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Production and Characterization of Exopolysaccharide from Novel *Bacillus* sp. M3 and Evaluation on Development Sub-Chronic Aluminum Toxicity Induced Alzheimer's Disease in Male Rats

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Abstract: The number of patients suffering from Alzheimer's Disease (AD) all over the world is rising continually and becomes one of the biggest challenges for most societies throughout the world. The potential of peripheral biochemical markers as complementary tools in the neuron-phsychiatric evaluation of these patients has claimed further attention. The aims of our study were to isolate the bacteria that able to produce exo-polysaccharides and to characterize then exopolysaccharide producing strains by 16S rDNA sequencing method. The exopolysaccharide (MEPS) produced from a newly isolated *Bacillus* sp. M3 was obtained by ethanol precipitation (6.5 g L^{-1} growth medium). The molecular masse of the MEPS was 1.45×10^4 g/mol wherein FT-IR, UV-Vis spectral analyses revealed prevalence of characteristic primary belonged to α-type exopolysaccharide with a pyran ring. Further, HPLC analysis revealed its two monosaccharide constituents galacturonic acid and glucuronic acid at molar ratio of 1:1. The second stage was the evaluation of anti-intoxicated effect of MEPS against aluminum chloride induced Alzheimer in rat relevant to its effect on oxidative stress, antioxidant brain status, cholinergic markers and serum level of S100B protein together with polysaccharide sub-chronic toxicity at 1/10 LD_{50} in both AD and control experimental animal groups to find their diagnostic value in this disease. MEPS ameliorated antioxidant status and reduced all oxidative stress parameters in brain tissue with decreasing S100B as compared to aluminum toxicant group with significant acetyl cholinesterase inhibition which increase acetylcholine concentration in brain tissue. No toxicity was observed for MEPS in sub-chronic toxicity study for 90 days in all rat organs or liver and kidney function.

Keywords: *Bacillus* sp. M3, Exopolysaccharides, Oxidative Stress, Alzhei-mer's Disease

Introduction

Dysregulated activation of inflammation and oxidative stress has been recognized as one principal causes of inflammatory diseases such as rheumatoid arthritis, diabetes, Alzheimer's disease and even cancers (Balkwill and Mantovani, 2012; Schacter and Weitzman, 2002). Exopolysaccharides (EPSs) contribute to various physiological activations in human beings as antitumor, antiviral, anti-inflammatory agents, anticardiovacular disease and anti-neurodegenerative disease specific Alzheimer's disease (Calazans *et al*., 1997; Mahmoud *et al*., 2014).

Recently, major attention has been focused on polysaccharides from marine due their structural and functional diversity beside it is natural source compounds (Gutierrez *et al*., 2012). The novel active EPSs from marine bacteria hold a great potential

application in biology and pharmacology such as antioxidant activity (Luo and Fang, 2008), immune stimulating effects (Xu *et al*., 2009), antitumor effects (Tong *et al*., 2009) and antiviral activity (Wang *et al*., 2007). Owing to the various functions of EPSs in marine ecosystem such as adhesion of bacteria, stabilization of biofilms and maintenance of symbiotic association with different species, investigation of EPSs producing marine bacteria was reviewed (Christensen *et al*., 1985; Decho, 1990; Holmstrom and Kjelleberg, 1999). The EPSs have many advantages, including non-toxic and safe, unique physical and chemical properties, simply separated from bacteria and can be produced at large scale (Czaczyk and Myszka, 2007). Alzheimer's Disease (AD) is a devastating neuron-degenerative disorder manifested by deterioration in memory and cognition, impairment in performing activities of daily living and many behavioral and neuro-psychiatric illnesses (Cummings, 2004). AD is the most common form of dementia in the old age. The percentage of persons with Alzheimer disease increases by a factor of two with every five years of age, so 1% of 60 year old and 30% of 85 year old have the Disease (Upadhyaya *et al*., 2010). S100B is a calcium binding protein that in the nervous system is mainly concentrated in glial cells. It has both trophic and potentially toxic effects on neurons and neuritis, suggesting that S100B over expression plays an important role in the genesis of neuritic changes in amyloid-β plaques, in the progression of diffuse non-fibrillar amyloid deposits to neuritic forms and consequently in the progression of disease itself (Mark and Griffin, 2001). It is hypothesized that high concentrations of S100B act in the pathogenesis of neurodegenerative processes, possibly though oxidative stress mechanisms (Emanuele *et al*., 2011). Oxidative stress has been shown to be a prominent and early feature of vulnerable neurons in AD. Exposure to oxidative stress induces the accumulation of intracellular Reactive Oxygen Species (ROS), which in turn causes cell damage in the form of protein, lipid and DNA oxidations. Elevated ROS levels are associated with increased deposition of amyloid β and formation of senile plaques, a hallmark of AD brain. If enhanced ROS exceeds the basal level of cellular protective mechanisms, oxidative damage and cell death will result. Hence, substances that can reduce oxidative stress are thought as possible drug candidates for treatment or preventive therapy of neurodegenerative diseases such as AD (Nelson *et al*., 2009). Our study aims to isolate, partially characterize the exopolysa-ccharide from a newly isolated *Bacillus* sp. M3 (MEPS) and to evaluate anti-Alzheimer effect of MEPS in intoxicated animals with aluminum chloride with investigation of its sub-chronic toxicity in intoxicant and normal animals.

