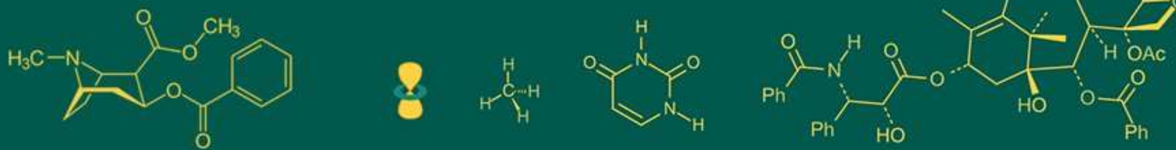

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The beneficial effects of anthocyanin on D-galactose induced cardiac muscle aging in rats

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Abstract
Background and Aim: This study investigated whether anthocyanins, a class of anti-inflammatory flavonoid compounds abundant in fruits and vegetables, could protect against heart aging in rats. The researchers focused on the potential of anthocyanins to inhibit apoptosis and autophagy triggered by D-galactose, a substance known to accelerate aging.
Materials and Methods: Seventy-five adult albino rats weighing between 120 and 140 grams were randomly assigned to five groups of fifteen each. The groups were designated as follows: control group (group I), anthocyanin group (group II), D-galactose group (group III), D-galactose and anthocyanin group (group IV), and anthocyanin pretreatment group (group V).
Results: Anthocyanin pretreatment improved many biochemical changes, induced autophagy, suppressed apoptosis and protected against damaging effects of D- galactose. However, the delay in its administration to be given with the onset of D -galactose administration resulted only in partial improvement. The histopathological findings confirmed the laboratory results.
Conclusions: Anthocyanin administration markedly alleviated inflammation in cardiac muscle and damage induced by D – galactose through inhibition of inflammation, induction of autophagy and suppression of apoptosis.

Keywords: Anthocyanin, d-galactose, cardiac muscle, aging, rats

Introduction

As most living organisms age, their physiological functions naturally decline [1]. This has led some researchers to propose classifying aging itself as a disease. Others, however, view aging as a normal consequence of life, albeit one that increases the risk of developing major diseases like cancer, diabetes, heart problems, and neurodegenerative conditions [2]. Researchers have found that administering D-galactose can accelerate aging in the heart. The reason behind this is that D-galactose causes an increase in the generation of reactive oxygen species (ROS) within cardiac cells. Free radicals (ROS) are dangerous chemicals that may break down proteins, fats (Lipids), and DNA, ultimately leading to cell death (Cardiomyocyte damage) and impaired heart function [3-5].
D-galactose administration has been shown to worsen cell death (Apoptosis) and the breakdown of cellular components (Autophagy) within the heart. This occurs because D-galactose triggers two main apoptotic pathways in heart muscle cells (Cardiomyocytes): the intrinsic pathway, initiated within the mitochondria, and the extrinsic pathway, activated by external signals. As a result, the number of apoptotic cells increases significantly [6]. Anthocyanins, water-soluble pigments found abundantly in colorful fruits like blueberries and grapes, belong to the flavonoid class of compounds. They possess anti-inflammatory properties and have shown promise in improving various inflammatory diseases. Notably, anthocyanins can neutralize harmful molecules called ROS [7].
Studies have shown that anthocyanins possess anti-aging properties. This is evidenced by a decrease in malondialdehyde (MDA) levels in the blood, a marker of oxidative stress. Superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) are antioxidant enzymes that anthocyanins boost, which further protects cells from harm [8]. Anthocyanins also exhibit anti-apoptotic effects. They have the ability to block mitochondria from releasing a protein known as apoptosis-inducing factor (AIF), which would otherwise trigger DNA

fragmentation and cell death via a caspase-independent pathway. In addition, the PI3K/Akt/mTOR signaling pathway is essential for controlling cell proliferation, death, autophagy, and aging; anthocyanins have been demonstrated to block this pathway [9]. Treatment with anthocyanin seems to enhance autophagic flow, even in D-galactose-induced senescent cells, which is surprising. Autophagic flux refers to the overall rate of autophagy, the cellular process of breaking down and recycling damaged components. This finding suggests that anthocyanin may work via blocking the PI3K/Akt/mTOR signaling triplet, as this pathway is known to decrease autophagic flux when activated [9].

A number of cellular functions, such as protein synthesis, cell proliferation, autophagy (cellular recycling), metabolism, and stress responses, are controlled by the mTOR protein. Interestingly, studies have shown that inhibiting mTOR can extend lifespan in mammals. Additionally, it may offer therapeutic benefits for heart conditions. Inhibiting mTOR has been linked to reduced pathological hypertrophy (Abnormal heart muscle thickening) and heart failure Caused by increased workload or genetic mutations (Cardiomyopathies). Furthermore, it may help alleviate heart problems associated with metabolic disorders [10]. Supplemental material one protein that is essential for several physiological processes is glomerulopathy (C3G). For proteins in the Ras family in particular, it functions as an exchange factor for guanine nucleotides. These Ras proteins are molecular switches that control cell proliferation, cell death (Apoptosis), and the cytoskeleton's organization-a web of filaments that affects cell adhesion and migration [11]. Advanced glycation end-products (AGEs) are irreversible modifications that occur to proteins, nucleic acids (like DNA), or fats (lipids) through a process called non-enzymatic glycation. This essentially means sugars can bind to these molecules without the involvement of enzymes, creating stable but potentially harmful end-products [12, 13].

This study investigated the potential benefits of anthocyanins in mitigating age-related heart failure in rats caused by D-galactose. The researchers focused on anthocyanin's ability to suppress cell death (Apoptosis) and the breakdown of cellular components (Autophagy) within the heart, processes believed to contribute to age-related decline.

Materials and Methods

Chemicals

D-galactose was purchased from Oxford Chemical Company (Navghar, India). Anthocyanins were obtained from Sciyu Biotech Company (Shaanxi, China).

Animals

Seventy-five adult male albino rats weighing between 120 and 140 grams were housed in standard galvanized metal cages under controlled room temperature. They were provided with free access to drinking water and food for one week prior to the commencement of the experiment to acclimate them to the laboratory environment.

