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Bioactive Exopolysaccharides (EPS) Synthesized From *Exiguobacterium aurantiacum* and *Brevundimonas diminuta* with Myeloid Cancer Cells Inhibiting and Flocculating Activity

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Original Research Article

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Abstract: Exopolysaccharides (EPS) are polysaccharides secreted by certain bacteria. *Exiguobacterium aurantiacum* and *Brevundimonas diminuta*, isolated from Nador's Marchica lagoon from soil and water samples were found to be capable of producing exopolysaccharides in YPM (Yeast-Peptone-Mineral salts) and YPMG (Yeast-Peptone-Glucose- Mineral salts) media. The partial characterization of the exopolysaccharides was carried out by an analysis combining infrared (FTIR) and thin layer chromatography (TLC). These exopolysaccharides would contain homopolymers composed of glucose. The crude EPS produced by *E. aurantiacum* showed a 35% antiproliferative activity against myeloid cancer and a flocculating activity of 68%. The EPS produced by *B. diminuta* demonstrated a 20% antiproliferative activity against myeloid cancer and a flocculating activity of 66%. This yield is better than that observed with the current industrial flocculants, suggesting the use of these EPS as a new biodegradable bioflocculant in water treatment.
Keywords: Exopolysaccharides, bacteria, Nador Marchica lagoon, antiproliferative activity, myeloid cancer, bioflocculant

INTRODUCTION

Nowadays, the study of bacteria and their potential role in the production of bioactive compounds is becoming a new topic for research [1]. Among the bioactive compounds produced by these microorganisms, EPS are high molecular weight polymers composed of saccharides subunits and are secreted by a microorganism into the surrounding environment. Microorganisms synthesize large spectrum multifunctional polysaccharides including intracellular polysaccharides, structural polysaccharides and extracellular polysaccharides (EPS). Exopolysaccharides generally consist of monosaccharides and some non carbohydrate substituents (such as protein, nucleic acids, lipids, acetate, pyruvate, succinate, and phosphate) [2].

Microbial polymers offer more advantages in their application than those obtained from seaweeds and plants, since they present a greater range of structures and properties which can be used in different applications [3]. Due to their interesting physicochemical properties, the EPS have found applications in many industries like textile, adhesives, paint, food, beverage, pharmaceutic, among others [4].

Among the many human health problems caused by environmental pollution and poor nutrition, cancer is one of the leading causes of death worldwide for women and men [5, 6]. Treatments such as chemotherapy and radiotherapy have adverse effects in patients. Recently, a wide variety of natural products have been recognized to have the ability to induce apoptosis in various tumor cells of human origin and many of these substances are from plants, animals and microorganisms sources with low or no toxicities to human body [7].

In the environmental field, flocculants are widely used in wastewater treatment, drinking water treatment, and industrial downstream processing [8-10]. They can be classified into three groups, namely, inorganic coagulants such as hydrolysing aluminium and iron salts, and their pre-hydrolysed forms; synthetic organic flocculants such as polyacrylamide derivatives and polyethyleneamine; and naturally occurring bioflocculants such as sodium alginate and microbial flocculants. Since synthetic organic polyelectrolytes are effective flocculants with lower costs they have been used widely as coagulants aids and in sludge dewatering, however, their usage has been restricted mainly in European countries because they are not readily biodegradable and some of their degraded monomers such as acrylamide are neurotoxic and exhibit strong carcinogenicity [11,12]. Because of the limitations of these synthetic flocculants, novel bioflocculants produced by microorganisms are needed as alternatives.

In view of these interesting properties of bacterial exopolysaccharides, the discovery of a new ecological niche with a very high concentration of bacteria leads to the identification of the species of EPS-producing bacteria. This work has therefore focused on the production of exopolysaccharides by two bacteria isolated from the Marchica lagoon of Nador in order to evaluate their biological activity. This study was undertaken to investigate the antiproliferative and the bioflocculating activities of EPS produced by *Exiguobacterium aurantiacum* and *Brevundimonas diminuta* isolated from Nador's Marchica lagoon in Morocco. The strain of *E. aurantiacum* was reported as an EPS producer microorganism for the first time by our research group [13] and *B. diminuta* produced a high amount of exopolysaccharide but these results have not yet been published. The present study aimed to evaluate the antiproliferative effects of EPS against myeloid cancer (P3 cells). The bioflocculating activity was compared to other commercially available polymers, namely alginate and polyéthylen to evaluate its potential industrial applications.

