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Suppression of isoproterenol induced apoptosis in H9c2 cardiomyoblasts cells by aescin

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Abstract

Medicinal herbs have been utilized for centuries to alleviate a spectrum of drugs. Horse chestnut (Aesculus hippocastanum) is used in phytomedicine to prevent and treat a wide range of conditions, and its seed extracts contain phytocompounds such as flavonoids, polyphenols, and triterpenoids (aescin). This study aims to investigate the potential mechanisms associated with the cardioprotective effect of aescin on isoproterenol (ISO) induced cardiotoxicity in H9c2 cell lines. The effect of the drug on cell morphology was studied by using a phase contrast microscope, and cell viability was studied by using 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining, and reactive oxygen species (ROS) production was estimated by 20-7'dichlorofluorescein diacetate staining. H9c2 cells were treated with ISO to cause cell damage and the effect of the aescin on cell morphology, mitochondrial membrane potential, intracellular ROS generation, cell viability, and apoptosis was studied. The results of this study showed that pre-administration of aescin significantly reduced the ISO-induced toxic effects on cell morphology and enhanced the number of viable cells in a dose-dependent manner. This study also demonstrates that ROS generation was significantly increased in ISO-administered cells and ISO-induced ROS production was significantly reduced in the aescin-pre-administered H9c2 cells. ISO-induced changes in the mitochondrial membrane potential of H9c2 cells were remarkably improved with aescin pretreatment. These results clearly suggest that pretreatment of aescin protects the cells against ISO-induced damage by resuming mitochondrial function and regulating apoptosis.

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Introduction

Cardiovascular disease (CVD) is the leading cause of mortality as such 17.9 million people died in 2019, representing 32% of all global deaths (WHO, 2021). A few of the cardiac dysfunctions associated with CVD include inflammation, hypertrophy, fibrosis, and apoptosis (Khan *et al.,* 2020). H9c2 cardiomyoblasts originated from rat heart ventricular tissue, and have been used *in vitro* as a model due to their morphological features and biochemical/electrophysiological properties (Suhaeri *et al.,* 2015). The differentiation potential of H9c2 cardiomyoblasts makes this cell line an attractive tool for investigating the cardiotoxicity of several agents (Dallons *et al.,* 2020). The vast majority of cardiotoxicity studies have only been conducted with cells in their undifferentiated and proliferative state. In order to test this possibility, the susceptibility of H9c2 cells to the toxicity of the beta-adrenergic agonist ISO was investigated and correlated with alterations in pro-apoptotic and beta-adrenergic signaling pathways (Branco *et al.,* 2015). ISO, a synthetic catecholamine, has the capacity to auto-oxidize, and produces quinones, thereafter adrenochrome will be formed. Heart conditions associated with catecholamines are brought on by adrenochrome's excessive production of ROS (Costa *et al.,* 2011), cardiac hypertrophy is a result of ISO-induced DNA damage in cardiomyoblasts, which also promotes apoptosis via the generation of free radicals and mitochondrial dysfunction in cardiac cells.

Natural substances high in antioxidants are essential for the treatment of several diseases. The prevention of CVD is linked to the use of antioxidant-rich foods such as fresh fruits, medicinal herbs, and vegetables in recent years (Wilson *et al.,* 2017). These natural protective properties of the foods may be due to the presence of flavonoids, terpenes, and phenolic compounds (Tungmunnithum *et al.,* 2018). Horse chestnut (*Aesculus hippocastanum*) contains a mixture of naturally occurring triterpene called aescin, which has antioxidant, anti-oedematous (Sirtori, 2001), and anti-inflammatory (Gallelli, 2019) activities. However, there is no report on the effect of its protection against ISO-induced inflammation and apoptotic cell death in H9c2 cells. Therefore, we investigated the cardioprotective efficacy of aescin against oxidative stress, and inflammatory and apoptotic signaling induced by ISO in the H9c2 cells.

Materials and methods

Chemicals

Dulbecco modified Eagle medium (DMEM), glutamine, fetal bovine serum (FBS), streptomycin, penicillin- and trypsin neutralizer solution were all purchased from Hi Media (Mumbai, India). ISO, aescin, 2,7 diacetyl dichlorofluorescein (DCFHDA), 3(4, 5 dimethylthiazol2yl) 2,5 diphenyl tetrazolium bromide (MTT), and7-Aminoactinomycin D(7-AAD) dye were all provided by Sigma Aldrich. All other chemicals utilized in this experiment were of high analytical grade and were purchased from Hi Media and E. Merck, India.

H9c2 cardiomyoblast cell line

H9c2 cells were purchased from NCCS, Pune. They were placed in an incubator with 5% CO2 at 37°C with DMEM containing 1% penicillin-streptomycin, 10% FBS, and 1% glutamine. At 37°C the cells were trypsinized for 2-3 minutes. After trypsinization, a monolayer of the cells was washed with phosphate-buffered saline (PBS) and then dispersed into fresh culture flasks containing the medium for subculturing. Cell cultures with passages 3 to 5 were employed for each experiment.

