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ARTICLE

Chemical structure and antioxidant activity of a new exopolysaccharide produced from *Micrococcus luteus*

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Abstract An exopolysaccharide (**EPS**) reaching a maximum of 13 g/L was isolated from *Micrococcus luteus* by ethanol precipitation. The crude **EPS** was purified by chromatography on DEAE-cellulose and Sephacryl S-200, affording a polysaccharide active fraction (**AEP**) with a molecular weight of ~137 kDa. **AEP** was investigated by a combination of chemical and chromatographic methods including FTIR, HPLC, periodate oxidation, methylation and GC–MS. Data obtained indicated that **AEP** was composed of mannose, arabinose, glucose and glucuronic acid in a molar ratio of 3.6:2.7:2.1:1.0, respectively. The main backbone consists of mannose units linked with (1→6)-glycosidic bonds and arabinose units linked with (1→5)-glycosidic bonds. There is a side chain consisting of mannose units linked with (1→6)-glycosidic bonds at C3, when all glucose and most of glucuronic acid are found in the side chain. The *in vitro* antioxidant assay showed that **AEP** possesses DPPH radical-scavenging activity, with an EC_{50} value of 180 μg/mL.

1. Introduction

Microbial exopolysaccharides (EPS) are soluble or insoluble polymers secreted by microorganisms [1]. The diversity in chemical composition of microbial polysaccharides results in a variety of properties that cannot be found in plant polysaccharides [2]. EPS are widely used in the food industry as viscosifying, stabilizing, gelling, or emulsifying agents [3]. New areas for the functional application of microbial polysaccharides include their use as bioflocculants, bioabsorbents and drug delivery agents [4]. In the recent years, there has been increasing interest in their biological activities including antitumor, antiviral, antioxidant and anti-inflammatory activities [5]. Oxidation is essential to many microorganisms for the production of energy to fuel biological

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processes. However, uncontrolled production of oxygen-derived free radicals is involved in the onset of many diseases [6]. Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion radical ($O_2^{\cdot}$), and hydroxyl radical ($OH^{\cdot}$) are inevitably generated during normal and/or aberrant consumption of molecular oxygen. Free radical-mediated modification of DNA, proteins, lipids and small cellular molecules is associated with a number of pathological processes, including atherosclerosis, cancer and rheumatoid arthritis [7]. Therefore, antioxidants may play an essential role in protecting our bodies from various oxidative damages linked to cancer, diabetes, cardiovascular disease and neurodegenerative disease, including Parkinson's and Alzheimer's disease [8]. The synthetic antioxidants most commonly used in industrial processing are suspected to have cytotoxicity [9]. An increasing pile of evidences highlights that some polysaccharides isolated from fungus have antioxidant activities [10]. Among various natural substances, polysaccharides from some microorganisms may harbor antioxidant activity. Traditionally, these important polysaccharides have been obtained from plant or algae sources. The novel active extracellular polysaccharides from microorganisms hold a great potential application in biology and pharmacology [11,1]. The bioactivities of polysaccharides can be affected by many factors including chemical components, molecular mass, structure, conformation, even the extraction and isolation methods [12]. In this study, the isolation, purification, the structural characteristics of an exopolysaccharide from *Micrococcus luteus* and its *in vitro* antioxidant character were investigated.

