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ORIGINAL ARTICLE

The discovery of antioxidants in marine microorganisms and their protective effects on the hepatic cells from chemical-induced oxidative stress

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ABSTRACT

The marine environment is emerging as a biodiversity resource for the discovery of natural molecules or chemical scaffolds with pharmaceutical activity. Marine microbes have a tremendous ability to sense and respond to their surroundings to survive in a stressful environment by producing different molecules. As oxidative stress is directly or indirectly involved in various pathological conditions in humans, we believe that marine-derived antioxidant molecules will have a great prospect as a novel antioxidant molecule. We, in this work, explored the marine microbial resources from the Gulf of Mannar, Bay of Bengal, India. High-throughput screening of antioxidant molecule producing marine microbes has revealed that extract from *Kocuria marina* CDMP 10, can effectively reduce the DPPH free radical. Methanolic crude extract obtained by the freeze-thawing was fractionated and purified by using activity guided purification with the help of reverse phase HPLC and analysed through UPLC-MS. Chemical analysis, as well as MS-spectra, indicated that marine bacteria *K. marina* CDMP 10 derived antioxidant fraction contains the short peptides. The antioxidant activity of the three highly hydrophobic peptides, (Ser–Ser–Gln, Phe–Glu, Asp–Ile and Leu–Glu) was confirmed by *in vitro* as well as a cell-based assay. These small peptide molecules are noncytotoxic and can prevent the human cells from chemical-induced oxidative stress. Ser–Ser–Gln peptide demonstrated a potent free radical scavenging activity in Hepatocellular carcinoma cell lines. This study suggests that these short peptides from *K. marina* CDMP 10 may serve as a potential pharmaceutical candidate with antioxidant activity.

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Introduction

For centuries, plants/lower invertebrates extracts, usually abundant in the primary and secondary metabolites are being used as therapeutic agents for the treatment of various human diseases. Despite the availability of a variety of molecules from plants, there is currently renewed interest in microbial sources for secondary metabolites due to the inherent problem associated with plants such as dependence on the season, geographical variation and also inability to produce in large quantities. Among the diverse microbial sources, the sea remains an unexplored area in search of many pharmacologically active substances.

Different studies have indicated that antioxidant therapy could be one of the best strategies to counteract the physiological effects and reduce the symptoms in various diseases such as insulin resistance, diabetes mellitus [1], mitochondrial dysfunction [2], obesity and obesity-associated malfunction [3], cancer [4], neurodegenerative diseases [5], sepsis [6], inflammation [7] etc. Some of the molecules like Edaravone [8], idebenone [9], N-acetylcysteine [10], etc. have shown promising bioactivity against the oxidative stress in various diseases. It is interesting to observe that not all antioxidant molecules have shown very conclusive results in clinical trials [11]. The failure of an antioxidant molecules in clinical trials has been attributed to the reasons like, low bioavailability [12], poor target specificity [13], toxicity of reaction products [14], lower potency [15], physiological mechanisms [16] etc. As the problem lies not in the therapy, but in the molecules used, there is an urgent need to discover new antioxidant molecules without any drawbacks [17].

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Supplemental data for this article can be accessed here.

Hence, in this context, this work presents the identification of novel antioxidant producer bacterial strain from Gulf of Mannar, India, the activity based fractionation of antioxidant molecules through RP-HPLC, ability to inhibit the chemical-induced oxidative stress in human cells and the characterisation of antioxidants with the help of UPLC-MS.

Materials and methods

Cell culture and conditions

HepG2 cells ATCC HB8065 were cultured in Dulbecco's Modified Eagle Medium (DMEM), 2 mM glutamine, 0.2% sodium bicarbonate and 10% FBS at 37 °C with 5% CO_2.

Collection of marine sample

Seawater sample collection was performed as reported elsewhere [18]. Briefly, seawater from the Gulf of Mannar was collected in the sterile tubes consisting of transport media (g/L NaCl 28.32; $MgCl_2$ 5.14; $CaCl_2$ 1.14; KCl 0.69; KBr 0.1; H_3BO_3 0.027; $SrCl_2$ 0.026; NH_4Cl 0.0064; NaF 0.003 $NaSiO_3$ 0.002; $FePO_4$ 0.001; Yeast extract or beef extract 1.0) and packed in sterile polythene bags. The samples were transported to the lab on the ice and processed immediately to avoid change in microbial composition.

Isolation of bacteria

Isolation of bacteria was performed as reported elsewhere with minor modification [19], before designing the minor modifications, all suggestions reported by Joint et al. in the review were considered [20]. Serial dilutions of the collected samples were carried out in 0.22 μm filter sterilised sea water. The diluted samples (10^{-6}) were spread on Zobell's marine growth medium [21] and incubated at 37 °C for 2 days. Colonies of different morphology had been isolated and grouped. The grouped bacteria were purified to a single progeny level, documented and stored at −80 °C in 30% glycerol for future use.

Extraction and screening for antioxidant molecules from marine bacteria

Extraction of metabolites from marine isolates was performed according to the methods described elsewhere with some modifications [22,23]. All marine bacteria have been cultured in 96 well plates at 37 °C for 72 hrs. After, the bacterial growth reached the optimum absorbance at 620 nm plates were centrifuged at 4500 rpm for 10 min. Culture medium had been removed without disturbing the cells and freeze-dried the pellet to facilitate the extraction of metabolites without degradation. Furthermore, 250 μL of methanol was added to each well and incubated for 3 hrs at 40 °C in water-bath. Again, centrifuged the plate at 4500 rpm for 10 min. The extracted supernatant was taken out in the fresh multiwell plate and performed the high-throughput DPPH free radical scavenging activity assay according to the method discussed elsewhere with some minor modification [24,25]. Later, the free radical scavenging activity of the crude extract was measured regarding hydrogen donating or radical-scavenging ability using the radical DPPH [26]. Two microlitres of extracts were added to the 98 μL of DPPH (0.1-mM solution in ethanol) and incubated for 30 min in room temperature at dark. After incubation, the absorbance was measured at 517 nm. The lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH free radical scavenging activity } (\%) = \frac{(\text{Abs cont-Abs test})}{\text{Abs cont}} \times 100$$

where Abs cont is the absorbance of the control reaction, and Abs test is the absorbance in the presence of the extracts. The antioxidant activity of the extract was expressed as IC_{50}. The IC_{50} value was defined as the concentration (in μg/mL) of extracts that can scavenge the DPPH radicals by 50%.