MTT test for cytotoxicity

Aescin and ISO stock solutions were mixed with 0.05% dimethyl sulfoxide (DMSO) and stored at 4°C. To get the right concentrations, further media dilutions were done after the cells had reached around 80% confluence. After the cells were treated with different dosages of ISO (10, 20, 40, 80,100, 200, and 400µM), the cytotoxicity was assessed using the MTT assay (Mosmann, 1983). The inhibitory concentration 50 (IC50) value was calculated and the effective dose was selected for further study. The MTT assay was used to assess the viability of cells treated with aescin at various concentrations (5, 10, 15, 20,

25, 50, 100, and 200 µM). The IC50 value was calculated and, the effective dose was used for the experiments. After receiving treatment for 24 hours, the whole plate was examined using an inverted phase contrast tissue culture microscope (Olympus CKX41 with an Optika Pro5 CCD camera) and photos of the microscopic findings were captured.

Experimental design

H9c2 cells were pretreated with aescin (15µM) for two hours before exposure to ISO (100 µM). Following a 24-hour incubation period, cellular modifications were examined. The following four groups were formed from the cultured cardiomyoblast cells.

Group 1: Normal cardiomyoblasts
Group 2: Normal cardiomyoblasts with 15 µM of aescin
Group 3: ISO-exposed cardiomyoblast cells (100 µM)
Group 4: ISO exposed (100 µM) and aescin-pretreated (15 µM) cardiomyoblast cells.

Evaluation of the levels of intracellular ROS

Using a multimode reader and fluorescent probe DCFH-DA, the production of intracellular ROS was quantified by the method of Kim & Xue 2020. Briefly, the cells were incubated with 10 µM DCFH-DA in PBS for 15 minutes at 37°C in the dark after being treated for 24 hours with aescin and ISO. A multimode reader measured the DCF fluorescence intensity at 488/525 nm (Teccan, Austria).

Detection of mitochondrial membrane potential (MMP)

Using the fluorescent dye 7-AAD, the changes in MMP were identified. A volume of 1 L of 7-AAD was added to the treated and control H9c2 cells, and the mixture was incubated for 30 minutes (Vishnu *et al.,* 2018). The samples were then examined by a flow cytometer.

Detection of apoptotic nuclei by ethidium bromide/acridine orange staining

Before receiving ISO (100 µM) treatment and a 24-hour incubation, the cells underwent aescin (15 µM) pretreatment for two hours. The cells were stained with ethidium bromide/acridine orange (EB/AO) following the treatment, and images were taken with a fluorescence microscope (40x) (Liu *et al.,* 2015).

Alkaline single-cell gel electrophoresis (comet assay)

According to Singh *et al.,* 1988, alkaline single-cell gel electrophoresis (comet test) was employed to assess DNA damage in the H9c2 cells. Aescin-treated H9c2 cells were combined with 0.5% low-melting-point of agarose and pipetted onto percolated slides. The slides were incubated for one hour at 4°C in a cold lysis solution with a pH of 10. The slides were then submerged for 25 minutes in an alkaline electrophoresis buffer with a pH of 13 to enable the DNA to become denaturized. The slides were then subjected to electrophoresis at 25 V for 25 minutes at 4°C in an electrophoresis tank containing a new alkaline electrophoresis buffer. The slides were stained with ethidium bromide after being neutralized in 0.4 M Tris (pH 7.5) for 5 minutes. An epifluorescent microscope was used to observe the DNA damage. The slides were analyzed using an inverted epifluorescent microscope from Olympus that was connected to an Opitka Pro5 CCD.

Statistical analysis

Mean and Standard Deviation was used to represent every value. Using a statistical package software, the group means were examined using Duncan's multiple range test (DMRT) and the data were statistically evaluated using a one-way analysis of variance (SPSS 11.0 for Windows). Statistical significance was defined as a P-value of 0.05.

Results

Effect of aescin against ISO-induced cytotoxicity

In order to examine the cytotoxic effect of various concentrations (5, 10, 15, 20, 25, 50, 100, and 200 µM) of aescin on H9c2 cells, cell viability was determined using MTT assay. The results showed that aescin, in concentrations from 0 to 15 µM, had no cytotoxic effect on H9c2 cells (figure 1A). From 5 to 15µM concentrations of aescin showed over 70 % of cell viability in H9c2 cells.

