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Thraatchathi Chooranam, protects cardiomyocytes against oxidative stress

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1. ABSTRACT

Thraatchathi Chooranam (TC), is a polyphenol-rich Indian traditional medicine. Present study was undertaken to investigate the effects of TC against H_2O_2 induced oxidative stress and apoptotic damage in H9C2 cardiomyocytes. Cell viability assay indicated relative safety (IC50= 488.10±12.04 μg/ml) of TC. Pretreatment of cells with TC upregulated anti-apoptotic Bcl2, and anti-oxidants TRX1 and TRXR and downregulated Bax and HIF-α and inflammatory genes iNOS and TNF-α. Together, these findings show that TC has both anti-oxidant and anti-apoptotic properties. Further studies may be considered to identify the bioactive principle(s) and precise mechanisms of action of TC.

2. INTRODUCTION

Experimental and clinical data have clearly ndicated the involvement of uncontrolled production of reactive oxygen species (ROS) in various metabolic disorders, including cardiovascular diseases (1). Exposure of cardiomyocytes to oxyradicals results in oxidative stress, leading to massive cellular damage, intracellular Ca^{2+} overload, mitochondrial dysfunction, and finally apoptosis, which describes the pathophysiology of cardiomyocytes death. Downstream signaling of antiapoptotic factors and over-activation of proapoptotic proteins, caspases (2), and various intracellular signal transduction proteins activate apoptosis (3), thereby leading to irreversible cardiac dysfunction. The ideal strategy to prevent cellular

degeneration caused by extreme oxidative stress is the control of cardiomyocyte damage through the suppression of ROS and the so on apoptosis cascades. As current interventional therapies, especially administration of modern medicines belongs to calcium and sodium channel blockers, anti-anginal, etc., cause significant adverse effects. Hence, novel therapeutic regimens or adjuvant treatment with minimal side effects are essential to reinstate cardiac function.

Over decades, Siddha system of medicine has gained considerable attention, and its role in different metabolic disorders, including heart diseases, has already well recognized. In India, although the practice of various traditional medical systems such as Ayurveda, Siddha, homeopathy and Unani have long history of treatment successes with better life quality and lesser side effects, a major setback exists with regard to standardization and modern experimental evidences.

Thraatchathi Chooranam (TC) is a polyphenol-rich herbal Siddha formula, which is traditionally used by Siddha practitioners for curing all three "dosha" imbalances and, specifically, for the treatment of heart diseases. Reports have indicated that TC contains many dietary antioxidants, predominantly polyphenols, which play a key role in fighting against free radicals induced damages *in vitro* (4-6). Recently, we reported the various polyphenols such as gallic acid, ellagic acid, quercetin, naringenin, and galangin contents in TC using using high performance thin layer chromatography technique (7). Although TC has been widely prescribed by Siddha practitioners as cardiotonic, its mode of action in cardiac cells is still needs to be studied. To evaluate the cardioprotective effects of new chemical entities, H_2O_2-induced oxidative stress in H9C2 cardiomyoblast cells is widely used as preliminary *in vitro* model system (8). In the present study, we examined the effects of TC against H_2O_2-induced oxidative stress, cytokines and apoptotic insults in H9C2 cardiomyoblast cell line and analyzed the molecular mechanism of action.

3. MATERIALS AND METHODS

3.1. Chemicals

TC was procured from M/s. Arogya Health Care Pvt. Ltd Chennai, India. H9C2 rat cardiomyoblast cells were purchased from NCCS, Pune, India. Prime RT-PCR Premix (2X) was purchased from Genet Bio, USA. Primers for RT-PCR were supplied by Eurofins Genomics, Bangalore, India. All other chemicals and reagents used were analytical grade and obtained from SISCO Research Laboratories Pvt Ltd. Mumbai, India.

3.2. Thraatchathi Chooranam

TC contains equal proportion dried powdered of 32 herbs such as *Vitis vinifera, Phoenix dactylifera, Cyperus rotundus, Piper wallichi, Santalum album, Oryza sativa, Curcuma anguistifolia, Elattaria cardamomum, Cuminum cyminum,Vetiveria zizonoides, Zingiber officinale [dried], Piper nigrum, Piper longum, Terminalia chebula, Terminalia bellarica, Embilica officinalis, Pavonia odorata, Costus speciosus, Glyzhirrizha glabra, Pavonia zeylanica, Tinospora cordifolia, Gmeliana asiatica, Tribulus terrestris, Plectranthus vittiviroides, Coccinium fenestratum, Nymphaea pubaecens, Syzigium aromaticum, Curcuma aromatic, Crocus sativus*, *Kaempferia galangal, Neliumbo nucifera* and *Sitramalli*. This is a Siddha sastric formula and being used for more than ten decades. Siddha verse indicates its use in diabetes mellitus, heart diseases, giddiness, palpitation etc. Interpreting the photochemistry behind the formula it is formulated to deliver various types of polyphenols to combat ailments.

