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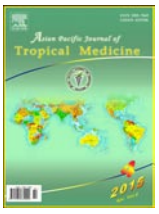

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Trigonelline protects the cardiocyte from hydrogen peroxide induced apoptosis in H9c2 cells

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ABSTRACT

Objective: To elucidate the key parameters associated with hydrogen peroxide induced oxidative stress and investigates the mechanism of trigonelline (TG) for reducing the H_2O_2 induced toxicity in H9c2 cells. **Methods:** Cytotoxicity and antioxidant activity of TG was assessed by EZ-CYTOX kit. RNA extraction and cDNA synthesized according to the kit manufacture protocol. Apoptosis was measured by the Flowcytometry, general PCR and qPCR. **Results:** It was found that the TG significantly rescued the morphology of the H9c2 cells. Treatment of cells with TG attenuated H_2O_2 induced cell deaths and improved the antioxidant activity. In addition, TG regulated the apoptotic gene caspase-3, caspase-9 and anti-apoptotic gene Bcl-2, Bcl-XL during H_2O_2 induced oxidative stress in H9c2 cells. These results were comparable with quercetin treatment. For evident, flow cytometer results also confirmed the TG significantly reduced the H_2O_2 induced necrosis and apoptosis in H9c2 cells. However, further increment of TG concentration against H_2O_2 could induce the necrosis and apoptosis along with H_2O_2. **Conclusions:** It is suggested that less than 125 μM of TG could protect the cells from H_2O_2 induced cell damage by down regulating the caspases and up regulating the Bcl-2 and Bcl-XL expression. Therefore, we suggest the trigonelline could be useful for treatment of oxidative stress mediated cardiovascular diseases in future.

1. Introduction

Cardiovascular diseases are a world threatening problem and cause many deaths. Many researchers reported that the oxidative stress is the main reason of pathogenesis of various forms of cardiovascular diseases including myocardial ischemia-reperfusion injury, congestive heart failure, arteriosclerosis, and drug-induced cardiomyopathy[1]. Apoptosis is a complex and fundamental mechanism which clear irrelevant cells during cell development, normal homeostasis and disease conditions. It plays an important role in fetal development and to maintain adult tissue homeostasis[2]. Any problem in regulation of apoptosis process leads to many diseases including cancer, restenosis, and neruodegradation[3]. The loss of cardiocyte through apoptosis causes a continuous decline of ventricular function. A little level of apoptosis of cardiocyte is adequate to cause lethal dilated cardiomyopathy. The progression of this syndrome can be restricted by inhibiting apoptosis[4]. Oxidative stress, accumulation of calcium and mitochondrial dysfunctions are the main reason for cardiocyte apoptosis, among these, oxidative stress is important role in the apoptosis[5].

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Oxidative stress is considered as a major apoptotic stimulus in many cardiovascular diseases, which trigger the myocyte apoptosis by up regulating pro-apoptotic genes which are inhibited by antioxidants[6]. Medicinal plants and their secondary metabolites are used as medicines for many diseases in worldwide. These products exert more pharmacological activity in the therapeutic treatment of different disorders. Trigonelline (TG) is an alkaloid which is found in *Trigonella foenum graecum* (fenugreek) Fabaceae and other edible plants such as onions, peas, soybeans cantaloupe, corn and coffee. It exhibited many medicinal applications such as inhibition of adipocyte differentiation[7], Anti-carcinogenic[8], anti-migraine, anti-hypercholesterolemia[9], anti-diabetic activities[10], anti-oxidative activity[11], anti-hyperlipidemic and hypo-cholesterolemic activity[12]. From above literature, we are planning to investigate the cardio protective activity of trigonelline against H_2O_2 induced apoptosis in H9C2 cell line model.

2. Materials and methods

2.1. Cell line and chemicals

The H9c2 cell line, derived from embryonic BD1X rat heart tissue was purchased from American Type Culture Collection (ATCC-CRL-1446, USA). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM-ATCC-30-2002) containing 4 mM L-glutamine, 4 500 mg/L glucose, 1 mM sodium pyruvate, and 1 500 mg/L sodium bicarbonate. According to ATTC guideline the cells were subculture. Cell viability assay kit (EZ-CYTOX, Daeillab Service Co. Ltd.), mRNA extraction kit and RT-PCR kit was obtained from the Daeil Lab services Co., Ltd and Invitrogen Life technology. Trigonelline (TG) and other analytical grade chemicals were purchased from Sigma (USA).

