Sardine oil loaded vanillic acid grafted chitosan microparticles, a new functional food ingredient: attenuates myocardial oxidative stress and apoptosis in cardiomyoblast cell lines (H9c2)

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Abstract Fish oil has been widely recognized as an excellent dietary source of polyunsaturated n-3 fatty acids such as EPA and DHA. However, it can undergo oxidation easily resulting in the formation of toxic off flavor compounds such as hydroperoxides. These compounds adversely affect the nutritional quality and may induce several stress reactions in body. To solve this problem, a new antioxidant bio-material, vanillic acid-grafted chitosan (Va-g-Ch), was synthesized and used as a wall material for microencapsulation of fish oil. The sardine oil loaded Va-g-Ch microparticles could be a potential functional food ingredient considering the numerous health benefits of fish oil, chitosan, and vanillic acid. The current study aimed to investigate the possible protective effect of sardine oil-loaded Va-g-Ch microparticles against doxorubicin-induced cardiotoxicity and the underlying mechanisms. In vitro cytotoxicity evaluation was conducted using H9c2 cardiomyocytes. MTT assay revealed that effective cytoprotective effect was induced by a sample concentration of 12.5 µg/mL. Results of apoptosis by double fluorescent staining with acridine orange/ethidium bromide and caspase-3 evaluation by ELISA substantiated the above findings. Further, flow cytometric determination of membrane potential, relative expression of NF-κB by PCR, and ROS determination using DCFH-DA also confirmed the protective effect

of encapsulated sardine oil against doxorubicin-induced cardiotoxicity. NF-κB expression was down-regulated nearly by 50% on cells treated with encapsulated sardine oil. Altogether, the results revealed that sardine oil-loaded Va-g-Ch microparticles demonstrated potential cell protection against doxorubicin-induced oxidative stress

Keywords Sardine oil · Cardiotoxicity · Doxorubicin · Microencapsulated fish oil · ROS scavenging · NF-κB

Introduction

Occurrence of cardiovascular diseases (CVDs) is ever increasing and is considered as one of the deadliest diseases in world, with 17.3 million deaths per year (Heron et al. 2009). Numerous epidemiological studies have showed the relationship between CVDs and the consumption of fish oil, emphasizing reductions in the risk of many CVDs (Lavie et al. 2009). Fish oil is associated with a lower incidence of CVDs mainly because of high content of polyunsaturated fatty acids, i.e., eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Taking into consideration of the health benefits of fish oil, many health organizations have recommended the consumption of fish oil either directly or in the form of fortified food. The American Heart Association (AHA) recommends consumption of fish oil at 1 g per day, which may lower the CVD mortality rate by 29% (Covington 2004; Shukla et al. 2010).

Recognizing the potential, many food science laboratories and food processors have come forward with novel functional food products, fortified with PUFA-rich oils. Microencapsulation has been considered as a promising technique for such fortification of highly unsaturated marine fish oils in food products, as it imparts

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controlled release behavior and protects against oxidation during processing, handling, and storage (Ghorbanzade et al. 2017; Cáceres et al. 2008; Hermida et al. 2015). Chitosan and its derivatives have often been used for microencapsulation of various functional food ingredients including oils. Particularly, synthesis of antioxidant-chitosan conjugates by grafting of antioxidant molecules onto chitosan has received much attention. Recently, application of gallic acid-grafted chitosan in delivery of bioactive components has been reported (Hu et al. 2015, 2016). Our previous publication also reported application of ferulic acid-grafted chitosan for microencapsulation and controlled release of thiamine and pyridoxine (Chatterjee et al. 2016).

Researchers have opined that the phenolic acid-grafted chitosan derivatives could potentially be used as new food additives or even as functional foods (Xie et al. 2014). It has been reported that phenolic acid-grafted chitosan derivatives demonstrate various bio-activities, such as antioxidant, antimicrobial, anti-diabetic, etc. (Chatterjee et al. 2015; Liu et al. 2013; Lee et al. 2014). Hence, application of phenolic acid-grafted chitosan derivatives for microencapsulation and delivery of bioactive lipids may result in development of new food additives or novel functional foods.

