



Bacterial Fucose-Rich Polysaccharide Stabilizes MAPK-Mediated Nrf2/Keap1 Signaling by Directly Scavenging Reactive Oxygen Species during Hydrogen Peroxide-Induced Apoptosis of Human Lung Fibroblast Cells

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Abstract

Continuous free radical assault upsets cellular homeostasis and dysregulates associated signaling pathways to promote stress-induced cell death. In spite of the continuous development and implementation of effective therapeutic strategies, limitations in treatments for stress-induced toxicities remain. The purpose of the present study was to determine the potential therapeutic efficacy of bacterial fucose polysaccharides against hydrogen peroxide (H₂O₂)-induced stress in human lung fibroblast (WI38) cells and to understand the associated molecular mechanisms. In two different fermentation processes, *Bacillus megaterium* RB-05 biosynthesized two non-identical fucose polysaccharides; of these, the polysaccharide having a high-fucose content (~42%) conferred the maximum free radical scavenging efficiency *in vitro*. Structural characterizations of the purified polysaccharides were performed using HPLC, GC-MS, and ¹H/¹³C/2D-COSY NMR. H₂O₂ (300 μM) insult to WI38 cells showed anti-proliferative effects by inducing intracellular reactive oxygen species (ROS) and by disrupting mitochondrial membrane permeability, followed by apoptosis. The polysaccharide (250 μg/mL) attenuated the cell death process by directly scavenging intracellular ROS rather than activating endogenous antioxidant enzymes. This process encompasses inhibition of caspase-9/3/7, a decrease in the ratio of Bax/Bcl2, relocalization of translocated Bax and cytochrome c, upregulation of anti-apoptotic members of the Bcl2 family and a decrease in the phosphorylation of MAPKs (mitogen activated protein kinases). Furthermore, cellular homeostasis was re-established via stabilization of MAPK-mediated Nrf2/Keap1 signaling and transcription of downstream cytoprotective genes. This molecular study uniquely introduces a fucose-rich bacterial polysaccharide as a potential inhibitor of H₂O₂-induced stress and toxicities.

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Introduction

Reactive oxygen species (ROS), which are derivatives of cellular metabolic reactions, modulate the fundamental physiological functions of aerobic life. Normally, aerobic organisms use oxygen as the terminal electron receptor during oxidative phosphorylation, which is likely the greatest source of free oxygen radicals for cells under normal circumstances. Oxidative stress is a specific cellular stress in which the physiological ratio between oxidants and reductants stands in favor of the oxidant, creating species such as free oxygen radicals. At times, excessive generation of these radicals due to a high specific-growth rate, a surge in the level of respiration or from exogenous factors may trigger degenerative cellular disorders in eukaryotes. There is increasing evidence

indicating that ROS and other oxygen-derived free radicals may contribute to a variety of pathological effects and induce diseases, including aging, cancer, neurodegenerative disorders, atherosclerosis, lung damage, diabetes and rheumatoid arthritis [1,2].

The mechanism by which oxygen exerts toxicity has been extensively studied. Free radicals, which are formed by a one-electron reduction of molecular oxygen (O₂), tend to stabilize themselves by retracting electrons from biological macromolecules such as proteins, lipids and DNA. ROS accelerate the peroxidation of membrane lipids (phospholipids and lipoproteins), thus damaging the cell membrane in a chain reaction [3–5]. In aerobic life, endogenous antioxidants shield against the ill effects of oxidative stress. However, various pathological processes inhibit these protective mechanisms. Therefore, supplementation of

antioxidants becomes necessary at times. Cellular oxidative damage is challenged by both synthetic and natural antioxidants. Different antioxidants of synthetic origin are commercially available, although in recent studies, many were found to have deleterious side effects [2,6,7]. Hence, natural antioxidants with the fewest side effects and acceptable biocompatibility remain in demand. Literature review reveals that many polysaccharides of plant and microbial origin stimulate a range of biological effects [8,9], including free radical scavenging and antioxidant activity [10].

Fucose is found only in its L-conformation in vertebrate glycoconjugates. Phylogenetically, fucose first appeared in algal and fungal polysaccharides. Later, fucose also appeared in bacterial and plant glycoconjugates. L-fucose, as well as some other monosaccharides, exhibits several interesting biological properties independent from its metabolic fate. Recently, researchers have demonstrated that L-fucose and fucose-rich oligosaccharides are potent free radical scavengers during ascorbate- and ROS-induced toxicity [11,12].

Apoptosis, unlike necrosis, is a tightly regulated type of cell death in which a cell effectively executes its own demise in a programmed manner. Recent evidence confirmed that ROS and the resulting oxidative stress play a critical role in apoptosis [13,14]. Apoptotic cell death is usually associated with the activation of the extrinsic or intrinsic pathway and occasionally with both. The extrinsic pathway is initiated by the activation of death receptors, which leads to cleavage of caspase-8. The intrinsic pathway involves mitochondrial dysfunction, release of cytochrome c and subsequent activation of caspase-9. Mitochondria arguably play a pivotal role in the induction and control of unregulate-mediated apoptosis. The pro- and anti-apoptotic proteins of the Bcl-2 (B-cell lymphoma 2) family are reported to be the crucial regulators of cell signaling in the mitochondria-dependent intrinsic pathway of apoptosis [15–19]. The Bcl-2 family, together with downstream proteins, maintains a dynamic balance between cell death and cell survival [19]. Several synthetic and natural antioxidants are reported to influence Bcl-2 family proteins. N-acetyl cysteine and Chaga extract effectively upregulated Bcl-2 against glucose/glucose oxidase (G/GO) and hydrogen peroxide treatments, respectively [20,21].

In redox-sensitive signaling pathways, the Nrf2/Keap1 (nuclear factor [erythroid-derived 2]-like 2/Kelch-like ECH-associated protein 1) axis plays a crucial role as a regulator of cellular response against endogenous or exogenous electrophilic assaults. Under normal conditions without any predominant electrophilic stress, Nrf2 remains associated with Keap1 primarily in the cytoplasm and is later subjected to proteasomal degradation [22–24]. The Nrf2/Keap1 pathway effectively regulates the downstream transcription of genes encoding phase II detoxifying enzymes such as heme oxygenase-1 (OH-1/HMOX1), glutathione S-transferase 2 (GASTA2), nicotinamide adenine dinucleotide phosphate quinone oxidoreductase 1 (NQO1), and glutathione peroxidase 1 (GPX1), among others [25]. However, when their components interact with the Nrf2/Keap1 complex, some major signaling pathways may affect the regulation of antioxidant response element (ARE)-responsive genes. In some previous reports, the mitogen-activated protein kinases (MAPKs), which are important mediators of stress-induced apoptosis, were also reported to be the mediators of the Nrf2/Keap1/ARE signaling pathway [26,27]. The MAPK family primarily consists of three relatively well-studied kinases: the extracellular signal-regulated protein kinases (ERKs), the c-Jun N-terminal kinases (JNKs), and the p38 kinases, all of which trigger the signaling cascade by phosphorylation on either serine or threonine residues flanking a

proline residue [16,28]. ERK2 and p38 MAPK positively regulate Nrf2 activity to initiate the transcription of antioxidant genes [29].

In this study, two non-identical, bacterially synthesized fucose polysaccharides were identified and compared as potent free radical scavengers *in vitro*. Furthermore, the polysaccharide showing greater antioxidant potential was challenged with hydrogen peroxide-induced stress in a human embryonic lung fibroblast cell line (WI38). In addition to initial structural characterization, experiments were performed to identify the underlying cell signaling mechanisms during H₂O₂-induced stress and polysaccharide treatment. This study uniquely highlights composition-based antioxidant efficiency of bacterial fucose polysaccharides and the mechanism of combating stress to attain cellular homeostasis via direct ROS scavenging rather than activating endogenous antioxidant enzymes.

Results

Bacterial exopolysaccharides (EPSs)

An EPS-producing bacterial strain, *Bacillus megaterium* RB05, was isolated from riverine sediments and identified by 16S rRNA gene sequencing. Two EPSs were biosynthesized, one in glucose mineral salts medium (GMSM) and one in GMSM-supplemented jute culture (JC). The EPSs were duly purified as described previously [30,31]. The apparent average molecular weight of the JC-derived EPS was 1.28×10^5 Da, whereas an apparent molecular weight of 1.7×10^5 Da was observed for the EPS synthesized in GMSM. The carbohydrate and protein contents of the EPS produced in GMSM were 95.7 ± 3.5 and 2.5 ± 0.4 wt.%, respectively, whereas the corresponding values for the JC-derived EPS were 93.9 ± 3.1 and 3.5 ± 0.8 wt.%, respectively. After hydrolysis and anthranilic acid derivatization, the purified EPSs were analyzed for sugar composition by HPLC. Galactose (37.6%) was found to be the major monosaccharide by weight for the GMSM-derived EPS, followed by arabinose (20.2%), mannose (19.3%) and glucose (14.0%); fucose (4.9%) and N-acetyl glucosamine (4.0%) were present as minor fractions. For the JC-derived EPS, fucose was identified as the primary functional monomer (41.9%), followed by glucose (26.6%), mannose (15.8%), galactose (12.2%), and N-acetyl glucosamine (3.5%). Based on the weight percentage of fucose content, the two EPSs were classified as a low-fucose-content (LFC) polysaccharide and a high-fucose-content (HFC) polysaccharide [30–33].

Free radical scavenging potential of the polysaccharides *in vitro*

The free radical scavenging potential of the polysaccharides was tested chemically before introduction into human cell lines. Free radicals and electrophiles were generated chemically in a system and scavenged by the added polysaccharides. The *in vitro* free radical scavenging potential of the polysaccharides in terms of their IC₅₀ values are shown in Table S1. In most cases, the values were found to be similar to the positive control values. Figures 1A and B show the ability of the polysaccharides to scavenge hydroxyl radicals in site-specific and non-specific reactions. In the non-specific cases, the LFC and HFC polysaccharides displayed similar scavenging performances at 1 mg/mL (95% and 93%), whereas for the site-specific reactions, the activity of the HFC polysaccharide was statistically superior ($p < 0.05$) at the same concentration. Both the LFC and HFC polysaccharides scavenged H₂O₂ almost as well as the standard control sodium pyruvate (Figure 1C), although the IC₅₀ values were comparatively higher; this might be due to the concentration of H₂O₂ used during the experiment. The polysaccharides showed a moderate dose-dependent scav-

enging effect against the singlet oxygen species, with IC_{50} values of 0.425 ± 0.024 mg/mL and 0.175 ± 0.028 mg/mL, respectively, for the LFC and HFC polysaccharides (Figure 1D). Figures 1E and F show the quenching of superoxide radicals in non-enzymatic and enzymatic reactions. The scavenging potential of the polysaccharides was found to be superior to that of the reference compound. In the enzymatic reaction, administration of these polysaccharides inhibited the process of free radical generation throughout their lifespan. As depicted in Table S1 and Figure 1G, the HFC (69%) and LFC (59%) polysaccharides successfully scavenged DPPH radical, but to a lower degree than the standard ascorbic acid (92%). The total antioxidant activity of these polysaccharides was evaluated in two different systems, one based on scavenging performance against $ABTS^{\cdot+}$ and the other via bleaching of β -carotene in the β -carotene-linoleate model system. In the first model, the total antioxidant capacity of the polysaccharides was calculated from the decolorization of $ABTS^{\cdot+}$. The polysaccharides were found to be more effective than the standard Trolox at suppressing $ABTS^{\cdot+}$ at the same concentrations. The results, which are expressed as percentage inhibition of absorbance, are shown in Figure 1H. In the β -carotene-linoleate model system (Figure 1I), the antioxidant performance of the LFC and HFC polysaccharides was found to be 82% and 83.5%, respectively, at 0.1 mg/mL. The antioxidant behavior of the reference compound butylated hydroxyanisole (BHA) increased in a concentration-independent manner in the present system. From the above results, one could easily consider these polysaccharides to be potent free radical scavengers *in vitro*. However, in most cases, the HFC polysaccharide was found to perform better than the LFC polysaccharide. The HFC polysaccharide was therefore subjected to further experiments to understand the structure and the cellular biology of the antioxidant during oxidative stress.

