obstructs various DNA \ processes such as transcription, replication, recombination, and repair. Plant consumption for medicinal purposes has been a long-standing human tradition in many parts of the world. *Hibiscus sabdariffa* (*H. sabdariffa*) is one of the plants that is considered medicinal. The calyces of *H. sabdariffa* have been found to be high in polyphenols,

particularly anthocyanins (anth), whose bioactive potential has been extensively researched [5]. Anthocyanins are considered as the most abundant and important water-soluble pigments in nature [6]. The term anthocyanin consists of two Greek words: anthos means flowers and kyanos which means dark blue [7]. They are members of the flavonoid family of compounds [8]. Numerous studies have revealed that anth have numerous biological activities [9] as antioxidant [10], and anti-inflammatory properties [11]. In addition, anth have been shown to have chemoprotective properties and may reduce the risks of cancer [12]. Furthermore, it was reported that anth-induced autophagy is the key player of anth anti-tumor effect on various cancer cell lines [13]. Autophagy is an essential, homeostatic cellular process of "self-eating". Autophagy is highly conserved pathway in eukaryotic cells that occurs in response to a variety of conditions, including deprivation of nutrients [14], growth factors withdrawal, energetic- [15] and oxidative-stresses [16]. Autophagy is the process by which intracellular components as organelles and long-lived proteins are degraded. Firstly, the double-membrane vesicles (autophagosomes) are formed enclosing organelles and part of cytoplasm. Finally, autophagosomes fuse with lysosomes forming autolysosomes where the degradation occurs, releasing amino acids and fatty acids that can be reused by cells [17] Autophagy is induced by DNA damage [18], however, its role in the DNA damage response is still obscure. Using ionizing radiation as DNA damaging agents revealed that cells induced autophagy after exposure to ionizing radiation followed by cell cycle arrest [19]. In the present study, the relationship between anth-induced autophagy and its role in protection against UVB-induced DNA damage are investigated.

MATERIALS AND METHODS

Chemicals

Growth medium DMEM with L-glutamine, FBS (fetal bovine serum) and penicillin and streptomycin were from Lonza, Belgium. 3-Methyladenine, Bafilomycin A1, Acridine orange, Leupeptin, pepstatin, aprotinin, RNase A, PMSF, Rapamycin and MTT reagent; (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) were from Sigma (St. Louis, MO, USA). Protein molecular weight marker from Jena bioscience, Germany. Enhanced chemiluminescent (ECL) substrate was obtained from (Thermo

Scientific, Rockford, USA). Coomassie blue and Genomic DNA Extraction Kit were from Abcam. Any other reagents- if not mentioned- will be denoted elsewhere.

Anthocyanin preparation

Anthocyanins were prepared from dry petals of *Hibiscus sabdariffa* which obtained from the farmers directly. Petals were soacked in methanol overnight, and then separated from other water-soluble inclusions by using methanol, chloroform and water at 1:1:0.5 (v/v), the organic phase was removed, and the process was repeated. The resulted solution was left to dry over a clean glass slap in dark at room temperature and the pigment was scratched out and immediately kept in sealed tubes at 4ºC. Stock solution was prepared as 1 mg/ml in DMSO. Anthocyanins content was determined by high performance liquid chromatography-diode array detection analysis as previously reported [20].

Cell Culture and treatments

Human cellular carcinoma cell line (HepG2) was purchased from VACSERA – Cell Culture Unit (Dokky, Giza, Egypt) which originally obtained from American type culture collection. All experiments were performed using mycoplasma-free cells. Cells were cultured in DMEM growth medium supplemented with 10% (v/v) FBS, and 1% antibiotic mix (10.000 u penicillin/ml, and 10.000 u streptomycin/ml) at 37°C in a humidified atmosphere with 5% CO_2. Cells were harvested 48h after inculcation, and then seeded onto 12-well plates in suitable cell density and left overnight for attachment. For induction of DNA damage, cells were treated with 0.5 µg/ml anth (for 24 h) alone or in combination with 20 nM Bafilomycin A1 (BafA1) or 10 mM 3-Methyladenine (3Ma). Autophagy inducer; Rapamycin (Rap) (5 µM) was used to compare its results with anth treatment. Then cells were subjected to 0.36 J/cm^2 UVB irradiation and left in 37°C incubator for 3 h, DNA extraction was carried out for DPCs and Thymidine dimer (Td) analysis. For detection of autophagy, cells were treated with Acridine orange (AO) for examination of autophagy vacuolar formation.