Materials and Methods

Isolation of Exopolysaccharides Producing Bacterial Strain

Marine sediment samples (5 g) collected from mangrove (Alexandra, Egypt) were suspended in 95 mL sterile water. Serial dilutions of water samples were plated on marine nutrient agar plates. After incubation at 30°C for 72 h, cultural logogriphic bacterial colony was obtained. Purification of single colonies was done by dilution streaking on marine nutrient agar plates. Single colony cultures were maintained on marine nutrient agar. Pure colonies of each facultative logographic isolates (capable of forming mucous and ropy colonies) were then inoculated into 50 mL of screening marine nutrient medium in 250-mL Erlenmeyer flask, incubated at 37°C in a rotary shaker at 150 rpm for 48 h. After centrifugation at 5000 rpm for 20 min, the supernatant was mixed with three volumes of chilled ethanol. The precipitate was collected by centrifugation at 5000 rpm for 20 min and the pellets were dried at 40°C under vacuum. EPS production was determined by quantifying the carbohydrate content of the pellets as glucose equivalents using the phenol-sulfuric acid method (Dubois *et al*., 1956).

Strain Identification

Morphological and Biochemical Studies

Morphological, physiological and biochemical characterization for the high EPS producing bacterium NRC27 were carried out. Characteristics of the isolate were compared with data from (Holt, 1986).

Phylogenic Studies

The identification was confirmed with phylogenic analysis. Genomic DNA from the NRC27 was isolated and quality was evaluated on 1.2% agarose gel, a single band of high Mw DNA has been observed. A Polymerase Chain Reaction was performed using ITS1-5′TCCGTAGGTGAACTTTGCGG3′ and ITS4-5′TCCTCCGCTTATTGATATGC3′ primers (Gardes and Bruns, 1993). A single discrete PCR amplicon band was observed when resolved on agarose gel. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried and then performs 35 amplification cycles at 94°C or 45 sec, 55°C for 60 sec and 72°C for 60 sec. DNA fragments are amplified about 1,400 bp in the case of bacteria. Include a positive control (*E. coli* genomic DNA) and a negative control in the PCR. Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied Bio-systems model 3730XL automated DNA sequencing system (Applied BioSystems, USA). Data were submitted to

GenBank database. The DNA sequence was compared to the GenBank database in the national Center for Biotechnology Information (http: //www.ncbi.nlm.nih. gov) using the BLAST program (Tamura *et al*., 2011).

Production and Isolation of Exopolysaccharide

Inoculums was prepared by transferring one loop full of culture from marine nutrient slant to an 250 mL conical flask containing 50 mL seed medium consisting of (g/L) glucose 20, yeast extract 0.1, $CaCO_3$ 1, NH_4NO_3 0.8, K_2HPO_4 0.6, KH_2PO_4 0.5, $MgSO_4.7H_2O$ 0.05, $MnSO_4.4H_2O$ 0.1 and dissolved in 75% seawater (Kim *et al*., 1998). The seed culture was grown at 37°C on a rotary shaker incubator at 150 rpm for 18 h. After incubation, 3 mL of the seed culture was transferred into a 250 mL Erlenmeyer flask containing 50 mL of fermentation medium consisting of (g/L) sucrose 20, peptone 4, yeast extract 2 and dissolved in 75% seawater, pH 7.0. The fermentation cultures were then incubated at 37°C with shaking at 150 rpm for 3 days. The EPS sample was prepared from strain NRC-27 culture in the fermentation medium. The fermented broth was collected and centrifuged at 5000 rpm at 4°C for 20 min. Trichloroacetic acid (50 g) was added to the 1000 mL supernatant, 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) against flowing tap-water using dialysis tubing (MWCO 2000) for 24 h. The deproteinated solution through precipitation with 1, 2, 3 and 4 volume chilled ethanol, the major fraction precipitate was collected and re-dissolved in distilled water, dialyzed with distilled water and precipitated by chilled absolute ethanol washed by acetone, diethyl ether and dried at 60°C until constant weight and coded MEPS (Asker *et al*., 2009).

Analysis of Monosaccharide Composition

For monosaccharide composition analysis, MEPS (200 mg) was hydrolyzed with 2 mL of 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) (Sudhamani *et al*., 2004). The monosaccharides contents were quantified by HPLC on a Shimadzu Shim-Pack SCR-101N column (7.9×300 mm) using deionized water as the mobile phase (flow rate 0.5 mL/min), as described by El-Sayed *et al.* (2005).

Molecular Weight Determination

The molecular weight of MEPS was determined on an Agilent 1100 HPLC system equipped with a Refractive Index Detector (RID) and FPl gel particle size (5 µm), 3 columns of pore type (100, 104, 105 A°) on series, length 7.5×300 mm (1000,5000000) for DMF solvent Styrogel HR-DMF, 3 µm (7.8×300 mm), Water Company Ireland. One column (5000-600000) for water solvent (polyethylene oxide/glycol standard) PL aquagel-OH 7.5 mm and 30 um pore type 8 um particle size. PL aquagel-OH 7.5 mm, 50 um pore type, 8um particle size, in series Mw from 100-1250000 g moL^{-1}. The sample 0.01 g was dissolved in 2 mL of solvent and then it filtrated by siring filter 0.45 then the sample but in GPC device (Jun *et al*., 2009). The polydispersity index calculated from the Mw/Mn ratio (You *et al*., 2013).