H9c2 cell line is widely regarded as the best in vitro model to study cardioprotection activity due to its high resemblance to primary cardiomyocytes (Pelloux et al. 2006). Doxorubicin (DOX) is one of the anthracycline compounds with potent anticancer activity; however, long-term use of this compound can cause severe cardiac dysfunction leading to irreversible congestive heart failure (Zhang et al. 2009). DOX-induced cardiotoxicity will increase cardiac oxidative stress and lipid peroxidation, along with decreased levels of antioxidants. In addition, it downregulates the genes responsible for contractile proteins, and p53 mediated apoptosis (Chatterjee et al. 2009). The possible protective effect of encapsulated fish oil with various biopolymers against DOX-induced cardiotoxicity and the underlying mechanisms are not known (Covington 2004; McLennan et al. 2007; Lavie et al. 2009). So, the present study attempts to evaluate the cardioprotective potential of sardine oil-loaded vanillic acid grafted chitosan (Va-g-Ch), as well as elucidate the underlying mechanisms of cardioprotection.

Materials and methods

H9c2 (cardiomyoblast cell line) was procured from National Centre for Cell Sciences (NCCS), Pune, India. Doxorubicin, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), 2,5 dichlorofluorescein diacetate (DCF-DA), vanillic acid, and Tween 20 were bought from Sigma Aldrich (St Louis, MO, USA). Dulbecco's modified Eagles medium (DMEM), penicillin–streptomycin, and fetal bovine serum (FBS) were purchased from Gibco (Gibco, Grand Island,

NY, USA). High purity RNA isolation kit was from Invitrogen (Carlsbad CA). RT-PCR kit was bought from Thermo Scientific, USA. Chitosan from shrimp shells (MW = 100 kDa, 88% degree of deacetylation) was prepared in the pilot plant facility of Central Institute of Fisheries Technology, Cochin, Kerala, India. Acetic acid, hydrogen peroxide (H_2O_2), and ascorbic acid were obtained from Merck Millipore (Germany). All the ingredients used for the preparation of microcapsules were of food grade.

Synthesis and characterization of Va-g-Ch

Va-g-Ch was synthesized, as previously described in our publication (Chatterjee et al. 2015). Briefly, 10 g of chitosan was dissolved in 2 L of 2% acetic acid solution (v/v) in a 5-L three necked round bottom flask. Twenty milliliters of 1 M H_2O_2 containing 1.08 g of ascorbic acid was added dropwise to the chitosan solution, followed by addition of 10 g vanillic acid dissolved in 100 mL ethanol. The reaction was maintained under nitrogen environment for 24 h at 25 °C with constant stirring. The reaction mixture was dialyzed against distilled water for 72 h to remove unreacted phenolic acids. The reaction mixture and free vanillic acid were developed in silica-coated TLC plates, to confirm complete removal of free vanillic acid. Finally, the dialysate was freeze dried to yield Va-g-Ch derivative in solid form. Structural characterization was carried out using various spectroscopic techniques.

Microencapsulation of sardine oil with Va-g-Ch

Va-g-Ch (0.8% wt/v) was dissolved in acetate buffer solution (2 mM sodium acetate and 98 mM acetic acid in water, pH 3.0) with overnight stirring on a magnetic stirrer. Sardine oil in water emulsion was prepared by blending sardine oil (30 wt% of total solid), Tween 20 (0.25% wt/v), and the Va-g-Ch solution using a high speed blender (Bio-Gen PRO-250 High speed homogenizer, Scientific Inc., USA) at 15,000 rpm for 30 min. The optimized emulsion was spray-dried using a pilot-plant spray dryer (S M Scientech, Kolkata) to yield sardine oil-loaded microparticles (SO-M). The inlet and outlet temperatures of the spray dryer were 140 and 77 °C, respectively. The SO-M obtained was immediately transferred into a cold glass jar. The spray-dried powder was then used for further studies.

