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Exopolysaccharide from *Advenella kashmirensis* strain 4MA with Antioxidant, anti-inflammatory, and anticancer activities

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Abstract

The unique characteristics of marine microbial polysaccharides make them a valuable source of bioactive chemicals that may be employed in a variety of disciplines, including food, feed, anti-tumor, antiviral, antioxidant, and anticoagulant treatments. The strain*Advenellakashmirensis* 4MA was isolated and identified from sand samples of the Red Sea marine environment using both phenotypic including (morphological biochemical) properties, and genotypic analysis by amplification of its 16S rRNA sequences. The isolate showed the ability to produce EPS with a composition of 40.9.9% sulfate and no uronic acid. The EPS component consists of glucose, xylose, arabinose, and rhamnose in the following molar ratios: 1.0:2.0:4.0:3.0 with molecular weight number-average 4.9×10^4 g/mol and molecular weight is 5.4×10^4 g/mol. The obtained EPS was biologically evaluated, and it showed a pronounced antioxidant activity up to 98.97 ± 1.9% at a concentration of 2000 µg/ml after 60 minutes. The IC_{50} value against the DPPH radical was approximately 500 µg/ml after 15 minutes. While the scavenging activity of H_2O_2 was 75.91± 2.1 at a concentration of 2000 µg/ml after 60 minutes. The IC_{50} value for the radical of H_2O_2 was determined after 15 minutes to be approximately 1500 µg/ml. furthermore, the anticancer activity of EPS was determined towards A-549, HepG-2, HCT-116, PC-3, MCF-7, HEP-2 proliferation and the IC_{50} values was 940.9±45.3, 485.1 ± 23.5, 841.2 ±34.6, 925.4 ±38.2, 1552 ± 59.4, and 988.3± 49.1 µg/ml respectively. Finally, the EPS showed a good anti-inflammatory with inhibition activity toward LOX and COX2 with IC_{50} reaching 14.5±0.92µg/ml and 28.6±1.8 µg/ml respectively.

Keywords: Exopolysaccharide, Antioxidant, anti-inflammatory, Anticancer, *Advenella kashmirensis*

1. Introduction

Recently, there has been a renewed focus on bacterial extracellular polymeric substances [EPS] due to their impact on human health. This is particularly important because certain bacteria with probiotic characteristics and their EPS may play a role in maintaining the health of the host [1].

Reactive oxygen species [ROS] have significant functions in the immune system and the redox equilibrium, including apoptosis, cell signaling, and ion transport[2]. Nevertheless, an overabundance of reactive oxygen species [ROS] can result in harmfuleffectson the body, including the development of conditions such as diabetes, cancer, and atherosclerosis. Lipid oxidation is a significant process in both biological and food systems and is seen as a deleterious response in lipid-containing food[2]. Several investigations have endeavored to hinder the process of lipid oxidation in food items, and including antioxidants during food processing is regarded as one of the more efficacious approaches. Antioxidants can hinder lipid oxidation by employing many processes, such as scavenging free radicals, breaking down lipid peroxides, and inhibiting the creation of peroxides [3].

Inflammation is a natural biological reaction in which cells with inflammation protect the human body from tissue damage and infections while simultaneously maintaining tissue balance. These cells produce pro-inflammatory chemicals called cytokines in response to inflammatory stimuli, which start and amplify the acute phase of the reaction [2, 3]. Endogenous anti-inflammatory chemicals are often created at this stage to mitigate the intensity and duration of the process. When

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inflammation reaches its height, neutrophils are recruited to help bring it under control. Neutrophils that have been transported to the harm site must be eliminated during the resolution phase, either by apoptosis or by migrating in the opposite direction from the inflammatory area [4]. Although the inflammatory response has benefits, chronic, uncontrolled inflammation can cause significant tissue damage and aid in the onset of illness. This may include conditions such as asthma, rheumatic disorders, cardiovascular diseases, and malignancy [5]. Osteoarthritis [OA] is a degenerative illness that causes gradual joint inflammation, degeneration of articular components, functional restrictions, and pain [6]. In contrast, prolonged use of synthetic anti-inflammatory medications, such as cortisol analogs, might have negative consequences.

