Research Article

Carboxymethylated Sulfated Heteroexopolysaccharide from a Haloarchaeal Strain as Potential Biomolecule for Harmless Adjuvant Therapy in Cancer Treatment

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This study explored the possible use of a microbial carboxymethylated sulfated heteroexopolysaccharide (CS-hEPS) as a potential anticancer agent. The investigation was carried out through antioxidant, antifatigue, and antiproliferative activities. Antioxidant potential including scavenging DPPH and hydroxyl radical activities and reducing power was evaluated. Antifatigue activity was determined by assessing the endurance of mice using the forced swimming test. Following 30 days of CS-hEPS oral treatment at different doses, biochemical parameters related to fatigue such as lactic dehydrogenase (LDH), serum urea nitrogen (SUN), and hepatic glycogen (HG) contents were measured. Antitumor activities were investigated against human cancer liver and myelogenous leukemia cells. Results showed that CS-hEPS possesses notable antioxidant, antifatigue, and antitumor effects. CS-hEPS significantly inhibited the proliferation of leukemia (86.6 ± 0.32%) and cancer liver (58.6 ± 0.43%) cells. CS-hEPS are promising natural antioxidant, antifatigue, and antitumor harmless adjuvant materials that could be applied in human cancer therapy.

1. Introduction

Extreme environments are valuable reservoir for rugged microorganisms with the ability to produce unusual macromolecules with particular biological properties. Microbial exopolysaccharides (mEPS), composed of numerous monosaccharide units linked by glycosidic bonds in linear or branched chains, are considered as one class of these active biomacromolecules. mEPS are recognized as important biological response modifiers [1, 2]. Their biological activities are greatly influenced by their characteristics such as molecular weight, charge density, monosaccharide composition, acidic fraction, types of glycosidic linkage, and substitution groups [3–5]. Specifically, sulfated polysaccharides have been proved to possess potent antitumor and antioxidant activities that made them gain increasing attention as potential new natural therapeutic materials and good substitutes for synthetic agents [1, 4]. It has been found that mEPS produced by Bacillus amyloliquefaciens show significant therapeutic activities against gastric tumors [4]. Similarly, mEPS of Lactobacillus casei 01 exhibited an antiproliferation effect on human colon cancer cells HT-29 with a significant dose-dependent effect [5]. Murofushi et al. [6] noticed that the acidic fraction from Lactobacillus plantarum N14 (LP14) strongly stimulated the NF-kB pathway in transfectant cells and decreased the production of proinflammatory cytokines. The presence of high content of uronic acid gave mEPS interesting properties to be used in regenerative medicine and tissue engineering. Priyanka et al. showed that mEPS produced by Nitratireductor sp. with 7.08% uronic acid and 2.68% sulfate functional group
constitute a promising drug for brain tumors as they can inhibit Akt/PI3K pathway through their anionic charged EPS by binding to the epidermal growth factor (EGF) secreted by the tumor. This correlates with the findings of Liu et al. that anionically charged EPS can prevent EGF receptor phosphorylation [7, 8]. Using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, Priyanka et al. [7] demonstrated that mEPS produced cytotoxicity against U87MG glioblastoma cells and showed an IC\textsubscript{50} value of 234.04 μg·mL\textsuperscript{−1}.

Additionally, side effects and symptoms are frequent challenges occurring during cancer chemoradiotherapy treatment of patients. One of the most frequently reported symptoms is fatigue [9, 10]. As defined by Miller et al., fatigue is a state of decreased energy level fluctuating from tiredness to exhaustion that can interfere with the ability to accomplish daily tasks and negatively impacts quality of life [11]. Patients undergoing cancer treatment have significantly higher fatigue levels when compared to patients without a chronic health condition [12]. There is scientific evidence that chemotherapy (platinum coordinating complexes, 5-fluorouracil, alkylating agents), hormonotherapy (tamoxifen, aromatase inhibitors), and radiotherapy (ionizing radiation) used in cancer treatment cause substantial oxidative stress (OS) in nontargeted tissues [13, 14]. This OS plays a pivotal role in many pathological processes, mainly fatigue. OS occurs when the balance between reactive oxygen species (ROS) and antioxidant defenses is disrupted due to an accumulation of ROS or a depletion of antioxidant defenses. This disruption initiates a chain of reactions that cause cell death [15]. Antioxidants may alleviate the adverse effects of ROS and fatigue caused by chemo-hormonoradiotherapies.

Synthetic antioxidants such as butylated hydroxytoluene and butylated hydroxy anisole are commonly authorized for use in food to cope with OS. However, because of the controversy surrounding their safety and carcinogenic potential [16, 17], there is a growing demand for natural molecules with similar antioxidant and antifatigue effectiveness [18, 19]. In this context, some mEPS have demonstrated an important role as natural free radical scavengers for the prevention of oxidative damage and fatigue [1].