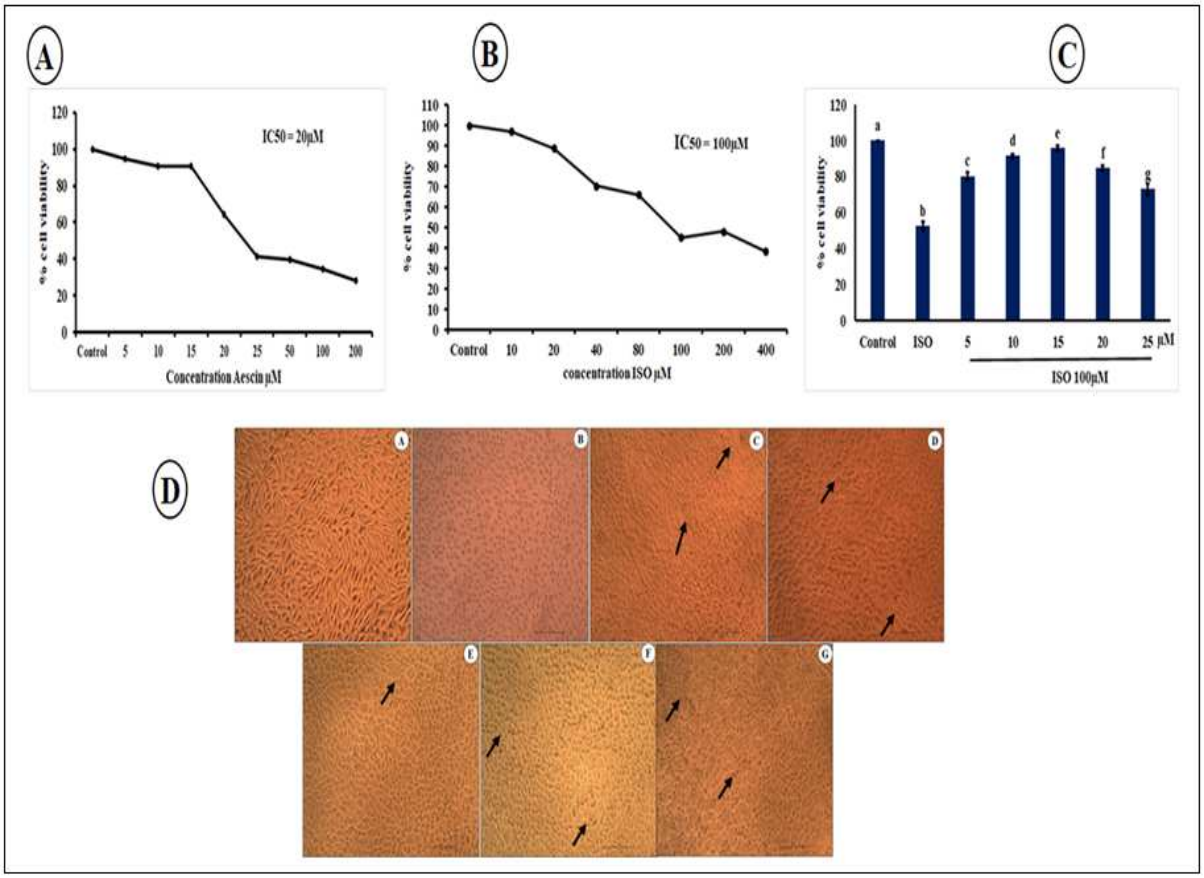

Fig. 1. Effect of aescin on ISO-treated cytotoxicity in H9c2 cells by MTT assay.

A: Effect of aescin on cell viability in H9c2 cells. B: Effect of different concentrations of ISO on cell viability in H9c2 cells. C: Effect of aescin on ISO-induced cytotoxicity in H9c2 cells by the MTT assay. The cells were treated with different concentrations of aescin (5, 10, 15, 20, 25µM) and 100µM of ISO, and the cell viability was determined by the MTT assay after 24-hour incubation. D: Effect of aescin on ISO-induced morphological changes in H9c2 cells. Cells were viewed by phase contrast microscopy and photographed at a magnification of x100. The black arrows indicate apoptotic cells.

The IC 50 value for aescin was found at 20 µM. Based on the results, 5 to 25µM concentrations of aescin were selected for further studies.

To determine the cytotoxic effect of ISO on H9c2 cells, cell viability was observed with various concentrations of ISO (10 to 400µM) for 24 hours. As shown in figure 1B, cell vitality was significantly inhibited ($P \leq 0.05$) in a dose-dependent manner, and IC50 was observed at 100µM concentration of ISO. Hence, the concentration of 100µM of ISO has been chosen as an effective dose for further study in H9c2 cells. To assess the protective effect of aescin on ISO-induced cytotoxicity, the H9c2 cell were treated with aescin (5 to 25µM) for 12 hours, and then ISO (100µM) was added and incubated for the next 12 hours. Subsequently, the cell viability has been detected by the MTT assay and the results (figure 1C) showed that aescin significantly enhanced the survival rate of the cells (5 to 25µM) after exposure to ISO, however, the increase in the concentration of aescin ranging from 50 to 200µM lowered the survivability of the cells.

We have also monitored the morphological changes of H9c2 cells, using a phase contrast inverted microscope. As shown in figure 1D, the treatment with ISO (100µM) and different concentrations (5 to 25µM) of aescin resulted in obvious morphological changes in H9c2 cells. We observed the cell damage as well as cell shrinkage in the ISO-induced cells, however, the aescin-treated cells alleviated the

morphological changes dramatically. This finding suggests that aescin significantly protects the H9c2 cells from the toxicity caused by ISO.

On the basis of these assessments, 15µM of aescin was selected as the optimum and nontoxic dose for further experiments.

