

Morphological and Functional Analysis of Cardiac Ameliorations in Elderly Rats Supplemented with a Magnolol Extract Complex

Análisis Morfológico y Funcional de las Mejoras Cardíacas en Ratas Ancianas Suplementadas con un Complejo de Extracto de Magnolol

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SUMMARY: Magnolia bark extract supplementation has an anti-oxidative role in mammals. However, its role in physiological aged-associated heart insufficiency is not known yet. Therefore, we investigated the effects of a magnolia bark complex, including magnolol and honokiol components (MAHOC), in elderly rat hearts (24-month-old aged group). One group of aged rats was supplemented with MAHOC (400 mg/kg/d, for 12 weeks) besides the standard rat diet while the second group of elderly rats and adult rats (to 6-month-old adult-group) were only fed with the standard rat diet. The morphological analysis using light microscopy has shown marked myofibrillar losses, densely localized fibroblasts, vacuolizations, infiltrated cell accumulations, and collagen fibers in the myocardium of the elderly rats compared to the adults. We also detected a markedly increased amount of degenerated cardiomyocytes including the euchromatic nucleus. The MAHOC supplementation of the elderly rats provided marked ameliorations in these abnormal morphological changes in the heart tissue. Furthermore, electrophysiological analysis of electrocardiograms (ECGs) in the supplemented group showed significant attenuations in the prolonged durations of P-waves, QRS-complexes, QT-intervals, and low heart rates compared to the unsupplemented elderly group. The biochemical analysis also showed significant attenuations in the activity of arylesterase and total antioxidant status in the myocardium of the supplemented group. We further determined significant attenuations in the activity of a mitochondrial enzyme succinate dehydrogenase, known as a source of reactive oxygen species (ROS), and the decreased level of ATP/ADP in the heart homogenates of the supplemented group. Moreover, under in vitro conditions by using an aging-mimicked cardiac cell line induced by D-galactose, we demonstrated that MAHOC treatment could provide prevention of depolarization in mitochondria membrane potential and high-level ROS production. Overall, our data presented significant myocardial ameliorations in physiological aging-associated morphological alterations parallel to the function and biochemical attenuations with MAHOC supplementation, at most, through recoveries in mitochondria.

KEY WORDS: Heart; Oxidative stress; Antioxidants; Nutritional ingredients; Biphenolic compounds.

INTRODUCTION

The global population of elderly humans has been growing in all over the world exponentially in the last two decades and it is today widely accepted that aging is the main risk factor for the development of various chronic diseases. Among chronic diseases, the prevalence of age-related cardiovascular diseases (CVDs) are gradually increasing and the contribution of risk factors is known to be complex (Lakatta *et al.*, 2001). Aging, being a combination of physiological and pathophysiological

processes in the body, is characterized by continuously increasing oxidations and reduction of antioxidant activity (Dillin *et al.*, 2014). Also, susceptibility to disease includes, at least in part, the increase of oxidative stress, which arises damages cellular components such as proteins, DNA, and lipids.

Various molecular and cellular changes are contributing to cardiac aging besides genetic factors

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(Rodgers *et al.*, 2019). Both clinical and experimental data point out that cardiac aging is mainly characterized by a reduction in the maximum heart rate, and a significant decrease in the contractile activity of the heart, as well as prolonged QT-intervals calculated from the electrocardiograms (ECGs). Moreover, impairments in organelle dynamics and metabolic flexibility such as dysfunctional mitochondria are important alterations in elderly hearts (Lesnefsky *et al.*, 2016). Some clinical observations demonstrated parallelism between the marked functional decline in the heart and the induction of insulin resistance, although individuals have normal blood glucose levels and body weight (Chason *et al.*, 2018). Indeed, various experimental studies support this interesting but important events related to the correlation between insulin resistance and cardiometabolic disturbances (Ormazabal *et al.*, 2018). These types of disturbances in the heart seems to underline the impairment of electrophysiological activities of cardiomyocytes, at most, associated with mitochondria-related increases in the amounts of oxidants (Boudina, 2013; Olgar *et al.*, 2020).

There is a mutual interplay between oxidative stress and antioxidants which play a crucial role in the development of age-related diseases including CVD (Izzo *et al.*, 2021). An imbalance between antioxidant and/or antioxidant-like species induces cellular redox imbalance further resulting in serious molecular and cellular damage (Conti *et al.*, 2016). An alteration of the redox status and increase in oxidants of cardiomyocytes during aging lead further to multiple cellular dysfunctions including structural changes, and alterations in excitation-contraction coupling more likely triggered by the decreases in antioxidative defense systems (Lennicke *et al.*, 2015). Therefore, elderly humans can be highly susceptible to oxidative stress through a decline in their endogenous antioxidant defences.

There is a great effort to use antioxidants in the prevention of aging-related diseases. As general acceptance, external antioxidant supplementations have been shown to reduce oxidative stress in the heart (Goszcz *et al.*, 2015). In this regard, there are important numbers of experimental studies on the benefits of antioxidant supplementations in cardiovascular diseases, however, there is yet no consensus associated with their benefits in clinical therapies, partly on account of the heterogeneity among study groups as well as the different antioxidant supplements (Lennicke *et al.*, 2015). In this subject, traditional Chinese medicines are demonstrated potential resources for natural antioxidants, including the bark of *Magnolia Officinalis* to treat a variety of disorders, while magnolol and honokiol are isomers of hydroxylated biphenolic compounds and are the major bioactive components of this bark (Zhang *et al.*, 2010; Xie

et al., 2020). Zhao and Liu demonstrated a comparable antioxidant ability of magnolol and honokiol to scavenge radicals and protect DNA (Zhao & Liu, 2011). Some animal studies are performed to demonstrate the benefits of *magnolia officinalis* bark extract supplementation on growth performance and disease resistance (Oh *et al.*, 2018). Likely, Zhang *et al.* (2010) examined the antihypertensive effects of honokiol *in vivo* in spontaneously hypertensive rats. Furthermore, even in early studies, under some *in vitro* conditions, magnolol and/or honokiol incubation provided a significant antioxidant effect in isolated rat heart mitochondria lipid peroxidation (Lo *et al.*, 1994), as well as protected the myocardium against ischaemic injury and suppressed ventricular arrhythmia during ischemia and reperfusion (Tsai *et al.*, 1996).