The present study illustrates for the first time the benefits of using a natural carboxymethylated sulfated polysaccharides, produced by a haloarchaeal strain, Halogeometricum borinquense 52, isolated from a Tunisian atypical environment, as antioxidant, antifatigue, and antitumor adjuvants in cancer therapy.

2. Materials and Methods

2.1. Inoculum Preparation and Microorganism Cultivation. 
\textit{H. borinquense} 52 was isolated from hypersaline sediments collected from Chott el-Djerid (Southern Tunisia). It was identified according to its 16S rDNA sequences analysis [20]. 
\textit{H. borinquense} was grown and maintained on an optimized DSC-97 medium containing (g/L) 7.32 g glucose, 1.25 g casamino acid, 150 g NaCl, 3.0 g trisodium citrate, 2.0 g KCl, 20.0 g MgSO\textsubscript{4}.7H\textsubscript{2}O, 0.023 g FeCl\textsubscript{2}, and 15 g agar-agar. The pH was adjusted to 7.4. The archaeon was grown at this medium at 45°C for 48 hours (h).

2.2. CS-hEPS Production and Purification. The optimized DSC-97 medium (250 ml) was inoculated with 1.5 ml of pregrown \textit{H. borinquense} 52. After six days of incubation at 45°C, culture samples were centrifuged (12,000 revolutions per minute (rpm), 20 min). Three volumes of cold (4°C) ethanol were added to one volume of culture supernatants and the mixtures were stored overnight at 4°C. The precipitate in the centrifuging tube was rinsed with distilled water, filtered, and then dried at 45°C using rotavap. Proteins were removed from CS-hEPS as described in our previous study [21]. For \textit{in vivo} activities, lyophilized CS-hEPS were further purified using a Sephadex G-100 column (2.6 cm × 50 cm), and the column was eluted with 0.01 M NaCl solution at a flow rate of 1 mL/min [22].

2.3. Functional Groups and Carbohydrate, Uronic Acid, Sulfate, and Monosaccharide Contents of Purified CS-hEPS. Purified CS-hEPS were checked for their carbohydrate content as described by Chaplin and Kennedy [23] with some modifications. A CS-hEPS solution (0.2 mL) was mixed with 0.2 mL phenol solution (5.0%, w/v), and 1.0 mL concentrated H\textsubscript{2}SO\textsubscript{4} was added to the reaction mixture. Distilled water and glucose were used as a blank and a standard, respectively, for plotting a calibration curve. The total carbohydrate in CS-hEPS was then estimated, as glucose equivalents, by reading the absorbance of the obtained solution against the glucose standard curve (supplementary data, Figure 1(A)). The absorbance was measured at 492 nm with a spectrophotometer (Analytic Jena SPEKOL 2000) after incubation at room temperature for 30 min.

Uronic acid content was determined by the method of Blumenkrantz and Asboe-Hansen [24] with modifications. A CS-hEPS solution (200 μL) was mixed with 1.2 mL of 0.0125 M sodium tetraborate solution in concentrated H\textsubscript{2}SO\textsubscript{4}. The mixture was homogenized by vortex and then cooled in ice. After incubation at 100°C for 5 min, the mixture was cooled quickly and then added to 20 μL of metahydroxydiphenyl at 0.15% in NaOH (0.5%). It is worth noting that the colorimetric reaction is sensitive to interference from neutral sugars which are brown in the presence of H\textsubscript{2}SO\textsubscript{4}. This interference was minimized by treating CS-hEPS with sulfamate (sulfamic acid/potassium sulfamate 4 M pH 1.6) before acid hydrolysis. The uronic acid content in CS-hEPS was estimated, as glucuronic acid equivalents, by reading the absorbance of the obtained solution against the glucuronic acid standard curve (supplementary data, Figure 1(B)). The absorbance was measured at 520 nm with a spectrophotometer (Analytic Jena SPEKOL 2000) after incubation at room temperature for 30 min.

The turbidimetric method [25] was applied to evaluate the sulfate content of CS-hEPS after HCl hydrolysis (5 mg of CS-hEPS with 0.2 mL of 1 M HCl for 6 h at 110°C). The obtained hydrolysate was added to 3.8 mL trichloroacetic acid (3.0% w/v) and 1.0 mL of a barium chloride-gelatin
solution and mixed. HCl was used as a blank. Sulfate content was estimated, as K$_2$SO$_4$ equivalents, by reading the absorbance against a standard curve prepared with a solution of K$_2$SO$_4$ (supplementary data, Figure 1(C)). The absorbance was measured at 360 nm after incubation at room temperature for 15 min.

For the assessment of the monosaccharides content, 20 mg of purified CS-hEPS was dissolved in trifluoroacetic acid (TFA) (1 mL, 3 M) and incubated for 2 h and 30 min at 110°C. After cooling, the reaction mixture was centrifuged and evaporated to remove TFA.