2.2. Experiment schedule

The water soluble tetrazolium (WST); 2(2-methoxy-4-nitrophenyl)-3(4-nitrophenyl)-5-(2, 4- disulfophenyl)-2-H-tetrazolium monosodium salt was used for analysis of the cell viability. The H9c2 cells were seeded in the 96 well at a density of 1×10^5 cells/well. The cells were treated with different concentrations of TG (25-150 μM) and hydrogen peroxide (25-125 μM). It was incubated at the 37 ℃ in 5% CO_2 incubator for 24 h and 6 h respectively and then the culture was treated with WST reagent incubated for 2 h to 4 h. The living cells absorbed the WST then it was converted into an orange colour product. Then, the intensity of colour was measured at 450 nm using spectra count ELISA reader. For cardio protective activity, the cells were seeded and separated into six groups control, H_2O_2 alone, the rest of groups' initially exposed to different concentration (25-150 μM) of TG for 48 hours. Then, 100 μM of H_2O_2 was added and incubated for 4 hours, after, read the absorbance at 450 nm for cell viability assay and then morphology changes of cardiocyte and apoptosis were analyzed using EVOS® XL microscope and flow cytometer respectively. Further, we conducted another set of experiment for assay of antioxidant activity, apoptotic and anti-apoptotic gene expressions. The cells were separated into control, H_2O_2 alone, the rest of groups' initially exposed to different concentration (75, 100 μM) of TG and positive control quercetin 100 μM for 48 hours. Then 100 mM of H_2O_2 was added and incubated for 4 hours.

2.3. Flow cytometer analysis

Cells were treated with different concentration of trigonelline for 24 h and then cells were harvested. The harvested cells were resuspended in binding buffer (10 mM HEPES pH7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1.8 mM $CaCl_2$) containing FITC-annexin Ⅴ (1 g/mL) and then further incubated for 20 min. 10 minutes before the end of incubation, PI (10 g/mL) was added to this cell suspension in order to stain necrotic cells. Cells were analyzed with a FACS can flow cytometer (Becton Dickenson Biosciences, San Jose, CA, USA) equipped with an excitation laser line at 488 nm. The PI was collected through a 575 15 nm band pass filter.

2.4. Caspase-3 quantification

The caspase-3 in experimental cells was quantified using a commercial kit (ApoTarget- Invitrogen). Briefly, the experimental cells were harvested and resuspended in 50 μL of cell lysis buffer and incubated on ice for 10 minutes. After that centrifuged for 1 minute at 10 000 *g*. The supernatant was collected and estimated the protein concentration in each sample and taken equal concentration of protein from each sample and added 50 μL of 2 reaction buffer and 5 μL of 4 Mm DEVD-pNA substrate, further it, incubated at 37 ℃ for 2 h. Then absorbance of samples was measured at 400 nm using microplate reader. The percentage of caspase-3 expression was calculated from caspase-2 in control cells. Caspase-3 in control cells considered as 100%.

2.5. Antioxidant and lipid peroxide assay

The activity superoxide dismutase (SOD), catalase, content of reduced glutathione and malondialdehyde (MDA) was performed according to the manufacture protocol (Cell bio-labs INL-USA).

2.6. Apoptotic and anti-apoptotic gene expression analysis using qPCR and general PCR

The total RNA was extracted according to the manufacture instruction (RNA lipid tissue mini kit, Qiagen USA). The extracted RNA was measured using UVS-99 Micro-volume UV/Vis Spectrophotometer-ACT Gene. A quantity of 1 μg RNA was