Aescin prevents oxidative stress induced by ISO

The intracellular ROS generation was measured by DCFH-DA staining. Fig.2. (A-D) illustrates the levels of ROS generation in control and experimental cells. ISO-treated H9c2 cells show a bright DCF fluorescence, which indicates higher levels of ROS production when compared to the control. Aescin and ISO treated with H9c2 cells show decreased fluorescence, which indicates a reduced level of ROS when compared to the ISO alone treated cells, which show an increased percentage of green fluorescence indicating increased ROS intensity. From these results, it is evident that aescin has a significant effect on the ROS production induced by ISO on H9c2 cells.

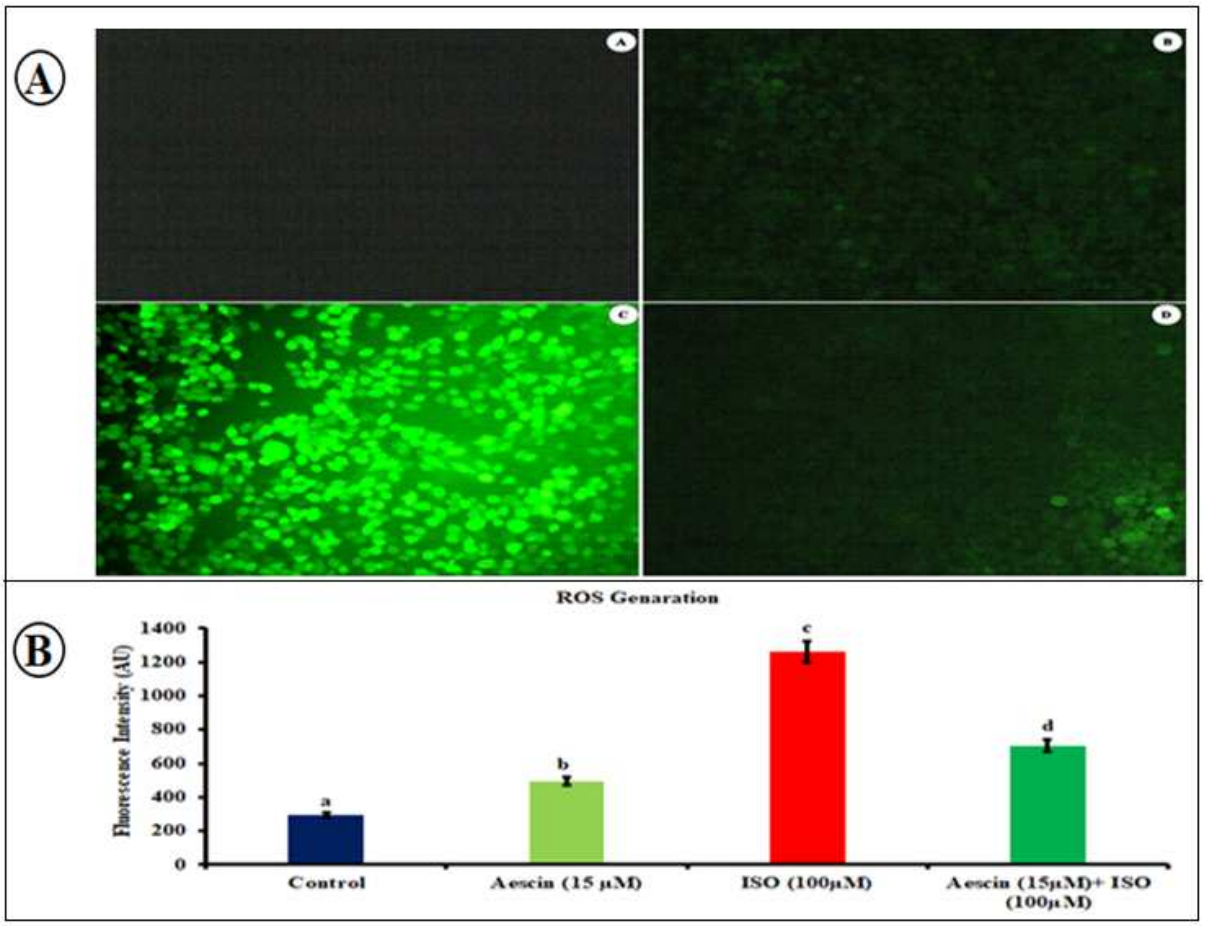

Fig. 2. Effect of aescin on ISO-induced intracellular ROS generation using DCFH-DA in H9c2 cells.

A: ROS generation was examined with a fluorescence microscope (Olympus CKX41 with optika pro5 CCD camera). B: Cells depicted fluorescence intensity was detected by spectrofluorometer. The values are given as mean ± SD of five experiments in each group. Values not sharing a common marking (a, b, c) differ significantly at p≤ 0.05 (Duncan's multiple range test).