3.3. Cytotoxicity assay (9)

Toxicity potential of TC to H9C2 cells was analysed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Pre-confluent H9C2 cells were seeded in 96 well plate configuration (8×10^3 cells/200 µl/well) and incubated at 37°C for 24 h. Then cells were treated with different concentrations of TC (ranging from 1 - 1×10^6 ηg) and for next 20 h. Then, 4µl of MTT (5 mg/ml) was added to the cells and incubated for 4 h in 37°C. After the final incubation period, culture media was aspirated and the formed insoluble formazan was dissolved in DMSO (200 µl) and kept in the dark for 15 min. MTT reduction was quantified by measuring the absorbance at 570 nm (Multiskan Spectrophotometer, USA).

3.4. Detection of apoptosis by DAPI staining (10)

Nuclear damage produced triggered by H_2O_2 was analyzed by DAPI (4',6'-diamidino-2-phenylindole) staining method with minor modifications. H9C2 cells were seeded in 12-well plates at a density of 2.5×10^4 cells/ml/well and incubated at 37°C for 24 h. Cells were pre-treated with different concentrations of TC (10, 30, and 100 µg/ml) and incubated at 37°C for 1 h, then washed with phosphate buffered saline (PBS), fixed with 4% paraformaldehyde for 15 min. This is then washed with PBS and stained using DAPI (5µl; 100µM) and incubated in dark for 30 min. Finally, the cells were washed thrice with PBS and examined under fluorescence microscope (Motic, China).

3.5. Measurement of intracellular oxidative stress using DCFH-DA fluorescence staining

Pre-confluent H9C2 cells were seeded into 12-well plates at a density of 2.5×10^4 cells/ml/well and incubated at 37°C for 24 h. Subsequently, the cells were treated with TC (10, 30, and 100 µg/ml) and incubated at 37°C for 1 h, and then 100 µM of H_2O_2 was added

Table 1. Primers used in the RT-PCR assay

Gene	Forward primer	Reverse primer
β-Actin	5'-GACATGGAGAAAATCTGGCA-3'	5'-AATGTCACGCACGATTTCCC-3'
Bax	5'-TTTTGCTTCAGGGTTTCATC-3'	5'-GACACTCGCTCAGCTTCTTG-3'
BCl2	5'-ATGTGTGTGGAGAGCGTCAACC-3'	5'-TGAGCAGAGTCTTCAGAGACAGCC-3'
HIF-α	5'-CCAGTTACGTTCCTTCGATCAGT-3'	5'-TTTGAGGACTTGCGCTTTCA-3'
TNFα	5'-ATGAGCACAGAAAGCATGATC-3'	5'-ACAGGCTTGTCACTCGAATT-3'
iNOS	5'-AATGGCAACATCAGGTCGGCCATCACT-3'	5'-GCTGTGTGTCACAGAAGTCTCGAACTC-3'
TR-1	5'-CGATCTGCCCGTTGTGTTTG-3'	5'-CAAGTAACGTGGTCTTTCACCAGTG-3'
TRX1	5'-GAGCAAGACTGCTTTCAGG-3'	5'-GGTCCAGAAAATTCACC-3'

to induce oxidative stress. After 1 h of incubation, the media was removed, the cells were washed thrice with 1X PBS, and 5µl (10 µM) of dichloro-dihydro-fluorescein diacetate (DCFH-DA) was added to the wells. The plates were then incubated at 37°C for 60 min and observed under fluorescence microscope at an excitation/emission wavelength of 485/535 nm (11).

3.6. Apoptotic, inflammatory and oxidative stress markers genes expression by RT-PCR

Preconfluent H9C2 cells were seeded into 60-mm petri plates (1×10^5 cells/plate) and incubated at 37°C for 24 h. Then, the cells were treated with different concentrations of TC (10, 30, and 100 µg/ml) at 37°C for 1 h. Subsequently, the medium was removed and 100 µM of H_2O_2 was added to induce oxidative stress. After 1 h of incubation, the media was removed and the cells were trypsinized with trypsin-EDTA and centrifuged at 1500 rpm for 5 min.

Reverse transcriptase-PCR was performed to determine the mRNA expression of Bax, Bcl2, HIF-α, iNOS, TNF-α, TRX1, and TRXR in H9C2 cells (primers sequence shown in Table 1). Briefly, total RNA was isolated by homogenizing the cells with TRIzol reagent (Sigma, USA). After homogenization, the samples were incubated for 10 min and centrifuged at 1000 rpm for 5 min. Subsequently, 200 µl of chloroform was added to the supernatant, and the samples were incubated for 5 min at room temperature and centrifuged at 12,000 rpm for 20 min. Then, 500 µl of isopropyl alcohol was added to the supernatant and incubated for 10 min, followed by centrifugation at 12,000 rpm for 15 min to precipitate total RNA. The pellet obtained was washed thrice with 75% ethanol, centrifuged at 12,000 rpm for 15 min, dried, resuspended in 20 µl of RNAase-free water, and stored at −80°C until further use. For RT-PCR, 200 ng of RNA was used according to the manufacturer's instructions (Genet Bio, Korea).

3.7. Data analysis

Results were expressed as mean ± standard error mean (SEM). Cytotoxicity data was analyzed by linear regression method. Mean difference in mRNA expression between groups was determined by one-way ANOVA, followed by Tukey's multiple comparison as posthoc test. P value ≤ 0.05 was considered as statistically significant. Graph Pad Prism 5.0 software (San Diego, USA) was used for statistical analyses.

