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Original article

Design and characterization of a novel *Arthrospira platensis* glutathione oxido-reductase-derived antioxidant peptide GM15 and its potent anti-cancer activity via caspase-9 mediated apoptosis in oral cancer cells

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ABSTRACT

Glutathione oxido-reductase (GR) is a primary antioxidant enzyme of most living forms which protects the cells from oxidative metabolism by reducing glutathione (GSH) from its oxidized form (GSSG). Although the anti-oxidant role of the enzyme is well characterized, the specific role of conserved N' peptide sequence in antioxidant mechanism remains unclear. In this study, we have identified an RNA sequence encoding GR enzyme from spirulina, *Arthrospira platensis* (*Ap*) and the changes in its gene expression profile was analysed during H_2O_2 stress. Results showed that H_2O_2 (10 mM) stimulated the expression of *Ap*GR throughout the timeline of study (0, 5, 10, 15 and 20 days) with highest expression at 5th day post-exposure which confirmed the antioxidant role of *Ap*GR in spirulina during H_2O_2 induced oxidative stress. A dithiol containing short antioxidant peptide, ^{39}GGTCVIRGCVPKKLM^{53} (GM15) from *Ap*GR was predicted and its radicals (superoxide and hydroxyl radical) scavenging potential was confirmed by *in vitro* cell-free assays. GM15 (12.5 μM) reduced the intracellular generalized oxidative stress level, as measured using DCFDA assay in H_2O_2 exposed leucocytes without affecting any of the cellular population. Further, the biomedical application of the radical scavenging property of GM15 was validated in oral carcinoma (KB) cells where GM15 exhibited significant cytotoxicity. Also, GM15 exhibited heterogenous effects on intracellular oxidative stress level in KB cells: at lower concentration (6.25 μM), the peptide reduced oxidative stress whereas, at higher concentration (25 μM) it increased the intensity of oxidative stress. GM15 (25 μM) induced caspase-9 mediated apoptosis in KB cells along with membrane disruption and DNA degradation which are confirmed by propidium iodide (PI) internalization and comet assays, respectively. Overall, the study shows that GM15 peptide i) scavenges superoxide, hydroxyl radicals, and influences intracellular oxidative stress, and ii) has anti-cancer effect in oral cancer cells.

1. Introduction

Role of oxidants in carcinogenesis is well documented and the redox imbalance is a signature event in almost all types of cancers which play crucial roles in both tumour development and progression [39]. In normal cells, a delicate equilibrium exists in the redox signaling pathway where the rate and magnitude of oxidant production is balanced by the endogenous antioxidant defense system like superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase. These endogenous antioxidants play a crucial role in protecting cells from DNA damage by free radicals generated during inflammation and carcinogens, thus preventing tumorigenesis [24]. Previous study [53]

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reported that the glutathione (GSH) and glutathione related enzymes protect tissues from the detrimental effect of free radicals, radiotherapy and chemotherapy attacks in human tissues. Moreover, it also been stated that the drug resistance pattern of cells is proportionate to the level of intracellular GSH. A typical example for this is that the elevation of GSH reduces the effects of platinum in ovarian cancer cells [8]. However, the functional importance of the GSH-linked antioxidant activity in drug resistance among cancer is least concerned by the research community. Among the cascade of GSH-associated enzymes, glutathione reductase and glutathione peroxidase are the major enzymes involved in the redox metabolic cycle.

Glutathione oxido-reductase (GR) is a homodimeric flavoprotein disulfide oxido-reductase which mainly involved in the prevention of oxidative damage within the cell by helping to maintain adequate levels of intracellular GSH. Glutathione reductase in concert with its co-factor, NADPH catalyzes the reduction of oxidized glutathione (glutathione disulfide, GSSG) to glutathione [57]. Apart from glutathione homeostasis, dithiol-containing GR involved in redox homeostasis as well as regulation of apoptosis in cancer cells [1]. Apoptosis controls the cancerous cell growth by inducing cell death without causing damage to the neighbour cells where pro-apoptotic enzymes such as caspases play an important role. Levels of free radical species linked to apoptotic activity can be correlated with regulation of caspases. Excess of intracellular reactive oxygen species such as superoxide anion, hydroxyl radicals, hydrogen peroxide and singlet oxygen may attack the macromolecules such as DNA, RNA, protein and lipids leading to many physiological disorders including cancer, cardiovascular, neurodegenerative, diabetes, inflammatory, aging and atherosclerosis diseases. Antioxidant may have an optimistic effect on human health while they can defend human body against deterioration by free radical species [10,41].

So far, several antioxidant compounds have been generated that target oxygen free radicals in cancer cells. Recently, use of peptides as therapeutic option is also being explored. The activity of antioxidant peptides are closely related to their molecular mass, amino acid compositions, structure, sequences and hydrophobicities [55]. Especially, peptides derived from immunologically relevant proteins received maximum attention because such drugs exhibit unique mode of action and target specificity. Therefore, the current study aims to generate peptide derived from glutathione reductase and its effect on cancer cells.

In this study, a full-length cDNA sequence of glutathione oxido-reductase gene was identified (*Ap*GR) from the previously constructed transcriptome database of spirulina [34]. H_2O_2 induced expression modulation of *Ap*GR gene was analyzed by qRT-PCR technique to understand the antioxidant role of the protein during oxidative stress. During multiple sequence alignment, a short peptide sequence at the N-terminal of *Ap*GR protein remained evolutionarily conserved between algae and human which is very intriguing. Physico-chemical parameters such as molecular mass, amino acid compositions, structure, sequences, hydrophobicity nature of the peptide region as well as the location of the peptide in GR domain denoted that the peptide might involve in oxidant scavenging mechanism. Therefore, the peptide (GM15) was synthesized chemically and its antioxidant mechanisms were evaluated by various functional assays. In addition, cytotoxicity of the peptide against normal cells was analyzed by exposing human blood leucocytes to the peptides. Moreover, changes in intracellular redox equilibrium in human blood leucocytes and oral cancer cells (KB) by GM15 peptide was analyzed by flow cytometry and fluorescence microscopy. Further the anticancer role of GM15 against KB cells was determined by MTT assay and its involvement in apoptosis process was confirmed by caspase-9 gene expression up-regulation patterns. Propidium Iodide (PI) internalization assay was performed to confirm the effect of GM15 on KB cell membrane integrity. The comet assay was performed to confirm the GM15 induced DNA damage in KB cells.

2. Materials and methods

2.1. Spirulina cultivation

Fresh healthy spirulina cells were collected from Potheri Lake, Chennai (12.825527°N 80.039606°E) and single spirulina cell was isolated by dilution method and the isolate was grown in modified Zarrouk's medium at 30 °C under illumination conditions as mentioned in our previous report [33,48]. Further, the isolated spirulina sample was confirmed as *A. platensis* by 16S rRNA sequencing and phylogenetic analysis and the gene sequence was submitted to GenBank NCBI repository (Accession No. KY393096).

2.2. H_2O_2 challenge and spirulina cells collection

Spirulina cells were acclimatized for 30 days by culturing as described in section 2.1. To determine the effect of H_2O_2 stress on spirulina, algal cells were grown in two different conditions: i) 10 mM concentration of 30% H_2O_2 stress (30% w/w in H_2O, Sigma-Aldrich) and ii) control cells were maintained without H_2O_2 stress. Further at five various time points (day 0, 5, 10, 15 and 20) of post challenge, algal cells were collected from challenged spirulina culture. For each experiment, spirulina cultures were taken in triplicate along with control at different time points. All the spirulina cells were flash frozen in liquid nitrogen and subsequently stored at −80 °C until further examination.

2.3. Validation of ApGR sequence and its expression modulation during H_2O_2 stress

From the culture medium, the spirulina cells were collected and the total RNA was isolated from both control and challenged spirulina cells using TRIzol reagent (Life Technologies, Rockville, MD, USA) as mentioned by the manufacturer. Further, cDNA was synthesized from the template RNA by using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics GmbH, Germany) according to the manufacturer's protocol with oligo (dT) as primer. The targeted gene expression of *Ap*GR was analysed using Fast SYBR® Green Master Mix (Roche Diagnostics GmbH, Germany) in Light Cycler 96 Real Time PCR system as mentioned in our previous studies [4,5,42]. Using 16S rRNA as internal control, the relative quantification of *Ap*GR gene was performed and the change in the folds of expression were recorded. For this study, to amplify the *Ap*GR mRNA, Primer Quest tool was used to design the following primers: *Ap*GR F1: CAT CGC TAC AGC GGT ATT CTC (Sense) and *Ap*GR R2: AAC ACA GCC CGA TAG CAT TTA (Antisense). For internal control, the primers from 16S rRNA gene of *A. platensis* was used (GenBank Accession No. KY393096): 16S rRNA F3: CGT AAA CCT CTC CTC AGT TCA G (Sense) and 16S rRNA R4: GAA CGG ATT CAC CGC AGT AT (Anti sense). Melting curve analysis was performed to verify the purity of the amplification product. The gene expression quantification was analysed by $2^{-\Delta\Delta CT}$ method using the Light Cycler 96 software (Version 1.1.0.1320). All the reactions were performed in triplicates and the Cq values were recorded for each reaction. The primer efficiency of the qRT-PCR primers of *Ap*GR and 16S rRNA have been calculated as 98.55% and 99.07%, respectively.