Infrared Spectroscopy

The Fourier-Transform Infrared (FTIR) spectrum of MEPS was measured on a Bucker scientific 500-IR Spectrophotometer. The MEPS was mixed with KBr powder, ground and pressed into a 1 mm pellets for FTIR measurements in the range of 400-4000 cm^{-1} (Ray, 2006).

Biological Activity of MEPS

Acute Toxicity Study

The acute toxicity test for the MEPS was carried out to evaluate any possible toxicity. Male albino mice (n = 8) were obtained from the Animal House of National Research Centre, Egypt. Mice were tested by administering MEPS at different doses in an oral route by increasing or decreasing the dose, according to the response of animal (Bruce, 1985). The dosing patron was 50, 100, 200, 400, 800, 1600, 2000 and 2500 mg kg^{-1} body weight. All groups were observed for any gross effect or mortality during 48 h. Death of half of examined animals was observed at 2 g kg^{-1} body weight. This study was approved by Medical Research Ethics Committee, National Research Centre, Egypt, under registration no. 11015.

Experimental Design

Adult male *spargue dawely* rats (150-180 g) were obtained from the Animal House of National Research Centre, Egypt. Rats were fed on standard diet and maintained under laboratory conditions, temperature controlled at 23±2°C, relative humidity 60±5% and light/dark cycles (12/12 h). Animal were housed in polypropylene cages, each cage was contained eight or seven rats adapted for one week before starting the experiment. Animals were divided into four groups each contained 15 rats and they were treated as follows:

Group I: Rats received 1 mL saline solution for 120 days in an oral route, control group for Alzheimer and sub-chronic toxicity experiments.

Group II: Rats received $ALCl_3$ orally at 17 mg kg^{-1} body weight for 30 days, served as positive intoxicant control group (Krasovskii *et al*., 1979).

Group III: Rats received saline for 30 days and then received MEPS orally at 200 mg kg^{-1} body

weight for 90 days, served as vehicle control group and as sub-chronic toxicity group.

Group IV: Rats received $ALCl_3$ orally at 17 mg kg^{-1} body weight for 30 and then treated with MEPS (200 mg kg^{-1} body weight) for 90 days.

At the end of experiment, rats fasted overnight and were subjected to anesthesia to facilitate collection of blood samples while whole brain of each rat was rapidly dissected and washed with isotonic saline and dried on filter paper. Brain of each rat was weighed and homogenized to give 10% (w/v) homogenate in ice cold medium containing 50 mM Tris-HCl and 300 mM sucrose at pH, 7.4. The homogenate was centrifuged at 4°C. The supernatant was stored at -80°C and were used in biochemical analyses including oxidative stress biomarker (nitric oxide concentration by method of Montgomery and Dymock (1961), hydrogen peroxide concentration, reduced glutathione concentration (Griffith, 1980) and malondialdehyde (MDA) concent-ration (Ohkawa *et al*., 1979), antioxidant biomarker (total antioxidant capacity according t*o* Koracevic *et al.* (2001), Superoxide Dismutase activity (SOD) (Kakkar *et al*., 1984), Catalase Activity (CAT) (Beers and Sizer, 1952), cholinesterase biomarker (acetylcholine conce-ntration and cholinesterase activity, kits were purchased from Quimica Clinica Aplicada S.A.) and S100B in sera was estimated by Enzyme Linked Immunoassay (ELISA), the kit was derived from Dia Sorin, USA according to Gao *et al.* (1997). Brain total protein conc. was measured for calculation of enzyme specific activity (Sedlack and Lindsay, 1968). The assessments were done by ELISA reader (Dynatech laboratories MRW micro plate reader, 2CXB2445).

Biochemical Assessment of Sub-Chronic Toxicity Study

Blood samples were collected from the retro orbital plexus, centrifuged at 3000 rpm for 10 min to separate sera. The liver enzymatic activity was determined according to the colorimetric method described by Hannig *et al.* (2009). Reduced glutathione concentration was measured spectrophotometrically at 405 nm by the method of Plancarte and Hernandez (2004), while protein concentration (g dL^{-1}) was determined according to Okutucu *et al.* (2007). Total lipid concentration (mg dL^{-1}) was estimate by method of Vatassery *et al.* (1981). In addition, kidney function was assessed by measuring creatinine concentration (mg dL^{-1}) according to Demirovic *et al.* (2009), uric acid concentration (mg dL^{-1}) by the method of Carolina *et al.* (2005) also urea concentration (mmol L^{-1}) was estimated according to Yoneyame *et al.* (2001).

Effect on Vital Organs

At the termination of polysaccharide treatment on 90th day, vital organs (heart, lungs, liver, kidneys, spleen and testis, male sex organs, were harvested from sacrificed rats. These were carefully examined for gross lesions and weighed (Precisa digital weighing balance, Type 300-9213/E 125A, Switzerland). The weight of each organ was standardized to 100 g body weight of each animal.

Hematological Assessment

Blood samples were collected from rats into Ethylene Diamine-Tetra Acetate (EDTA) bottles after super- ficially anaesthetized. Collected samples were analyzed for determination of Packed Cell Volume (PCV) (Dacie and Lewis, 1975), Red Blood Cell (RBC) count, hemoglobin (Anonymous, 1965), total and differential White Blood Cell (WBC) count percentage using standard methods (Ghai, 1995).

