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Research Article
Biological Evaluation of Golden Delicious Apples Exposure to UV Lights in Rats

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Abstract

Background and Objective: Anthocyanin is responsible for the red color of apple. Ultraviolet light plays a key role in activating the genes responsible for anthocyanin biosynthesis. However, the most important concern is using UV light irradiation on fruit to increase anthocyanins level and its nutritional quality. In this study, the accumulation of anthocyanin in green apple using UV-B and UV-C was investigated and its biological influence was evaluated in rats. **Material and Method:** Green Golden delicious apples were irradiated with doses of UV-C and UV-B light for a period of 3 h/day each for 3 days. Two Groups of rats were fed on balanced diet or balanced diet supplemented with 10% apple exposure to UV (AP-UV) for a month. **Results:** The HPTLC and spectrophotometric determination of anthocyanin revealed that color development was significantly increased by 90% in treated apple compared to the control apples. Histological difference was observed between the 2 groups. Plasma levels of uric acid, the activity of transaminases (ALT and AST) as well as malondialdehyde (MDA) were significantly elevated in AP-UV rats. Plasma total cholesterol, triglycerides and creatinine level did not differ among the 2 groups. Liver MDA and catalase levels were eminent in AP-UV rats compared to control. Gene expression of selected inflammatory cytokines (TNF-α, IL-6 and IL-1β) was significantly up-regulated in liver of AP-UV rats in comparison to control rats. **Conclusion:** The result revealed that there is a health-hazard linked to feeding rats on diet containing irradiated-apple with UV-B and UV-C, which represented by body weight reduction, inflammation development, liver function and oxidative stress elevation.

Key words: Anthocyanin, UV light irradiation, apple, oxidative stress markers, cytokines gene expression

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Anthocyanins are water soluble pigments in a wide range of plants species that are responsible for leaves, stems, flowers and fruits coloration (red, blue or purple)[1]. In higher plants, anthocyanins are protective secondary metabolites belong to the flavonoid family and are biosynthesized via the phenylpropanoid pathway[1-3]. Anthocyanins protect plant from ultraviolet (UV) overexposure, biotic stress, high temperature, able to attract pollinators and seed dispersers[4-6]. Anthocyanins play an important role in human health-promoting benefits as antioxidants with anti-diabetic, anti-cancer, anti-inflammatory and in degenerative effects[7-9].

Apple is one of the most important fruit crops worldwide. The color of apple peel is an imperative factor determining apple market value. Anthocyanin is responsible for red pigments in apple peel[10,11]. Environmental factors such as water stress, some nutrients, pathogen infection, UV light able to induce accumulation of anthocyanin. Among these environmental factors, UV-B is a major factor for accumulation of anthocyanin[12]. The UV-B (280-315 nm) irradiation, which is an integral part of the solar radiation reaching the surface of the Earth, induces a wide-ranging of physiological responses in plants[13]. Dong *et al.*[14] reported that red pigment accumulated effectively in apple skin using a combination of white light and UV-B light (fluency of 150 μW cm^{-2}) for 48 h with low temperature. Apple exposure to UV-B light at level equal to half of UV-B light on sunny day (fluency of 0.16-0.2 W m^{-2} for 72 h) led to increase the concentration of anthocyanin in apple skin[15]. Smetanska *et al.*[16] reported that application of visible-UV-B light for 72 h strongly elevated red color of harvested apples. At the same time, ultraviolet C (UV-C) radiation prevents fungal decay and enhances flavonoids content in fruits when irradiated post and after- harvest[17,18]. Effect of UV-C treatment on the anthocyanin accumulation has been studied in several fruits such as strawberries[19,20], blueberries[21], red cabbage[22]. However, no study has been conducted regarding the effect of UV-C treatment on accumulation of anthocyanin in apple skin.

The US food and drug administration and US department agriculture have stated that the use of low-pressure mercury lamp for UV-C irradiation is safe in disinfection of fresh juice and handling of food[23,24]. As many studies investigated the accumulation of anthocyanin under UV light exposure especially UV-B in plant[14-16,25]; the concerns about the use of it is raised. There are no available data regarding feeding animals with plants irradiated by UV-B treatment. Hence, the aim of the current research was to study the influence of apple irradiated with UV-B and UV-C light consumption on biochemical, molecular and nutritional biomarkers in rats. Additionally, study the effects of UV-B and UV-C radiation on anthocyanin accumulation in green apple fruit.