Cell viability by MTT assay

In 96-well plates, 2×10^3 cells /well were incubated with or without anth (1,5,10 and 20 μg/ml) for 24 h. The medium was removed and 2 mg/ml MTT in 200 μl of fresh medium was added in each well and incubated for 3 hours at 37°C. The formed crystals of formazan were dissolved in 200 μl of DMSO and the resulted color intensity was quantified with absorbance at 570 nm and a reference wavelength of 630 nm. Data were expressed as the average percentage of viable cells compared to control; experiments were carried out in triplicate for each treatment.

Acridine orange staining for detection of autophagy

Autophagy acidic vacuolar formation was detected by acridine orange staining as described earlier [21] with little modifications. Briefly, after treatment of cells with or without anth, or anth combined with autophagy inhibitors as mentioned above, the medium was aspirated, and cells washed by PBS at room temperature. AO solution (1 μg/ml) in DMEM was added for 15 minutes and cells were washed briefly with PBS and examined immediately under a Zeiss fluorescence microscopy at excitation/ emission, 575/640 nm.

DNA-protein cross-links detection

After exposure to UVB, 1.5×10^6 cells/mL were washed with ice cold PBS and suspended in 300 μl PBS. For isolation of nuclei, equal volume of lysis buffer (20 mM Tris-HCl, 10 mM $MgCl_2$, 2% Triton-X100, 600 mM sucrose) was added. The solution was left on ice briefly and centrifuged (2000× g for 10 min at 4 °C). The pellets containing nuclei were suspended in second lysis buffer (24 mM EDTA, 75 mM NaCl, 1% SDS, pH 8) with protease inhibitor cocktail (1mM PMSF, 1 μg/mL pepstatin, 0.5 μg/mL leupeptin and 1.5 μg/mL aprotinin) and RNase A (10 μg/mL) and then incubated at 37 °C for 2 h. DNA from nuclear lysates were extracted with Tris-buffer saturated phenol and chloroform to remove DNA-unbounded proteins, and then the wanted DPC-containing DNA was precipitated using cold ethanol. To ensure the purity of DNA and exclude any DNA-unbounded protein, 10 μg of the resulted DNA was subjected to neutral thermal hydrolysis at 70 °C for 1 h and then DNA aliquots were separated by 12%

SDS-PAGE. The obtained DPCs bands were visualized by staining with Coomassie blue Protein stain.

Thymidine dimers detection

Thymidine dimers were detected according to the published protocol [22] with some modifications. DNA was extracted by genomic DNA extraction kit and the resulted DNA concentration was quantified by measuring absorbance at 260 nm. DNA (10 ng) was subjected to alkaline thermal denaturation by 1 M NaOH, at 80°C for 30 min. Samples were blotted onto nitrocellulose membrane with BioRad Dot-blot apparatus and the membrane was left to dry in 80 °C. Blocking of non-specific sites by incubating the membrane in PBS-T buffer with 5% BSA for 1 h at ambient temperature. Then, membrane was incubated with mouse anti Td primary antibody (Kamiya Biomedical; 1:3000 dilution). Then, the membrane was washed and incubated with goat anti-mouse HRP conjugated for 1 h (Santa-Cruze biotech, 1:1000). The membrane was washed and the immunoreactive dots were visualized with ECL substance. The intensity of each dot was measured by the ImageJ freeware.

Statistical analysis

Quantitative results are expressed as means ± SD of three independent experiments. Statistical significance of differences was analyzed with one-way analysis of variance followed by Newman-Keuls post-test. Results were considered statistically significant at $p < 0.05$.