4. RESULTS

4.1. Effects of TC on the viability of H_2O_2-treated cells

To determine whether TC produces any toxic effect on cardiomyocytes, we treated the H9C2 cells with 10 different concentrations of TC (from 1 pg to 1 mg) for 24 h. IC_{50} of TC was found to be 488.10±12.04µg/ml (Figure 1). This indicates that TC is non-toxic at lower concentration and might posse's wider safety window.

4.2. Effects of TC on the formation of apoptotic bodies produced by H_2O_2

To examine the effect of TC against H_2O_2 intoxication induced nuclear morphology changes in H9C2 cells DAPI staining was performed. Cells treated with H_2O_2 for 24 h showed condensed chromatins, fragmented nuclei, and increased number of apoptotic bodies. Whereas, the cells pretreated with TC (10, 30, and 100 µg) priorure to H_2O_2 exhibited a decrease in the apoptotic bodies formation in a dose-dependent manner (Figure 2). Cells treated with high dose of TC (100 µg) but no H_2O_2 showed normal nuclear morphology.

4.3. Effects of TC on the intracellular ROS level in H_2O_2-treated cells

To examine the effect of H_2O_2 on intracellular ROS production, H9C2 cells were subjected to DCFH-DA fluorescence staining. Cells treated with H_2O_2 showed high fluorescence intensity, revealing the increased production of intracellular ROS. Conversely, pretreatment with TC (10, 30, and 100 µg) significantly attenuated the high fluorescence intensity caused by H_2O_2 treatment in a dose-dependent manner (Figure 3).

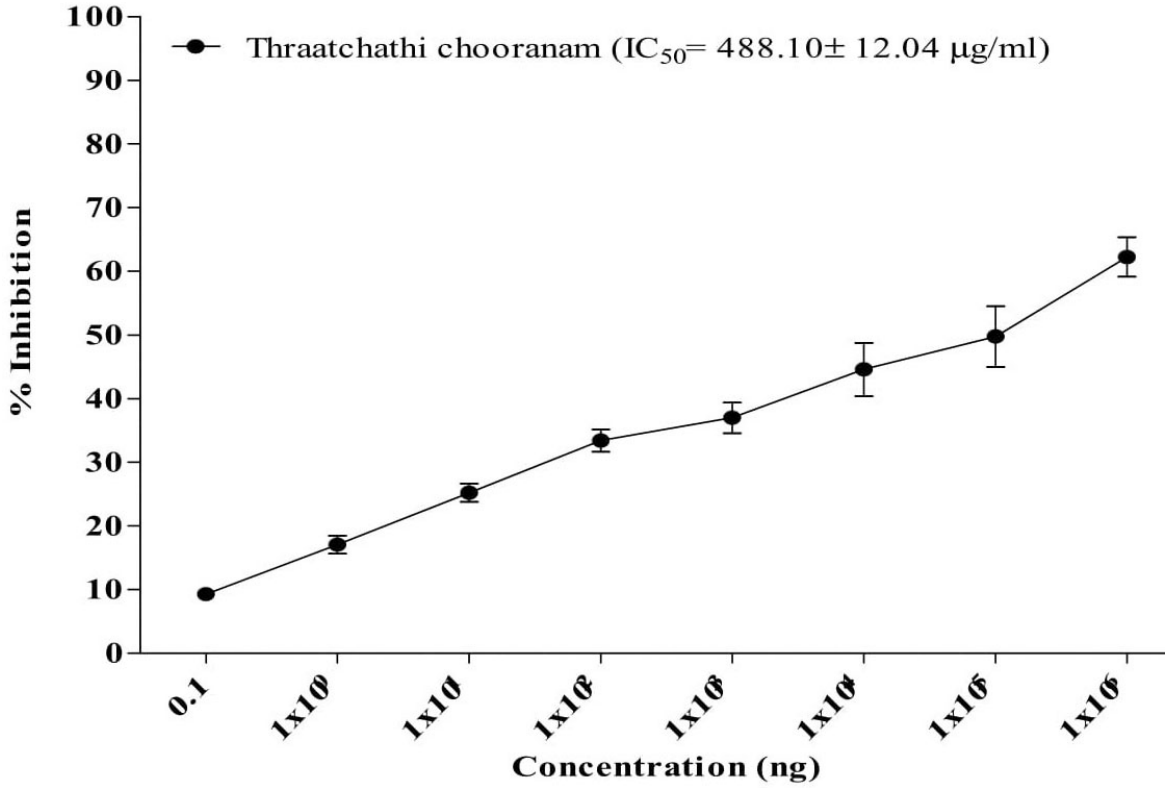

Figure 1. Cytotoxicity of Thraatchathi Chooranam.

4.4. Effects of TC on the mRNA expression of apoptotic, inflammatory and oxidative stress markers

Apoptotic (Bax and HIF-α) (Figure 4A and 4B) and inflammatory (iNOS and TNF-α) (Figure 5A and 5B) markers were significantly upregulated in the H_2O_2-treated cells, when compared with normal cells ($p<0.01$). Conversely, TC pretreatment reversed the effects due to H_2O_2-induced stress in a dose-dependent manner. Furthermore, concurrent downregulation of the antiapoptotic (Bcl2) and oxidative stress markers (TRX-1, TRXR) (Figure 6A, 6B and 6C) was observed in the cells treated with H_2O_2, when compared with normal cells; however, pretreatment with TC reduced the effects of H_2O_2 and increased the expression of antiapoptotic and oxidative stress markers, and this effect was significant in cells treated with high dose of TC ($p<0.01$).