Structural elucidation of the HFC polysaccharide

The sugar linkages in alditol acetates of the methylated sugars derived from the HFC polysaccharides were elucidated using GC-MS (Table S2). The linkages between the monomers were analyzed according to the description by Bjorndal et al. [34]. The 1H NMR spectrum (Figure 2A) of the HFC polysaccharide at $40^\circ C$ showed three signals at δ 5.11, 5.24, and 5.23 ppm for three anomeric protons corresponding to α -linked glycopyranose residues and five signals at δ 4.62, 4.54, 4.64, 4.55, and 4.68 ppm for five anomeric protons corresponding to β -linked residues. The sugar residues were designated as **A–H** according to their decreasing anomeric proton chemical shifts (Table S3). In the ^{13}C NMR spectrum (Figure 2B) at $40^\circ C$, three signals appeared at δ 100.94, 102.02, and 100.19 ppm for three anomeric carbons corresponding to α -linked glycopyranose residues and five signals at δ 102.59, 105.17, 101.82, 103.60, and 95.75 ppm for five anomeric carbons corresponded to β -linked residues. The sugar residues were designated as **A–H** according to their decreasing anomeric carbon chemical shifts (Table S3). The anomeric proton chemical shift for residue **A** at δ 5.11 ppm and a carbon chemical shift of 100.94 ppm indicated that it is an α -linked anomer. The downfield shift of C-1 (δ 100.94 ppm), C-2 (δ 77.40 ppm) and C-4 (δ 74.76 ppm) with respect to the standard values [35,36] and the characteristic $J_{H=1, H=2}$ coupling constant value (3.4 Hz) indicated that residue **A** is a (1 \rightarrow 2,4)-linked- α -D-mannopyranosyl moiety. The anomeric proton chemical shift for residue **B** at δ 5.24 ppm and a carbon chemical shift of 102.02 ppm indicated that it is an α -linked anomer. The downfield shift of C-1 (δ 102.02 ppm), C-4 (δ 74.84 ppm) and C-6 (δ 69.79 ppm) and the characteristic $J_{H=1, H=2}$ coupling constant value (3.5 Hz) indicated that residue **B** is a (1 \rightarrow 4,6)-linked- α -D-mannopyr-

anosyl moiety. Residue **C** displayed an anomeric proton signal at δ 5.23 ppm and a carbon chemical shift at δ 100.19 ppm. The downfield shift of C-1 (δ 100.19 ppm) and C-3 (δ 74.24 ppm) along with a characteristic $J_{H=1, H=2}$ coupling constant value of 3.7 Hz signified residue **C** as (1 \rightarrow 3)-linked- α -D-fucopyranosyl moiety. Additionally, a slight downfield shift of C-4 (δ 74.07 ppm) also suggested the presence of a sulfate group (SO_3^-) within this moiety. Residue **D** was found to be a (1 \rightarrow 2,4)-linked- β -D-galactopyranosyl moiety showing anomeric proton and carbon signals at δ 4.62 and 4.54 ppm, respectively, and residue **E** was identified as a (1 \rightarrow 4)-linked- β -D-galactopyranosyl moiety with anomeric proton and carbon signals at δ 102.59 and 105.17 ppm, respectively. Downfield shifts were observed at C-1 (δ 102.59 ppm), C-2 (δ 79.57 ppm), and C-4 (δ 78.61 ppm) for residue **D** and at C-1 (δ 105.17 ppm) and C-4 (δ 78.63 ppm) for residue **E** with a $J_{H=1, H=2}$ coupling constant value of \sim 8.2 Hz. Residue **F** was found to be a (1 \rightarrow 2,4)-linked- β -D-glucopyranosyl moiety showing anomeric proton and carbon signals at δ 4.64 and 4.55 ppm, respectively, and residue **G** was identified as a (1 \rightarrow 4)-linked- β -D-glucopyranosyl moiety with anomeric proton and carbon signals at δ 101.82 and 103.60 ppm, respectively. Downfield shifts were observed at C-1 (δ 101.82 ppm), C-2 (δ 81.14 ppm), and C-4 (δ 79.41 ppm) for residue **F** and at C-1 (δ 103.60 ppm) and C-4 (δ 79.66 ppm) for residue **G** with a characteristic $J_{H=1, H=2}$ coupling constant value of \sim 8 Hz. Residue **H** displayed an anomeric proton signal at δ 4.68 ppm and a carbon chemical shift at δ 95.75 ppm. The downfield shift of only C-4 (δ 74.24 ppm) along with the characteristic $J_{H=1, H=2}$ coupling constant value of \sim 9 Hz signified residue **H** as a terminal β -D-GlcNAc β . Figure 3 shows the postulated structure of the HFC polysaccharide.

Effect of H_2O_2 and polysaccharides on the growth of human lung fibroblast cells

MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assays were performed for primary dose selection. WI38 cells were treated with H_2O_2 at different doses (100–500 μ M) for the indicated period of time (0–24 h). As shown in Figure S1, cell growth was inhibited by 76% after treatment with 300 μ M H_2O_2 for 24 h. The cells were pre-treated with the LFC and HFC polysaccharides for 2 h prior to H_2O_2 administration. After 24 h of treatment, the inhibition of cell growth by H_2O_2 in the presence of the LFC and HFC polysaccharides was found to be reduced to 27.3 and 13.4%, respectively, of the inhibition of cell growth by H_2O_2 alone at 250 μ g/mL. However, at higher concentrations (500 μ g/mL), the polysaccharides further inhibited growth compared with H_2O_2 treatment alone. Henceforth, selection of the optimal dose is critical to achieve the maximum response. Collectively, these data indicated that both polysaccharides displayed protective effects against the growth inhibition caused by oxidative stress over a range of concentrations, although the performance index of the HFC polysaccharide was comparatively superior. Thus, the HFC polysaccharide was preferentially adopted for treatments in the future experiments.

The viability of the cells during both the stress and polysaccharide treatment period was also determined via optical imaging by phase-contrast microscopy (Figure 4). During the stress period (300 μ M H_2O_2), fewer live cells were found, and the cells appeared to be morphologically misshapen and distorted as time progressed. The number of viable cells increased with polysaccharide treatment in a time- and dose-dependent manner, and the normal cell shape and size was observed after a treatment of at least 12 h.

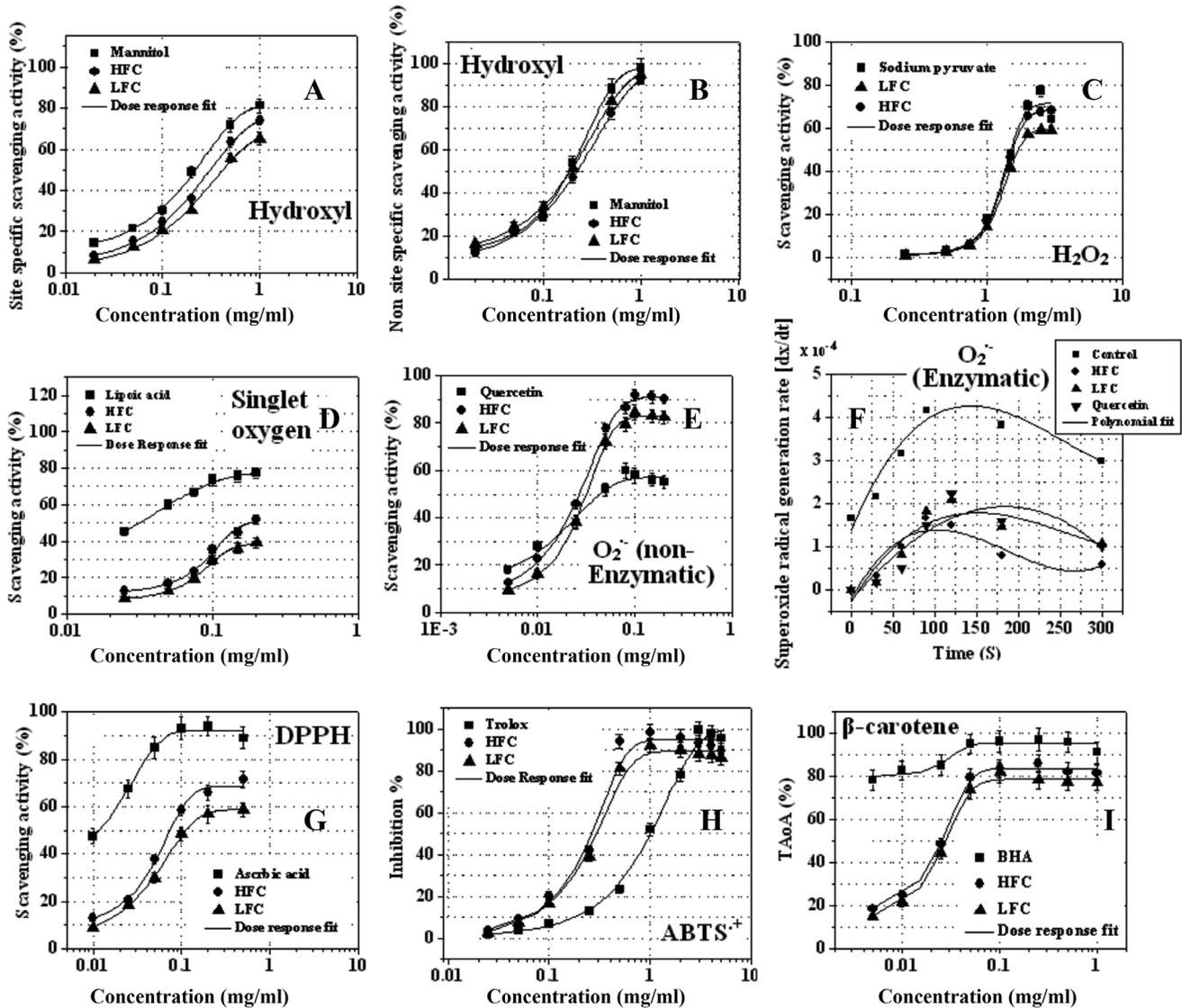


Figure 1. Scavenging performance of purified LFC and HFC polysaccharides against chemically-generated free radicals *in vitro*. A) hydroxyl radical (site specific); B) hydroxyl radical (non- specific); C) hydrogen peroxide; D) singlet oxygen; E) superoxide radical anion (non-enzymatic); F) superoxide radical anion (enzymatic); G) DPPH radical, H) total antioxidant capacity (ABTS^{•+} scavenging); and I) total antioxidant capacity (β -carotene linoleate model). Results are representative of three independent experiments performed in triplicate and are represented as mean \pm SD. Inhibition concentration (IC_{50}) values were calculated from pharmacological dose-response curve fit (sigmoidal) the equation: $Y = Bottom + (Top-Bottom) / [1 + 10^{-(\text{Log}[C50-X] * Hill slope)}]$. doi:10.1371/journal.pone.0113663.g001

Polysaccharide inhibited H₂O₂-induced apoptosis

To determine whether H₂O₂-induced toxicity involves alteration in the cell cycle, the DNA content of the cells was analyzed by flow cytometry. Figure 5 shows that H₂O₂ (300 μ M) significantly increased ($p < 0.05$) the sub-G₀/G₁ cell population with time. The cell population in this phase was found to be 60% of the total cells after 12 h exposure to H₂O₂. The HFC polysaccharide efficiently reduced the accumulation of cells in the sub-G₀/G₁ phase in a dose- and time-dependent manner. A polysaccharide dose of 250 μ g/mL induced a significant decrease ($p < 0.05$) in the percentage of cells in sub-G₀/G₁ phase after 24 h. This value decreased to 7.4%, which is similar to the value of the control cells (3.1%). However, H₂O₂ stress also disrupted the arrangement of phospholipids in the cell membrane [37]. The externalization of phosphatidylserine on the plasma membrane was detected by

annexin V-FITC staining in WI38 cells. Annexin V staining of the cells (Figure 6) indicated that H₂O₂-induced cell death was apoptotic and significantly increased ($p < 0.05$) with time. During the polysaccharide treatment, the number of annexin V-positive cells was found to be significantly decreased ($p < 0.05$). The HFC polysaccharide (250 μ g/mL) reduced the percentage of annexin V-positive cells from 42.7% to 6.8% after 24 h. Similarly, a considerable reduction in the necrotic population was also observed during the polysaccharide treatment period.

Effect of ROS and polysaccharide treatment on H₂O₂-induced apoptosis

A fluorescent probe, H₂DCF-DA (2',7'-dichlorodihydrofluorescein diacetate), was used to measure the generation of intracellular ROS within WI38 cells during H₂O₂ stress. As

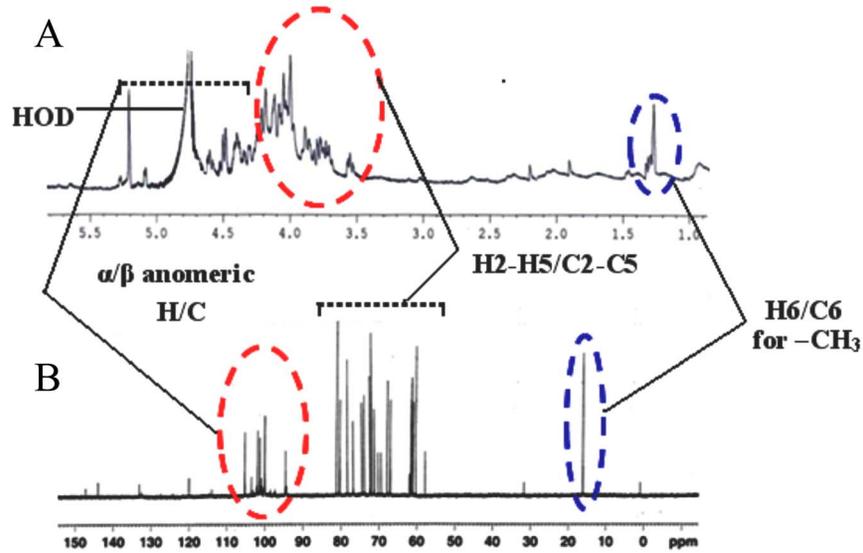


Figure 2. Partial structure elucidation of HFC polysaccharide by NMR spectroscopy. The purified samples were exchanged with deuterium by lyophilizing several times with D₂O. The polysaccharide was dissolved in 0.7 mL of D₂O (99.96%) at concentrations of 15 mg/mL (for ¹H NMR) and 30 mg/mL (for ¹³C NMR). Spectra were run at a probe temperature of 40°C. **A**) in the ¹H NMR spectrum, black dotted line indicating α and β proton anomers, and dotted circles displaying H2–H5 and H6 (–CH₃ group) of the polysaccharide directed from downfield to upfield. **B**) in the ¹³C NMR spectrum, downfield dotted circle stands for α and β carbon anomers, black dotted line indicating C2–C5, and upfield dotted circle signifying C6 (–CH₃ group) of the polysaccharide.
doi:10.1371/journal.pone.0113663.g002

shown in Figure 7, the intensity of the DCF-liberated fluorescent signal from the stress-treated cells was found to increase gradually with time (maximum after 3 h), indicating an elevated level of intracellular ROS compared with the control basal level of ROS. Pre-treatment with the HFC polysaccharide significantly decreased (p<0.05) the cell fluorescence with time. This decrease in the fluorescence signal was dose specific. Treatment with the polysaccharide inhibited fluorescence to a greater degree than treatment with N-acetyl cysteine (NAC), which was used as positive control.

