Spirulina platensis prevents high glucose-induced oxidative stress mitochondrial damage mediated apoptosis in cardiomyoblasts

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Abstract The current study was undertaken to study the effect of *Spirulina platensis* (Spirulina) extract on enhanced oxidative stress during high glucose induced cell death in H9c2 cells. H9c2 cultured under high glucose (33 mM) conditions resulted in a noteworthy increase in oxidative stress (free radical species) accompanied by loss of mitochondrial membrane potential, release of cytochrome c, increase in caspase activity and pro-apoptotic protein (Bax). Spirulina extract (1 µg/mL), considerably inhibited increased

ROS and RNS levels, reduction in cytochrome c release, raise in mitochondrial membrane potential, decreased the over expression of proapoptotic protein Bax and suppressed the Bax/Bcl2 ratio with induced apoptosis without affecting cell viability. Overall results suggest that Spirulina extract plays preventing role against enhanced oxidative stress during high glucose induced apoptosis in cardiomyoblasts as well as related dysfunction in H9c2 cells.

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Graphical Abstract

Keywords H9c2 · High glucose · Oxidative stress · Apoptosis · *Spirulina platensis*

Cu–Zn Copper–Zinc-superoxide dismutase
SOD
L-NAME NG-nitro-L-arginine methyl ester

Abbreviations

NG	Normal glucose
HG	High glucose
SP	<i>Spirulina platensis</i>
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SO_2^-	Superoxide
H_2O_2	Hydrogen peroxide
NO	Nitric oxide
ONOO ⁻	Peroxynitrite
X + XO	Xanthine plus xanthine oxidase
SNAP	S-nitroso-n-acetylpenicillamine
SIN-1	3-morpho-linosydnonimine-N-ethylcarbamide

Introduction

Hyperglycemia or high glucose conditions leading to oxidative stress mediated cell injuries are an important cause of cardiovascular complications arising during diabetes (Brownlee 2001; Yu et al. 2008). Generation of reactive oxygen species (ROS), reactive nitrogen species (RNS) and apoptotic cell death induced by hyperglycemia, play the critical role in cardiac pathogenesis (Smogorzewski et al. 1998; Cai and Kang 2001; Tsai et al. 2012). Cardiac apoptosis induced by high glucose condition is a vital cause of cardiac remodelling due to loss of contractile units and related

complications (Cai and Kang 2001). Studies have shown that hyperglycemia, as an autonomous risk aspect, directly leads to cardiac damage and diabetic cardiomyopathy. According to established reports, hyperglycemia results in the generation of ROS and RNS, which causes oxidative myocardial injury (Smogorzewski et al. 1998; Cai and Kang 2001; Cai et al. 2002). The heart is a susceptible target because it contains low levels of free radicals scavengers (Chen et al. 1995; Desagher and Martinou 2000).

Spirulina is an edible, photosynthetic, multicellular filamentous microbe. The emergence of this natural product as therapeutics agent is a result of its nutritional composition (Khan et al. 2005; Kulshreshtha et al. 2008; Sanghvi and Lo 2010). It contains ample of nutrients that comprise of proteins, vitamins, B-complex, minerals, gamma-linolenic acid, carotene, *C-phycoerythrin* (C-PC) and other unexplored bioactive compounds (Desagher and Martinou 2000; Khan et al. 2005; Kulshreshtha et al. 2008). The aqueous extract of *Spirulina platensis* or C-PC is known to possess anti-oxidative and anti-inflammatory properties (Romay et al. 1999; Wu et al. 2016). A well known active compound of aqueous extract of Spirulina is sulfated polysaccharide and *phycobiliprotein* (Khan et al. 2005; Zheng et al. 2013). One of the important *phycobiliproteins* of *S. platensis*, *C-Phycocyanin* (C-PC) is known for antioxidant and free radicals scavenging activity (Kaul et al. 1996). Spirulina could, therefore, be a good source for prevention of high glucose-induced oxidative stress and related cardiovascular complications. Though, the mechanism of protection against apoptosis in cardiac myocytes is not well understood. High glucose (HG) generates reactive oxygen species (ROS) as a result of glucose auto-oxidation. Since glucose oxidase catalyses the oxidation of D-glucose in vitro, we exposed H9c2 cardiac myoblast cells to high glucose (33 mM) (Cai et al. 2002), and tested the hypothesis that Spirulina extract may play protective role against high-glucose-mediated cardiac cell apoptosis through its antioxidant properties.

