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RESEARCH

β-(1 → 3) Linked Sulfated Polygalactan from a Seaweed-associated *Bacillus velezensis* MTCC 13097: A Potential Lead Against Human Hepatocellular Adenocarcinoma

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Abstract

Among tumors, liver cancer has an inferior prognosis. Therefore, exploring alternative strategies to improve the effectiveness of treatment for this ailment is of utmost urgency. In this study, we focused on analyzing the anti-cancer properties of bacterial exopolysaccharide from *Bacillus velezensis* associated with the seaweed *Sargassum wightii* against hepatocellular adenocarcinoma. A culture-dependent method was used to isolate heterotrophic *B. velezensis,* which was then evaluated for its antioxidant and anti-cancer properties. A β-(1 → 3) linked sulfated polygalactan exopolysaccharide (BVEP-2) was isolated from the bacterial extract and characterized by spectroscopic analysis. The anti-cancer property was analyzed through assays involving 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), neutral red uptake (NRU), and apoptosis (by annexin V-FITC/PI staining) against the human hepatocellular adenocarcinoma cell line (HepG2). BVEP-2 demonstrated potential cytotoxicity in the MTT assay (IC_{50} 65.05 µg/mL) and 23.02% cell viability in the NRU assay at a 100 µg/mL concentration of BVEP-2 against HepG2, compared to the standard doxorubicin. Potential antioxidant properties of BVEP-2 (IC_{50} 112–117 µg/mL) corroborated the anti-cancer activities, and the attenuation of free radicals could play a significant role in its anti-cancer potential. BVEP-2 induced approximately 9% early apoptosis and 39% late apoptosis in the HepG2 cell line, whereas the standard drug resulted in around 38% early apoptosis and 37% late apoptosis, along with 6% necrotic cells. The β-(1 → 3) linked sulfated polygalactan exopolysaccharide (BVEP-2) of *B. velezensis* MTCC13097 showed potential antioxidant and anti-cancer activities, and thus, could be developed as a promising pharmacophore lead against human hepatocellular adenocarcinoma.

Keywords Heterotrophic bacterium · *Bacillus velezensis* MTCC13097 · β-(1 → 3) linked sulfated polygalactan · Anti-cancer agent · Human hepatocellular adenocarcinoma

Abbreviations

BVEP	*Bacillus velezensis* exopolysaccharide
HCC	Hepatocellular carcinoma
HepG2	Hepatocellular carcinoma cell line
MCF-7	Metastatic adenocarcinoma cell line
HPLC	High-performance liquid chromatography
NMR	Nuclear magnetic resonance
NCBI	National center for biotechnology information
MTCC	Microbial-type culture collection
MeOH	Methanol
NaCl	Sodium chloride
KH_2PO_4	Potassium dihydrogen phosphate
K_2HPO_4	Dipotassium hydrogen phosphate
$CaCO_3$	Calcium carbonate
Na_2NO_3	Sodium nitrate

Sumayya Asharaf and Kajal Chakraborty have equally contributed to the outcome of the paper.

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DEAE-Cellulose	Diethylaminoethyl cellulose
ABTS	2,2′-Azino-bis-(3-ethylbenzothaizolin-6-suifonic acid)
DPPH	2,2-Diphenyl-1-picryl-hydrazyl-hydrate
MTT	3-(4,5-Dimethyl thiazol-2-yl)-2,5-diphenyltettrazolium bromide)
NRU	Neutral red uptake
FITC	Fluorescein isothiocyanate
PI	Propidium iodide
FTIR	Fourier transform infra-red

Introduction

Liver cancer is the world's fourth leading cause of cancer-associated deaths, recording more than 78,000 cases annually (Mak and Kramvis 2021). Hepatocellular carcinoma (HCC), accounting for over 80% of primary liver malignancies, is connected to several factors, including infection by the hepatitis C/B virus, excessive alcohol intake, and non-alcoholic fatty liver disease. These factors cause persistent liver inflammation leading to HCC in the long run (Suresh et al. 2020). Recently, marine microbial polysaccharides have become significant bio-resources for developing anti-malignancy agents owing to several advantages, such as biocompatibility, biodegradability, non-toxicity, and abundance (Wang et al. 2019). Natural products have been the cornerstone of tumor chemotherapy for the past years. They could provide many lead structures, which can be used as templates to produce new compounds with improved chemical characteristics. Asker et al. (2018) reported that exopolysaccharides (EPS) isolated from marine sediment bacteria of the Mediterranean and Red Seas have cytotoxic activity against HepG2 cells. It has been reported that marine exopolysaccharide inhibits the invasion of liver cancer cells (Liu et al. 2021). Abdelnasser et al. (2017) discovered that exopolysaccharides of the marine bacterial strains exhibited cytotoxic activity on HepG2 cells. A heteropolysaccharide produced by a halophilic strain of *Salipiger mucosus* $A3^T$ was reported to possess potential cytotoxic activity (Llamas et al. 2010).

The exopolysaccharide extracted from the heterotrophic *Bacillus* was reported to have greater yields than other marine bacteria (Finore et al. 2014). Conspicuously, the EPS derived from various *Bacillus* sp. are widely used in food, pharmaceuticals, and chemical industries, such as bioabsorbents, bioflocculants, and drug delivery agents on account of their scavenging properties of reactive oxygen species (ROS) (Yassin et al. 2020a; Mostafa et al. 2023). The EPS produced from bacteria that can synthesize xanthan and dextran are the two additives of microbial origin used in the food industry in the EU and USA (Petrova et al. 2021). The structural attributes of EPS, including monosaccharide compositions, sequence of glycosyl residues, linkage pattern of glycosides, and branching, play prominent roles in the biological functions (Wang et al. 2016). *Bacillus* sp. is a principal source of $(1\rightarrow3)/(1\rightarrow6)$-$\beta$-D-glucan polysaccharides, and previous works of literature reported that the proteoglycan isolated from *Bacillus* sp. demonstrated strong anti-inflammatory activity by suppressing cytokine production (Wang et al. 2020). The exopolysaccharides of the endophytic bacterium *Bacillus amyloliqufecians* were extracted from the endophyte, showing antitumor activity against gastric carcinoma cell lines (Chen et al. 2013).