Regulation of mitochondrial function and expression of Bcl-2 family proteins

Mitochondrial membrane potential (ΔΨ_m) regulates mitochondrial permeability, which may be critical for inducing or arresting the stress-induced apoptotic pathways [37,38]. The effect of H₂O₂-induced stress on ΔΨ_m of WI38 cells was examined using flow cytometry following the DiOC6 (3,3'-dihexyloxycarbocyanine iodide) staining method. As shown in Figure 8A, when WI38 cells were exposed to 300 μM H₂O₂, ΔΨ_m decreased, resulting in a significant drop in potential (p<0.05). Although the membrane regained potential with time, this change was not significant (p>0.05). The HFC polysaccharide (250 μg/mL) induced a gradual

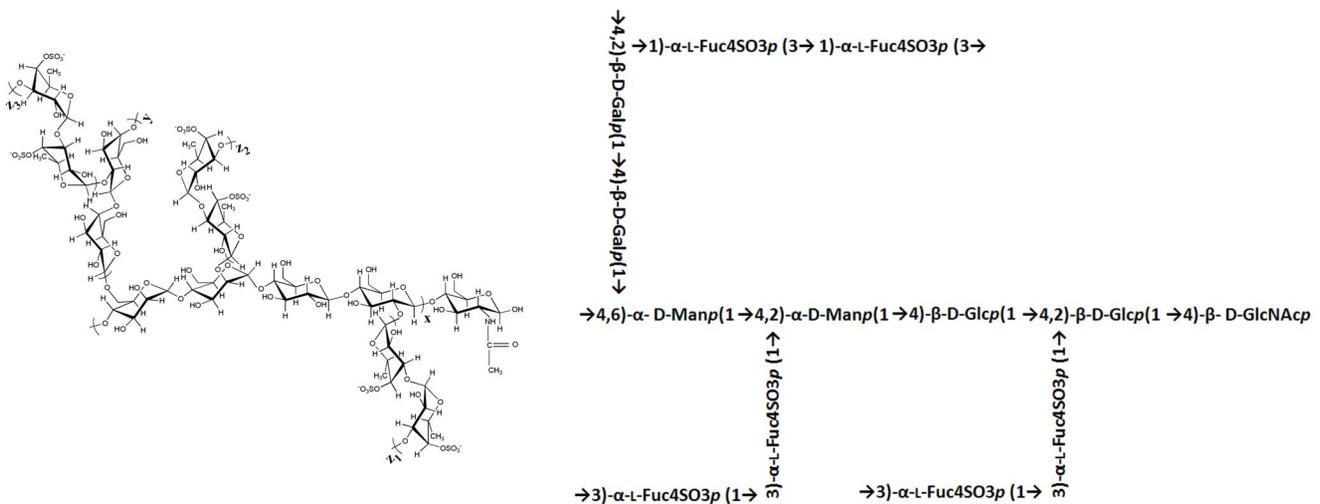


Figure 3. The possible structure interpreted for HFC polysaccharide.
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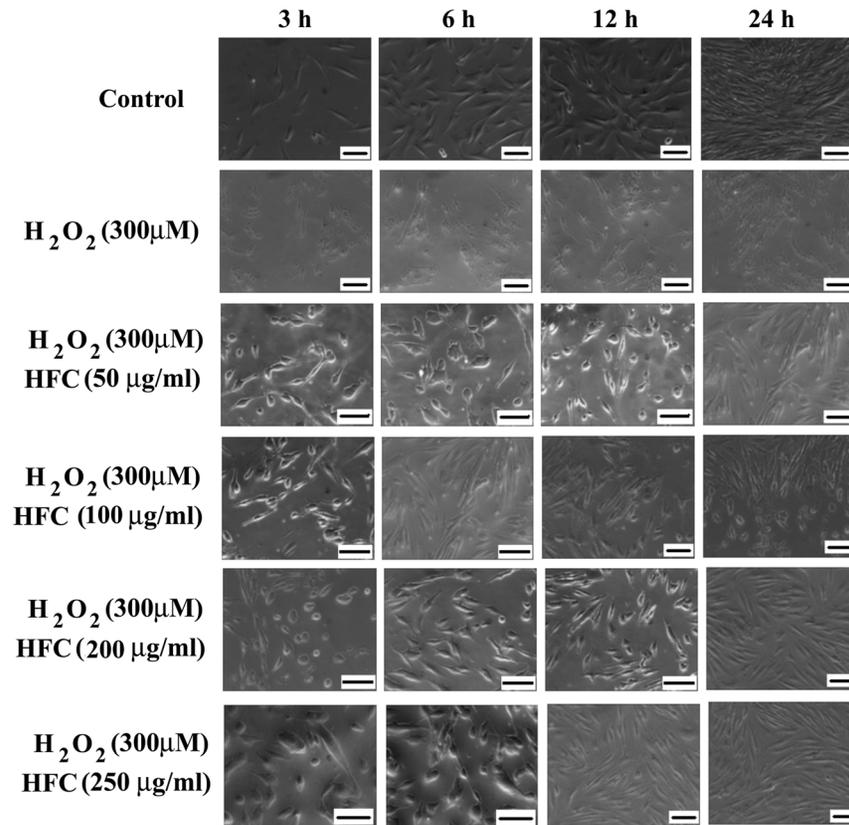


Figure 4. The effect of HFC polysaccharide on H₂O₂-induced morphological changes in WI38 cells. The cells were incubated in presence (100, 200 and 250 μg/ml) or in absence of HFC polysaccharide for 1 h followed by the treatment with 300 μM H₂O₂ in both the cases for varying periods of time (0–24 h). Cell morphology was observed under microscope in phase contrast mode. Results are representative of three independent experiments performed in triplicate. Indicated scale bars signify 50 μm distance and photographs were taken at 10× zoom. doi:10.1371/journal.pone.0113663.g004

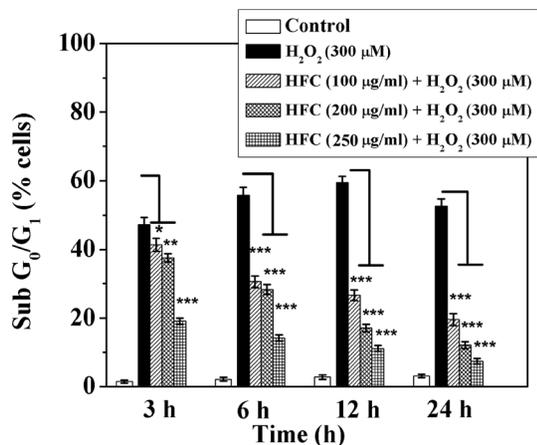


Figure 5. The protective effect of HFC polysaccharide on H₂O₂-induced DNA damage during cell cycle progression. WI38 cells were incubated in presence (100, 200 and 250 μg/ml) or in absence of HFC polysaccharide for 1 h followed by the treatment with 300 μM H₂O₂ in both the cases for varying periods of time (0–24 h). The cell cycle progression was assayed by PI staining with flow-cytometry. The corresponding data are shown as bar graphs. Results are representative of three independent experiments performed in triplicate and are represented as mean ± SD. A one-way analysis of variance (ANOVA, Bonferroni corrections for multiple comparisons) was performed, where significant level stands for *p<0.05, **p<0.001, ***p<0.0001. doi:10.1371/journal.pone.0113663.g005

increase in mitochondrial membrane potential in a time- and dose- dependent manner. No significant loss or gain in potential was observed after the administration of the polysaccharide without H₂O₂ exposure. The Bcl-2 family proteins have been reported to regulate $\Delta\Psi_m$ [19]. Therefore, the expression of Bcl-2 family proteins and their corresponding genes was measured in H₂O₂- and HFC polysaccharide-treated WI38 cells.

Immunoblotting (Figure 8B and Figure S2) revealed that the expression of anti-apoptotic proteins such as Bcl-2 and Bcl-xl was found to be suppressed in the cells where stress was induced (the levels were 44% and 48%, respectively, of those of the control after 24 h). Bad was the least responsive against the applied stress ($p > 0.05$). The HFC polysaccharide relieved the stress by arresting the apoptotic pathway in a time-dependent manner. The ratio between Bax (Bcl2-associated × protein) and Bcl-2 was measured using band-densitometry. As shown in Figure 8C, H₂O₂-induced stress increased the ratio (4.8-fold increase compared with the control after 24 h), whereas polysaccharide treatment for 24 h decreased the ratio to a level that was not significantly different from that of the control cells ($p > 0.05$).

The expression of the Bcl-2 family proteins was quantified using real-time reverse transcriptase (RT-PCR) (Figure 8D). Even at the mRNA level, significant alterations ($p < 0.05$) in expression pattern, in terms of fold change ($2^{\Delta\Delta Ct}$), were observed for Bcl-2, Bcl-xl, and Bax during the stress and treatment period. The mRNA expression of Bcl-2 and Bcl-xl was downregulated (5.9- and 5.2-fold decreases, respectively, compared with the control after 24 h),

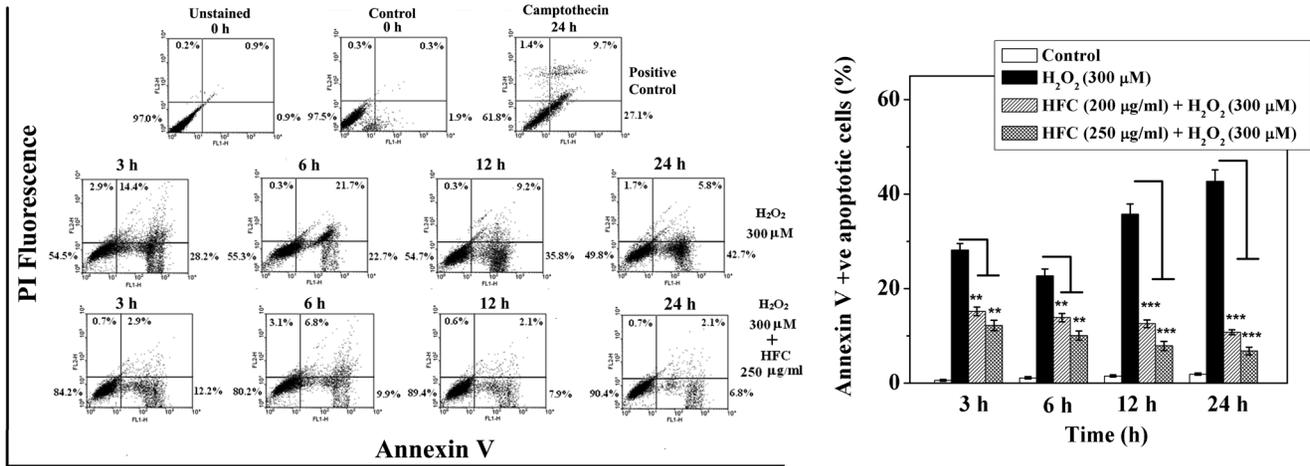


Figure 6. Inhibitory effects of HFC polysaccharide on H₂O₂-induced apoptosis of WI38 cells. WI38 cells were incubated in presence (200 and 250 μg/ml) or in absence of HFC polysaccharide for 1 h followed by the treatment with 300 μM H₂O₂ in both the cases for varying periods of time (0–24 h). Cellular apoptosis was assayed by annexin V-FITC and PI counterstaining and analyzed with flow cytometry. Camptothecin was used as positive control. Dual parameter dot plot of FITC fluorescence (x-axis) versus PI fluorescence (y-axis) is represented as logarithmic fluorescence intensity. Quadrants: upper left necrotic cells, lower left live cells, lower right apoptotic cells, and upper right necrotic or late phase of apoptotic cells. The corresponding data are shown as bar graphs. Results are representative of three independent experiments performed in triplicate and are represented as mean ± SD. A one-way analysis of variance (ANOVA, Bonferroni corrections for multiple comparisons) was performed, where significant level stands for ** p<0.001, *** p<0.0001. doi:10.1371/journal.pone.0113663.g006

whereas the expression of Bax was upregulated (5.7-fold increase compared with the control after 24 h) with H₂O₂ treatment. For Bad and cytochrome c, the fold changes were moderate (2.8- and 1.9-fold increases, respectively, compared with the control after 24 h). The HFC polysaccharide actively regulated the aforementioned genes. After 24 h of treatment, a significant decrease (p<

0.05) in the mRNA expression was observed, and the values were observed to be similar to those of untreated cells.

Translocation of Bax and cytochrome c

Mitochondrial damage as a result of stress-induced apoptosis is often accompanied by the release of apoptotic factors into the cytosol [19]. Evidence indicates that the release of cytochrome c into the cytosol induces cleavage of caspase-9. This, in turn, activates caspase-3, which plays an important role in the execution of stress-induced apoptosis in different cell types [39]. Therefore, the translocation of cytochrome c and Bax was studied using western blotting (Figure 9A and Figure S2). The localization of proteins during the time-course of stress and polysaccharide treatment was also determined by immunofluorescence using specific antibodies. In the untreated control cells, cytochrome c immunoreactivity (green fluorescence) colocalized with the Mito-Tracker red fluorescence, indicating the mitochondrial association of cytochrome c (Figure 9B). After H₂O₂ exposure, the stressed cells exhibited diffusion of the green fluorescence from the mitochondria to the cytoplasm. A visual decrease in the fluorescence intensity was observed for the mitochondria-associated cytochrome c, thus indicating a release of cytochrome c from the mitochondria to the cytoplasm. However, with HFC polysaccharide treatment, cytochrome c successfully translocated back to and re-localized in the mitochondria in a time-dependent manner.