The most promising medical applications of these polysaccharides are anti-cancer and immune-modulating applications. It is postulated that the polysaccharides in question may enhance both in vitro and in vivo cell-mediated immune responses, while also serving as biological response modifiers[7]. Furthermore, research has been conducted in recent years on the anti-inflammatory properties of polysaccharides, evaluating the anti-inflammatory characteristics of a particular kind of polysaccharide called fungal beta-glucan [7, 8].

2. Experimental

2.1. Chemical

All chemicals used in this investigation were bought from the Sigma Chemical Company, which is based in the United States. Merck, a German-based business, supplied the nutritional agar media used in the research. The solvents and other chemicals used were analytical grade and obtained from Sigma to assure the greatest degree of purity and uniformity in the findings.

2.2. Marine samples collection and isolation of bacteria

Marine water samples were gathered from a variety of places in the, Hurghada, Red Sea, primarily from marine sources. Bacteria were isolated from the medium at specific concentrations [gm/l]. Following the mothodsescribed by [9] with the following medium ingredients: glucose [20], KH_2PO_4 [0.05], NH_4NO_3 [0.8], K_2HPO_4 [0.6], $CaCO_3$ [1.0], $MgSO_4.7H_2O$ [0.05], yeast extract [0.1], $MnSO_4.4H_2O$ [0.1], and agar [15.0]. The pH of the medium was kept between 7.0 and 7.4. The media were diluted in 750 mL of seawater before being brought to a total volume of 1 L using the serial dilution procedure [10].

2.3. Separation of exopolysaccharide (EPS) from culture broth

The isolates were tested for exopolysaccharide synthesis in a broth medium consisting of 20 g/l sucrose, 4 g/l peptone, along 2 g/l yeast extract, at a pH of 7.0-7.4. [11]. After being prepared in 750 milliliters of saltwater, the medium was concentrated to a final amount of one liter. After incubation, 10% Trichloroacetic acid was added to the broth, which was then chilled at 4°C for the entire night. to remove protein content, the mixture was centrifuged at 5000 rpm. The pH of the solution was adjusted to 7.0 with NaOH solution. The supernatant was diluted with 95% ethanol to four times its original volume and incubated overnight at 4°C. After being separated by centrifugation for 20 minutes at 5000 rpm, the exopolysaccharides were dehydrated with diethyl ether and then went through two acetone washes [12].

2.4. Identification of the most potent bacteria

The potent bacterial isolate that exhibited the greatest quantity of EPSs with potent antioxidant properties was determined through an analysis of its morphological including colony morphology and pigmentation [13], The confirmation of identification involved phylogenetic analysis [14]. After the DNA was extracted from the bacterial isolate, an agarose gel of 1.2% was used to evaluate its quality. The presence of a singular band of high molecular weight DNA was confirmed. The Polymerase Chain Reaction [PCR] utilized the forward primer 5'-TCCGTAGGTGAACTTTGCGG-3' and reverse 5'-TCCTCCGCTTATTGATATGC-3', The acquired data were then uploaded to the GenBank database, and the BLAST program was used to align the obtained DNA sequence with the known sequences at the GenBank database at the NCBI [15&16]. The alignment concentrated on a subset of sequences that shared the greatest similarity with the bacterial isolate's 16S rRNA gene. The nucleotide sequences corresponding to the 16S rRNA gene in bacteria were incorporated into the DDBJ/EMBL/GenBank databases, and a phylogenetic tree was constructed.

