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Exoplysaccharide Produced by *paenibacillus lactes* NRC1: Its characterization and anti-inflammatory activity via cyclooxygenases inhibitory activity and modulation of inflammation related cytokines

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ABSTRACT

Prostaglandin (PG) secreted is characteristic for most inflammatory diseases. The committed step in the form of free arachidonic acid into PG products is supported by cyclooxygenase (COX) which exists as two genetically distinct iso forms, COX-1 and COX-2. This study was conducted using expolysacchaide produced by isolated strain paenibacillus, named PLEPS. Strain NRC1 was isolated from mangroves and identified as belonging to paenibacillus lactes NRC1 species by 16SrRNA and it produced 9.5 g of PLEPS per liter of broth medium. The PLEPS was a heteropolysaccharide composed of arabenose, fructose, glucose and galactouronic acid at molar ration 3.0: 1.0: 0.25: 3.0, respectively with molecular mass of 2.55×10^4 g/mol. To verify the claims of PLEPS on the anti-inflammatory activity and possibly deduce its activities, through testing its effect as cycoloxygenases 1 and 2 inhibitor at five concentrations was tested. It inhibited each of them concentration dependently to reach maximum activity, 98.82 %, for COX-2 and 55.18% for COX-1 at 400 µg/mL. However, celecoxibe inhibited them by 100% and 69.33%, respectively at the same concentration. PLEPS scavenged 50% of nitric oxide radical at 50.03 µg/mL, DPPH radical at 25 µg/ ml and superoxide radicals at 17.73 µg/mL. In in-vivo study, PLEPS inhibited production of interleukin-1 (IL-1), TNF-α and nitric oxide in sera of animals pretreated with PLEPS at 200 mg/ kg body weight before carrageenan injection in acute inflammation model. It is evident from recorded activities that it can be used as a potential antioxidant and Non-steroidal anti-inflammatory material through enhances adaptive immune responses.

Keywords: Cyclooxygenase, inflammation, cytokines, carrageenan, *paenibacillus lactes*, exopolysaccharides

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INTRODUCTION

Inflammatory diseases, such as chronic asthma, rheumatoid arthritis (RA), multiples sclerosis, psoriasis and inflammatory bowel disease are widespread in the world on large scale [1]. To alleviate pain and stiffness in patients with inflammatory diseases, physician often applied drugs like cyclooxygenase inhibitors [2]. Most used drugs are expensive and show the side effects [3]. Thus, there is an urgent need to develop new drugs with minimal side effects and cost reduction [4]. Mangroves are tropical or subtropical inertial forested wetlands containing a great

diversity of organisms. The second largest ecological group of the marine bacteria is mangrove organisms, and many of them are new or adequately enough described species and may produce chemical substances with novel functions and structures [5]. Bacteria often produce exopolysaccharides (EPS) which are secreted into the growth media or still tightly attached to the cell surface. Some of these EPS have shown useful biological activities [6-9]. The production EPS by attached microorganisms are a very complicated process, which is affected by many unique parameters. It is also considered that the mechanisms of biofilm development process are vastly different from species to species [10]. Thus, it is necessary to develop and use of new natural antioxidants which can protect the human body from free radicals and delay the progress of many chronic diseases [11]. Many natural substances have attracted attention in the search for bioactive compounds for the development of new drugs and health foods. Sugars play important roles in many biological processes, and they can work delimiters virulence in pathogens. Occurrence in the nature of the biological activities of EPS attracting more and more attention in biochemistry and medicine [12]. Some of polysaccharides have proven to play an important role as a scavenger of free radicals in the laboratory (*in-vitro*) and antioxidants to prevent oxidative damage in living organisms [13]. Some of these exopolysaccharides have developed into new drugs [14]. Generally, the biological activity associated with the polysaccharide from marine bacteria is related to molecular size and characteristics of anticoagulant properties [15], as well as anti-inflammatory activities [16], which make it applicable for pharmaceutical applications. It is well known that reactive oxygen species (ROS), such a hydroxyl radicals ($OH^{\bullet}$), superoxide anion ($O_2^{\bullet-}$), singlet oxygen ($1O_2$) and hydrogen peroxide (H_2O_2), play a key role in the development of oxidative stress can lead to many diseases including inflammation, cardiovascular diseases, degenerative diseases, diabetes, cancer, anemia, and ischemia [17]. Inflammation, a part of the non-specific immune response, It involves the variations in blood flow, and increased permeability of blood vessels and tissue destruction through the activation and migration of leukocytes with the synthesis of reactive oxygen derivatives [18]. Peripheral inflammation includes increase in the (COX-2) mediated (PG) synthesis in the central nervous system [(CNS inflammation involves an increase in (COX-2)-mediated (PG) synthesis in the central nervous system (CNS)], which contributes to hyperalgesia and Leather pain [19]. The inflammatory process is a temporal phenomenon, characterized by intense leaching of the muscles in a variety of mediators such as histamines, serotonin, nitric oxide, bradykinins and prostaglandins (PGs) [20-22]. There is a strong relationship between the inflammatory process and the development of pain. Accompanying inflammatory pain by increasing the excitability of sensory fibers reasoned peripheral pain produced by the mediators of the inflammatory actions [23]. This study was conducted to explore the characterization of PLEPS from marine and the identification of by 16SrRNA. The mechanism of action of PLEPS as anti-inflammatory agent relevant to its effect on (COX-2), (COX-1) activities in *In-vitro* model with verification of its role in nitric oxide (NO) production, proinflammatory cytokines including interleukins-1β (IL-1β) and Tumor necrosis factor-α (TNF-α) production and anti-inflammatory cytokines involving transforming growth factor-β (TGf- β) and interleukin-4 (IL-4) in sera of rats with carrageenan-induced hind paw edema.

MATERIALS AND METHODS

Chemicals

Peroxidase, 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid diammonium salt, phenazine methosulphate (PMS)-nicotinamide adenine dinucleotide (NADH), nitroblue tetrazolium (NBT), Tris-HCl buffer, Greiss reagent, Sodium nitroprusside Ascorbic acid (Vc), butylated hydroxytoluene (BHT), Leuco-2,7-dichlorofluorescien diacetate, hematin, arachidonic acid, Tris-buffer, Cyclooxygenase enzyme (COX-1 or COX-2) were purchased from Sigma-Aldrich, USA.

Isolation of Bacteria

Soil samples were collected from mangroves (Marsa Alam, Egypt). The soil samples were diluted and a known aliquot was plated on marine nutrient agar media incubated at 37 °C for 3 days. Some strains exhibited mucoid surface on the growth media. It indicates the production of EPS by bacteria [24]. The strains were routinely sub-cultured and maintained in nutrient agar slants as stock.