MATERIAL AND METHODS

Plant material: Green Golden delicious apples were field-grown in Bayern Region (Germany), harvested at a mature green stage in 2017 and treated with UV-B and UV-C light (September 2017).

UV irradiation treatment conditions: UV treatment was carried out used the irradiation chamber BS-02 (Opsytec Dr. Groebel, Germany) according to method of Smetanska *et al.*[16] with modification. The irradiation experiment was carried out at 17°C and the UV light lamps (Philips and Osram, Germany) was 100 cm from apples. Green apples were irradiated with UV-C (254 nm) and UV-B (312 nm) with density of 0.37 and 0.38 W m^{-2}, for a period of 3 h per day each for 3 days. The UV dose rate was measured with a digital radiometer. The total radiation doses were 1.21 and 1.23 J cm^{-2}. Apple fruits exposure to UV-B and UV-C were homogenized, freeze-dried to powder form and keep in deep freeze till used in the animal experiment. The UV dose was calculated using the following equation:

$$\text{UV dose (J m}^{-2}\text{)} = \text{Irradiance (W m}^{-2}\text{)} \times \text{exposure time (sec)}$$

Total anthocyanins determination in the apple fruit: Total anthocyanin was measured according to Sims and Gamon[26] with minor modification. Apple powder (0.1 g) was homogenized with 1 mL cold acidified methanol (methanol: HCl, 99:1, v/v) and then incubated overnight in dark at 4°C with moderate shaking. The extracts were centrifuged at 10000 ×g for 10 min, at 4°C. Absorption of the extracts at 530 and 650 nm was recorded using spectrophotometer (Specord®250, Analytik Jena, Germany). Anthocyanins concentrations were calculated as μg cyanidin-3-O-glucoside/g.

HPTLC systems: The apple extracts were carefully applied with capillaries on High-performance thin-layer chromatography (HPTLC) silica gel plates F_{254} (10×20 cm, Merck, Germany) as 25 mm from the side edges, 15 mm from the bottom and 10 mm apart using syringe. The plate was saturated for 20 min in CAMAG glass twin trough chamber with the mobile phase of ethyl acetate:formic

acid:acetic:water 10:1.1:1.1:2.6 mL (v/v/v/v). Plate was developed up to a migration distance of 7.5 cm at 25±5°C. After drying, the chromatograms were documented using the TLC visualizer (CAMAG). The chromatographic and the integrated data were recorded using computer-based software vision CATS.

Rat experimental

Animals and diets: Male albino rats (89.9±6.703) were obtained from the animal house of National Research Centre, Cairo, Egypt. The animals were kept individually in stainless steel cages at room temperature. Water and food were given *ad-libitum*. The animals were fed on a basal diet for 7 days as an adaptation period. Diet composition prepared according to AIN-93M diet[27]. Rats were fed a balanced diet composed of 61.5% wheat starch, 15% casein, 4% sunflower oil, 10% sucrose, 5% cellulose, 3.5% mineral mixture and 1% vitamin mixture. In the case of the diet supplemented with apple exposure to UV (AP-UV), 10% apples powder was added to the balanced diet at the expense of starch.

Experimental design: Rats were divided randomly into 2 groups, 7 rats for each group. Group 1 where rats fed on balanced diet and set as control group. Group 2 where rats fed on balanced diet supplemented with 10% of AP-UV powder. After 4 weeks from treatments, rats were fasted and subsequently anaesthetized and blood samples were collected on heparinized tubes. Plasma was separated by centrifugation (3000 rpm for 5 min 4°C) and used for biochemical analysis. All organs were weighted. Indices of the liver were frozen in liquid nitrogen and stored at -80°C for RT-PCR analysis. Other indices of the liver were used for oxidative stress analysis. The rest of the liver and kidney were immersed in 10% formalin solution for histological examination. This study has been carried out according to the Ethics Committee, National Research Centre, Cairo, Egypt and followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

Biochemical analysis in blood: Hemoglobin was determined using Drabkin's method [28]. All assays were carried out using a commercial kit (Bio-diagnostic kit, Giza, Egypt) according to the manufacturer's instructions. Total cholesterol and triglyceride were measured using standard procedures as described by Watson[29] and Fossati and Prencipe[30], respectively. The safety of AP-UV was studied through liver and kidney function. The activity of alanine amino transaminase (ALT) and aspartate amino transaminase (AST) were carried as indicator of liver function according to the method of Reitman and Frankel[31]. Plasma levels of creatinine[32] and uric acid[33] acid was determined as indicator of kidney function.