2.5. Analysis of EPS

FTIR spectra of EPS done by the FTIR-UNIT Bruker Vector 22 Spectrophotometer [17]. For quantifying uronic acids at a wavelength of 525 nm, the m-hydroxyphenyl colorimetric method was employed [18]. Sulfate was measured using the

turbidimetric approach [19]. Monosaccharide composition was investigated using the Agilate Pack, serics1, 200 system, which included an Aminex carbohydrate HP-87C column [300 x 7.8 mm]. The mobile phase consisted of 0.5 mL/min of deionized water [20]. The mean molecular weight [Mw] was calculated using high-performance chromatography [HPLC] with refractive index [RI] detection on an Agilent 1100 Series System produced by Hewlett-Packard in Germany. The polydispersity index [PI] was determined by computing the Mw/Mn ratio [21].

2.6. Biological evaluation of EPS

2.6.1. Assessment of Antioxidant Activity of EPS

2.6.1.1. DPPH assay

EPS was assessed for its antioxidant activity using the DPPH assay. Different conc. of EPS [100, 300, 500, 1000, 1500, and 2000 µg/ml] were used in combination with a 2 ml DPPH solution. The blend underwent vigorous shaking and was left to stand for various durations [30, 60, 90, and 120 minutes] in the absence of light. Subsequently, the absorbance at 517 nm was gauged, and the scavenging ability was determined through the following calculation method: Scavenging ability [%] = [A517 of control - A517 of sample / A517 of control] ×100.

2.6.1.2. Hydrogen peroxide scavenging [H2O2] assay

The technique outlined by [22] can be used to determine how well EPS removes hydrogen peroxide. Various concentrations of EPS [ranging from 200 to 2000 µg/ml] were suspended in distilled water mixed with H2O2, and the resulting absorbance was determined at a wavelength of 230 nm. The calculation for determining the percentage of hydrogen peroxide [H2O2] scavenging is as follows: Scavenging ability [%] = [A230 of control – A230 of sample / A 230 of control] ×100.

2.6.2. Anticancer activity

To assess cytotoxicity, several cell lines included laryngeal cancer [HEP-2 cells], prostate cancer [PC3 cells], human breast cancer [MCF-7 cells], colon cancer [HCT-116 cells], human hepatocellular carcinoma [HepG-2 cells], and human lung cancer [A549 cells] were used. By the MTT method described by [23], the viable cell production was assessed after a full day of incubation with the colorimetric technique [23]. The number of viable cells and the viability % were calculated using: [1-[ODt / ODc]] ×100

The mean optical density of wells exposed to the tested sample is indicated by ODt, while the mean optical density of cells that have not been treated is indicated by ODc.

2.6.3. Evaluation of the anti-inflammatory activity of EPS

2.6.3.1. In vitro lipoxygenase [LOX] inhibition

Sample with a final concentration range of 125-0.98 µg/ml were tested. Using a BIOTEK [USA] microplate reader, the increase in absorbance at 234 nm suggested inhibitory action [24]. The following formula was used to obtain the inhibitory percentages:

$$\text{Inhibitory activity [\%]} = [1 - \text{As} / \text{Ac}] \times 100,$$

"As" denotes the absorbance while the test chemical is present, and "Ac" denotes the absorbance of the control. IC50 values were ascertained by creating graphs depicting enzyme inhibition [%] against sample concentrations. The IC50 value indicates the concentration at which the enzyme's activity is decreased by 50%.

2.6.3.2. Inhibition of cyclooxygenase [COX-2] in vitro:

Analysis was performed on samples that fell within 125-0.98 µg/ml to determine whether or not they may inhibit the COX-2 enzyme and cause an anti-inflammatory reaction [25&26]. Using a microplate reader [BIOTEK; USA], the absorbance rise at 611 nm was used to measure inhibitory activity. The following formula was used to obtain the inhibitory percentages:

$$\text{Inhibitory activity [\%]} = [1 - \text{As} / \text{Ac}] \times 100,$$

When the test material is present, the absorbance measurement is represented by A, and when it is not, the reading is represented by Ac. The COX-2 isoenzyme was tested for extracts' ability to inhibit it, along with the effectiveness of a reference chemical [celescoxib], at concentrations that resulted in a 50% enzyme inhibition [IC50].