Statistical Analysis

The results obtained were presented as mean ± SD while analysis of variance was performed by one way ANOVA procedure (SPSS 09.05).

Results and Discussion

Screening for Bacterial Strains Producer of Exopolysaccharides

Marine bacteria have become ever more popular and novel sources of EPSs. Although many known marine bacteria can produce EPSs, few of the EPSs are of biotechnological importance, so the search of EPSS that might have innovative applications is still of potential interest (Llamas *et al*., 2010). The promising strain was selected based on the development of mucoid morphology because it was one of the fundamental screenings for isolation of EPS producing bacteria (Inmaculada *et al*., 2010; Parthiban *et al*., 2014). A total of thirteen bacterial isolates collected from various marine samples and exhibiting mucoidal morphology on marine agar media were inoculated into shake flasks containing 50 mL of fermentation broth medium. Marine bacterial isolates were screened for their capacity to produce EPS. The highest yield of EPS (6.01 g L^{-1} growth medium) was obtained by a marine bacterium isolated from a mangrove sample.

Taxonomical Studies

Morphological Characterization

Identification of bacterial isolate NRC27 was carried out according to a great variety of morphological, cultural, physiological and biochemical features. The

isolate NRC27 had short rod shape and was Gram-positive, aerobic, catalase and oxidase-positive. The bacterial diameter was ranged from 0.3 to 0.5 and 0.9 to 3 μm. The organism was able to grow over a range of pH (from 5.0 to 7.0). It grew at temperatures ranging from 25 to 50°C and the optimal temperature was 40°C. It had the ability to utilize many carbohydrates as a sole carbon source including lactose, sucrose, glucose, fructose, glycerol (Table 1). It was sensitive to Cephalothin and Mecillinam. The identification was confirmed by molecular analyses based on 16S rDNA.

Molecular Characterization

A molecular technique was used to prove and further confirm the identification of the isolate NRC27 to the species level. The partial 16S rDNA sequence was determined and was compared to the GenBank databases in the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) using the BLASTN 2.2.6 program. This isolate was found belonging to the genus *Bacillus* sp. with 99% homology level as depicted in the phylogenetic tree analysis. The isolate was identified as *Bacillus* sp. M3 (Fig. 1). The sequence was submitted to GenBank in NCBI (http://www.ncbi.nlm.nih.gov/nuccore/JQ425073) with the accession number KP09417.

Isolation and Chemical Structural of MEPS

Many marine bacteria could produce EPSs, such as *Paenibacillus polymyxa*, *Edwardsiella tarda* and *Alteromonas* (Guo *et al*., 2010). EPS production from *Bacillus* sp. M3 reached a maximum of 6.5 g of crude product per liter of growth medium after 3 day. The main fraction MEPS was obtained after fractionation with ethanol precipitation from the crude exopolysaccharide. The MEPS was collected for further analysis of structure and biological activity. It appeared as a white powder, with a negative response to the Bradford test. The fact that no absorption was detected by the UV spectra at both 260 and 280 nm indicated the absence of nucleic acids and protein. The monosaccharide of MEPS hydrolysate was determined by HPLC, wherein galacturonic acid: Glucuronic acid was identified in the hydrolysate and their molar ratios were 1:1, respectively. FT-IR analysis showed a broadly stretched intense peak at around 3428 cm^{-1} characteristic of COO^- and a weak C–H band at around 2928 cm^{-1}. The relatively strong absorption peak at a ~1646 cm^{-1} indicated the characteristic IR absorption of polysaccharides (Bremer and Geesey, 1991). The band at 343.53 cm^{-1} region was attributed to the stretching vibration of O-H in the constituent sugar residues (Kanmani *et al*., 2011). The band at 2927.41 cm^{-1} was associated with the stretching vibration of C-H in the sugar ring.

Table 1. Morphological, cultural and physiological characteristics of the bacterial isolate

Characteristics	Bacterial isolate
Morphology	Gram-positive, short rods, non-spore forming
Motile	Non-motile
Cultural	Circular, smooth, mucous, white
Physiological	Aerobic, catalase positive, halophilic

The band at 1649.8 cm^{-1} was due to the stretching vibration of C = O and COO^-. The absorptions around 1456.69 cm^{-1} represented CH_2 and O-H bonding. The strong absorption at 1068.08 cm^{-1} was dominated by glycosidic linkage ν(C–O–C)-stretching vibration (Sun *et al*., 1998). In addition, the band at 836.95 cm^{-1} indicated the α-pyranose form of the glucosyl residue. Therefore, the IR analysis suggested that it was highly likely that the MEPS belonged to α-type heteropolysaccharide with a pyran ring (Cheng *et al*., 2008). The weight-average (Mw) and number-average (Mn) molecular weights and polydispersity (Mw/Mn) of the MEPS was analyzed by GPC. The MEPS in the GPC chromatogram (Fig. 2) was widely dispersed molecules polydispersity index (Mw/Mn = 2.5, the ratio of weight average molecular weight to number average molecular weight) and had an overall weight average molecular weight (Mw) of 1.45×10^4 g moL^{-1} and number average molecular weight (Mn) of 5.7×10^3 g moL^{-1}. The molecular weight of MEPS from *Bacillus* sp.1-450 was 2.2×10^6 Da and the functional groups in the molecular chains of the EPS are important determinants for biological activity (Kumar *et al*., 2004).