Biochemical analysis in liver tissue: Liver tissue was homogenized using ice-cold phosphate buffer (pH 7.4). The ratio of tissue weight to homogenization buffer was 1:5 (w/v). After centrifugation at 15,000 ×g for 15 min, the supernatant was collected for determination of malondialdehyde (MDA) as indicator of lipid peroxidation and catalase (CAT) as antioxidant enzyme according to the methods of Satoh[34] and Aebi[35], respectively.

Determination of inflammatory cytokines by RT-PCR: Total RNA was isolated from <50 mg of liver with Pure Link® RNA Mini Kit (ambion® Life technologies™), according to the manufacturer's instructions. The cDNA was synthesized from 2.0 µg of total RNA in 20 µL reaction with the high capacity cDNA reverse transcription kit (ambion® Life technologies™) according to the manufacturer's instructions.

Real-time PCR was performed with a Rotor-Gene® MDx instrument. The RT-PCR reaction mix (25 µL) contained 1 µL template cDNA, 1×the SYBR® Green PCR master mix (ambion® Life technologies™) and 0.2 µM of the primer pairs. Primers pairs sequence used for tumor necrosis factor-α (TNF-α); interleukin-6 (IL-6) and interleukin-1β (IL-1β) genes expression analysis were adapted from the literature[36], sequences were as follow: TNF-α-FW (5-ACT GAA CTT CGG GGT GAT TG-3), TNF-α-RW (5-GCT TGG TGG TTT GCT ACG AC-3), IL-6-FW (5-TGA TGG ATG CTT CCA AAC TG-3), IL-6-RW (5-GAG CAT TGG AAG TTG GGG TA-3), IL-1β-FW (5-CAC CTT CTT TTC CTT CAT CTT TG-3), IL-1β-RW (5-GTC GTT GCT TGT CTC TCC TTG TA-3), GAPDH-FW (5-GTA TTG GGC GCC TGG TCA CC-3) and GAPDH-RW(5-CGC TCC TGG AAG ATG GTG ATG G-3). RT-PCR reactions were performed using the following protocol: 50°C for 2 min, 95°C for 10 min, 45 cycles of 15 sec at 95°C, 60 sec at 60°C, 15 sec at 72°C. The PCR water was used instead of cDNA templates as a negative. $2^{-\Delta\Delta CT}$ was used to calculate the relative expression ratio of the target gene[37]; the target gene expression was normalized to the expression of the house-keeping gene GAPDH.

Statistical analysis: The results of experiments were expressed as mean±SD and they were analyzed statistically using Student's t-test. In all cases $p<0.05$ was used as the criterion of statistical significance.

RESULTS

Anthocyanin analysis: It was observed that irradiation of green apple by UV-B and UV-C supports accumulation of anthocyanins in the apple fruits and change its color from green to red as seen in Fig. 1a. The anthocyanin content in the UV treated apple (154.01±3.46 μg g^{-1}) fruits has been increased by 90% compared with the control green apple (16.32±0.81 μg g^{-1}) (Fig. 1b, c).

HPTLC analysis: The HPTLC fingerprint of un-irradiated and irradiated apple extract is presented in Fig. 2. There were 9 bands with different Rf values detected at 366 nm (Fig. 2a). At 254 nm, 4 bands were detected in UV irradiated apple (Fig. 2b). The un-irradiated apple shows the same patent at 366 nm but just one band appeared at 254 nm. The HPTLC chromatogram showed that out of nine detected bands, the bands with Rf value 0.68 and 0.96 were found to be more predominate as they covered maximum percentage of area (Fig. 2c, d). However, the height of bands with Rf values 0.68 and 0.96 was higher in UV irradiated apple (0.0856, 0.1380) than un-irradiated apple (0.0345 and 0.1196), respectively.

Biological evaluation of feeding rats on diet containing apple irradiated with UV

Histological analysis: Histological examination of liver and kidney of rats feeding on apple irradiated with UV and normal control rats are present in Fig. 3. Normal histological structure of hepatic lobule and renal parenchyma was showing in liver and kidney tissue of control rats. The liver tissue of rat from AP-UV group shown lipidosis of hepatocytes, Kupffer cells activation, congestion of hepatic sinusoids and portal edema. In the kidney tissue of rat from AP-UV group was observed a cytoplasmic vacuolation of renal tubular epithelium and congestion of glomerular tuft.