About 5×10^{-4} billion marine microbial bioactives were characterized, and more than 8000 were endowed with potential antibiotic and antitumor activities (Villarreal-Gomez et al. 2010). Marine *Bacillus* species were recognized for biosynthesizing different classes of bioactive metabolites with anti-cancer and antimicrobial potential (Kizhakkekalam et al. 2020; Yassin et al. 2020b). Previous work on the culture-dependent study from our laboratory developed an antibacterial strain of *Bacillus altituidinis* MTCC13046 isolated from an intertidal seaweed *Sargassum wightii* with bioactivities against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecalis* (Asharaf et al. 2022). As there were reports of antibacterial metabolites possessing potential anti-cancer activities (Villarreal-Gomez et al. 2010; Pham et al. 2019; Francis and Chakraborty 2021; Mikaili et al. 2013), the present study envisaged evaluating the anti-cancer action of the β-$(1\rightarrow3)$ linked sulfated polygalactan isolated from *Bacillus velezensis* MTCC13097 associated with seaweed *Sargassum wightii* by cytotoxicity analyses and induction of apoptosis by in vitro studies on human hepatocellular adenocarcinoma (HepG2) cell lines. *B. velezensis* was previously recognized as a biocontrol agent (Chen et al. 2018), but has lately been found to possess remarkable anti-cancer properties against MCF-7 (breast cancer) cells (Francis and Chakraborty 2021). Noticeably, oxidants can further the pathophysiology leading to carcinogenesis, while antioxidants can impede the advancement of cancer. Therefore, assessing antioxidant potential is essential to corroborate the anti-cancer properties (Kizhakkekalam and Chakraborty 2021; Zimmermann et al. 2001). Thus, the prospect of β-$(1\rightarrow3)$ linked sulfated polygalactan to scavenge the oxidants was evaluated by several in vitro systems.

Materials and Methods

Isolation of Heterotrophic Bacteria Associated with Seaweed

Heterotrophic *B. velezensis* MTCC13097 associated with the intertidal seaweed *S. wightii* was isolated. The algal thalli (8

g) were homogenized in 10 mL of sterile seawater. The suspension was serially diluted, and each dilution was spread-plated on nutrient agar (NA) and Zobell marine agar (ZMA) supplemented with sodium chloride (1% w/v) before being incubated at 30°C (in the dark) for 1 week (Chakraborty et al. 2021a). Pure cultures were separated by the streak plate method. Then, the cultures were characterized using morphological, biochemical, matrix-assisted laser desorption time of flight mass spectral analysis, 16S rRNA gene sequencing, and Biolog characterization (Chakraborty et al. 2022). Polymerase chain reactions were used to amplify the fragmented DNA using the universal primers AGAGTTTGA TCCTGGCTCAG (forward) and ACGGCTACCTTGTTA CGACTT (reverse) (Weisburg et al. 1991; Chakraborty et al. 2021b). The fragmented DNA products were sequenced and submitted to the NCBI GenBank database.

Purification, Spectroscopic Characterization, and In Vitro Antioxidant Assays

Isolates were tested for the generation of EPS using media containing the following components (g/L): glucose 20, $CaCO_3$ 0.1, KH_2PO_4 0.05, K_2HPO_4 0.6, $MnSO_4.H_2O$ 0.1, NH_4NO_3 0.8, and yeast extract 1.0. The seaweed-associated *B. velezensis* MTCC13097, producing polysaccharide, was grown on nutrient agar plates, and incubated at 37°C for 48 h. The surface colonies were scraped and boiled in water for 3 h, followed by the addition of ice-cold ethanol and refrigeration for 24 h. The culture was centrifuged at 5000 *g* for 30 min at 4°C to remove bacterial cells. The supernatant was subjected to depotentization by trichloroacetic acid (5% w/v) before being centrifuged. The supernatant was collected, mixed with five times its volume of ice-cold ethanol, and refrigerated for 12 h before centrifugation. The precipitate was washed with acetone and dried under an infra-red lamp to yield the crude polysaccharide, BVEP (Sahana and Rekha 2020).

Anion exchange chromatography utilizing diethyl aminoethyl (DEAE)-cellulose on a glass column (25 cm × 4 cm) was used to fractionate the crude polysaccharide isolated from *B. velezensis* MTCC13097. Initially, a slurry of DEAE-cellulose (15 g) in tris buffer (30 mL, 50 mM, pH 7.4) was prepared and allowed to swell for 1 h before being packed onto the column and equilibrated with tris buffer. The crude exopolysaccharide (1.5 g) dissolved in distilled water was passed through packed DEAE-cellulose resin and eluted with an increasing sodium chloride gradient (0.1 to 0.4 M) to produce the sub-fractions BVEP-1 through BVEP-4 that were analyzed for the positive chemical reaction of polysaccharide (Wu et al. 2016). Monosaccharide composition was analyzed by HPLC connected with an evaporative light scattering detector (ELSD-SofTA 300S, Teledyne Technologies Inc., Lincoln NE) attached to an amino column (25 × 0.46 cm, 5 µm) after hydrolyzing with trifluoroacetic acid (TFA, 2.0 M). Spectroscopic analysis of BVEP-2 was performed by using the proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy on a Bruker ADVANCE III 500 MHz spectrometer and Fourier transform infrared (FTIR) spectroscopy. Peak analysis of NMR spectral data was analyzed by MestReNova (version 7.1.1–9649, Mestrelab Research S.L) using the global spectrum deconvolution (GSD) technique. The exopolysaccharide BVEP-2 fraction (~ 60 mg) was evaluated for its capacity to scavenge the oxidants 2, 2′-diphenyl-1-picrylhydrazyl (DPPH•) and 2, 2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^+$) in addition to its metal-chelating potential (Kizhakkekalam and Chakraborty 2019). The percentage inhibition of the radicals was computed, and the results were expressed as IC_{50}, the concentration at which the bacterial exopolysaccharide could inhibit 50% of the radical activity.