RESULTS

1. Effect of anth on HepG2 cell viability

Cells were treated with variable concentrations (1, 5 10 and 20 µg/ml) of anth for 24 h and cell viability was checked with MTT assay (fig. 1). Cell viability was gradually reduced with increasing concentrations of anth. Anthocyanins at concentration of 1 µg/ml reduced cell viability to 90.5±3.7 % compared to control untreated cells whereases, 20 µg/ml of anth reduced cell viability to 71± 2.4 % compared to control untreated cells. In

the next experiments, we used low concentration of anth (0.5 µg/ml) to avoid its cell death effect and at the same time protect cells against DPCs and Td induced by UVB radiation.

Figure 1

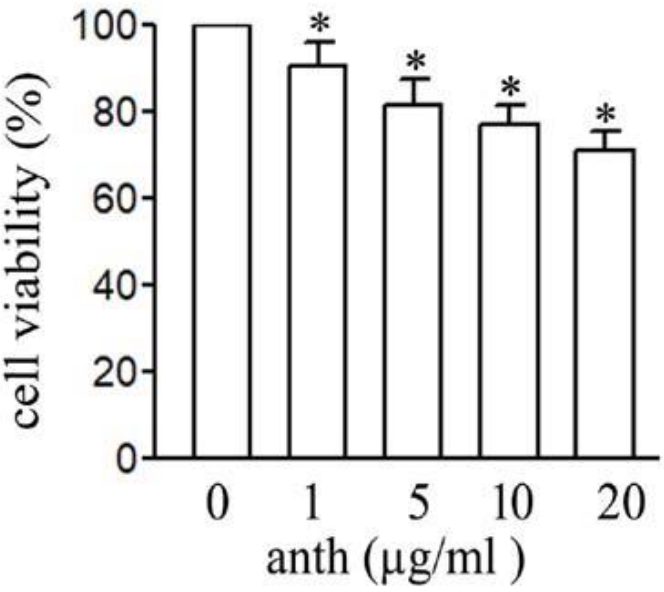

Figure 1: MTT cell viability assay. HepG2 cells were exposed to different concentrations of anth as shown for 24 h and then cell viability was tested by MTT assay. Data are means of three independent experiments ± SD (* $p < 0.05$).

2. Induction of autophagy by anth

Autophagy induction by anth was studied by AO pH-dependent R/G color shifting. Acridine orange color shifts from green in neutral pH to red in acidic pH, which enabled to detect the acidic autophagy vacuolar formation (specially autolysosomes). Anth (0.5 µg/ml) treated cells for 24 h induced autophagy (fig. 2A) as detected from the heavy red vacuoles inside cells compared to the untreated cells which have green to

yellow vacuoles. Autophagy formation is confirmed by calculation of R/G color intensity (fig. 2B). Inhibition of autophagy by anth and 3Ma co-treatment resulted in great and significant reduction of R/G color intensity (fig. 2A and B). A well-known autophagy inducer-Rap- was used to compare its autophagy induction with anth. It was found that autophagy induced by anth is more intense compared to that induced by Rap (fig. 2A and B).