Biochemical analysis: Biochemical parameters of different experimental groups are presented in Table 1. Hemoglobin level did not change between the 2 groups. Rats feeding on diet containing UV-irradiated apple showed significant elevation ($p<0.05$) in plasma AST and ALT activities compared to the normal control. Correspondingly, plasma levels of uric acid showed significant elevation ($p<0.001$) in AP-UV rats compared with control rats. Plasma creatinine, total cholesterol and plasma triglycerides levels did not differ

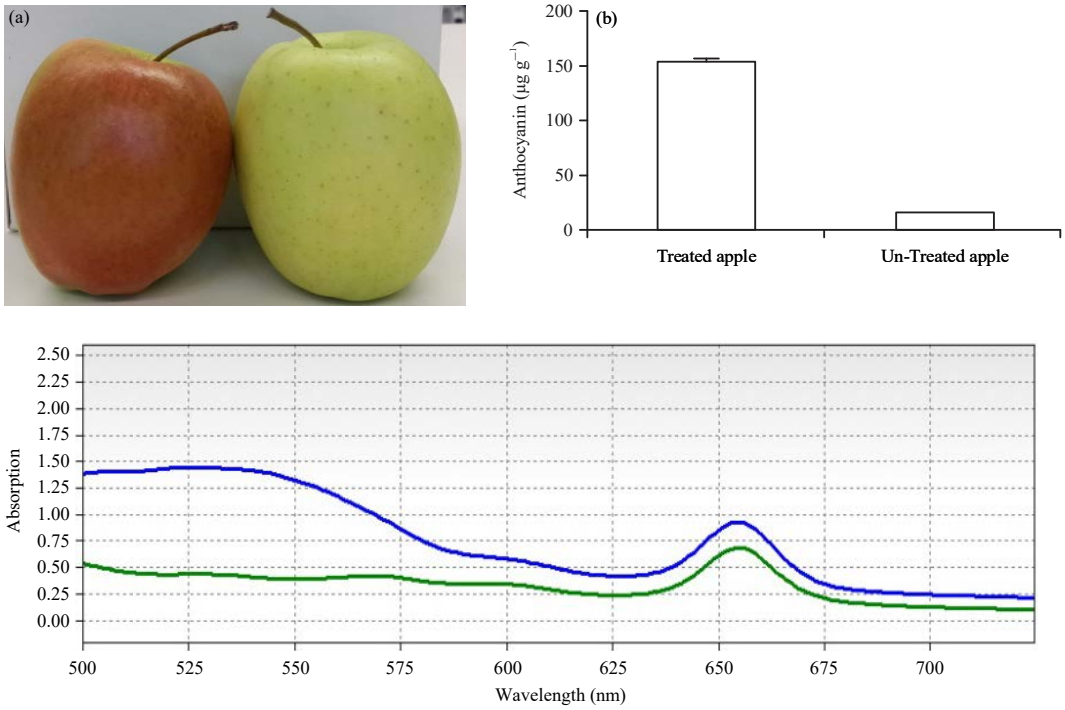

Fig. 1(a-c): Effect of UV lights on anthocyanin accumulation in green apple, (a) Image of green apple before and after UV exposure, (b) Anthocyanin content in green apple extracts before and after UV exposure and (c) Absorption spectra of green apple extracts before (green line) and after (blue line) UV exposure