MATERIAL AND METHODS

Materials

The strains *E. aurantiacum* (NR_113666.1) and *B. diminuta* (NR_040805.1) were isolated from the lagoon Marchica (Soil and water) in Morocco. These bacteria were isolated in the context of the project of the 7th PCRD research ULIXES « Unraveling and exploiting Mediterranean Sea microbial diversity and ecology for the xenobiotics and pollutants cleanup » in Morocco. Bacterial identification was done by sequence method using ***GenElute™ Bacterial Genomic DNA Kit*** and ***ABI 3130xl*** Genetic Analyzer. The identification of isolated strains was performed by direct sequencing of PCR amplified 16S rRNA gene fragments. The bacteria have been purified and maintained in glycerol at -20 ° C.

Human myeloid cancer P3 cells were obtained from the Laboratory of experimental medicine and biotechnology of the Faculty of medicine and pharmacy at Casablanca (Morocco). All chemicals and solutions used in this study were supplied by Sigma (USA), Difco (USA) and Fluka (Switzerland).

Isolation and purification of EPS

Two media were used for the production of EPS by *E. aurantiacum* and *B. diminuta.* First, the inoculum of each bacterium was prepared by transferring a single colony from the slant culture to the screw-top glass tube containing 5ml of the sterile nutrient broth and subsequently incubated at 37°C for 16h. Fermentation was carried out in 250 ml Erlenmeyer flask with a working volume of 100ml consisted of Yeast-Peptone-Mineral salts-Glucose medium (YPMG) composition per liter: Glucose : 20g ; Peptone: 5g; yeast extract: 5g; $(NH_4)_2S0_4$:0,6g ; KH_2PO_4 :3,18g ; K_2HPO_4 :5,2g ; $MgSO_4$:0,3g ; $CaCl_2$:0,05g ; $ZnSO_4$:0,2mg ; $CuSO_4$: 0,2mg ; $MnSO_4$:0,2mg ; $FeSO_4$:0,6mg) and 100ml of yeast-peptone-mineral salts medium (YPM), the same previous medium without glucose. The pH of the culture medium was adjusted to 8.0 and sterilization was done by autoclaving at 121°C for 30 min and incubated for 4 days at 37°C at 180rpm with 1 ml of initial inoculum. The fermented culture broth after 4 days was centrifuged at 8,600 g for 30 min at 4°C for each bacterium. The culture supernatant was added with two volume of ice cold ethanol (95%,v/v) and kept overnight at 4°C to precipitate. The mixture was then centrifuged at 8,000g for 20min at 4°C to recover the precipitation. EPS was extracted and purified according to the method followed by castellane *et al.* [14].

Chemical analysis

The exopolysaccharide samples (10 mg), were dissolved with 2 mol/L trifluoroacetic acid (10 mL) in a sealed flask, respectively. The flask was placed in an oven at 110 ◦C for 3 h. After being cooled to room temperature, the flask was opened and methanol was added into it. Then the reaction mixture was evaporated to dryness under a reduced pressure. After that, the same amount of methanol was added and dried again by the same method above, and the procedure was repeated thrice for trifluoroacetic acid to be removed. The dried hydrolyzed polysaccharide samples were dissolved in 1,5mL of water. Total carbohydrates [15], proteins [16] and sulfates [17] were analyzed. Samples of purified EPS were prepared for I.R. analysis using Perkin-Elmer FT-IR instrument. FTIR is an instrument competent to detect the functional groups of purified EPS. One part

of extract was mixed with ninety nine parts of dried potassium bromide (KBr) separately and then compressed to prepare a salt disc of 3 mm diameter. These discs were subjected to IR-spectra measurement in the frequency range of 400 and 4000 cm^{-1} [18]**.** The qualitative analysis of the isolated EPS was done by thin layer chromatography (TLC). TLC was performed for the monosaccharide components using a mobile system consist of butanol-acetic acid-water (2:1:1, v/v/v). The resultant spots on the TLC plates were visualized by spraying with a solution of sulphuric orcinol (0, 1% (v/v) of orcinol in 20% of sulfuric acid) followed by heating at 120°C.