Influence of aescin on the mitochondrial membrane potential (MMP)

MMP depends on the charge difference across the membrane, which is permeable to ions and produces an electric potential difference. This electrochemical gradient drives the synthesis of ATP. In our study, we analyzed the levels of healthy and apoptotic cells in the control and treatment groups. In the control group, we observed that the ratio of 7-AAD dye aggregates in the live cell region seemed to be higher

than in the ISO-treated group shown in the figure. 3 (A & B). The 7-AAD dye aggregates in the live region only if the cells have normal ΔΨm, negatively charged mitochondria, and normal membrane permeability. When the cells are treated with ISO, a higher percentage (83.26%) of cells have been observed in the depolarized region and this indicates the loss ΔΨm, increased membrane permeability, and less negative charge inside the mitochondrial membrane. However, the aescin treatment prevented the excessive loss of ΔΨm and decreased the excessive membrane permeability as it showed a higher percentage (31.10 %) of 7-AAD aggregates in the live cell region than the ISO-alone treated group (7.70 %).

Hence, we can assume that aescin has a significant effect on preventing the loss of membrane potential in the mitochondria of H9c2 cells.

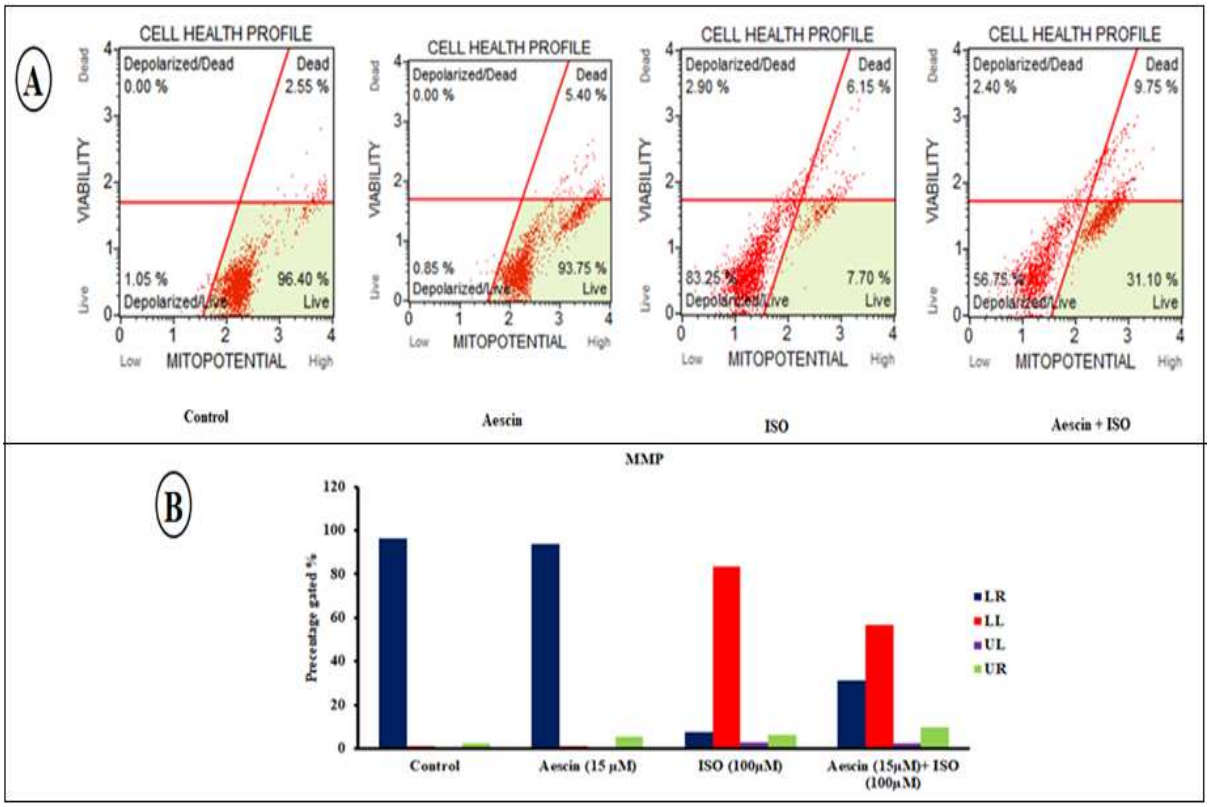

Fig. 3. Effect of aescin on MMP was evaluated with H9c2 cells using 7-AAD via flow cytometry.
A: Cell profile of living and depolarization on H9c2 cells. B: The % of cell rate was measured by flow cytometry following mitopotential 7-AAD. (LL) lower left, (LR) lower right, (UR) upper right, (UL) upper lift. The values are given as mean ± SD of five experiments in each group. Significant compared to the control (p≤ 0.05), and significant compared to ISO (p≤ 0.05).

Efficacy of aescin against ISO-induced DNA damage

The comet assay was used to determine the effect of aescin on DNA damage generated by ISO. Using a cell image station, a red fluorescence lamb, fluorescent microscopic images were captured. The software CAPS was used to determine the % of head DNA and tail length. Figure 5 depicts the level of DNA damage in H9c2 cells following ISO and aescin treatment. The proportions of head DNA and tail DNA were significantly changed in cells treated with ISO. On the other hand, aescin therapy prior to ISO induction significantly (P≤0.05) prevented comet formation in the ISO-exposed H9c2 cells.

Effect of aescin on ISO-induced apoptosis in H9c2 cells

To investigate whether aescin is able to protect the cardiomyocytes from ISO-induced nuclear damage, the apoptotic cells in the cardiomyocytes were examined by using AO/EB double staining method.