2.6.4. Membrane stabilization

An erythrocyte suspension was prepared from rat whole blood obtained via cardiac puncture using heparinized syringes. The samples' ability to stabilize the membrane was evaluated [27].Using 96-well plates, the absorbance of the supernatant was determined at 540 nm. The following formula was used to get the % inhibition of membrane stabilization or hemolysis:

%Inhibition of hemolysis [membrane stabilization%] = 100 x {OD1-OD2/OD1}

The sign OD1 denotes the optical density of a saline solution that has been buffered to a hypotonic level, whereas OD2 represents the optical density of the test sample in a hypotonic solution. The sample concentration needed to prevent 50% red blood cell hemolysis under the specified test conditions is indicated by the IC50 value.

2.7. Statistical analysis

The results were statistically evaluated using the computer application SPSS software, version "20" for Windows. Data were provided as mean ± SE.

3. Results and discussion

3.1. Isolation, purification, and screening of the bacteria that Produce EPS

Marine microorganisms create bioactive chemicals with structures and functionalities because of their unique living environments [28]. Exopolysaccharides [EPSs] generated from these microorganisms have a major impact on the discovery of new drugs [29&30]. After three days of culture, the isolated bacteria were screened for the synthesis of the extracellular polymer [EPS]. After fermentation, The cultures were then dried at 80 °C, and four volumes of ethanol were used for EPS precipitation. The EPSs were produced via acetone washing and ether dehydration. As antioxidants and free radical scavengers, polysaccharides are essential for reducing oxidative damage in living things [31]. Strong biological activity is demonstrated by EPSs, which also have antiviral, antioxidant, and anticancer effects and affect immunological regulation, cell division, and differentiation. Studies on EPSs have demonstrated their strong antioxidant activity, pointing to a possible physiological underpinning for their function in reducing inflammation and atherosclerosis [32]. Twenty bacterial strains were isolated from the Red Sea, and ten EPSs were screened for antioxidant activity using DPPH. The highest antioxidant activity was observed in RS10 [95.85%] at 90 minutes. Further characterization of strain RS10 revealed its Gram-negative coccus morphology with a creamy white colony surface, smooth texture, entire edge, and opaque large colony. Positive results were obtained for the Catalase test, oxidase, urease, and nitrate reduction. Phylogenetic analysis based on the 16S rDNA sequence identified strain RS10 as *Advenellakashmirensis* with the accession number MZ021519 in the GenBank sequence database. The phylogenetic tree of the partial 16S rRNA sequence for the local isolate *Advenellakashmirensis* strain 4MA is shown in Figure [1] with closely similar sequences that are stored in GenBank databases. Many marine bacteria produce EPSs, aiding bacterial communities in surviving extreme conditions of salinity, temperature, and nutrient availability [33]. *Advenellakashmirensis* strain NRC7, isolated from marine sediments, can produce EPS [34].

Figure 1 shows the evolutionary tree of the local isolate *Advenella kashmirensis* strain 4MA partial 16S rRNA sequence about closely similar sequences found in Gen Bank databases.

3.2. EPS composition, isolation, and partial purification

The EPS generated from RS10 peaked at 8.89 g/L following three days of accelerated fermentation. To partially filter and fractionate the crude EPS extract, it was reconstituted in deionized water and then put through a full day of dialysis in the same deionized water. After dialysis was finished, the solution was precipitated by gradually adding 1, 2, 3, and 4 liters of cold 100% ethanol. Following fractionation, three volumes of ethanol were precipitated to yield the primary 75% EPS fraction, known as EPS. With a sulfate content of 40.9% and no uronic acid, the EPS fraction was classified as a heteropolysaccharide because of its monosaccharide composition, which included glucose, xylose, rhaminose, and arabinose in a molar ratio of 1.0:2.0:3.0:4.0.