An HPLC-RID (Agilent Technologies 1200 series) equipped with a phenomenex Luna aminated (NH$_2$) column (5 μm, 250 × 4.6 mm) was used with acetonitrile : water (3 : 1) as the mobile phase. Hydrolysed CS-hEPS and 20 mg of each standard (arabinose, fructose, galactose, glucose, mannose, rhamnose, ribose, xylose, maltose, saccharase, and lactose) were dissolved in 1 mL of distilled water. A flow of 1 mL/min and 20 μL injections were used at 25°C.

To determine the functional groups of purified CS-hEPS, Bruker Vertex 70 FTIR spectrometer was used to obtain a Fourier Transform InfraRed (FTIR) spectrum over a wave range of 400–4000 cm$^{-1}$ at 64 scans, 4 cm$^{-1}$ wave number, and resolution transmission mode.

2.4. CS-hEPS In Vitro Antioxidant Activity. For determining the CS-hEPS in vitro antioxidant activity, ascorbic acid, at various concentrations, was used as a reference. Experiments were performed in triplicate and averaged.

2.4.1. CS-hEPS DPPH Radical Scavenging Activity. The free radical scavenging activity of the CS-hEPS was evaluated by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) test according to the method described by Zhang et al. [26]. Different concentrations of CS-hEPS (0.5–3 mg/mL) were mixed with DPPH (0.2 mM in 95% ethanol, 2 mL). The mixture was then shaken at 120 rpm vigorously and kept at room temperature for 30 min. Equation (1) was used to determine the DPPH scavenging rate (DSR).

$$DSR(\%) = \left(1 - \frac{A_i - A_j}{A_0}\right) \times 100\%,$$

where $A_i$ is the absorbance of the sample with DPPH, $A_j$ is the absorbance of the sample without DPPH, and $A_0$ is the absorbance of pure DPPH at 517 nm.

2.4.2. CS-hEPS Hydroxyl Radical Scavenging Activity. The protocol of Wu et al. [27] was used to evaluate the hydroxyl radical (HO•) scavenging activity of haloarchaeal CS-hEPS. Equal volume (0.5 mL) of 1,10-phenanthroline (7.5 mM) and FeSO$_4$ (7.5 mM) was mixed with 0.5 mL of sodium phosphate buffer (0.2M). Then, 1 mL of the CS-hEPS at various concentrations (0.5–3 mg/mL) and H$_2$O$_2$ (0.1%, 0.5 mL) were added. The mixture was incubated for 60 min at 37°C. Equation (2) was used to evaluate the hydroxyl radical scavenging rate (HSR).

$$HSR\% = \left(\frac{H_i - H_0}{H_c - H_0}\right) \times 100\%,$$

where $H_i$ is the absorbance of the sample, $H_0$ is the absorbance of the blank solution using distilled water, and $H_c$ is the absorbance of a control solution in the absence of H$_2$O$_2$. All absorbances were measured at 510 nm using an analytic Jena SPEKOL 2000 spectrophotometer.

2.4.3. CS-hEPS Superoxide Radical Scavenging Activity. The scavenging activity of CS-hEPS against superoxide radical (O$_2$•$^-$) was determined according to the protocol described by Qiao et al. [28]. All needed solutions (nicotinamide adenine dinucleotide (NADH), nitroblue tetrazolium (NBT), phenazine methosulphate (PMS), and EPS samples) were dissolved in 0.1 M PBS (pH 7.4). Then, equal volumes (1.0 mL of 156 μmol/L NBT solution and 468 μmol/L NADH solution were mixed with CS-hEPS at various concentrations (0.5–3 mg/mL). The addition of 1.0 mL of 60 μmol/L PMS to the mixture indicated the initiation of the reaction. After 5 min incubation at room temperature, absorbance at 560 nm was read against PBS (0.1M, pH 7.4) used as a blank. Equation (3) was used to evaluate the superoxide radical scavenging rate (SSR):

$$SSR(\%) = \frac{S_0 - (S_1 - S_2)}{S_0} \times 100\%,$$

where $S_0$ is the absorbance of the control (PBS instead of sample solution), $S_1$ is the absorbance of the sample, and $S_2$ is the absorbance of the sample under identical conditions.

2.5. CS-hEPS In Vivo Antifatigue Effect. One hundred mice were randomly divided into five groups based on body weight after one week accommodation ($n = 20$ mice/group; equal numbers of male and female) and orally treated with 0.5 mL of saline solution (serving as a negative control group), 0.5 mL of ascorbic acid (serving as positive control group), and 0.5 mL of purified CS-hEPS, dissolved in saline solution, at doses of 100, 150, and 200 mg/kg once daily for 30 days.