Bacterial identification through 16S rRNA gene amplification and sequencing

Genomic DNA was isolated from marine bacteria according to the manufacturer's protocol (GenElute™ Bacterial genomic DNA kit mini, Sigma, Catalogue No. NA2110). The quality of the DNA isolated was analysed by using the agarose gel electrophoresis method. Amplification of 16S rRNA gene segment was carried out with the help of a set of primers, 27 F: AGAGTT TGATCMTGGCTCAG M = A and C; 63 F: CAGGCCTAACAC ATGCAAGTC; 1387 R: GGGCGGWGTGTACAAGGC W = A and T; 1492 R: TACGGYTACCTTGTTACGACTT, Y = C and T; 1525 R: AGGAGGTGWTCCARCC, W = A and T; with working concentration 1 μM [27]. Briefly, 25-μL reaction volume was set up consisting of 10 ng of genomic DNA, 150 ng of each primer and 12.5 μL of Master mix (EmeraldAmp GT PCR Master Mix TaKaRa RR310). The amplification was carried out in the Applied Biosystems veriti 96 well thermal cycler. The PCR cycles were preset

to denaturation 180 sec at 95 °C, and 30 cycles of denaturation for 60 sec at 95 °C, annealing for 60 sec at 55 °C, an extension for 90 sec at 72 °C, then a final extension for 10 min at 72 °C, 30 min at 4 °C. After gel electrophoresis of amplified 16S rRNA gene segment, it was eluted from agarose gel according to the manufacturer's protocol (GenElute^{TM} gel extraction kit, Sigma, NA1111). DNA sequencing was done by using ABI 3730 XL sequencer. The obtained sequence was trimmed and cleaned by using the software DNA baser with default settings (parameters: Good bases >75%, Base window = 20, QV >26, Bases with QV equal or higher than 30 were considered trusted). Chimaera sequences were checked using the web-based tool DECIPHER [28] with default settings. The processed sequence was submitted to NCBI gene bank for the global record. By using the processed sequence BLAST analysis was performed with the help of BLASTn programme available at the NCBI site. The evolutionary history was inferred using the Neighbor-Joining method [29]. The optimal tree with the sum of branch length parameter used was 0.05. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches [30]. The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site [31]. The analysis involved 13 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were total of 502 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [32].

Extraction of active components and sample preparation

Kocuria marina CDMP10 was inoculated in Zobell's medium, incubated in rotator incubator shaker at 180 rpm for 48 hrs at 37 °C. After incubation, the culture was centrifuged to pellet the cells, removed the supernatant, followed by the single cycle of freeze–thaw, i.e. freezed the cells at −80 °C and thawed at 40 °C to break the cells and extract the bioactive molecules. Methanol was added to the pellet after the single cycle of freeze–thaw, vortexed and incubated at 40 °C for 10 min in water bath. Further, the samples were centrifuged to pellet the cells. The red colour supernatant was evaporated in rota vapour to get it concentrated. The concentrated extract was washed with n-hexane to remove the oily and nonpolar substance. After washing, the crude extract was dissolved (50 mg/mL) in methanol: water (20% water) and filtered through the 0.45 μm filter for the injection in waters HPLC to fractionate the sample.

Antioxidant activity-guided RP-HPLC based fractionation of crude extract

Initially, crude extract fractionation was performed by semipreparative RP-HPLC. Methanol: water gradient mobile phase was used on Waters Spherisorb ODS2 C18 (5 μm) 4.6 × 250 mm reverse phase column using water (A) and methanol (B) as an organic modifier at a flow rate of 3.5 mL/min in gradient elution mode. The column was equilibrated with 10 column volumes of mobile phase at the initial gradient composition before sample injection. For sample run a continue gradient of 30% water and 70% methanol was used up to 5 min, followed by 20% water gradient up to 15 min, then 10% water till 25 min and again ended with 5% water up to 35 min of the total run. The column compartment was at 25 °C and a photodiode array (PDA) detector was employed throughout the analysis. Injection volume used in chromatography was 50 μL, and the sample was analysed using a PDA (200–600 nm) detector. In chromatogram, peak purity was determined with the help of Diode Array detector Spectral Data Asia discussed elsewhere [33]. Each collected peak was subjected to the DPPH free radical scavenging activity according to the protocol discussed earlier.

Purification of antioxidant fraction

Acetonitrile (ACN) and water gradient was used in purification of an antioxidant fraction with the help of Waters Spherisorb ODS2 C18 (5 μm) 4.6 × 250 mm reverse phase column using water (A) and ACN (B) as an organic modifier at a flow rate of 2.6 mL/min in gradient elution mode. The column was equilibrated with 10 column volumes of mobile phase at the initial gradient composition before sample injection. For sample run a continue gradient of 95% water and 5% ACN was used up to 10 min, followed by 5% water gradient up to 20 min, and again ended with 95% water up to 35 min of the total run. The column compartment with a 25 °C temperature and a photodiode array (PDA) detector was employed throughout the analysis. Injection volume used in chromatography was 50 μL, and the sample was analysed using a PDA (200–600 nm) detector. After purification, compound(s) peak was screened for DPPH free radical scavenging activity according to the protocol discussed earlier.