Strain Identification

Morphological, physiological and biochemical characterization for the promising bacterium NRC1 were carried out. Characteristics of the isolate were compared with data from Bergey's Manual of Determinative Bacteriology [25]. The identification was confirmed with phylogenic analysis. Genomic DNA from the NRC1 was isolated and quality was evaluated on 1.2 % agarose gel, a single band of high Mw DNA has been observed. The forward primer was 5′TCCGTAGGTGAACTTTGCGG3′ and the reverse primer was 5′TCCTCCGCTTATTGATATGC3′ [26]. 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 988 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. The sequence was compared with 16S rRNA sequences in GenBank and aligned with close relatives using the BLAST program (http://www.ncbi.nlm.nih.gov) [27].

Production 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 [28]. The seed culture was grown at 35°C on a rotary shaker incubator at 130 rpm for 24 h. After incubation, 5 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, yeast extract 0.2, K_2HPO_4 0.25, $MgSO_4$ $7H_2O$ 0.1 and dissolved in 750 mL seawater, pH 7 [29]. The fermentation cultures were then incubated at 35°C with shaking at 130 rpm for 3 days. The EPS sample was prepared from strain NRC1 culture in the fermentation medium.

Isolation and fractionation of the PLEPS

The culture medium was separated by centrifugation at 5000 rpm for 20 min. The supernatant was collected and mixed with four-volumes of absolute ethanol, and left overnight at 4 °C for. The precipitate in the centrifuging tube was rinsed carefully with water, filtered and then dried at 50°C in an oven to get the biomass dry weight [30]. The crude EPS isolated was dissolved in distilled water and further treated with Sevage reagent (Chloroform: Butanol, 5:1, v/v) for three times to remove the residual protein. The EPS, which was in supernatant, was purified again by absolute ethanol and left overnight. The resulting precipitate was re-dissolved in distilled water and dialyzed (2 kDa cut-off membrane) against distilled water to remove low molecular weight compounds [31]. The dialyzed solution was concentrated and fractionation according precipitation using 1, 2, 3 and 4-volume absolute ethanol[32]. The major fraction **(PLEPS)** solution was sited at −5°C overnight thawed rapidly and centrifuged at 5000 rpm for 30 min to examine the precipitation. If no precipitation occurred, the UV absorption spectrum was recorded using a spectrophotometer UV-visible 2401PC (Shimadzu, Japan) between 190 and 550 nm, in order to examine the existence of proteins and nucleic acids [33].

Monosaccharide analysis

PLEPS (~ 200 mg) was hydrolyzed with 2 mL of TFA (1 M) at 100°C in a sealed tube for 12 h. Excess acid was removed by flash evaporation on a water bath at a temperature of 50°C and co-distilled with water (1 mL×3) and then evaporated to dryness [34]. The monosaccharides contents were quantified by HPLC on a Shimadzu Shim-Pack SCR-101N column (7.9 mm × 30 cm), using deionized water as the mobile phase at flow rate 0.5 mL/min [35, 36]. Sugar identification was done by comparison with authentic sugars. Uronic acid content of the PLEPS was determined by the *m*-hydroxybiphenyl method [37, 38], using glucuronic acid as the standard. Sulfate was measured using the turbidimetric method [39] with sodium sulfate as standard.

Molecular Weight Determination

Homogeneity and average molecular weight (M_w) of PLEPS was determined on an Agilent 1100 HPLC system equipped with a RI Detector 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 M_w from 100-1250000 g/moL. The sample 10 mg was dissolved in 2 mL of solvent and then it filtrated by siring filter 0.45 then the sample but in GPC device. The polydispersity index (*PI*) calculated from the M_w/M_n ratio [40, 41].

Fourier-Transform Infrared Spectroscopy (FTIR)

The FTIR spectrum of PLEPS was determined using a Bucker scientific 500-IR FTIR spectrophotometer (Bucker Co., Ettlingen, Germany) for the detection of various functional groups. The purified PLEPS was ground with KBr powder and pressed into pellets for FTIR measurement in the frequency range of 4000–400 cm^{-1} [42].

Cyclooxygenases inhibitory activity
The oxidation of leuco-dichlorofluorescein (L-DCF) in the presence of phenol by the hydroperoxide formed in the cyclooxygenase reaction can be used as a sensitive spectro-photometric assay for PGH synthase activity [43]. Leuco-2,7-dichloro-fluorescein diacetate (5 mg) was hydrolyzed at room temperature in 1 M NaOH (50 µL) for 10 min, then 1M HCl (30 µL) was added to neutralize excess NaOH before the resulting L-DCF was diluted in 0.1 M Tris-buffer, pH 8. Cyclooxygenase enzyme (COX-1 or COX-2) was diluted in 0.1 M Tris-buffer, pH 8, so that a known aliquot gave an absorbance change of 0.05/ min in the test reaction. 20 µL of test samples or methanol were pre-incubated with enzyme at room temperature for 5 min in the presence of hematin. Premixed phenol, L-DCF and arachidonic acid were added to the enzyme mixture to begin the reaction, and to give a final reaction mixture of arachidonic acid (50 µM), phenol (500 µM), L-DCF (20 µM) and hematin (1 µM) in 1 mL final volume of 0.1 M Tris-buffer, pH 8. The reaction was recorded spectrophotometrically over 1 min at 502 nm. A blank reaction mixture was analyzed in the spectrophotometer reference cell against each test reaction to account for any non-enzymatic activity attributed to the test sample. This blank consisted of the reaction mixture without the addition of enzyme. Celecoxib was used as standard compound.

Nitric Oxide radical scavenging activity
$NO^{\bullet}$ radical scavenging activity of PLEPS at 25, 50, 100, 200 and 400 µg/mL was determined using a Sodium nitroprusside (SNP) generating $NO^{\bullet}$ system. $NO^{\bullet}$ generated from SNP in aqueous solution at physiological pH reacts with oxygen to produce nitrite ions which were measured by the Greiss reagent [44]. which constitutes 1% Sulfanilamide in 5% ortho-H_3PO_4 and 0.1% Naphthylethylene diamine dihydrochloride. The reaction mixture (2 mL) containing various concentrations of the LPEPS and standard compounds and SNP (final concentration, 10 mM) in phosphate buffered saline (PBS) pH 7.4 were incubated at 25ºC for 150 min. After incubation, 1ml samples of reaction mixtures were removed and diluted with 1 mL Greiss reagent. The absorbance of these solutions was measured at 540 nm against the corresponding blank solution.