Taking into consideration the marked increase in oxidative stress in advanced-age humans, particularly with CVD, here, we aimed to evaluate the cardioprotective effects of long-term administration of a magnolia bark extract with active component magnolol and honokiol complex in elderly rats via analyzing the structure and function of hearts as well as biochemical properties of the hearts. Therefore, we evaluated the status of oxidants and antioxidants as well as mitochondrial enzyme activities in these heart tissues. To confirm the heart-targeting effects of MAHOC, particularly through targeting mitochondria, we performed *in vitro* investigations. Our *in vitro* data further implied that the beneficial effect of MAHOC supplementation on an aging heart is mostly via recoveries in mitochondria. Overall, here, we have demonstrated that this magnolia extract bark complex supplements to elderly rats could significantly ameliorate aging-associated insufficiencies in the heart via the mediation of augmentation in the antioxidant defense system and mitochondria.

MATERIAL AND METHOD

Experimental design. The animals were housed as 2-3 animals per cage and were fed with standard chow *ad libitum*, daily. All experiments were carried out according to the guidelines given by the animal welfare committee of the local institution.

Elderly male Wistar rats with an age of 24-months-old were used in comparison to those of male adult rats with an age of 6-months-old. One group of elderly rats was supplemented with a magnolia extract with active component magnolol and honokiol complex (+MAHOC group; 12-week with 400 mg/kg/day, intragastrically)

besides their standard animal chow, while the second group of elderly rats and adult rats were only fed with the standard animal chow for the same duration. The substance is a pure powder bulk supplement standardized to contain ≥ 2 % honokiol and does not include sugar, soy, dairy, yeast, gluten, and other additives.

***In situ* arterial pressure measurements.** The tail-cuff measurements were made in rats that had not undergone any invasive procedures without the stresses of heating and restraint required during the tail-cuff procedure, as described previously (Olgar *et al.* 2020). Briefly, animal tails were passed through a cuff and the pressure transducer was tied around the tail distal to the cuff under light ketamine-xylazine anesthesia (as intraperitoneal administration; 10 mg/kg). Therefore, the blood pressure was measured by a noninvasive blood pressure meter (NIBM200-A, BIOPAC Systems Inc. USA). From these recordings, systolic and diastolic blood pressures as well as heart rates were determined.

Light microscopy analysis. Removed left ventricular heart tissues from the animals were performed routine histological procedures. Tissue samples were fixed in phosphate-buffered 10 % formalin for 96 hours and then washed in running tap water, then passed through a series of 75 %, 96 %, and 100 % ethanol for dehydration. After dehydration, these tissues were clarified in xylene and then embedded in paraffin wax. After the routine follow-up stages, 5- μm -thick sections were cut from paraffin blocks with a microtome (Leica RM 2125RT) and transferred onto microscope slides. These slides were deparaffinized with xylene at 60 °C overnight, and then they were rehydrated by using graded series of ethanol. The slides were stained with either Hematoxylin-Eosin (H-E) or Masson's trichrome (M-T). All histological examinations were performed under the Axioscope A-II photomicroscope (Carl Zeiss Oberkochen-Germany). Sections stained with H-E were used for histopathological evaluation, and sections stained with M-T were used for observation in terms of fibrosis.

Surface electrocardiogram recording. The surface electrocardiograms (ECG) were recorded *in situ* in the anesthetized animals with ketamine-xylazine administered intraperitoneally. The recordings were performed by using bipolar limb leads (lead I, II, III) placed 20 gauge needles to the forearms and hind limb. The ECG recordings were continued for 10 min from individual animals. The recorded ECG data were acquired by using an analog-to-digital converter BIOPAC MP35 (Goleta California) and processed with a high-cut (low-pass) filter at 50–500Hz. The ECG parameters such as P-wave duration, PQ-duration, QRS-duration, and QT-duration were calculated from every ECG recording.

Biochemical analysis in heart tissues

Heart tissue preparation: Following animals were anesthetized with ketamine-xylazine at a dose of 30 mg/kg of the animal body weight applied intraperitoneally, hearts were rapidly excised and stored at -80 °C. To prepare tissue homogenates, the frozen hearts were crushed at liquid nitrogen and then were homogenized with 3 volumes of 50 mM K_2PO_4 buffer, pH 7.4, with an Ultra-Turrax homogenizer. The homogenized suspensions were centrifuged first at 22,000 rpm/min and again at 14,000 rpm/min for 20 min at 4 °C with a Sorvall RC-5B centrifuge. The final supernatants were used for the biochemical assays.

Determination of total oxidant and antioxidant statuses (TOS and TAS): The TOS levels in the heart supernatants were measured by using commercially available kits (RL0024, Rel Assay Diagnostics, Turkey) as described previously (Olgar *et al.* 2020). Briefly, the assay is calibrated with H_2O_2 and the oxidation reaction is enhanced by glycerol molecules. The color intensity of the medium is presenting the total amount of oxidant molecules present in the sample which is determined spectrophotometrically. The changes in the intensities are expressed in terms of μM H_2O_2 equivalent/L. The TAS levels were also determined by using the commercially available kit (RL0024, Rel Assay Diagnostics, Turkey) as described previously (Olgar *et al.* 2020). The results are expressed as mmol Trolox equivalent/L.

Determination of paraoxonase and arylesterase activities: Paraoxonase and arylesterase activities were measured by using commercially available kits (RL0031 and RL0055, Rel Assay Diagnostics, Turkey). The rate of paraoxon hydrolysis was determined by monitoring the increase of absorption at 412 nm at 37 °C. The amount of generated p-nitrophenol was calculated from the molar absorption coefficient at $18.290 \text{ M}^{-1} \text{ cm}^{-1}$. Paraoxonase activity was expressed as IU/mg protein. To determine arylesterase activity, it has been used phenylacetate, and the enzymatic activity was calculated from the molar absorption coefficient of the produced phenol at $1310 \text{ M}^{-1} \text{ cm}^{-1}$. One unit of arylesterase activity was defined as 1 μmol phenol generated per min under the above conditions and the results were expressed as IU/mg protein.

Determination of succinate dehydrogenase activity: Succinate dehydrogenase (SDH; mitochondrial complex II) activity was measured as described elsewhere (Jespersen *et al.*, 2020). Briefly, the homogenized heart tissue samples were prepared for SDH activity measurements using a Complex II Enzyme activity assay kit (Abcam, ab109908), and a colorimetric measurement was performed. A competitive inhibitor of SDH was used as a negative control. The results are presented as IU/mg protein.