The GPC chromatogram for EPS molecules exhibits notable dispersion, featuring a polydispersity index [PI] of 1.1, a total molecular weight [Mw] of 5.4×104 g/mol, and a number-average molecular weight [Mn] of 4.9×10^4 g/mol. The expansion vibration of O-H in the components of the sugar residue was identified as the cause of the noticeable, broad distinctive peak observed in the fraction's FTIR spectra at around 3286.22 cm^{-1}**[35]**. Furthermore, a unique band at 1651.76 cm^{-1} was observed in the EPS fraction, which was mostly linked to circular vibrations **[36]**. According to [**37]**, absorptions at 1334.80 cm^{-1} indicated CH_2, a band at 1100.80 cm^{-1} indicated SO_3, and a strap at 833.14 cm^{-1} suggested β-pyranose **[37].**

To assess these bacterial extracellular polymers' possible uses in biotechnology and environmental preservation, an investigation into their chemical characteristics was conducted **[38&39]**. Environmental and nutritional factors have a significant impact on the nature and composition of bacterial extracellular polymers [EPSs] **[40]**. Most EPSs produced by marine microbes are heteropolysaccharides, which are composed of different monosugar units arranged in repeating units **[41]**.

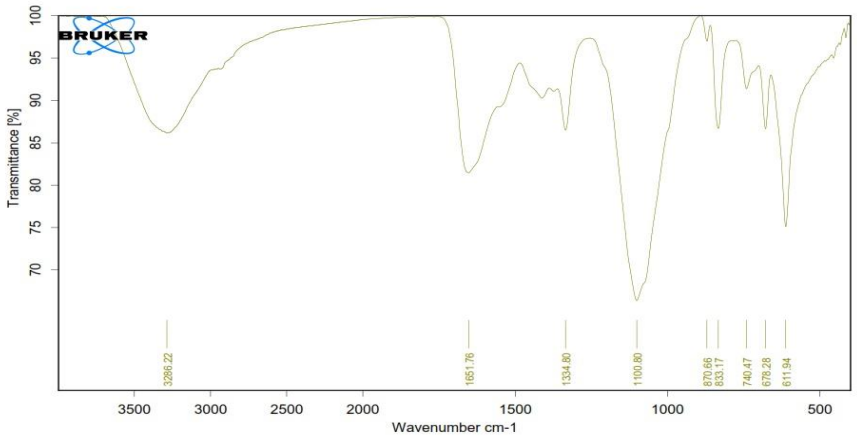

Figure 2. FTIR Spectrum of EPS

3.3. Biological evaluation of EPS

3.3.1. Antioxidant activity of EPS

The molecular weight and chemical makeup of EPSs determine their biological efficacy. Low molecular weight polymers and unusual sugars like ribose and arabinose are important indicators of the antioxidant capability of extracellular polymers [EPSs] [42]. Antioxidant activity was quantitatively assessed at different intervals [15, 30, 45, and 60 minutes]. Table 1 illustrates that the compound's total antioxidant activity increased as concentrations increased, from 100, 300, 500, 1000, 1500, and 2000 µg/ml. After 60 minutes, the highest concentration of antioxidant activity that was highest, measuring 98.97 ± 1.9%, was found at 2000 µg/ml.

The IC_{50} value against the DPPH radical was approximately 500 µg/ml after 15 minutes. Furthermore, at various doses [100, 300, 500, 1000, 1500, and 2000 µg/mL], the H_2O_2 scavenging activity of EPS exhibited its highest activity, reaching 75.91 ± 2.1% at 2000 µg/ml after 60 minutes. The IC50 value against the H_2O_2 radical was roughly 1500 µg/ml after 15 minutes [Table 2].