Free radical scavenging activity
The free radical scavenging activity of PLEPS was measured using the method of [45]. 0.1 mM solution of $DPPH^{\bullet}$ in methanol was prepared. Then, 1ml of this solution was added to 3 mL of PLEPS and standards solution at 25, 50,100, 200 and 400 µg/mL. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then the 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. The $DPPH^{\bullet}$ radical concentration in the reaction medium was calculated from the following equation:

$DPPH^{\bullet}$ scavenging effect (%) = 100 − $[(A_0\text{-}A_1)/A_0)\ 100]$

Where A_0 was the absorbance of the control reaction and A_1 was the absorbance in the presence of the sample of PLEPS.

Superoxide anion scavenging activity
Superoxide radicals are generated in phenazine methosulphate (PMS)-nicotinamide adenine dinucleotide (NADH) systems by oxidation of NADH and were assayed by the reduction of nitroblue tetrazolium (NBT). In this experiment, the superoxide radicals were generated in 3 mL of Tris-HCl buffer (16 mM, pH 8.0) containing 1 mL of NBT (50 µM) solution, 1 mL NADH (78 µM) solution and 1ml sample solution of PLEPS at different concentrations were mixed. The reaction was started by adding 1 mL of PMS solution (10 µM) to the mixture. The reaction mixture was incubated at 25ºC for 5 min, and the absorbance at 560 nm in a spectrophotometer was measured against blank samples [46, 47]. Ascorbic acid and BHT was used as controls. Decrease in absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated using the following formula:

$\% = [(A_0\text{-}A_1)/A_0] \times 100$

Where A_0 was the absorbance of the control, and A_1 was the absorbance of PLEPS or standards.

Total antioxidant activity
Exactly 0.2 mL of peroxidase (4.4 Units/mL), 0.2 mL of H_2O_2 (50 µM), 0.2 mL of ABTS (2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic cid, diammonium salt, 100 µM) and 1ml methanol were mixed, and were kept in the

dark for 1 h to form a bluish green complex after adding of 1 mL PLEPS of different concentrations, ascorbic acid and BHT, used as a control.[30,31] All were tested in triplicates. The absorbance at 734 nm was measured to represent the total antioxidant activity and then was calculated as follows:

Total antioxidant activity (%) = [1- (A sample/A control)] × 100

Animals and experimental design

Acute toxicity

The acute toxicity test for PLEPS was carried out to evaluate any possible toxicity. Male albino mice obtained from animal house of National Research Centre (n=8) were divided into six groups then, administered different doses of PLEPS in an oral route by increasing or decreasing the dose, according to the response of animal [48]. The dosing patron was 250, 500, 1000, 1500, 2000 and 2500 mg/kg body weight while the control group received only the normal saline. All groups were observed for any gross effect or mortality during 48h. Death of half of examined animals was observed at 2000 mg/ kg body weight.

Animals and experimental design of anti-inflammatory activity in Carrageenan induced paw oedema model

Adult male *sapargue dawely* rats weighing between 150-180 g were maintained under normal laboratory conditions and kept in standard polypropylene cages at room temperature of 25-30°C, 60 to 65% relative humidity and provided with standard diet and water *ad libitum.* The diurnal lighting and darkness cycle is 12 h. After acclimation for 5 days, animals were randomly divided into four groups each of eight animals to conduct the experiments. Rat paw edema was induced by carrageenan injection using a modified method [49]. After pre-treatment of PLEPS at one tenth of LD_{50} for 7 days, except control animals, each male rat was injected with 0.1 mL of carrageenan (1% in normal saline) at the plantar side of right hind paw. Paw edema values were measured with a digital caliper before and 1, 2, 3, 4 or 5 h after carrageenan injection [50]. Effect of inhibition on paw edema was calculated using the following formula:

Percent Inhibition = [(C– T) / C] × 100

Where T_0 is the thickness of paw before carrageenan injection, T_t presents the paw thickness after carrageenan injection, t =1,2,3,4,5 h. Celecoxibe was used at recommended dose (100 mg/kg). After last measurement of the paw thickness, rats were sacrificed by cervical dislocation. Blood was collected and the serum was isolated for the determination of inflammation biomarkers, NO level, Interleukin-1β, interleukin-6 and tumor necrosis factor–α. The inflammation biomarkers (IL-1β and IL-4) levels were determined using enzyme linked immune adsorbent assay kits in accordance with the manufacturer's recommendations (Quantikine, USA), tumoral necrosis factor-α was carried as manufacturer's by Hycult biotech, Netherelands while transforming growth factor-β1 was assessed as manufacturer's by Abnova, Taiwan. The assessment was done by ELISA reader (NJ 2000; Nihom Inter Med. Co), the sensitivity of assay was 20 pg/ml. NO was measured according to the method of [51]. For nitrite determination, NO_3^- was converted into nitrite after enzymatic conversion by nitrate reductase; $NO2^-$ was measured by using the Griess reaction [52]. Values obtained by this procedure represented the sum of nitrite and nitrate.

Statistical analysis

All data are expressed as means ± S.D., where analysis of variance was used for statistical evaluation of significant differences among the multiple groups with One-way ANOVA, Tukey's Multiple Comparison test. Differences were considered significant when P <0.05. The analyses was performed using SPSS 9 program

RESULTS AND DISCUSSION

Isolation, Screening, and Identification of the EPS producing bacteria

A total of 65 morphologically well-formed single colonies were selected from different marine samples on the basis of their morphological differences in nutrient agar plates. Among them 30 strains were found to be producer EPSs. Finally, NRC1 strain was selected as the best EPSs producer according to among of yield per liter. Were examined marine bacterial isolates its ability to produce EPS. It has been getting the highest return of EPS (7.1 g L^{-1}) by type of marine bacteria isolated from a sample of mangroves **(Table 1).** Moreover, the implementation of the process of determining the molecular identity of the isolated bacteria using 16S ribosomal way out. It revealed evolutionary analysis and development on the basis of recombinant DNA sequencing 16S is a close relationship (99%) for