Measurement of ATP to ADP ratio level: The ATP to ADP ratio in the left ventricular tissue homogenates was determined by using an ADP/ATP Ratio Assay Kit (ab65313). Briefly, luciferase catalyzed the conversion of ATP and lucifer into light, which was subsequently measured using a luminometer. ADP level was measured by its conversion to ATP which was subsequently detected using the same reaction. Absorbances were read via SpectraMax Plus384 microplate reader at 570 nm and the results are expressed as fold-change among groups.

Determination of mitochondrial membrane potential and total cellular reactive oxygen species ROS production by using confocal microscopy. In these groups of examinations, we used an embryonic rat heart H9c2 cell line which was purchased from the American Type Culture Collection (CRL 1446) and grown at 37 °C in 5 % CO₂ in Dulbecco's modified Eagle's medium (DMEM) in the presence of 5.5 mM glucose with a density of about 10⁵ cells/cm² as described previously (Olgar *et al.* 2020). They were cultured as a monolayer supplemented with 10 % fetal calf serum, 50 U/mL penicillin-G, and 50 µg/mL streptomycin at 37 °C.

To obtain aging-mimicking cells, the sub-confluent cells were treated with D-galactose (D-Gal, 100 mg/L for 48 h incubation). One group of D-Gal treated cells was incubated with MAHOC (10 µM for 24 h) while another group of the cells was incubated with saline.

All cells were loaded with a mitochondria membrane potential (MMP) specific fluorescence dye (JC-1, 5 µM for 30 min incubation) to determine MMP status by using a confocal microscope (Leica TCS SP5), as described previously (Olgar *et al.* 2020). To obtain fluorescence intensity changes, the cells were first excited at 488 nm, and then the fluorescence responses were determined at 535 nm. While imaging the fluorescence changes, we calibrated all fluorescence signals with carbonylcyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP; 5 µM).

To examine the reactive oxygen species (ROS) level, we used a fluorescence-based method as described elsewhere (Olgar *et al.* 2020). The cells were loaded with a ROS indicator chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (DCFDA, 5 µM for 1 h incubation), and then were examined with the confocal microscope. The DCFDA-loaded cells were excited at 488 nm and emissions were collected at 560 nm wavelengths. For maximal fluorescence intensity, the cells were exposed to a HEPES-buffered solution supplemented with hydrogen peroxide H₂O₂ (100 µM). The peak fluorescence changes ($\Delta F/F_0$, where $\Delta F = F - F_0$; F identified as the local maximum elevation of fluorescence intensity over basal level, F₀) were calculated from the confocal images, and results were presented as percentage changes in the fluorescence intensities.

Statistical analysis. To analyze the data, we used Graph Pad Prism (Prism 5 for Windows, GraphPad Software, USA). For nonparametric statistical data, we used Mann-Whitney U-test. The significance level was considered p<0.05 as statistically significant. Data were presented as mean ± standard error of the mean (X±SEM).

RESULTS

General parameters of animals. The initial body weights of elderly animals were 393±27 g while this value was 344±31 g for adult rats. Following 2 weeks of MAHOC supplementation besides their standard feeding, the average body weight of the supplemented elderly rats was 415±23 g while it was 364±12 g in the unsupplemented elderly rats (Table I). The MAHOC supplementation to the elderly rats could not affect the blood glucose level, as well (Table I). We did not observe any drug toxicity reactions in the livers and kidneys of the elderly animals following the supplementation (data not shown).

Table I. Effects of a magnolia extract complex supplementation on general parameters of elderly animals.

Groups/ Parameters	Adult Rat Group	Elderly Rat Group	+MAHOC Group
Body weight (g)	364±12	415±23	445±16
Blood glucose (mg/dL)	90±4	82±8	86±7
Systolic pressure (mmHg)	122±6	144±8*	143±6*
Diastolic pressure (mmHg)	86±3	102±5*	105±5*
Heart rate (min ⁻¹)	382±8	285±6*	336±12#

One group of elderly Wistar male rats (24-month-old, n=8) was administered with standard animal chow (12 weeks) while the second group of elderly rats was standard animal chow plus a magnolia bark extract (+MAHOC groups; 12 weeks with 400 mg/kg/day). The adult male Wistar rats (6-month-old) were kept as the control group. All values are given as mean (±SEM). Statistical comparison is accepted as *p<0.05 vs Adult group, #p<0.05 vs Elderly group.

The final average systolic and diastolic pressure levels of the supplemented elderly rats (144±8 mmHg vs 102±5 mmHg) were not significantly different compared to their initial values (139±6 mmHg and 104±4 mmHg). Similarly, these values for the unsupplemented elderly rats (143±6 mmHg vs 105±5) were also not significantly different compared to their initial levels (138±8 mmHg vs 101±5 mmHg). These systolic and diastolic pressure values of the

elderly rats were significantly high than those of the adult rats while the MAHOC supplementation could not attenuate these values, significantly (Table I).

The heart rate of the unsupplemented elderly group was significantly slowed down compared to the adult group (Table I). The MAHOC supplementation of the elderly rats could slightly but significantly augment the depressed heart rates of the elderly animals.

Effects of MAHOC supplementation on the structure of the elderly rat hearts. This group of examinations was performed elderly and MAHOC supplemented elderly rats compared to the adult rats. We examined the left ventricular heart sections subjected to hematoxylin and eosin staining (H-E). In the H-E staining tissue sections from elderly rats (Fig. 1A), myofibrillar loss (red arrow) was observed in the cross-section of the cardiomyocytes (left). As can be seen on the

right, there was a normal myofibrillar appearance (black arrow) in the treated elderly-group (Fig. 1, right row, A). There was also marked vacuolization in the cytoplasm (thin arrow), dark-eosinophilic stained degenerated cardiomyocytes (arrowhead) and active fibroblasts with euchromatic nucleus (tailed white arrow) were significant in most areas in elderly-group tissue sections (Fig. 1, middle row, B). Furthermore, there were fewer degenerated cardiomyocytes in the treated group, and the fibroblast nuclei (tailed white arrow) were mostly inactive and had heterochromatin nuclei (Fig. 1, right row, B). Moreover, we detected the marked fibroblasts and mononuclear cell (blue arrow) infiltration in the elderly-group (Fig. 1, middle row, C) with less amount of infiltrated cells in the treated elderly-group (Fig. 1, right row, C). Overall, the MAHOC supplementation of the elderly rats provided marked recovery in the myocardium from the left ventricle of the elderly animal hearts. The normal tissue appearances are given in Fig. 1, left, A-C.