Anti-cancer Properties and Cytotoxicity Analysis of *B. velezensis* Exopolysaccharide BVEP-2

The anti-cancer assay was performed against the human hepatocellular adenocarcinoma cell line (HepG2). This study used doxorubicin as a standard drug and untreated cell lines as a control, compared to the BVEP-2 exopolysaccharide. HepG2 cells were preserved in Dulbecco's modified Eagle's (DME) medium (Invitrogen, Carlsbad, CA, USA). Briefly, the cells (0.2 mL) were poured into 96-well microplates (1×10^6 cells $well^{-1}$) before incubating in a carbon dioxide incubator (at 37°C) for 24 h until reaching 90% confluence. After replacing the medium, the cells were treated with BVEP-2 (12.5, 25, 50, and 100 µg/mL) and incubated for 48 h. After the incubation period, the spent media was washed with phosphate-buffered saline (PBS, pH 7.4) and 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT, 500 µg/mL) was added. Following storage of the plates at 37°C for 3 h in the dark, formazan crystals were dissolved in dimethylsulfoxide (100 µL) before measuring the absorbance at 570 nm (Price and McMillan 1990). In the neutral red uptake assay (NRU), a neutral red staining solution (100 µL) was added, and the optical density of formazan crystals was estimated at 570 nm (Francis and Chakraborty 2021). Cell viability was calculated by comparing the absorbance of treated cells to that of control and calculating the IC_{50} value using the linear regression equation.

Apoptosis Assay with Annexin-V and Propidium Iodide

The cells (0.5×10^6 cells in 2 mL) were grown in a 96-well plate and incubated for a day at 37°C (in a CO_2 incubator). The medium-free cells were treated with the BVEP-2 bacterial exopolysaccharide (2% w/v) before incubating for 2

days. EDTA/trypsin solution (0.2 mL) was supplemented with PBS-washed spent medium before being incubated at 37°C for 4 min, and the culture medium (2 mL) was added. The cells were harvested by centrifugation (6000 *g*, 25°C for 10 min). Later, annexin-V (5 µL)/fluorescein isothiocyanate (FITC) was added to the PBS-washed cells in the 96-well microplate, which was vortexed before incubating for 20 min at 25°C in the dark, and added with propidium iodide (PI, 5 µL) and 1 × binding buffer (400 µL). Following that, flow cytometry analysis was performed (Koopman et al. 1994).

Data Analysis

The data were analyzed using the statistical program for social sciences (SPSS Inc, CA; version 10.0). The experiments were performed in triplicate, and the means of both parameters were analyzed using ANOVA (analysis of variance) for significance ($p < 0.05$).

Results

Isolation and Characterization of Seaweed-associated *B. velezensis* MTCC13097

The heterotrophic bacteria *B. velezensis* MTCC13097 was isolated to homogeneity from the intertidal seaweed *S. wightii*. The bacterium was characterized by morphological/microbiological, KOH screening/gram staining analyses, which were followed by comprehensive phenotypic and genotypic analysis (Fig. 1). *B. velezensis* was characterized as white undulate colonies and short-chained, gram-positive, and spore-forming. Biochemical characterization showed positive reactions for catalase, starch hydrolysis, oxidase activity, nitrate reduction, Voges-Proskauer's, and citrate/carbohydrate/arginine utilization, wherein negative results were obtained using malonate, *β*-galactosidase, indole processing, and lysine decarboxylation. Optimal growth of the heterotrophic bacterium was found to occur at 37°C, and at a mildly alkaline condition (pH 7.0) with a salt concentration of 0–4%. Finally, the molecular characterization was carried out by 16S rRNA gene sequencing before submitting to the GenBank (accession number MT122835). The hemolytic activity of *B. velezensis* was assessed through genetic-level screening of toxicity-producing genes (hemolysin BL (A-D)) as well as on blood agar plates, as reported previously (Asharaf et al. 2022). The results suggested that *B. velezensis* is negative in amplifying hemolysin-producing genes compared to the control organism *B. cereus*. *B. velezensis* showed negative results on blood agar plates as well. Furthermore, we have performed hemolysis activity of BVEP-2 on blood agar plates and observed negative results, demonstrating their biosafety and hemocompatibility.

Extraction, Purification of Exopolysaccharide, and In Vitro Antioxidant Assays

Anion exchange chromatography of crude exopolysaccharide, BVEP (with a 5.22% yield) from *B. velezensis* MTCC13097, resulted in four sub-fractions (BVEP-1 to BVEP-4). Among these, BVEP-2 (eluted with 0.2 M NaCl) with greater carbohydrate content (74.8%) was selected for