5. DISCUSSION

Uncontrolled oxyradicals activity might combine with other factors to cause apoptosis, leading to cell death. The results of the present study showed that H_2O_2 treatment caused a significant decrease in the viability of H9C2 cells, whereas pretreatment with TC protected against it, suggesting that TC is capable of protecting H9C2 cells from oxidative stress.

Oxidative stress triggers apoptosis (12) and it is implicated in the pathogenesis of various CVDs (10). Cardiomyocyte apoptosis causes loss of contractile functions and reparative mechanisms, which might contribute to worsen whole cardiac function (13). Therefore, protection against cardiomyocyte cell death has gained significant clinical interest, and it is important to identify the signaling pathways that mediate cell survival. Many physiological and chemical inducers of oxidative stress cause apoptosis (14). The principal oxygen species responsible for oxidative stress are H_2O_2, free radical superoxide anion ($O2^-$), and hydroxyl radical (OH^-). In the present study, we chose H_2O_2 for the induction of stress in H9C2 cells because it is a well-established agent for the evaluation of oxidative stress induced cardiomyocyte cell death. Furthermore, DAPI staining was used to assess the nuclear morphology (15). Our results showed that pretreatment of the H9C2 cells with TC protected the cells from the formation of apoptotic bodies caused by H_2O_2.

Free radicals are known to cause oxidative damage to the critical cellular components and membranes of the cardiac tissue, and attenuation of increased ROS level to normalcy is an important and frequently neglected therapeutic target. 2', 7'-Dichlorofluorescein (DCF) is a non-fluorescent derivative that emits fluorescence after being oxidized by H_2O_2. As the emitted DCF fluorescence is directly

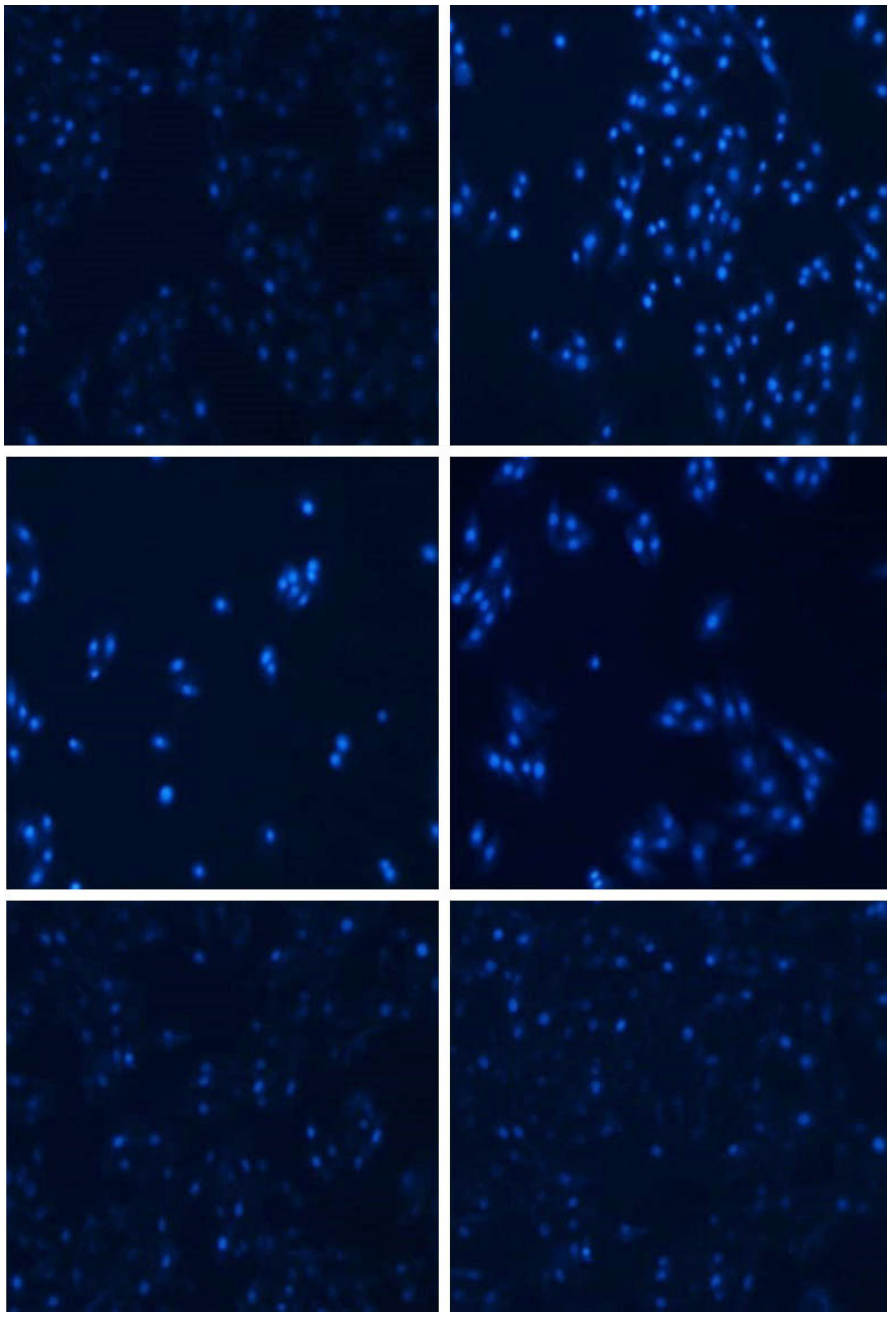

Figure 2. A-F: DAPI stained H9C2cells. Groups: 2A- Control; 2B- H_2O_2; 2C- TC (10 μg/ml) + H_2O_2; 2D- (30μg/ml) + H_2O_2; 2E- (100μg/ml) + H_2O_2 and 2F- (100μg/ml).