2.4. ApGR cDNA sequence analysis

A full length *Ap*GR cDNA sequence was identified from spirulina transcriptome which was obtained by Illumina NextSeq500 technology as provided in our earlier study [34]. The identified *Ap*GR cDNA sequence was submitted to the European Nucleotide Archive (EMBL Nucleotide Sequence Database). The protein sequence was derived from identified *Ap*GR cDNA sequence by using ExPASy Translate tool (https://web.expasy.org/translate/). Further, the physico-chemical properties of cDNA and its protein sequence including open reading

frame (ORF), molecular weight, instability index, aliphatic index and theoretical isoelectric point (pI) was determined using ExPASy ProtParam tool (http://web.expasy.org/protparam/). Homologous analysis, conserved domain identification, primary, secondary and tertiary structures, multiple sequence alignment (MSA), putative cleavage site of the signal peptide, subcellular location of *Ap*GR were analysed as reported in the earlier study [6].

2.5. Prediction and synthesis of antioxidant peptide

During multiple sequence alignment, a short peptide sequence remained evolutionarily conserved between algae, fish, birds and human. Therefore, the peptide sequence was subjected to multi-factor screening such as amino acid compositions, amino acids sequence, molecular weight, evolutionary conserved regions and hydrophobicity. After screening, the conserved peptide sequence from the glutathione reductase N-terminal region (GM15: GGTCVIRGCVPKKLM) was determined with potential antioxidant properties. To calculate the hydrophobic moments (μH) of GM15, the HeliQuest online tool (http://heliquest.ipmc.cnrs.fr/cgi-bin/ComputParams.py) was used and the helical wheel structure of GM15 was predicted by online tool (http://rzlab.ucr.edu/scripts/wheel/wheel.cgi?sequence). PEP-FOLD (ver. 3.5) *de novo* peptide structure prediction server (http://mobyle.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py#forms::PEP-FOLD3) was used to determine the three-dimensional structural conformation of GM15 peptide. Followed by the prediction, the GM15 peptide as well as the scrambled peptide (reverse sequenced) was synthesized at Zhengzhou Peptides Pharmaceutical Technology Co. Ltd. (China) and the peptide sequence was confirmed by MALDI-TOF MS analysis.

2.6. In vitro evaluation of free radical scavenging potential of GM15

To evaluate the antioxidant activity exhibited by the peptide, the following antioxidant assays namely DPPH assay, ABTS assay, superoxide anion radical scavenging assay and hydroxyl radical scavenging assay were performed.

2.6.1. DPPH radical scavenging activity assay

2,2-Diphenyl-1-picrylhydrazyl (DPPH) was used to determine the free radical scavenging activity of the peptide using colorimetric method [38] with slight modification [48]. Briefly, an aliquot of 100 μL of peptide solution at various concentrations (6.25 μM–200 μM) was mixed with 100 μL of DPPH solution (0.1 mM) dissolved in 95% ethanol. Further, the reaction mixture was incubated for 30 min at room temperature and the absorbance (A) of the reaction mixture was evaluated at 517 nm using a UV–Vis spectrophotometer (UV1800, SHIMADZU, Kyoto, Japan). Ethanol was used as negative control. All the experiments were performed in triplicates. The radical scavenging efficacy of the peptide was determined using the following formula:

$$\text{DPPH Radical scavenging activity (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100\%$$

2.6.2. Trolox equivalent antioxidant capacity (TEAC) (or) ABTS assay

The Trolox equivalent antioxidant capacity of the peptide was determined based on the protocol detailed elsewhere [44] with slight modifications [48]. Briefly, 7 mM ABTS stock solution was mixed with 2.45 mM potassium persulfate to produce ABTS radical cation ($ABTS^{\cdot}+$). The reaction mixture was diluted to an absorbance of 0.70 ± 0.02 at 734 nm using 0.2 M PBS (pH 7.4) at 30 °C. Twenty μL of the sample or Trolox at various concentrations (6.25 μM–200 μM) was added to 180 μL of diluted $ABTS^{\cdot}+$ solution and incubated at 30 °C for 6 min. The decrease in absorbance at 734 nm was monitored using a spectrophotometer (UV1800, SHIMADZU, Kyoto, Japan) which is equivalent to the efficacy of scavenging of radicals by the peptides. All the experiments were conducted in triplicates.

The concentration of the peptide (at mM) giving the similar decrease of $ABTS^{\cdot}+$ cation as that of 1 mM of trolox was considered as 'Trolox equivalent antioxidant capacity'.

$$\text{ABTS Radical scavenging activity (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100\%$$

2.6.3. Superoxide anion radical scavenging activity assay

The superoxide anion radical scavenging activity of the peptide was analyzed using the method suggested by Chi et al. [14] with slight modification [48]. Briefly, superoxide anion radical was generated in 50 μL of nitrotetrazolium blue chloride (2.52 mM), 50 μL of NADH (624 mM), and 50 μL of various concentrations of peptide (6.25 μM–200 μM). The reaction was started by adding 50 μL of phenazine methosulfate solution (120 μg/mL) to the reaction mixture. The absorbance of the solution was measured at 560 nm (UV1800, SHIMADZU, Kyoto, Japan) against to correlate with blank after 5 min incubation at 25 °C. The ability of scavenging superoxide anion radical was calculated using the following formula:

$$\text{Superoxide anion radical scavenging activity (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100\%$$

2.6.4. Hydroxyl radical scavenging activity assay

The hydroxyl radical scavenging activity assay was performed following to the method described by Li et al. [37]; with slight modification [48]. Briefly, A mixture of 30 μL of 1,10-phenanthroline (5.0 mM), 30 μL of EDTA (15 mM) and 30 μL of $FeSO_4$ (5.0 mM) was mixed with 30 μL of sodium phosphate buffer (0.2 M, pH 7.4). Further, 40 μL of peptides or trolox at different concentrations (6.25 μM–200 μM) and 40 μL of H_2O_2 (0.03%) were added and incubated for 60 min at 37 °C. Finally, the absorbance of the solution was measured at 536 nm (UV1800, SHIMADZU, Kyoto, Japan). The antioxidant activity was calculated accordance with the trolox calibration curve and converted to the TEAC value.

$$\text{Hydroxyl radical scavenging activity (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100\%$$

2.7. Determination of intracellular redox state of human leucocytes by DCFDA

To determine the intracellular redox state, DCFDA oxidation sensitive dye was used [7]. Generalized intracellular oxidative stress, rather than any particular reactive species, was evaluated using cell permeant probe 2′, 7′-dichlorofluoresceindiacetate (DCFDA) dye by measuring the quantity of fluorescent dichlorofluorescein (DCF) formed within the human blood leucocytes using Cytomics FC 500 (BECKMAN COULTER Life Sciences, Indiana, USA). Briefly, leucocytes seeded in black 96-well plate at a density of 1×10^6cells/well were incubated with 10 μM DCFDA and 2 mM concentration of 30% H_2O_2 was added into each well according to the concentrations that were decided to be used for 30 min at 37 °C followed by incubation with desired treatments of GM15 (6.25, 12.5, 25, 50, 100, 200 μM). After 2 h, the extent of intracellular oxidative stress was measured by flow cytometry while the cells were gated on FSC/SSC to remove debris and mean fluorescence intensity from 1×10^4 cells was acquired [48]. Simultaneously, the reduction in intracellular fluorescence level, as an indication of reduction of cellular oxidative stress, in peptide-treated blood leucocytes was visually captured using confocal fluorescence microscope (ZEISS™, Jena, Germany) under 20× objective.

2.8. Cytotoxic activity of GM15 on human leucocytes

To evaluate the toxicity of the GM15 in normal cells, cytotoxicity analysis was conducted against blood leucocytes from human (Ethical Clearance No. CDRI/IEC/2014/A1). Human blood leucocytes (1×10^7 cells) were separated from whole blood using gradient centrifugation (1500 × *g* for 15 min at 4 °C) with RBC lysis buffer (Sigma-Aldrich) as provided in the manufacturer's protocol with slight modification [48]. The separated blood leucocytes were treated with GM15 at a concentration of 100 μM and incubated for 12 h in DMEM media. The cytotoxic effect of GM15 at a concentration of 100 μM on monocytes, granulocytes and lymphocytes population of human blood was determined by Cytomics FC 500 (BECKMAN COULTER Life Sciences, Indiana, USA). Triton X-100 was used as positive control and 1X PBS was used as negative controls. All the reactions were performed in triplicates and the results were represented as mean ± standard deviation.