Sub-Chronic Toxicity Study

Owing to the extensive use of aluminum in treatment of drinking water and industrial purposes accidental and/or prolonged exposure of both animals and human can lead to great economic losses in the animal wealth and toxicological hazards for human health. Heavy metal when consumed in considerable amount can result in damage or reduce mental and central nervous function, damage to blood composition, lung, kidneys, liver and other vital organs. Long term exposure may result in muscular and neurological disorder that mimic the AD, Parkinson's disease and muscular dystrophy (Shafii *et al*., 2011). The safety and toxicity information of herbal medicine or natural are required prior to expanded clinical studies and to support the registration of herbal and/or natural product with drug control agency. Sub-chronic toxicity study was carried out to observe any progressive effect of MEPS administration through the experimental period as compared to negative control rats and $AlCl_3$-intoxicant rats. Administration of $AlCl_3$ showed significant enlargement in heart (0.42 g/100 g), liver (3.75 g/100 g) and spleen (0.39 g/100 g) as compared to control group (0.37, 3.10 and 0.3 g/100 g, respectively) while other organs, lung, testis and brain were shrank (0.50, 0.57

and 0.30 g/100 g, respectively) (Table 2). Administration of MEPS at 1/10 of LD_{50} didn't produce any adverse effect on heart, lung, liver, kidney, testis or brain (Table 2), they remained near to controls. However, treating $AlCl_3$-intoxicante animals for 90 days improved organs weight. It decreased the enlargement levels produced by $ALCl_3$ intoxication (0.38, 3.31 and 0.32 g/100 g for heart, liver and spleen, respectively). These findings are compatible with those of recorded hematological parameters. $ALCl_3$ administration produced significant increments in hemoglobin, PCV and white blood cell count also it was observed in augmentation of neutrophiles count, basophiles and eosinophiles. These increments were accompanied with significant decrease in red blood cell count, monocyte and eosinophiles (Table 3).

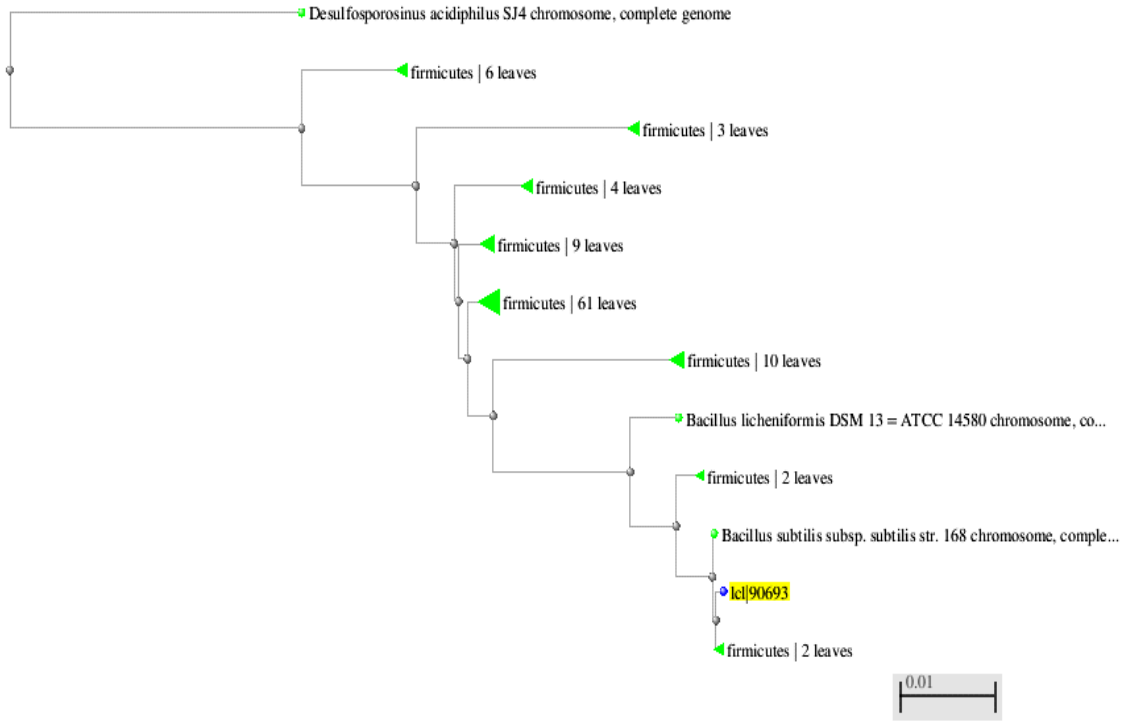

Fig. 1. Phylogenetic tree of the partial sequence of 16S rDNA of the local isolate NRC27 with respect to closely related sequences available in GenBank databases