2. Materials and methods

2.1. Screening and identification

Soil samples (10 g) collected from Zagazig (Egypt) were suspended in 90 mL sterile water. Serial dilutions and the spread-plate method were used for obtaining different microorganisms. The screening medium contained, 30 g sucrose, 1.0 g beef extract, 0.5 g $(NH_4)_2SO_4$, 2.5 g $K_2HPO_4{\cdot}3H_2O$, 2.5 g KH_2PO_4,1.0 g NaCl, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.001 g $FeSO_4{\cdot}7H_2O$ and 20 g agar in 1000 mL of water at pH 7.0 [13]. The first screening was conducted by the selection of a smooth, humid, and mucoid colony on solid plates. The isolates were cultured in a liquid screening medium at 30 °C with shaking at 180 rpm for 48 h. After centrifugation at 5000 rpm for 10 min, the supernatant was mixed with three volumes of chilled ethanol. The precipitate was collected by centrifugation at 5000 rpm for 10 min and the pellets were dried at 50 °C under vacuum. EPS production was determined by quantifying the carbohydrate content of the pellets as D-glucose equivalents using the phenol–sulfuric acid method [14]. Strain BA-1, which produces high amounts of EPS, was identified based on morphological and physiological characteristics determined using Biolog GP2 MicroPlate™, as well as a characteristic reaction pattern called a ''metabolic fingerprint''. The metabolic fingerprint was identified using the MicroLog™ database software [15]. The isolate has been placed in the culture collection of the Microbial Biotechnology Department, National Research Center, Cairo (Egypt).

2.2. Production, isolation and purification of polysaccharide

M. luteus was grown in a liquid medium containing 30 g sucrose, 1.0 g beef extract, 0.5 g $(NH_4)_2SO_4$, 2.5 g $K_2HPO_4{\cdot}3H_2O$, 2.5 g KH_2PO_4,1.0 g NaCl, 0.2 g $MgSO_4{\cdot}7H_2O$, and 0.001 g $FeSO_4.7H_2O$ in 1000 mL of water at pH 7.0 [13]. This medium was distributed in 250 mL Erlenmeyer flasks containing 50 mL and sterilized at 121 °C for 20 min. The flasks were inoculated aseptically by a standardized volume of *M. luteus* suspension to give an optical density of 0.2 at 600 nm. Incubation was carried out at 30 °C for three days on a 150 rpm rotary shaker. The culture broth was diluted with water and centrifuged at 5000 rpm for 30 min to remove bacterial cells. Trichloroacetic acid (TCA) 5% was added to the culture broth, left overnight at 4 °C and centrifuged at 5000 rpm. The pH of clear solution was adjusted to 7.0 with 0.1 M NaOH and dialyzed three times (1000 mL × 3). The supernatant was concentrated under reduced pressure, and precipitated with three volumes of absolute ethanol at 4 °C overnight. The crude polysaccharide was recovered by centrifugation, and dried at 45 °C under reduced pressure after washing successively with ethanol and ether. The polysaccharide was dissolved in 100 mL distilled water, vortexed and centrifuged at 5000 rpm for 20 min to remove insoluble materials. Higher molecular weight polysaccharides were precipitated with 1, 2, 3 and 4-volumes of absolute ethanol, and the supernatant was recovered by centrifugation. The crude polysaccharide fraction (**EPS**) was re-dissolved in deionized water and forced through a filter (0.45 mm), then applied to a column (2.5 × 30 cm) of DEAE-cellulose. After loading with sample, the column was eluted with gradient NaCl solution (0–1.0 M), and the procedure was monitored by the phenol-sulfuric acid method mentioned above. The collected fractions were further purified on a Sephacryl S-200 column (2.6 cm × 70 cm) eluted with 0.1 M NaCl at a flow rate of 0.5 mL/min. Total sugar content of each tube was measured at 490 nm by Dubois's method, and protein absorption at 280 nm recorded for each fraction. One polysaccharide active fraction (**AEP**) was collected, dialyzed and lyophilized. **AEP** was used for activity assessment and structural analysis [16].

2.3. Molecular weight determination

The molecular weight of the **AEP** was determined by gel permeation chromatography (GPC) on a Sephacryl S-200 column (2.6 cm × 70 cm). Dextrans 40, 500 and 2000 kDa (Fluka Chemical Co., Buchs, Switzerland) and glucose, then the elution volumes were plotted against the logarithm of their respective molecular weights. The elution volume of the purified polysaccharide (**AEP**) was plotted in the same graph, and the molecular weight was determined [17].