2.9. MTT assay

MTT assay was performed to study the effect of GM15 peptide on the viability of KB cells. The KB cells (1×10^6 cells/well) were seeded in 96-well plate and incubated with 20 μl of peptides at different concentrations (6.25, 12.5, 25, 50 and 100 μM) for 24 h in complete DMEM media. Cells treated with Doxorubicin (2 μM) were maintained as positive control whereas, DMSO (0.1%) treated cells were maintained as vehicle control and PBS treated cells were maintained as negative control. After the incubation, 100 μL of MTT (2 mg/mL) reagent was added into each well and allowed to react for 3 h. Further, excess MTT was washed off and 100 μL of DMSO was added to solubilise the blue formazan crystals. The absorbance was measured at 570 nm with a microplate reader.

2.10. Determination of intracellular redox state in oral cancer cells (KB cells)

To determine the generalized intracellular oxidative stress in KB cells, DCFDA dye was used. KB cells were counted by trypan blue exclusion test using the haemocytometer. In each well, 2×10^6 cells/ml was seeded with 20 μM of DCFDA solution and H_2O_2 (50 μM) was added to the cells and incubated for 30 min for staining. The GM15 peptide (25 μM) was treated with stained cells and incubated for 4 h. After incubation, the treated cells were collected in the round bottomed flow cytometry tube. Hydrogen peroxide treated cells were used as the positive control. DCFDA fluorescence was measured in FL-1 filter in flow cytometer (BD Biosciences). Simultaneously, the reduction in fluorescence level, as an indication of ameliorated intracellular oxidative stress, on peptide treated KB cells were analysed and the images were captured using confocal fluorescence microscope (ZEISS™, Jena, Germany) under 20× objective.

2.11. ApGR induced caspase expression in KB cells

To know the involvement of GM15 peptide in caspase mediated apoptosis in oral cancer cells, the gene expression analysis was performed. KB cells were cultured and seeded for 24 h for adherence. The cells were treated with GM15 (25 μM) for 6 h. After total RNA isolation and cDNA synthesis, qRT-PCR analysis was performed in 96 well plate using GAPDH as internal control (housekeeping gene) in Light Cycler 96 Real Time PCR machine (Roche Diagnostics GmbH, Germany). The following primers were synthesized for the expression study: *Hs*CASP9 F1, TTC CCA GGT TTT GTT TCC TG (Sense) and *Hs*CASP9 R2: CCT TTC ACC GAA ACA GCA TT (Anti sense); *Hs*GAPDH F3, CTC GCT TCG GCA GCA CA (Sense) and *Hs*GAPDH R4: AAC GCT TCA CGA ATT TGC GT (Anti sense).

2.12. Propidium iodide assay

The effect of GM15 peptide on KB cell membrane integrity was confirmed by using a DNA intercalating dye, propidium iodide (PI). PI can intercalate with the DNA only if the cell membrane is disrupted and/or damaged but cannot permeabilize the intact cell membranes. KB cells were counted by trypan blue exclusion test using the haemocytometer. Briefly, 2×10^6 cells/ml was seeded in the wells then the cells were treated with GM15 (25 μM) or Doxorubicin (2 μM - Positive control) and incubated for 24 h. Cells treated with scrambled peptide (25 μM) were maintained as negative control. After incubation, the treated cells were trypsinized and collected in the round bottomed flow cytometry tube. Five μl of PI solution was added to the cells 5 min prior to the analysis and incubated in the dark. PI fluorescence was measured in FL-2 filter in fluorescence assisted cell sorter flow cytometry (BD Biosciences, USA) with CELL-QUEST software.

2.13. Comet assay

To detect the DNA damage induced by GM15 on KB cells, the comet assay was performed. KB cells were cultured and seeded for 24 h for adherence. The cells were treated with the GM15 peptide (25 μM) for 6 h. Slides were prepared by dipping into melted agar solution with the cell suspension and kept for solidifying. Further the slide was dipped in cold lysis buffer (2.5 M NaCl, 10 mM Tris-base, 100 mM EDTA, pH 10, with 10% DMSO and Doxorubicin 2 μM added fresh) for 2 h at 4 °C. Then the slide was gently removed from lysis buffer and electrophoresis was run at 24 V, 300A for 30 min at 4 °C. Then, the slides were neutralized with 0.4 M Tris and washed with absolute ethanol. Ethidium bromide (2 μg/ml in water) was added on to the slide for staining for 10 min. The slides were rinsed with distilled water and kept in a moist chamber at 4 °C. Finally, the slide was visualized under the fluorescence microscopy (ZEISS™, Jena, Germany).

2.14. Statistical analysis

All the data given in the study is a mean of triplicate ± standard deviation. For all the activity assay and gene expression study, we have performed a one-way ANOVA followed by Tukey's Multiple Range Test using SPSS 11.5 at the significance level 5%.

3. Results

3.1. H_2O_2 induced modulation of ApGR expression

In spirulina cells, the mRNA expression of *Ap*GR was determined in different time points (0, 5, 10, 15 and 20 days) post-challenge with H_2O_2. The highest expression of *Ap*GR was found at day 5 during 10 mM H_2O_2 exposure (*i.e.*) at the early stage of exposure when compared to the later stage where a constant decrease in expression was observed which is an indication that the *Ap*GR is getting back to the basal level of expression (Fig. 1).

3.2. ApGR identification and molecular characterization

*Ap*GR full length cDNA sequence was identified from the spirulina transcriptome and the sequence was submitted to the EMBL nucleotide archive under the accession number LT667404. The full length of the *Ap*GR cDNA sequence contained 1344 nucleotides along with an ORF which encoded a protein of 447 amino acid residues with a predicted molecular weight 48360 Da and theoretical isoelectric point (pI) 5.40. The putative protein sequence has a total number of 46 positively charged residues (Arg + Lys) and 59 negatively charged residues (Asp + Glu). The aliphatic index of the protein was calculated as 91.43 which represent that the protein exhibits better thermostability. Cellular location analysis revealed that the protein is located in

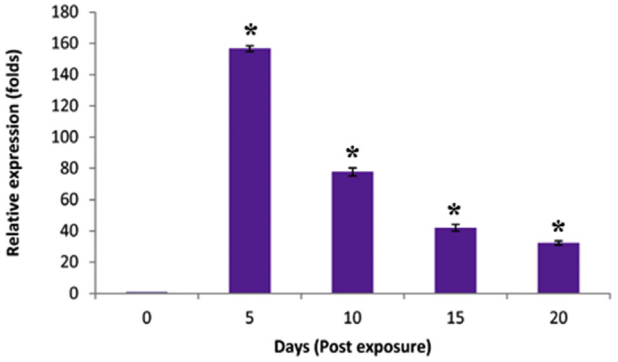

Fig. 1. H_2O_2 induced gene expression modulation in *A. platensis*. The modulated expression of glutathione oxido-reductase (*Ap*GR) post H_2O_2 challenge in *A. platensis* quantified by qRT-PCR at different time points (0, 5, 10, 15 and 20 days). The highest expression of *Ap*GR mRNA was observed at 5th day of post-treatment (p.t.) and the expression level declined to the basal level at 10th, 15th followed by 20th day p. t. Data shown is the mean ± SD, n = 3. The asterisk (*) denotes the significant different between control (day 0) and treatments (day 5, 10, 15 and 20) at $p < 0.05$ level by one-way ANOVA followed by Tukey's multiple range test.

mitochondria and the signal peptide prediction tool showed that no signal sequence in the protein.

From the BLAST analysis, protein showed more similarities when aligned with other homologous and orthologous sequences of GR particularly, *Lyngbya aestuarii* (83%), *Aspergillus ibericus* (37%), *Arabidopsis thaliana* (48%), *Callorhincus milii* (43%), *Mus musculus* (41%) and *Homo sapiens* (49%). *Ap*GR sequence remained conserved with other higher organisms including human specifically at the N-terminal region. The similarity pattern between *Ap*GR sequence and other homologous, orthologous sequences are depicted in Fig. 2A. This showed the functional importance of N terminal region of *Ap*GR.

3.3. *ApGR structure analysis and peptide prediction*

Domains and functional analysis showed that *Ap*GR contained 12 N-Myristoylation sites, 8 protein kinase C phosphorylation sites, 11 Casein kinase II phosphorylation sites, 2 cAMP and cGMP dependent protein kinase phosphorylation sites (^{92}KRLS^{95} and ^{127}RKIT^{130}) and 1 putative N− glycosylation site (^{227}NTTI^{230}) present in the N-terminal domain. Further, conserved domain database (CDD) of protein annotation resource revealed that *Ap*GR has two distinct functional domains. The *Ap*GR N-terminal domain (pfam00070) is located between ^{168}R and D^{247} followed by *Ap*GR C-terminal domain (pfam02852) which is located between ^{338}A and T^{445} with class I and class II oxido-reductases and also a small NADH binding domain wherein a larger FAD binding domain that belongs to pyridine nucleotide-disulphide oxido-reductase dimerization domain superfamily. I-TASSER (Iterative Threading ASSEmbly Refinement) online tool was used to predict the structure of the *Ap*GR protein sequence, based on the previously solved GR protein structures. *Ap*GR resulted that 34.67% amino acids are located in α-helix region, 15.43% in β-sheet region and 49.88% in coil region. Fourteen distinct α-helices were found in the *Ap*GR protein which is a conserved feature among class I and class II oxido-reductases where FAD binding sites were distributed in both helix and coil region (Fig. 2B).