reverse transcribed using oligo (dT) and ||| reverse transcriptase is a version of M-MLV RT (superscript ||| first stand synthesis system for RT-PCR - Invitrogen Life technology). Real-time PCR was carried out with an ABI 7500 Real-Time PCR System. Target cDNA levels were determined by SYBR green-based real-time PCR in 20 μL reactions containing 10 μL Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA), 1 μL cDNA, 1 μL 10 pmole forward (FP) and reverse primers (RP). Caspase3- FP: ATG TCG ATG CAG CTA ACC TC RP: TCC TTT TGC TGT GAT CTT CC, Caspase9 - FP: TCC TGC TTA GAG GAC ACA GG, RP: TGC TCC TTT GAT TTG AGT CC, Bcl-2- FP: GAC TCA CTA TAG GCG GGA GAT CGT G, RP: CAC TAT AGA GAA GGG CGT CAG GTG C, Bcl-XL- FP: GAG CCA GAT CAT GTT TGA AGC CTT, RP: GGT GAC CGT AAC ACT ACC TGA G, Beta actin FP: AGG TCA TCA CTA TTG GCA AC, RF : ACT CAT CGT ACT CCT GCT TG. The general PCR (Gene Amp PCR system 9700) was performed according to the Maxime PCR Mix Kit (i-*Taq*) manufacture protocol with slight modification. The protocol as follows 5 min at 94 ℃, 29 to 32 cycles of 95 ℃ for 45 seconds, 57-60 ℃ for 45 seconds, 72 ℃ for 1 min, 72 ℃ for 5 min and hold at 4 ℃ The PCR products were resolved in a 1.2% agarose gel and the amplified DNA was visualized by the Safe view nucleic acid staining (Applied Biological material). Expression was normalized against the β-actin transcript signal.

3. Results

Figure 1 showed cytotoxic activity of the trigonelline (25-150 μM) on the H9c2 cells. Treatment of cells with different concentration of TG for 24 h did not disturb the normal cell growth. However, subsequent increase of TG concentration could influences the cytotoxic effects on the cardiocyte (125-150 μM). Hence, concentration of TG (25-100 μM) could be considered as good and safe compound for the cardio protective activity analysis.

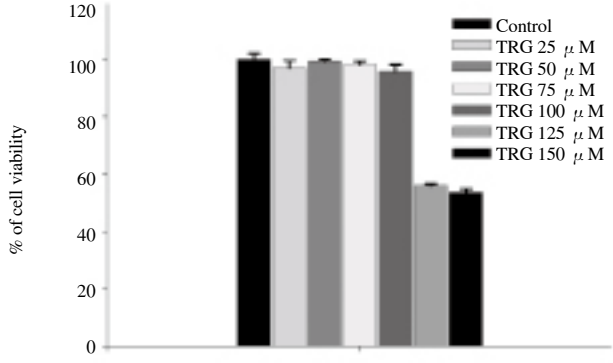

Figure 1. Effect of different concentration of TG on cardiocyte for 24 h.
Cell viability was measured using EZ-cytox kit. Different concentration of TG incubation for 24 h did not influences on the cell viability upto 100 μM, further increment of TG (125 and 150 μM) shows decreased the cell viability (P<0.05).

For determination of optimum concentration H_2O_2 for experiment, we treated H9c2 cells with different concentrations of H_2O_2 (25-125 μM) for 6 h exhibited the dose dependent cytotoxicity. Figure 2 showed decreased the cell viability with increased H_2O_2 concentration. High concentration of H_2O_2 induces the severe cell damage and reduced the cell viability. Treatment of cells with 100 and 125 μM of H_2O_2 exhibited the 50% and 70% of cell deaths respectively. In our study, 100 μM of H_2O_2 was considered as an optimum concentration for further cardio protective activity.

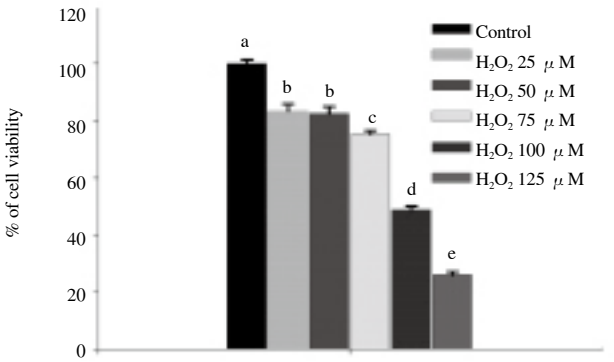

Figure 2. Effect of different concentration (25-125 μM) of hydrogen peroxide on cardiocyte.
The cells treated with H_2O_2 treatment for 6 h decreased the cell viability in a concentration dependent manner. Different letters within treatment represent significant difference (P<0.05).