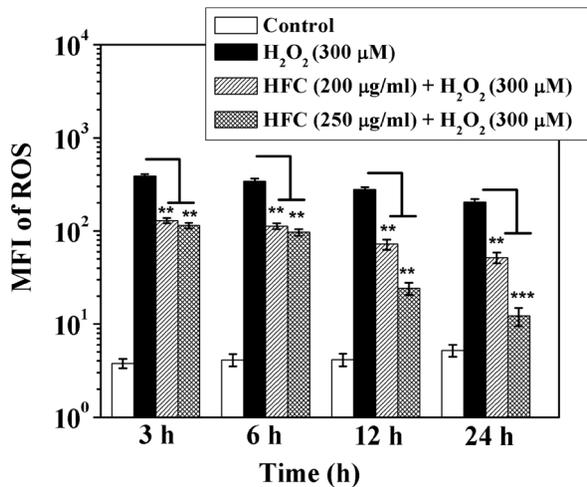


Figure 7. Stabilization of H₂O₂-induced intracellular ROS by HFC polysaccharide. WI38 cells were incubated in presence (200 and 250 μg/ml) or in absence of HFC polysaccharide for 1 h followed by the treatment with 300 μM H₂O₂ in both the cases for varying periods of time (0–24 h). ROS levels were monitored by flow cytometry using H₂DCF-DA. N-acetyl cysteine (NAC) was used as positive control. The mean fluorescence indices (MFI) are shown as bar graphs. Results are representative of three independent experiments performed in triplicate and are represented as mean ± SD. A one-way analysis of variance (ANOVA, Bonferroni corrections for multiple comparisons) was performed, where significant level stands for ** p<0.001, *** p<0.0001. doi:10.1371/journal.pone.0113663.g007

The Bax protein was immunostained using a Bax-specific antibody and a green fluorescent secondary antibody (Figure 9B). In the untreated control cells, Bax was found to be localized throughout the cytosol, but H₂O₂ treatment triggered Bax localization in the mitochondria. This mitochondrial association of Bax decreased with time as the mitochondrial membrane regained potential after polysaccharide administration. Western blots (Figure 9A) demonstrated an increase in cytosolic and mitochondrial pro-apoptotic Bax (4.2- and 3.4-fold increases, respectively, compared with the control after 24 h) during the

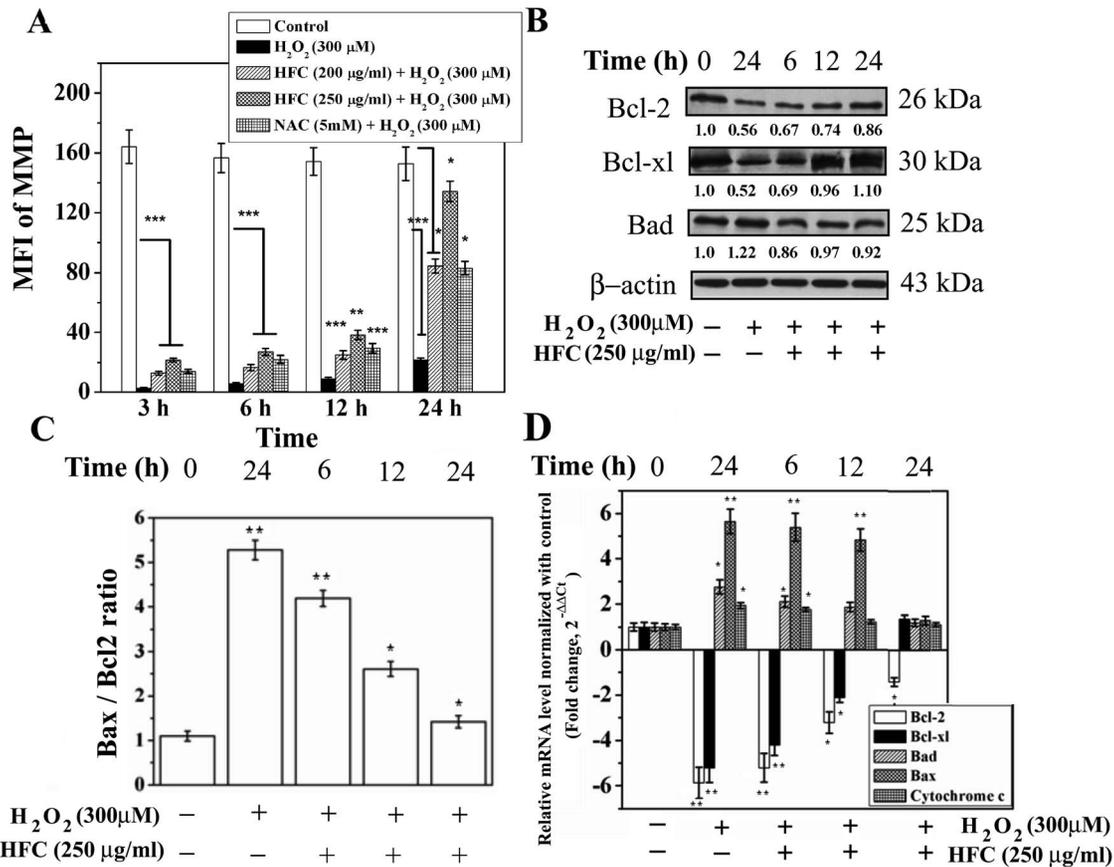


Figure 8. The effects of HFC polysaccharide treatment on H₂O₂-induced regulation of mitochondrial functions. The polysaccharide prevented H₂O₂-induced changes in the expression of Bcl2 family at both mRNA and protein level. WI38 cells were incubated in presence (200 and 250 μg/mL) or in absence of HFC polysaccharide for 1 h followed by the treatment with 300 μM H₂O₂ in both the cases for varying periods of time (0–24 h). **A**) Mitochondrial membrane potential (MMP) was monitored by DiOC6 staining with flow cytometry. The mean fluorescence indices (MFI) are shown as bar graphs. **B**) Protein level expression of Bcl2, Bcl-xl, and Bad was evaluated by immunoblotting. β-actin was used as loading control. Fold changes are represented as relative values of band densitometries normalized to control and are shown as numbers below the immunoblots. Results are representative of three independent experiments performed in triplicate and are represented as mean value. **C**) The ratio between Bax and Bcl2 were calculated from band densitometries of corresponding protein level expressions and are shown as bar graphs. **D**) Fold changes of Bcl2, Bcl-xl, Bad, Bax, and cytochrome c at mRNA level were calculated using real-time RT-PCR (SYBR green method). Fold changes are represented as relative values normalized to control and quantified in the terms of $2^{-\Delta\Delta Ct}$. GAPDH was used as internal control. Results are representative of three independent experiments performed in triplicate and are represented as mean \pm SD. A one-way analysis of variance (ANOVA, Bonferroni corrections for multiple comparisons) was performed, where significant level stands for * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$. doi:10.1371/journal.pone.0113663.g008

stress period. At the same time, cytochrome c level in the cytosol was significantly increased (2.1-fold increase compared with the control after 24 h), although the mitochondrial level of cytochrome c was found to be significantly decreased (68% decrease compared with the control after 24 h). Treating the cells with the HFC polysaccharide reversed the translocation process over time. As a result, the mitochondrial level of cytochrome c increased with time (12% decrease compared with the control after 24 h), with an effective post-treatment increase in $\Delta\Psi_m$. The Bax level was decreased in both the cytosol and the mitochondria as a part of the polysaccharide treatment against stress (24% and 12% decreases, respectively, compared with the control after 24 h).

Caspase-dependent signaling pathway

The involvement of caspase proteins during the stress and the polysaccharide treatment periods was investigated by western blotting analysis. The ratio of pro and active caspases was calculated from band-densitometry. As depicted in Figure 10 and Figure S2, H₂O₂ downregulated the expression of procaspase-9

and significantly increased active caspase-9 (5.4-fold decrease in the ratio of pro/active caspase-9 compared with the control after 24 h). Similarly, a significant increase ($p < 0.05$) in the levels of active effector caspase-3 and -7 (7.4- and 5.3-fold decreases in the ratio of pro/active caspase-3 and -7, respectively, compared with the control after 24 h), as well as cleavage of PARP (5.4-fold increase compared with the control after 24 h), was observed. In contrast, treatment with the HFC polysaccharide markedly decreased ($p < 0.05$) the expression of active caspases, effector caspases, and cleavage of PARP in a time-dependent fashion (1.5-, 1.7-, and 1.3-fold decreases in the ratio of pro/active caspase-9, -3 and -7, respectively, and 1.4-fold increase in PARP cleavage compared with the control after 24 h).

In caspase inhibition assays, WI38 cells were pre-treated with the pan-caspase inhibitor Z-VAD-fmk (50 μM) for 1 h and then treated with the polysaccharide and H₂O₂ for 24 h. As shown in Figure 11A and B, Z-VAD-fmk effectively inhibited the H₂O₂-induced apoptosis of WI-38 cells (81.5% decrease in the percentage of apoptotic cells) and significantly reduced the level

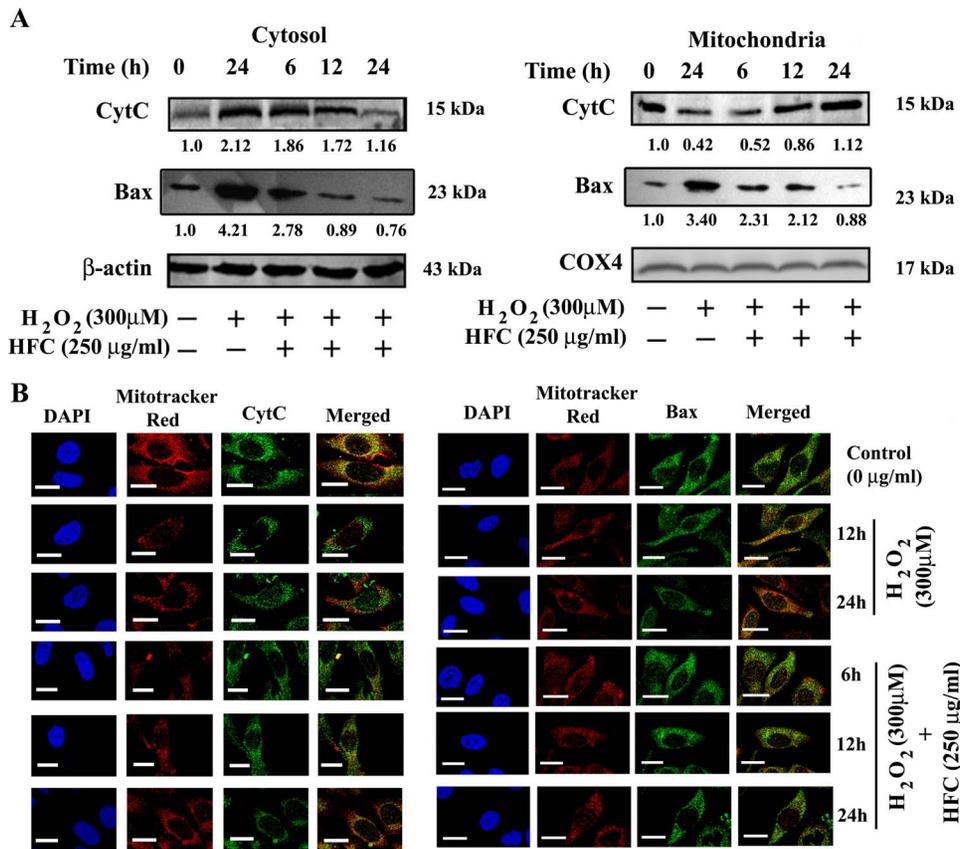


Figure 9. Translocation of Bax and cytochrome c induced by H_2O_2 and HFC polysaccharide during the stress and polysaccharide treatment period, respectively. WI38 cells were incubated in presence (250 μ g/ml) or in absence of HFC polysaccharide for 1 h followed by the treatment with 300 μ M H_2O_2 in both the cases for varying periods of time (0–24 h). **A**) Protein level expression of Bax and cytochrome c in both cytosolic and mitochondrial fractions was observed by immunoblotting. β -actin and COX4 were used as loading control. Fold changes are represented as relative values of band densitometries normalized to control and are shown as numbers below the immunoblots. Results are representative of three independent experiments performed in triplicate and are represented as mean value. A one-way analysis of variance (ANOVA, Bonferroni corrections for multiple comparisons) was performed, where significant level stands for * $p < 0.05$, ** $p < 0.001$. **B**) H_2O_2 -induced release of cytochrome c from mitochondria to cytosol and re-localization into mitochondria again during the polysaccharide treatment were monitored under fluorescence microscope using fluorescence-tagged (green fluorescence) specific antibodies. Similarly, mitochondrial translocation of Bax and their cytosolic re-localization was also tracked following the same procedure. The cells were treated with 100 nM MitoTracker Red (red fluorescence) for 30 min before cell-fixation for mitochondrial staining. Each image shown is representative of 20 random fields observed. Indicated scale bars signify 10 μ m distance and photographs were taken at 100 \times zoom. doi:10.1371/journal.pone.0113663.g009

of active caspase-9 ($p < 0.05$). For further confirmation, the effects of the caspase-9-specific inhibitor Z-LEHD-fmk and the caspase 3/7-specific inhibitor Ac-DEVD-CHO were evaluated using flow cytometry (annexin V-PI staining). The inhibitors attenuated H_2O_2 -induced apoptotic cell death (82.2% and 78.4% decreases for Z-LEHD-fmk and Ac-DEVD-CHO, respectively, compared with the cells treated with H_2O_2 only). Additionally, together with the HFC polysaccharide, the inhibitors further reduced the apoptotic cell numbers (a 90.4% decrease compared with the cells treated with H_2O_2 only).