Materials and methods

Reagents

Fetal Bovine Serum (FBS), Dulbecco's Modified Eagle Medium (DMEM), and non essential amino

acids were purchased from GIBCO, Invitrogen (Carlsbad, CA, USA). Propidium iodide (PI), hydrogen peroxide (H_2O_2), 4',6-diamidino-2-phenylindole (DAPI), S-nitroso-nacetyl penicillamine (SNAP), D-glucose, xanthine, xanthine oxidase (XO), dimethyl sulfoxide (DMSO), hydrogen peroxide (H_2O_2), catalase, 3-morpho-linosydnonimine-N-ethylcarbamide (SIN-1), ebselen and Copper–Zinc-superoxide dismutase (Cu–Zn SOD), NG-nitro-L-arginine methyl ester (L-NAME), Staurosporine (STS), 3,3'-dihexyloxacarbocyanineiodide ($DiOC_6$), dihydroethidium (DHE), diaminofluorescein 2-diacetate (DAF-2DA), dichlorofluoresceindiacetate (DCF-DA) and dihydrorhodamine-123 (DHR-123), were purchased from Sigma-Aldrich (St. Louis, MO, USA). RNAase A was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Fluorescein Isothiocyanate (FITC)-labeled Annexin V antibody, annexin binding buffer were purchased from BD Biosciences (Franklin Lakes, NJ, USA). All other chemicals used were of the highest grade available

Spirulina preparation

Axenic culture of *S. platensis* (Spirulina) was maintained in Zarrouk's culture medium as described previously (Gupta et al. 2008). Biomass was recovered by filtration, dried at 40 °C for 48 h (Silveira et al. 2007) and aqueous extract was prepared as described earlier (Hwang et al. 2013).

Cell culture

Rat cardiomyoblast H9c2 cells (ATCC CLR-1446; Rockville, MD, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS (Gibco, USA). 40–50% confluent cultures were treated with high glucose 33 mM (HG) and normal glucose 5.5 mM (NG) to serve as control (Cai et al. 2002). All experiments were performed with the cells between passages 10 and 15 and regularly new cultures were re-established from frozen stocks. In all sets of experiments cells were in native phenotypic state. Mannitol (30 mM) was used in the control cultures in order to rule out the possibility of hyperosmolarity effect.

Treatment in H9c2 cells was given as NG, HG, and HG with Spirulina extract (1 µg/mL), for 48 h.

Previous studies showed that maximum increase in oxidative stress was detected at 48 h during HG treatment (Kumar et al. 2012). HG treatment for 48 h was, therefore, given in all experiments. Spirulina extract as an antioxidant was added 2 h before treatment of high glucose. In some experiments, scavengers of ROS and RNS have also been added 2 h before treatment of high glucose and positive controls.

Intracellular ROS and RNS

Intracellular ROS generation as superoxide (SO_2^-) was detected through MitoSOXTM red (5 μM) (Kumar et al. 2012). While hydrogen peroxide (H_2O_2) was detected by using DCF-DA (10 μM) and RNS types, nitric oxide (NO) and peroxynitrite (ONOO^-) were detected through DAF-2DA (2 μM), and DHR-123 (5 μM), respectively (Kumar et al. 2012; Felix et al. 2001). In brief, H9c2 cells were seeded at the density of 1×10^5 cells per 60-mm dish and after treatment for 48 h were analyzed by FACS. Cells were trypsinized, washed and suspended in respective probes in the dark for 30 min at 37 °C. Cells were washed after incubation and suspended again in $1 \times \text{PBS}$ followed by filtration through nylon mesh and acquisition through FACS Calibur (Beckton Dickinson). CELL QuestTM software was used for data analysis. In each sample, ten thousand cells were examined.