Experimental design

All experiments with animals were carried out in compliance with the regulations established by the Tanta University, Egypt, Faculty of Medicine's Ethics Committee (Approval code 30977/5/16). Briefly, seventy-five adult male albino rats weighing between 120 and 140 grams were randomly assigned to five groups (n=15/group). The control group (group I) received daily intraperitoneal (IP) injections of 1 mL normal saline (0.9%) for eight weeks [14], Group II (Anthocyanin group): Rats in this group received a daily oral gavage of anthocyanin extract (150 mg/kg body weight) for eight weeks [15], Group III (D-galactose group): To induce cardiac aging, rats in this group received daily intraperitoneal (IP) injections of D-galactose (600 mg/kg body weight) dissolved in normal saline (0.9%) for eight weeks [14, 15], Group IV (D-galactose and anthocyanin group): Rats in this group received daily co-administration of D-galactose (600 mg/kg body weight) and anthocyanin extract (150 mg/kg body weight) via intraperitoneal (IP) injection for eight weeks, following the same route and dosage regimen as previously described. Group V (Anthocyanin pretreatment group): Rats in this group received a daily oral gavage of anthocyanin extract (150 mg/kg body weight) for two weeks, followed by co-administration of D-galactose (600 mg/kg body weight) and anthocyanin extract (150 mg/kg body weight) via daily IP injection for six weeks [15].

Blood Sampling

Following the eight-week treatment period, all animals were euthanized under anesthesia. Blood samples were collected in dry, sterile citrate-containing centrifuge tubes. The blood samples were centrifuged at 5,000 rpm for 10 minutes after being allowed to clot at room temperature for 30 minutes. To prepare for analysis, the resultant plasma was divided into portions and kept at a temperature of -70 °C. Prior to quantification in the assays, the samples were allowed to defrost at room temperature.

Tissue sampling

Following the eight-week treatment period, rats were euthanized after an overnight fast. Tissue samples were collected for further analysis. Hearts were dissected, and portions of cardiac tissue were immediately stored at -20 °C for subsequent biochemical assays. For the purpose of histopathological analysis, extra tissue slices were cut out and preserved in 10% formalin.

Homogenization of the heart tissue

Sample collection and storage (Tissue homogenates): One gram of tissue was rinsed with 10 mM PBS (pH 7.4) to remove any extraneous material. The tissue was then homogenized in 10 mL of 10 mM PBS (pH 7.4) to create a 10% (w/v) homogenate. The homogenate was stored overnight at -20 °C. To further lyse the cells, two freeze-thaw cycles were performed. Subsequently, the homogenates were centrifuged at 5000 x g for 5 minutes at 4 °C to separate cellular components [16].

Determination of C3g level ELISA (SunRed, Shanghai):

A commercially available enzyme-linked immunosorbent assay (ELISA) kit was used to quantify rat C3G protein levels in accordance with the manufacturer's instructions. Briefly, the wells pre-coated with capture anti-C3G antibody were incubated with tissue homogenates or standards. After washing, biotinylated anti-C3G antibody was added, followed by incubation with streptavidin-conjugated horseradish peroxidase (HRP). Following additional washes, a chromogenic substrate was added, resulting in a

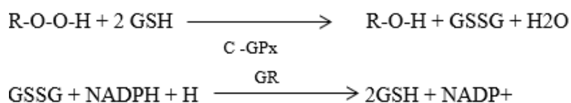
colorimetric change. The color intensity, measured at a specific wavelength, is directly proportional to the concentration of C3G in the samples. Tissue samples were weighed, homogenized in ice-cold PBS (pH 7.4) using a grinder, and centrifuged at 2000-3000 rpm for 20 minutes at 4 °C. The supernatant was collected for further analysis.

Determination of AGEs level ELISA (SunRed, Shanghai)

Similar to the C3G ELISA, the levels of AGEs in the samples were determined using ELISA kit according to the manufacturer's instructions. Briefly, wells pre-coated with capture anti-AGE antibody were incubated with tissue homogenates or standards. After washing, biotinylated anti-AGE antibody was added, followed by incubation with streptavidin-conjugated horseradish peroxidase (HRP). Subsequent washes removed unbound materials. Finally, a chromogenic substrate was introduced, leading to a color change. The intensity of this color, measured at a specific wavelength, is directly proportional to the concentration of AGEs in the samples Determination of MDA level [17]

We measured the malondialdehyde (MDA) content using a colorimetric assay kit from Biodiagnostic Company (Egypt) in accordance with the manufacturer's instructions. Briefly, this assay utilizes the reaction of thiobarbituric acid (TBA) with MDA under acidic conditions at 95 °C for 30 minutes. We measured the absorbance at 534 nm of the pink-colored product, a thiobarbituric acid reactive substance (TBARS). Higher absorbance values indicate increased MDA levels, a marker of lipid peroxidation.

Determination of glutathione oxidase activity [18]



GPx activity was assessed spectrophotometrically by monitoring the oxidation of NADPH to NADP+ at 340 nm (A340) using a commercial kit (please specify the company and kit name) or following a published protocol [reference citation]. Briefly, the assay mixture contains glutathione, glutathione reductase, NADPH, and the sample (Serum or tissue homogenate). Adding hydrogen peroxide as a substrate starts the process, and the reduction in A340 is tracked over time. The rate at which A340 decreases is inversely related to the sample's GPx activity, as the enzymatic reduction of hydrogen peroxide with glutathione consumes NADPH. This assay utilizes the high molar extinction coefficient of NADPH (6220 M^-1 cm^-1 at 340 nm), allowing for the quantification of GPx activity based on the rate of NADPH disappearance.

Assessment of mTOR relative gene expression by quantitative real time PCR (qRT-pcr)

Total RNA extraction was conducted by a commercially available total RNA purification kit (Thermo Scientific, Fermentas, #K0731) according to the manufacturer's instructions. Briefly, this kit utilizes a column-based purification method. The protocol involves lysing and homogenizing samples in the presence of guanidinium isothiocyanate, a chaotropic salt that inactivates endogenous RNases and protects RNA. The lysate is then mixed with ethanol to facilitate RNA binding to the silica membrane within the purification column. Subsequent centrifugation allows the lysate to pass through the column while RNA remains bound. Impurities are removed by washing the column with specific buffers. Finally, pure RNA is eluted from the column using nuclease-free water under low ionic strength conditions.