2.4. Analysis of monosaccharide composition

The polysaccharide **AEP** was hydrolyzed with 90% formic acid at 100 °C in a sealed tube for 5 h. Excess acid was removed by flash evaporation on a water bath at a temperature of 40 °C and co-distilled with water (1 mL × 3) [18]. The mono-

saccharide contents were quantified by HPLC on a Shimadzu Shim-Pack SCR-101 N column (7.9 mm × 30 cm), using deionized water as the mobile phase (flow rate 0.5 mL/min), as described by El-Sayed et al., [19]. Uronic acid content was determined according to *m*-hydroxydiphenyl method using glucuronic acid as standard [20].

2.5. Infrared spectroscopy

The Fourier-transform infrared (FTIR) spectrum of the **AEP** fraction was measured on a Bucker scientific 500-IR Spectrophotometer. The polysaccharide was mixed with KBr powder, ground and pressed into a 1 mm pellets for FTIR measurements in the range of 400–4000 cm^{-1} [21].

2.6. Periodate oxidation and Smith degradation

The sample (30 mg) dissolved in 12.5 mL of distilled water was mixed with 12.5 mL of 30 mmol/L $NaIO_4$. The solution was kept in the dark at room temperature; 0.1 mL aliquots were withdrawn at 24 h intervals, diluted to 25 mL with distilled water and read in a spectrophotometer at 223 nm [22]. Periodate consumption was calculated on the basis of the change of the absorbance at 223 nm. The solution of periodate product (2 mL) was used to assess the amount of formic acid by titration with 0.005 mol/L NaOH. Ethylene glycol (2 mL) was added, then the experiment of periodate oxidation was over. The solution of periodate product was extensively dialyzed against tap water and distilled water for 48 h, respectively. The content inside was concentrated and reduced with $NaBH_4$ (100 mg), and the mixture was left for 24 h at room temperature, neutralized to pH 6.0 with acetic acid (50 mL/100 mL), dialyzed as described above, and re-concentrated to 10 mL. One-third of the solution mentioned above was freeze-dried, fully hydrolyzed and analysed by HPLC [19]. Two-thirds of the solution was added to the same volume of 1 mol/L sulfuric acid, kept for 40 h at 25 °C, neutralized to pH 6.0 with $BaSO_4$, and filtered for analysis by smith degradation. The filtrate was dialyzed (molecular weight cut off of 3 kDa), and the content out of the dialysis bag was analyzed by HPLC; whereas the contents inside the dialysis bag were mixed with four volumes of absolute ethanol and centrifuged. The supernatant and precipitate were also analyzed by HPLC [23].

2.7. Partial hydrolysis

The AEP (100 mg) was partially hydrolyzed with 0.05 M TFA (3 mL) for 16 h, at 80 °C. The hydrolysate was mixed with four volumes of ethanol absolute and kept at 4 °C overnight. The precipitate was removed by centrifugation at 5000 rpm for 20 min (**AEP-1**), and the supernatant was dialyzed against distilled water for 48 h in a dialysis bag (molecular weight 3 kDa cut off). Each of solutions in and out of the dialysis bag was collected for further analysis. Ethanol was added to the solution in the bag after dialysis, and the precipitate and supernatant designated as **AEP-2** and **AEP-3**, respectively were recovered after centrifugation. The fraction out of dialysis bag (**AEP-4**) and all other fractions were analyzed by HPLC [16].

2.8. Methylation analysis

Prior to methylation, **AEP** was reduced to the corresponding neutral sugars [24]. Original **AEP** and reduced-**AEP** were methylated separately using the method of Ciucanu and Kerek [25]. The methylated products were extracted with $CHCl_3$, washed with distilled water (three times) and evaporated to dryness. The product was then hydrolyzed with TFA (2 mol/L) at 100 °C for 6 h. The methylated products were converted into their corresponding alditol by reduction with $NaBH_4$ and acetylated [26]. The resulting product was subjected to linkage analysis by GLC-MS on DB-5 capillary column (30 m × 0.25 mm) with a film thickness of 0.25 μm [27]. The GLC temperature was isothermal at 140 °C for 2 min, followed by a 4 °C/min gradient up to 280 °C. The components were identified by a combination of the main fragments in MS and relative retention times in GLC, and the molar ratios for each sugar were calibrated using the peak areas and the response factors.