proportional to the concentration of ROS, it is used as an index to determine the overall oxidative stress in cells (16). In the present study, pretreatment with TC reduced the DCF fluorescence intensity in the H_2O_2-treated cells in a dose-dependent manner, when compared with the normal cells, suggesting that TC has potent antioxidant effect against H_2O_2-induced oxidative damage.

The present findings showed that TC inhibited the characteristic apoptotic bodies formation induced by H_2O_2 in H9C2 cells, which might be related to its anti-apoptotic effect. Therefore, we focused to investigate the anti-apoptotic mechanism of TC in H_2O_2-intoxicated cells. mRNA analyses demonstrated that TC pretreatment downregulated HIF-α and Bax expression with synchronized up regulation of Bcl2 expression in H9C2 cells exposed to H_2O_2. HIF-α is a transcription factor that maintains cellular homeostasis in response to hypoxia. Evidences show that HIF-1 dysregulation can also trigger apoptosis, possibly when cellular responses are inadequate to meet the energy demands under hypoxic conditions. HIF-α is critical for the

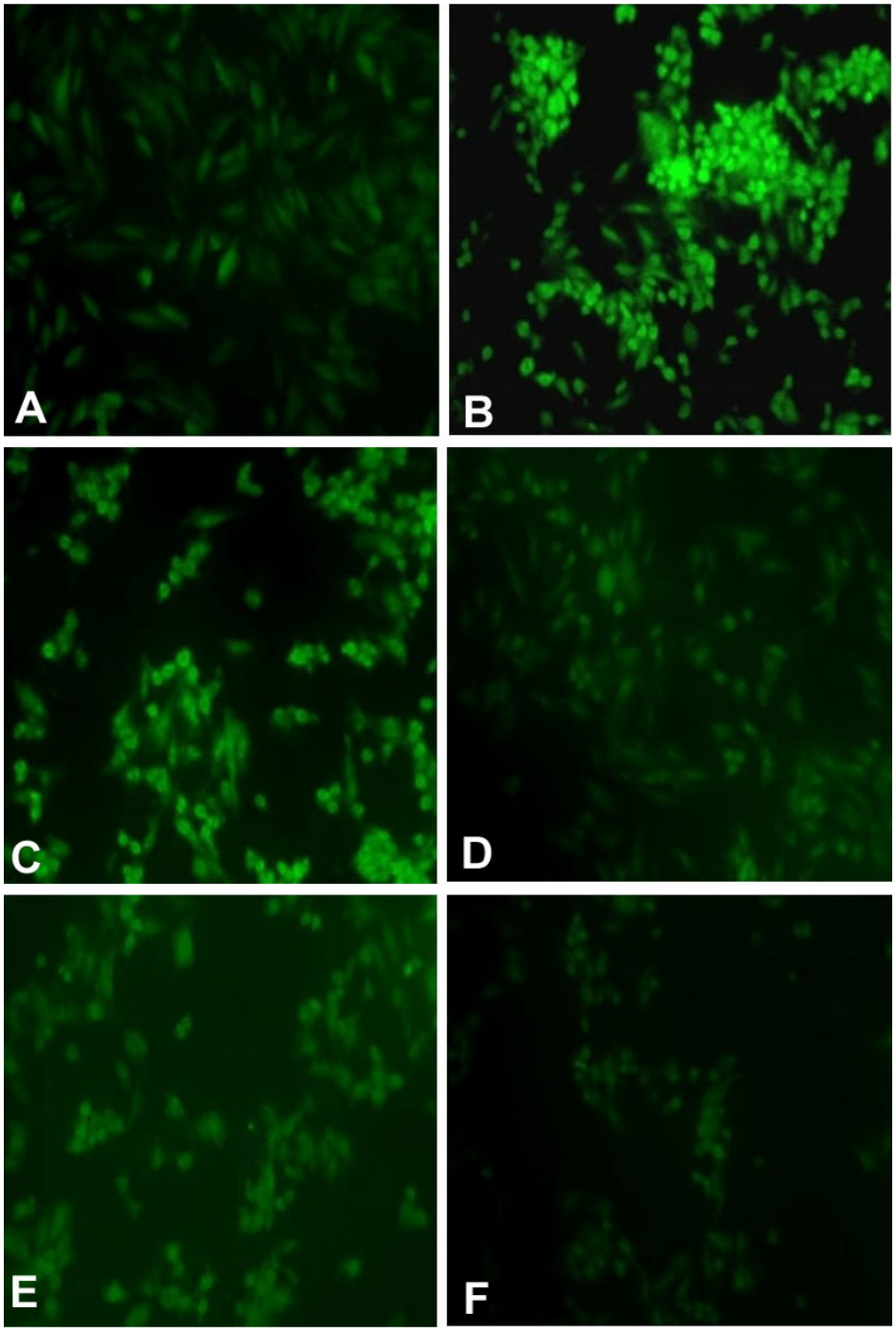

Figure 3. A-F: DCF-DA stained H9C2 cells. Groups: 3A- Control; 3B- H_2O_2; 3C- TC (10 μg/ml) + H_2O_2; 3D- (30μg/ml) + H_2O_2; 3E- (100μg/ml) + H_2O_2 and 3F- (100μg/ml).