As shown in figure 4, the ISO-induced cardiomyocyte apoptosis has been indicated by the distinctive red-orange fluorescence. When treated with aescin (15 µM), no apoptotic injury has been observed and it didn't induce red fluorescence, showing that aescin has non-toxic nature. Treatment with aescin along with ISO prevented the apoptotic injury caused by ISO evidenced by the decrease in the red fluorescence. When compared to the ISO-alone treated group, we observed more viable cells (green fluorescence) which denotes that aescin protects the cardiomyocytes from apoptotic cell damage.

Discussion

Myocardial infarction (MI) is a severe health concern in developed and developing countries, affecting a large proportion of the global population, and it has a significant impact on mortality numbers. Despite the fact that safe and efficient medications and therapies are still lacking, MI has emerged as one of the primary disorders that threaten human health and life (Hausenloy and Yellon, 2018). Plant-derived antioxidants have the ability to reduce the detrimental effects of ROS, reducing the risk of chronic oxidative stress-related illnesses (Witaicenis *et al.,* 2014). Aescin, a significant active component of the horse chestnut tree *Aesculus hippocastanum* (Hippocastanaceae), has demonstrated significant clinical activity in the treatment of hemorrhoids, post-operative edema, and chronic venous insufficiency (CVI) (Sirtori, 2001).

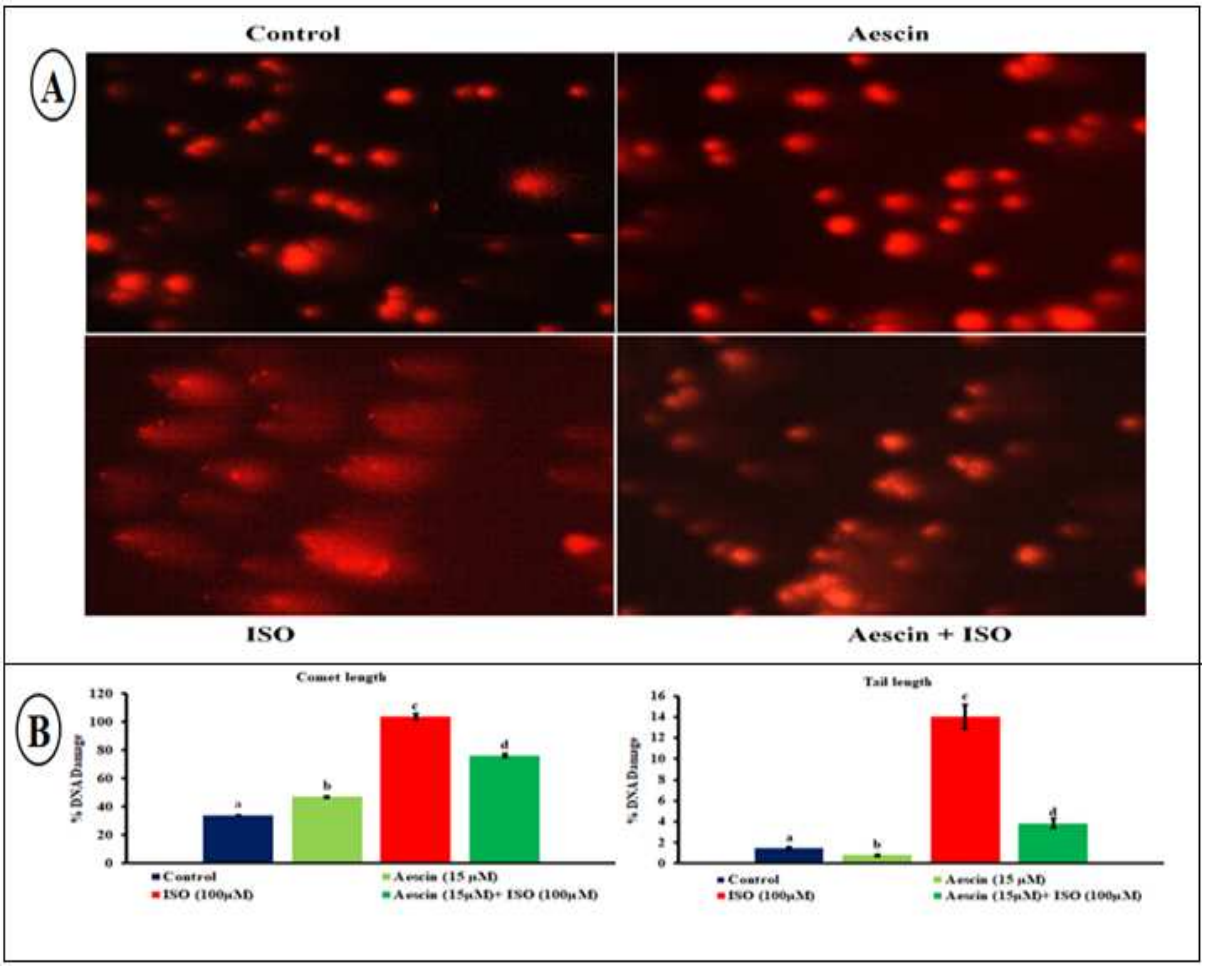

Fig. 4. Effect of aescin on ISO-induced DNA damage by comet assay in H9c2 cells.