Experimental procedure of flocculation

The flocculating activity was tested using kaolin remblend as the suspend solid [19, 20] at 50 gm/ L. EPS and commercial polysaccharides were tested at different concentrations (7; 10; 13 ; 17 et 20 gm/L). The flocculating activity was done according to ASTM D2035 norm. The test was also carried out using aqueous solutions of alginate (Sigma) and polyethylene with the same concentration. All tests were performed in triplicate.

Cell proliferation viability assay

MTT assay was uses to measure viability of P3 myeloid cancer. Briefly, P3 cells were seeded in 96-well plates at a density of 3.104 cells/well and incubated at 37°C under CO_2. After 3 days, the supernatant was aspirated and 100 µl of RPMI (Roswell Park Memorial Institute) medium containing various final concentrations of EPS (200- 600 µg/ml) were added and incubated for 72h. Three replicate wells were used at each point in this experiment. To each well was added 20 µl (5mg/ml) MTT, followed by incubation for another 3h. Then the supernatant was discarded and 100 µl of DMSO was added to each well. The absorbance was detected at 540 nm by microplate ELISA reader. Relative cell viability was presented as a percentage relative to the control group.

RESULTS

Chemical composition of EPS

The EPS produced in both media (YPM and YPMG) were hydrolyzed and the glycosidic residues were assayed. The results (Table 1) showed that in all the tested media, the EPS of both bacteria are composed of carbohydrates in variable amounts. The EPS of the bacteria are composed of 66% and 60% of carbohydrates in *E. aurantiacum* and *B. diminuta* respectively in the YPMG medium. In the YPM medium, the amounts of carbohydrates are low compared to the YPMG medium (15% in *E. aurantiacum* and 20% in *B. diminuta*). The absence of proteins is observed in the YPM medium in *E. aurantiacum* unlike *B. diminuta* which contains 6% protein and traces of sulfates. Sulfates are also present in the EPS produced by E.. Aurantiacum (1.5%) in the YPM medium (Table 1).

Table-1: Comparison of carbohydrate, protein and sulfate content of EPS produced by *E. aurantiacum* (EA) and *B. diminuta* (BD) in YPM (Yeast-Peptone-Mineral salts) and YPMG (Yeast-Peptone-Mineral salts-Glucose) media

	EA-YPMG EPS	EA-YPM EPS	BD-YPMG EPS	BD-YPM EPS
% carbohydrates	66	15	60	20
% proteins	0,123	0	0,09	6
% sulfates	3	1,5	1	1

After hydrolysis of EPS, the monomer contents were identified by thin layer chromatography TLC (Figure 1). The results (Figure 1) showed that EPS were qualitatively more or less the same. The monosaccharide which could be identified by thin layer chromatography in EPS produced by *E. aurantiacum* and *B. Diminuta* in both media YPM and YPMG is glucose.