For the purpose of assessing whether the TG protects the cardiocyte from H_2O_2 induced oxidative stress. Cell pretreated with different concentration of TG and then added 100 μM of H_2O_2 for 4 h. Pre-treatment with TG protects the cells from H_2O_2 induced cell damage as compared with H_2O_2 alone treated cells (Figure 3). Further, it protects the % of cell viability as compared with H_2O_2 alone treated cells. However, increases of TG concentration against H_2O_2 could induce the cell deaths. Therefore, 25-100 μM of TG exhibited the potent cardio protective activity against H_2O_2 (Figure 4).

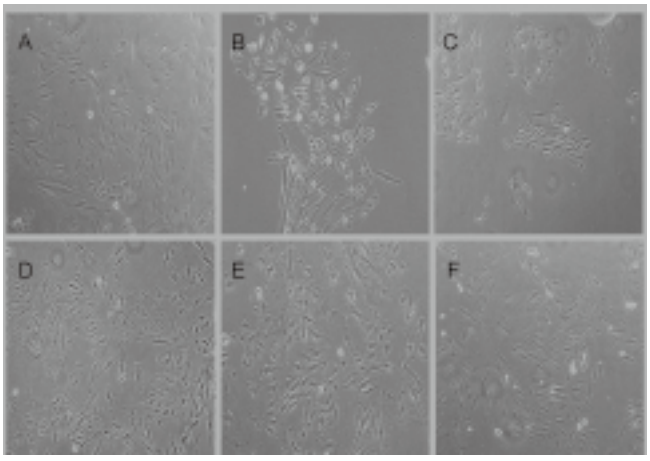

Figure 3. Effect of TG on hydrogen peroxide induced oxidative damage in H9c2 cells.
A-Control, B - H_2O_2 alone, C - H_2O_2+ 25 μM, D- H_2O_2+ 50 μM, E- H_2O_2+ 75 μM, F- H_2O_2+ 100 μM.

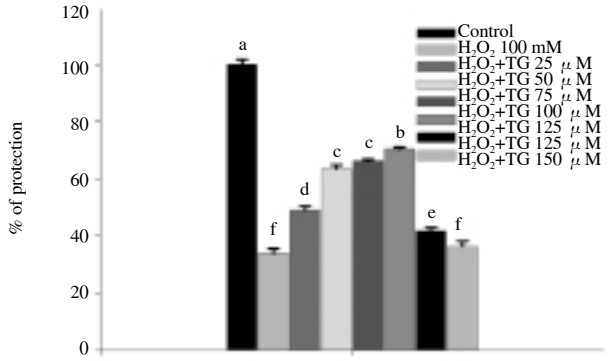

Figure 4. Percentage of protection of TG on hydrogen peroxide induced oxidative damage in H9c2 cells.
Different letters within treatment represent significant difference (P<0.05).

Flow cytometer analysis results revealed that the TG (25-100 μM) significantly reduced the percentage of necrosis and apoptosis as compared with H_2O_2 treated cells. However, further increment of TG concentration (125-150 μM) against H_2O_2 could increases the necrosis and apoptosis. Hydrogen peroxide alone treated cell showed necrosis (0.4%), late apoptosis (66.1%) and early apoptosis (32.1%). Hence, 25-100 μM of TG significantly recues the cardiocyte from H_2O_2 induced necrosis and apoptosis (Figure 5).