Cytotoxicity assay

The viability of cells against exposure to extract was analysed by using MTT assay. Five milligrams of MTT (3-(4,5-dimethylthiazolyl)-2,5- diphenyltetrazolium bromide; Sigma-Aldrich, St. Louis, USA) was dissolved in 1 mL of PBS and filter sterilised in brown colour Eppendorf tubes before storing at −20 °C. Two hundred microlitres of HepG2 cells (2.5×10^5 cells/mL) were plated in 96 well plates with DMEM (Sigma), 2 mM glutamine, 0.2% sodium bicarbonate and 10% Foetal Bovine Serum (FBS). Cells were incubated with 50 and 100 μg/mL of complete purified extract and incubated for 18–24 hrs at 37 °C and 5% CO_2. After incubation, 20 μL of MTT solution (5 mg/mL) was added and further incubated for 1 hr at 37 °C and 5% CO_2. Medium containing the MTT was removed, and 100 μL of DMSO was added to dissolve the formazan formed from MTT by the enzymes associated with the metabolic activity. The plates were gently swirled at room temperature to dissolve the precipitate, for 10 min, and the absorbance was monitored at 550 nm. For LDH assay, HepG2 cells (2.5×10^5 cells/mL) were plated in 96 well plates with DMEM (Sigma), 2 mM glutamine, 0.2% sodium bicarbonate and 10% Foetal Bovine Serum (FBS) and allowed to rest for 2 hrs. Cells were further incubated with 50 and 100 μg/mL of purified extract and incubated for 18–24 hrs at 37 °C and 5% CO_2. After incubation, 100 μL of the cell-free medium was transferred into a fresh tube, and the LDH release from the cells was quantified by using cytotoxicity Detection Kit (Roche, cytotoxicity Detection Kit, Cat # 11 644 793 001) as per manufacturer's instructions. The mean values represent here are from 3 independent experiments and are given as fractional LDH release compared to the positive control consisting of 1% Triton X-100 (yielding 100% LDH release). Triton X-100 is used as a control, and dimethyl sulfoxide was used as the vector control.

Cellular antioxidant activity assay

DCFH-DA (20 mM in methanol) was prepared, aliquoted in multiple tubes and stored at −20 °C. Quercetin in dimethyl sulfoxide (DMSO) at a stock concentration of 50 mg/mL was used as positive control. *Tert*-butyl hydroperoxide (TBHP) to a final concentration 50 μM was used as a reactive oxygen species generator in HepG2 cell lines. Purified extract was dissolved in DMSO, and 25 μM DCFH-DA was prepared freshly in serum-free culture media. Human hepatocellular carcinoma HepG2 cells were seeded at a density of 6×10^4 cells per well on a 96-well microplate in 100 μL of growth medium. Upon full confluence, growth medium was removed, and the cells were washed with PBS to remove any nonadherent and dead cells. Next, 50 μL of 25 μM DCFH-DA working solution was added to each well, followed by 50 μL of the completely purified antioxidant extract in triplicate wells. Fifty microlitres of DCFH-DA and serum-free culture media (no antioxidant included) was used as a control in triplicate wells. After the DCFH-DA and antioxidant treatments, the plate was incubated for one hours at 37 °C. The cells were washed three times with PBS to ensure that any antioxidant effect observed later in the assay was only due to the compounds internalised by the cells. After washing, 100 μL of the free radical generator, *tert*-butyl hydroperoxide (50 μM) was added to the wells. The fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 538 nm. Further, cells were visualised and imaged with the help of EVOS™ Fluorescence cell imaging system at 40X.

UPLC-MS analysis of the purified fraction

Chromatographic separation of samples was carried out on Surveyor UPLC system (Thermo Fisher Scientific, Germany) consisting of a quaternary gradient pump, an autoinjector and an in-line degasser. The chromatographic separation was achieved on an Acquity UPLC® BEH C18 column (2.1 × 100 mm, 1.7 μm; Waters) using 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) as an organic modifier at a flow rate of 0.3 mL/min in gradient elution mode. The column was equilibrated with 10 column volumes of mobile phase at the initial gradient composition before sample injection. For sample run a continue gradient of 95% water and 5% acetonitrile was used up to 5 min, followed by 50% water gradient up to 15 min, then 90% water till 25 min and again ended with 95% acetonitrile up to 32 min of the total run.

The column compartment with a 25 °C temperature and a photodiode array (PDA) detector were employed throughout the analysis. Injection volume used in chromatography was 10 μL, and the sample was analysed using a PDA (200–600 nm) detector.

The mass spectral analysis was carried out on Thermo Orbitrap Velos Pro™ mass spectrometer (Thermo Fisher, Bremen, Germany). The effluent of UPLC was directed into Mass spectrometer through electrospray ionisation (ESI) interface and operated in the positive ionisation mode. The mass spectrometer was calibrated before analysis using the manufacturer's calibration solution consisting of caffeine, methionine-arginine-phenylalanine-alanine acetate (MRFA) and Ultramark® to obtain mass accuracies within a 2.5 ppm

range in external calibration mode. Parameters of the ion source were as follows: ion spray voltage + 4.25 kV, capillary temperature 350 °C, S-Lens RF Level 67.5%, sheath gas flow 30 (arbitrary units), auxiliary gas flow 8 (arbitrary units) and sweep gas 0 (arbitrary units). Nitrogen was used as the sheath and auxiliary gas in the ion source. The instrument was operated in full – scan FTMS over a range of *m/z* 100–1000 Da at a resolving power of 60 000 (full width at half maximum). PDA and mass spectral data were processed using Thermo Xcalibur software (v. 2.2).

Fragment matching search by METLIN

After the spectra acquisition from UPLC-MS, fragment matching search was performed with METLIN Metabolomics Database, maintained by the Siuzdak laboratory at the Scripps Research Institute [34]. In the METLIN database search programme, the tolerance was 5 ppm, the mode was positive, and filtered out fragments with intensity less than 10%.

Statistics

All the values are mean ± standard deviation. ANOVA (analysis of variance), followed by post hoc testing using the Holm-Sidak method was carried out using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla, CA, USA, (www.graphpad.com) to determine significance in readings. Significance at $p < 0.05$ was taken as relevance.

Results

Marine sample collection and isolation of bacteria

Marine samples collection was performed as previously reported elsewhere [18]. Seawater and sediment from the Gulf of Mannar (Supporting information S1) were collected in the sterile tube consisting of Zobell Marine Broth 2216 or transport media and packed in sterile polythene bags. The samples were transported to the lab on the ice and processed immediately to avoid any significant change in microbial composition. Bacteria isolation and selection method was done as previously reported [20].