Table 1. Scavenging activity [%] of EPS using DPPH at different times

Concentration [µg/mL]	Time [min.]			
	15	30	45	60
100	32.32±1.2	39.42±1.6	45.56±1.7	48.31±1.8
300	44.94±1.5	53.26±1.8	61.9±1.4	66.78±1.6
500	52.54±1.9	64.36±2.1	78.05±1.4	81.39±1.5
1000	76.61±2.1	83.54±1.9	90.42±2.0	95.91±0.8
1500	79.81±1.1	85.31±1.0	91.53±1.7	98.25±0.7
2000	80.92±0.9	87.78±1.2	93.42±1.0	98.97±1.0

Numerous investigations have investigated the bacterial EPS's potential as an antioxidant. For instance, research was done on the antioxidant characteristics of partly purified EPSR3 from Bacillus cereus strain AG3. At a dosage of 1500 µg/ml, the results showed an overall antioxidant efficacy of 90.4 ± 1.6% after 120 minutes. After 60 minutes, the predicted IC_{50} value against the DPPH radical was 500 µg/ml. When the scavenging activity against H_2O_2 was measured during the same period, it came out to be 75.1 ± 1.9% at a concentration of 1500 µg/ml. After a 15-minute break, the IC_{50} value against the H_2O_2 radical was found to be roughly 1500 µg/ml [43].

Table 2. Scavenging activity [%] of EPS at different times by H_2O_2

Concentration [µg/mL]	Time [min.]			
	15	30	45	60
100	14.21±1.5	27.45±1.0	30.78±1.3	32.51±2.2
300	19.43±1.3	28.57±1.2	39.12±0.8	43.66±1.3
500	27.74±2.0	38.31±2.3	47.52±1.8	51.76±1.9
1000	36.81±1.3	45.92±0.9	58.43±1.4	64.82±1.6
1500	51.49±0.9	59.51±1.4	66.62±1.7	70.18±1.4
2000	58.24±1.7	64.52±1.5	71.78±0.9	75.91±2.1

3.3.2. Antitumor action directed toward different cancer cell lines

Using the MTT assay, the influence of the EPS fraction on the proliferation of HepG-2, A-549, HCT-116, MCF-7, HEP-2, and PC-3 cells was examined, as illustrated in Figure 3. The calculated IC_{50} for the HepG-2 cell line was 485.1 ± 23.5 µg/ml. A-549 exhibited a cytotoxic effect with a relatively high calculated IC_{50} value of 940.9 ± 45.3 µg/ml. For HCT-116, the calculated IC_{50} was 841.2 ± 34.6 µg/ml, while MCF-7 showed an IC_{50} of 1552 ± 59.4 µg/ml. The IC_{50} for the HEP-2 cell line was 988.3 ± 49.1 µg/ml, and for the PC-3 cell line, it was 925.4 ± 38.2 µg/ml.TheDoxorubicin was used as a control and has varied IC50 values among tested cancer cell lines. The HepG-2 cell line has an IC50 of 1-10 µg/ml. The A-549 cell line has an IC50 of 1-10 µg/ml. The HCT-116 cell line has a somewhat lower IC50, ranging from 0.1-1 µg/mL. The IC50 values for the MCF-7 and PC-3 cell lines vary from 1 to 10 µg/mL.

Previous research has established the known apoptotic anti-cytotoxic action of EPS [44]. However, we are concerned with the controversial effect of molecular weight [Mw] on EPS's anti-tumor properties. According to certain research, high Mw EPS is more effective against cancer because it interacts with cancer cell receptors that control signaling and transduction instead of infiltrating the cell [45&46]. On the other hand, some contend that low Mw makes it possible for EPS to cross the cell membrane

barrier more quickly, promoting biological processes including cell cycle arrest [47]. An IC_{50} of 389 µg/ml was obtained for the anti-cancer efficaciousness of EPSs derived from *Advenellakashmirensis* NRC-7 against the hepatocellular carcinoma HepG2 cell line. The ability of living cells to assimilate and bind the survival dye-neutral red in lysosomes served as the basis for this evaluation [34]. It has been observed that the marine bacterial strain GWS-BW-H8hM inhibits the growth of the liver cell line HepG2, the breast cell line MCF7, and the gastric cell line HM02 [48].