Figure 2

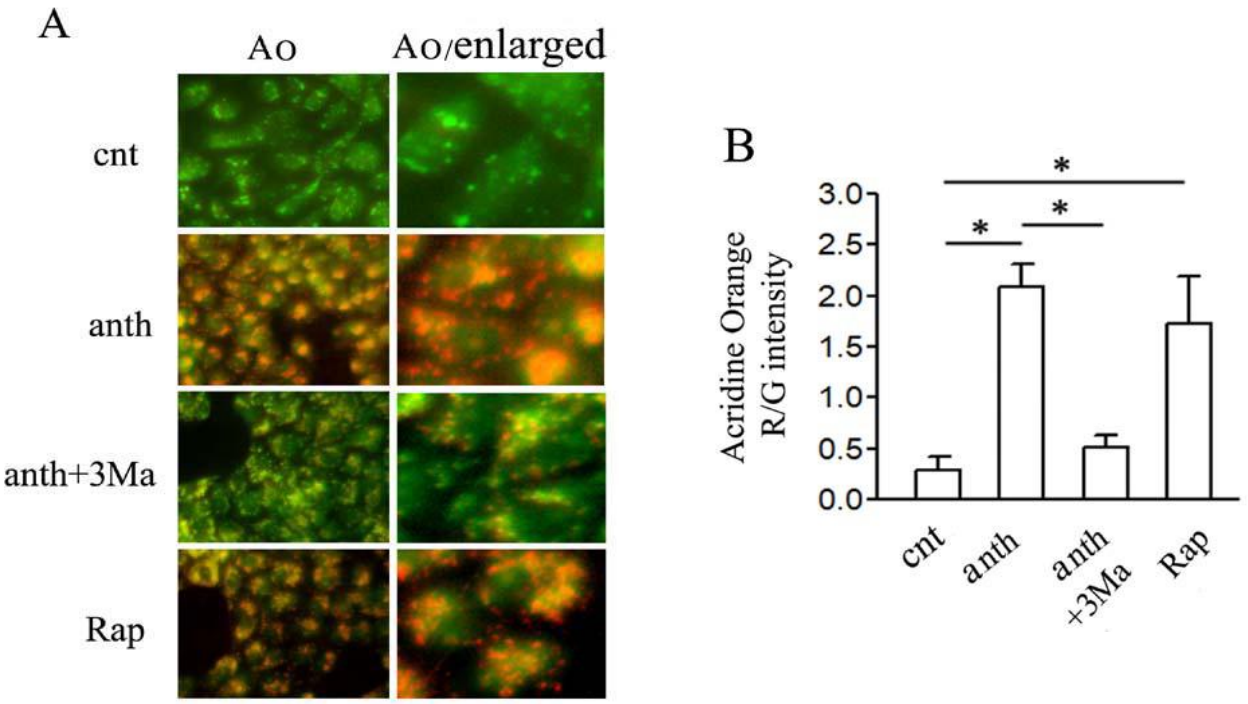

Figure 2: AO staining of acidic autophagy vacuoles. Cells were treated or not with 0.5 µg/mL anth for 24 h, anth combined with 3Ma or Rap alone. Then cells were stained by AO and examined under fluorescent microscope, representative images are shown (A) left panel, X400, right panel is a 3X digital magnification of a part of the corresponding left images. Red to green intensity indicating autophagy status (B). Data are means of three independent experiments ± SD (* $p < 0.05$).

3. Autophagy mediates the protection effect of anth against UVB-induced DPCs formation

Cells treated or not with anth was exposed to UVB radiation and then DNA was extracted, and electrophoresis of covalently linked proteins was carried out. The exposure

of anth untreated cells to UVB radiation resulted in obvious DPCs formation compared with the control unexposed cells (fig. 3A). Also, the effect of UVB on DPCs formation is clearly obvious in calculations of relative band density (fig. 3B). Anth treatment protects cells from UVB-induced DPCs formation. To test if anth-induced autophagy has a role in this protection, autophagy was inhibited by two different inhibitors: Baf-A1 and 3Ma. Co-treatment of anth and autophagy inhibitors reduced the protection effect of anth against UVB-induced DPCs (fig.3 A and B). Whereas Rap treatment alone has almost the same protection effect of anth against UVB-induced DPCs formation.