cellular response to hypoxia because it transactivates a number of genes responsible for cellular survival (17). Conversely, HIF-α can also stimulate the mitochondrial apoptotic pathway and cell death during hypoxia (18-22). Nevertheless, the role of HIF-1 in hypoxia-induced apoptosis remains controversial. Bcl2 and Bax proteins are known to modulate the cell survival signals of various apoptotic stimuli (23). In the present study, treatment of the H9C2 cells with H_2O_2 markedly up-regulated the expression of apoptotic markers such as Bax and HIF-α and down-regulated the expression of anti-apoptotic protein, Bcl2; however, pretreatment of the cells with TC reversed these effects.

Free radicals, pro-inflammatory cytokines, nitric oxide (NO), and antioxidants play an important

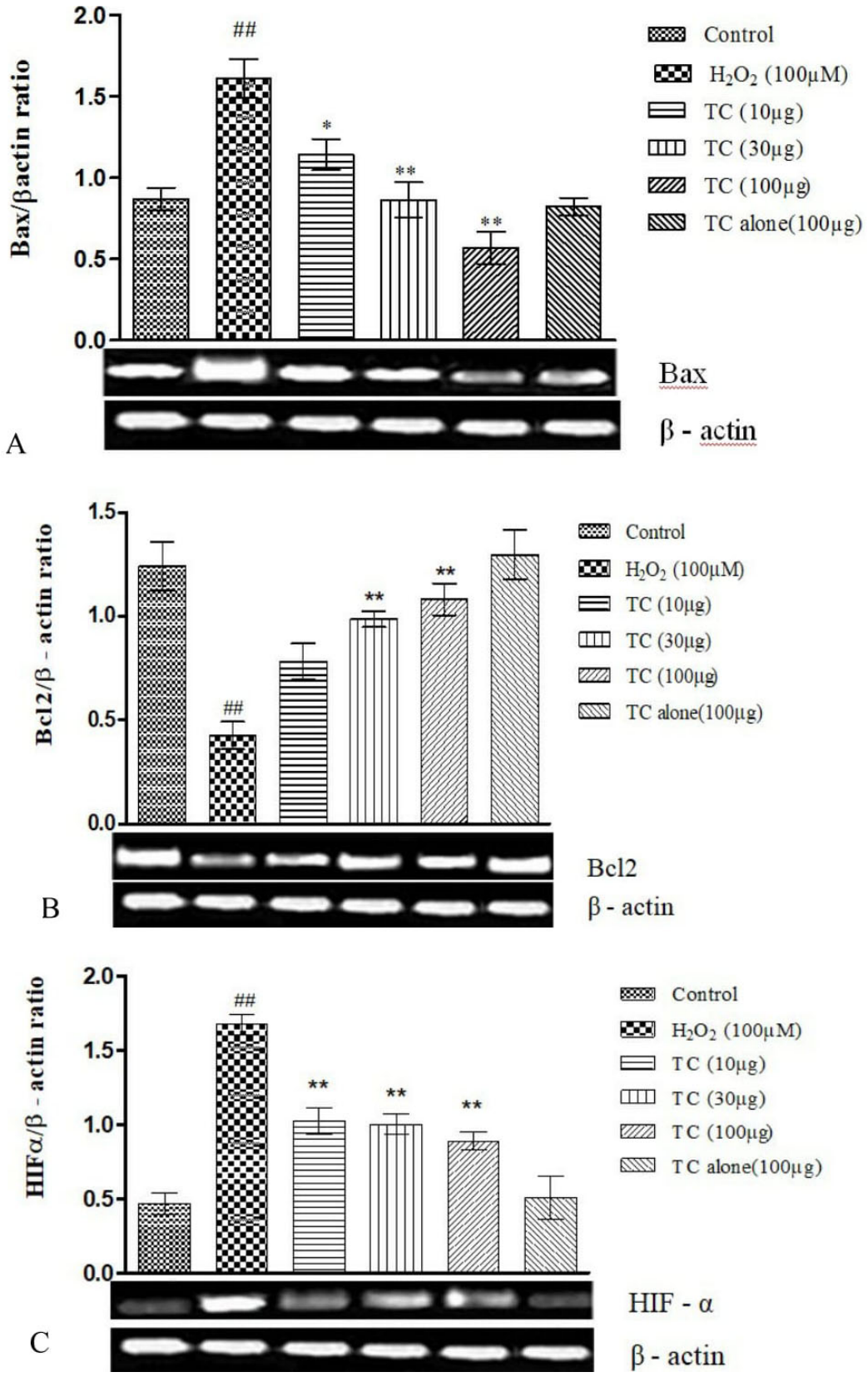

Figure 4. A-C: Effects of TC on BAX (4A), BCl2 (4B) and HIFα (4C) mRNA expression in H9C2 cells. Data represented as mean±SEM; ## indicates p<0.01 vs control group; *& ¨- indicates p<0.05 and 0.01, respectively, vs H_2O_2 group. Mean difference between the groups was analyzed using one-way ANOVA, followed by Tukey's multiple comparison as posthoc test. P value ≤ 0.05 was considered as statistically significant.