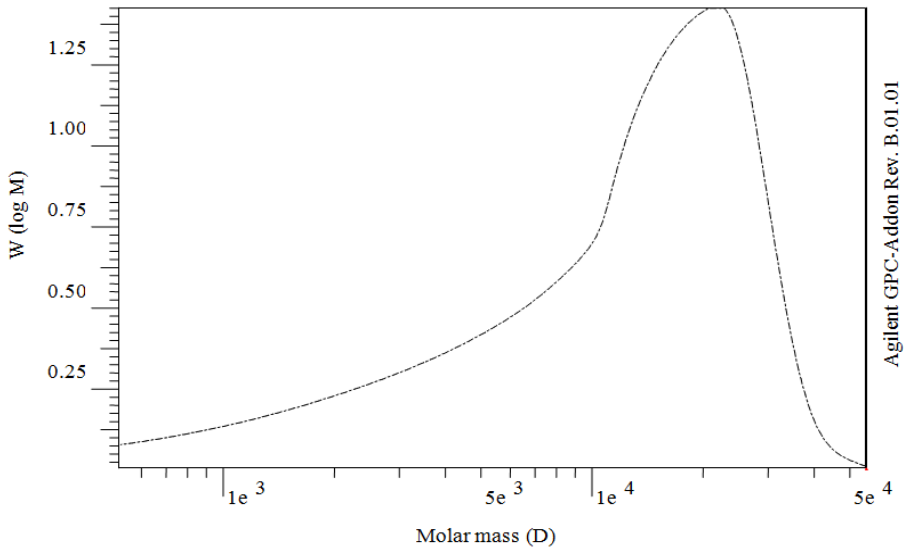

Fig. 2. Molecular weight distributions of MEPS production by *Bacillus* sp. M3

Table 2. Effect of MEPS on rat organs weights (per 100 g body weight)

Group	Heart	Lung	Liver	Kidney	Spleen	Testis	Brain
Control	0.37±0.03^{b}	0.60±0.14^{c}	3.10±0.75^{d}	0.38±0.06^{f}	0.30±0.06^{g}	0.70±0.18^{h}	1.00±0.12^{I}
$ALCl_3$ intoxicant	0.42±0.01	0.50±0.02	3.75±0.95	0.38±0.04^{f}	0.39±0.01	0.57±0.03	0.30±0.02
MEPS group	0.35±0.06^{b}	0.62±0.10^{c}	3.18±0.87^{d}	0.37±0.04^{f}	0.31±0.01^{g}	0.70±0.01^{h}	0.99±0.03^{I}
Treated intoxicant	0.38±0.05^{b}	0.62±0.08^{c}	3.31±0.86	0.37±0.06^{f}	0.32±0.05^{g}	0.69±0.02^{h}	0.75±0.01

Data are presented as mean of 15 animals ± SD. Groups have the same letter have insignificant difference p<0.05

Table 3. Effect of MEPS on hematological parameters in male rats

					Deferential white blood cell (%)				
Group	Hemoglobin (g dL^{-1})	PCV (mm)	RBC ($\times10^3$/mm^3)	WBC ($\times10^3$/mm^3)	Monocyte	Eosinophile	Neutrophile	Lymphocyte	Basophile
Control	13.32±2.1	39.87±3.14	7.45±1.30^{b}	9.65±0.99	2.60±0.04	1.60±0.03^{d}	22.09±1.40^{e}	72.01±2.85^{f}	1.70±0.01^{g}
$ALCl_3$ intoxicant	14.45±2.56^{a}	43.68±3.22	6.20±1.11	11.00±2.41	1.71±0.08	1.00±0.09	42.42±3.11	52.33±4.11	2.68±0.08
MEPS	14.00±2.01^{a}	40.11±2.77	7.65±1.64^{b}	10.00±1.85^{c}	2.10±0.02	1.54±0.04^{d}	23.00±2.64^{e}	71.67±3.52^{f}	1.69±0.07^{g}
Treated intoxicant	14.21±1.33^{a}	41.37±3.16	7.20±1.05^{b}	10.22±2.10^{c}	1.92±0.01	1.20±0.05	30.72±3.01	65.08±2.87	1.08±0.03

Data are presented as mean of 15 animals ± SD. Groups have the same letter have insignificant difference p<0.05

Data presented in Table 3 showed that administration of MEPS as positive control did not affect differential white blood cell with increasing of hemoglobin concentration while administration of aluminum chloride for 30 days increased white blood cell count (11×10^3 mm^{-3}) that was observed in significant increment in neutrophiles with significant reduction in lymphocyte. As well as enhancing hemoglobin concentration (14.45 g dL^{-1}) (Table 3). The ameliorative effect of MEPS administration for 90 days after aluminum intoxication was recorded. It reduce the increment level in PCV to be insignificant as compared to negative control (39.87 and 40.11 mm for treated and control groups, respectively) also increased the red blood cell count to reach the control group (7.20 and 7.45×10^6 mm^{-3}, respectively) and saving the white blood count around the control group (10.22 and 9.65×10^3 mm^{-3} for treated and control, respectively) with plausible effect on differential count, it reduced the neutrophile production (30.72 and 42.42 for treated and intoxicant group, respectively) and induced lymphocyte production (65.08 and 52.33% for treated and intoxicant group, respectively) to rearrange the differentials count to reach nearly levels to control.

On the other hand, administration of aluminum chloride significantly increased AST and ALT activity (210.74 and 180.66 U mL^{-1}, respectively) and protein and lipid production (9.02 g dL^{-1} and 206.23 mg dL^{-1}, respectively) with augmentation of creatinine level, uric acid and urea concentration (18.11 mg dL^{-1}, 3.02 mg dL^{-1} and 5.15 mmol L^{-1}) as well as decreasing glutathione concentration (0.88 mg dL^{-1}) (Table 4).