Determination of in vitro cardioprotective effect by MTT assay

H9c2 cell culture and seeding in 96-well plate

The cell lines (H9c2) were cultured in DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate, penicillin (100 U/mL), streptomycin (100 µg/mL), and amphotericin B

(2.5 µg/mL). Cultured cell lines were kept at 37 °C in a humidified 5% CO₂ incubator (NBS Eppendorf, Germany). Confluent monolayer cells were then trypsinized and suspended in 10% growth medium, and the cells were seeded with a seeding density of 5000 cells/well in a 96-well tissue culture plate and incubated at 37 °C in a humidified 5% CO₂ incubator. Sample stock was prepared by dissolving 1 mg of encapsulated fish oil powder in 1 mL of DMEM.

Cardio protective activity of SO-M

Doxorubicin was used to induce toxicity as per methods described by Xiao et al. 2012. After 24 h of cell seeding, the growth medium was removed; doxorubicin was added at a final concentration of 20 mM to induce toxicity and incubated for an hour. After doxorubicin treatment, freshly prepared SO-M in 5% DMEM was added at different concentrations (25, 12.5, 6.25, and 3.1 µg in 100 µL of 5% DMEM) and incubated at 37 °C in a humidified 5% CO₂ incubator. Experiment was conducted in triplicate for each concentration. After 24 h, the entire plate was observed in an inverted phase contrast microscope (Olympus CKX41 with Optika Pro5 CCD camera), and microscopic observation was recorded as images. Changes in the morphology of the cells, such as shrinking of cells, formation of cytoplasmic vacuoles, and rounding of cells, were considered as indicators of cell cytotoxicity.

Cytotoxicity assay by MTT method

The sample content in the wells was kept for 24-h incubation period. After incubation period, the samples were removed, and 30 µL of reconstituted MTT (15 mg of MTT was reconstituted in 3 mL PBS solution) was added to all test and control wells; the plate was gently shaken and incubated at 37 °C in a humidified 5% CO₂ incubator for 4 h. After the incubation period, the supernatant was removed, and 100 µL of MTT solubilization solution was added, and the wells were mixed gently for solubilizing the formazan crystals. The absorbance values were measured at 570 nm (Talarico et al. 2004).

The percentage of growth inhibition was calculated using the Eq. 1

$$\text{Percentage of viability} = \left(\frac{\text{Mean OD of sample}}{\text{Mean OD of control}} \right) \times 100 \quad (1)$$

Determination of apoptosis by acridine orange (AO) and ethidium bromide (EB) double staining

Acridine orange and ethidium bromide (Sigma, USA) are major DNA binding dyes mainly used in molecular biology for differentiating apoptotic and necrotic cells (Zhang et al. 1998).

Acridine orange stains both viable and non-viable cells. The intercalation of this dye into double-stranded nucleic acid (DNA) will emit green fluorescence whereas ethidium bromide stains only non-viable cells and emits red fluorescence. The cells were cultured in DMEM and grown to 70–80% confluency and treated for 24 h; the cells were washed by cold PBS and then stained with a mixture of acridine orange (100 µg/mL) and ethidium bromide (100 µg/mL) at room temperature for 10 min. The stained cells were washed with 1× PBS and observed by a fluorescence microscope (Olympus CKX41 with Optika Pro5 camera).

Relative expression of caspase-3 levels in H9c2 cells by indirect ELISA

One hundred microliters of cell lysate was added to the 96-well plate and kept in overnight incubation at 37 °C. The next day, the wells were drained and washed with PBS for 3 to 5 times. Then, 200 µL of freshly prepared blocking buffer (0.2% gelatin in 0.05% Tween-20 containing PBS) was added to the wells and incubated for 1 h at room temperature and washed two times with washing buffer (0.05% Tween-20 containing PBS) at room temperature. Following, 100 µL of primary antibody (Anti Caspase, Santhacruz, USA) was added and incubated for 2 h at room temperature. The 1° antibody was washed in washing buffer. Sequentially, secondary antibody (anti-HRP conjugated secondary antibody—100 µL) was added and left for 1 h at room temp. Finally, the wells were washed with PBS-Tween 20 for two times and incubated with 200 µL of chromogen for 30 min at room temperature in dark conditions. The color was developed using O-dianizidine (composition—1 mg/100 mL methanol + 21 mL citrate buffer pH 5 + 60 mL H₂O₂) hydrochloride, and the reaction was stopped by adding 5 N HCL (50 µL). OD was read at 415 nm in an ELISA reader.