For confocal microscopy, in brief, H9c2 cells were seeded onto glass coverslips in 6-well plates and after treatment for 48 h, cells were incubated with appropriate probes in the dark for 30 min with Mitosox, DCF-DA, DAF 2-DA and DHR123, as described above. Subsequently, after washing with PBS, cells were fixed with 3.7% paraformaldehyde in PBS and examined by the confocal laser-scanning microscope (Zeiss CLSM 510, Oberkochen, Germany).

ROS generators, 0.1 mM X + 0.01 U XO (O_2^- generator) for 6 h, 15 μM H_2O_2 (H_2O_2 generator) for 6 h, 10 μM SNAP (NO generator) for 6 h, and 50 μM SIN-1 (ONOO^- generator) for 6 h, respectively, were used as positive control. 100 U Cu–Zn SOD, 250 U catalase and 100 μM ebselen (scavenger of peroxynitrite) and L-NAME (100 μM) served as scavenger/inhibitor of ROS and RNS in the experiment.

Mitochondrial membrane potential ($\Delta\Psi\text{m}$)

DiOC6, the carbocyanine dye was used for measuring mitochondrial membrane potential and acquisition of images at λ_{ex} 488 nm and λ_{em} 525 nm by Zeiss 510 confocal laser scanning microscope (Kumar et al. 2012). Cells incubated with xanthine in combination with xanthine oxidase treatment (0.1 mM X + .01UXO) for 6 h served as experimental positive control (Kumar et al. 2012).

Cytochrome c release

Confocal microscopy was used to assess the subcellular localization of cytochrome c with double-labeled molecular probe mitotracker red (Molecular Probes, Eugen, OR, USA) and a cytochrome c antibody (Cell Signaling Technology, Danvers, MA, USA) (Kumar et al. 2012). Cells treated with staurosporine (100 nm) treatment for 12 h were taken as positive control for the experiment.

Assessment of Caspases activation

Cytochrome c leakage is followed by the dissipation of Ψm resulting in the activation of caspases cascade leading to the formation of the apoptosome. Caspase activity was determined by flow cytometry by employing Calbiochem caspase detection kit based on manufacturer's protocol (Kumar et al. 2012). The assay utilizes a caspase inhibitor (VAD-FMK) conjugated to FITC as the fluorescent in situ marker. This probe binds covalently and irreversibly to the active site of the active caspase heterodimer emitting green fluorescent signal detected by the FL-1 which is a direct detection of activated caspases in an apoptotic cell by flow cytometry (Kumar et al. 2012). The expression of Bax and Bcl-2 was also analyzed, cells were probed for the protein levels of Bax, Bcl-2 (Santa Cruz Biotechnology, USA) using specific primary antibodies followed by an HRP-conjugated appropriate secondary antibody (BioRad, Hercules, CA, USA). Flow cytometry data were evaluated as relative mean fluorescence intensities (MFIs), calculated as the ratio from the mean value of each treatment as fold change compared to the normal glucose control. The results

were expressed as an index (bax/bcl-2), obtained by dividing MFI bax and MFI bcl-2.

Apoptosis analysis

Apoptosis analysis was carried out by AnnexinV-FITC/PI staining by flow cytometry (Kumar et al. 2012). Annexin V-FITC binding analysis was performed by FITC signal detector (FL-1) and PI staining analysis by using the phycoerythrin emission signal detector (FL-2) by BD FACS Vantage (Becton–Dickinson, San Jose, CA). Total 10,000 events were acquired for determination of fluorescence intensity and CellQuestTM software was used for data analysis.