RNA Analysis: The extracted RNA was quantified and assessed for purity using a NanoDrop spectrophotometer (Analytik Jena, model ND-ScanDrop, Germany).

Synthesis of cDNA via Reverse Transcription

The RevertAid First Strand cDNA Synthesis Kit (#K1622, Thermo Scientific) was used for complementary DNA (cDNA) synthesis in accordance with the instructions provided by the manufacturer. Briefly, this kit utilizes reverse transcriptase to convert isolated RNA templates into cDNA. The resulting cDNA was then amplified using polymerase chain reaction (PCR) for further analysis.

PCR amplification for cDNA of GAPH, mTOR genes

The expression levels of target gene mRNAs in heart tissue from rats were quantified using quantitative real-time PCR (qRT-PCR) with SYBR Green dye. GAPDH was employed as an internal reference gene for normalization.

PCR reagents: In order to amplify the target genes, quantitative real-time PCR (qRT-PCR) was employed. The reaction mixture contained SYBR Green master mix (TOP real™ qPCR 2X PreMIX, Enzynomics, Korea) and gene-specific primers (Thermo Fisher Scientific, USA).

Primers: Primer sequences for mTOR and GAPDH mRNA were designed based on published rat sequences and validated using BLAST software [refer to the appropriate citation for the BLAST tool used, if applicable]. The specific primer sequences are presented in Table 1.

Table 1: The specific primer sequences are presented

Gene	Forward primer (5'to 3')	Reverse primer (5'to 3')
GA PDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
mTOR	AGAAGGGTCTCCAAGGACGACT	GCAGGACACAAAGGCAGCATTO

Total protein assessment – Biuret method [19]

A colorimetric assay developed from the Lowry technique was used to measure the content of protein. Briefly, this method utilizes the reaction of copper sulfate with protein in an alkaline medium to form an intensely colored violet-blue complex. Iodide is included in the reaction mixture as an antioxidant. The sample's total protein content is directly proportional to the color intensity, allowing for quantification through absorbance measurement at a specific wavelength.

Statistical analysis

Statistical analysis was done by SPSS v26 (IBM Inc., Chicago, IL, USA). Quantitative variables were presented as

mean and standard deviation (SD) and compared between the two groups utilizing ANOVA (F) test with post hoc test (Tukey). Qualitative variables were presented as frequency and percentage (%) and were analysed utilizing the Chi-square test. A two tailed P value < 0.05 was considered statistically significant.

Results

Plasma MDA concentration was significantly increased in Galactose group ($p<0.001$) and galactose – anthocyanin group ($p<0.01$) when compared to control and anthocyanin groups. On other hand, plasma MDA concentration was significantly decreased in galactose – anthocyanin group ($p<0.01$) and galactose – anthocyanin group pretreated ($p<0.001$) groups as compared to Galactose group. MDA concentration in tissue was significantly increased in Galactose group as compared to control ($p<0.001$), anthocyanin ($p<0.001$) and G&A pretreated ($p<0.01$) groups.

Table 2: Comparison of MDA concentration in plasma and tissue in different groups

	MDA conc. (nmol/ml) (n=10)		**P**
	Plasma	**Tissue**	
Group I (Control)	1777.1±120.1	21.7±4	<0.001*
Group II (Anthocyanin)	1580.1±180.2	19.99±5	
Group III (Galactose)	4323.9±190.5	63.57±5.4	
Group IV (G&A)	3022±170.2	39.95±4.2	
Group V (G&A pratreated)	2409.75±180.1	34.57±5.1	
	P1<0.001*, P2<0.01*, P3<0.01*, P4<0.001*, P5<0.001*	P 6<0.001*, P7<0.001*, P8<0.001*	

Data are presented as mean ± SD. *significant p value <0.05. MDA: malondialdehyde. P1: significance between I&III, P2: significance between I&IV, P3: significance between II&IV, P4: significance between III&IV, P5: significance between III&V, P6: significance between I&III, P7: significance between II&III, P8: significance between III&V.

Plasma AGEP concentration was significantly increased in Galactose group ($p<0.001$) as compared to another groups, AGEPs level also is significantly decreased in group IV, V compared to group III. Tissue AGEP concentration was significantly increased in Galactose group as compared to control ($p<0.01$), anthocyanin ($p<0.01$) and G and A pretreated ($p<0.05$) groups, also AGEPs level is decreased in group IV and group V compared to group III. There was no significant difference between groups in plasma glutathione peroxidase activity, however enzyme activity was elevated in group IV, V compared to group III. Activity of glutathione peroxidase in tissue was significantly decrease in Galactose group as compared to control ($p<0.01$) and anthocyanin ($p<0.001$) groups Also, G&A ($p<0.01$) and G&A pretreated ($p<0.05$) groups were significantly decrease in tissue glutathione peroxidase activity as compared to anthocyanin group. Table 3

Table 3: Comparison of AGEPs concentration in plasma and tissue and glutathione peroxidase activity of different groups

	AGEPs conc. in (ng/L) (n=10)		**P**
	Plasma	**Tissue**	
Group I (Control)	0.42±0.02	3.25±1.6	**<0.001***
Group II (Anthocyanin)	0.4±0.02	2.89±1.4	
Group III (Galactose)	1.87±0.04	6.18±2.3	
Group IV (G&A)	0.7±0.05	4.74±1.8	
Group V (G&A pratreated)	0.2±0.04	3.49±1.3	
	P1<0.001*, P2<0.001*, P3<0.001*, P4<0.001*	P1<0.01*, P2<0.001*, P4<0.05*	
	Glutathione peroxidase activity (mU/mL)		
	Plasma		
Group I (Control)	20.07±2.3		0.9
Group II (Anthocyanin)	25.7±2.1		
Group III (Galactose)	18.98±2.33		
Group IV (G&A)	24.18±2.5		
Group V (G&A pratreated)	25.89±2.34		
	Tissue		
Group I (Control)	26.5±3.1		<0.001*
Group II (Anthocyanin)	29.48±4.98		
Group III (Galactose)	19.3±3.6		
Group IV (G&A)	22.26±2.2		
Group V (G&A pratreated)	23.88±3.2		
	P1<0.001*, P2<0.001*, P5<0.001*, P6<0.001*		

Data are presented as mean ± SD. *significant p value <0.05. P1: significance between I&III, P2: significance between II&III, P3: significance between III&IV, P4: significance between III&V, P5: significance between II&IV, P6: significance between II&V, MDA: malondialdehyde.