Even though there is an immense interest among the researchers, approximately, less than 10% of the marine species have been identified by using Zobell's marine agar or synthetic seawater medium or by artificial sea salt medium [35]. We, in this study, used Zobell's marine broth to culture the microbes and were able to culture only twenty-four marine isolates with a high rate of reproducibility under *in-vitro* conditions. The major problem in the study of marine microbes is the inability to grow the marine-derived bacteria on artificial nutrient mediums [36]. For the culture of a single strain, a loop full of bacteria from the glycerol stock was streaked on Zobell marine growth medium and further incubated for 24–48 hrs at 37 ± 2 °C.

Screening for potential antioxidant producer strain

High throughput screening was employed as it was a robust and straightforward way to screen antioxidant activity of marine bacterial extracts. All twenty-four bacterial strains were tested for DPPH free radical scavenging activity, some of them showed the potent antioxidant activity, with low cell density against the α-Tocopherol (100 μg/mL) as positive control, e.g. CDMP 10, CDMP 3 were showing the intense activity with small cell number (blue circled area of Supporting information S2). The low absorbance at 620 nm showed that these bacteria are less in number but producing sufficient quantity or active antioxidant molecules to reduce the DPPH free radical more effectively, in comparison to other microbes with high cell density. Hence, we preferred to select those bacteria which were able to produce highly active antioxidant molecules during the log phase.

Molecular and physical identification of bacterial species

Natural product drug discovery programme has many hurdles in their way. One of the most common is the replication of discovery efforts regarding the discovered molecules [37]. To avoid redundancy and repetition of the work, we decided to identify the marine microbes or the active ingredients which had shown promising antioxidant activity as early as possible. For achieving our goals, all the positive strains had been characterised through morphological (Gram-staining and scanning electron microscopy (SEM)), and molecular (16S rRNA) identification. The 16S rRNA gene amplicon obtained after PCR amplification was found to have the size of 1.5 kb (Supporting information S3). The final processed 16S rRNA sequencing data were submitted to NCBI (as mentioned in Supporting information S4, S5) and then used for blast analysis. Identification of potential antioxidant producer strains (Supporting information S4) and reported literature survey had shown that some of the bacterial strains are already reported for their antioxidant activity. Hence, we proceeded with the marine

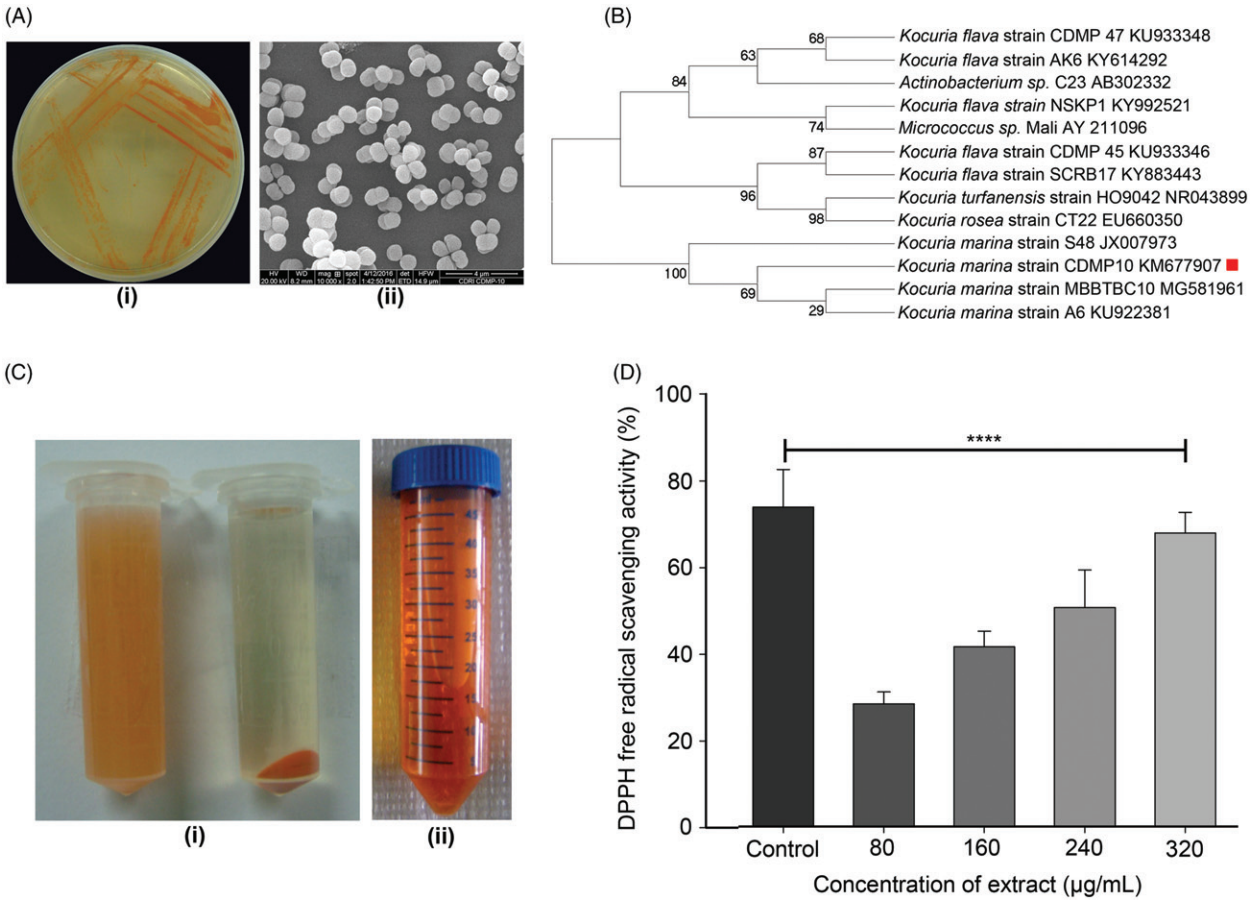

Figure 1. (A) Photographs show the CDMP 10 bacterial strain: streak plate culture of bacterial strain CDMP 10, Scanning electron microscopy image of bacteria. (B) Evolutionary relationship of marine bacterial isolate *K. marina* CDMP 10 (marked: red coloured square) with some other bacteria. (C) (i) *K. marina* CDMP 10 cultured and peleted bacteria. (ii) Red pigmented methanolic crude extract (D) DPPH free radical scavenging activity of crude methanolic extract from *K. marina* CDMP 10 at various concentrations with α-tocopherol (100 µg/mL) as a control. One way ANOVA analysis showed $p < 0.001$ with significance difference between mean values ($p < 0.05$).

bacteria *Kocuria marina* CDMP 10 isolate, which was not reported earlier.