Figure 3

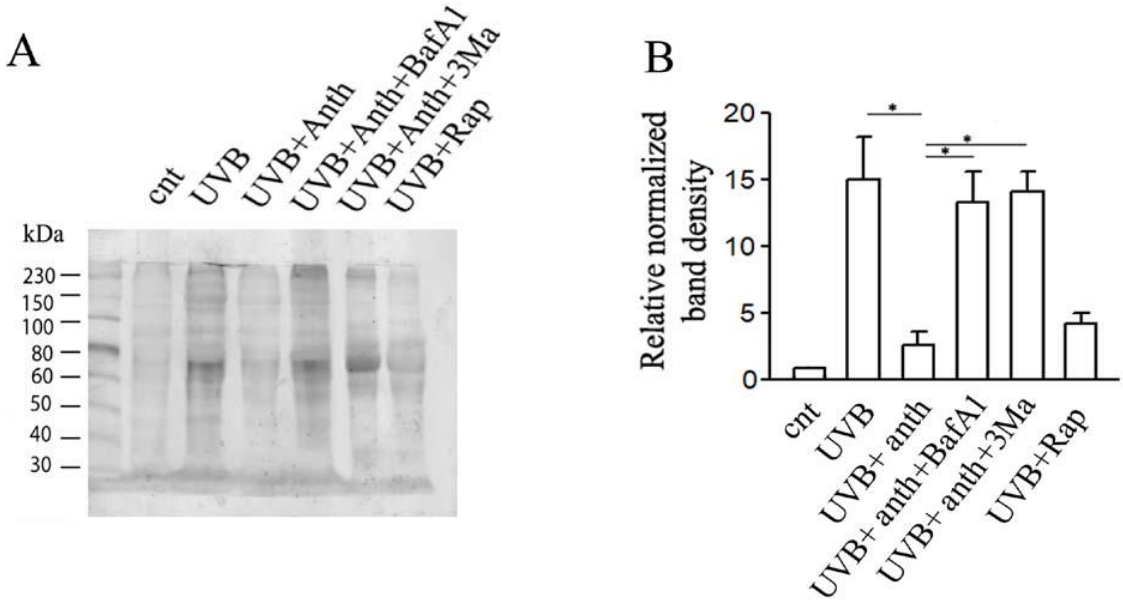

Figure 3: DPCs formation after exposure to UVB. Cells treated or not with 0.5 µg/mL anth for 24 h, anth combined with 3Ma, BafA1 or Rap alone and then exposed to UVB. DNA was extracted as shown in material and method section and subjected to 12% SDS-PAGE electrophoresis to show the covalently linked proteins, a representative image is shown (A). Band density of the corresponding gel was estimated (B). Data are means of three independent experiments ± SD (* $p < 0.05$).

4. Autophagy mediates the protection effect of anth against UVB-induced Td

formation of Td was analyzed by dot blot of DNA using specific antibody for Td (fig. 4A) and then calculations of dot intensity (fig. 4B). It was found that anth protects cells against UVB-induced Td formation. This protection is mediated by anth-induced autophagy, because Baf-A1 and 3Ma reduced the protection effect of anth upon co-treatments. To confirm that autophagy is the main key player in protection of cells against UVB-induced Td formation, cells was treated alone with rap which resulted in a comparable protection against UVB-induced Td as anth (fig. 4A and B).