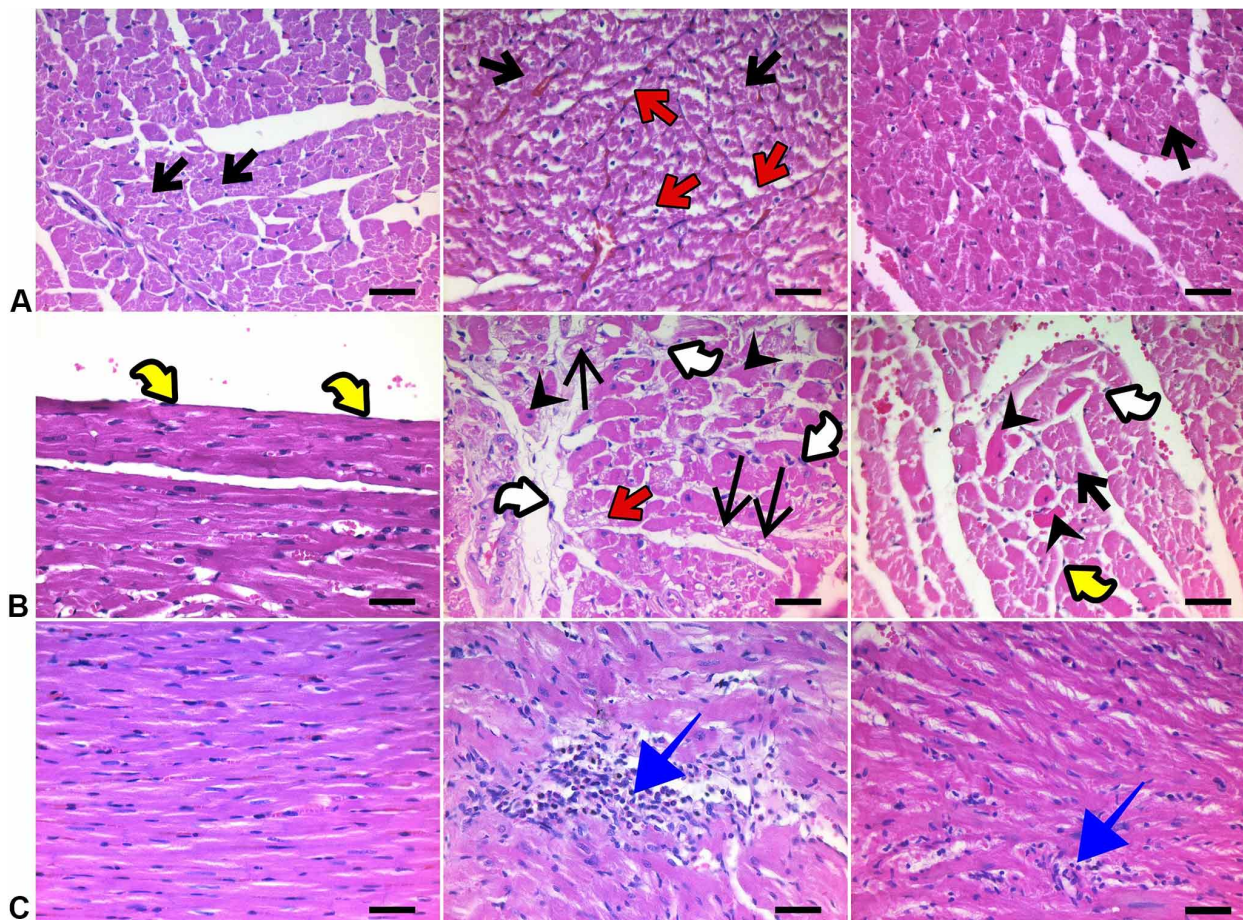


Fig. 1. Light microscopy photomicrographs of left ventricular heart tissues from elderly rats supplemented with or without MAHOC. In the Hematoxylin-Eosin (H-E) staining tissue sections the left, middle, and right rows are representing the 6-mo-old rat (adult) group, elderly (24-mo-old) rat group, and MAHOC supplemented elderly rat group, respectively. The middle row is the (A) Red arrow: myofibrillar loss and the black arrow: normal myofibrillar appearance. (B) Thin arrow: marked vacuolization, arrowhead: dark-eosinophilic stained degenerated cardiomyocytes, and tailed white arrow: active fibroblasts with euchromatic nucleus (middle). Tailed white arrow: degenerated cardiomyocytes (right), and tailed white arrow: fibroblast nuclei (right). (C) Blue arrow: mononuclear cell infiltration (left), and with less amount of infiltrated cells (right). Normal appearance of tissue sections from adult rats are given in the left parts. Magnification: $\times 400$ and the bar represents $50 \mu\text{m}$.

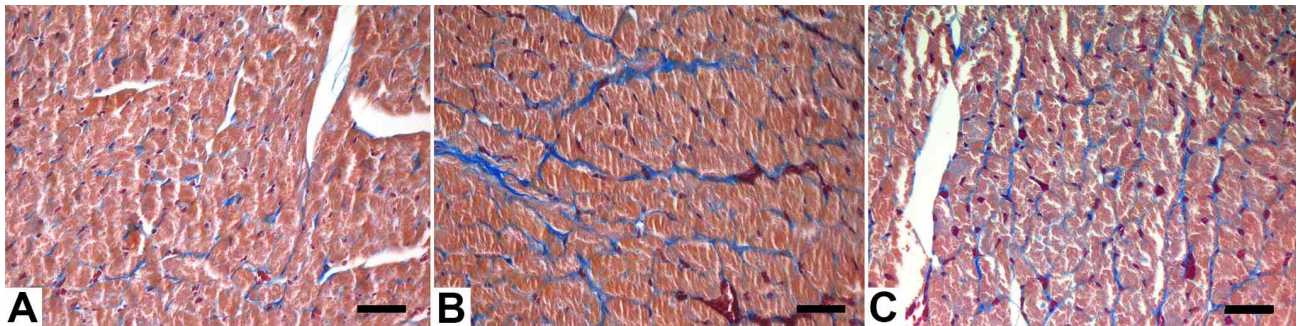


Fig. 2. Light microscopy photomicrographs of left ventricular heart tissue stained with Masson's trichrome (M-T) in elderly rats supplemented with or without MAHOC. (A) M-T staining tissue sections from adult rats, while (B) in elderly rats. The blue color indicates the collagen fibers and the red indicates the muscle fibers. (C) Less amount of blue color with a normal muscle fiber normal appearance in the MAHOC supplemented rats (magnification: 400). In all photomicrographs, the bar represents 50 μm .