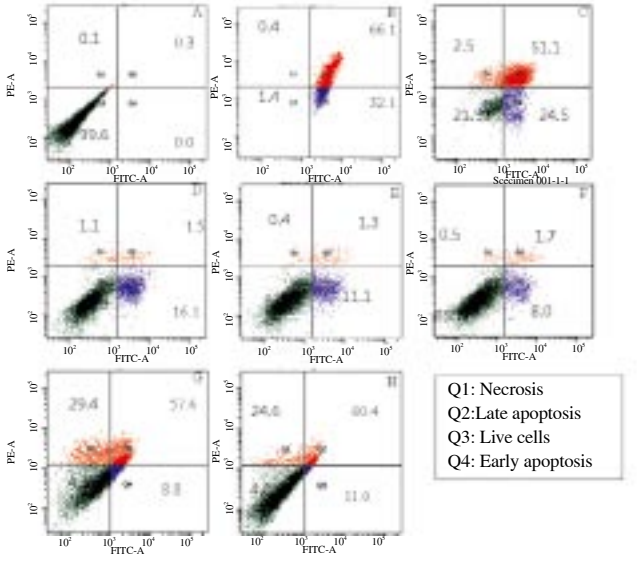

Figure 5. Flow cytometer analysis of experimental cells.
A- Control, B - H_2O_2 alone, C - H_2O_2+ 25 μM, D- H_2O_2+ 50 μM, E- H_2O_2+ 75 μM, F- H_2O_2+ 100 μM, G- H_2O_2+ 125 μM, H- H_2O_2+ 150 μM.

Figure 6 showed quantification of caspase-3 expression in control and experimental cells by spectrophotometric method. H_2O_2 treatment increased the caspase-3 expression as compared with control cells. However, pre-treatment with TG significantly decreased the caspase-3 expression as compared with H_2O_2 alone treated cells.

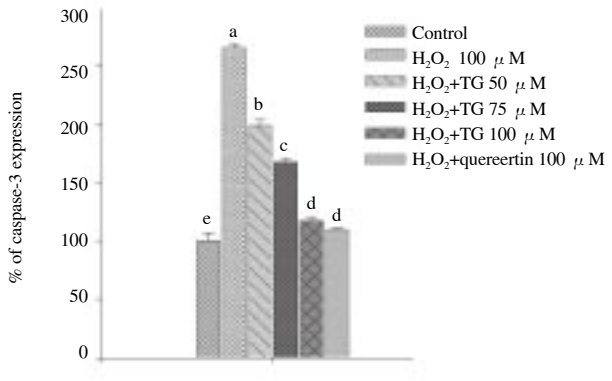

Figure 6. Effect of TG treatment on caspase-3 expression as compared with H_2O_2 alone treated cells.
Different letters within treatment represent significant difference (P<0.05).

Figure 7 showed the level of lipid peroxide and antioxidant status in control and experimental cells. MDA indicated the membrane lipid per-oxidation level during oxidative damage. Treatment of cells with 100 μM H_2O_2 increased the intracellular MDA levels as compared with control cells. However, cells pre-treated with TG reduced the formation of MDA as compared with 100 μM H_2O_2 treatment alone. Treatment of cells with different concentration of TG against H_2O_2 enhanced the SOD, catalase and total GSH level in a concentration dependent manner. These results suggest that the antioxidant activity of TG which helps to protect the cells from H_2O_2 induced cytotoxicity.

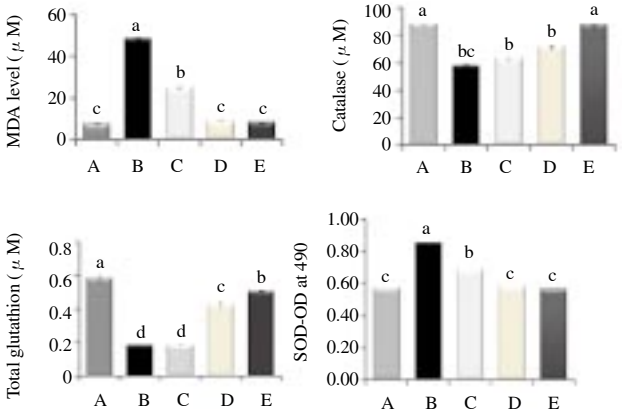

Figure 7. Level of lipid peroxide and antioxidant status in control and experimental group of cells.
A: Control; B: H_2O_2 100 μm; C:H_2O_2+ Trigonelline 75 μM; D: H_2O_2+ Trigonelline 100 μm; E: H_2O_2+ quercertin 100 μM.
Different letters within treatment represent significant difference (P<0.05).

Figure 8A&B showed apoptotic and anti-apoptotic mRNA expression in experimental cardiocyte. Treatment of cells with H_2O_2 alone showed down regulation of anti-apoptotic mRNA such as Bcl-2 and Bcl-XL expression and up regulation of caspase-3 and 9 as compared with control cells. The cells pre-treatment with different

concentrations of TG significantly increased the expression of Bcl-2, Bcl-XL and decreased the expression of caspase-3 and 9.