Analysis of cell cycle

Flow cytometry is employed for cell cycle analysis. In brief, after treatment for 48 h, cells were harvested, fixed and permeabilization with 70% ethanol, followed by washing with PBS. Subsequently, cells were incubated with 0.5 mL of 50 µg/mL PI solution containing 20 U/mL RNase A (Kumar et al. 2012). Through FL-2A channel, the sub-G0/G1 hypodiploid apoptotic population was determined. CellQuest ProTM software was used for cell cycle analysis.

Statistical analysis

All experiments were performed at least three times for each determination. Data were expressed as mean $\pm$ standard error (SE) and were analyzed using one-way analysis of variance (ANOVA) and secondary analysis for significance with the Turkey–Kramer post hoc test. A *P* value <0.05 was considered statistically significant.

Results

Experiments were performed to look for the protective effect of Spirulina extract in HG-induced cell death. The experimental condition was established from an initial study examining the effect of Spirulina extract on cell viability. H9c2 cells were treated with various concentrations of Spirulina extract (0–20 µg/mL) for

48 h and cell viability was determined by MTT assay. Spirulina extract showed no cytotoxicity from 0 to 8 µg/mL with more than >98% cell viability. However, cell viability was decreased significantly (87%) in HG challenge cells compared with the corresponding control (100%). Treatment with Spirulina extract significantly attenuated HG reduced viability by 99.8% (*P* < 0.05) compared to HG-treated cells. As Spirulina extract provided the maximum protective effect in H9c2 at 1 µg/mL in high glucose-induced cell death (Fig. 1a, b), therefore, was selected for rest of the study.

ROS generation was markedly increased in HG-treated H9c2 cells detected by the appropriate probes. HG treated H9c2 cells for 48 h resulted from the increase in Mitosox (159.5%) and DCF fluorescence (134.9%) as a contrast with corresponding control NG value (100%) (Fig. 2a, b). However, pretreatment of HG treated H9c2 cells with Spirulina extract resulted in a noteworthy decrease in intracellular ROS, to 77.48 and 98.4%, respectively. Similarly, there was a significant enhancement in H9c2 cells treated with HG for 48 h in DAF-2DA (123.2%) and DHR 123 (122.4%) fluorescence as a contrast to control NG cells value (100%). Spirulina extract pre-treatment reduced the value by 96 and 100.3%, respectively (Fig. 2c, d). Further, these results were complemented with confocal microscopic images where the generation of O₂^{•−}, H₂O₂, NO and ONOO[−] were increased in HG-treated cells, and Spirulina extract pre-treatment was able to abrogate this fluorescence (Fig. 2e) against appropriate positive controls. These findings reveal that Spirulina extract suppressed intracellular ROS and reactive RNS by attenuating free radical generation in HG-treated H9C2 cells by playing a crucial role against cardiac cell damage. Oxidative stress leads to loss of mitochondrial membrane potential and release of cytochrome c. Our confocal microscopy observation deduced that HG-treated cells exhibited a reduction in mean fluorescence intensities of Dioc6, upon treatment, indicating loss of Ψ m. Pretreatment of Spirulina extract restored similar profile as that of NG control cells in HG-treated cells (Fig. 3a).

HG-induced cytochrome c release was ascertained from mitochondria by measuring cytochrome c immunoreactivity in the cell. A diffused appearance throughout the cytoplasm and a decrease in mitochondria associated cytochrome c, indicated that there was a noteworthy release of cytochrome c from

mitochondria to the cytoplasm. Cytochrome *c* immunoreactivity was co-localized in untreated NG control cells, with Mitotraker red fluorescence, indicating that the cytochrome *c* was confined to the mitochondria (Fig. 3b). Cells pre-treated with Spirulina extract did not exhibit any such cytochrome *c* release pattern.