We also performed an analysis in the Masson's trichrome (M-T) staining samples and observed highly dense collagen fibers in untreated aged rat samples (blue-stained collagen fibers; Fig. 2B) with a markedly decreased amount of blue-stained collagen fibers in the treated aged rat samples (Fig. 2C). The normal tissue appearance in the adult rat heart is shown in Fig. 2A. Overall, the MAHOC treatment of the elderly rats provided marked protection against aging-associated damage in the heart tissues.

The effects of MAHOC supplementation on the ECG parameters in elderly rats. We first analyzed the parameters of surface recorded ECGs in the treated and untreated elderly rats, such as P-wave duration, PQ duration, QRS duration, and QT interval. The original representative ECG recordings of the rats are given in Figure 3A. As can

be seen in Figure 1 (B-E), following the supplementation period, the prolonged durations in ECGs of the elderly animals such as the durations of P-waves (B), QRS-complex (D), and QT-interval (E) were significantly recovered with no significant effect on the duration of PR-interval (C). In addition, there was no effect on the amplitude of the QRS-complex, significantly.

Beneficial effects of MAHOC on oxidative stress and antioxidant status of elderly rat hearts.

To examine the systemic oxidative status in elderly rats, we determined the heart tissue levels of total oxidant status (TOS) and total antioxidant status (TAS). As can be seen in Figure 4 (A-B), The TOS levels were significantly lower in the MAHOC-treated elderly rats than in unsupplemented elderly rats (Aged group; A) while the TAS level in the

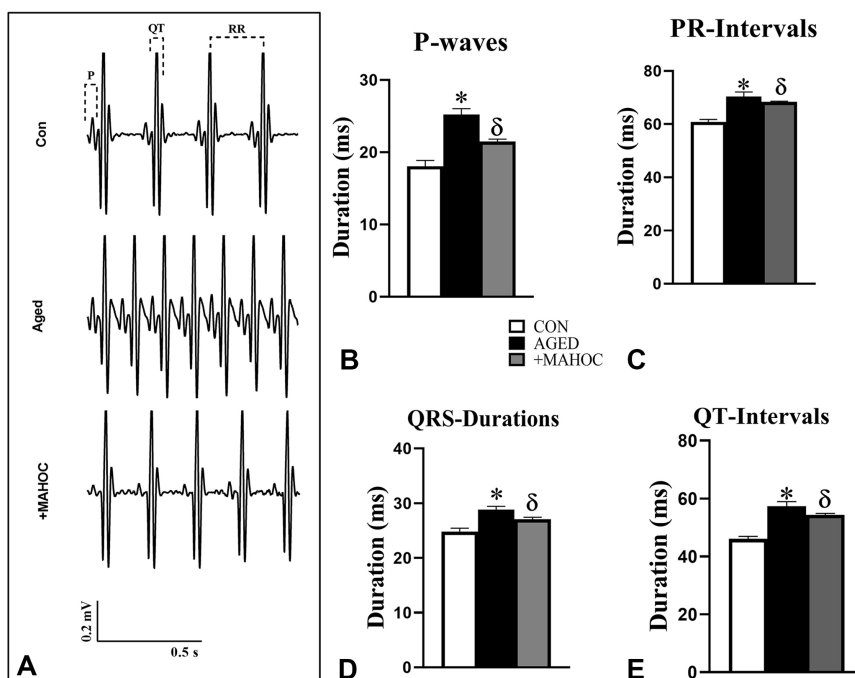


Fig. 3. The representative surface *in situ* electrocardiograms and the effects of MAHOC supplementation on ECG parameters. (A) The representative *in situ* surface electrocardiograms (ECGs) in the adult rats (CON group upper part), elderly rats (Aged group, middle part) and supplemented with a magnolia bark extract with active component magnolol and honokiol complex (MAHOC) (lower parts). The effects of MAHOC supplementation in elderly rats (+MAHOC groups) on the durations of P-waves (B), PR-intervals (C), QRS-complex (D), and QT-intervals (E). All values in bar graphs are presented as a mean (\pm SEM) from 6-7 rats per group. The significance levels are at * $p < 0.05$ vs. Adult group, $\delta p < 0.05$ vs. Aged group.

supplemented group was significantly higher than the unsupplemented elderly group (Aged group) (B). We also determined the paraoxonase and arylesterase activities in the heart samples from both supplemented and unsupplemented rats as well as from the adult rats. As seen in Figure 4C and D, the arylesterase activity but not paraoxonase activity in the supplemented group rats was significantly higher than those of the unsupplemented group.

We further examined the activity of mitochondrial enzyme activity, and the activity of succinate

dehydrogenase (SDH; mitochondrial complex II) in the heart homogenates, which is closely associated with the production level of ROS. As can be seen in Figure 4E, the SDH activity was found about 2-fold less in the supplemented elderly group compared to the unsupplemented group. In the last group of examinations, to determine the ATP production level in the heart homogenates, we also determined the level of ATP to ADP ratio. As can be seen in Figure 4F, the ATP to ADP ratio in the supplemented elderly group was about 2-fold higher than in the unsupplemented elderly rats, which is almost the same as that of the adult ratio.