G&A and G&A pretreated groups were significantly increased when compared to control ($p<0.01$) and anthocyanin ($p<0.001$) groups. Also, C3G was significantly increased in G&A and G&A pretreated as compared to Galactose ($p<0.001$) group. mTOR concentration was significantly decreased in G&A pretreated group when compared to control ($p<0.001$), anthocyanin ($p<0.01$) and G&A group groups. Galactose group was significantly increased in mTOR concentration as compared to control and anthocyanin groups ($p<0.001$). G&A and G&A pretreated groups were significantly decreased in mTOR concentration as compared to Galactose ($p<0.001$) group. Table 4, Figure 1

Table 4: ELISA C3G and mTOR PCR result in different groups

	ELISA C3G	mTOR PCR	
Group I (Control)	230.9±56.4	0.057±0.01	<0.001*
Group II (Anthocyanin)	238.2±49.8	1.33±0.12	
Group III (Galactose)	47.85±34.3	2.21±0.2	
Group IV (G&A)	560.8±45.5	1.48±0.11	
Group V (G&A pratreated)	603.98±55.5	0.36±0.09	
	P1<0.01*, P2<0.01*, P3<0.001*, P4<0.001*, P5<0.001*, P6<0.001*	P7<0.01*, P8<0.01*, P9<0.001*, P10<0.001*, P11<0.001*, P12<0.05 *	

Data are presented as mean ± SD. *significant p value <0.05. P1: significance between I&IV, P2: significance between I&V, P3: significance between II&IV, P4: significance between II&V, P5: significance between III&IV, P6: significance between III&V, P7: significance between I&III, P8: significance between I&V, P9: significance between II&III, P10: significance between II&V, P11: significance between III&V, P6: significance between IV &V, MDA: malondialdehyde, ELISA: enzyme-linked immunoassay, mTOR: mammalian target of rapamycin, PCR: polymerase chain reaction.