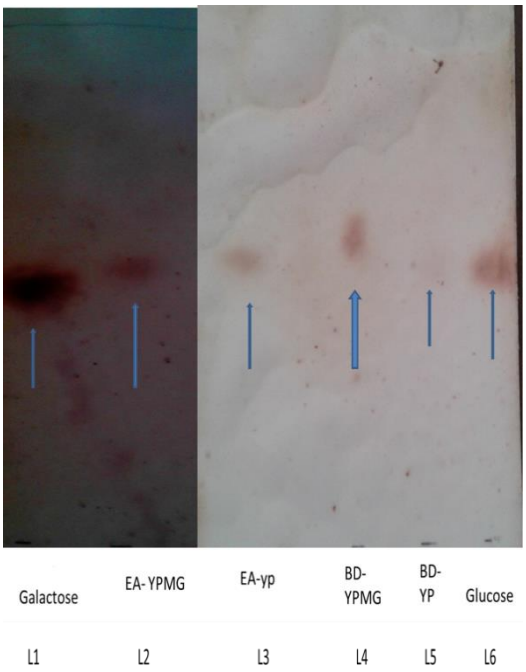


Fig-1: TLC plate with Lane 1: Galactose, lane 2: YPMG hydrolyzed EPS produced by *E. aurantiacum*, lane 3: YPM hydrolyzed EPS produced by *Exiguobacterium aurantiacum*, lane 4: YPMG hydrolyzed EPS produced by *Brevundimonas diminuta*, lane5: YPM hydrolyzed EPS produced by Brevundimonas diminuta and lane 6: Glucose

Mobile system: BuOH/ CH3COOH/ H2O, 2 /1/ 1 ; révélation : sulfuric orcinol

The FTIR spectrum of *E. aurantiacum* and *B.diminuta* EPS in YPM medium revealed characteristics of functional groups (Figure 2). The results demonstrate that a difference could be observed in I.R. spectra of the EPSs produced by both bacteria. H-bonded hydroxyl groups which are typical for exopolysaccharide were found (3600-3200 and 1050-1100 cm $^{-1}$) [21] in both bacteria. The list of the bands at 500-900 cm^{-1} is also present. Polysaccharides C-O-C and C-O-P was at 1100 cm^{-1}. The *E. aurantiacum* EPS spectrum showed bands around 1000, 1200, 1400, 1500 and 1600 cm $^{-1}$ revealed the (1,3) -β- glucan linkages. The FTIR spectrum of *B. diminuta* EPS revealed characteristics of functional groups such as –C=O acetate deformation at 1250 cm^{-1} and a carboxylate asymmetric stretching at 1400, 32 cm^{-1} [21]. Other functional groups such as O-H stretching peak of free hydroxyl group at 3650 cm^{-1} was found. The absorption at 1671 cm^{-1} also present indicates the pyruvate group.

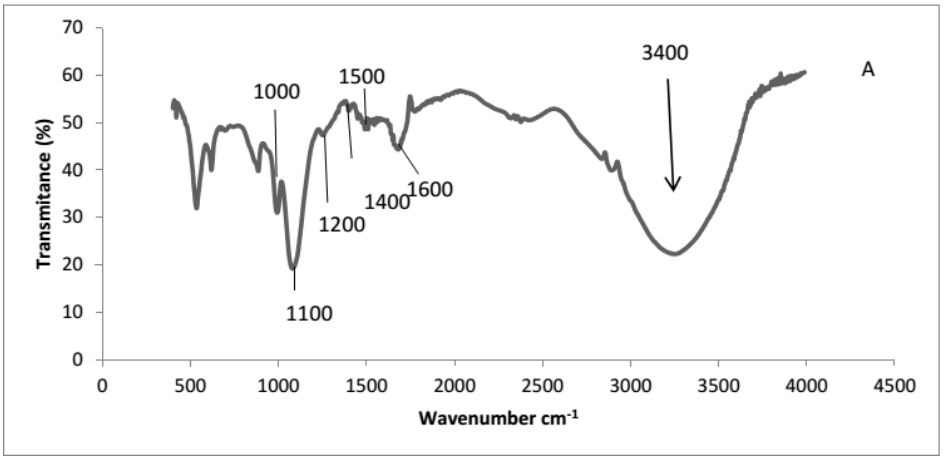


Fig-2A: Comparative FT-IR spectra of polysaccharides: *Exiguobacterium aurantiacum* (A) in YPM medium