In vitro ROS measurement using DCFDA

Total ROS activities in the cells were determined by a fluorogenic dye dichlorodihydrofluorescein diacetate, 2,7-Dichlorofluoresceindiacetate (DCFDA). Measurement of ROS was done using DCFDA-Cellular Reactive Oxygen Species Detection Assay Kit, Sigma Aldrich, according to the manufacturer's instructions. Briefly, cells were seeded on to 96-well culture plates at a cell density of 5000 cells/well and allowed to adhere overnight. Following treatment with samples, culture media was removed, and cells were incubated with 50 µL of DCFDA for 30 min, and excess dye was washed off with PBS. The fluorescence was measured at 470 nm excitation and emission at 635 nm (Qubit 3.0, Life technologies, USA) by a microplate fluorimeter (Qubit 3.0, Life technologies, USA).

Determination of mitochondrial membrane potential by flow cytometry

H9c2 cell was seeded on to T25 flasks at a cell density of 5000 cells/well and allowed to reach 70% confluency. The cells were then treated with 20 mM doxorubicin followed by 12.5 µg/mL of sample. Doxorubicin-treated cells were served as positive control. After 24-h incubation, the cells were harvested and in cells were incubated with Muse™ MitoPotential Dye according to manufacturer's protocol. After staining, cells were washed with cold PBS and subjected to flow cytometric analysis using a flow cytometer (Millipore, USA).

Relative expression of NF- κ B in H9c2 cells and isolation of total RNA

H9c2 cells seeded on T25 flasks were grown to 70% confluency, and one flask was treated with 20 mM doxorubicin (Sigma-Aldrich, USA) followed by 12.5 µg/mL of samples. Doxorubicin (20 mM) alone was kept as positive control, and the samples were incubated for 24 h. After incubation, the RNA isolation was carried out using trizol reagent. Briefly, total RNA was isolated using the total RNA isolation kit according to the manufacturer's instruction (Invitrogen, USA). The RNA pellet was dried and dissolved in TE buffer. The purity of extracted RNA was determined using fluorometer Qubit 3.1 (Life Technologies, USA).

Reverse transcriptase PCR analysis

Verso One step RT PCR kit of ThermoScientific, USA, was used for the cDNA synthesis and amplification. About 5 µL of RNA, 1 µL of enzyme mix, 2.5 µL of RT Enhancer, 2 µL of forward primer, and reverse primer were added to an RNase-free tube (forward 5'-CCCACACTATGGATTTCCTA CTTATGG-3' and reverse 5'-CCAGCAGCATCTTC ACGTCTC-3'). To this mixture, 25 µL of primer RT-PCR premix was added. Then, the total reaction volume was made up to 50 µL with the addition of sterile distilled water. The solution was mixed by pipetting gently up and down. The thermal cycler (Eppendorf Master cycler) was programmed to undergo cDNA synthesis and amplification. The stained gel was visualized using a gel documentation system (E gel imager, Invitrogen), and the mean density was determined using ImageJ analysis software.

Statistical analysis

All the experiments were carried out in triplicate, and results were reported as the mean and standard deviation of these measurements. Independent *t* test was carried out to compare the means of caspase-3 level and NF- κ B expression. One-way analysis of variance was carried out for MTT assay. Means

were compared by Tukey's test at 5% level of significance. All the statistical analysis were carried out using SPSS version 16 software.