Figure 1(A and C), shows the pigmentation, morphology of bacterial colony and electron microscopy image of bacteria CDMP 10. These Gram-positive bacteria are cocci and having red pigmented colonies. Phylogenetic tree of CDMP 10 bacterial isolate from the Bay of Bengal (Figure 1(B)) shows that isolate has a very close evolutionary relationship with the *K. marina* strain S48 RNA sequence. The growth curve of *K. marina* CDMP 10 was studied in Zobell's medium (Supporting information S6). The bacterium achieved the stationary phase by 48 hrs with optimum cell density.

Extraction and antioxidant activity of the crude extract

The freeze lysed bacterial pellet of *K. marina* CDMP 10 was subjected to extraction with methanol as a solvent (Figure 1(C(ii))). In the DPPH free radical scavenging activity assay, the crude methanolic extract of *K. marina* CDMP 10 had shown the concentration-dependent inhibition of DPPH free radical with EC_{50} value at approximate 240 µg/mL (Figure 1(D)). The antioxidant activity of the extract was approximately 2.5 fold lower than α-tocopherol, which was having the EC_{50} value as 100 µg/mL. We believe that the higher concentration of extract requirement is due to the presence of other components as impurities and a comparatively lower level of the bioactive molecule.

Activity-guided HPLC fractionation of the crude extract

Upon evaporation of methanol, we observed that extract had an oily solid appearance which could be due to the presence of nonpolar residues. Hence *n*-hexane washing was done to remove the fatty fraction. The *n*-hexane washed extract was fractionated with the help of preparative RP-HPLC (Waters Spherisorb® S10

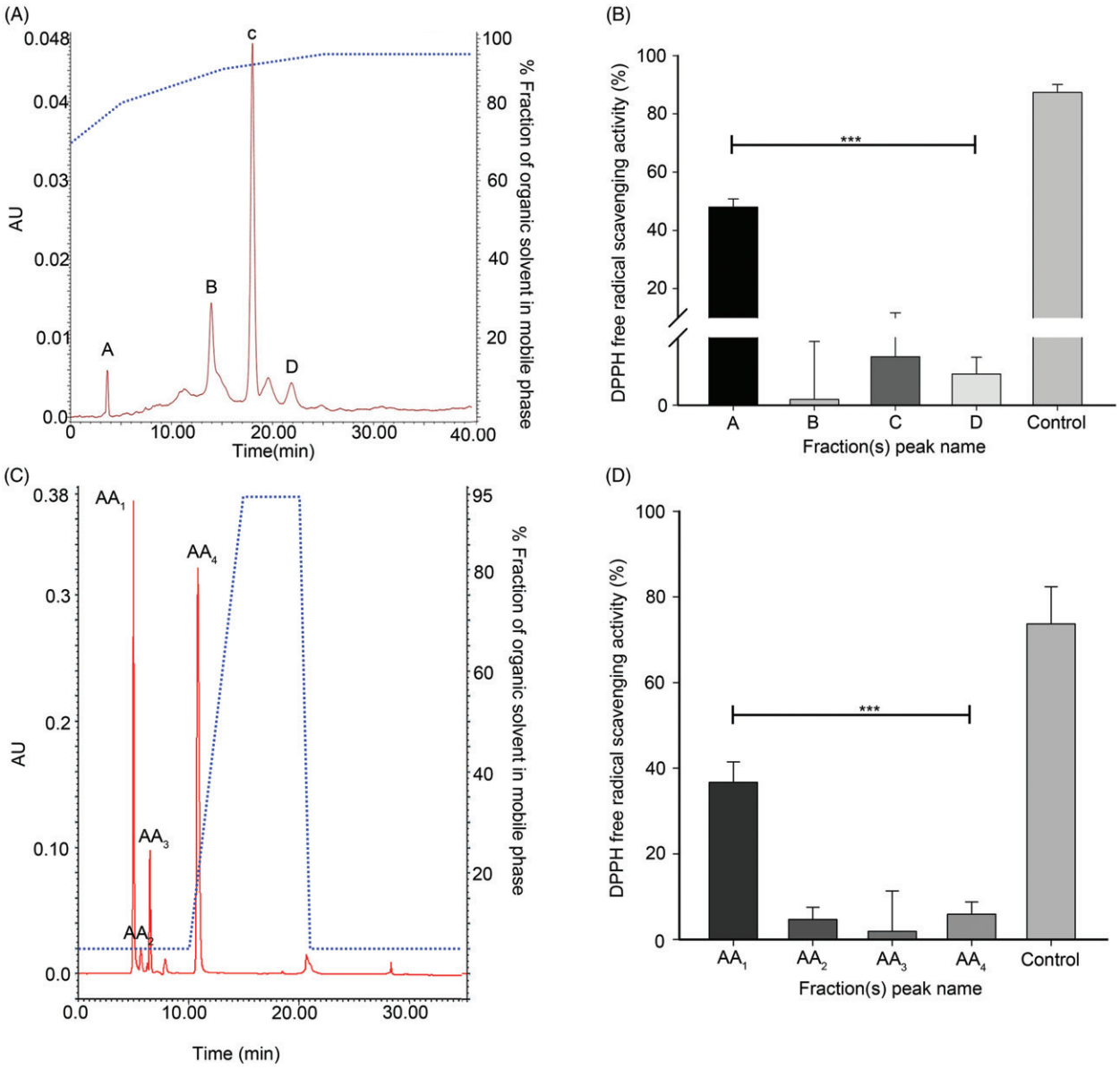

Figure 2. (A) Preparative RP-HPLC Chromatogram of methanolic crude extract; chromatogram represents the elution time and absorption unit of particular fractions of compounds, the blue line represents the concentration of organic solvent. (B) DPPH free radical scavenging activity of collected fractions peaks from preparative RP-HPLC. Statistical analysis by one way ANOVA revealed $p < 0.001$ with significance difference between mean values ($p < 0.05$). (C) HPLC chromatogram of antioxidant fraction purification through RP-HPLC. Chromatogram represents the elution time and absorption unit of particular fractions of compounds, the blue line represents the concentration of organic solvent. (D) DPPH free radical scavenging activity of purified peaks from preparative RP-HPLC. One way ANOVA analysis of all group showed $p < 0.001$ with significance difference between mean values ($p < 0.05$).