The effect of Spirulina extract on Bcl-2 proteins expression and caspase activation was further examined and results obtained suggested that there was a trend of reduced Bcl-2 protein expressions under the HG condition, while, expressions of Bax protein significantly increased at 48 h treatment. Cells treated with Spirulina extract showed a significant increase in the expression of Bcl-2, along with a reduced expression of Bax, compared to HG and consequently, a reduced Bax/Bcl-2 ratio (Fig. 4a–c). The relative Bax/Bcl-2 ratio in H9c2 treated HG was increased to 4.76 compared to the NG control cells (1.0). The relative Bax/Bcl-2 ratio in Spirulina extract pre-treated H9c2 cells in high glucose condition were reduced to less than 1, respectively, indicating Spirulina extract significantly altered relative Bax/Bcl-2 ratio, in HG-treated cells.

Fig. 1 Spirulina extract and cell viability. **a** Effect of Spirulina extract (0, 1, 2, 4, 8 and 20 $\mu\text{g/mL}$) on cell viability was determined by MTT assay. **b** Effect of Spirulina extract (1 $\mu\text{g/mL}$) on HG (33 mM) induced cell death in H9c2 cells. Mannitol

Fig. 2 Effect of Spirulina extract on ROS and RNS generation: **a** Bar graphs depict flow cytometry results showing percentage of fluorescence positive cells: **a** MITOSOX Red (Mitochondrial superoxide detection), **b** DCF-DA (H_2O_2 detection), **c** DAF-2DA (nitric oxide detection) and **d** DHR 123 (peroxide detection). Appropriate positive controls and scavengers were used in the experiment as discussed in materials and methods. Data represent the mean $\pm$ SE of at least three separate experiments. * $P < 0.05$ versus Normal Glucose (NG), # $P < 0.05$ versus High Glucose (HG), ## $P < 0.05$ versus positive control. **e** Images of cells fluorescently marked with Mitosox (red signal), DCF-DA (green signal), DAF-2DA (green signal) and DHR 123 (red signal)—as described in materials and methods. The image group is representative of the three best independent experiments. Confocal imaging was done with standard magnification 63×1.4 NA Oil objective. Scale bar of the figure indicates a size of 50 μm . (Color figure online)

The involvement of caspase-dependent pathway in HG-induced cell apoptosis was examined by measuring caspase activities by flow cytometry analysis. Results obtained led us to speculate that HG treatment clearly resulted in activation of caspase, a contrast to the NG control, demonstrating the HG-treated H9c2 cells instigated to apoptosis. However, pre-treatment with Spirulina extract altered the caspase activity in

(30 mM) was taken as osmotic control. Values are mean $\pm$ SE of three independent experiments. * $P < 0.05$ versus NG, # $P < 0.05$ versus HG. ns not significant versus NG

Fig. 2 continued

HG-treated cells that indicate exposure of cell to the extract was able to inhibit this activation (Fig. 4d).

The effect of Spirulina extract during HG-induced apoptosis was investigated by annexinV/PI staining. Results showed that cells were distributed in the different phases as early apoptosis 3.40%, late apoptosis 13.93% and the necrosis 3.14% when they were exposed to HG for 48 h. However, pretreatment of HG-treated cells with Spirulina extract resulted in a different phase distribution with 2.6% as early apoptotic cells and 6.61% late apoptosis cells, though the change was not significant in early apoptosis and necrosis stage (Fig. 5a). Further, cell cycle analysis revealed that an increased accumulation of the apoptotic population in the sub-G0 phase (as M1 marker) notably enhanced from 2.13% in NG control to 8.13% ($P < 0.05$) in HG-treated cells for 48 h, respectively (Fig. 5b). Pretreatment of Spirulina extract in HG-treated cells resulted in a reduction in sub-G0 phase significantly to 3.71%.