3.4. *Characterization of antioxidant peptide*

The purity of the synthesized peptide GM15 was determined to be 95.455% by HPLC technique and the synthesized GM15 peptide sequence was confirmed by MALDI-TOF MS analysis. HeliQuest analysis showed that GM15 has 3 net charged amino acids (Z) with 0.549 hydrophobicity (H) and 0.091 hydrophobic moments (μH) on the same surface. The molecular weight and theoretical isoelectric point (pI) of the peptide is 1561 Da and 9.50, respectively. PEP-FOLD prediction of peptide folding showed that the GM15 peptide is folded onto itself (Fig. 2C) and form helical structure which is in line with our structure

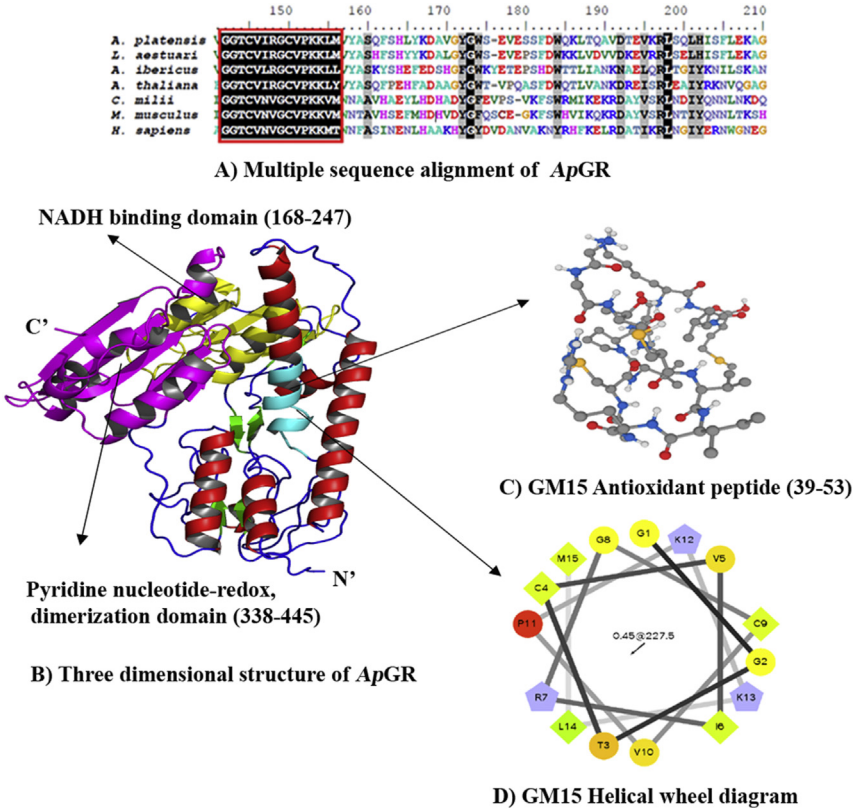

Fig. 2. Structural features of *Ap*GR protein. A) Multiple sequence alignment of *Ap*GR protein sequences with other GR homologs from *Lyngbya aestuarii*, *Aspergillus ibericus*, *Arabidopsis thaliana*, *Callorhincus milii*, *Mus musculus* and *Homo sapiens*. The conserved residues are highlighted in black background whereas the variable residues are represented in multiple colors. The highly conserved antioxidant peptide region (GM15) is highlighted in red box. B) Three-dimensional structure of *Ap*GR highlights the antioxidant peptide from the N-terminal region. The red spirals denote the helix region, blue lines represent the coil region and green arrows denote the beta sheet. In addition, the small NADH binding N-terminal domain is highlighted in yellow and the pink colour denotes pyridine nucleotide-disulphide oxidoreductase dimerization C-terminal domain. The antioxidant peptide residues are highlighted as cyan colored coils. C) The predicted structure of antioxidant peptide, GM15 with numbers representing the location (G_{39}-M_{53}). D) The green color denotes the highly hydrophobic amino acid and the color proportionally decreases to yellow based on the degree of hydrophobicity. The red colour indicates hydrophilic amino acid. Similarly, the pentagons represent positively charged amino acid whereas the triangles denote negatively charged amino acid. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

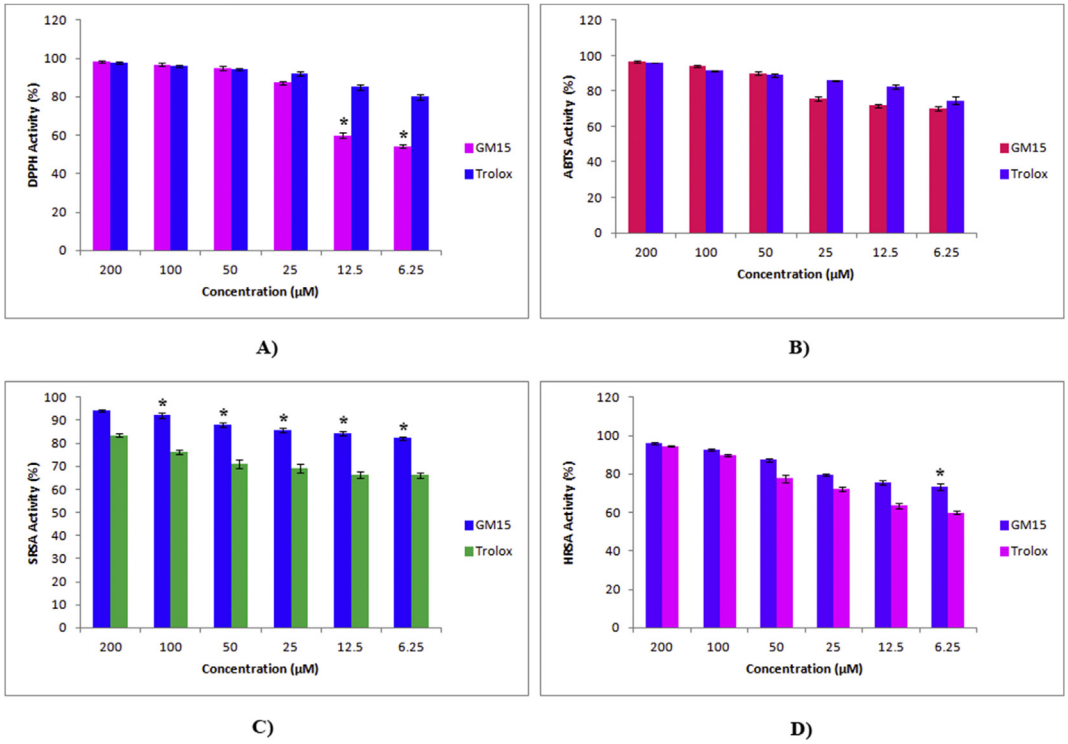

Fig. 3. Radical scavenging activity of the GM15 peptide. A) The DPPH radical scavenging ability of GM15 peptide was compared with that of standard antioxidant, Trolox by DPPH radical scavenging assay where higher concentrations of GM15 exhibited better radical scavenging activity equivalent to that of the standard. The asterisk (*) denotes the significant different between control (Trolox) and treatments (12.5 and 6.25 μM) at $p < 0.05$ level by one-way ANOVA followed by Tukey's multiple range test. B) TEAC assay reveals the concentration-dependent trolox equivalent capacity of GM15 in scavenging the ABTS radicals. C) The superoxide radical scavenging ability of GM15 peptide was compared with that of Trolox where GM15 exhibited superior radical scavenging activity than the standard. The asterisk (*) represents the significant better peptide activity compared with control (Trolox), the peptide treatments (100, 50, 25, 12.5 and 6.25 μM) at $p < 0.05$ level by one-way ANOVA followed by Tukey's multiple range test. D) HRSA assay exhibits the ability of GM15 in scavenging of hydroxyl ions. In all the tested concentrations, GM15 exhibit higher activity than the standard. The asterisk (*) denotes the significant different between control (Trolox) and treatment (6.25 μM) at $p < 0.05$ level by one-way ANOVA followed by Tukey's multiple range test. All the experiments were performed in triplicates and the values are represented in mean ± standard deviation, n = 3.

prediction where GM15 peptide sequence was found in alpha helix region of *Ap*GR. This indicated the amphipathic nature of GM15 with two faces exhibiting difference in hydrophilicity (Fig. 2D).

3.5. Antioxidant mechanism of GM15 peptide

3.5.1. DPPH radical scavenging activity of GM15

To evaluate the antioxidant ability of GM15 peptide to act as free radical scavengers or hydrogen donors, the stable radical DPPH was used. The GM15 peptide showed concentration dependent antioxidant activities, the lower concentration of GM15 peptide showed a significant ($P < 0.05$) radical scavenging activity (54.22 ± 0.96 at 6.25 μM) whereas, the higher concentration of peptide exhibited highest radical scavenging activity (98.55 ± 0.51 at 200 μM) (Fig. 3A).