2.9. Radical scavenging activity (RSA) of AEP fraction toward DPPH radical

The free radical scavenging activity of **AEP** was measured against 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radicals using the method of Asker, et al., [28]. Five mL of DPPH ethanol solution (freshly prepared at a concentration of 0.1 mmol/L) was added to 1 mL of **AEP** solution of different concentrations (40–240 μg) in water. After 30 min, absorbance was measured at 517 nm using Spectrophotometer UV-visible 2401PC (Shimadzu, Japan). Lower absorbance of the reaction mixture indicated higher free radical scavenging activity, which was analyzed from the graph (inhibition percentage plotted against concentration of compound). Ascorbic acid was used as positive control. The experiment was carried out in triplicate and averaged. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\text{Scavenging ability } (\%) = \left[\left(\Delta A_{517 \text{ of control}} - \Delta A_{517 \text{ of sample}}\right) / \Delta A_{517 \text{ of control}}\right] \times 100.$$

The EC_{50} value is the effective concentration (μg) of **AEP** at which the DPPH radicals were scavenged by 50%.

2.10. Statistical analysis

The obtained data were subjected to a One-way ANOVA and the differences between means were found to be at the 5% probability level using Duncan's new multiple range tests. The software SPSS, version 10 (SPSS, Richmond, USA) was used as described by Dytham [29].

3. Results and discussion

3.1. Screening and identification of the strain

Twenty-two bacterial strains were isolated from the soil samples. Nine strains were obtained based on colony morphology, and the carbohydrate content of their supernatant broth. Most of the isolates produced exopolysaccharides, but strain **BA-1** was selected for further study because of its highest

exopolysaccharide production (8.14 g/L). Colonies of strain **BA-1** showed mucous appearance on solid medium. The cells were gram-positive, coccus-shaped, and non-spore forming. The strain is therefore identified as *M. luteus* according to the metabolic fingerprint identified using the MicroLog™ database software.

3.2. Isolation, purification and composition of exopolysaccharide

Polysaccharide production reached a maximum of 13.6 g/L crude polysaccharide (**EPS**) after 5 days. The main active fraction (**AEP**) was purified with DEAE-cellulose and Sephacryl S-200 gel-filtration columns. The main fraction was collected, lyophilized for further structure characterization and antioxidant activity. The average molecular weight of **AEP** was determined as 137 kDa by the GPC technique. The GPC profile (Fig. 1) also demonstrated that **AEP** had a single and symmetrically sharp peak revealing that **AEP** was a homogeneous polysaccharide. The purified **AEP**, a white powder, was used for subsequent analysis. It had a negative response to the Bradford test and no absorption at 280 nm or 260 nm in the UV spectrum, indicating the absence of protein and nucleic acid. As determined by *m*-hydroxydiphenyl method, the polysaccharide contains uronic acid. Analysis by HPLC indicated that AEP was composed of mannose, arabinose, glucose and glucuronic acid with a molar ratio of 3.6:2.7:2.1:1.0, respectively.

3.3. Structural characterization of AEP

The infrared spectrum of **AEP** (Fig. 2) revealed a typical major broad stretching peak around 3423 cm^{-1} for the hydroxyl group, and a weak band at 2925 cm^{-1} showing the C–H stretching vibration. The absorbance at 1739.5 cm^{-1} indicated the presence of uronic acid. The broad band at 1618 cm^{-1} was due to the bound water. The band at 841 cm^{-1} was ascribed to α-pyranoses in the polysaccharide [30,31].