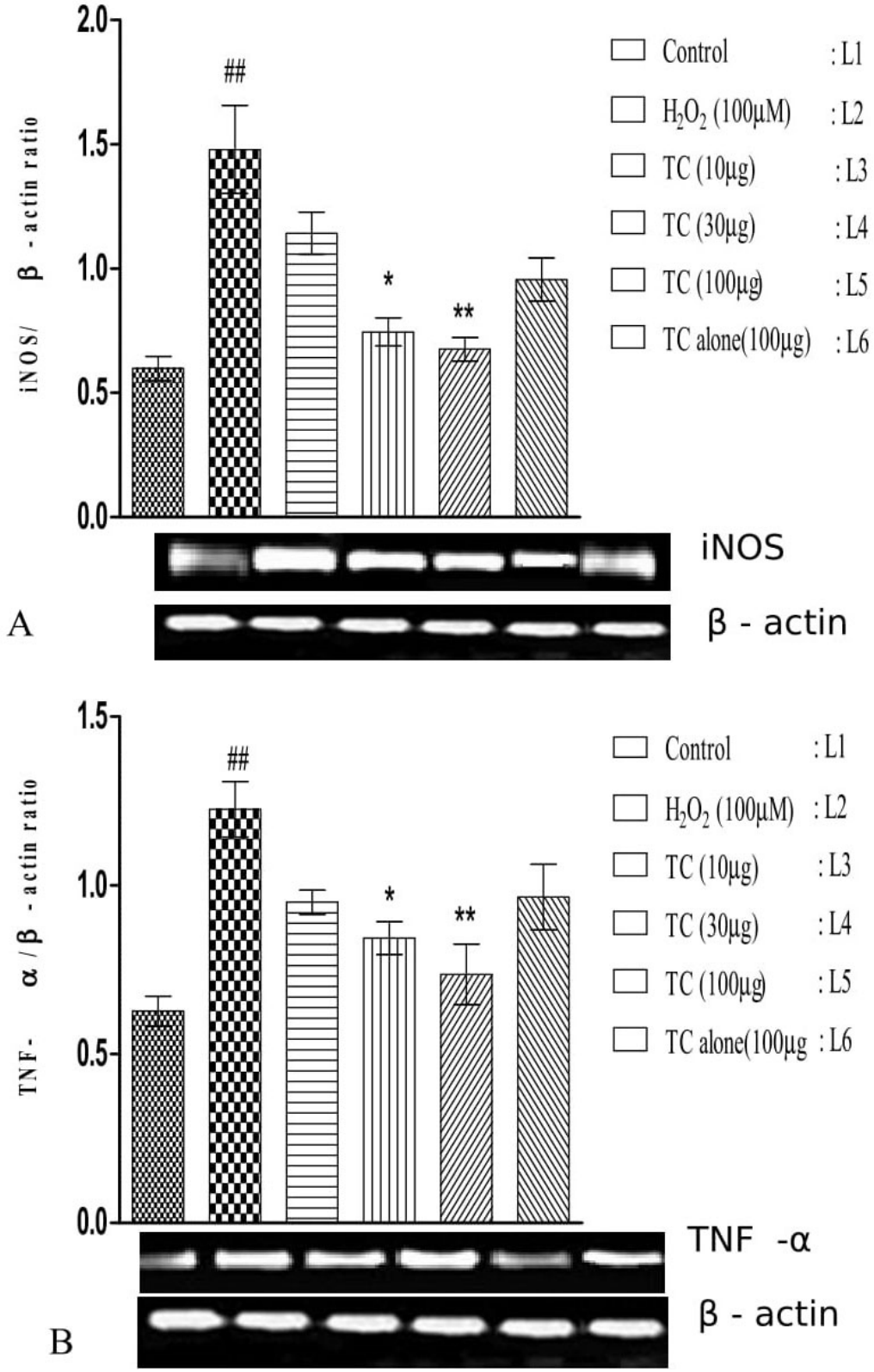

Figure 5. A-B: Effects of TC on iNOS (5A), and TNFα (5B) mRNA expression in H9C2 cells. Data represented as mean±SEM; ## indicates p<0.01 vs control group; *& ¨- indicates p<0.05 and 0.01, respectively, vs H_2O_2 group. Mean difference between the groups was analysed using one-way ANOVA, followed by Tukey's multiple comparison as posthoc test. P value ≤ 0.05 was considered as statistically significant.