The exhaustive swimming test (EST) was carried out in a cubic container (30 cm deep water) at room temperature as described by Huang et al. [29]. At the end of experiments, mice were left to rest for one day. As an adaptive training before EST, mice were made to swim for 2 min twice per week. The exhaustive swimming time was used as an index of the increase in exercise tolerance. The mice were considered to be exhausted when they failed to rise to the surface to breathe after 10 s. After swimming, blood was collected from mice orbit to determine LDH and SUN contents. The livers of the mice were taken to determine HG levels. Biochemical parameters were assayed by using lactate dehydrogenase (ELISA Kit (Abcam)), blood urea nitrogen (ELISA Kit, Abbott) and Glycogen Assay Kit (Abcam) according to the manufacturer’s protocols.
2.6. CS-hEPS In Vitro Antitumoral Activity. To study the antitumor activity of CS-hEPS from the haloarchaeal strain *H. borinquense*, two types of human cancer cells were used (liver cancer HepG and myelogenous leukemia cells K562). They were both cultured in DMEM medium supplemented with 10% (v/v) fetal bovine serum. One hundred U/mL penicillin and streptomycin were added to the culture medium maintained at 37°C in a 5% CO₂ atmosphere. The antitumor activities of various concentrations of CS-hEPS (0, 50, 100, 200, 400, and 600 μg/mL) on HepG-2 and K562 cells in vitro were evaluated using the MTT assay according to Chen et al. [30].

2.7. Statistical Analyses. Data were analyzed using SPSS software. One-way analysis of variance and multiple comparisons were used to evaluate differences among groups. *P* value <0.05 was considered to indicate a statistically significant difference.

3. Results

3.1. Preliminary Characterization of Purified CS-hEPS. Based on the calibration curve OD = f (concentration of glucose) already drawn (supplementary data, Figure 1(A)), we estimated the total carbohydrate content of the purified CS-hEPS. The absorbance of the solution was 0.320 which corresponds to a concentration of 0.038 mg/mL glucose equivalent. The value obtained was converted with the initial concentration of CS-hEPS (45 mg/L) giving a total carbohydrate content of 84.2%. Using the same approach and the corresponding calibration curves (supplementary data, Figures 1(B) and 1(C)), the amounts of uronic acid and sulfate in CS-hEPS (45 mg/L) were determined as 12.5% and 3.2% with OD = 0.04 and 0.13, respectively.

Purified CS-hEPS showed a negative response to the Bradford test and no absorption at 260 or 280 nm in the UV spectrum, which indicates the absence of nucleic acids and proteins.

HPLC-RID analysis (Figure 1) showed that CS-hEPS from *H. borinquense* was a heteropolysaccharide composed of the three monosaccharides, rhamnose, fructose, and glucose.

The FTIR spectrum at 4000–400 cm⁻¹ of CS-hEPS is represented in Figure 2. All obtained peaks were in agreement with typical absorption data of a polysaccharide. The large absorption peak observed at around 3160 cm⁻¹ indicated the presence of intensive hydroxyl groups –OH. In addition, the absorption bands at 1598 and 1310 cm⁻¹ indicated the existence of two functional carboxymethyl groups of –COOH and –CH₂–. The band within the 1124 cm⁻¹ region was attributed to the vibration of C-O-C band. The peaks around 1066–902 cm⁻¹ were related to methoxyl groups, typical group of sugar derivatives, and uronic acid. This was in accordance with the results of uronic acid analysis. Moreover, bands found at 842–790 cm⁻¹ and 1216–1266 cm⁻¹, which represented functional groups of C-S-O and S-O, indicated the presence of sulfated groups in the polysaccharide. Bands at 694 and 486 cm⁻¹ represented absorption peaks for the aromatic –CH bending vibration.

In summary, analyses demonstrated that the polysaccharide from the haloarchaeal strain is a carboxymethylated sulfated heteroepoxypolysaccharide (CS-hEPS) with abundant functional groups.

3.2. In Vitro Antioxidant Activities of *H. borinquense* CS-hEPS. The *in vitro* antioxidant ability of CS-hEPS was evaluated through the detection of the scavenging rates of DPPH, (HO•), and (O₂−•) radicals. Results are summarized in Figure 3. The DPPH free radical scavenging activity of the CS-hEPS is shown in Figure 3(a) and compared with ascorbic acid as a positive control. The scavenging activity increased with the concentration from 0.5 to 2.5 mg/mL of the CS-hEPS. It was slightly lower than that of ascorbic acid at the concentration of 2.5 mg/mL. When the concentration was higher than 1.5 mg/mL, the DPPH radical scavenging activity increased slightly. The IC₅₀ of DPPH radical scavenging for the EPS was 2.5 mg/mL. Additionally, from Figures 3(b) and 3(c) we observed that CS-hEPS exhibited a concentration-dependent scavenging activity, for both hydroxyl and superoxide radicals, with CS-hEPS IC₅₀ equal to 1.5 and 0.5 mg/mL, respectively. CS-hEPS was more active against superoxide than hydroxyl radicals; however, its activity was weaker than that of ascorbic acid.