The HFC polysaccharide downregulates H_2O_2 -mediated phosphorylation of MAP kinases

Similar to caspase activation, the MAPK family is involved in a process that induces stress-mediated cell death [19]. In view of this evidence, the regulation of the MAPK signaling pathway (JNK, p38, and ERK) was studied during the stress and polysaccharide treatment periods. As shown in Figure S3, the phosphorylation level of all three kinases was found to be maximal after 6 h of

H_2O_2 treatment (83%, 114%, and 112% increases for JNK, p38, and ERK, respectively, compared with the control). The phosphorylation level significantly ($p < 0.05$) decreased in response to the polysaccharide treatment after 6 h (25%, 33.6% and 28.3% decreases for JNK, p38, and ERK, respectively, compared with the cells treated with only H_2O_2 after 6 h). But at the later time periods, the corresponding rates of decrease was found not to be statistically significant ($p > 0.05$) [data not shown].

Involvement of MAPK-mediated Nrf2/Keap1 signaling and transcriptional regulation of downstream genes

Nrf2 has been demonstrated to be a transcription factor that is usually sequestered in the cytoplasm by Keap1, but during oxidative stress, Nrf2 translocates to the nucleus, resulting in transcriptional activation of cytoprotective genes encoding phase II detoxifying enzymes. Therefore, in the current effort, the protein levels of Nrf2 and Keap1 in the cytoplasm and the nucleus were determined using western blotting, and the mRNA expression was examined using real-time RT-PCR. The translo-

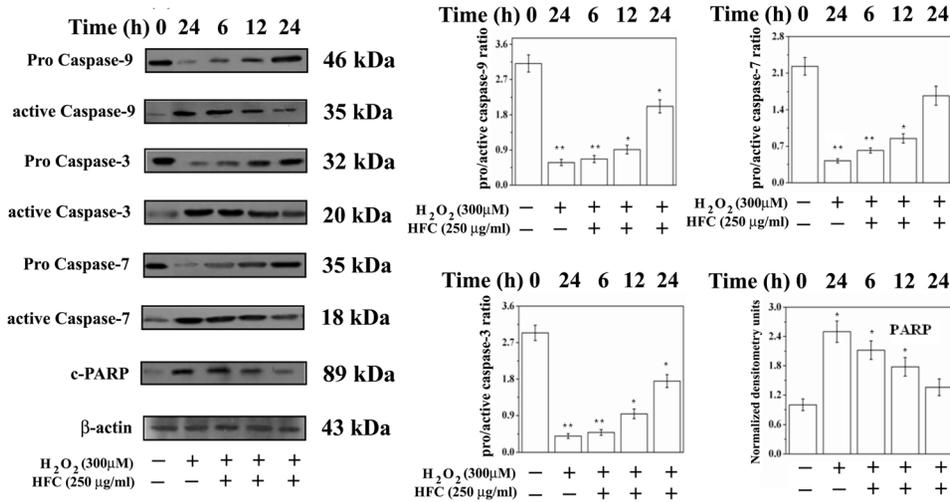


Figure 10. Polysaccharide imposed protection against H₂O₂-induced intrinsic apoptosis of WI38 cells via caspase inhibition at protein level. WI38 cells were incubated in presence (250 μg/ml) or in absence of HFC polysaccharide for 1 h followed by the treatment with 300 μM H₂O₂ in both the cases for varying periods of time (0–24 h). Protein level expression of various pro- and active forms of caspases (caspase-9, -3, and -7) and cleaved PARP was evaluated by immunoblotting. β-actin was used as loading control. Band densitometries are represented as a ratio between pro and active caspases in the form of bar graphs, Results are representative of three independent experiments performed in triplicate and are represented as mean ± SD. A one-way analysis of variance (ANOVA, Bonferroni corrections for multiple comparisons) was performed, where significant level stands for * p<0.05, ** p<0.001. doi:10.1371/journal.pone.0113663.g010

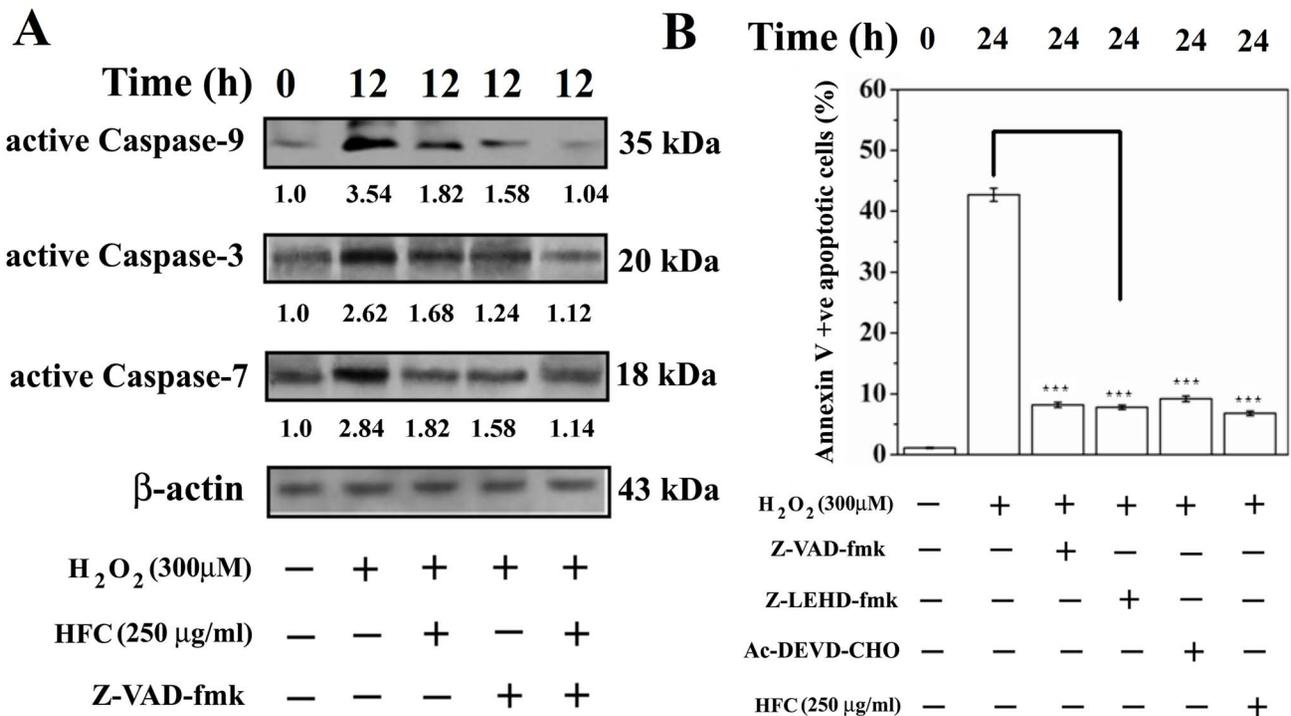


Figure 11. Caspase inhibition assay using pan- and specific caspase inhibitors for intrinsic pathway. The cells were pre-incubated for 1 h individually with 100 μM pan caspase-inhibitor Z-VAD-fmk, caspase-9-specific inhibitor Z-LEHD-fmk (50 μM), caspase-3 and -7-specific inhibitor Ac-DEVD-CHO (50 μM). Then the cells were incubated in presence (250 μg/ml) or in absence of HFC polysaccharide for 1 h followed by the treatment with 300 μM H₂O₂ in both the cases for varying periods of time (0–24 h). **A**) The expression of active forms of caspases (caspase-9, -3, and -7) in the presence of the inhibitors was evaluated by immunoblotting. **B**) Apoptosis was quantified by flow cytometry as described earlier. β-actin was used as loading control. Fold changes are represented as relative values of band densitometries normalized to control and are shown as numbers below the immunoblots. Results are representative of three independent experiments performed in triplicate and are represented as mean value. A one-way analysis of variance (ANOVA, Bonferroni corrections for multiple comparisons) was performed, where significant level stands for * p<0.05, *** p<0.0001. doi:10.1371/journal.pone.0113663.g011

cation was viewed microscopically using immunofluorescence. As depicted in Figure 12A and Figure S2, H₂O₂ induced a significant decrease ($p < 0.05$) in the levels of Nrf2 and Keap1 in the cytosol (66% and 61% decreases, respectively, compared with the control after 24 h) and simultaneously facilitated a significant increase ($p < 0.05$) in their nuclear levels (2.3- and 2.6-fold increases, respectively, compared with the control after 24 h). Immunofluorescence (Figure 12B) also supported significant nuclear translocation of Nrf2 and Keap1 after H₂O₂ treatment. In accordance with protein expression, mRNA expression of Nrf2 and Keap1 was also upregulated (8.6- and 7.5-fold increases, respectively, compared with the control after 24 h) with the applied stress in a time-dependent manner (Figure 13B). The nuclear translocation of Nrf2 subsequently triggered the transcription of a downstream battery of genes. As shown in Figure 13C, H₂O₂ significantly upregulated ($p < 0.05$) the mRNA expression of HMOX1, NQO1, GSTA2, SOD1, and GPX1 (11.5-, 12.8-, 9.8-, 11.8-, and 10.6-fold increases, respectively, compared with the control after 24 h). However, the polysaccharide treatment successfully decreased the intracellular ROS level, which in turn inhibited the nuclear translocation of Nrf2 (Figure 12B), resulting in a time-dependent stabilization of the Nrf2/Keap1 signaling pathway. Therefore, the increase in the nuclear Nrf2 and Keap1 was found to be insignificant ($p > 0.05$) as compared with the control (1.5- and 1.2-fold increases, respectively, after 24 h), which occurred in parallel to the significant increase ($p < 0.05$) in cytosolic levels. As shown in Figure 13B, the mRNA expression of Nrf2 and Keap1 decreased gradually with time back to a level similar to that of the control values after 24 h of polysaccharide treatment. As a result, the post-treatment increase in the mRNA level expression of cytoprotective genes (Figure 13C) was found to be statistically insignificant ($p > 0.05$) (1.4-, 2.2-, 1.9-, 1.9-, and 1.7-fold increases for HMOX1, NQO1, GSTA2, SOD1, and GPX 1, respectively, compared with the control after 24 h), thus passively indicating a decrease in the intracellular level of ROS.

At times, ROS-generating oxidants may confer a relative cooperation between major death signals and cellular defense mechanisms. Hence, to detect whether the nuclear translocation of Nrf2 was mediated by MAPK, the cells were pre-treated with MAPK-specific inhibitors 1 h before any other treatment. As shown in Figure 13A, SP600125 (a JNK-specific inhibitor) and SB203580 (a p38-specific inhibitor) effectively attenuated the phosphorylation and nuclear translocation of Nrf2. The cytosolic level of Nrf2 increased significantly ($p < 0.05$) compared with the cells treated with only H₂O₂ (increases of 74% and 62% for SP600125 and SB203580, respectively, after 6 h). The combined treatment with inhibitor and polysaccharide further reduced the phosphorylation level of JNK and p38 and increased the level of Nrf2 in the cytosol (increases of 144% and 126% for SP600125 and SB203580, respectively, after 12 h). The results suggest that phosphorylation of the MAPKs JNK and p38 may be partially responsible for transducing signals involved in Nrf2 translocation in H₂O₂-treated WI38 cells.

Discussion

The present study was primarily performed to identify the utility of functional bacterial polysaccharides as therapeutic agents against cellular injury during oxidative stress. Experiments were also performed to understand the underlying mechanism of cellular response and signal coordination within human lung fibroblasts during hydrogen peroxide-induced stress and subsequent polysaccharide treatment against stress. *Bacillus megaterium* RB-05, a fresh-water bacterial isolate, biosynthesized two non-

identical EPSs, one in GSM and one in GSM-supplemented JC. Structural elucidation revealed the differences in the compositions of the polysaccharides, especially in fucose content. In earlier reports, fucose-rich polysaccharides from algae, fungi and bacteria were effectively used in therapeutics. The role of fucose-rich polysaccharides as antioxidants has been widely accepted and has consistently been employed for healing critical diseases. Both polysaccharides, the HFC and LFC, were found to have antioxidant potential and effectively scavenged free radicals *in vitro*, although performance of the HFC polysaccharide was found to be superior.

The oxidative stress induced by an overproduction of ROS in the lung causes many clinical conditions including cancer, asthma, cystic fibrosis, ischemia-reperfusion injury, and aging [40]. During this investigation, a fucose-rich bacterial polysaccharide exhibited cytoprotective effects against H₂O₂-induced oxidative stress and the apoptosis of WI38 cells by scavenging intracellular ROS and regulating mitochondrial membrane potential, the Bcl-2 family proteins, the caspase cascade, and the phosphorylation status of MAPKs. The effective decrease in intracellular ROS resulted in the stabilization of Nrf2/Keap1-mediated redox signaling, leading to cellular homeostasis.

Dosage selection remains a critical issue when supplementing natural antioxidants. These exogenous materials may also act as pro-oxidants depending on concentration and the buffering capacity of the cell [13]. In some previous studies, it was shown that fucoidans induce ROS-mediated apoptosis of MCF-7 cells at concentrations of 1 mg/mL [19,41] and 820 µg/mL [19] via caspase-dependent and caspase-independent pathways, respectively, and exhibit antitumor activity in Huh7 cells by downregulating chemokine expression [19,42]. Considering these results, the present investigation optimized the IC₅₀ values to determine the free radical scavenging potential and the cellular cytotoxicity of the polysaccharides *in vitro*. Both the polysaccharides displayed a significant cytoprotective effect during H₂O₂ exposure in WI38 cells at the dose of 250 µg/mL. Flow cytometry provided evidence for the apoptotic death of WI38 cells in response to H₂O₂ administration, with significant changes in the percentages of annexin V-positive and sub-G0/G1 cells; these characteristics were considerably decreased upon HFC polysaccharide treatment. Interestingly, at a similar dose, the polysaccharide had minimal effects on the cell cycle progression of untreated WI38 cells. Usually, during the treatment period, ROS is capable of degrading and depolymerizing polysaccharides, presumably leading to their dysfunction. Prolonged treatment with polysaccharides such as dextran has been shown to limit exogenous and endogenous ROS levels, resulting in smaller fragmentation products of polysaccharides (~10 kDa) [43–46]. Hence, it can be concluded that the external application of these biomacromolecules may passively shield cellular components from oxidative assaults triggered by ROS.