Discussion

Oxidative stress and apoptosis of cardiac cells are early measures in diabetic cardiomyopathy.

Hyperglycemia, as an autonomous factor, directly lead to cardiac injury and diabetic cardiomyopathy. The current consensus is that hyperglycemia results in the production of ROS and RNS species, which leads to oxidative myocardial damage (Cai et al. 2002; Hwang et al. 2013). Spirulina has a unique blend of nutrients that no single source can exhibit on the regulation of lipid and carbohydrate metabolism in diabetes patients by protection of low-density lipoprotein, protein and lipid oxidation (Khan et al. 2005; Kulshreshtha et al. 2008; Felix et al. 2001; Samuels et al. 2002). Phycocyanin and β -carotene are important molecules in Spirulina playing a key role in protecting cells against oxidative stress (Khan et al. 2005; Kulshreshtha et al. 2008). Thus, the antioxidant, immunomodulatory, and anti-inflammatory activities of Spirulina may play an important role in human health (Wu et al. 2016). The present investigation was designed to assess the effect of Spirulina extract directly against enhanced ROS-RNS species and mitochondria dependent key components of apoptosis pathway during high glucose condition in H9c2 cardiomyoblasts. The embryonic rat heart-derived H9c2 cell line resembles some characteristics of cardiac myocytes (Cai et al. 2002). It was,

Fig. 3 **a** Effects of Spirulina extract on mitochondrial membrane potential and Cytochrome c release during HG treatment: **a** Representative images of cells of confocal microscopy stained with DiOC6. Xanthine (X) + xanthine oxidase (XO) (O_2^- , generator) treated cells for 6 h was used as positive control in the given experiment. **b** Images showing mitotracker red

fluorescence (red), cytochrome c immunoreactivity (green), DAPI staining (blue) and merged images (yellow indicates sites of co-localization) in H9c2 cells. Staurosporine (100 nM) treatment for 12 h was used as positive control for the experiment. The *image* group is representative of the three best independent experiments. (Color figure online)

therefore, used to study the consequences of high glucose condition in the current investigations as an *in vitro* model. One of the early outcomes of high glucose treatment is the enhanced generation of ROS and RNS causative to the development of diabetic complications (Brownlee 2001; Yu et al. 2008; Smogorzewski et al. 1998; Hand and Menze 2008; Jin et al. 2013; Kumar and Sitasawad 2009). High blood glucose may auto-oxidize, generate free radicals and induce apoptosis in cardiac cells. Free radicals and reactive oxygen species (ROS) such as hydroxyl radical ($HO^\bullet$), superoxide radical ($O_2^{\bullet-}$), peroxy radical ($ROO^\bullet$), nitric oxide radical ($NO^\bullet$) and hydrogen peroxide (H_2O_2) are highly reactive molecules produced from aerobic metabolism. Such oxidants can damage the cellular membrane or intracellular molecules (especially DNA) if not efficiently removed by the antioxidant defense mechanisms of the cell (Cooke et al. 2003; Chu et al. 2010). The cells during high glucose incubation exhibit enhanced ROS and RNS levels which are an imperative constituent of cell signaling pathways (Yu et al. 2008; Jin et al. 2013; Kumar

and Sitasawad 2009). Spirulina has a protective effect against apoptotic cell death due to free radicals. The oxidative stress develops and induces apoptosis when intracellular antioxidants are unable to neutralize pro-oxidants such as ROS (Chu et al. 2010; Mates 2000). Spirulina could reduce significantly apoptotic cell death induced by the free radicals. The covalently-linked tetrapyrrole chromophore phycocyanobilin is suggested to be involved in the scavenging activity of phycocyanin (Zhou et al. 2005). Phycocyanin has been shown to protect normal human erythrocytes and plasma against oxidative damage in *in vitro* studies (Benedetti et al. 2004). In addition, phycocyanin protects pancreatic beta cells against apoptotic cell death by attenuating oxidative stress (Li et al. 2009). The synergistic action of a wide spectrum of antioxidants present in Spirulina beside phycocyanine and phycoerythrin may be more effective than the activity of a single antioxidant.