Results

Synthesis and characterization of Va-g-Ch

The yield of Va-g-Ch varied between 58 and 65%. No spots corresponding to vanillic acid were observed on the developed TLC plates for Va-g-Ch, confirming absence of free vanillic acid and successful grafting on chitosan (Supplementary material Fig. S1). FTIR spectra of chitosan exhibited major characteristic bands at around 3428 cm⁻¹ (OH), 2883 cm⁻¹ (C-H stretching), 1650 cm⁻¹ (amide I), 1550 cm⁻¹ (amide II), 1072 cm⁻¹ (COC), and 899 cm⁻¹ (pyranose ring) (Supplementary material Fig. S2). As compared to chitosan, intensity of amide I (at around 1644 cm⁻¹) and amide II (at around 1549 cm⁻¹) bands in Va-g-Ch increased, indicating formation of new amide linkage. It was also observed that integral ratio of the CH stretching band (at around 2933 cm⁻¹, belonging to chitosan and vanillic acid) to the pyranose band (at around 896 cm⁻¹, belonging to chitosan), i.e., I2933/I896, was higher in Va-g-Ch as compared to chitosan. UV-Vis absorption spectra (Supplementary material Fig. S3) was recorded between 200 and 500 nm and compared to that of chitosan. Chitosan in 1% acetic acid (v/v) showed no absorption peak between 200 and 500 nm. Va-g-Ch showed primary absorption peak at 258 nm which was the same as that of vanillic acid. The proton nuclear magnetic resonance (¹H NMR) spectra of chitosan and Va-g-Ch have been presented in Supplementary material Fig. S4. The ¹H NMR assignments of chitosan were as follows: ¹H NMR (deuterium [D₂O], 400 MHz) δ = 2.00 ppm (NH [CO] CH₃); δ = 3 ppm (H-2); δ = 3.2–3.9 ppm (H-3-H-6); δ = 4 ppm (H-1); δ = 5 ppm (D₂O). The ¹H NMR assignments of Va-g-Ch were as follows: at δ = 2 ppm (NH [CO] CH₃); δ = 3.5–3.6 ppm (H-2, -OCH₃ substitution in benzene ring); δ = 3.6–3.9 ppm (H-3-H-6); δ = 4 ppm (H-1); δ = 5 ppm (D₂O); δ = 6.9 ppm (e, methine protons of vanillic acid); δ = 7–7.6 (f, b, methine protons of vanillic acid).

Characterization of SO-M

Size distribution by number of the re-dispersed SO-M has been presented in Supplementary material Fig. S5. The average diameter of the particles was found to be 2.3 µ. Increase in particle size after spray drying as compared to oil in water emulsion micelle size was expected due to formation of larger droplets in the spray dryer nozzle. ζ potential value of 16.5 was observed, which was similar to that of oil in water emulsion. However, a lower PDI value of 0.345 was recorded as

compared to the oil in water emulsion. This indicates comparatively better homogeneity in particle size distribution. SEM images of SO-M showed spherical to irregular morphology (Supplementary material Fig. S6) with a few small pores on the smooth surface. Broken particles at higher magnification clearly showed cavity inside the particle indicating encapsulation of the oil inside the wall material. The EE value of the sardine oil in Va-g-Ch microparticles was $84 \pm 0.84\%$. The LE value was determined as $67 \pm 0.51\%$. Hence, around 33% of the initial oil was lost during spray drying process.

MTT assay

Cell viability, after treatment with different concentrations of SO-M (3.1, 6.25, 12.5, 25 $\mu\text{g/mL}$), is shown in Table 1. It is evident that there is no cytotoxicity up to a sample concentration of 25 $\mu\text{g/mL}$, and hence, further experiments were carried out using 12.5- $\mu\text{g/mL}$ concentration. Simulated cardiotoxicity by doxorubicin for 1 h caused a change in morphology of the H9c2 cells. The cells showed shrinkage, change in nuclear morphology, and membrane blebbing which are characteristics of apoptotic cell death (Fig. 1). IC_{50} was found to be 25 $\mu\text{g/mL}$, and it indicates the protective effect of SO-M on H9c2 cell lines.

Determinations of apoptosis by acridine orange (AO) and ethidium bromide double staining

If the sample is cytotoxic, the cells will undergo cell death following necrotic pathway. MTT assays cannot differentiate between the mechanisms of apoptosis and necrosis. It is possible to detect basic morphological changes in apoptotic cells by acridine orange/ethidium bromide fluorescent staining. AO/EB staining is one of the reliable methods to distinguish normal, early apoptotic, late apoptotic, and necrotic cells (Biffi et al. 1996). Acridine orange is a membrane permeable dye which can stain normal as well as apoptotic cells and has a characteristic green fluorescence. On the other hand, only

dead cells and late apoptotic cells with a damaged cell membrane are permeable to ethidium bromide which fluoresce orange-red (Ribble et al. 2005). H9c2 cells were stained with AO/EB after 24-h treatment with SO-M. No cell death was observed in the SO-M-treated group on visualization under fluorescent microscope. Necrotic cells increased in volume and showed uneven orange-red fluorescence (Fig. 2). The cells appeared to be in the process of disintegrating.