Mitochondria play a pivotal role in the ROS-mediated apoptotic process [20]. It has been found that H₂O₂ induces mitochondrial dysfunction followed by a rapid efflux of intracellular ROS, which further triggers apoptosis of the cells. Literature reports also suggest that the exposure to low doses of H₂O₂ promotes apoptosis rather than necrosis [13]. During an early event of apoptosis, ROS increase the permeabilization and depolarization of the mitochondrial membrane to facilitate a rapid loss of membrane potential [28]. Similarly, in this study, the intracellular ROS led to mitochondrial dysfunction in WI38 cells after H₂O₂ exposure. The HFC polysaccharide successfully suppressed the formation of intracellular ROS, leading to a substantial regaining of mitochondrial membrane potential.

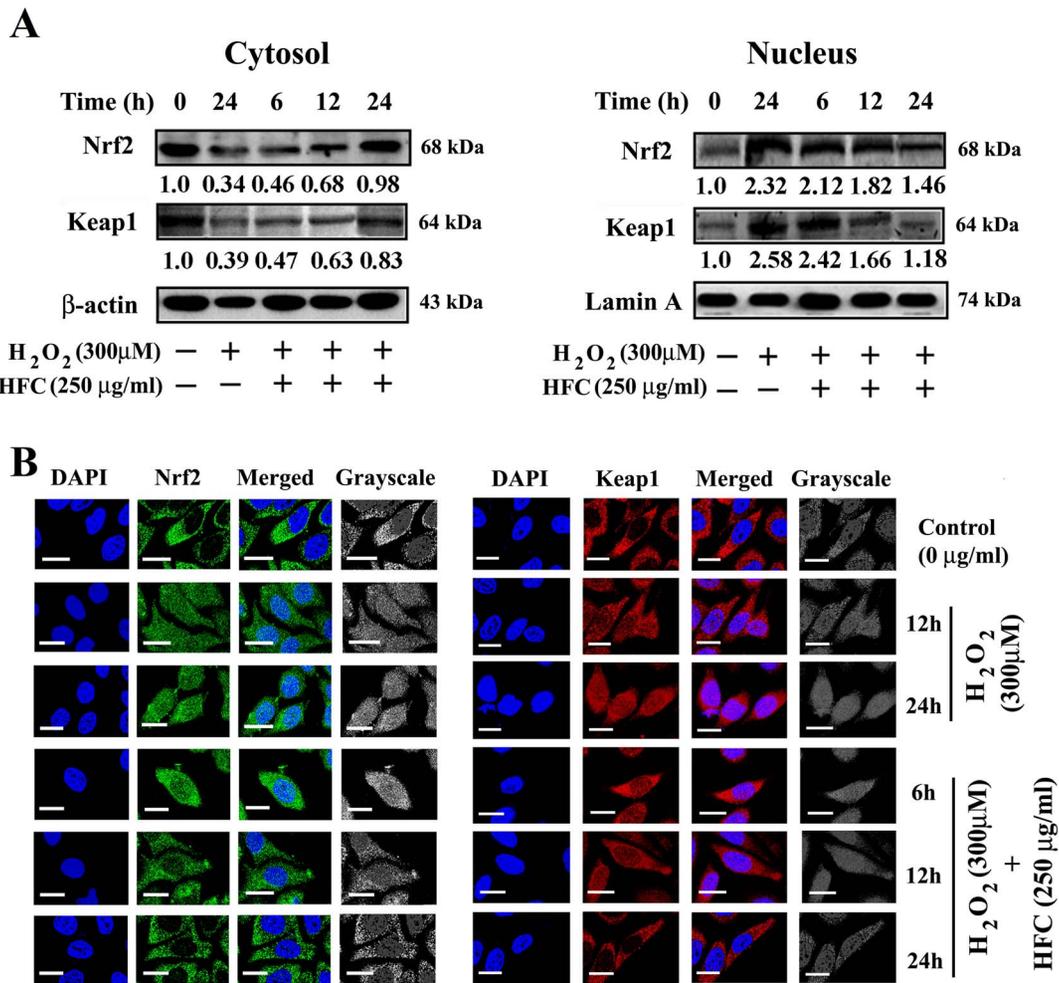


Figure 12. Stabilization of Nrf2/Keap1 signaling and their nuclear translocation induced by HFC polysaccharide. WI38 cells were incubated in presence (250 μ g/ml) or in absence of HFC polysaccharide for 1 h followed by the treatment with 300 μ M H_2O_2 in both the cases for varying periods of time (0–24 h). **A**) Protein level expression of Nrf2 and Keap1 in both cytosolic and nuclear fractions was observed by immunoblotting. β -actin and Lamin A were used as loading control. Fold changes are represented as relative values of band densitometries normalized to control and are shown as numbers below the immunoblots. Results are representative of three independent experiments performed in triplicate and are represented as mean value. A one-way analysis of variance (ANOVA, Bonferroni corrections for multiple comparisons) was performed, where significant level stands for $*p < 0.05$. **B**) H_2O_2 -induced translocation of Nrf2 and Keap1 from cytosol to nucleus and re-localization into cytosol once again during the polysaccharide treatment were monitored under fluorescence microscope using fluorescence-tagged (red and green fluorescence, respectively, for Nrf2 and Keap1) specific antibodies. The cells were counterstained with DAPI (blue fluorescence) to visualize nuclear morphology. Each image shown is representative of 20 random fields observed. Indicated *scale bars* signify 10 μ m distance and photographs were taken at 100 \times zoom.
doi:10.1371/journal.pone.0113663.g012

Thus far, the best-studied regulator of apoptosis is the Bcl-2 family, which contains the integral outer-membrane oncoproteins of the mitochondria. The HFC polysaccharide significantly upregulated the expression of anti-apoptotic proteins and down-regulated the apoptotic proteins, which further attenuated ROS-induced cellular injury and likely inhibited the peroxidation of lung lipids, including pulmonary surfactants, and also reduced membrane permeability and surfactant activity [47]. The Bax/Bcl-2 ratio, a determinant of cell susceptibility to death signals, was found to be decreased in a time-dependent manner with the polysaccharide treatment. ROS-induced imbalances in protein expression during apoptosis form mitochondrial permeability transition pores (PTPs), through which cytochrome c is released to the cytosol from the intermembrane space. However, controversy remains regarding the timing of this phenomenon. As per one opinion, cytochrome c is released before any detectable loss of

$\Delta\Psi_m$, whereas other researchers have postulated that Bax plays a direct role in membrane permeability and promotes loss of $\Delta\Psi_m$ after translocating to the mitochondria, which is followed by the release of cytochrome c. The third group has proposed that the loss of $\Delta\Psi_m$ precedes the mitochondrial translocation of Bax and cytochrome c is released concurrently with membrane collapse [28,48–51]. In the present work, it was shown that the ROS-mediated translocation of cytochrome c and Bax occurred simultaneously and concurrently with $\Delta\Psi_m$ loss. The release of cytochrome c facilitates the formation of the apoptosome complex, thus activating the binding of procaspase-9 and Apaf-1 in the presence of ATP [52]. HFC polysaccharide treatment in stressed cells inhibited the formation of the apoptosome complex and the cleavage of procaspase-9, which is known to be a mediator of caspase-3 activation. This further suppressed the signal that

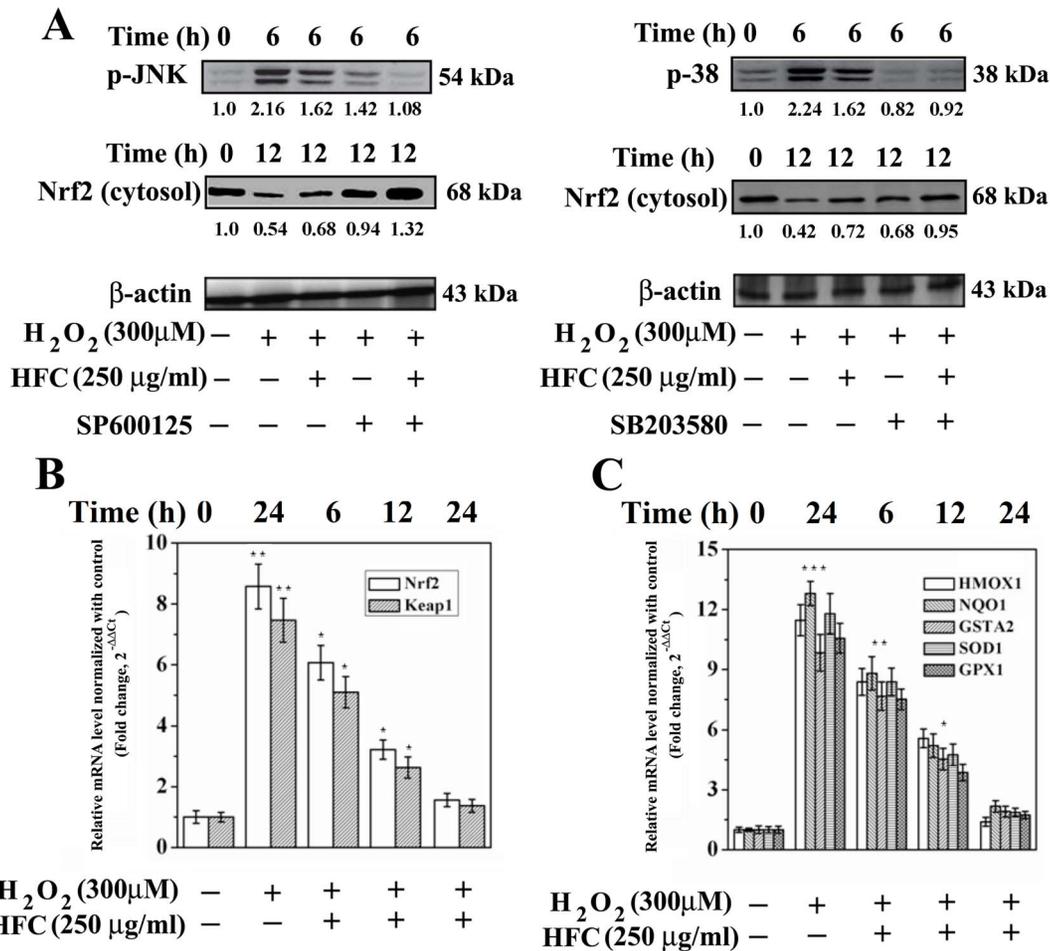


Figure 13. MAPK-mediated activation of Nrf2/Keap1 signaling during H₂O₂-induced apoptosis of WI38 cells. The cells were incubated in presence (250 μ g/mL) or in absence of HFC polysaccharide for 1 h followed by the treatment with 300 μ M H₂O₂ in both the cases for varying periods of time (0–24 h). **A**) Protein level expression of phosphorylated JNK and p38, and cytosolic Nrf2 in the presence of JNK- and p38- specific inhibitors, SP600125 and SB203580, respectively. Before any other treatment, WI38 cells were pre-incubated with 10 μ M inhibitors for 1 h separately. β -actin was used as loading control. Fold changes are represented as relative values of band densitometries normalized to control and are shown as numbers below the immunoblots. Results are representative of three independent experiments performed in triplicate and are represented as mean value. **B**) The mRNA level expression of Nrf2 and Keap1 was quantified using real-time RT-PCR. RNA was extracted from the treated and untreated WI38 cells and after enzymatic reverse transcription, the cDNA content was analyzed by electrophoresis in 2% agarose gel containing 0.1% ethidium bromide. Fold changes were calculated using real-time RT-PCR (SYBR green method). **C**) Similarly, the expression of HMOX1, NQO1, SOD1, GPX1, and GASTA2 at mRNA level was quantified. Fold changes are represented as relative values normalized to control and quantified in the terms of $2^{-\Delta\Delta Ct}$. GAPDH was used as internal control. Results are representative of three independent experiments performed in triplicate and are represented as mean \pm SD. A one-way analysis of variance (ANOVA, Bonferroni corrections for multiple comparisons) was performed, where significant level stands for * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$. doi:10.1371/journal.pone.0113663.g013

induces cleavage of polyadenosine diphosphate ribose polymerase (PARP), which may inhibit apoptosis [53].