By partial acid hydrolysis **AEP** was fractionated giving four fractions termed **AEP-1**, **AEP-2**, **AEP-3** and **AEP-4**. The monosaccharide components of these fractions are shown in Table 1. It is evident that **AEP-1** was composed of mannose and arabinose at a molar ratio of 1.0:1.2, respectively, **AEP-2** was composed of mannose and arabinose at a molar ratio of 1.0:0.53, respectively and may be the backbone structure of **AEP**. On the other hand, **AEP-3** was composed of mannose, glucose and glucuronic acid at a molar ratio of 1.0:1.52:1.21, respectively. **AEP-4** was only composed of glucose and glucuronic acid at a molar ratio of 0.32:1.0, respectively and may be the branched structure of **AEP**. The polysaccharide of **AEP** showed abundant periodate uptake, while it was oxidized.

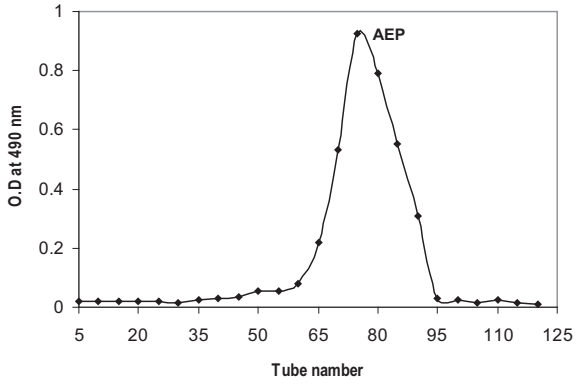

Figure 1 Gel permeation chromatography of polysaccharide fraction (AEP) on Sephacryl S-200.

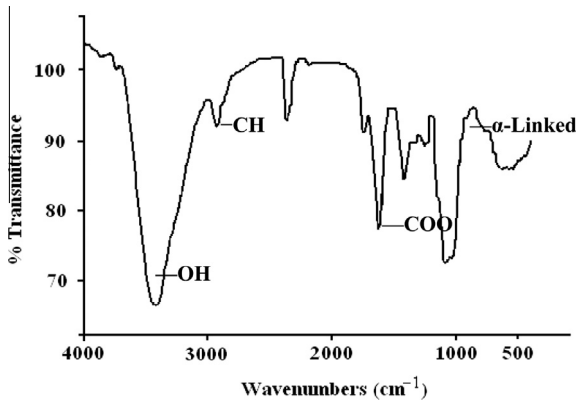

Figure 2 Infrared spectrum of polysaccharide fraction (AEP) from *Micrococcus luteus*.

Table 1 HPLC analysis results of fractions from partial acid hydrolysis.

Fractions	Molar ratios			
	Arabinose	Mannose	Glucose	Glucuronic acid
AEP-1	1.20	1.00	0.00	0.10
AEP-2	0.53	1.00	0.00	0.00
AEP-3	0.00	1.00	1.52	1.21
AEP-4	0.00	0.00	0.32	1.00

The consumption of periodate (0.97 mol) was four-times more than the amount of formic acid (0.23 mol), that was produced after periodate treatment, indicating the presence of small amounts of monosaccharides, which are (1→4)-linked or (1→6)-linked. The fact that the amount of periodate consumption was more than the amount of formic acid demonstrated other linkages oxidized by periodate, such as (1→4) or (1→2). The periodate-oxidized products were fully hydrolyzed and analyzed by HPLC (Table 2). The presence of mannose and glucuronic acid revealed that some residues of mannose and glucuronic acid were (1→3)-linked, (1→2, 3)-linked, (1→2, 4)-linked, (1→3, 4)-linked, (1→3, 6)-linked or (1→2, 3, 4)-linked that cannot be oxidized, respectively. No arabinose and glucose were observed and large amounts of glycerol and erythritol were obtained, demonstrating that arabinose and glucose were with linkages which can be oxidized by periodate. HPLC analysis for Smith-degradation is shown in Table 2, and indicated that there was no precipitation in the dialysis bag and this demonstrated that the backbone of **AEP** should be oxidized completely by HIO_4. Hence, it may be concluded that the linkages of backbone are (1→), (1→2), (1→6), (1→2, 6), (1→4) and (1→4, 6) that may be oxidized producing glycerol and erythritol detected out of the dialysis bag.