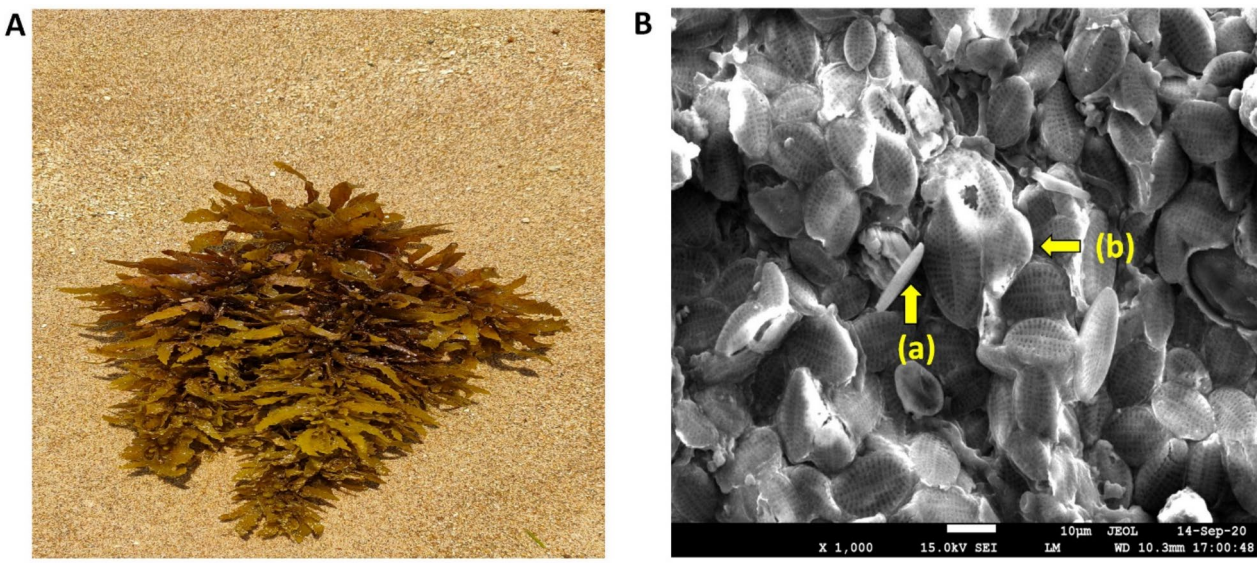

Fig. 1 **A** Representative photograph of the intertidal seaweed *Sargassum wightii*. **B** Scanning electron microscopic image of seaweed-associated bacteria depicting their symbiotic association ((a) rod-shaped heterotrophic bacteria *B. velezensis* MTCC13097 attached to the seaweed thallus and (b) seaweed thalli)

further spectroscopic characterization. Monosaccharide composition analysis of BVEP-2 revealed the presence of galactose as the major sugar component. The BVEP-2 fraction was further tested for antioxidant properties and displayed potential scavenging activities against DPPH and $ABTS^+$ (IC_{50} 117 and 112 µg/mL, respectively). Noticeably, the exopolysaccharide fraction BVEP-2 of *B. velezensis* displayed significantly greater antioxidative potential than that exhibited by α-tocopherol (IC_{50} 660–750 µg/mL) (Table 1).

In Vitro Evaluation of Anti-cancer Properties of β-(1 → 3) Sulfated Polygalactan Exopolysaccharide (BVEP-2)

The experiments used the liver cancer cell line HepG2 and doxorubicin as the standard drug and untreated cells as control.

MTT and NRU Assay

The BVEP-2 fraction extracted from *B. velezensis* MTCC13097 demonstrated substantial cytotoxicity in the MTT assay (IC_{50} 65.05 µg/mL) against HepG2 (Fig. 2A–F). We have identified that BVEP-2 induced a considerable cytotoxic effect on the HepG2 cell line compared to the lesser effects of the standard drug doxorubicin. In contrast to the standard, the BVEP-2 displayed more significant cytotoxicity to HepG2 cancer cells and more secondary toxicity to normal cell lines. Briefly, the cytotoxicity property of BVEP-2 was tested against normal human dermal fibroblast (HDF) via MTT assay. The results suggested that BVEP-2 does not trigger a cytotoxic effect on normal HDF cell lines. Additionally, the BVEP-2 showed 70–89% cell viability in the HDF cell line. Likewise, BVEP-2 demonstrated dose-dependent cytotoxicity against HepG2 cells, in response to NRU assay (Fig. 2G), at concentrations of 12.5–100 µg/mL (Table 1). The cancer cells showed only 23.02% cell viability at 100 µg/mL of BVEP-2, while the standard doxorubicin had cytotoxicity that reduced cancer cell viability to 36.06% at a much lower concentration of 5 µg/mL.

Table 1 Percentage of cell viability and pharmacological properties induced by BVEP-2 from *B. velezensis* MTCC13097 assessed by in vitro methods

Percentage of cell viability		
Treatments	Hepatocellular adenocarcinoma cell line (HepG2)	
Untreated	100.0	
Doxorubicin	36.06	
BVEP-2 from *B. velezensis* at various concentrations (µg/mL)		
12.5	94.03	
25.0	77.12	
50.0	62.24	
100	23.02	
Pharmacological properties {IC_{50} (µg/mL)}		
Antioxidant property	BVEP-2 from *B. velezensis*	Standards[§]
DPPH radical scavenging	$117^b \pm 0.03$	$660^a \pm 0.07$
ABTS radical scavenging	$112^b \pm 0.08$	$750^a \pm 0.02$
Ferrous iron chelating	$3926^a \pm 0.23$	$8.4^b \pm 0.99$

The exopolysaccharide fraction BVEP-2 is significantly cytotoxic in nature against human hepatocellular adenocarcinoma cell line (HepG2 cells) in a dose-dependent manner, with doxorubicin as the standard for cell viability.

The samples were analyzed in triplicates ($n=3$) and expressed as mean ± standard deviation. Means followed by the different superscripts (a, b) within the same row indicate a significant difference ($p<0.05$).

Other notations were as described in the text.

[§] α-tocopherol was used as a standard antioxidant.

Apoptosis Assay

The effect of BVEP-2 inducing apoptosis in cancer cells was evaluated using annexin V-FITC/PI staining and quantified by clustering the cells into live, dead late apoptosis, and early apoptotic stages. At its IC_{50} concentration, the bacterial exopolysaccharide induced approximately 9% early apoptosis (annexin-V positive, PI negative) and 39% late apoptosis (annexin-V positive, PI positive) in the HepG2 cell line. In contrast, the standard drug resulted in about 37% late apoptosis, 38% early apoptosis, and 6% necrotic cells in HepG2 (Fig. 3). Table 2 summarizes the percentage distribution of the apoptosis assay.