Polysaccharide administration didn't produce any progressive effect on all determined liver and kidney functions when animals treated for 90 days to serve as vehicle control group. On the other hand, it repaired the liver and kidney dysfunctions observed on AL-intoxicant animals. It reduced AST and ALT activities (156.24 and 100 U mL^{-1}, respectively) and lipid peroxide production (180.66 mg dL^{-1}) as well as elevation in glutathione from 0.88 mg dL^{-1} in intoxicant group to 2.11 mg dL^{-1} in treated intoxicant group. It also decreased creatinine production from 18.11 in intoxicant animal to 12.41 mg dL^{-1} in treated intoxicated animals which accompanied with decreasing concentration of uric acid and urea from 3.02 and 5.15 mg dL^{-1} in intoxicated rats, respectively, to 2.10 and 3.25 mg dL^{-1} in treated group. Generally, administration of polysaccharide after aluminum toxicity ameliorated all determined liver and kidney functions as a part of sub-chronic toxicity study.

Our data are in accordance with those of Sallam *et al.* (2005) who reported that treatment with $AlCl_3$ resulted in significant decrease in body weight, feed intake, drinking water, nitrogen balance, digestibility coefficients. Also, treatment had significant effects on the activities of Aspartate Aminotransferase (AST), Alanine Amino Transferase (ALT), Alkaline Phosphatase (AlP), Acid Phosphatase (AcP) and the concentration of Thiobarbituric Acid-Reactive Substances (TBARS) plasma enzymes activity.

Anti-Alzheimer Effect of MEPS on Male Rats

Oxidative stress parameters were decreased by MEPS administration as vehicle group. It reduced production of malondialdehyde (3.61 nmol mg^{-1} protein), hydrogen peroxide (3.61 nmol mg^{-1} protein) and nitric oxide (1.11 µmo lmg^{-1} protein) also it stimulated antioxidant parameters as catalase activity (6.99 U mg^{-1} protein), superoxide dismutase (4.38 U mg^{-1} protein) and total antioxidant capacity (14.99 mmol mg^{-1} protein) determined in brain tissue while aluminum chloride adminis tration enhance all oxidative stress parameters. It augmented production of malonedialdehyde (11.26 $nmolmg^{-1}$ protein) and hydrogen peroxide (10.41 nmol mg^{-1} protein) and nitric oxide conc. (9.23 $\mu molmg^{-1}$ protein) as well as depression in catalase activity (3.26 U mg^{-1} protein) and SOD activity (2.81 U mg^{-1} protein) which highly lowered total antioxidant capacity (6.48 $mmolmg^{-1}$ protein) (Table 5). When intoxicant animals treated with MEPS, it inhibited the production of oxidative stress molecules in cells, therefore malon-

dialdehyde were decreased to reach 5.99 nmol mg^{-1} protein, hydrogen peroxide to be 5.13 nmol mg^{-1} protein and nitric oxide to1.61 µmol mg^{-1} protein with stimulation of antioxidant parameters. The MEPS enhanced the activity of catalase (5.46 U mg^{-1} protein) and SOD (3.77 U mg^{-1} protein) with amelioration in total antioxidant capacity (10.58 mmol mg^{-1} protein). Tissue damage resulting from an imbalance between reactive oxygen species generating and scavenging systems (oxidative stress) has been implicated in the pathogenesis of a variety of disorders, including dege-nerative disorders of the CNS such as Alzheimer's disease (Harman, 1993).

Animals intoxicated with aluminum chloride have high acetyl cholinesterase activity (915.34 U mg^{-1} protein) with low acetylcholine concentration (5.76×10^{-2} mmol mg^{-1} protein) with elevation in calcium binding protein accumulation (52.77 ng mL^{-1}) determined in these animals sera. On the other hand, oral administration of polysaccharide showed inhibitory effect on acetyl cholinesterase activity (306.21 U mg^{-1} protein) which increased acetylcholine concentration in brain cell (8.87×10^{-2} mmol mg^{-1} protein) with reduction calcium binding protein accumulation in serum (23.43 ng mL^{-1}) (Table 6). Treating intoxicant animals with polysaccharide showed positive effect on acetylcholine biomarkers. It inhibited acetyl cholinesterase activity (510.55 U mg^{-1} protein) that increases accumulation of acetylcholine in brain cells (7.36×10^{-2} nmol mg^{-1} protein). This plausible effect was accompanied with decreasing in Ca-binding protein concentration 33.92 ng mL^{-1}. The bioactivities of polysaccharides can be affected by many factors including chemical comp-onents, molecular weight, configuration and isolation methods (Xu *et al*., 2011). The research on chemical characterization and antioxidant properties of polysacch-arides from *Sargassum fusiforme* found that a relatively low molecular weight and a relatively high uronic acid content could increase the antioxidant activity (Zhou *et al*., 2008). Likewise, it had been reported that there was a direct relationship between the uronic acid contents and the radical scavenging effects of tea polysaccharide conjugates (Chen *et al*., 2004; 2008).

Inhibition of Acetyl Cholinesterase (AChE), the key enzyme in the breakdown of Acetylcholine (ACh), is considered as a promising strategy for the treatment of neurological disorders such as AD, senile dementia, ataxia and myasthenia gravis. Principal role of AChE is the termination of nerve impulse transmission at the cholinergic synapses by rapid hydrolysis of ACh. Inhibition of AChE serves as a strategy for the treatment of AD, senile dementia, ataxia, myasthenia gravis and Parkinson's disease (Rahman and Choudhary, 2001).