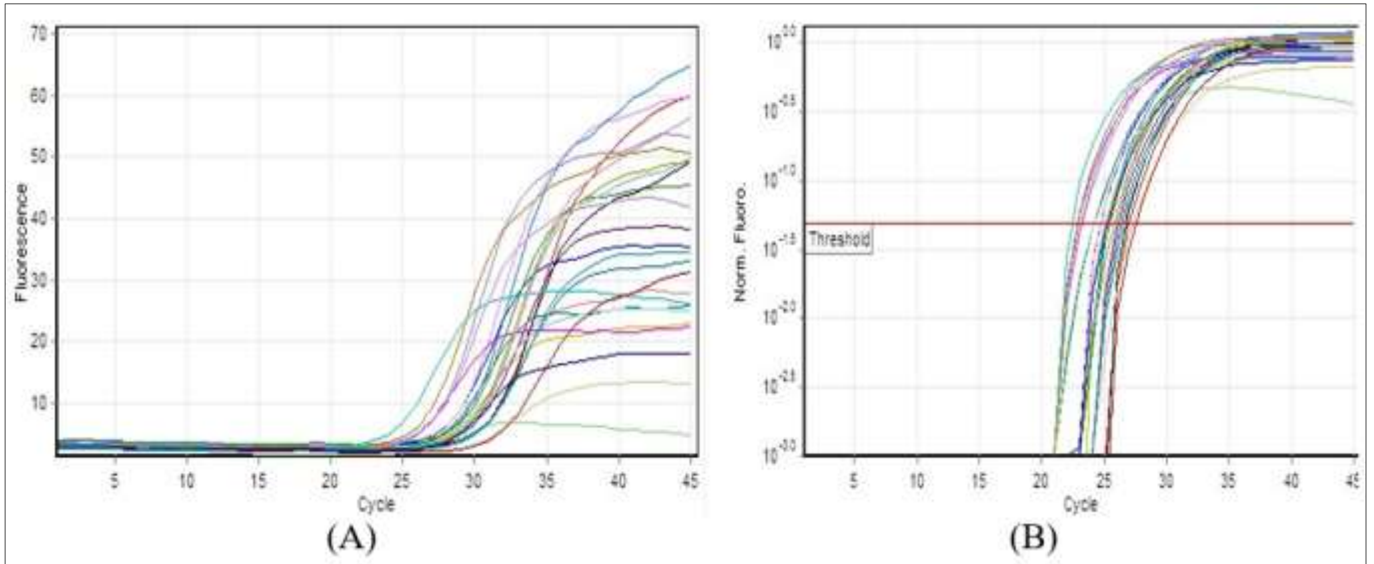
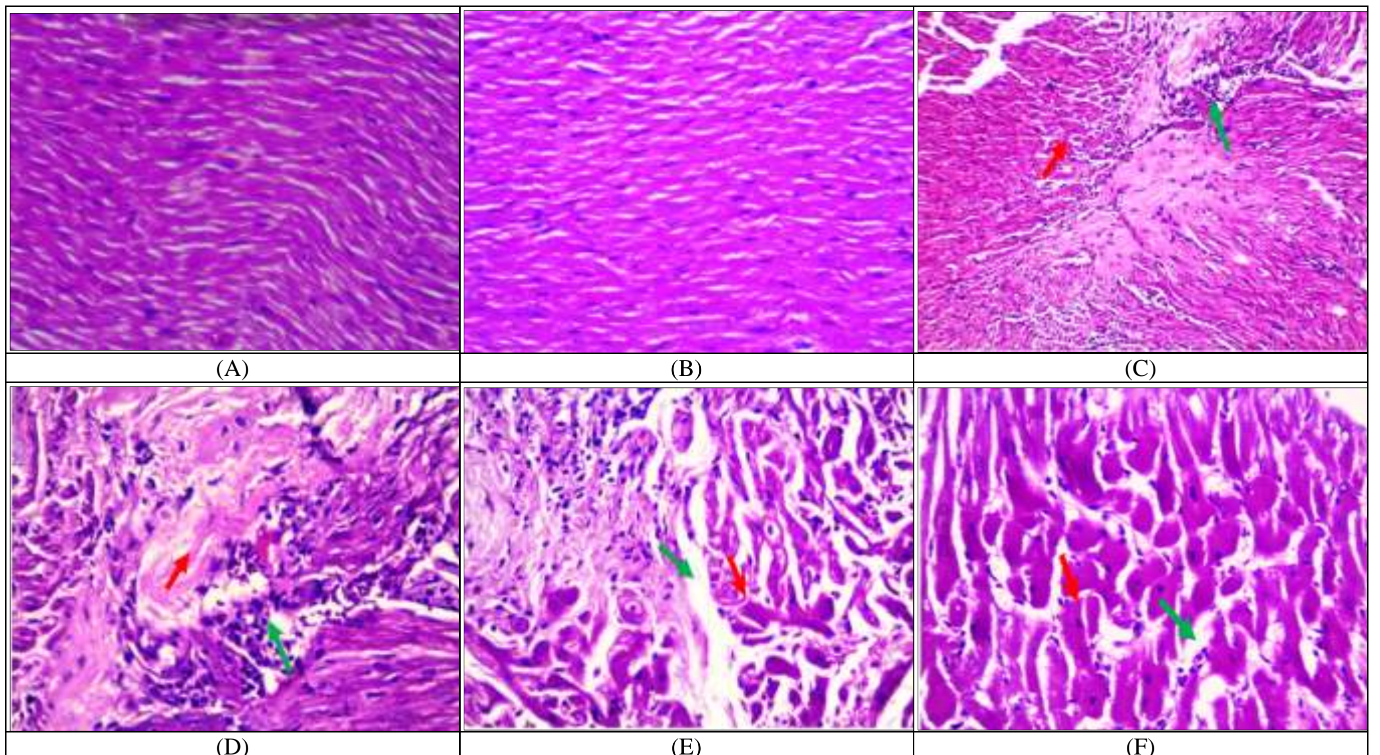
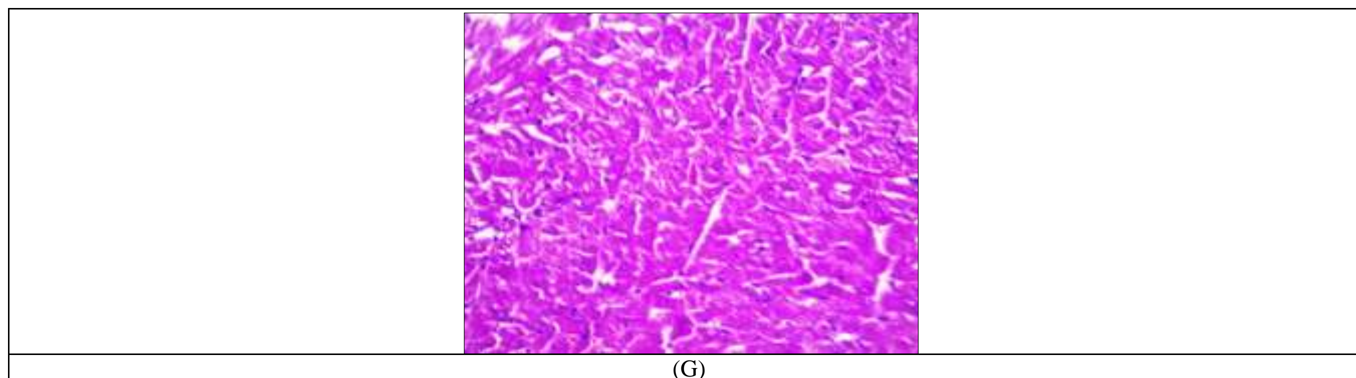


Fig 1: (A) Real-time PCR mTOR gene expression in a samples

Histopathological changes: Representative photomicrograph of cardiac muscle using hematoxylin and eosin for studied groups showed different changes as in Figure 2.





(G)

Fig 2: Representative photomicrograph of cardiac muscle using hematoxylin and eosin from (A) control (group I), (B) treated control (group II) showing normal cardiac muscle fibers, (C) galactose group (group III) showing fibrosis(red arrow) and chronic inflammatory cellular infiltrate(green arrow) caused by D-Galactose, (D) with higher magnification from galactose group (group III) showing fibrosis (red arrow) and chronic inflammatory cellular infiltrate (green arrow) caused by D-Galactose, (E) galactose group (group III) showing degenerated cardiac muscle fibers (red arrow) with disrupted normal arrangement and increased intercellular spaces (green arrow) (H&E X400), (F) treated group (group IV) showing mildly degenerated cardiac muscle fibers (red arrow) with mild chronic inflammation and widened intercellular spaces (green arrow) (H&E X400), (G) pretreatment group (group V) showing lack of degeneration, fibrosis and inflammation (H&E X400)

Discussion

Cardiovascular aging is a natural biological process that disrupts cardiovascular homeostasis. This disruption leads to the gradual deterioration of the heart and blood vessels, both structurally and functionally. As a consequence, individuals become more susceptible to developing various cardiovascular diseases [20].