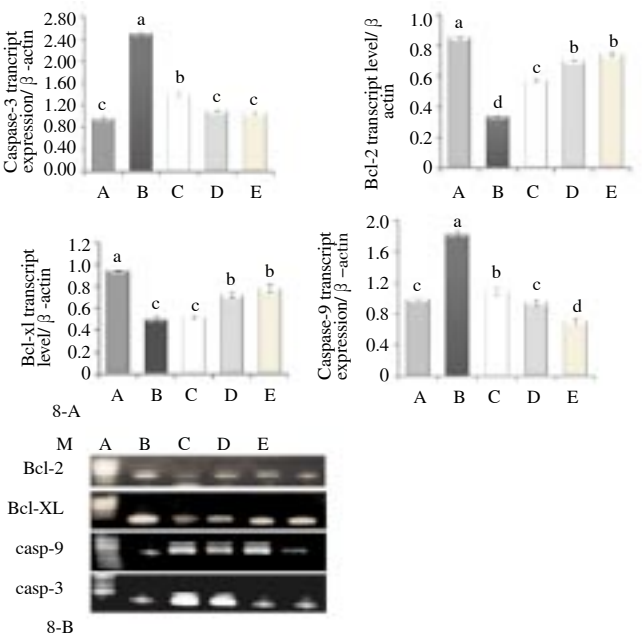

Figure 8. Apoptotic and anti-apoptotic mRNA transcripts expression in experimental cardiocyte.

Different letters within treatment represent significant difference (P<0.05).

M: Marker; A: Control; B: H_2O_2 100 μM; C: H_2O_2+ Trigonelline 75 μM; D: H_2O_2+ Trigonelline 100 μM; E: H_2O_2+ Quercetin 100 μM.

4. Discussion

Cell death and cell injury is one of the most critical events in the evaluation of many diseases in any organ. Two important events are involved in cell death such as necrosis and apoptosis, which differ in their morphology, molecular mechanism and role in physiology and disease[13]. Oxidative stress is an important apoptotic stimulus in many cardiovascular diseases. Reactive oxygen species (ROS) stimulates apoptosis by increasing the pro-apoptotic gene expression. Generally, this kind of apoptosis was inhibited by the antioxidants[6]. The H_2O_2 and OH^{*-} enhance the intracellular oxidative stress which damage the various intracellular bimolecules and results in apoptosis and necrosis of the cells. H_2O_2 may induce the generation of ROS at mitochondria which has been widely used as a model exogenous oxidative stress mediated experiment in cardiocyte apoptosis[14]. Similarly our study, exposing of different concentration of H_2O_2 to cardiocyte increased the apoptosis of cells in a concentration dependent manner. Around 50% cells were died at a concentration of 100 μM H_2O_2. It was considered as optimum dose for further experiment. For cardio protective activity, TG significantly protects the cells from H_2O_2 induced oxidative damage; it rescues the morphology of cells. Further, TG increased the % of cell viability against H_2O_2 induced stress. For evident, flow cytometry results revealed that the TG significantly reduced the necrosis, late apoptosis and early apoptosis in a dose dependent manner. However further increment of TG concentration (125-150 μM) against H_2O_2 induced toxicity could increases the necrosis and apoptosis. It indicated that the more concentration of TG may stimulate the necrosis and apoptosis signaling pathway. Arlt *et al*[15] reported that TG inhibits the nuclear factor E2 related factor-2 (Nrf2) which is essential role in cancer development and chemo resistance. They concluded that the TG enhances the apoptosis via down regulation of Nrf2 expression. Here we found TG could enhance the % of necrosis and apoptosis at concentration of 125 and 150 μM. However, previous concentration of TG might protect the cells from H_2O_2 induced necrosis and apoptosis.

Antioxidant such as SOD, CAT, GPx, GST, and GSH protect the cells from oxidative stress[16]. The SOD catalyzes the dismutation of O_2 to H_2O_2 in the extracellular compartment. This is the major pathways for the removals of H_2O_2 in cells are catalyzed by GSH-Px and CAT, converting H_2O_2 into water and oxygen. In our study, cells exposed with 100 μM H_2O_2 alone increased level of MDA and decreased antioxidant status. When cells pre-treated with TG significantly maintains the enzymatic antioxidant activity such as SOD, Catalase and non enzymatic antioxidant like reduced glutathione content as compared with100 μM H_2O_2 alone exposed cells. From that result suggesting the TG has ability to scavenge the H_2O_2 mediated free radical productions and to protect the cardiocyte by preserving the antioxidants. Our finding concurrent with[17] Yen *et al*, who reported that trigonelline significantly increased the free radical scavenging activity in roasted coffee residues. In addition, TG significantly improved the biochemical alteration and increased the antioxidant status against streptozotocin induced neonatal diabetic mellitus in rats[18].