Spectroscopic Characterization of β-(1 → 3) Linked Sulfated Polygalactan Exopolysaccharide (BVEP-2)

The exopolysaccharide fraction BVEP-2 was partially characterized as (1 → 3) linked sulfated polygalactan by spectroscopic analyses, such as proton nuclear magnetic resonance (^{1}H-NMR) and Fourier transform infrared spectroscopic analyses (FTIR) (Fig. 4). The ^{1}H NMR spectrum (500 MHz) exhibited proton signals in the region δ_H 4.40 – 5.35 corresponding to the envelope of anomeric protons and ascribed to H-1 of pyranose sugar moieties. The chemical shifts of anomeric protons of the glycosyl residues lesser than δ_H 4.90 indicates the presence of β-configuration, thus the linkage to be β-glycosidic (Zhang et al. 2013). Besides the anomeric signals, the other signals at the region of δ_H 3.40–4.00 were accounted for the H-2 to H-6 pyranoid ring protons of galactose sugar residues supported by the monosaccharide composition data. Further, the coupling constants recorded for the anomeric signals in the region δ_H 4.40 – 4.60 were

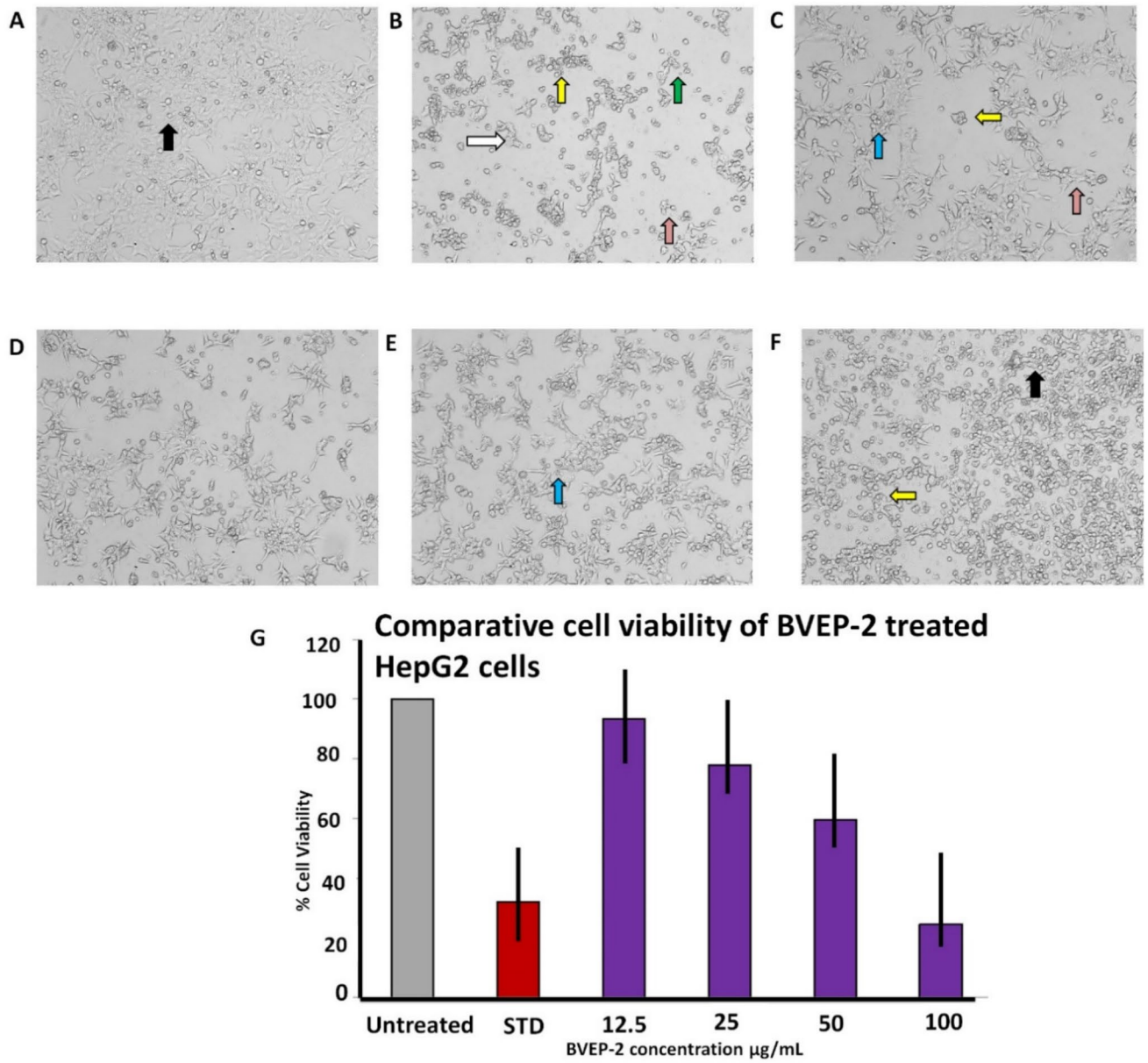

Fig. 2 Morphological changes in carcinoma cell lines treated with BVEP-2 exopolysaccharide isolated from *B. velezensis* MTCC13097. Direct microscopic images of the cell lines HepG2 in response to BVEP-2 (MTT assay for cytotoxicity against cancer cell line). **A** Microscopic images showing results of MTT assay on untreated HepG2 cell line (National Centre for Cell Sciences, Pune, India). **B** Standard control on HepG2 cell line. **C** BVEP-2 on HepG2 cell line (12.5 µg/mL). **D** BVEP-2 on HepG2 cell line (25 µg/mL). **E** BVEP-2 on HepG2 cell line (50 µg/mL). **F** BVEP-2 on HepG2 cell line (100 µg/mL). Microscopic images of alteration of cell morphology with HepG2 cells after MTT staining demonstrated that the cells treated with BVEP-2 samples displayed a decrease in cell density as well as viability, cell shrinkage, loss of cell-to-cell contact, and formation of apoptotic bodies. The arrows with different colors indicate the following: black-normal cells, yellow-apoptotic bodies, blue-cell shrinkage, white-membrane blebbing, green-echinoid spikes, and light red-bubbling. **G** Graph showing the percentage of viable cells after administering BVEP-2 to HepG2 cells