Malondialdehyde (MDA) levels, a marker of lipid peroxidation, were significantly increased in the D-galactose group (group III) compared to both the control and anthocyanin groups (groups I and II, respectively). Conversely, groups receiving anthocyanin treatment (groups IV and V) displayed significantly lower MDA levels in both tissue and plasma samples. MDA is a toxic by-product of lipid peroxidation, a biomarker of oxidative stress and ROS accumulation, and an indicator of aging [21]. Danyang Tian *et al.* [22] found that chronic oxidative stress induced by excess D-galactose exposure is likely responsible for the observed decrease in Cu,Zn-SOD activity in our study. This decline in antioxidant defense mechanisms may contribute to the enhanced oxidative damage in myocardial tissue, a recognized hallmark of aging. Similar to MDA levels, AGE (advanced glycation end product) concentrations were significantly elevated in the D-galactose group (group III) compared to the control group (group I). Conversely, groups receiving anthocyanin treatment (groups IV and V) exhibited significantly lower AGE levels. The observed elevation of AGEs in the D-galactose group (group III) is likely due to their formation via the Maillard reaction with excess D-galactose. These AGEs can interact with their receptors, leading to the activation of NADPH oxidase and subsequent increased production of reactive oxygen species (ROS). Furthermore, NADPH oxidase activation has been linked to the upregulation of p38 MAP kinases, promoting the translocation of NF-κB to the nucleus. This translocation enhances the transcription of TNF-α, a potent pro-inflammatory cytokine. Collectively, these events contribute to the increased inflammation and deleterious effects on cardiac myocytes, which are hallmarks of aging [23]. Wang *et al.* [3] found that advanced glycation end products (AGEs) contribute to the pathogenesis of various diseases by binding to their receptor, RAGE. This interaction triggers a cascade of detrimental events, including oxidative stress, inflammation, and the accumulation of extracellular matrix. Notably, RAGE also serves as a receptor for HMGB1 (High Mobility Group Box 1), a protein released by necrotic cells that further amplifies inflammatory responses.

Anthocyanins exhibit protective effects against AGEs primarily through their interaction with proteins, which can occur in two main ways. Firstly, anthocyanins compete for glycation sites by binding to protein surfaces, thereby potentially impeding the formation of AGEs during the Maillard reaction. Secondly, these compounds can stabilize protein structure via non-covalent bonds like hydrogen bonds and van der Waals forces, which may help maintain the native α-helical structure of proteins, reducing their vulnerability to glycation and consequent AGE formation [24]. V.M. Totlani *et al.* [25] found that anthocyanins likely exert their protective effects against AGEs through multiple mechanisms. One mechanism involves inhibiting aldose reductase, an enzyme that contributes to the formation of AGEs by converting glucose to sorbitol. Additionally, anthocyanins may help regulate blood sugar levels, indirectly reducing the precursor substrate available for AGE formation. Furthermore, anthocyanins can act as scavengers, trapping reactive dicarbonyl compounds. These dicarbonyl compounds, with their adjacent carbonyl functional groups, are highly reactive precursors of AGEs. By binding to these dicarbonyls, anthocyanins prevent them from participating in AGE formation.

The observed decrease in GPx activity in the D-galactose group (group III) might be attributed to a depletion of the reducing equivalent NADPH. NADPH serves as a crucial cofactor for GPx, enabling its antioxidant function in redox reactions. However, in our study, excess D-galactose may have diverted NADPH towards the aldose reductase pathway, which utilizes NADPH to convert galactose to galactitol. This competition for NADPH could have limited its availability for GPx, thereby compromising its antioxidant capacity [26]. E Ali H *et al.* [27] who reported that reduced Nrf-2 activity can contribute to this effect. Nrf-2 is a transcription factor that regulates the expression of antioxidant genes, including GPx. However, D-galactose administration has been shown to increase NF-κB and TNF-α levels, which can suppress Nrf-2 activity. This suggests that the elevated levels of NF-κB and TNF-α observed in

our D-galactose group (group III) might be responsible for the decreased GPx activity. These results are consistent with Milton Packer *et al.* [28] who demonstrated elevated mTOR activity in senescent cells. Upregulated mTOR signaling has been linked to resistance to apoptosis and a propensity for cells to enter senescence. This resistance is thought to occur through the activation of the p53/p21 or p16 pathway by mTOR, leading to cell cycle arrest – a hallmark characteristic of senescence.

Anthocyanins trigger autophagy via activating AMPK, which in turn inhibits mTOR signaling. One possible mechanism that helps eliminate damaged organelles and prevents their buildup, along with accumulating misfolded proteins, is an increase in autophagic flux [29], protein is responsible for disruption of cellular function, another mechanism by which anthocyanin inhibit mTOR signaling is indirectly through its anti – inflammatory effect, decreasing ROS with concomitant inhibition of mTOR [30]. Orjalo A.V. *et al.* [31] suggest that mTOR complex 1 (mTORC1) inhibition can suppress the expression and secretion of pro-inflammatory cytokines in senescent cells. This suppression occurs through two mechanisms: 1) blocking the translation of IL-1α, and 2) reducing the transcriptional activity of NF-κB, which subsequently leads to decreased expression and secretion of IL-6 and IL-8. Given the upregulated mTOR signaling observed in our D-galactose group, it is possible that this dysregulation contributes to the elevated inflammatory response observed in this group.

Our findings revealed a significant decrease in C3G (presumably referring to Cyanidin-3-glucoside) levels in the D-galactose group (group III) compared to the control groups (groups I and II). Conversely, C3G levels were significantly elevated in the anthocyanin-treated groups (groups IV and V). Notably, C3G possesses direct anti-apoptotic properties, acting to reduce apoptosis through a caspase-independent pathway [32]. C3G, also known as RapGEF1 or RAP1 guanine nucleotide exchange factor 1 (RAPGEF1), is a protein with a specific role in regulating cellular signaling pathways. C3G functions as a guanine nucleotide exchange factor (GEF). GEFs play a critical role in activating small GTPases by promoting the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP). This exchange from the inactive GDP-bound state to the active GTP-bound state allows small GTPases to transduce signals within the cell [33]. Tracy E. *et al.* [34] demonstrated that the mitogen-activated protein kinase (MAPK) pathway is an essential signaling cascade in heart remodeling, and C3G deficiency can cause deregulation of this mechanism.

The c-Abl, a cellular tyrosine kinase, plays a complex role in regulating cell death pathways. Originally identified as the counterpart of the oncogenic v-Abl protein in the Abelson murine leukemia virus, c-Abl is now known to be conserved across various species, including humans, mice, fruit flies (Drosophila), and nematodes. Interestingly, the activation of c-Abl appears to be dependent on the phosphorylation of Cyanidin-3-glucoside (C3G). However, C3G itself exerts a dual effect on cell death depending on the stressor. While C3G can mediate cell death in response to oxidative stress, it can also promote cell survival during serum starvation [35]. C3G behaves as a proapoptotic molecule, Arturo carabias *et al.* [36] demonstrated that C3G depletion, either through knockdown or knockout, can enhance cell survival. This enhanced survival appears to be mediated by the upregulation of p38α activity. Notably, p38α can exhibit an anti-apoptotic role under conditions of oxidative stress.