ODS2, C18, 10 × 250 mm column) with gradient mobile phase 70–100% methanol in water. We have collected each of the peaks (at 470 nm) and subjected to DPPH free radical assay for the assessment of antioxidant activity (Figure 2(A,B)).

As shown in Figure 2(B), peak A fraction (100 μg/mL) showed the antioxidant activity as compared to α-tocopherol (100 μg/mL). Peak A was soluble in water. Due to polar nature of compounds, peak A retention time was less than 5 min. Peak purity determination revealed that antioxidant (Peak A) fraction of extract contains a group of very polar compounds. Hence, to purify the active molecule we used RP-HPLC with a different solvent system.

Purification of an Anti-oxidant fraction with the help of RP-HPLC

The antioxidant fraction (Peak A fraction) was repurified by RP-HPLC with different solvent (Figure 2(C)). Various peaks have been acquired at various time points. The chromatogram showed four to five absorption spectra

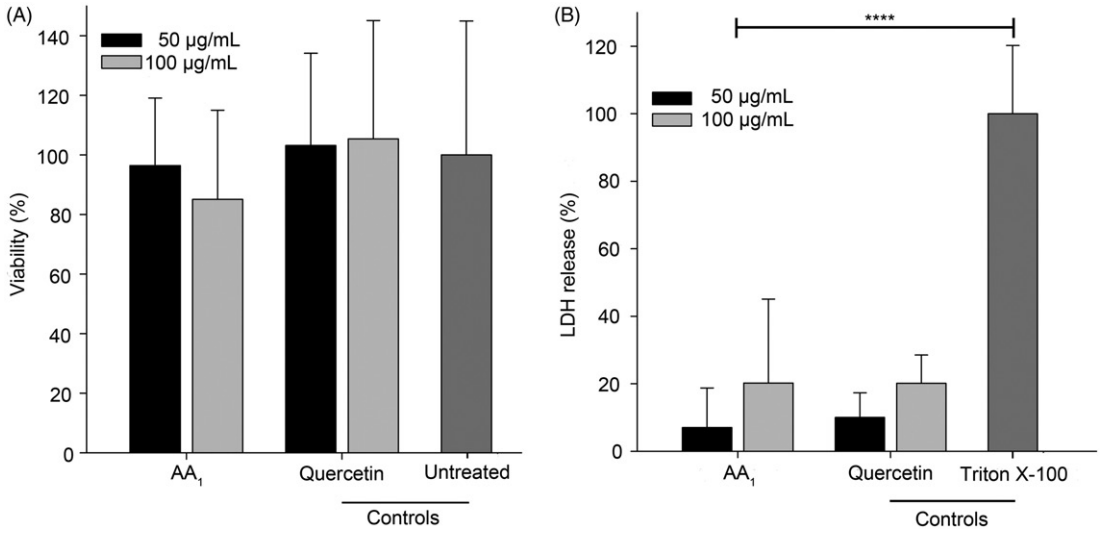

Figure 3. Cellular toxicity of fraction AA_1 against the Hepatocellular carcinoma cell line (HepG2) with the help of (A) MTT cell viability assay and (B) LDH assay. The values represent mean of triplicate samples, and a representative experiment (of three) is shown here, one way of ANOVA was done on raw values, $p < 0.001$ with significance difference between mean values ($p < 0.05$).

at the same retention time point, which indicates the presence of four to five different compounds with the same polarity. All the peaks from RP-HPLC (Figure 2(C)) were collected and tested for DPPH free radical scavenging activity (Figure 2(D)). Among all the peaks obtained, only the Peak AA_1 has shown antioxidant activity (100 µg/mL).

Cellular toxicity of antioxidant molecules against the Hepatocellular carcinoma cell line (HepG2)

The golden rule in the drug discovery process is "false positive and toxic compounds should be eliminated as early as possible". For developing the lead molecule for antioxidant therapy, the molecule should not have the toxicity towards the eukaryotic cells; we, therefore, checked the cellular toxicity of fraction AA_1 against the human hepatocellular carcinoma cell line with the help of MTT cell viability assay and LDH assay.

As demonstrated by MTT assay (Figure 3(A)), fraction AA_1 treated cells had shown normal cell viability in comparison to control quercetin (50 or 100 µg/mL) treated or untreated cells at the concentration of 50 or 100 µg/mL. For MTT, LDH and cellular antioxidant assay quercetin is the most favoured control in comparison to the α-tocopherol as well as quercetin has maximum antioxidant activity in ROS stressed cells [38]. This outcome from MTT assay is quite comparable to the LDH assay (Figure 3(B)). These results indicated that fraction AA_1 has no toxic effect on Hep G2 cells. Based on above-mentioned properties of this extract, it is tempting to speculate that the compounds isolated from *K. marina* CDMP 10 might be involved in the protection of bacterial cells from the reactive oxygen species as they show antioxidant activity. To check whether these molecules can prevent the human cell lines from tert-butyl hydroperoxide generated ROS, we performed the cellular antioxidant assay on HepG2 cells.

Cellular antioxidant activity (CAA) assay

Cellular antioxidant activity assay was performed to check whether fraction AA_1 can quench the intracellular induced reactive oxygen species level. As it is quite visible from the Figure 4(A and B) that *tert*-butyl hydroperoxide treated cells has the highest fluorescence intensity. However, the *tert*-butyl hydroperoxide treated cells when supplemented with antioxidant molecules (fraction AA_1 or quercetin) was having lesser fluorescence intensity comparable to the nonsupplemented (Control).