greater than $J = 7.0$ Hz, supporting the β-configuration (Roslund et al. 2008). The information regarding the functional moieties of the exopolysaccharide fraction (BVEP-2) was further confirmed using the FTIR data (Fig. 4). The FTIR spectrum displayed a strong and broad band at 3268 cm^{-1} ascribed to pyranose hydroxyl stretching vibrations of the exopolysaccharide. The band at 2929 cm^{-1} was due to aliphatic C–H stretching vibrations, and those at 1451, 1063 cm^{-1} were due to the stretching vibrations of C–O–C linkages of the pyranoid rings (Jiang et al. 2011). The absorption at 957 cm^{-1} could be correlated to β-glycosidic linkage in the titled exopolysaccharide (Hong et al. 2021), and that

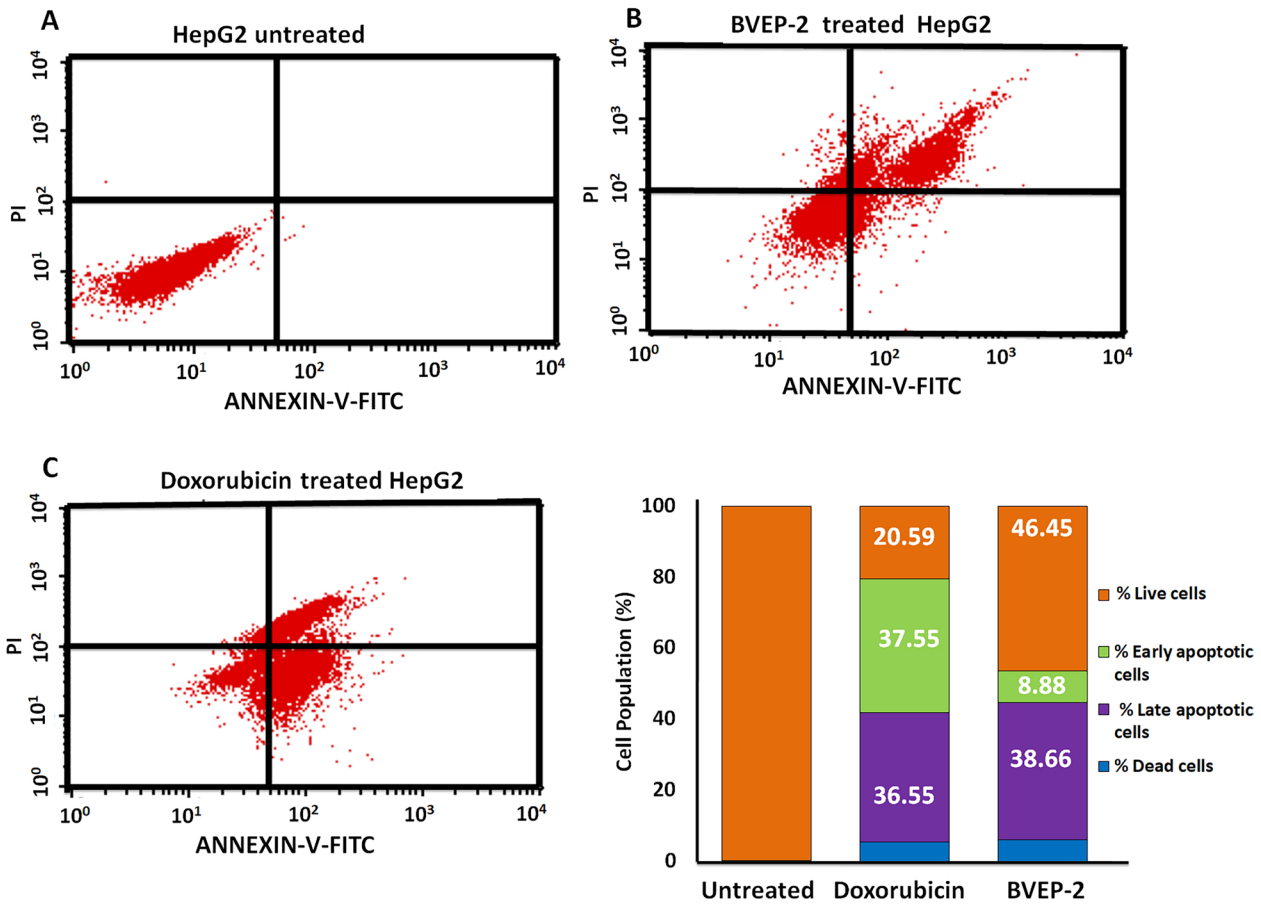

Fig. 3 Annexin V-FITC-propidium iodide expression in the HepG2 cell line (human hepatocellular adenocarcinoma cells) showing the proportion of cells undergoing apoptosis. **A** Untreated cells, **B** cells treated with the BVEP-2, and **C** cells treated with the standard drug, doxorubicin. The lower left quadrant shows the viable cells while the lower right shows early apoptotic cells. The upper right quadrant gives the late apoptotic cells and the upper left shows necrotic or apoptotic cells. **D** Graphical representation with a percentage distribution of early apoptotic, late apoptotic and necrotic or apoptotic cells

Table 2 Induction of apoptosis by BVEP-2 exopolysaccharide of *B. velezensis* MTCC13097 against human hepatocellular adenocarcinoma cell line (HepG2)

Treatments	% Necrotic cells	% Late apoptotic cells	% Viable cells	% Early apoptotic cells
Human hepatocellular adenocarcinoma cell line (HepG2)				
Cell control[†]	0.01	ND	99.94	0.05
Standard control[‡]	5.31	36.55	20.59	37.55
BVEP-2 IC_{50}[§]	6.01	38.66	46.45	8.88

ND not-detectable.