3.5.2. Trolox equivalent antioxidant capacity of GM15

ABTS radical cation decolorization assay was widely used to determine the GM15 peptide radical scavenging activity. ABTS radical is both lipid-soluble and water-soluble compound which is used to evaluate the antioxidant activity. In this research, ABTS assay was considered as hydrogen atom transfer action (HAT). The GM15 peptide showed concentration dependent ABTS radical scavenging activities, the lower concentration of GM15 peptide showed a similar ABTS radical scavenging activities (70.18 ± 1.11 at 6.25 μM) when compared with standard antioxidant trolox (74.68 ± 2.34 at 6.25 μM). Increasing the concentration of the peptide increased the ABTS radical scavenging activities. The highest ABTS radical scavenging activities were determined as 96.39 ± 0.70 at 200 μM which is higher than trolox (95.86 ± 0.21 at 200 μM) (Fig. 3B).

3.5.3. Superoxide anion radical scavenging activity of GM15

To evaluate antioxidant activity of the GM15 peptide, superoxide anion radical scavenging activity assay were investigated in comparison with standard antioxidant trolox as positive control. The lower concentration of GM15 peptide showed a significantly ($P < 0.05$) higher superoxide anion radical scavenging activities (82.18 ± 0.89 at 6.25 μM) compared to the standard antioxidant trolox (66.16 ± 1.08 at 6.25 μM). Overall, the results showed that the activity is concentration dependent; increasing the concentration of the peptide increased the superoxide anion radical scavenging activities. The highest superoxide anion radical scavenging activities were evaluated at 200 μM (94.29 ± 0.51) and its respective activity in control is (83.54 ± 0.67) (Fig. 3C).

3.5.4. Hydroxyl radical scavenging activity of GM15

Hydroxyl radicals are the most biologically active among the oxygen radicals and can be formed from superoxide anion and hydrogen peroxide, in the presence of metal-ions, such as iron or copper. In this experiment, o-phenanthroline/H_2O_2 system was used to evaluate the hydroxyl radical scavenging activity of GM15 peptide. GM15 peptide exhibited significantly ($P < 0.05$) a greater hydroxyl radical scavenging activity (73.18 ± 1.74 at 6.25 μM) when compared with its standard antioxidant trolox (59.86 ± 0.82 at 6.25 μM); while increasing the concentration of the peptide, it exhibited higher hydroxyl radical scavenging activity (94.95 ± 0.58 at 200 μM) (Fig. 3D).

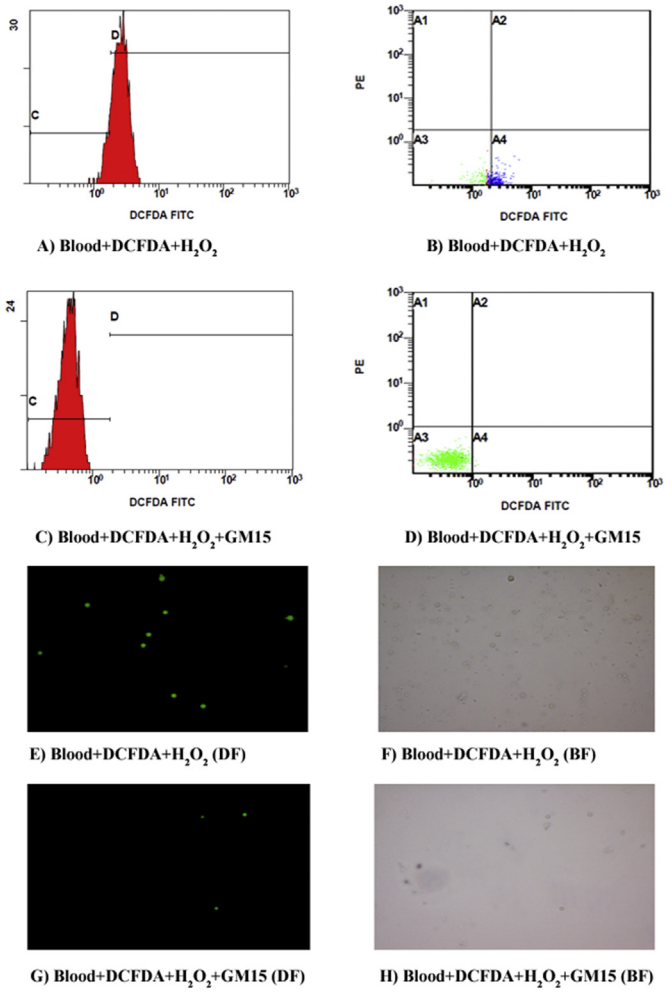

Fig. 4. GM15 peptide influences intracellular generalized oxidative stress in human leucocytes. Intracellular oxidative stress level in leucocytes were determined by fluorescence dye DCFDA using FACS: A) Histogram showing the shift in fluorescence indicating the increased oxidative stress in 2 mM concentration of 30% H_2O_2 treated leucocytes; B) Fluorescence shift encapsulation of high concentration of DCFDA due to high intracellular oxidative stress; C) Histogram showing no shift in fluorescence peak due to minimum level of oxidative stress in leucocytes post-peptide treatment; D) Reduced fluorescence level due to oxidant scavenging activity of GM15 peptide. Data shown is the mean ± SD, n = 3. Fluorescence imaging showed the real time oxidative stress captured by confocal microscopy: E) Dark field image showing fluorescence exhibited by oxidant-DCFDA complex in H_2O_2 treated leucocytes; F) Bright field image showing the actual cells; G) Dark field image showing reduced oxidative stress level in GM15 treated cells; H) Bright field image showing the actual cells.

3.6. GM15 alters the intracellular redox state in blood leucocytes on exposure to H_2O_2

In this experiment, using the fluorescence probe DCFDA, GM15-induced changes in the intracellular redox state was measured in blood leucocytes. During labeling, non-fluorescent DCFH-DA dye that easily penetrates into the cells gets hydrolysed by intracellular esterase to become DCFH, and this compound is trapped inside the cells and gets oxidized by H_2O_2. GM15-induced changes in generalized intracellular oxidative stress in leucocytes were measured at 2 h post treatment with 30% of H_2O_2. FACS analysis showed that all the tested doses of GM15 peptide (6.25, 12.5, 25, 50, 100, 200 µM), reduced the extent of oxidative stress; however, significant reduction was detected above 12.5 µM concentrations (Fig. 4A-D). This confirmed that the GM15 peptide exhibited concentration dependent reduction in intracellular oxidative stress in all the populations of leucocytes. In addition, fluorescence micrographs of DCFH-DA stained peptide-treated cells displayed a significantly lower level of DCF-fluorescence, compared with the control cells which confirmed the above fluorometric findings (Fig. 4E-4H).

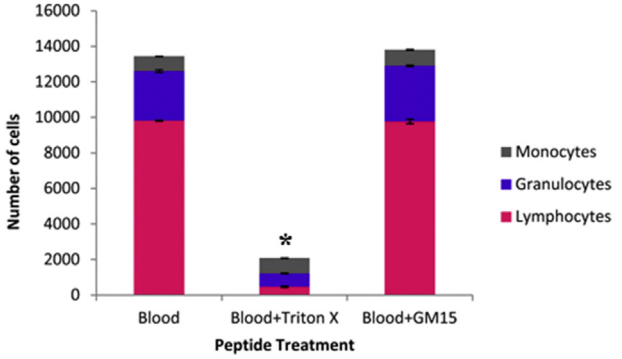

Fig. 5. Cytotoxicity of GM15 on human leucocytes. Cytotoxicity of GM15 was determined against blood leucocytes in the presence or absence of 100 µM GM15 peptide. Phosphate Buffered Saline (PBS) was used as negative control and Triton X used as positive control. GM15 exhibited no significant cytotoxic activity against human blood leucocytes. Data shown is the mean ± SD, n = 3. The asterisk (*) denotes the significant difference between positive control (Triton X-100) and treatment (100 µM) at $p < 0.05$ level by one-way ANOVA followed by Tukey's multiple range test.

3.7. Cytotoxic activity of GM15 on human leucocytes

The cytotoxicity of GM15 peptide was checked against human blood leucocytes by analysing the change in population pattern of blood cells before and after exposure of peptide. Results showed that GM15 (100 µM) peptide induced slight increase in granulocytes population (12.6%) than the untreated cells and there is no significant difference in other leucocytes population whereas, Triton X-100 (positive control) significantly ($P < 0.05$) reduced the cell numbers of all the leucocytes population of human blood (Fig. 5).