Apoptosis may be activated by the intrinsic or by the extrinsic pathway[19]. Caspases are a group of aspartate specific cysteine protease, which plays a key role in regulating the apoptosis induced by different kind of stimuli including oxidative stress[20]. Functionally, caspase-3 is an important effector in the apoptotic process and caspase-9 is an initiator of caspase-3 in the mitochondria-dependent pathway[21]. Caspases execute the apoptosis through DNA degradation, chromatin condensation and nuclear fragmentation. The activation of caspase-3 can be initiated by upstream protease either by caspases-9 in the intrinsic pathway (mitochondria) or by caspases-8 in the extrinsic pathway in death receptors[19]. Treatment of H_2O_2 to the H9c2 cells increased the caspases-9 expression in cardiocyte. It indicating that the mitochondrial pathway plays an important function in H_2O_2 induced apoptosis in cells. Our study, H9c2 treated with 100 μM H_2O_2, up regulates the expression of caspases-3 and caspases-9, which indicated that the apoptosis induced by H_2O_2 through activation of Caspases cascade. However, cardiocyte pre-treated with two different concentration of TG significantly attenuates the expression of caspases. It indicated that the TG suppresses the H_2O_2 induced caspases activation.

Bcl-2 proteins are major regulators of mitochondrial cytochrome c and caspases activation. It plays an important role in the regulation of cardiocyte apoptosis[22]. This family contains both pro-apoptotic and anti-apoptotic proteins (Bcl-2 and Bcl-XL). Bcl-2 is an important cellular component which can protect against apoptotic cell death. It prevents the release of cytochrome c and some other apoptosis inducing factor. The Bcl-XL expressions prevent the mitochondrial cytochrome c release and protecting the cells from apoptosis by inhibiting the availability of cytochrome c in the cytosol[23]. The

present study, we investigated the effect of TG on H_2O_2 induced alteration in anti-apoptotic proteins expression. When H_2O_2 treated cells exhibited the down regulation of anti-apoptotic protein such as Bcl-2 and Bcl-XL as compared with control cells, whereas cells pre-treated with different concentrations of TG prevents the H_2O_2 induced down regulations of Bcl-2 and Bcl-XL. These results are comparable with quercetin treatment. It indicated that the trigonelline significantly protect the cells from oxidative stress mediated apoptosis via activation of anti-apoptotic pathway.

Oxidative stress involved in many abnormalities related with different cardiovascular diseases including hypertension, hypercholesterolemia, atherosclerosis, heart failure and type Ⅱ diabetes[24]. Protection of heart from the oxidative stress is an important consequence process. The medicinal plants and plants derived lead compounds given much valuable source for the natural antioxidant products, which reduced the oxidative stress. Generally, antioxidants might be decreased the cellular injury and apoptosis via scavenging of free radical mechanism[25]. The present study, TG significantly maintains the antioxidant activities such as SOD, CAT, reduced GSH and decreased the MDA content during H_2O_2 treatment and also it maintains the structural integrity of H9c2 cells. Flow cytometer results also strongly supports the TG significantly attenuates the H_2O_2 induced necrosis and apoptosis up to certain concentration. In addition, pre-treatment of trigonelline regulates the apoptotic (Caspase-3 caspase-9) and anti-apoptotic (Bcl-2 and Bcl-XL) mRNA transcripts. From the results we concluded the TG significantly protect the H9c2 cells from H_2O_2 induced necrosis and apoptosis via improvement of antioxidant activities and regulation of apoptotic and anti-apoptotic pathway, which could be useful for treatment of oxidative stress mediated diseases and further need to investigate the cardio protective mechanism of TG in animal model system in future.

Conflict of the interest statememt

We declare that we don't have any conflict of interest.

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