Relative expression of caspase-3 levels in H9c2 cells

To investigate the effect of SO-M on the intrinsic pathway of apoptosis, caspase-3 activity in both treated and untreated H9c2 cells was measured (Fig. 3). It has been well documented that the activation of caspase-3 is essential for the occurrence of apoptosis in cardiomyocytes (Harrington et al. 2008). In the present study, the SO-M demonstrated to inhibit the subsequent activation of caspase-3. However, in doxorubicin-induced control group, caspase-3 activation was markedly increased. A high magnitude of decrease in the level of caspase-3 activity was observed in the presence of SO-M, suggesting protective effects.

In vitro ROS measurement using DCFDA

Reactive oxygen species (ROS) are special type of molecules that can damage DNA and RNA. It can oxidize proteins and lipids also. It includes H_2O_2 (hydrogen peroxide), NO (nitric oxide), O_2^- (oxide anion), and hydroxyl radical ($\text{OH}^\cdot$). Mainly, this oxidative species are produced in the conditions of cancers, neurologic, cardiovascular, infectious, and inflammatory conditions (Lei et al. 2015). To investigate whether the encapsulated fish oil powder triggers ROS production in H9c2 cells lines, we analyzed the ROS status using fluorescent dye, DCFDA. As expected, doxorubicin treatment increased ROS production in H9c2 cell lines whereas the addition of SO-M decreased ROS generation less than that of control (Fig. 4).

Mitochondrial membrane potential by flow cytometry

Inner mitochondrial membrane potential (MMP) is one of the indicators of mitochondrial dysfunction, and it is very important in the study of apoptosis. Mitochondrial changes especially its MMP and viability are highly sensitive indicators of cell health (Landes and Martinou 2011). MMP is an indicator of doxorubicin-induced apoptosis (Green and Leeuwenburgh 2002). To determine whether doxorubicin induced apoptosis through disrupting MMP and how SO-M affected this process, changes in MMP were also observed. The mitopotential dye detects the changes in mitochondrial membrane potential and 7-AAD acts as a dead cell marker. Four populations of cells could be distinguished from the data, namely live cells with depolarized

Table 1 Percentage of viability at different sample concentration

Sample concentration ($\mu\text{g/mL}$)	Average OD at 540 nm	Percentage viability
Control	0.82	00 ^a
Doxorubicin	0.35	43.0 ^b
3.1 μg sample	0.45	54.9 ^c
6.25 μg sample	0.69	83.5 ^d
12.5 μg sample	0.70	84.9 ^d
25 μg sample	0.71	86.0 ^d

Values were calculated from the mean OD of samples and control group

*A different alphabet superscript indicates a significantly different percentage viability value than others by a Tukey test at $p \leq 0.05$

Fig. 1 MTT images explaining the cellular morphological changes following treatment of doxorubicin and sample treatments. *Arrows* highlight cellular rounding and detachment, reflecting apoptotic cell death. **a** Treated with 3.1 $\mu\text{g/mL}$ concentration of sample. **b** 6.25 $\mu\text{g/mL}$ concentration of sample. **c** 12.5 $\mu\text{g/mL}$ concentration of sample. **d** 25 $\mu\text{g/mL}$ concentration of sample. **e** Control H9c2 cell lines. **f** Doxorubicin (20 mM)-treated cells

mitochondrial membrane, live cells with intact mitochondrial membrane, dead cells with depolarized mitochondrial membrane, and dead cells with intact mitochondrial membrane.