Paenibacillus elgii n *Paenibacillus ehimen* is. Nucleotide sequence deposited in a sequence database GenBank, because of the number of join KP145013. And shows the relationship of evolution and development of the mammary Paenibacillus NRC1 DNA sequencing with 16S recombinant of *Bacillus* and *Paenibacillus* others. Strain NRC1 beyond that characterization, plates morphological and physiological and biochemical standard that NRC1 strain was spore forming Gram-positive and non-bacillus, which grows in both aerobic environments showed. According to the morphological and physiological property, it was found NRC1 strain belonging to *Paenibacillus* sp.. Moreover, the implementation of the process of determining the molecular identity of the isolated bacteria using 16S ribosomal is way out. He revealed evolutionary analysis and development on the basis of recombinant DNA sequencing 16S is a close relationship (99%) for *Paenibacillus elgii* and *Paenibacillus ehimensis*. Deposited the sequence of nucleotides in a sequence database GenBank, and given the number of join KP145013. And shows the relationship of evolution and development of the mammary *Paenibacillus* NRC1 DNA sequencing with 16S recombinant of *Bacillus* and *Paenibacillus* others is shown in **Figure (1).**

Table 1. Morphological, cultural and physiological characteristics of NRC1 isolate

Characteristics	Isolate bacteria
Morphology	Gram-positive, short rods, non-spore forming
Motile	Non-motile
Cultural	Circular, smooth, viscous, white yellowish
Physiological	Aerobic, catalase-positive, halophilic

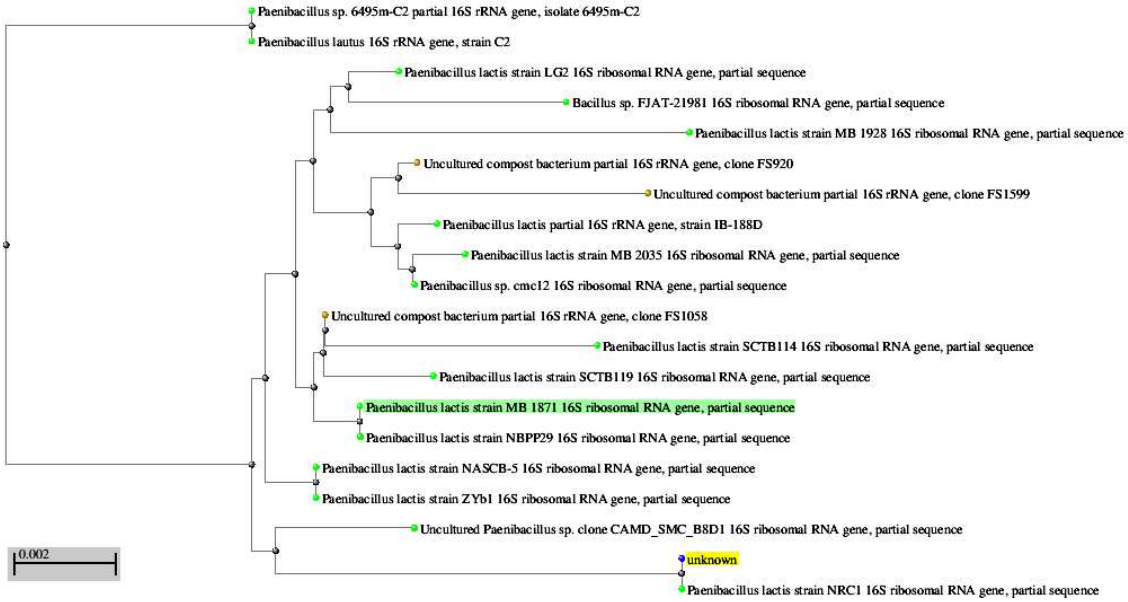

Figure 1. Phylogenetic dendogram obtained by distance matrix analysis showing the position of Paenibacillus lactis NRC1 among 16S rDNA of the highest 16 similar Paenibacillus elgii and Paenibacillus ehimensis

Many marine bacteria can produce EPSs, such as, *Edwardsiella tarda Alteromonas* and *Paenibacillus polymyxa* [53] but the hydroxyl radical scavenging activity of the EPS of *Pseudomonas* PF-6 reached 90% in much lower concentration level, 0.6 mg/mL. Accidentally, [40] reported that the scavenging ability on superoxide anion radical of an EPS from *Paenibacillus polymyxa* EJS-3 was about 80% at 1 mg/mL. EPS producing bacteria strain (S9) was isolated from marine soil sample on zobell agar medium at 32°C for 7 days. A convex colony was observed with tenure and orange colony. Strain promising S9 has been characterized as gram positive, rod shaped bacteria moving. The 16SrRNA sequence analysis showed a high percentage of similarity to the genus *Exiguobacterium* sp.

Isolation, purification and defined chemical composition of PLEPS

The maximum of exopolysaccharide production was 7.1 gL^{-1} of growth medium after 3-days and the relationship between cell growth and product formation of *Paenibacillus lactis* NRC1 was 2.95 g EPS per gram cell dry weight. The main fraction PLEPS (71%) was obtained after fractionated with ethanol precipitation from the crude EPS. **PLEPS** was yellow light, in the form of colorless powder smell. It was soluble in water but not soluble in ethanol

and other organic solvents, such as ether, acetone, which is consistent with the general characteristics of the EPS. The solution of PLEPS was a uniform liquid with light colored yellow, and no precipitation occurred after centrifugation. So it was thought that PLEPS be pure.. The lack of absorption was detected by UV spectrum in both the 260 and 280 nm refers to the absence of nucleic acids and proteins. PLEPS contained 18.4 % uronic acid as evaluated by *m*-hydroxydiphenyl colorimetric method and sulfate (11.3%). These indicate that the PLEPS is an acidic exopolysaccharide. The HPLC analysis indicated that PLEPS was consisted of arabinose, fructose and glucose and galacturonic acid with a relative molar ratio of 3.0: 1.0: 0.25: 3. The molecular weight average (M_w) , number average of molecular weight (M_n) and polydispersity (M_w/M_n) of the **PSEPS** was determined by GPC. The PSEPS was widely dispersed molecules polydispersity index (*PI*) was M_w/M_n = 1.16 and had molecular weight (M_w) of 2.55×10^4 g/mol and molecular number (M_n) of 2.19×10^4 g/mol. The range of molar masses (0.57 to 15.8 MDa), M_w (4.66 MDa), M_n (2.48 MDa) and PI (1.9) were agreement with the available data Earnings per share purified from different bacterial strains isolated from the Southern Ocean and Antarctic sea ice [54]. We describe the chemical and biological function of the **PLEPS** synthesized by *Paenibacillus lactis* NRC1, this polymer has anti-inflammatory activity via cyclooxygenases inhibitory activity. EPS produced by *Paenibacillus* sp. TKU023 contains glucose and maltose showed the highest antioxidant activity [55]. [56] was reported that the EPS contains any type of lactose, rhamnose, galactose, glucose, mannose, xylose and raffinose could possibly be utilized for medicinal purposes. On the other hand, the EPS1, EPS2 and EPS3 displayed an immune modulatory effect as measured using interleukin 6 (1L6) and tumor necrosis factor alpha (TNFα) and these three EPS had very different composition, with EPS1 and EPS2 composed of a single but different sugars, putatively fucose and galactose, while EPS3 had a composition with two sugars putatively mannose and galactose from *bacillus licheniformis* SVD1 with different molecular weight 70 to 200 KD [57]. The IR of the PLEPS was displayed in There were many peaks ranged from 3430.85 to 615.86 cm^{-1}. The band at 3430.85 cm^{-1} region was due to the stretching vibration of O–H in the constituent sugar residues[58]. The infrared rang at 2933.43 cm^{-1} was associated with the stretching vibration of C–H in the sugar ring. The band at 1642.05 cm^{-1} was attributed to the stretching vibration of C=O and COO group. The absorptions at 1456.69 cm^{-1} represented CH_2 and OH bonding. The strong absorption at 1068.08 cm^{-1} was controled by glycosidic linkage [59]. Moreover, the band at 927.49 cm^{-1} indicated the β-pyranose form of the glucosyl residue. Therefore, the infrared spectrometry analysis proposed that it was highly likely that the PLEPS belonged to a β-type hetero-polysaccharide with a pyran group [60].