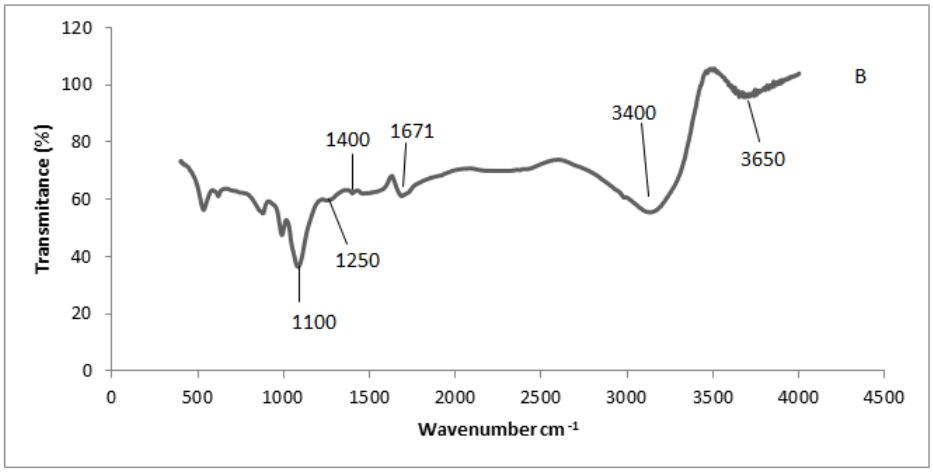
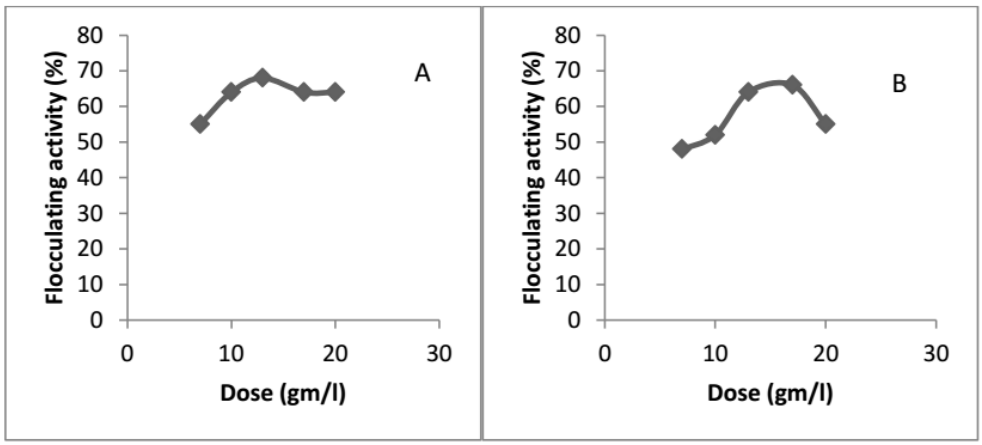


Fig-2A: Comparative FT-IR spectra of polysaccharides: *Brevundimonas diminuta* (B) in YPM medium

Flocculating activity

A preliminary test of the flocculating activity of *E. aurantiacum* and *B. diminuta* EPS was performed in a kaolin suspension and compared with other commercial flocculants, namely polyethylene and alginate (Figure 3). These preliminary tests were performed at room temperature and the initial pH of Kaolin suspension (7, 6), for flocculants concentration of 7, 10, 13, 17 et 20 gm/L. EPS produced in YPMG medium by *E. aurantiacum* had the highest flocculating activity (68%) at 13gm/l while EPS of *B. diminuta* had flocculating activities of 66% (at 17gm/l in YPMG), 59% (at 17gm/l in YPM). The lower flocculating activity was obtained by EPS produced in YPM medium by *E. aurantiacum* (57% at 17gm/l). Alginate and polyethylene had flocculating activities of 57% (13gm/l) and 68% (17gm/l) respectively. Polyethylene, the flocculant often used in industry, had the same flocculating activity (68% at 17gm/l) as YPMG EPS by *E. aurantiacum* (68% at 13gm/l), which is a very promising result (Figure 3).