3.8. Anticancer potential of GM15 on KB cells

To determine whether GM15 inhibits the proliferation of oral carcinoma cell lines (KB), we treated KB cells with GM15 peptide, scrambled peptide or doxorubicin (positive control). MTT assay was performed to analyse the change in the proliferation of KB cells. Results showed that the GM15 (25 µM) exhibited better cytotoxic effect against KB cells and the activity is comparable with doxorubicin (2 µM) which is a well-known anticancer drug isolated from the bacterium *Streptomyces peucetius* var. *caesius*. But the scrambled peptide did not induce any significant cytotoxicity even at higher concentration (100 µM) (Fig. 6A). Also, the concentration of GM15 peptide was correlated inversely with the viability of KB cells indicating that the peptide had strong inhibitory activity against KB cells in a concentration-dependent manner. Further, the results provoked few queries about the role of GM15 peptide on KB cells such as the mode of action, apoptosis mechanism and specific intracellular targets which were explained in the sub heads 3.9–3.11.

3.9. GM15 alters the intracellular redox state in KB cells and up-regulates caspase-9 gene expression

To understand the influence of GM15 peptide on intracellular oxidative stress, DCFDA stained KB cells were utilized for FACS analysis. We observed that cells treated with GM15 peptide (6.25 µM) and H_2O_2 (50 µM) showed a reduction in the fluorescence intensity than the untreated cells. But at higher concentrations (25 µM-100 µM), the peptide

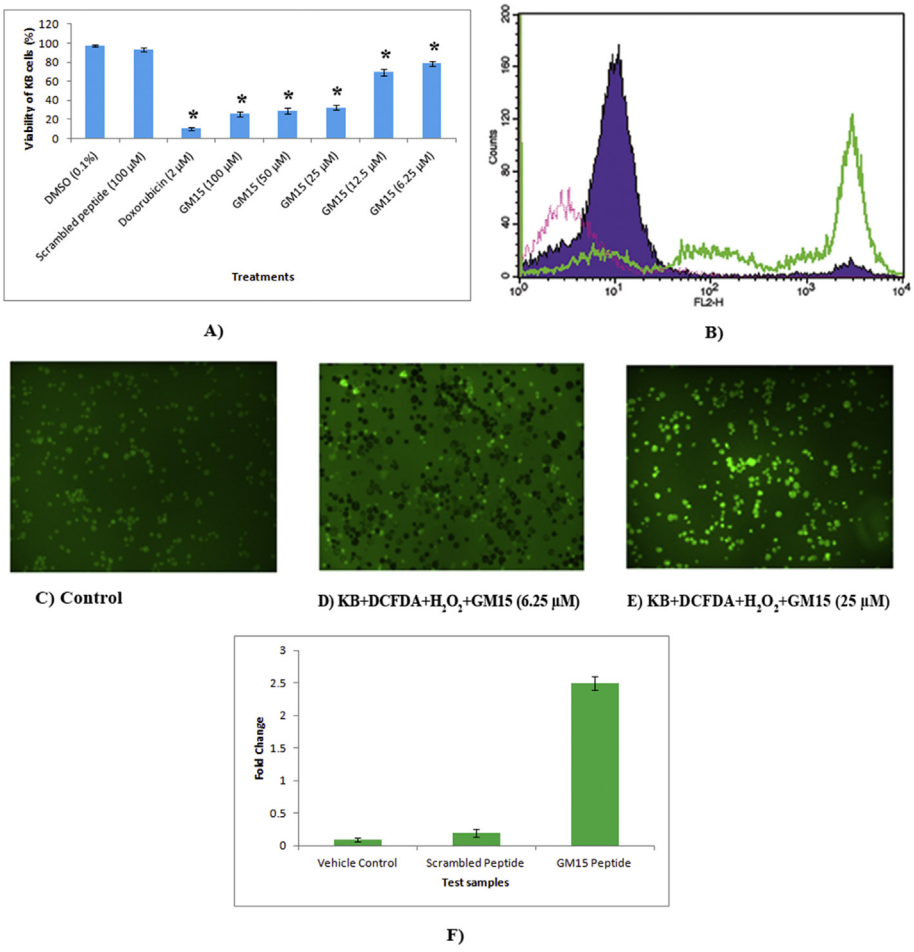

Fig. 6. Effect of GM15 peptide on KB cells. (A) The KB cells were incubated with GM15 peptide at five different concentrations and PBS was used as negative control. GM15 peptide induced significant toxic effect among KB cells in a concentration dependent manner. The percentage of viability of the cells was compared to the positive control doxorubicin. Data shown is the mean ± SD, n = 3. The asterisk (*) denotes the significant difference between control (DMSO) and treatments (100 µM) at $p < 0.05$ level by one-way ANOVA followed by Tukey's multiple range test. (B) Intracellular oxidative stress level in KB cells were determined by fluorescence dye DCFDA using flow cytometry; Pink line represents the effect of GM15 peptide (6.25 µM) which decreased the oxidative stress but did not affect the cell counts. Green line indicates that the higher concentrations of GM15 peptide (25 µM) increased the intracellular oxidative stress level as well as decreased the cell counts. (C) Intracellular oxidative stress measurement of KB cells was determined using fluorescence microscope; untreated cells (D) cells treated with GM15 peptide at a lower concentration 6.25 µM showed the reduced intracellular oxidative stress. (E) Cells treated with GM15 peptide at a higher concentration 25 µM showed the increased oxidative stress. (F) qRT-PCR analysis exhibited an up-regulation pattern of caspase-9 gene (2.66 folds) in GM15 (25 µM) treated cells than the untreated cells. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

showed increased oxidative stress by up-regulating the fluorescence intensity (Fig. 6B-E). Also, there is a significant ($P < 0.05$) reduction in the number of KB cells, which emphasizes the heterogeneity of the peptide in regulating intracellular oxidative stress. Therefore, to understand the mechanism of action of the peptide in KB cancer cells, the involvement of caspase-9 was analysed. qRT-PCR was used to analyse the changes in gene expression pattern of caspase-9 in KB cells treated with GM15 (25 µM); the results showed an up-regulation pattern of caspase-9 gene (2.66 folds) than the untreated cells which illustrates the critical involvement of caspase-9 in GM15 mediated apoptosis activity in KB cells (Fig. 6F).

3.10. GM15 exhibits membrane disruption activity in KB cells

Since the composition and arrangement of amino acids indicated that GM15 might interact with the membrane of cancer cells, the membrane disrupting ability of the GM15 peptide on KB cells were investigated by analyzing the propidium iodide (PI) internalization using FACS. Results showed that GM15 (25 µM) induced cell death where 31.95% cells remained alive among which 55.11% cells exhibited fluorescence (Table .1). However, the scrambled peptide exhibited negligible toxicity on cancer cells (2.81%) which strongly indicated that the activity of GM15 is specific for the arrangement of the sequence. Doxorubicin (positive control) treated cells induced maximum cell death (86.49%) where only 13.51% of cells remained viable after treatment. In this experiment, PBS treated cells were used as negative control and the survival percentage of treated cells was compared with that of negative control (Fig. 7A-C).

3.11. Comet test on KB cells

Apart from membrane disrupting activity, the effect of GM15 peptide on DNA targets in KB cells was analyzed by the DNA degradation assay where the degradation of the cells could be inferred by the appearance of cells like elongation of the tail in a comet. Results showed that a significant level of degradation up to 60% was observed in GM15 treated cells whereas, the positive control doxorubicin induced 89% of DNA degradation (Fig. 7D-G).

4. Discussion

Glutathione oxido-reductase is a ubiquitous enzyme containing highly conserved dithiol motif, CXXC catalytic redox active site that has been identified in bacteria, yeast, plants and mammals which is

Table 1
Cell count of peptide-treated KB cells by FACS. The assay was performed in three replicates and the data shown is mean value ± SD.

No	Peptide Treatment	Total Events/15S	PI Stained	% cells in comparison with PBS treated cells
1	KB cells + Doxorubicin	13,458 ± 187	11,751 ± 128 (87.31%)	13.51
2	KB cells + GM15 peptide	31,821 ± 387	17,538 ± 212 (55.11%)	31.95
3	KB cells + Scrambled peptide	98,989 ± 243	2791 ± 145 (2.81%)	99.39