Biological activities of PLEPS

The inflammation, characterized by swelling, redness, heat, and pain, is one of the most important host defense mechanisms against infection by any pathogens. Pro-inflammatory cytokines (*e.g.*, TNF-α), activate cellular responses, overproduces of many cytokines as prostaglandins as well as production of NO, during the response to inflammation [61]. In many inflammatory diseases, Inducible COX-2 may be accountable for the high levels of PGs [62]. Non-steroidal anti-inflammatory drug (NSAID) is a form of COX-2 selective inhibitor that directly targets COX-2 which is responsible for inflammation progression and pain. Selectivity for COX-2 minimizes the risk of peptic ulceration; celecoxib and rofecoxib are members of this drug class. Inducible nitric oxide synthase, iNOS, produces much amounts of NO and play a central role in inflammatory diseases [63]. Repression of NO and PGs production by the inhibition of iNOS and COX-2 expression plays important and beneficial role in inflammatory disorder treatment [64].

In an *in-vitro* study, PLEPS strongly inhibited COX-2 in a dose dependent manner. It reached 98.82% inhibition percentage at 400 µg/mL with IC_{50} value at 27.21 µg/mL while standard nonsteroidal NSAIDs, celecoxibe, reached 100% inhibition percentage at the same concentration with IC_{50} value 80.44 µg/mL which means polysaccharide was potent inhibitory effect than reference drug **(Table 2).**

Table (2): COX-1 and COX-2 inhibitory effect of PLEPS and standard compound

Concentration (µg/ mL)	COX-1		COX-2	
	PLEPS	Celecoxibe	PLEPS	Celecoxibe
25	13.11±2.11*	7.67±1.17	48.03±1.24*	12.79± 0.98
50	13.11±2.11*	7.67±1.17	48.03±1.24*	12.79± 0.98
100	13.11±2.11*	7.67±1.17	48.03±1.24*	12.79± 0.98
200	13.11±2.11*	7.67±1.17	48.03±1.24*	12.79± 0.98
400	13.11±2.11*	7.67±1.17	48.03±1.24*	12.79± 0.98

*Data are expressed as mean of triplicates ± S.D. *means significant difference as compared to celecoxibe (P< 0.05). There is insignificant difference between groups have the same letter.*

On the other hand, both PLEPS and celecoxibe produced inhibitory effect against COX-1. There is no significant difference ($P<0.05$) between them at 50 and 100 µg/mL while the difference between their inhibitory effect was significant at 200 and 400µg/mL. Polysaccharide recorded 52.27 and 55.18% at 200 and 400 µg/mL, respectively, whereas celecoxibe showed 60.46 and 69.33% at the same concentrations. PLEPS produced low effect against COX-1 as compared to reference drug; the IC_{50} value was higher than reference compound 191.31 and 170.29 µg/mL, respectively **(Table 2).** It is evidence from our findings that PLEPS is more selective to COX-2 than reference drug, celecoxibe, with minimum effect on COX-1. Considering these findings, suppression of COX-2, lead to the lowering of COX-2 activity is potential mechanism answerable for the anti-inflammatory activity of **PLEPS**. Many exopolysaccharides were reported for their anti-inflammatory activities through their inhibitory effect on COX-2 and iNOS like β-glucan polysaccharide isolated from mushroom inhibited gene expression of each of them concentration dependently in cell culture technique **(Table 2).** EPSs from *Paenibacillus* spp. were reported as structurally diverse class of biological macromolecules with many applications in pharmacy, cosmetics and biomedications[66]. *Paenibacillus* spp. EPSs are safe than any other synthetic polymer and they showed beneficial role in treating many diseases as inflammation and its related disease like skin rejuvenation and wound healing as well as tumor [67, 68].

Presence and/or production of free radicals in vital cells means increasing of hazard materials for many danger diseases as cancer, liver damage and inflammation related diseases. In order to obtain the natural antioxidant for reduction of these harmful molecules, a lot of researches were achieved by many scientists[69].

Inflammation excited by oxidative stress is the cause of much, perhaps even most, chronic human disease including human aging. The oxidative stress creates mainly in mitochondria from reactive oxygen and reactive nitrogen species (ROS/RNS) and can be identified in most of the key steps in the pathophysiology of many inflammatory diseases. The recognition of the high importance of oxidative stress has led to the passionate use of antioxidants in the curing and prevention of these diseases[70]. Regarding the scavenging effect of PLEPS, it was efficient in scavenging nitric oxide with IC_{50} at 50.03 µg/mL with insignificant differences between polysaccharide and tested standards BHT and Vc which recorded 53.25 and 53.83 µg/mL, respectively**.** This effect was supported with DPPH radical scavenging effect. It was more potent than standards; IC_{50} of polysaccharide was 25 µg/mL while IC_{50} BHT and Vc were 44.52 and 41.51 µg/mL, respectively **(Table 3).** The same trend of data was observed in case of superoxide radical scavenging effect. PLEPS was superior to standards in scavenging superoxide radicals, IC_{50} was 17.73 µg/mL while BHT and Vc recorded lower effect than PLEPS, IC_{50} were 48.38 and 52.32 µg/mL, respectively. These effects were reflected in increasing total antioxidant capacity, PLEPS IC_{50} was 25.22 µg/mL. *Paenibacillus* spp. exopolysaccharide was reported as radical scavenger against DPPH and superoxide radical with reduction capability and metal chelation effect [71]. *P. polymyxa* was reported as superoxide scavenger with moderate inhibition of lipid peroxidation and showed reducing power effect [72].