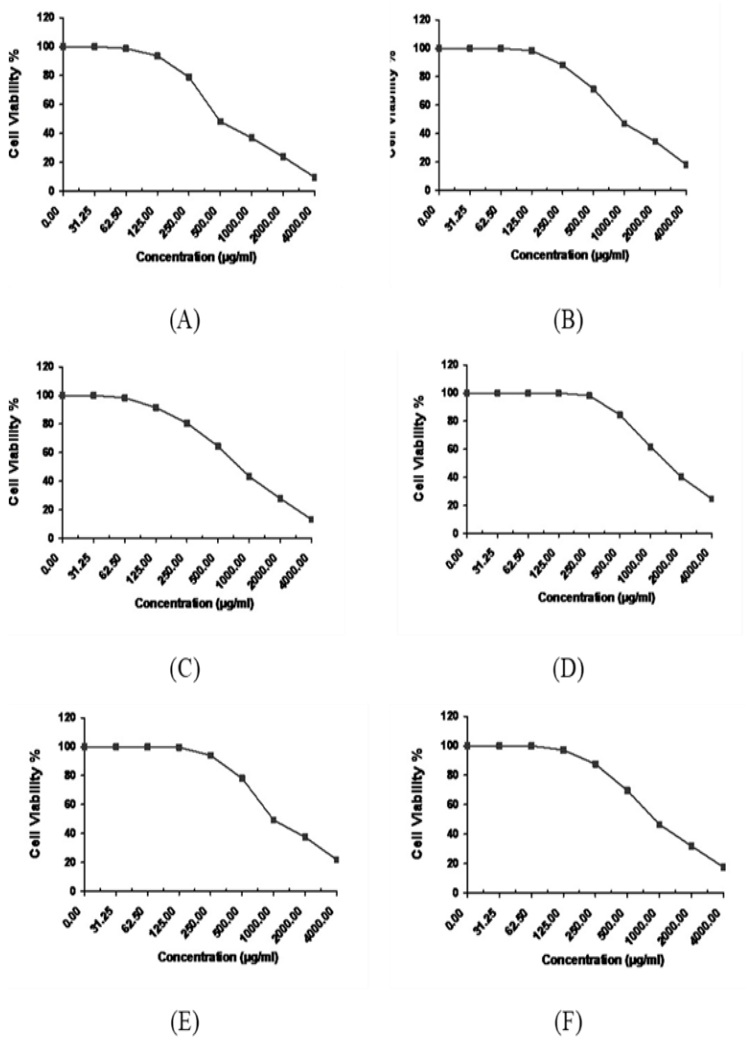

Figure 3. Cytotoxicity activity against different cell lines [A]: HepG2 & [B]: A-549 & [C]: HCT & [D]: MCF7 & [E]: HEP-2 & [F] : PC-3

3.3.3. Anti-inflammatory activity

The anti-inflammatory effect of EPS may be attributed to its structural composition and its inhibitory activity on cyclooxygenases [49]. Additionally, it is believed that the primary impact of exopolysaccharides is to modulate cytokines and their associated transcription factors [50]. The assessment of anti-inflammatory activity for EPS involved various methods, including the Lipoxygenase [LOX] inhibitory assay, as depicted in Figure 4, revealing an IC_{50} of 14.5 ± 0.92 µg/ml. The control sample [ibuprofen] exhibited an IC_{50} of 1.5 ± 1.3 µg/ml in the same assay. Moreover, the evaluation of cyclooxygenase [COX2] inhibitory activity resulted in an IC_{50} of 28.6 ± 1.8 µg/ml, with the control [Celecoxib] showing an IC_{50} of 0.28 ± 1.7 µg/ml. In the membrane stabilization inhibitory assay, EPS demonstrated an IC_{50} of 84.6 ± 0.82 µg/ml, while the control [Indomethacin] exhibited an IC_{50} of 17.02 ± 0.82 µg/ml. Pro-inflammatory cytokines TNF-α, IL-1, and IL-6, together with the anti-inflammatory cytokine IL-10, are important mediators in the actions of these natural products [51]. Microbial metabolites play a crucial role in triggering macrophages, leading to the secretion of cytokines, phagocytosis of bacteria, and regulation of immunity. Additionally, they contribute to driving phagocytosis and delivering bacterial antigens to helper T cells [52].