Identification of purified fraction through UPLC-MS

We have tried to purify the AA_1 fraction through different purification methodologies, but we were unsuccessful in separating the fraction with high resolution, probably due to high polar nature of the compounds. Hence, peak AA_1 from RP-HPLC was analysed through UPLC-MS. The resultant mass spectral peaks (Supporting information S7) were analysed with the help of METLIN Metabolomics Database, maintained by the Siuzdak laboratory at the Scripps Research Institute (https://metlin.scripps.edu) (Figure 5). Fragment similarity search had the tolerance value of 0–5 ± 2 ppm and

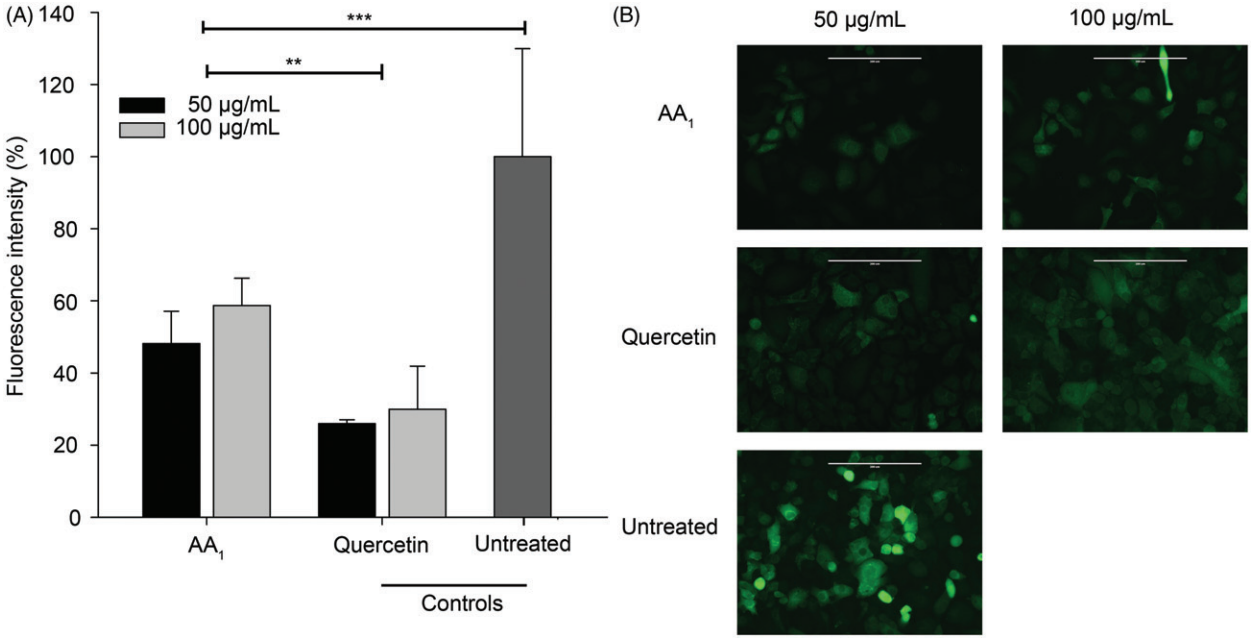

Figure 4. (A) Cellular antioxidant activity (CAA) assay against DCFH-DA fluorescence dye. One way ANOVA analysis based on raw values, $p < 0.001$ with significance difference between mean values ($p < 0.05$). (B) Fluorescence image of HepG2 cells in a stressed condition (negative control), Stressed cells supplemented with quercetin (drug control) or fraction AA_1 (antioxidant molecules).

mode was kept positive (Supporting information S8). The analysis indicated the presence of small fragments of peptides, i.e. Ser–Ser–Gln, Phe–Glu, Asp–Ile and Leu–Glu, in the AA_1 fraction. Based upon the data obtained from UPLC-MS, we first decided to rule out the possibility of the false positive results; we tested the RP-HPLC purified fraction for the presence of amine group/amide bond. Our results indicated that there is the presence of amine group/amide bond in the fractions. This positive result made us to believe that active fraction constituted of amino acid or amine group. We believe that these short peptides were able to reduce the DPPH free radical and chemical induced oxidative stress in human cells.

Discussion

Previously, many compounds such as mycosporine-like amino acids [39], carotenoids [40], some polyphenolic compounds [41], isobenzofuranone derivative [42], exopolysaccharides [43] etc. have been reported from marine microbial isolates to show promising antioxidant activity. Hence, we tried to explore the marine bacteria antioxidant properties through high-throughput screening method.

Activity-based purification and fractionation is a very effective way in natural product drug discovery. Earlier W. Dunlap has used microtitre plate technology and xanthene dye reagent to screen the marine organisms for their antioxidant potential [44]. We had chosen the DPPH free radical assay over xanthene dye-based assay because DPPH (purple-blue) free radical become colourless in the presence of antioxidant molecule whereas the xanthine dye firstly get oxidise by certain oxidising agents and then oxidised dye get reduces by the action of test antioxidant molecules. Hence, DPPH free radical requires direct action of antioxidant on DPPH molecule itself. In this study, we have optimised the DPPH free radical to screen the antioxidant molecule from various marine bacteria.

Previously, Kim et al. reported *K. marina* strain from sea sediment taken from the Troitsa Bay of the Gulf of Peter the Great, East Siberian Sea [45]. We here report the presence of *K. marina* strain in the Bay of Bengal, India and establish the antioxidant property of this strain for the first time.