D-galactose treatment (group III) resulted in a significant upregulation of Bax and cleaved caspase-3 protein levels in cardiac tissue compared to control groups (groups I and II). In contrast, there was a significant reduction in the expression of Bcl-2, an anti-apoptotic protein. These findings suggest increased apoptotic activity in the D-galactose group, on the other hand Pengxiao Li *et al.* [37] reported that it is possible that anthocyanins enhance apoptosis, which in turn aids in the clearing of aged cells. An increase in the percentage of healthy cells in the tissue would result from this clearing.

Histopathological analysis revealed minimal evidence of cardiac muscle degeneration in control groups (groups I and II). In contrast, group III, treated with D-galactose, displayed significant fibrosis and chronic inflammatory cell infiltration. Additionally, this group exhibited degenerated cardiac muscle fibers with disrupted normal arrangement and increased intercellular space. These findings suggest substantial structural damage to the myocardium in the D-galactose group. Conversely, groups receiving anthocyanin treatment (groups IV and V) displayed a marked improvement in cardiac health compared to group III. While group IV showed mild signs of degenerated muscle fibers, chronic inflammation, and widened intercellular space, these changes were significantly less severe compared to the D-galactose group. Notably, group V, receiving the highest anthocyanin dose, exhibited no detectable evidence of cardiac muscle degeneration, chronic inflammation, or widened intercellular space [38]. Geum - Hwa *et al.* [39] reported that D-galactose treatment in their model resulted in severe disarrangement of cardiomyocytes (heart muscle cells) and the presence of large interstitial spaces. These observations suggest a detrimental effect of D-galactose on cardiac structure, potentially contributing to impaired function.

This study recommended combining mTOR inhibition with cell cycle arrest inhibitors to alleviate aging related disease. Further investigation of mTORC2, mTORC2 expression and their role in aging. Further investigation of MTOR localization and their role in aging. Compare effect of different types of anthocyanins on cardiac muscle aging induced by D – galactose. Measurement of more specific aging marker in cardiac muscle e.g. telomer length to study more aspect of anthocyanin action.

Conclusions

The treatment with anthocyanin markedly alleviated D-galactose induced inflammation and damage in experimental rats as evidenced by alterations in the biochemical indices and heart histopathological changes. In this investigation, we found that anthocyanin can protect heart muscle against D-galactose-induced inflammation and injury. The effect that may occur through anti-inflammatory, antioxidant action antiapoptotic, anti-autophagic effect. However, evaluation of it's of the underlying mechanisms of actions deserve further studies.