From Fig. 5, it is evident that there is cell death due to disruption of MMP in doxorubicin-treated group as indicated by increase in cell density in upper right quadrant. In SO-

Fig. 2 AO/EB fluorescence staining for apoptotic assays. **a** 20 mM doxorubicin-treated cells showing uneven orange-red fluorescence and signs of disintegration which indicates non-viable cells by necrosis or

late apoptosis. **b** 12.5 $\mu\text{g/mL}$ SO-M-treated group; nucleus of the encapsulated oil-treated cells showed yellow-green fluorescence by acridine orange after 24 h

Fig. 3 Relative expression of caspase-3 levels in H9c2 cells when treated with 20 mM doxorubicin and/or 12.5 $\mu\text{g/mL}$ SO-M as determined by indirect ELISA

M-treated samples, the red fluorescence is very less in the right quadrant whereas sharp red fluorescence was observed in the lower left quadrant indicating the viability. The treatment with the SO-M samples increased the MMP to near normal levels, thereby confirming the attenuating effect of encapsulated fish oil powder on MMP integrity (Fig. 5).

Relative expression of NF-kB in H 9C2 cells

According to Wang et al. (2002), DOX-induced apoptosis in cardiac muscle cells may be mediated through activation of NF-kB. (Fig. 6a). We observed that NF-kB activity in H9c2 cells was sharply increased by incubation in the presence of doxorubicin. However, treatment with the SO-M samples markedly attenuated NF-kB activation induced by doxorubicin (Fig. 6b).

Discussion

Doxorubicin is an anti-cancer drug and is proven to induce cardiotoxicity by damaging the cardiac muscle tissues (Wencker et al. 2003; Xin et al. 2009). Our findings showed that doxorubicin increased cell death, ROS generation, and induced apoptotic cell death. Doxorubicin-

induced cardiotoxicity is an excellent model to elucidate the cardio protection activity of various bioactive molecules. In our study, we observed that 12.5 $\mu\text{g/mL}$ of sardine oil-loaded vanillic acid-grafted chitosan microparticles decreased cytotoxic effects of doxorubicin in cardiomyoblast cell lines (H9c2). Around 84.9% cardiomyocytes cells were recovered from doxorubicin-induced cardiotoxicity. It indicates the effectiveness of sardine oil-loaded vanillic acid-grafted chitosan microparticles against cardiotoxicity. Modern world lifestyle diseases such as cardiotoxicity due to various stress are becoming common, so it is highly recommended to include such functional food in diet to provide protection against cardio toxicity (Das et al. 2016).

Identification of apoptotic stages and necrosis can be better done by AO/EB staining which clearly differentiate morphological changes of the cell to confirm apoptosis and necrosis. AO is permeable to both viable and apoptotic cells and emits green fluorescence, whereas EB is taken up only by non-viable cells and emit orange-red fluorescence (Cury-Boaventura et al. 2004; Ribble et al. 2005). In our study, AO/EB staining of SO-M and doxorubicin-treated cells has shown a clear indication of necrotic and apoptotic cells. Uniform orange-red fluorescence was evident in the major portion of doxorubicin-treated sample; simultaneously, late apoptotic signals were also observed. It is the confirmation of cell death due to necrotic mechanism to a large extent and apoptotic involvement to a small extent. On the other hand, SO-M-treated sample could resist and survive cell death caused by necrosis and apoptosis and is proven by green fluorescence cells observed after 24-h incubation (Ferreira et al. 2013).

Caspases are the cysteine proteases widely considered as a marker of apoptosis during cell death. Elevated levels of caspases-3 clearly indicate the apoptosis-mediated cell death (Hanahan and Weinberg 2011). In this study, increase in the caspase-3 level was observed in doxorubicin-treated cell lines, and decreased level was recorded in SO-M-treated sample. The results suggest involvement of apoptosis and higher cell death in doxorubicin-treated cell lines and proved

Fig. 4 Investigation of ROS potential in H9c2 cell lines. **a** 20 mM doxorubicin-treated cardiomyoblast cell lines showing bright green fluorescence due to excess presence of ROS. **b** 12.5 $\mu\text{g/mL}$ SO-M-treated cells showing less fluorescence because of lower content of ROS

Fig. 5 Mitochondrial membrane potential by flow cytometry with 7-AAD. **a** Doxorubicin-treated (20 mM) cells. **b** SO-M (12.5 μ g/mL)-treated cells