3.3. In Vivo Antifatigue Effects of CS-hEPS from *H. borinquense*. The data obtained from various tests measuring the antifatigue activity of CS-hEPS is represented in Figure 4. As shown in Figure 4(a), the average exhaustive swimming time of mice group at doses of 100, 150, and 200 mg/kg, respectively, was significantly (*P* < 0.01) prolonged compared to mice of the negative control group (saline solution). Exhaustive swimming time of every treatment group of CS-hEPS at doses of 100, 150, and 200 mg/kg was 161.75 ± 4.25, 175.83 ± 5.75, and 194.50 ± 6.35 seconds (sec), respectively. CS-hEPS groups of mice had extended exhaustive swimming time by 36.90, 48.81, and 64.62%, respectively, compared to mice treated with saline solution (negative control group).

Then, the exhaustive swimming fatigue-related biochemical parameters in mice (SUN, LDH, and HG) were measured. As demonstrated in Figure 4(b), SUN levels of CS-hEPS groups (100, 150, 200 mg/kg) were 27.25 ± 3.06, 22.59 ± 3.13, and 23.75 ± 2.52 mmol/L, respectively, which were reduced significantly (*P* < 0.01) by 58.09, 65.26, and 63.47%, respectively, compared to the negative control group (saline solution).

The data from Figure 4(c) indicated that HG contents of two treatment CS-hEPS groups, CS-hEPS-150 and CS-hEPS-200, were significantly higher (*P* < 0.01) than the negative control group. Contents of HG in CS-hEPS-150 and CS-hEPS-200 groups were 5.66 ± 0.90 and 5.81 ± 0.75 mg/g, which increased by 56.35 and 60.49%, respectively, compared to the group treated with saline solution (negative control group) (3.62 ± 0.66 mg/g). However, HG level of CS-hEPS-100 group was 3.28 ± 0.48 mg/g remaining lower than that observed in the negative control group. It appears that
the 100 mg/kg dose of CS-hEPS would remain insufficient to cover the hepatic glycogen deficiency of mice group CS-hEPS-100. Moreover, Figure 4(d) indicated that 30-day CS-hEPS treatment reduced by 5.57% (at a dose of 100 mg/kg), 7.81% (at a dose of 150 mg/Kg), and 11.25% (at a dose of 200 mg/kg) the serum LDH activity in mice (P < 0.05).

3.4. In Vitro Antitumor Activity of CS-hEPS from H. borinquense. The antitumor activity of CS-hEPS against myelogenous leukemia K562 and liver cancer Hep G cells at different incubation periods and concentrations is summarized in Figures 5(a) and 5(b). As shown in Figure 5(a), inhibition effects of CS-hEPS on myelogenous leukemia K562 cells significantly (P < 0.05) increased along with the increasing concentrations and treatment time. At the lowest concentration (50 μg/mL) and treatment period (24 h), the inhibition rate was 17.2 ± 0.26%. Following the treatment with the highest concentration (600 μg/mL) and at the longest treatment period (72 h), the inhibition rate significantly (P < 0.05) improved (86.6 ± 0.32%) and became very close to the positive control 5-FU (91.4 ± 0.14%). The antitumor activity of CS-hEPS against liver cancer HepG cells was also demonstrated in terms of dose and incubation time (Figure 5(b)). The highest inhibition rate was 58.6 ± 0.43% obtained at 600 μg/mL and 72 h of incubation.

4. Discussion

Although very diversified with a great number of species, luckily, no member of the domain Archaea has been described as a pathogen for humans, animals, or plants [31, 32]. The CS-hEPS produced by the archaean H. borinquense was similar to other microbial EPS with respect to crude chemical and mainly monosaccharide composition. Interestingly, it exhibited peculiar chemical characteristics, such as high uronic acid and sulfate contents, and importantly specific functional units, such as carboxymethyl, methoxyl,
and sulfated groups. Sulfated and/or carboxymethylated EPS have attracted attention in the field of pharmacology for their valuable biological activities [30, 33, 34]. Moreover, carboxymethylation and sulfation were applied to chemically modify polysaccharides for enhancing their bioactivities [33, 34].

In this study several in vitro and in vivo methods have been used to evaluate the bioactivity (antioxidant, antifatigue, and antitumoral activities) and to assess the potential of CS-hEPS of an archaeon to be applied as harmless adjuvants in human cancer therapy. In order to obtain a candidate antioxidant that would be of benefit to human health [35, 36], CS-hEPS antioxidant potential was investigated through hydrogen atom transfer and single electron transfer methods [3, 37].