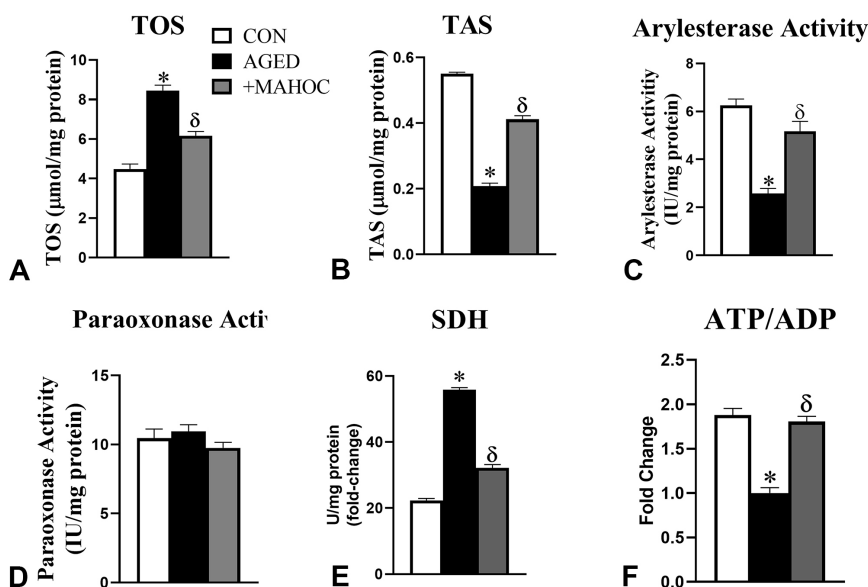


Fig. 4. The effects of MAHOC supplementation on antioxidant defense parameters of the hearts of elderly rats. The effects of MAHOC administration on either aged rats (+MAHOC groups) on the levels of TOS (A) and TAS (B), and the activities of arylesterase (C) and paraoxonase (D). The activity of SDH (E) and the ATP/ADP level (F) in the tissue homogenates. All values in bar graphs are presented as a mean (\pm SEM) from 6-7 rat hearts per group. The significance levels are at * $p < 0.05$ vs. Adult group, $\delta p < 0.05$ vs. Aged group.

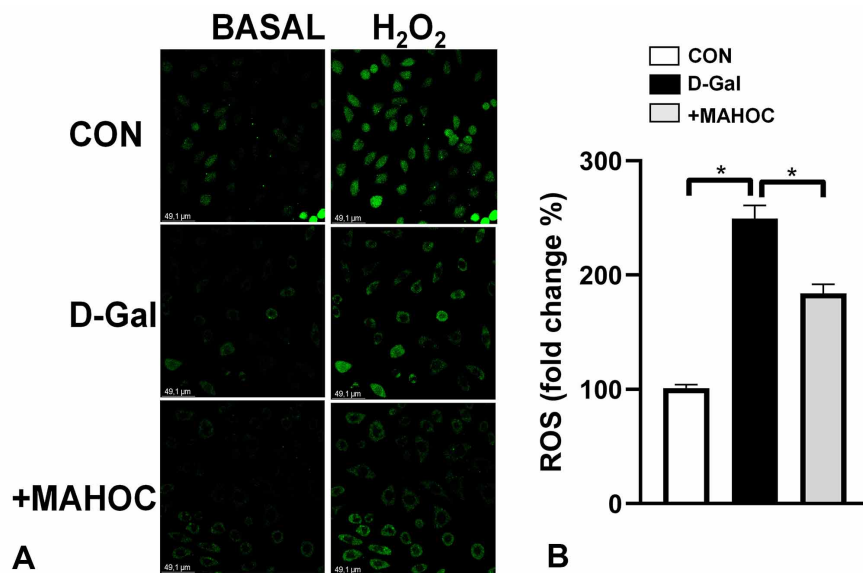


Fig. 5. *In vitro* validation of antioxidant-like action of MAHOC treatment in the aging-mimicked cell line. (A) Representative confocal images of chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (DCFDA, 10-µM for 60-min incubation) loaded H9c2 cells (upper part). To monitor the ROS level, the DCFDA-loaded cells were calibrated with H₂O₂ (100-µM). (B) Results are given as percentage changes in the fluorescence intensities and the estimated ROS levels of all groups as presented as bar graphs. Scale bar: 10-µm. Data were presented as Mean \pm SEM. The significance level is accepted as * $p < 0.05$ vs. untreated H9c2 cells or D-Gal treated H9c2 cells.

In vitro validation of antioxidant-like action of MAHOC in aging-mimicked cell line. To confirm a heart-targeting antioxidant-like action of MAHOC administration, we determined the attenuation levels in the total cellular reactive oxygen species (ROS) production and depolarization of mitochondrial membrane potential (MMP) in H9c2 myoblasts via incubation with MAHOC. As can be seen in Figure 5 (A-B), there was a significant increase (about 2.5-fold) in ROS production of D-Gal treated cells (aging-mimicked), whereas this level significantly decreased in the MAHOC-treated aging-mimicked cells comparison to those of untreated ones. In the last part of this group examination, we determined the effect of MAHOC supplementation on the depolarized MMP in the aging-mimicked cells (Fig. 6 A-B). The depolarization in MMP was significantly recovered in the MAHOC-treated cells.

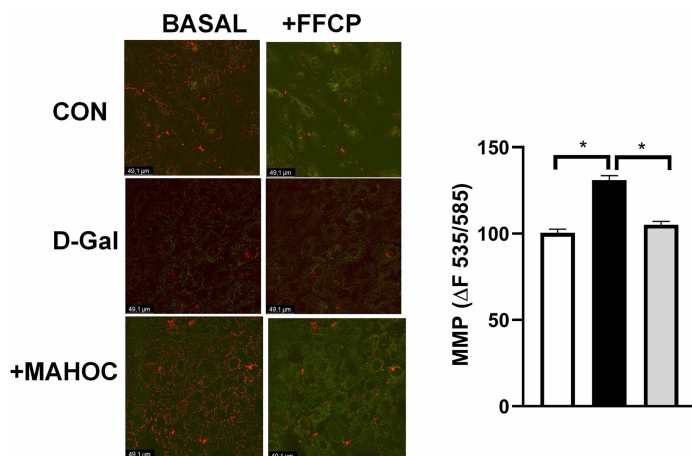


Fig. 6. *In vitro* validation of antioxidant-like action of MAHOC treatment on mitochondria function in the aging-mimicked cell line. (A) The representative confocal images of JC-1 (5- μ M, 30-min) loaded H9c2 cells. The probes were excited at 488 nm, and the red fluorescence image was detected at both 535 and 585 nm. To calibrate the changes in MMP, cyanide 4-(trifluoromethoxy)phenylhydrazon (FCCP, 5- μ M) was used (lower part). Scale bar: 10- μ m. (B) Results are given as percentage changes in the fluorescence intensities and the estimated MMP levels of all groups as presented as bar graphs. Scale bar: 10- μ m. Data were presented as Mean \pm SEM. The significance level is accepted as * p < 0.05 vs. untreated H9c2 cells or D-Gal treated H9c2 cells.