Fig. 6 **a** (i) Higher magnitude of expression of NF- κ B in doxorubicin-treated (20 mM) samples indicates cardiotoxicity and (ii) decreased expression of NF- κ B in SO-M-treated (12.5 μ g/mL) cell lines indicates cardioprotection. **b** Relative expression of NF- κ B in H9c2 cells treated with SO-M

its toxicity on cardiomyocytes, whereas reduction in caspase-3 level in SO-M-treated cells proved its efficiency to resist cardiotoxicity exerted by doxorubicin. Hence, sardine oil-loaded vanillic acid-grafted chitosan microparticles could possibly inhibit the apoptosis and necrosis activation in cardiomyocytes.

ROS are the major agents which lead to pathological symptoms associated with cardiomyopathy (Penna et al. 2009). ROS originate in the body mainly by damaging the membrane permeability of mitochondria and in turn cause severity to cells such as cardiomyocytes (Maack and Bohm 2011). When ROS level is elevated, a series of signaling cascades are activated which in turn trigger the apoptotic pathway and ultimately leads to apoptosis. ROS mainly cause depolarization, and bulging of mitochondria therefore accelerates apoptotic mechanism through mitochondrial involvement (Yamamoto et al. 1999; Suhara et al. 1999). Elevated levels of ROS and caspase-3 were observed in doxorubicin-treated cells. Therefore, we assumed that doxorubicin administration may have an association with elevated ROS production and activated caspase-mediated apoptosis (Ghosh et al. 2011). However, decreased presence of ROS was recorded in SO-M-treated cell line, and the observation is well supported by decreased caspase-3 production. Hence, SO-M treatment could possibly reduce the ROS production and in turn facilitated reduced cardiotoxicity during oxidative stress condition.

Doxorubicin induces damage to cardiomyocytes by NF- κ B activation, the extensively discussed transcription factor in mammals (El-Bakly et al. 2012). NF- κ B mediates cardiomyocyte damage by controlling the expression of apoptotic proteins such as Bcl-2 family proteins and caspases (Karin and Ben-Neriah 2000). TNF- α is known to exert anti-apoptotic effects through the activation of NF- κ B signaling and in turn prevent heart failure. Decreased expression of NF- κ B in SO-M-treated cardiomyocytes can be due to the TNF- α -regulated mechanism which could have been inhibited activation of caspase expression and resulted in decreased production of reactive oxygen species (McGowan et al. 2003). Hence, the results suggest a positive effect of SO-M treatment in alleviating cardiac damage by reducing the NF- κ B, and this can be correlated with the decreased caspase-3 and ROS production. The potential difference across the inner mitochondrial membrane contribute to the mitochondrial membrane potential, $\Delta\psi_{\text{mt}}$ or $\Delta\psi_{\text{mt}}$ which rely on the motive electron across the transmembrane (Gnaiger E 1993). Cardiomyocyte health is directly related to intact and proper mitochondrial potentials, substrate concentration, and energy levels of mitochondrial membranes. Damage of the cardiomyocytes readily reflect on mitochondrial membrane potential and ion flow through protein channel state (Ashruf et al. 1999; Riess et al. 2004). A higher magnitude of membrane potential restoration was observed in SO-M-treated sample which indicates the ability of the new functional food ingredient to maintain mitochondrial potentials and hence cardioprotection (Li et al. 2015).

Conclusion

Cardioprotective functional foods are in high demand nowadays due to increasing incidence of cardiovascular diseases. Fish oil is conventionally used as a cardioprotection nutrient but undesirable if oxidized and it causes adverse effect rather than beneficial effects. Chemical preservatives added in order to prevent oxidation could be harmful and mask protective effect of fish oils. Encapsulation with chitosan and natural antioxidants could be efficiently preventing undesirable oxidation of fish oils to a great extent. Hence, proven cardioprotection effect of fish oils is better exerted or enhanced by micro encapsulation and grafting of chitosan with natural antioxidant agents such as vanillic acid. Effects exerted by the encapsulated sardine oil microparticles protect cardiomyocytes through a series of mechanisms namely alleviated ROS actions, suppression of apoptosis, and reduced expression of NF- κ B.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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