Figure 4

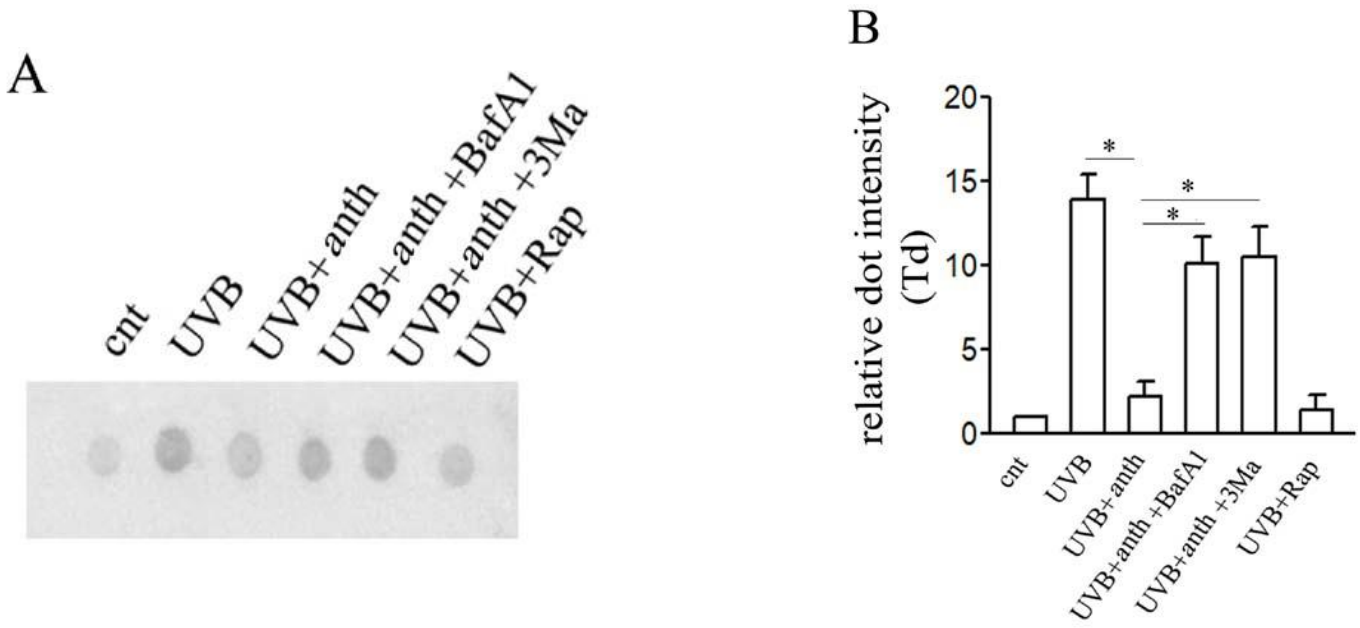

Figure 4: Td immune-dot blot: Cells were treated as in figure 3 and then DNA was extracted as shown in materials and methods section. DNA was blotted onto nitrocellulose and immunodetection of Td was carried out, a representative image is shown (A). Relative dot intensity was calculated compared to control (B). Data are means of three independent experiments ± SD (* $p < 0.05$).

DISCUSSION

UVB (280–315 nm) is absorbed by cellular DNA causing adverse damages including DPCs and Td [2]. Anthocyanins are belonging to flavonoids family [٨], which have vast majority of biological activities [٩] including antioxidant [١٠] and autophagy induction [١٣]. Autophagy in general has a pivotal role in DNA damage response and repair [١٩, 2٣]. In the present study, the relationship between anth induced autophagy and

protection of DNA damages induced by UVB was investigated. We used a low concentration (0.5 µg/mL) of anth extracted from *Hibiscus sabdariffa*, this concentration has a negligible effect on cell viability as detected by MTT assay but still has a great autophagy induction ability. This low anth concentration induced autophagy as detected by AO red to green color shifting. To confirm the induction of autophagy, cells were treated by anth combined by autophagy inhibitor; 3Ma, which resulted in an obvious decline of red vacuoles stained with AO. Also, autophagy inducer; Rap was used to compare its induction of autophagy with the AO red autophagic vacuole formation by anth. Anthocyanins were able to reduce the DPCs and Td formed after exposure to UVB compare with the cells exposed to UVB and not treated with anth. After that we tested the hypothesis that anth-induced autophagy is the main key-player for the protection ability of anth against UVB-induced DPCs and Td. To test this hypothesis cells were treated either with autophagy inhibitors; 3Ma and BafA1 or autophagy inducer; Rap. When anth-induced autophagy was inhibited the ability of anth to protect cells against DPCs and Td was abolished. Whereas Rap alone protected cells against UVB-induced DPCs and Td, which confirm the role of anth-induced autophagy in the protection of these DNA damages induced by UVB. Hence autophagy functions as an important mechanism protecting cells from DNA damage [١٩, 2٣]. In addition, most agents causing DNA damages activated autophagy, so autophagy may be a critical cellular defense against DNA damaging agents. Reactive oxygen species (ROS) are highly active molecules, generated as byproducts of normal metabolic processes. It is well-known that excess ROS levels and low levels of its detoxification threaten many cellular molecules including genetic material. Direct effects of ROS on DNA causing the formation of DNA damages is also reported [٢٤]. Interestingly, in many cases, loss or inhibition of autophagy causes DNA damage and loss of genomic stability. As example, in knock out mice models in autophagic genes (atg5 and atg7) there is accumulation of ROS in different tissues which followed by DNA damage and cell death [٢٥, ٢٦]. In autophagy compromised cells, have no longer ability to eliminate the damaged mitochondria and accordingly have increased DNA damages. In breast cancer, Beclin-1 heterozygosity resulted in autophagy deficiency and ROS accumulation followed by DNA damage [٢٧]. Anthocyanins-

induced autophagy is the key-player of anth protection ability against DPCs and Td formed by exposure to UVB, the subcellular mechanism which enables autophagy to do so may be according to crosstalk between autophagy and ROS signaling pathways.

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