[†]Untreated cell line without any compound or drug.

[‡]Standard drug, doxorubicin administered with a concentration of 10 µg/mL.

[§] BVEP-2 was administered at IC_{50} 65.05 µg/mL against HepG2.

at 1234 cm^{-1} corresponds to sulfate ester linkages (Chen et al. 2011). Previous literature reported that the exoploysaccharides from *Bacillus* sp. are composed of glucose, galactose, and mannose consisting mainly of (1→3) D-glycosidic linkages (Asker et al. 2018). Monosaccharide composition analysis of the BVEP-2 fraction revealed galactose as the main structural building block. Based on spectroscopic and monosaccharide attributions, the structural motif of the main component in BVEP-2 was characterized as β-(1→3) linked sulfated polygalactan.

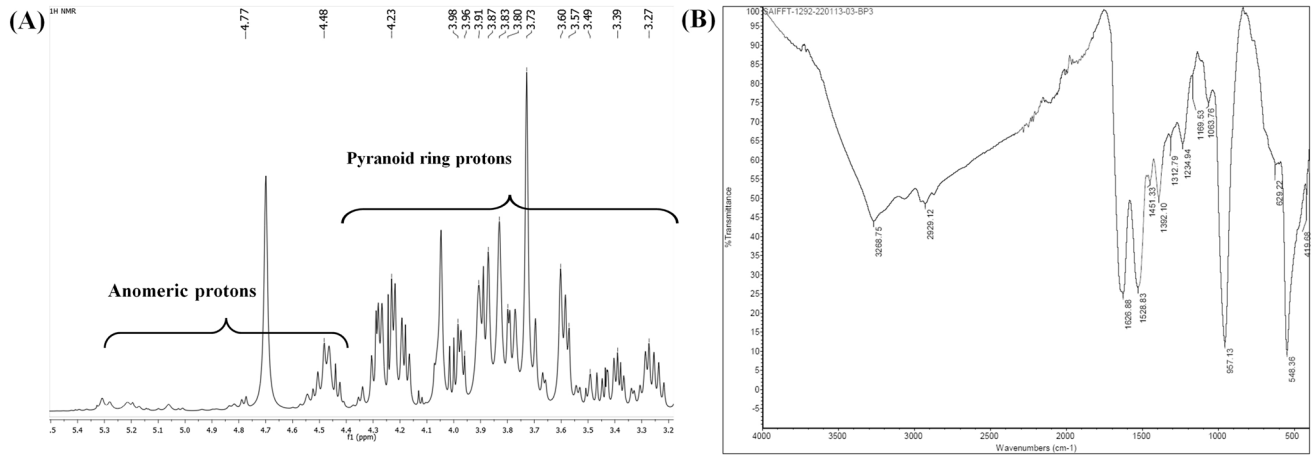

Fig. 4 **A** ^{1}H NMR spectrum of the hydrolyzed sulfated polygalactan exopolysaccharide (BVEP-2). **B** FTIR spectrum of BVEP-2

Discussion

The seaweed-associated heterotrophic bacteria are rich sources of biologically active molecules holding promising pharmacological potentials (Thilakan et al. 2016). As early bacteria could be used as potential anti-cancer agents, there has been a tremendous progress in bacterial immunotherapy to treat several forms of cancer coupled with developments of new chemotherapeutic drugs (Sedighi et al. 2019). In the present study, the heterotrophic *Bacillus velezensis* isolated from intertidal seaweed *S. wightii* was found to possess potent antioxidant and anti-cancer properties. *B. velezensis* has a morphology similar to *B. safensis*, displaying optimum growth at 37 °C, and in 1–8% NaCl concentration (Fig. 1). Furthermore, hot water extraction was used in the development of exopolysaccharides, which retained the structural integrity and stability of the EPS. DEAE cellulose-52 column fractionation was effective because the cellulosic ion exchange groups were sparsely arranged. The adsorption of polysaccharides to DEAE cellulose was weak. Thus, the polysaccharides were eluted using an increasing salt concentration (Wu et al. 2016). Among the four fractions (BVEP-1 to BVEP-4), BVEP-2 had the highest carbohydrate content and bioactivities. Hence, it was chosen for further analysis. Spectroscopic experiments were used to define the fraction BVEP-2 based on carbohydrate content and monosaccharide compositional analysis with galactose as the main sugar component.

The bacterial exopolysaccharide, BVEP-2 extracted from the studied bacterium displayed potential antioxidant and anti-cancer activities. HepG2 cells treated with various concentrations of BVEP-2 showed morphological dissimilarities and apoptosis-like characteristics. As the concentration of BVEP-2 increased, the results showed reduced cell density, displaying nuclear condensation, membrane bleeding, and contraction of cell volume. The highest concentration of BVEP-2 led to the projection of the cells, wherein 30–40% of those displayed membrane blebbing/ballooning. Broadly vacuolated cell cytoplasm and autophagosome structured apoptotic bodies implying autophagy-like progression of cell death were noted in treated cells (Fig. 2). At the highest concentration of EPSs (100 µg/mL), the cells were shrunken and detached from the cell surface, leading to cell death. Thus, the changes in morphological features result in the induction of apoptosis, following the administration of studied BVEP-2 on the HepG2 cells. It was reported that an exopolysaccharide isolated from marine sediment bacteria of the Mediterranean and Red Sea showed cytotoxic activity on HepG2 cells (Asker et al. 2018). It has been reported that marine exopolysaccharide inhibits migration and invasion of liver cancer cells by directly targeting collagen I (Liu et al. 2021).