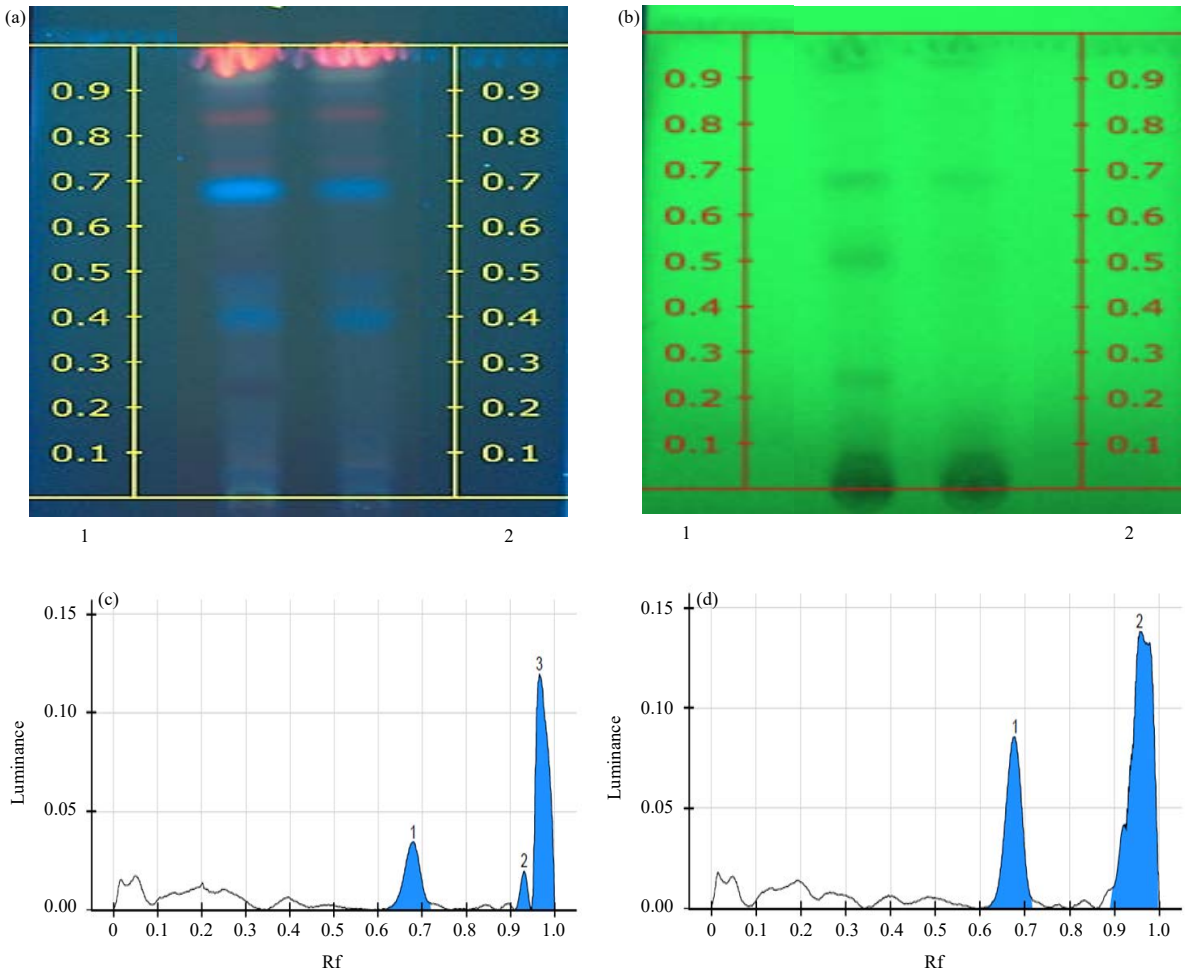

Fig. 2(a-d): HPTLC chromatogram of apple extracts, (a) HPTLC fingerprint of apple extracts at UV 366 nm, (b) HPTLC fingerprint of apple extracts at UV 254 nm, 1: Green apple after UV irradiation, 2: Green apple without irradiation, (c) Densitogram of green apple without irradiation and (d) Densitogram of green apple after UV irradiation

Table 1: Biochemical parameters in rats after treatment with irradiated-apple

Parameters	Control	Apple-UV
Blood Hb (g dL^{-1})	12.860±0.022	12.660±0.041
Plasma		
ALT (U L^{-1})	55.830±4.555	72.000±2.363*
AST (U L^{-1})	49.480±4.008	69.880±9.18*
Creatinine (mg dL^{-1})	0.283±0.011	0.285±0.011
Uric acid (mg dL^{-1})	1.340±0.143	2.440±0.034***
Total cholesterol (mg dL^{-1})	78.400±3.143	81.500±6. 367
Triglyceride (mg dL^{-1})	61.400±9.673	62.800±10.122
MDA (nmol mL^{-1})	0.470±0.041	0.840±0.159*
Liver		
MDA (nmol g^{-1} tissue)	42.050±2.70	61.570±1.50**
Catalase (U g^{-1} tissue)	0.550±0.07	0.730±0.03**

Values presented as Mean±SD, values significantly differ from the control, *p<0.05, **p<0.01, ***p<0.001

between AP-UV and control rats. AP-UV rats was observed a significant increase (p<0.05) in plasma MDA. Tissue MDA level and catalase activity were significantly increased (p<0.01) in liver of AP-UV rats compared to the control rats (Table 1).