Among these methods, the DPPH free radical has been widely adopted as a tool for estimating the free radical scavenging activities of antioxidants [38, 39]. A lower absorbance of the reaction mixture indicates a higher DPPH radical scavenging activity. In this work, the radical scavenging activities on DPPH exhibited an obvious concentration dependency within the range of tested concentrations (Figure 3(a)). These findings indicated that CS-hEPS had significant scavenging activity with an IC50 equal to 2.5 mg/mL. Previous studies evaluated radical scavenging activities of EPS from different species. CS-hEPS showed similar activity to that of C. pyrenoidosa EPS (IC50, 2.14 mg/mL) and better activities than that of Lactobacillus plantarum C88 EPS (52.2% at 4.0 mg/mL) and Pseudomonas fluorescens EPS (30.0% at 1.0 mg/mL) [40–42].

Additionally, hydroxyl and superoxide radicals as primary ROS are considered as the most contributors of oxidative stress. They are the most harmful species of free radicals that may cause severe damage to cells, even DNA and protein, and lead to various disorders such as cancer and fatigue. Therefore, scavenging these radicals is important for protecting physiological systems. Interestingly, CS-hEPS exhibited considerable scavenging activities against hydroxyl and superoxide radicals in a concentration-dependent manner and could be explored as a potential antioxidant.

![Figure 3: (a) DPPH radical scavenging activity, (b) hydroxyl radical scavenging activity, and (c) superoxide radical scavenging activity of the CS-hEPS from H. borinquense and control standards. Results are expressed as a mean ± SD (n = 3).](attachment:image.jpg)
agent. By comparing IC50 values, CS-hEPS showed better activities on hydroxyl and superoxide radicals than that on DPPH (Figures 3(a)–3(c)). IC50 values for these radicals were 1.5 and 0.5 mg/mL, respectively. Additionally, CS-hEPS demonstrated similar hydroxyl radical scavenging capacity to *Lactobacillus gasseri* FR4 EPS (IC50, 1.58 mg/mL) [43] and lower capacity than *Scenedesmus* sp. EPS (IC50, 0.38 mg/mL) [40].

**Figure 4:** Effect of administrations of *H. borinquense* purified CS-hEPS on (a) swimming exercise performance. The endurance of mice was measured after 30 days of CS-hEPS administration by an exhaustive swimming exercise, (b) SUN, (c) hepatic glycogen, (d) LDH levels at the end of the experiments. Data are presented as mean ± SD (n = 20).

**Figure 5:** Antitumor effects of *H. borinquense* purified CS-hEPS on myelogenous leukemia cells K562 (a) and liver cancer Hep G (b) cells. All values were expressed as means ± SD of three replications.
It has been reported that the antioxidant activity of polysaccharides is closely linked to their physiochemical properties and functional groups, mainly the uronic acid and sulfate contents [3, 21, 44, 45]. In the present study, CS-hEPS contained a significant proportion of uronic acid and sulfate. The presence of sulfate groups could activate the hydrogen atom of the anomic carbon and lead to stronger atom donating capacities [46, 47]. Additionally, hydrogen atoms from all ring CH bonds on carbohydrate contribute to the radical scavenging ability and act as electron or hydrogen donors to scavenge free radicals and consequently exhibit an antioxidant activity [48, 49].

Taking into account the above results, which suggested that CS-hEPS had the potential to be an effective scavenger for hydroxyl and superoxide radicals, our results are also concordant with a previous report that showed that a polysaccharide from Bacillus licheniformis BLP2 [47], with more sulfate groups (6.17%), had higher in vitro scavenging abilities. Thus, it is legitimate to suggest that sulfate groups play an important role in the scavenging of free radicals. CS-hEPS had higher scavenging ability due to its carboxymethyl, methoxyl, and sulfated groups, which is in agreement with the FTIR analysis and the sulfate content. These groups were described in previous studies as free radical scavengers [50–52]. However, all these studies described the effect of chemically modified polysaccharides and so none of these works examined the combined effect of carboxymethyl, methoxyl, and sulfated groups on the bioactivity of polysaccharides. Moreover, interestingly, all these three types of functional groups are naturally available in CS-hEPS.

With regard to the antifatigue activity, an exhaustive weight loaded swimming test was performed to evaluate the degree of physical fatigue in mice following 30 days of oral treatment by CS-hEPS at different doses. Swimming time to exhaustion is an appropriate parameter to reveal the endurance capacity of mice and gives a high reproducibility to measure the degree of fatigue [29]. CS-hEPS groups of mice had extended exhaustive swimming time at doses of 100, 150, and 200 mg/kg by 36.90, 48.81, and 63.47%, respectively, compared to the control group treated with saline solution (Figure 4(a)), which indicated that CS-hEPS exhibited an antifatigue effect. Previous studies have attempted to identify antifatigue polysaccharides to delay fatigue and accelerate the elimination of tiredness. CS-hEPS showed better effects of relieving fatigue (64.62%) than Abelmoschus esculentus EPS (30.5%) [53]. These effects are similar to Gynostemma pentaphyllum polysaccharide (GPP) which significantly prolonged exhaustive exercise time of mice. According to Chi et al. [54], the underlying mechanisms by which GPP exerts its antifatigue effect may be associated with the role of this polysaccharide in scavenging excessive free radicals produced during physical exercise.