Table (3): antioxidant properties of PLEPS and standards

Tested material	IC_{50} (µg/mL)			
	NO scavenging	DPPH Radical scavenging	NO_2 cavenging	Total antioxidant capacity
PLEPS	50.03±1.20*,**	25.00±0.97*,**	17.73±1.08*,**	25.22±1.88*,**
Vitamin C	53.83±2.00[a]	41.51±1.99**	52.32±2.14**	45.81±1.33**
BHT	53.25±1.58[a]	44.52±1.58*	48.38±1.96*	36.79±2.10*

*Data are expressed as mean of triplicates ±S.D. * means significant difference as compared to Vc and ** means significant difference as compared to BHT (P< 0.05). There is insignificant difference between groups have the same letter.*

Effect of PLEPS on acute inflammation parameters

Induction of acute inflammation using carrageenan produced oedema in rat hind paw which magnified time dependently in vehicle group. Administration of PLEPS at 200 mg/kg reduced oedema at the first hour with 8.79% inhibition percentage while it reduced by celecoxibe by 5.15%. This effect was significantly increased to the second hour (11.21% inhibition) to reach the same effect of reference drug (10.64). PLEPS produced the maximum inhibitory effect at the last hour to give 28.91% oedema inhibition. At fourth and fifth hrs, both polysaccharide and celecoxibe showed nearly the same level of inhibition **(Table 4).** Orchestration of immune and inflammatory restraints depends upon connection between cells by soluble molecules given the generic term cytokines including chemokine, interleukin, growth factors, and interferon. IL-1 is an important regulator of the inflammatory cascade. Enhancing secretion of IL-1 has been reported for a growing number of inherited, chronic auto inflammatory syndromes. IL-1 affects several targets that account for the manifestations of systemic disease. These are recurrent

fevers, neutrophilia, thrombocytosis, elevated serum amyloid A and C-reactive protein, anemia, Skin rashes and urticaria. The endothelium is a prime target for IL-1-mediated inflammation because IL-1 receptors on the endothelium can be triggered by systemic IL-1, which results in prostaglandin E production, bone marrow release of neutrophils, and the production of IL-6 [73].

Table (4): Effect of PLEPS on the hind paw Oedema (%) in inflammatory rats induced by carrageenan

Groups	Time (hours)					
	0	1	2	3	4	5
Control	2.70±0.08$^{\#}$	3.30±0.12$^{\#}$	3.57±0.10	3.95±0.11	4.00±0.15	4.22±0.13
PLEPS	2.87±0.30$^{\# a}$ (6.29%)	3.01±0.87^{f} (8.79%)	3.17±0.14*^{d} (11.21%)	3.25±0.18*^{e} (19.72%)	3.10±0.11*^{b} (22.50%)	3.00±0.88*^{c} (28.91%)
Celecoxibe	2.90±0.09^{a} (7.41%)	3.13±1.23^{f} (5.15%)	3.19±1.41*^{d} (10.64%)	3.26±1.16*^{e} (17.4%)	3.13±0.99*^{b} (21.75%)	3.08±1.01*^{c} (27.01%)

*Results are expressed as mean ±S.D. (n = 10). *mea ns significant as compared to carrageenan, groups have the same letter have insignificant difference (P <0.05).*

Carrageenan administration significantly induced production of pro-inflammatory cytokines; IL-1 was elevated by carrageenan administration (458.37 pg/mL) with magnification of TNF-α (407.81 pg/mL) and significant reduction in anti-inflammatory cytokines. IL-4 was decreased by carrageenan administration to 15.39 pg/mL also TGF- β1 was reduced to 10.82 pg/mL. On the other hand, Administration of PLEPS induced anti-inflammatory cytokines production; it maximizes IL-4 production to reach 32.93 pg/ ml and TGF-β1 to be 25.40 pg/mL **(Table 5).**

In parallel, tumor necrosis factor alpha (TNF-α), proinflammatory cytokine which was originally characterized as a circulating factor, can cause necrosis of tumors, and also identified as a key regulator of the inflammatory response. TNF is produced predominantly by stimulated macrophages and T lymphocytes. TNF is not usually detectable in healthy individuals, but high serum and tissue levels are found in inflammatory and infectious conditions. TNF receptors are differentially expressed on a broad range of tissues and cells. In response to TNF, endothelial cells promote inflammation by displaying, in a discrete temporal, spatial and anatomical pattern, in combination with the release of chemokines [74].

Table (5): Effect of PLEPS on inflammatory cytokines and nitric oxide production

Group	IL-1β (pg/mL)	TNF-α (pg/mL)	IL-4 (pg/mL)	TGF-β1 (pg/mL)	NO (µmol/mL)
Control	185.21±0.63*	110.80±2.55*	45.1±0.87*	28.32±3.54*	8.13±1.35*^{c}
Carrageenan	458.37±2.11	407.81±2.81	15.38±1.01	10.82±2.69	21.36±1.76
PLEPS	200.41±1.95*	155.34±3.11*	32.93±1.13*^{b}	25.40±1.87*^{a}	10.15±2.44*^{c}
Celecoxibe	239.60±3.10*	179.72±2.48*	30.81±1.62*^{b}	24.28±1.94*^{a}	12.37±3.01*
Control	185.21±0.63*	110.80±2.55*	45.1±0.87*	28.32±3.54*	8.13±1.35*^{c}

*Results are expressed as mean ±S.D. (n = 10). *mea ns significant difference as compared to carrageenan. Groups have the same letter have insignificant difference (P <0.05).*

Increasing in anti-inflammatory cytokines when animal treated with PLPES was accompanied with decreasing in pro-inflammatory cytokines, IL-1β (200 pg/mL) and TNF-α (155.34 pg/mL), as well as decreasing the nitric oxide production (10.15 µmol/mL) to be nearly normal control group (8.13 µmol/mL). Therefore, PLEPS reduced oedema produced by carrageenan administration at 200 mg/kg. The maximum inhibitory effect was observed at the last hour; both polysaccharide and celecoxibe showed nearly the same level of inhibition. NO is a short-lived small molecule which plays an important role in a variety of physiological progresses. Over production of NO can activate nuclear factor (NF)-kappa B to induce the expression of pro-inflammatory mediators, and can raise inflammation by increasing cGMP level and vascular permeability [75]. NO can also stimulate COX which in turn drives to the production of pro-inflammatory mediators, such as PGs [50]. EPSs were also reported for their inhibitory effect on nitric oxide production by inhibiting iNOS gene through the inhibition of its mRNA expression, COX-2 gene expression and inhibited secretion of some inflammation related interleukin by inhibiting their gene expression [76][77]. The mentioned results of our work clearly indicated that PLEPS may have potential application as anti-inflammatory agent. The mechanisms of anti-inflammatory of PLEPS appear to be connected to the supperssion of COX-2 activity, NO release; pro-inflammatory cytokine release with induction of anti-inflammatory cytokines and it may be exerted through its antioxidant properties.