DISCUSSION

The present study, performed by using an administration of a magnolol bark extract (MAHOC) to elderly rats, for the first time, provides significant new information related to its important relevance on physiological aging-related cardiac insufficiency. Two major findings were observed in these elderly animals in this study: (1) The first important finding is to demonstrate marked

ameliorations with MAHOC supplementation on morphology, electrical function, and biochemical parameters of the heart from elderly rats as well as their systemic parameters such as decreased heart rates. (2) Second, we determined significant attenuations with MAHOC supplementation in the activity of a directly associated with mitochondria, an enzyme succinate dehydrogenase (SDH), and the level of ATP/ADP in the heart homogenates. Furthermore, our *in vitro* data by using the aging-mimicking cardiac cell line, demonstrated that MAHOC application provided significant preventive effects on depolarization of mitochondrial membrane potential and high-level ROS production. Therefore, taking into consideration these findings, our present data confirmed that all these attenuations in elderly rats with this antioxidant-like nutrient are through its heart-targeting antioxidant-like action in the

heart, at most, via its mitochondria-targeting properties. Our results, therefore first, strengthen the concept of high-level induction of oxidative stress during physiological aging as a potential mechanism for physiological aging-associated cardiac insufficiency/complications. Furthermore second, our findings strongly imply the induction of oxidative stress in cardiomyocytes, at most, through alterations in mitochondria in terms of electrical, biochemical, and structural properties. Moreover, third, the present data overall demonstrated that a magnolol supplementation provides structural and functional myocardial amelioration in elderly rats, at least, via augmentation in the alterations of the mitochondria.

Literature data pointed out oxidative stress has been implicated in the physiopathology of aging, and age-associated organ dysfunction including heart, and antioxidants supplementation has provided prevention and/or therapy for cardiovascular dysfunction (Bout-Teen *et al.*, 2021). Although aging-associated cardiac insufficiency/dysfunction has been reported to be associated with an increase in oxidative stress, and the antioxidant benefits observed in most animal studies the exact mechanism has not been fully understood, however, clinical studies have not fully agreed with the benefits of antioxidants in the primary and secondary prevention/therapy of cardiovascular disease (Leopold, 2015). In the present study, treatment of elderly rats with MAHOC besides their standard feeding could recover the abnormal heart rhythms and the altered parameters of ECGs, particularly, prolongations in durations of P-waves, QRS-complexes, and QT-intervals. Supportingly, previous studies also demonstrated similar prolongations, particularly in the duration of either QRS-complex and/or QT-interval in elderly

mammalians (Luo *et al.*, 2022). Importantly, there was a significant recovery in the prolonged P-wave duration of elderly animals administered with MAHOC. Indeed, this is the first data to demonstrate the beneficial effect of antioxidant-like nutrients on this parameter. This finding can be important for the prevention/attenuation of aging-associated humans because previously, the authors demonstrated the relationship between aging and P-wave dispersion in elderly humans, which seems associated with cardiovascular and all-cause mortality outcomes through its possible role in the reflection of subclinical disease and/or its merit elucidation as a marker of risk for adverse outcomes (Magnani *et al.*, 2011). Consequently, here, we demonstrated that the effectiveness of antioxidant supplementation can not only recover and/or modify plasma biochemistry but also they can provide organ-targeting benefits against increases in oxidative metabolism, depending on the basal endogenous antioxidant defenses. Present results emphasize that awareness of the importance of antioxidant-like nutrient administration might be advisable to elderly humans starting the beginning of the aging period to prevent the population of elderly cardiovascular patients, who need special precautions worldwide.

Although it has not exactly demonstrated the mechanisms of this nutritional ingredient in the heart, it seems that there is an important action on the antiaging mechanisms. There are some but not many experimental animal studies with either magnolol, honokiol, or their combination in heart studies. In these studies, the authors demonstrated the antihypertensive effect of alone honokiol supplementation together with attenuations in high-level liver malondialdehyde as well as the attenuation with a complex of magnolol and honokiol on the acute phase of coronary ligation of rats (Zhang *et al.*, 2010), ischemia- and reperfusion-induced ventricular arrhythmias (Lee *et al.*, 2001), and cardiac inflammatory responses and oxidative stress in mice subjected to doxorubicin via reduction of apoptosis (Huang *et al.*, 2017). In another study, it has been demonstrated the blockage and reverses of cardiac hypertrophy in mice with honokiol treatment of primary cultures of cardiac myocytes from neonatal rat hearts (Pillai *et al.*, 2015). These *in vivo* beneficial effects were even demonstrated by early studies performed *in vitro* studies, at most, via recoveries in the level of heart mitochondria lipid peroxidation (Lo *et al.*, 1994).

Aging is associated with an increased incidence of abnormal heart rhythms (cardiac arrhythmias) and heart failure, at least, related to increases in oxidants and induction of dysfunction in mitochondria (Yan *et al.*, 2021). Studies have shown that not only magnolol but also honokiol is 1,000 times more potent than α -tocopherol in inhibiting lipid