Nrf2, a transcription factor that is part of the redox homeostatic gene regulatory network, is activated under any oxidative or electrophilic stress to enhance the expression of the phase II detoxifying enzymes [54]. Under basal conditions, Nrf2 remains an integral part of an E3 ubiquitin ligase complex (the Nrf2-Keap1-Cul3-Rbx1E3 ubiquitin ligase complex) and destined for proteasomal degradation in the cytosol. During any electrophilic assault, a conformational change may take place in the cysteine-rich domain of Keap1 that dissociates Nrf2 from the E3 ubiquitin ligase complex, thus leading to nuclear translocation of Nrf2. Accumulating Nrf2 in the nucleus binds to its transcriptional associate, Maf, forming a heterodimer that binds to the ARE binding site on DNA and activates the transcription of a battery of

cytoprotective genes. Therefore, the expression status of genes such as HMOX1, NQO1, GSTA2, SOD1 and GPX1 can provide an indication of the intracellular ROS level [54–56]. As evident from the present study, the upregulation of the above genes provides the primary signal for increased ROS level after H₂O₂ exposure, whereas after polysaccharide administration, mRNA expression of the cytoprotective genes decreased to baseline with time. Here, it could be hypothesized that the HFC polysaccharide directly scavenged H₂O₂-generated ROS rather than implementing phase II enzymes for the detoxification of ROS, resulting in cellular homeostasis via stabilization of Nrf2/Keap1 signaling. Keap1 plays a post-inductive repressive role in the stabilization of the Nrf2/Keap1 signaling system. A nuclear export sequence (NES) present in Keap1 has been reported to terminate Nrf2/Keap1/ARE signaling. During stress recovery, Keap1 also

translocates into the nucleus to dissociate Nrf2 from the ARE. The Nrf2/Keap1 complex then traffics out of the nucleus and reassociates with the Cul3-E3 ubiquitin ligase in the cytosol. Simultaneously, supplemented antioxidants lower the intracellular ROS and free radical levels and eventually create a reduced intracellular environment that maintains Keap1 in a reduced configuration. With a reduction in the amount of oxidized Keap1, Nrf2 undergoes regular ubiquitin-mediated degradation, which inhibits the nuclear translocation of Nrf2. This subsequently reduces the transcription of cytoprotective genes, and the levels of endogenous phase II enzymes lead to cellular homeostasis [25,54–56].

Earlier reports suggest that oxidative stress triggers the phosphorylation of MAPKs. The most studied kinases, such as p38, ERK1/2 and JNK1/2, play important roles in regulating the pathways involved in H₂O₂-induced cell death [28,39,57–59]. Therefore, the expression status of MAPKs and crosstalk between the MAPK pathway and other signaling pathways were investigated during the stress and polysaccharide treatment periods. An earlier study described the importance of the duration and intensity of JNK activation during cell death [19]. In alveolar epithelial cells, the phosphorylation of JNK begins within a few minutes of H₂O₂ exposure and persists for at least 1–6 h. Levels of both the p-JNK1 and p-JNK2 isoforms were reportedly increased [60]. In the present study, WI38 cells showed a marked difference ($p < 0.05$) in the phosphorylation status of MAPKs between the stress and treatment periods.

MAPK signaling has been implicated in the induction of the Nrf2/Keap1 pathway by many previous reports; this may occur via phosphorylation of Nrf2 or through the nuclear translocation of Nrf2 [26]. The overexpression of JNK2 enhances site-specific phosphorylation of Nrf2 *in vivo*, although this phosphorylation has only a limited role in regulating Nrf2. In another study, the administration of chemical stimulants only slightly altered the ARE-dependent transcription and the protein levels of Nrf2 [26]. In the present investigation, the JNK and p38 MAPK inhibitors SP 600125 and SB 203580 significantly inhibited the nuclear translocation of Nrf2 after H₂O₂ exposure. This suggests that JNK and p38 MAPKs are important mediators of the Nrf2 signaling network in H₂O₂-treated WI38 cells. Simultaneous treatment with the HFC polysaccharide and JNK and p38 MAPKs inhibitors attenuated the phosphorylation of JNK and p38 and relocalized Nrf2 in the cytosol. Figure 14 highlights the evident crosstalk between the death-regulatory signaling system and cellular defense mechanisms.

Conclusions

In the eukaryotic system, antioxidants function by directly by scavenging free radicals and/or inducing the activity of antioxidant enzymes. The present study most likely displayed the first. Both the HFC and LFC polysaccharides showed efficient free radical scavenging activities *in vitro*, although the activity of the HFC polysaccharide was comparatively better. The HFC polysaccharide readily scavenged H₂O₂-induced intracellular ROS, which decreased the oxidative stress in WI38 cells through stabilization of the Nrf2/Keap1 signaling system and regulation of cytoprotective genes involving the MAPK and mitochondria-mediated pathways. In future studies, this molecular mechanism needs to be validated *in vivo*; a positive result, if confirmed, would provide priceless information to the development of new approaches for effective stress-responsive antioxidants.

Materials and Methods

Materials

Human embryonic fibroblast lung cell line (WI38) was purchased from the National Centre for Cell Science (Pune, India). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS) and antibiotic/antimycotic solution and gentamycin were purchased from HyClone, Thermo Fisher Scientific (Waltham, MA). All primary and secondary antibodies were procured from Santa Cruz Biotechnology (Santa Cruz, CA) and Cell Signaling Technology (Beverly, MA). Alexa fluor 660-conjugated anti-goat secondary antibody and FITC-conjugated anti-rabbit, anti-mouse, and anti-goat secondary antibodies were from Invitrogen (Carlsbad, CA) and Millipore (Billerica, MA, USA). The pan caspase inhibitor (Z-VAD-fmk) and the caspase-3/7 inhibitor (Ac-DEVD-CHO) were purchased from Promega (Madison, WI). The caspase-8 inhibitor (Ac-IETD-CHO) and the caspase-9 inhibitor (Z-LEHD-fmk) were purchased from BD Biosciences (San Diego, CA). The JNK inhibitor SP600125 and the p38 inhibitor SB203580 were purchased from Enzo Life Sciences International, Inc. (Plymouth, PA). DiOC6, H₂DCF-DA, MitoTracker Red, SYBR Green Master Mix, and TRIzol reagent were procured from Invitrogen, Carlsbad, CA. All other chemicals were from Merck (Mumbai, India and Darmstadt, Germany), Sigma Aldrich (St. Louis, MO), and Himedia (Mumbai, India) unless indicated otherwise.

Extracellular polysaccharide (EPS) production in different fermentation media

Bacillus megaterium RB-05 biosynthesized EPSs in glucose mineral salts medium (GMSM) and jute culture (JC). The isolation of the crude EPSs from the cultures and their subsequent purification were performed as described previously [30,31]. Carbohydrate content was spectrophotometrically determined at 490 nm (750 Lambda Double Beam UV-Vis Spectrophotometer, Perkin Elmer) using the phenol-sulfuric acid method [61], and protein content was analyzed at 595 nm according to the Lowry method [62]. Molecular weights of the purified EPSs were derived from the standard plot of the reference dextran.

Analysis of monosaccharide composition

Lyophilized polysaccharide (10 mg) was hydrolyzed with 2 mol/L trifluoroacetic acid (TFA) for 2 h at 121°C. TFA was removed using a rotary vacuum evaporator. The monosaccharide composition was identified by HPLC separation of their anthranilic acid derivatives, which were obtained as described by Anumula [63].

Structural elucidation

A solution of 5 mg of purified EPS in 0.5 mL of DMSO (dimethyl sulfoxide) was methylated by adding finely powdered NaOH (20 mg) and methyl iodide (0.1 mL) prior to sonication for 15 min. The methylated products were extracted with CHCl₃ and H₂O (5:2, v/v). The CHCl₃ phase was separated and dried under N₂ and hydrolyzed in 2 M TFA at 100°C for 6 h. The hydrolyzed EPS was reduced with 50 mM sodium borohydride at room temperature for 4 h, evaporated three times from a mixture of acetic acid/methyl alcohol (1:1), and acetylated in 50:50 acetic anhydride/pyridine at 100°C for 90 min. Alditol acetates of the methylated sugars were analyzed using a Shimadzu GCMS-QP2010. NMR spectra were obtained using a Bruker Avance DPX-500 MHz NMR Spectrometer (Bruker Co., Billerica, MA).

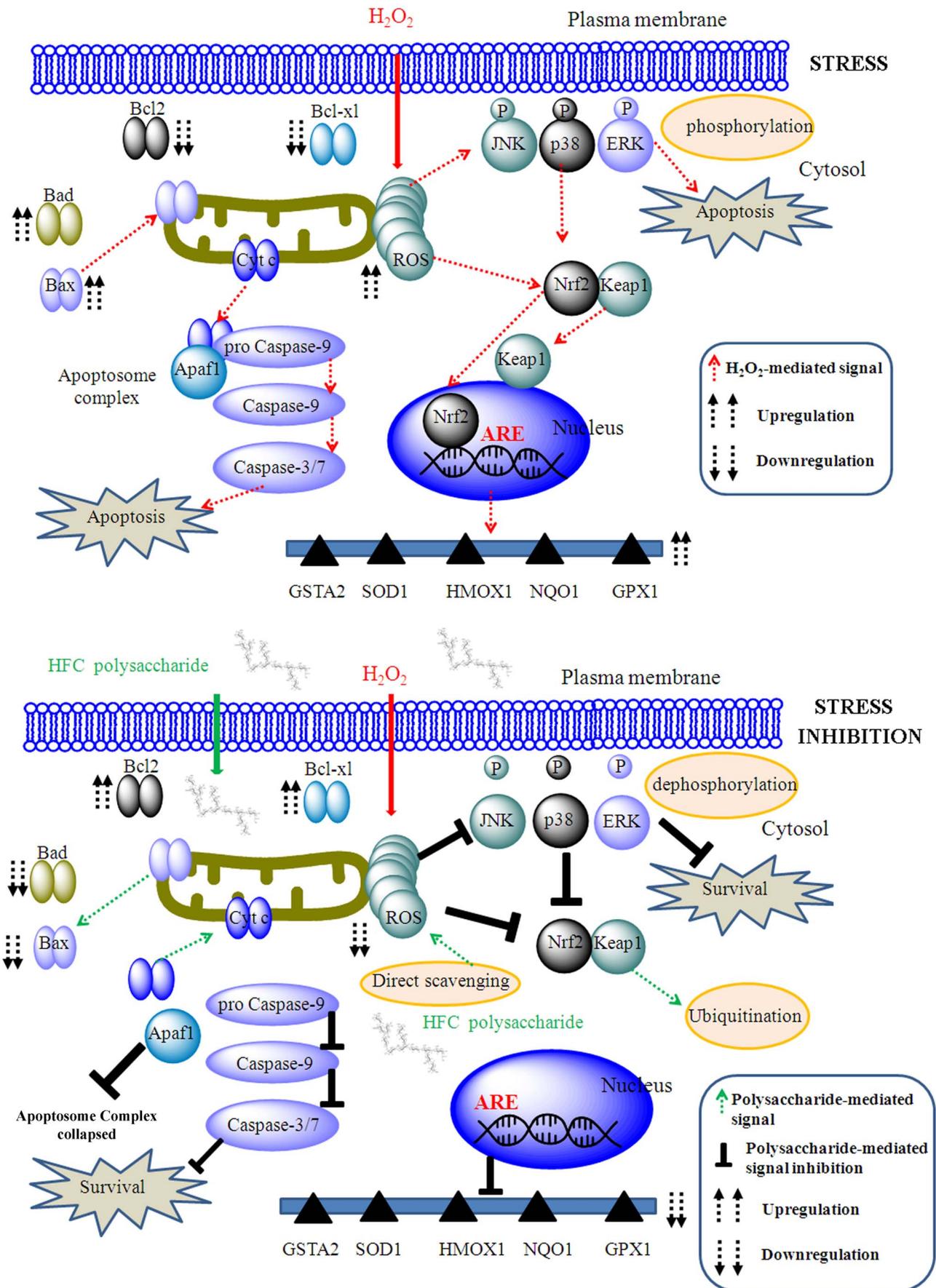


Figure 14. Plausible signaling cross-talk involved in the HFC polysaccharide treatment against H_2O_2 -induced apoptosis of WI38 cells.
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The proton and ^{13}C spectra were run at a probe temperature of 40°C . The purified samples were dried in a vacuum over P_2O_5 and then exchanged with deuterium by lyophilizing several times with D_2O . The EPS was dissolved in 0.7 mL of D_2O (99.96%) at concentrations of 15 mg/mL (^1H NMR) and 30 mg/mL (^{13}C NMR). The 2D COSY experiment was recorded at a mixing time of 150 ms.

Free radical scavenging activity of the EPSs

Non-specific hydroxyl radical scavenging activity was assayed as described by Elizabeth and Rao [64] with slight modifications. The procedure for measuring site-specific hydroxyl radical scavenging activity was identical to that detailed above, but EDTA was replaced by an equal volume of buffer as reported previously [65]. Hydrogen peroxide scavenging activity was determined according to Hazra et al. (2008) [2]. The production of singlet oxygen ($^1\text{O}_2$) was determined by monitoring *N,N*-dimethyl-4-nitrosoaniline (RNO) bleaching following Hazra et al. (2008) [2]. Superoxide radical scavenging activity was measured by the reduction of nitro blue tetrazolium (NBT) [66,67]. In enzymatic assays, superoxide radicals were generated by the xanthine/xanthine oxidase (XO) system [66,67]. The effect of the tested samples on XO activity was evaluated by measuring the formation of uric acid from xanthine at 295 nm. The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging was performed following Hazra et al. [2]. Antioxidant capacity was measured based on the scavenging of ABTS^+ by the test samples in comparison to a Trolox standard [2]. The antioxidant activity of the polysaccharides was also evaluated using the β -carotene linoleate model system.

Cell culture

The WI38 cell line was cultured in DMEM supplemented with 10% FBS and antibiotic/antimycotic solution (100 units) and gentamycin (50 $\mu\text{g}/\text{mL}$) with Na pyruvate (1 mM) in a humid incubator at 37°C in 5% CO_2 . After 24 h, the medium was replaced with fresh medium containing various concentrations of GMSM- and JC-derived fucose polysaccharides for the initial screening. Cells grown in medium containing phosphate-buffered saline (PBS) without polysaccharide served as control.

MTT assays

WI38 cells were seeded in 96-well plates at a density of 3000 cells/well in 200 μl medium. The cells were incubated in presence (50–500 $\mu\text{g}/\text{mL}$) or in absence of HFC polysaccharide for 1 h followed by the treatment with H_2O_2 (0–500 μM) in both the cases for varying periods of time (0–24 h). After completion of the treatments, 10 μl of MTT (5 mg/mL) was added to each well. The formazan complex was dissolved in 100 μl dimethyl sulfoxide (DMSO) after 3 h of incubation. The absorbance of each well was measured at 570 nm with a microplate reader.