REFFERENCES

[1] R Gautam , SM Jachak, *Medicinal Research Reviews*, **2009**, 29, 767–820.
[2] SN Willich, K Rossnagel, S Roll, A Wagner, O Mune, J Erlendson, A Kharazmi, H Sorensen, K Winther, *Phytomedicine*, **2010**,17, 87–93.
[3] D Mukherjee, SE Nissen, EJ Topol, *JAMA*, **2001**, 286, 954–959.
[4] CA Shearer, E Descals, B Kohlmeyer, J Kohlmeyer, L Marvanová, D Padgett, D Porter, HA Raja, JP Schmit, HA Thorton, H Voglymayr, *Biodiversity and Conservation*, **2007**, 16, 49–67.
[5] J Kobayashi, M Tsuda, *Phytochemistry Reviews*, **2004**, 3, 267–274.
[6] Z Wang, D Luo, *Carbohydr. Polymers*, **2007**, 68 (1), 54–58.
[7] CCBO Miranda, RFH Dekker, JM Serpeloni, EAL Fonseca, IMS Cólus, AM Barbosa, *Int. J. Biol. Macromol.*, **2008**, 42, 172–177.
[8] CL Xu, YZ Wang, ML Jin, XQ Yang, *Bioresour. Technol*. **2009**, 100, 2095–2097.
[9] XJ Du, JS Zhang, Y Yang, LB Ye, QJ Tang, W Jia, YF Liu, S Zhou, RX Hao, CY Gong, YJ Pan, *Carbohydr. Res.*, **2009**, 344, 672–678.
[10] WM Dunne, Review. *Clin. Microbiol. Rev*., **2002**, 15, 155-166
[11] S Nandita, PS Rajini, *Food Chem.*, **2004**, 85, 611-616.
[12] GW Peng, G Hongjie, Y Wen, K Jing, *Curr. Topics Med. Chem*., **2008**. 8(2),141-151.
[13] QB Zhang, N Li, XG Liu, ZQ Zhao, ZE Li, ZH Xa, *Sci. Res. Technol*., **2004**, 339, 105-111.
[14] G Zhou, Y Sun, H Xin, Y Zhang, Z Li, Z Xu, *Pharmacology Research*, **2004**, 50, 47–53.
[15] A Cumashi, NA Ushakova, ME Preobrazhenskaya, A D'Incecco, A Piccoli, L Totani, N Tinari, GE Morozevich, AE Berman, MI Bilan, AI Usov, NE Ustyuzhanina, AA Grachev, CJ Sanderson, M Kelly, GA Rabinovich, S Iacobelli, NE Nifantiev, *Glycobiology*, **2007**, 17, 541–552.
[16] ACC Rocha, E Fernandes, NLM Quint, MM Campos, JB Calixto, *British J. of Pharmacology*, **2006**, 148, 688–695.
[17] Y Cai, Q Luo, M Sun, H Corke, *Life Sci*., **2004**, 74, 2157–2184.
[18] L Ferrero Miliani, OH Nielsen, PS Andersen, SE Girardin, *Clin. Exp. Immunol*., **2007**, 147, 227-35.
[19] M Vazirian, S Dianat, A Manayi, R Ziari, A Mousazadeh, E Habibi, S Saeidnia, Y Amanzadeh, *Research J. of Pharmacognosy (RJP)*, **2014**, 1, 15-21
[20] JTC Carvalho, JRM Teixeira, PJC Souza, JK Bastos, DS Filho, SJ Sarti, *J. of Ethnopharmacology*, **1996**, 53, 175–178.
[21] K Srinivasan, S Muruganandan, J Lal, S Chandra, SK Tandan, VR Prakash, *J. of Ethnopharmacology*, 2001, 78, 151–157.
[22] SW Hajare, S Chandra, J Charma, SK Tandan, J Lal, AG Telang, *Fitoterapia*, **2001**, 72, 131–139.
[23] JE Linley, K Rose, L Ooi, N Gamper, *European Journal of Physiology*, **2010**, 459, 657–669.
[24] TK Ng, WS Hu, *Appl. Microbiol. Biotechnol*., **1989**, 31,480-485
[25] JG Holt, Bergey's Manual of Systematic Bacteriology. 1st Edn., Williams and Wilkins, Baltimore U.A., ISBN-10: 0683078933, **1986**, pp: 2648.
[26] M Gardes, TD Bruns, Mol. Ecol., **1993**, 2,113-118.
[27] K Tamura, D Peterson, N Peterson, G Stecher, M Nei, S Kumar, *Molecular Biol. Evolut*., **2011**, 28, 2731-2739.
[28] S Kim, S Ahu, W Seo, G Kwan, Y Park, *J. Microb. Biotechnol*., **1998**, 8, 178-181.
[29] H Mokaddem, Z Sadaoui, N Boukhelata, N Azouaou, Y Kaci, *J. Hazard. Mater*, **2009**, 172, 1150–1155.
[30] HL Po, Z Shuna, PH Kwok, YW Jian, *Food Chemistry*, **2009**, 114, 1251–1256.
[31] C Liu, J Lu, L Lu, Y Liu, F Wang, M Xiao, *Bioresou. Techn*. **2010**, 101, 5528-5533.
[32] RL Whistler, LJ Sannella, Methods Carbohydrate Chemistry, Acadimic press, New York and London, **1965**, 5, 34-46,
[33] H Wang, EV Ooi, PO Ang, *Sci. China*, **2007**, 50, 611-618.
[34] SR Sudhamani, RN Tharanathan, MS Prasad, *Carbohydrate Polymers*, **2004**, 56, 423-427.
[35] OH El-Sayed, SA Ismail, YM Ahmed, M Abd El-Samei, MMS Asker, *Egyptian Pharmaceutical J.*, **2005**, 4, 439-452.
[36] MMS Asker, YM Ahmed, MF Ramadan, *Carbohydrate Polymers*, **2009**, 77, 563–567.
[37] N Blumenkrantz, G Asboe-Hansen, *Anal. Biochem*., **1973**, 54, 484–489.
[38] TMCC Filisetti-Cozzi, NC Carpita, *Anal. Biochem*., **1991**, 197, 157–162.
[39] KS Dogson, RG Price, *Biochem J*., **1962**, 84, 106-110.
[40] L Jun, L Jiangguang, Y Hong, S Yi, L Zhaoxin, Z Xiaoxiong, *Carbohydrate Polymers*, **2009**, 78, 275–281.
[41] L You, Q Gao, M Feng, B Yang, J Ren, L Gu, C Cui, M Zhao, *Food Chem.*, **2013**, 138(4), 2242-9.