Gene expression analysis: The mRNA expression level of inflammatory cytokines (TNF-α, IL-1β and IL-6) in liver tissue is presented in Fig. 4. The gene expression of TNF-α was significantly up-regulated in liver of AP-UV rats by 2.17 fold-changes. Interleukins (IL-1β and IL-6) gene expression were significantly up-regulated by 4.14 and 2.79 fold-change in liver of rats feeding on diet containing apples irradiated with UV compared with control.

Nutritional parameters: All determined nutritional parameters showed insignificant changes in rats feeding on diet containing apples irradiated with UV compared with

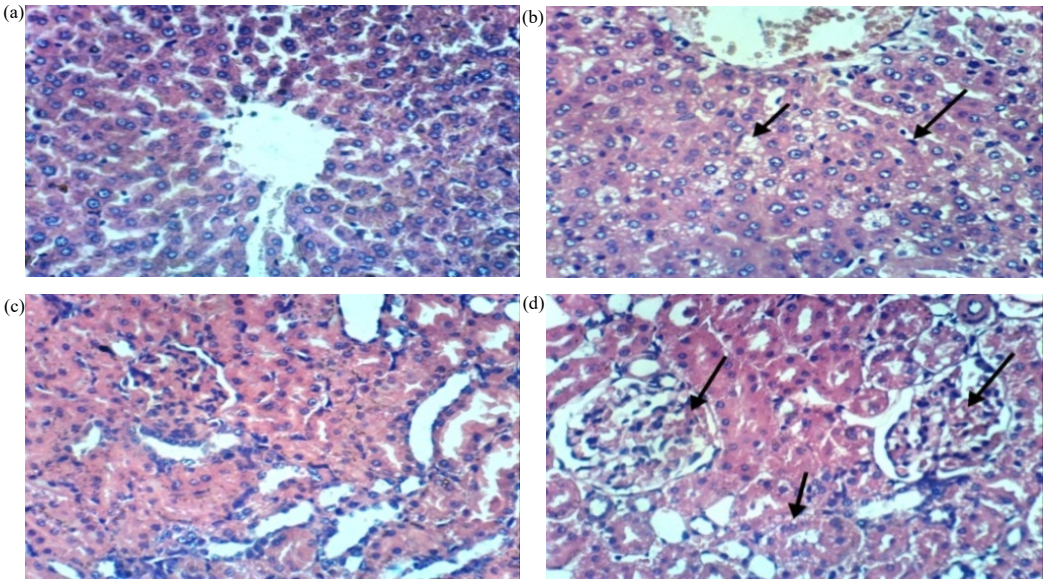

Fig. 3(a-d): Histological examination of liver and kidney of rat fed on irradiated-apple, (a) Liver of rat from control group, (b) Liver of rat from AP-UV, (c) Kidney of rat from control group and (d) Kidney of rat from AP-UV group (H and E ×400)