Superoxide ($O_2^{\bullet-}$) is the product of a one-electron reduction of oxygen; the precursor of most ROS and a mediator in oxidative chain reactions. $O_2^{\bullet-}$ may react

Fig. 4 Spirulina altered Bax and Bcl-2 protein expression in HG treated cells. **a** Bar chart represents effect of pre-treatment Spirulina extract on down regulation of Bax protein expression. **b** Bar graph represents effect of pre-treatment with Spirulina extract on rise of Bcl-2 expression after treatment with Spirulina. **c** Graph shows Bax/Bcl-2 protein ratio. Values are mean $\pm$ SE from at least three independent experiments.

* $P < 0.05$ versus Normal Glucose, # $P < 0.05$ versus High Glucose (HG). **d** Spirulina extract prevented high glucose-induced activation of caspases cascade in HG-treated H9c2 cells. **i** Representative overlay showing caspase associated immunofluorescence. **ii** Bar graph represents reduction in caspase activity

with NO and generate a very powerful and toxic oxidant, peroxynitrite (ONOO^-), if not scavenged properly. We, therefore, determined the intracellular levels of superoxide using MitosoxTM red (molecular probe). H_2O_2 production was monitored by using probes Carboxy-DCF-DA while DHR 123 and DAF-2DA probes were used to distinguish intracellular RNS, NO, and ONOO^- radical generation. Spirulina extract was able to reduce the generation of ROS and RNS competently ascertaining the role of Spirulina extract as an antioxidant. It is known that the antioxidants delay or prevent the oxidation of cellular oxidizable substrates (Chu et al. 2010; Budihardjo et al. 1999). Various apoptotic signals are integrated and transduced by mitochondria leading to membrane permeabilization (Desagher and Martinou 2000). The disruption of mitochondrial transmembrane potential

is the key event in the apoptosis process. The rate limiting manifestation of mitochondrial cell death and release of apoptogenic protein caused by the opening of the permeability transition pore through the collapse of $\Delta\Psi_m$ leading to increase of the release of cytochrome c from the damaged mitochondria are the key events in the apoptosis process (Mayer and Oberbauer 2003; Li et al. 1997; Chen-Levy and Cleary 1990; Oltvai et al. 1993; Yin et al. 1995). In this study, pretreatment with Spirulina extract reduced the levels of mitochondrial $\text{O}_2^{\cdot-}$, H_2O_2 , NO, and NOO^- . Furthermore, restored Ψ_m and inhibited cytochrome c release suggest that Spirulina extract worked through its antioxidant potential. These results are very alike to the other reports where Spirulina pre-treatment provided near to complete protection in terms of serum and tissue biochemical changes and oxidative stress

Fig. 5 Effect of Spirulina extract on Caspase activity and apoptosis: **a** The dot plots illustrate annexin-FITC acquired on FL1-H (X axis) versus PI staining acquired on FL2-H (Y axis) by flow cytometry. Representative best of three acquisitions

(b) Cell cycle analysis on FL-2A channel was used to determine the sub-G0/G1 hypodiploid apoptotic population by flow cytometry. Representative best of three acquisitions

(Wu et al. 2016; Zheng et al. 2013; Chu et al. 2010). The effect of Spirulina extract on apoptosis varies with the type of cells used for the test (Yang et al. 2003; Chen et al. 2009).