[42] RG Zhbankov, VM Andrianova, MK Marchewka, *J. Mol. Struct*., **1997**, 436, 637-654.
[43] LN Larsen, E Dahl, J Bremer, *Biochim. Biophys. Acta.*, **1996**, 1299, 47–53.
[44] I Marcocci, JJ marguire, MT Droy-lefaiz, L packer, *Biochemical and biophysical Research communication*, **1994**, 201, 748-755.
[45] T Yamaguchi, H Takamura, T Matoba, J Terao, *Biosci. Biotechnol. Biochem.*, **1998**, 62(6), 1201-4.
[46] F Liu, VE Ooi, ST Chang, *Life Sci.*, **1997**, 60, 763-71.
[47] I Gülçin, MT Uguz, M Oktay, S Beydemir, OI Küfrevioglu, *Journal of Food Technology*, **2003**, 1: 9-17.
[48] R Bruce, *Fundamental and Applied Toxicology*, **1985**, 5, 151-157.
[49] D Salvemini, ZQ Wang, PS Wyatt, DM Bourdon, MH Marino, PT Manning, MG Currie, *British J. of Pharmacology*, **1996**, 118, 829–838.
[50] D Guo, L Xu, X Cao, Y Guo, Y Ye, CO Chan, DKW Mok, Z Yu, S Chen, *Journal of Ethnopharmacology*, **2011**, 138: 717– 722.
[51] H Moshage, B Kok, JR Huizenga, PL Jansen, *Clinical Chemistry*, **1995**, 41, 892–896.
[52] LC Green, DA Wagner, J Glogowski, PL Skipper, JS Wishnok, SR Tannenbaum *Analytical Biochemistry*, **1982**, 126, 131–138.
[53] SD Guo, WJ Mao, Y Han, *Bioresource Technology*, **2010**, 101, 4729–4732.
[54] C Mancuso-Nichols, SG Lardière, JP Bowman, PD Nichols, J Gibson, J Guézennec, *Microb. Ecol.*, **2005**, 49, 578–589.
[55] CL Wang, TH Huang, TW Liang, CY Fang, SL Wang, *New Biotechnol*., **2011**, 28: 559–565.
[56] T Ramanathan, A Ahmed, AS Ahmed, M Kalimutho, *J. of Sustainability Science and Management*, **2011**, 6: 2-9.
[57] JS Van Dyk, LAN Kee, CL Frost, BI Pletschke, *Bio Resources*, **2012**, 7(4), 4976-4993.
[58] P Kanmani, RS Kumar, N Yuvaraj, KA Parri, V Pattukumar, C Zhang. *Bioresource Technol*., **2011**, 102, 4827-4833.
[59] RC Sun, JM Fang, A Goodwin, JM Lawther, AJ Bolton, *Carbohydrate Polymers*, **1998**, 37: 351-359.
[60] A Cheng, F Wan, J Wang, X Xu, *J. Ethnopharmacol*., **2008**, 118, 59-64.
[61] G Cirino, *Biochemical Pharmacology*, **1998**, 55, 105–111.
[62] DL Simmons, RM Botting, T Hla, *Pharmacological Reviews*, **2004**, 56: 387–437.
[63] Y Vodovotz, PK Kim, EZ Bagci, GB Ermentrout, CC Chow, I Bahar, TR Billiar, *Current Molecular Medicine*, **2004**, 4, 753–762.
[64] C Bogdan, *Nature Immunology*, **2001**, 2, 907–916.
[65] BR Lee, SY Kim, DW Kim, JJ An,Y Song Ha, KY Yoo, TC Kang, MH Won, KJ Lee, KH Kim, JH Joo, HJ Ham, JH Hur, SW Cho, KH Han, KS Lee, J Park, SY Choi, WS Eum, BMB reports, **2009**, http://bmbreports.org.
[66] TW Liang, SL Wang, *Marine drugs*, **2015**, 13, 1847-1863.
[67] TW Liang, CC Wu, WT Cheng, YC Chen, CL Wang, IL Wang, SL Wang, *Appl. Biochem. Biotechnol.*, **2014**, 172, 933–950.
[68] IL Shih, *Mini Rev. Med. Chem.*, **2010**, 10, 1345–1355.
[69] BT Ashok, R Ali, *Exp. Gerontol*., **1999**, 34:293–303.
[70] T Münzel, T Gori, RM Bruno, S Taddei, *European Heart Journal*, **2010**, 31, 2741–2749.
[71] MB Arnao, A Cano, M Acosta, *Food Chem*., **2001**, 73, 239–244.
[72] W Raza, K Makeen, Y Wang, Y Xu, S Qirong, *Bioresour. Technol.*, **2011**, 102, 6095–6103.
[73] CA Dinarello, *Am. J. Clin. Nutr.*, **2006**, 83, 447S–55S.
[74] SL Montgomery, WJ Bowers, *J. of Neuroimmune Pharmacology*, **2012**, 7, 42–59.
[75] V Adams, B Nehrhoff, U Spate, A Linke, PC Schulze, A Baur, S Gielen, R Hambrecht, G Schuler, *Cardiovascular Res*., **2002**, 54, 95–104.
[76] YY Wu, CC Chen, CC Chyau, SY Chung, YW Liu, *Acta Pharmacol. Sin.*, **2007**, 28, 258–267.
[77] L Wang, S Gao, W Jiang, C Luo, M Xu, L Bohlin, M Rosendahl, W Huang, *Int. J. Mol. Sci.*, **2014**, 15, 16226-16245.