Marine bacteria are known to produce many short peptides (Di or Tri peptides) to accomplish multiple functions. It was reported earlier that marine bacteria *Chromohalobacter* sp. produces an extracellular antioxidant molecule which is peptide [46]. Apart from above-mentioned activities, some peptides such as cyclo-D-pipecolinyl-L-isoleucine isolated from the cell-free culture supernatant of the Antarctic psychrophilic marine bacterium *Pseudoalteromonas haloplanktis* TAC125, has shown DPPH free radical scavenging activity [47]. Recently, Yang J et al., have isolated new antioxidant dipeptides as Tyr–Leu (YL) and Phe–Tyr (FY), from

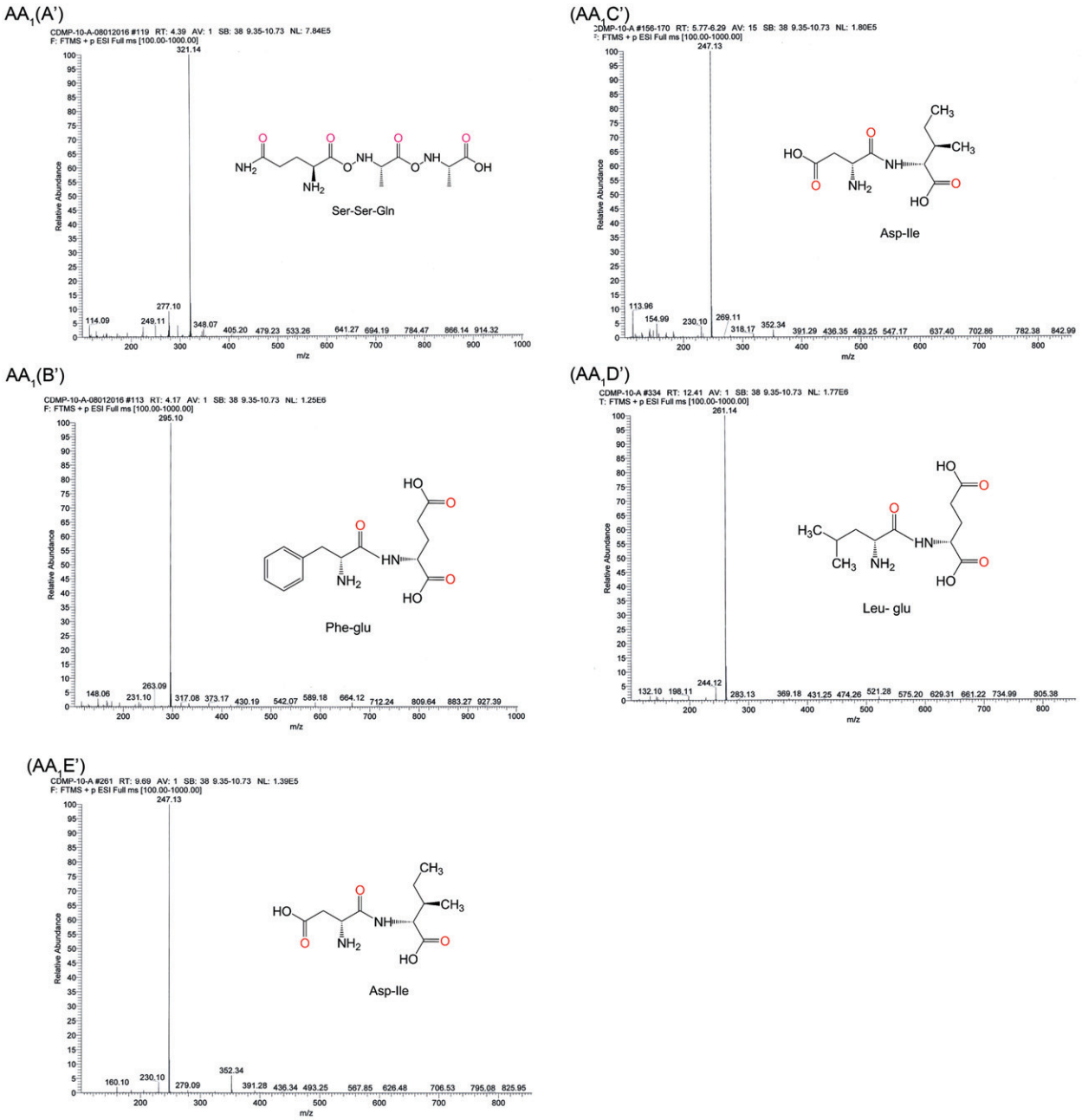

Figure 5. ESI-MS spectra acquired from UPLC-MS at different elution time and analysed through METLIN.

perilla (*Perilla frutescens* L. Britton) seed protein hydrolysate [48]. The antioxidant dipeptide FY induced HepG-2 cell apoptosis at 100 μg/mL concentration. In comparison to FY dipeptide, the di and tri peptides from *K. marina* CDMP 10 has no cytotoxicity at the concentration of 50 and 100 μg/mL on HepG2 cell lines. Furthermore, isolated small peptides could be directly linked with the environmental adaptation of bacteria, as the dipeptide N-acetyl glutaminyl glutamine amide (NAGGN) have been isolated from the high osmolarity grown bacteria *Sinorhizobium meliloti*. It has been shown that this dipeptide accumulates in some other bacteria when they are grown in the high osmotically challenging environment [49]. These present studies also confirm that the bioactive peptide-based molecule could have antioxidant activity.

Nowadays, many other synthetic antioxidants small molecules such as butylated hydroxyanisole, butylated hydroxytoluene, *tert*-butylhydroquinone and propyl gallate [50] are being used for this purpose in the cosmetics and cosmeceutical industry. However, these molecules are associated with toxicity. We believe that these di or tri peptides could be used in cosmetic formulations to prevent oxidation of ingredients or protecting the skin from oxidation induced by UV radiation and loss of moisture in skin.

Targeted drug delivery is emerging as a promising method to achieve better clinical efficacy or deliver cytotoxicity drugs to the hard to hit target site [51]. Recently, peptides especially (<10 amino acids), are being used as the one of the three building blocks used to make drug conjugates. Peptide ligands are advantageous over other classes of targeting ligands due to their accessibility of high-throughput screening, ease of synthesis, high specificity and affinity, etc. [52]. Tripeptide (RGD) and GnRH peptide analogues constitute an emerging class of tumour homing peptides for treating different aggressive cancers [53,54].

In line with these studies, our study has revealed that *K. marina* CDMP 10, methanol extract has multiple short peptides i.e. Ser–Ser–Gln, Phe–Glu, Asp–Ile and Leu–Glu with free radical scavenging activity. Probably these peptides are preventing the bacterial cells from ROS-induced-stress. Given the small size, biological in origin, following the Lipinski's rule, we believe that these short peptides can be used as a lead molecule for antioxidant therapy in various diseases including potential commercial dermatological applications or target delivery approaches.

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Disclosure statement

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