Table 2 HPLC results of Smith-degradation of polysaccharide fraction AEP from *M. luteus*.

Fractions	Molar ratios			
	Glycerol	Erythritol	Mannose	Glucuronic acid
Full acid hydrolysis	4.0	2.6	1.0	0.7
Smith degradation (out of bag)	3.7	2.9	1.0	0.6

Table 3 GC–MS results of methylation analysis of AEP and reduced-AEP.

Methylation product	Type of linkage	Molar ratios	
		AEP	Reduced-AEP
2,3,4,6-Tetra-*O*-methyl-glucose	(1→) Glc	1.3	1.3
2,3-Di-*O*-methyl-arabinose	(1→5) Ara	2.4	2.5
2,3,4,6-Tetra-*O*-methyl-mannose	(1→) Man	0.2	0.2
2,4,6-Tri-*O*-methyl-glucose	(1→3) Glc	1.5	2.9
2,3,4-Tri-*O*-methyl-mannose	(1→6 Man	1.6	1.5
2,3,6-Tri-*O*-methyl-mannose	(1→4) Man	1.0	1.0
2,4-Di-*O*-methyl-mannose	(1→3,6) Man	0.9	0.9

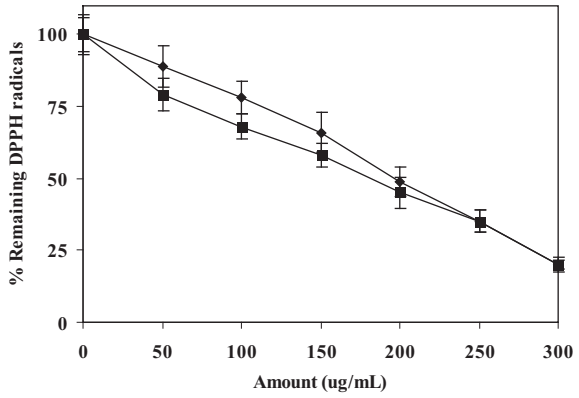

Figure 3 Scavenging effect of polysaccharide fraction AEP (—■—), vitamin C (—♦—) during DPPH test by changes in absorbance at 517 nm. Each value expressed as mean ± standard deviation (n = 3).

The fact that no erytheric acid was detected suggested that there were (1→3) and (1→2, 3) linkages in glucuronic acid [32,33]. The fully methylated **AEP** and reduced-**AEP** were hydrolyzed with acid, converted into alditol acetates, and analyzed by GC–MS. As summarized in Table 3, **AEP** and reduced-**AEP** showed the presence of seven derivatives, namely 2,3,4,6-tetra-*O*-methyl-glucose; 2,3-di-*O*-methyl-arabinose; 2,3,4,6-tetra-*O*-methyl-mannose; 2,4,6-tri-*O*-methyl-glucose; 2,3,4-tri-*O*-methyl-mannose; 2,3,6-tri-*O*-methyl-manose; and 2,4-di-*O*-methyl-mannose in molar ratios of 1.3:2.4:0.2:1.5:1.6:1.0:0.9 and 1.3:2.5:0.2:2.9:1.5:1.0:1.0, respectively. According to the difference in molar ratio of 2,4,6-tetra-*O*-methyl-glucose, and 2, 4-dii-*O*-methyl-glucose in **AEP** and reduced-**AEP** (Table 3), it could be deduced that the linkage of glucuronic acid is (1→3)-linkage [18,21].

The results of GC–MS analysis, which were consistent with the results from partial acid hydrolysis, periodate oxidation and Smith degradation, indicated that 2,3-di-*O*-methyl-arabinose (1→5)-linked arabinose and 2,3,4-tri-*O*-methyl-mannose (1→6)-linked mannose were major components of the backbone structure with 3 branches attached to O-3 of (1→3)-linked glucose; all glucose and glucuronic acid were distributed in branches; and residues of branches terminated with either glucose and were composed of (1→3)-linked glucuronic acid (1→4)-linked mannose, and (1→3)-linked glucose [33]. In brief, the monomer of **AEP** was evaluated as below according to GC–MS analysis, partial acid hydrolysis; periodate oxidation and Smith-degradation.