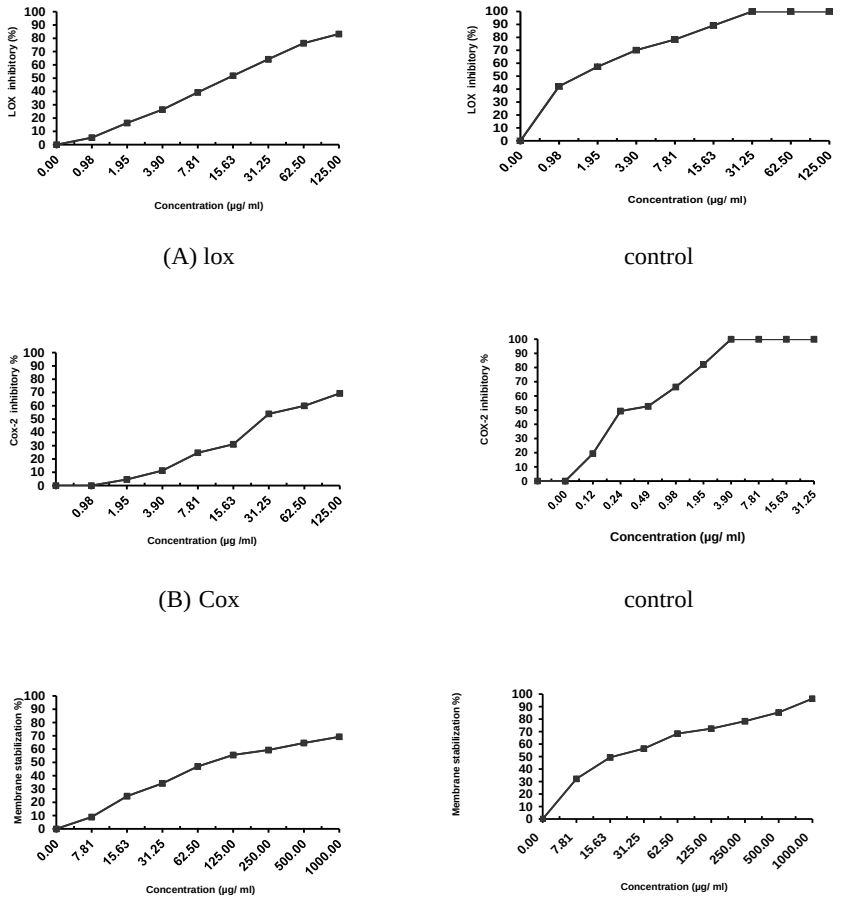

Figure 4. Anti-inflammatory activity of EPSRS [A]: LOX & [B]: COX & [C]: Membrane stabilization

Conclusion

In this study, EPSs 8.9 g/l were produced and partially purified from marine bacteria *Advenella kashmirensis* strain 4MA. According to its physical characteristics, EPSRS was soluble in water and insoluble in typical organic solvents. Its Mn was 4.9×104 g/mole and its Mw was 5.4×10^4 g/mole. EPSRS has very promising activity as an antioxidant against DPPH and H_2O_2 radical which was increased by increasing the concentrations of the EPSRS. Furthermore, the EPSRS exerted a significant protective effect against different cancer cell lines. According to this study, EPS may have use in the future as a novel therapeutic agent for the treatment of malignancies as well as a natural antioxidant.

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