A: Aescin on ISO-induced DNA damage was analyzed by comet assay. Fluorescence microscopic images were recorded using a cell imaging station, under a red fluorescence lamp. B: The % comet DNA, and % tail length were calculated by CAPS software. The values are given as mean ± SD of five experiments in each group. Significant compared to the control ($p \leq 0.05$), and significant compared to ISO ($p \leq 0.05$).

The role of aescin in MI treatment remains undefined. Hence, we have planned to study the effect of aescin on ISO-induced abnormalities in H9c2 cells. H9c2 cells can accurately mimic the hypertrophic responses of primary cardiomyocytes, thus they have been used as the standard experimental model *in vitro* system for prospective molecular studies in heart development and diseases (Watkins *et al.,* 2011). Both experimentally and clinically, the formation of ROS by oxidative stress is crucial to the development of cell damage (D'Oria *et al.,* 2020). In our study, we observed that H9c2 cells are vulnerable to cytotoxicity when treated with 10 to 100 µM doses of ISO in a dose-dependent manner. In spite of the fact that catecholamines are responsible for regulating cardiac function, a higher level can cause ischemic heart disease, including MI, cardiac hypertrophy, and heart failure (Severino *et al.,* 2020).

The experimental findings of the study reveal that aescin may prevent cardiac toxicity in ISO-exposed H9c2 cells. We observed pretreatment with aescin affirmed the cardioprotective actions, by enhancing cardiac cell viability, declining DNA damage, decreased myocardial apoptosis, and significantly lowered oxidative stress.ROS may cause apoptosis via various methods depending on the cell type and stimulus (Redza-Dutordoir & Averill-Bates, 2016). DCFH – DA stain was commonly used to investigate the level of ROS, which binds to ROS radicals and transforms them into the fluorescently active esters 2′,7′-dichlorofluorescein.

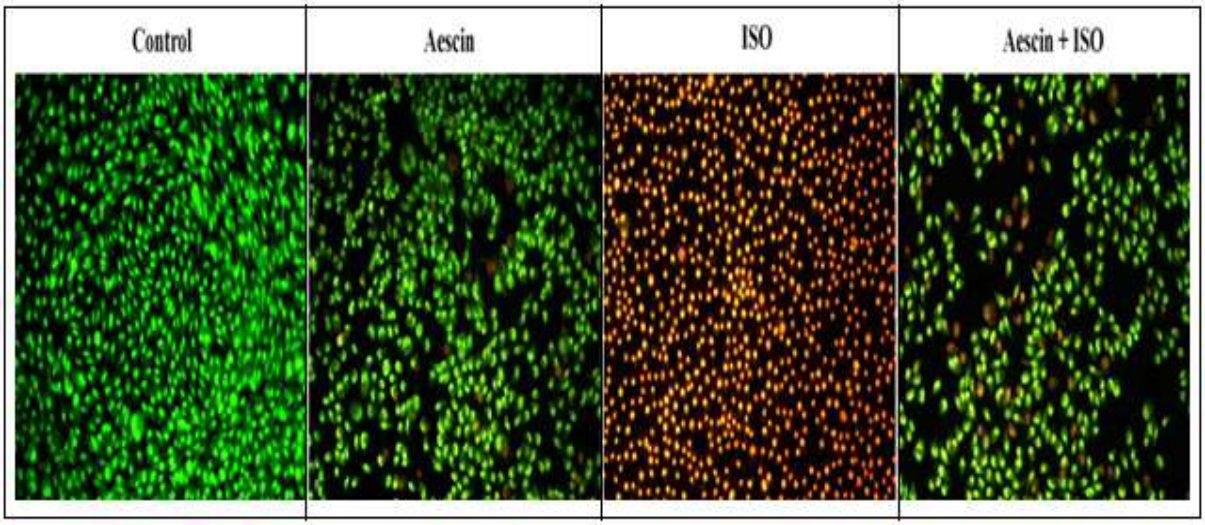

Fig. 5. Effect of aescin on ISO-induced apoptosis using AO/EB staining on H9c2 cells.
Cellular apoptotic morphological changes were observed under a fluorescence microscope using OA/EtBr staining (20×). Control and aescin alone group: green fluorescent nuclei-intact cells; ISO: orange-stained nuclei-apoptotic cells. Aescin 15µM and ISO-treated cells show less orange stained nuclei.

In our study, we analyzed the level of ROS production by ISO and its inhibition by aescin. The results demonstrated that ISO treatment greatly increased ROS generation in the cells, as indicated by the higher fluorescence intensity, which is consistent with the findings of prior research (Soetikno *et al.,* 2013). While cells pre-treated with aescin in ISO-exposed cells displayed lower levels of ROS compared to the cells treated with ISO alone. This study demonstrated that aescin significantly prevented ISO-induced cytotoxicity, resulting in an increase in cell viability which may be attributable in part to aescin capacity to neutralize the free radicals generated by ISO.