The Bcl-2 family proteins have been localized to the nuclear membrane, mitochondrial membrane and endoplasmic reticulum and their interaction with cytochrome c initiates the mitochondrial pathway (Kinoshita et al. 1995; Susin et al. 1996, 1999; Nishikawa et al. 2000; Mates 2000; Desagher and Martinou 2000; Carmody and Cotter 2001; Chandra et al. 2003; Shih et al. 2004; Allen et al. 2003; Verzola et al. 2004). Therefore, Bcl-2 family proteins and

cytochrome c are measured that symbolize a critical checkpoint within apoptotic pathways (Budihardjo et al. 1999; Allen et al. 2003; Gustafsson and Gottlieb 2007). The intracellular elevated Bax/Bcl-2 ratio might add to decrease in mitochondrial membrane potential and consequential release of cytochrome c from mitochondria to cytoplasm matrix as cytochrome c binds to Apaf-1 and procaspase-9 cleave resulting activate caspase (Susin et al. 1996, 1999). Bax protein appears to be a determinant of whether Bcl-2 inhibits apoptosis. The expression of both proteins determines whether the cell will undergo apoptosis (Oltvai et al. 1993). When Bcl2 is over expressed, it forms

homodimers inhibiting apoptosis and excess expression of Bax protein makes the cell susceptible to apoptosis (Yin et al. 1995). Factors modulating the expression of intracellular Bcl-2 or the Bax protein change the ratio of the two proteins leading to inhibition or promotion of apoptosis (Kinoshita et al. 1995; Susin et al. 1996). Pretreatment of Spirulina extract reduced Bax expression and enhanced Bcl-2 expression.

We further examined caspases activation through flow cytometry which demonstrated that there was an increase in the activated caspase during HG treatment in H9c2 cells for 48 h and treatment with Spirulina extract was able to decrease it, suggesting that Spirulina extract exerted protection through modulation in mitochondrial dysfunction. Several studies have established the fact that oxidative stress induces apoptosis (Susin et al. 1996, 1999; Carmody and Cotter 2001; Chandra et al. 2003). Apoptosis is programmed cell death accompanied by a number of sequential events; mitochondria liberate apoptosis inducing factors which trigger DNA fragmentation in nuclei (Susin et al. 1999; Chandra et al. 2003). Our results revealed that HG treated H9c2 cells showed a noteworthy enhancement in Annexin V/PI positive cells, a sign of apoptotic cell death with an accumulation of cells in sub-G0 phase (Abdel-Daim et al. 2013). The results further showed that the extract has a protective effect against cell death due to apoptosis. The extract might exert its effect by scavenging the free radicals leading to reduction of activation of the apoptotic pathway. In a study it has been shown that phycocyanin from Spirulina reduces apoptotic cell death of pancreatic beta cells by preventing the overproduction of reactive oxygen species and enhancing the activities of enzymes like superoxide dismutase and glutathione peroxidase (Li et al. 2009). While in different studies it has been demonstrated that antioxidant administration reduces functional or morphological injury to diabetic hearts through ROS mediated inhibition of apoptosis against high glucose condition in H9c2 cells (Cai et al. 2002; Kumar and Sitasawad 2009; Kaul et al. 1996; Rosen et al. 1995). Thus, in accordance with the above studies, our results also suggest that Spirulina extract containing bioactive components with antioxidant potential exert a protective effect on high glucose induced apoptosis in cardiac cells via inhibition of reactive oxygen species.

Conclusion

Spirulina is used as a nutraceutical food supplement, although its other potential health benefits have attracted much attention. *Spirulina platensis* is the most common and extensively studied cyanobacterial species in the field of medicine and by the food industry as a functional food. Spirulina extract considerably enhanced viability and decreased apoptosis in HG treated cells. These outcomes provide a probable mechanism of action of Spirulina extract to inhibit oxidative stress during high glucose induced cell death as shown in graphical abstract. Spirulina extract therefore has immense potential to prevent diabetic cardiac injury and related complications. This is the first study where we provide evidence on the role of Spirulina extract during high glucose induced apoptosis in H9c2 cells. However, further in vivo verification is necessary to prove their biological effect.

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Compliance with ethical standards

Conflict of interest We declare that there is no conflict of interest.

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