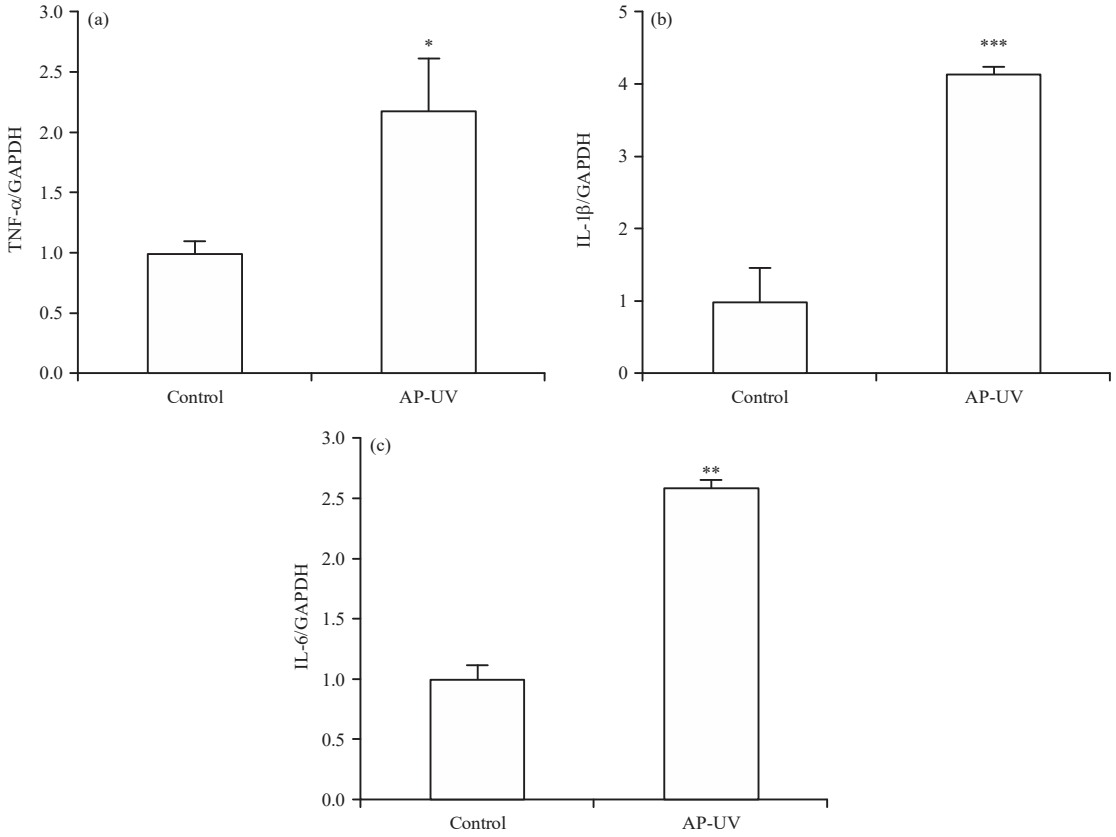

Fig. 4(a-c): Levels of mRNA expression of selected inflammation cytokines genes in liver of rat treated with irradiated-apple, (a) TNF-α expression level, (b) IL-1β expression level and (c) IL-6 expression level

GAPDH: Gene expression is normalized with housekeeping gene, values presented as Mean±SD, values significantly differ from the control, *p<0.05, **p<0.01, ***p<0.001

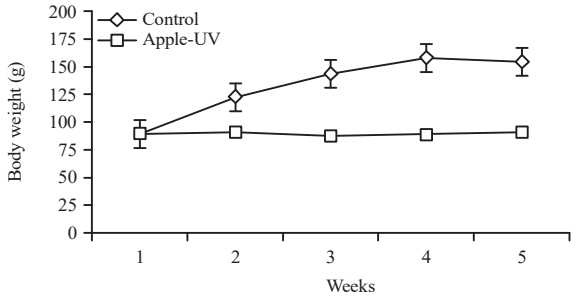

Fig. 5: Growth curve of different experimental groups (n = 7)

Table 2: Nutritional parameters in rats after treatment with irradiated-apple

Parameters	Control	AP-UV
Initial body weight (g)	89.80±14.27	90.20±9.28
Final body weight (g)	154.60±19.53	90.80±7.73***
Body weight gain (g)	64.80±28.62	0.60±8.64***
Total food intake(g)	382.40±2.68	253.20±3.348
Liver weight, percentage of body weight	2.49±0.30	2.80±0.08
Kidney weight, percentage of body weight	0.601±0.07	0.738±0.12
Heart weight, percentage of body weight	0.394±0.04	0.404±0.06
Lung weight, percentage of body weight	0.751±0.05	0.855±0.12
Spleen weight, percentage of body weight	0.376±0.05	0.339±0.04

Values presented as Mean±SD, values significantly differ from the control, ***p<0.001

normal control except for final body weight and body weight gain, which reduced significantly compared with normal control (Table 2). AP-UV rats showed significant reduction in growth from 1st week till 5th week of the study compared with normal control rats (Fig. 5).