Table 4. Liver and kidney functions of rats administered MEPS for 90 days

Group	AST (U mL^{-1})	ALT (U mL^{-1})	Protein (g dL^{-1})	Total lipid (mg dL^{-1})	Glutathione (mg dL^{-1})	Creatinine (mg dL^{-1})	Uric acid (mg dL^{-1})	Urea (mmol L^{-1})
Control	100.56±2.44	79.20±2.83	7.23±0.29	154.40±3.11	3.74±0.43	09.34±0.56	1.68±0.15	2.23±0.83
$ALCl_3$ intoxicant	210.74±3.01[a]	180.66±4.67[a]	9.02±1.06[a]	206.23±2.94[a]	0.88±0.08[a]	18.11±1.13[a]	3.02±0.24[a]	5.15±0.09[a]
MEPS	106.00±2.87	82.33±3.01	7.31±1.02	160.18±2.54	4.12±0.19[a]	09.02±1.02	1.59±0.28	2.21±0.07
Treated intoxicant	156.24±1.0[ab]	100.00±1.02[ab]	8.14±1.10[ab]	180.06±3.06[ab]	2.11±0.04[ab]	12.41±2.06[ab]	2.10±0.11[ab]	3.25±0.13[ab]

Data are presented as mean of 15 replicates ± SD
a; significant change at $p<0.05$ for control group
b; significant with Al-intoxicant control group

Table 5. Effect of MEPS on brain oxidative stress biomarkers and brain antioxidant status in normal and AL-intoxicant groups

Groups	MDA (nmol mg^{-1})	H_2O_2 (nmol mg^{-1})	NO_2 (µmol mg^{-1})	CAT (U mg^{-1})	SOD (U mg^{-1})	T-AOC (mmol mg^{-1})
Control	04.11±1.37	04.86±1.61	1.91±1.13	5.46±0.65	3.11±0.91	12.46±1.22
$AlCl_3$-intoxicant	11.26±2.63[a]	10.41±1.97[a]	9.23±1.52[a]	3.26±2.11[a]	2.81±0.99[a]	06.48±1.94[a]
MEPS	03.61±0.91[a]	3.42±1.42[a]	1.11±0.86[a]	6.99±1.04[a]	4.38±0.79[a]	14.99±0.05[a]
Treated intoxicant	05.99±1.94[ab]	5.13±1.18[ab]	1.61±0.31[ab]	5.46±0.97[ab]	3.77±0.81[ab]	10.58±1.23[ab]

Data are presented as mean of 15 replicates ± SD
a; significant change at $p<0.05$ for control group
b; significant with Al-intoxicant control group

Table 6. Effect of MEPS on Ca binding protein and acetylcholine biomarkers

Groups	Ach (nmol mg^{-1})	AChE (U mg^{-1})	S100B (ng mL^{-1})
Control	7.69×10^{-2}±3.11	498.56±2.27	24.75±1.01
$AlCl_3$- intoxicant	5.76×10^{-2}±1.53[a]	915.34±3.41[a]	52.77±3.21[a]
MEPS	8.87×10^{-2}±2.43[a]	306.21±2.38[a]	23.43±2.42
Treated intoxicant	8.26×10^{-2}±1.76[b]	510.55±2.15[ab]	33.92±3.16[ab]

Data are presented as mean of 15 replicates ± SD
a; significant change at $p<0.05$ for control group
b; significant with Al-intoxicant control group

There are a few synthetic medicines, *e.g*., tacrine, donepezil and the natural product-based rivastigmine for treatment of cognitive dysfunction and memory loss associated with AD (Oh *et al*., 2004). These compounds have been reported to have their adverse effects including gastrointestinal disturbances and problems associated with bioavailability (Schulz, 2003) which necessitates the interest in finding better AChE inhibitors from natural resources. The EPS attenuated the LPS-induced release of pro-inflammatory factors possibly via suppressing the activation of NF-kB and ASK1-p38/JNK signaling and reduced the LPS-induced intracellular ROS accumulation which may possibly at least in part contribute to the suppression of NF-kB and ASK1-p8/JNK and then to the reduced productions of pro-inflammatory cytokines (Diao *et al*., 2014). Generally, MEPS showed potent anti-Alzheimer effect as well as it didn't have any chronic toxicity through the experim-ental period. These findings suggest that polysaccharide isolated from *Bacillus* sp. M3 may be a good natural source for Alzheimer disease therapy.

Conclusion

The water soluble exopolysaccharide coded as MEPS isolated from a newly *Bacillus* sp. M3 contained galacturonic acid and glucuronic acid with molecular masse of 1.45×10^4 g/mol. FT-IR, UV-Vis spectral analyses revealed prevalence of characteristic primary belonged to α-type with a pyranose ring. The MEPS ameliorated antioxidant status and reduced all oxidative stress parameters in brain tissue with decreasing S100B as compared to aluminum toxicant group with significant acetyl cholinesterase inhibition which increase acetyl choline concentration in brain tissue.

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Author's Contributions

MMSA, MGM and SSM designed the study. Isolation, screening, identification of bacterial strain and MEPS was prepared by bacteria. Chemical charact erization of MEPS was carried by MMSA and MGM. Animal study experiments were carried out by AYI. The data were analyzed by MMSA, MGM, AYI and SSM. The manuscript was written by MMSA with contributions from MGM and AYI.

Ethics

This study was approved by Medical Research Ethics Committee, National Research Centre, Egypt, under registration no. 11015.

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