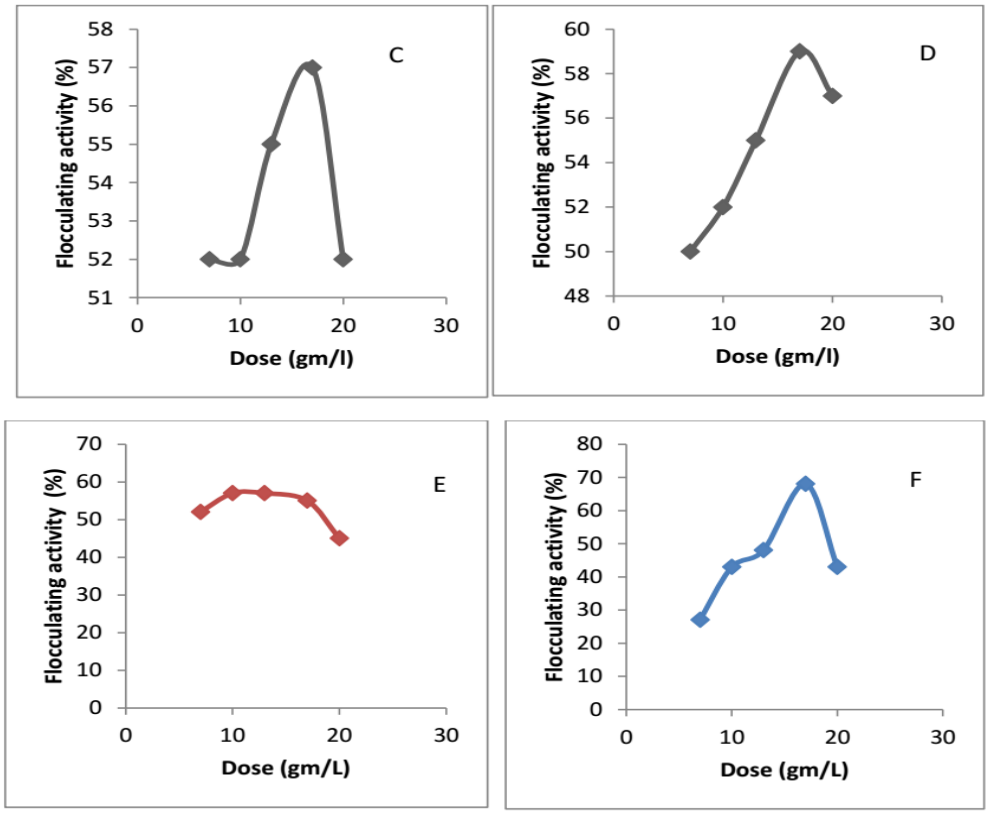


Fig-3: Flocculating activity of the exopolysaccharide produced by *Exiguobacterium aurantiacum* (A : EPS-YPMG medium ; C : EPS-YPM medium) and *Brevundimonas diminuta* (B : EPS-YPMG medium ; D : EPS- YPM medium) in comparison with alginate (E) And polyethylene (F) pH: 7.6

Antiproliferative activities

It had been reported that exopolysaccharides played a certain role in anticancer activity [22]. In this study, MTT assay was employed to evaluate the antiproliferative effect of four EPS on myeloid cancer cell lines P3. The cells were treated with various concentrations of EPS produced by *E. aurantiacum* et B. Diminuta in YPMG and YPM media. As shown in figure 4, all four EPS exhibited concentration-dependent inhibition activities to myeloid cancer cells. EPS produced in YPM medium by *E. aurantiacum* had the highest antiproliferative activity (35%) while EPS of *B. diminuta* had antiproliferative activity of 20% at 600 µg/ml. EPS produced by both bacteria in YPMG medium had lower antiproliferative activities (18% for *E. aurantiacum* and 12% of *B. diminuta*) at 600µg/l than EPS isolated in YPM medium (Figure 4).