DISCUSSION

A good color in apple cultivation and production is important[15]. Anthocyanins are plant pigment (red, blue, purple) belongs to phenolic compounds and are responsible for the colors of flowers, fruits and vegetables[38]. Red coloration in apple fruit is due to the activity of the MYB transcription factor which relates to induction of anthocyanin biosynthesis[39]. Furthermore, anthocyanins possess anti-oxidant, anti-mutagenic, anti-cancer and anti-obesity properties and reduce the risk of cardiovascular diseases[38,40]. Anthocyanins play a valuable role in plant defense against pathogens, UV and drought affects[41]. Anthocyanin level increases in response to the temperature and light as well[1]. It was observed that exposure of apple, peach, blueberry fruit, buckwheat and radish sprouts to UV-B light led to increase anthocyanin biosynthesis[42-46]. Furthermore, studies reported that treatment with UV-C light elevated the level of anthocyanin in strawberries[19,20] and blueberries[21,47]. Wu *et al.*[22] reported induction of anthocyanin biosynthesis gene expression level in fresh-cut red cabbage after treatment with UV-C. Effect of UV-C on anthocyanin accumulation in apple skin has been not studied, also the influence of consuming apple exposure to UV lights in rats. So, the present study was aimed to investigate the effect of UV-B and UV-C irradiation on the accumulation of anthocyanin in the green apple and evaluate the effect of consuming UV irradiated apple in rats.

UV-B radiation and low temperature (17°C) synergistically induced the expression of anthocyanin biosynthetic genes in apple, while high temperature (27°C) treatment with UV-B irradiation had less effect on stimulation[11]. Ban *et al.*[45] reported that UV-B irradiation time affect the anthocyanin accumulation. Anthocyanin concentration in apple skin was increased after 48 and 94 h of UV-B exposure compared to untreated apple but the anthocyanin level didn't change between untreated and treated apple skin with UV-B exposure for 24 h[45]. Combination of UV-C with UV-B under low temperature (17°C) was successfully accumulated anthocyanin in less than 24 h in the current study. Anthocyanin concentration in apple exposure to UV light in the current study was in the same range of published result of anthocyanin content in natural grown red apple[48].

There are no available data regarding feeding animals with plants irradiated by UV treatment. So, the present research is original in this field. In presented study was shown that feeding rats on diet containing irradiated apples elevated ALT and AST activity and that means a disturbance of liver function. Uric acid level was elevated in plasma of rats feeding on irradiated-apple. Studies reported that uric acid is a risk factor for renal disease development and progression[49,50]. Likewise, MDA level in plasma and liver were increased indicating of lipid peroxidation induction. Lipid peroxidation is a biomarker for oxidative stress[51,52]. Oxidative stress can cause damage of lipids, DNA, protein and contributes to development diseases including cardiovascular diseases, diabetes, cancer, chronic pulmonary disease and liver diseases[53,54]. However, cells manifest effective antioxidant defense such superoxide dismutase, catalase and glutathione peroxidase against reactive oxygen species generated through oxidative stress[55]. In current study, catalase activity was elevated in liver tissue of rats feeding on diet containing irradiated apples which can support an activation of cellular defense agent against oxidative stress. Development of oxidative stress may induce increasing of inflammatory cytokines[56]. The production of inflammatory cytokines is connected to the immune dysfunction and damage of tissue and organ[57]. Reactive oxygen species and inflammatory cytokines activate NF-κB

which induces genes expression involved in cell proliferation and apoptosis[58]. Apple-irradiated with UV cause inflammatory effect shown by the up-regulation of inflammatory cytokines (TNF-α, IL-1β and IL-6) gene expression.

All described biochemical changes can relate to significant changes in liver and kidney tissues which were observed in the histological examination. Kupffer cells in liver associated with numerous liver diseases such as steatohepatitis, intrahepatic cholestasis and liver fibrosis[59]. In present study, the Kupffer cells were activated in AP-UV rats. Also, renal tubules and glomeruli were affected by the treatment of apples irradiated with UV in AP-UV rats. Such effect can cause a renal injures as reported Frazier *et al*.[60].

CONCLUSION

The present results revealed that combination of UV-B and UV-C increased anthocyanin accumulation in green apples. At the same time, the feeding diets containing irradiated apple increased liver function and uric acid level in rats. Elevation of MDA level and inflammatory cytokines level were indicator for development of inflammation process and oxidative stress in the rats feeding diets containing irradiated apple.

SIGNIFICANCE STATEMENT

A combination of UV-B and UV-C for increased anthocyanin accumulation in apples under the condition of use is a harmful technique for animal health. Future *in vivo* studies must be done in current field to obtain results for the healthy optimal dose of UV lights that may be used for increasing anthocyanin or any other phytochemicals in the plant object.

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