3.4. Radical scavenging activity (RSA) of AEP fraction

The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of natural compounds [34]. In the DPPH test, the antioxidants were able to reduce the stable DPPH radical to the yellow-colored diphenyl picryl hydrazine. The effect of antioxidants on DPPH radical scavenging was conceived to be due to their hydrogen-donating ability. The DPPH radical scavenging activities of **AEP** and vitamin C, as a positive control, were determined [35], and the results are shown in Fig. 3. As illustrated, **AEP** exhibited the scavenging activity toward DPPH radicals in a concentration-dependent manner, with an EC_{50} value of 180 μg/mL. Under the same conditions, ascorbic acid, a free radical scavenger, showed a slightly weaker effect on the hydroxyl radicals, with an EC_{50} value of 200 μg/mL. Both of them presented approximately identical change trend of antioxidant activity. These results indicated that the **AEP** had a noticeable effect on scavenging free radical, especially at high concentration. It is well known that reactive oxygen species (ROS), such as hydroxyl radicals, super oxide anion and hydrogen peroxide, are related to the pathogenesis of various diseases [36]. Hydroxyl radical is the most reactive among the oxygen radicals and induces severe damage to the adjacent biomolecules [37,38]. However, the scavenging activity of **AEP** against the DPPH-radical was less than that of ascorbic acid at the same concentration. Further elucidation of possible mechanisms and evaluation of the bioactivities of the polysaccharide will be important for their application in the food and medicinal fields [39,40].

4. Conclusion

The structure elucidation of a water-soluble exopolysaccharide **AEP** from *M. luteus*, by means of chemical and spectroscopic analyses was studied. The structure study demonstrated that **AEP** had a backbone composed of (1→6) linked mannose and (1→5) linked arabinose with branches attached to O-3

terminated with (1→3) linked glucose, (1→3) glucuronic acid. The results of DPPH radical scavenging activity showed that **AEP** possesses radical scavenging effects in a concentration-dependent manner with an IC_{50} value of 180 μg/mL. Further, studies are necessary to focus on the detailed antioxidant mechanism of **AEP**.