peroxidation in rat heart mitochondria (Hong *et al.*, 1996). A variety of pharmacological activities of magnolol have been reported, including antioxidant effects besides other effects (Tsai *et al.*, 2010). Supporting this statement, magnolol application in mouse embryonic stem/embryoid body-derived endothelial-like cells by regulating ROS-mediated apoptosis and the PI3K/AKT/mTOR signaling pathway (Kim *et al.*, 2013). This ingredient also mediated cardioprotection through its effect on neutrophil inhibition in myocardial ischemia/reperfusion by its antioxidant activity (Lee *et al.*, 2001). In the present study, MAHOC treatment of the elderly rats provided a strong antioxidant action in the heart samples via decreasing TOS and increasing TAS and activity of an antioxidant enzyme arylesterase. Correspondingly, Zhao & Liu (2011) documented the antioxidant ability of magnolol and honokiol by comparing their actions on scavenging radicals. Epidemiological and clinical studies suggest that a polyphenol-rich diet may protect against cardiovascular disease. Therefore, there is a growing body of evidence that these two poly-phenolic compounds exert their cardiovascular-modulating effects action via a complex signal transduction cascade reaction. Most of their effects are through their antioxidant actions in various types of cells, at most, their reaction with peroxy radicals (Yuan *et al.*, 2020). Their data emphasized that they are uncommon antioxidants with complex redox chemistry via clarifying the influence of intramolecular and intermolecular interactions in fine-tuning their chain-breaking antioxidant behavior and in preventing any generation of superoxide radicals by reaction with molecular oxygen. We, in the present study, demonstrated that the antioxidant-like protective action of MAHOC supplementation is including its directly not only targeting heart but also mitochondria. Indeed, mitochondrial redox signaling is associated with the ROS level in the cells. More importantly, in our previous studies, we have shown that a mitochondria-targeting antioxidant MitoTEMPO provides an antiarrhythmic effect in elderly rats through attenuation of mitochondrial ROS and attenuation in the mitochondrial membrane potential (Olgar *et al.*, 2020). Consequently, here with not only the recoveries in mitochondrial SDH activity and ATP/ADP ratio but also in our *in vitro* data, a decrease in high-level ROS production in MAHOC-treated cells is closely associated with a direct recovery in the mitochondria function. In this regard, it is well accepted that SDH, a mitochondrial enzyme, has an important role in the production of ATP via a link between the Krebs cycle and the electron transport chain. Therefore, it is responsible for high-level ROS production. Consequently, a well-controlled regulation of it can contribute to cardioprotection against increased ROS production associated with oxidative stress (Du *et al.*, 2022). Since physiological aging can contribute to adverse left ventricular and mitochondrial dysfunction, a

magnolol bark complex can help to improve heart function against aging-associated insufficiency. In these regards, there are some supporting studies on the antioxidant-like effects of a magnolol bark, particularly associated with its modulatory effect on mitochondrial dysfunction. The authors examined the protective effects of magnolol or honokiol in not heart but in neurons such as either oxidant-injured nerve cells of hypertrophic mice cells through mitochondria targeting the beneficial effects of these molecules (Pillai *et al.*, 2015).

Altogether the present study for the first time investigated to explain the cardioprotective effect of magnolia bark, MAHOC on physiological aging associated with both structural and functional alterations of the heart in elderly rats. Although the protective action of this extract is mostly mediated by its antioxidant capacity by prevention of increases in oxidant production, here, we have demonstrated its recovery effect on both structure and function of the heart by using electrophysiological, histological, and biochemical analysis together with cell level *in vitro* investigations. Overall, a chronic MAHOC supplementation to elderly mammals could provide important cardio-recovery action in physiological aging-associated insufficiencies in the heart. Therefore, our present data have provided further information about the role of MAHOC in the possible therapeutic potential to control aging-associated insufficient heart function and/or heart dysfunction in elderly humans as a novel regulator via affecting mitochondria.

ETHICAL APPROVAL. All experimental protocols were performed following the standards of the European Community guidelines on the care and use of laboratory animals according to the relevant regulations. This study is exempt from the approval requirement of the Institutional Animal Care and Use Committee of Ankara University with reference number 2019-100-10. All animals received humane care under an institutionally approved experimental animal protocol with an ethical license in Turkey.

BILLUR, D.; AKTAY, I.; BAYRAM, P.; BITIRIM, C. V. & TURAN, B. Análisis morfológico y funcional de las mejoras cardíacas en ratas ancianas suplementadas con un complejo de extracto de magnolol. *Int. J. Morphol.*, 41(3):915-925, 2023.

RESUMEN: La suplementación con extracto de corteza de magnolia tiene un papel antioxidante en los mamíferos, sin embargo, su rol en la insuficiencia cardíaca asociada al envejecimiento fisiológico aún no se conoce. Por lo anterior, investigamos los efectos de un complejo de corteza de magnolia, incluidos los componentes magnolol y honokiol (MAHOC), en corazones de ratas seniles (grupo de edad de 24 meses). La alimentación de grupo de

ratas seniles se complementó con MAHOC (400 mg/kg/d, durante 12 semanas) además de la dieta estándar, mientras que el segundo grupo de ratas seniles y ratas adultas (hasta el grupo de adultos de 6 meses) solo recibió la dieta estándar para ratas. El análisis morfológico mediante microscopía óptica ha mostrado marcadas pérdidas miofibrilares, fibroblastos densamente localizados, vacuolizaciones, acumulaciones de células infiltradas y fibras de colágeno en el miocardio de las ratas seniles en comparación con las adultas. También detectamos una cantidad notablemente mayor de cardiomiocitos degradados, incluido el núcleo eucromático. La suplementación con MAHOC de las ratas seniles proporcionó mejoras marcadas en estos cambios morfológicos anormales en el tejido cardíaco. Por otra parte, el análisis de los electrocardiogramas (ECG) en el grupo suplementado mostró atenuaciones significativas en las duraciones prolongadas de las ondas P, los complejos QRS, los intervalos QT y las frecuencias cardíacas bajas, en comparación con el grupo de ratas seniles sin suplementación alimenticia. El análisis bioquímico también mostró atenuaciones significativas en la actividad de la arilesterasa y el estado antioxidante total en el miocardio del grupo suplementado. Determinamos además atenuaciones significativas en la actividad de la enzima mitocondrial succinato deshidrogenasa, conocida como fuente de especies reactivas de oxígeno (ROS), y la disminución del nivel de ATP/ADP en los homogeneizados de corazón del grupo suplementado. Además, en condiciones *in vitro* mediante el uso de una línea de células cardíacas, imitando el envejecimiento inducido por D-galactosa, demostramos que el tratamiento con MAHOC podría prevenir la despolarización en el potencial de membrana de las mitocondrias y la producción de ROS de alto nivel. En general, nuestros datos presentaron mejoras miocárdicas significativas en alteraciones morfológicas asociadas con el envejecimiento fisiológico paralelas a la función y atenuaciones bioquímicas con la suplementación con MAHOC, como máximo, a través de recuperaciones en las mitocondrias.

PALABRAS CLAVE: Corazón; Estrés oxidativo; Antioxidantes; Ingredientes nutricionales; Compuestos bifenólicos.

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