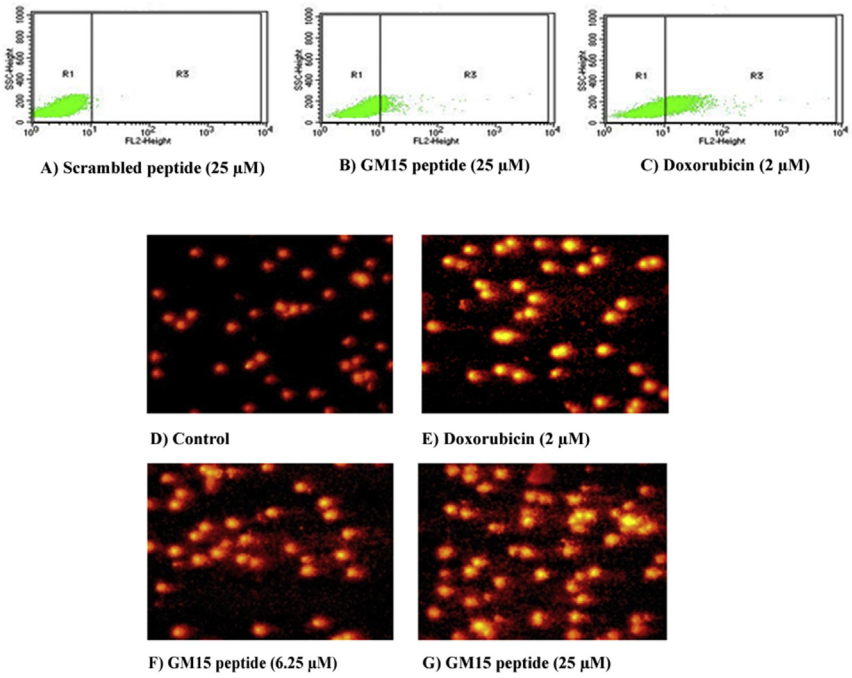

Fig. 7. Mode of action of GM15 peptide on KB cells. Internalization of PI by GM15-treated KB cells as assessed by flow cytometry. (A) KB cells treated with scrambled peptide did not show any significant membrane disruption activity. (B) GM15 peptide (25 μM) treated KB cells were stained with PI which is observed by a significant shift from the M1 to M2 region. (C) KB cells treated with doxorubicin also showed a similar pattern of GM15 peptide (2 μM) treated KB cells. The representative comet tails for KB cells; (D) untreated cells showing no degradation of DNA (E) cells treated with doxorubicin (positive control, 2 μM) exhibited strong DNA degradation (F) cells treated with GM15 peptide at a lower concentration 6.25 μM showed notable DNA degradation (G) cells treated with GM15 peptide at a higher concentration (25 μM) exhibited strong DNA degradation.

responsible for maintaining cellular redox homeostasis in all living organism [50]. Recently, spirulina has been considered as a source of antioxidant and are used as supplements for human and animals especially in cattle farms and aquaculture. In this study, we have reported the radical scavenging activities of *Ap*GR enzyme from *Arthrospira platensis* and its involvement during H_2O_2 cells. qRT-PCR analysis was performed to understand the involvement of the *Ap*GR gene in spirulina cells at different time points (0, 5, 10, 15 and 20 days) post challenge with H_2O_2 and the highest expression of *Ap*GR was found at day 5 during 10 mM H_2O_2 exposure which suggest the involvement of the gene during environment-induced oxidative stress. Earlier findings suggested that the mRNA level of GR gets up-regulated in plants and algae during oxidative stress induced by various environmental factors [35,54]. It is basically the environmental stress-induced production of free radicals at higher concentrations, which consequently activate the gene expression by the antioxidant enzymes in plant cells [28]. Li et al. [36] reported that GR is an important enzyme of the antioxidant system required for the detoxification of oxidants in plants by converting GSSG to reduced GSH and maintaining the redox state in the intracellular environment against oxidation. This finding clearly suggested that *Ap*GR gene may have an intrinsic antioxidant role in *A. platensis* to protect the cells from damage during H_2O_2 induced oxidative stress.

The full length of the *Ap*GR mRNA sequence encoded a protein of 447 amino acid residues with theoretical molecular weight (MW) of 48.36 kDa which was close to the molecular mass of GR from *Chlamydomonas* sp which is 54.6 kDa [19]. Stevens et al. [52] reported that the GR genes have been identified by two forms, a cytosolic isoform and a plastidic isoform dually-targeted to mitochondria and chloroplasts and our cellular location analysis revealed that the protein is localized at mitochondria which suggest that *Ap*GR might involve in mitochondrial-derived oxidants scavenging mechanism of *A. platensis*. Collinson and Dawes [15] stated that GR has two distinct functional domains. In *Ap*GR, there are two domains where the N-terminal domain (pfam00070) is located between ^{168}R and D^{247} followed by *Ap*GR C-terminal domain (pfam02852) which is located between ^{338}A and T^{445} with class I and class II oxido-reductases and also a small NADH binding domain wherein a larger FAD binding domain. These findings confirmed that *Ap*GR can use NADPH or NADH as an electron donor. The *Ap*GR signature sequence, ^{39}GGTCVIRGCVPKK^{51} contains two conserved cysteine residues, ^{42}Cys and Cys^{47} which forms the redox-active disulfide bridge in *Ap*GR. These cysteine residues are responsible for the helical conformation of the signature domain. Although the FAD binding function of the signature sequence was well characterized, the antioxidant roles of this sequence were not studied so far. The overall analysis revealed that the N-terminal peptide sequence, ^{39}GGTCVIRG-CVPKKLM^{53} (GM15) along with the conserved catalytic dithiol active residues, ^{42}Cys and Cys^{47} might act as a potential antioxidant peptide and therefore, we analyzed the antioxidant activity of the short peptide and its possible use in maintaining the redox balance in human oral cancer.

The GM15 peptide contains 3 net charged amino acids (Z) with 0.549 hydrophobicity (H), 0.091 hydrophobic moments (μH) on the same surface and the molecular weight, theoretical isoelectric point (pI) of the peptide is 1561 Da and 9.50, respectively. It is a known fact that the lower molecular weight peptides (1000–3000 Da) exhibit strong free radical scavenging activities by terminating the propagation cycles of lipid peroxidation [47]. Moreover, GM15 peptide sequence was folded onto itself and it was found in alpha helix region of *Ap*GR. This indicated the amphipathic nature of GM15 with two faces exhibiting difference in hydrophilicity. Generally, amphipathic peptides are known for its membrane binding ability as well as membrane-crossing potential that are necessary for the intracellular functions of those peptides [49,58]. Chen et al. [11] reported that the sulphur containing amino acids Cys and Met and aromatic amino acids Tyr, Trp and Phe exhibit greatest antioxidant activity. Also, hydrophobic amino acids Val or Leu at the N-terminal region of the peptide showed highly potent inhibitory activity [11]. Kawashima et al. [29] reported that peptides with Ala, Tyr, His, and Met at N-terminal end exhibit higher antioxidant activities. GM15 fulfils all the above-mentioned properties and showed its potential antioxidant properties. Also, the physico-chemical parameters and structural characteristics suggested that GM15 peptide might interact with the membrane of mammalian cells.

Antioxidant activity assays showed that the GM15 peptide exhibited radical scavenging activities in concentration dependent manner. This might be because of the presence of Cys in the peptide which act as a direct free radical scavenger [43]. Also, the DPPH radical scavenging activity of GM15 was higher than the earlier reported hydrolysates of *A. platensis* (85.21 ± 1.59% at 10 mg/ml) [27,30]. Chi et al. [13] reported that peptide with lower molecular weight (432.52 Da) showed a

highest radical scavenging activity on DPPH (IC50 0.118 mg/ml) which coincides with our observations. In addition, our finding is also in agreement with the reports of Zhuang et al. [60] who stated that hydrolysates or peptides with lower molecular weight showed higher free-radical scavenging activity.

In ABTS radical scavenging assay, ABTS radical reacts very quickly with antioxidant compounds [2]. The lower concentration of GM15 peptide showed a similar ABTS radical scavenging activity (70.18 ± 1.11 at 6.25 μM) when compared with trolox (74.68 ± 2.34 at 6.25 μM). Increasing the concentration of the peptide increased the ABTS radical scavenging activity and the highest ABTS radical scavenging activity were determined as 96.39 ± 0.70 at 200 μM which is higher than trolox (95.86 ± 0.21 at 200 μM). Sheih et al. [51] reported that the peptide (VECTGPNRPGP) derived from pepsin hydrolysate of a microalgae (*Chlorella vulgaris*), exhibited greatest ABTS radical scavenging activity (IC50 9.8 ± 0.5) when compared with trolox (IC50 32.5 ± 1.3). In addition, Cys and Tyr amino acid residues were crucial for free radical elimination which has been reported by Liu et al. [40]. These results strongly suggested that GM15 might exhibit free radical scavenging activity by hydrogen atom transfer.