The mechanism of action of exopolysaccharides against tumor cells occurs in two different ways, immune stimulation of host defense by activating B/T lymphocytes (indirect action) and induction of apoptosis (direct action) (Bao et al. 2001). During the initial stage of the host defense mechanism, the exopolysaccharides, such as β-glucans stimulate the secretion of cytokines, interleukins (ILs), and interferons (IFNs) (Córdova-Martínez et al. 2021). Anti-cancer activity displayed by *Lactobacillus kefiri* exopolysaccharides on HT-29 cancerous cells showed an apoptotic mechanism (Rajoka et al. 2019), and that produced by *Lactobacillus strain* SB27 showed G0/G1 cell cycle arrest and anti-proliferative activity on HT-29 cells (Di et al. 2018). Recent reports have proposed that exopolysaccharides augment the capacity of immune cells to distinguish tumor cells as foreign bodies, and thereby improving the efficacy of host defense mechanisms.

The antioxidant activity of BVEP-2 revealed its potential as both an antioxidant and anti-cancer agent. The radical quenching property of BVEP-2 varied with IC_{50} values of 117 and 112 µg/mL against DPPH and ABTS radicals, respectively. Noticeably, the oxidation of biomolecules leads to the development of oxidative stress associated diseases, such as arteriosclerosis, type-2 diabetes, and various forms of cancer (Scott 2004). Hence, *S. wightii*-associated *B. velezensis* MTCC13097 could be developed to produce microbial products with antioxidant and anti-cancer potentials. Previous reports described the pharmacological potential of bioactive heterotrophs *B. amyloliquefaciens* MTCC12716 and *Shewanella algae* MTCC12715 against oxidative stress-induced inflammation (Kizhakkekalam and Chakraborty 2021).

The ferrous ion chelating ability of the studied BVEP-2 exopolysaccharide (IC_{50} 3926.0 µg/mL) was relatively lesser compared to conventional metal ion chelators. Mainly, the oxidants are produced as the result of metabolic reactions, such as the electron transport chain. The reactive oxygen species function as critical intermediates in metal-catalyzed reactions. Fe^{2+} can propagate free radicals' formation through losing/gaining electrons (reductants) (Adjimani and Asare 2015). Afterwards, a chelating metal ion may comprehend the attenuation of the advancement of free radical species or oxidants. Fe^{2+} ion cheating action significantly contributes to the efficient antioxidant property of Fe^{2+}-associated pathways (Sudan et al. 2014). Noticeably, the potential antioxidant activity of the exopolysaccharide of *B. velezensis* MTCC13097 (IC_{50} 112–117 µg/mL) could be corroborated to its capacity to attenuate the pathophysiology leading to carcinogenesis. Villarreal-Gomez et al. (2010) reported that the heterotrophic bacteria *Centrocera sclavulatum* isolated from *Sargassum muticum* displayed anti-cancer activity. In the present study, the BVEP-2 revealed cytotoxicity against human hepatocellular adenocarcinoma (HepG2) cancer cells (IC_{50} 65.05 µg/mL). Annexin V-FITC/PI studies for apoptosis assay further quantified the apoptotic, viable, and necrotic cells. The cell lines administered with the BVEP-2 exhibited considerably lesser viability for cancer cells, displaying up to approximately 9% cells at the early apoptosis stage (Fig. 3). When compared to a standard drug, in vitro cell line tests of anti-cancer activity showed that the studied exopolysaccharide was substantially cytotoxic to cancer cell lines. The BVEP-2 induced apoptosis in cancer cell lines at a faster rate, causing most of the cells to enter an early apoptotic stage. As a result, the BVEP-2 could have therapeutic potential against human liver cancer, which can be verified by more pre-clinical research. In previous studies, the exopolysaccharide isolated from marine bacterial strains *Bacillus subtilis* subsp. *spizizenii*, *Bacillus megaterium*, *Bacillus flexus*, and *Bacillus subtilis* subsp. *subtilis* exhibited cytotoxicity activity on HepG2 cells (Abdelnasser et al. 2017). Exopolysaccharides from *Bacillus* sp. showed cytotoxicity against MCF-7 breast cancer cells (Vidhyalakshmi and Vallinachiyar 2013). Noticeably, marine organisms have recently appeared as potential sources of anti-cancer agents, with some of them currently undergoing clinical trials (Wali et al. 2019).

A significant source of microbial communities is associated with intertidal seaweeds, and thus, heterotrophic bacteria can be the largest reservoir of potential bioactive agents. *B. velezensis* MTCC13097, a symbiotic unicellular bacterium found in seaweed, could be a promising therapeutic agent with anti-cancer and antioxidant properties. The present study demonstrated that the β-(1 → 3) linked sulfated polygalactan exopolysaccharide (BVEP-2) of *B. velezensis* MTCC13097 possessed potential antioxidant and anti-cancer activities, and thus, could be developed as a promising pharmacophore lead against human hepatocellular adenocarcinoma.

Conclusions

Numerous polysaccharides derived from natural sources have recently been found to impede the proliferation of cancer cells and have gained significant interest in the medical community. The current study successfully identified a promising pharmacophore lead against human hepatocellular adenocarcinoma using the β-(1–3) linked sulfated polygalactan exopolysaccharide (BVEP-2) from a seaweed-associated heterotrophic bacterium *B. velezensis* MTCC13097.

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Author contributions K.C. has secured the funds. S.A. and K.C. hypothesized the work and planned the experiments. K.C. contributed to the samples/reagents/materials/analysis tools. S.A. and C.V. performed experiments and analyzed the data. S.K.P., S.D., and S.A. analyzed the spectral data. S.A. and S.K.P. wrote the main manuscript text. All authors reviewed the manuscript.

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Data Availability All data generated or analyzed during this study are included in this published article and its supporting information files. The whole genome sequence of the candidate bacterium was submitted in GenBank with an accession number of JAKYLL000000000 (Biosample code: SUB10967896; link for publicly available data is as follows: https://www.ncbi.nlm.nih.gov/search/all/?term=JAKYLL000000000.

Declarations

Ethics Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Competing Interests The authors declare no competing interests.

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