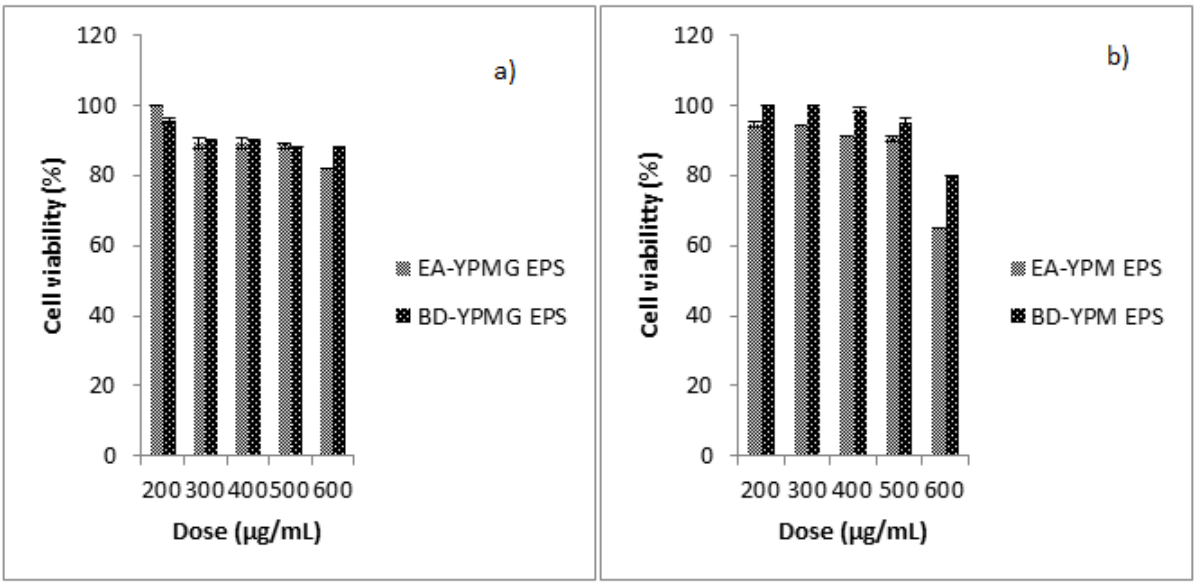


Fig-4: The effect of Exiguobacterium aurantiacum and *Brevundimonas diminuta* exopolysaccharides produced in YPMG medium (a) and YPM medium (b) on the viability of P3 myeloid cancer cells at different concentrations of EPS (200-600 µg/ml) EA: Exiguobacterieum aurantiacum; BD: *Brevundimonas diminuta*

DISCUSSION

We report here for the first time the antiproliferative and the bioflocculating activities of EPSs produced by *E. aurantiacum* and *B. diminuta*, isolated from Nador's Marchica lagoon. The EPS are produced in two media YPM (Yeast-Peptone- Mineral salts) and YPMG (Yeast-Peptone-Mineral salts – Glucose). The different media assayed change the chemical composition of the EPS. With regard to the chemical composition of our EPS, there's sulfate content, is particularly interesting. In general, EPS is composed of polysaccharides, proteins, nucleic acids and inorganic compounds. Sulfates are not commonly found in microbial EPSs, although they are present in all the EPSs produced by the halophilic bacteria and also in many marine bacteria and in cell wall polysaccharides from red and brown macroalgae. Sulfated EPSs are of great potential interest in medicine since they have a number of bioactive properties: anticoagulant, antiangiogenic, antiproliferative, antiviral, etc. [23, 24]. After hydrolysis of EPS, the monomer contents were identified by thin layer chromatography TLC. The monosaccharide which could be identified by thin layer chromatography in EPS produced by *E. aurantiacum* and B. diminuta in both media YPM and YPMG is glucose. EPS exopolysaccharides are either homopolysaccharides or heteropolysaccharides. The homopolysaccharides are generally neutral and composed of only one monosaccharide type such as D-glucose or L-fructose [25,26, 27, 28] The I.R. spectra of the purified EPS in *E. aurantiacum* proved the presence of (1, 3) -β- glucan linkages indicates that the EPS produced by *E. aurantiacum* would be a homopolymer. Similar finding was also recorded by vijayabaskar et al [18]. The identification of bands corresponding to acid groups in the EPS of B. diminuta spectrum is consistent with the presence of acyl groups. The IR spectrum of the polymer proved the presence of carboxyl group, which may serve as binding sites for divalent cations. The carboxyl group may also work as functional moieties to generate new or modified polymer variants using different approaches like novel [2].