References

[1] Y. Kimura, M. Sumiyoshi, T. Suzuki, M. Sakanaka, Antiviral Res. 26 (2006) 4131–4141.
[2] M. Kambourova, R. Mandeva, D. Dimova, A. Poli, B. Nicolaus, G. Tommonaro, Carbohydr. Polym. 77 (2009) 338–343.
[3] A. Laws, Y. Gu, V. Marshall, Biotechnol. Adv. 19 (2001) 597–625.
[4] Y. Wang, Z. Ahmed, F. Wu, C. Li, S. Song, Int. J. Biol. Macromol. 43 (2003) 283–288.
[5] A. Arena, T.L. Maugeri, B. Pavone, D. Iannello, C. Gugliandolo, G. Bisignano, Int. Immunopharmacol. 6 (2006) 8–13.
[6] J.L. Mau, H.C. Lin, S.F. Song, Food Res. Int. 5 (2002) 519–526.
[7] B. Hallowell, J. Gutteridge, Biochem. J. 219 (1984) 1–14.
[8] M.T. Lin, F.M. Beal, Clin. Neurosci. Res. 2 (2003) 305–315.
[9] H.Q. Zhang, T. Zhao, R. Chen, R. Zhang, X. Niu, Z. Li, Int. J. Biol. Macromol. 37 (2005) 195–199.
[10] Y.H. Jiang, X.L. Jiang, P. Wang, X.K. Hu, J. Food Biochem. 29 (2005) 323–335.
[11] X.J. Du, J.S. Zhang, Y. Yang, L.B. Ye, Q.J. Tang, W. Jia, et al, Carbohydr. Res. 344 (2009) 672–678.
[12] X. Rihua, S. Qian, D. Xuelong, G. Wengeng, L. Pinglan, Eur. Food Res. Technol. 232 (2011) 231–240.
[13] M.A. Ronald, in: C. Parks Lawrence (Ed.), Handbook of Microbiological Media Second Edition by Ronald, CRC Press, Boca Raton, New York, London, Tokyo, 1997.
[14] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Anal. Chem. 28 (1956) 350–356.
[15] M.P. De Suza, A. Amint, M.A. Dojka, I.L. Pickering, S.C. Dawson, N.R. Pace, N. Terry, Appl. Environ. Microbiol. 67 (2001) 3785–3794.
[16] M.M.S. Asker, B.T. Shawky, Food Chem. 123 (2010) 315–320.
[17] D. Luo, Carbohydr. Polym. 74 (2008) 544–549.
[18] S.R. Sudhamani, R.N. Tharanathan, M.S. Prasad, Carbohydr. Polym. 56 (2004) 423–427.
[19] O.H. El-Sayed, S.A. Ismail, Y.M. Ahmed, M. Abd, Pharm. J. 4 (2005) 439–452.
[20] T.M.C.C. Filisetti-Cozzi, N.C. Corpita, Anal. Biochem. 197 (1991) 157–162.
[21] B. Ray, Carbohydr. Polym. 66 (2006) 408–416.
[22] A. Linker, L.R. Evans, G. Impollomeni, Carbohydr. Res. 335 (2001) 45–54.
[23] W.J. Zhang, Technology of Biochemical Research on Compound Polysaccharide, Zhejiang University Press, Hang Zhou, 1987.
[24] W.S. York, A. Darvill, M.O. Neill, T. Stevenson, P. Albersheim, Methods Enzymol. 118 (1985) 3–40.
[25] I. Ciucanu, F. Kerek, Carbohydr. Res. 131 (1984) 209–217.
[26] L.S. Guilherme, I. Marcello, A.J.G. Philip, An. Acad. Bras. Cienc. 77 (2005) 223–234.
[27] Y. He, C. Liu, Y. Chen, A. Ji, Z. Shen, T. Xi, Q. Yao, J. Biosci. Bioeng. 104 (2007) 111–116.
[28] M.M.S. Asker, Y.M. Ahmed, M.F. Ramadan, Carbohydr. Polym. 77 (2009) 563–567.
[29] C. Dytham, Choosing and Using Statistics: A Biologist's guide, Blackwell Science Ltd., London UK, 1999.
[30] F.S. Park, Application of I.R. Spectroscopy in Biochemistry, Biology, & Medicine, Plenum, New York, 1971.
[31] J. Bao, X. Fang, J. Fang, Carbohydr. Res. 336 (2001) 127–140.
[32] M. Abd-El-Akher, J.K. Hamilton, R. Montgomeny, F. Smith, J. Am. Chem. Soc. 74 (1952) 4970.
[33] Z.J. Wang, D.H. Luo, Z.Y. Liang, Carbohydr. Polym. 57 (2004) 241–247.
[34] S.E. Lee, H.J. Hwang, J.S. Ha, H.S. Jeong, J.H. Kim, Life Sci. 73 (2003) 167–179.
[35] K. Shimada, K. Fujikawa, K. Yahara, T. Nakamura, J. Agric. Food Chem. 40 (1992) 945–948.
[36] J. Abe, B.C. Berk, Trends Cardiovasc. Med. 8 (1998) 59–64.
[37] B. Chance, H. Sies, A. Boveris, Physiol. Rev. 59 (1979) 527–605.
[38] K. Jahanbina, A.R. Goharib, S. Moinic, Z. Emam-Djomehc, P. Masid, Int. J. Biol. Macromol. 49 (2011) 567–572.
[39] W. Li, J. Ji, X. Chen, M. Jiang, X. Rui, M. Dong, Carbohydr. Polym. 102 (2014) 351–359.
[40] J.-H. Hong, H.K. Jung, Biol. Chem. 57 (2014) 105–112.