Fatigue-related biochemical parameters in mice, notably SUN, LDH, and HG, were measured to validate and elucidate the underlying mechanisms of the antifatigue activity of CS-hEPS. Metabolic dysregulation involves the exhaustion of energy sources, such as HG. Consequently, the accumulation of metabolites, such as SUN, and the generation of free radicals contribute to fatigue. Moreover, the accumulation of lactic acid during intensive exercise causes decrease in pH of muscles and blood, which inhibits muscle contraction and induces fatigue [18, 55, 56]. Under normal conditions, muscular LDH catalyzes the mutual transformation of lactic acid and pyruvate. A high serum LDH level is also a marker of muscle damage; it is therefore a relevant indicator of fatigue after exercise. When energy sources derived from carbohydrates and fats are depleted, the organism invokes the catabolism of proteins and amino acids [35, 57]. Consequently, the excess production of SUN will reflect the protein decomposition which will attenuate the muscle contraction and induce fatigue. SUN level will significantly increase when body is poorly adapted for exercise tolerance [33, 56]. In this study, after the exhaustive swimming (Figure 4(b)), SUN levels of CS-hEPS groups (100, 150, 200 mg/kg) were reduced significantly ($P < 0.01$) by 58.09, 65.26, and 63.47%, respectively, compared to the group treated with saline solution. CS-hEPS reduced SUN level better than LEP-1b polysaccharide from Lachnum (19.77% at 200 mg/kg) [33]. Two mechanisms could explain these results: (1) CS-hEPS decrease protein catabolism and therefore maximize the exhaustive swimming time and/or (2) CS-hEPS improve hepatic glycogenesis which in turn controls protein catabolism. It is widely accepted that the endurance of the body depends on the level of energy source including HG and rapid consumption of hepatic glycogen resulting in a decrease in the body’s workload capacity. Thus, restoring the level of HG is essential to counteract fatigue and improve physical performance. Results from Figure 4(c) indicated that, after the swimming test, HG contents of two treatment groups, CS-hEPS-150 and CS-hEPS-200, were significantly higher ($P < 0.01$) than saline solution-treated group. Contents of HG increased by 56.35 and 60.49%, respectively. These results argue in favor of the second above-mentioned proposed mechanism in which CS-hEPS from H. borinquense could reduce the catabolic metabolism of proteins by restoring the liver energy reservoir. Similarly, Surhio et al. [33] demonstrated that EPS from Lachnum LEP-1b increased the content of hepatic glycogen in mice (21.89% at 200 mg/kg). However the glycogenesis activity of CS-hEPS was significantly higher than that of LEP-1b at the same doses. This is due to functional groups of CS-hEPS, particularly, carboxymethyl, methoxyl, and sulfated groups (Figure 2).

LDH is recognized as a reliable parameter of muscle physiological activity, and, normally, it is localized in the cytosol. An increase in its serum level indicates that muscle damage has occurred. LDH oxidizes lactic acid, restores the pH value, and heals lactic acid damage [29].

In this study, daily oral administration of CS-hEPS for 30 days significantly ($P < 0.05$) reduced serum LDH activity in mice (11.25% at 200 mg/kg; Figure 4(d)) as compared with the saline solution-treated group.

In summary an exhaustive swimming test was applied to study the antifatigue activity which demonstrated that each treatment group CS-hEPS extended exhaustive swimming time in mice. In addition oral treatment with CS-hEPS increased the exercise tolerance, boosted the HG level by activating the energy metabolism, and reduced the...
accumulation of SUN and LDH contents during exercise, which could prolong the exercise durability. Due to the particularities of its chemical structure, CS-hEPS showed better antifatigue effects than those of previously reported polysaccharides [33, 53, 54, 58].

The antitumor activity of CS-hEPS has also been tested against two types of cancer cell lines, myelogenous leukemia K562 and liver cancer Hep G cells, at different incubation periods and doses using 5-fluorouracil molecule (5-FU) as a positive control. The inhibition rate against K 562 cell line significantly improved at the highest concentration of CS-EPS (86.6 ± 0.32% at 600 μg/mL and 72 h of treatment) and became very close to the positive control 5-FU (91.4 ± 0.14%) (Figure 5(a)). In an earlier report, Chen et al. [30] reported three fractions, OCAP-2-2, OCAP-3-1, and OCAP-3-2, of polysaccharides from Ornithogalum caudatum that significantly inhibited the growth of K562 cells, when treated for 24 h, at concentrations from 0.01 to 100 μg/mL. OCAP-3-2 exhibited significantly higher inhibition activity than other fractions at all concentrations. The highest inhibition activity of OCAP-3-2 was 47.83 ± 6.15% at the concentration of 0.1 μg/mL. At concentration of 100 μg/mL, inhibition activities of OCAP-3-1 and OCAP-3-2 were 14.69 ± 3.57 and 24.61 ± 12.43%, respectively. These results suggest that CS-hEPS has significant inhibition effects on leukemia K562 cells.