The flocculating and antiproliferative activities against myeloid cancer cells were tested. These results indicate that EPS produced by *E. aurantiacum* in YPMG medium has high flocculating activity, which envisages its potential use for colloid and cell aggregation in several applications, such as water treatment, food and mining industries. Inorganic and synthetic organic flocculating agents (e.g. polyethylene) are inexpensive products, but have a low biodegradability and are not shear resistant [29]. On the other hand, some of them are dangerous for human health, namely polyacrilamides, whose monomers are neurotoxic. To overcome these environmental and public health problems, naturally occuring flocculants, including several polysaccharides have been suggested as safe alternatives [19, 30, 31, 32]. The EPS produced by *E. aurantiacum* and *B. diminuta* may be included amongst these bioflocculants. The crude EPS produced by *E. aurantiacum* in YPM medium showed a 35% antiproliferative activity against myeloid cancer and the EPS produced by *B. diminuta* demonstrated a 20% antiproliferative activity. These results are similar to those obtained by Kothari *et al.* [33]. The oligasaccharide produced by *Leuconostoc mesenteroides* would have an antiproliferative activity of 34% at 600 µg / ml against the colon cancer cells

after 36 h of incubation. Li *et al.* [34] also proved the antiproliferative activity of the crude and fractionated EPS produced by *Lactobacillus helveticus* MB2-1. After 24 h of treatment, the results showed an antiproliferative activity of 31.84% at 600 µg / ml. It was reported that the anticancer activity of polysaccharides could be influenced by monosaccharide composition, molecular weight, form, degree of branching, and solubility [34]. In fact, anticancer properties of polysaccharides have been described in the case of complexes of β-glucan-protein, α-manno-β-glucans, complexes of α-glucan-protein and complexes of heteroglycan-protein [34]. In the present study, chemical composition revealed that EPS produced by *E. aurantiacum* in YPM contained a homopolymer (glucose) with (1,3) -β- glucan linkages. Taken together, our results implied that the novel YPM EPS of *E. aurantiacum* could be developed as potential natural product for myeloid cancer prevention. However, the anticancer mechanisms and chemical characterization of this polysaccharide remain to be investigated in the future.

CONCLUSION

Exiguobacterium aurantiacum and *Brevundimonas diminuta*, isolated from Nadir's Marchica lagoon from soil and water samples produced exopolysaccharide in YPM (Yeast-Peptone-Mineral salts) and YPMG (Yeast-Peptone-Glucose- Mineral salts) media. The EPS produced by *Exiguobacterium aurantiacum* in YPMG medium is composed of 66% carbohydrates, 0.123% protein and 3% sulfates whereas the EPS produced by *Brevundimonas diminuta* contains 60% carbohydrates, 0.09% protein and 1% sulfates. In YPM medium, the amounts of carbohydrates are low compared to the YPMG medium (15% in *Exiguobacterium aurantiacum* and 20% in *Brevundimonas diminuta*). The absence of proteins is observed in *Exiguobacterium aurantiacum* unlike *Brevundimonas diminuta* which contains 6% protein and traces of sulfates. Sulfates are also present in the EPS produced by *Exiguobacterium aurantiacum* (1.5%). The crude EPS produced by both bacteria showed an antiproliferative activity against myeloid cancer and a flocculating activity. The flocculating activity is better than that observed with the current industrial flocculants, suggesting the use of these EPS as a new biodegradable bioflocculant in water treatment. Our results implied also that the novel YPM EPS of *Exiguobacterium aurantiacum* could be developed as potential natural product for myeloid cancer prevention. However, the anticancer mechanisms and chemical characterization of this polysaccharide remain to be investigated in the future.

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