In superoxide anion radical scavenging assay, lipid oxidation is initiated by perhydroxyl radicals in the form of releasing protein-bound metals by the action of superoxide radical which can promote oxidative reaction due to its ability and reducing transition metals [20]. Superoxide anion is one of the crucial free radicals which is formed by addition of an electron to molecular oxygen. Superoxide radical easily react with nucleic acids, amino acids, lipids and cell membrane, to exert a sturdy cytotoxic effect. Therefore, it is very crucial to scavenge superoxide anion radical. The lower concentration of GM15 peptide showed a significantly ($P < 0.05$) higher superoxide anion radical scavenging activity (82.18 ± 0.89 at 6.25 μM) compared to the standard antioxidant trolox (66.16 ± 1.08). Increasing the concentration of the peptide increased the superoxide anion radical scavenging activity. The highest superoxide anion radical scavenging activity were evaluated at 200 μM (94.29 ± 0.51) and its respective activity in control is (83.54 ± 0.67). In antioxidant activity, glycine contribution is mainly considered to quench unpaired electrons or radicals by supporting protons; glycine is a potential target site of free radicals because it has hydrogen atom in the side chain [56]. Wang et al. [56] also reported that antioxidant peptides containing glycine (GFRGTIGLVG and GPAGPAG) from hydrolysate of the croceine croaker (*Pseudosciaena crocea*) showed good superoxide radical scavenging activity (IC50 = 0.463, 0.099 mg/ml). Interestingly, to correlate with our study the GM15 peptide containing tri glycine amino acids displayed a greatest superoxide radical scavenging activity. In addition, it was reported that arginine, lysine, histidine amino acid residues have a crucial role in the effects of metal chelation activity [46]. Moreover, jumbo squid skin hydrolysate MW < 3 kDa fraction (inhibition value = 82%) inhibited lipid oxidation more efficiently than the MW > 10 kDa fraction (inhibition value = 39%) [21]. Overall, the highest superoxide radical scavenging activity of GM15 suggested that the peptide derived from the N-terminal region of *Ap*GR showed a potent antioxidant activity.

In hydroxyl radical scavenging assay, hydroxyl radicals are directly reacting with almost all the biological macromolecules such as nucleic acids which exhibit proteins and polyunsaturated fatty acids and cause severe cellular damage [59]. Therefore, antioxidants are necessary for balancing the redox state in the cells to deactivate the hydroxyl radical. GM15 peptide exhibited significantly ($P < 0.05$) a greater hydroxyl radical scavenging activity (73.18 ± 1.74 at 6.25 μM) when compared with its standard antioxidant trolox (59.86 ± 0.82); while increasing the concentration of the peptide, it exhibited higher hydroxyl radical scavenging activity (94.95 ± 0.58 at 200 μM). Recently, Jie et al. [27] reported that spirulina peptide showed good hydroxyl radical scavenging activity (54.01 ± 0.82 at 10 mg/ml), in this study it is reported that GM15 peptide exhibited greatest hydroxyl radical scavenging activity even at a lower concentration. In addition, Ko et al. [31] reported that peptide (LNGDVW) from *Paralichthys olivaceus* showed a significant antioxidant activity (1.42 ± 0.08 at 0.01 mg/ml) against hydroxyl radical. Moreover, another study [26] reported that tuna backbone protein hydrolysate showed a strong hydroxyl radical scavenging activity (80.91% at a concentration of 1.3 mg/ml), which is similar to our findings. Interestingly, thiol containing peptides showed highest hydroxyl radical scavenging activity has been reported by Han et al. [25]; similarly in our study, GM15 peptide contained dithiol active site showed highest antioxidant activity. This suggested that the presence of thiol containing amino acid in GM15 may involve in the hydroxyl radical scavenging activity.

Since the peptide has been identified to scavenge free radical species such as superoxide and hydroxyl radical through cell-free assays, we analyzed the efficacy of the peptide in mitigating intracellular generalized oxidative stress in different mammalian cells and to implicate its function in cancer therapy. Initially, the oxidative stress-mitigating effect of the GM15 peptide was tested in the H_2O_2 induced human blood leucocytes. Earlier studies reported that extracellular H_2O_2 at higher concentration (> 1 μM) has a major impact over the antioxidant mechanism and increases the level of oxidants in blood leucocytes which causes serious damage to those cells [22]. Therefore, we analyzed whether GM15 peptide can influence the intracellular redox balance in human healthy blood leucocytes using DCFDA. FACS analysis showed that all the tested doses of GM15 peptide (6.25, 12.5, 25, 50, 100, 200 μM), reduced the intracellular generalized oxidative stress level; however, significant scavenging effect were detected above 12.5 μM concentrations. In addition, fluorescence microscopy images showed that GM15 peptide reduced the intracellular oxidative stress significantly by lower level of DCF-fluorescence. Overall, these findings highlight the effect of GM15 peptide in decreasing the excessive intracellular oxidative stress and inhibition of radical mediated oxidation in healthy blood leucocytes. Also, we have analysed the cytotoxicity of GM15 peptide on H_2O_2 induced human blood leucocytes. Results showed that GM15 (100 μM) peptide induced slight increase in granulocytes population (12.6%) than the untreated cells and there is no significant difference in other leucocytes population. Host cytotoxicity is one of the major limiting factors for using peptides as a therapeutic agent [23]. Thus, the findings indicate that GM15 did not exhibit cytotoxicity against healthy human blood leucocytes in any of the experimented concentrations. Therefore, the peptide is human compatible, and it can be used as therapeutic agent without any cytotoxic effects to the normal cells.

It is a well-known fact that molecules that scavenge oxidants are potential anticancer agents because excessive oxidative stress contributes to tumor growth and the molecules with high oxidant reducing activity decrease the incidence of cancer development [39]. Therefore, we analyzed the inhibitory effect of GM15 on oral carcinoma cell lines (KB). GM15 (25 μM) exhibited better cytotoxic effect against KB cells and the activity is comparable with doxorubicin (2 μM). Also, the concentration of GM15 peptide was correlated inversely with the viability of KB cells indicating that the peptide had strong inhibitory activity against KB cells in a concentration-dependent manner. Notably, the GM15 peptide can have heterogenous effect in cancer cells; at lower concentration, the peptide (6.25 μM) reduced the intracellular generalized oxidative stress and at higher concentration, the peptide (25 μM) induced caspase-9 mediated apoptosis in KB cells. Based on the cell specific activity observed in our study, the difference in generalized oxidative stress and cytotoxic activity could be due to the fact that the leucocytes are from healthy volunteer while the KB cells are cancer cell lines. Moreover, the leucocytes are isolated from blood while the KB cells have an oral origin. Hence, the peptide shows potent cytotoxicity against KB oral cancer cells while simultaneously does not cause any adverse effect on healthy leucocytes. It is known that most anticancer agents such as doxorubicin, carboplatin and cisplatin cause

disproportionate increase in intracellular oxidants concentrations and induce cancer cell cycle arrest, senescence and apoptosis by triggering the cytochrome c/Apaf-1/Caspase-9-dependent pathway [16,18,32]. The intrinsic initiator caspase, caspase-9 is an essential mediator of apoptosis which induce apoptosis as well as supports the degradation of cellular structures. Therefore, we have analyzed the modulation of caspase-9 expression pattern in KB cells treated with GM15 (25 μM) and the qRT-PCR results showed an up-regulation pattern of caspase-9 gene (2.66 folds) which indicated that GM15 induced apoptosis in KB cells by caspase-9-mediated pathway. Previous report [17] stated that glutathione is usually targeted for oxidant-inducing therapy strategies. Therefore, up-regulating oxidant levels, either by decreasing the radical scavenging potential or increasing the production of oxidants, could be a way to selectively kill or arrest cancer cells without causing significant toxicity to normal cells. Thus, we confirmed that GM15 peptide potentially involve in apoptosis intrinsic pathway.

Membrane disruption activity analysis by FACS showed that GM15 peptide (25 μM) exhibited significant PI internalization in KB cells (31.95%) which confirmed the membrane disrupting ability of the peptide on KB cells which means apart from oxidative stress-mediated pathway, the peptide also involved in membrane disruption. Chen et al. [12] reported that Tumour-targeting peptide (TTP) containing Arg-Gly-Asp (RGD motif) has ability to be internalized into the human glioma cell. Further, DNA targets for GM15 in KB cells was analyzed by the comet assay and the results exhibited that GM15 (25 μM) significantly degrades the DNA up to 60%. This finding infers that along with oxidative stress-mediated cytotoxicity, the peptide also induces cell damage via DNA degradation and membrane disruption. It is an usual observation that oxidants-mediated caspase-9 activation and DNA damage activates apoptosis where both the pathways converge to induce cytotoxicity in cancer cells.

5. Conclusion

In conclusion, the differential gene expression analysis exhibited that the *A. platensis* glutathione oxido-reductase plays a vital role in antioxidant function against oxidative stress induced by higher concentration of H_2O_2. Also, the activity assays confirmed the oxidant scavenging potential of the peptide GM15 which is derived from evolutionarily conserved region of *Ap*GR. Further, the oxidant scavenging ability of the peptide had positive implications in reducing free radicals in leucocytes during oxidative stress which is a remarkable biomedical application of the peptide. In addition, the GM15 peptide exhibited significant inhibitory effect against oral cancer cells.

Authors contributions

Conceived and designed the experiments: AS, VK, MP, JA.
Ethical clearance: MP.
Performed the experiments: AS, VK, SA, MP.
Analysed the data: AS, VK, MRG, KM, BAP, MKA-S, MFA.
Contributed reagents/materials/analysis tools: JA, BAP, MKA-S, MFA.
Wrote the paper: AS, VK, JA.

Conflicts of interest

The authors have declared that no competing interests exist.

Informed consent

For Blood collection, informed consent was obtained from all the participated adults in written form.

Author agreement statement

The authors declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere. We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us. We understand that the corresponding author is the sole contact for the editorial process. He is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs.

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Appendix A. Supplementary data

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