Mitochondria regulate crucial cell death processes including apoptosis and maintain the vital energy balance of the cell (Wang and Youle, 2009). Abnormalities in the mitochondria are highly sensitive markers of cell death. In healthy cells, the energy produced during mitochondrial respiration is stored as an electrochemical gradient across the membrane of the mitochondria. This accumulation of

energy generates a mitochondrial trans-membrane potential that allows the cell to drive the synthesis of ATP (Johannsen and Ravussin, 2009). Necrotic cell death, apoptosis, and caspase-independent cell death have all been linked to alterations in the mitochondrial membrane potential, which may affect the function of mitochondria thereby reducing the formation of ATP.

In the majority of mammalian cell types, mitochondria are the predominant source of endogenous ROS. Mitochondria generate large quantities of ROS during ischemia. Nevertheless, excessive ROS generation also harms mitochondria (Zorov *et al.,* 2014). Mitochondrial changes are strongly linked to ROS production, and the degradation of mitochondrial integrity is linked to the Δψm. Additionally, it has been proven that altered mitochondrial membrane permeability may result in higher intracellular ROS production (Marchi *et al.,* 2012). Mitochondria are therefore a key therapeutic target in MI.

Depolarization of the inner mitochondrial membrane potential is thus a reliable indicator of mitochondrial dysfunction and cellular health (Sakamuru *et al.,* 2016). This study used a specific cationic, lipophilic dye (7-AAD) to detect the changes in the mitochondrial membrane potential and as an indicator of cell death. In this study, ISO significantly reduced the MMP level in H9c2 cells compared with the control. In treatment with aescin (20μM), the MMP in ISO-induced H9c2 cells was significantly improved. Since the loss of mitochondrial potential is caused by the mitochondrial permeability transition pore opening caused by ISO. The present results demonstrated that ISO caused depolarization and dissipation of ΔΨm in cardiomyocytes, however, the loss of ΔΨm has been reduced with the treatment of aescin as it reduces the excessive ROS formation into the mitochondrial membrane. This can be supported by the evidence that ROS and mitochondrial Ca^{2+} overload opens the mPTP in the inner membrane, causing the depolarization and loss of ΔΨm, and this can be prevented by attenuation of ROS generation and inhibition of upstream signaling (sedlic *et al.,* 2010). From our study it is evident that aescin has the ability to attenuate ROS generation, hence it can prevent the loss of ΔΨm.

Oxidative stress has the ability to alter cellular macromolecules including DNA, lipids, and proteins, and it is a significant contributor to complex CVD (Pizzino *et al.,* 2017). In the end, it can result in apoptosis by damaging DNA and altering biological processes. According to the results of the current investigation, ISO caused H9c2 cells to considerably increase their DNA damage. Antioxidants and free radical scavengers have been shown to be able to shield cellular DNA damage brought on by various factors (Lu *et al.,* 2013). In addition to reducing intracellular ROS levels and the rate of ISO-induced apoptotic cell death, triterpenoids have substantial inhibitory effects against superoxide production (Ling *et al.,* 2020). In accordance with earlier results, more DNA damage was seen in the ISO-induced cardiomyocytes compared to the control in the comet assay evaluated by an increased percentage of tail length (Ye *et al.,* 2016). Comparing the ISO-administered group with the aescin-ISO-treated group, it is evident that aescin treatment prevented DNA damage since the percentage ratio of the tail length is reduced when comparing the ISO-treated groups. Thus, aescin has the ability to defend against DNA damage brought on by ISO, therefore being in part attributed to its antioxidant action.

Under a fluorescence microscope, the dual AO/EB fluorescent staining method can be utilized to spot apoptosis-related alterations in cell membranes (Kasibhatla *et al.,* 2006). As a result, the AO/EB staining technique was employed to investigate apoptosis in ISO-induced H9c2 cells. AO may enter both healthy and early-stage apoptotic cells with intact membranes and, when bound to DNA, fluoresces green. Only cells with broken membranes, including late apoptotic and dead cells, could be accessed by EB, which only emitted orange-red fluorescence when bound to concentrated DNA fragments or apoptotic bodies. Dual AO/EB staining

can also detect mild DNA damage. Additionally, it permits the distinction of necrotic cells from normal cells as well as early and late apoptotic cells. In our study, the ISO-induced cardiomyocyte apoptosis has been indicated by the distinctive red-orange fluorescence and the treatment with aescin and ISO showed a significantly reduced apoptotic injury in cells when compared to the ISO-treated group, more viable cells (green fluorescence) has observed, which denotes recovery of the cardiomyocytes. It's evident that aescin is effective against ISO-induced cardiac injury.

Conclusion

Aescin has cardioprotective properties against ISO-induced abnormalities in H9c2 cells, as per the results of the current study. From the results of this study, we conclude that aescin can enhance cell viability by scavenging ROS and balancing mitochondrial membrane potential in order to prevent apoptosis. These results suggested that aescin may have a protective impact against oxidative stress and apoptosis in H9c2 cells induced by ISO. However, further research is required to determine the cardioprotective action in animal models.

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Conflict of interests

The authors declare no conflicts of interest.

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