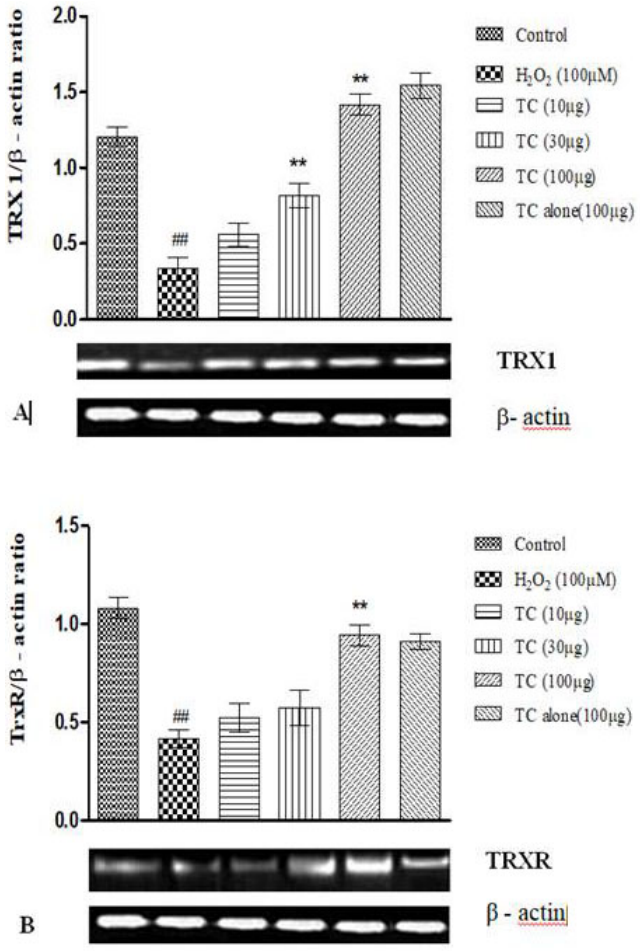

Figure 6. A-B: Effects of TC on TRX1 (6A), and TRXR (6B) mRNA expression in H9C2 cells. Data represented as mean±SEM; ## indicates p<0.01 vs control group; *& **- indicates p<0.05 and 0.01, respectively, vs H_2O_2 group. Mean difference between the groups was analysed using one-way ANOVA, followed by Tukey's multiple comparison as posthoc test. P value ≤ 0.05 was considered as statistically significant.

role in myocardial damage and preservation. It has been reported that TNF-α augments the apoptosis of cardiomyocytes in decompensated human heart despite the enhanced expression of Bcl2, a proto oncogene, which protects the cells from apoptosis (24). Excess production of NO by iNOS has been found to be involved in myocardial damage, including myocardial apoptosis in rats with myocardial infarction (25, 26). Myocardial TNF-α and NO production has been demonstrated to increase in acute myocardial infarction and heart failure (27-30). In the present study, exposure of H9C2 cells to H_2O_2 produced up-regulation of TNF-α and iNOS mRNA expression, whereas TC reversed these changes significantly. These findings suggest that TC has potential anti-inflammatory action.

Redox homeostasis is essential for normal cellular functions, and an extensive network of antioxidant defense systems has evolved to attenuate various ROS/RNS. Antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, TRX1, and TRXR, and small molecules such as vitamin E and C play a major role in maintaining the redox equilibrium (31-33). Thus, an intricate network of enzymes (and other biomolecules) is dedicated to the generation, utilization, and diminution of ROS/RNS to maintain intracellular redox, and any disturbance in the redox status produces distinctive effects (34-37). Till date more than 8000 phenolic principles have been identified from plant sources such as curcumin, resveratrol, gallic acid, naringenin, etc. and are reported to possess various pharmacological actions such as anti-inflammatory, anti-aging, anti-oxidant, cardioprotective, and anticancer activities (38). Most phenols exist as ester forms and are poorly absorbed from intestine; following polyphenols intake they are first hydrolyzed by intestinal enzymes or by colonic microflora and then are absorbed (39). These factors may be the reasons for recommending high dose of polyphenols in order to achieve an appreciable plasma concentration. Interestingly, this may be corroborated

for having such a huge number of ingredients in TC in order to deliver different polyphenols. In this line, many *in vitro* and *in vivo* experiments suggested that polyphenol dietary intake may serve as therapeutic regimen or as adjuvant, at least partly, in the prevention or treatment of various chronic diseases. The findings of the present study indicated that exposure to H_2O_2 reduced the expression of TRX1 and TRXR in the H9C2 cells, whereas pretreatment with TC reversed these changes remarkably.

6. CONCLUSION

In summary, Thraatchathi Chooranam, a Siddha traditional medicine, exerted cardioprotective effects through the regulation of apoptotic (HIF-α and Bax), anti-apoptotic (Bcl2), inflammatory (TNF-α and iNOS), and antioxidant (TRX1 and TRXR) markers. The findings of the present study suggest that TC can be considered as a potential candidate for the further investigations.

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Abbreviations: Thraatchathi Chooranam (TC), cardiovascular diseases (CVDs), reactive oxygen species (ROS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), DAPI (4',6'-diamidino-2-phenylindole), phosphate buffered saline (PBS), dichloro-dihydro-fluorescein diacetate (DCFH-DA), 2', 7'-Dichlorofluorescein (DCF)

Key Words: Thraatchathi Chooranam, Polyphenols, Cardiovascular Disease, Hydrogen peroxide, Antiapoptotic, Oxidative stress, Cytokine

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