Phase contrast microscopy

WI38 cells were seeded onto 96-well plates (5×10^3 cells/well). The cells were incubated in presence (50, 100, 200 and 250 $\mu\text{g}/\text{mL}$) or in absence of HFC polysaccharide for 1 h followed by the treatment with 300 μM H_2O_2 in both the cases for varying periods of time (0–24 h). Cell growth and morphology were captured throughout the treatment period using a U-TVO 63 \times C microscope fit with an Olympus CAMEDIA digital wide-zoom camera, model C-7070 (Olympus Corp., Tokyo, Japan).

Cell cycle analysis and determination of apoptotic cells by flow cytometry

WI38 cells were seeded in a 6-well culture plate at a density of 5×10^4 cells/well and incubated in DMEM containing 10% FBS. The cells were incubated in presence (50, 100, 200 and 250 $\mu\text{g}/\text{mL}$) or in absence of HFC polysaccharide for 1 h followed by the treatment with 300 μM H_2O_2 in both the cases for varying periods of time (0–24 h). Evaluation of the cell cycle distribution and sub- G_0/G_1 peaks by flow cytometry was performed by measuring PI-fluorescence with a BD FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA) through an FL-2 filter (585 nm). For each sample, 1×10^4 events were recorded. Flow cytometry data were analyzed using Cell Quest.

The presence of apoptotic cells was determined by the ability of cells in suspension to bind to annexin V. Control and treated WI38 cells were adjusted to 5×10^5 cells/mL in binding buffer (10 mM HEPES [(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] [pH 7.4], 140 mM NaCl, 2.5 mM CaCl_2). Then, 10 μl of FITC-annexin V was added to 190 μl of cell suspension. The mixture was incubated for 10 min at room temperature. After centrifugation, the cells were resuspended in 190 μl binding buffer, and 10 μl PI (1 mg/mL) solution was added. The cells were then analyzed in a FACS Calibur flow cytometer; for each sample, 1×10^4 events were acquired. The cells positive for both PI and annexin V were considered to be necrotic cells and thus excluded from the analysis.

Determination of intracellular ROS and mitochondrial membrane potential (MMP)

The generation of intracellular ROS was analyzed with the oxidation-sensitive fluorescent probe $\text{H}_2\text{DCF-DA}$ using a FACS Calibur flow cytometer at excitation and emission wavelengths of 488 nm and 544 nm, respectively. The loss of mitochondrial transmembrane potential was verified by flow cytometry at the single-cell level. Control and WI38 cells treated for different periods of time were incubated with DiOC6 (50 nM) for 15 min at 37°C in the dark. Loss of DiOC6 fluorescence indicates disruption of the mitochondrial inner transmembrane potential. The probe was excited at 488 nm, and emission was measured through a 530 nm band pass filter with the FACS Calibur.

Preparation of sub-cellular fractions

For determination of Nrf2 and Keap1 protein levels in cytosolic and nuclear compartments, control and treated WI38 cells of different time periods were lysed in a hypotonic buffer (10 mM HEPES [pH 7.9], 1.5 mM MgCl_2 , 10 mM KCl, 0.5 mM DTT, and 1 \times Complete Protease Inhibitor Cocktail [Roche, Molecular Biochemicals, Indianapolis, IN]). After centrifugation ($20,000 \times g$), the cytosolic proteins in the supernatant were collected, and the nuclear pellets were extracted with a high salt buffer (20 mM HEPES, pH 7.9, 1.5 mM MgCl_2 , 20% glycerol, 0.2 mM EDTA, 300 mM KCl, 0.5 mM DTT, 1 \times Complete Protease Inhibitor Cocktail) by placing it into the ice for 30 min, which was followed by centrifugation ($20,000 \times g$) to collect the nuclear extracts.

Mitochondrial fraction was isolated following Frezza et al., [68] to determine the expression levels of cytochrome c and Bax protein in the control and treated cells of different time periods. PBS-washed cells (120×10^6) were suspended in ice-cold isolation buffer (0.1 M Tris-MOPS [3-(*N*-morpholino) propanesulfonic acid], 0.1 M EGTA/Tris, and 1 M sucrose, pH 7.4) for 30 min, homogenized, and centrifuged at $600 \times g$ for 10 min at 4°C . Collected supernatant was centrifuged at $7000 \times g$ for 10 min at 4°C . The pellet was washed with isolation buffer, resuspended in

200 μ l of ice-cold isolation buffer, and then again centrifuged at 7000 \times g for 10 min at 4°C. The supernatant was discarded to obtain the pellet containing mitochondria.

To measure the expression levels of Bcl-2, caspase and MAPK family proteins, PBS-washed cells were lysed in ice-cold RIPA lysis buffer (150 mM sodium chloride, 1.0% TritonX-100, 50 mM Tris pH 8.0, 0.01% SDS, 0.5% sodium deoxycholate) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) (SRL, Mumbai, India), 1 μ g/mL of aprotinin, leupeptin and pepstatin. The samples were incubated on ice for 30 min and centrifuged at 20,000 \times g for 15 min at 4°C and protein containing supernatant was collected. Protein concentration of all the fractions was determined using the Bradford reagent (Sigma-Aldrich, St. Louis, MO) and subsequent measurement of absorbance was done at 595 nm in a UV-1700 PharmaSpec, Shimadzu spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD). The remaining supernatant was stored at -20°C.

Western blot analysis

Cell lysates were diluted in sample buffer (0.312 mM Tris-HCl (pH 6.8), 50% glycerol, 10% SDS, 25% β -mercaptoethanol, and 0.25% bromophenol blue) at a final protein concentration of 5 μ g/ μ l, and were then boiled at 100°C for 5 min. Aliquots of each sample (10 μ l containing 50 μ g protein) were loaded into dedicated wells of 9–12% polyacrylamide gels and separated by electrophoresis for 3 h at 100 V. Proteins were transferred to polyvinylidene difluoride membrane (Amersham Biosciences, Piscataway, NJ) for 1.5 h at 300 mA. After blocking of nonspecific binding with 5% nonfat dry milk in TBST (Tris-buffered saline-Tween), the membranes were then probed with primary antibodies (Text S1) and incubated overnight at 4°C. The membranes were washed with Tris-buffered saline-0.01% (v/v) containing Tween-20 at room temperature for 15 min and then incubated with alkaline phosphatase (AP)- or horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-rabbit, anti-goat and anti-mouse IgG; 1:1000 dilution) in TBST for 2 h at room temperature. The membranes were developed according to the laboratory protocols. The band intensity of the detected protein was measured by densitometry (Gel Doc XR+ System, Bio-Rad Laboratories, Berkeley, CA). β -actin, Lamin A, and COX 4 were chosen as loading controls for constitutive expression in cytosolic, nuclear and mitochondrial fraction, respectively.

Inhibitors

WI38 cells were treated with different caspase inhibitors (Z-VAD-fmk, Ac-DEVD-CHO, Z-IETD-fmk and Z-LEHD-fmk), MAPK inhibitors (SP600125, JNK specific inhibitor and SB203580, p38 specific inhibitor) for 1–2 h prior to polysaccharide and H₂O₂ treatment.

RNA isolation, reverse transcription (RT), and real-time PCR

RNA was extracted using the TRIzol reagent and first-strand cDNA was synthesized from 1 μ g total RNA using Random Hexamer (Promega, Madison, WI) and MMLV high performance reverse transcriptase enzyme (Epicentre Biotechnologies, Madison, WI) according to the manufacturer's instructions. The reverse transcriptase PCR was performed using the 2720 Thermal Cycler from Applied Biosystems (Foster City, MA). The PCR program involved subsequent incubations at 25°C for 10 min, 37°C for 60 min, 85°C for 5 min, and 4°C for 1 min. The cDNA content was analyzed by electrophoresis in 2% agarose gel containing 0.1% ethidium bromide. Real-time PCR amplifications were

performed in triplicate using the SYBR Green I assay and were carried out using ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, MA). The reactions were carried out in a 96-well plate in 20- μ l reactions containing 2 \times SYBR Green Master Mix, 2 pmol each of forward and reverse primer, and a cDNA template corresponding to 25 ng total RNA. The real time PCR program included activation at 50°C for 2 min, initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 1 min. Presence of <200 bp amplicons was checked in 2% agarose gel. The threshold cycle (C_t) was obtained from the PCR curves and expression levels of the target mRNA were quantified in terms of the C_t values of the untreated and treated samples and were normalized with the C_t values of GAPDH (internal control). The mRNA expression was quantified in terms of 2^{- $\Delta\Delta$ C_t}. Primer sequences (Table S4) were designed using the NCBI-Primer BLAST online tool and synthesized commercially.

Immunofluorescence analysis

WI38 cells were grown in poly-L-lysine (0.1 mg/mL) coated sterile cover slip. Control and treated WI38 cells of different time periods were incubated with 100 nM MitoTracker Red for 30 min for mitochondrial staining. Then the cells were rinsed three times with PBS, fixed for 10 min in 4% *p*-formaldehyde, and permeabilized for 10 min with 0.1% Triton X-100, followed by rinsing with PBS containing 1.0% bovine serum albumin for three times. The permeabilized cells were incubated with primary antibodies for 1 h at 37°C in a moist chamber. After PBS wash, the cells were incubated with Alexa fluor 660- or FITC-conjugated secondary antibodies for 1 h at 37°C in a moist chamber. DAPI (4',6-diamidino-2-phenylindole, dihydrochloride) was used as a nuclear stain. The cells were visualized using U-IVO 63 \times C microscope fit with Olympus CAMEDIA digital wide zoom camera, Model C-7070 (Olympus Corp., Tokyo, Japan).

Statistical analysis

The experiments were performed in triplicates. Data shown are representative of at least three experiments and are represented as mean \pm SD. Inhibition concentration (IC₅₀) values were calculated from pharmacological dose-response curve fit (sigmoidal) following the equation:

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / [1 + 10^{\wedge}\{(\text{LogIC}_{50} - X) * \text{Hill slope}\}]$$

ANOVA (Bonferroni corrections for multiple comparisons) was employed to assess the statistical significances of differences among pair of data sets with a p<0.05 considered to be significant. The statistical analysis was performed using Origin 8 software.

GenBank

16 rRNA gene sequence of *Bacillus megaterium* RB-05: GenBank Accession Number HM371417

Supporting Information

Figure S1 Protective effect of LFC and HFC polysaccharides on H₂O₂-induced cytotoxicity. Initially, WI38 cells were treated with various concentrations of H₂O₂ alone for 24 h and cell viability was determined by MTT assay for initial dose optimization. The cells were incubated in presence (50–500 μ g/mL) or in absence of HFC polysaccharide for 1 h followed by the

treatment with H₂O₂ (0–500 μM) in both the cases for varying periods of time (0–24 h). Cell viability was determined by MTT assay. Results are representative of three independent experiments performed in triplicate and are represented as mean ± SD.

(TIF)

Figure S2 The protective effect of HFC polysaccharide on H₂O₂-induced apoptosis of WI38 cells by regulating protein level expression of Bcl-2 family, caspase family and translocation of Bax, cytochrome c, Nrf2 and Keap1.

The cells were incubated in presence (250 μg/mL) or in absence of HFC polysaccharide for 1 h followed by the treatment with 300 μM H₂O₂ in both the cases for varying periods of time (0–24 h). Band densitometries of the immunoblots were compared between only H₂O₂ treatment and combining treatment of H₂O₂ and HFC polysaccharide. β-actin, COX4 and Lamin A were used as loading control.

(TIF)

Figure S3 The protective effect of HFC polysaccharide on H₂O₂-induced apoptosis of WI38 cells by regulating phosphorylation of MAPKs.

The cells were incubated in presence (250 μg/mL) or in absence of HFC polysaccharide for 1 h followed by the treatment with 300 μM H₂O₂ in both the cases for varying periods of time (0–24 h). Protein level expression of total and phosphorylated JNK, p38, and ERK was evaluated by immunoblotting. β-actin was used as loading control. Fold changes are represented as relative values of band densitometries normalized to control and are shown as numbers below the immunoblots. Results are representative of three independent experiments performed in triplicate and are represented as mean value. A one-way analysis of variance (ANOVA, Bonferroni corrections for multiple comparisons) was performed, where significant level stands for * p<0.05, ** p<0.001.

(TIF)

Table S1 Antioxidant and free radical scavenging potential of HFC and LFC polysaccharides with respect

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to standard materials. Inhibition concentration (IC₅₀) values were determined from the fitted sigmoidal dose-response curves as derived from the equation mentioned in the 'materials and methods'.

(DOCX)

Table S2 Partially methylated alditol acetate derivatives of HFC polysaccharide. Calculated from peak areas and response factors obtained using a flame ionization detector.

(DOCX)

Table S3 ¹³C NMR^d and ¹H NMR^c chemical shifts for HFC polysaccharide recorded in D₂O at 40°C.

(DOCX)

Table S4 Designed primers for real-time PCR. The primers were designed using online database from National Center for Biotechnology Information (NCBI), NCBI-BLAST, and online PrimerQuest tool of Integrated DNA Technologies (IDT) as discussed in the 'Materials and methods' section.

(DOCX)

Text S1 List of used primary antibodies and the sources.

(DOCX)

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Author Contributions

Conceived and designed the experiments: SRC SS RKB RS BA AB. Performed the experiments: SRC SS SB RKB TKS. Analyzed the data: SRC SS SB RKB TKS. Contributed reagents/materials/analysis tools: RS BA AB. Wrote the paper: SRC SS SB RKB RS BA AB.

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