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References

1. Bulterijs S, Hull RS, Björk VC, Roy AG. It is time to classify biological aging as a disease. Front Genet. 2015;6:205.
2. Stallone G, Infante B, Prisciandaro C, Grandaliano G. mTOR and Aging: An Old Fashioned Dress. Int J Mol Sci. 2019;20:210-214.
3. Wang SS, Zhang X, Ke ZZ, Wen XY, Li WD, Liu WB, *et al*. D-galactose-induced cardiac ageing: A review of model establishment and potential interventions. J Cell Mol Med. 2022;26:5335-5359.
4. Lin HJ, Ramesh S, Chang YM, Tsai CT, Tsai CC, Shibu MA, *et al*. D-galactose-induced toxicity associated senescence mitigated by alpinate oxyphyllae fructus fortified adipose-derived mesenchymal stem cells. Environ Toxicol. 2021;36:86-94.
5. Yang X, Jia J, Ding L, Yu Z, Qu C. The role of Nrf2 in D-galactose-induced cardiac aging in mice: Involvement of oxidative stress. Gerontol. 2021;67:91-100.
6. Lay IS, Kuo WW, Shibu MA, Ho TJ, Cheng SM, Day CH, *et al*. Exercise training restores IGFIR survival signaling in d-galactose induced-aging rats to suppress cardiac apoptosis. J Adv Res. 2021;28:35-41.
7. Dong Y, Wu X, Han L, Bian J, He C, El-Omar E, *et al*. The potential roles of dietary anthocyanins in inhibiting vascular endothelial cell senescence and preventing cardiovascular diseases. Nutrients. 2022;14:106-115.
8. Xue H, Gao Y, Wu L, Cai X, Liao J, Tan J. Research progress in extraction, purification, structure of fruit and vegetable polysaccharides and their interaction with anthocyanins/starch. Crit Rev Food Sci Nutr. 2023;170:1-26.
9. Hu X, Yang Y, Tang S, Chen Q, Zhang M, Ma J, *et al*. Anti-aging effects of anthocyanin extracts of sambucus canadensis caused by targeting mitochondrial-induced oxidative stress. Int J Mol Sci. 2023;24:88-102.
10. Dumas SN, Lamming DW. Next generation strategies for geroprotection via mTORC1 inhibition. J Gerontol A Biol Sci Med Sci. 2020;75:14-23.
11. Sasi Kumar K, Ramadhas A, Nayak SC, Kaniyappan S, Dayma K, Radha V. C3G (RapGEF1), a regulator of actin dynamics promotes survival and myogenic differentiation of mouse mesenchymal cells. Biochim Biophys Acta. 2015;1853:2629-2639.
12. Campbell DJ, Somaratne JB, Jenkins AJ, Prior DL, Yii M, Kenny JF, *et al*. Diastolic dysfunction of aging is independent of myocardial structure but associated with plasma advanced glycation end-product levels. PLoS One. 2012;7:49-58.
13. Simm A. Protein glycation during aging and in cardiovascular disease. J Proteomics. 2013;92:248-259.
14. El-Far AH, Elewa YHA, Abdelfattah EA, Alsenosy AA, Atta MS, Abou-Zeid KM, *et al*. Thymoquinone and curcumin defeat aging-associated oxidative alterations induced by d-galactose in rats' brain and heart. Int J Mol Sci. 2021;22:45-62.
15. Herawati ERN, Santosa U, Sentana S, Ariani D. Protective effects of anthocyanin extract from purple sweet potato (*Ipomoea batatas* L.) on blood mda levels, liver and renal activity, and blood pressure of hyperglycemic rats. Prev Nutr Food Sci. 2020;25:375-379.
16. Uzelac JJ, Stanić M, Krstić D, Čolović M, Djurić D. Effects of homocysteine and its related compounds on oxygen consumption of the rat heart tissue homogenate: the role of different gasotransmitters. Mol Cell Biochem. 2018;444:143-8.
17. Lovrić J, Mesić M, Macan M, Koprivanac M, Kelava M, Bradamante V. Measurement of malondialdehyde (MDA) level in rat plasma after simvastatin treatment using two different analytical methods. Periodicum biologorum. 2008;110:63-68.
18. Bahrami S, Shahriari A, Tavalla M, Azadmanesh S, Hamidinejat H. Blood Levels of Oxidant/Antioxidant Parameters in Rats Infected with Toxoplasma gondii. Oxid Med Cell Longev. 2016;2016:80-96.
19. Zaias J, Mineau M, Cray C, Yoon D, Altman NH. Reference values for serum proteins of common laboratory rodent strains. J Am Assoc Lab Anim Sci. 2009;48:387-390.
20. Evans MA, Sano S, Walsh K. Cardiovascular disease, aging, and clonal hematopoiesis. Annu Rev Pathol. 2020;15:419-438.
21. Liang Z, Miao Y, Teng X, Xiao L, Guo Q, Xue H, *et al*. Hydrogen sulfide inhibits ferroptosis in cardiomyocytes to protect cardiac function in aging rats. Mol Front J. 2022;9:94-100.
22. Zhang H, Dai J, Tian D, Xiao L, Xue H, Guo Q, *et al*. Hydrogen sulfide restored the diurnal variation in cardiac function of aging mice. Oxid Med Cell Longev. 2021;2021:1-10.
23. Mumtaz S, Ali S, Qureshi MZ, Muhammad A, Manan A, Akbar Mughal T. Antioxidant and anti-aging role of silk sericin in D-galactose induced mice model. Saudi J Biol Sci. 2023;30:103-117.
24. Daveri E, Cremonini E, Mastaloudis A, Hester SN, Wood SM, Waterhouse AL, *et al*. Cyanidin and delphinidin modulate inflammation and altered redox signaling improving insulin resistance in high fat-fed mice. Redox Biol. 2018;18:16-24.
25. Totlani VM, Peterson DG. Epicatechin carbonyl-trapping reactions in aqueous maillard systems: Identification and structural elucidation. J Agric Food Chem. 2006;54:7311-18.
26. Hadzi-Petrushev N, Stojkovski V, Mitrov D, Mladenov M. D-galactose induced changes in enzymatic antioxidant status in rats of different ages. Physiol Res. 2015;64:61-70.
27. El-Far AH, Mohamed HH, Elsabagh DA, Mohamed SA, Noreldin AE, Al Jaouni SK, *et al*. Eugenol and carvacrol attenuate brain D-galactose-induced aging-related oxidative alterations in rats. Environ Sci Pollut Res Int. 2022;29:47436-47447.
28. Packer M. Longevity genes, cardiac ageing, and the pathogenesis of cardiomyopathy: implications for understanding the effects of current and future treatments for heart failure. Eur Heart J. 2020;41:3856-3861.
29. Fan H, Ji Y, Wang Y, Liu D, Wei T, Cao X, *et al*. Anthocyanins from lycium ruthenicum murray Inhibit HepG2 cells growth, metastasis and promote apoptosis and G2/M phase cycle arrest by activating the AMPK/mTOR autophagy pathway. Evid Based Complement Alternat Med. 2022;2022:960-1000.
30. Wallace TC. Anthocyanins in cardiovascular disease. Adv Nutr. 2011;2:1-7.

31. Orjalo AV, Bhaumik D, Gengler BK, Scott GK, Campisi J. Cell surface-bound IL-1alpha is an upstream regulator of the senescence-associated IL-6/IL-8 cytokine network. Proc Natl Acad Sci U S A. 2009;106:17031-17036.
32. Sequera C, Bragado P, Manzano S, Arechederra M, Richelme S, Gutiérrez-Uzquiza A, *et al*. C3G Is Upregulated in Hepatocarcinoma, Contributing to Tumor Growth and Progression and to HGF/MET Pathway Activation. Cancers (Basel). 2020;12:150-5.
33. Kilian LS, Voran J, Frank D, Rangrez AY. RhoA: A dubious molecule in cardiac pathophysiology. J Biomed Sci. 2021;28:33-40.
34. Tracy E, Rowe G, LeBlanc AJ. Cardiac tissue remodeling in healthy aging: the road to pathology. Am J Physiol Cell Physiol. 2020;319:166-182.
35. Gutiérrez-Uzquiza A, Arechederra M, Molina I, Baños R, Maia V, Benito M, *et al*. C3G down-regulates p38 MAPK activity in response to stress by Rap-1 independent mechanisms: involvement in cell death. Cell Signal. 2010;22:533-542.
36. Carabias A, Gómez-Hernández M, de Cima S, Rodríguez-Blázquez A, Morán-Vaquero A, González-Sáenz P, *et al*. Mechanisms of autoregulation of C3G, activator of the GTPase Rap1, and its catalytic deregulation in lymphomas. Sci Signal. 2020;13:98-102.
37. Li P, Ma Y, Wang X, Li X, Wang X, Yang J, *et al*. The protective effect of PL 1-3 on D-galactose-induced aging mice. Front Pharmacol. 2023;14:13-48.
38. Lin J, Lopez EF, Jin Y, Van Remmen H, Bauch T, Han HC, *et al*. Age-related cardiac muscle sarcopenia: Combining experimental and mathematical modeling to identify mechanisms. Exp Gerontol. 2008;43:296-306.
39. Lee GH, Hoang TH, Jung ES, Jung SJ, Han SK, Chung MJ, *et al*. Anthocyanins attenuate endothelial dysfunction through regulation of uncoupling of nitric oxide synthase in aged rats. Aging Cell. 2020;19:13-27.