For the second cell line, liver cancer Hep G cells, the highest inhibition rate by CS-hEPS was 58.6 ± 0.43% obtained at 600 μg/mL and 72 h of incubation (Figure 5(b)). From previous literature, CS-hEPS inhibition rate was better than that obtained by polysaccharides from Cordyceps militaris (57.11% at 8 mg/mL and 72 h of incubation) [59]. The inhibition rate is also close to that obtained by polysaccharide from Phellinus baumii which suppresses the HepG-2 cells proliferation by 61.2% at 400 μg/mL and 48 h of treatment period [60]. It is quite clear from our findings that CS-hEPS are more effective against myelogenous leukemia K562 cells, with similar antiproliferative effect of 5-FU, but with doses 8 to 12 times higher than that of 5-FU. Chemical modifications of polysaccharides by carboxymethylation or sulfation were applied in previous works for boosting their antiproliferative activities [61, 62]. Chen et al. [63], interested in the chemical modification by simultaneous carboxymethylation and sulfation processes of a native polysaccharide isolated from Poria cocos, obtained a modified polysaccharide with significantly higher inhibition ratio to Sarcoma 180 tumor in BALB/c mice compared to the nonmodified polysaccharide. These data confirmed that the antiproliferative efficacy of CS-hEPS against cancerous cell lines could be attributed to their structural characteristics, in particular carboxymethyl and sulfated groups.

Previous studies demonstrated that some microbial polysaccharides fight cancer cells through different mechanisms such as cell cycle interruption, induction of apoptosis, collapse of membrane potential, poly-ADP-ribose polymerase (PARP) cleavage, and expression of many tumor suppressor genes such as Bax, Cas-3, Bcl-2, and p53 [64–66]. In this context, Ruiz-Ruiz et al. [64] explored the influence of the sulfate group on the antitumoral activity of EPS by modifying them up to saturation and then by depriving them of their constitutive native sulfates. The authors have found that the sulfated EPS induced high apoptosis hematopoietic tumor T cell lines. Apoptosis induction has been confirmed through activation and inhibition of the caspase gene and change in the mitochondrial membrane potential [64]. Li et al. [66] investigated the molecular mechanism involved in the antitumor activity of EPS from Ganoderma atrium against tumor growth in S180-bearing mice. They found that the induction of apoptosis was achieved through mitochondrial pathways and immunoenhancement properties. With regard to the CS-hEPS antitumoral mechanism, taking into account its structural properties, it might be pertinent to suggest that functional groups would allow CS-hEPS to bind and interact with cell targets, hence the induction of apoptosis probably through the cross-linking of multiple intracellular signals. Based on our results, a substantive work should be done to elucidate the antitumor mechanism of CS-hEPS.

Altogether, it was demonstrated that CS-hEPS from the archaeal strain H. borinquense exhibit valuable biological activities (antioxidant, antifatigue, and antitumoral activities) that could alleviate physiological disturbances in cancer patients including fatigue, ROS, stress, and even tumor cell suppression.

5. Conclusion

In this study, we have confirmed that H. borinquense CS-hEPS induce fatigue recovery via retarding the accumulation of SUN, restoring the level of LDH, and enhancing the hepatic glycogen level. The in vitro antioxidant activity demonstrated that CS-hEPS could suppress oxidative stress, which could be another action pathway of its antifatigue effects. These findings with consideration of its antitumor activity argue in favor of a possible application of CS-hEPS as a novel natural adjuvant against cancer. In-depth structural characterization (e.g., NMR) and studies on animal cancer models will be conducted to validate the antitumor mechanism of CS-hEPS.

Abbreviations

- Akt/PI3K: Protein kinase B (PKB), also known as Akt/
  phosphatidylinositol-3-kinase
- Bax, Cas-3, Bcl-2, and p53: Tumor suppressor genes encoded proteins that normally function to prevent tumor development
- CS-hEPS: Carboxymethylated sulfated heteropolysaccharide
- DPPH: 1,1-Diphenyl-2-picrylhydrazyl
- DSR: DPPH scavenging rate
- EGF: Epidermal growth factor
- EST: Exhaustive swimming test
- FTIR: Fourier Transform InfraRed
- GPP: Gynostemma pentaphyllum polysaccharide
- HepG: Liver cancer cells
**Data Availability**

The data used to support the findings of this study are available from the corresponding author upon request.

**Conflicts of Interest**

No potential conflicts of interest were reported by the authors.

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**Supplementary Materials**

Figure 1: (A) glucose standard curve, (B) glucuronic acid standard curve, and (C) potassium sulfate standard curve plotted to estimate total carbohydrate, uronic acid, and sulfate contents in CS-EPS, respectively. (Supplementary Materials)

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